Thesis

Tamoxifen pharmacokinetics and pharmacogenetics in endocrine sensitive breast cancer patients

DAHMANE, Elyes Ben Ali

Abstract

Tamoxifen is used for the treatment of estrogen-sensitive breast cancer. It is a prodrug that is bioactivated into endoxifen, the active metabolite mostly responsible for tamoxifen efficacy. Endoxifen levels show large inter-individual variability. Cytochrome (CYP)2D6 activity was presumed to play a role in predicting this variability and treatment efficacy. The objectives of the thesis were to assess the influence of pharmacogenetic and non-genetic factors on this variability and study the tamoxifen/endoxifen dose-concentration relationship. Based on a population pharmacokinetic study, we confirmed that CYP2D6 phenotype and inhibitors were major factors affecting endoxifen levels. We demonstrated the effectiveness of doubling tamoxifen dose in correcting low endoxifen levels, except for CYP2D6 poor metabolizers or patients under potent CYP2D6 inhibitors. We showed that CYP2D6 phenotype is a modest predictor of endoxifen exposure and demonstrated the rational of endoxifen concentration monitoring for detecting patients at risk of suboptimal treatment and for guiding tamoxifen dosage optimization.

Reference

DAHMANE, Elyes Ben Ali. *Tamoxifen pharmacokinetics and pharmacogenetics in endocrine sensitive breast cancer patients*. Thèse de doctorat : Univ. Genève, 2013, no. Sc. 4617

URN : urn:nbn:ch:unige-334293

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FACULTÉ DE BIOLOGIE ET MÉDECINE
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Tamoxifen Pharmacokinetics and Pharmacogenetics in Endocrine Sensitive Breast Cancer Patients

THÈSE

présentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention sciences pharmaceutiques

par

Elyes Dahmane

De

Tunis,Tunisie

Thèse N° 4617

Genève
2013

UNIVERSITÉ DE GENÈVE

FACULTÉ DES SCIENCES

Doctorat ès sciences
Mention sciences pharmaceutiques

Thèse de *Monsieur Elyes DAHMANE*

intitulée :

" Tamoxifen pharmacokinetics and pharmacogenetics in endocrine sensitive breast cancer patients "

La Faculté des sciences, sur le préavis de Madame C. CSAJKA, professeure associée et directrice de thèse (Section des sciences pharmaceutiques), Messieurs L. DECOSTERD, professeur et codirecteur de thèse (Service de biomédecine, Laboratoire de pharmacologie clinique, Faculté de médecine, Université de Lausanne, Suisse), S. RUDAZ, professeur associé (Section des sciences pharmaceutiques) et M. JOERGER, docteur (Medizinische Onkologie und Klinische Pharmakologie, Department Innere Medizin, Kantonsspital, St Gallen, Schweiz), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 29 novembre 2013

Thèse - 4617 -

Le Doyen, Jean-Marc TRISCONE

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

REMERCIEMENT

Je tiens à exprimer ma profonde reconnaissance au Professeur Chantal Csjaka qui m'a offert l'opportunité d'effectuer cette thèse au sein de son équipe et qui m'a ouvert les portes pour enrichir mes connaissances et acquérir des compétences dans le domaine de la pharmacologie clinique et la pharmacocinétique. Je la remercie pour sa grande confiance, son soutien, ses conseils et sa grande disponibilité.

J'aimerais également et tout particulièrement remercier le Professeur Laurent Arthur Decosterd qui m'a ouvert son laboratoire, m'a intégré dans son équipe et m'a permis de m'initier et de développer mes connaissances en bioanalyse médicamenteuse par spectrométrie de masse. Je le remercie également pour son encadrement, son soutien et ses conseils durant ma thèse, aboutissant à une fructueuse collaboration. Par la même occasion, j'adresse mes sincères remerciements à toute l'équipe du laboratoire : Thomas et Boris, avec qui j'ai étroitement collaboré, et Sandra, Nicole et Beatrice pour leur aide pour les dosages du tamoxifène et pour leur soutien logistique.

J'adresse mes vifs remerciements au Professeur Thierry Buclin pour tous les échanges et discussions enrichissantes et motivantes, ses conseils, son enthousiasme et l'intérêt particulier porté à mon projet de thèse permettant ainsi de stimuler et améliorer la qualité de ce travail.

Je suis aussi très reconnaissant au Dr. Khalil Zaman, l'investigateur principale de l'étude clinique dans le cadre de laquelle s'est découlé mon travail de thèse.

J'adresse aussi mes sincères et chaleureux remerciements au Dr. Bertrand Rochat avec qui j'ai collaboré et qui m'a permis d'avoir un bon aperçu sur la spectrométrie de masse à haute résolution.

Je remercie tous mes collègues de la Division de Pharmacologie Clinique - Aline, Ali, Alice, Catherine, Delphine, Eric, Etienne, Fady, Françoise, Haithem, Hélène, Laura, Léonore, Manuel, Monique, Nicolas, Ursula et Verena – pour leur accueil, amitié et leur soutien concernant des questions scientifique et non scientifiques liés a l'activité de cette division. Un remerciement tout particulier à Monia pour sa grande amitié, sa disponibilité, ses conseils et son encadrement pour la pharmacocinétique de population.

Finalement, je tiens à exprimer toute ma gratitude envers ma famille pour tous leurs encouragements et soutiens.

ABSTRACT

Background: Tamoxifen, a standard treatment for estrogen-sensitive breast cancer, is a pro-drug that needs to be activated into its major active metabolite – endoxifen – to fully exert its pharmacological activity. CYP2D6 is the key enzyme responsible for endoxifen formation and patients with impaired CYP2D6 activity, due for instance to *CYP2D6* gene polymorphism or some potent CYP2D6 inhibiting co-medications, display low endoxifen plasma concentrations and thus could achieve less benefit from their tamoxifen treatment. Endoxifen levels are characterized by a large inter-individual variability. *CYP2D6* polymorphism has been presumed to play the major role in explaining and predicting endoxifen levels variability and exposure and has been proposed as a potential indirect marker for tamoxifen efficacy.

Objectives: The objectives of this thesis have been addressed within the framework of a clinical trial aimed to study the effect of doubling tamoxifen dose on the pharmacokinetics of tamoxifen and its major metabolites, notably endoxifen, in breast cancer patients classified according to their CYP2D6 phenotype. Furthermore, this trial aimed at studying the population pharmacokinetics of tamoxifen and its metabolites, in order to quantify the inter- and intra-individual variability in their plasma levels and identify the major (pharmaco)genetic and non-genetic factors influencing and predicting this variability.

Methods and results: We developed and validated a sensitive and selective liquid chromatography-tandem mass spectrometry bioanalytical method for the measurement of tamoxifen and three of its metabolites in the plasma of breast cancer patients. The joint population pharmacokinetics of tamoxifen and its metabolites of clinical interest have been analyzed and performed using a non-linear mixed effects modeling approach. This first population pharmacokinetic model allowed us to identify and/or to confirm the influence of demographic and environmental factors (age, compliance, CYP2D6 inhibitors) as well the influence of pharmacogenetic factors (CYP2D6 phenotype, CYP3A4 activity) on the disposition of tamoxifen and its metabolites. This model particularly enabled us to confirm that the major identified contributors impacting CYP2D6 activity explained together only a third of the large inter-individual variability in endoxifen levels. Besides, the results from this study suggested that CYP2D6 phenotype is a modest predictor of endoxifen levels and confirmed the inferiority of *CYP2D6* genotyping over direct endoxifen levels measurement in indentifying patients with low endoxifen levels. Our analysis also demonstrated the effectiveness of doubling tamoxifen dose in correcting endoxifen levels above a specific concentration threshold (> 6 ng/mL, reported in literature) in a majority of patients with the exception of some patients (eg. some CYP2D6 poor

metabolizers patients and some patients under potent CYP2D6 inhibitors) that may benefit from higher tamoxifen doses.

Conclusions: The observations from our study demonstrate the strong rational and superiority of direct endoxifen monitoring for detecting patients at risk of suboptimal tamoxifen treatment and driving tamoxifen dosage optimization. Our developed population pharmacokinetic model contributed to a general insight, a better understanding and quantification of the impact of various sources of variability on tamoxifen and its metabolites disposition, particularly endoxifen. This model is useful to support large therapeutic drug monitoring studies, throughout the development of reference pharmacokinetic curves and model-based simulations to derive dose optimization strategies.

RÉSUMÉ

Introduction : Le tamoxifène est un traitement standard du cancer du sein hormono-dépendant. Le tamoxifène est considéré comme une 'prodrogue' qui nécessite d'être métabolisée en endoxifène, son principal métabolite actif, afin d'exercer pleinement son activité anti-œstrogènique. Le CYP2D6 est l'enzyme clé intervenant dans la formation de l'endoxifène. Les patientes ayant une activité altérée (réduite ou nulle) du CYP2D6, suite par exemple à un polymorphisme génétique du *CYP2D6* ou à une interaction avec certains médicaments puissant inhibiteurs du CYP2D6, présentent des taux plasmatiques faibles d'endoxifène se traduisant par une diminution de l'efficacité de ce traitement. De surcroît, les concentrations plasmatiques d'endoxifène présentent une grande variabilité interindividuelle. Le polymorphisme génétique du *CYP2D6* est supposé jouer un rôle prépondérant dans cette variabilité et a été proposé par certaines études comme un marqueur indirect de l'efficacité du tamoxifène.

Objectifs : Les objectifs de cette thèse ont été de caractériser le profil pharmacocinétique du tamoxifène et de ses principaux métabolites actifs afin de quantifier la variabilité interindividuelle et d'identifier les sources génétiques et non génétiques associés à cette variabilité. D'autre part, une étude clinique évaluant l'effet du doublement de la dose sur la pharmacocinétique du tamoxifène et de ses principaux métabolites, notamment l'endoxifène a été conduite afin de déterminer si une modification de la posologie était susceptible de compenser pour une déficience en CYP2D6 associée à des concentrations sous-optimales d'endoxifène.

Méthodes et résultats : Dans le cadre de cette étude, nous avons développé et validé une méthode analytique, par chromatographie-liquide couplée à la spectrométrie masse en mode tandem, permettant le dosage du tamoxifène et trois de ses métabolites dans le plasma. Les données collectées lors de cette étude nous ont permis d'établir, en utilisant une approche de régression non linéaire à effets mixtes, la pharmacocinétique de population pour ce médicament, ainsi que pour celle de trois de ses métabolites, notamment l'endoxifène. Ce modèle de pharmacocinétique de population, qui n'a jamais été reporté auparavant dans la littérature, a permis d'identifier et/ou de confirmer l'influence de certains facteurs démographiques et environnementaux (âge, adhérence thérapeutique, médicaments inhibiteurs du CYP2D6), ainsi que l'influence de facteurs pharmacogénétiques (phénotype CYP2D6 et du CYP3A4) sur la pharmacocinétique du tamoxifène et ses métabolites. Ce modèle a notamment permis de confirmer que les principaux facteurs affectant l'activité du CYP2D6 expliquent uniquement un tiers de la large variabilité interindividuelle observée pour les concentrations d'endoxifène. Par ailleurs, nous avons aussi pu démontrer que le génotypage du *CYP2D6* ne permet pas de prédire quelles patientes seraient à risque de présenter des

concentrations sous-thérapeutiques d'endoxifène et par conséquent de tirer un moindre bénéfice de leur traitement. La comparaison des concentrations plasmatiques d'endoxifène mesurées avant et après le doublement de dose du tamoxifène a permis également de démontrer l'efficacité et la faisabilité de l'adaptation posologique du tamoxifène comme moyen permettant la correction des taux d'endoxifène au-dessus d'un seuil thérapeutique critique (> 6 ng/mL, rapporté dans la littérature) chez la majorité des patientes. Toutefois, chez certains patients métaboliseurs lents du CYP2D6 ou recevant de puissants inhibiteurs du CYP2D6 en comédication, le doublement de la dose du tamoxifène ne permet pas d'atteindre les concentrations cibles d'endoxiféne.

Conclusion : Les observations de notre étude démontrent le rationnel et la supériorité du suivi thérapeutique des taux d'endoxifène d'une part pour détecter les patientes à risque d'une réponse non optimale au traitement par le tamoxifène et d'autre part pour l'optimisation de la posologie du tamoxifène. Le modèle de pharmacocinétique de population du tamoxifène et de ses métabolites a contribué à une meilleure compréhension et à la quantification de l'impact des différentes sources de variabilité sur les concentrations des différentes substances actives. Ce modèle pharmacocinétique permettra le développement de courbes de pharmacocinétiques de références utiles pour guider le suivi thérapeutique et l'individualisation du traitement par le tamoxifène.

PUBLICATIONS

PUBLICATIONS IN PEER REVIEWED JOURNALS

ORIGINAL RESEARCH ARTICLES

Dahmane E, Mercier T, Zanolari B, Cruchon S, Guignard N, Buclin T, Leyvraz S, Zaman K, Csajka C, Decosterd LA. An ultra performance liquid chromatography-tandem MS assay for tamoxifen metabolites profiling in plasma: first evidence of 4'-hydroxylated metabolites in breast cancer patients. Journal of Chromatography. B, Analytical Technologies In the Biomedical and Life Sciences 2010 Dec 15;878(32):3402-14. Epub 2010 Oct 30.

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Zaman K, **Dahmane E**, Perey L, Bodmer A, Wolfer A, Galmiche M, Buclin T, Eap C.B, Decosterd LA, Csajka C and Leyvraz S. Endoxifen levels after tamoxifen dose escalation: a prospective trial with genotyping, phenotyping and pharmacokinetics over 4 months. [In preparation].

BOOK CONTRIBUTIONS

Decosterd LA, **Dahmane E**, Neeman M, Buclin T, Csajka C, Haouala A and Widmer N. Therapeutic Drug Monitoring of Targeted Anticancer Therapy. Tyrosine Kinase inhibitors and Selective Estrogen Receptor Modulators: A Clinical Pharmacology Laboratory Perspective. In: LC-MS in Drug Bioanalysis. Edited by Xu QA, Madden TL: Springer US; 2012: 197-250. [DOI: 10.1007/978-1-4614-3828-1_9].

SCIENTIFIC COMMUNICATIONS

POSTERS

Dahmane E, Zaman K, Perey L, Bodmer A, Leyvraz L, Eap C.B, Galmiche M, Decosterd LA, Buclin T, Guidi M, Csajka C. Population pharmacokinetics of tamoxifen and three of its metabolites in breast cancer patients. In: 22st Annual Meeting of the Population Approach Group Europe (PAGE), Glasgow Scotland, 11-14 June, 2013.

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LIST OF ABREVIATIONS

20 md BID	20 mg twice daily
20 mg QD	20 mg once daily
4OHTam or 4-OH-Tam	4-hydroxy-tamoxifen
AI	Aromatase inhibitor
AS	Activity score
BC	Breast cancer
BCRP	Breast cancer resistance protein
CL/F	Apparent clearance
CV	Coefficient of variation
CYP	Cytochrome P450
DCIS	Ductal carcinoma in situ
DM/DX	Dextromethorphan/Dextrorphan ratio
EHC	Enterohepathic circulation
EM	Extensive metabolizer
ESI	Electrospray ionisation
F	Bioavailability
HR	Hazard ratio
IIV	Inter-individual variability
IM	Entermediate metabolizer
k_{23}	Tam to NDTam metabolic rate constant
k_{24}	Tam to 4OHTam metabolic rate constant
k_{35}	NDTam to endoxifen metabolic rate constant
k_{45}	4OHTam to endoxifen metabolic rate constant
k_a	Absorption rate constant
LCIS	Lobur carcinoma in situ

LC-MS/MS	Liquid chromatography tandem mass spectrometry
MR	Midazolam metabolic ratio
MRP2	Multidrug resistance associated-protein 2
MS	Mass spectrometry
NDTam or N-D-Tam	N-desmethyl-tamoxifen
PD	Pharmacodynamics
PG	pharmacogenetics
P-gp	P-glycoprotein
PK	Pharmacokinetics
PM	Poor metabolizer
POR	P450 oxidoreductase
RSE	Relative standard Error
SD	Standard deviation
SE	Standard Error
SNRI	Selective serotonin and norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
SULT	Sulfotransferase
Tam	Tamoxifen
TDM	Therapeutic drug monitoring
UGT	UDP-glucuronosyltransferases
UM	Ultrarapid metabolizer
UPLC	Ultra-high-performance liquid chromatography
V/F	Apparent volume of distribution
VPC	Visual predictive check
ΔOFV	Objective functions difference

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CHAPTER I - INTRODUCTION

I.1. EPIDEMIOLOGY

Breast cancer is the most commonly diagnosed malignancy and is the leading cause of cancer mortality in women worldwide. It accounted for 23% of all cancer diagnoses (1.38 million) and 14% of cancer deaths (458,400) in 2008 [1]. Male breast cancer is rare and account for approximately 1% of all breast cancer cases. Approximately 70 to 80% of breast cancers express estrogen and/or progesterone receptors and thus are estrogen and/or progesterone sensitive.

In general, incidence rates are higher and mortality rates are lower in industrialized than non-industrialized countries. However, breast cancer incidence has been rapidly increasing along with the westernization of the developing countries and about 50% the breast cancer cases and 60% of the deaths are now thought to occur in developing regions [1]. Reduced death rates in developed countries are likely due to the early detection of breast cancers through established mammography screening programs (~ 20% relative reduction in mortality in 50 to 70 year-old women that followed the program) and the improvements in treatment options [2].

In Europe, it is estimated that 1 in 9 women will be diagnosed with breast cancer during their lifetime (Table 1). In 2012, the absolute incidence and mortality of breast cancer in Europe was ~ 464,000 and ~ 131,000 new cases, respectively [3]. In Switzerland, 9% of women (1 in 11 women) would be expected to develop a breast cancer and 1.9% (1 in 50 women) would be expected to die from breast cancer before the age 75 (Table 1). In 2012, the estimated new cases and deaths from breast cancer were ~ 5750 new cases and ~ 1200 deaths [3-5].

Table 1: Breast cancer incidence and mortality statistics [3]

	Age-standardized annual incidence	Age standardized annual mortality
Europe	94.2 per 100,000	23.1 per 100,000
Switzerland	111.3 per 100,000	19.8 per 100,000

I.2. RISK FACTORS

Female gender and age are the major determinants for breast cancer incidence [6]. Breast cancer incidence increases steadily and rapidly between the ages of 35 and 39 then slows around the age of 50 years but continue to increase and flatten by the age of 70 [4, 7]. Breast cancer is predominantly a postmenopausal malignancy. In Switzerland, approximately 75% of cases develop in postmenopausal women and nearly half of breast cancer cases (47%) are diagnosed between the ages of 50 and 69

years [4]. Apart from sex and age, several other factors (Table 2) have been associated to an increased risk for the development of breast cancer [6, 8-10].

Table 2: Breast cancer risk factors

Risk factors	Relative Risk (RR)		
	RR ≤ 2	**2 < RR < 4**	**RR ≥ 4**
Personal and family medical history:			
▪ Age (50 to 70 years old).			++
▪ Sex (female).			++
▪ *BRCA1* or *BRCA2* gene mutation.			+
▪ One or two first-degree relatives (mother, sister, daughter) with diagnosed breast cancer.			+
▪ History of non-invasive carcinoma: lobur or ductal carcinoma in situ (DCIS or LCIS).			+
▪ History of atypical lobur or ductal hyperplasia.			+
▪ Personal history of breast cancer.			+
▪ Breast density on mammography i.e. more glandular and fibrous tissue than fat tissue (postmenopausal).		+	
Reproductive history:			
▪ Early age at menarche (< 12 years).	+		
▪ Late age of menopause (>55 years).		+	
▪ Nulliparity and older age at first pregnancy (> 30 years).		+	
▪ No breastfeeding.	+		
Medication history:			
▪ High-dose radiation therapy to the chest before the age of 30.		+	
▪ Combination hormone therapy (estrogen - progestin) current and recent use (last 5 years).	+		
▪ Current or recent use of oral contraceptives (last 10 years).	+		
Lifestyle factors:			
▪ Obesity and weight gain (postmenopausal women).	+		
▪ No physical activity.	+		
▪ Excess of alcohol consumption.	+		

I.3. BREAST CANCER TREATMENT

Breast cancer treatment consists in various combinations of surgery, radiation therapy and neo-adjuvant or adjuvant systemic therapy including: cytotoxic chemotherapy, anti-hormonal (endocrine) therapy, targeted anti-HER2 treatments, or combination of these. For early breast cancer and according to the 2013 St Gallen International Expert Consensus [11], the choice of the appropriate adjuvant systemic treatments should be based on the surrogate early breast cancer intrinsic subtypes (Figure 1).

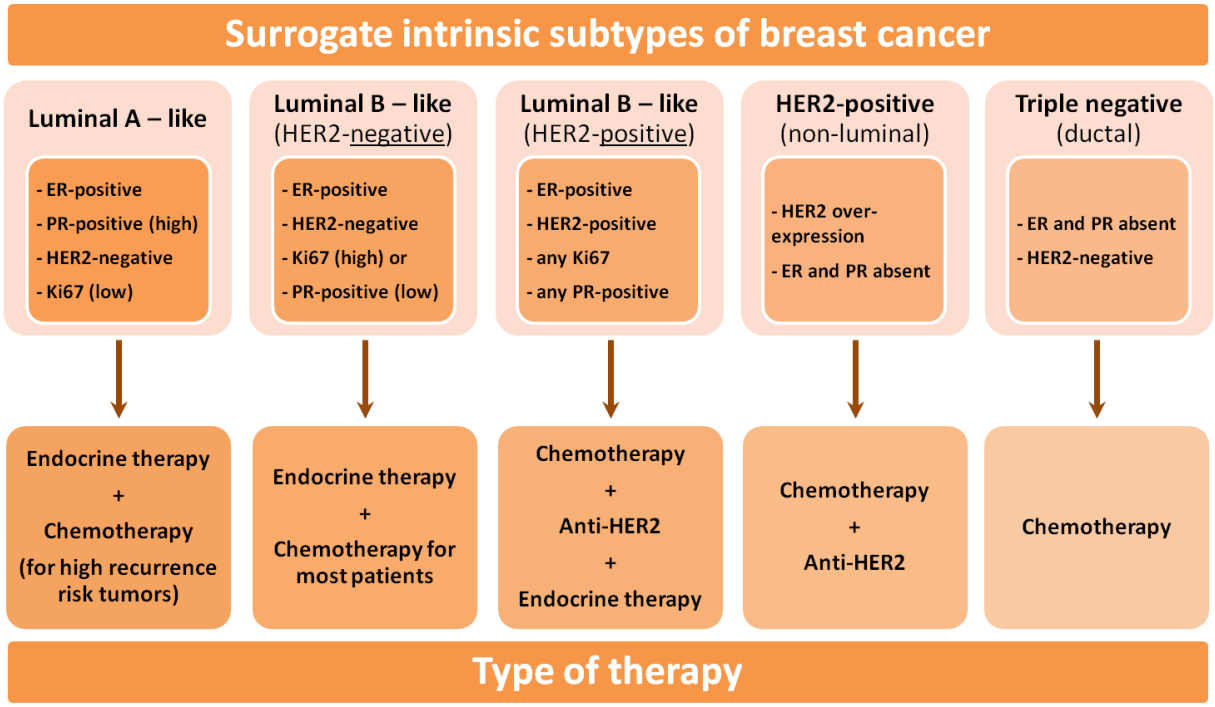

Figure 1: Adjuvant systemic treatment recommendations for early breast cancer subtypes. Derived from [11, 12]. ER: estrogen receptor, PR: progesterone receptor, HER2: Human Epidermal growth factor Receptor 2, Ki-67: proliferating cell nuclear antigen, Endocrine therapy (tamoxifen or aromatase inhibitors), anti-HER2 (trastuzumab).

Endocrine therapy (tamoxifen, aromatase inhibitors or fulvestrant) remain also the preferred option as initial therapy in advanced ER-positive breast cancer, in case of slow progression and less aggressive metastatic disease (i.e limited/asymptomatic visceral involvement) and in the absence of suspected endocrine resistance [12-14].

I.4. ADJUVANT ENDOCRINE TREATMENT OF BREAST CANCER: THE SPECIFIC CASE OF TAMOXIFEN

Tamoxifen is a milestone in the adjuvant treatment of early and advanced ER and/or PR positive breast cancer. Tamoxifen is also indicated for the prevention of breast cancer in healthy women at high risk of developing an invasive cancer (i.e women with LCIS, atypical hyperplasia or an absolute risk ≥ 1.66 according to Gail model [15]).

In the adjuvant setting, tamoxifen is the gold standard adjuvant treatment for premenopausal women. Tamoxifen remains also a valid choice over aromatase inhibitors (AIs), either in upfront use or in switching strategy, for postmenopausal women at lower to intermediate risk of relapse or presenting specific contraindications to AIs or severe adverse effects [16].

A 5-year course of tamoxifen (at 20 mg/day) reduces breast cancer recurrence by 39% throughout the first 15 years (50% rate reduction during the first 5 years of treatment and 30% during the 5 years following the end of therapy) with an absolute recurrence reduction of 13% (46.2 vs 33%). Breast cancer mortality is reduced by almost 30% throughout the first 15 years (24 reduction the first 5 years and a 30% extra reduction in mortality during years 5-9 and 10-15) with an absolute mortality reduction of 9% (33.1% vs 23.9%) compared to the non tamoxifen control arm [17-19].

Recently, the ATLAS (Adjuvant Tamoxifen-Longer Against Shorter) trial suggested that the extended adjuvant tamoxifen treatment, with 5 additional years, significantly further reduces breast cancer recurrence by 25% and breast cancer mortality by 29% during years 5 to 15 (absolute reduction of 2.8% and 3.7% respectively) [20]. In the preventive setting, tamoxifen (20mg per day for 5 years) reduces breast cancer incidence (invasive and DCIS) in pre- and postmenopausal women by ~ 33% over 10 years of follow-up [21].

I.4.1. Mechanism of action

The expression of ER in breast tumors provided the first target to breast cancer treatment and is the critical factor predicting the response to anti-hormonal therapy. Tamoxifen was the first targeted anticancer agent used in the treatment for estrogen sensitive breast cancer.

Selective estrogen receptor modulators, such as tamoxifen, display tissue-selective estrogen agonist or antagonist effects. In breast tissues, tamoxifen exerts an antiestrogenic activity mediated by the competitive inhibition of 17-beta-estradiol (E2) binding to estrogen receptors alpha and beta (ERα and ERβ), resulting in the suppression of ERα transcriptional activity and inhibition of estrogen-dependent growth and proliferation of malignant breast epithelial cells [22]. However, several lines of evidence indicate that the overall anti-proliferative effects of tamoxifen depend on the formation of the pharmacologically active metabolites 4-hydroxy-tamoxifen and notably 4-hydroxy-*N*-desmethytamoxifen (named: endoxifen) which have up to 100 fold greater affinity to ERs and 30 to 100 fold greater potency in suppressing breast cancer cell proliferation as compared to the parent drug [22].

Of these active metabolites, endoxifen is suggested to be the primary active metabolite responsible for the majority of tamoxifen clinical efficacy, as endoxifen plasma concentrations are about 5–10 fold higher than those of 4-hydroxy-tamoxifen [22]. Endoxifen may have additional mechanisms of action than 4-hydroxy-tamoxifen by targeting Erα for degradation by proteasome [22] and through the promotion of ERα/ERβ heterodimerization, blocking ERα transcriptional activity [23].

I.4.2. Tamoxifen metabolism

The metabolism of tamoxifen is complex and undergoes extensive phase I and phase II reactions (Figure 2). Various potentially polymorphic cytochrome P450 (CYP) enzymes including CYP3A4, 3A5, 1A2, 2B6, 2C9, 2C19 and 2D6 catalyze, to different extent, the hepatic biotransformation of tamoxifen into active and inactive primary and secondary metabolites [24-27].

Tamoxifen is primary converted to N-desmethyl-tamoxifen by CYP3A4 and to 4-hydroxy-tamoxifen mainly by CYP2D6 and CYP2C9. Both N-desmethyl-tamoxifen and 4-hydroxy-tamoxifen are secondly metabolized to form endoxifen through CYP2D6 and CYP3A4/5 enzymes, respectively.

N-desmethyl-tamoxifen is quantitatively the major tamoxifen metabolite. It accounts approximately for 92% of primary tamoxifen oxidation [27]. In women receiving a daily dose of 20 mg tamoxifen, steady-state plasma concentrations of NDTAM are 1.5 to 2 fold higher than those of tamoxifen. 4-hydroxy-tamoxifen and endoxifen constitute relatively minor tamoxifen metabolites with plasma concentrations of 4-hydroxy-tamoxifen ~ 5 to 10 fold lower than those of endoxifen [28-31]. Steady-state plasma concentration of tamoxifen is achieved after 1 month with terminal elimination half-life of about 5 to 7 days [28, 29]. *N*-desmethyl-tamoxifen (elimination half-life of about 10 to 14 days), 4-hydroxy-tamoxifen, endoxifen have longer elimination half-life than tamoxifen and their steady-state plasma concentrations were reported to achieved within 2 to 4 months after treatment initiation [28, 29].

Tamoxifen and its metabolites undergo further glucuronidation and sulfation. Different hepatic and extrahepatic UDP-glucuronosyltransferases (UGTs) exhibited in-vitro glucuronidation activities towards tamoxifen and its metabolites leading to inactive metabolites [32]. The hepatic enzyme UGT1A4 is considered the major enzyme responsible – *in vitro* – for the *N*-glucuronidation of tamoxifen and 4-hydroxy-tamoxifen [33-36]. Hydroxylated active tamoxifen metabolites (i.e. *Z*-4-hydroxy-tamoxifen and *Z*-endoxifen) equally go through O-glucuronidation involving mainly UGT2B7 and the extra-hepatic glucuronidating enzymes UGT1A10 and 1A8 [37, 38]. Sulfotransferase (SULT) 1A1 is the major phase II metabolizing enzyme involved in the sulfation of 4-hydroxy-tamoxifen and endoxifen [39-42]. These sulfated and glucuronidated metabolites are further eliminated in urine, bile and undergo enterohepathic circulation (EHC) [43-45].

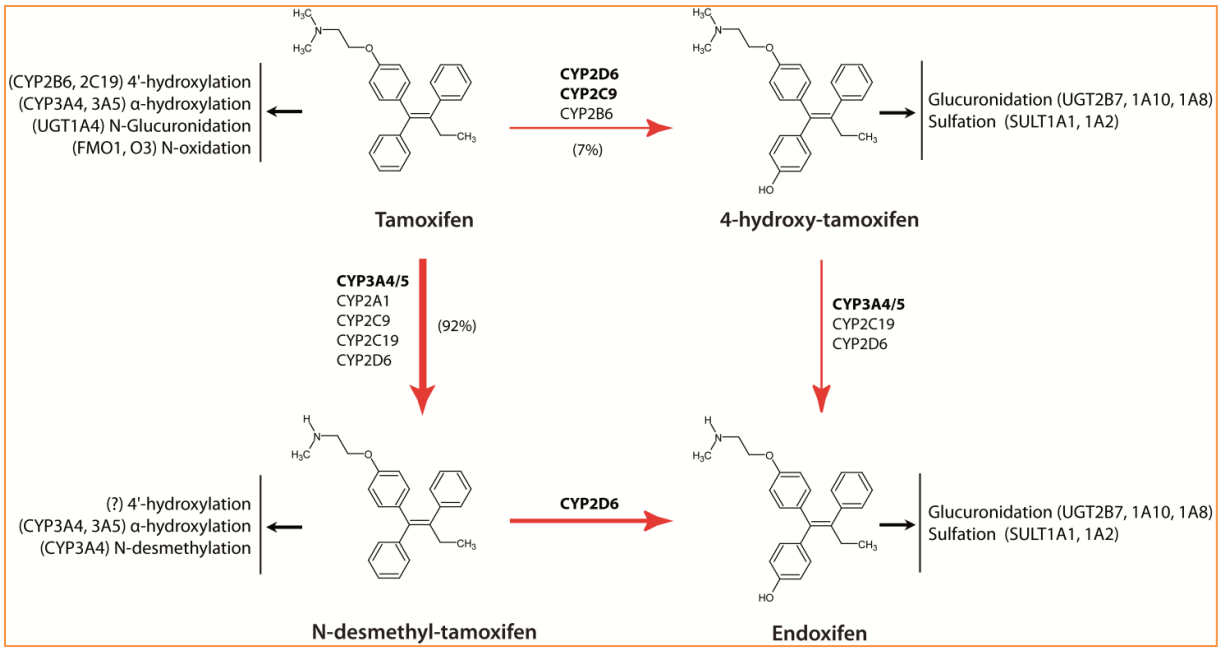

Figure 2: Principal tamoxifen metabolism pathways.

I.5. PHARMACOGENETICS AND PHARMACOKINETICS DETERMINANTS OF TAMOXIFEN EFFICACY

Despite the obvious benefits of this tamoxifen in the different treatment settings, the clinical outcomes of tamoxifen treatment in terms of efficacy and side effects are incomplete and inconstant, and almost 30 to 40% of patients either fail to respond or become resistant to tamoxifen [46]. One of the proposed mechanisms that may account for the impaired response to tamoxifen therapy is an altered bio-activation of the parent drug into endoxifen, and this either by genetic or environmental (non-genetic) factors [46, 47].

I.5.1. CYP2D6 genetic variability

CYP2D6 is the key enzyme responsible for the generation of endoxifen [27]. The metabolizing activity of this enzyme is highly polymorphic and varies considerably within a population and between ethnic groups. This large variability is partly determined by genetic polymorphisms in the *CYP2D6* gene, with over 100 allelic variants identified to date, resulting in different phenotypic patterns [48, 49]. Currently, on the basis of CYP2D6 activity, the population is usually categorized into 4 phenotypes including ultrarapid metabolizers (UMs), extensive metabolizers (EMs), intermediate metabolizers (IMs) and poor metabolizers (PMs). Actually, *CY2D6* gene polymorphisms, associated with null or reduced enzyme activity, have been reported to negatively influence (in a gene-dose manner) the blood level of endoxifen in numerous prospective pharmacokinetic studies [28, 29, 50-54]. Some

retrospective and prospective studies have shown that *CYP2D6* polymorphism was associated with worse clinical outcomes in PMs and IMs patients in terms of recurrence, disease free survival and overall survival or breast cancer development in the prevention setting [50, 52, 53, 55-63].

Several studies sought for the relevance of *CYP2D6* genotyping as a biomarker of tamoxifen efficacy. As much as 25 published reports, essentially retrospectives studies and retrospective analyses of prospective cohort trials, have addressed this issue. The report by Schroth et al. [60] is one of the largest cohort study, in postmenopausal women with early breast cancer and under adjuvant tamoxifen monotherapy, that observed a significantly higher risk of breast cancer recurrence in patient with impaired CYP2D6 activity (HR of 2.12 for PM and 1.49 for IM for time to recurrence, $P < 0.006$) than in EM patients. Other studies in Caucasians and Asian populations have reported similar worse clinical outcomes in PM and IM than EM patients in terms of recurrence- and disease-free survival and overall survival [52, 53, 59, 61, 62, 64-67]. Conversely, several studies [68-74] and most notably two recent retrospective analyses of large prospective trials, the ATAC (Arimidex®, Tamoxifen, Alone or in Combination) [75] and the BIG 1-98 (Breast International Group 1-98) [76] trials failed to show any significant relationship between CYP2D6 phenotypic groups and recurrence, however the validity of genotype data in these studies have been questioned.

In the metastatic treatment setting, 2 studies observed a shorter time to progression and worse overall survival in IM CYP2D6 patients heterozygous for the reduced *CYP2D6* allele *10 [50] and in patients poor *CYP2D6* metabolizers or under strong CYP2D6 inhibitors [77]. In the prevention setting, an Italian prevention trial showed a higher breast cancer incidence in women with the PM phenotype [63]. However, 2 other larger prevention trials failed to demonstrate an impact of CYP2D6 metabolism or potent CYP2D6 inhibitor on tamoxifen efficacy [78, 79].

The great heterogeneity and inconsistency in study results have been attributed to different confounding factors and critical errors in experimental and study designs [80-82]. The major identified factors are inherent to the quality genotype data including:

- The lack of comprehensive *CYP2D6* genotyping with limited allelic coverage and consequently misclassification of patients [83].
- Heterogeneity in alleles grouping and phenotypes definition or scoring.
- The use of low-quality genetic DNA extracted from somatic tissues and especially breast tumor tissues frequently affected (~ 35% of ER + tumors) by a loss of heterozygosity (LOH) at the chromosome 22q13 harboring the *CYP2D6* gene. This deletion in the *CYP2D6* genes leads to strong allelic imbalance, deviation for Hardy-Weinberg Equilibrium (a measure of expected allele and genotype frequencies in the population and an indicator of genotyping quality and

uniformity of the population under investigation) and erroneous *CYP2D6* genotyping and misinterpretation of pharmacogenetics study results.

Other confounders identified from study comparisons may explain theses discrepant data. These confounders include lack of information or adjustment for CYP2D6 inhibitors co-administration, lack of information on tamoxifen adherence and the use of combination therapy. Actually, most of the studies that failed to show any association between *CYP2D6* genotype polymorphism and tamoxifen efficacy, included patients with concomitant use of tamoxifen and chemotherapy. Besides, heterogeneity in size of the study population, disease stage, end-point definition (i.e recurrence-free survival, event-free survival, disease-free survival, overall survival) and length of follow-up (particularly in case of a switch to an aromatase inhibitor) may contribute to explain controversial findings of these studies [80, 81, 84].

I.5.2. Influence of other genetic variations of metabolizing enzymes and transporters

As previously indicated, other CYPs (CYP2C9, 2C19, 3A4, 3A5), UGTs and SUTs are also involved in the metabolism of tamoxifen. For CYP2C19, Schroth et al. [57] found that carriers of the CYP2C19*17 variant (homozygous or heterozygous) had a lower risk for relapse than patient carriers of *1 (wild type), *2 or *3 (defectives) alleles. Other group either failed to confirm such results or found opposite results suggesting a longer breast cancer survival in carriers of the defective CYP2C19*2 variant [63, 71, 85, 86]. Conversely, for CYP3A5 (*3 null variant), 2B6, 2C9 (*2, *3 null variant), SULT1A1 (gene copy number and *2 reduced activity variant), UGT1A2B7 (*2 reduced activity allele) and UGT2B15 (*2, increased activity alleles) no association between genotype and clinical outcome have been demonstrated [55, 60, 68-70, 75, 87, 88]. In addition, tamoxifen and its active metabolites 4-hydroxy-tamoxifen and endoxifen are substrate of P-glycoprotein (P-gp), however, this enzyme do not seem to play a significant role in the drug pharmacokinetics and pharmacodynamics [66, 89, 90]. The study by Kiyotani et al. [53] have shown no effect of *ABCB1* gene polymorphism (coding for the P-gp) on tamoxifen outcome, however, they observed that patient with reduced activity of the transporter MRP2 (multidrug resistance associated-protein 2 coded by the *ABCC2* gene) have shorter recurrence-free survival.

I.5.3. Effect of non-pharmacogenetics factors

I.5.3.1. CYP2D6 inhibitors

Amongst patient under tamoxifen treatment, approximately 14 to 30% receive an antidepressant, such as selective serotonin reuptake inhibitors (SSRIs) or selective serotonin and norepinephrine reuptake inhibitors (SNRIs), to treat depression or to alleviate tamoxifen-induced vasomotor

symptoms (i.e. hot flushes) [91-93]. Some of these SSRIs, such as paroxetine and fluoxetine, are strong CYP2D6 inhibitors resulting in a reduced to null CYP2D6 activity and impairment in tamoxifen metabolism with a significant reduction (up to 70%) in the main active tamoxifen metabolite, endoxifen concentration to levels comparable to those in CYP2D6 PM patients [28, 29, 94].

Such phenocopying to apparent CYP2D6 PM status, is expected to hamper treatment effectiveness. However, the epidemiologic studies that have attempted to correlate concomitant CYP2D6 inhibitor use to tamoxifen outcome have also reported mixed conclusions. One large cohort study by Kelly et al. [95] reported an increase in breast cancer mortality risk in patient using paroxetine and this increase in mortality was closely related to the duration of overlap use with tamoxifen. They estimated that for a 41% overlap-time use with tamoxifen, 1 additional breast cancer death over 20 women occur within 5 years after tamoxifen cessation. This study failed to demonstrate the same association for fluoxetine, sertraline, fluvoxamine, venlafaxine and citalopram. Other studies reported an increased risk of recurrence and lower overall survival in patients under strong and moderate CYP2D6 inhibitors [55, 77, 96]. By contrast, in a population-based case-control study in Denmark, Lash et al. find no evidence of a relationship between concurrent use of (es)citalopram and tamoxifen and risk of breast cancer recurrence [97]. The same observation was also reported for more potent CYP2D6 inhibitors (paroxetine, fluoxetine, sertraline) [74, 98-101].

As for pharmacogenetics studies, results from different retrospective heterogeneous cohort and case-control studies were conflicting and failed to confirm the observed association even between strong CYP2D6 inhibitors and tamoxifen outcome. Although, these findings were inconclusive, present recommendations are to avoid whenever possible the use of potent CYP2D6 inhibitors in tamoxifen treated breast cancer patients and to consider rather the use of medications with little CYP2D6 inhibitory potential such as venlafaxine and citalopram [12, 81, 102, 103].

I.5.3.2. Compliance

Non-adherence to tamoxifen may have played a non negligible role as confounding factor in pharmacogenetics studies as well as in trials investigating the effect of CYP2D6 inhibitors on tamoxifen effectiveness. Early treatment discontinuation and non-adherence to treatment are a major concern for adjuvant endocrine therapies. Among patients under tamoxifen, 15 to 20% discontinued their treatment by the 1st year and 31 to 61% at the end of 5 years. Among patients who continued their treatment, adherence to tamoxifen declined over-time and ranged from 41 to 88% [104-107]. Most notably, poor adherence to tamoxifen has been linked to a lower breast cancer recurrence or event-free time [65, 101]. Poor adherence and early discontinuation have also been linked to an increase in all-cause mortality in breast cancer patients [108, 109]. It has been reported

that tamoxifen side effects such as hot flashes are major determinant for adherence and persistence to the adjuvant hormonal therapy [110, 111]. Rae et al. [111] also observed that patients with higher CYP2D6 activity presented increased rate of treatment discontinuation and postulated that this could be related to the reported higher burden of side effects such as hot flashes in CYP2D6 EM and IM than PM patients [55].

I.6. CLINICAL RATIONAL FOR A THERAPEUTIC DRUG MONITORING (TDM) AND METABOLITE PROFILING OF TAMOXIFEN

The association between *CYP2D6* genetic status and treatment outcome has prompted the consideration of a potential role for *CYP2D6* genotype testing in patients' management and choice of alternative adjuvant therapy. Whether genotype-guided tamoxifen administration is a valuable and useful option to optimize anti-hormonal adjuvant therapy remains, however, controversial and no clear consensus has yet been reached regarding the insufficient and somewhat conflicting retrospective clinical data relating *CYP2D6* genotype to tamoxifen efficacy [70, 71, 112-114]. Moreover, large inter-patient variability in endoxifen levels still subsists even after correcting for CYP2D6 status (Table 3).

In fact, *CYP2D6* genotype explained roughly 30 to 40% of endoxifen levels variability in univariate analysis [31, 54, 115]. The remaining unexplained variability may depend on environmental factors such as treatment adherence [101, 107-109, 116, 117] and particularly, interacting co-medications that do modulate drug exposure independently of genetic traits [28, 29, 118]. In fact, it is estimated that 20 to 30% of patients under tamoxifen therapy are also taking antidepressants. Pharmacokinetic studies evaluating the impact of administered comedication on tamoxifen and its metabolites exposure showed, that potent CYP2D6 inhibitors such as paroxetine and fluoxetine reduced endoxifen plasma concentrations by 64% to 72% in CYP2D6 extensive metabolizers (EM) patients and by 24% in CYP2D6 intermediate metabolizers (IM) patients. Coadministration of such potent CYP2D6 inhibitors brought endoxifen concentrations in EM to levels comparable to those observed in CYP2D6 poor metabolizers (PM) patients [28, 29]. Moderate CYP2D6 inhibitors such as citalopram and sertraline reduced endoxifen concentrations by 25% in CYP2D6 EM patients but this inhibition did not reached significance in the study of Borges et al. [29] and Barginear et al. [119]. Unlike endoxifen, CYP2D6 inhibitors were not associated to a reduction in plasma concentrations of 4-hydroxy-tamoxifen [28, 29].

Nonetheless, both *CYP2D6* genetic variability and CYP2D6 inhibitory medications have been identified to explain at maximum ~ 46% of endoxifen levels variability [115] and patient with null CYP2D6 activity (CYP2D6 PM, phenotype) are still able to generate this main tamoxifen active

metabolite (Table 3). Moreover, tamoxifen metabolism and pharmacokinetics are complex and involve many other CYP (CYP3A4/5, 2C9, 2C19, 2B6) and phase II reaction (SULT1A1, UGT1A4, 2B7, 1A10, 1A8, 2B15) enzymes and possibly drug transporters such as P-gp [89, 90].

Several pharmacokinetics studies have assessed the role of such factors on endoxifen systemic concentrations. Mürdter et al . [54] showed that carriers of reduced CYP2C9 activity variant alleles (CYP2C9*2, *3) have significantly lower 4-hydroxy-tamoxifen and endoxifen levels. Teft et al. [120] failed, however, to confirm this observation for endoxifen levels, while the effect on 4-hydroxy-tamoxifen have not been reported. Patients with reduced expression of the CYP3A4 enzyme, harboring the variant allele CYP3A4*22, have significantly higher plasma levels of tamoxifen and its major primary and secondary metabolites of interest i.e. N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen. The major impact of the presence of such a variant allele (CYP3A4*22) was observed in CYP2D6 PM patients, in whom, endoxifen concentrations were increased to levels higher than the sub-therapeutic levels (of 6 ng/mL) usually observed in this CYP2D6 phenotypic group. The putative underlying mechanism of such an increase may be a reduction of the first pass metabolism and the increased bioavailability of the parent drug [120]. Fernández-Santander et al. [121] reported that patients featuring the SULT defective gene variants SULT1A2*2 and SULT1A2*3 alleles (null alleles) showed higher 4-hydroxy-tamoxifen and endoxifen exposure. Other studied polymorphisms in CYP3A5 [121-123], P450 oxidoreductase (POR), CYP2B6 [120], CYP2C19 [122, 123], P-gp [120], multidrug resistance associated-protein 2 (MRP2), breast cancer resistance protein (BCRP) [53, 120] and SULT1A1 genes [51, 121, 123] have not been associated to variations in active tamoxifen metabolites and seem to have no or limited impact on tamoxifen and metabolites systemic exposure. All together, this suggests that the remaining unexplained variability in endoxifen concentrations could be related to other genetic and non-genetic factors that have not been accounted for by solely focusing on the pharmacogenetics of the *CYP2D6* gene.

The monitoring of plasma concentration of tamoxifen active metabolites (mainly endoxifen) may therefore constitute a better predicting tool for tamoxifen efficacy than genotype testing. In fact, endoxifen levels correspond to the final phenotypic trait of patients' drug exposure, accounting for the combined effects of all genetic polymorphisms, physiological (age, body-mass index) [115, 124] and environmental factors that may affect drug disposition and bioactivation. However, whether the therapeutic monitoring of endoxifen plasma concentrations in breast cancer patients would constitute a valid approach to optimize individual dosage and improve treatment effectiveness remains to be demonstrated. So far, only one study has been recently published that sought for the association between endoxifen concentrations and breast cancer outcomes [31]. This pioneering study by Madlensky et al. [31] suggested a probable non linear dose-response relationship for

tamoxifen effect and identified a threshold concentration for endoxifen, of about 6 ng/mL, above which approximately 26% lower disease recurrence rate was observed. All CYP2D6 PM patients for this largest cohort study, have endoxifen levels lower than this identified threshold. Two in vitro studies confirmed this threshold, as they observed, respectively, that concentrations of 5 ng/mL correspond to the endoxifen levels required to reach 90% ER inhibition (IC90) [54] and to achieve 50% inhibition of a xenograft tumor cells growth rate [125].

Early attempts that examined the feasibility and usefulness of tamoxifen dose-adjustment strategy were based exclusively on *CYP2D6* genotype. Genotype-guided dose-adjustment studies have shown that tamoxifen dose increase to 30 mg or 40 mg/day significantly increases 4-hydroxy-tamoxifen and endoxifen concentrations in IM and even in PM patients carrying two null alleles (reflecting metabolism by other enzymes), without any significant difference in adverse effects. However, an important variability is still observed in 4-hydroxy-tamoxifen and endoxifen levels between the genotypic groups [126, 127] and this would be a strong argument for considering TDM of tamoxifen and its active metabolites levels as a valuable strategy for tamoxifen dose-adjustment further reducing the residual variability within *CYP2D6* genotype groups. In addition, Barginear MF, et al. [119] investigated, in another prospective study, the effect of tamoxifen dose increase on the concentrations of tamoxifen, 4-hydroxy-tamoxifen, endoxifen and their position isomers (4'-hydroxylated) and proposed an "antiestrogenic activity score" (AAS) based on the concentrations of these metabolites and their respective antiestrogenic activities. According to Barginear et al. this AAS score would constitute a better approach to estimate the biologic effectiveness of tamoxifen and therefore to guide future tamoxifen dose optimization. However, this approach has yet to be validated by larger studies.

In conclusion, the plasma concentration of tamoxifen active metabolites and mainly endoxifen concentrations may represent a better predicting tool for tamoxifen efficacy than genotype testing. In fact, endoxifen levels is to the final phenotypic trait reflecting patients' drug exposure, accounting for the combined effects of all genetic polymorphisms, physiological and environmental factors that may affect drug pharmacokinetics. Whether the monitoring of endoxifen plasma concentrations in breast cancer patients would constitute a valid approach in individualizing dosage and improve treatment efficacy is under scrutiny and remains to be demonstrated.

Table 3: Variability in tamoxifen and metabolites concentrations (in ng/mL) stratified by patients *CYP2D6* genotype or phenotype

	n	Design	Tam (tamoxifen)	NDTam (N-desmethyl-tamoxifen)	4-OH-Tam (4-hydroxy-tamoxifen)	Endoxifen	Comments
Jin et al. 2005 [28] **Mean (95% CI)** **vt/vt (PM/PM)** **wt/vt (EM/PM)** **wt/wt (EM/EM)** **P**	 3 29 48	20mg/day for at least 4 months Alleles: *1, *3, *4, *5, *6 Patients : mainly caucasians. CYP2D6 inhibitors: Potent: Paroxetine, fluoxetine, amiodarone, metoclopramide; Weak: sertraline, citalopram.	 107 (64 - 150) 131 (112 - 150) 138 (119 - 157) 0.92	 237 (107 - 368) 245 (204 - 287) 233 (188 - 266) 0.62	 2.7 (0.5 - 5.0) 3.2 (2.6 - 3.8) 3.7 (3.3 - 4.1) 0.86	 7.5 (4.1 - 10.8) 16.1 (12.4 - 19.7) 29.1 (24.6 - 33.6) <0.001	Endoxifen levels were 1.8 fold (45%, p=0.003) lower in EM/PM and 3.8 fold (74%, p=0.003) lower in PM/PM compared to EM/EM patients. Steady state reached within 4 months for NDTam, 4OHTam and Endoxifen and after 1 month for Tam. Use of calcium channel blockers (CYP3A inhibitors) in 5 patients significantly reduced tamoxifen levels by 1.6 fold (p= 0.04) without impact on the other metabolites. No association between Tam /metabolites and polymorphisms in CYP2C9 genotype (at least one variant allele: *2, *3), CYP3A5 genotype (*1/*3 vs. *3/*3) and SULT1A1*2.
Borges et al. 2006 [29] **Mean (SD)** **All** **PM/PM, IM/PM** **EM/PM, EM/IM** **EM/EM, UM/EM and IM/*41xN** **P**	 94 9 - -	20mg/day for at least 4 months Alleles: 33 alleles Patients : mainly caucasians; early breast cancer. CYP2D6 inhibitors: Potent: Paroxetine, fluoxetine; Weak: sertraline, citalopram.	 125 (58)	 228 (117)	 3.0 (1.5)	 26.7 (15.4) 8.2 (2.5) 23.9 (14.2) 33 (14.8) < 0.05	Potent CYP2D6 inhibitors reduced endoxifen levels by 72% (3.6 fold) in CYP2D6 EM/EM patients (84.1 ± 39.4 vs. 23.5 ± 9.5, p < 0.0001) and were comparable to CYP2D6 PM/PM patients (19.4 ± 6.1). Potent CYP2D6 did not affected 4OHTam levels. Weak CYP2D6 inhibitors non significantly reduced endoxifen levels by 24% (1.3 fold) in CYP2D6 EM/EM patients (84.1 ± 39.4 vs. 63.9 ± 36.9, p = 0.15).

	n	Design	Tam (tamoxifen)	NDTam (N-desmethyl-tamoxifen)	4-OH-Tam (4-hydroxy-tamoxifen)	Endoxifen	Comments
Lim et al. 2007 [50] **Mean (95% CI)** ***10/*10** **wt/*10** **wt/wt** **P**	 49 89 64	20 mg/day for at least 8 weeks Alleles: *5, *10, *2xN Patients: Asian; Metastatic breast cancer No CYP2D6 inhibitors or inducers			 1.5 (1.3 - 1.6) 2.5 (2.4 - 2.7) 2.8(2.5 - 3.1) < 0 .0001	 7.9 (7.1 - 8.8) 18.1 (16.8 - 19.5) 19.9 (18.0 - 21.9) < 0 .0001	CYP2D6 *10/*10 vs. other genotypes: ~ 1.5 and 2 fold lower 4OHTam and endoxifen. CYP2D6 *10/*10 vs. other genotypes: lower time to progression (5 vs. 21.8 months, P = 0.0032) with 50% vs. 100% non responders.
Gjerde et al 2008 [51] **Median (range)** **PM** **IM** **EM** **UM** **P**	 11 49 86 5	 20 mg/day for at least 80 days	 82 (31–149) 89 (27–302) 90 (34–291) 97 (58–151) 0.828	 255 (142–461) 241 (156–691) 217 (90–596) 185 (127–369) 0.001	 5.1 (3.0–8.0) 5.7 (1.7–11.3) 5.8 (2.2–17.2) 5.9 (5.7–12.7) 0.044	 36.7 (30.7–68.6) 49.6 (27.3–108.2) 52.3 (24.3–184.8) 46.3 (37.6–141.4) 0.003	Lack of information on comedication
Barginear et al. 2011 [119] **Median (range)** **Score 0 (PM/PM)** **Score 0.5 (IM/PM)** **Score 1 (EM/PM), (IM/IM)** **Score 1.5 (EM/IM)** **Score 2 (EM/EM), (UM/PM)** **P**	 3 10 31 27 45	 20mg /day for at least 90 days	 72 (40 - 160) 86 (31 - 137) 115 (20 - 240) 101 (47 - 216) 105 (13 - 279) 0.44		 0.8 (0.8 - 1.5) 1.2 (0.4 - 1.9) 1.5 (0.4 - 47.6) 1.5 (0.8 - 2.7) 1.9 (0.4 - 5.8) 0.06	 2.2 (1.9 - 3.4) 3.4 (1.5 - 5.6) 6.7 (1.1 - 24.6) 7.5 (3.7 – 16.8) 10.1(2.2 – 32.5) <0.0001	

	n	Design	Tam (tamoxifen)	NDTam (N-desmethyl-tamoxifen)	4-OH-Tam (4-hydroxy-tamoxifen)	Endoxifen	Comments
Madlensky et al. 2011 [31]							
Mean (SD)		20 mg/day					
PM	27		142.3 (63.1)	312.7 (114.2)	1.7 (0.9)	5.6 (3.8)	
IM	1,097	for at least 4	142.9 (70.8)	295.7 (112.6)	1.7 (0.8)	8.1 (4.9)	
EM	164	months	136.4 (64.3)	242.1 (95.3)	2.3 (1.1)	15.9 (9.2)	
UM	82		143 (58.4)	230.8 (71.1)	2.7 (1.2)	22.8 (11.3)	
P			0.55	< 0.001	< 0.001	< 0.001	
Lim et al. 2011 [122]							
Median (range)							
***5/*10 (PM/IM)**	12	20 mg/day	169.9 (39.26– 452.29)	330.67 (78.46–757.13)	1.87 (0.47–3.17)	7.46 (1.79–13.77)	
***10/*10 (IM/IM)**	40		217.2 (84.27–599.91)	374.41 (84.77–802.98)	1.76 (0.72–3.82)	8.03 (1.74–34.68)	
***1/*5 (EM/PM)**	9	for at least 8 weeks	216.1 (93.56–325.56)	261.07 (206.94–464.25)	1.58 (1.25–3.25)	14.51 (10.73–26.04)	
***1/*10 (EM/IM)**	31		194.9 (51.80–421.16)	279.43 (115.41–502.13)	1.92 (0.86–4.51)	19.74 (7.26–33.24)	
***1/*1 (EM/EM)**	13		161.2 (50.06–369.89)	174.59 (40.82–448.65)	2.49 (0.97–3.36)	19.55 (4.18–39.47)	

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CHAPTER II

Therapeutic Drug Monitoring of targeted anticancer therapy. Tyrosine kinase inhibitors and selective oestrogen receptor modulators: a clinical pharmacology laboratory perspective

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Own Contribution: part of the chapter entitled: Tamoxifen as the first targeted anticancer agent.

In: LC-MS in Drug Bioanalysis. Edited by Xu QA, Madden TL: Springer US; 2012: 197-250

CHAPTER II - THERAPEUTIC DRUG MONITORING OF TARGETED ANTICANCER THERAPY: TYROSINE KINASE INHIBITORS AND SELECTIVE OESTROGEN RECEPTOR MODULATORS: A CLINICAL PHARMACOLOGY LABORATORY PERSPECTIVE

II.1. INTRODUCTION

During the past decades, it has been established that the therapeutic use of selected drugs could be optimized by an individualization of their dosage, based on blood concentrations measurement [1, 2]. Such a Strategy, termed Therapeutic Drug Monitoring (TDM), is now current practice for drugs such as antibiotics, antiepileptics, immunosuppressant drugs, antifungals [3], and more recently, anti-HIV drugs [4, 5]. TDM is generally considered for drugs with large inter-individual but limited intra-individual pharmacokinetic variability with both consistent concentration-efficacy and concentration-toxicity relationships. The sources of variability in drug response are multifactorial and apart from genetics, other factors, such as patient' pathophysiological conditions, environment, drug–drug interactions, food, drinking and smoking habits, medication errors and poor compliance, may have an important impact on drug pharmacokinetics and/or pharmacodynamics, thereby affecting the therapeutic outcome [1, 2]. Information provided by TDM is particularly useful for drugs with a narrow therapeutic index, subjected to physiologic, genetic, environmental influences and used for prolonged periods. In oncology patients, maintaining circulating drug concentrations over a given threshold appears to be crucial to ensure optimal pharmacological action as exposure to suboptimal drug levels during chronic therapy substantially increases the risk of therapeutic failure, due to the progressive selection of cancer cell clones. On the other hand, excessive drug concentrations may be associated with intolerance and adverse drug reactions (such as tamoxifen frequently induced gynecologic and vasomotor symptoms) leading in term to frequent therapeutic treatment interruption. In general, implementation of a routine TDM program necessitates the access to suitable instrumental technology, bioanalytical expertise, and definite knowledge in clinical pharmacokinetics for drug levels interpretation leading possibly to dosage adjustment. The analytical results, integrated with the clinical observations, may influence the therapeutic intervention and in turn, clinical outcome. Reliability of analytical methods is therefore a critical issue, justifying the efforts and time devoted to their thorough validation and extensive characterization of their performance (i.e. precision, accuracy, robustness, and turn-around time). A comprehensive review of mass spectrometry methods for tamoxifen and its metabolites is therefore presented in the context of the current growing interest for monitoring tamoxifen active metabolites, particularly endoxifen, as a potentially clinically useful tool to monitor tamoxifen treatment in breast cancer patients.

II.2. TAMOXIFEN AND METABOLITES IDENTIFICATION AND QUANTIFICATION:

To date, several quantitative analytical methods have been developed for the monitoring of tamoxifen and some of their metabolites in human biological fluids and tissues, including conventional [6-8] and micellar [9] liquid chromatography (LC) methods coupled to fluorescence detection, capillary electrophoresis-mass spectrometry (CE-MS) [10], gas chromatography-mass spectrometry (GC-MS) [11], as well as liquid chromatography methods hyphenated with mass spectrometry (LC-MS) [12] and tandem mass spectrometry (LC-MS/MS) [13-33]. Reports have also been published describing liquid chromatography method coupled to mass spectrometry or fluorescence detection for the study of tamoxifen metabolism *in vitro* and *in vivo* in animal models [34-41]. Most of these qualitative and quantitative LC, GC and CE methods have already been reviewed by Teunissen SF, et al. [42].

Various hyphenated LC-MS based assays, using either the electrospray ionisation (ESI) or the atmospheric pressure chemical ionization (APCI) interface, have been developed and applied in the clinical setting in order to support pharmacokinetic (PK), pharmacogenetic-pharmacokinetic (PG-PK), and pharmacokinetic-pharmacodynamic (PK-PD) studies in breast cancer (BC) patients under tamoxifen therapy (Table 3).

Among these, both LC-MS and LC-MS/MS approaches have been described using different mass analyzers operating in the positive ion mode scan such as triple stage quadrupole (TSQ) mass spectrometers [13, 17, 18, 21, 22, 24-27, 30-33] and hybrid quadrupole-linear ion trap (LTQ) [16, 19, 20, 23, 28] mass spectrometers working in selected reaction monitoring (SRM) mode as well as time-of-flight (TOF) [12] and hybrid quadrupole-TOF (Q-TOF) [14, 15] mass spectrometers working in the MS mode.

Table 1: Overview of LC-MS and LC-MS/MS developed methods for the quantification of tamoxifen and its metabolites in human blood samples.

Matrix (volume)	Analytes ‖	Internal standard	LOQ (ng/mL)	Sample preparation	Column (particle size, dimensions)	Ionisation and detection mode	Ref.
Plasma * (100 µL)	Tam 4-OH-Tam Others SERMS (raloxifene, nafoxidine, idoxifene)	Idoxifene-d5	5 -	LLE(hexane/isoamyl- alcohol (96:4 v/v)) Dilution (DMSO) Evaporation (hexane layer) Dilution (H20)	Luna C18 (3 µm, 30x1 mm)	ESI-TSQ	[13]
Plasma (250 µL)	Tam N-D-Tam 4-OH-Tam	Toremifene	-	PP (ACN) Dilution (0.5M ammonium acetate)	Hypersil BDS C18 (3 µm, 150x2.1 mm)	ESI-Q-TOF (MS mode)	[14, 15]
Serum § (75 µL)	Tam 4-OH-Tam N-D-Tam N-D-D-Tam Tam-NO Endoxifen ¶	Tam-d5	0.25 0.25 0.25 1.0 1.0 -	PP (ACN) On-line SPE (Oasis HLB, 50x1mm)	Chromolith Performance, RP-18e (100x4.6 mm)	ESI-LTQ	[16, 20]
Serum § (100 µL)	Tam N-D-Tam 4-OH-Tam Endoxifen ‡ Soy isoflavone (genistein, daidzein, equol)	Tam-$^{13}C_2$,^{15}N N-D-Tam-d5 4-OH-Tam-d5 Endoxifen-d5 ‡ (genistein-d4, daidzein-d3, equol-d4)	0.4 0.4 0.2 1.1	PP (ACN) Hydrolysis (glucuronidase/sulfatase) SPE (SPEC 96-WELL PLATE C18) Evaporation Reconstitution (MeOH/H2O (1:1 v/v))	Luna C18 (3 µm, 150x2 mm)	ESI-TSQ	[17, 18]
Serum (1 mL)	Tam N-D-Tam 4-OH-Tam	- - -	5 5 0.5	LLE (n-hexane/isoamyl alcohol (98:2)) Evaporation Reconstitution (MeOH)	Beckman C8 (5 µm, 50x4.6 mm)	ESI-LTQ	[19]
Serum	Tam 4-OH-Tam	Propranolol	10 1	LLE (hexane/isopropanol (95:5 v/v))	HiQ-Sil C18 (5 µm, 150x2.1 mm)	ESI-TSQ	[21]
Serum § (50 µL)	Tam Tam-NO ¶	Tam-d5	6.76 6.19	PP (ACN) Dilution (3.5mM ammonium	Synergi Hydro-RP (4 µm, 150x2 mm)	ESI-TSQ	[22]

Matrix (volume)	Analytes ‖	Internal standard	LOQ (ng/mL)	Sample preparation	Column (particle size, dimensions)	Ionisation and detection mode	Ref.
	N-D-Tam 4-OH-Tam Endoxifen ‡ Soy isoflavone (genistein, daidzein, glycitein)	N-D-Tam-d5 4-OH-Tam-d5 Endoxifen-d5 ‡ (genistein-d4, daidzein-d6)	6.72 1.13 2.69	formate buffer, pH 3.5)			
Plasma (100 µL)	Tam N-D-Tam 4-OH-Tam Endoxifen	Imipramine	20 20 1 3.75	PP (ACN) SPE (BOND ELUTE-C18 cartridges, 100mg/1mL)	XBridge C18 (3.5 µm, 150x3 mm)	ESI-TOF	[12]
Plasma § (1 mL)	Tam AIs (Anastrozole, letrozole)	Bunitrolol	25	PP (2% aqueous phosphoric acid) Polymer-based mixed-mode SPE (Strata X-C, 200mg/3mL)	Eurosphere Si-C18 (5 µm, 200x0.5 mm)	ESI-LTQ	[23]
Plasma (100µL)	Endoxifen	Anastrozole	-	PP (ACN)	Kromasil 100 C8 (5 µm, 150x4.6 mm)	ESI-TSQ	[24, 25]
Plasma § (100 µL)	Tam N-D-Tam 4-OH-Tam 4'-OH-Tam ¶ Endoxifen ‡ 4'-OH-N-D-Tam ¶	Tam-d5 N-D-Tam-d5 4-OH-Tam-d5 Endoxifen-d5 ‡	1 2 0.4 0.4 1 1	PP (ACN) Evaporation Reconstitution (MeOH/20mM ammonium formate buffer, pH 2.9, (1:1 v/v) Centrifugation	Acquity UPLC BEH C18 (1.7 µm, 30x2.1 mm)	ESI-TSQ	[26]
Plasma § (100 µL)	Tam Tam-NO N-D-Tam 4-OH-Tam ‡ 4'-OH-Tam Endoxifen ‡ 4'-OH-N-D-Tam	Tam-$^{13}C_2$,^{15}N N-D-Tam-d5 4-OH-Tam-d5 Endoxifen-d5 ‡	1.1 - 0.5 0.2 0.2 0.2 0.2	PP (MeOH) Filtration Dilution (H_2O)	Zobrax SB-C18 (1.8 µm, 50x2.1 mm)	ESI-TSQ	[27]

Matrix (volume)	Analytes ‖	Internal standard	LOQ (ng/mL)	Sample preparation	Column (particle size, dimensions)	Ionisation and detection mode	Ref.
Serum (200 µL)	Tam	Tam-d5	-	Polymer-based mixed-mode SPE (Oasis MCX 1mL cartridges)	XTerra MS C18 (3.5 µm, 100x2.1 mm)	ESI-LTQ	[28]
	N-D-Tam	N-D-Tam-d5	-				
	4-OH-Tam		-				
	Endoxifen	Endoxifen-d5	-				
Plasma (50 µL)	Tam	Tam-d3	0.5	PP (ACN+1% acetic acid) Dilution (H_2O +1% acetic acid)	Zobrax Eclipse plus C18 (1.8 µm, 100x2 mm)	ESI-TSQ	[29]
	Tam-NO		0.2				
	N-D-Tam	N-D-Tam-d5	1				
	N-D-D-Tam		0.2				
	4-OH-Tam ‡	4-OH-Tam-d5 ‡	0.1				
	3-OH-Tam		0.1				
	4'-OH-Tam ‡		0.1				
	α-OH-Tam		0.02				
	Endoxifen ‡	Endoxifen-d5 ‡	0.1				
	3-OH-N-D-Tam ¶		-				
	4'-OH-N-D-Tam ‡		0.05				
	α- OH-N-D-Tam ¶		-				
	Tam-N^+-Gluc	Tam-4-O-Gluc-d5 ‡	0.05				
	Tam-3-O-Gluc ¶		-				
	Tam-4-O-Gluc ‡		0.1				
	N-D-Tam-3-O-Gluc ¶		-				
	N-D-Tam-4-O-Gluc ‡		0.05				

Matrix (volume)	Analytes ‖	Internal standard	LOQ (ng/mL)	Sample preparation	Column (particle size, dimensions)	Ionisation and detection mode	Ref.
Serum § **(50 µL)**	Tam N-D-Tam 4-OH-Tam 4'-OH-Tam Endoxifen ‡ 4'-OH-N-D-Tam	Tam-d5 N-D-Tam-d5 4-OH-Tam-d5 Endoxifen-d5 ‡	5 5 0.4 0.2 1 1	PP (ACN) Evaporation Reconstitution (ACN/4mM ammonium formate buffer, pH 3.5, (3:7 v/v))	Kinetex C18 (2.6 µm, 150x2.1 mm)	APCI-TSQ	[30]
Plasma (250 µL)	Tam N-D-Tam 4-OH-Tam Endoxifen	Diphenhydramine	- - - -	LLE (ethyl acetate under pH 11.3) Evaporation Reconstitution (mobile phase)	Luna C18 (3 µm, 100x2 mm)	ESI-TSQ	[31]
Plasma § **(200 µL)**	Tam N-D-Tam 4-OH-Tam Endoxifen	Tam-d5 N-D-Tam-d5 4-OH-Tam-d5 Endoxifen-d5 ‡	1.86 1.78 0.194 0.187	PP (ACN/acetone (1:1)) LLE (n-hexane/isopropanol (95 :5)) Evaporation Reconstitution (ACN/H_2O/formic acid (40:60:0.1 v/v/v)) Centrifugation	Acquity UPLC BEH C18 (1.7 µm, 100x2.1 mm)	ESI-TSQ	[32]
Plasma (100 µL)	Tam N-D-Tam 4-OH-Tam Endoxifen	Imipramine	20 40 1 4	PP (ACN)	Acquity UPLC BEH C18 (1.7 µm, 100x2.1 mm)	ESI-TSQ	[33]

* Non- clinical samples.

§ Fully validated method.

¶ Method has not been validated for the quantification of the analyte.

‡ Reported E/Z isomers chromatographic resolution.

‖ Abbreviations (see Table 4).

II.2.1. Chromatographic conditions and tamoxifen metabolites separation:

Since the introduction of ionization sources working at atmospheric pressure such as ESI interface, LC–MS has become the gold standard in the field of quantitative bioanalysis due mainly to the selectivity, sensitivity and high-throughput detection in LC-MS systems. However, LC-MS features depend not only on the ionization technique and mass spectrometer unrivalled inherent selectivity, sensitivity and speed acquisition but are also challenged, notably in drug metabolism studies, by the availability of stable isotope labeled (SIL) version of metabolites (see below) and the need of efficient and adequate chromatographic resolution of multiple analytes from interfering metabolites or endogenous biological components in a minimum time frame.

Reversed-phase LC (RPLC) methods using conventional, microbore [23], narrow-bore [21, 22] and short [19] HPLC columns have been used for the separation of tamoxifen/metabolites either under isocratic or gradient elution conditions. Narrow-bore columns present the advantages of being solvent saving and by the need of low sample injection (or loading) volumes. These advantages were illustrated by Beer B, et al. [23] who developed an analytical method for the separation of tamoxifen, anastrozole and letrozole under gradient of 30µL/min of acetone in aqueous heptafluorobutyric acid solution and volumes as low as 2µL, from the processed samples, were injected into the system. Furlanut M, et al. [19] used a short analytical column for the separation of tamoxifen and two of its metabolites within almost 8 min under isocratic conditions at flow rate of 1mL/min. Although, the use of conventional short columns is a simple method for shortening analytical run times, these columns suffer from a loss in efficiency and resolution.

For enhanced throughput, fast RPLC methods using monolithic silica columns [16], small size particles (3µm) packed columns [12-15, 17, 18, 28, 31], ultra high pressure liquid chromatography (UHPLC) columns packed with sub-2µm particles [26, 27, 29, 32, 33] and 2.6 µm core-shell particles HPLC columns [30] have been proposed for the high-throughput separation and quantification of tamoxifen/metabolites.

Five UHPLC methods have already been described to improve speed, resolution, and sensitivity of HPLC assays for the quantification of tamoxifen phase I as well as phase II metabolites. These methods exclusively enabled, within run times of about 12 min or even less, to reach an excellent overall resolution for all considered metabolites including (E/Z) endoxifen isomers and position isomers of 4-hydroxy and 4-hydroxy-N-desmethyl-tamoxifen. Alternatively, Zweigenbaum J and Henion J [13] developed a high-throughput analysis technique for the separation of tamoxifen, 4-hydroxytamoxifen and other SERMs within only 30 s using a narrow-bore short analytical column packed with small (3µm) particles. Separation was performed under isocratic conditions at flow rate

of 500µL/min. Gjerde J, et al. [16] also described an on-line solid phase extraction (SPE)-LC-MS/MS procedure where chromatographic resolution of tamoxifen and five of its metabolites was achieved within 6 min using a monolithic silica column. (Separation was performed under a gradient program at a flow rate of 500µL/min). However this method, as probably other HPLC assays, clearly failed to resolve all the hydroxylated and N-desmethyl-hydroxylated tamoxifen metabolites.

Tamoxifen is metabolized to a plethora of N-desmethylated, hydroxylated and their corresponding glucurono- or sulfo-conjugated metabolites (supplementary data: Figure 1 and 2). Some of these hydroxylated metabolites are position isomers (such as 4-hydroxy-tamoxifen, 3-hydroxy-tamoxifen, 4'-hydroxy-tamoxifen; endoxifen and 4'-hydroxy-N-desmethyl-tamoxifen) and have similar molecular mass and fragmentation pattern (Table 4). Besides, E/Z isomerisation (around the ethylenic double bond of tamoxifen and its metabolites) may occur either in biological samples or as contaminants or degradation products in pure standards. Some pure standards are also best synthesized as an E/Z mixture. Therefore, the chromatographic resolution of these metabolites and their (E/Z) geometric isomers is of paramount importance to ensure reliable and accurate bioanalytical methods.

However, of the LC-MS and LC-MS/MS methods developed so far for the comprehensive and quantitative study of levels variability in tamoxifen metabolites, there is limited data with respect to the resolution of both 4-hydroxytamoxifen and endoxifen position isomers (notably 4'-hydroxylated metabolites) and their corresponding (E/Z) geometric isomers. In fact, apart from the most recently published articles [26-30, 32], no data have been provided regarding this issue. We were the first group that focused on method selectivity and on the effective separation on potentially interfering hydroxylated tamoxifen metabolites. This allow us to identify for the first time the occurrence of 4'-hydroxy-tamoxifen and 4'-hydroxy-N-desmethyl-tamoxifen and to estimate their plasma levels in a subset of BC patients [26]. Such differences in chromatographic performances, between assays, can thus affect the selectivity, the accuracy and reliability of some of the proposed bioanalytical methods, potentially leading to discrepant data (or results) between the PK, PG-PK and PK-PD studies. Actually, Mürdter TE, et al. [29] reported twice or even higher differences in median concentrations of (Z)-endoxifen between studies conducted in the United States, Japan and Norway. They also found a plausible explanation for these discrepancies in method selectivity problems. Madlensky L, et al. [28] compared the performance of their assay to that of another laboratory performing similar measurements of tamoxifen metabolites in human serum. They found discordant results for 4-hydroxy-tamoxifen levels measured in the same serum samples.

Table 2: Molecular masses and SRM transitions for tamoxifen and some of its metabolites of interest.

Analytes	Abbreviation	Molecular weight	Precursor ion * $[M+H]^+$	Product ion
Tamoxifen	Tam	371	372	72
N-desmethyl-tamoxifen	N-D-Tam	357	358	58
N,N-didesmethyl-tamoxifen	N-D-D-Tam	343	344	44
4-Hydroxy-tamoxifen	4-OH-Tam	387	388	72
3-Hydroxy-tamoxifen	3-OH-Tam	387	388	72
4'-Hydroxy-tamoxifen	4'-OH-Tam	387	388	72
α-Hydroxy-tamoxifen	α-OH-Tam	387	388	72
Tamoxifen-N-oxide	Tam-NO	387	388	72
4-Hydroxy-N-desmethyl-tamoxifen	Endoxifen	373	374	58
3-Hydroxy-N-desmethyl-tamoxifen	3-OH-N-D-Tam	373	374	58
4'-Hydroxy-N-desmethyl-tamoxifen	4'-OH-N-D-Tam	373	374	58
α-Hydroxy-N-desmethyl-tamoxifen	α- OH-N-D-Tam	373	374	58
Tamoxifen-N^+-glucuronide	Tam-N^+-Gluc	548	548	372
Tamoxifen-4-O-glucuronide	Tam-4-O-Gluc	563	564	388
Tamoxifen-3-O-glucuronide	Tam-3-O-Gluc	563	564	388
N-desmethyl-tamoxifen-4-O-glucuronide	N-D-Tam-4-O-Gluc	549	550	374
N-desmethyl-tamoxifen-3-O-glucuronide	N-D-Tam-3-O-Gluc	549	550	374

* Molecule protonation occurs on the amino group.

Another drawback, challenging the applicability of some of these LC-MS and LC-MS/MS assays, in the routine, for measuring exposure to tamoxifen and its active metabolites is that for some assays no data have been provided concerning the validation process. Other methods have only been partially validated and have not or limitedly addressed matrix effects (ME) issues.

II.2.2. Handling matrix effects

Matrix effects, caused by co-eluting endogenous and exogenous matrix components, significantly affect the efficiency and reproducibility of the ionization process of target analytes. This phenomenon represents a major concern for LC-MS bioanalytical methods precision, accuracy,

sensitivity and robustness. Amongst the atmospheric pressure ionization interfaces used in LC-MS systems, ESI source is more prone to signal alteration (ion suppression or enhancement) due to matrix. Therefore, careful evaluation and correction for ME must be considered particularly with ESI-MS.

The use of stable isotope labeled (SIL) version of the target analyte as an internal standard (IS) is theoretically considered to be the best approach to compensate or correct for ME and minimize their influence on the accuracy and precision of ESI-MS quantitative assays.

With the exception of the LC-MS/MS methods recently published, previous assays were using either no IS [19], structurally related IS [12, 14, 15, 21, 23-25, 31, 33] or a single SIL-IS [13, 16, 20] as a surrogate IS for the quantification of tamoxifen/metabolites.

Since SIL-ISs are not always available and their use rather expensive, especially in the case of multiple analytes analysis, the use of structurally related compounds or analogue IS with different mass and with close or similar chromatographic behaviour to that of the analytes can represent an acceptable alternative. Nevertheless, in these latter instances, ME variability between different sources of plasma (relative matrix effect variability) must be investigated and quantified. From the assays operating with either no IS or a unique analogue IS, only three methods quantitatively assessed for ME variability. Zweigenbaum J and Henion J [13] reported a significant ion suppression which approximately halved 4-hydroxytamoxifen signal. This ion suppression was not corrected by the IS and affected the precision and accuracy of the method that failed to meet the acceptance criteria for 4-hydroxy-tamoxifen quantification. Furlanut M, et al. [19] monitored Tam, N-D-Tam and 4-OH-Tam in serum and tissue of BC patients, employing external standard calibration and reported no ion suppression problem after quantitative evaluation of ME. Unfortunately, no detailed information was available regarding the extent of matrix effects variability and the number of plasma lots tested. Only the recent method described by Beer B, et al. [23] thoroughly examined ME effect variability using the quantitative approach proposed by Matuszewski BK, et al. [43, 44].

It is noteworthy that ME variability should be investigated even when using SIL-ISs. In fact, SIL-IS may not fully correct for matrix effects, obviously when they do not completely co-elute with their corresponding analyte. This phenomenon has been particularly observed with deuterated SIL-IS that were found to be less lipophilic than their corresponding non deuterated analogues, causing a slightly earlier elution on a reversed phase column [45].

Although most recent developed assays used SIL-IS, only few methods quantitatively investigated potential ME variability on tamoxifen and its metabolites quantification [26, 27].

In our proposed assay [26], we thoroughly investigated ME both qualitatively using the post-column infusion system proposed by Bonfiglio R, et al. [46] and quantitatively using the recommendations of Matuszewski BK, et al. [43, 44] and the 2007 Washington workshop/conference report [47]. Although the qualitative examination of ME did not show any signal alteration, probably due to the infusion of high concentration of analytes, quantitative ME examination showed an ion suppression of approximately 40% for the signal of N-D-Tam. We observed a similar extent of ion suppression with the deuterated N-D-Tam (N-D-Tam-d5) and ascertained that SIL-IS effectively corrected for the absolute and relative ME (or ME effect variability among 6 different lots of plasma). Therefore, this was a good illustration of the value of SIL-IS use for an efficient control of residual matrix effects.

Besides the use of SIL-IS, another upstream and primordial approach that allow to anticipate and drastically reduce matrix effects is the optimization of sample preparation procedure.

Plasma protein precipitation (PP) with either acetonitrile (ACN) or methanol (MeOH) was the most frequently used sample clean-up technique in the described bioanalytical methods [14, 15, 22, 24-27, 29]. Of these ACN was the prevalent precipitant used, as it was considered to be an optimal choice for protein removal than methanol (MeOH) [48-50]. Although PP is a simple and fast way for preparing samples, it does not result in a very clean extract, as it fails to remove endogenous components such as lipids, phospholipids (such as glycerophosphocholines) and fatty acids, etc. However, if necessary, the elimination of most endogenous lipidic compounds from PP extracts can be performed by subjecting the PP extracts to an additional step of evaporation under nitrogen (or, even better, by submitting them to speed-vac technology) followed by the reconstitution of dried residues with medium polarity solvent system (e.g. MeOH-buffer mixture) wherein lipids would not be resolubilized.

Solid phase extraction (SPE) allows yielding a much cleaner extract than PP, since it significantly lowers phospholipids levels which represent the major endogenous compounds causing significant matrix effects [50-52].

Different reversed phase [12, 17, 18], mixed mode (ion exchange and reversed phase) SPE cartridges [23, 28] and on-line SPE column [16, 20] have been also been reported for samples preparation and extraction. Some of these assays combined both PP and SPE in order to achieve an extensive sample clean-up [12, 16-18, 20]. Likewise SPE, liquid-liquid extraction (LLE) provides cleaner plasma extracts than PP. Nevertheless, LLE procedure does not always provide satisfactory results with regard to extraction recovery and selectivity, especially with polar analytes and particularly in the case of multicomponent analysis such as in drug-metabolism studies, where analytes polarity varies widely. This issue was addressed by Zweigenbaum J and Henion J [13] and extraction solvent optimization,

using isoamyl alcohol, to achieve acceptable extraction selectivity and recovery for polar analytes have been discussed.

II.3. CONCLUSION

To sum up, there is a great heterogeneity in the described methods that have so far been developed and, for the great majority of them, used in the clinical setting to support pharmacogenetic-pharmacokinetic-pharmacodynamic (PG-PK-PD) studies. Of these methods, only the most recent fully validated ones that have proven enough accuracy, precision, robustness and selectivity seems to be reliable and suitable for measuring exposure of tamoxifen and its metabolites in tamoxifen-treated breast cancer patients.

Whether the monitoring of endoxifen plasma concentrations in breast cancer patients would constitute a valid approach to optimize individual dosage and improve treatment efficacy is under scrutiny and remains to be demonstrated. In that purpose large prospective studies relating endoxifen plasma levels to clinical outcomes are as yet needed. In this perspective, it is critical to settle analytical and selectivity discrepancies between methods and laboratories and to insure reproducible quantification results between laboratories. These concerted harmonization efforts can be carried-out within the frame of an international external quality control program, which as yet, remains to be organized.

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II.5. SUPPLEMENTARY DATA

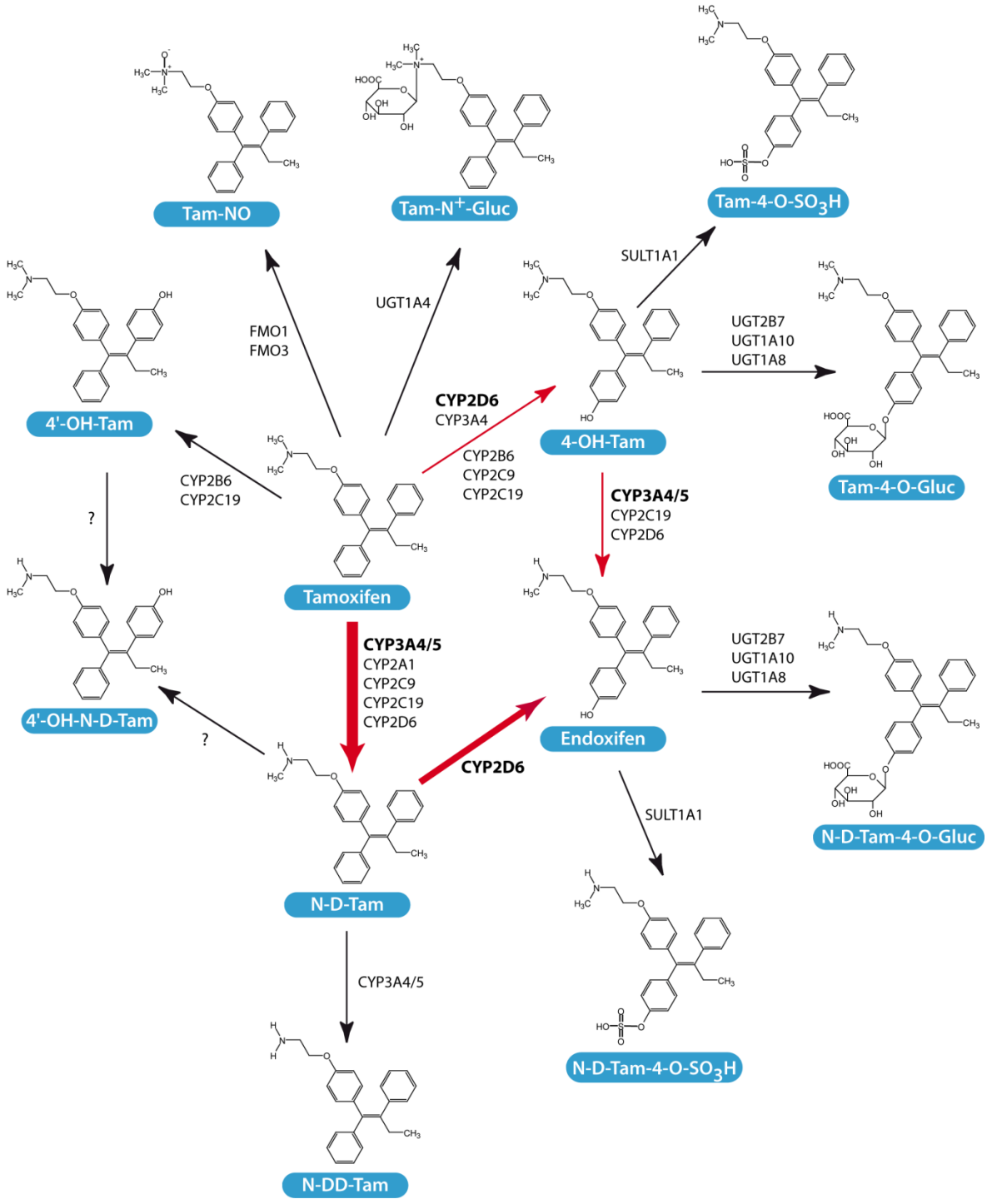

Figure 1. Principal tamoxifen metabolic pathways of clinical interest. Abbreviations: Tam (Tamoxifen), N-D-Tam (N-desmethyl-tamoxifen), N-DD-Tam (N,N-didesmethyl-tamoxifen), 4-OH-Tam (4-Hydroxy-Tamoxifen), 4'-OH-Tam (4'-Hydroxy-tamoxifen), Tam-NO (Tamoxifen-N-oxide), Endoxifen (4-Hydroxy-N-desmethyl-tamoxifen), 4'-OH-N-D-Tam (4'-Hydroxy-N-desmethyl-tamoxifen), Tam-N^+-Gluc (Tamoxifen-N^+-glucuronide), Tam-4-O-Gluc (Tamoxifen-4-O-glucuronide), N-D-Tam-4-O-Gluc (N-desmethyl-tamoxifen-4-O-glucuronide), Tam-4-O-SO_3H (Tamoxifen-4-O-sulfate), N-D-Tam-4-O-SO_3H (N-desmethyl-tamoxifen-4-O-sulfate).

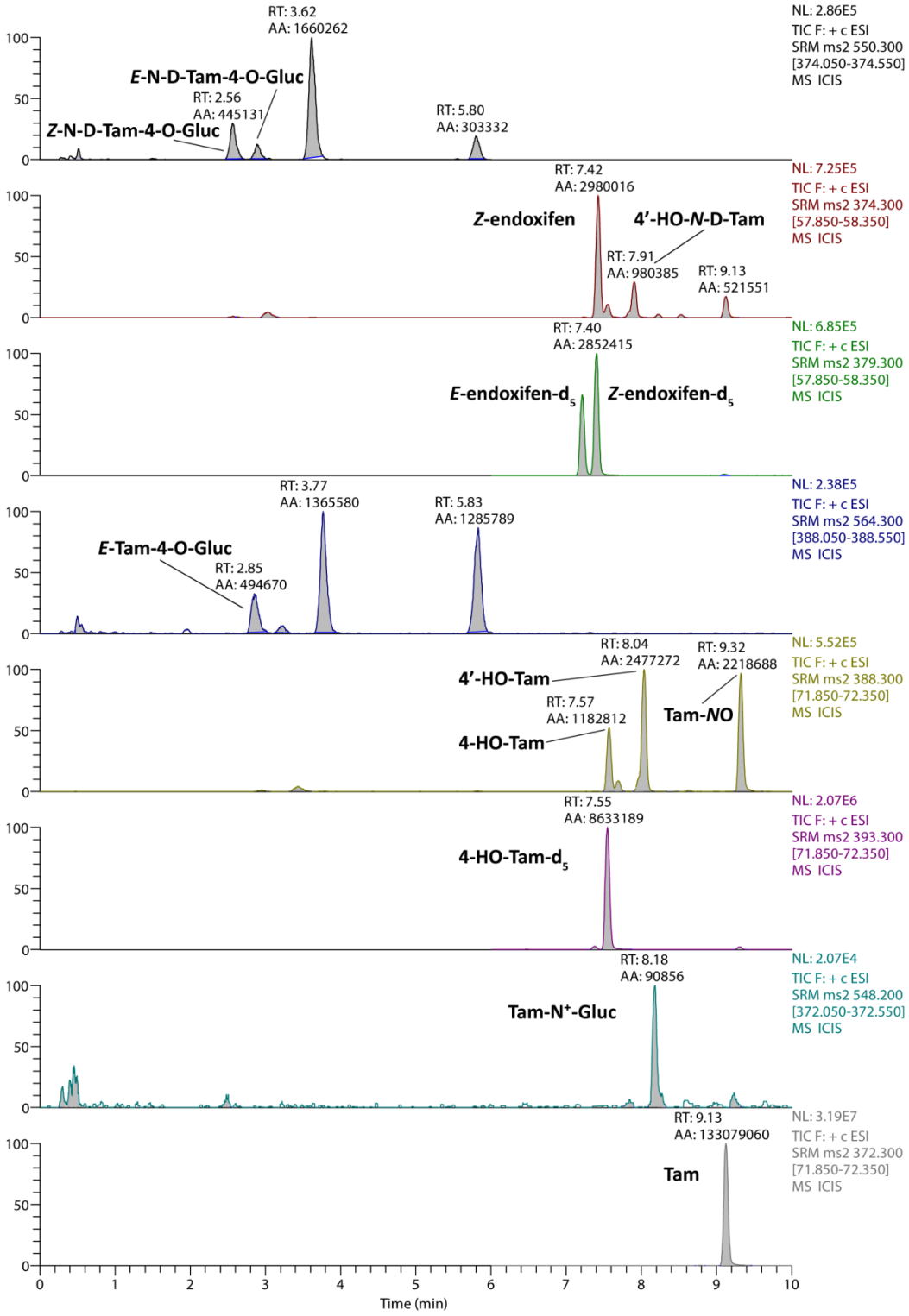

Figure 3. Chromatographic profiles of main tamoxifen phase I metabolites and some of its identified glucuronidated metabolites in a plasma sample from a breast cancer patient receiving tamoxifen 20 mg BID (modified elution gradient from reference [26]. Mobile phases consisted of: buffer A (10 mM NH_4 formate + 0.1% formic acid) and solvent B (Acetonitrile + 0.1% formic acid). The elution gradient was programmed as follows: 0 to 1min, 25% B; 30% B at 5 min; 60% B at 9 min and 30% B from 9.5 to 13 min.

An Ultra Performance Liquid Chromatography - Tandem MS assay for tamoxifen metabolites profiling in plasma: first evidence of 4'-hydroxylated metabolites in breast cancer patients

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Journal of chromatography B, Analytical technologies in the biomedical and life sciences 2010,878(32):3402-3414.

CHAPTER III - AN ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MS ASSAY FOR TAMOXIFEN METABOLITES PROFILING IN PLASMA.

III.1. ABSTRACT

There is increasing evidence that the clinical efficacy of tamoxifen, the first and most widely used targeted therapy for estrogen-sensitive breast cancer, depends on the formation of the active metabolites 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethyl-tamoxifen (endoxifen). Large inter-individual variability in endoxifen plasma concentrations has been observed and related both to genetic and environmental (i.e. drug-induced) factors altering CYP450s metabolizing enzymes activity. In this context, we have developed an ultra performance liquid chromatography–tandem mass spectrometry method (UPLC–MS/MS) requiring 100 µL of plasma for the quantification of tamoxifen and three of its major metabolites in breast cancer patients. Plasma is purified by a combination of protein precipitation, evaporation at room temperature under nitrogen, and reconstitution in methanol / 20 mM ammonium formate 1:1 (v/v), adjusted to pH 2.9 with formic acid. Reverse-phase chromatographic separation of tamoxifen, *N*-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethyl-tamoxifen is performed within 13 min using elution with a linear gradient of 10 mM ammonium formate and acetonitrile, both containing 0.1% formic acid. Analytes quantification, using matrix-matched calibration samples spiked with their respective deuterated internal standards, is performed by electrospray ionization–triple quadrupole mass spectrometry using selected reaction monitoring detection in the positive mode. The method was validated according to FDA recommendations, including assessment of relative matrix effects variability, as well as tamoxifen and metabolites short-term stability in plasma and whole blood. The method is precise (inter-day CV%: 2.5 – 7.8 %), accurate (-1.4 to +5.8 %) and sensitive (lower limits of quantification comprised between 0.4 and 2.0 ng/mL). Application of this method to patients' samples has made possible the identification of two further metabolites, 4'-hydroxy-tamoxifen and 4'-hydroxy-*N*-desmethyl-tamoxifen, described for the first time in breast cancer patients. This UPLC-MS/MS assay is currently applied for monitoring plasma levels of tamoxifen and its metabolites in breast cancer patients within the frame of a clinical trial aiming to assess the impact of dose increase on tamoxifen and endoxifen exposure.

III.2. INTRODUCTION

Tamoxifen (*Z*-isomer) (Figure 1) is a standard hormonal therapy currently used for the secondary treatment of hormone-responsive breast cancer [1-6] and for the prevention in women at high risk of developing the disease [7]. Tamoxifen is a non-steroidal selective oestrogen receptor modulator (SERM), which competitively binds to estrogen receptors (ERs) and inhibits estrogen-dependent growth and proliferation of malignant breast epithelial cells [1, 6]. However, several lines of evidence indicate that the overall anti-proliferative effects of tamoxifen depends notably on the formation of the clinically active metabolites 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethytamoxifen (endoxifen) (B and E in Figure 1) which have 100 fold greater affinity to ERs and 30 to 100 fold greater potency in suppressing breast cancer cell proliferation as compared to the parent drug [8-12].

Tamoxifen can thus be considered a quasi-prodrug that is extensively metabolised by several polymorphic cytochrome P450 (CYP) enzymes into its active metabolites 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethytamoxifen (endoxifen) [1]. Briefly, tamoxifen is primarily oxidized to *N*-desmethyl-tamoxifen (the most abundant metabolite in human plasma) and 4-hydroxy-tamoxifen predominantly by CYP3A4/5 and CYP2D6, respectively, followed by endoxifen formation from *N*-desmethyl-tamoxifen, exclusively catalyzed by CYP2D6 and from 4-hydroxy-tamoxifen by CYP3A4/5. Tamoxifen and its metabolites undergo further glucuronidation and sulphation [13, 14].

Endoxifen is considered to be responsible for an important part of the *in vivo* pharmacological activity of tamoxifen, as endoxifen plasma concentrations are about 5–10 fold higher than those of 4-hydroxy-tamoxifen, with a different mode of action for endoxifen being suggested [8, 10, 15].

The clinical outcomes of tamoxifen treatment in terms of efficacy and side effects are inconstant, and some patients either fail to respond or become resistant to tamoxifen therapy [14, 16, 17]. One of the proposed mechanisms explaining the impaired response to tamoxifen therapy is an altered bio-activation into endoxifen by genetic or environmental factors. A polymorphism in CY2D6 enzymes that catalyze this conversion has been reported to influence the blood level of endoxifen [14, 18-21] and, in some retrospective studies, to predict clinical outcomes in patients [14, 21-25]. This has prompted the consideration of a potential role for *CYP2D6* genotype/phenotype testing in patients' management, which remains controversial, however [26-34]. In fact, large inter-patient variability in endoxifen levels still subsists even after correcting for CYP2D6 status [18, 27]. The remaining variability may depend on the activity of other cytochromes (CYP3A4/5, 2C9, 2C19), some of them known to be polymorphic, and by the influence of environmental factors such as interacting co-medications, among others. Of importance are some selective serotonin reuptake inhibitors (SSRIs)

antidepressants with strong CYP2D6 inhibiting activity, such as paroxetine and fluoxetine advised formerly to treat tamoxifen-induced hot flashes or depression are known to influence tamoxifen bioactivation [10, 28, 29].

The plasma concentration of the active metabolites of tamoxifen (mainly endoxifen and 4-hydroxy-tamoxifen) corresponding to the final phenotypic trait, may therefore represent a better predictor of tamoxifen efficacy than the patients' *CYP2D6* genotype. However, whether the monitoring of endoxifen plasma concentrations in breast cancer patients would constitute a valid approach to optimize individual dosage remains to be demonstrated. In that context, several analytical methods have been published for the monitoring of tamoxifen and its metabolites in human biological fluids, including GC-MS [35], CE-MS [36], conventional and micellar liquid chromatography methods coupled to fluorescence detection [37-40] and LC-MS/MS methods [41-46]. Reports have also been published describing liquid chromatography method coupled to mass spectrometry or fluorescence detection for the study of tamoxifen metabolism in vitro and in vivo [47-54]. For mass spectrometry techniques, conventional HPLC [42, 45, 46] and fast liquid chromatography coupled to tandem MS method using monolithic [41] or small particles (3µm) packed columns [43, 44] has been proposed for the quantification of tamoxifen and/or its metabolites. With the exception of the HPLC-MS/MS methods recently published [42, 46], the potential impact of biological matrix effects variability on tamoxifen metabolites quantification was only scarcely addressed, as previous assays were using either no I.S. [45], or only a single labeled I.S. [41, 43] as a surrogate I.S. for the quantification of tamoxifen and/or its metabolites.

Herein, we describe the development and validation of an UPLC-MS/MS method for the sensitive quantification in human plasma of tamoxifen, *N*-desmethyl-tamoxifen, and the active metabolites 4-hydroxy-tamoxifen and endoxifen within 13 minutes. The influence of matrix effects on tamoxifen and its metabolites quantification has been thoroughly investigated. The chromatographic profile of known (tamoxifen-*N*-oxide, α-hydroxy-tamoxifen) and previously unreported tamoxifen metabolites (4'-hydroxy-tamoxifen, 4'-hydroxy-*N*-desmethyl-tamoxifen, 3-hydroxy-tamoxifen) has also been studied in detail to exclude the risk of interferences during the comparatively short duration of the UPLC MS/MS analysis.

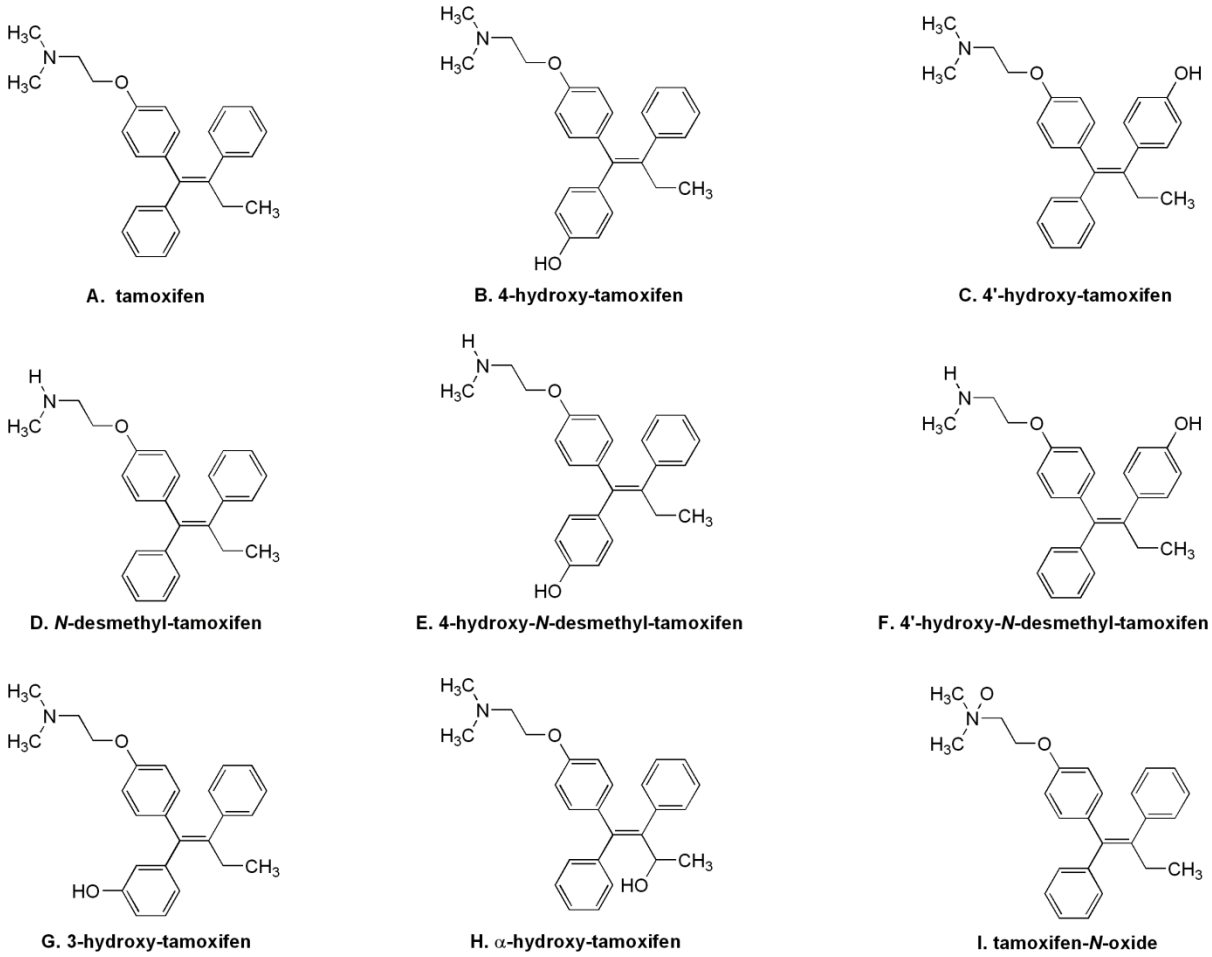

Figure 1: Chemical structures of the tamoxifen and its three major metabolites studied: **A.** tamoxifen; **B.** 4-hydroxy-tamoxifen; **C.** 4'-hydroxy-tamoxifen; **D.** *N*-desmethyl-tamoxifen; **E.** 4-hydroxy-*N*-desmethyl-tamoxifen (endoxifen); **F.** 4'-hydroxy-*N*-desmethyl-tamoxifen; **G.** 3-hydroxy-tamoxifen; **H.** α-hydroxy-tamoxifen; **I.** tamoxifen-*N*-oxide.

III.3. EXPERIMENTAL

III.3.1. Chemicals and reagents

Tamoxifen (Tam) and *Z*-4-hydroxy-tamoxifen (4-OH-Tam) were purchased at Sigma-Aldrich (Schnelldorf, Germany). *N*-desmethyl-tamoxifen (*N*-D-Tam) hydrochloride, 4-hydroxy-*N*-desmethyl-tamoxifen 1:1 E/Z mixture (4-OH-*N*-D-Tam), 4'-hydroxy-tamoxifen (4'-OH-Tam), 4'-hydroxy-*N*-desmethyl-tamoxifen (4'-OH-*N*-D-Tam), α-hydroxy-tamoxifen (α-OH-Tam), 3-hydroxy-tamoxifen (3-OH-Tam), tamoxifen-*N*-oxide (Tam-*N*O), and the internal standards (I.S.): tamoxifen-ethyl-d5 (Tam-d5), *N*-desmethyl-tamoxifen-ethyl-d5 (*N*-D-Tam-d5), 4-hydroxy-tamoxifen-ethyl-d5 (4-OH-Tam-d5) and 4-hydroxy-*N*-desmethyl-tamoxifen-ethyl-d5 (endoxifen-d5), were purchased from Toronto Research Chemicals Inc. (North York, Canada).

Chromatography was performed using Lichrosolv® HPLC-grade acetonitrile (MeCN) purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). Ammonium formate was purchased from Fluka (Buchs, Switzerland). Formic acid (98%) and methanol for chromatography Lichrosolv® (MeOH) were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Different sources of blank plasma used for the assessment of matrix effects and for the preparation of calibration and control samples were isolated (1850 g, 10 min, +4 °C, Beckman Centrifuge, Model J6B) from outdated blood donation units from the Hospital Blood Transfusion Centre (CHUV, Lausanne, Switzerland) or from citrated blood withdrawn from patients with Vaquez's Disease (polycythemia vera).

III.3.2. Equipment

The liquid chromatography system consisted of Rheos 2200 quaternary pumps, equipped with an online degasser and a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) controlled by Janeiro-CNS software (Flux Instruments, AG, Thermo Fischer Scientific Inc, Waltham, MA). Separations were done on a 2.1 mm x 30 mm Acquity UPLC® BEH C18 1.7 μm analytical column (Waters, Milford, MA, USA) placed in a thermostated column heater at 40°C (Hot Dog 5090, Prolab, Switzerland). The chromatographic system was coupled to a triple quadrupole (TSQ) Quantum Ultra mass spectrometer (MS) from Thermo Fisher Scientific, Inc. equipped with an Ion Max electrospray ionization (ESI) interface and operated with Xcalibur software package (Version 2.0.7, Thermo Fischer Scientific Inc, Waltham, MA).

III.3.3. Solutions

III.3.3.1. Mobile phase and extracts reconstitution solutions

The mobile phase used for chromatography was composed of 10 mM ammonium formate in ultrapure water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid (FA). A solution of MeOH / 20 mM ammonium formate 1:1 (v/v), adjusted to pH 2.9 with FA, was used for the reconstitution of the extracted plasma samples prior to their analysis.

III.3.3.2. Working solutions, internal standard, calibration standards and quality controls (QCs) solutions

Stock solutions of deuterated internal standards (I.S.) (0.5 mg/mL in MeOH) were diluted with acetonitrile (ACN) to obtain a single working I.S. solution containing 25 ng/mL of tamoxifen-d5, *N*-

desmethyl-tamoxifen-d5 and 4-hydroxy-tamoxifen-d5 at a concentration of 25 ng/mL, and 50 ng/mL of endoxifen-d5 (1:1 E/Z mixture) .

Standard stock solutions of tamoxifen base, *N*-desmethyl-tamoxifen hydrochloride, 4-hydroxy-tamoxifen base and endoxifen (1:1 E/Z mixture) base each at 1 mg/mL were prepared in MeOH and stored at -20°C. Appropriate volumes of stock solutions were serially diluted with H_2O/MeOH (3:1) as indicated in Table 1 to obtain single working solutions of analytes at concentration ranging from to 0.008 to 20 µg/mL. These working solutions were diluted 1:20 with blank citrated plasma to obtain for tamoxifen/metabolites the calibration samples ranging from 0.4 to 1000 ng/mL and their corresponding three quality control (low (L), medium (M) and high (H) QCs) samples ranging from 1.2 to 750 ng/mL. All spiked plasma samples were prepared according to the recommendations for bioanalytical methods validation stating that total added volume must be ≤ 10 % of the biological sample [55]. The calibration standard and control plasma samples were stored as 100 µL aliquots at -80 °C. Of note, the accuracy of calibration and QC samples is subsequently verified by comparison with another batch of calibration and QCs samples prepared with freshly made stock solutions (at the occasion of plasma calibration batch renewal). The response of both series (i.e. new and previous) of calibration samples are compared, and analytes' levels in the two series of QC samples calculated using the calibration curve established with both series of calibrations samples. Residuals for newly and previous calibration standards and quality controls have to meet the acceptance criteria for precision and accuracy.

Table 1: Preparation of working solutions

Drug	Stock solution solvent	Stock solution concentration	Working solution concentration (obtained by dilution of stock solution with H_2O / MeOH 3:1)	Calibration range (obtained by dilution of working solution with plasma 1/20)	QCs controls
Tam	MeOH	1 mg/mL	0.02 - 10 µg/mL	1 - 500 ng/mL	3; 50; 375 ng/mL
4-OH-Tam	MeOH	1 mg/mL	0.008 - 4 µg/mL	0.4 - 200 ng/mL	1.2; 20; 150 ng/mL
***N*-D-Tam**	MeOH	1 mg/mL	0.04 - 20 µg/mL	2 - 1000 ng/mL	6; 100; 750 ng/mL
***E*-endoxifen**	MeOH	0.5 mg/L	0.02 - 10 µg/mL	1 - 500 ng/mL	3; 50; 375 ng/mL
***Z*-endoxifen**	MeOH	0.5mg/mL	0.02 - 10 µg/mL	1 - 500 ng/mL	3; 50; 375 ng/mL

All stock solutions are mixed together to give a single working solution.

III.3.4. LC-MS/MS conditions

The mobile phase was delivered using the stepwise gradient elution program reported in Table 2. The thermostated column heater was set at +40 °C and the autosampler was maintained at +4°C. The injection volume was 10 µL.

Table 2: Gradient elution program

Time (min)	Buffer A* (%)	Solvent B* (%)	Flow rate (µL/min)
0.00	70.0	30.0	300
9.00	48.0	52.0	300
9.01	48.0	52.0	300
9.50	70.0	30.0	350
13.00	70.0	30.0	350

* Buffer A: 10 mM NH_4 formate + 0.1% formic acid. Solvent B: Acetonitrile + 0.1% formic acid. Temperature (°C): 25. Injection volume (µL): 10 µL.

The MS conditions were as follows: ESI in positive mode, capillary temperature: 350°C; in source collision induced dissociation): 4 V; tube lens voltages range: 101 to 126 V; spray voltage: 4 kV; sheath gas pressure: 60 psi and auxiliary gas (nitrogen) pressure: 10 (arbitrary units). The Q2 collision gas (argon) pressure was 1.5 mTorr (0.2 Pa); Q2 collision induced dissociation (CID): 10 V. MS is acquired in selected reaction monitoring (SRM). The optimal parameters and MS/MS transitions were determined by direct infusion of tamoxifen, its metabolites and I.S. solutions separately into the MS/MS detector at a concentration of 1 µg/mL in MeOH / 20 mM ammonium formate 1:1 (volume/volume, v/v), adjusted to pH 2.9 with FA. The selected *m/z* transitions and the collision energy for each analyte and I.S. are reported in Table 3.

The first (Q1) and third (Q3) quadrupoles were set at 2.8 amu mass resolution (Full-Width Half-Maximum = 2 Da). Scan time and scan width were 0.02 s and 0.5 m/z, respectively. MS acquisitions were done in centroid mode. Two segments of data acquisition were programmed in the positive mode: the first acquisition segment from 0 to 6 min, and the second one from 6 to 12 min.

Chromatographic data acquisition, peak integration and quantification were performed using the QUAL and QUAN browser of Xcalibur software package (version 2.0.7, ThermoQuest, Thermo Fischer Scientific Inc, Waltham, MA).

Table 3: Instrument method for the LC-MS/MS analysis of tamoxifen/metabolites with deuterated analogs as internal standards

Drug	Parent (m/z)	Product (m/z)	CE (eV)	Tube Lens (V)	Mean RT (min)	Polarity mode
Tamoxifen (Tam)	372.3	72.10	23	122	7.7	Positive
***N*-desmethyl-tamoxifen (*N*-D-Tam)**	358.3	58.10	21	122	7.4	Positive
***Z*-4-hydroxy-tamoxifen (4-OH-Tam)**	388.3	70.10	38	126	4.3	Positive
		72.10	25	126		Positive
		129.10	28	126		Positive
Endoxifen (1:1 E/Z mixture)	374.3	58.10	22	122	4.0	Positive
		129.10	28	122		
		223.10	20	122		
Tamoxifen –d5 (Tam-d5)	377.3	72.10	24	122	7.7	Positive
***N*-desmethyl-tamoxifen (*N*-D-Tam-d5)**	363.3	58.10	21	122	7.4	Positive
4-hydroxy-tamoxifen (4-OH-Tam-d5)	393.3	72.10	25	126	4.3	Positive
Endoxifen-d5 (1:1 E/Z mixture)	379.3	58.10	22	122	4.0	Positive

CE = collision energy; Mean RT = Retention Time; MS acquisition time (min) = 12.00 ; Q2 Collision gas pressure (mTorr) = 1.5.

III.3.5. Clinical blood samples collection

Blood samples were obtained from consenting breast cancer patients enrolled in the study protocol "Tamoxifen metabolism and the impact of tamoxifen dose on the level of the active metabolites in endocrine sensitive breast cancer patients" (ClinicalTrials.gov Identifier: NCT00963209), approved by the Ethics Committee of the University Hospital. Written informed consent was obtained from all patients. Blood samples (5.5 mL) from breast cancer patients treated with tamoxifen were collected at random time after last drug intake in Monovettes® (Sarstedt, Nümbrecht, Germany) containing K-EDTA as anticoagulant. According to study protocol, blood samples were collected in patients receiving 20 mg tamoxifen once daily, at two occasions at baseline (e.g. on day 0 and day 1, i.e. after inclusion and before dose escalation), and after 1, 3 and 4 months of continuous treatment at a regimen of 20 mg tamoxifen twice daily (BID).

III.3.6. Plasma sample extraction procedure

A 100 µL aliquot of plasma was mixed with 100 µL of I.S. solution (25 ng/mL of tamoxifen-d5, *N*-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5, and 50ng/mL of endoxifen-d5 1:1 E/Z mixture, in ACN) and with acetonitrile (300 µL), carefully vortex-mixed and sonificated for 30 seconds. (Branson Ultrasonics Corporation, Danbury, CT, USA). The mixture was centrifuged at 4°C for 10 min at 16000g (12000 rpm) on a benchtop Hettich® Centrifuge (Benchtop Universal 16R centrifuge, Bäch, Switzerland). A 400 µL aliquot of the supernatant was transferred into a polypropylene tube and

evaporated to dryness under nitrogen at room temperature. Of note, SpeedVac® concentrator may also be used, presenting the advantage of organic solvent recuperation. The solid residue was reconstituted in 600 µL of a solution of MeOH / 20 mM ammonium formate 1:1 (v/v), adjusted to pH 2.9 with FA, vortex-mixed and centrifuged again under the above-mentioned conditions. A 400 µL of the supernatant was introduced into 1.5 mL glass HPLC microvials maintained at +4°C in the autosampler rack during the entire LC-MS/MS analysis.

III.3.7. Calibration curves

Quantitative analysis of tamoxifen and its three main metabolites (*N*-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and *Z*-endoxifen) in plasma was performed using the internal standard method. Deuterated compounds of each target analyte were used as I.S. Each level of the calibration curve was measured with two sets of calibrators: the first at the beginning and the second at the end of the run. Calibration curves were established with calibration standards prepared in citrated plasma.

Calibration standard curves have been calculated and fitted by quadratic log-log regression [56] of the peak-area ratio of tamoxifen and its metabolites to its respective I.S., versus the nominal concentrations of each analyte in each standard sample. To determine the best weighting factor, concentrations were back-calculated and the model with the lowest total bias across the concentration range was considered the best suited. The seven-point calibration curves for tamoxifen and its three metabolites were established over the range reported in Table 1. The ranges of calibration were selected to cover the range of concentrations expected in patients according to previously published pharmacokinetic studies [18-20, 42].

III.3.8. Analytical method validation

The method validation was based on the recommendations published on-line by the Food and Drugs Administration (FDA) [55] as well as on the recommendations the Conference Report on "Quantitative Bioanalytical Methods Validation and implementation: Best Practice for Chromatographic and Ligand Bindings Assays" [57], the Arlington Workshop "Bioanalytical Methods Validation – A revisit with a Decade of Progress" [58]. Recommendations from Matuszewski to assess matrix effects were also considered [59, 60].

III.3.8.1. Selectivity

The assay selectivity was assessed by analysing plasma extracts from ten batches of blank plasma from different sources.

III.3.8.2. Accuracy and precision

The concentrations for the quality control (QC) samples were selected to encompass the whole range of the calibration curve corresponding to the drug levels anticipated to occur in most patient samples: low (L), medium (M) and high (H). The concentration selected for the low QC sample corresponds to 3 times the respective lower limit of quantification (i.e. the lowest calibration level) kept in the finalized method, in accordance to the FDA recommendations [55]. Replicate analysis (n=6) of three QC samples were used for the intra-assay precision and accuracy determination. Inter-assay accuracy and precision were determined by duplicate analysis of the three QC repeated on six different days. The precision was calculated as the coefficient of variation (CV %) and the accuracy was calculated as the bias or percentage of deviation between the nominal and measured concentrations.

After the completion of the above validation procedure, for the routine analysis of patient samples, duplicate QC samples at the three concentration levels (L, M and H) were used.

III.3.8.3. Matrix effects, extraction yield and overall recovery

In the initial step of method validation, matrix effect was examined qualitatively by the simultaneous post-column infusion of tamoxifen/metabolites and I.S. into the MS/MS detector during the chromatographic analysis of 6 different blank plasma extracts. The standard solution of all analytes and their corresponding deuterated I.S. at 5 µg/mL was infused at a flow-rate of 20 µL/min during the chromatographic analysis of blank plasma extracts from 6 different sources. The chromatographic signals of each selected MS/MS transition were examined to check for any signal perturbation (drift or shift) of the MS/MS signal at the analytes' retention time (data not shown).

Subsequently, the matrix effects were also quantitatively assessed. Three series of QC samples at L, M and H concentrations were processed in duplicate as follows:

(A) Pure stock solutions dissolved in the reconstitution solvent (MeOH - buffer (Ammonium formate 20 mM, pH adjusted to 2.9 with FA) 1:1) and directly injected onto column.

(B) Plasma extracts samples from 6 different sources, spiked after extraction with tamoxifen/metabolites and I.S. (from pure stock solutions in the reconstitution solvent).

(C) Plasma samples from 6 different sources (same as in B) spiked with tamoxifen/metabolites standard solutions and I.S. before extraction.

The recovery and ion suppression/enhancement of the MS/MS signal of drugs in the presence of plasma matrix (i.e. matrix effects) was assessed by comparing the absolute peak areas of the analytes either dissolved in the reconstitution solvent: MeOH-buffer (Ammonium formate 20 mM, pH

adjusted to 2.9 with FA) 1:1 (A), or spiked after plasma extraction (B) or spiked before plasma extraction (C), using 6 different batches of plasma, based on the recommendations proposed by Matuszewski et al. [59, 60].

The *extraction yield* of tamoxifen/metabolites and I.S. was calculated as the absolute peak-area response in processed plasma samples spiked with the standard analytes *before* extraction (C) expressed as the percentage of the response of the same amount of analytes spiked into blank plasma *after* the extraction procedure (B) (C/B ratio in %). The *matrix effect* was assessed as the ratio of the peak areas of the analytes spiked into blank plasma *after* the extraction procedure (B) to the peak areas of the analytes solubilised in MeOH-Ammonium formate 20 mM pH 2.9 1:1 (A) (B/A ratio in %). The *overall recovery* of tamoxifen/metabolites and I.S. was calculated as the ratio of absolute peak-area responses of tamoxifen/metabolites spiked in processed plasma samples *before* extraction (C) to the peak areas of the analytes solubilised in MeOH/Ammonium formate 20 mM pH 2.9 1:1 (A) (C/A ratio %). Recovery studies were performed with plasma from 6 different sources spiked with tamoxifen, its metabolites and their respective I.S. at the concentrations reported in Table 4. The results normalized with the signal of I.S. (i.e. B2 and C2), used as an index of the effective injection volume, are also reported in Table 4.

III.3.8.4. Carry-over

Memory effect has been investigated by the injection during an analytical run of 2 or 3 blank plasma after the highest calibration standard. Peak area response of the blank plasma sample, at each expected retention time, was compared to the peak area of the corresponding analyte at the lowest limit of quantification (LLOQ).

III.3.8.5. Dilution effect

During the course of patients' samples analyses, one patient sample was found to have tamoxifen concentration exceeding the highest level of the calibration curve (see Table 1). To ascertain whether the dilution of this sample could affect the accuracy of the drug or its metabolites determination, a blank plasma sample was spiked with pure standards (tamoxifen/metabolites) at a concentration exceeding by two-fold the highest calibration level. The sample was thereafter analysed in duplicate after a three, four, five and six fold dilution to bring the concentration within the calibration range. Dilution was carried out with blank plasma. Calculated and expected concentrations were compared.

III.3.8.6. Stability of tamoxifen and its metabolites

Stability studies of tamoxifen and its three metabolites at different storage conditions included:

Stability in plasma spiked with tamoxifen/metabolites (i.e. QCs at L, M and H concentrations) over time at room temperature (RT) and at +4°C up to 48 h. Variations of tamoxifen/metabolite concentrations were expressed as percentages of the initial concentration measured immediately after preparation, i.e. T_0. Analyses were performed in triplicate at T_0 and at each subsequent time point.

Stability of tamoxifen/metabolites in whole blood at +4°C and at RT assessed by calculating the percent deviation of the I.S. normalized peak area of each analyte in the collected plasma from the initial peak area ratio measured at T_0. Two batches of whole blood samples spiked with analytes at the L, M and H levels (1 ml final volume) were prepared in triplicate and kept for 0, 1, 2, 4, 8, 24 and 48 h before plasma separation at +4 °C and at RT. All plasma samples collected from centrifuged blood aliquots were stored at -80°C and subsequently analysed in the same analytical sequence.

Stability in plasma samples after multiple freeze-thaw cycles: plasma QCs at low, medium and high levels of tamoxifen/metabolites underwent three freeze-thaw cycles. Frozen samples were allowed to thaw at RT for 2 h and were subsequently refrozen at -80°C during approximately 24 h. Tamoxifen/metabolites levels were measured in aliquots from the three consecutive freeze-thaw cycles.

Stability in plasma samples kept frozen at -80°C: QCs samples at the L, M and H concentrations were stored at –80°C during 4 months and measured using fresh plasma calibration samples.

III.3.8.7. Identification of other tamoxifen metabolites

Next to tamoxifen, *N*-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen analysis, additional phase I tamoxifen metabolites were identified in patients samples by comparison of the retention times and product-ion mass spectra of authentic standard compounds spiked into blank plasma, or added to patients' plasma samples. The full-scan mass spectra were acquired over a scan range of 40 to 400 m/z at scanning speed of 0.08 sec/scan.

In the present analytical work, the concentrations of the newly identified metabolites 4'-hydroxy-tamoxifen and 4'-hydroxy-*N*-desmethyl-tamoxifen have also been estimated using 4-OH-Tam-d5 and endoxifen-d5 as I.S. in a separate series of analysis of 20 patients' samples.

III.4. RESULTS AND DISCUSSION

III.4.1. Chromatograms

The proposed ultra performance-liquid chromatography coupled with tandem MS method enables the simultaneous quantification within 13 min of tamoxifen and three metabolites: *N*-desmethyl-

tamoxifen, 4-hydroxy-tamoxifen and *Z*-endoxifen (4-hydroxy-*N*-desmethyl-tamoxifen), in 100 µL plasma aliquots. A chromatographic profile of the highest calibration plasma sample containing tamoxifen/metabolites is shown in Figure 2 in the positive ionization mode, during the two acquisition segments (0-6 and 6-12 min), using the selected reaction monitoring (SRM) detection mode; the proposed gradient program is described in Table 2. Tamoxifen and its metabolites were eluted in less than 9 min, followed by approx. 4 minutes of column re-conditioning step with 70% of buffer A (Ammonium formate 10 mM + 0.1% FA) and 30% of solvent B (Acetonitrile + 0.1% FA) at a flow rate of 0.35 mL/min (Table 2). The respective retention times and mass spectrometry conditions for tamoxifen/metabolites and their corresponding stable isotope labeled I.S. are reported in Table 3. Three m/z transitions were selected for 4-hydroxy-tamoxifen (*m/z* 388) with product ions at *m/z* 70, 72 and 129, and for endoxifen (*m/z* 374) with product ions at *m/z* 58, 129 and 223, in order to increase the detection sensitivity for these metabolites. The fragment ions at *m/z* 72 and 58 are the major signals visible on the product ion spectrum of 4-hydroxy-tamoxifen and endoxifen, respectively.

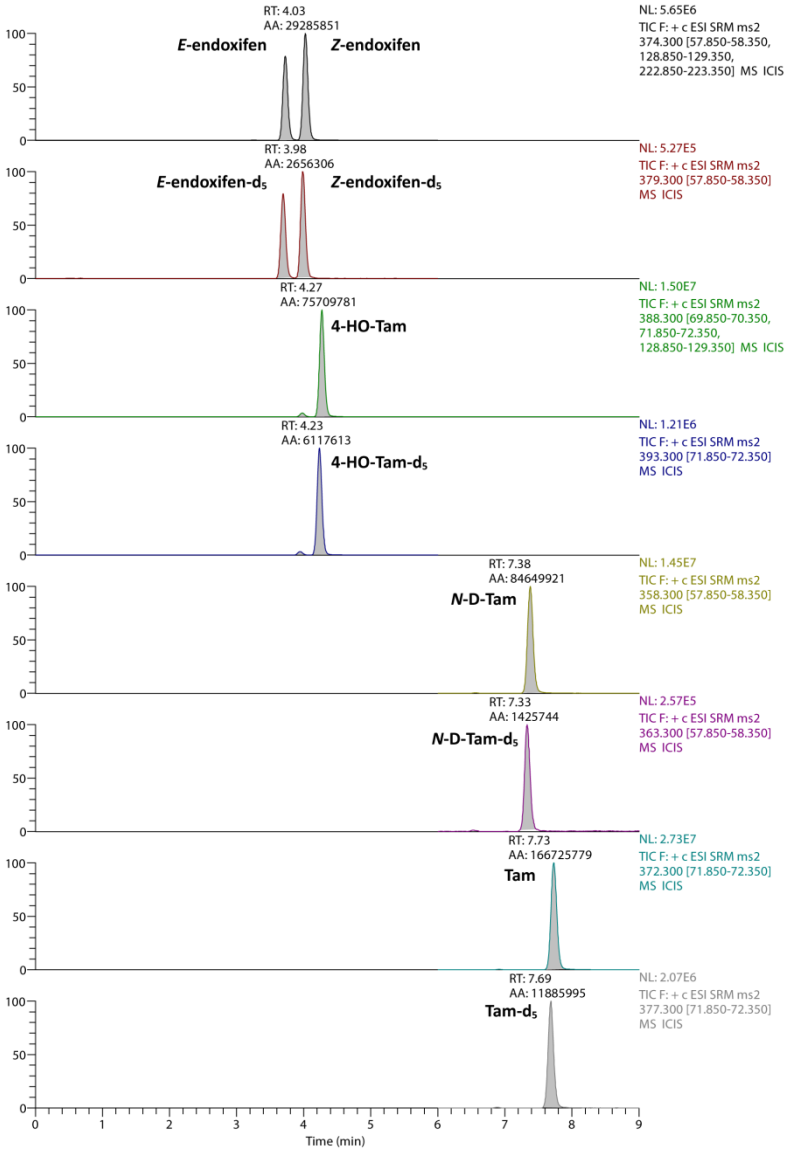

Figure 2: Chromatogram of the highest calibration sample containing tamoxifen and its three major metabolites. Corresponding deuterated analogs are used as internal standards (details in the text).

A satisfactory separation was achieved for all considered analytes, especially for (*E*-) and (*Z*-) isomers of endoxifen and endoxifen-d5 obtained as a racemic mixture (Figure 2). Data from blank plasma samples spiked with the deuterated I.S., obtained throughout the method validation procedure and during patients' plasma samples analysis, revealed no interfering "cross-talk" signals arising from the isotopically-labeled I.S. on the transition of the corresponding target analyte, thus testifying the isotopic purity of these isotope labeled I.S.

Moreover, the proposed UPLC method provides an excellent chromatographic separation of tamoxifen-*N*-oxide from tamoxifen, preventing therefore analytical bias due to potential *in-source* dissociation of tamoxifen-*N*-oxide into tamoxifen that would give rise to spuriously elevated levels of

tamoxifen. Of note, it was rather unexpected that tamoxifen-*N*-oxide, intuitively more polar, elutes later than tamoxifen on a reverse phase column, in line with previous reports [41, 42, 49]. Alterations of intra- or inter-molecular bindings, or pH–dependent changes in molecular lipophilicity, (i.e. Log D) [50, 52] might be involved.

Figure 3a shows the chromatographic profile of a plasma sample collected in a hormone sensitive breast cancer patient having received tamoxifen for 1 month at a regimen of 20 mg twice a day. The plasma levels of tamoxifen, *N*-desmethyl-tamoxifen, *Z*-4-hydroxy-tamoxifen and *Z*-endoxifen measured 7.5 hours after last drug intake were 666.6, 929.4, 15.2 and 217.9 ng/mL respectively). As reported in the literature, only the (*Z*) isomers of 4-OH-Tam and endoxifen were observed in plasma, thus excluding any *E-Z* interconversion of tamoxifen metabolites during sample preparation [42, 44, 61, 62].

Figure 3b shows the chromatographic profile of a plasma obtained from a hormone sensitive breast cancer patient receiving tamoxifen for 1.5 year at the standard regimen of 20 mg once daily. The plasma levels of tamoxifen, *N*-desmethyl-tamoxifen, *Z*-4-hydroxy-tamoxifen and *Z*-endoxifen measured 13.25 hours after last drug intake were 207.6, 445.2, 1.4 and 6.2 ng/mL, respectively).

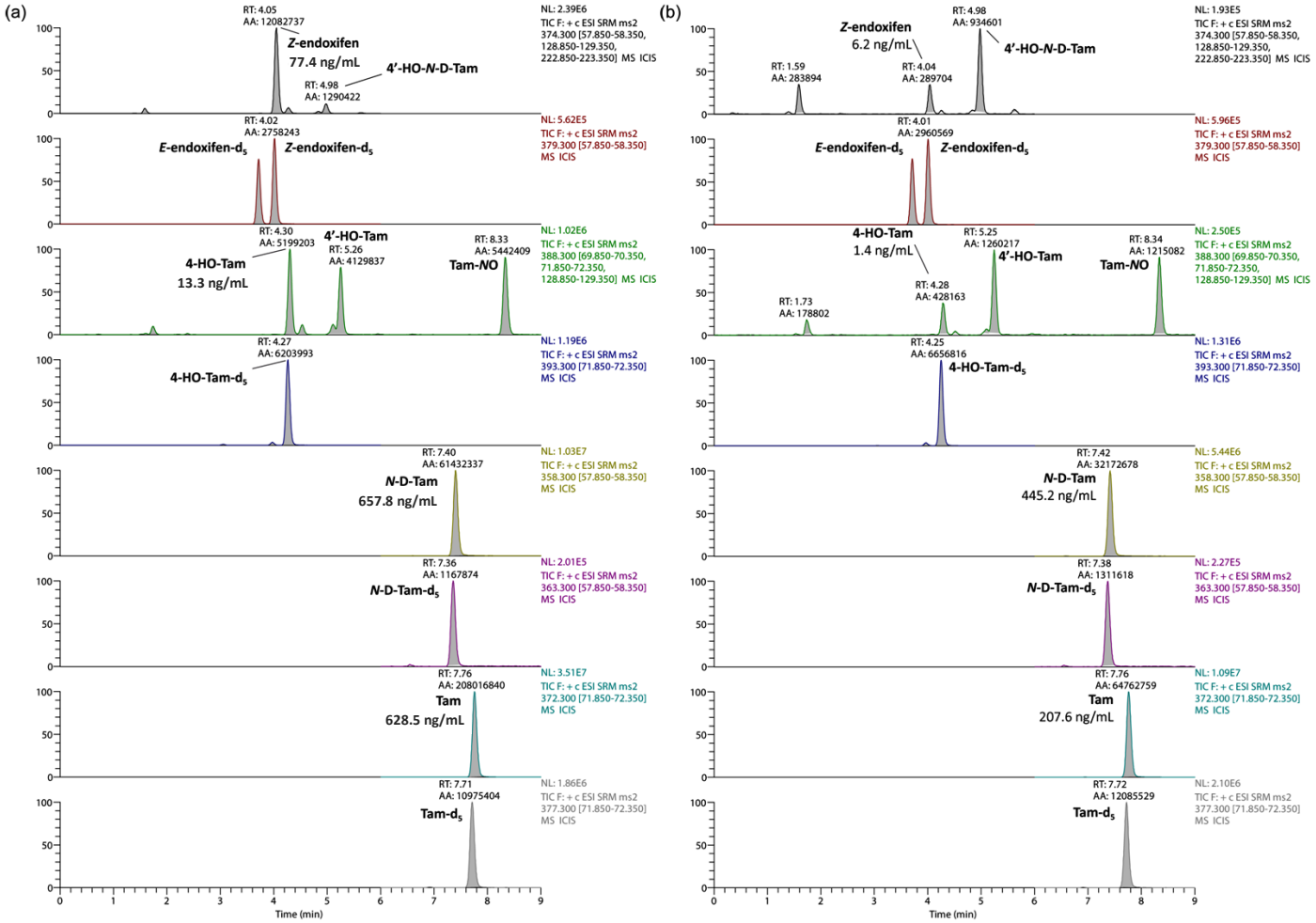

Figure 3: Chromatographic profile of plasma samples from two patients (a and b) receiving tamoxifen (details in the text).

III.4.2. Method Validation

III.4.2.1. Selectivity

No peaks from endogenous compounds were observed at the drugs retention time in any of the blank plasma extracts. The product ion monitoring was selected, based on its relative abundance, while avoiding possible structural analogies with the other analysed drugs or metabolites. All channels were simultaneously observed, and no selectivity issue as well as no crosstalk were detected across the acquisition channels.

III.4.2.2. Internal standard and calibration curve

The use of stable isotope-labeled internal standards is considered to be the best approach to minimize the influence of matrix effects on the accuracy and precision of a quantitative method, of particular importance when using electrospray mass spectrometry [59, 60, 63].

Therefore, deuterated analogues of tamoxifen and the metabolites to be quantified, have been used throughout our analytical method validation procedure (i.e. tamoxifen-d5, *N*-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5 and endoxifen-d5 (1:1 *E/Z* mixture). No problems regarding the isotopic purity, *E* to *Z* interconversion, and chemical stability of the I.S. (either in stock solution or in biological fluid and during sample processing), neither any "cross-talk" between MS/MS channels used for monitoring tamoxifen and the considered metabolites and the I.S. were identified throughout method validation procedure.

Calibration curves over the entire ranges of concentrations delineated in Table 1 were satisfactorily described by quadratic log-log regression of the peak-area ratio of tamoxifen and its metabolites to their I.S., versus the concentrations of the respective analytes in each standard sample. This model of calibration described by Singtoroj et al. [56] was found well suited to best fit the criteria of homoscedasticity (homogeneity of variance over the entire calibration range) and minimum bias for each single calibrator. The determination coefficients (R^2) of all calibration curves were higher than 0.99 with back-calculated concentrations of the calibration samples within ±15% of nominal values (±20% at LLOQ).

There was originally some concern that the calibration samples prepared with citrated plasma collected from blood from outdated transfusion bag or from Vaquez patients may not fully reflect the plasma matrix from patients collected on EDTA. However, getting blood on EDTA from volunteers solely for the purpose of calibration samples preparation would be unpractical and difficult to justify from an ethical point of view. For the sake of validation, a cross-validation was performed by performing replicate analysis (n=3) of QC samples at the three levels, prepared either in citrated and in EDTA plasma. The QC samples were assayed using the calibration curve established with citrated plasma samples. Head-to-head comparison shows that the anticoagulant does not influence significantly the analytical results for tamoxifen and its metabolites. No statistically significant differences ($p < 0.05$) in concentrations were found for QCs samples prepared in EDTA and citrated plasma using calibration curves established with citrated plasma (p values comprised within 0.07 to 0.92 for tamoxifen, *N*-desmethyl-tamoxifen, *Z*-4-hydroxy-tamoxifen, *Z*-endoxifen and *E*-endoxifen (Student *t*-test).

III.4.2.3. Precision, accuracy, and LLOQ

Precision and accuracy determined with the L, M and H QC samples are summarized in Table A.1 (on-line supplementary data). The mean intra-assay precision was similar over the entire concentration range and always less than 6.8%. Overall, the mean inter-day precision was within 2.5 and 7.8 %. The

intra-assay and inter-assay deviation (bias) from the nominal concentrations of QCs ranged between -5.3 and +7.4 %, and -1.4 and +5.8%, respectively.

Of note, the chosen ranges of calibration were selected initially to cover the clinical range of tamoxifen/metabolites concentrations previously reported in the literature [18-20, 42]. In fact, we observed during the method's validation that the responses attained at the LLOQs levels would be sufficient so that it may be possible to validate this method at even lower levels (ca. 0.1 - 0.75 ng/mL) if desired in the future.

III.4.2.4. Matrix effect and recovery

Matrix effect was examined quantitatively by the simultaneous post-column infusion of tamoxifen/metabolites and I.S. into the MS/MS detector during the chromatographic analysis of six different batches of blood donors. During the chromatography of blank matrices, the signals at all the *m/z* transitions selected showed a remarkably similar pattern, with all traces being essentially superimposable. No noticeable matrix effect (no drifts or shifts of the signals) was observed at the respective retention time of tamoxifen and its metabolites and their deuterated I.S. (data not shown).

The inter-subject variations in suppression/enhancement profiles have been studied also quantitatively (Table 4). The results reported in Table 4 (column B/A) indicate that co-eluting plasma matrix components appear to have a minimal effect on the considered analytes, except for *N*-D-Tam whose signal was approximately halved (mean ratio B/A = 62%). As expected, a similar extent of ion suppression was observed with *N*-D-Tam labeled internal standard *(N*-D-Tam-d5) (B/A ratio = 57.5%). Thus overall, the mean B/A ratios for *N*-D-Tam when normalized with those of deuterated I.S. was 1.1 (i.e. at or slightly above unity), demonstrating the value of stable isotope-labeled I.S. use for an efficient control of the relative matrix effect [64]. Plasma matrix does not appear to significantly interfere with Tam, 4-OH-Tam and both endoxifen isomers ionisation (B/A ratio ranged between 96.7 and 104.7%).

Using the proposed protein precipitation, supernatant evaporation and dissolution in appropriate buffer provided a good extraction recovery (C/B, column REext) always higher than 95%, resulting in an excellent sensitivity.

As indicated in Table 4, the *analytical recovery values* were always higher than 89.9%. The *process efficiency* (i.e. overall recovery) given in Table 4 (column PE, C/A ratio) was comprised within 92.4-108.6 % except for *N*-D-Tam, which gives a process efficiency around 61%. As reported above, matrix components do influence to some extent *N*-D-Tam ionisation and consequently the overall process efficiency, requiring therefore the preparation of calibration and control samples in a plasma matrix

reflecting at best the composition of the samples to be analysed. Most importantly, this is not so much the absolute matrix effect, but rather its variability (relative matrix effect) that must be reduced. As shown in Table 4, the variability of the matrix effect of 6 different plasma matrix were close to 20% for *N*-D-Tam at all QCs and never exceeded 5.7% for all other analytes, which indeed demonstrates that the proposed extraction procedure is able at least to normalize these matrix effects, even in the absence of the correcting effect of labeled I.S. In fact, the use of isotope-labelled internal standards in our UPLC-tandem MS method seems to effectively control most of the residual relative matrix effect variability. This has been experimentally verified notably for *N*-D-Tam for which the observed matrix effect variability in 6 plasma lots never exceeded 4% when *N*-D-Tam peak areas where normalized to those from its deuterated I.S. (N-D-Tam-d5).

Table 4: Matrix effects, extraction yield, overall recovery and process efficiency of tamoxifen/metabolites

Component	*Nominal conc. (ng/mL)*	*Mean peak area*			*Mean peak area ratio*		*ME (%)*		*extRE (%)*		*Analysis RE (%)*			*PE (%)*	
		A (n = 6)	*B (n = 6)*	*C (n = 6)*	*B2*	*C2*	*B/A*	*CV (%)*	*C/B*	*CV (%)*	*C2/B2*	*Mean*	*CV (%)*	*C/A*	*CV (%)*
Tam	3	1263441	1255380	1372710	0.072	0.076	99.4	3.7	109.3	4.3	105.0	96.4	8.1	108.6	1.6
	50	28878341	27938705	27393059	1.606	1.513	96.7	2.0	98.0	4.0	94.2			94.9	3.5
	375	228978707	226034897	211505317	12.997	11.683	98.7	1.0	93.6	2.3	89.9			92.4	1.8
***N*-D-Tam**	6	804396	497408	613605	0.243	0.304	61.8	18.0	123.4	6.9	124.8	109.9	11.9	76.3	14.2
	100	17796321	10574145	10929594	5.169	5.406	59.4	21.0	103.4	8.9	104.6			61.4	18.0
	750	125333845	80869169	80226329	39.529	39.685	64.5	18.4	99.2	6.7	100.4			64.0	16.2
4-OH-Tam	1.2	537944	545444	559305	0.062	0.067	101.4	2.9	102.5	5.0	107.8	104.1	3.3	104.0	3.5
	20	10730921	10607311	10417567	1.202	1.241	98.8	1.5	98.2	3.0	103.3			97.1	3.4
	150	79332011	79252260	76170959	8.980	9.076	99.9	1.9	96.1	2.1	101.1			96.0	1.1
Z-endoxifen	3	227307	235540	230538	0.059	0.063	103.6	5.2	97.9	8.1	106.5	105.2	1.2	101.4	4.7
	50	4467005	4597862	4431075	1.149	1.205	102.9	0.9	96.4	4.6	104.9			99.2	4.8
	375	32717609	33369469	31924813	8.339	8.682	102.0	1.5	95.7	2.5	104.1			97.6	1.1
E-endoxifen	3	154699	162010	160857	0.055	0.059	104.7	5.7	99.3	2.8	106.6	103.6	2.5	104.0	3.7
	50	3048595	3146053	2988009	1.074	1.095	103.2	2.3	95.0	3.1	102.0			98.0	4.1
	375	22805748	23258002	22122028	7.939	8.109	102.0	1.5	95.1	0.9	102.1			97.0	1.3
Tam-d5	25	17793384	17391055	18104182			96.1	2.8	104.1	1.7				101.7	2.8
***N*-D-Tam-d5**	25	3404892	1959036	1937111			57.5	17.3	98.9	6.8				56.9	15.5
4-OH-Tam-d5	25	8825185	8825420	8392730			100.0	3.0	95.1	3.1				95.1	2.4
***Z*-endoxifen-d5**	25	3796772	4001590	3677241			105.4	2.7	91.9	3.1				96.9	3.8
***E*-endoxifen-d5**	25	2881493	2929682	2728140			101.7	2.0	93.1	2.6				94.7	3.9

A = peak area of standard solutions without matrix and without extraction (MeOH/buffer A 1:1), B = peak area of analytes spiked after extraction, C = peak area of analytes spiked before extraction, B2 = ratio of the peak area of the analyte and the I.S. spiked after extraction, C2 = ratio of the peak area of the analyte and the I.S. spiked before extraction, ME=matrix effect expressed as the ratio of the mean peak area of the analytes spiked after extraction (B) to the mean peak area of the same standard solution without matrix (A) multiplied by 100. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression. ext RE = extraction procedure recovery calculated as the ratio of the mean peak area of the analytes spiked before extraction (C) to the mean peak area of the analytes spiked after extraction (B) multiplied by 100. Analysis RE = analysis recovery calculated as the ratio of the mean peak-area ratio of the analytes spiked before extraction (C2) to the mean peak-area ratio of the analytes spiked after extraction (B2) multiplied by 100. PE = process efficiency expressed as the ratio of the mean peak area of the analyte spiked before extraction (*C*) to the mean area of the same analyte standards (*A*) multiplied by 100.

III.4.2.5. Memory effect

No major carry-over was observed with our method. The highest memory effect was observed for tamoxifen, the most lipophilic analyte. This carry-over effect was successfully eliminated by programming the injection of three blank samples after the highest calibration samples, prior to the analysis of patients' samples. The peak intensity visible in the third blank matrix sample corresponds to less than 20% of those of the LLOQ sample. In fact, during routine plasma analysis, it has prudently been decided to program a single blank plasma injection after each patient's sample which was found sufficient to reduce the memory effect to an extent unlikely to affect the accuracy of tamoxifen and its metabolites measurements in the following patients' plasma samples.

III.4.2.6. Dilution effect

After the three, four, five and six-fold dilutions of the spiked plasma with tamoxifen/metabolites at a concentration exceeding by two-fold the high calibration level, the deviation (bias) from the expected concentrations of all compounds was less than 8.2, 7.5, 4.6 and 8% respectively. This indicates that plasma samples containing tamoxifen/metabolites above the highest level of calibration can be adequately diluted with blank plasma prior to the LC–MS/MS analysis, to bring down concentration within the calibration range.

III.4.2.7. Stability of tamoxifen/metabolites in plasma and whole blood

a) The stability of tamoxifen/metabolites in human plasma samples was ascertained with QC samples left at room temperature (RT) and at +4°C up to 48 h. The variation over time of the concentrations of tamoxifen and its metabolites in plasma remained comprised within ±15% of initial (T_0) concentrations (see Table A.2 in on-line supplementary data), indicating that tamoxifen and its metabolites are stable in plasma at RT and at +4°C.

b) During the clinical study, which prompted this analytical development, some blood samples had to be stored temporally at +4°C before being shipped to our laboratory and centrifuged for plasma collection. Given the absence of information on the stability of tamoxifen and its principal metabolites in blood, we have studied the evolution of their concentrations over time in whole blood. The results of stability studies in whole blood are summarized in Table A.3 (on-line supplementary data), indicating that tamoxifen and its metabolites can reliably be considered as stable in whole blood, up to 8 hours storage either at +4°C or at RT.

c) Variations of tamoxifen/metabolites concentrations were always less than –15% from nominal levels after three freeze-thaw cycles (Table A.2, in on-line supplementary data), indicating no significant loss of drug upon this procedure.

d) QCs samples prepared in batches, distributed as 100 µL aliquots and stored at -80°C in 1.5ml Eppendorf vials were analysed 4 months later. All QCs (L, M and H) were analysed in duplicate. Variations of tamoxifen/metabolites concentrations were less than -11.9% from their nominal concentrations, indicating the long term stability of tamoxifen and its metabolites in plasma samples stored at -80°C.

III.4.3. Metabolites profiles studies and metabolites identification.

Given the reduced elution time of analytes with UPLC, it was critical for this analytical development to verify that tamoxifen metabolites would not potentially perturb the quantification. The chromatographic elution pattern of reported or putative tamoxifen metabolites was therefore studied thoroughly.

Three additional peaks were observed in patients samples at 1.7, 5.2 and 8.3 min on the SRM transition (*m/z* 388 → 70, 72, 129) selected for 4-hydroxy-tamoxifen (itself eluted at 4.2 min) (Figure 3b, third chromatogram from top, and Figure 4a). These metabolites were identified in patients (Figure 4a) as α-hydroxy-tamoxifen, 4'-hydroxy-tamoxifen and tamoxifen-*N*-oxide, respectively (H, C, I, respectively in Figure 1) [13, 49, 51, 65] by comparison to the retention times (Figure 4a, lower chromatogram) and/or product-ion spectra of authentic standards spiked into blank plasma or added to patients' plasma samples (data not shown). The fragmentation pattern of the 4'-hydroxy-tamoxifen standard spiked into blank plasma (Figure 5a) was equivalent to that observed for the putative endogenous 4'-hydroxy-tamoxifen. The product ions (72, 129, 145, 223, 316 m/z) were invariably observed in all product ion scans determined at the retention time of the metabolite observed in patients samples.

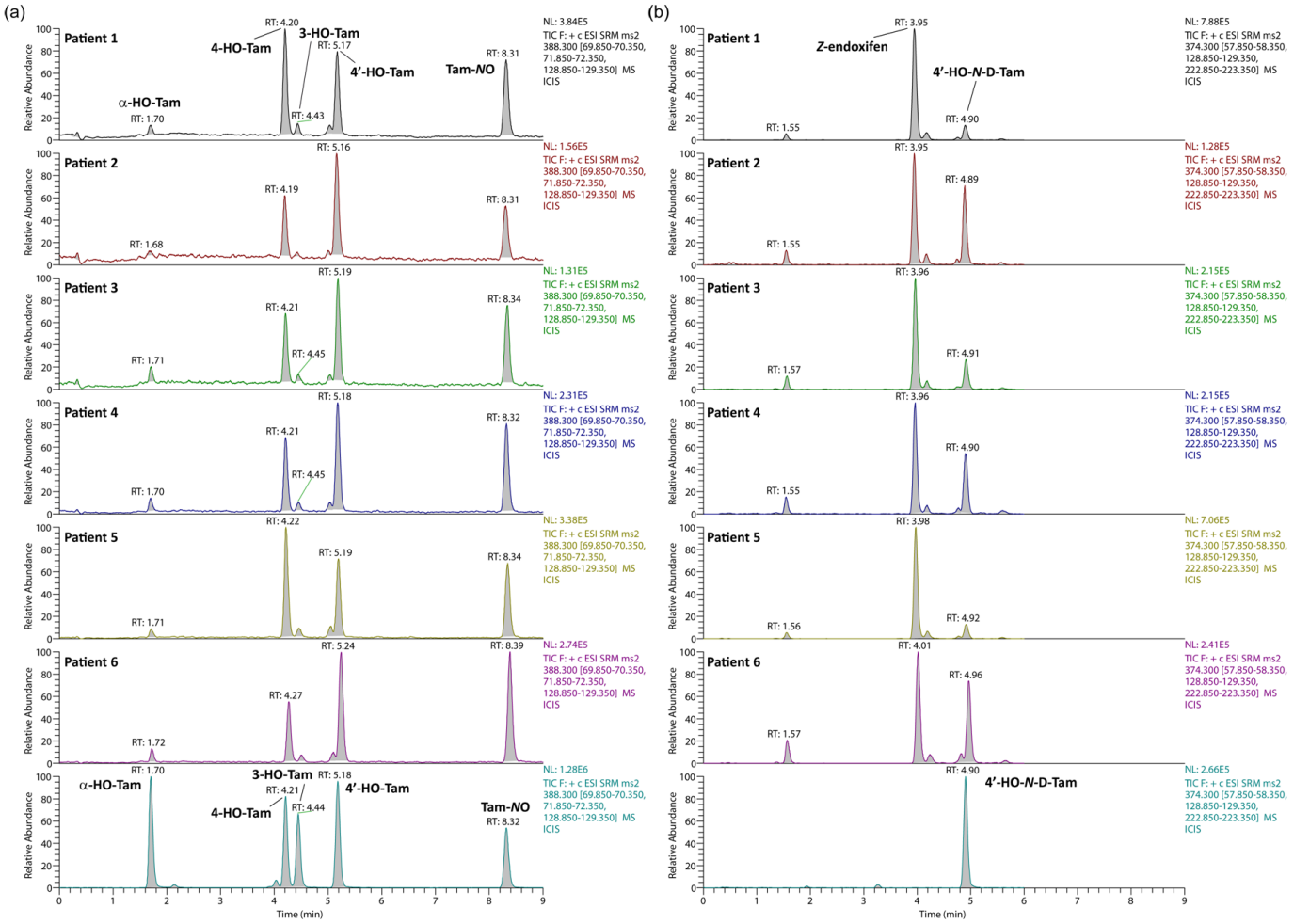

Figure 4: Chromatographic profiles (a) at SRM transition (m/z 388 → 70, 72, 129) and (b) at SRM transition (m/z 374 → 58, 129, 223) in plasma from 6 unselected patients receiving tamoxifen. Last chromatograms (bottom traces) are blank plasma spiked with: (a) pure standard of α-hydroxy-tamoxifen, 4-hydroxy-tamoxifen, 3-hydroxy-tamoxifen, 4'-hydroxy-tamoxifen and tamoxifen-N-oxide and (b) pure standard of 4'-hydroxy-N-desmethyl-tamoxifen (details in the text).

Interestingly, the UPLC gradient program also allows the base-line separation of 4-hydroxy-tamoxifen and 3-hydroxy-tamoxifen eluted at 4.2 and 4.4 min, respectively (Figure 4a, lower chromatogram of standard compounds spiked in plasma). The compound 3-hydroxy-tamoxifen is a metabolite reported to be produced *in vitro* upon incubation of tamoxifen with human liver microsomes (HLMs) [13]. In patients' plasma however, there was only a very small peak, if any, visible at the retention time of 3-hydroxy-tamoxifen. (Figure 4a, metabolites profiles in patients).

Finally, inspection of the transition (*m/z* 374 → 58, 129, 223) selected for monitoring *Z*-endoxifen (eluted at 4.0 min) revealed the presence in patients samples of two additional peaks at 1.5 and 4.9 min (Figure 3b, upper chromatographic profile, and Figure 4b). The first eluted peak at 1.5 min was tentatively identified as α-hydroxy-*N*-desmethyl-tamoxifen based on literature (no available reference material). The latest peak visible in this m/z transition at 4.9 min was identified as 4'-

hydroxy-*N*-desmethyl-tamoxifen, which has the same retention time (Figure 4b, lower trace) and a comparable product-ion mass spectrum as the synthetic compound (Figure 5b) either spiked into blank plasma or patients' plasma samples. The product ions (58, 129, 145, 223 and 316 m/z) were observed during the fragmentation of the 4'-hydroxy-*N*-desmethyl-tamoxifen pure compound and were likewise detected in all product ions scans at the retention time of the putative endogenous metabolite. As recently described, the fragment at 129 m/z was reported to be indicative of the tamoxifen structure [54] and was detected in product ion spectra of both metabolites 4'-hydroxy-*N*-desmethyl-tamoxifen and 4'-hydroxy-tamoxifen.

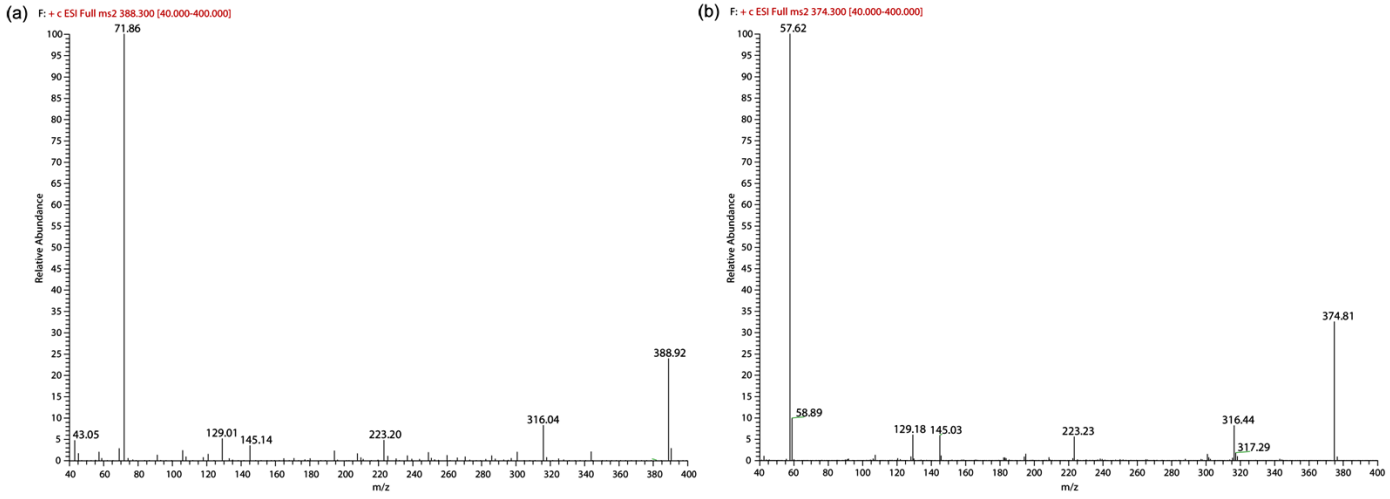

Figure 5: Product ion spectra of the pure standards (a) 4'-hydroxy-tamoxifen and (b) 4'-hydroxy-*N*-desmethyl-tamoxifen spiked into blank plasma.

The metabolite 4'-hydroxy-tamoxifen, whose formation might be catalyzed by the polymorphic CYP2B6 [13, 61], has been previously detected in rat and mouse liver microsomes [13, 52, 61, 65] and in recent *in vitro* studies (using Human Cytochrome P450 Systems) as primary metabolite of tamoxifen [13, 51], but its occurrence had never been formally reported in humans. Similarly, 4'-hydroxy-*N*-desmethyl-tamoxifen has been previously detected in mouse liver microsomal incubates [52]. Neither metabolite has yet been identified so far in patients.

This is the first report of the occurrence of 4'-hydroxy-tamoxifen and 4'-hydroxy-*N*-desmethyl-tamoxifen in plasma from patients under tamoxifen therapy. Typical metabolites profiles in 6 unselected patients receiving tamoxifen are shown in Figure 4a and 4b: 4'-hydroxy-tamoxifen and 4'-hydroxy-*N*-desmethyl-tamoxifen are detected in patients' samples at 5.1 and 4.9 min in their respective m/z transition channel. So far, both metabolites were found in all patients' samples analyzed (n = 70), with substantial variability in plasma levels.

Although our method has not been formally validated for the quantification of these newly identified metabolites, their plasma levels have been estimated in a separate analysis of 20 unselected patients' samples. The concentrations of 4'-hydroxy-tamoxifen and 4'-hydroxy-*N*-desmethyl-tamoxifen ranged between 2.2 to 5.5 ng/mL, and 4.4 to 11.8 ng/mL, respectively, in patients under tamoxifen 20 mg QD, and between 3.3 to 9.5 ng/mL, and 6.2 to 20.6 ng/mL, respectively, in patients under 20 mg BID tamoxifen regimen. The clinical importance of these new metabolites, and their potential contribution to the clinical effects of tamoxifen remain to be determined [13]. Limited studies available from the literature suggest that 4'-hydroxy-tamoxifen might have higher affinity for the estrogen receptor than tamoxifen itself [13, 66, 67].

III.5. CONCLUSION

We have developed and validated a specific and sensitive UPLC-MS/MS method enabling reliable and sensitive monitoring of tamoxifen and three clinically relevant metabolites in patients' plasma. Our method provides an excellent chromatographic separation of tamoxifen and seven known and previously unreported metabolites in a relatively short gradient program of 13 min. The method was developed using deuterated I.S. for all target analytes, which further strengthen our analytical assay for selective and sensitive quantification of tamoxifen and its metabolites by electrospray ionisation mass spectrometry.

During the course of these chromatographic investigations, we have been able to identify for the first time the two metabolites 4'-hydroxy-tamoxifen and 4'-hydroxy-*N*-desmethyl-tamoxifen in plasma from breast cancer patients. Our estimation of 4'-hydroxy metabolites plasma levels in a subset of patients indicates that the range of 4'-hydroxy-tamoxifen plasma concentrations was similar to that measured for 4-hydroxy-tamoxifen. Conversely, 4'-hydroxy-*N*-desmethyl-tamoxifen plasma levels were two to three times lower than the endoxifen levels determined in these 20 unselected patients. The clinical importance of these previously unreported metabolites and their potential contribution to the clinical effects of tamoxifen has yet to be determined. Finally, we could show that 3-hydroxy-tamoxifen is very limitedly, if not at all, found in the blood of patients on tamoxifen therapy.

In conclusion, This UPLC–MS/MS method has been shown suitable for measuring exposure of tamoxifen and its metabolites in tamoxifen-treated breast cancer patients. In this context, the present analytical methodology is currently applied in a population pharmacokinetic study of tamoxifen and its metabolites, helping us primarily at characterizing the influence of pharmacogenetic and environmental factors (including interacting medications) on plasma concentrations.

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III.7. SUPPLEMENTARY DATA

Table A.1: Precision and accuracy of L, M and H QC samples determined by repeated analysis performed on six different days (inter-assay) and within the same day (intra-assay).

Nominal concentrations (ng/mL)		Intra-assay (n=6)				Inter-assay (n=6)			
		Conc. found (ng/mL)	SD	Precision CV (%)	Accuracy biais (%)	Conc. found (ng/mL)	SD	Precision CV (%)	Accuracy biais (%)
Tam	***3***	3.2	0.2	5.0	7.4	3.2	0.2	5.1	5.8
	50	49.3	1.5	3.1	-1.4	49.8	1.3	2.5	-0.3
	375	368.1	11.3	3.1	-1.9	382.6	13.9	3.6	2.0
***N*-D-Tam**	***6***	6.4	0.3	4.2	7.4	6.2	0.2	4.0	4.0
	100	100.0	2.1	2.1	0.0	99.9	5.6	5.6	-0.1
	750	748.1	21.0	2.8	-0.3	749.6	24.7	3.3	-0.1
4-OH-Tam	***1.2***	1.3	0.1	5.9	6.3	1.3	0.1	5.1	5.0
	20	18.9	0.4	2.1	-5.3	19.9	0.8	4.1	-0.4
	150	148.8	4.3	2.9	-0.8	151.7	5.7	3.8	1.1
***Z*-endoxifen**	***3***	3.0	0.2	6.8	0.8	3.0	0.1	4.7	0.2
	50	50.3	1.9	3.8	0.5	50.1	1.5	3.0	0.1
	375	358.5	10.4	2.9	-4.4	375.2	11.7	3.1	0.0
***E*-endoxifen**	***3***	3.1	0.2	5.5	2.0	3.1	0.2	7.8	2.4
	50	47.7	1.6	3.3	-4.7	49.9	1.8	3.5	-0.2
	375	359.5	11.0	3.1	-4.1	369.6	19.4	5.2	-1.4

Table A.2: Stability of tamoxifen/metabolites at QCs levels in human plasma.

Drug	Tam			*N*-D-Tam			4-OH-Tam			*Z*-endoxifen			*E*-endoxifen		
Nominal conc. (ng/mL)	*3*	*50*	*375*	*6*	*100*	*750*	*1.2*	*20*	*150*	*3*	*50*	*375*	*3*	*50*	*375*
Room temperature															
1h	2.2	-2.2	-0.1	-0.4	-0.4	1.5	-7.2	-2.4	1.6	0.1	-2.9	5.2	-3.3	-5.6	0.2
2h	7.7	-1.4	-2.8	7.9	4.2	-2.4	2.0	1.5	1.3	-5.2	0.6	2.0	-2.7	-2.3	-4.0
4h	6.4	0.7	1.6	7.3	8.3	-2.0	-3.3	1.6	3.3	0.4	1.0	4.0	4.7	0.2	-0.5
8h	8.4	1.3	-0.2	2.4	9.8	-6.0	-1.5	2.9	0.6	-0.4	0.8	3.0	-1.2	4.0	-2.2
24h	10.8	-1.8	0.3	3.3	2.0	-5.7	1.2	1.4	2.4	0.8	0.3	5.0	3.8	-1.5	-2.1
48h	6.1	-0.1	0.8	3.1	3.8	-9.9	-2.8	2.6	1.4	-2.3	0.3	0.4	0.3	1.3	-4.2
At +4°C															
1h	-4.9	-1.6	3.2	1.4	2.3	-3.2	-1.0	0.1	0.9	-0.4	-1.8	4.4	1.6	-2.8	1.1
2h	-3.3	0.3	-0.2	3.7	3.2	-6.0	-3.4	3.5	0.7	2.1	2.3	4.6	-0.4	2.8	-3.2
4h	1.2	-1.0	1.2	6.9	4.7	-3.3	-3.0	2.8	1.8	-0.0	0.6	2.6	8.2	-1.4	0.4
8h	-4.2	-1.7	-0.8	1.5	2.9	-5.4	-4.0	0.4	0.3	2.7	1.2	2.0	-2.0	0.3	-2.2
24h	-1.2	-0.2	0.5	-1.3	1.6	-5.1	-0.5	0.8	0.1	-5.9	-0.2	2.5	0.0	-2.7	-2.9
48h	-2.1	-2.1	1.3	2.7	-1.1	-5.5	-0.6	0.6	2.2	-1.8	-2.4	3.4	5.60	-0.4	-1.1
Freeze -thaw															
cycle 1	2.2	0.0	0.1	-3.0	2.5	-1.45	2.7	-1.7	3.3	3.4	0.1	1.5	-11.5	-1.1	0.5
cycle 2	-1.0	-0.1	0.5	-2.6	0.6	-3.50	1.8	-2.5	2.6	0.5	-0.9	0.1	-9.2	-4.3	-0.1
cycle 3	1.5	-0.7	2.0	0.4	1.0	1.46	0.2	-1.0	2.0	-0.3	1.9	0.8	-8.2	1.3	-1.7

Results are given as the deviation from initial (T_0) concentrations (%).

Table A.3: Stability of tamoxifen/metabolites at QCs levels in citrated whole blood.

Drug	Tam			*N*-D-Tam			4-OH-Tam			*Z*-endoxifen			*E*-endoxifen		
Nominal conc. (ng/mL)	*3*	*50*	*375*	*6*	*100*	*750*	*1.2*	*20*	*150*	*3*	*50*	*375*	*3*	*50*	*375*
Room temperature															
1h	-0.8	-4.2	-5.6	-8.0	-10.9	-5.8	-7.2	0.0	-2.5	-3.5	-9.2	-5.7	18.3	-0.9	3.6
2h	-5.3	-4.5	-8.5	-4.9	-11.7	-10.0	-11.5	-3.6	-5.8	-7.8	-11.6	-7.0	2.4	0.8	4.8
4h	-2.1	-4.7	-4.7	-10.1	-14.5	-12.8	-8.3	-4.0	-6.9	-5.8	-11.2	-8.2	14.5	6.7	4.6
8h	-2.9	-6.8	-12.1	-8.8	-14.1	-15.0	-10.5	-4.8	-8.7	-6.2	-13.5	-11.1	11.1	1.2	1.4
24h	-9.4	-9.1	-11.1	-17.8	-14.5	-18.3	-15.1	-4.4	-9.9	-12.1	-15.5	-16.8	9.1	2.6	2.0
48h	-14.2	-10.2	-15.1	-24.5	-27.1	-17.3	-23.3	-9.7	-14.5	-19.1	-17.8	-17.6	14.4	8.0	11.8
At +4°C															
1h	0.2	3.6	0.3	0.1	3.2	3.3	-5.5	6.6	5.1	5.0	0.9	7.9	-1.7	-5.2	-6.0
2h	2.3	0.5	2.6	4.9	2.3	4.1	1.6	6.7	7.0	11.1	-1.7	7.3	9.4	-7.7	-4.6
4h	-0.5	3.2	2.9	3.3	0.7	3.9	-1.9	7.3	4.1	1.3	-0.9	5.5	-4.0	-12.5	-3.7
8h	-1.1	-2.6	0.0	-1.2	-3.8	-2.5	-8.4	0.7	1.4	4.7	-1.5	0.2	-0.2	-9.0	-7.1
24h	1.6	-4.7	-1.5	4.0	-5.0	0.0	-3.0	-1.9	2.7	10.8	-5.5	4.7	-6.0	-19.9	-16.2
48h	-3.5	-1.5	-1.7	-1.9	-0.5	-1.2	-13.0	2.8	0.0	-5.2	-5.3	1.5	-13.0	-24.3	-21.4

Results are given as the deviation from initial (T_0) concentrations (%).

Quantitative monitoring of tamoxifen in human plasma extended to forty metabolites using liquid-chromatography-high resolution-mass spectrometry: New investigation capabilities for clinical pharmacology

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Submitted to: Analytical and Bioanalytical Chemistry, on the 17th of October 2013

CHAPTER IV - QUANTITATIVE MONITORING OF TAMOXIFEN IN HUMAN PLASMA EXTENDED TO FORTY METABOLITES USING LIQUID-CHROMATOGRAPHY-HIGH RESOLUTION-MASS SPECTROMETRY: NEW INVESTIGATION CAPABILITIES FOR CLINICAL PHARMACOLOGY

IV.1. ABSTRACT

LC-high-resolution (HR)-MS analysis can record HR-full scans, a technique of detection that shows comparable selectivity and sensitivity to ion transitions (SRM) performed with triple-quadrupole (TQ)-MS, but that allows *de facto* determination of "all" ions including drug metabolites. This could be of potential utility in *in vivo* drug metabolism and pharmacovigilance studies in order to have a more comprehensive insight in drug biotransformation profile differences in patients.

This simultaneous quantitative and qualitative (Quan/Qual) approach has been tested with 20 patients chronically treated with tamoxifen (TAM). The absolute quantification of TAM and 3 metabolites in plasma was realized using HR- and TQ-MS and compared. The same LC-HR-MS analysis allowed the identification and relative quantification of 37 additional TAM metabolites. A number of new metabolites were detected in patients' plasma including metabolites identified as didemethyl-tri-hydroxy-TAM-glucoside and didemethyl-tetrahydroxy-TAM-glucoside conjugates corresponding to TAM with six and seven biotransformation steps, respectively.

Multivariate analysis allowed relevant patterns of metabolites and ratios to be associated with TAM administration and *CYP2D6* genotype. Two hydroxylated metabolites, α-OH-TAM and 4'-OH-TAM, were identified as putative CYP2D6 substrates.

The relative quantification was precise (<20%) and the semi-quantitative estimation suggests that metabolite levels are non-negligible. Metabolites could play an important role in drug toxicity, but their impact on drug-related side effects has been partially neglected due to the tremendous effort needed with previous MS technologies. Using present HR-MS, this situation should evolve with the straightforward determination of drug metabolites, enlarging the possibilities in studying inter- and intra-patients drug metabolism variability and related effects.

IV.2. INTRODUCTION

Adverse drug reactions (ADR) are a major concern in term of the number of people exposed (millions), the number of deaths (a few thousands) and financial costs (10-100 billions USD in a country such as the USA) [1-9]. Moreover, the absolute number of ADR is steadily increasing due to i) an increased number of prescriptions, ii) the increased number of available drugs, and iii) polymedication [2,10].

Recently, the importance of drug metabolites in ADR have been specifically underscored with the release of Authority Guidance for industry about the safety of drug metabolites (MIST Metabolites In Safety Testing in 2008) and the International Conference on Harmonization (ICH Guideline M3(R2) in 2009/2010) [11-13]. Today, drug developers must address drug metabolite toxicology when their levels represent more than 10% of the total drug-related exposure or if they are present in disproportionate higher levels in humans than in the tested animals. [14-17]

The mechanisms of toxicity of a drug and its metabolites are diverse and can be i) on-target, ii) off-target with the binding of the drug/metabolites to an alternate target, or iii) related to the covalent binding of reactive metabolites to proteins, nucleic acids or membranes [18,19,20]. Strong side-effects can also be observed with very low levels of metabolites or at a very low frequency (<1 case in 1,000 patients). These are rare events involving (alone or in combination) rare alleles (single nucleotide polymorphisms, SNPs), or drug-drug/-herbal/-food interactions (induction and/or inhibition) able to alter the drugs/metabolites' pharmacokinetics [21,22]. Eventually, the realistic safety profile of a new approved drug can only be appraised after a number of years of public use [2,23]. This underscores the huge significance of pharmacovigilance [24].

One main example of a drug whose metabolic pathways and related pharmacodynamics has raised interest through the last decades, is Tamoxifen (TAM), a selective estrogen receptor modulator, used for the prevention and adjuvant treatment of estrogen-sensitive breast cancer. TAM is extensively metabolized into active, inactive and reactive metabolites though different metabolic pathways involving different enzymes (mainly cytochrome P-450 -CYP- and conjugation enzymes) whose activities can vary intra- and inter-individually [18,25-29]. Two metabolites, 4-hydroxy-TAM and endoxifen (4-hydroxy-N-demethyl-tamoxifen), show much higher activity than the parent drug with up to 100 fold greater affinity and potency in inhibiting estrogen receptors [25,26]. Patients with lower endoxifen levels, as a consequence of the reduced CYP2D6 activity, are less likely to achieve benefit from TAM treatment [27]. Conversely, patients with higher endoxifen concentrations have higher frequency of side effects such as hot flashes. Other TAM metabolites could be related to the occurrence of other ADR such as cancer via the formation of reactive carbocation or benzoquinones

which can damage DNA [18]. Thus, the extensive TAM biotransformation underscores the importance of comprehensive *in vivo* drug metabolism studies [29-35].

In the present work, our objective was to show that nowadays, the determination of a drug and tens of its metabolites in plasma is feasible with recent high-resolution mass spectrometers (HR-MS; time-of-flight- and Orbitrap-MS) [36-42]. In liquid-chromatography (LC) coupled to HR-MS analysis, the detection of a drug and its metabolites can record high-resolution full-scan (HR-FS) with comparable selectivity and sensitivity to SRM performed with triple-quadrupole MS (TQ-MS) [43,44]. HR-FS acquisition records virtually all ions and allows the determination of known or unexpected compounds that can possibly be identified retrospectively [36-40]. The selectivity of HR-FS takes place post-acquisition by the construction of an accurate mass extracted ion chromatogram (XIC) on the analyte theoretical m/z with a narrow mass extraction window (MEW) [41]. For productive laboratories, the robustness, sensitivity, ease of use and level accuracy of LC-HR-MS analyses are comparable to TQ-MS analyses performing SRM acquisition [36-42]. This is why, there is a replacement of TQ-MS by HR-MS in most laboratories [38,45,46].

As a noteworthy consequence for clinical pharmacologists, HR-MS technology i) offers the possibility to identify and survey the exposure to a drug *and* tens of its metabolites and ii) allow, whenever needed, to extend drug monitoring to many drug metabolites in patients' samples, in order to relate their concentrations with toxicity or efficacy. This is in line with recent Authority's demands about pharmacovigilance [24,47-48].

In the present study, we have tested a quantitative and qualitative (Quan/Qual) approach on 20 patients treated with TAM. In parallel to the absolute quantification of TAM and 3 known metabolites by HR-MS and TQ-MS, the relative quantification of 37 other identified TAM metabolites was performed together with their provisional elucidation.

IV.3. MATERIALS AND METHODS

IV.3.1. Materials and reagents

(*Z*)-Tamoxifen (TAM) was purchased from Sigma–Aldrich (Germany). (*Z*)-4-hydroxy-tamoxifen (4-OH-TAM), (*Z*)-N-demethyl-tamoxifen (N-demethyl-TAM), N-demethyl-4-hydroxy-tamoxifen 1:1 *E/Z* mixture (N-demethyl-4-OH-TAM) and the deuterated internal standards (IS): tamoxifen-ethyl-D_5 (TAM-IS), N-demethyl-tamoxifen-ethyl-D_5 (N-demethyl-TAM-IS), 4-hydroxy-tamoxifen-ethyl-D_5 (4-OH-TAM-IS) and 4-hydroxy-N-demethyl-tamoxifen-ethyl-D_5 (endoxifen-IS), were purchased from Toronto Research Chemicals Inc. (Canada). Chromatography solvents and other chemicals were of analytical grade.

IV.3.2. Patients' blood withdrawal, plasma extraction and CYP2D6 status

Blood samples were obtained from breast cancer patients enrolled in a study protocol (ClinicalTrials.gov Identifier: NCT00963209) approved by the local Ethics Committee and randomly collected in Monovettes® containing K-EDTA (Sarstedt, Germany). Patients received 20mg TAM once or twice daily for ≥ 3 months. Blood samples were centrifugated (1,850 g, 10 min, +4°C). Plasma were transferred and frozen at -20°C.

As previously described [49], the extraction was as follows: 100µL of plasma samples were mixed with 400 µL MeCN containing the 4 IS (5 ng/mL). The mixture was centrifuged (16,000xg, 10 min, 4°C). Supernatants were transferred into polypropylene tubes and evaporated at RT under a N_2 flux. The dried residues were reconstituted in 600µL MeOH/20 mM ammonium formate 1:1 (v/v) adjusted to pH 2.9, and re-centrifuged and supernatants were transferred into injection vials.

Patients were classified according to their *CYP2D6* genotype as poor (PM, N=1), intermediate (IM, N=7), extensive (EM, N=11) or ultra-rapid metabolizers (UM, N=1).

IV.3.3. LC-MS methods and parameters

The analyses used 2 UHPLC systems coupled to a triple quadrupole Quantum Ultra MS (Thermo, USA) or an Exactive Plus Orbitrap MS (Thermo, Germany) and included a Rheos Allegro pump (Flux Instruments, Switzerland) and a HTS PAL autosampler (CTC analytics, Switzerland) set at 10°C. Heated electrospray ionization (H-ESI) was operated in positive mode. All H-ESI and MS parameters were usual values including spray voltage, 3.8-4.7 kV; sheath gas and auxiliary nitrogen pressures, 40-60 and 10-20 respective arbitrary units, declustering potential, 4-10 V; capillary temperature, 300-350 °C and tube lens voltages, 60 to 180 V.

The mobile phase was 10 mM ammonium formate + 0.1% FA (A) and MeCN + 0.1% FA (B) and was delivered at 300 µl/min using the following stepwise gradient: T = 0 min: 20% B; T= 11 min maintained for 1.4 min: 55% B; T= 12.5 min: initial conditions for 3.5 min. The analytical column, 2.1 × 30 mm (i.d. x L) Acquity UPLC BEH C18 (Waters, USA), was placed in an oven set at +40°C. The injection volume was 10 µL. Data acquisition, peak integration, and quantification were performed using *Xcalibur* software (Thermo, USA).

TQ-MS settings: quadrupole resolution, 0.7 u at FWHM; collision gas (Arg) pressure, 1.5 mTorr , and transitions with 0.5 u and 0.02 s scan width and scan time, respectively. Ion transitions (SRM) were recorded in centroid mode with the following precursor → product m/z and collision induced dissociation (see Table 1 for abbreviations and chemical compositions). TAM: 372.3 → 72.1@23eV; N-demethyl-TAM: 358.3 → 58.1@21eV; 4-OH-TAM: 388.3 → 70.1 + 72.1 + 129.1@38/25/25eV;

Endoxifen : 374.3 → 58.1 + 129.1 + 223.1@22/28/20eV; Endoxifen_IS: 377.3 → 72.1@24eV; 4-OH-TAM_IS: 363.3 → 58.1@21eV; N-demethyl-TAM_IS: 393.3 → 72.1@25eV; TAM_IS: 379.3 → 58.1@22eV.

Exactive Plus-HR-MS settings: HR-full scan (HR-FS) alternating with "all-ion fragmentation" MS (MS^{ALL}; high-energy collisional dissociation = 40eV) scan: from *m/z* 200 to 800 and *m/z* 55 to 800, respectively. MS^{ALL} is the fragmentation of all precursor ions entering the C-trap (no ion selections). C-trap capacity: 10^6 charges; maximum injection time: 250 ms; H-ESI probe temperature: 300°C. External mass calibration of the Exactive Plus-MS was performed when mass accuracy (MA) was ≤ ± 4 ppm. Resolution: 70,000 and 17,500 FWHM for HR-FS and MS^{ALL} acquisition, respectively. Extracted ion chromatograms (XIC) were based on a ± 5ppm mass extraction window (MEW).

IV.3.4. LC-MS parameters for metabolite confirmation with MS^2 acquisition

For confirmation and structure elucidation attempt of some TAM metabolites, additional product scan acquisitions were performed (MS^2 with the precursor ion selection within a m/z unit) on a Q-Exactive-MS (Thermo, Germany). UHPLC conditions were similar to the analysis on the Exactive Plus-MS but sample extracts were 10x more concentrated.

IV.3.5. Quantitative Analysis

As previously described [49], calibration curves were prepared with TAM, 4-OH-TAM, N-demethyl-TAM, and Endoxifen (Z-isomers) at 8 different levels ranging from 0.5-500, 0.2-200, 1-1000 and 0.5-500 ng/mL whereas quality control samples (QCs) were spiked at 3, 50, 375 and 1.2, 20, 150 and 6, 100, 750 and 3, 50, 375 ng/mL, respectively. Patients' plasma extracts were analyzed with the LC-TQ-MS system and a few months later re-extracted and analyzed with the LC-HR-MS system. Absolute quantifications were compared between the two analyses. Detection sensitivity, robustness and convenience (operability) were assessed.

IV.3.6. Qualitative analysis (metabolite identification)

The exact same HR-MS data used for the absolute quantification were reprocessed with *Metworks* 1.3 (Thermo, USA) for metabolite identification (Qual analysis). *MassFrontier* 6.0 (Thermo, USA) was used for the structure elucidation with the identification of fragment ions.

Two main strategies were employed for the identification of TAM metabolites. First, extracted ion chromatograms (XIC) with a MEW = 5 ppm around theoretical m/z values from 50 biotransformations predicted by Metworks software 1.3 SP2, were constructed (see also published

lists of biotransformation [50,51]). XIC of patients' plasma extracts were compared with blank plasma and calibrant extracts. Secondly, a mass defect filtering (MDF) using *Metworks* software was applied and all ions that did not enter the MDF limits were removed. This MDF delimitation considered the mass defect and nominal m/z values of TAM metabolites discovered by the first strategy and was defined as a square between m/z 0.165 at m/z 250 (lower limits) and m/z 0.270 at m/z 600 (upper limits). The MDF-cleaned spectra of a patient's chromatogram were checked visually scan by scan to reveal unexpected potential TAM metabolites and their m/z constructs were compared in patients' and control plasma chromatograms.

For structure elucidation attempt of some identified TAM metabolites, additional LC-MS analyses were performed with a Q-Exactive HR-MS recording (true) MS^2 product ion spectra. Different collision energies were applied (see Results).

IV.3.7. Semi- and relative quantification of tamoxifen metabolites

After metabolite identification and confirmation, semi- and relative quantifications of TAM metabolites in the 20 patients' plasma extracts were assessed. Semi-quantification is the estimation of metabolite levels in universal units (e.g. ng/mL) but within a large range (e.g. 0.1- to 10-fold accuracy) whereas relative quantification is the more or less precise determination of metabolites in arbitrary units.

With no pure standards of TAM metabolites, it is difficult to evaluate accurately the absolute levels due to unpredictable ionization yields. Nevertheless, the semi-quantification of TAM metabolites was estimated using the following equation applied to mean values: TAM metabolite level (ng/mL) = [(metabolite/TAM_IS peak area ratio) / (TAM/TAM_IS peak area ratio)] x TAM absolute level.

The relative quantification was done with LC peak area ratios of TAM metabolites and internal standard (IS). Metabolite/IS peak area ratios were calculated to reduce variability. The IS selection for each TAM metabolite has been chosen based on chemical structure similarities and retention times (see Table 1).

IV.3.8. Statistics and Bioinformatics data treatment

Passing-Bablok regression (PB) and Bland-Altman test (BA) have been used to assess the agreement between the two analytical methods for the absolute quantification. Assumptions of linearity and normal distribution of differences, for respectively PB and BA, have been assessed. For BA analysis, due to the presence of proportional linear bias for some analytes, mean difference (bias) between measurement and limits of agreements were expressed as percentages of the absolute analytes

concentrations and percent difference plots were used. Statistical analyses were performed using the MedCalc software, version 12.7 (MedCalc Software, Ostend, Belgium).

Multivariate analysis, including Principal Component Analysis (PCA) and Partial Least Squares (PLS) regression, was performed using SIMCA-P© v.13 (Umetrics, Sweden). PLS aims at building a linear multivariate model by determining an appropriate compromise between a synthetic description of the variables and a good correlation with the response. A leave-one-out cross-validation procedure was used to ensure the robustness of the PLS model and its generalization ability. For that purpose, the model was computed with data from 19 patients, leaving one subject out. The unseen sample was then predicted by the model. The process was repeated 20 times, once per subject. Model validity was further verified using permutation tests and CV-ANOVA.

IV.4. RESULTS

IV.4.1. Absolute Quantification of tamoxifen and 3 metabolites

The absolute plasma levels of TAM and N-demethyl-TAM, 4-OH-TAM and endoxifen was determined in 20 patients with the LC-HR-MS analysis (HR-FS) and compared to those obtained with the validated LC-TQ-MS assay [49].

Passing-Bablok regression analysis performed on measured TAM levels, revealed the presence of a linear proportional bias (supplementary data 1A and 1B). Bland-Altman analysis shows the estimated bias (expressed as % of mean absolute concentrations of TQ and HR-MS) between the mehods and the lower and upper limits of agreements (LOA) for TAM, N-demethyl-TAM, 4-OH-TAM and endoxifen levels were 5.6% (LOA: -12.3% and 25.5%), -4.3% (-25.4% and 10%), -7.7% (-27.9% and 19.2%) and -6% (-27.9% and 19.2%), respectively (supplementary data 1C).

Statistical methods comparison revealed a lack of agreement between the methods used for the LC-HR-MS. Our experiment was not optimally designed for a reliable comparison between the instruments. In fact, different elution gradients have been used for the two LC-MS systems, the extracted samples and calibrators for the LC-HR-MS systems have been freezed and thawed at multiple occasions and analytes quantifications have not been done on freshly extracted calibrators and samples for the HR-MS. All these conditions could putatively explain this acceptable bias between the analyses. Robust method-comparison experiment should be conducted under similar method conditions with the same extracts and using a higher number of samples in replicates.

The sensitivity of the two technologies was also compared. The lowest levels detected were the first calibrants, 0.5, 0.2, 1, and 0.5 ng/mL, with the TQ-MS, and 1, 0.8, 2, and 1 ng/mL with the HR-MS for TAM, 4-OH-TAM, N-demethyl-TAM, and endoxifen, respectively. This indicates that in our analysis,

the Exactive Plus-HR-MS was 2- to 4-times less sensitive than the TSQ-Ultra-TQ-MS. In both analyses, QCs and calibrants accuracies were comparable for the two technologies. Calibration curves recorded by the LC-Exactive Plus-HR-MS analysis and depicted in the Supplementary data 1B, show a behavior similar to the LC-TQ-MS analysis. No co-eluting peaks were observed on the chromatograms using SRM or HR-FS detection.

In conclusion, ours results confirm previous published data [37-42] showing that LC-HR-MS is fully capable of performing robust quantitative determinations in a productive environment (ease of use, robustness, sensitivity and level accuracy).

IV.4.2. Identification of tamoxifen metabolites

Using the software dedicated to drug metabolism and its integrated list of predicted metabolites (m/z) to be extracted (XIC with MEW = ± 5ppm), we rapidly identified most TAM metabolites by comparing XIC from treated patients' and control samples (Figure 1). We focused our comparison on two plasma samples showing the highest TAM levels (344 and 446 ng/mL). Over a day, we identified more than 40 potential TAM metabolites (LC peaks). With MS^{ALL} acquisitions, we confirmed most of our identifications by detecting TAM fragment ions that co-eluted with the metabolite (precursor ion). The tolerance on mass accuracy (MA) of the fragment ions were ± 15ppm (MS^{ALL} acquisition was set at a lower resolution = 17,500). The fragmentation of TAM in the HCD cell at 40eV is presented in the Supplementary data 2A-B.

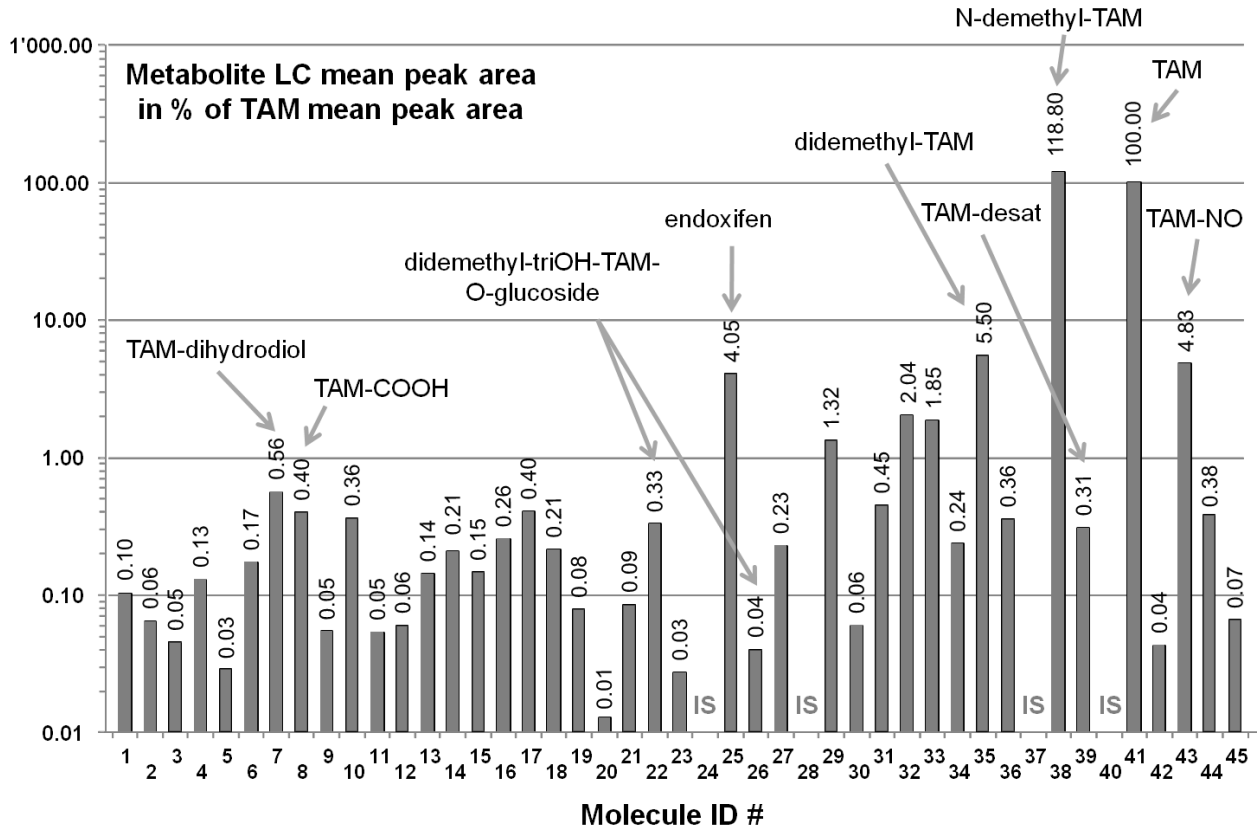


Figure 1: Mean peak area of TAM metabolites relatively to TAM mean area (in %; N = 8 to 20 patients). Molecules are defined by their ID number presented in Table 1. Whereas the mean absolute concentration of TAM was 208 ng/mL, the lowest metabolite (ID#20) represented 1/10,000 of TAM peak area. Log scale representation.

The most intense marker ion of TAM and its metabolites was m/z = 72.08078 corresponding to $[C_4H_{10}N]^+$ and was found in most metabolite MS^{ALL} spectra. Using chromatograms and spectra cleaned by mass defect filtering, a few additional ions were detected as potential metabolites whereas they were not predicted by the *Metworks* software or published lists of biotransformation (see thereafter) [50,51], The last step of metabolite identification, consisting in measuring the correlation between TAM/IS and metabolite/IS LC peak area ratios, is given in Table 1 (see examples in Figure 2 for ID#7, 8, 22, 25, 38 and 39). A metabolite that was not correlated to TAM/IS candidate ratio ($R^2 < 0.1$) was considered as a false positive and discarded. In contrast, a metabolite candidate with a R^2 was > 0.25 and was identified with additional evidence. The best correlation was obtained with TAM-desat (ID#39; $R^2 = 0.968$) whereas the poorest value was observed with (*Z*)-endoxifen (ID#25; $R^2 = 0.305$) (Table 1; Figure 2), a metabolite whose formation is known to be controlled by CYP2D6 activity.

Identification of TAM metabolites were considered as "confirmed" only if the 3 following criteria were fulfilled : i) mass accuracy (MA) between measured and theoretical m/z < ± 3ppm; ii) positive correlation ($R^2 > 0.1$) between TAM metabolite/IS and TAM/IS ratios; and iii) presence of ≥ 2 fragment ions (MS^{ALL} or MS^2 acquisition), with a mass accuracy < ± 15ppm, identical to TAM or bearing the metabolic change and coeluting with the precursor ion and/or iv) a fine isotopic distribution corresponding to the metabolite chemical composition (e.g. ^{34}S versus $^{13}C_2$).

Summarizing the results of the above investigations, Table 1 shows all identified TAM metabolites detected in plasma extracts and present in at least 8 patients (≥ 40%). Similarly, Figure 1 shows the mean of 40 metabolite LC peak area relatively to TAM mean area (in %) on a log scale representation. Additional putative metabolites were detected (e.g. hydroxy-methoxy-TAM, demethyl-TAM-COOH and additional isomers of some metabolites depicted in Table 1) but were below the detection limit in most patients (> 12/20) with our sample preparation and therefore not considered further.

Table 1: List of compounds determined in this study. Compound identification number (ID#), retention time (RT), monoisotopic m/z used to construct extracted ion chromatograms (XIC), chemical composition, abbreviated name, metabolite generation number (F), internal standrad (IS) (id#) used for metabolite/IS ratio (relative quantification), determination coefficient (R2) between metabolite/IS and TAM/IS ratios and metabolite mean peak area (AA) expressed in % of TAM mean peak area, are given.

ID #	RT (min)	m/z	Chemical composition	Identification name (*)	F	IS # used	R^2 (◊)	AA: % TAM	
1	2.62	550.24354	C31H35NO8	demethyl-TAM-O-Gluc_1	3	#24	0.84	0.10	●/◙
2	2.81	564.25919	C32H37NO8	TAM-OH-Gluc_1	2	#24	0.69	0.06	●/◙
3	3.44	578.23846	C32H35NO9	TAM-carboxy-Gluc	4	#24	0.40	0.05	◙
4	3.57	580.25411	C32H37NO9	diOH-TAM-O-Gluc_1	4	#24	0.82	0.13	◙
5	3.64	570.23406	C30H35O10N	didemethyl-tetraOH-TAM-glucoside	7	#24	0.67	0.03	○
6	4.21	388.19072	C25H25NO3	demethyl-TAM-COOH	4	#24	0.57	0.17	◙
7	4.36	406.23767	C26H31NO3	TAM-dihydrodiol	2	#24	0.46	0.56	●
8	4.43	402.20637	C26H27NO3	TAM-COOH	3	#24	0.35	0.40	◙
9	4.44	550.24354	C31H35NO8	demethyl-TAM-OH-Gluc_2	3	#24	0.35	0.05	●/◙
10	4.54	374.21146	C25H27NO2	demethyl-OH-TAM_1	2	#24	0.93	0.36	●/◙
11	4.59	564.25919	C32H37NO8	TAM-OH-Gluc_2	2	#24	0.55	0.05	●/◙
12	4.78	388.22711	C26H29NO2	α-OH-TAM	1	#24	0.58	0.06	●/◙
13	4.92	550.24354	C31H35NO8	demethyl-TAM-OH-Gluc_3	3	#24	0.43	0.14	●/◙
14	4.97	418.20128	C26H27NO4	OH-TAM-COOH	4	#24	0.57	0.21	○
15	5.00	564.25919	C32H37NO8	TAM-OH-Gluc_3	2	#24	0.58	0.15	●/◙
16	5.35	404.22202	C26H29NO3	diOH-TAM_1	2	#24	0.68	0.26	●/◙
17	5.57	404.22202	C26H29NO3	diOH-TAM_2	2	#24	0.72	0.40	●/◙
18	5.80	564.25919	C32H37NO8	TAM-OH-Gluc_4	2	#24	0.42	0.21	●/◙
19	5.82	550.24354	C31H35NO8	demethyl-TAM-OH-Gluc_4	3	#24	0.38	0.08	●/◙
20	5.90	468.18392	C26H29NO5S	TAM-OH-sulfate	2	#24	0.36	0.01	●
21	5.92	580.25411	C32H37NO9	diOH-TAM-O-Gluc_2	4	#24	0.39	0.09	◙
22	6.33	554.23846	C30H35O9N	didemethyl-triOH-TAM-glucoside_1	6	#24	0.57	0.33	○
23	7.05	360.19581	C24H25NO2	didemethyl-OH-TAM	3	#24	0.47	0.03	◙
24	7.25	379.24284	C25H22D5NO2	*Z*-endoxifen_IS			NA		
25	7.27	374.21146	C25H27NO2	*Z*-Endoxifen (OH-demethyl-TAM_2)	2	#24	0.31	4.05	◙
26	7.29	554.23846	C30H35O9N	didemethyl-triOH-TAM-glucoside_2	6	#28	0.74	0.04	○
27	7.46	374.21146	C24H25NO2	OH-demethyl-TAM_3	2	#28	0.49	0.23	●/◙
28	7.50	393.25849	C26H24D5NO2	4-OH-TAM_IS			NA		
29	7.50	388.22711	C26H29NO2	4-OH-TAM	1	#28	0.48	1.32	●/◙
30	7.60	388.22711	C26H29NO2	3-OH-TAM	1	#28	0.51	0.06	●/◙
31	7.64	404.22202	C26H29NO3	diOH-TAM_3	2	#28	0.39	0.45	●/◙
32	8.02	374.21146	C24H25NO2	demethyl-OH-TAM_4	2	#28	0.63	2.04	●/◙
33	8.23	388.22711	C26H29NO2	4'-OH-TAM	1	#28	0.79	1.85	●/◙
34	9.60	372.23219	C26H29NO	*E*-TAM	?	#37	0.84	0.24	○
35	9.69	344.20089	C24H25NO	Didemethyl-TAM	2	#37	0.84	5.50	●/◙
36	9.69	356.20089	C25H25NO	demethyl-TAM-desat	2	#37	0.85	0.36	○
37	9.92	363.24792	C25H22D5NO	N-demethyl-TAM_IS			NA		
38	9.95	358.21654	C25H27NO	N-demethyl-TAM	1	#37	0.90	118.80	●/◙
39	9.95	370.21654	C26H27NO	TAM-desat	1	#40	0.97	0.31	○

ID #	RT (min)	m/z	Chemical composition	Identification name (*)	F	IS # used	R^2 (◊)	AA: % TAM	
40	10.20	377.26357	C26H24D5NO	*Z*-TAM_IS	NA				
41	10.20	372.23219	C26H29NO	***Z*-TAM (parent drug)**	0	#40	1.00	100.0	●/◙
42	10.43	386.21146	C26H27NO2	OH-TAM-desat_1	2	#40	0.52	0.04	○
43	10.70	388.22711	C26H29NO2	TAM-NO	1	#40	0.60	4.83	●/◙
44	11.77	374.21146	C24H25NO2	demethyl-OH- TAM_5	2	#40	0.65	0.38	○
45	12.89	386.21146	C26H27NO2	OH-TAM-desat_2	2	#40	0.44	0.07	○

Abbreviations: desat = desaturated; OH = hydroxylated; NO = N-oxygenated; Gluc = glucuronide.
(○) never reported, (●) reported in animals or incubations only (◙) reported in human plasma.

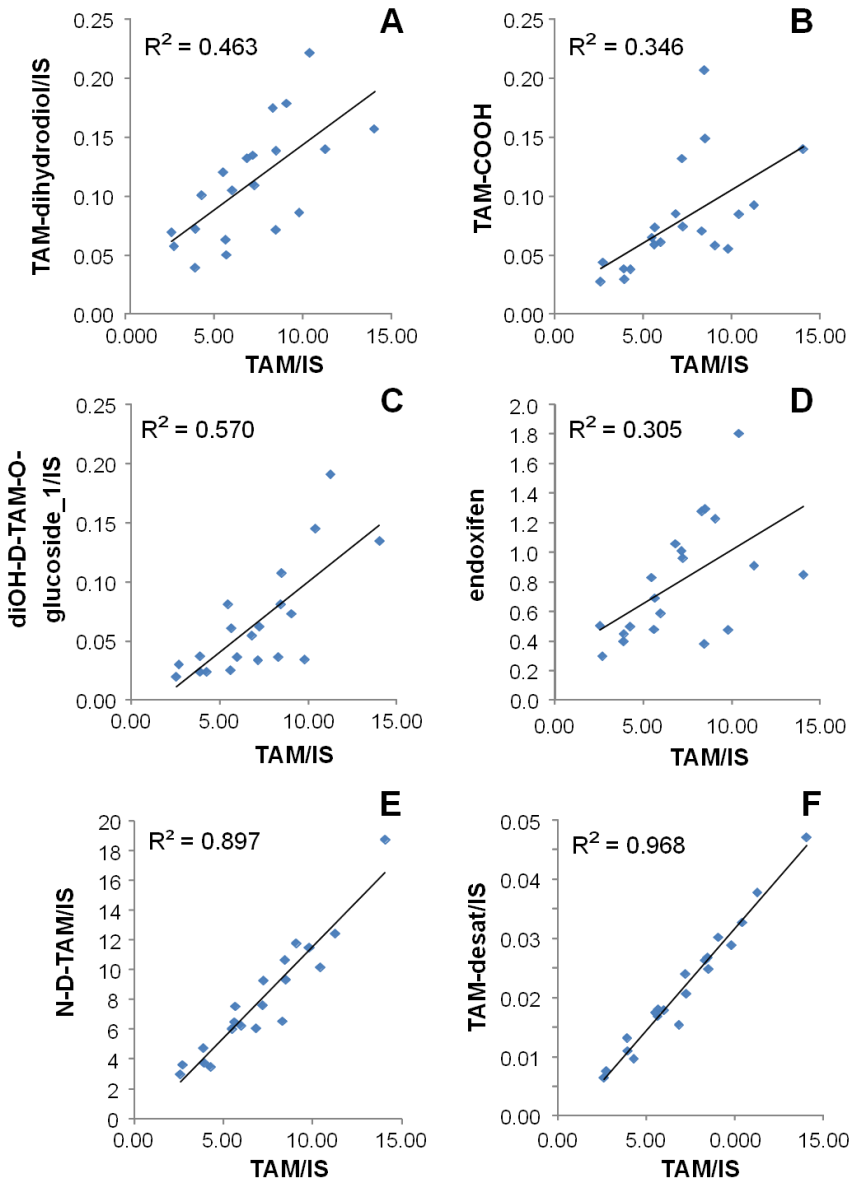


Figure 2: Determination coefficient (R^2) between six metabolites/IS (ID#7, 8, 22, 25, 38 and 39: A to F) and TAM/IS peak area ratios (N ≥ 8). Each diamond represents a metabolite/TAM ratio in a single patient. Correlations of endoxifen or TAM-desat with TAM show the lowest and highest R^2 values of all TAM metabolites. Table 1 depicts the R^2 values for all identified metabolites.

Examples of metabolite identification with the Exactive Plus and Q-Exactive analyses are depicted in the Supplementary data 3A-E. MS^2 spectra with fragment ions and metabolite structure proposals of TAM-COOH (ID#8), TAM-desat (ID#39) and TAM-dihydrodiol (ID#7) as well as the fine isotopic distribution of OH-TAM-sulfate at A+2 ($m/z_{monoisotopic}$ + 2u = m/z 470; ID#20) confirming the presence of a sulfur atom [52], are depicted. In Figure 3A-B, we show the identification of three putative glucose conjugates, namely two didemethyl-trihydroxy-TAM-glucosides (ID#22 and 26) and one didemethyl-tetraOH-TAM-glucoside (ID#5).

These glucosides have never been described and were revealed in MDF cleaned spectra (Supplementary data 4A-B). Key information to identify their chemical composition was the capability to establish the absence of a sulfur atom using the fine isotopic distribution (Figure 3A). This information reduced significantly the chemical composition possibilities. Taking metabolite #22 as a case in point, Figure 3A challenges two possible chemical compositions, with or without a sulfur atom (which would derive from an initial glutathione conjugate): $[C_{37}H_{34}ON_2S]^+$ and $[C_{30}H_{36}O_9N]^+$. The theoretical isotopic distribution of these two compositions is depicted for A+2 ($m/z_{monoisotopic}$ + 2u). A resolution > 75,000 is sufficient to dissociate the two chemical compositions (m_1 and m_2) with a partial overlapping (resolution = 1.5x $[m_1/|(m_1-m_2)|]$; see [41]). Figure 3B shows the fragmentation pathways of one of the possible regioisomeric structures of metabolite #22. Its fragmentation is very different from that of TAM (Supplementary data 2A) due to the proton location: on the tertiary amine for TAM and on the glucosyl moiety for the glucoside. While neither their positions of hydroxylation nor the point of attachment of the glucose moiety (on the $-NH_2$ or an -OH group) could be ascertained with the methodologies used, one can nevertheless note that metabolites #22 and #26 correspond to 6 biotransformation steps, whereas the more polar metabolite #5 (Supplementary data 3B) corresponds to 7 steps.

To the best of our knowledge, many of the TAM metabolites reported herein have never been detected before in patients' plasma even if some were described in *in vitro* or *in vivo* animal studies or different biomatrices, e.g. TAM-dihydrodiol and OH-TAM-sulfate (see ID#7 and in ID#20 in Table 1).

From our previous established retention time with pure standards [49] and from literature [30,35,53,54], we have fully identified 8 metabolites whereas in most cases absolute configuration of identified TAM metabolites could not be established (Table 1).

Other metabolites were never reported, namely: OH-TAM-COOH (ID#14), N-demethyl-TAM-desat (ID#36), TAM-desat (ID#39), OH-TAM-desat (ID#42, 45) and the three glucosides (ID#5, 22 and 26).

The characterization of these new metabolites in patients' plasma owes much to the high sensitivity and selectivity of HR-FS and the ease to trace potential metabolites with XIC constructions.

The four desaturated metabolites were identified from their loss of m/z = 2, most likely at the end of the -CH_2CH_3 side-chain, a CYP-catalyzed reaction energetically favorable when the double bond so created is conjugated with an aromatic system, as is the case here [54]. As for glucosyl conjugates, they are seldom reported compared to glucuronides, but their existence has been known since decades [55,56]. Their formation involves the cofactor uridine-diphosphate-glucose (UDP-glucose) and is catalyzed by UDP-glucuronosyltransferases (UGTs) [57,58]. Glucosylation has been shown to occur at alcoholic, phenolic and carboxylic -OH groups to yield O-glucosides, [55,56,58-63] and amido and amino groups to yield N-glucosides [55,56,64-66]. In our case, glucosylation could have occurred at one of the hydroxy groups or at the primary amino group. It is also remarkable that the glucosides were produced by 6, respectively 7 metabolic steps while remaining well above detection limit.

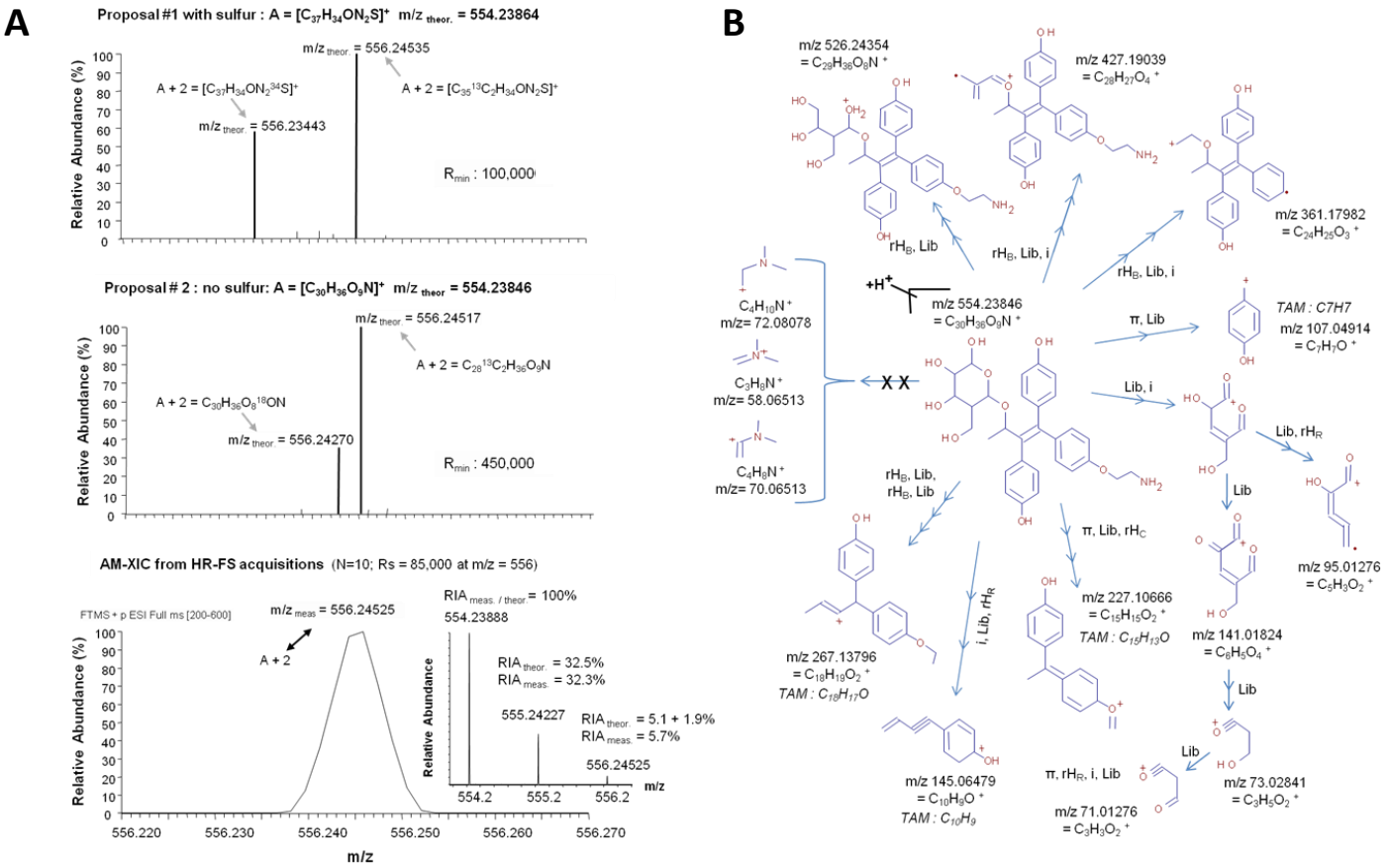


Figure 3: Identification of an unknown metabolite (m/z 554.23888). **A** Two possible compositions, $[C_{37}H_{34}ON_2S]^+$ and $[C_{30}H_{36}O_9N]^+$, with MA = + 0.4ppm and + 0.8 ppm, respectively. MA = $([m/z_{measured} - m/z_{theoretical}] / m/z_{theoretical}) \times 10^6$. The presence or not of a sulfur atom in the chemical composition was elucidated with the fine isotopic distribution of (A + 2) corresponding to the theoretical distributions depicted as Proposals 1 and 2. R_{min} = minimum resolution to resolve $[C_{37}H_{34}ON_2{}^{34}S]^+$ and $[C_{35}{}^{13}C_2H_{34}ON_2S]^+$ or $[C_{28}{}^{13}C_2H_{36}O_9N]^+$ and $[C_{30}H_{36}O_8{}^{18}ON]^+$, respectively. Bottom panel: HR-MS resolution were setup at 140,000 (at m/z=200) that corresponds to 85,000 at m/z 556 (measured resolution). This is sufficient to conclude to the absence of a sulfur atom in the chemical composition of this unknown metabolite (no m/z at 556.23443 detected). The insert (bottom right) shows the measured $m/z_{monoisotopic}$ (A), A+1 and A+2 of this unknown metabolite in the full scan spectra. The measured and theoretical relative isotopic abundance (RIA) values are given. **B** One of the possible regioisomers (sites of hydroxylation and site of glucose attachement) corresponding to the composition $C_{30}H_{36}O_9N$ and to the proposed fragmentation pathway from HR-MS^2 product scan. Due to the absence of the tertiary amino group (TAM), the H^+ is located on the glucoside and drives the fragmentation. Abbreviations: i = inductive cleavage, Lib = fragmentation predicted using a library reaction; π = ionization on pi bonds; rH_B = charge site rearrangement (α,β); rH_C = charge site rearrangement (γ); rH_R = charge remote rearrangement. Additional information in the Supplementary data 4A-C).

IV.4.3. Semi- and relative quantification of tamoxifen metabolites.

The absolute quantification of 40 metabolites by LC-MS analysis appears to be unrealistic and to a large extend useless. However, a relative quantification of TAM metabolites (in arbitrary units), if precise (< ± 30%), can be used to establish various associations between metabolite levels and side effects or treatment efficacy. Such associations are key when one or a few metabolites are suspected to contribute to clinical events. This is the main goal of these metabolite relative quantifications.

In LC-MS analysis, the detection intensity of a compound is depending on its ionization yield and on the matrix effect at its retention time, both depending on sample cleanup and LC-MS. Using constant extraction procedure and LC-MS parameters, the ionization yield can be considered as similar between samples especially when weighted by internal standards.

Matrix effect was evaluated in our method with post-column infusion of TAM, 4-OH-TAM, N-demethyl-TAM, and endoxifen [49]. Water and different plasma extracts showed a low difference and low variability of matrix effects for TAM, 4-OH-TAM and endoxifen with ≤ ± 20% during the entire chromatogram (1 to 13 min). When internal standards (IS) were permuted in our relative quantification (metabolite/IS ratios), no significant impact on relative quantification was observed (40 linear regressions gave determination coefficients (R^2) ≥ 0.977; mean = 0.990 ± 0.005; see typical correlations in Supplementary data 5A). The precision of the relative quantification (determined area ratios versus predicted ratios from the correlations; N= 380), remains between 85 – 117%. Previous results [67] obtained with similar sample preparation and LC conditions showed low impact of matrix effects on relative quantification. Taking into account the precision (< ± 20%) of our relative determination of TAM metabolites, data were processed further.

Relations between relative levels of TAM metabolites and TAM (TAM metabolite/IS against TAM/IS peak area ratios) for each metabolite gave various information. Indeed, R^2 values decreased with the number of biotransformation steps (= metabolite generation, F, see Figure 4A) indicating increased inter-patient variability in metabolite production with the implication of additional metabolizing enzymes. The decrease of R^2 values was less pronounced when considering F1-F4 (linear equation; slope = – 0.064) rather than F1-F2 or F1- F3 (slope = -0.124 and -0.121, respectively). This decrease was almost abolished when all metabolite generations, F1 to F7, were taken into account (linear equation; slope = – 0.015; Figure 4A). This observation could be explained by the fact that F4-F7 metabolites were produced by the same enzymes than the F1, F2 and F3 metabolites, and/or that F4-6 metabolites were produced from previous metabolite generations in a sort of production line of various metabolizing enzymes associated on the endoplasmic reticulum membrane. When the relative concentrations of TAM metabolites, expressed in % of TAM levels ([TAM metabolite/IS mean

peak area ratios] / [TAM/IS mean peak area ratios]), were plotted against metabolite generation, they decreased from F1 to F3 but remained constant from F3 to F4 (Figure 4B). This is consistent with the previously proposed explanation.

In a global perspective, Figure 4A-B underscores the importance to study *in vivo* drug metabolism and therapeutic monitoring beyond the usual first- or second-generation metabolites, since the formation to toxic/reactive metabolites is by far not restricted to the first metabolic steps [33,68].

Without pure standards, the semi-quantitative estimation of drug metabolites can be realized by comparing UV and MS signals [14,15,16,17]. This can be difficult when small amounts of metabolites prevent good UV detection, or when biotransformation strongly modifies UV absorbance. In this study, we have estimated the absolute level of TAM metabolites based on peak areas of TAM and TAM metabolites and TAM absolute mean level of this study, 240 ng/mL (see *Materials and methods* for the equation). In the Supplementary data 5B, eight metabolite levels were given in % of TAM absolute levels (this work and [53]) or in % of TAM peak area. The maximum difference between these two calculations was observed with didemethyl-TAM (ID#35). In this case, didemethyl-TAM mean level was 5-fold underestimated when considering the TAM metabolite/TAM peak area ratios rather than the absolute levels. This is due to a lower attraction of H^+ for the primary amino group (didemethyl-TAM) compared to the tertiary amino group (TAM) and a lower ionization yield. The absolute levels of the 7 other metabolites were only slightly underestimated by their peak area: mean = -40% (from -80 to -10%) (Supplementary data 5B). Since most new metabolites detected in our samples show peak area between 0.1 and 1% of TAM peak area (Figure 1 and Table 1), we can estimate that these metabolites are probably in the range of 0.2 to 2 ng/mL of plasma (corresponding to 0.1 - 1% of TAM absolute mean level in this study). According to the blood concentrations of known toxic compounds listed by the International Association of Forensic Toxicologists (http://www.tiaft.org/) [69,70], the TAM metabolite levels estimated in this work are comparable to those of many compounds that show toxicity at very low concentrations (clenbuterol, digoxin, fentanyl, etc …). Such a comparison underscores that drug metabolites, even at low levels, can have an impact on human body homeostasis and that, when needed, clinical investigations should associate plasma metabolite concentrations with drug toxicity or efficacy (esp. pharmacovigilance).

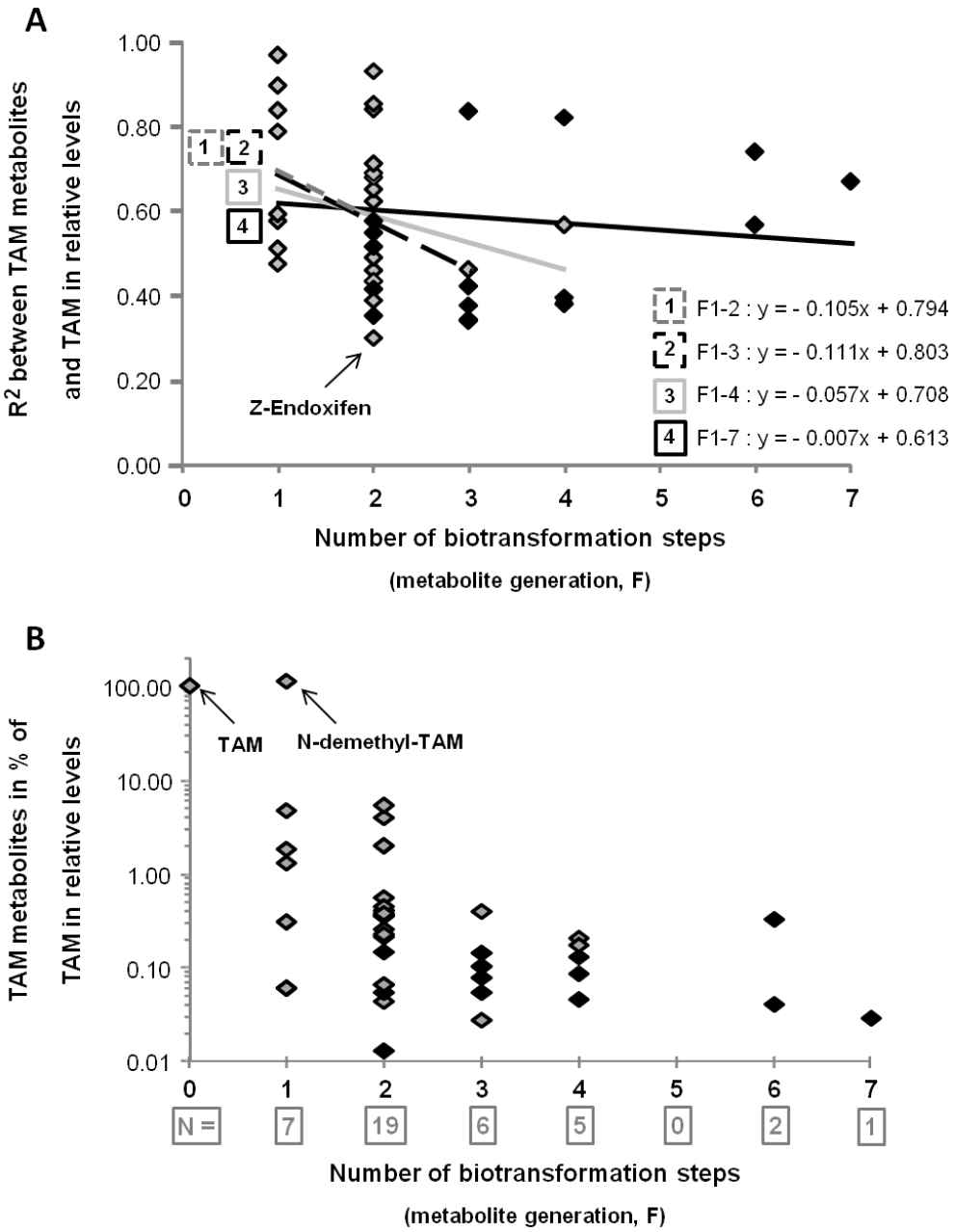


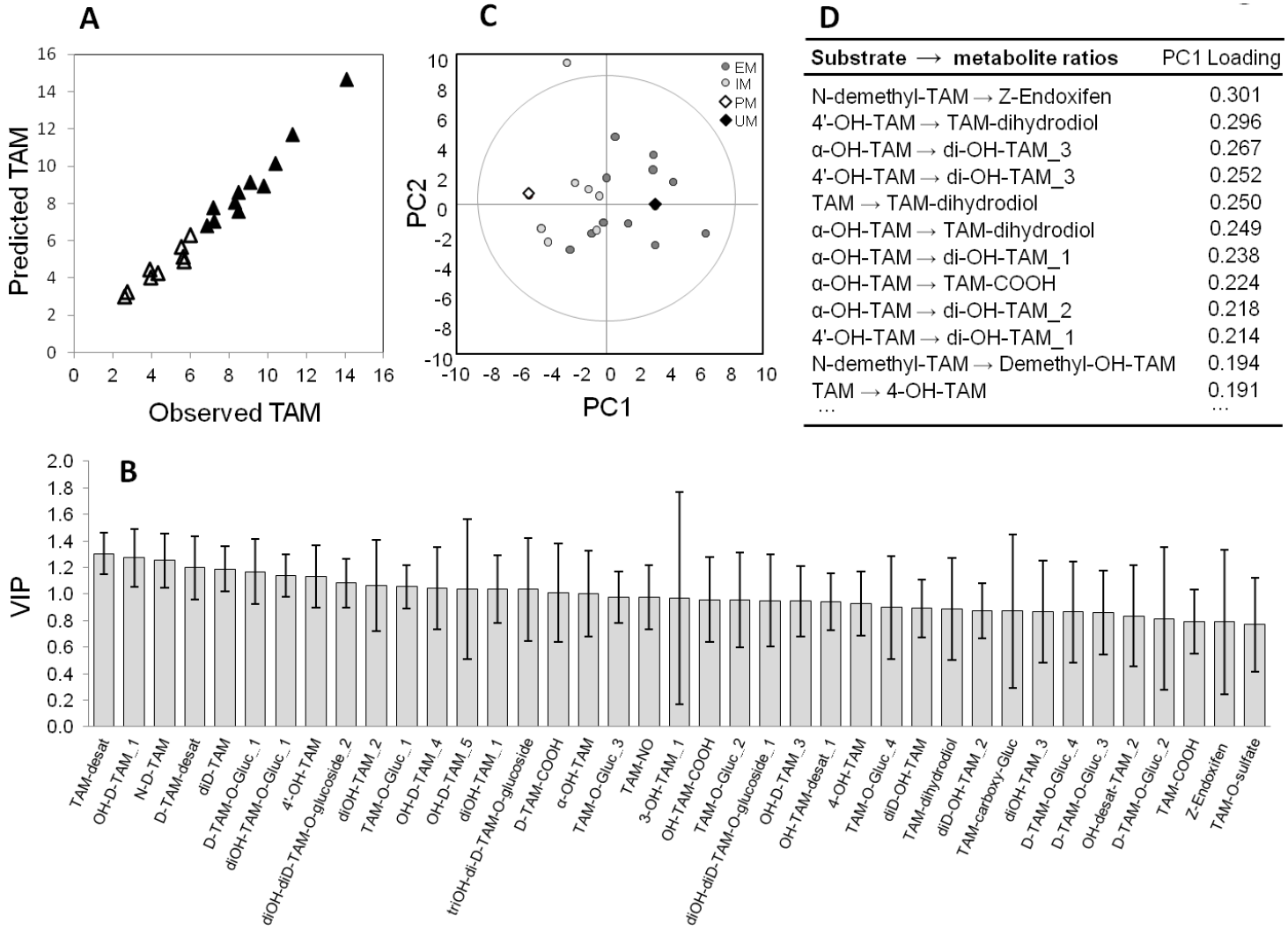
Figure 4: A. Determination coefficient (R^2) between relative levels of TAM metabolites and TAM (TAM metabolite/IS versus TAM/IS peak area ratios; see Figure 2) against metabolite generation (F). Each diamond represents the R^2 value for a given metabolite. Trend line equations taking into account different F are depicted. **B.** TAM metabolite relative levels for each metabolite ([TAM metabolite/IS peak area mean in percent of and TAM/IS peak area mean]) *versus* metabolite generation, F. Log scale representation. The number of TAM metabolites per generation is shown in the boxes beneath the plot. Open (◊) and filled (♦) diamonds stand for phase I and II metabolites, respectively. (*E*)-Tam is not depicted because it could be produced by chemical or enzymatic isomerization (F0 or F1, respectively).

IV.4.4. Statistics and Bioinformatics data treatment.

Multivariate analysis was performed to provide an overall picture of the drug metabolism events occurring after TAM administration, by accounting for all the information provided by the 40 measured metabolites. In order to highlight characteristic metabolic patterns related to TAM plasma values, the TAM relative concentration was used as a response vector to be predicted as a linear combination of its metabolites using a PLS regression model. A PLS model with 3 latent variables fitted the dataset well, as shown by the high R^2 value (R^2 = 0.97), and was selected as the optimal model size according to prediction ability evaluated by leave-on-out cross-validation (Q^2 = 0.92, CV-ANOVA $p<0.01$). The distribution of the samples on the PLS score plot revealed a clear separation of the patients on the first latent variable according to the TAM dose administered, *i.e.* patients with a single (20 mg) or double daily dose (40 mg) (data not shown). This underlines the strong effect of the dose on the measured metabolites relative concentrations (see also the correlation coefficients in Table 1). This influence was confirmed by the loadings examination, as all metabolite concentrations were increased for patients receiving a double dose compared to the single dose group (data not shown). The observed vs. predicted plot (Figure 5A) highlighted a clear trend caused by the TAM dose but additional inter-individual variabilities resulted in a well-balanced continuous distribution of TAM plasma values. The Variables Importance in Projection (VIP) scores were then investigated to highlight characteristic metabolites related to the TAM level response. A VIP score greater than 1 indicates a potentially important variable in the model considered. As shown in Figure 5B, the highest VIP scores were obtained for TAM_desaturated (1.31), OH-Demethyl-TAM_1 (1.27) and N-demethyl-TAM (1.25). Therefore, the most characteristic metabolites of the TAM plasma concentrations could be highlighted (see also R^2 values in Table 1).

To circumvent the major source of variability related to the dose when investigating the whole set of measured metabolites, a subset of 32 metabolites ratios was selected based on possible sequential biotransformation. This biologically-driven selection aimed at uncovering metabolic patterns associated with CYP2D6 enzymatic activity. Thus, a PCA model was then computed to assess patients' groupings with respect to their *CYP2D6* genotypes. A trend separating poor, intermediate, extensive and ultra metabolizers is depicted on the first principal component, as presented on Figure 5C. Even if principal components correspond to directions maximizing the variance without any objective of class separation, the selection of biologically relevant metabolic ratios allowed the effects of the *CYP2D6* genotypic diversity to be highlighted as the major source of variability in the reduced dataset (30.3% of the initial variance). Metabolites ratios were then associated with either poor/intermediate or extensive/ultra genotypes according to their position on the loading plot (data not shown). Interestingly, the 12 most distinctive ratio characterizing extensive and ultra metabolizers were

related to 4 compounds, namely TAM, N-demethyl-TAM, α-OH-TAM and 4'-OH-TAM (Figure 5D). While the two first are known CYP2D6 substrates, these results suggest that α-OH-TAM and 4'-OH-TAM may also be biotransformed *in vivo* by CYP2D6. Therefore, the extended profiling of TAM metabolites proposed in this study constitutes a promising way to gain knowledge about TAM metabolism. Moreover, this example with TAM shows that extended drug-metabolite profiling could be applied to most drug deserving similar investigations (e.g. when a metabolite-related ADR is suspected).



D.

Substrate ⟶ metabolite ratios	PC1 Loading
N-demethyl-TAM → Z-Endoxifen	0.301
4'-OH-TAM → TAM-dihydrodiol	0.296
α-OH-TAM → di-OH-TAM_3	0.267
4'-OH-TAM → di-OH-TAM_3	0.252
TAM → TAM-dihydrodiol	0.250
α-OH-TAM → TAM-dihydrodiol	0.249
α-OH-TAM → di-OH-TAM_1	0.238
α-OH-TAM → TAM-COOH	0.224
α-OH-TAM → di-OH-TAM_2	0.218
4'-OH-TAM → di-OH-TAM_1	0.214
N-demethyl-TAM → Demethyl-OH-TAM	0.194
TAM → 4-OH-TAM	0.191
…	…

Figure 5: PLS regression model relating plasma levels TAM to the 40 measured metabolites. **A.** Observed vs. predicted plot. **B.** VIP plot. Patients with a single dose (20 mg) are symbolized by white triangles (Δ) and patients with a double dose (40 mg) by black triangles (▲). PCA Model based on 32 selected metabolic ratios (PC1 30.3% vs. PC2 19.8%). **C.** Score plot. Symbols are related to *CYP2D6* genotypes: poor, intermediate, extensive and ultra-rapid metabolizers (PM, IM, EM and UM, respectively). **D.** List and values of the most contributing variables to PC1.

IV.5. DISCUSSION AND PERSPECTIVES

The advantage of HR-MS allowed us to study in greater depth and with better convenience tamoxifen metabolites in patients in parallel with the quantification of TAM and its metabolites. Such a Quan/Qual analysis could be of potential utility in *in vivo* drug metabolism and pharmacovigilance studies in order to reach broader insights in inter-patient drug biotransformation profile and relate metabolite concentrations with toxicity.

Some metabolites were detected for the first time in patients' plasma whereas others had never been described before. Thus, putative didemethyl-trihydroxy- and didemethyl-tetrahydroxy-TAM-glucoside conjugates were identified, implying 6 and 7 biotransformation steps, respectively. It is noteworthy to mention that glucosides are seldom reported in the literature compared to glucuronides. This finding underscores the potential need to determine metabolites formed by more than the usual one, two or three biotransformation steps or in specific sub-population [70].

Metabolites are known to play an important role in drug toxicity, particularly chemically reactive ones, but the assessment of their impact in drug-related side-effects has been somewhat neglected due to the considerable time and effort needed with previous MS technologies. Using the latest HR-MS techniques, this situation should evolve and allow an easier access to the production of late-generation metabolites with a view to reaching a more comprehensive understanding of the relation between metabolites and adverse effects.

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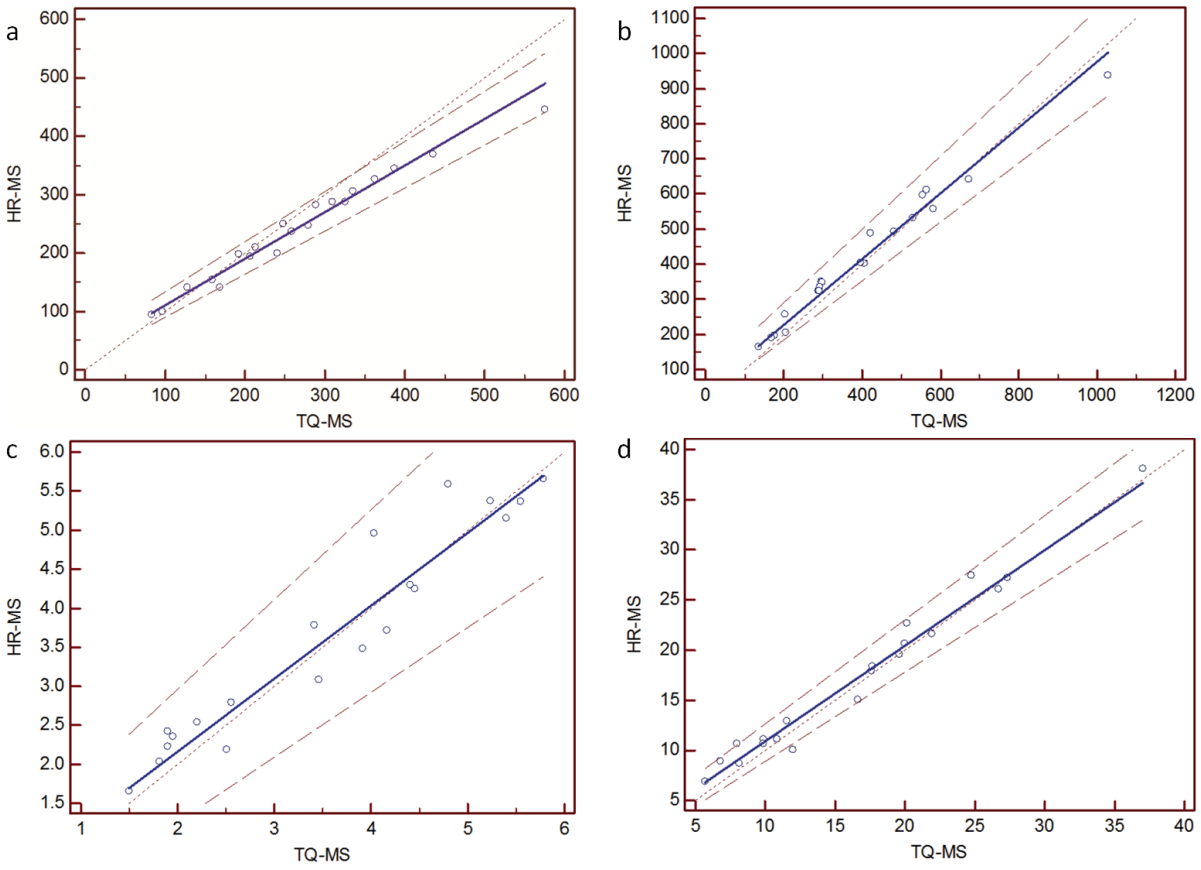
IV.7. SUPPLEMENTARY DATA

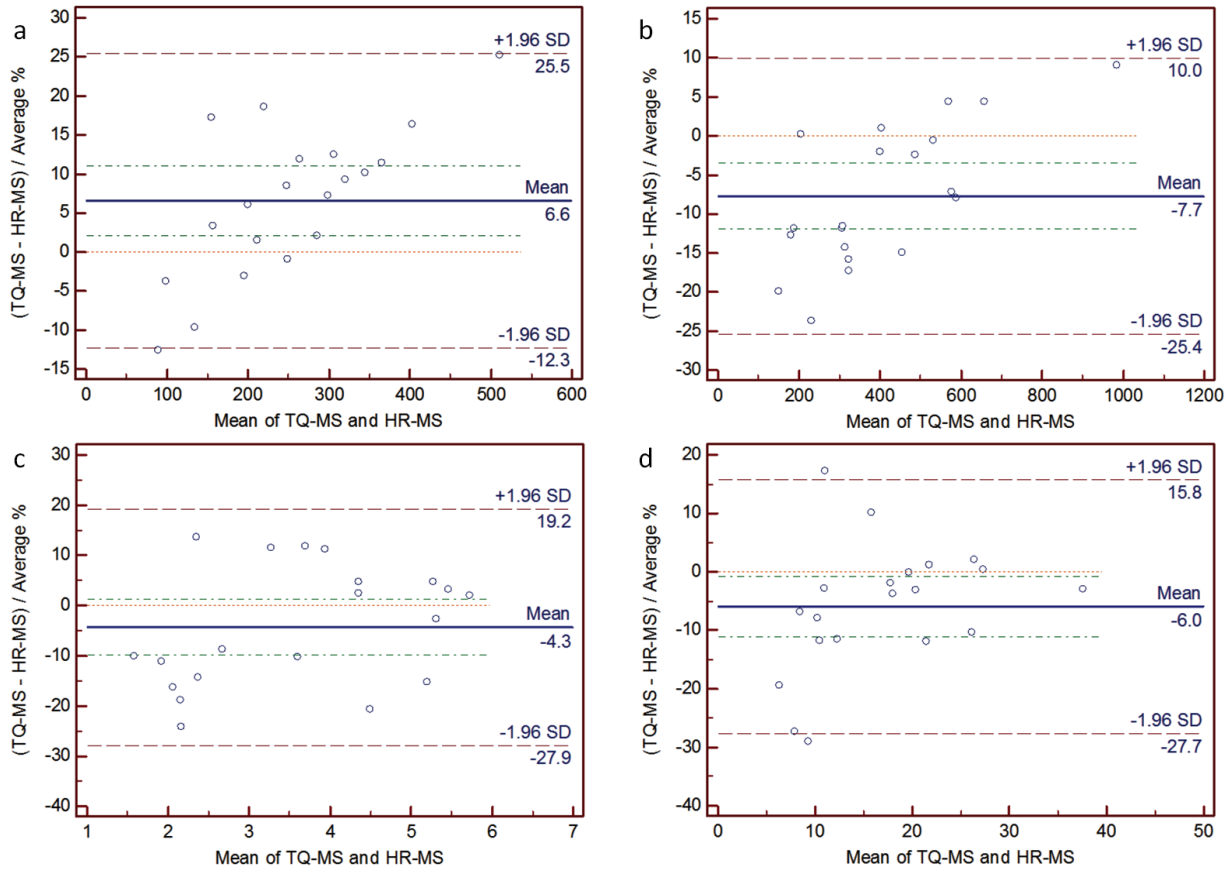
IV.7.1. Supplementary Data 1: Absolute Quantification

A. TQ-MS and HR-MS methods comparison, using Passing-Bablok regression approach.

Compound	Number of samples	Slope (95% CI)	Intercept (95% CI)
TAM	20	0.8 (0.7 – 0.9)	29.8 (16.7 – 47)
N-desmethyl-TAM	20	0.9 (0.8 – 1.1)	40.5 (16.4 – 82.1)
4-OH-TAM	20	0.9 (0.8 – 1.2)	0.3 (-0.4 – 0.7)
Endoxifen	20	0.95 (0.9 – 1.1)	1.4 (-0.04 – 2.3)

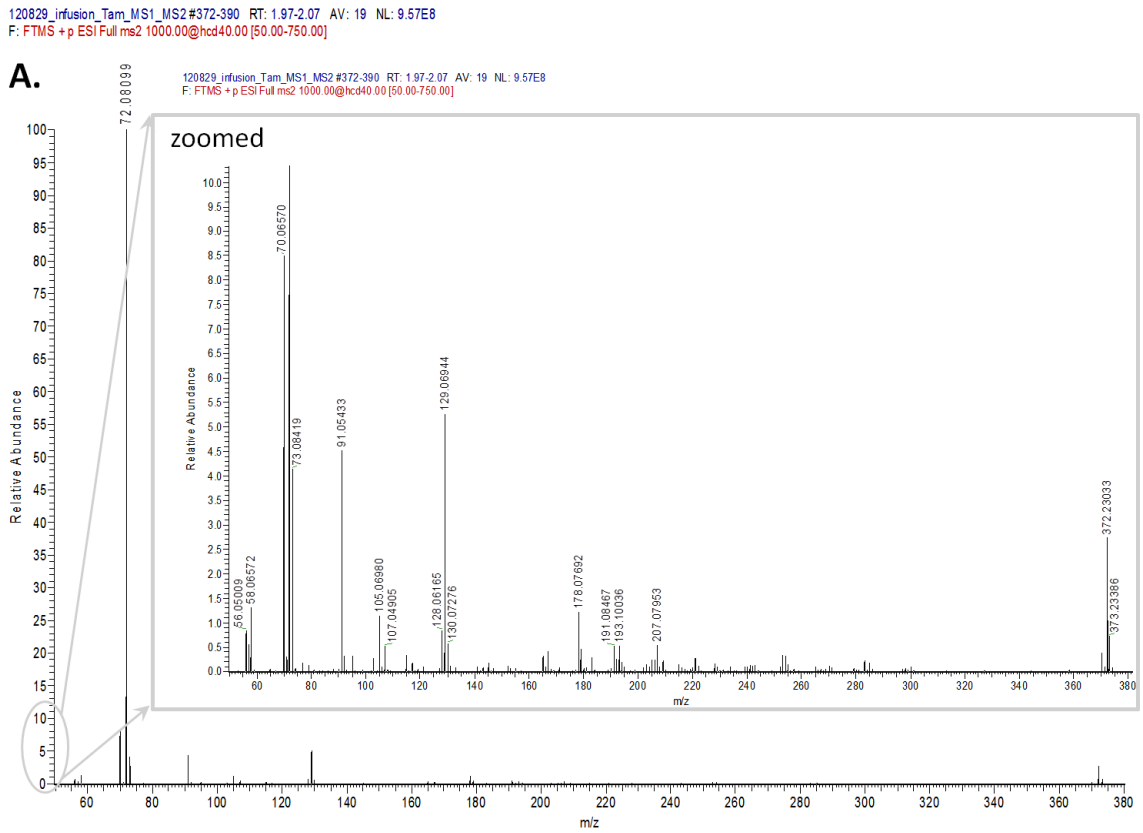


B. Passing-Bablok regression plots for : TAM (a), N-demethyl-TAM (b), 4-OH-TAM (c) and endoxifen (d). (– – – –) are the 95% CI for the regression line and (.....) is the identity line (slope=1).

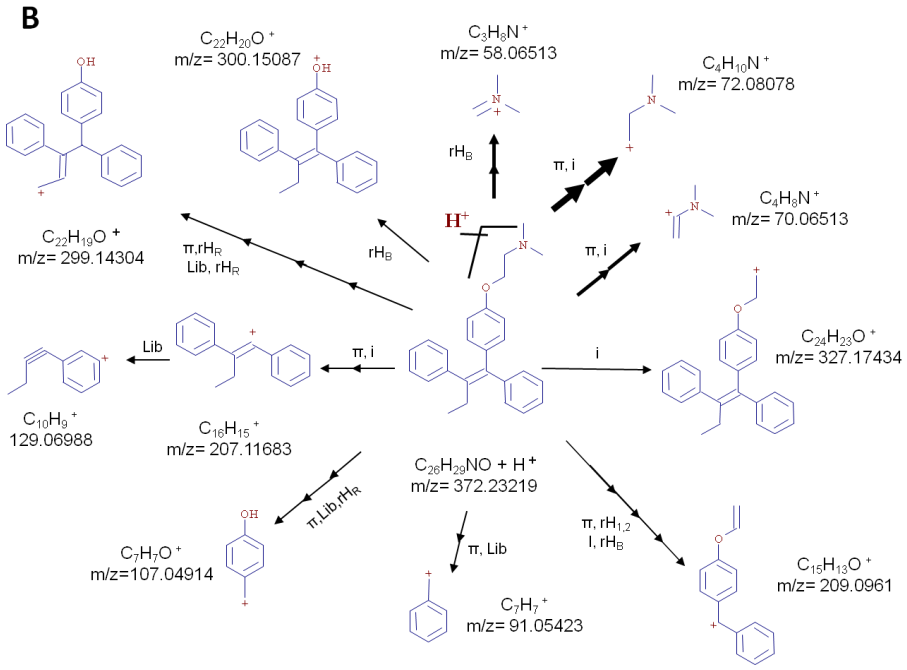


C. Bland-Altman plots (difference plots as %) for : TAM (a), N-demethyl-TAM (b), 4-OH-TAM (c) and endoxifen (d). (_ . _ . _) corresponds to 95% CI of the % mean difference (% mean difference ± 1.96 SE); (_ _ _ _) corresponds to the Limits of agreement (% mean difference ± 1.96 SD); (.....) mean bias equal 0.

IV.7.2. Supplementary Data 2. Tamoxifen HCD fragmentation

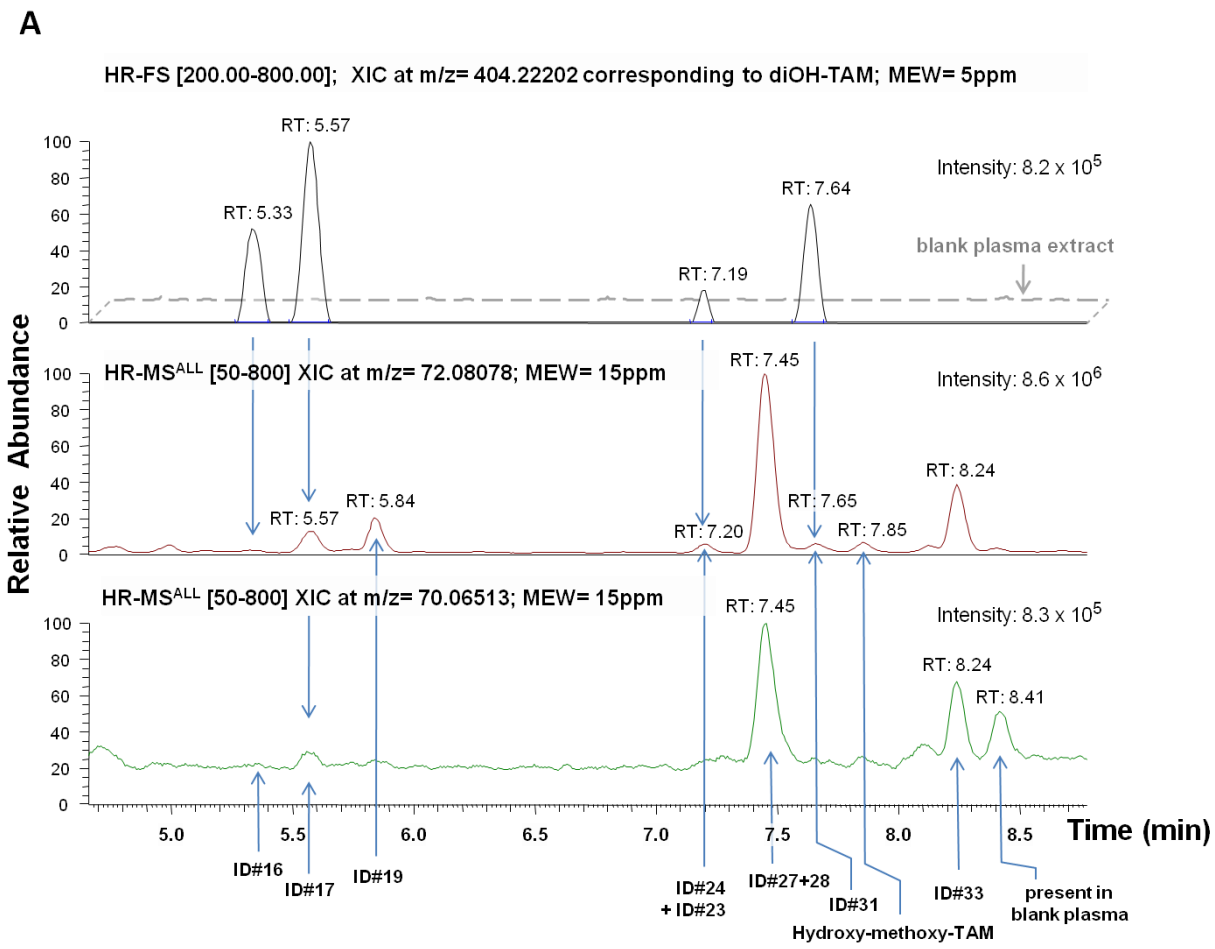


A. Fragmentation of TAM by high-energy collisional dissociation (HCD cell) at 40eV on the Exactive Plus HR-MS.

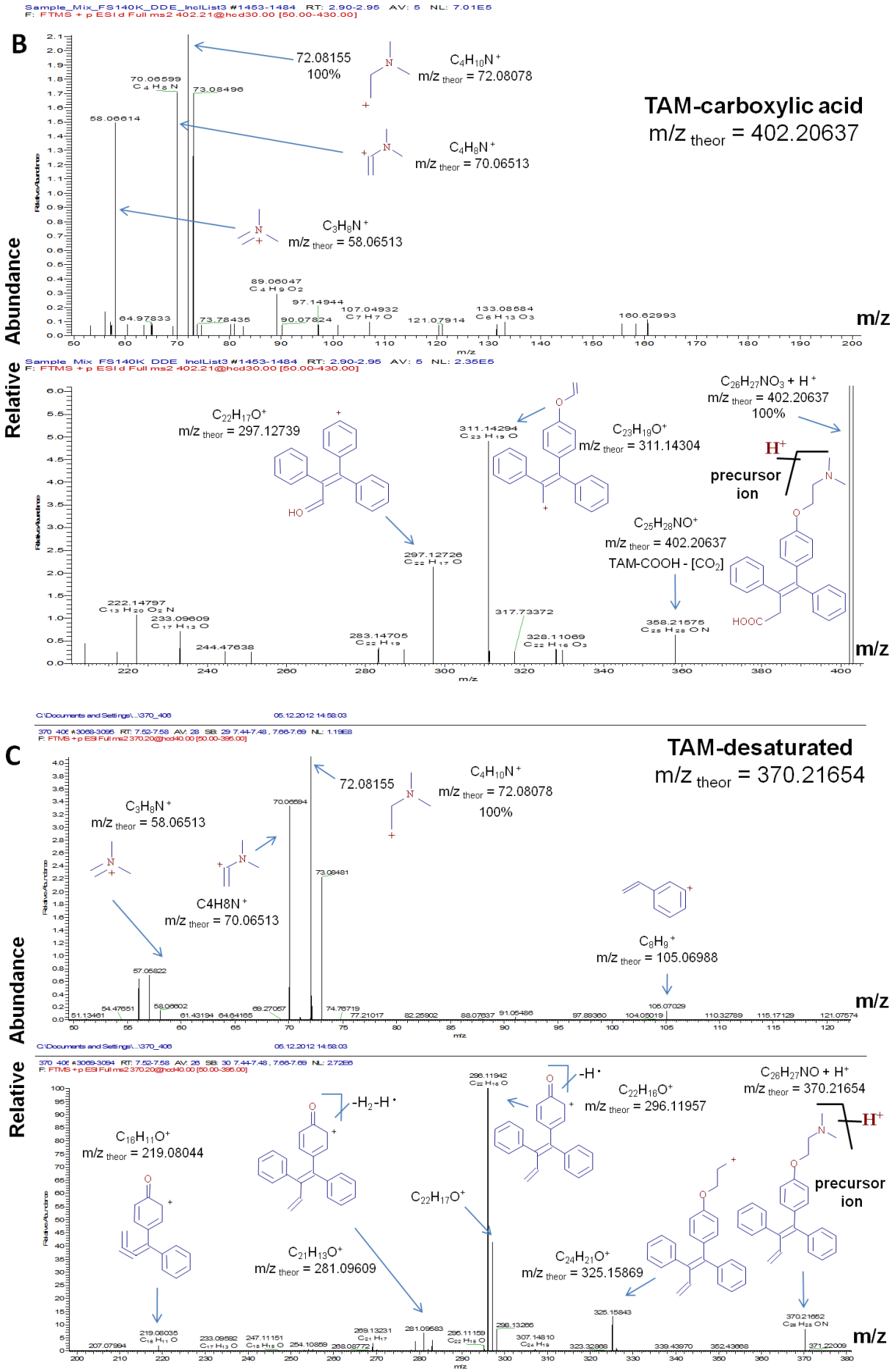


B. Proposed fragmentation of TAM with ion composition, structure and theoretical m/z. Abbreviations: i: inductive cleavage, Lib: fragmentation predicted using a library reaction; π: ionization on Pi bonds; rH1,2: hydrogen shift to an adjacent position; rHB: charge site rearrangement (α,β); rHC: charge site rearrangement (γ); rHR: charge remote rearrangement.

IV.7.3. Supplementary Data 3: Identification of some metabolites



A. Extracted ion chromatograms (XIC) in LC-Exactive Plus-HR-MS analysis. Top panel: From HR-FS acquisition (resolution = 70,000), XIC is constructed around the theoretical m/z at 404.22202 (MEW = 5ppm) corresponding to diOH-TAM. Middle and bottom panels: From HR-MS^{ALL} acquisition (resolution = 17,500), XIC is constructed around the theoretical m/z at 72.08078 and 70.06513 (MEW = 15ppm) corresponding to 2 intense fragment ions of TAM (see **Supplementary Data #2**). Fragment ions confirm the identification of TAM metabolites (ID#17 and 31) or suggest other metabolites (ID#19 and 33). In some cases, the coelution of 2 metabolites avoid the identification only based on MS^{ALL} spectra (ID#23-24) whereas some fragmentation does not produce the 2 marker ions (ID#16) suggesting that other strategies have to be used.


B
TAM-carboxylic acid
m/z theor = 402.20637
72.08155
100%
C4H10N+
m/z theor = 72.08078
C4H8N+
m/z theor = 70.06513
C3H8N+
m/z theor = 58.06513
Abundance
m/z
Relative
C22H17O+
m/z theor = 297.12739
C23H19O+
m/z theor = 311.14304
C26H27NO3 + H+
m/z theor = 402.20637
100%
precursor ion
C25H28NO+
m/z theor = 402.20637
TAM-COOH - [CO2]
C
TAM-desaturated
m/z theor = 370.21654
C8H9+
m/z theor = 105.06988
C16H11O+
m/z theor = 219.08044
C22H16O+
m/z theor = 296.11957
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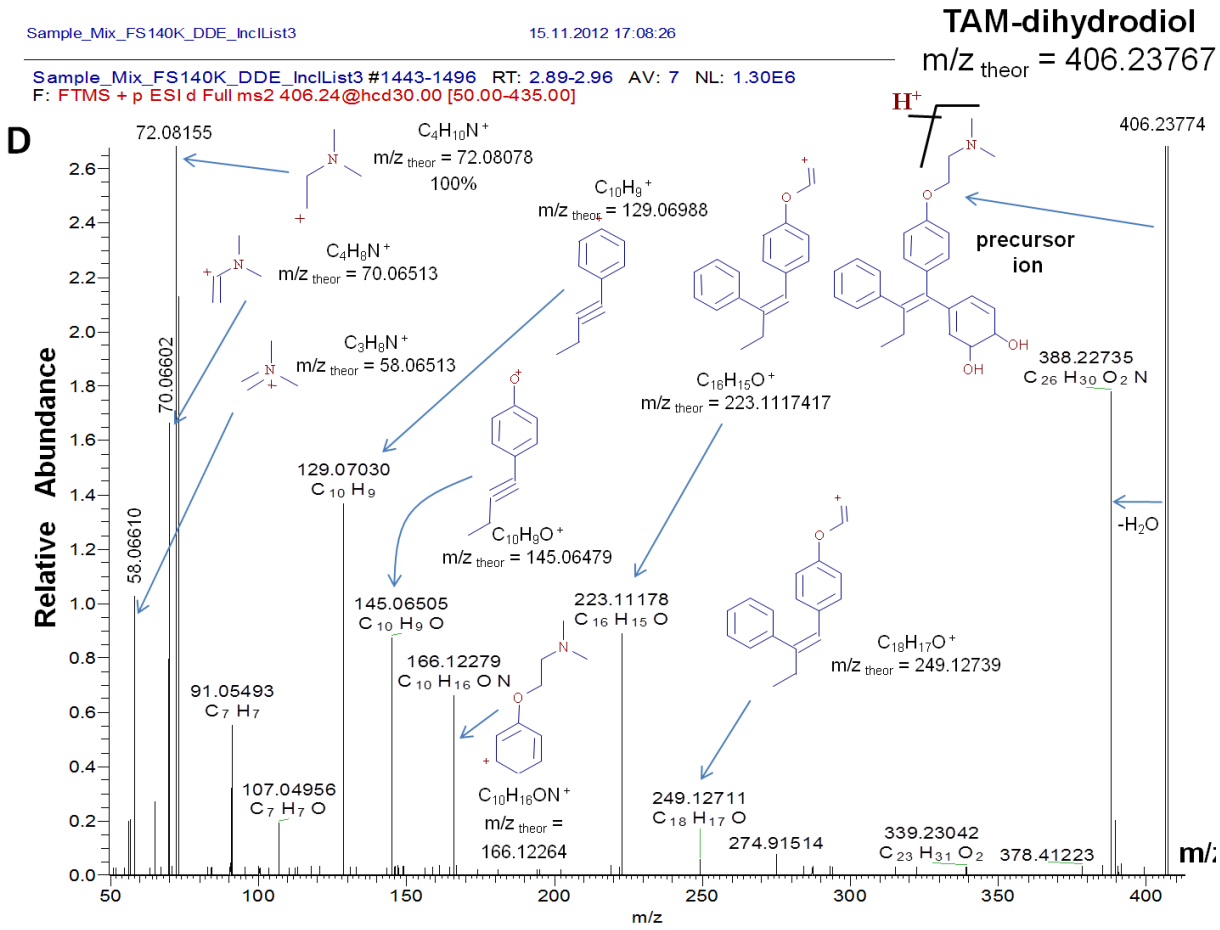
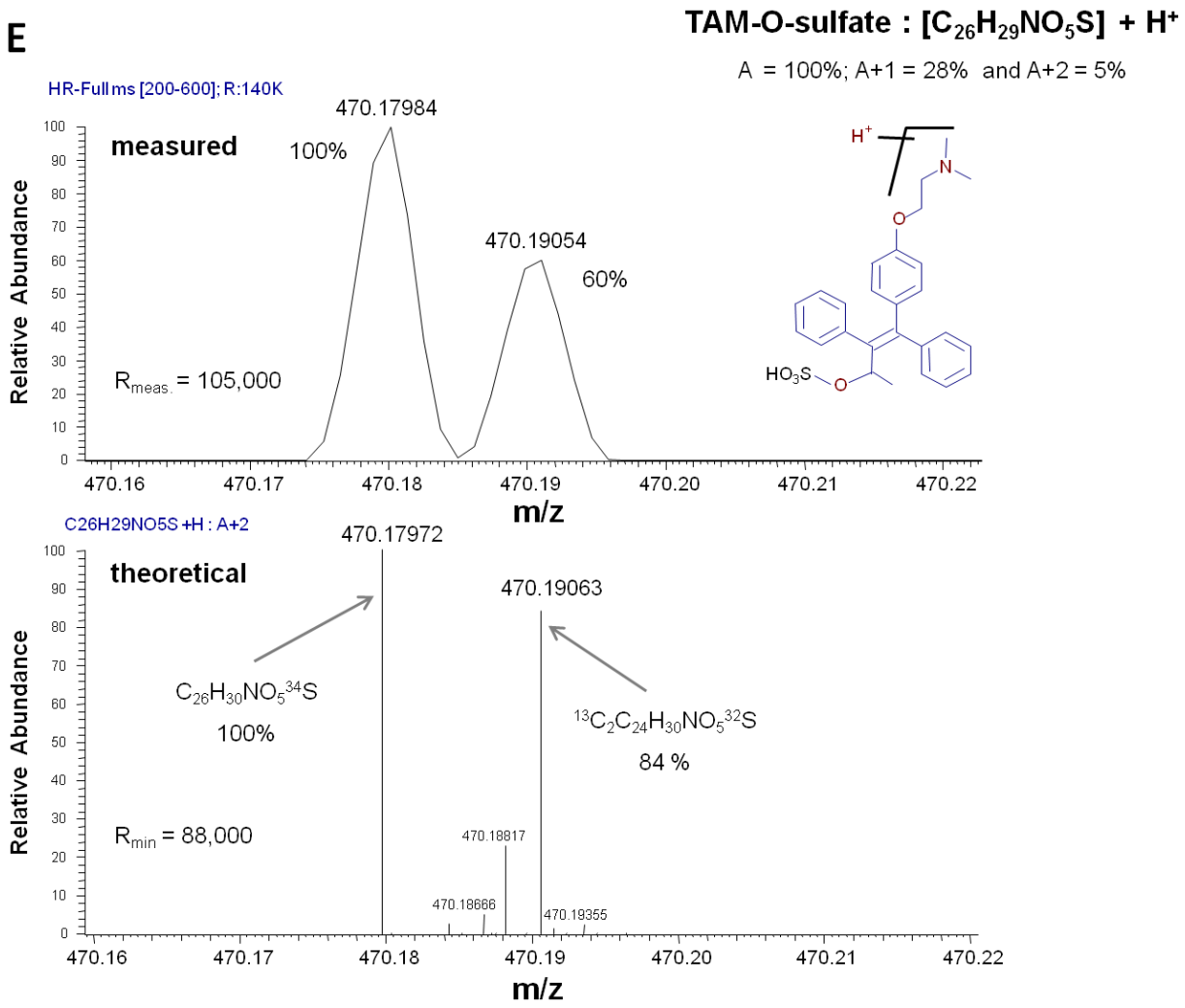
m/z theor = 370.21654

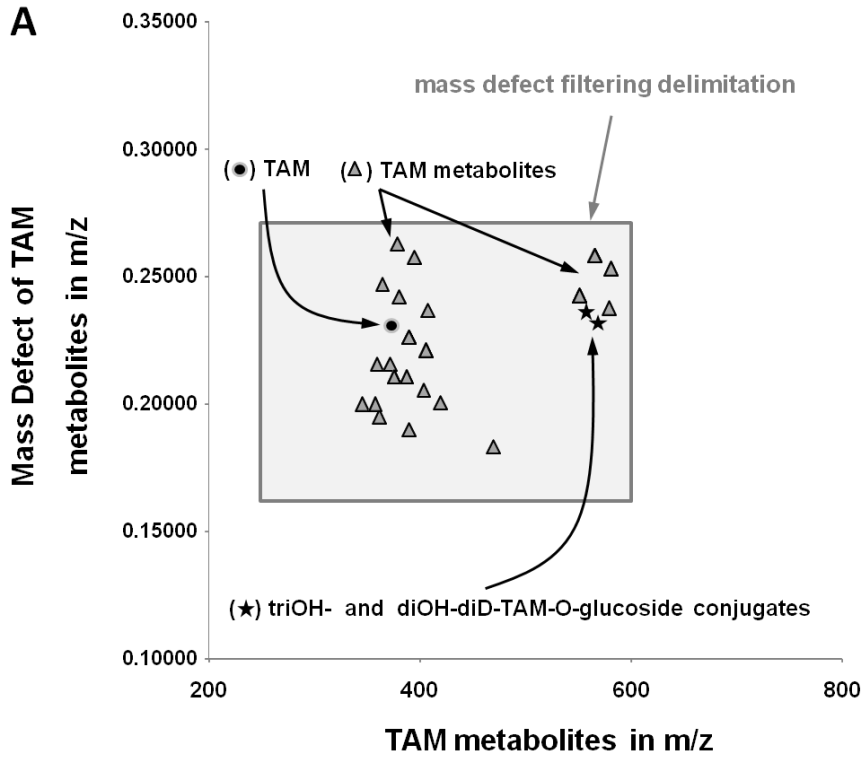


B-D. MS^2 product spectra (Q-Exactive-HR-MS) with fragment and metabolite structure proposal of TAM-carboxylic acid (TAM-COOH; ID#8), TAM-desaturated (TAM-desat; ID#39) and TAM-dihydrodiol (ID#7), respectively.

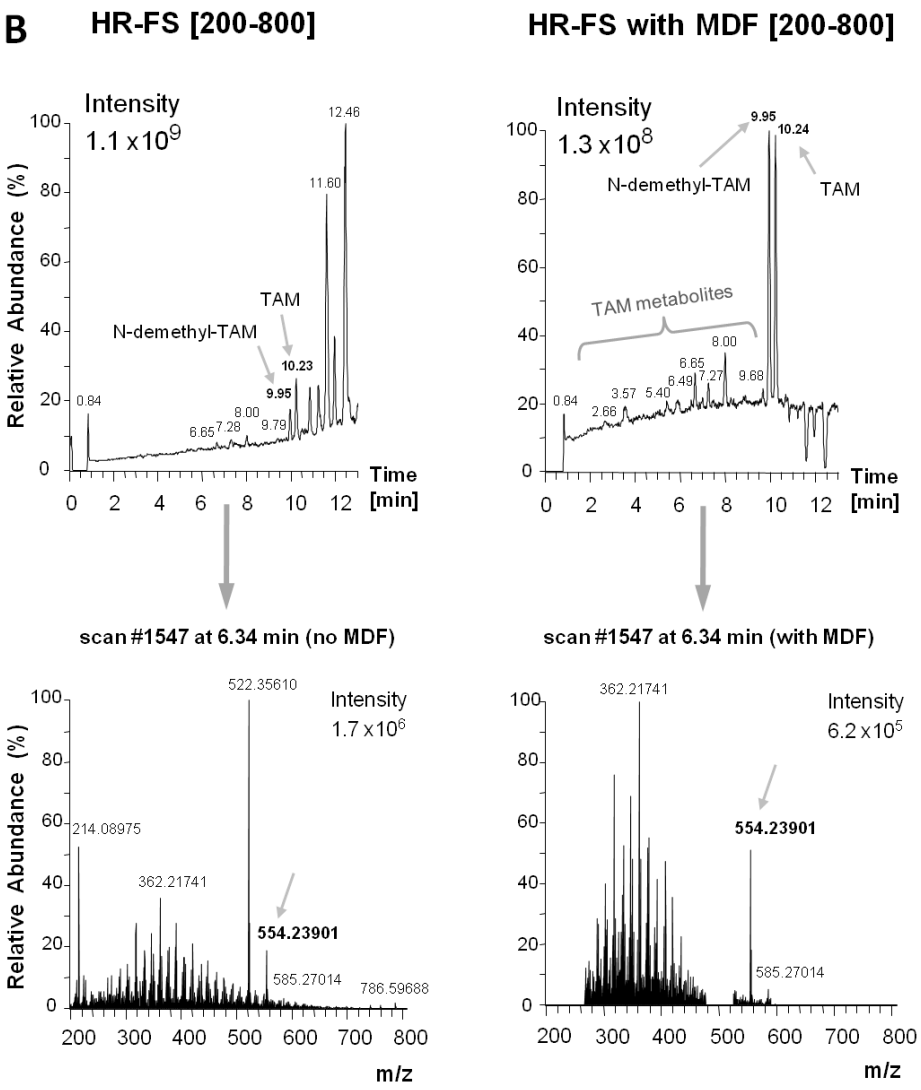


E. TAM-O-sulfate (C26H29NO5S; ID#20) is depicted at A+2 with the measured and theoretical isotopic distribution of $C_{26}H_{30}NO_5{}^{[34]}S$ and $^{[13]}C_2H_{30}NO_5{}^{[32]}S$. The minimum resolution (R_{min}) to fully resolve both ions is 88,000 whereas the mesured resolution (R_{meas}) is 105,000. The presence of a [S] in the metabolite is confirmed.

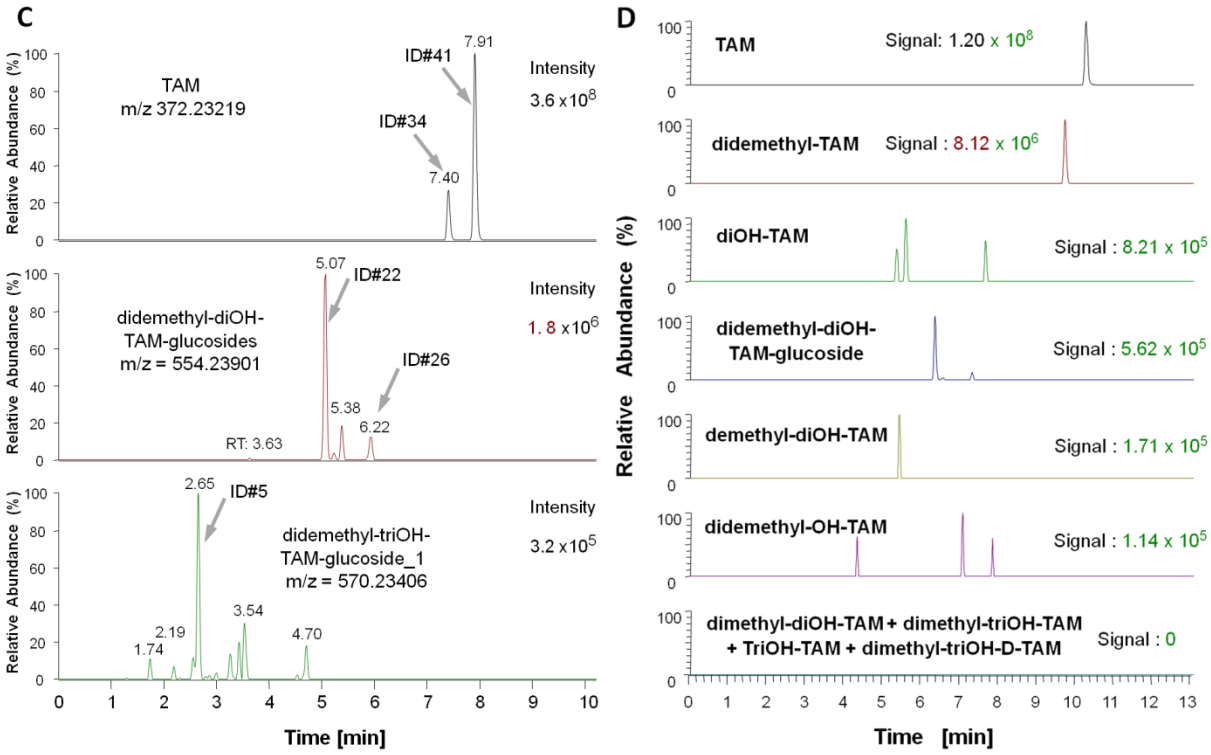
IV.7.4. Supplementary Data 4: Discovery and identification of new tamoxifen metabolites at m/z 554 and 570



A. Representation of mass defect filtering (MDF). MDF delimitation considered the mass defect and nominal m/z values of TAM metabolites identified by the first strategy for metabolite discovery (XIC of predicted metabolite m/z). MDF was defined as a square between m/z 0.165 at mass 250 (lower limits) and m/z 0.270 at mass 600 (upper limits). Ions outside the MDF delimitation were removed in the MDF cleaned spectra.

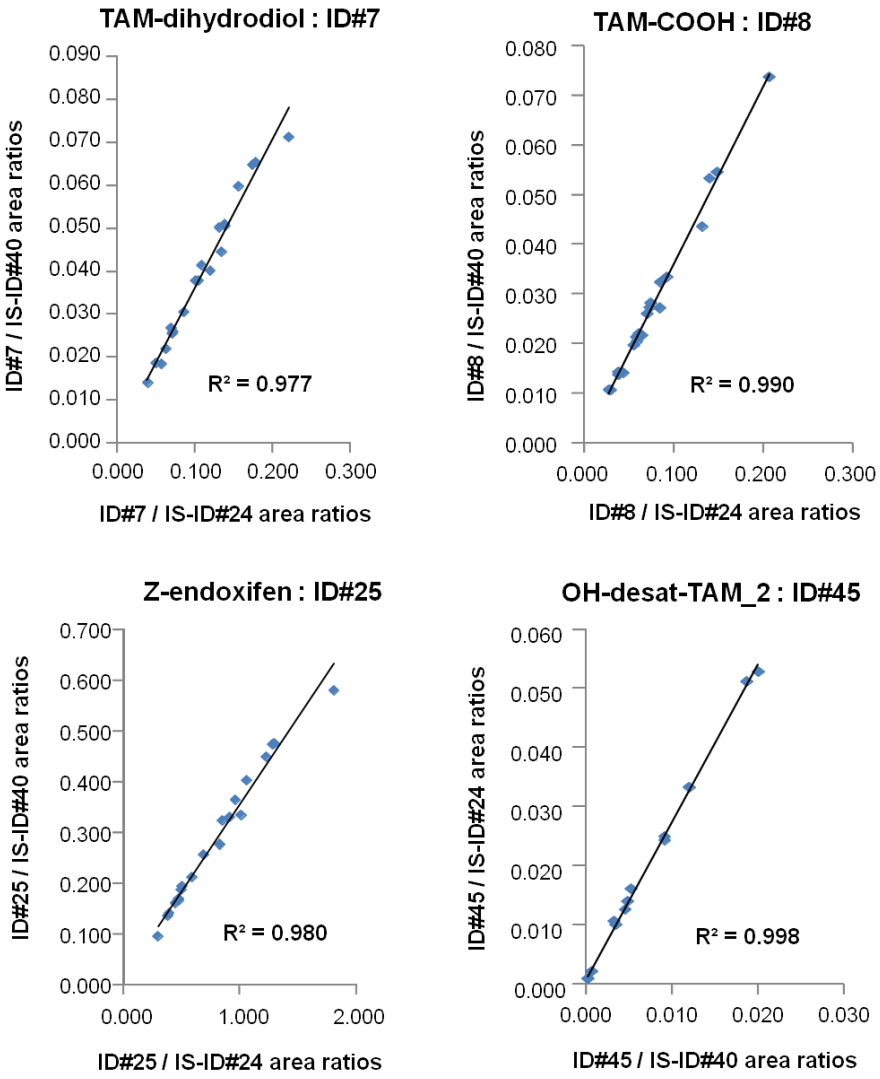


B. Total ion current chromatogram (top left; HR-FS acquisition) and a specific full scan spectrum at 6.34 min (bottom left) is depicted from the raw acquisition. The same total ion current chromatogram (top right) and same specific full scan spectrum (bottom right) are shown after MDF. Most ions have been removed showing more predominantly some possible unpredicted metabolites. In this case, m/z 554.23901 appears and can be used to trace XIC in patients' and control samples.



C. XIC (MEW = 5ppm) at m/z 554.23901 and 570.23406 identified as diOH- and triOH-didemethyl-TAM-O-glucoside conjugates, respectively. **D.** XIC (MEW = 5ppm) at various m/z corresponding to intermediate metabolites of diOH-didemethyl-TAM-O-glucoside conjugates (F6 generation). Some potential metabolite intermediates (F3 to F5 generation) are not detected on our chromatograms (bottom chromatogram). Additional data of identification are presented in Figure 3.

IV.7.5. Supplementary Data 5: Relative and semi-quantification of tamoxifen metabolites



A. Evaluation of the precision in relative metabolite quantification. Examples of correlations between different pairs of metabolite/IS peak area ratios. TAM-dihydrodiol, TAM-COOH, endoxifen and OH-desat-TAM_2 divided by different internal standard (IS) are depicted and show excellent correlation coefficients. It underscores the insignificant difference between plasma extracts of matrix effect and argues in favor of a precise relative quantification of TAM metabolites.

Metabolite plasma levels [in % of TAM] (*)					
		% TAM peak area (*)	% of TAM absolute levels (*)		Fold diff. (**)
Metabolite	ID#	this work	this work	Mürdter et al. [53]	
α-OH-TAM	ID#12	0.06	-	0.23	0.3
Z-Endoxifen	ID#25	4.05	7.18	6.78	0.6
4-OH-TAM	ID#29	1.32	1.51	1.36	0.9
3-OH-TAM_1	ID#30	0.06	-	0.16	0.4
4'-OH-TAM	ID#33	1.85	-	2.13	0.9
di-D-TAM	ID#35	5.50	-	22.90	0.2
N-D-TAM	ID#38	118.8	173.7	178.3	0.7
TAM-NO	ID#43	4.83	-	7.24	0.7

(*) Metabolite levels were expressed in % of TAM calculated from absolute levels (in ng/mL) or from peak area (relative quantification in arbitrary units). Tamoxifen (TAM) mean level is 240 and 159 ng/mL in this work and in Mürdter et al. (Supplementary Table 1), respectively.

(**) fold difference in metabolite plasma levels determined by relative and absolute quantifications ratios. Values underlined were considered as absolute levels for this calculation.

B. Semi-quantitative estimation of TAM metabolite levels. The semi-quantitative estimation of TAM metabolites in plasma have been calculated using peak area and absolute quantification of TAM and some metabolites. When comparing the metabolite levels expressed in % of TAM levels, the difference between the absolute levels extrapolated from peak area and the absolute quantification was 0.2 to 0.9 fold. The under-estimation (up to 5x) of TAM metabolite levels (ng/mL) is consistent with the fact that H^+ has a better affinity for the tertiary amine (TAM) then the secondary and primary amines (demethyl- and didemethyl-TAM).

CHAPTER V

Population pharmacokinetics of tamoxifen and three of its metabolites in breast cancer patients

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In preparation

CHAPTER V - POPULATION PHARMACOKINETICS OF TAMOXIFEN AND THREE OF ITS METABOLITES IN BREAST CANCER PATIENTS

V.1. ABSTRACT

Background: Tamoxifen (Tam) is a pro-drug transformed via cytochromes (CYP) 3A4 and 2D6 pathways into inactive and active metabolites, particularly endoxifen. Patients with null or reduced CYP2D6 activity display lower endoxifen concentrations and might experience lower treatment benefit. High variability in Tam and its active metabolites levels has been reported and partially attributed to *CYP2D6* polymorphism. The aim of this analysis was to characterize the population pharmacokinetics of Tam and its major metabolites, to quantify the inter- and intra-individual variability and to explore the influence of genetic and non-genetic factors on their disposition.

Methods: Patients under Tam 20mg/day were genotyped and phenotyped for CYP2D6 and CYP3A4/5. Plasma levels of Tam, N-desmethyl-Tam (NDTam), 4-hydroxy-Tam (4OHTam) and endoxifen were measured at baseline (20mg/day), then at 30, 90 and 120 days after a dose escalation to 20 mg twice daily. A population pharmacokinetic model for Tam and its metabolites was build using a non-linear mixed effects modeling approach.

Results: A total of 457 samples were collected from 97 patients. The full model consisted in a 4-compartment model with first-order absorption and elimination and linear conversion to the three metabolites. Average Tam apparent clearance (CL/F_{Tam}) was 5.8 L/h (CV 25%) and apparent volume of distribution 724 L with an absorption constant rate fixed to 0.7 h^{-1}. Estimated Tam to NDTam (k_{23}), Tam to 4OHTam (k_{24}), NDTam to endoxifen (k_{35}) and 4OHTam to endoxifen (k_{45}) metabolic rate constants were 0.007 (16%), $5.5x10^{-5}$ (26%) and $3x10^{-4}$ h^{-1} (59%) and 0.015, respectively. NDTam, 4OHTam and endoxifen apparent clearances were 3.4 L/h, 2.9 L/h and 6.2 L/h. Age reduced CL/F_{Tam} by 50%; increasing CYP3A4 activity was associated with an increased CL/F_{Tam} and k_{23} by 16% and 7%, respectively; *CYP2D6* genotype and CYP2D6 drug inhibitors have the most significant impact on k_{24} and k_{35}. CYP2D6 PM and IM have a reduced k_{35} by 96% and 56%. Potent and moderate CYP2D6 inhibitors reduced k_{35} by 85% and 41%. Reduced CYP2D6 activity (PM and IM) decreased k_{24} by 26%.

Conclusion: Metabolites formation rates were subject to an important inter-individual variability, in particular regarding endoxifen. CYP2D6 phenotype and CYP2D6 inhibiting comedications, were found to be the most influential ones. However, both of these factors explained only a third of total variability in endoxifen exposure. Due to this large unexplained variability, direct endoxifen monitoring seems to be a better approach to evaluate endoxifen exposure.

V.2. INTRODUCTION

Tamoxifen (Tam) is a standard therapy for estrogen-sensitive breast cancer both in the adjuvant and in the preventive settings. Five years of adjuvant tamoxifen treatment reduces breast cancer recurrence by 39% and reduces 15-year breast cancer mortality by one third [1, 2]. Despite the obvious benefits of this drug, 20 to 30% of patients either fail to respond or become resistant to tamoxifen [1-3]. Tamoxifen hepatic metabolism is of particular clinical interest as many studies support that tamoxifen efficacy is closely related to the extent of its bioactivation into more active metabolites, in particular 4-hydroxy-tamoxifen and 4-hydroxy-N-desmethyl-tamoxifen, commonly known as endoxifen [4-6]. Both metabolites are equipotent with up to 100 fold greater affinity to estrogen receptors (ERs) and potency in suppressing breast tumor cells proliferation than the parent drug and its major metabolite N-desmethyl-tamoxifen. Endoxifen is considered as the main therapeutically active contributor to oestrogen effects antagonism as it is present at around five to ten fold higher concentrations than 4-hydroxy-tamoxifen [7-9].

Tamoxifen (Tam) is a basic and highly lipophilic drug that is readily absorbed and largely distributed into biological fluids and peripheral tissues [10-15]. Tam is indeed metabolized via several cytochrome P450 (CYP) as well as UDP-glucuronosyl- and sulfo-transferase (UGT and SULT) enzymes. It is primary converted to N-desmethyl-tamoxifen (NDTam) by CYP3A4 and to 4-hydroxytamoxifen mainly by CYP2D6 and CYP2C9 [16]. Both NDTam and 4OHTam are secondly metabolized to form endoxifen through CYP2D6 and CYP3A4/5 enzymes, respectively. NDTam is quantitatively the major Tam metabolite with steady-state concentrations 1.5 to 2 fold higher than Tam, while 4OHTam constitutes a minor Tam metabolite. NDTam hydroxylation is the major metabolic pathway leading to endoxifen and thus CYP2D6 enzyme is considered as a rate-limiting enzyme involved in Tam bioactivation into endoxifen [5, 17]. Tam, 4OHTam and endoxifen undergo further glucuronidation and sulfation leading to inactive metabolites (Figure 1). The hepatic enzyme UGT1A4 is considered the major UDP-glucuronosyltransferase responsible for the *N*-glucuronidation of tamoxifen [18-21]. 4OHTam and endoxifen O-glucuronidation involve mainly UGT2B7 and the extra-hepatic enzymes UGT1A10 and 1A8 [22, 23]. Sulfotransferase (SULT) 1A1 is the major phase II metabolizing enzyme involved in the sulfation of 4OHTam and endoxifen [24-27]. These sulfated and glucuronidated metabolites are eliminated in urine (< 20%), bile, feces (~ 20 to 50 %) and undergo enterohepathic circulation (EHC) [10, 13, 28].

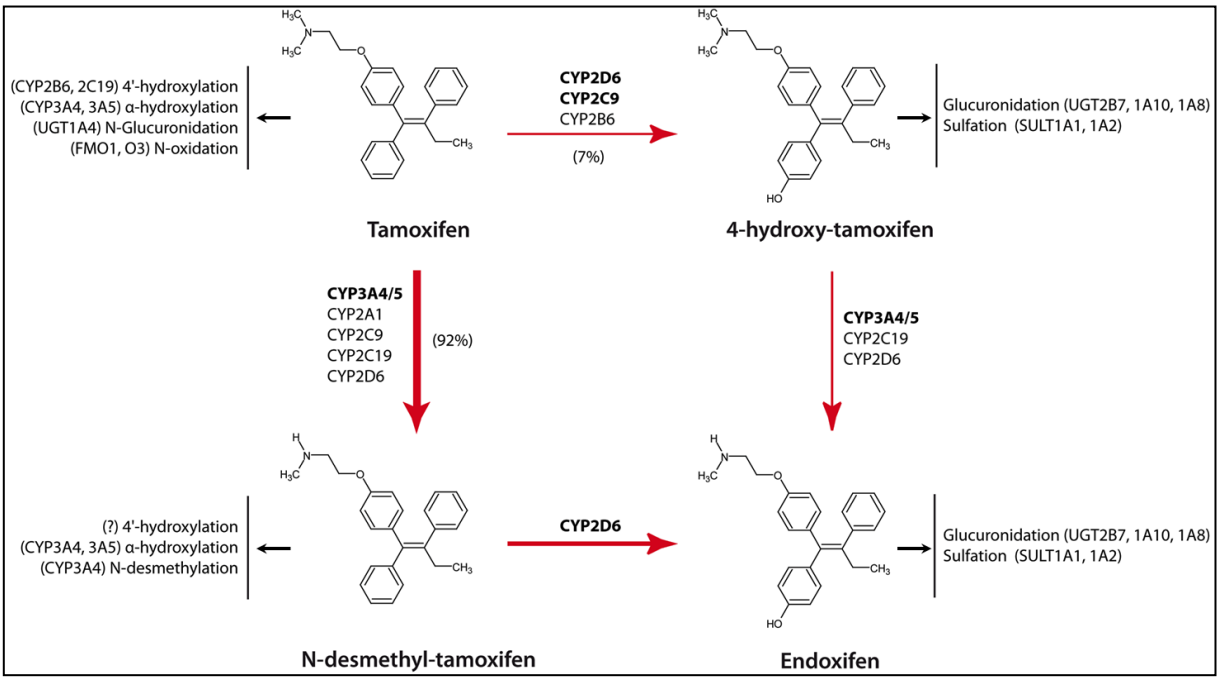

Figure 1: Principal tamoxifen metabolism pathways.

Tam, NDTam, 4OHTam and endoxifen plasma concentrations are highly variable between individuals [29]. Part of this variability could be explained by the complex Tam metabolism pathways engaging highly inter- and intra-ethnic polymorphic enzymes, most notably CYP2D6 [4, 30]. *CYP2D6* gene polymorphisms have been shown in several pharmacokinetics-pharmacogenetics studies to influence 4OHTam and endoxifen plasma concentrations in a gene-dose manner. Patient who are poor (PM) or intermediate (IM) CYP2D6 metabolizers have lower endoxifen and 4OHTam exposure than patient extensive (EM) and ultra-rapid (UM) CYP2D6 metabolizers [4, 29]. Other studies, have investigated the association between *CYP2D6* polymorphism and Tam treatment outcomes. Some retrospective and prospective studies have shown that *CYP2D6* polymorphism was associated with worse clinical outcomes in PM and IM patients in terms of recurrence and disease free survival or breast cancer development in the chemoprevention setting [31-33]. These findings brought to consider the potential use of *CYP2D6* genotyping either to individualize anti-hormonal therapy or to study the feasibility of a genotype-guided dose optimization strategy in order to improve tamoxifen efficacy [34-36]. However, these pharmacogenetics-pharmacodynamics studies have yielded conflicting results [37, 38]. Heterogeneity in study designs, important issues with the quality of DNA, the coverage of genotyped alleles, the correct genotype-phenotype assignment and the presence of other confounders such as interacting comedications, non-adherence and the presence of combination tamoxifen chemotherapy are probable factors explaining these discrepancies [39-41].

Actually, *CYP2D6* genotype polymorphism explained only 20 to 40% of endoxifen levels variability and large inter-individual variability in plasma levels still subsists even after adjusting for CYP2D6 status [29, 42, 43]. This remaining variability may depend on the activity of other cytochromes (CYP3A4/5, 2C9, 2C19, 2B6) [29, 44-46], phase II conjugation enzymes (SULT1A1, UGT1A4, 2B7, 1A10, 1A8, 2B15) [47] or drug transporters [48]. Treatment adherence and particularly, interacting co-medications have also shown to modulate drug exposure independently of genetic traits. Selective serotonin reuptake inhibitors (SSRIs) with strong or moderate *CYP2D6* inhibiting activity that are frequently prescribed to treat depression or to alleviate tamoxifen-induced hot flushes, are known to reduce endoxifen plasma concentration and have been associated with poorer tamoxifen efficacy [49, 50]. Recently, Madlensky et al. [29] found a probable non linear dose-response relationship for tamoxifen effect and identified a threshold concentration for endoxifen (of about 6 ng/mL) above which approximately 30% reduction in disease recurrence rate was observed.

Therefore, direct endoxifen levels monitoring seem to be a straightforward and a better approach addressing the issues of pharmacogenetics and non-pharmacogenetics confounding factors and would represent a better predictor of tamoxifen outcomes. Accordingly, the objectives of our study was to perform a population pharmacokinetics analysis of tamoxifen and its principal metabolites in order to quantify the inter-individual variability and the influence of genetic and non-genetic factors on Tam and its metabolites levels. The established population models will be useful for Tam dose optimization studies and strategies.

V.3. MATERIAL AND METHODS

V.3.1. Study population and design

Patients receiving tamoxifen (Tam) as an adjuvant therapy for hormone-sensitive breast cancer were enrolled in a multicenter, prospective, open-label trial (ClinicalTrials.gov NCT00963209). This study protocol was approved by the Institutional Research Ethics Committees and all participants provided written informed consent. Exclusion criteria were: less than 4 months of standard Tam treatment, history of deep venous thrombosis, pulmonary embolism, endometrial carcinoma, vaginal bleeding, endometriosis, endometrial hypertrophy and/or polyps, pregnancy, breast feeding women, allergy to midazolam and/or dextromethorphan. Study design (Figure 2) was as follow: patients under a standard Tam dose of 20 mg per day (20 mg QD) were genotyped for *CYP2D6* and phenotyped for CYP2D6 and CYP3A4/5, respectively at inclusion date and on day 0 (baseline treatment conditions). Baseline (steady-state) plasma levels of Tam and three of its metabolites of interest were also measured on these two different occasions. Tam dosage was then increased, in all patients, to 20 mg twice a day (20 mg BID) for up to 4 months. During this period, patients were followed every month

and plasma levels of Tam and its metabolites were measured at the end of months 1, 3 and 4. Blood chemistry was also assayed at inclusion and at the end of months 1 and 4. Patients demographic and clinical characteristics, concomitant medications, compliance (self-reported through a drug intake frequency questionnaire), side effects (tolerance, hot flashes and night sweating) were recorded and graded at inclusion and at each follow-up visit.

V.3.2. Tamoxifen and metabolites measurements in plasma

Blood samples (5.5 mL) were collected, at random time after last drug intake, in potassium-EDTA Monovettes® (Sarstedt, Nümbrecht, Germany). Samples were centrifuged and plasma was stored at -80°C until analysis. According to study protocol, blood samples were collected in patients receiving TAM 20 mg QD, on two occasions at baseline (e.g. at inclusion and on day 0) and after 1, 3 and 4 months of continuous treatment at a regimen of 20 mg BID. Tam, N-desmethyltamoxifen (NDTam), 4-hydroxytamoxifen (4OHTam), and endoxifen concentrations were measured simultaneously by a validated ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method [51]. This developed assay is precise (inter-day CV%: 2.5 – 7.8 %), accurate (-1.4 to +5.8 %), sensitive (lower limits of quantification comprised between 0.2 and 1 ng/mL) and selective (mass spectrometry selectivity and good chromatographic resolution of possible interfering isomers). All measured Tam and metabolites levels, in patients, were above the lower limits of quantifications of the assay.

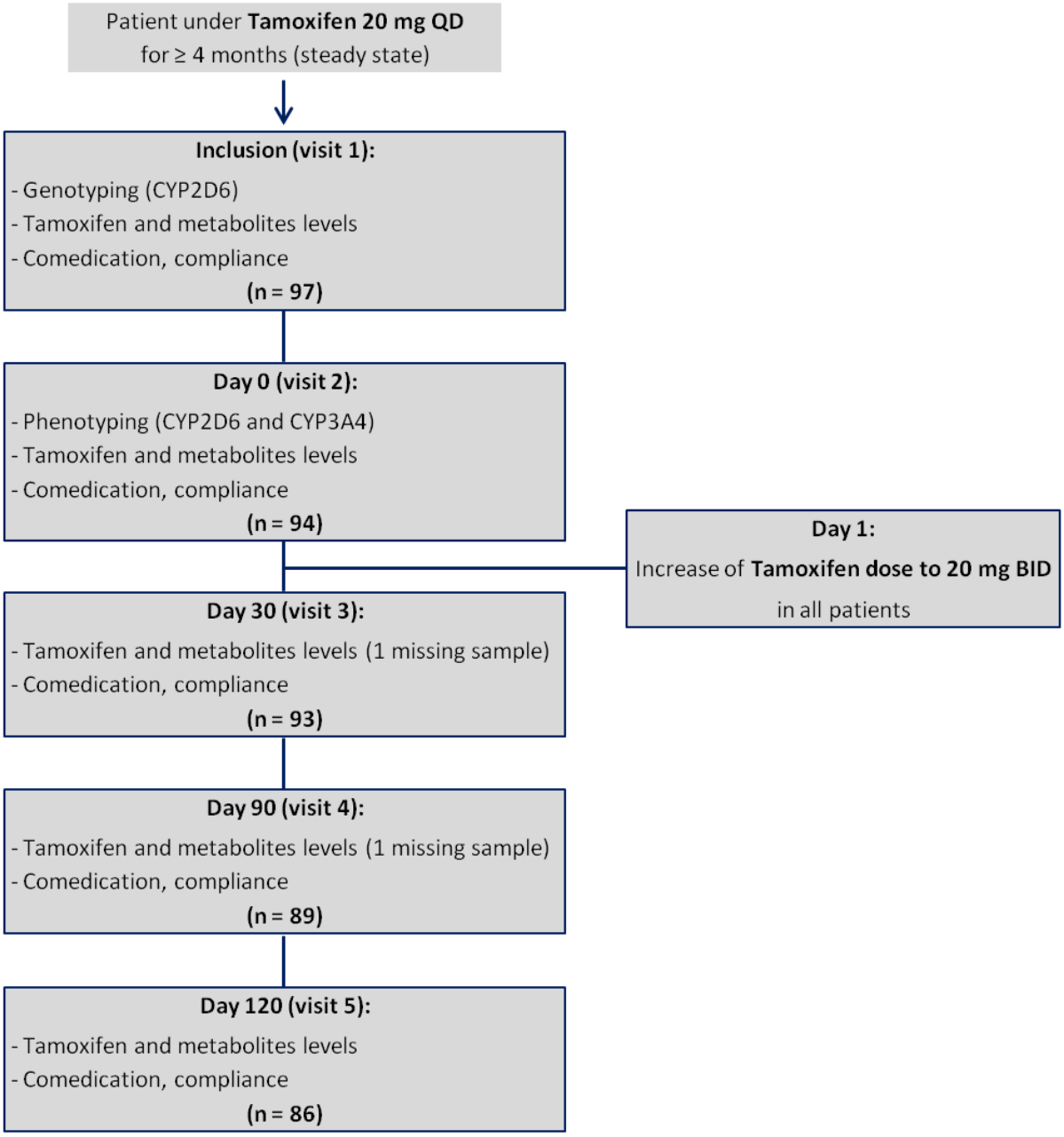

Figure 2: Schematic diagram of the study design

V.3.3. Genotyping and phenotyping assays

V.3.3.1. CYP2D6 genotype

Genomic DNA was extracted from the EDTA blood samples using the FlexiGene DNA Kit (Qiagen, Hombrechtikon, Switzerland). Single-nucleotide polymorphisms were analyzed by real-time polymerase chain reaction (PCR) with the 5'-nuclease allelic discrimination assays according to r the manufacturer's instructions (ABI PRISM 7000 Sequence Detection System; Applied Biosystems, Rotkreuz, Switzerland). The following SNPs were analyzed: *CYP2D6*: allele *3, *4, *5, *6; The *CYP2D6* *XN gene duplication was analyzed by long PCR. Internal quality control samples of known genotype were included in all analyses. Patients were categorized according to the number of functional alleles as CYP2D6 poor (PM=homozygous loss of functional allele), intermediate (IM=heterozygous loss (*1/*4)), extensive (EM=homozygous reference allele), or ultrarapid metabolizer (UM=multiple functional alleles). Heterozygous genotypes (*4/*XN) have been scored according to the dextromethorphan metabolic ratio test. Finally, a CYP2D6 activity score of 0 was assigned to PM, 1 for IM, 2 for EM and 3 for UM.

V.3.3.2. CYP2D6 phenotype

Dextromethorphan metabolic ratio test was used for the determination of CYP2D6 activity, as previously described [52] . Each patient was asked to take a single 25 mg oral dose of dextromethorphan hydrobromide after voiding at bedtime and to collect their urine for the next 8 hours or overnight. Urine samples were then analyzed by gas chromatography-mass spectrometry [53]. Patients were classified according to their dextromethorphan/dextrorphan (DM/DX) ratio as: PM (DM/DX ≥ 0.3), IM (0.03 ≤ DM/DX < 0.3), EM (0.003 ≤ DM/DX < 0.03) and UM (DM/DX < 0.003).

V.3.3.3. CYP3A4/5 phenotype

Midazolam metabolic ratio (MR) test was used to evaluate CYP3A4 activity as previously described [54]. Briefly, a single dose of 0.075 mg of midazolam (0.075 mL of a 0.1% midazolam solution in 100 mL of water) was administered to each participant. A blood sample was drawn 30 minutes later in potassium-EDTA Monovettes® (Sarstedt, Nümbrecht, Germany). Midazolam and 1'-OH-midazolam plasma levels were measured by gas chromatography-mass spectrometry. CYP3A4/5 activity expressed as a midazolam metabolic ratio (1'-OH-midazolam/midazolam) was used as a continuous variable.

V.3.4. Population Pharmacokinetic Modeling

Plasma concentrations of Tam and its measured metabolites, converted to nanomoles per liter (nM), were modeled using the NONMEM computer program version 7.2 (NM-TRAN version II) with the PsN-Toolkit version 3.5.3. The program uses mixed (fixed and random) effects regression to estimate population means and variances of the pharmacokinetic parameters and to identify factors that influence them.

V.3.4.1. Structural model

A stepwise procedure with sequential addition of metabolites was used to find the full structural model that best fitted the observed data. One- and two compartment models with first-order absorption were first compared for the parent drug Tam. Tam model was then sequentially expanded by an additional compartment for each metabolite and the data were sequentially fitted for each added compartment. The final parameters estimated were Tam and metabolites apparent clearances (CL_{Tam}/F, CL_{NDTam}/F, CL_{4OHTam}/F, $CL_{Endoxifen}/F$) and the apparent volume of distribution of the central compartment (V_c/F). Due to structural identifiability problems, the volume of distribution of Tam (V_c) and those of its metabolites were assumed to be equal. The metabolic rate constant from Tam to NDTam (k_{23}), Tam to 4OHTam (k_{24}), NDTam to endoxifen (k_{35}) and NDTam to endoxifen (k_{45}) were also estimated. Tam absorption rate constant (k_a) cloud not be adequately estimated in the parent-metabolites model; therefore k_a was fixed to the value estimated from the Tam model without metabolites. A schematic representation of the parent-metabolites model is depicted in Figure 3.

V.3.4.2. Statistical model

Assuming that pharmacokinetic (PK) variables follow a log-normal distribution, inter-individual variability (IIV) of the PK parameters were characterized by exponential errors models, as illustrated by the equation $\theta_j = \theta \cdot \exp(\eta_j)$, where θ_j is the pharmacokinetic (PK) random variable of the jth individual, θ is the population value (geometric mean) of the PK parameter, and η_j is the random effect or deviation for the jth individual from θ, with η_j assumed to be independent across individuals and normally distributed with mean 0 and variance ω^2 equal to 1. Proportional and combined proportional-additive error models were compared to describe the intra-individual (residual) variability for both Tam and its metabolites. Correlations between residual errors for measured levels of TAM, NDTam, 4OHTam and endoxifen were investigated using the L2 function in NONMEM®. Inconsistencies in concentration-time data (e.g. due to unreported non-compliance) that could bias parameters estimates have been detected by introducing a random effect on the residual error of the parent drug (Tam) as follows $Y_{obs} = Y_{pred} + Y_{pred} \cdot \varepsilon \cdot \exp(\eta_\varepsilon)$ where Y_{obs} is the observed

concentration of the parent drug for the ith individual at the jth obsenvation, Y_{pred} the predicted concentration from the model, ε is the residual error (independent, ~ $N(0, \sigma^2)$) and η_ε is the random effect associated to the residual error [55, 56]. Correlations between PK parameters were also investigated and integrated after finalizing the covariates model.

V.3.4.3. Covariate Model

Relevant covariates investigated were: age, menopausal status, body weight (BW), body mass index (BMI), Compliance (self-reported through a semi-quantitative questionnaire), CYP2D6 activity score (predicted from genotype or DM/DX ratio test), CYP3A4 phenotype and concomitant medications. Putative interacting co-medications with Tam metabolizing enzymes such as CYP2D6, 2C19 or 2C9 inhibitors were coded as strong (score=3), moderate (score=2), weak (score=1) or null (score=0) inhibitors. The covariate analysis was performed using a stepwise insertion/deletion approach. Visual inspection of the correlation between individual random effects estimates and the available covariates was first conducted by graphical exploration. Potentially influential covariates were then sequentially incorporated into the PK model and tested for significance on the PK parameters. For continuous covariates, the typical value of a given parameter θ was modeled to depend on the tested covariate (X) either linearly with $\theta = \theta_a \cdot (1 + \theta_b \cdot (X\text{-}MX/MX))$ or allometricly as a power function model $\theta = \theta_a \cdot (X/MX)^{\theta_b}$, where θ_a is the mean population value, θ_b is the relative deviation from θ_a due to the covariable and MX is the population median of X. Dichotomous covariates (e.g. sex and menopausal status), coded as 0 and 1, were entered as linear model $\theta = \theta_a \cdot (1 + \theta_b \cdot X)$. Categorical covariates, such as CYP2D6 activity score or CYP2D6 inhibitors, were first dummy coded and implemented in the model by assigning a fixed effect (θ_i) to each category, using $\theta = \sum \theta_i \cdot I_i$, where θ_i is the typical value of a PK parameter for the ith category and I is the dummy variable taking the value of 1 for the ith category, and 0 otherwise. The contributions of the categorical groups were further expressed relatively to a reference group as follows: $\theta = \theta_{ref} \prod (1 + \theta_i \cdot I_i)$ where θ_{ref} is the typical value of a PK parameter for the reference category (e.g. common CYP2D6 activity score or null CYP2D6 inhibitor). The full model was also compared to reduced models where covariate categorical groups were merged. For correlated covariables (e.g. BW and BMI) or those yielding similar information (e.g. CYP2D6 activity score predicted on genotype or DM/DX ratio phenotype), only the most significant, relevant or the covariable with no missing data was considered.

V.3.4.4. Selection of the model and parameter estimation

Drugs and metabolites were fitted using the first-order conditional estimation with interaction (FOCE-I) method in NONMEM® and the subroutine ADVAN5. The log likelihood ratio test (LRT) was used to assess the differences in the objective function values (ΔOFV) and discriminate between

hierarchical models. ΔOFV (which approximates a χ2 distribution) between nested models is considered statistically significant if it exceeded, for one additional parameter, 3.84 ($p < 0.05$; df=1) in forward model-building or 6.63 ($p < 0.01$; df=1) in the backward-deletion approach. Additional criteria for model selection were goodness-of-fit plots, precision of the pharmacokinetic parameters estimations (RSE %), and the reduction of the inter-individual variability of the population PK parameters.

V.3.4.5. Validation of the model

Model validation was performed by visual predictive checks (VPC), simulating data for 1000 individuals based on the final model estimates and generating 2.5^{th}, 50^{th} and 97.5^{th} percentiles. The observed concentrations-time data were plotted against the 95% prediction interval (95 % PI) of the simulated dataset and visually compared. Figures were generated with GraphPad Prism® (Version 6.00 for Windows, GraphPad Software, San Diego California USA, http://www.graphpad.com/).

V.4. RESULTS

V.4.1. Study data

The data for population analysis consisted in a sparse data set with 457 samples collected from a total 97 patients. Blood samples were drawn on two occasions at baseline, with assumed steady-state concentrations under Tam 20 mg QD (inclusion date and day 0 before dose escalation), and at the end of months 1, 3 and 4 after doubling Tam dose to 20 mg BID. During the course of the study, 10 patients discontinue their participation. Three patients withdraw from study just after the inclusion date, 1 patient at the end of month 1 and 6 patients at the end of month 3. The number of samples available from each aforementioned visit was 97, 94, 92, 88, and 86 samples respectively. Patient's characteristics and study data are summarized in Table 1.

Table 1: Summary of patients demographics and clinical characteristics

Characteristics	Number or median	Range or %
nb patients / samples:		
Inclusion	97/97	
Day 0	94/94	-
Day 30	93/92	
Day 90	89/88	
Day 120	86 /86	
Sex (female/male)	97/1	-
Median age (years)	50	32-78
Median body weight (kg)	65	47-116
Median height (cm)	165	151-183 (11 missing data)
BMI (kg/cm2)	24	(18-43)
Menopausal status (yes/no)	47/50	-
Ethnicity:		
Caucasian	94	
North African	2	-
Indian	1	
CYP2D6 activity score:		
PM (*4/*4)	4	4%
IM (*1/*4, *4/XN)	30	31%
EM (*1/*1, *4/XN)	60	62%
UM (*1/XN or *XN/XN or *4/XN)	3	3%
CYP3A4 phenotype (midazolam metabolic ratio)	4.81	0.37-17
Compliance (per visit date):		
>95%	93/91/81/78/76	
80 – 95%	4/3/9/7/8	-
80 – 60%	0/0/2/3/2	
Comedication:		
- CYP2D6 inhibitors:		
Paroxextine (potent)	2	
Fluoxetine(potent)	1	
Citalopram/Escitalopram (moderate)	6	
Sertraline (moderate)	1	-
Risperidone (weak to null)	1	
Venlafaxine (weak to null)	8	
- CYP2C19 inhibitors:		
Omeprazole (weak to moderate)	8	-
Pantoprazole (weak to null)	3	
- CYP2C9 inhibitors:		
Pantoprazole (moderate to weak)	3	-
Cotrimoxazole(weak to null)	1	
ASAT (UI/L)	24	11-70
ALAT (UI/L)	18	10-68
BT (µmol/L)	10	2-29
GGT (UI/L)	22	5-255
AP (UI/L)	48	17-228

V.4.2. Population pharmacokinetics analysis

V.4.2.1. Base model

The simultaneous disposition of Tam and its three metabolites (NDTam, 4OHTam and endoxifen) was best characterized by a joint four-compartment model with first-order absorption, elimination and linear conversion to metabolites. Tam dose is absorbed into the central compartment at a rate ka, the parent drug is then either eliminated with a clearance CL_{Tam} or partially converted to NDTam and 4OHTam at metabolic rate constants k_{23} and k_{24}, respectively. NDTam and 4OHTam are, in turn, either cleared with CL_{NDTam} and CL_{4OHTam} or partially transformed to endoxifen at metabolic rates k_{35} and k_{45}, respectively. Endoxifen is further eliminated from the central compartment with a clearance $CL_{Endoxifen}$ (Figure 3).

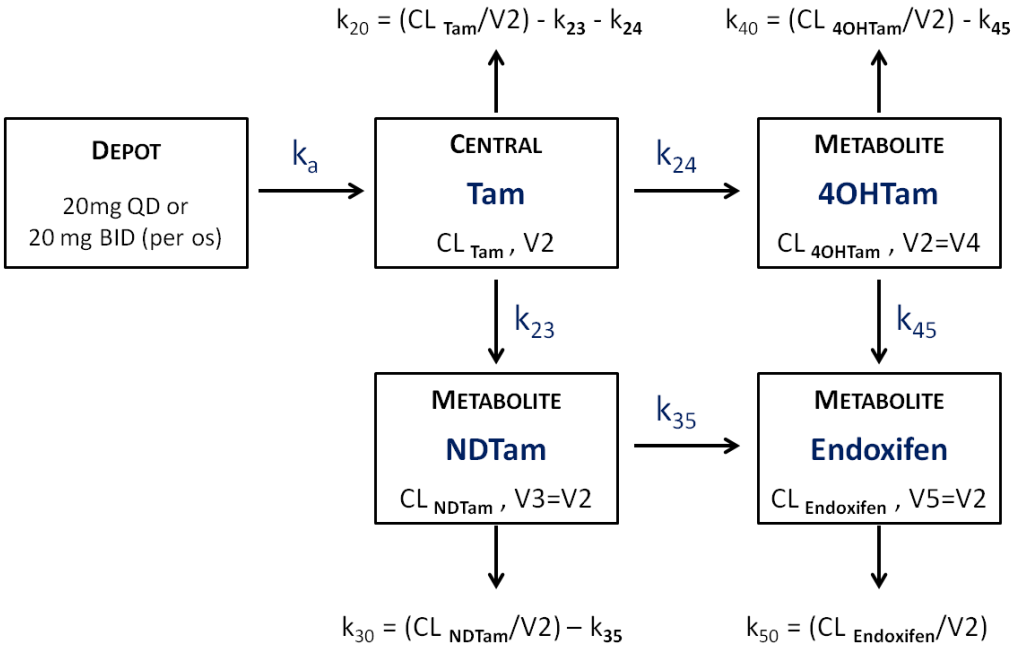

Figure 3: Schematic model used to describe the combined pharmacokinetic of tamoxifen and three of its metabolites: 4-hydroy-tamoxifen (4OHTam), N-desmethyl-tamoxifen (NDTam) and endoxifen. CL and V2 are apparent parameters conditional on Tam bioavailability.

The final base model was parameterized in term of the apparent clearance of Tam (CL_{Tam}/F), NDTam (CL_{NDTam}/F), 4OHTam (CL_{4OHTam}/F) and endoxifen ($CL_{Endoxifen}/F$), the apparent volume of distribution for the central compartment of Tam (V_2/F), the metabolic rates constants k_{23}, k_{24}, k_{35} and k_{45}. The apparent volumes of distribution for Tam metabolites were unknown and unidentifiable. Hence, for identifiability purposes, the apparent volumes of distribution of Tam (V_2/F) and its metabolites (V_3/F, V_4/F, V_5/F) were assumed to be equal. Likewise, Tam absorption rate constant (k_a) cloud not be adequately estimated in the parent-metabolites joint model, therefore, k_a was fixed to the value (0.7

h^{-1}) estimated from the one-compartment Tam model (i.e. prior metabolites inclusion). Proportional residual error models best described intra-individual variability for Tam and its three metabolites. Correlations between residual errors on Tam and NDTam ($\sigma_{Tam,\ NDTam}$) and between 4OHTam and endoxifen ($\sigma_{4OHTam,\ Endoxifen}$) levels were identified. During the course of the study, two patients presented inconsistent changes in the levels of the parent drug and its metabolites, given their reported compliance, dose regimen and the unchanged co-medications or clinical settings. The introduction of a random effect (η_ε) on the residual error associated to Tam (ε_1) showed that these 2 patients have the highest η_ε variance. A sensitivity analysis performed by removing these 2 patients showed significant changes in the parameters estimates, their precisions (RSE), as well as a decrease in their variability. The unreliable data from these 2 patients were, therefore, discarded from the dataset for the population analysis. Inter-individual variability was successfully estimated on the apparent clearance of Tam (CL_{Tam}/F) and on metabolic rate constants k_{23}, k_{24} and k_{35}. Correlation between random effects on k_{24} and k_{35} ($\omega_{k24,\ k35}$) has also been identified. ETA shrinkage for all model parameters was lower than 6%. Final baseline model population estimates were: CL_{Tam}/F = 5.8 L/h (IIV: 29%); V_2/F = 708 L; k_a fixed to 0.7 h^{-1}; k_{23} = 0.007 L/h (17%); k_{24} = 4.7x10^{-05} L/h (29%) and k_{35} = 0.01 L/h (84%), CL_{NDTam}/F = 3.4 L/h; CL_{4OHTam}/F = 2.7 L/h; $CL_{Endoxifen}/F$ = 5.6 L/h.

V.4.2.2. Covariate model

Univariate analysis showed that age (ΔOFV = -12, p = 0.0004), CYP3A4 phenotype (ΔOFV = -8, p = 0.004), menopausal status (ΔOFV = -8, p = 0.004) and compliance (ΔOFV = -10, p =0.001) significantly influenced CL_{Tam}/F. In multivariate analysis, only age, CYP3A4 phenotype (expressed as a midazolam metabolic ratio: MR) and compliance remained significant since menopausal status is correlated to age. Tam apparent clearance increased linearly with increasing CYP3A4 activity (MR) and decreased linearly with age. A 9% increased CL_{Tam}/F was associated with lower compliance (≤ 80%). Age, CYP3A4 activity and compliance explained 8%, 4% and 1% of the inter-individual variability (IIV) in CL_{Tam}/F, respectively. In multivariate analysis, 14% of the IIV was explained by these covariates.

Univariate analysis identified CYP2D6 activity score predicted from genotype (ΔOFV = -22, p < 0.00001) as an influencing factor on Tam transformation to 4OHTam (k_{24}). Patients with null and reduced CYP2D6 activity (i.e. PM or IM phenotype) have a reduced 4OHTam formation by about 25% compared to patients with CYP2D6 EM and UM phenotypes. No significant difference in k_{24} was observed between IM and PM as well as between EM and UM groups. Even though not statistically significant, CYP2D6 UM patients tend to have a higher k_{24} (39%) than EM patients (ΔOFV = -4, p = 0.05). CYP2D6 activity explained 12% of the inter-individual variability on k_{24}. In contrast to CYP2D6 phenotypic groups, no significant impact on k_{24} has been found for CYP2D6 inhibiting co-medications. In univariate analysis, the presence of CYP2C19 inhibitors (proton pump inhibitors), showed an effect

on k_{24} (ΔOFV = -10, p = 0.006). However, the effect of these medications was not retained since patients under pantoprazole (a weak to null CYP2C19 inhibitor) showed a higher impact on k_{24} than omeprazole/esomeprazole (a moderate CYP2C19 inhibitor). Moreover, of the three patients under pantoprazole, two patients have an occasional use of the drug. A sensitivity analysis (by ignoring these two patients) showed that the identified effect was only due to the patient with long term pantoprazole prescription.

CYP2D6 activity score (ΔOFV = -60, p < 0.00001), CYP2D6 inhibiting drugs (ΔOFV = -50, p < 0.00001), age (ΔOFV = -5, p = 0.03) and BW (ΔOFV = -8, p = 0.004) significantly influenced NDTam to endoxifen formation rate (k_{35}) in univariate analyses. All remained significant in the multivariate analyses, except for age. Patients with CYP2D6 PM and IM have a reduced k_{35} compared to normal CYP2D6 metabolizers (EM) by 98% and 55%, respectively. The increase of k_{35} in CYP2D6 UM (35%) was not found to be significantly different from the reference EM group. Potent and moderate CYP2D6 inhibitors reduced endoxifen formation rate compared to non-CYP2D6 inhibitors by 87 and 45%, respectively. CYP2D6 phenotype and interacting co-medication explained, respectively, 17% and 6% of the inter-individual variability in endoxifen formation rate constant. Both covariates explained 28% of the inter-individual variability. Finally Tam to NDTam transformation rate (k_{23}) linearly increased with midazolam metabolic ratio (MR) increase (ΔOFV = -7, p = 0.01). CYP3A4 activity explained 4% of the IIV on k_{23}.

The final covariates model retained for Tam and its three metabolites were age, CYP3A4 activity (MR) and compliance on CL_{Tam}/F, CYP3A4 activity on k_{23}, CYP2D6 activity score on k_{24} and CYP2D6 activity score and CYP2D6 inhibitors on k_{35}. The introduction of a covariance between random effects on k_{24} and k_{35} ($\omega_{k24,\ k35}$) significantly improved the model fit (ΔOFV = -19, p = 0.00002). Parameters estimates of the final model are given in Table 2. VPC plots of the final PK model for Tam, NDTam, 4OHTam and endoxifen are given in Figure 4.

Table 2: Final population parameters estimates of tamoxifen and its metabolites

Parameters	Population mean			
	Estimate	RSE [a] (%)	IIV [b] (%)	RSE [c] (%)
CL_{Tam}/F (l/h)	5.8	3	25	8
θ_{Age}	0.5	26		
θ_{MR}	0.16	41		
$\theta_{Compliance}$	0.09	43		
V2/F (l)	724	17		
k_a (h^{-1}) (FIX)	0.7	-		
k_{23} (h^{-1})	$7.07x10^{-03}$	14	16	8
θ_{MR}	0.07	38		
k_{24} (h^{-1})	$5.49x10^{-05}$	36	26	12
$\theta_{CYP2D6\ PM/IM}$	0.26	17		
k_{35} (h^{-1})	$2.84x10^{-04}$	70	59	10
$\theta_{CYP2D6\ PM}$	0.96	4		
$\theta_{CYP2D6\ IM}$	0.56	12		
$\theta_{potent\ 2D6\ inhibitor}$	0.85	12		
$\theta_{moderate\ 2D6\ inhibitor}$	0.41	30		
k_{45} (h^{-1})	0.015	72		
CL_{NDTam}/F (l/h)	3.4	19		
CL_{4OHTam}/F (l/h)	2.9	48		
$CL_{Endoxifen}/F$ (l/h)	6.2	85		
σ_{Tam} (CV%)	17	4 [c]		
σ_{NDTam} (CV%)	17	5 [c]		
σ_{4OHTam} (CV%)	18	4 [c]		
$\sigma_{Endoxifen}$ (CV%)	19	5 [c]		
	ρ (%)	RSE (%)		
ω (k_{24} ,k_{35})	51	19		
$\sigma_{Tam,\ NDTam}$	87	2		
$\sigma_{4OHTam,\ Endoxifen}$	75	3		

[a] Relative standard error (RSE) of the estimate θi derived from the covariance matrix and defined as SE of estimate/estimate, expressed as %.
[b] Inter-individual variability, expressed as a coefficient of variation (CV %).
[c] Relative standard error (RSE) of the coefficient of variation, derived from the correlation matrix and defined as SE of estimate/estimate, expressed as %.

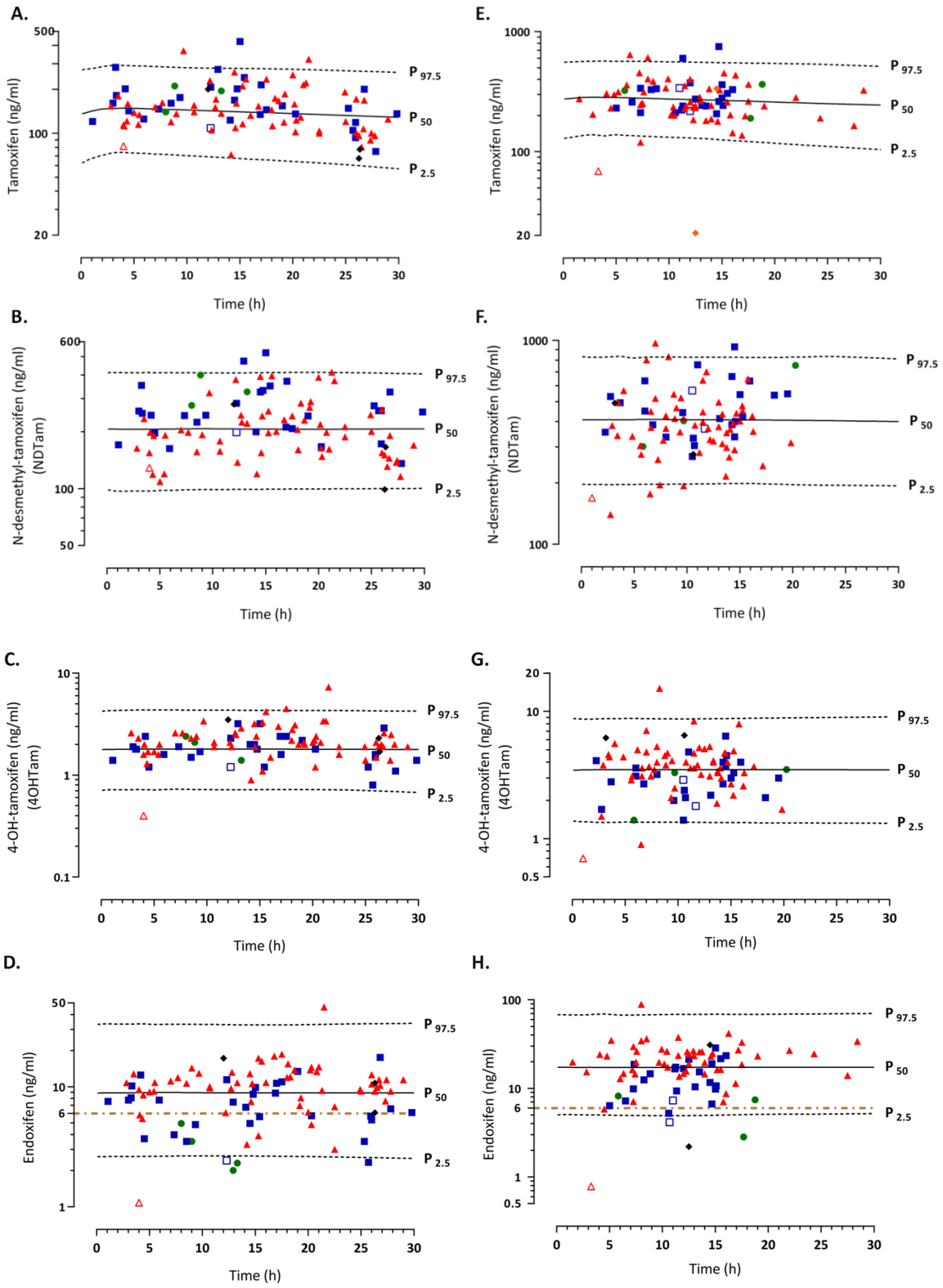

Figure 4: VPC plots for Tam and metabolites steady-state levels under a Tam dose of 20 mg qd (**A.; B.; C.; D**) and under 20 mg bid (**E.; F.; G.; H.**) comparing observations (symbols) with their predictive distribution according to the model (percentiles: P2.5, P50, P97.5). Green circles: CYP2D6 PM patients, blue rectangles: IM, red triangles: EM, diamonds: UM. Open symbols: patients under potent a CYP2D6 inhibitor. The value 6 ng/mL, in the VPC plots of endoxifen, corresponds to a reported threshold from literature [29] that has been correlated to treatment efficacy.

V.5. DISCUSSION

This is the first report that characterizes the joint population pharmacokinetics and explores the factors influencing the exposure to tamoxifen and its major metabolites of clinical interest: N-desmethyl-tamoxifen (NDTam), 4-hydroxy-tamoxifen (4OHTam) and endoxifen. Our results are in good accordance with a population pharmacokinetics analysis of tamoxifen reported by the manufacturer [57]. Their population estimates for Tam absorption rate constant (median: 1.28 h^{-1}; range: 0.7 – 1.9), apparent clearance (6.5 L/h; 2.97 – 14.5) and volume of distribution (422 L; 201-1071) are also in line with our findings. These results contrasted from early published PK studies in a limited number of patients where biphasic elimination and a higher final distribution volume of 50-60 L/kg has been reported [58]. More recently, a physiologically based PK modeling of Tam and its three metabolites estimated the volume of distribution of Tam in a woman of 65 kg to be approximately 34 L/kg [59]. The estimated endoxifen clearance is in accordance with the average apparent clearance of 5 L/h calculated from a recent pharmacokinetic study where different single oral doses of the main active metabolite, endoxifen, were administered in healthy subjects [60].

Plasma concentrations of tamoxifen and its major metabolites, notably endoxifen, are highly variable. The fluctuations in plasma levels can be attributed to different genetic and non genetic factors such metabolic capacities differences, co-medications and compliance. The covariates analysis in this population pharmacokinetics study allowed identifying and quantifying the contribution of these factors in the exposure of Tam and its principal phase I metabolites. Tam is metabolized to NDTam by CYP3A4/5 enzyme. This is considered as the main metabolic route of Tam that accounted in vitro for 92% of Tam metabolism. CYP3A4/5 catalyses, further, the formation of other metabolites from Tam and NDTam such as 4OHTam and N-didesmethyl-tamoxifen. Hence, increasing CYP3A4 activity, measured form midazolam metabolism, was found to be significantly associated with Tam total clearance and NDTam formation. Age, as a covariate, was associated to decreased Tam clearance. In the current study, a 10 years increase in age was associated with a 9% decrease in CL_{Tam}/F. This is in line with previously published pharmacokinetic data where higher Tam and NDTam levels were recorded in older patients [61, 62]. Compliance is a major concern in breast cancer patients under Tam and under-compliance has been linked to poorer treatment response. Measured compliance in our study was based on patients' self-reported compliance [63]. Patients with an average of 20% lower self reported-compliance had lower Tam plasma concentrations resulting in about 9% higher apparent Tam clearance. Due to the tendency of patients to exaggerate their treatment adherence when self-reporting [64], our result could under-estimate the effect of under-compliance on Tam and thus metabolites exposure.

4OHTam formation accounted, in vitro, for 7% of Tam primary metabolism. CYP2D6 and 2C9 are the main enzymes implicated in 4OHTam formation from Tam [16, 17]. In our study, we estimated that patient with null or reduced CYP2D6 phenotype (PM or IM) have 25% lower 4OHTam formation than normal or ultra-rapid CYP2D6 metabolizers. A trend toward a higher 4OHTam formation was observed in UM group. Possible explanations for the non observed differences between PM and IM and between EM and UM CYP2D6 phenotype groups could be on one hand the low number of patients in PM and UM groups and on the other hand the involvement of other CYP enzymes that compensate for the reduced CYP2D6 enzyme in PM patients [46]. CYP2C9 genotypes were not available and no major CYP2C9 interacting co-medications were identified. The use of pantoprazole or omeprazole/esomepazole did not show significant interaction with 4OHTam formation route. Besides, CYP2D6 inbibitors did not influence 4OHTam formation rate and this is an accordance with previous published reports indicating no impact of potent CYP2D6 inhibiting comedication of 4OHTam [6, 65].

CYP2D6 activity is the key and rate limiting enzyme involved in the formation of endoxifen form NDTam. As expected and previously reported [29, 46, 49, 66], impaired CYP2D6 activity and CYP2D6 inhibitors are the main factors influencing endoxifen formation. NDTam to endoxifen metabolic rate constant showed a *CYP2D6* gene-dose effect, with PM patients (*4/*4 genotype) having on average a 96% decrease in the endoxifen formation rate constant (k_{35}) and IM patients (29 out of 30 patients displaying *1/*4 genotype) having on average a 56 % lower k_{35}. The effects on k_{35} of potent and moderate CYP2D6 inhibitors use were comparable to those observed in patient with *CYP2D6* homozygous loss of functional allele (PM) and heterozygous loss of functional allele (IM), respectively. In our study, CYP2D6 phenotype explained 17% of the variability in endoxifen formation rate and CYP2D6 inhibitors 6% of this inter-individual variability.

Endoxifen levels depicted in the VPC plots, under Tam doses of 20 mg QD (Figure 4 D.) and 40 mg BID (Figure 4 H.) show that, in the majority of patients, doubling Tam dose could correct for endoxifen levels (above the 6 ng/mL threshold reported by Malensky et al. [29]). However, doubling Tam dose seems to be insufficient for some patients, either under potent CYP2D6 inhibitors or with a CYP2D6 PM phenotype. These patients may probably need higher Tam doses to reach suitable endoxifen exposure.

In conclusion, our joint population pharmacokinetics model showed that endoxifen levels vary widely between tamoxifen-treated patients (Table 1) and this variability was the highest observed in comparison to Tam and the other metabolites (precursor of endoxifen). Amongst the studied pharmacogenetics and non-genetic factors that could potentially explain overall variability in tamoxifen pharmacokinetics and endoxifen exposure, CYP2D6 phenotype and CYP2D6 inhibiting

comedications, were found to be the most influential ones. However, both of these factors explained only a third of total variability in endoxifen exposure, suggesting that other unaddressed variables either alone or in conjunction are likely to be involved in this inter-individual variability. In fact as demonstrated by other groups, polymorphisms affecting other Phase I and Phase II reaction enzymes, such as CYP3A4*22, CYP2C9*2 and *3 and SULT1A2*2 and 3, could modify endoxifen levels. It is of interest to test for the effect of these polymorphisms in this developed pharmacokinetic method in order to thoroughly understand and identify important sources of viability and quantify their impact on endoxifen levels. Nonetheless, the observed between-subject variability in tamoxifen and its metabolites and particularly endoxifen concentrations are most likely not only due to a single gene polymorphism but to the concurrency of multiple factors. Accounting for all of these sources of variability is difficult. Therefore, direct endoxifen levels measurement would be a better marker to identify patients with lower exposure (< 6 ng/mL) and thus at higher risk of suboptimal response to Tam therapy. Moreover, given the small intra-individual variability (< 20%), and the presence of a threshold for endoxifen predictive of Tam efficacy, endoxifen therapeutic drug monitoring constitute a good candidate for driving Tam dosage optimization strategy.

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Endoxifen levels after tamoxifen dose escalation: a prospective trial with genotyping, phenotyping and pharmacokinetics over 4 months.

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This report is part of the poster presented in Thirty-Fifth Annual CTRC-AACR San Antonio Breast Cancer Symposium-- Dec 4-8, 2012; San Antonio, TX

Own Contribution: bioanalytical measurements of plasma concentrations, data management, processing and statistical analysis, graphing, and interpretation of the results. Writing of the following report.

Cancer Res 2012;72(24 Suppl):Abstract nr P6-04-05.

CHAPTER VI - ENDOXIFEN LEVELS AFTER TAMOXIFEN DOSE ESCALATION: A PROSPECTIVE TRIAL WITH GENOTYPING, PHENOTYPING AND PHARMACOKINETICS OVER 4 MONTHS.

VI.1. ABSTRACT

Background: Retrospective studies assessing the impact of tamoxifen (Tam) metabolism and its active metabolite, endoxifen, on the efficacy of the treatment produced conflicting results. In the present study we assessed if the level of Tam metabolites could be improved by doubling Tam dose in breast cancer patients with any CYP2D6 phenotype.

Methods: This multicenter, prospective, open-label trial included patients treated with Tam for > 4 months. CYP2D6 activity was determined centrally by genotyping and phenotyping (dextromethorphan test). Liquid chromatography-tandem mass spectrometry assay was used to measure Tam, N-desmethyl-tamoxifen (N-D-Tam), 4-hydroxy-tamoxifen (4-OH-Tam) and endoxifen twice at baseline (Tam 20 mg/day), then at days 30, 90 and 120 after having increased the dose to 20 mg twice daily. Endoxifen increase and the differences between genotype-predicted phenotype groups were analyzed.

Results: 84 patients were analyzed. Steady-state concentrations for Tam and its metabolites were reached 30 days after doubling the dose. A range of 1.7 to 1.9 fold increase in plasma levels was observed with geometric mean plasma concentrations in ng/mL (CV%) were: at baseline and day 30 respectively 144 (41) and 269 (38) for Tam; 215 (41) and 387 (36) for N-D-Tam; 2.0 (45) and 3.5 (51) for 4-OH-Tam; 8.3 (74) and 14.8 (83) for endoxifen. The level of endoxifen increased 1.5 to 2 folds in all phenotype groups with geometric mean plasma concentrations in ng/mL (CV%): 3.1 (59) to 4.6 (63) in PMs ($p = 0.01$); 6.7 (47) to 12.4 (52) in IMs ($p < 0.0001$); and 10.0 (57) to 19.8 (60) in EMs ($p < 0.0001$). *CYP2D6* genotyping and phenotyping tests explained respectively up to 30% and 45 % of the variability in endoxifen levels and failed to identify EM and UM patients with low endoxifen exposure, corresponding to the lowest quintle of endoxifen concentrations in our study.

Conclusions: This is the first trial reporting the impact of the increase of Tam dose in all *CYP2D6* genotypes, including EMs. Dose escalation of Tam increased significantly the plasma level of endoxifen by similar ratio in all genotype subgroups. Because of a huge inter-individual variability genotyping and phenotyping are poor markers of endoxifen level. Very low endoxifen levels are observed even in patients classified as EM. Future trials aiming to improve the plasma level of endoxifen should consider direct measurement of endoxifen in plasma and adjust Tam dose according to the initial level of endoxifen independently of the genotype.

VI.2. INTRODUCTION

Tamoxifen is a widely used endocrine therapy in the treatment of early and advanced stage breast cancer (BC) in pre- and postmenopausal women and men. A 5-year adjuvant Tam treatment reduces BC recurrence by almost 39% and BC mortality by almost a third throughout 15 years of follow-up. It have been recently suggested that an extended adjuvant treatment to 10 years, further reduces recurrence and mortality [1]. However, almost 20 to 30% of patients either relapse or die from their disease [1-3]. One of the underlying reasons for a worse benefit to Tam treatment is an altered bioactivation of the parent drug into endoxifen [4, 5].

Actually, Tam is a pro-drug metabolized to 4-OH-Tam and mainly endoxifen, which have 30 to 100 times higher affinity and anti-estrogenic potency toward the estrogen receptors. Cytochrome 2D6 (CYP2D6) plays a key and rate-limiting role in the metabolism of Tam into endoxifen [6]. *CYP2D6* gene is highly polymorphic, with more than 100 allelic variants identified to date, and patients with null (PM) or reduced (IM) CYP2D6 activity display lower endoxifen concentrations and thus might experience lower benefit from their treatment [5, 7-9]. CYP2D6 is also subject to inhibition by some frequently used drugs such as selective serotonin reuptake inhibitors, frequently used to treat depression and relief hot flashes, the most-common Tam side effect. Some of these potent inhibitors have been shown to drastically decrease endoxifen concentrations to levels comparable to those observed in CYP2D6 PM patients [7, 10, 11]. Moreover, endoxifen levels are highly variable and *CYP2D6* genotyping alone or when adjusted to concomitant use of CYP2D6 inhibitors explained, respectively, only 40% and 46% of the total variability [12].

Therefore, genotyping may not be an optimal method to predict and to catch all sources of endoxifen level variably in patients. The assessment of CYP2D6 activity could also be performed with the use of the dextromethorphan phenotyping test. Dextrometorphan is indeed a substrate of CYP2D6 and the measurement of the dextromethorphan to dextrorphan metabolic ratio provides the level of the enzyme activity. This phenotyping test could be regarded as a surrogate marker to endoxifen exposure and is expected to better predict endoxifen concentrations variability.

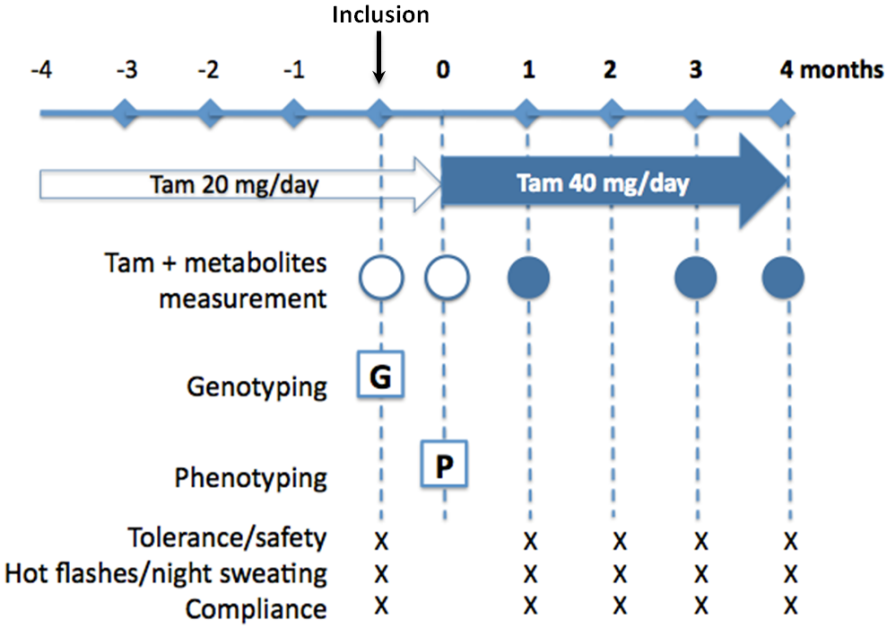
In this study, we sought to determine whether doubling Tam dose in all breast cancer patients, regardless of their CYP2D6 phenotype, would increase or correct low endoxifen concentrations that could be observed in the different *CYP2D6* genotype groups. We also compared the performances of *CYP2D6* genotyping and phenotyping in explaining and predicting endoxifen level variability.

VI.3. MATERIAL AND METHODS

VI.3.1. Patients and study design

Patients (men or women) diagnosed with hormone receptor-positive breast cancer and who were taking Tam 20 mg/day for at least 4 months (ensuring steady-state concentrations), as adjuvant therapy, were prospectively recruited in the study (ClinicalTrials.gov NCT00963209). Exclusion criteria were pregnancy, breastfeeding patient with known allergy for dextromethorphan and patients with history of deep venous thrombosis, pulmonary embolism, vaginal bleeding, endometriosis, endometrial hyperplasia / hypertrophy and/or polyps. This study protocol was approved by the Institutional Research Ethics Committees and all participants provided written informed consent. Enrolled patients were genotyped and phenotyped for CYP2D6 and their steady-state plasma levels of Tam and three of its metabolites were measured at inclusion date and on day 0 (i.e. baseline levels, before dose escalation). Tam dosage was then increased, in all patients, to 20 mg twice daily (40 mg /day) for up to 4 months. During this period, patients were followed every month for tolerance and side effects (hot flashes and night sweating), compliance (self-reported through a semi-quantitative questionnaire) and concomitant medications. Plasma concentrations of Tam and metabolites were also measured at months 1, 3 and 4 (Figure 1).

Figure 1: Study design



VI.3.2. CYP2D6 genotyping

Genomic DNA was extracted from EDTA blood samples with FlexiGene DNA extraction kit and QIAmp DNA Blood Mini Kit (QIAGEN, Hombrechtikon, CH). Genotyping of *CYP2D6* alleles*3, *4, *5, *6, *XN was performed by real-time polymerase chain reaction (rtPCR) according to the manufacturer's instructions (ABI PRISM 7000 Sequence Detection System; Applied Biosystems, Rotkreuz, Switzerland). Patients were categorized according to the number of functional alleles as CYP2D6 poor (PM=homozygous loss of functional allele), intermediate (IM=heterozygous loss of functional allele (*1/*3; *1 /*4; *1/*5 or *1/*6), extensive (EM=homozygous wild-type allele), or ultrarapid metabolizer (UM=multiple functional alleles). Heterozygous genotypes (*4/*XN) have been scored according to the dextromethorphan metabolic ratio test. Finally, a CYP2D6 activity score of 0 was assigned to PM, 1 for IM, 2 for EM and 3 for UM. CYP2D6 activity score was adjusted to 0 in patients under a potent CYP2D6 inhibitor.

VI.3.3. CYP2D6 phenotyping

Dextromethorphan metabolic ratio test was used for the determination of CYP2D6 activity, as previously described [13]. The night preceding the test, the patient was asked to take a pill of dextromethorphan hydrobromide (25 mg), before going to bed and to collect all his/her first urine of the morning (urine of 8 hours) and/or overnight. Urine samples were then analyzed by gas chromatography-mass spectrometry [14]. Patients were classified according to their dextromethorphan/dextrorphan (DM/DX) ratio as: PM ($DM/DX \geq 0.3$), IM ($0.03 \leq DM/DX < 0.3$), EM ($0.003 \leq DM/DX < 0.03$) and UM ($DM/DX < 0.003$).

VI.3.4. Tamoxifen and Metabolites measurement in plasma

Blood samples (5.5 mL) were collected, at random time after last drug intake, in potassium-EDTA tubes. Samples were centrifuged and plasma was stored at -80°C until analysis. Tamoxifen (Tam), N-desmethyl-tamoxifen (NDTam), 4-hydroxy-tamoxifen (4OHTam), and Endoxifen concentrations were measured simultaneously by a validated ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method [15]. The validated assay showed good precision (inter-day CV%: 2.5 – 7.8 %), accuracy (-1.4 to +5.8 %), sensitivity (lower limits of quantification comprised between 0.2 and 1 ng/mL) and selectivity (resolution of possible interfering isomers and metabolites). All measured Tam and metabolites levels, in patients, were above the lower limits of quantifications of the assay.

VI.3.5. Statistical analyses

Only patients with trough plasma concentrations, drawn 6 to 31 hours after last drug intake, were selected for the analysis. Tam and metabolites concentrations were log-transformed to normalize their distribution. Endoxifen levels were grouped into quintiles, independently of the CYP2D6 phenotype (CYP2D6 activity score). The relative increase in endoxifen levels after a doubling of Tam dose as well as the difference in increase according to the CYP2D6 activity scores or the endoxifen levels quintiles were evaluated either by a paired t-test and ANOVA. Exact McNemar's test was used to compare the effectiveness of achieving endoxifen levels above the defined threshold of 6 ng/mL based either on the genotyping or on the percentile strategy. All data analyses were conducted with Stata statistical software (StataCorp. 2011. *Stata Statistical Software: Release 12*. College Station, TX: StataCorp LP).

VI.4. RESULTS

VI.4.1. Patient's characteristics

A total of 96 patients were recruited in the study at the time of analysis. Only 84 patients, with Tam and metabolites trough plasma levels, drawn 6 to 31 hours after last dose intake, were eligible for the analysis (Table 1). One patient with an IM genotype-predicted phenotype was under paroxetine (potent CYP2D6 inhibitor) during the whole study course. The dextromethorphan test for this patient predicted a PM phenotype, therefore, the CYP2D6 activity score (AS) was corrected accordingly. A second IM (genotype predicted phenotype) patient started paroxetine at the last month (month 4) of the study protocol. Therefore, plasma measurements from this month were discarded. The results from one UM patient with low and inconsistent changes in Tam and its metabolites levels (probably due to unreported non-compliance) had not been considered for the analysis. All patients reported good treatment adherence (> 80%).

Four patients (5%) were classified as PM (CYP2D6 activity score of 0), 25 patients (30%) as IM (AS of 1), 53 patients (63%) as EM (AS of 2) and 2 patients (2%) as UM (AS of 3).

Table 1: Characteristics of patients

Parameters		Values
Median age (range)		50 (33-78)
Origin	Caucasian North African Indian	82 1 1
Gender	Male Female	1 83
Histology	ductal invasive lobular invasive ductal in situ others	54 15 5 10
Co-medication	weak CYP2D6 inhibitor (venlafaxine) moderate CYP2D6 inhibitor (es/citalopram) potent CYP2D6 inhibitor (paroxetine)	7 6 2
Adherence to tamoxifen at baseline	≥ 95% 80-95%	81 3
Phenotype (activity score*)	PM (0) IM (1) EM (2) UM (3)	3 (4) 26 (25) 53 2

*CYP2D6 phenotype predicted from genotyping test and corrected for potent CYP2D6 inhihitors.

VI.4.2. Plasma concentrations of tamoxifen and its metabolites

VI.4.2.1. Time to reach steady-state after increasing tamoxifen daily dose to 40 mg

Total mean plasma concentrations of Tam and its metabolites, at baseline (day 0 under 20 mg daily dose) and at the end of month 1 (day 30), 3 (day 90) and 4 (day 120) after doubling Tam dose were compared. Over the 84 patients available for the analysis, only 60 patients had complete plasma concentrations data for at least each of the follow-up visits at day 0, day 30 and 90. The data collected from these 60 patients were used to compare changes in Tam and metabolite levels before and throughout the study course after dose escalation.

Data selected from 60 patients (over 84) with successive plasma levels, from at least day 0, day 30 and day 90, showed that Tam and metabolites concentrations significantly increased after dose escalation (Table 2 and Figure 2). At the 1st month, concentrations already increased by 1.7 to 1.8 fold in all patients, regardless of their CYP2D6 phenotype.

There were no significant difference between mean plasma levels for Tam and metabolites, and most notably endoxifen levels, at month 1, 3 and 4. This could suggest that, in the majority of patient, steady-state levels are almost reached after the 1st month under 40 mg/day Tam (Figure 2).

Table 2: Tamoxifen and metabolites levels after dose increase

Drug and metabolites	Follow-up date (Day)	Geometric mean (ng/mL)	CV (%)	Concentration ratio: day 0 / day30 (CV%)
Tamoxifen	(d0)	144	41	1.9 (22)
Tamoxifen	(d30)	269	38	
N-D-Tam	(d0)	215	41	1.8 (21)
N-D-Tam	(d30)	387	36	
4-OH-Tam	(d0)	2.0	45	1.7 (23)
4-OH-Tam	(d30)	3.5	51	
Endoxifen	(d0)	8.3	74	1.8 (28)
Endoxifen	(d30)	14.8	83	

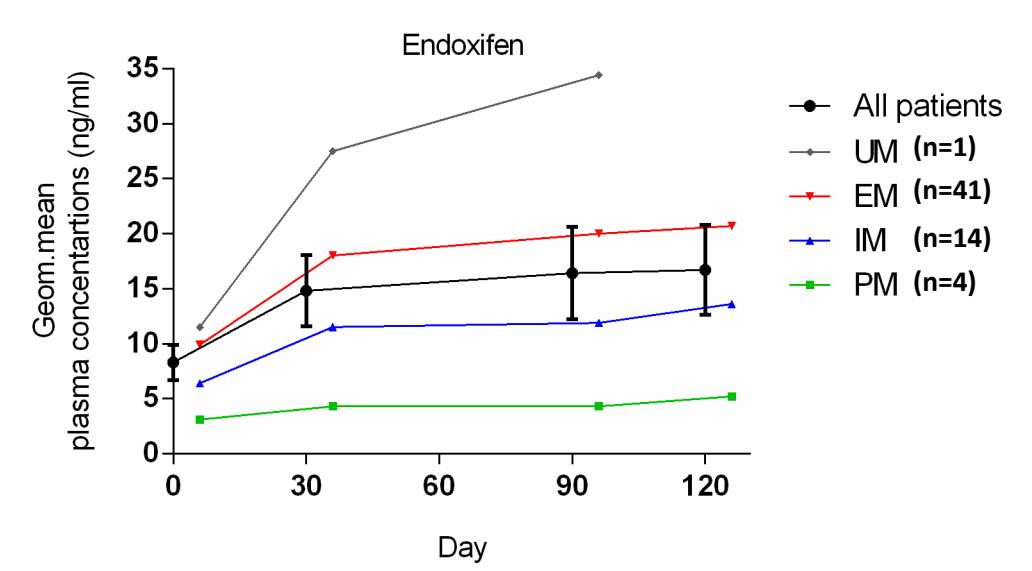


Figure 2: Changes in endoxifen concentrations before (day 0) and after 1, 3 and 4 months of doubling Tam dose. Data are expressed as geometric mean, 95%CI bars.

VI.4.2.2. Effect of tamoxifen dose increase on endoxifen levels

Endoxifen steady-state concentrations seem to be achieved after the 1st month of therapy at the daily Tam dose of 40 mg. Therefore, all the available data on endoxifen plasma levels measured after day 30 were pooled and compared again to baseline concentrations (20 mg/day regimen).

Comparison of baseline (20 mg) and pooled steady-state endoxifen levels (under 40 mg), measured in the whole study population (n=84 patients), showed that endoxifen concentrations increased significantly in all CYP2D6 phenotype groups (Table 3) by 47 % in PM (AS = 0), 85 % in IM (AS=1), 99 %

in EM (AS =2), 27 % in UM (AS = 3). Mean endoxifen concentration consistently increased in all CYP2D6 phenotype groups and almost doubled in all phenotype groups but not in PM patient, where the increase was significantly lower than 2 fold (p = 0.02). Comparison of endoxifen levels in EM patients under baseline treatment to those observed in IM and PM patients after doubling Tam dose (Figure 3), showed that the difference in mean endoxifen levels between IM patients (40 mg dose) and EM patients (20 mg dose) was not longer statistically significant (p = 0.3).

Table 3: Endoxifen level at steady-state (Css) in each phenotype group with Tam 20 mg/day and 40 mg/day.

	Geometric mean (CV %)				Ratio: Css 40 mg / Css 20 mg (CV %)					$P_{(anova)}$
Activity Score (n)	0 (n=4)	1 (n=25)	2 (n=53)	3 (n=2)	All patients	0	1	2	3	
Endoxifen (Css 20mg)	3.1 (59%)	6.7 (47%)	10 (57%)	13.3 (23%)	1.92 (28)	1.47 (19)	1.85 (27)	1.99 (27)	2.27 (27)	0.07
Endoxifen (Css 40mg)	4.6 (63%)	12.4 (52%)	19.8 (60%)	30.3 (3%)						
P values	0.01[a]	<0.0001[a]	<0.0001[a]	0.06[a]	0.4[b]	0.02[b]	0.15[b]	0.8[b]	0.5[b]	-

[a] *P value* associated to a paired t-test.

[b] *P value* associated to a one-sample t-test (H_0 : mean ratio Css 40 mg/ Css 20mg equals 2).

VI.4.2.3. Endoxifen levels variability explained by genotyping and phenotyping tests

At both Tam regimens, highly variable steady-state endoxifen plasma concentrations were observed between and within each CYP2D6 phenotype groups. Endoxifen concentrations were statistically different between PM, IM and EM patients, except for EM and UM. CYP2D6 phenotype expressed as CY2D6 activity score explained only 30 % and 38 % of endoxifen levels variability under daily Tam doses of 20 mg and 40mg, respectively (Figure 3).

The correlation between endoxifen levels and the dextromethorphan metabolic ratio as a continuous variable performed better than the genotype-based CYP2D6 AS, and explained 45% of endoxifen variability. Comparison of the genotyping and the phenotyping tests showed that there is a good agreement between both methods in predicting PM phenotype. However, for the IM, EM and UM phenotypes prediction, the two test yielded discordant results (Figure 4), with IM (n=25 from genotyping vs. n=8 from phenotyping test), EM (53 vs. 31) and UM (2 vs. 41).

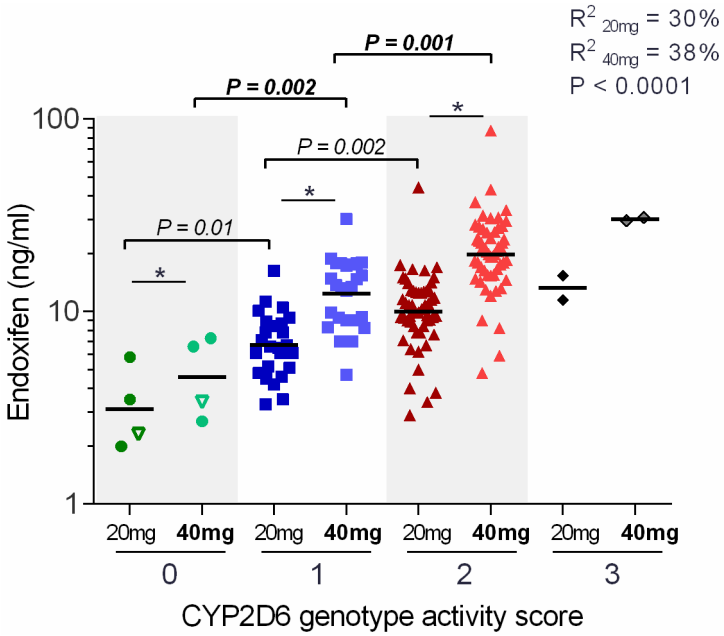


Figure 3: Steady state endoxifen levels in 84 patients stratified by CYP2D6 activity score, before and after Tam dose escalation. (Horizontal bars correspond to the geometric means plotted on a semi-logarithmic scale; green circles: PM, inverted open triangle: IM patient under paroxetine, blue rectangles: IM, red triangles: EM, diamonds: UM).

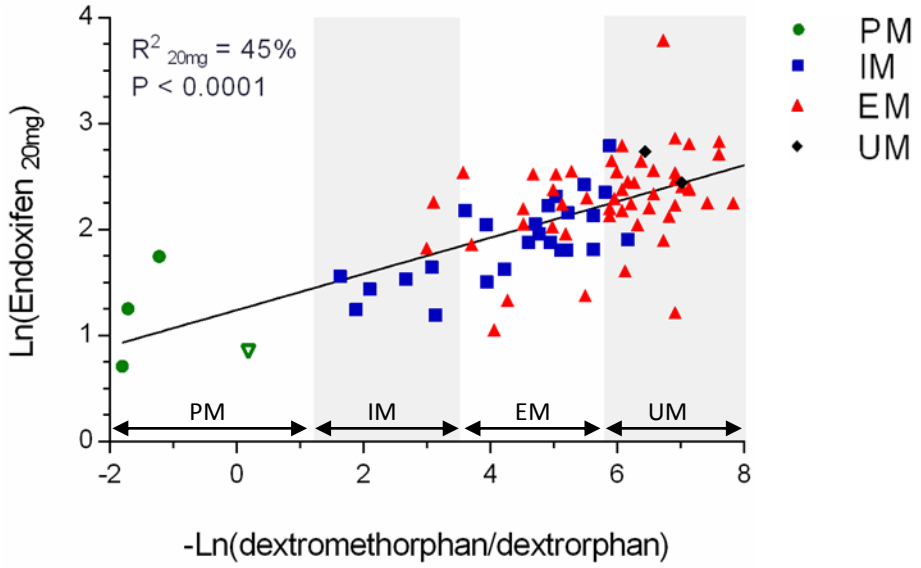


Figure 4: Correlation between endoxifen levels and dextromethorphan metabolic ratios. Patients' points are depicted according to the genotype-based CYP2D6 activity score with green circles: PM, inverted open triangle: IM patient under paroxetine, blue rectangles: IM, red triangles: EM, diamonds: UM. The alternate shaded and unshaded areas correspond to the defined intervals of the DM/DX ratios for phenotype classification.

VI.4.2.4. Endoxifen levels stratified by percentiles

All PM patients (or with AS = 0) had endoxifen concentrations within the lowest endoxifen levels percentile (≤ percentile 20) with concentrations ranging from 2.0 to 5.8 ng/mL (Table 4). Endoxifen levels showed higher variability in the lowest and higher percentiles (≤ P20 and ≥ P80). Increasing Tam daily dose from 20 mg to 40 mg almost doubled mean endoxifen levels in the different percentile groups. This mean increase in the active metabolite concentrations was constant and ranged from 1.8 to 2.1 fold.

Table 4: Changes in endoxifen levels per percentile after doubling tamoxifen dose

Tamoxifen 20 mg /day			Ratio Css 40mg / Css 20mg (CV %)
Endoxifen	**Median (Range) ng/mL**	**Geometric mean (CV%) ng/mL**	
≤Percentile 20	3.9 (2.0-5.8)	3.9 (26)	1.8 (31%)
P20 – P40	6.7 (6.1-8.4)	6.9 (10)	2.1 (18%)
P40 – P60	9.2 (8.4-9.9)	9.2 (5)	1.9 (32%)
P60 – P80	11.1 (10-12.6)	11.2 (7)	2.0 (33%)
≥P80	15.1 (12.7-44.2)	16.5 (44)	1.8 (23%)
P values	-	-	$P_{(anova)} = 0.3$

VI.4.3. Genotype-guided versus therapeutic drug monitoring-guided tamoxifen dose optimization

As reported by Madlensky et al. [5], patients with endoxifen plasma levels lower than 6 ng/mL (i.e ≤ P20) have a higher chance of breast cancer recurrence. We sought to assess the effectiveness of genotyping/phenotyping strategy in detecting and correcting low endoxifen levels (< 6 ng/mL) in patients with reduced CYP2D6 activity (PM and IM patients) and compared this strategy to the direct measurement of endoxifen concentrations before dose adjustment.

In the genotype/phenotype-guided Tam dose increase approach (Figure 5), 76 over the 84 patients of the study (91 %) would have their endoxifen concentrations above the defined 6 ng/mL threshold (i.e. ≥ P20). 5 patients in the EM and UM groups with low endoxifen groups would have been missed. 17 patients would have an unjustified increase in Tam dose. With the direct endoxifen levels measurement approach (Figure 6), 80 over 84 patients (95%) would have their endoxifen concentrations above the 6 ng/mL threshold after dose adjustment to 40 mg daily. Although the difference between both approaches did not reached significance (95% success vs 91%, p = 0.1), direct monitoring of endoxifen levels avoided unjustified dose increase and allowed to identify all patients with low endoxifen levels.

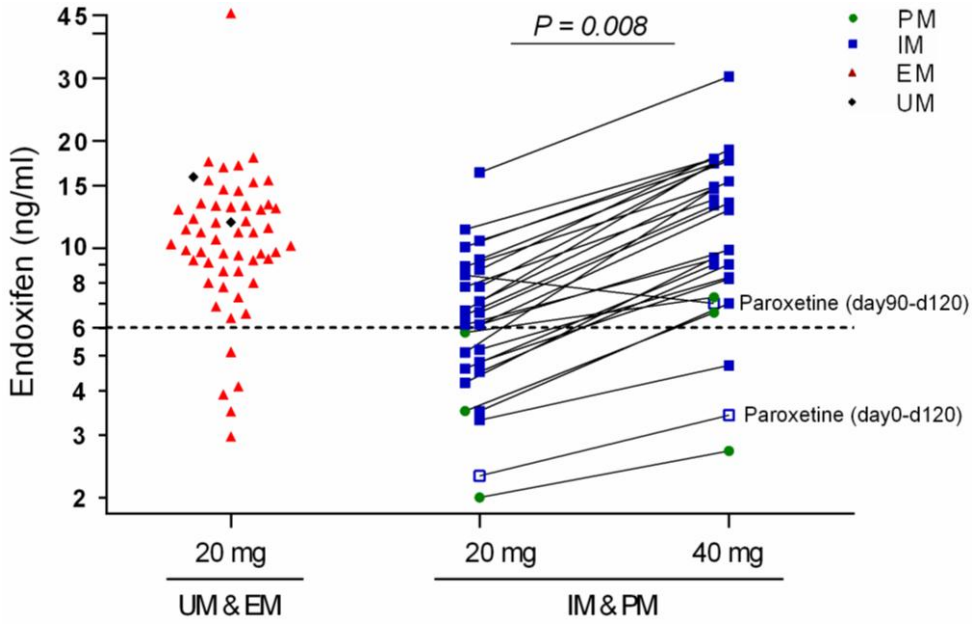


Figure 5: Genotype/phenotype-guided Tam dose increase: genotype/phenotype misses some EM patients with very low endoxifen level (< 6 ng/mL threshold). An increase of Tam dose would be offered only to patients classified as IM and PM. (green circles: PM, open square: IM patient under paroxetine, blue square: IM, red triangles: EM, black diamond: UM).

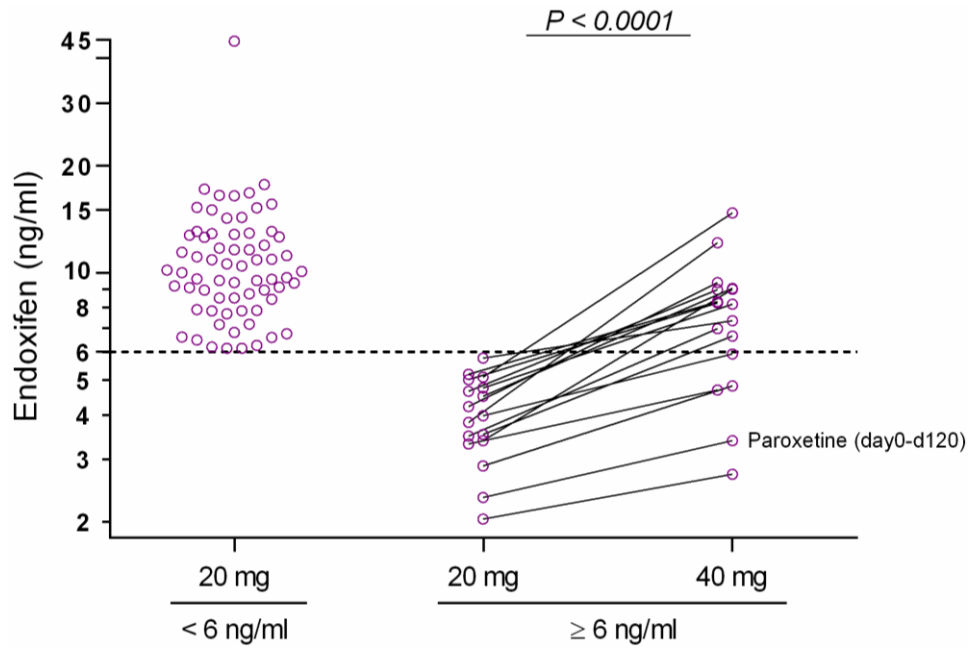


Figure 6: Monitoring endoxifen levels approach: identify all patients who may benefit from an increase of Tam dose.

VI.5. DISCUSSION

Endoxifen concentrations vary widely between and within each CYP2D6 phenotype groups and CYP2D6 phenotype, either through genotyping or phenotyping (dextromethorphan test) tests, explains only a minor part of this variability (30 to 45%). Our results are in agreement with previously published data where *CYP2D6* genotyping has been reporter to explain roughly 20 to 40 % of the variability in endoxifen levels [5, 9, 12, 16] Dextromethorphan metabolic ratio test, used as a continuous variable, performed better that *CYP2D6* genotyping but its contribution as a predictor of endoxifen concentrations remained modest and explained only 45% of the total endoxifen inter-individual variability. This observation is concordant with a recent reported data where dextrompthorphan levels have been found to explain 50% of endoxifen exposure [17].

Increase in Tam daily dosage to 40 mg significantly increases mean endoxifen plasma levels by almost 2 fold, in all phenotype groups, with the exception of PM patients. In IM patients, doubling Tam dose resulted in mean endoxifen concentrations comparable to those observed in EM patient under the standard 20 mg daily Tam dose. This suggests the feasibility of dose adjustment in patient with reduced CYP2D6 activity and confirms the observations from two recent genotype-guided dose optimization studies [18, 19].

However, *CYP2D6* genotyping and or phenotyping may not be the most appropriate surrogate markers of endoxifen exposure for individual patients as they have been shown to be modest predictor of endoxifen levels. Moreover, we have demonstrated from the preliminary results of our study that *CYP2D6* genotyping do not seem to be the optimal approach for the detection and correction of endoxifen plasma levels (above the 6 ng/mL threshold) as it fails to identify EM and UM patients with low endoxifen exposure and therefore at higher risk of suboptimal response despite their full CYP2D6 activity. In addition to identifying all patient with problematic exposure to endoxifen, direct endoxifen monitoring has the advantage to avoid unjustified dose increase in some patients and thus to avoid overtreatment of patients with the risk of exacerbating some adverse event and possibly disfavoring treatment adherence.

All PM patients in our study had endoxifen concentrations within the lowest percentile (≤ percentile 20) where concentrations ranged from 2.0 to 5.8 ng/mL. The range of endoxifen concentrations observed in this lowest quintile was in good agreement with those reported from a larger cohort study, suggesting that the levels measured in our study are representative of a larger population [5].

In conclusion, the observations from the preliminary results of our study indicated that direct endoxifen monitoring might represent a better approach to evaluate actual endoxifen exposure in an individual, and to adjust dosage regimen consequently. Doubling Tam dose seems to be insufficient

to correct endoxifen plasma levels in some patients. These patients may therefore benefit from higher Tam doses to reach suitable endoxifen exposure. Larger therapeutic monitoring studies are, therefore, needed to confirm the superiority of endoxifen monitoring,establish therapeutic endoxifen thresholds associated with optimal treatment outcomeds and define Tam doses that could be safely used for adjusting treatment.

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CHAPTER VII - DISCUSSION AND PERSPECTIVES

The non-steroidal selective estrogen receptor modulator (SERM), tamoxifen (Tam), was the first targeted cancer drug approved since 1973 and 1998, respectively, for the treatment and prevention of hormone-sensitive breast cancer [1, 2]. Even with the recent advent of aromatase inhibitors that proven superiority in post-menopausal women, Tam is still a gold standard therapy in pre-menopausal patients and remain a valid option, in post-menopausal women [3, 4]. Tam has been shown effective in reducing breast cancer (BC) recurrence and mortality in the adjuvant and metastatic settings and to reduce the incidence of primary BC in high risk women, in the preventive setting. However, the clinical response to Tam varies widely among patients, with 30 to 40% of patient relapse and/or become resistant to Tam [5-7]. One of the proposed mechanisms underlying resistance is an altered metabolic bioactivation of Tam into endoxifen. In fact, since the recent findings of the last decade demonstrating that endoxifen is the most abundant Tam active metabolite with 30 to 100 fold more potent anti-estrogenic activity, Tam has been considered as a pro-drug, which full pharmacological activity is thought to be dependent on its bioconversion by CYP2D6 into endoxifen [8].

CYP2D6 is a rate-limiting enzyme catalyzing the conversion of tamoxifen into endoxifen and impaired CYP2D*6* activity, either through *CYP2D6* gene polymorphism and/or interaction with potent CYP2D6 inhibitors, has been reported to decrease the levels of endoxifen and to predict suboptimal response in Tam treated patients. The evident crucial role of CYP2D6 enzyme in endoxifen formation and the results from early retrospective pharmacogenetic (PG) studies, reporting an increased risk of BC recurrence under Tam in patient carrying variant *CYP2D6* alleles, has brought many researchers and clinicians to consider the potential role for *CYP2D6* genotyping either for individualizing anti-estrogen therapy with the choice of alternative therapy for PM and IM patients or to guide Tam dosage optimization [9]. However, endoxifen levels are highly variable between patients and *CYP2D6* genotyping explains only part (20 to 40%) of this variability as a large inter-patient variability in endoxifen concentrations is still observed even within the different genotype-predicted CYP2D6 phenotype groups [10, 11]. In fact, this residual variability may depend on the activity of other genetic (e.g. other CYPs, SULTs and UGTs, transporters) and non genetic factors (e.g. age, BMI, compliance and interacting comedications). Therefore *CYP2D6* genotyping does not seem to be an optimal choice for predicting endoxifen levels, driving Tam dosage optimizations and predicting Tam treatment efficacy.

In that purpose, we sought, in the frame of a prospective, open-label trial, to first determine how the increase of Tam dose influences the levels of endoxifen in the different CYP2D6 phenotype groups (PM, IM, EM and UM) in order to evaluate the feasibility and impact of dose adjustment on the level of the active metabolites, notably endoxifen and in a second time we tried to characterize the population pharmacokinetic (PK) of Tam, endoxifen and its precursor metabolites in order to quantify the inter- and intra-individual variability affecting their plasma concentrations and study the influence of different pharmacogenetic and non-genetic factors that may predict this variability.

In that perspective we first developed and fully validated a specific and sensitive analytical method, based on a high-performance liquid chromatography coupled to tandem mass spectrometry, that enabled reliable and sensitive monitoring in patients' plasma of Tam and three of its clinically relevant metabolites, namely N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen (4-OH-Tam) and endoxifen. Our method provided an excellent chromatographic resolution of Tam and seven known and previously unreported metabolites in a relatively short gradient program of 13 min. Given the extensive metabolism of Tam in hydroxylated metabolites, we focused on method selectivity and on the effective separation on potentially interfering Tam metabolites. Actually, some of these metabolites are either geometric isomers (Z and E isomers) or position isomers (such as 3-hydroxy-tamoxifen, 4'-hydroxy-tamoxifen and 4'-hydroxy-N-desmethyl-tamoxifen) of the measured hydroxylated metabolites, 4-OH-Tam and endoxifen, and could thus interfere with their plasma measurement. This allowed us to report for the first time the occurrence of 4'-hydroxy-tamoxifen and 4'-hydroxy-N-desmethyl-tamoxifen in patients' plasma and to estimate their levels in a subset of BC patients [12]. These metabolites, unlike 4-OH-Tam and endoxifen, have been demonstrated later to be devoid from anti-estrogenic activity. Therefore, the chromatographic resolution of such interfering metabolites is of paramount importance to ensure reliable and accurate bioanalytical methods and could explain the discrepant laboratories data from some PK studies with twice or even higher fold differences in reported median concentrations of endoxifen [11]. Our measured Tam and metabolites levels in patients from our prospective trial, are similar to recent reports from larger studies [11, 13].

Results from our prospective trial were also in concordance with the major published data from the different PK-PG studies confirming the limited contribution of *CYP2D6* genotyping in describing the large variability in endoxifen concentrations [10, 11, 14]. Indeed, our results from univariate and multivariate population PK analysis showed that genotype-predicted CYP2D6 activity either alone or when considering for major confounders, such as CYP2D6 inhibiting comedications, explained roughly 20% to 30% of the inter-patient variability in endoxifen levels. A significant variability in endoxifen levels still exist within CYP2D6 phenotype groups. Dextromethorphan metabolic ratio test,

used as a continuous variable, performed better that *CYP2D6* genotyping but its contribution as a predictor of endoxifen concentrations remained also modest and explained only 45% of the total variability. This observation was concordant with a recent reported data where dextrompthorphan levels have been found to explain 50% of endoxifen exposure [15].

Our results pertaining to the feasibility and impact of Tam dose escalation to a daily dose of 40 mg (20 mg twice daily) showed that Tam dose increase was associated to a significant change, within 1 month, in mean steady-state endoxifen plasma levels by almost 2 fold, in all CYP2D6 phenotype groups, with the exception of PM patients. Of interest, we observed that doubling Tam dose in IM patients (with heterozygous loss of *CYP2D6* functional allele) resulted in similar endoxifen concentration to those observed in EM patient (CYP2D6 normal metabolizers) on the standard 20 mg daily Tam dose. This suggests the feasibility and effectiveness of dose adjustment to correct endoxifen exposure in patients with reduced CYP2D6 activity and was in agreement to recent genotype-guided dose escalation studies [16, 17]. However, *CYP2D6* genotyping is a modest predictor of endoxifen levels and an overlap in endoxifen levels have been observed between the different CYP2D6 phenotype groups.

On the light of a recent study by Madlensky et al. [13] suggesting a threshold concentration for endoxifen (of about 6 ng/mL) above which lower disease recurrence was observed, we assessed the effectiveness of *CYP2D6* genotype testing in detecting and correcting low endoxifen levels (< 6 ng/mL) in patients with reduced CYP2D6 activity (PM and IM patients) and compared this genotype-guided strategy to the direct measurement of endoxifen concentrations before dose adjustment. We found that *CYP2D6* genotyping was, indeed, not the optimal choice to predict endoxifen exposure as it fails to identify EM and UM patients with low endoxifen exposure and therefore at higher risk of suboptimal response despite their full CYP2D6 activity. In addition to identifying all patient with problematic exposure to endoxifen (e.g. in PM patients, due to drug-drug interaction, noncompliance or other unexplored mechanism), direct endoxifen monitoring had the advantage to avoid unjustified dose increase in some patients and thus to avoid overtreatment of patients with the risk of exacerbating some adverse event and possibly disfavoring treatment adherence.

All PM patients in our study had endoxifen concentrations within the lowest endoxifen levels percentile (≤ percentile 20) with concentrations ranging from 2.0 to 5.8 ng/mL and this was in total agreement and confirm the results from the larger cohort study by madlensky et al. [13]. Besides, we have observed in some these patients within the lower percentile (< 6 ng/mL), that doubling Tam dose seems to be insufficient to correct their endoxifen plasma levels. These patients may therefore benefit from higher Tam doses to reach suitable endoxifen exposure.

In conclusion, the observations from our study in conjunction with the published results from other groups, definitely demonstrate and confirm the strong rational and superiority of direct endoxifen monitoring for detecting patients at risk of suboptimal Tam treatment and driving Tam dosage optimization.

Future perspectives

Increasing Tam dose to 30 mg/day or 40 mg/day in IM and PM, in genotype-guided dose-adjustment studies, have been proven to be safe and well tolerated with no significant increase in frequent side effects of Tam such as hot flashes, diaphoresis, vaginal discharge or other more severe and less frequent side effects such as thromboembolic events and vaginal bleeding [16-18]. However, all these studies have followed patients over a limited period of time (4 months at maximum) and could not inform on the cumulative toxicity and safety of higher than 20 mg Tam dose over a 5 years of adjuvant Tam treatment. Moreover, the optimal dose needed for some patients to achieve therapeutic levels of endoxifen is not known. So far, only one study has studied the association between endoxifen concentration and disease outcome in Tam-treated BC patients and identified an endoxifen therapeutic threshold level (6 ng/mL).

Therefore, large randomized controlled therapeutic drug monitoring (TDM) studies are warranted to replicate and confirm these finding and establish therapeutic endoxifen thresholds associated with optimal response and in order to define Tam doses that could be safely used for optimizing treatment outcomes.

Our developed population pharmacokinetic model could be used to support such (TDM) studies, throughout model-based forecasting to derive dose optimization strategies. Data from these large TDM studies would also allow to update our population pharmacokinetic model and integrate other possible sources of pharmacokinetics variability (polymorphisms in CYP3A4, CYP2C9, CYP2B6, UGTs, SULTs, P-gp) to bring a comprehensive insight into Tam and its metabolites levels variability.

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APPENDIX

Journal of Chromatography B, 878 (2010) 3402–3414

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

An ultra performance liquid chromatography–tandem MS assay for tamoxifen metabolites profiling in plasma: First evidence of 4′-hydroxylated metabolites in breast cancer patients

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ARTICLE INFO

Article history:
Received 19 July 2010
Accepted 26 October 2010
Available online 30 October 2010

Keywords:
Ultra performance liquid chromatography
Tandem mass spectrometry
Tamoxifen
N-Desmethyl-tamoxifen
4-Hydroxy-tamoxifen
4-Hydroxy-*N*-desmethyl-tamoxifen (endoxifen)
4′-Hydroxy-tamoxifen
4′-Hydroxy-*N*-desmethyl-tamoxifen
Breast cancer

ABSTRACT

There is increasing evidence that the clinical efficacy of tamoxifen, the first and most widely used targeted therapy for estrogen-sensitive breast cancer, depends on the formation of the active metabolites 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethyl-tamoxifen (endoxifen). Large inter-individual variability in endoxifen plasma concentrations has been observed and related both to genetic and environmental (i.e. drug-induced) factors altering CYP450s metabolizing enzymes activity. In this context, we have developed an ultra performance liquid chromatography–tandem mass spectrometry method (UPLC–MS/MS) requiring 100 μL of plasma for the quantification of tamoxifen and three of its major metabolites in breast cancer patients. Plasma is purified by a combination of protein precipitation, evaporation at room temperature under nitrogen, and reconstitution in methanol/20 mM ammonium formate 1:1 (v/v), adjusted to pH 2.9 with formic acid. Reverse-phase chromatographic separation of tamoxifen, *N*-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethyl-tamoxifen is performed within 13 min using elution with a gradient of 10 mM ammonium formate and acetonitrile, both containing 0.1% formic acid. Analytes quantification, using matrix-matched calibration samples spiked with their respective deuterated internal standards, is performed by electrospray ionization–triple quadrupole mass spectrometry using selected reaction monitoring detection in the positive mode. The method was validated according to FDA recommendations, including assessment of relative matrix effects variability, as well as tamoxifen and metabolites short-term stability in plasma and whole blood. The method is precise (inter-day CV%: 2.5–7.8%), accurate (−1.4 to +5.8%) and sensitive (lower limits of quantification comprised between 0.4 and 2.0 ng/mL). Application of this method to patients' samples has made possible the identification of two further metabolites, 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen, described for the first time in breast cancer patients. This UPLC–MS/MS assay is currently applied for monitoring plasma levels of tamoxifen and its metabolites in breast cancer patients within the frame of a clinical trial aiming to assess the impact of dose increase on tamoxifen and endoxifen exposure.

1. Introduction

Tamoxifen (*Z* isomer) (Fig. 1) is a standard hormonal therapy currently used for the secondary treatment of hormone-responsive breast cancer [1–6] and for the prevention in women at high risk of developing the disease [7]. Tamoxifen is a non-steroidal selective estrogen receptor modulator (SERM), which competitively binds to estrogen receptors (ERs) and inhibits estrogen-dependent growth and proliferation of malignant breast epithelial cells [1,6]. However, several lines of evidence indicate that the overall anti-proliferative effects of tamoxifen depends notably on the formation of the clinically active metabolites 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethytamoxifen (endoxifen) (B and E in Fig. 1) which have 100-fold greater affinity to ERs and 30–100-fold greater potency in suppressing breast cancer cell proliferation as compared to the parent drug [8–12].

Tamoxifen can thus be considered a quasi-prodrug that is extensively metabolised by several polymorphic cytochrome P450 (CYP)

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doi:10.1016/j.jchromb.2010.10.027

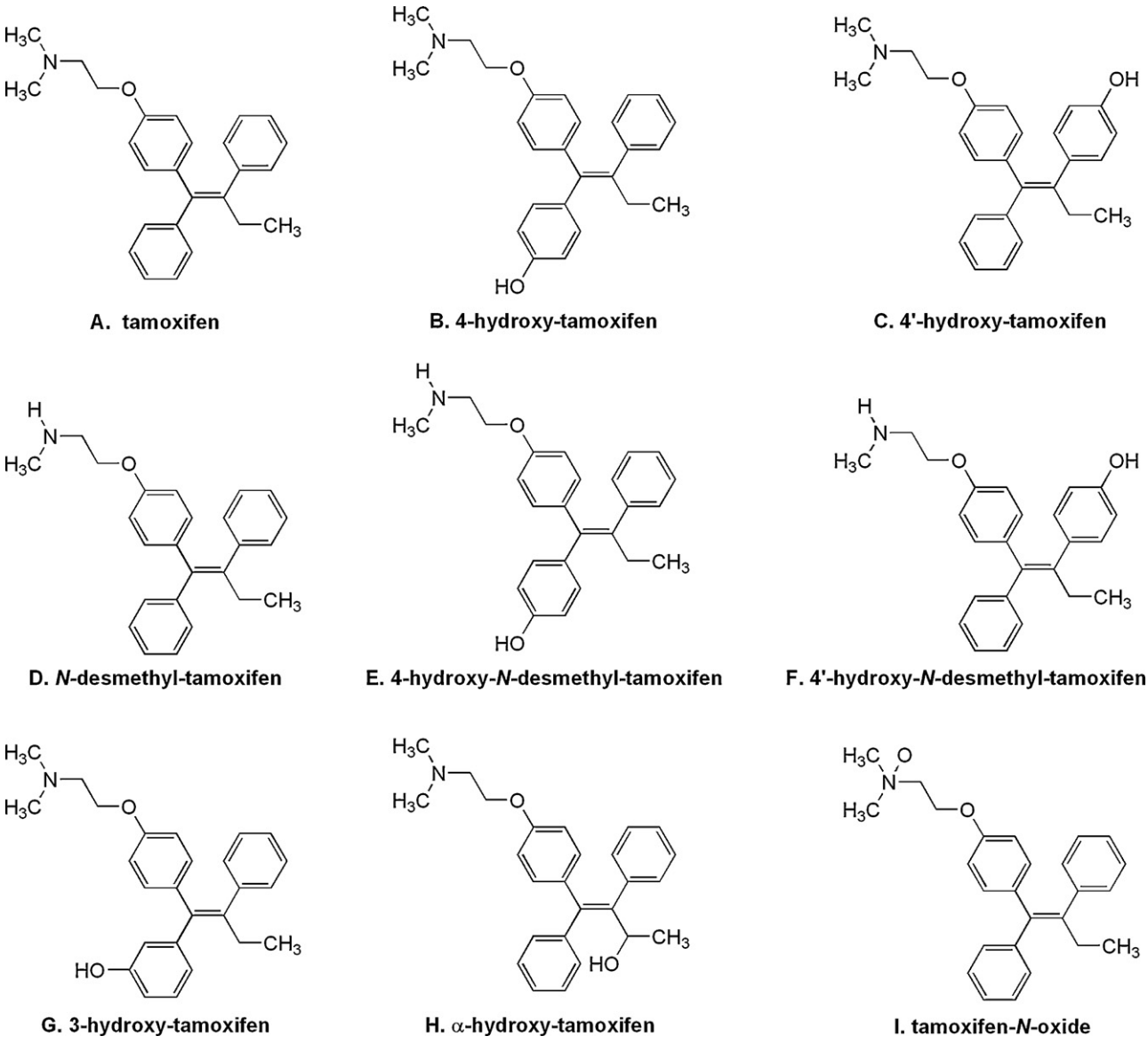

Fig. 1. Chemical structures of the tamoxifen and its three major metabolites studied: (A) tamoxifen; (B) 4-hydroxy-tamoxifen; (C) 4′-hydroxy-tamoxifen; (D) *N*-desmethyl-tamoxifen; (E) 4-hydroxy-*N*-desmethyl-tamoxifen (endoxifen); (F) 4′-hydroxy-*N*-desmethyl-tamoxifen; (G) 3-hydroxy-tamoxifen; (H) α-hydroxy-tamoxifen; (I) tamoxifen-*N*-oxide.

enzymes into its active metabolites 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethytamoxifen (endoxifen) [1]. Briefly, tamoxifen is primarily oxidized to *N*-desmethyl-tamoxifen (the most abundant metabolite in human plasma) and 4-hydroxy-tamoxifen predominantly by CYP3A4/5 and CYP2D6, respectively, followed by endoxifen formation from *N*-desmethyl-tamoxifen, exclusively catalyzed by CYP2D6 and from 4-hydroxy-tamoxifen by CYP3A4/5. Tamoxifen and its metabolites undergo further glucuronidation and sulphation [13,14].

Endoxifen is considered to be responsible for an important part of the *in vivo* pharmacological activity of tamoxifen, as endoxifen plasma concentrations are about 5–10-fold higher than those of 4-hydroxy-tamoxifen, with a different mode of action for endoxifen being suggested [8,10,15].

The clinical outcomes of tamoxifen treatment in terms of efficacy and side effects are inconstant, and some patients either fail to respond or become resistant to tamoxifen therapy [14,16,17]. One of the proposed mechanisms explaining the impaired response to tamoxifen therapy is an altered bio-activation into endoxifen by genetic or environmental factors. A polymorphism in CY2D6 enzymes that catalyze this conversion has been reported to influence the blood level of endoxifen [14,18–21] and, in some retrospective studies, to predict clinical outcomes in patients [14,21–25]. This has prompted the consideration of a potential role for CYP2D6 genotype/phenotype testing in patients' management, which remains controversial, however [26–34]. In fact, large inter-patient variability in endoxifen levels still subsists even after correcting for CYP2D6 status [18,27]. The remaining variability may depend on the activity of other cytochromes (CYP3A4/5, 2C9, 2C19), some of them known to be polymorphic, and on the influence of environmental factors such as interacting co-medications, among others. Of importance are some selective serotonin reuptake inhibitors (SSRIs) with strong CYP2D6 inhibiting activity, such as paroxetine and fluoxetine advised formerly to treat tamoxifen-induced hot flashes or depression are known to influence tamoxifen bioactivation [10,28,29].

The plasma concentration of the active metabolites of tamoxifen (mainly endoxifen and 4-hydroxy-tamoxifen) corresponding to the final phenotypic trait, may therefore represent a better predictor of tamoxifen efficacy than patients' CYP2D6 genotype.

However, whether the monitoring of endoxifen plasma concentrations in breast cancer patients would constitute a valid approach to optimize individual dosage remains to be demonstrated. In that context, several analytical methods have been published for the monitoring of tamoxifen and its metabolites in human biological fluids, including GC–MS [35], CE-MS [36], conventional and micellar liquid chromatography methods coupled to fluorescence detection [37–40] and LC–MS/MS methods [41–46]. Reports have also been published describing liquid chromatography method coupled to mass spectrometry or fluorescence detection for the study of tamoxifen metabolism in vitro and in vivo [47–54]. For mass spectrometry techniques, conventional HPLC [42,45,46] and fast liquid chromatography coupled to tandem MS methods using monolithic [41] or small particles (3 μm) packed columns [43,44] have been proposed for the quantification of tamoxifen and/or its metabolites. With the exception of the HPLC-MS/MS methods recently published [42,46], the potential impact of biological matrix effects variability on tamoxifen metabolites quantification was only scarcely addressed, as previous assays were using either no I.S. [45], or only a single labeled I.S. [41,43] as a surrogate I.S. for the quantification of tamoxifen and/or its metabolites.

Herein, we describe the development and validation of an UPLC–MS/MS method for the sensitive quantification in human plasma of tamoxifen, *N*-desmethyl-tamoxifen, and the active metabolites 4-hydroxy-tamoxifen and endoxifen within 13 min. The influence of matrix effects on tamoxifen and its metabolites quantification has been thoroughly investigated. The chromatographic profile of known (tamoxifen-*N*-oxide, α-hydroxy-tamoxifen) and previously unreported tamoxifen metabolites (4′-hydroxy-tamoxifen, 4′-hydroxy-*N*-desmethyl-tamoxifen, 3-hydroxy-tamoxifen) has also been studied in detail to exclude the risk of interferences during the comparatively short duration of the UPLC–MS/MS analysis.

2. Experimental

2.1. Chemicals and reagents

Tamoxifen (Tam) and *Z*-4-hydroxy-tamoxifen (4-OH-Tam) were purchased at Sigma–Aldrich (Schnelldorf, Germany). *N*-desmethyl-tamoxifen (*N*-D-Tam) hydrochloride, 4-hydroxy-*N*-desmethyl-tamoxifen 1:1 *E/Z* mixture (4-OH-*N*-D-Tam), 4′-hydroxy-tamoxifen (4′-OH-Tam), 4′-hydroxy-*N*-desmethyl-tamoxifen (4′-OH-*N*-D-Tam), α-hydroxy-tamoxifen (α-OH-Tam), 3-hydroxy-tamoxifen (3-OH-Tam), tamoxifen-*N*-oxide (Tam-*N*O), and the internal standards (I.S.): tamoxifen-ethyl-d5 (Tam-d5), *N*-desmethyl-tamoxifen-ethyl-d5 (*N*-D-Tam-d5), 4-hydroxy-tamoxifen-ethyl-d5 (4-OH-Tam-d5) and 4-hydroxy-*N*-desmethyl-tamoxifen-ethyl-d5 (endoxifen-d5), were purchased from Toronto Research Chemicals Inc. (North York, Canada).

Chromatography was performed using Lichrosolv® HPLC-grade acetonitrile (MeCN) purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). Ammonium formate was purchased from Fluka (Buchs, Switzerland). Formic acid (98%) and methanol for chromatography Lichrosolv® (MeOH) were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Different sources of blank plasma used for the assessment of matrix effects and for the preparation of calibration and control samples were isolated (1850 g, 10 min, +4 °C, Beckman Centrifuge, Model J6B) from outdated blood donation units from the Hospital Blood Transfusion Centre (CHUV, Lausanne, Switzerland) or from citrated blood withdrawn from patients with Vaquez's Disease (polycythemia vera).

2.2. Equipment

The liquid chromatography system consisted of Rheos 2200 quaternary pumps, equipped with an online degasser and a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) controlled by Janeiro-CNS software (Flux Instruments, AG, Thermo Fischer Scientific Inc., Waltham, MA). Separations were done on a 2.1 mm × 30 mm Acquity UPLC® BEH C18 1.7 μm analytical column (Waters, Milford, MA, USA) placed in a thermostated column heater at 40 °C (Hot Dog 5090, Prolab, Switzerland). The chromatographic system was coupled to a triple quadrupole (TSQ) Quantum Ultra mass spectrometer (MS) from Thermo Fisher Scientific, Inc. equipped with an Ion Max electrospray ionization (ESI) interface and operated with Xcalibur software package (Version 2.0.7, Thermo Fischer Scientific Inc., Waltham, MA).

2.3. Solutions

2.3.1. Mobile phase and extracts reconstitution solutions

The mobile phase used for chromatography was composed of 10 mM ammonium formate in ultrapure water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid (FA). A solution of MeOH/20 mM ammonium formate 1:1 (v/v), adjusted to pH 2.9 with FA, was used for the reconstitution of the extracted plasma samples prior to their analysis.

2.3.2. Working solutions, internal standard, calibration standards and quality controls (QCs) solutions

Stock solutions of deuterated internal standards (I.S.) (0.5 mg/mL in MeOH) were diluted with acetonitrile (ACN) to obtain a single working I.S. solution containing 25 ng/mL of tamoxifen-d5, *N*-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5 and 50 ng/mL of endoxifen-d5 (1:1 *E/Z* mixture).

Standard stock solutions of tamoxifen base, *N*-desmethyl-tamoxifen hydrochloride, 4-hydroxy-tamoxifen base and endoxifen (1:1 *E/Z* mixture) base each at 1 mg/mL were prepared in MeOH and stored at −20 °C. Appropriate volumes of stock solutions were serially diluted with H_2O/MeOH (3:1) as indicated in Table 1 to obtain single working solutions of analytes at concentration ranging from to 0.008 to 20 μg/mL. These working solutions were diluted 1:20 with blank citrated plasma to obtain for tamoxifen/metabolites the calibration samples ranging from 0.4 to 1000 ng/mL and their corresponding three quality control (low (L), medium (M) and high (H) QCs) samples ranging from 1.2 to 750 ng/mL. All spiked plasma samples were prepared according to the recommendations for bioanalytical methods validation stating that total added volume must be ≤10% of the biological sample [55]. The calibration and control plasma samples were stored as 100 μL aliquots at −80 °C. Of note, the accuracy of calibration and QC samples is subsequently verified by comparison with another batch of calibration and QCs samples prepared with freshly made stock solutions (at the occasion of plasma calibration batch renewal). The response of both series (i.e. new and previous) of calibration samples are compared, and analytes' levels in the two series of QC samples calculated using the calibration curve established with both series of calibrations samples. Residuals for newly and previous calibration standards and quality controls have to meet the acceptance criteria for precision and accuracy.

2.4. LC–MS/MS conditions

The mobile phase was delivered using the stepwise gradient elution program reported in Table 2. The thermostated column heater was set at +40 °C and the autosampler was maintained at +4 °C. The injection volume was 10 μL.

Table 1
Preparation of working solutions.

Drug	Stock solution solvent	Stock solution concentration	Working solution concentration (obtained by dilution of stock solution with H_2O/MeOH 3:1)	Calibration range (obtained by dilution of working solution with plasma 1/20)	QCs controls
Tam	MeOH	1 mg/mL	0.02–10 μg/mL	1–500 ng/mL	3; 50; 375 ng/mL
4-OH-Tam	MeOH	1 mg/mL	0.008–4 μg/mL	0.4–200 ng/mL	1.2; 20; 150 ng/mL
N-D-Tam	MeOH	1 mg/mL	0.04–20 μg/mL	2–1000 ng/mL	6; 100; 750 ng/mL
E-endoxifen	MeOH	0.5 mg/mL	0.02–10 μg/mL	1–500 ng/mL	3; 50; 375 ng/mL
Z-endoxifen	MeOH	0.5 mg/mL	0.02–10 μg/mL	1–500 ng/mL	3; 50; 375 ng/mL

All stock solutions are mixed together to give single working solutions.

Table 2
Gradient elution program.

Time (min)	Buffer A (%)	Solvent B (%)	Flow rate (μL/min)
0.00	70.0	30.0	300
9.00	48.0	52.0	300
9.01	48.0	52.0	300
9.50	70.0	30.0	350
13.00	70.0	30.0	350

Buffer A: 10 mM NH_4 formate + 0.1% formic acid. Solvent B: acetonitrile + 0.1% formic acid. Temperature (°C): 25. Injection volume (μL): 10.

The MS conditions were as follows: ESI in positive mode, capillary temperature: 350 °C; in source collision induced dissociation): 4 V; tube lens voltages range: 122–126 V; spray voltage: 4 kV; sheath gas pressure: 60 psi and auxiliary gas (nitrogen) pressure: 10 (arbitrary units). The Q2 collision gas (argon) pressure was 1.5 mTorr (0.2 Pa); Q2 collision induced dissociation (CID): 10 V. MS is acquired in selected reaction monitoring (SRM). The optimal parameters and MS/MS transitions were determined by direct infusion of tamoxifen, its metabolites and I.S. solutions separately into the MS/MS detector at a concentration of 1 μg/mL in MeOH/20 mM ammonium formate 1:1 (v/v), adjusted to pH 2.9 with FA. The selected *m/z* transitions and the collision energy for each analyte and I.S. are reported in Table 3.

The first (Q1) and third (Q3) quadrupoles were set at 2.8 amu mass resolution (Full-Width Half-Maximum = 2 Da). Scan time and scan width were 0.02 s and 0.5 *m/z*, respectively. MS acquisitions were done in centroid mode. Two segments of data acquisition were programmed in the positive mode: the first acquisition segment from 0 to 6 min, and the second one from 6 to 12 min.

Chromatographic data acquisition, peak integration and quantification were performed using the QUAL and QUAN browser of Xcalibur software package (version 2.0.7, ThermoQuest, Thermo Fischer Scientific Inc., Waltham, MA).

2.5. Clinical blood samples collection

Blood samples were obtained from consenting breast cancer patients enrolled in the study protocol "Tamoxifen metabolism and the impact of tamoxifen dose on the level of the active metabolites in endocrine sensitive breast cancer patients" (ClinicalTrials.gov Identifier: NCT00963209), approved by the Ethics Committee of the University Hospital. Written informed consent was obtained from all patients. Blood samples (5.5 mL) from breast cancer patients treated with tamoxifen were collected at random time after last drug intake in Monovettes® (Sarstedt, Nümbrecht, Germany) containing K-EDTA as anticoagulant. According to study protocol, blood samples were collected in patients receiving 20 mg tamoxifen once daily, at two occasions at baseline (e.g. on day 0 and day 1, i.e. after inclusion and before dose escalation), and after 1, 3 and 4 months of continuous treatment at a regimen of 20 mg tamoxifen twice daily (BID).

2.6. Plasma sample extraction procedure

A 100 μL aliquot of plasma was mixed with 100 μL of I.S. solution (25 ng/mL of tamoxifen-d5, *N*-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5, and 50 ng/mL of endoxifen-d5 1:1 *E/Z* mixture, in ACN) and with acetonitrile (300 μL), carefully vortex-mixed and sonificated for 30 s. (Branson Ultrasonics Corporation, Danbury, CT, USA). The mixture was centrifuged at 4 °C for 10 min at 16,000 × *g* (12,000 rpm) on a benchtop Hettich® Centrifuge (Benchtop Universal 16R centrifuge, Bäch, Switzerland). A 400 μL aliquot of the supernatant was transferred into a polypropylene tube and evaporated to dryness under nitrogen at room temperature. Of note, SpeedVac® concentrator may also be used, presenting the advantage of organic solvent recuperation. The solid residue was reconstituted in 600 μL of a solution of MeOH/20 mM ammonium formate 1:1 (v/v), adjusted to pH 2.9 with FA, vortex-mixed and centrifuged again under the above-mentioned conditions. A 400 μL of the supernatant was introduced into 1.5 mL glass HPLC microvials maintained at +4 °C in the autosampler rack during the entire LC–MS/MS analysis.

Table 3
Instrument method for the LC–MS/MS analysis of tamoxifen/metabolites with deuterated analogs as internal standards.

Drug	Parent (*m/z*)	Product (*m/z*)	CE (eV)	Tube lens (V)	Mean RT (min)	Polarity mode
Tamoxifen (Tam)	372.3	72.10	23	122	7.7	Positive
N-desmethyl-tamoxifen (*N*-D-Tam)	358.3	58.10	21	122	7.4	Positive
Z-4-hydroxy-tamoxifen (4-OH-Tam)	388.3	70.10	38	126	4.3	Positive
		72.10	25	126		Positive
		129.10	28	126		Positive
Endoxifen (1:1 *E/Z* mixture)	374.3	58.10	22	122	4.0	Positive
		129.10	28	122		
		223.10	20	122		
Tamoxifen-d5 (Tam-d5)	377.3	72.10	24	122	7.7	Positive
N-desmethyl-tamoxifen (*N*-D-Tam-d5)	363.3	58.10	21	122	7.4	Positive
4-Hydroxy-tamoxifen (4-OH-Tam-d5)	393.3	72.10	25	126	4.3	Positive
Endoxifen-d5 (1:1 *E/Z* mixture)	379.3	58.10	22	122	4.0	Positive

CE, collision energy; RT, retention time; MS acquisition time (min) = 12.00. Q2 Collision gas pressure (mTorr) = 1.5.

2.7. Calibration curves

Quantitative analysis of tamoxifen and its three main metabolites (*N*-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and *Z*-endoxifen) in plasma was performed using the internal standard method. Deuterated compounds of each target analyte were used as I.S. Each level of the calibration curve was measured with two sets of calibrators: the first at the beginning and the second at the end of the run. Calibration curves were established with calibration standards prepared in citrated plasma.

Calibration standard curves have been calculated and fitted by quadratic log–log regression [56] of the peak-area ratio of tamoxifen and its metabolites to its respective I.S., versus the nominal concentrations of each analyte in each standard sample. To determine the best weighting factor, concentrations were back-calculated and the model with the lowest total bias across the concentration range was considered the best suited. The seven-point calibration curves for tamoxifen and its three metabolites were established over the range reported in Table 1. The ranges of calibration were selected to cover the range of concentrations expected in patients according to previously published studies [18–20,42].

2.8. Analytical method validation

The method validation was based on the recommendations published on-line by the Food and Drugs Administration (FDA) [55] as well as on the recommendations of the Workshop/Conference Report "Quantitative Bioanalytical Methods Validation and implementation: Best Practice for Chromatographic and Ligand Bindings Assays" [57] and the Arlington Workshop "Bioanalytical Methods Validation – A Revisit with a Decade of Progress" [58]. Recommendations from Matuszewski to assess matrix effects were also considered [59,60].

2.8.1. Selectivity

The assay selectivity was assessed by analysing plasma extracts from ten batches of blank plasma from different sources.

2.8.2. Accuracy and precision

The concentrations for the quality control (QC) samples were selected to encompass the whole range of the calibration curve corresponding to the drug levels anticipated to occur in most patient samples: low (L), medium (M) and high (H). The concentration selected for the low QC sample corresponds to 3 times the respective lower limit of quantification (i.e. the lowest calibration level) kept in the finalized method, in accordance to the FDA recommendations [55]. Replicate analysis ($n = 6$) of three QC samples was used for the intra-assay precision and accuracy determination. Inter-assay accuracy and precision were determined by duplicate analysis of the three QC repeated on six different days. The precision was calculated as the coefficient of variation (CV %) and the accuracy was calculated as the bias or percentage of deviation between the nominal and measured concentrations.

After the completion of the above validation procedure, for the routine analysis of patient samples, duplicate QC samples at the three concentration levels (L, M and H) were used.

2.8.3. Matrix effects, extraction yield and overall recovery

In the initial step of method validation, matrix effects were examined qualitatively by the simultaneous post-column infusion of tamoxifen/metabolites and I.S. into the MS/MS detector during the chromatographic analysis of 6 different blank plasma extracts. The standard solution of all analytes and their corresponding deuterated I.S. at 5 μg/mL was infused at a flow-rate of 20 μL/min during the chromatographic analysis of blank plasma extracts. The chromatographic signals in each selected MS/MS transition were examined to check for any signal perturbation (drift or shift) at the analytes' retention time (data not shown).

Subsequently, the matrix effects were also quantitatively assessed. Three series of QC samples at L, M and H concentrations were processed as follows:

(A) Pure stock solutions dissolved in the reconstitution solvent (MeOH-buffer (ammonium formate 20 mM, pH adjusted to 2.9 with FA) 1:1) and directly injected onto column.
(B) Plasma extracts samples from 6 different sources, spiked after extraction with tamoxifen/metabolites and I.S. (from pure stock solutions in the reconstitution solvent).
(C) Plasma samples from 6 different sources (same as in B) spiked with tamoxifen/metabolites standard solutions and I.S. before extraction.

The recovery and ion suppression/enhancement of the MS/MS signal of drugs in the presence of plasma matrix (i.e. matrix effects) was assessed by comparing the absolute peak areas of the analytes either dissolved in the reconstitution solvent: MeOH-buffer 1:1 (A), or spiked after plasma extraction (B) or spiked before plasma extraction (C), using 6 different batches of plasma, based on the recommendations proposed by Matuszewski et al. [59,60].

The *extraction yield* of tamoxifen/metabolites and I.S. was calculated as the absolute peak-area response in processed plasma samples spiked with the standard analytes *before* extraction (C) expressed as the percentage of the response of the same amount of analytes spiked into blank plasma *after* the extraction procedure (B) (C/B ratio in %). The *matrix effect* was assessed as the ratio of the peak areas of the analytes spiked into blank plasma *after* the extraction procedure (B) to the peak areas of the analytes solubilised in MeOH-buffer 1:1 (A) (B/A ratio in %). The *overall recovery* of tamoxifen/metabolites and I.S. was calculated as the ratio of absolute peak-area responses of tamoxifen/metabolites spiked in processed plasma samples *before* extraction (C) to the peak areas of the analytes solubilised in MeOH-buffer 1:1 (A) (C/A ratio %). Recovery studies were performed with plasma from 6 different sources spiked with tamoxifen, its metabolites and their respective I.S. at the concentrations reported in Table 4. The results normalized with the signal of I.S. (i.e. B2 and C2), used as an index of the effective injection volume, are also reported in Table 4.

2.8.4. Carry-over

Memory effect has been investigated by the injection during an analytical run of 2 or 3 blank plasma after the highest calibration standard. Peak area response in the blank plasma sample, at each expected retention time, was compared to the peak area of the corresponding analyte at the lowest limit of quantification (LLOQ).

2.8.5. Dilution effect

During the course of patients' samples analyses, one patient sample was found to have tamoxifen concentration exceeding the highest level of the calibration curve (see Table 1). To ascertain whether the dilution of this sample could affect the accuracy of the drug or its metabolites determination, a blank plasma sample was spiked with pure standards (tamoxifen/metabolites) at a concentration exceeding by two-fold the highest calibration level. The sample was thereafter analysed in duplicate after a three, four, five and six fold dilution to bring the concentration within the calibration range. Dilution was carried out with blank plasma. Calculated and expected concentrations were compared.

Table 4
Matrix effects, extraction yield, overall recovery and process efficiency of tamoxifen/metabolites.

Component	Nominal conc. (ng/mL)	Mean peak area			Mean peak area ratio		ME (%)	CV (%)	extRE (%)	CV (%)	Analysis RE (%)	Mean	CV (%)	PE (%)	CV (%)
		A ($n=6$)	B ($n=6$)	C ($n=6$)	B2	C2	B/A		C/B		C2/B2			C/A	
Tam	3	1,263,441	1,255,380	1,372,710	0.072	0.076	99.4	3.7	109.3	4.3	105.0	96.4	8.1	108.6	1.6
	50	28,878,341	27,938,705	27,393,059	1.606	1.513	96.7	2.0	98.0	4.0	94.2			94.9	3.5
	375	228,978,707	226,034,897	21,1505,317	12.997	11.683	98.7	1.0	93.6	2.3	89.9			92.4	1.8
N-D-Tam	6	804,396	497,408	613,605	0.243	0.304	61.8	18.0	123.4	6.9	124.8	109.9	11.9	76.3	14.2
	100	17,796,321	10,574,145	10,929,594	5.169	5.406	59.4	21.0	103.4	8.9	104.6			61.4	18.0
	750	125,333,845	80,869,169	80,226,329	39.529	39.685	64.5	18.4	99.2	6.7	100.4			64.0	16.2
4-OH-Tam	1.2	537,944	545,444	559,305	0.062	0.067	101.4	2.9	102.5	5.0	107.8	104.1	3.3	104.0	3.5
	20	10,730,921	10,607,311	10,417,567	1.202	1.241	98.8	1.5	98.2	3.0	103.3			97.1	3.4
	150	79,332,011	79,252,260	76,170,959	8.980	9.076	99.9	1.9	96.1	2.1	101.1			96.0	1.1
Z-endoxifen	3	227,307	235,540	230,538	0.059	0.063	103.6	5.2	97.9	8.1	106.5	105.2	1.2	101.4	4.7
	50	4,467,005	4,597,862	4,431,075	1.149	1.205	102.9	0.9	96.4	4.6	104.9			99.2	4.8
	375	32,717,609	33,369,469	31,924,813	8.339	8.682	102.0	1.5	95.7	2.5	104.1			97.6	1.1
E-endoxifen	3	154,699	162,010	160,857	0.055	0.059	104.7	5.7	99.3	2.8	106.6	103.6	2.5	104.0	3.7
	50	3,048,595	3,146,053	2,988,009	1.074	1.095	103.2	2.3	95.0	3.1	102.0			98.0	4.1
	375	22,805,748	23,258,002	22,122,028	7.939	8.109	102.0	1.5	95.1	0.9	102.1			97.0	1.3
Tam-d5	25	17,793,384	17,391,055	18,104,182			96.1	2.8	104.1	1.7				101.7	2.8
N-D-Tam-d5	25	3,404,892	1,959,036	1,937,111			57.5	17.3	98.9	6.8				56.9	15.5
4-OH-Tam-d5	25	8,825,185	8,825,420	8,392,730			100.0	3.0	95.1	3.1				95.1	2.4
Z-endoxifen-d5	25	3,796,772	4,001,590	3,677,241			105.4	2.7	91.9	3.1				96.9	3.8
E-endoxifen-d5	25	2,881,493	2,929,682	2,728,140			101.7	2.0	93.1	2.6				94.7	3.9

A, peak area of standard solutions without matrix and without extraction (MeOH/buffer A 1:1); B, peak area of analytes spiked after extraction; C, peak area of analytes spiked before extraction; B2, ratio of the peak area of the analyte and the I.S. spiked after extraction; C2, ratio of the peak area of the analyte and the I.S. spiked before extraction; ME, matrix effect expressed as the ratio of the mean peak area of the analytes spiked after extraction (*B*) to the mean peak area of the same standard solution without matrix (*A*) multiplied by 100. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression; ext RE, extraction procedure recovery calculated as the ratio of the mean peak area of the analytes spiked before extraction (*C*) to the mean peak area of the analytes spiked after extraction (*B*) multiplied by 100; Analysis RE, analysis recovery calculated as the ratio of the mean peak-area ratio of the analytes spiked before extraction (C2) to the mean peak-area ratio of the analytes spiked after extraction (B2) multiplied by 100; PE, process efficiency expressed as the ratio of the mean peak area of the analyte spiked before extraction (*C*) to the mean area of the same analyte standards (*A*) multiplied by 100.

2.8.6. Stability of tamoxifen and its metabolites

Stability studies of tamoxifen and its three metabolites at different storage conditions included:

(a) Stability in plasma spiked with tamoxifen/metabolites (i.e. QCs at L, M and H concentrations) over time at room temperature (RT) and at +4 °C up to 48 h. Variations of tamoxifen/metabolite concentrations were expressed as percentages of the initial concentration measured immediately after preparation, i.e. T_0. Analyses were performed in triplicate at T_0 and at each subsequent time point.
(b) Stability of tamoxifen/metabolites in whole blood at +4 °C and at RT assessed by calculating the percent deviation of the I.S. normalized peak area of each analyte in the collected plasma from the initial peak area ratio measured at T_0. Two batches of whole blood samples spiked with analytes at the L, M and H levels (1 ml final volume) were prepared in triplicate and kept for 0, 1, 2, 4, 8, 24 and 48 h before plasma separation at +4 °C and at RT. All plasma samples collected from centrifuged blood aliquots were stored at −80 °C and subsequently analysed in the same analytical sequence.
(c) Stability in plasma samples after multiple freeze-thaw cycles: plasma QCs at low, medium and high levels of tamoxifen/metabolites underwent three freeze-thaw cycles. Frozen samples were allowed to thaw at RT for 2 h and were subsequently refrozen at −80 °C during approximately 24 h. Tamoxifen/metabolites levels were measured in aliquots from the three consecutive freeze–thaw cycles.
(d) Stability in plasma samples kept frozen at -80 °C: QCs samples at the L, M and H concentrations were stored at–80 °C during 4 months and measured using fresh plasma calibration samples.

2.8.7. Identification of other tamoxifen metabolites

Next to tamoxifen, *N*-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen analysis, additional phase I tamoxifen metabolites were identified in patients samples by comparison of the retention times and product-ion mass spectra of authentic standard compounds spiked into blank plasma, or added to patients' plasma samples. The full-scan mass spectra were acquired over a scan range of 40–400 *m*/*z* at scanning speed of 0.08 s/scan.

In the present analytical work, the concentrations of the newly identified metabolites 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen have also been estimated using 4-OH-Tam-d5 and endoxifen-d5 as I.S. in a separate series of analysis of 20 patients' samples.

3. Results and discussion

3.1. Chromatograms

The proposed ultra performance-liquid chromatography coupled with tandem MS method enables the simultaneous quantification within 13 min of tamoxifen and three metabolites: *N*-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and *Z*-endoxifen (4-hydroxy-*N*-desmethyl-tamoxifen), in 100 μL plasma aliquots. A chromatographic profile of the highest calibration plasma sample containing tamoxifen/metabolites is shown in Fig. 2 in the positive ionization mode, during the two acquisition segments (0–6 and 6–12 min), using the selected reaction monitoring (SRM) detection mode; the proposed gradient program is described in Table 2. Tamoxifen and its metabolites were eluted in less than 9 min, followed by approx. 4 min of column re-conditioning step with 70% of buffer A (ammonium formate 10 mM + 0.1% FA) and 30% of solvent B (acetonitrile + 0.1% FA) at a flow rate of 0.35 mL/min (Table 2). The respective retention times and mass spectrometry conditions for tamoxifen/metabolites and their corresponding stable isotope labeled I.S. are reported in Table 3. Three *m*/*z* transitions were selected for 4-hydroxy-tamoxifen (*m*/*z* 388) with product ions at *m*/*z* 70, 72 and 129, and for endoxifen (*m*/*z* 374) with product ions at *m*/*z* 58, 129 and 223, in order to increase the detection sensitivity for these metabolites. The fragment ions at *m*/*z* 72 and 58 are the major signals visible on the product ion spectrum of 4-hydroxy-tamoxifen and endoxifen, respectively.

A satisfactory separation was achieved for all considered analytes, especially for (*E*-) and (*Z*-) isomers of endoxifen and endoxifen-d5 obtained as a racemic mixture (Fig. 2). Data from blank plasma samples spiked with the deuterated I.S., obtained throughout the method validation procedure and during patients' plasma samples analyses, revealed no interfering "cross-talk" signals arising from the isotopically-labeled I.S. on the transition of the corresponding target analyte, thus testifying the isotopic purity of these isotope labeled I.S.

Moreover, the proposed UPLC method provides an excellent chromatographic separation of tamoxifen-*N*-oxide from tamoxifen, preventing therefore analytical bias due to potential *in-source* dissociation of tamoxifen-*N*-oxide into tamoxifen that would give rise to spuriously elevated levels of tamoxifen. Of note, it was rather unexpected that tamoxifen-*N*-oxide, intuitively more polar, elutes later than tamoxifen on a reverse phase column, in line with previous reports [41,42,49]. Alterations of intra- or inter-molecular bindings, or pH-dependent changes in molecular lipophilicity, (i.e. Log D) [50,52] might be involved.

Fig. 3a shows the chromatographic profile of a plasma sample collected from a hormone sensitive breast cancer patient having received tamoxifen for 1 month at a regimen of 20 mg twice a day. The plasma levels of tamoxifen, *N*-desmethyl-tamoxifen, *Z*-4-hydroxy-tamoxifen and *Z*-endoxifen measured 7.5 h after last drug intake were 666.6, 929.4, 15.2 and 217.9 ng/mL respectively). As reported in the literature, only the (*Z*) isomers of 4-OH-Tam and endoxifen were observed in plasma, thus excluding any *E*-*Z* interconversion of tamoxifen metabolites during sample preparation [42,44,61,62].

Fig. 3b shows the chromatographic profile of a plasma obtained from a hormone sensitive breast cancer patient receiving tamoxifen for 1.5-year at the standard regimen of 20 mg once daily. The plasma levels of tamoxifen, *N*-desmethyl-tamoxifen, *Z*-4-hydroxy-tamoxifen and *Z*-endoxifen measured 13.25 h after last drug intake were 207.6, 445.2, 1.4 and 6.2 ng/mL, respectively).

3.2. Method validation

3.2.1. Selectivity

No peaks from endogenous compounds were observed at the drugs retention time in any of the blank plasma extracts. The product ion monitoring was selected, based on its relative abundance, while avoiding possible structural analogies with the other analysed drugs or metabolites. All channels were simultaneously observed, and no selectivity issue as well as no crosstalk were detected across the acquisition channels.

3.2.2. Internal standard and calibration curve

The use of stable isotope-labeled internal standards is considered to be the best approach to minimize the influence of matrix effects on the accuracy and precision of a quantitative method, of particular importance when using electrospray mass spectrometry [59,60,63].

Therefore, deuterated analogs of tamoxifen and the metabolites to be quantified, have been used throughout our analytical method validation procedure (i.e. tamoxifen-d5, *N*-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5 and endoxifen-d5 1:1 *E*/*Z* mixture). No problems regarding the isotopic purity, *E* to *Z* interconversion,

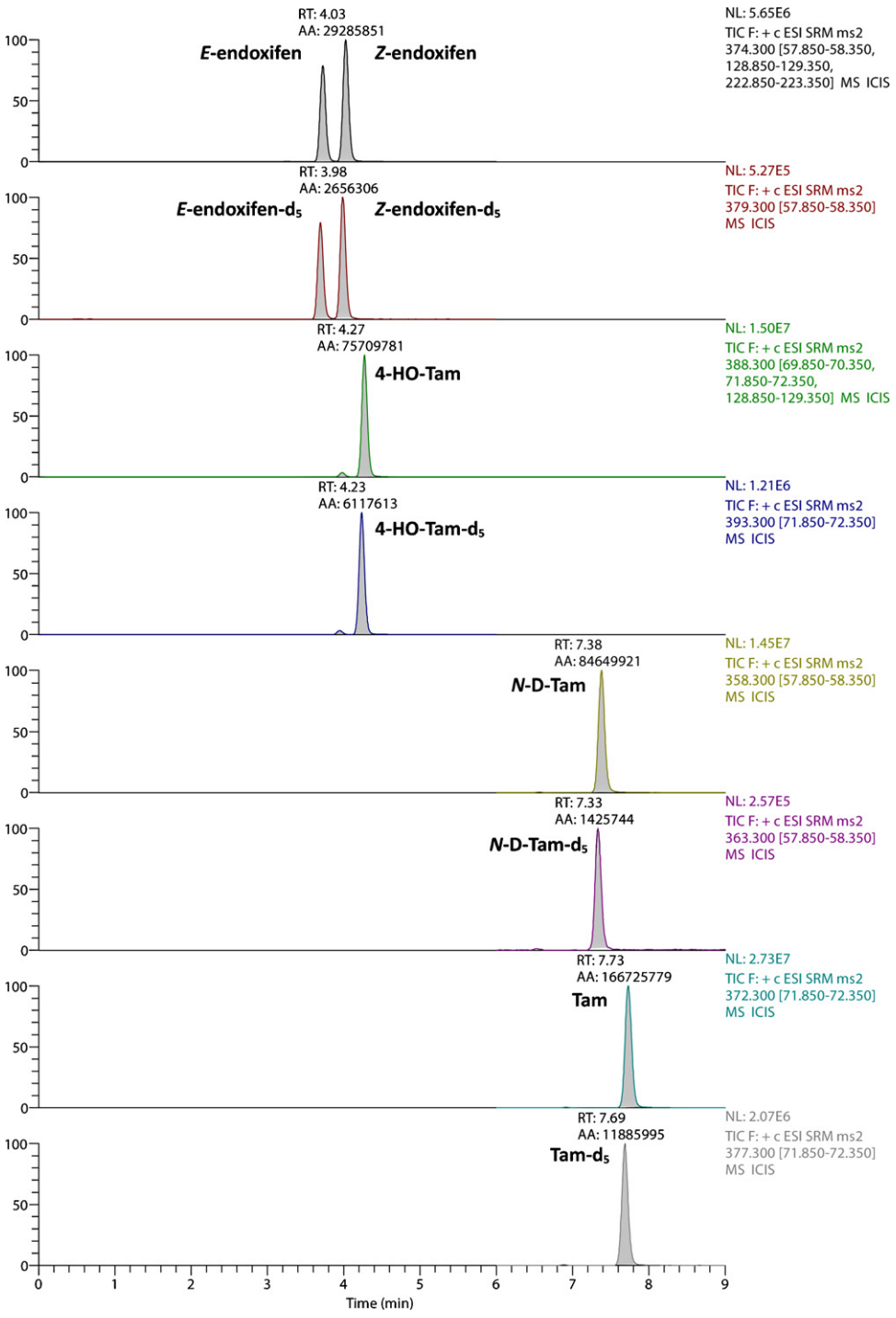

Fig. 2. Chromatogram of the highest calibration sample containing tamoxifen and its three major metabolites. Corresponding deuterated analogs are used as internal standards (details in the text).

and chemical stability of the I.S. (either in stock solution or in biological fluid and during sample processing), neither any "cross-talk" between MS/MS channels used for monitoring tamoxifen and the considered metabolites and the I.S. were identified throughout method validation procedure.

Calibration curves over the entire ranges of concentrations delineated in Table 1 were satisfactorily described by quadratic log-log regression of the peak-area ratio of tamoxifen and its metabolites to their I.S., versus the concentrations of the respective analytes in each standard sample. This model of calibration described by Singtoroj et al. [56] was found well suited to best fit the criteria of homoscedasticity (homogeneity of variance over the entire calibration range) and minimum bias for each single calibrator. The determination coefficients (R^2) of all calibration curves were higher than 0.999 with back-calculated concentrations of the calibration samples within ±15% of nominal values (±20% at LLOQ).

There was originally some concern that the calibration samples prepared with citrated plasma collected from blood from outdated transfusion bags or from Vaquez patients may not fully reflect the plasma matrix from patients collected on EDTA. However, getting blood on EDTA from volunteers solely for the purpose of calibra-

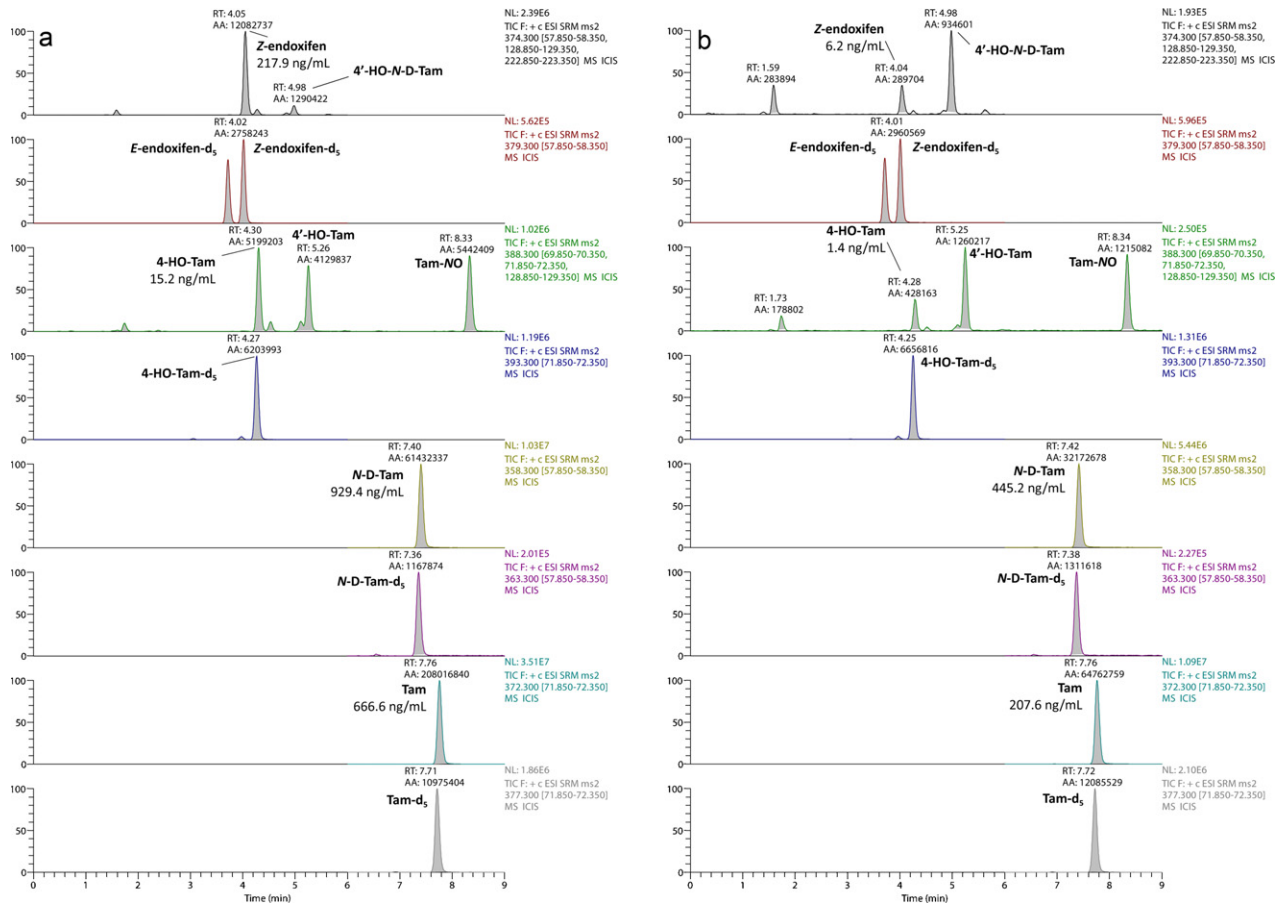

Fig. 3. Chromatographic profiles of plasma samples from two patients (a and b) receiving tamoxifen (details in the text).

tion samples preparation would be unpractical and difficult to justify from an ethical point of view. For the sake of validation, a cross-validation was performed by replicate analysis ($n = 3$) of QC samples at the three levels, prepared both in citrated and in EDTA plasma. The QC samples were assayed using the calibration curve established with citrated plasma samples. Head-to-head comparison shows that the anticoagulant does not influence significantly the analytical results for tamoxifen and its metabolites. No statistically significant differences ($p > 0.05$) in concentrations were found for QCs samples prepared in EDTA and citrated plasma using calibration curves established with citrated plasma (p values comprised within 0.07–0.92 for tamoxifen, *N*-desmethyl-tamoxifen, *Z*-4-hydroxy-tamoxifen, *Z*-endoxifen and *E*-endoxifen (Student *t*-test).

3.2.3. Precision, accuracy, and LLOQ

Precision and accuracy determined with the L, M and H QC samples are summarized in Table A.1 (on-line supplementary data). The mean intra-assay precision was similar over the entire concentration range and always less than 6.8%. Overall, the mean inter-day precision was within 2.5 and 7.8%. The intra-assay and inter-assay deviation (bias) from the nominal concentrations of QCs ranged between −5.3 and +7.4%, and −1.4 and +5.8%, respectively.

Of note, the chosen ranges of calibration were selected initially to cover the clinical range of tamoxifen/metabolites concentrations previously reported in the literature [18–20,42]. In fact, we observed during the method's validation that the responses attained at the LLOQs levels would be sufficient so that it may be possible to validate this method at even lower levels (ca. 0.1–0.75 ng/mL) if desired in the future.

3.2.4. Matrix effects and recovery

Matrix effects were examined qualitatively by the simultaneous post-column infusion of tamoxifen/metabolites and I.S. into the MS/MS detector during the chromatographic analysis of six different batches of blank plasma. During the chromatography of blank matrices, the signals at all the *m/z* transitions selected showed a remarkably similar pattern, with all traces being essentially superimposable. No noticeable matrix effects (no drifts or shifts of the signals) were observed at the respective retention time of tamoxifen and its metabolites and their deuterated I.S. (data not shown).

The inter-subject variations in suppression/enhancement profiles have also been studied quantitatively (Table 4). The results reported in Table 4 (column B/A) indicate that co-eluting plasma matrix components appear to have a minimal effect on the considered analytes, except for *N*-D-Tam whose signal was approximately halved (mean ratio B/A = 62%). As expected, a similar extent of ion suppression was observed with *N*-D-Tam labeled internal standard (*N*-D-Tam-d5) (B/A ratio = 57.5%). Thus overall, the mean B/A ratios for *N*-D-Tam when normalized with those of deuterated I.S. was 1.1 (i.e. at or slightly above unity), demonstrating the value of stable isotope-labeled I.S. use for an efficient control of the relative matrix effect [64]. Plasma matrix does not appear to significantly interfere with Tam, 4-OH-Tam and both endoxifen isomers ionisation (B/A ratio ranged between 96.7 and 104.7%).

Using the proposed protein precipitation, supernatant evaporation and dissolution in appropriate buffer, our plasma extraction procedure provided a good extraction recovery (C/B, column extRE) always higher than 95%, resulting in an excellent sensitivity.

As indicated in Table 4, the *analytical recovery values* were always higher than 89.9%. The *process efficiency* (i.e. overall recovery) was comprised within 92.4-108.6% except for *N*-D-Tam, which

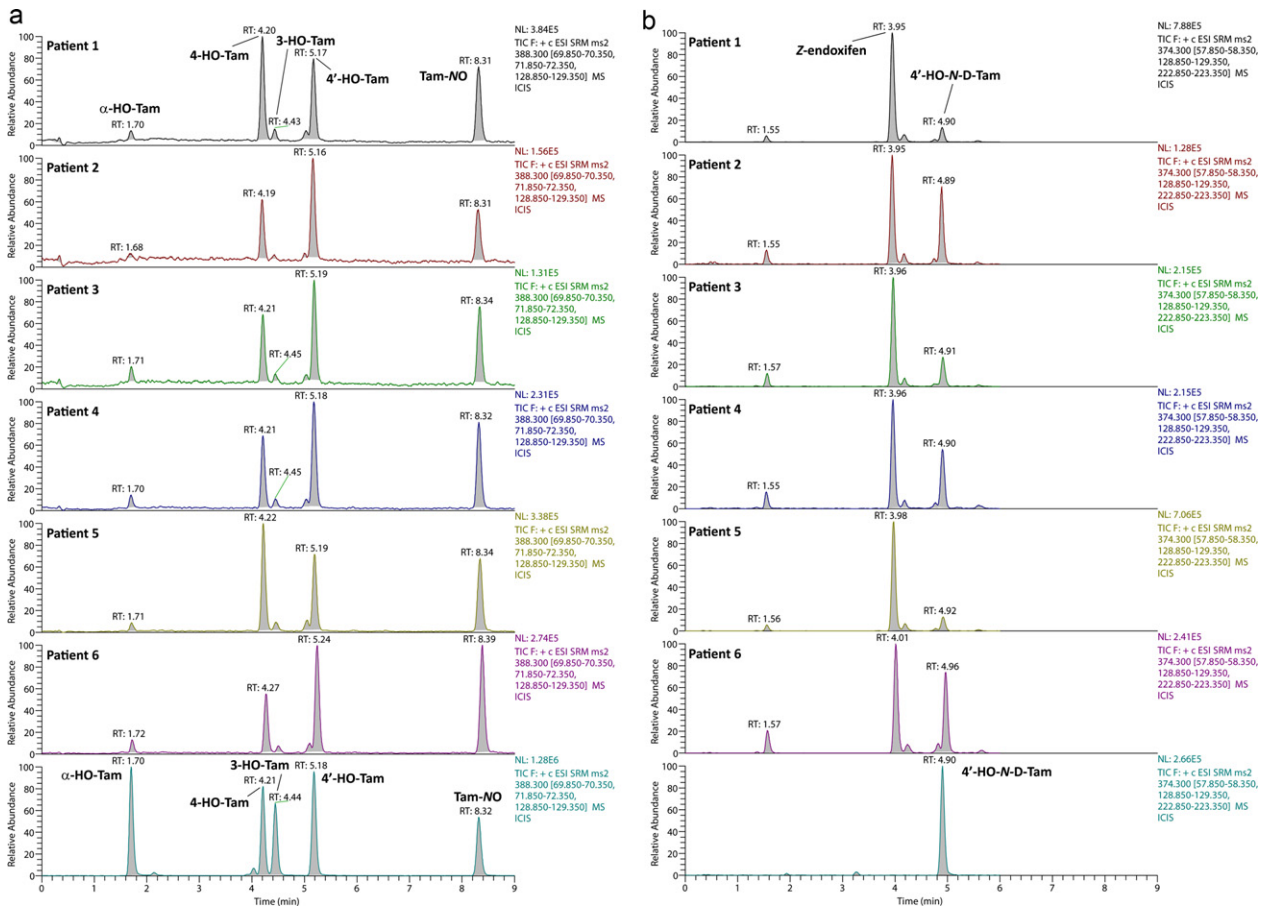

Fig. 4. Chromatographic profiles (a) at SRM transition (m/z 388 → 70, 72, 129) and (b) at SRM transition (m/z 374 → 58, 129, 223) in plasma from 6 unselected patients receiving tamoxifen. Last chromatograms (bottom traces) are blank plasma spiked with: (a) pure standards of α-hydroxy-tamoxifen, 4-hydroxy-tamoxifen, 3-hydroxy-tamoxifen, 4′-hydroxy-tamoxifen and tamoxifen-*N*-oxide and (b) pure standard of 4′-hydroxy-*N*-desmethyl-tamoxifen (details in the text).

gives a process efficiency around 67%. As reported above, matrix components do influence to some extent *N*-D-Tam ionisation and consequently the overall process efficiency, requiring therefore the preparation of calibration and control samples in a plasma matrix reflecting at best the composition of the samples to be analysed. Most importantly, this is not so much the absolute matrix effect, but rather its variability (relative matrix effect) that must be reduced. As shown in Table 4, the variability of the matrix effect in 6 different plasma matrix were close to 20% for *N*-D-Tam at all QCs and never exceeded 5.7% for all other analytes, which indeed demonstrates that the proposed extraction procedure is able at least to normalize these matrix effects, even in the absence of the correcting effect of labeled I.S. In fact, the use of isotope-labelled internal standards in our method seems to effectively control most of the residual relative matrix effect variability. This has been experimentally verified notably for *N*-D-Tam for which the observed matrix effect variability in 6 plasma lots never exceeded 4% when *N*-D-Tam peak areas where normalized to those from its deuterated I.S. (N-D-Tam-d5).

3.2.5. Memory effect

No major carry-over was observed with our method. The highest memory effect was observed for tamoxifen, the most lipophilic analyte. This carry-over effect was successfully eliminated by programming the injection of three blank samples after the highest calibration standard, prior to the analysis of patients' samples. The peak intensity visible in the third blank matrix sample corresponds to less than 20% of that of the LLOQ sample. In fact, during routine plasma analysis, it has prudently been decided to program a single blank plasma injection after each patient's sample which was found sufficient to reduce the memory effect to an extent unlikely to affect the accuracy of tamoxifen and its metabolites measurements in the following patients' plasma samples.

3.2.6. Dilution effect

After the three, four, five and six-fold dilutions of the spiked plasma with tamoxifen/metabolites at a concentration exceeding by two-fold the high calibration level, the deviation (bias) from the expected concentrations of all compounds was less than 8.2%. This indicates that plasma samples containing tamoxifen/metabolites above the highest level of calibration can be adequately diluted with blank plasma prior to the LC–MS/MS analysis, to bring down concentration within the calibration range.

3.2.7. Stability of tamoxifen/metabolites in plasma and whole blood

(a) The stability of tamoxifen/metabolites in human plasma samples was ascertained with QC samples left at room temperature (RT) and at +4 °C up to 48 h. The variation over time of the concentrations of tamoxifen and its metabolites in plasma remained comprised within ±15% of initial (T_0) concentrations (see Table A.2 in on-line supplementary data), indicating that tamoxifen and its metabolites are stable in plasma at RT and at +4 °C.

(b) During the clinical study, which prompted this analytical development, some blood samples had to be stored temporarily at +4 °C before being shipped to our laboratory and centrifuged for plasma collection. Given the absence of information on the stability of tamoxifen and its principal metabolites in blood, we

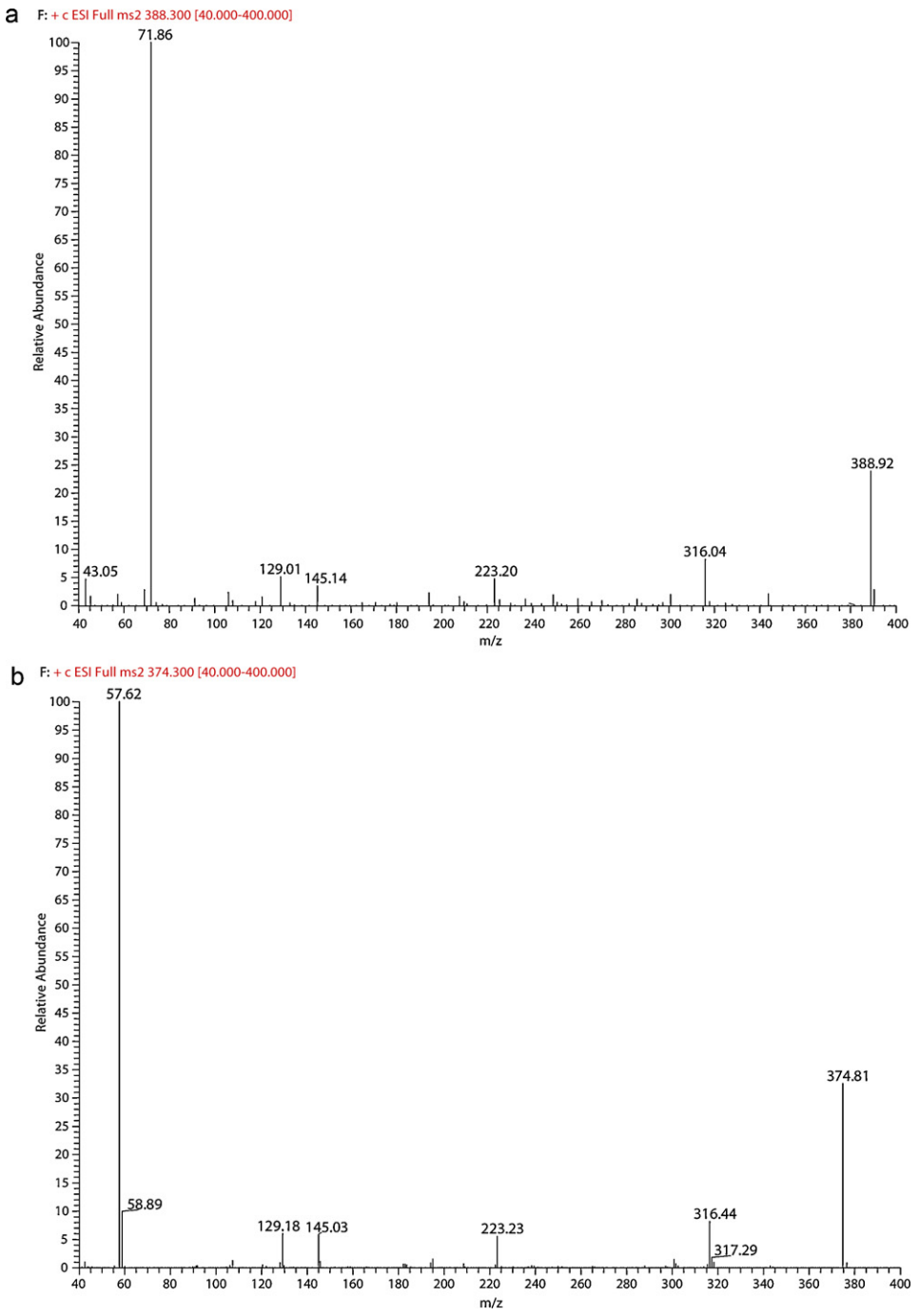

Fig. 5. Product ion spectra of the pure standards (a) 4′-hydroxy-tamoxifen and (b) 4′-hydroxy-*N*-desmethyl-tamoxifen spiked into blank plasma.

have studied the evolution of their concentrations over time in whole blood. The results of stability studies in whole blood are summarized in Table A.3 (on-line supplementary data), indicating that tamoxifen and its metabolites can reliably be considered as stable in whole blood, up to 8 h storage either at +4 °C or at RT.

(c) Variations of tamoxifen/metabolites concentrations were always less than −15% from nominal levels after three freeze-thaw cycles (Table A.2, in on-line supplementary data), indicating no significant loss of drug upon this procedure.

(d) QCs samples prepared in batches, distributed as 100 μL aliquots and stored at −80 °C in 1.5 ml Eppendorf vials were analysed 4 months later. All QCs (L, M and H) were analysed in duplicate. Variations of tamoxifen/metabolites concentrations were less than -11.9% from their nominal concentrations, indicating the long term stability of tamoxifen and its metabolites in plasma samples stored at −80 °C.

3.3. Metabolites profiles studies and metabolites identification

Given the reduced elution time of analytes with UPLC, it was critical for this analytical development to verify that tamoxifen metabolites would not potentially perturb the quantification. The chromatographic elution pattern of reported or putative tamoxifen metabolites was therefore studied thoroughly.

Three additional peaks were observed in patients samples at 1.7, 5.2 and 8.3 min on the SRM transition (*m/z* 388 → 70, 72, 129) selected for 4-hydroxy-tamoxifen (itself eluted at 4.2 min)

(Fig. 3b, third chromatogram from top, and Fig. 4a). These metabolites were identified in patients (Fig. 4a) as α-hydroxy-tamoxifen, 4′-hydroxy-tamoxifen and tamoxifen-*N*-oxide, respectively (H, C, I, respectively in Fig. 1) [13,49,51,65] by comparison to the retention times (Fig. 4a, lower chromatogram) and/or product-ion spectra of authentic standards spiked into blank plasma or added to patients' plasma samples (data not shown). The fragmentation pattern of the 4′-hydroxy-tamoxifen standard spiked into blank plasma (Fig. 5a) was equivalent to that observed for the putative endogenous 4′-hydroxy-tamoxifen. The product ions (72, 129, 145, 223, 316 *m*/*z*) were invariably observed in all product ion scans determined at the retention time of the metabolite observed in patients samples.

Interestingly, the UPLC gradient program also allows the baseline separation of 4-hydroxy-tamoxifen and 3-hydroxy-tamoxifen eluted at 4.2 and 4.4 min, respectively (Fig. 4a, lower chromatogram of standard compounds spiked in plasma). The compound 3-hydroxy-tamoxifen is a metabolite reported to be produced *in vitro* upon incubation of tamoxifen with human liver microsomes (HLMs) [13]. In patients' plasma however, there was only a very small peak, if any, visible at the retention time of 3-hydroxy-tamoxifen. (Fig. 4a, metabolites profiles in patients).

Finally, inspection of the transition (*m*/*z* 374 → 58, 129, 223) selected for monitoring *Z*-endoxifen (eluted at 4.0 min) revealed the presence in patients samples of two additional peaks at 1.5 and 4.9 min (Fig. 3b, upper chromatographic profile, and Fig. 4b). The first eluted peak at 1.5 min was tentatively identified as α-hydroxy-*N*-desmethyl-tamoxifen based on literature (no available reference material). The latest peak visible in this *m*/*z* transition at 4.9 min was identified as 4′-hydroxy-*N*-desmethyl-tamoxifen, which has the same retention time (Fig. 4b, lower trace) and a comparable product-ion mass spectrum as the synthetic compound (Fig. 5b) either spiked into blank plasma or patients' plasma samples. The product ions (58, 129, 145, 223 and 316 *m*/*z*) were observed during the fragmentation of the 4′-hydroxy-*N*-desmethyl-tamoxifen pure compound and were likewise detected in all product-ion scans at the retention time of the putative endogenous metabolite. As recently described, the fragment at 129 *m*/*z* was reported to be indicative of the tamoxifen structure [54] and was detected in product ion spectra of both metabolites 4′-hydroxy-*N*-desmethyl-tamoxifen and 4′-hydroxy-tamoxifen.

The metabolite 4′-hydroxy-tamoxifen, whose formation might be catalyzed by the polymorphic CYP2B6 [13,61], has been previously detected in rat and mouse liver microsomes [13,52,61,65] and in recent *in vitro* studies (using Human Cytochrome P450 Systems) as primary metabolite of tamoxifen [13,51], but its occurrence had never been formally reported in humans. Similarly, 4′-hydroxy-*N*-desmethyl-tamoxifen has been previously detected in mouse liver microsomal incubates [52]. Neither metabolite has yet been identified so far in patients.

This is the first report of the occurrence of 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen in plasma from patients under tamoxifen therapy. Typical metabolites profiles in 6 unselected patients receiving tamoxifen are shown in Fig. 4a and b: 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen are detected in patients' samples at 5.1 and 4.9 min in their respective *m*/*z* transition channel. So far, both metabolites were found in all patients' samples analysed ($n = 70$), with substantial variability in plasma levels.

Although our method has not been formally validated for the quantification of these newly identified metabolites, their plasma levels have been estimated in a separate analysis of 20 unselected patients' samples. The concentrations of 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen ranged between 2.2 to 5.5 ng/mL, and 4.4 to 11.8 ng/mL, respectively, in patients under tamoxifen 20 mg QD, and between 3.3 to 9.5 ng/mL, and 6.2 to 20.6 ng/mL, respectively, in patients under 20 mg BID tamoxifen regimen. The clinical importance of these new metabolites, and their potential contribution to the clinical effects of tamoxifen remain to be determined [13]. Limited data available from the literature suggest that 4′-hydroxy-tamoxifen might have higher affinity for the estrogen receptor than tamoxifen itself [13,66,67].

4. Conclusion

We have developed and validated a specific and sensitive UPLC–MS/MS method enabling reliable and sensitive monitoring of tamoxifen and three clinically relevant metabolites in patients' plasma. Our method provides an excellent chromatographic separation of tamoxifen and seven known and previously unreported metabolites in a relatively short gradient program of 13 min. The method was developed using deuterated I.S. for all target analytes, which further strengthen our analytical assay for selective and sensitive quantification of tamoxifen and its metabolites by electrospray ionisation mass spectrometry.

During the course of these chromatographic investigations, we have been able to identify for the first time the two metabolites 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen in plasma from breast cancer patients. Our estimation of 4′-hydroxy metabolites plasma levels in a subset of patients indicates that the range of 4′-hydroxy-tamoxifen plasma concentrations was similar to that measured for 4-hydroxy-tamoxifen. Conversely, 4′-hydroxy-*N*-desmethyl-tamoxifen plasma levels were two to three times lower than the endoxifen levels determined in 20 unselected patients. The clinical importance of these previously unreported metabolites and their potential contribution to the clinical effects of tamoxifen has yet to be determined. Finally, we could show that 3-hydroxy-tamoxifen is very limitedly, if not at all, found in the blood of patients on tamoxifen therapy.

In conclusion, This UPLC–MS/MS method has been shown suitable for measuring exposure of tamoxifen and its metabolites in tamoxifen-treated breast cancer patients. In this context, the present analytical methodology is currently applied in a population pharmacokinetic study of tamoxifen and its metabolites, helping us primarily at characterizing the influence of pharmacogenetic and environmental factors (including interacting medications) on plasma concentrations.

Acknowledgments

The Swiss National Science Foundation (REQUIP Grant No 326000-121314/1 to LAD) and a matching fund from UNIL-CHUV have made possible the acquisition of the UPLC-MS/MS instrumentation. The Swiss National Science Foundation (Switzerland) (Grant No 324730-124943/1 to LAD) has supported in part the salary of TM. The School of Pharmaceutical Sciences, University of Geneva is acknowledged for having supported the salary of ED.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.10.027.

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Therapeutic Drug Monitoring of Targeted Anticancer Therapy. Tyrosine Kinase Inhibitors and Selective Estrogen Receptor Modulators: A Clinical Pharmacology Laboratory Perspective

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Abstract In the last decade, a new era of cancer therapy has emerged, and the treatment of several cancers has shifted from cytotoxic and nonspecific chemotherapy to chronic oral treatment with targeted molecular therapies. Most oral anticancer-targeted drugs approved at present are tyrosine kinase inhibitors (TKIs) and some of them are accompanied with diagnostic test aiming at preselecting patients who are more likely to respond to anticancer treatment, constituting vivid examples of the emerging field of personalized medicine. In that context, since most TKIs are also characterized by an important interindividual variability in their pharmacokinetics, renewed efforts for treatment optimization should be made for targeting adequate drug exposure in patients, increasing thereby the likelihood of optimal clinical response and tolerability of anticancer treatment. This can be done through the Therapeutic Drug Monitoring (TDM) approach, whereby the careful selection

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Q. Alan Xu and Timothy L. Madden (eds.), *LC-MS in Drug Bioanalysis*,
DOI 10.1007/978-1-4614-3828-1_9,

of TKI dosage is adapted to each patient according to individual plasma levels, contributing to minimize the risk of major adverse reactions and to increase the probability of efficient, long-lasting, therapeutic response. This chapter reviews the bioanalytical developments by chromatography and mass spectrometry in the field of targeted anticancer therapy, across the growing family of recent FDA-approved oral TKIs as well as for tamoxifen and its active metabolites, being in fact the most widely used targeted anticancer agent. The text also provides an introduction to existing pharmacokinetics–pharmacodynamics knowledge in the field of targeted anticancer therapy, and the rationale for a TDM program for TKIs.

1 Introduction

Cancer has become one of the most common diseases in developed countries. It is the leading cause of death among men and women aged younger than 85 years in the United States, above cardiovascular problems [1]. Fortunately, considerable medical advances have been achieved in the field of cancer chemotherapy in the last few decades, notably via the progressive optimization and improved management of toxicities of approved anticancer drugs, or by the continuous discovery of novel agents. Nowadays, treatment of many cancers relies on cytotoxic chemotherapy regimens, sometimes in combination with radiation therapy and surgery. Standard cytotoxic drugs used for cancer therapy have generally a narrow therapeutic index, are nonspecific, as they target ubiquitous cell division mechanisms. In that context, considerable research efforts have been pursued for finding specific treatments of cancer [2], with limited success until 2000s, with the notable exception of all-trans retinoic acid in Acute Promyelocytic Leukemia, and tamoxifen in breast cancer. These two drugs, designed for binding to retinoic acid receptors (RAR) and estrogen receptors (ER), respectively, can actually be considered the first clinically used targeted anticancer agents that have been associated with high rates of treatment success and control of the disease for prolonged period of time.

In the last decade, a new era of cancer therapy has emerged, and the treatment of several cancers has shifted from cytotoxic and nonspecific chemotherapy to chronic oral treatment with targeted molecular therapies. These treatments are characterized by unique mechanisms of action and are highly specific for single or multiple key cellular biological pathways responsible per se or implicated in the cancer process [3]. Targeted therapy via protein kinase inhibitors is directed against (onco) proteins allowing the modulation of various signaling pathways and is therefore characterized by more limited nonspecific toxicities. At present, 12 new oral targeted anticancer agents have been approved by FDA (Table 1), over 20 compounds are in Phase I and II trials, and many more at various stages of preclinical development [3]. Except for one agent (vemurafenib), all oral anticancer-targeted drugs approved by FDA are tyrosine kinase inhibitors (TKIs), and will be collectively designated thereafter as the generally accepted acronym TKIs.

Table 1 FDA-approved targeted tyrosine kinase inhibitors

DCI name (trade name)	Year of approval	Target	Indication, cancer
Imatinib (Gleevec®, Glivec®)	2001	BCR-ABL, c-KIT, PDGRF	Philadelphia-positive-chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL) Myelodisplasic syndrome-myeloproliferative disorders (MDS/MPD) Aggressive systemic mastocytosis (ASM) Hypereosinophilic syndrome (HES), chronic eosinophilic leukemia (CEL) Dermato-fibrosarcoma protuberans (DFSP) CD17-positive gastrointestinal stromal tumors (GIST)
Gefitinib (Iressa®)	2003	EGFR	Non-small cell lung cancer (NSCLC)
Erlotinib (Tarceva®)	2004	EGFR	NSCLC Pancreatic cancer
Sorafenib (Nexavar®)	2005	VEGFR, PDGRF, RAF, Mek, Erk	Hepato-cellular carcinoma (HCC) Renal cell carcinoma (RCC)
Sunitinib (Sutent®)	2006	FLT3, PDGFR, VEGFR, KIT	RCC, GIST Pancreatic NET
Dasatinib (Sprycel®)	2006	Src, ABL	Philadelphia-positive CML, ALL
Lapatinib (Tyverb®)	2007	EGFR, HER2	HER2-positive breast cancer
Nilotinib (Tasigna®)	2007	BCR, ABL	Philadelphia-positive CML
Pazopanib (Votrient®)	2009	VEGRF 1,2,3	RCC
Vandetanib (Caprelsa®)	2011	VEGFR, EGFR	Thyroid cancer
Vemurafenib (Zelboraf®)	2011	B-RAF	Melanoma with B-RAF V600E mutation
Crizotinib (Xalkori®)	2011	ALK, hepatocyte growth factor receptor (HGFR; cMet)	Anaplastic lymphoma kinase (ALK)-positive NSCLC

These therapeutic agents are about to revolutionize cancer treatment, and some of them have allowed to transform deadly malignancies into chronically manageable conditions. Nevertheless, primary or secondary drug resistance, persistence of cancer stem cells, and drug adverse effects still limit their ability to stabilize or even cure malignant diseases in the long term. In addition, poor tolerance and therapeutic failure are not uncommon, and relapse is a nearly inevitable consequence of treatment interruption. The appropriate management of oncologic patients therefore requires careful monitoring of these novel treatments [4], for which most clinicians have at present a limited experience.

All these drugs, some of them accompanied with diagnostic tests aiming at preselecting patients who are more likely to respond to anticancer treatment [5], constitute vivid examples of the emerging field of personalized medicine [6, 7]. In that context, since most TKIs are also characterized by an important interindividual variability in their pharmacokinetics (PK), increasing efforts for treatment optimization should be made for targeting adequate drug exposure in patients, increasing thereby the likelihood of optimal clinical response and tolerability of anticancer treatment. This can be done through the Therapeutic Drug Monitoring (TDM) approach, whereby the careful selection of TKI dosage is adapted to each patient according to individual plasma levels, contributing to minimize the risk of major adverse reactions and to increase the probability of efficient, long-lasting, therapeutic response [8, 9].

Conversely, although clinically used for more than 30 years, it has been less than a decade ago that several publications have reported that the clinical efficacy of tamoxifen, the first and most widely used targeted therapy for estrogen-sensitive breast cancer, may depend on the formation of the active metabolites 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethyl-tamoxifen (endoxifen) [10]. Large interindividual variability in endoxifen plasma concentrations has been observed and related both to genetic and environmental (i.e., drug-induced) factors altering CYP450s metabolizing enzymes activity [11]. Since endoxifen is considered to be responsible for an important part of the in vivo pharmacological activity of tamoxifen [10], there is a growing interest for monitoring endoxifen plasma concentrations in breast cancer patients. Whether this would constitute a valid approach to optimize individual tamoxifen dosage remains however to be formally demonstrated in randomized clinical trials (RCTs).

Implementation of a routine TDM program for both TKIs and tamoxifen/endoxifen necessitates the access to suitable instrumental technology, bioanalytical expertise, and definite knowledge in clinical pharmacokinetics for drug level interpretation leading possibly to dosage adjustment. The analytical results, integrated with the clinical observations, may influence the therapeutic intervention and in turn, clinical outcome. Reliability of analytical methods is therefore a critical issue, justifying the efforts and time devoted to their thorough validation and to extensive characterization of their performance (i.e., precision, accuracy, robustness, and turnaround time). Initially, high performance liquid chromatography techniques coupled to ultraviolet detection (HPLC-UV) have been developed for the measurement of imatinib [12] and other TKIs in biological fluids. At present, however, because of its unsurpassed selectivity and sensitivity, HPLC or Ultra performance liquid chromatography (UPLC) coupled to tandem triple quadrupole Mass Spectrometry (LC-MS/MS) has become the method of choice for drug plasma level measurements and is extensively applied for early and more recent TKIs. These powerful analytical technologies are becoming accessible to an increasing number of Academic Hospital Centers for TDM clinical service and research projects. These LC-MS/MS assays can bring invaluable information on patients' drug exposure and contribute, in conjunction with patient's pharmacogenetic tests as well as tumor genetic profiles determination, to the reinforcement and refinement of the personalized anticancer drug prescription.

This chapter reviews the bioanalytical developments by mass spectrometry in the field of targeted anticancer therapy, across the growing family of recent FDA-approved oral TKIs as well as tamoxifen and its active metabolites. The text also provides an introduction to existing pharmacokinetics–pharmacodynamics knowledge in the field of targeted anticancer therapy.

2 New Targets, New Drugs, and New Strategies for Improved Tolerability and Enhanced Clinical Response of the Anticancer Therapy

2.1 New Targets, News Drugs

The first prominent example of TKIs, imatinib, has revolutionized the treatment and prognosis of chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors (GIST) [13, 14]. However, imatinib treatment is not devoid of toxicity, and resistance occurs. It is becoming increasingly recognized that the response is influenced not only by the genetic heterogeneity of drug target determining the tumor's sensitivity (BCR-ABL for CML, and c-KIT for GIST) but also by patient's genetic background and environmental factors that influence drug disposition and overall exposure in the body. Indeed, imatinib drug exposure was found to be a predictor of clinical response in CML [15, 16] and in GIST [17–20].

Following imatinib, other TKIs, including sunitinib, nilotinib, dasatinib, sorafenib, and lapatinib have been developed and are now used for treating various hematological malignancies, solid tumors including GIST [21], advanced renal cell carcinoma (RCC), hepatocellular carcinoma (HCC), and breast cancer [22], and have shown promising activity in other tumors as well [23]. In addition, there was a renewed interest for the EGRF inhibitors gefitinib and erlotinib for the treatment of non-small cell lung cancer (NSCLC), when it was discovered that a patient subgroup, with tumors harboring specific activating mutations of the EGFR genes, was likely to respond better [24, 25]. In 2011, three additional TKIs have been approved by the FDA: vandetanib for the treatment of thyroid cancer [26], vemurafenib against melanoma with B-RAF V600E mutation [27], and crizotinib for anaplastic lymphoma kinase (ALK)-positive NSCLC [28] (Table 1).

Despite their important specificity, toxicity and side effects similar to those of the standard cytotoxic chemotherapeutic approaches can also occur with signal transduction inhibitors.

Whereas toxicities encountered with TKIs treatment are generally less severe than those encountered with conventional cytotoxic approaches, they can, however, significantly impact the safety and quality of life in the long term, jeopardizing treatment adherence. The clinical responses for TKIs may also not always be optimal, calling for a renewed effort for exploring novel avenues and strategies to improve tolerability and therapeutic response.

2.2 New Strategy: Therapeutic Drug Monitoring, General Criteria

During the past decades, it has been established that the therapeutic use of selected drugs could be optimized by an individualization of their dosage, based on blood concentration measurement [29, 30]. As previously mentioned, such a feedback strategy, termed TDM, is now current practice for drugs such as antibiotics, antiepileptics, immunosuppressant drugs, antifungals [31], and, more recently, anti-HIV drugs [32, 33].

TDM is generally considered for drugs with large interindividual but limited intraindividual pharmacokinetic variability with both consistent concentration–efficacy and concentration–toxicity relationships. The sources of variability in drug response are multifactorial, and apart from genetics, other factors such as patient's pathophysiological conditions, environment, drug–drug interactions, food, drinking and smoking habits, medication errors, and poor compliance, may have an important impact on drug pharmacokinetics and/or pharmacodynamics, thereby affecting the therapeutic outcome [29, 30]. Information provided by TDM is particularly useful for drugs with a narrow therapeutic index, subjected to physiologic, genetic, and environmental influences and used for prolonged periods.

In oncology patients, maintaining circulating drug concentrations over a given threshold appears to be crucial to ensure optimal pharmacological action as exposure to suboptimal drug levels during chronic therapy substantially increases the risk of therapeutic failure, due to the progressive selection of cancer cell clones. On the other hand, excessive drug concentrations may be associated with intolerance and adverse drug reactions, leading in term to frequent therapeutic treatment interruption.

While careful monitoring is normally recommended for any type of treatment, their interest varies according to the clinical situations. Short-term treatments generally require little, if any, blood drug level monitoring. For long-term treatments, the interest of TDM is probably limited if all patients respond similarly to the standard regimen. Alternately, in the presence of a significant interindividual variability in response to treatment, the determination of circulating drug concentration in patients' blood may provide clinically useful information for patients' tailored treatment optimization [34].

Like any diagnostic test, the measurement of drug plasma level is, however, justified only when the information provided is of potential therapeutic benefit and has been demonstrated in clinical trials. The clinical value of plasma level monitoring depends on how precisely the treatment outcome can be defined. On the other hand, when a precise therapeutic end point is difficult to define, monitoring of drug levels may be of considerable therapeutic assistance for clinicians [35].

2.3 Therapeutic Drug Monitoring in Conventional Cytotoxic Chemotherapy

In oncology, drug dosage individualization for conventional cytotoxic anticancer therapy is performed according to mg/m^2 or mg/kg. However, even after dose adjustment, the pharmacokinetic variability observed for many cytotoxic

chemotherapeutic agents remains important. The TDM approach is still limitedly used for conventional cytotoxic therapy [36], notably because of the lack of established therapeutic ranges for drug plasma concentrations. Pharmacokinetics-pharmacodynamics studies have shown that TDM of some cytotoxic drug improves the management of therapeutic response and hematological toxicity. For a few drugs (busulfan, cytosine-arabinoside, 5-fluorouracile, and methotrexate), minimal concentration (C_{min}) levels have been found to be predictive of clinical response [37]. Nowadays, in high-dose methotrexate chemotherapy regimen, methotrexate plasma levels are monitored in order to adjust the tetrahydrofolate (i.e., leucovorin) dose administered in prevention of methotrexate renal and systemic toxicity (i.e., methotrexate overdosing prevention by "leucovorin rescue") [30, 37].

2.4 Therapeutic Drug Monitoring Program for Targeted Anticancer Therapy

Fixed dosing is still standard practice for TKIs in the medical oncologist community. While most standard anticancer chemotherapy regimens are administered through short *i.v.* cycles, targeted drugs such as imatinib and the more recent TKIs are orally administered and must be taken in the long term, if not indefinitely. Moreover, they are metabolized mostly by cytochromes P450, in particular the isoenzymes CYP3A4/5, whose activity is known to present a large interindividual variability and influenced by co-medications, organs diseases, diet, and environmental factors, as well as genetic background. Some TKIs are also substrates of drug transporters, such as efflux pumps (e.g., P-glycoprotein; P-gp) or uptake pumps (e.g., human organic cation transporter 1; hOCT1) [18, 22, 38–41]. Finally, as they are extensively bound to circulating proteins in plasma (such as for instance, imatinib on α1-acid glycoprotein [42]), only a small fraction of drug as free drug [43] is likely to enter cells to exert its pharmacological action. The wide interindividual pharmacokinetic variability of TKIs has been clearly demonstrated in several studies [42, 44–52] and there are some preliminary evidences of its consequences on treatment response (see also Sect. 2.5) [15, 16, 19, 20, 47, 48, 53–55]. Identified factors affecting drug disposition include genetic polymorphisms of drug metabolizing enzymes and efflux and influx transporters, age, gender, weight, diet, smoking habit, alcohol consumption, renal and liver function, concomitant diseases, and co-medications. A given dose can therefore yield very different circulating concentrations between patients, favoring the selection of resistant cellular clones in case of subtherapeutic drug exposure, or increasing the risk of adverse drug reactions at excessive plasma levels.

There are therefore several strong lines of arguments for monitoring plasma levels of current and probably other newer TKIs drugs to come [48]. The initiation of such a TDM program for TKIs must also comprise a comprehensive investigation on their concentration–effect relationships, which is still lacking for a majority of them. At present, although not yet formally validated, the TDM for TKIs should

probably be considered in special clinical situations such as in case of less-than-expected initial clinical response, disease recurrence, adverse drug reactions, drug interaction problems, doubt on patient compliance, and in further defined clinical conditions (pediatrics, renal and hepatic failure, etc.). However, further extensive evaluation should be carried out in well-conducted clinical trials before systematic TDM can be integrated into cancer patient's standard of care [56]. The recent experience with the TDM of anti-HIV drugs that has been adopted in the current medical practice without a rigorous evaluation by RCTs of its impact on clinical response and toxicity should be avoided. Most TKIs have just been introduced in the clinical practice, and such window of opportunity should not be missed. The formal demonstration of the clinical usefulness of TDM in RCT for the first major TKI imatinib may therefore constitute the initial step opening the way of a generalized TDM program for all subsequent TKIs for the optimal management of anticancer-targeted therapy [16, 17, 19, 20].

2.5 Pharmacokinetic Variability, Concentration–Efficacy and Concentration–Toxicity Relationships for TKIs

This section presents an overview of the existing pharmacokinetics–pharmacodynamics knowledge in the field of targeted anticancer therapy for the TKIs approved or in late phase of clinical development. An excellent comprehensive review of the clinical pharmacokinetics of the first eight TKIs has been already published [57]. Addressing these aspects is relevant because besides significant variability in pharmacokinetics, relationships between concentrations and efficacy and/or toxicity are amongst the principal characteristics that must be met for considering a formal TDM program.

2.5.1 Imatinib

Pharmacokinetic Variability

Imatinib is characterized by an important interpatient pharmacokinetic variability, yielding trough plasma concentrations spreading over between 40 and 80 % under standard dosing regimens [15, 16, 19, 20, 42, 50, 58]. The intraindividual variability is lower and does not exceed 30 % [42]. The high interindividual variability in pharmacokinetics has been mostly related to differences in the distribution and metabolism of this drug. Imatinib distribution is mainly influenced by plasma protein concentrations, as approximately 95 % of the drug binds to albumin and α-1 acid glycoprotein [18, 59]. The levels of α-1 acid glycoprotein are known to be altered (i.e., increased) in case of infections, and in acute and chronic conditions (cancer, etc.). Active transport mechanisms are also responsible for imatinib tissue uptake (via the carrier human organic cation hOCT-1) and efflux from tissues and

cells (P-glycoprotein [P-gp]) [18, 60, 61]. The expression and activity of these drug transporters is modulated both by genetic factors (polymorphisms affecting function and expression) and environmental influences. As previously mentioned, imatinib is metabolized by CYP3A4, whose activity is genetically determined, as well as likely to be inhibited or induced by various environmental factors (coadministered drugs [62, 63] or food). Low concentrations have been described with the CYP3A4-inducing agents rifampicin, antiepileptics drugs, and St. John's Wort [63]. Alternately, excessive plasma concentration of imatinib, associated with clinical toxicity, has been reported during the coadministration of voriconazole, a known CYP3A4 inhibitor [64]. Finally, poor adherence has been recognized as an important additional source of pharmacokinetic variability [8].

Concentration–Effect Relationship

Several studies have described a relationship between imatinib trough plasma concentrations and clinical response. Initially, Picard et al. [16] have shown that trough plasma concentrations of imatinib are significantly higher in patients with complete cytogenetic response (CCR, defined by the complete disappearance of the Philadelphia positive cells) and a major molecular response (MMR, defined by a 3 log decrease of *BCR-ABL* transcripts). A trough level above 1,002 ng/ml was then recommended for CML patients [16]. In the landmark IRIS study [65], Larson et al. [15] have retrospectively observed higher imatinib trough concentrations 1 month after treatment initiation in patients who showed a complete cytogenetic response and major molecular response, in comparison to patients without cytogenetic or molecular response (cut-off: 1,000 ng/ml). These findings were confirmed in other studies. Whereas target values may differ between studies, most authors acknowledged the potential clinical value of TDM of imatinib in CML patients [66–70].

In GIST, important findings are also emerging from studies examining the relationships between imatinib PK and response to treatment [9]. A pharmacokinetic analysis from a clinical trial of imatinib in patients with unresectable or metastatic GIST reveals a correlation between imatinib total exposure and clinical response. Trough levels over 1,100 ng/ml predicted a better overall benefit rate [17]. Widmer et al. [19, 20] showed that free trough level was correlated with a clinical benefit in GIST patients, with responders having higher free levels than non-responders. Target levels might further depend on tumor genetics [20].

The threshold of effective concentrations of imatinib for optimal clinical response remains to be clearly defined and validated in a prospective clinical trial. It remains also possible that different thresholds exist for different levels of response and cancer cell genetic profiles [58].

Widmer et al. [19, 20] demonstrated that both total (in GIST) and free drug exposure (in CML and GIST) correlated with the occurrence and number of side effects. Moreover, the study of Larson et al. [15] showed that during the first 3 months of imatinib treatment, the types and grades of emerging adverse events were similar

among patients, except for fluid retention, nausea, musculoskeletal pain, rash, myalgia, and anemia, which were more frequently reported by patients with higher imatinib concentrations. Based on the overall 5-year data, only fluid retention, rash, myalgia, and anemia were more frequently reported by the patients with higher imatinib concentrations. These studies therefore suggest that some, but not all, adverse events may be related to elevated imatinib plasma concentrations [34].

TDM for imatinib probably represents therefore a clinically useful tool for providing valuable information for clinicians to investigate the absence of expected clinical response, the occurrence of toxicity, drug–drug interaction problems, and to assess patients' short-term adherence. At present, the level of proof for imatinib TDM varies between "recommended" and "potentially useful" [34]. Whether TDM is also beneficial for the other TKIs remains to be established, but can be anticipated considering their pharmacokinetics characteristics and metabolic pathways and the drug interaction potentials (see below).

2.5.2 Nilotinib

Pharmacokinetic Variability

The interpatient variability in exposure to nilotinib is 32–64 % for exact reasons remaining yet to be explained [44, 57]. In the phase I dose escalation study, a saturation of nilotinib serum levels was observed with doses ranging from 400 to 1,200 mg daily. With the administration of daily doses at the steady-state level, the peak concentration and the area under the concentration–time curve increased among patients receiving 50–400 mg of the drug and reached a plateau among patients receiving more than 400 mg. A possible explanation might be that nilotinib gastrointestinal absorption saturates at doses exceeding 400 mg [71].

Concentration–Effect Relationship

The relationships between nilotinib plasma concentration and clinical efficacy (or toxicity) have not been studied yet. Irrespective of nilotinib PK-PD per se, Saglio et al. [72] showed that nilotinib at a dose of either 300 or 400 mg twice daily was superior to imatinib in patients with newly diagnosed chronic-phase Philadelphia chromosome-positive CML. At 12 months, the rates of major molecular response for nilotinib were nearly twice that observed for imatinib. The rates of complete cytogenetic response by 12 months were also significantly higher for nilotinib than for imatinib [72]. No data have been published for nilotinib concentration–toxicity relationships nor plasma target values to be achieved for optimal clinical response.

The limited PK-PD information available at present for nilotinib does not exclude, however, that this drug may be a good candidate for TDM.

2.5.3 Dasatinib

Pharmacokinetic Variability

Dasatinib interpatient and inter-occasion variability is important and ranges from 32 to 118 %. A substantial proportion of the inter-occasion variability is supposedly related to the drug bioavailability. The origin of the interpatient variability has not been elucidated yet, but is presumably related to dasatinib CYP3A-mediated metabolism, characterized by high variability in activity and expression [46].

Concentration–Effect Relationship

Preclinical and clinical investigations have demonstrated that dasatinib is active against imatinib-resistant BCR-ABL variants CML and has further improved the treatment of CML [73]. Moreover, dasatinib, as compared with imatinib, induced significantly higher and faster rates of complete cytogenetic response and major molecular response. Since achieving complete cytogenetic response within 12 months has been associated with better long-term, progression-free survival, dasatinib may improve the long-term outcomes among patients with newly diagnosed chronic-phase CML [54]. However, no clear concentration–efficacy relationship has been proposed yet, which again does not exclude a role for TDM in the future.

In the study of Wang et al., dasatinib trough levels appear to correlate strongly with toxicity but not with efficacy. The lowest trough concentration was achieved with the lowest dose regimen (100 mg once daily) which has been shown to have the optimal therapeutic index among the regimens tested [74].

2.5.4 Sunitinib

Pharmacokinetic Variability

Sunitinib interpatient variability in pharmacokinetics is also significant, of approximately 40 %, which is unexplained yet [47].

Concentration–Effect Relationship

The results of a meta-analysis [47] indicate that increased exposure to sunitinib in patients with advanced solid tumors, including patients with GIST and metastatic RCC, is associated with improved clinical outcomes, as well as some increased risks of adverse effects. This analysis indicates that increased exposure to sunitinib is associated with longer time to tumor progression, longer overall survival, a higher probability of a response, and greater tumor-size decreases. A sunitinib 50-mg starting

dose has been proposed to provide clinical benefit with acceptably low risk of adverse events [47]. Based on preclinical data [75] and a phase I study [76], a target plasma concentration of 50 ng/ml (parent drug *plus* metabolite SU12662) was defined for sunitinib, even though no formal TDM study has, to the best of our knowledge, been initiated yet for this latter drug.

Houk et al. [47] have shown that increased exposure to sunitinib is associated with increased risk of adverse effects generally mild to moderate in severity. Faivre et al. [76] have found dose-limiting toxicities at plasma concentrations of sunitinib *plus* SU12662 higher than 100 ng/ml.

2.5.5 Sorafenib

Pharmacokinetic Variability

Sorafenib pharmacokinetics shows a large interpatient variability [49, 57]. The large interpatient variability is supposed to be the result of slow dissolution of the drug in the gastrointestinal tract and of the existence of an entero-hepatic circulation [51].

Concentration–Effect Relationship

No information on sorafenib concentration–toxicity relationships is available at present. Again, the absence of any PK-PD data does not preclude any interest for a formal TDM for sorafenib.

A study has shown in patients with metastatic RCC and hepatocarcinoma, given the standard regimen of sorafenib (800 mg daily), that toxicity occurrence may be related to high plasma sorafenib exposure [77]. However, an upper plasma level was not determined.

2.5.6 Lapatinib

Pharmacokinetic Variability

Lapatinib variability is large (68 %) and not significantly reduced by the coadministration of food (52 %) [45, 57]. M. Ratain and E. Cohen [78] have suggested that a lower dose of lapatinib could be administered if taken with food, to take advantage of the increased absorption of lapatinib in the presence of high fat meals, or if taken with grapefruit juice, a known CYP3A inhibitor, which should result in an overall reduction in treatment cost. However, they strongly recommended that this approach should not be done without a formal pharmacokinetic assessment.

Concentration–Effect Relationship

Burris et al. [53] showed in patients with metastatic solid tumors treated with lapatinib at doses ranging from 500 to 1,600 mg once daily that clinical responses were generally associated with doses in the middle of the range examined. Clinical response was more often associated with doses of 900–1,200 mg daily. However, due to the limited response data, it was not possible to adequately characterize the relationship between clinical response and drug exposure, which would be a prerequisite before assessing the potential role of TDM in lapatinib dosage individualization.

Finally, relationships between lapatinib plasma concentration and clinical toxicity have not been yet formally studied.

2.5.7 Miscellaneous TKIs

Pharmacokinetic Variability

Very large variations have been demonstrated for gefitinib exposures and for the recent TKI axitinib (evaluated for metastatic melanoma, renal cell and thyroid cancer, and NSCLC), with variation in drug exposure ranging between 113 % and 39–94 % for gefitinib and axitinib, respectively [48]. In that context, an assay for phenotyping patients' CYP3A activity has been proposed for predicting gefitinib systemic exposure and helping at drug dosage selection [79]. Erlotinib interpatient pharmacokinetic variability is also important (60 %) and, as yet, unexplained [52].

Information on the clinical pharmacokinetics is also available of the recently approved TKIs. Vandatenib pharmacokinetics, studied in healthy volunteers and patients [80, 81] was found to be both influenced by patient's renal function and vulnerable to drug–drug interactions [82, 83]. An important pharmacokinetic variability is noticeable in the mean steady state PK profiles published for verumafenib (formerly PLX4032) [27].

Several new TKIs are in advanced stage of clinical development, including bosutinib [84] and bafetinib [85], the third-generation TKIs against imatinib-, nilotinib-, and dasatinib-resistant CML; the multi-targeted kinase inhibitor pazopanib, approved for advanced or metastatic RCC [86]; and neratinib with antitumor activity in HER2–positive breast cancer. Bosutinib, pazopanib and neratinib are all substrates of the CYP3A enzymatic system, and their plasma exposure is increased when coadministered with potent CYP3A inhibitors [86–88], potentially requiring dose adjustment for neratinib [87].

Finally, pharmacokinetics and metabolic studies are also available for TKIs at various stages of clinical development, including vatalanib [89–91], cediranib [92–94], and motesanib [95].

Concentration–Effect Relationship

Statistically significant associations were demonstrated between the 5 and 10 h post-dosing plasma concentrations of erlotinib and survival in patients receiving 150 mg erlotinib daily for advanced recurrent and/or metastatic squamous cell cancer of the head and neck [55]. Based on in vitro data, a target plasma concentration of erlotinib higher than 420 ng/ml has been proposed [96]. Moreover, vatalanib trough plasma concentration, AUC and maximal concentration were positively correlated with likelihood of response in metastatic liver lesions on imaging [48]. Finally, target efficacious plasma levels based on in vitro data have been proposed for crizotinib, a TKI recently approved for locally advanced or metastatic NSCLC positive for anaplastic lymphoma kinase (ALK) [97, 98].

Erlotinib area under the curve was positively correlated with the occurrence of skin toxicity in two independent studies, in patients with non-small cell lung cancer [99, 100]. Moreover, Mohamed et al. [101] showed that the occurrence of skin rash was associated with significantly improved survival for advanced NSCLC patients who failed prior chemotherapy, upon treatment with gefitinib. Hypertension is another example of group effect of VEGF inhibitors, which might be due to inhibition of vascular relaxation, decreased production, and rarefaction of nitric oxide [102]. Indeed, the rise in diastolic blood pressure during treatment with the VEGF1/2/3 inhibitor axitinib was a predictor of longer survival in patients with various malignancies [48].

In conclusion, most recent TKIs share with imatinib the same large interindividual pharmacokinetic variability with, at least for a few of them, some reports of concentration–efficacy and concentration–toxicity relationships, calling for further extensive evaluation of the TDM approach. The development of analytical methods allowing to confidently quantifying TKIs in biological fluids is a prerequisite prior to the implementation of any clinically useful TDM Service.

2.6 Analytical Methods by LC Tandem MS for the Bioanalysis of TKIs

This section reviews the analytical methods using liquid chromatography coupled to tandem mass spectrometry that has been developed for measuring the concentration of TKIs in various human biological samples.

2.6.1 Methods of Quantification for Single TKIs

Quantification of TKIs in patients' plasma samples is at present principally performed by liquid chromatography-mass spectrometry (LC-MS) after suitable plasma pretreatment, which implies most generally a protein precipitation with an organic

solvent, or a liquid–liquid extraction (LLE) using a non-miscible phase (sometimes adjusted at a pH that takes advantage of the mostly basic nature of TKIs) or, alternately, using either an off-line or online solid phase extraction (SPE) step.

Most analytical methods published to date using liquid chromatography coupled to mass spectrometry (LC-MS) have focused on the assay in human biological fluids, generally plasma, of a single TKI, namely imatinib [103–110] (see Table 2). Most proposed methods use generic protein precipitation by acetonitrile (ACN) [104–106, 108, 109] of whole blood [107] or plasma [106, 108, 109], prior to imatinib quantification in supernatants. A semiautomated protein precipitation step within a 96-well plate format is also described [104]. A methodology using LLE with hexane-ethylacetate (30:70, v/v), followed by evaporation and reconstitution in acetonitrile/water/formic acid (30:70:0.1 %, v/v/v) [103] or in 4 mM ammonium formate buffer/methanol (1:1, v/v) [110], is an alternative method for sample preparation that was performed by two other groups. The described sample extraction procedures for the bioanalysis of the closely related TKI nilotinib involved plasma protein precipitation by acetonitrile [111], as well as LLE with methyl *tert*-butyl ether, evaporation and reconstitution in acetonitrile/0.2 % formic acid (1:9, v/v) [112].

The two methods published for the assay of sunitinib have used LLE of plasma with methyl *tert*-butyl ether solvent, evaporation and reconstitution in acetonitrile/water/formic acid (20:80:0.1, v/v/v) [113] and acetonitrile [114].

Two articles have described analytical methods for the determination of sorafenib plasma concentrations, involving a similar protein precipitation step with acetonitrile [115, 116]. There is at present only one published method for the quantification of lapatinib in human plasma after off-line SPE onto C18 cartridge, followed by evaporation and reconstitution in ACN/5.0 mM ammonium formate pH 3/formic acid (1,000:50:1, v/v/v) [117].

An assay has been described for the determination of vandetanib in human plasma and in cerebrospinal fluid. The assay consists in an LLE with *tert*-butyl methyl ether in the presence of ammonium hydroxide, followed by evaporation of the top organic layer and reconstitution in ACN/10 mM ammonium formate pH 5, prior to reversed-phase LC tandem MS [118].

Analytical methods have been published also for the more recent TKIs in early or late phase of clinical development, which comprise vatalanib [119] and axitinib [120]. Abbas et al. have developed an analytical method for measurement of the anti-CML bosutinib in plasma of healthy subjects [88]. Samples were extracted from plasma by LLE with carbonate buffer pH 10 and 1-chlorobutane (1:10, v/v) prior to evaporation and reconstitution in water/methanol solution (50:50, v/v).

The assays of these TKIs in plasma involve mostly reversed-phase liquid chromatography. The chromatographic principles and separation mechanisms are the same for High Performance Liquid Chromatography (HPLC) and Ultra Performance Liquid Chromatography (UPLC), while speed, sensitivity, and resolution are improved from UPLC [121]. The main advantage of UPLC is a significant reduction of analysis time, resulting in a decrease in solvent consumption, turnaround time

Table 2 Description and performance of the reported methods for quantification of TKIs

Drugs analyzed	Volume of biological sample	Analytical method	LOQ (ng/ml)	Accuracy at LOQ (% bias)	Precision at LOQ (CV%)	Reference
Imatinib	200 µl Human plasma	ESI-TSQ	10	−14.7	5.27	Titier et al. [110]
Imatinib	200 µl Human plasma	ESI-TSQ	10	1.68	5.75	Awidi et al. [103]
Imatinib	250 µl Human red blood cells plasma	ESI-TSQ	2.1 1.8	−4.3 to 0.8 0.2 to 2	3.78 to 5.02 5.74 to 2.39	Guetens et al. [106]
Imatinib	200 µl Whole human blood and leukemia cells	Turbo ion spray-TSQ	0.03	0.9 −1.9	8.8 13.2	Klawitter et al. [107]
Imatinib and CGP 74588	200 µl human plasma	ESI-TSQ	1 2	–	–	Boddy et al. [105]
Imatinib and CGP 74588	200 µl human plasma	ESI-single quadrupole	30	–	–	Parise et al. [108]
Imatinib and CGP 74588	100 µl human plasma	ESI-TSQ	1	–	–	Rochat et al. [109]
Imatinib and CGP 74588	200 µl human plasma	ESI-TSQ	4	−0.8 to 4	4.19 to 5.64	Bakhtiar et al. [104]
Nilotinib	200 µl Human plasma Serum	ESI-single quadrupole	5	−7.9 1.6	3.6 7.8	Parise et al. [111]
Nilotinib	100 µl Human serum	ESI-TSQ	2.5	–	–	Tanaka et al. [112]
Dasatinib	100 µl Human plasma	ESI-linear trap	–	–	–	Christopher et al. [126]
Bosutinib	100 µl Human plasma	Turbo ion spray-TSQ	1	–	–	Abbas et al. [88]
Sunitinib	200 µl Human plasma	ESI-TSQ	0.6	–	–	Minkin et al. [114]
Sunitinib	100 µl Human plasma	ESI-TSQ	0.2	9.5	11.7	De Bruijn et al. [113]
Sorafenib	50 µl Human plasma	ESI-TSQ	5	−4.33	7.45	Jain et al. [115]
Sorafenib	100 µl Human plasma	ESI-TSQ	7.3	2.3	3.3	Zhao et al. [116]
Lapatinib	100 µl Human plasma	Turbo ion spray-TSQ	15	–	11	Bai et al. [117]

Vatalanib	50 µl Human plasma	ESI-TSQ	10.2	6.25	8.38	Lankheet et al. [119]
Axitinib	100 µl Human plasma	ESI-TSQ	0.2	–	–	Sparidans et al. [120]
Neratinib	250 µl Human plasma	ESI-TSQ	3			Abbas et al. [87]
Vandetanib	100 µl Human plasma 100 µl Human CSF	Turbo ion spray-TSQ	1 0.25	8.5 –0.2	3.4 2.6	Bai et al. [118]
Imatinib, dasatinib, nilotinib	250 µl Human plasma	ESI-single quadrupole	78.1 62.5 62.5	–	–	De Francia et al. [133]
Imatinib Erlotinib Gefitinib	100 µl Human plasma	ESI-TSQ	4.4 4.5 4.8	–3.8 to 10.6	2.1 to 3.2	Chahbouni et al. [135]
Imatinib, dasatinib, nilotinib, sunitinib, sorafenib, and lapatinib	100 µl Human plasma	ESI-TSQ	1–10	–6.3 to 6.7	3.4 to 9.9	Haouala et al. [122]
Erlotinib, imatinib, lapatinib, nilotinib, sorafenib, and sunitinib	100 µl Human plasma	ESI-TSQ	2.2 for sunitinib 3.8–12.6 for other TKI			Gotze et al. [137]
Bosutinib, erlotinib, gefitinib, and vemurafenib		ESI-TSQ	1, 10, 10, and 80, resp.			Neeman [139]
Dasatinib and lapatinib	5×10^4 cells/mL lung cancer cell-lines	ESI-TSQ	50 100	–	8 13	Roche et al. [141]

(continued)

Table 2 (continued)

Drugs analyzed	Volume of biological sample	Analytical method	LOQ (ng/ml)	Accuracy at LOQ (% bias)	Precision at LOQ (CV%)	Reference
Gefitinib, erlotinib, sunitinib and sorafenib	20 µl Human whole blood, serum, plasma and cell culture medium	Turbo ion spray-TSQ	5	3.7 to 13.5	1.9 to 12	Honeywell et al. [136]
Imatinib	PBMC	ESI-TSQ	10	–	–	Widmer et al. [42]
Nilotinib, dasatinib, and bosutinib	2×10^6 K562 cells	ESI-TSQ	0.1–1	–	–	Hegedus et al. [142]
Imatinib, nilotinib, dasatinib, sunitinib, and sorafenib	9×10^5 K562 cells	ESI-TSQ	1–10	−12.6 to 10.1 %	9.1 to 11.3 %	Haouala et al. [143]
Imatinib, dasatinib, and nilotinb	PBMC	HPLC-MS	0.25 ng			D'Avolio et al. [144]

and costs [121]. However, UPLC has been applied only for quantification of the TKIs sunitinib [113] and axitinib [120].

A publication describes a column switching procedure for an imatinib assay, involving a C8 extraction column, followed after activation of the switching valve by the back-flushing of imatinib onto a C18 analytical column [107]. The run time was 10 min. The other published methods use HPLC with C18 columns with analytical times of 2 min [106], 3 min [103], 6 min [110], 10 min [107], 14 min [108], and 20 min [109]. C8 columns were used by two groups with analytical times varying between 2.5 min [104] and as much as 40 min [105].

Nilotinib was analyzed by HPLC onto a C18 column [111, 112], with a reported run time of 15 min [111].

Interestingly, the two methods published for quantification of sunitinib used either HPLC [114] or UPLC [113] but the analytical time periods were of similar duration (run time of 3 min and 4 min, respectively). Of note, we observed during the course of our own method development [122] the presence of two peaks with the same molecular mass/signal transition for sunitinib, which, to the best of our knowledge, has not been reported elsewhere [113, 114, 123]. The phenomenon was known, however, and is due to a *Z–E* isomerization reaction of sunitinib [124]. Previous studies by the sunitinib manufacturer have shown that *E* isomer can be generated from the *Z* isomer in a reversible manner in solution [124]. The rate of interconversion between the *Z–E* configurations in solution is dependent on a number of factors, most notably exposure to light. In our studies [122], we found that both isomers could be detected in the pharmaceutical preparation (tablet) at ratios of about 1:2, as well as in patients' plasma samples (variable ratios).

The determination of sorafenib plasma concentrations was performed by HPLC onto a C8 column (4 min run time) [115] and C18 column (6 min run time) [116]. Similarly, HPLC C18 columns have been used for the quantification in plasma of lapatinib [117], vatalanib [119] (3 and 8 min run time, respectively), and also for bosutinib [88], whereas UPLC was used for the TKI in development of axitinib (1.2 min run time) [120].

Detection of imatinib is performed by triple quadrupole mass spectrometer with an electrospray ionization (ESI) interface operated in positive ion mode [103, 104, 106, 107, 109, 110]. Except the methods published by Parise et al. for imatinib and its main metabolite [108], and for nilotinib [111], where a single quadrupole mass spectrometer was used, most TKIs are analyzed in plasma by atmospheric pressure ionization (electrospray or turbo ion spray) coupled to triple stage mass spectrometer. Expectedly, higher limit of quantifications for imatinib (30 ng/ml) [108], and nilotinib (5 ng/ml) [111], are obtained for the assays using single quadrupole MS (see Table 2).

In general, triple stage quadrupole mass spectrometer with ESI in positive ion mode is perceived as the most appropriate MS technique available at present for small—mostly basic—molecules and was used for the assay of nilotinib [112], sorafenib [115, 116], lapatinib [117], sunitinib [113, 114], bosutinib [88], vatalanib [119] axitinib [120], vandetanib [118] neratinib [87], and crizotinib [125]. The latter assay for crizotinib was developed for preclinical experiments and does not contain

information on its analytical performance, and was subsequently adapted for pharmacokinetic studies in cancer patients [125].

So far, validated LC-MS/MS methods published for the assay of dasatinib in human plasma also comprise the analysis of metabolites [46, 126, 127].

Finally, to the best of our knowledge, there are, as yet, no analytical method validation reports for the assay of the latest TKIs pazopanib, bafetinib, cediranib, and motesanib in human biological samples.

2.6.2 Methods for Quantification of TKIs and Metabolites

Up to now, most investigations on the pharmacokinetic–pharmacodynamic aspects of TKIs therapies have focused almost exclusively on concentrations of the parent TKI drug in plasma, considering it as the best pharmacokinetic marker of anticancer drug exposure and, in case of higher levels, of toxicity. However, drug metabolites resulting from complex mutual genetic and environmental influences can also contribute to treatment outcome. The metabolite profile can be considered as a snapshot on the phenotypic pattern of the metabolizing activity in a patient at a given time. Unfortunately, integration of this aspect with pharmacokinetics has attracted little attention so far in the field of TKIs therapy. Distinct metabolite profiling patterns per se could play an important role in the toxicity, tolerability, and outcome of targeted anticancer therapy.

In that context, the LC–MS/MS technology makes it possible to determine in patients' plasma not only the parent drug but also metabolites. Such an approach has been applied for the quantification of imatinib and its main active *N*-desmethyl metabolite CGP 74588 in plasma [104, 108], for monitoring imatinib metabolites profile in patients' plasma [109], and for metabolism studies on dasatinib [46, 126, 127]. Assays enabling the quantification of sunitinib and its n-desethyl metabolite SU12662 [113] and, more recently, dasatinib and two active metabolites [127] have also been published. Overall, exposures of pharmacologically active metabolites in patients suggested that they are not expected to contribute significantly for the in vivo activity.

2.6.3 Methods for Multiplex Quantification of TKIs

Plasma Measurements

Mass spectrometry detection qualifies for the simultaneous measurement of arrays of structurally unrelated anticancer-targeted agents in a single analytical run. Multiplex analyses offer, therefore, the advantage of the establishment of calibration curves for several TKIs simultaneously, resulting in an overall reduction in analytical time, turnaround time, and costs [128, 129]. Analytical methods using a simplified extraction procedure followed by simultaneous quantification of multiple TKIs are more efficient for rapidly providing TDM results allowing real-time

processing of blood samples from patients receiving different single-drug or combined regimens and for maximizing laboratory's resource utilization.

Thus, the development and validation of enhanced throughput methods with simple extraction procedure followed by LC-MS/MS are of high interest for the simultaneous analysis of every major anticancer-targeted agent [130, 131], which in the future may possibly be used also in combination therapy [132].

In that context, an assay limited to the antileukemic drugs imatinib, dasatinib, and nilotinib was proposed in 2009 implying plasma protein precipitation procedure followed by reversed-phase chromatography. TKIs detection was made by an ESI interface, coupled to positive SIM mode single quadrupole mass spectrometer [133]. However, single quadrupole MS analysis is probably not sensitive enough for the accurate quantification of very low plasma levels of dasatinib. In fact, the lower limit of quantification for dasatinib reported in this study is 62.5 ng/ml, which corresponds to peak plasma concentrations rather than trough dasatinib levels [134] (see Table 2). This suggests that such an assay, because of the insufficient sensitivity provided by a single quadrupole mass spectrometer, is of limited clinical usefulness for a formal therapeutic monitoring of dasatinib.

At about the same time, our laboratory has reported the development and validation of an LC tandem MS assay for as much as six TKIs simultaneously. The proposed LC-MS/MS method allows the simultaneous determination of clinically relevant ranges of concentrations for the six major TKIs currently in use imatinib, dasatinib, nilotinib, sunitinib, sorafenib, and lapatinib [122]. Plasma is purified by acetonitrile protein precipitation followed by reversed-phase chromatographic separation. Analyte quantification is performed by electrospray ionization–triple quadrupole mass spectrometry by selected reaction monitoring (SRM) detection using the positive mode. This was the first broad-range LC-MS/MS assay covering the major currently in-use TKIs.

Various methodologies have been proposed since then for multiple TKIs assays. The measurement of the first three marketed TKIs gefitinib, erlotinib, and imatinib was carried out by liquid–liquid extraction of human plasma, using hexane-ethyl acetate (30:70, v/v) as extracting solvent. The reconstituted extracts in the organic upper phase were subjected to reversed-phase HPLC and the TKIs were detected by electrospray triple quadrupole mass spectrometry, operated in the positive mode [135]. A multiplex analysis of TKIs used for the treatment of solid tumors, (gefitinib, erlotinib, sunitinib, and sorafenib) was also proposed using plasma protein precipitation with acetonitrile, supernatant injection into reversed-phase column, and TKIs detection/quantification by a triple quadrupole mass spectrometer equipped with a turbo-spray ionization operating in positive multi-reaction-monitoring-mode [136]. Just recently, Götze et al. have published a multiplex assay for the determination of erlotinib, imatinib, lapatinib, nilotinib, sorafenib, and sunitinib that was proposed for routine clinical application [137]. Finally, an assay allowing the determination of as much as nine TKIs simultaneously (imatinib, its metabolite, nilotinib, lapatinib, erlotinib, sorafenib, dasatinib, axitinib, gefitinib, and sunitinib) has been recently developed by Bouchet et al., using 96-well SPE plates and UPLC tandem MS [138].

Recently, we have adapted our previous methodology for the multiplex assay of TKIs [122] for the assay of additional current and newer TKIs possibly analyzed simultaneously. Briefly, a modification of the gradient program and the adjustment of the composition of the mixture (15 % of MeOH in Ammonium formate 20 mM pH 2) used for diluting the supernatants (obtained after plasma protein precipitation with acetonitrile) were carried out to account for the chromatographic behavior as well as different solubilities of early (bosutinib, gefinib) and late (verumafenib, formerly PLX4032) eluting drugs (Fig. 1) [139]. Such adaptation allowed the simultaneous measurement in plasma of gefitinib, erlotinib, the third generation anti-CML agent bosutinib, as well as vemurafenib. This latter drug attracted much interest lately, because of its impressive clinical effect against melanoma harboring the BRAF V600E mutation [27]. First applications of this methodology to patients samples confirmed the wide interindividual variability and—fairly unpredictable—pharmacokinetics of erlotinib and verumafenib, and gave preliminary insights on clinical consequences of its pharmacokinetic variability. For example, very high plasma concentrations of erlotinib were found in a female patient who developed a grade 2 rash, confirming the known relationships between erlotinib plasma levels and the incidence of cutaneous adverse drug reactions [140].

Cellular Measurements

Up to now, most investigations on the TDM of TKIs therapy have focused on the measurement of concentrations of the parent TKI drug in plasma. However, TKIs act intracellularly and their concentrations in cell cytoplasm, besides being determined by circulating blood levels, are also controlled by various transmembrane drug transporters influencing cellular uptake and release. TKIs' pharmacological activity in cells is modulated by complex mutual biological, genetic, and environmental influences, which remain poorly known, and efforts have been recently made to study their cellular disposition, i.e., at determining their "cellular" concentration that would closely reflect the intracellular environment of the therapeutic target, than the systemic blood concentrations that are currently measured.

In that context, Klawitter et al. [107] developed and validated an LC-MS/MS method using a turbo ion spray coupled to TSQ mass spectrometer for the quantification of imatinib in human leukemia cells, using a first step of protein precipitation followed by column switching.

A method has been proposed for the determination of cellular levels in lung cancer cell lines of the TKIs dasatinib and lapatinib [141]. Cellular samples were extracted with a mixture of *tert*-butyl methyl ether/ACN/ammonium formate pH 3.5 (6:2:1, v/v/v). The organic layer was subjected to evaporation and the samples were reconstituted in acetonitrile, followed by chromatographic separation on a C18 column. Dasatinib and lapatinib were monitored by tandem MS equipped with a positive electrospray ionization interface in positive ion mode. These cellular experiments showed that lapatinib is not actively expelled from P-gp over-expressing cancer cells, while P-gp activity significantly decreases cellular levels of dasatinib [141].

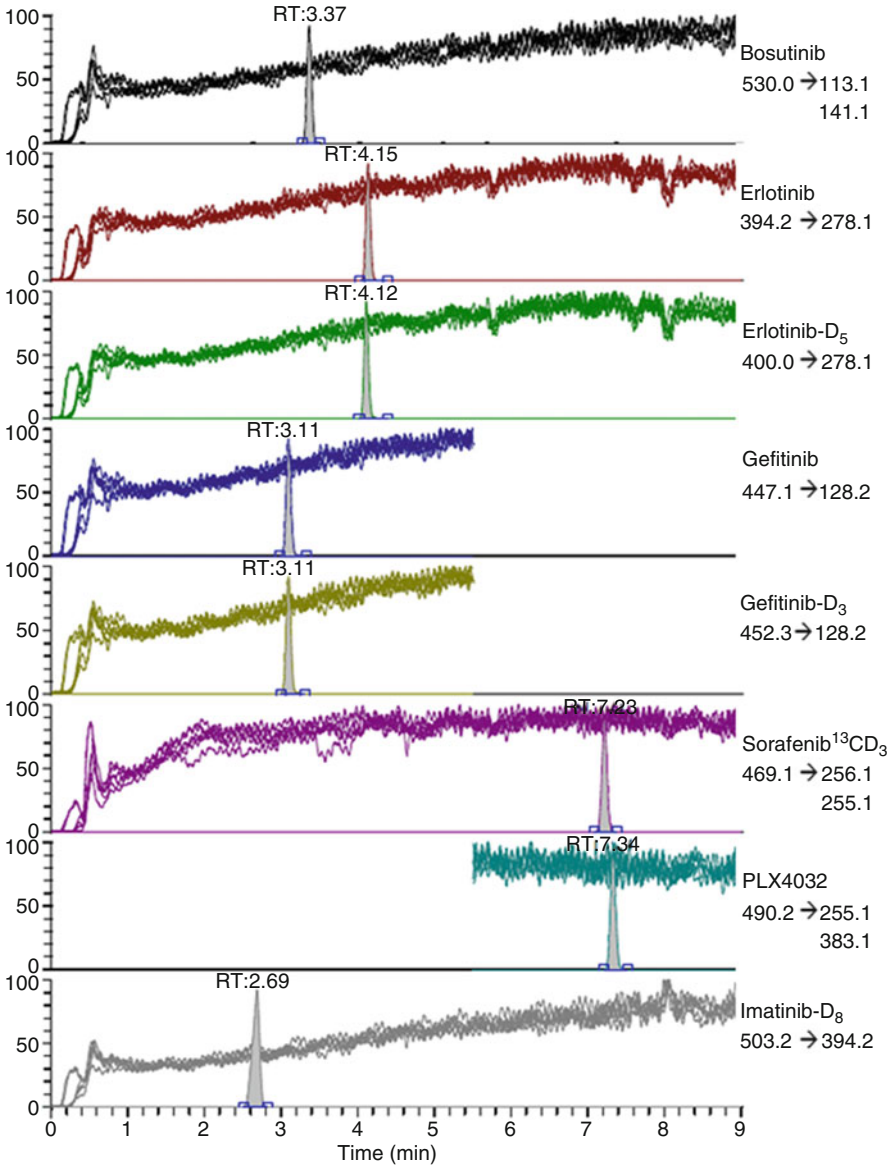

Fig. 1 A multiplex LC tandem MS for the quantification of the targeted anticancer agents bosutinib, gefitinib, erlotinib, and vemurafenib (formerly PLX4032). The chromatographic profile of a QC control containing 37.5 ng/ml of bosutinib, 375 ng/ml of gefitinib and erlotinib, and 3,000 ng/ml of vemurafenib is shown. Chromatographic separations were performed on a column Waters XTerra MS C18 2.1 × 50 mm. Solvent A consisted of 10 mM ammonium acetate containing 1.5 % formic acid (pH 2.3). Solvent B was acetonitrile with 1 % formic acid. The mobile phase was delivered at 0.3 ml/min according to the gradient elution program: 0–9 min, solvent B 5 % → 85 % B, followed by a re-equilibration step. Quantifications were done using the internal standards erlotinib-D_6 and gefitinib-D_3. The internal standards (I.S.) imatinib-D_8 and sorafenib-^{13}C-D_3 have similar retention times as bosutinib and verumafenib, respectively, and were used as I.S. for these latter drugs, because of the lack of labeled standards at the time the assay was developed. On the same chromatographic profiles are shown in offset the superimposed ionization traces of the selected transitions during the analysis of six blank plasma extracts with post-column infusion of a solution containing the four TKIs (1 μg/ml)

Quantification of nilotinib, dasatinib, and bosutinib was done in K562 leukemia cells by protein precipitation using acetonitrile, followed by triple quadrupole mass spectrometer analysis operated in positive ion electrospray mode [142]. Nilotinib and dasatinib were found to act both as transported substrates and, at high concentrations, inhibitors of *ABCB1* (gene coding for P-glycoprotein) and *ABCG2* (gene coding for BCRP) [142]. Whereas neither ABCB1 nor ABCG2 could confer bosutinib resistance; this TKI efficiently inhibited both transporters at higher concentrations [142].

In our laboratory, an assay has been developed for the determination of cellular concentration of imatinib in peripheral blood monocytes cells (PBMCs) isolated from patients [42]. Intracellular concentrations of imatinib were measured in PBMCs from five patients using validated LC MS/MS methods [42]. The intra/extracellular ratio appeared to be constant over the observation period indicating an average eightfold accumulation of imatinib in cells. More recently, as part of our in vitro studies on the consequence of drug transporters expression on TKIs disposition, we have developed a simplified methodology for the intracellular determination of several major TKIs (imatinib, nilotinib, dasatinb, sunitinib, and sorafenib) in K562 cell lines [143]. Incubated cells were first extracted with 0.5 ml MeOH/H_2O 50:50 by vortex mixing, ultrasonication, and centrifugation, yielding cellular extracts. TKIs were subsequently quantified over the relevant concentration range of 0.1–5,000 ng/ml with an adaptation of our validated multiplex LC-MS/MS method [122]. These experiments have revealed that the differential expression and/or function of P-gp was not affecting the cellular disposition of nilotinib, in contrast to the other tested TKIs.

Lately, the development of an assay by LC coupled to single quadrupole mass spectrometry has been recently published for the determination of cellular concentrations of imatinib, dasatinib, and nilotinib in PBMCs, but the authors did not give much details on the results obtained with patients samples [144].

In conclusion, the multiple-analytes LC-MS methods represent an improvement over previous single-analyte methods in terms of convenience (a single extraction procedure for several TKIs, reducing significantly the analytical time), sensitivity, selectivity, and throughput. The current facilitated access to LC-MS technology may contribute to filling our current knowledge gap in the pharmacokinetics–pharmacodynamics relationships of the latest TKIs developed following imatinib. It might better define therapeutic ranges of TKIs in various patient populations prior to the evaluation of a systematic TDM-guided dose adjustment of these anticancer drugs.

3 Tamoxifen as the First Targeted Anticancer Agent

Introduced into the clinic some 30 years ago, tamoxifen selectively modulates estrogen receptors and thus can be considered as one of the first examples of “targeted” anticancer therapy, years before the era of TKIs described in Section 2. This largely justifies that a section of the present review is devoted to tamoxifen, especially in

the light of recent findings —some of them made possible by the advent of new powerful mass spectrometry techniques— suggesting that tamoxifen pharmacological activity and clinical outcomes do not rely on the parent drug only, but also depends on the presence of several tamoxifen metabolites produced in patients via complex metabolic pathways. A comprehensive review of mass spectrometry methods for tamoxifen and its metabolites is therefore presented in the context of the current growing interest for monitoring tamoxifen metabolites as a potentially clinically useful tool to monitor tamoxifen treatment in breast cancer patients.

3.1 Clinical Rational for a TDM and Metabolites Profiling of Tamoxifen [145]

The non-steroidal selective estrogen receptor modulator (SERM), tamoxifen, was the first molecularly targeted cancer therapy approved by the U.S. Food and Drug Administration (FDA) since 1977 and 1998, respectively, for the treatment and prevention of estrogen-sensitive breast cancer (BC) [146–148]. Tamoxifen—the *Z* geometric isomer of a triphenylethylene derivative—has been for more than three decades the most widely used antihormonal therapy for premenopausal and postmenopausal women with metastatic breast cancer, for adjuvant and neo-adjuvant treatment of primary breast cancer and as a preventive agent for women at high risk of developing the disease [149–157].

Selective estrogen receptor modulators, such as tamoxifen, display tissue-selective estrogen agonist or antagonist effects. In breast tissues, tamoxifen exerts an anti-estrogenic activity mediated by the competitive inhibition of 17beta-estradiol (E2) binding to estrogen receptors alpha and beta (ERα and ERβ), resulting in the suppression of ERα transcriptional activity and inhibition of estrogen-dependent growth and proliferation of malignant breast epithelial cells [158–161]. However, several lines of evidence indicate that the overall anti-proliferative effects of tamoxifen depend on the formation of the pharmacologically active metabolites 4-hydroxy-tamoxifen and notably 4-hydroxy-*N*-desmethytamoxifen (endoxifen) which have up to 100-fold greater affinity to ERs and 30 to 100-fold greater potency in suppressing breast cancer cell proliferation as compared to the parent drug [10, 162–164].

Of these active metabolites, endoxifen is suggested to be the primary active metabolite responsible for the majority of tamoxifen clinical efficacy, as endoxifen plasma concentrations are about five to tenfold higher than those of 4-hydroxy-tamoxifen [11, 165]. Endoxifen may have additional mechanisms of action than 4-hydroxy-tamoxifen by targeting Erα for degradation by proteasome [166] and through the promotion of ERα/ERβ heterodimerization, blocking ERα transcriptional activity [167].

Tamoxifen could thus be considered a quasi-prodrug that requires metabolic bioactivation to exert its effects. The metabolism of tamoxifen is complex and undergoes extensive phase I and phase II transformation (Fig. 2). Various potentially

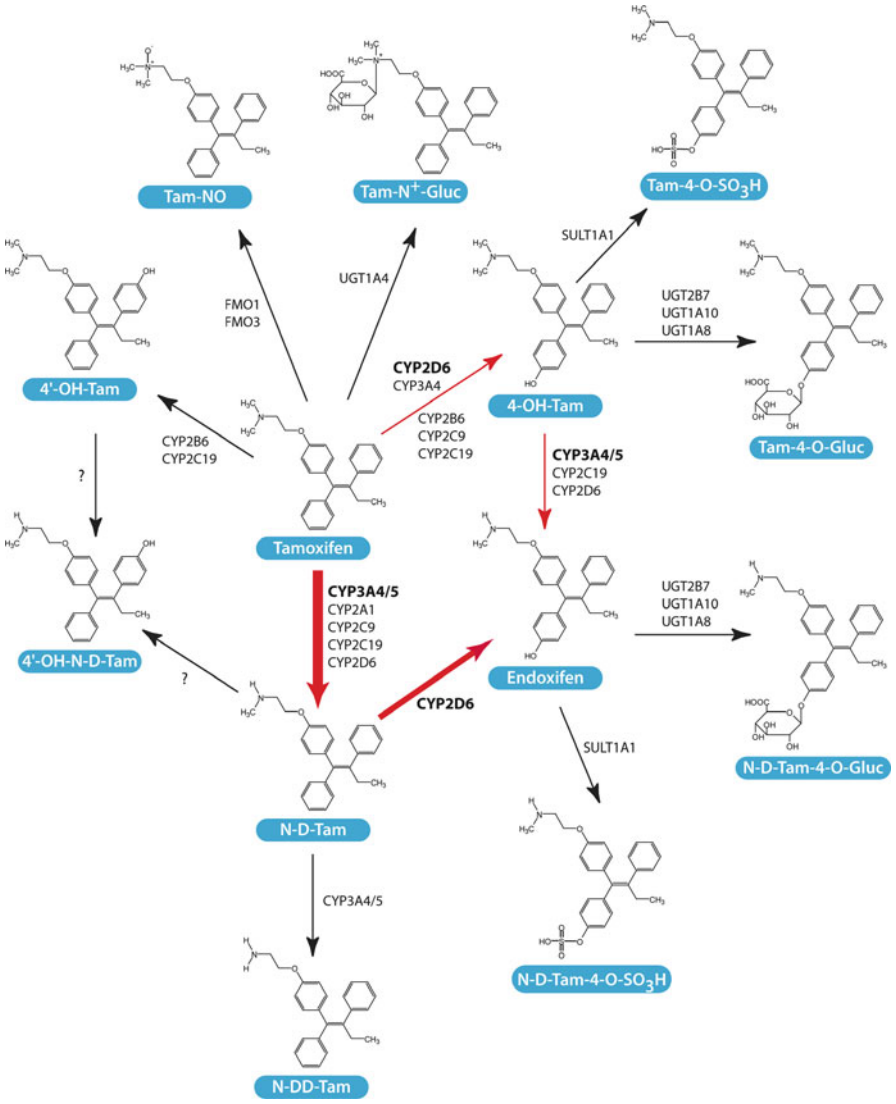

Fig. 2 Principal tamoxifen metabolic pathways of clinical interest. Abbreviations: Tam (Tamoxifen), *N*-D-Tam (*N*-desmethyl-tamoxifen), *N*-DD-Tam (*N*,*N*-didesmethyl-tamoxifen), 4-OH-Tam (4-Hydroxy-Tamoxifen), 4′-OH-Tam (4′-Hydroxy-tamoxifen), Tam-NO (Tamoxifen-*N*-oxide), Endoxifen (4-Hydroxy-*N*-desmethyl-tamoxifen), 4′-OH-*N*-D-Tam (4′-Hydroxy-*N*-desmethyl-tamoxifen), Tam-N^+-Gluc (Tamoxifen-N^+-glucuronide), Tam-4-*O*-Gluc (Tamoxifen-4-*O*-glucuronide), *N*-D-Tam-4-*O*-Gluc (*N*-desmethyl-tamoxifen-4-*O*-glucuronide), Tam-4-*O*-SO_3H (Tamoxifen-4-*O*-sulfate), *N*-D-Tam-4-*O*-SO_3H (*N*-desmethyl-tamoxifen-4-*O*-sulfate)

polymorphic cytochrome P450 (CYP) enzymes including CYP3A4, 3A5, 1A2, 2B6, 2 C9, 2 C19 and 2D6 catalyze, to different extents, the hepatic biotransformation of tamoxifen into active and inactive primary and secondary metabolites [168–171].

Briefly, tamoxifen is primarily oxidized to *N*-desmethyl-tamoxifen (the most abundant metabolite in human plasma) and 4-hydroxy-tamoxifen predominantly by CYP3A4/5 and CYP2D6, respectively, followed by endoxifen formation from *N*-desmethyl-tamoxifen, exclusively catalyzed by CYP2D6 and from 4-hydroxy-tamoxifen by CYP3A4/5 (Fig. 2).

N-desmethyl-tamoxifen is quantitatively the major metabolite found in patients' plasma and serum. It accounts approximately for 92 % of primary tamoxifen oxidation [171]. In women receiving a daily dose of 20 mg tamoxifen, steady-state plasma concentrations of *N*-desmethyl-tamoxifen are 1.5 to 2-fold higher than those of tamoxifen. Plasma levels of 4-hydroxy-tamoxifen are five to tenfold lower than those of endoxifen [11, 165, 172, 173]. Steady-state plasma concentration of tamoxifen is achieved after 1 month with terminal elimination half-life of about 5–7 days. *N*-desmethyl-tamoxifen (elimination half-life of about 10–14 days), 4-hydroxy-tamoxifen, endoxifen have longer elimination half-life than tamoxifen and their steady-state plasma concentrations are achieved 3–4 months after treatment initiation [11, 172].

Tamoxifen and its metabolites undergo further glucuronidation and sulfation. Different hepatic and extra-hepatic UDP-glucuronosyltransferases (UGTs) exhibited in-vitro glucuronidation activities towards tamoxifen and its metabolites leading to inactive metabolites [174]. The hepatic enzyme UGT1A4 is considered the major UDP-glucuronosyltransferase responsible – in vitro – for the *N*-glucuronidation of tamoxifen and 4-hydroxy-tamoxifen [175–178]. Hydroxylated active tamoxifen metabolites (i.e., *Z*-4-hydroxy-tamoxifen and *Z*-endoxifen) equally go through *O*-glucuronidation involving mainly UGT2B7 and the extra-hepatic glucuronidating enzymes UGT1A10 and 1A8 [179, 180]. Typical chromatographic profiles of tamoxifen phase I and phase II glucuronidated metabolites, observed in a plasma sample from a BC patient, are depicted in Fig. 3. Sulfotransferase (SULT) 1A1 is the major phase II metabolizing enzyme involved in the sulfation of 4-hydroxy-tamoxifen and endoxifen [181–184]. These sulfated and glucuronidated metabolites are further eliminated in urine and bile and undergo enterohepathic circulation (EHC) [185–187].

As an adjuvant therapy, in pre- and post-menopausal women with ER-positive BC, a standard 5 years treatment with tamoxifen has been demonstrated to almost half (43 %) the rate of disease recurrence and reduce the annual breast cancer death by a third (31 %). In the preventive setting, tamoxifen also reduces the risk of developing a new breast cancer by nearly one-half [150, 151, 154, 157].

Despite the obvious benefits of this drug in the different treatment settings, the clinical outcomes of tamoxifen treatment in terms of efficacy and side effects are incomplete and inconstant, and almost 30–50 % of patients either fail to respond or become resistant to tamoxifen [188]. One of the proposed mechanisms that may account for the impaired response to tamoxifen therapy is an altered bioactivation of the parent drug into endoxifen, either by genetic or environmental factors [188, 189].

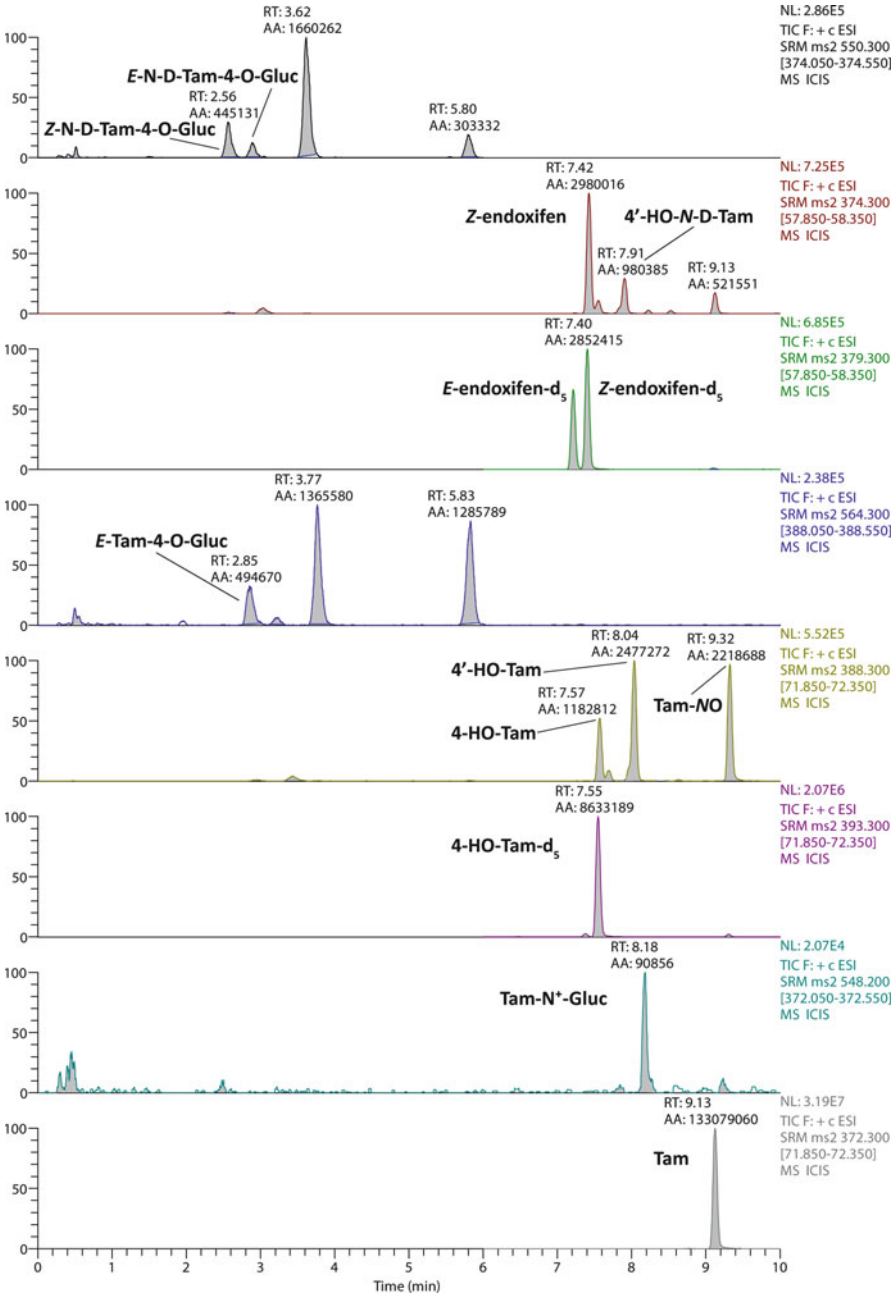

Fig. 3 Chromatographic profiles of main tamoxifen phase I metabolites and some of its identified glucuronidated metabolites in a plasma sample from a breast cancer patient receiving tamoxifen 20 mg BID (modified elution gradient from reference [145]). See legends in Fig. 2 for abbreviations

It appears that CYP2D6 is the key enzyme responsible for the generation of endoxifen [171]. The metabolizing activity of this enzyme is highly polymorphic and varies considerably within a population and between ethnic groups. This large variability is partly determined by genetic polymorphisms in the CYP2D6 gene, with over 100 allelic variants identified to date, resulting in different phenotypic patterns [190, 191]. Currently, on the basis of CYP2D6 activity, the population is usually categorized into four phenotypes including ultrarapid metabolizers (UMs), extensive metabolizers (EMs), intermediate metabolizers (IMs), and poor metabolizers (PMs).

Actually, CY2D6 gene polymorphisms, associated with null or reduced enzyme activity, have been reported to negatively influence (in a gene-dose manner) the blood level of endoxifen in numerous prospective pharmacokinetic studies [11, 172, 192–196]. Some retrospective and prospective studies have shown that CYP2D6 polymorphism was associated with worse clinical outcomes in PMs and IMs patients in terms of recurrence and disease free survival or BC development in the chemoprevention setting [192, 194, 195, 197–205].

This has prompted the consideration of a potential role for CYP2D6 genotype testing in patients' management and choice of alternative adjuvant therapy. Whether genotype-guided tamoxifen administration is a valuable and useful option to optimize antihormonal adjuvant therapy remains, however, controversial, and no clear consensus has yet been reached regarding the insufficient and somewhat conflicting retrospective clinical data relating CYP2D6 genotype to tamoxifen efficacy [206–210]. Moreover, large interpatient variability in endoxifen levels still subsists even after correcting for CYP2D6 status. This remaining variability may depend on one hand on the activity of other cytochromes (CYP3A4/5, 2 C9, 2 C19, 2B6), phase II conjugation enzymes (SULT1A1, UGT1A4, 2B7, 1A10, 1A8, 2B15) some of them known to be polymorphic [11, 173, 182, 196, 206, 207, 211], as well as transporters (other than P-glycoprotein or multidrug resistance-associated protein 2 [MRP2] that seems to have no or limited impact on tamoxifen and metabolites systemic exposure) [195, 212–214]. On the other hand, environmental factors such as treatment adherence [215–220] and particularly, interacting co-medications do modulate drug exposure independent of genetic traits [10, 11, 172]. In fact, it is estimated that 20–30 % of patients under tamoxifen therapy are also taking antidepressants. Of importance are some selective serotonin reuptake inhibitors (SSRIs) with strong CYP2D6-inhibiting activity, such as paroxetine and fluoxetine, that can be prescribed to treat depression or to alleviate tamoxifen-induced hot flushes. The latter drugs are known to reduce endoxifen plasma concentration and may therefore be associated with poorer tamoxifen efficacy [221–225].

The monitoring of plasma concentration of tamoxifen-active metabolites (mainly endoxifen) may therefore constitute a better predicting tool for tamoxifen efficacy than genotype testing. In fact, endoxifen levels correspond to the final phenotypic trait of patients' drug exposure, accounting for the combined effects of all genetic polymorphisms, physiological and environmental factors that may affect drug disposition and bioactivation.

However, whether the monitoring of endoxifen plasma concentrations in breast cancer patients would constitute a valid approach to optimize individual dosage and improve treatment effectiveness remains to be demonstrated. So far, only one study

has been recently published that sought for the association between endoxifen concentrations and breast cancer outcomes [173]. This pioneering study suggested a probable nonlinear dose–response relationship for tamoxifen effect and identified a threshold concentration for endoxifen (of about 6 ng/ml) above which approximately 30 % reduction in disease recurrence rate was observed.

Early attempts that examined the feasibility and usefulness of tamoxifen dose-adjustment strategy were based exclusively on CYP2D6 genotype. Genotype-guided dose-adjustment studies have shown that tamoxifen dose increase to 30 mg or 40 mg/day significantly increases 4-hydroxy-tamoxifen and endoxifen concentrations in IM and even in PM patients carrying two null alleles (reflecting metabolism by other enzymes), without any significant difference in adverse effects. However, an important variability is still observed in 4-hydroxy-tamoxifen and endoxifen levels between the genotypic groups [226, 227] and this would be a strong argument for considering TDM of tamoxifen and its active metabolites levels as a valuable strategy for tamoxifen dose adjustment further reducing the residual variability within CYP2D6 genotype groups.

Barginear et al. [228]. investigated in another prospective study the effect of tamoxifen dose increase on the concentrations of tamoxifen, 4-hydroxy-tamoxifen, endoxifen, and their position isomers (4′-hydroxylated) and proposed an "antiestrogenic activity score" (AAS) based on the concentrations of these metabolites and their respective antiestrogenic activities. According to Barginear et al. this AAS score would constitute a better approach to estimate the biologic effectiveness of tamoxifen and, therefore, to guide future tamoxifen dose optimization. However, this approach has yet to be validated by larger studies.

3.2 Tamoxifen and Metabolites Identification and Quantification

To date, several quantitative analytical methods have been developed for the monitoring of tamoxifen and some of its metabolites in human biological fluids and tissues, including conventional [229–231] and micellar [232] liquid chromatography (LC) methods coupled to fluorescence detection, capillary electrophoresis-mass spectrometry (CE-MS) [233], gas chromatography–mass spectrometry (GC-MS) [234], as well as liquid chromatography methods hyphenated with mass spectrometry (LC-MS) [195] and tandem mass spectrometry (LC-MS/MS) [145, 173, 193, 194, 196, 218, 226, 227, 235–247]. Reports have also been published describing liquid chromatography method coupled to mass spectrometry or fluorescence detection for the study of tamoxifen metabolism in vitro and in vivo in animal models [185, 248–254]. Most of these qualitative and quantitative LC, GC, and CE methods have already been reviewed by Teunissen et al. [255].

Various hyphenated LC-MS-based assays, using either the electrospray ionization (ESI) or the atmospheric pressure chemical ionization (APCI) interface, have been developed and applied in the clinical setting in order to support pharmacokinetic (PK), pharmacogenetic-pharmacokinetic (PG-PK), and pharmacokinetic–pharmacodynamic (PK-PD) studies in BC patients under tamoxifen therapy (Table 3).

Table 3 Overview of LC-MS and LC-MS/MS methods for the quantification of tamoxifen and its metabolites in human blood samples

Matrix (volume)	Analytes[e]	Internal standard	LOQ (ng/ml)	Sample preparation	Column (particle size, dimensions)	Ionization and detection mode	Reference
Plasma[a] (100 µl)	Tam 4-OH-Tam Others SERMS (raloxifene, nafoxidine, idoxifene)	Idoxifene-d5	5 –	LLE (hexane/ isoamyl-alcohol (96:4 v/v)) Dilution (DMSO) Evaporation (hexane layer) Dilution (H20)	Luna C18 (3 µm, 30×1 mm)	ESI-TSQ	[235]
Plasma (250 µl)	Tam N-D-Tam 4-OH-Tam	Toremifene	–	PP (ACN) Dilution (0.5 M ammonium acetate)	Hypersil BDS C18 (3 µm, 150×2.1 mm)	ESI-Q-TOF (MS mode)	[236, 237]
Serum[b] (75 µl)	Tam 4-OH-Tam N-D-Tam N-D-D-Tam Tam-NO Endoxifen[c]	Tam-d5	0.25 0.25 0.25 1.0 1.0 –	PP (ACN) On-line SPE (Oasis HLB, 50×1 mm)	Chromolith Performance, RP-18e (100×4.6 mm)	ESI-LTQ	[193, 238]
Serum[b] (100 µl)	Tam N-D-Tam 4-OH-Tam Endoxifen[d] Soy isoflavone (genistein, daidzein, equol)	Tam-$^{13}C_2$,^{15}N N-D-Tam-d5 4-OH-Tam-d5 Endoxifen-d5[d] (genistein-d4, daidzein-d3, equol-d4)	0.4 0.4 0.2 1.1	PP (ACN) Hydrolysis (glucuronidase/ sulfatase) SPE (SPEC 96-WELL PLATE C18) Evaporation Reconstitution (MeOH/H2O (1:1 v/v))	Luna C18 (3 µm, 150×2 mm)	ESI-TSQ	[239, 240]

(continued)

Table 3 (continued)

Matrix (volume)	Analytes[e]	Internal standard	LOQ (ng/ml)	Sample preparation	Column (particle size, dimensions)	Ionization and detection mode	Reference
Serum (1 ml)	Tam N-D-Tam 4-OH-Tam	– – –	5 5 0.5	LLE (*n*-hexane/ isoamyl alcohol (98:2)) Evaporation Reconstitution (MeOH)	Beckman C8 (5 µm, 50×4.6 mm)	ESI-LTQ	[241]
Serum	Tam 4-OH-Tam	Propranolol	10 1	LLE (hexane/ isopropanol (95:5 v/v))	HiQ-Sil C18 (5 µm, 150×2.1 mm)	ESI-TSQ	[194]
Serum[b] (50 µl)	Tam Tam-NO[c] N-D-Tam 4-OH-Tam Endoxifen[d] Soy isoflavone (genistein, daidzein, glycitein)	Tam-d5 N-D-Tam-d5 4-OH-Tam-d5 Endoxifen-d5[d] (genistein-d4, daidzein-d6)	6.76 6.19 6.72 1.13 2.69	PP (ACN) Dilution (3.5 mM ammonium formate buffer, pH 3.5)	Synergi Hydro-RP (4 µm, 150×2 mm)	ESI-TSQ	[242]
Plasma (100 µl)	Tam N-D-Tam 4-OH-Tam Endoxifen	Imipramine	20 20 1 3.75	PP (ACN) SPE (BOND ELUTE-C18 cartridges, 100 mg/1 ml)	XBridge C18 (3.5 µm, 150×3 mm)	ESI-TOF	[195]
Plasma[b] (1 ml)	Tam AIs (Anastrozole, letrozole)	Bunitrolol	25	PP (2 % aqueous phosphoric acid) Polymer-based mixed-mode SPE (Strata X-C, 200 mg/3 ml)	Eurosphere Si-C18 (5 µm, 200×0.5 mm)	ESI-LTQ	[218]

Plasma (100 µl)	Endoxifen	Anastrozole	–	PP (ACN)	Kromasil 100 C8 (5 µm, 150×4.6 mm)	ESI-TSQ	[243, 244]
Plasma[b] (100 µl)	Tam N-D-Tam 4-OH-Tam 4′-OH-Tam[c] Endoxifen[d] 4′-OH-N-D-Tam[c]	Tam-d5 N-D-Tam-d5 4-OH-Tam-d5 Endoxifen-d5[d]	1 2 0.4 0.4 1 1	PP (ACN) Evaporation Reconstitution (MeOH/20 mM ammonium formate buffer, pH 2.9 (1:1 v/v)) Centrifugation	Acquity UPLC BEH C18 (1.7 µm, 30×2.1 mm)	ESI-TSQ	[145]
Plasma[b] (100 µl)	Tam Tam-NO N-D-Tam 4-OH-Tam[d] 4′-OH-Tam Endoxifen[d] 4′-OH-N-D-Tam	Tam-$^{13}C_2$,^{15}N N-D-Tam-d5 4-OH-Tam-d5 Endoxifen-d5[d]	1.1 – 0.5 0.2 0.2 0.2 0.2	PP (MeOH) Filtration Dilution (H_2O)	Zobrax SB-C18 (1.8 µm, 50×2.1 mm)	ESI-TSQ	[245]
Serum (200 µl)	Tam N-D-Tam 4-OH-Tam Endoxifen	Tam-d5 N-D-Tam-d5 Endoxifen-d5	– – – –	Polymer-based mixed-mode SPE (Oasis MCX 1 ml cartridges)	XTerra MS C18 (3.5 µm, 100×2.1 mm)	ESI-LTQ	[173]

(continued)

Table 3 (continued)

Matrix (volume)	Analytes[e]	Internal standard	LOQ (ng/ml)	Sample preparation	Column (particle size, dimensions)	Ionization and detection mode	Reference
Plasma (50 µl)	Tam Tam-NO N-D-Tam N-D-D-Tam 4-OH-Tam[d] 3-OH-Tam 4′-OH-Tam[d] α-OH-Tam Endoxifen[d] 3-OH-N-D-Tam[c] 4′-OH-N-D-Tam[d] α- OH-N-D-Tam[c] Tam-N$^+$-Gluc Tam-3-O-Gluc[c] Tam-4-*O*-Gluc[d] N-D-Tam-3-O-Gluc[c] N-D-Tam-4-O-Gluc[d]	Tam-d3 N-D-Tam-d5 4-OH-Tam-d5[d] Endoxifen-d5[d] Tam-4-O-Gluc-d5[d]	0.5 0.2 1 0.2 0.1 0.1 0.1 0.02 0.1 – 0.05 – 0.05 – 0.1 – 0.05	PP (ACN + 1 % acetic acid) Dilution (H_2O + 1 % acetic acid)	Zobrax Eclipse plus C18 (1.8 µm, 100×2 mm)	ESI-TSQ	[196]
Serum[b] (50 µl)	Tam N-D-Tam 4-OH-Tam 4′-OH-Tam Endoxifen[d] 4′-OH-N-D-Tam	Tam-d5 N-D-Tam-d5 4-OH-Tam-d5 Endoxifen-d5[d] 	5 5 0.4 0.2 1 1	PP (ACN) Evaporation Reconstitution (ACN/4 mM ammonium formate buffer, pH 3.5, (3:7 v/v))	Kinetex C18 (2.6 µm, 150×2.1 mm)	APCI-TSQ	[246]
Plasma (250 µl)	Tam N-D-Tam 4-OH-Tam Endoxifen	Diphenhydramine	– – – –	LLE (ethyl acetate under pH 11.3) Evaporation Reconstitution (mobile phase)	Luna C18 (3 µm, 100×2 mm)	ESI-TSQ	[226]

Plasma[b] (200 µl)	Tam N-D-Tam 4-OH-Tam Endoxifen	Tam-d5 N-D-Tam-d5 4-OH-Tam-d5 Endoxifen-d5[d]	1.86 1.78 0.194 0.187	PP (ACN/acetone (1:1)) LLE (n-hexane/isopropanol (95:5)) Evaporation Reconstitution (ACN/H_2O/formic acid (40:60:0.1 v/v/v)) Centrifugation	Acquity UPLC BEH C18 (1.7 µm, 100×2.1 mm)	ESI-TSQ	[247]
Plasma (100 µl)	Tam N-D-Tam 4-OH-Tam Endoxifen	Imipramine	20 40 1 4	PP (ACN)	Acquity UPLC BEH C18 (1.7 µm, 100×2.1 mm)	ESI-TSQ	[227]

[a]Non-clinical samples
[b]Fully validated method
[c]Method has not been validated for the quantification of the analyte
[d]Reported E/Z isomers chromatographic resolution
[e]Abbreviations (see Fig. 2 and Table 4)

Among these, both LC-MS and LC-MS/MS approaches have been described using different mass analyzers operating in the positive ion mode scan such as triple stage quadrupole (TSQ) mass spectrometers [145, 194, 226, 227, 235, 239, 240, 242–247] and hybrid quadrupole-linear ion trap (LTQ) [173, 193, 218, 238, 241] mass spectrometers working in SRM mode as well as time-of-flight (TOF) [195] and hybrid quadrupole-TOF (Q-TOF) [236, 237] mass spectrometers working in the MS mode.

3.3 Chromatographic Conditions and Tamoxifen Metabolites Separation

Since the introduction of ionization sources working at atmospheric pressure such as ESI interface, LC–MS has become the gold standard in the field of quantitative bioanalysis due mainly to the selectivity, sensitivity, and high-throughput detection in LC-MS systems. However, LC-MS features depend not only on the ionization technique and mass spectrometer unrivaled inherent selectivity, sensitivity, and speed acquisition but are also challenged, notably in drug metabolism studies, by the availability of stable isotope labeled (SIL) version of metabolites (see below) and the need of efficient and adequate chromatographic resolution of multiple analytes from interfering metabolites or endogenous biological components in a minimum time frame.

Reversed-phase LC (RPLC) methods using conventional, microbore [218], narrow-bore [194, 242], and short [241] HPLC columns have been used for the separation of tamoxifen/metabolites either under isocratic or gradient elution conditions. Narrow-bore columns present the advantages of being solvent saving and by the need of low sample injection (or loading) volumes. These advantages were illustrated by Beer et al. [218], who developed an analytical method for the separation of tamoxifen, anastrozole, and letrozole under gradient of 30 μl/min of acetone in aqueous heptafluorobutyric acid solution and volumes as low as 2 μl, from the processed samples, were injected into the system. Furlanut et al. [241] used a short analytical column for the separation of tamoxifen and two of its metabolites within almost 8 min under isocratic conditions at flow rate of 1 mL/min. Although, the use of conventional short columns is a simple method for shortening analytical run times, these columns suffer from a loss in efficiency and resolution.

For enhanced throughput, fast RPLC methods using monolithic silica columns [238], small size particles (3 μm) packed columns [173, 195, 226, 235–237, 239, 240], ultra high pressure liquid chromatography (UHPLC) columns packed with sub-2 μm particles [145, 196, 227, 245, 247] and 2.6 μm core-shell particles HPLC columns [246] have been proposed for the high-throughput separation and quantification of tamoxifen/metabolites.

Five UHPLC methods have already been described to improve speed, resolution, and sensitivity of HPLC assays for the quantification of tamoxifen phase I as well as phase II metabolites. These methods exclusively enabled, within run times of

about 12 min or even less, to reach an excellent overall resolution for all considered metabolites including (*E*/*Z*) endoxifen isomers and position isomers of 4-hydroxy and 4-hydroxy-*N*-desmethyl-tamoxifen. Alternatively, Zweigenbaum J and Henion J [235] developed a high-throughput analysis technique for the separation of tamoxifen, 4-hydroxytamoxifen, and other SERMs within only 30 s using a narrow-bore short analytical column packed with small (3 μm) particles. Separation was performed under isocratic conditions at flow rate of 500 μl/min. Gjerde et al. [238] also described an online SPE-LC-MS/MS procedure where chromatographic resolution of tamoxifen and five of its metabolites was achieved within 6 min using a monolithic silica column (Separation was performed under a gradient program at a flow rate of 500 μl/min). However this method, like other HPLC assays, clearly failed to resolve all the hydroxylated and *N*-desmethyl-hydroxylated tamoxifen metabolites.

Tamoxifen is metabolized to a plethora of *N*-desmethylated, hydroxylated, and their corresponding glucurono- or sulfo-conjugated metabolites. Some of these hydroxylated metabolites are position isomers (such as 4-hydroxy-tamoxifen, 3-hydroxy-tamoxifen, and 4′-hydroxy-tamoxifen; endoxifen; and 4′-hydroxy-*N*-desmethyl-tamoxifen) and have similar molecular mass and fragmentation pattern (Table 4). Besides, *E*/*Z* isomerization (around the ethylenic double bond of tamoxifen and its metabolites) may occur either in biological samples or as contaminants or degradation products in pure standards. Some pure standards are also best synthesized as an equimolar *E*/*Z* mixture. Therefore, the chromatographic resolution of these metabolites and their (*E*/*Z*) geometric isomers is of paramount importance to ensure reliable and accurate bioanalytical methods.

However, of the LC-MS and LC-MS/MS methods developed so far for the comprehensive and quantitative study of levels variability in tamoxifen metabolites, there is limited data with respect to the resolution of both 4-hydroxytamoxifen and endoxifen position isomers (notably 4′-hydroxylated metabolites) and their corresponding (*E*/*Z*) geometric isomers. In fact, apart from the most recently published articles [145, 173, 196, 245–247], no data have been provided regarding this issue. We were the first group that focused on method selectivity and on the effective separation on potentially interfering hydroxylated tamoxifen metabolites. This has allowed us to identify for the first time the occurrence of 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen and to estimate their plasma levels in a subset of BC patients [145]. Such differences in chromatographic performances, between assays, can thus affect the selectivity, the accuracy, and the reliability of some of the proposed bioanalytical methods, potentially leading to discrepant data (or results) between the PK, PG-PK and PK-PD studies. Actually, Mürdter et al. [196] reported twice or even higher differences in median concentrations of (*Z*)-endoxifen between studies conducted in the United States, Japan and Norway. They also found a plausible explanation for these discrepancies in method selectivity problems. Madlensky et al. [173] compared the performance of their assay to that of another laboratory performing similar measurements of tamoxifen metabolites in human serum. They found discordant results for 4-hydroxy-tamoxifen levels measured in the same serum samples.

Table 4 Molecular masses and SRM transitions for tamoxifen and some of its metabolites of interest

Analytes	Abbreviation	Molecular weight	Precursor ion[a] $[M+H]^+$	Production
Tamoxifen	Tam	371	372	72
N-Desmethyl-tamoxifen	*N*-D-Tam	357	358	58
N,*N*-Didesmethyl-tamoxifen	*N*-D-D-Tam	343	344	44
4-Hydroxy-tamoxifen	4-OH-Tam	387	388	72
3-Hydroxy-tamoxifen	3-OH-Tam	387	388	72
4′-Hydroxy-tamoxifen	4′-OH-Tam	387	388	72
α-Hydroxy-tamoxifen	α-OH-Tam	387	388	72
Tamoxifen-*N*-oxide	Tam-NO	387	388	72
4-Hydroxy-*N*-desmethyl-tamoxifen	Endoxifen	373	374	58
3-Hydroxy-*N*-desmethyl-tamoxifen	3-OH-*N*-D-Tam	373	374	58
4′-Hydroxy-*N*-desmethyl-tamoxifen	4′-OH-*N*-D-Tam	373	374	58
α-Hydroxy-*N*-desmethyl-tamoxifen	α- OH-*N*-D-Tam	373	374	58
Tamoxifen-N^+-glucuronide	Tam-N^+-Gluc	548	548	372
Tamoxifen-4-*O*-glucuronide	Tam-4-*O*-Gluc	563	564	388
Tamoxifen-3-*O*-glucuronide	Tam-3-*O*-Gluc	563	564	388
N-Desmethyl-tamoxifen-4-*O*-glucuronide	*N*-D-Tam-4-*O*-Gluc	549	550	374
N-Desmethyl-tamoxifen-3-*O*-glucuronide	*N*-D-Tam-3-*O*-Gluc	549	550	374

[a]Molecule protonation occurs on the amino group

Another drawback, challenging the routine applicability of some of these LC-MS and LC-MS/MS assays for measuring exposure to tamoxifen and its active metabolites is that for some assays no data have been provided concerning the validation process. Other methods have only been partially validated and have not or limitedly addressed matrix effects (ME) issues.

3.4 Handling Matrix Effects

Matrix effects (ME), caused by co-eluting endogenous and exogenous matrix components, significantly affect the efficiency and reproducibility of the ionization process of target analytes. This phenomenon represents a major concern for LC-MS bioanalytical method precision, accuracy, sensitivity, and robustness. Amongst the atmospheric pressure ionization interfaces used in LC-MS systems, ESI source is more prone to signal alteration (ion suppression or enhancement) due to matrix. Therefore, careful evaluation and correction for ME must be considered particularly with ESI-MS.

The use of stable isotope labeled (SIL) version of the target analyte as an internal standard (IS) is theoretically considered to be the best approach to compensate or correct for matrix effects and minimize their influence on the accuracy and precision of ESI-MS quantitative assays.

With the exception of the LC-MS/MS methods recently published, previous assays were using either no IS [241], structurally related IS [194, 195, 218, 226, 227, 236, 237, 243, 244], or a single SIL-IS [193, 235, 238] as a surrogate IS for the quantification of tamoxifen/metabolites.

Since SIL-ISs are not always available and their use rather expensive, especially in the case of multiple analytes analysis, the use of structurally related compounds or analogue IS with different mass and with close or similar chromatographic behavior to that of the analytes can represent an acceptable alternative. Nevertheless, in these latter instances, ME variability between different sources of plasma (relative matrix effect variability) must be investigated and quantified. From the assays operating with either no IS or a unique analogue IS, only three methods quantitatively assessed for ME variability. Zweigenbaum J and Henion J [235] reported a significant ion suppression which approximately halved 4-hydroxytamoxifen signal. This ion suppression was not corrected by the IS and affected the precision and accuracy of the method that failed to meet the acceptance criteria for 4-hydroxy-tamoxifen quantification. Furlanut et al. [241] monitored Tam, *N*-D-Tam, and 4-OH-Tam in serum and tissue of BC patients, employing external standard calibration and reported no ion suppression problem after quantitative evaluation of ME. Unfortunately, no detailed information was available regarding the extent of matrix effects variability and the number of plasma lots tested. Only the recent method described by Beer et al. [218] thoroughly examined ME variability using the quantitative approach proposed by Matuszewski et al. [256, 257].

It is noteworthy that ME variability should be investigated even when using SIL-ISs. In fact, SIL-IS may not fully correct for matrix effects, obviously when they do not completely co-elute with their corresponding analyte. This phenomenon has been particularly observed with deuterated SIL-IS that were found to be less lipophilic than their corresponding non-deuterated analogues, causing a slightly earlier elution on a reversed-phase column [258].

Although most recent developed assays used SIL-IS, only few methods quantitatively investigated potential ME variability on tamoxifen and its metabolites quantification [145, 245].

In our proposed assay [145], we thoroughly investigated ME both qualitatively using the post-column infusion system proposed by Bonfiglio et al. [259] and quantitatively using the recommendations of Matuszewski et al. [256, 257] and the 2007 Washington workshop/conference report [260]. Although the qualitative examination of ME did not show any signal alteration, probably due to the infusion of high concentration of analytes, quantitative ME examination showed an ion suppression of approximately 40 % for the signal of *N*-D-Tam. We observed a similar extent of ion suppression with the deuterated *N*-D-Tam (*N*-D-Tam-d5) and ascertained that SIL-IS effectively corrected for the absolute and relative ME (or ME effect variability among six different lots of plasma). Therefore, this was a good illustration of the value of SIL-IS use for an efficient control of residual matrix effects.

Besides the use of SIL-IS, another upstream and primordial approach that allows to anticipate and drastically reduce matrix effects is the optimization of sample preparation procedure.

Plasma protein precipitation (PP) with either ACN or methanol (MeOH) was the most frequently used sample cleanup technique in the described bioanalytical methods [145, 196, 236, 237, 242–245]. Of these, ACN was the prevalent precipitant used, as it was considered to be an optimal choice for protein removal than methanol (MeOH) [261–263]. Although PP is a simple and fast way for preparing samples, it does not result in a very clean extract, as it fails to remove endogenous components such as lipids, phospholipids (such as glycerophosphocholines) and fatty acids. However, if necessary, the elimination of most endogenous lipidic compounds from PP extracts can be performed by subjecting the PP extracts to an additional step of evaporation under nitrogen (or, even better, by submitting them to speed-vac technology) followed by the reconstitution of dried residues with medium polarity solvent system (e.g., MeOH-buffer mixture) wherein lipids would not be resolubilized.

Solid phase extraction allows yielding a much cleaner extract than PP, since it significantly lowers phospholipids levels which represent the major endogenous compounds causing significant matrix effects [263–265].

Different reversed phase [195, 239, 240], mixed mode (ion exchange and reversed phase) SPE cartridges [173, 218] and online SPE column [193, 238] have been also reported for samples preparation and extraction. Some of these assays combined both PP and SPE in order to achieve an extensive sample cleanup [193, 195, 238–240]. Likewise SPE, LLE provides cleaner plasma extracts than PP. Nevertheless, LLE procedure does not always provide satisfactory results with regard to extraction recovery and selectivity, especially with polar analytes and particularly in the case of multicomponent analysis such as in drug-metabolism studies, where analytes polarity varies widely. This issue was addressed by Zweigenbaum J and Henion J [235] and extraction solvent optimization, using isoamyl alcohol, to achieve acceptable extraction selectivity and recovery for polar analytes has been discussed.

To sum up, there is a great heterogeneity in the described methods that have so far been developed and, for the great majority of them, used in the clinical setting to support pharmacogenetic-pharmacokinetic–pharmacodynamic (PG-PK-PD) studies. Of these methods, only the most recent fully validated ones that have proven enough accuracy, precision, robustness, and selectivity seems to be reliable and suitable for measuring exposure of tamoxifen and its metabolites in tamoxifen-treated breast cancer patients.

Whether the monitoring of endoxifen plasma concentrations in breast cancer patients would constitute a valid approach to optimize individual dosage and improve treatment efficacy is under scrutiny and remains to be demonstrated. In that purpose large prospective studies relating endoxifen plasma levels to clinical outcomes are as yet needed. In this perspective, it is critical to settle analytical and selectivity discrepancies between methods and laboratories and to ensure reproducible quantification results between laboratories. These concerted harmonization efforts can be carried out within the frame of an international external quality control program, which as yet, remains to be organized.

4 Discussion and Conclusions

An increasing body of evidence is accumulating for legitimating the blood monitoring of targeted anticancer TKIs and tamoxifen metabolites profiles, especially given their high interindividual pharmacokinetic variability, due to influences of co-medications, diet, and comorbidities, in addition to patients' genetic constitution.

Overall, the analytical developments by mass spectrometry have been instrumental (1) for the development of initial population pharmacokinetics–pharmacodynamics models for some targeted anticancer drugs (mostly imatinib), possibly also integrating the underlying patients' pharmacogenetic background and (2) for being able to respond to clinically relevant issues on drug interaction problems with first-generation targeted anticancer agents [63]. At present, however, it must be acknowledged that the information on the relationships existing between the pharmacokinetics, pharmacodynamics, and in some cases pharmacogenetics, is for most TKIs frequently lacking, or supported by a limited number of—often anecdotal—studies. Therapeutic intervals remain therefore to be determined for the majority of TKIs, and PK-PD studies are best suited to that endeavor. Renewed translational efforts integrating population pharmacokinetics analysis and patients' clinical responses should therefore be carried out in the field of targeted anticancer therapy. Once established, they should open the way to randomized clinical trials for formally validating the clinical usefulness of TDM for TKIs dosage adjustment, before being integrated into standard of care. This raises ethical concerns, as once analytical methods have been developed, clinicians are usually reluctant to deny control patients group to TDM service. Alternate study designs should thus be considered, such as comparison of "routine" TDM (i.e., done even in the absence of clinical problems) versus a "rescue" TDM (done in case of unsatisfactory clinical response or adverse events) [266].

Even though not yet validated by RCTs for approved or more recent anticancer-targeted agents, TDM can already be expected to bring clinically useful information for the optimal management of selected cancer patients, e.g., in case of less-than-optimal clinical response, occurrence of adverse side effects, treatment initiation in the presence of interacting agents, or questionable compliance. For example, TDM appears to be presently used to a rather large extent for the first TKI imatinib, based on recommendations about target plasma levels to maintain for optimal clinical response. Nevertheless, results from randomized controlled studies about TDM usefulness are still eagerly expected for this TKI [266]. This is no less the case for all the more recent TKIs as well as for tamoxifen and its metabolites.

The TDM of TKIs is thus likely to become a very rapidly evolving field, with new targeted anticancer agents approved at a regular pace. Further developments for the TDM of several new TKIs are therefore anticipated to occur within the next few years. In that context, not only a facilitated access to powerful mass spectrometry instruments, but also the availability of robust methodologies for TKIs and tamoxifen/metabolites analysis is a necessity for academic hospital centers that provide TDM service for targeted anticancer therapy. In particular, bioanalytical methods

cross-validation is a general problem that should be prioritized amongst the clinical pharmacology community working in the field of targeted anticancer therapy. Initially, external quality control program have been organized for imatinib at the Bordeaux University Hospital (France) within the frame European Treatment and Outcome Study (EUTOS) of the European Leukemia Net [267]. Some private laboratories currently provide external quality control samples for imatinib, nilotinib and dasatinib [268]. Given the growing armamentarium anticipated for targeted anticancer therapy in the next decade, a reinforced analytical collaboration must be deployed between laboratories for harmonizing the assays for current and new TKIs to come, as well as for tamoxifen and metabolites, and possibly also for other anticancer endocrine agents administered chronically (i.e., aromatase inhibitors) as well as the m-TOR inhibitor everolimus, increasingly used in oncology. Beyond working out analytical issues, collaborative research efforts should also be devoted to structuring the collection of data internationally, so that translational research aimed at understanding pharmacokinetics, pharmacodynamics, pharmacogenetics of newest anticancer-targeted therapy will allow without delay the return to clinicians of relevant measurements and their validated interpretations. The systematic and efficient collection of accurate clinical information along with TDM samples indeed represents no less challenging issues that the measurement of those samples.

In complement to the diagnostic tests already approved for selecting patients who are more likely to benefit from a given anticancer treatment [5], individualization of TKIs drug dosage by TDM represents the next step towards a further refinement for targeted anticancer therapies, aiming at administering "the right dose of the right drug to the right patient." In this emerging field of personalized medicine, the development of TDM for patient-tailored dose adjustment should allow to maximize both the therapeutic benefit and the tolerability of these new drugs. These issues are certainly relevant both to individual patients, given the frequency of suboptimal clinical responses, toxicities, intolerance, and treatment discontinuations, and to the society, given the elevated costs of TKIs treatments and of their shortcomings.

Acknowledgments This chapter has been realized within the frame of the research project "Integrative cellular pharmacokinetics/pharmacodynamics/pharmacoproteomics studies of anticancer TKIs in leukemia" (SNF grant no. 310030_138097/1 to LAD) supported by the Swiss National Science Foundation (SNF, Switzerland). It also benefited from the support of the SNF-funded initiative Nano-Tera (ISyPeM project [269] to TB).

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The American Journal of Pathology, Vol. 182, No. 2, February 2013

The American Journal of
PATHOLOGY
ajp.amjpathol.org

MUSCULOSKELETAL PATHOLOGY

The Anticancer Drug Tamoxifen Counteracts the Pathology in a Mouse Model of Duchenne Muscular Dystrophy

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Accepted for publication October 7, 2012.

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Duchenne muscular dystrophy (DMD) is a severe disorder characterized by progressive muscle wasting, respiratory and cardiac impairments, and premature death. No treatment exists so far, and the identification of active substances to fight DMD is urgently needed. We found that tamoxifen, a drug used to treat estrogen-dependent breast cancer, caused remarkable improvements of muscle force and of diaphragm and cardiac structure in the *mdx*^{5Cv} mouse model of DMD. Oral tamoxifen treatment from 3 weeks of age for 15 months at a dose of 10 mg/kg/day stabilized myofiber membranes, normalized whole body force, and increased force production and resistance to repeated contractions of the triceps muscle above normal values. Tamoxifen improved the structure of leg muscles and diminished cardiac fibrosis by ~50%. Tamoxifen also reduced fibrosis in the diaphragm, while increasing its thickness, myofiber count, and myofiber diameter, thereby augmenting by 72% the amount of contractile tissue available for respiratory function. Tamoxifen conferred a markedly slower phenotype to the muscles. Tamoxifen and its metabolites were present in nanomolar concentrations in plasma and muscles, suggesting signaling through high-affinity targets. Interestingly, the estrogen receptors ERα and ERβ were several times more abundant in dystrophic than in normal muscles, and tamoxifen normalized the relative abundance of ERβ isoforms. Our findings suggest that tamoxifen might be a useful therapy for DMD. *(Am J Pathol 2013, 182: 485–504; http://dx.doi.org/10.1016/j.ajpath.2012.10.018)*

Duchenne muscular dystrophy (DMD) is a common and fatal genetic disease that affects the striated muscles in boys. It is characterized by muscle wasting, starting at ~3 years of age, leading to progressive paralysis and loss of ambulation during the teenage years and cardiac dysfunctions that cause death in early adulthood.[1]

DMD results from the inability of muscles to express dystrophin, a large subsarcolemmal protein that bridges the extracellular matrix to the intracellular cytoskeleton. Dystrophin is essential for protecting muscle cells from contraction-induced mechanical damage and for regulating processes in the subsarcolemmal space, such as mechanotransduction, reactive oxygen species production, and cation channel activity. The absence of dystrophin causes calcium overload, oxidative stress, and impairment of mitochondrial functions, which, collectively, alter myofibrillar function and cause muscle cell death.[2,3] The ensuing chronic inflammation impairs muscle regeneration and renders the surviving fibers more susceptible to stress. The dystrophic muscles are progressively invaded by connective and adipose tissues,[1] resulting in a dramatic loss of muscle strength. Disease progression to the respiratory muscles [eg, the diaphragm (DIA)] and the heart greatly restricts the life expectancy of patients with DMD.[4,5]

Supported by Swiss National Science Foundation grant 3100A0-109981 (U.T.R.), REQUIP grant 326000-121314/1 (L.A.D.), the Duchenne Parent Project-The Netherlands (DPP-NL; S17474 DPP 2011-12), the Association Française contre les Myopathies (AFM; 13090, 13984, and 15093), and a Ph.D. student fellowship grant from the AFM (13091 to J.R.-P.).

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http://dx.doi.org/10.1016/j.ajpath.2012.10.018

So far, the only pharmacologic treatments that have been clinically validated for patients with DMD are the glucocorticoids, prednisolone, and deflazacort.[6] However, these drugs prolong muscle strength and ambulation of patients only for a short term,[6] and adverse effects lead some patients with DMD to discontinue treatment. The identification of additional pharmacologic compounds that would decrease the course of the disease remains a major goal for research.[7,8]

Estrogens have long been regarded as female sex hormones. The expression of the estrogen receptors (ERs) ERα and ERβ, which mediate most estrogen actions, and aromatase, the rate-limiting enzyme that produces estrogens from androgens, was found in skeletal muscle.[9–11] In fact, skeletal muscles are major sites of estrogen production in men and postmenopausal women. Overall, estrogens increase force output,[12] enhance muscle recovery from disuse atrophy,[13] protect skeletal muscle membrane from contraction-induced injury,[14] and reduce the risk of developing cardiovascular diseases.[15,16]

Selective estrogen receptor modulators (SERMs) are compounds that either mimic or antagonize estrogens in a tissue-dependent manner. Tamoxifen (TAM), a first-generation SERM with antiestrogenic activity on the mammary gland, has been used to prevent and treat breast cancers for >20 years. At the same time, its proestrogenic activity on bone has made it attractive for the treatment of osteoporosis.[17–19] TAM has shown efficacy in scavenging peroxyl radicals,[20] stabilizing biological membranes,[14] preventing apoptosis,[21] inhibiting fibrosis,[22,23] and modulating calcium homeostasis.[24–26] Because these features all contribute to the pathogenesis of DMD, we hypothesized that dystrophic muscles could benefit from chronic TAM treatment.

We found that oral administration of TAM at a dose of 10 mg/kg/day for 15 months to *mdx*^{5Cv} mice, a commonly used model for DMD, remarkably improved dystrophic muscle structure and function. Specifically, TAM improved the whole body force of living mice, increased the force of leg muscles above that of normal mice, rendered these muscles more resistant to fatigue, induced a shift toward a slower phenotype, stabilized muscle fiber membrane, and normalized their diameter. Importantly, TAM decreased the development of fibrotic tissue in the DIA and in the heart and considerably increased the amount of contractile muscle tissue in the DIA. All these effects were obtained with plasma and muscle concentrations of TAM and its active metabolites being in the low nanomolar range, well below the levels displayed by patients with breast cancer under standard TAM therapy (ie, 20 mg/day). ERα and ERβ proteins were both overexpressed several fold in dystrophic muscles, and TAM altered the relative abundance of the ERβ isoforms ERβ1 and ERβ2 at both the mRNA and at the protein levels. Because ERβ2 may function as an inhibitor of ERβ1 and the ERβ2-to-ERβ1 ratio partly controls ER signaling,[27,28] these alterations of ER levels are likely significant in the exceptional responsiveness of dystrophic muscles to TAM.

Because TAM has a good safety profile, not only in adults but also in children, our findings suggest that TAM might be helpful for the treatment of patients with DMD.

Materials and Methods

While this study was ongoing, we contributed to the elaboration of standard operating procedures for preclinical investigations in the dystrophic mouse.[29] Whenever possible, the present study was performed in accordance with the experts' recommendations.

Mice and Treatments

All procedures involving animals complied with the Swiss Federal Law on Animal Welfare. Colonies of dystrophic *mdx*^{5Cv} mice[30] (The Jackson Laboratory, Bar Harbor, ME), and wild-type (wt) C57BL/6J mice (Charles River France, Saint Germain sur l'Arbresle, France) were maintained at the School of Pharmaceutical Sciences. Mice were housed in plastic cages containing wood granule bedding, kept on a 12-hour dark/12-hour light cycle, and allowed unlimited access to food and water.

Tamoxifen [(Z)-tamoxifen, catalog number T-5648; Sigma-Aldrich, Buchs, Switzerland] was incorporated into standard rodent diet at 100 mg/kg (Provimi-Kliba, Kaiseraugst, Switzerland). Both control and TAM-containing pellets were stored at −20°C in 0.5-kg vacuum-sealed bags. Male pups were marked by microtattooing of the toes under slight ketamine-xylazine sedation. Three groups were treated for approximately 15 months (63 ± 1 week) starting on postnatal day 21, that is, at the time when necrosis starts in most leg muscles: 14 dystrophic males were given control diet (Dys group), 12 dystrophic males were given TAM-containing diet (TAM group), and 12 wt males were given control diet (wt group). Body weights and food consumption were monitored weekly. A group of 9 dystrophic females fed control diet (FEM group) was included for comparison of certain end points with the groups of male mice.

Wire Grip Test

After 58 to 60 weeks of treatment, a wire test was used to assess whole body force. The mice were allowed to grasp by their four paws a 2-mm diameter metal wire maintained horizontally 35 cm above a thick layer of soft bedding. The length of time until the mice fell from the wire was recorded. After each fall, the mice were allowed to recover for 1 minute. Each session consisted of three trials from which the scores were averaged. The final grid test score was calculated as the average value from three sessions performed at 1-week intervals.

Muscle Contraction Properties

At the end of the treatment period, mice were anesthetized, and muscle responses to electrical stimulations were recorded isometrically in the right triceps surae as previously

described.[31–36] At the end of the treatment period, mice were anesthetized by an i.p. injection of a mixture of urethane (1.5 g/kg) and diazepam (5 mg/kg). In brief, the knee joint was firmly immobilized, and the Achilles tendon was linked to a force transducer coupled to a LabView interface (National Instruments, Austin, TX). Two thin steel electrodes were inserted intramuscularly, and 0.5-ms pulses of controlled intensity and frequency were delivered. After manual settings of optimal muscle length (L_o) and optimal current intensity, five phasic twitches were recorded at a sampling rate of 3 kHz to determine the absolute peak twitch force (P_t), the time to peak twitch tension (TTP), the time for half relaxation from peak twitch tension ($RT_{1/2}$), the maximum rate of tension development (T_{dev}), and the maximum rate of tension loss (T_{loss}). After a 3-minute pause, muscles were subjected to a force-frequency test: 200-ms long stimuli of increasing frequencies (10 to 100 Hz by increments of 10 Hz) were delivered at intervals of 30 seconds. When necessary, further stimulations at 120, 150, and 200 Hz were delivered to obtain the maximum response, which was taken as the absolute optimal tetanic tension (P_o). After another 3-minute pause, the resistance of the triceps to repeated tetani was assayed. Frequency was set at 60 Hz, and muscle tension was recorded while stimulations were repeatedly delivered, each consisting of a 1-second burst and a 3-second rest. The responses were expressed as the percentage of the maximal tension. Absolute phasic and tetanic tensions were converted into specific tensions (in mN per mm^2 of muscle section) after normalization for the muscle cross-sectional area. The cross-sectional area values (in mm^2) were determined by dividing the triceps surae muscle mass (in mg) by the product of the optimal muscle length (in mm) and the density of mammalian skeletal muscle (1.06 mg/mm^3).

Tissue Collection and Plasma CK

Immediately after isometric force recording, heparin was injected into the heart (30 μL, 3000 IU/mL), the mice were bled, and plasma was prepared by centrifugation (1000 x *g*, 10 minutes, 4°C). Skeletal muscles and other selected organs were dissected and weighed. Plasma creatine kinase (CK) levels were determined with a commercial kit (Catachem; Investcare Vet, Middlesex, UK) according to the manufacturer's recommendations.

Quantification of TAM and of Its Metabolites

The concentrations of the TAM isomers (E)-TAM and (Z)-TAM, and the TAM metabolites (E)-4-hydroxytamoxifen (OH-TAM), (Z)-4-OH-TAM, (E)-*N*-desmethyl-TAM, (Z)-*N*-desmethyl-TAM, (E)-4-hydroxy-*N*-desmethyl-TAM, (Z)-4-hydroxy-*N*-desmethyl-TAM (endoxifen), in the plasma of the TAM-treated mice were determined by an ultra performance liquid chromatography–tandem mass spectrometry assay as described.[37] The levels of these compounds were also determined in the gastrocnemius (GAS) muscles from TAM-treated mice with the use of a modification of the method used for plasma. Briefly, the GAS muscles were pulverized in liquid nitrogen-cooled mortars. Twenty milligrams of the muscle powder was homogenized for 30 seconds with a tissue tearor (Omni International, Kennesaw, GA) in a mixture composed of 900 μL of absolute ethanol and 100 μL of deuterated internal standards solution (25 ng/mL TAM-d5, *N*-desmethyl-TAM-d5, 4-OH-TAM-d5, and 50 ng/mL endoxifen-d5, 1:1 E/Z mixture, in methanol). The tissue suspension was then centrifuged (4°C for 10 minutes at 16000 × *g*). Seven hundred microliters of the supernatant fluid was transferred into a propylene tube and dried under nitrogen at room temperature. The residue was reconstituted in 100 μL of acetonitrile, vortex-mixed, diluted with 200 μL of a buffer solution (10 mmol/L ammonium formate, containing 0.25% formic acid) and centrifuged again as above. Supernatant fluid (150 μL) was introduced in a high performance liquid chromatography glass microvial, and 20 μL was injected into the high performance liquid chromatography system. Ultra performance liquid chromatography–tandem mass spectrometry conditions (mobile phases, elution gradient, and mass spectrometer conditions) were identical to those described for plasma levels measurements.[37] Calibration curves for tissue samples, prepared in ethanolic matrix (20 mg tissue/mL), ranged from 0.05 to 3 ng/mL for (E)-endoxifen, 0.025 to 3 ng/mL for (Z)-endoxifen, and 0.013 to 3 ng/mL for (Z)-4-OH-TAM, (Z)-*N*-desmethyl-TAM, and (Z)-TAM. In this specific setting, the method was precise and accurate with the interassay precision (CV %) and accuracy (bias %) ranging between 1% and 13% and −8.9% and 6.1%, respectively.

For plasma and muscle sample, (E)-TAM, (E)-*N*-desmethyl-TAM, and (E)-4-OH-TAM levels were quantified with the calibration curves of their corresponding Z isomers. In plasma and tissue samples, E-TAM isomer was chromatographically identified by comparison of its retention time with that of the purchased pure standard (Toronto Research Chemicals Inc., North York, ON, Canada). (E)-*N*-desmethyl-TAM and (E)-4-OH-TAM isomers were tentatively identified by comparison of their retention times with those of E isomers produced *in vitro* by exposing methanolic solutions of the corresponding Z isomers to UV light (254 nm) for ∼3 hours.

The results are expressed as ng/mL of plasma, ng/g of tissue, and nmol/L. A qualitative analysis of the food pellets confirmed that (Z)-TAM was the only form of TAM in the TAM-containing diet and that the control diet was devoid of TAM and metabolites.

Histologic Examination of Skeletal Muscles and Morphometry

The extensor digitorum longus (EDL), GAS, soleus, and tibialis anterior (TA) muscles from the right leg and the right hemi-DIA were embedded in tragacanth gum, frozen in

liquid nitrogen-cooled isopentane, and stored at −80°C until processed further. Transverse sections 10 μm thick were stained with H&E according to standard procedures, and images covering the entire muscle sections were acquired either with a Spot Insight camera (Visitron Systems, Puchheim, Germany) mounted on an Axiovert 200M microscope (Zeiss, Feldbach, Switzerland) or with an Axiocam camera (Zeiss) fitted on a Mirax Midi automated microscope (Zeiss), at a final magnification of ×50 or ×200, respectively. In dystrophic mice, skeletal muscles undergo repeated cycles of necrosis and regeneration with progressive accumulation of adipose and connective tissues. In normal fibers, the nuclei are located close to the sarcolemma ("peripheral nuclei"), whereas in regenerated fibers the nuclei remain internalized. On the basis of these morphologic features, both normal and regenerated fibers were counted. Regenerated fibers are expressed as the percentage of the total muscle fibers.

Sections were incubated with 2 μg/mL wheat germ agglutinin conjugated to Alexa Fluor 488 (WGA-AF_{488}; Molecular Probes, Invitrogen, Basel, Switzerland) in phosphate-buffered saline for 1 hour at room temperature to stain the connective tissue as described.[36] Fluorescence images from the whole muscle surface were taken with a Mirax Midi microscope as described above. The area covered by the connective tissue was measured with the Metamorph software version 5.0r7 (Visitron Systems, Puchheim, Germany) and expressed as the percentage of the total muscle area. In addition, the minimum fiber diameter was determined in the GAS, DIA, EDL, TA, and soleus muscles with the use of the Metamorph software as described.[38] For each muscle >500 fibers were counted from four to six fields taken at a final magnification of ×200.

Fiber typing was performed by immunohistochemistry with the use of mouse monoclonal antibodies against specific myosin heavy chains (MyHCs), according to standard procedures. The primary monoclonal antibodies BA-D5, SC-71, BF-35, and BF-F3 (Developmental Studies Hybridoma Bank, Iowa City, IA) were used to reveal fibers expressing type I, type IIA, all types but IIX, and type IIB MyHCs, respectively. The BA-D5, SC-71, and BF-35 antibodies were detected with a goat anti-mouse IgG antibody conjugated to Alexa Fluor 594 (Molecular Probes), and the connective tissue was counterstained with WGA-AF_{488} as described above. The BF-F3 antibody was detected with a goat anti-mouse IgM antibody conjugated to Alexa Fluor 488 (Molecular Probes). Fibers of type I and of type IIA were counted on separate sections. Other sections were double-stained with the anti-type IIB and all anti-types but IIX as above, and the connective tissue was counterstained with WGA-AF_{594} (Molecular Probes). The negative fibers were classified as IIX, and the yellow fibers (resulting from the superimposition of the green and red staining) were classified as IIB. The number of fibers expressing a given MyHC was determined with ImageJ version 1.46r (NIH, Bethesda, MD) from the whole TA, EDL, and soleus muscles, from the right hemi-DIA, and from the lateral GAS muscle and was expressed as the percentage of the total fiber count.

Further morphometric analyses were performed on the right hemi-DIA after H&E staining as follows. Approximately 10 images at a final magnification of ×100 were needed to capture the whole surface. Each image was viewed with ImageJ software, and lines were drawn at three preset locations equally distributed perpendicularly to the long axis of the DIA. At these locations, the thickness of the DIA was measured, and the number of myofibers crossing these lines was counted. The adipose tissue was identified as unstained "empty" fibers demarcated by perimysial connective structures. The foci of adipose tissue were demarcated with Photoshop software version 7.0 (Adobe, San Jose, CA). Then, the corresponding areas were quantified with ImageJ software and expressed as the percentage of the total muscle area. An approximate value of the area occupied by muscle cells (both normal and regenerated fibers) in the DIA was obtained by subtracting the surfaces of adipose and connective tissues from the total muscle surface.

Determination of Cardiac Fibrosis

Hearts were fixed in 4% buffered paraformaldehyde. After inclusion in paraffin, 5-μm-thick sections across the ventricles were collected 1.50 mm, 2.25 mm, and 3.00 mm from the apex and stained with Masson trichrome. The entire cross-sections were microphotographed with a Mirax Midi microscope at a final magnification of ×200. Each virtually reconstructed section was divided into four images from which the area covered by fibrotic deposits (appearing as a blue staining on a red background) was quantified with ImageJ software and expressed as the percentage of the total tissue surface. Finally, the values obtained from the three sections were averaged.

ER mRNA Expression

The left GAS muscle was snap-frozen in liquid nitrogen and stored at −80°C until processed. The muscles were ground to a fine powder in mortars cooled in liquid nitrogen. RNA were extracted from 10 mg of muscle powder (RNeasy Fibrous Tissue mini kit; Qiagen, Hombrechtikon, Switzerland), and 100 ng of total RNA was reverse-transcribed with Super-Script II Reverse Transcriptase (Invitrogen). The cDNA corresponding to 1 ng of reverse-transcribed total RNA was subjected to quantitative PCR (qPCR) amplification with the use of SYBR detection. To quantify the overall ERα or ERβ variants, primers were designed in regions that are not affected by alternative splicing. The expression levels of ERα and ERβ relative to the levels in the Dys group were determined with the TATA box-binding protein (*TBP*) as the invariant housekeeping gene.

The identification of the ERβ mRNA variants encoding the ERβ1, ERβ2, ERβ5, ERβ5A, and ERβ6 isoforms (as defined under the Accession number O08537 of the

UniProtKB/Swiss-Prot database) in the GAS muscle was performed by PCR as described.[39] Briefly, 1 μL of GAS muscle cDNA was subjected to PCR amplification with the use of primers annealing to exons 5 and 10 and under the following conditions: 95°C for 60 seconds; 40 cycles consisting of 95°C for 20 seconds, 55°C for 30 seconds, and 72°C for 60 seconds; and a final elongation step at 72°C for 5 minutes. As positive controls, mRNA from ovaries and brain were run in parallel. Three microliters (GAS muscle) or 0.1 μL (ovary) of the PCR product was resolved on denaturing polyacrylamide-urea gels (5W, 2 hours, 57°C) as described.[39] After silver staining, the gels were air-dried between two sheets of cellophane and scanned, and densitometric analysis of the signals was performed with ImageJ software. Alternatively, 1000-fold dilutions of the PCR products were used as templates for a second round of PCR amplification with the use of primers hybridizing to exons 6 and 8. The conditions were as described above, except that 35 cycles were performed and the annealing temperature was set to 60°C. Ten microliters (GAS muscle), 3 μL (brain), or 0.3 μL (ovary) of the second PCR products were resolved on 1.2% agarose gels and stained with ethidium bromide before quantification of ERβ1 and ERβ2 signals.

The primers used are shown in Table 1. They were designed from the sequences published under the NCBI Accession numbers NM_007956.4 (mouse ERα), NM_010157.3 (mouse ERβ1), NM_207707.1 (mouse ERβ2), and NM_013684.3 (mouse TATA box-binding protein).

Protein Expression

Muscle extracts were prepared from the left GAS muscle powder as described.[34] The final protein concentration was adjusted to 3 μg/μL with reducing Laemmli buffer. Muscle extracts (30 to 60 μg/lane) were resolved by SDS-PAGE, and proteins were transferred onto nitrocellulose membranes according to standard procedures. Equal loading and transfer efficiency were verified by staining with Ponceau Red. Membranes were blocked for 1 hour in TBST (20 mmol/L Tris-base, 150 mmol/L NaCl, 0.1% Tween-20, pH 7.5) containing 5% nonfat dry milk and incubated overnight at 4°C with a primary antibody (see Table 2 for detailed information on the primary antibodies used, providers, clonality, working dilutions, and nature of the competing protein). After extensive washing, membranes were incubated for 1 hour with an appropriate horseradish peroxidase-conjugated secondary antibody in TBST containing 5% milk. The bound antibody against ERβ2[27] was detected with protein G—horseradish peroxidase in TBST-milk. Proteins were revealed by chemiluminescence (ECL plus kit; Amersham, GE Healthcare Europe, Glattbrugg, Switzerland) after exposure to Fuji X-ray films (Fujifilm Europe, Dusseldorf, Germany). The films were scanned, and densitometric analysis was performed with ImageJ software. Signals were normalized to the MyHC content (determined on separate gels stained with Coomassie Blue) and corrected for the intensity of a reference sample loaded several times on every gel for the purpose of intragel and intergel comparisons.[34]

Data and Statistical Analysis

One wt mouse and one Dys mouse died at 55 and 64 weeks of age, respectively. Two Dys mice, one TAM mouse, and one FEM mouse died on preterminal anesthesia. Thus, the data presented here were collected from 11 to 14 males and from 8 to 9 females and expressed as the means ± SEMs. GraphPad Prism software version 5.03 (GraphPad, San Diego, CA) was used for constructing the graphs and for performing the statistical analyses. The differences between groups were assessed by one-way analysis of variance followed by Tukey's multiple comparison posttest. Differences with P values ≤ 0.05 were considered significant.

Results

Effects of TAM Treatment on Mouse Behavior, Body Weight, and Food Intake

The mice did not show noticeable alterations of their behavior during the 15 months of TAM treatment. Overall,

Table 1 Primers used for analysis of estrogen receptor mRNA levels

Primer	Exon	Sequence	Amplicon size (bp)
Primers used for determination of ER levels by RT-qPCR			
ERα-forward	5	5′-TGCGCAAGTGTTACGAAGTG-3′	
ERα-reverse	6	5′-TTTCGGCCTTCCAAGTCATC-3′	109
ERβ-forward	2	5′-TCGCTTCTCTATGCAGAACC-3′	
ERβ-reverse	3	5′-AGAAGTGAGCATCCCTCTTG-3′	138
TBP-forward	6	5′-TGCTGCAGTCATCATGAG-3′	
TBP-reverse	7	5′-CTTGCTGCTAGTCTGGATTG-3′	115
Primers used for determination of ER levels by RT-qPCR			
ERβ-forward	5	5′-TGAAGGAGCTACTGCTGAAC-3′	
ERβ-reverse	10	5′-CCCACTTCTGACCATCATTG-3′	914, 860, 726, 721, 587*
ERβ-forward	6	5′-GCTGATGGTGGGGCTGATGT-3′	
ERβ-reverse	8	5′-ATGCCAAAGATTTCCAGAAT-3′	177, 123†

*Amplicons corresponding to ERβ2, ERβ1, ERβ6, ERβ5, and ERβ5A isoforms, respectively.
†Amplicons corresponding to ERβ2 and ERβ1 isoforms, respectively.

Table 2 Characteristics of the antibodies used for analysis of protein levels

Antigen	Host	Clonality	Clone	Dilution	Competitor	Company	Catalog no.
α7 Integrin*	Rat	M	Cy8	1:2000	BSA	NA	NA
αB-crystallin	Rabbit	P	NA	1:1000	BSA	Calbiochem	238702
Calcineurin	Rabbit	P	NA	1:1000	BSA	Cell Signaling Technology	2614
Calsequestrin 1	Mouse	M	VIIID12	1:3000	BSA	Thermo Scientific	MA3-913
Calsequestrin 2	Rabbit	P	NA	1:2000	BSA	Thermo Scientific	PA1-913
ERα	Rabbit	P	NA	1:400	Milk	Santa Cruz Biotechnology	sc-7207
ERβ1	Rabbit	P	NA	1:1000	BSA	Cell Signaling Technology	5513
ERβ2	Rabbit	P	Twoβer.1†	1:1000	BSA	NA	NA
Parvalbumin	Mouse	M	NA	1:2000	BSA	Millipore	MAB1572
SERCA1	Mouse	M	IIH11	1:2000	BSA	Thermo Scientific	MA3-911
SERCA2	Rabbit	P	NA	1:1000	BSA	Abcam	ab3625
Utrophin	Mouse	M	DRP3/20C5	1:1000	Milk	Novocastra	NCL-DRP2

*Kindly donated by Prof. Randall H. Kramer (University of California, San Francisco, CA).
†Described previously.[27]
BSA, bovine serum albumin; M, monoclonal; NA, not applicable; P, polyclonal.

the groups of untreated Dys males and of untreated wt males showed similar growth curves (Supplemental Figure S1A). The mice treated with TAM for 15 months (TAM) were significantly smaller throughout the study and, at sacrifice they weighed the same as the untreated dystrophic females (FEM; Supplemental Figure S1, A and B). From the food consumption curves (Supplemental Figure S1C), we calculated that TAM intake decreased from 14 to 10 mg/kg/day during the first 17 weeks of treatment and then remained at ∼10 mg/kg/day until the end of the study.

Effects of TAM Treatment on the Weight of Organs and Muscles

The Dys mice had larger livers and testes than the wt mice. TAM treatment fully normalized the relative weights of these organs (Supplemental Table S1). The mice in the TAM group had significantly less white fat and more brown fat than the untreated Dys mice (Supplemental Table S1). TAM treatment did not change the relative weights of the other organs examined, such as the heart and the kidneys.

The Dys mice exhibited a significant hypertrophy of all of the skeletal muscles examined (Supplemental Table S1), which is a common feature of the dystrophic mouse models. Hypertrophy of the GAS, plantaris, soleus, and TA muscles was partly rescued by TAM. The relative weight of the triceps surae was completely normalized (Table 3). Overall, TAM diminished the relative weights of the muscles close to those of FEM mice, which showed less hypertrophy than the Dys group. In marked contrast, TAM increased the weight of the EDL muscle and the DIA. The relative weights of the heart were similar in all groups.

Table 3 Effect of TAM treatment on the mechanical properties of the triceps muscle

	Dys	TAM	wt	FEM
Phasic and tetanic isometric tensions				
P_t, actual (mN)	902.0 ± 39.2	1001.5 ± 127.2	1192.8 ± 35.7**	516.6 ± 19.0***†††
P_t, specific (mN/mm^2)	81.9 ± 2.9	162.3 ± 16.6***	109.9 ± 3.3*†	66.1 ± 2.4†††
P_o, actual (mN)	3013 ± 160	2835 ± 85	4573 ± 197***†††	2108 ± 66***†††
P_o, specific (mN/mm^2)	273.9 ± 11.3	465.4 ± 9.3***	421.0 ± 16.8***†	269.3 ± 6.7†††
Kinetics of contraction and relaxation				
TTP (ms)	14.9 ± 0.3	23.2 ± 1.5***	16.4 ± 0.3*†††	15.1 ± 0.6†††
$RT_{1/2}$ (ms)	15.9 ± 0.5	27.3 ± 1.1***	16.9 ± 0.4†††	18.1 ± 1.2†††
T_{dev} (%max/ms)	14.75 ± 0.50	9.42 ± 0.77***	15.28 ± 0.31†††	13.83 ± 0.68†††
T_{loss} (%max/ms)	4.09 ± 0.25	2.53 ± 0.16***	4.15 ± 0.18†††	3.37 ± 0.26†
Structural characteristics of the triceps surae				
Mass, actual (mg)	190.2 ± 3.4	112.1 ± 4.3***	189.0 ± 3.8†††	131.7 ± 3.6***†
Mass, corrected (mg/g)	5.26 ± 0.11	4.43 ± 0.12***	4.70 ± 0.09***	4.47 ± 0.11***
L_o (mm)	16.40 ± 0.19	17.28 ± 0.20**	16.41 ± 0.20††	15.86 ± 0.17†††
CSA (mm^2)	10.95 ± 0.22	6.12 ± 0.20***	10.87 ± 0.19†††	7.84 ± 0.21***

Data represent means ± SEMs from 8 to 11 mice.
*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ compared with the Dys group.
†$P \leq 0.05$, ††$P \leq 0.01$, and †††$P \leq 0.001$ compared with the TAM group.
CSA, cross-sectional area; L_o, optimal muscle length; P_o, optimal tetanic tension; P_t, peak twitch tension; T_{dev}, tension development; T_{loss}, tension loss; TTP, time to peak twitch tension.

Effect of TAM Treatment on the Wire Test Performance

Within the last weeks of the treatment period, a wire test was used to assess whole body force. Typically, soon after the mice were allowed to grasp the horizontal wire, they started to move along the wire and tried to flip around their body's axis to explore the wire in the other direction (Figure 1A). The Dys animals rapidly lost the grip of their hind paws, causing them to hang onto the wire with their fore limbs only. From this position, the mice were rarely able to bring their hind paws back onto the wire and were unable to sustain their own body weight for more than a few seconds at each testing (Figure 1A). By contrast, both the wt and the TAM mice were able to move their fore limbs and chest into an extended position over the wire, to bring their hind limbs back onto the wire, and to turn around their body's axis to flip from one direction of the wire toward the other. The TAM group performed much better than the Dys group and equally well as the wt group (Figure 1B). The FEM group performed significantly better than the Dys group but remained significantly weaker than the TAM group. Given that the smaller body weight of the dystrophic females and TAM-treated males could increase their score at the wire test, we also expressed the physical impulse as the product of the wire test score (in seconds) and the mouse body weight (in g).[40] The improved performance of the TAM group compared with the Dys group was still marked, whereas the FEM group performed as poorly as the Dys group (Figure 1C).

Effect of TAM Treatment on Plasma CK Activity

Plasma CK activity was approximately three times higher in dystrophic males than in normal males, revealing an increased fragility of dystrophic muscle membrane (Figure 2). CK levels were much lower in dystrophic females than in dystrophic males, suggesting a role for estrogens in stabilizing muscle membranes. TAM treatment reduced plasma CK levels to values not significantly different from those of normal males and of dystrophic females (Figure 2).

Effects of TAM Treatment on Muscle Contractile Properties

The isometric contractile characteristics of the triceps surae muscle were determined at the end of the treatment period. The muscle mechanics data are summarized in Table 3 and the most remarkable findings are shown in Figure 3. After correction for the body weight, the dystrophic triceps presented a slight hypertrophy compared with wt triceps. This was normalized by TAM (Table 3). Both phasic tension (P_t) and tetanic tension (P_o) were reduced in dystrophic mice compared with normal mice. Although the actual size of the triceps of the TAM group was reduced compared with the Dys group, the P_t and P_o outputs were similar in both groups (Table 3). Correction for the muscle cross-sectional area showed that the P_t and P_o developed by the TAM-treated mice were considerably higher than those of the untreated Dys mice (Figure 3, A and C). In fact, TAM treatment for 15 months increased the specific P_t and P_o of the dystrophic triceps by 100% and 70%, respectively. Remarkably, the triceps of TAM-treated mice became significantly stronger per surface unit than those of normal mice (Table 3 and Figure 3, A and C).

The time required to achieve maximum contraction (TTP) was slightly longer in the wt group than in the Dys group, whereas the time for $RT_{1/2}$ was similar in both groups

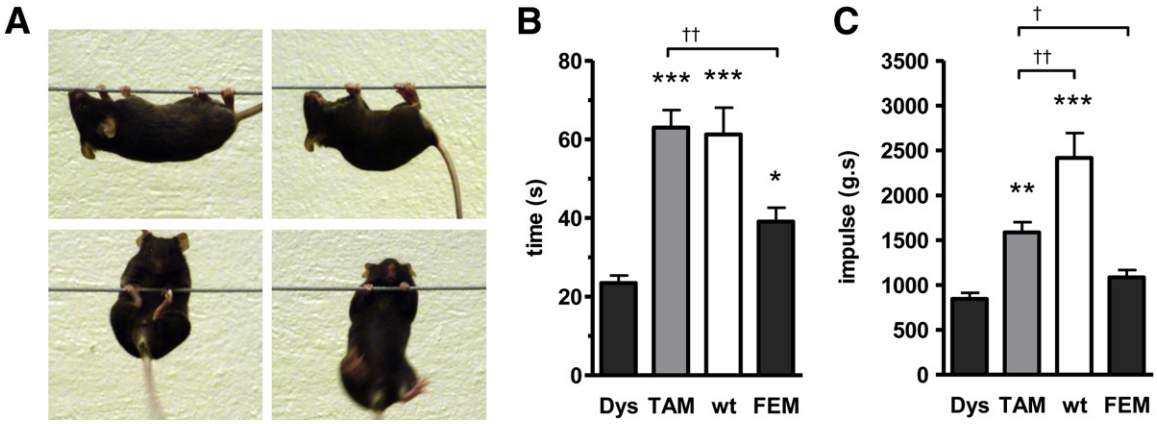

Figure 1 Effect of TAM treatment on the wire test score. A wire test was used to assess whole body force of male dystrophic mice (Dys), male dystrophic mice treated with 10 mg/kg per day of tamoxifen for 15 months (TAM), male wild-type mice (wt), and female dystrophic mice (FEM). **A:** Different views of a Dys mouse during the wire test. The mice were allowed to grasp a metal wire maintained horizontally above a thick layer of soft bedding. The Dys animals rapidly lost grip of their hind paws and hung onto the wire with their forelimbs only. From this position, they were unable to sustain their own body weight for more than a few seconds before falling. **B:** The length of time until the mice fell from the wire was recorded and showed that TAM normalized the ability of the dystrophic mice to maintain their grip. **C:** The physical impulse was calculated to take into account the smaller body weight of the dystrophic females and the TAM-treated males. The values represent means ± SEMs of 9 to 14 mice. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared with the Dys group; $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$ compared with the TAM group.

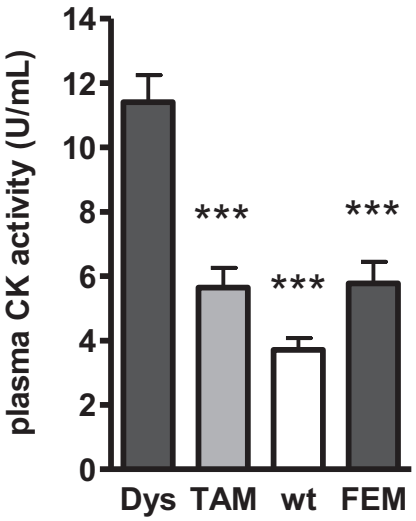

Figure 2 Effect of TAM treatment on plasma CK levels. Blood was collected from male dystrophic mice (Dys), male dystrophic mice treated with 10 mg/kg per day of tamoxifen for 15 months (TAM), male wild-type mice (wt), and female dystrophic mice (FEM). The plasma was prepared by centrifugation, and plasma CK levels were determined spectrophotometrically with the use of a commercial kit. The values represent means ± SEMs of 8 to 11 mice. ***$P \leq 0.001$ compared with the Dys group.

(Table 3 and Figure 3B). TAM treatment for 15 months conferred the triceps surae a much slower phenotype. Compared with the Dys group, the TTP value increased by 56% and the $RT_{1/2}$ value by 72% (Table 3 and Figure 3B). Accordingly, the rates of maximum tension development during contraction or of maximum tension loss during relaxation were 36% and 38% smaller, respectively (Table 3). Consistent with the slower contraction, the TAM group exhibited a marked leftward shift of the curve connecting the tension output to the frequency of stimulation (Figure 3D).

We next evaluated the resistance of the triceps surae against repeated tetanic contractions (Figure 3E). Previous studies from our laboratory suggested that this drastic assay showed the nonrecoverable fragility of the muscle toward damaging contractions rather than fatigue resulting, for instance, from limitation in oxygen supply or changes in the redox balance.[31] The tricep muscles of the Dys group showed a sharp loss of force as the tetani were repeatedly delivered. The normal mice were significantly more resistant during the first 15 tetani, until their force dropped and became similarly low as in the Dys group (Figure 3E). The dystrophic females exhibited increased resistance compared with the dystrophic males. TAM treatment for 15 months caused the triceps to lose force at a lower rate than in the untreated Dys group (Figure 3E). After a few tetani, the resistance of the TAM group to contraction-induced loss of force showed a significant improvement compared with the Dys group. Of note, the muscle resistance of the TAM group closely paralleled that of the FEM group (Figure 3E). The force drop index was calculated as the average difference between the experimental values and the response elicited by the first tetanus. The loss of force in the TAM and FEM groups was significantly less than that of the Dys and wt groups (Figure 3F).

Effects of TAM Treatment on Leg Muscle Structure

Examination of sections of EDL, soleus, and TA muscles stained with H&E or wheat-germ agglutinin showed in the Dys group well-known dystrophic changes, such as fibers with centrally located nuclei, indicative of muscle fiber regeneration, and excessive connective tissue. Exhaustive morphometric analyses showed that TAM caused diverse effects on different muscles with respect to centronucleated fibers, fibrosis, number of myofibers, and myofiber size (Supplemental Figures S2 and S3).

Effects of TAM Treatment on Diaphragm Morphology

H&E and wheat germ agglutinin staining revealed that long-term TAM treatment improved the quality of the DIA (Figure 4A and B, and Supplemental Figure S2, J–L). Fibrosis was significantly reduced (−21%) by TAM treatment (Figure 4, B and C). Interestingly, DIA from TAM-treated Dys mice were significantly heavier (76%) and thicker (59%) than those of untreated Dys mice (Figure 4, D and E). Further analysis demonstrated that these DIAs presented a significantly higher number of fibers (67%) and more fiber layers (48%) (Figure 4, F–H). The number of centronucleated fibers was significantly increased (33%), suggesting that more regeneration occurred (Figure 4I). In addition, the mean fiber diameter was increased close to that of the wt mice (Figure 4J), and the fraction of muscle cells to the total muscle surface was increased (21%) (Figure 4C). When combining the increased thickness of the DIA with the increased surface occupied by the myofibers, TAM augmented the amount of contractile tissue in the DIA by 72%.

Effect of TAM Treatment on Heart Fibrosis

Fibrosis was ∼3.5 times higher in Dys hearts than in the wt hearts (3.04% and 0.87% of the heart cross-sections, respectively) (Figure 5, A–C). Cardiac fibrosis was similar in the FEM group and in the Dys group. After TAM treatment, fibrosis was reduced to 1.86% of the ventricular surface, showing that TAM prevented the development of fibrosis in dystrophic hearts by ∼53% (Figure 5C).

Effects of TAM Treatment on Fiber Type Distribution

Fiber typing was performed with fluorescent antibodies directed against specific MyHC isoforms. We found subtle differences in the distribution of MyHCs between the Dys and wt groups (Supplemental Figure S4, A–E). In all leg muscles tested, except the GAS muscle, TAM caused an accumulation of the type I fibers (fatigue-resistant,

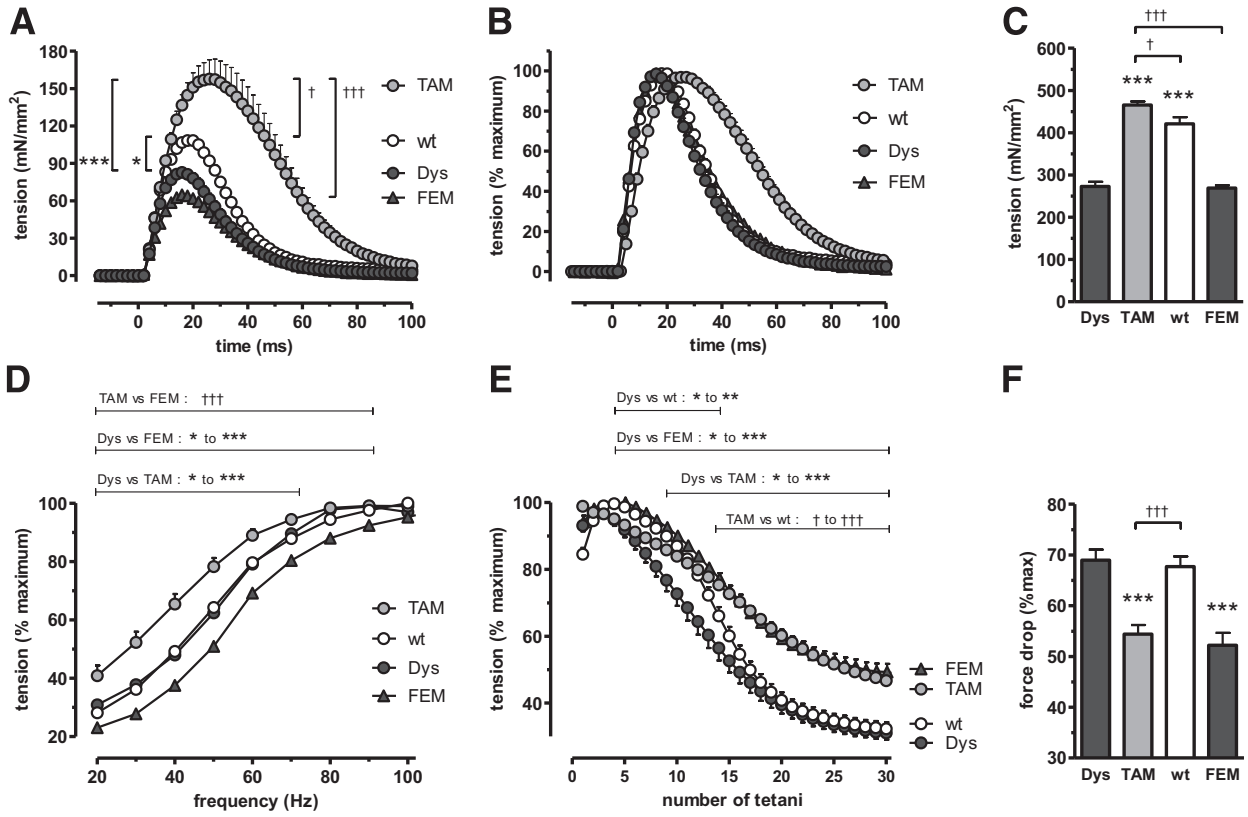

Figure 3 Effects of TAM treatment on the mechanical properties of the triceps muscle. Isometric force characteristics were determined on male dystrophic mice (Dys), male dystrophic mice treated with 10 mg/kg per day of tamoxifen for 15 months (TAM), male wild-type mice (wt), and female dystrophic mice (FEM). **A:** Phasic twitch traces normalized for muscle cross section showing that TAM-treated triceps developed much higher force than the other groups. **B:** Phasic twitch traces normalized to their maximum peak value, highlighting the slower kinetics of contraction and relaxation of TAM-treated triceps. **C:** Tetanic tensions normalized for muscle cross section showing that TAM-treated triceps were as strong as normal ones. **D:** Curves connecting the frequency of stimulation to muscle tension output, showing the slower contractile phenotype of the TAM-treated triceps. **E:** Loss of muscle tension on repeated tetanic contractions, showing that TAM made the triceps more resistant to fatigue. **F:** Average force drop calculated from the curves in E, showing that TAM prevented contraction-induced loss of force. The values represent means ± SEMs of 8 to 11 mice. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ compared with the Dys group; $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, and $^{\dagger\dagger\dagger}P \leq 0.001$ compared with the TAM group.

slow-contracting fibers) or type IIA fibers (fatigue-resistant, fast-contracting fibers), with a concomitant reduction in the type IIX and IIB fibers (fatigue-sensitive, fast-contracting fibers). By contrast, the DIA showed an opposite response to TAM. As a result of these fiber type shifts, the ratio of types (I + IIA) to IIB fibers was normalized in the EDL, the soleus, the TA, and the DIA muscles of TAM-treated mice (Supplemental Figure S4, F–J).

Effects of TAM Treatment on the Expression of Muscle Markers

Proteins from GAS muscle extracts were analyzed by Western blot analysis. Their levels were corrected for MyHC content and normalized to the levels in the Dys group (Figure 6). Compared with the wt mice, the GAS muscle of the Dys mice contained significantly more utrophin, calsequestrin 2, SERCA2, and calcineurin but less calsequestrin 1. Treatment of Dys mice for 15 months with TAM significantly enhanced the expression of utrophin (+27%), α7 integrin (+36%), αB-crystallin (+61%), calsequestrin 2 (+39%), and calcineurin (+38%) and reduced the levels of parvalbumin (−35%), calsequestrin 1 (−28%), SERCA1 (−25%), and SERCA2 (−18%) (Figure 6, A–J).

Effects of TAM Treatment on Expression of ERs

The expression levels of ERα and ERβ were explored in the GAS muscle. RT-qPCR showed that ERα mRNA levels were similar in all groups (Figure 7A). In contrast, total ERβ mRNA levels were 2.3 times more abundant in the Dys mice than in the wt mice. These levels were further increased (+40%) by TAM treatment, resulting in ERβ mRNAs being 3.2 times higher than in the wt mice (Figure 7B). As shown by nested PCR with the use of primers flanking exon 7 (Figure 7C), the levels of ERβ1 mRNA, encoding the physiologically active ERβ subtype, were unchanged on TAM treatment. We found that the increase in ERβ mRNAs was mostly caused by the accumulation of the mRNA encoding ERβ2, a variant having an extended ligand-binding domain with lower affinity for estrogens (Figure 7D).[28,39] The ERβ2/ERβ1 mRNA

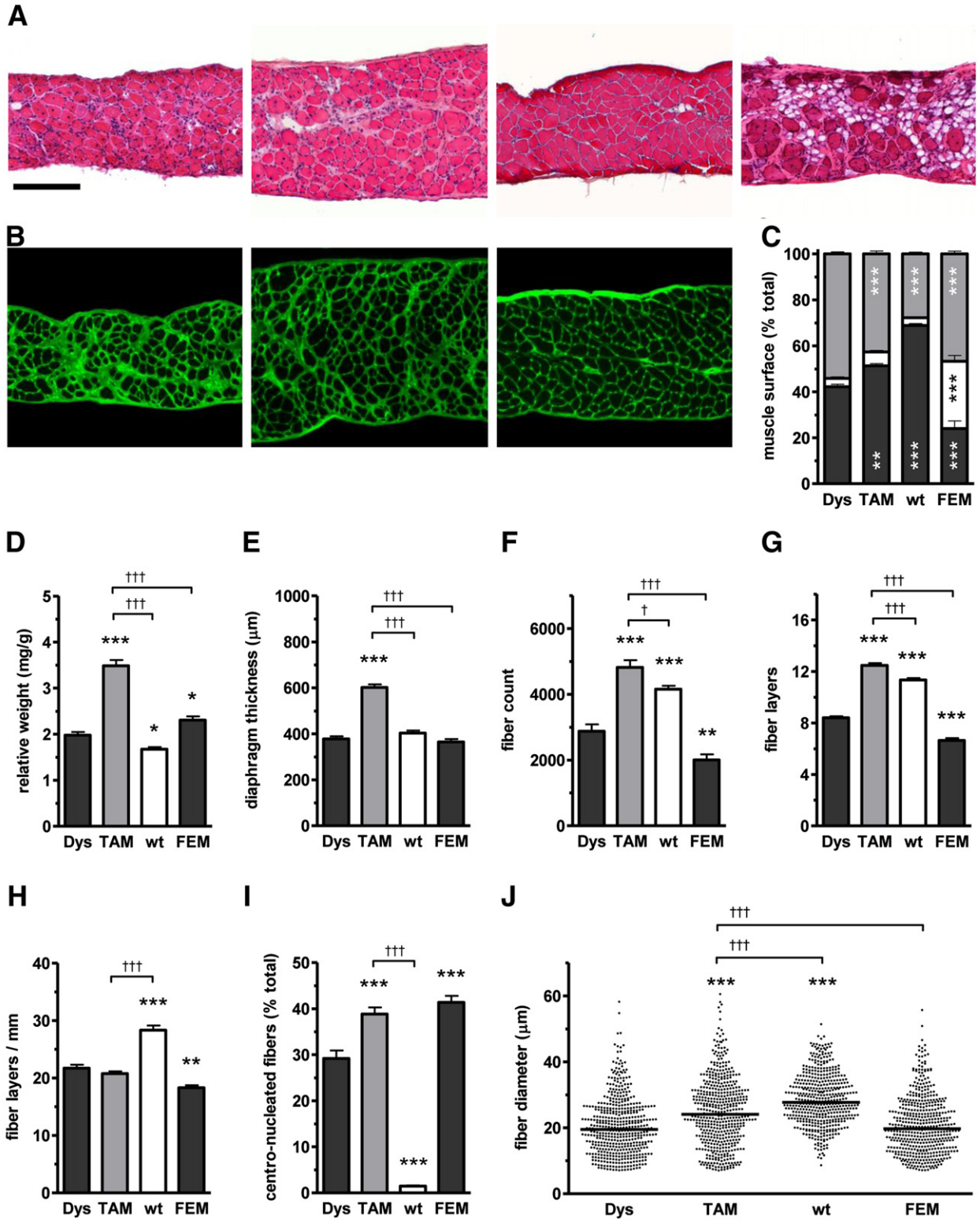

Figure 4 Effects of TAM treatment on diaphragm morphology. **A**: H&E-stained diaphragms from male dystrophic mice (Dys), male dystrophic mice treated with 10 mg/kg day of tamoxifen for 15 months (TAM), male wild-type mice (wt), and female dystrophic mice (FEM) (left to right). Scale bar = 200 µm. **B**: Fluorescent wheat germ agglutinin-stained diaphragms of the same groups as in **A**. The microphotograph of the FEM group was omitted. **C**: The surfaces occupied by myofibers (dark gray columns), connective tissue (light gray columns), and adipose tissue (white columns) were expressed as the percentage of the total diaphragm cross-sectional area. The relative weight (**D**), thickness (**E**), fiber number (**F**), and fiber layers (**G**) of the diaphragms were increased by TAM treatment. **H**: The number of fiber layers per millimeter was not changed by TAM, but an increase of centronucleated myofibers (**I**) and mean fiber diameter (**J**) was noted. For clarity, the scatter plots in J show the diameter of 500 individual fibers per group of >6000 fibers analyzed. The statistical analyses were performed on the total fiber populations. The values in **C**—**I** represent the means ± SEMs of 8 to 11 mice. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ compared with the Dys group; $^{\dagger}P \leq 0.05$, $^{\dagger\dagger\dagger}P \leq 0.001$ compared with the TAM group.

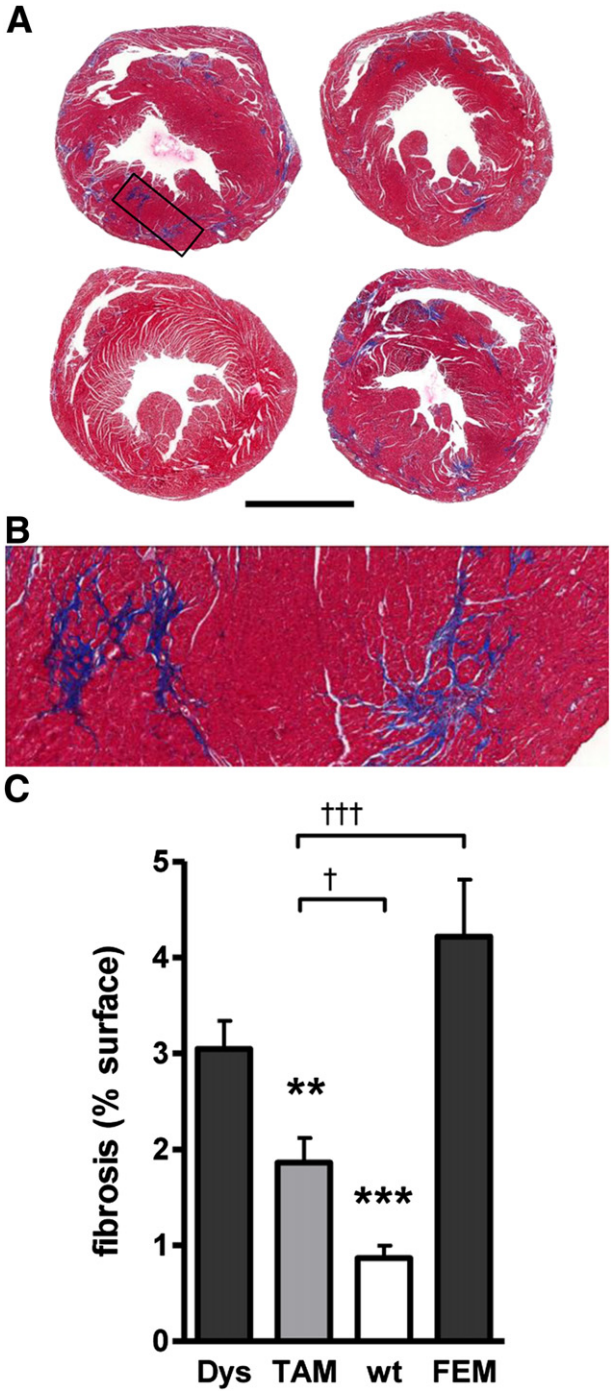

Figure 5 Effect of TAM treatment on heart fibrosis. Hearts were fixed in 4% buffered paraformaldehyde. After inclusion in paraffin sections (5 μm thick) across the ventricles were stained with Masson trichrome. The fibrotic deposits appear as a blue staining on a red background. **A**: Representative heart sections from a male dystrophic mouse (Dys) (**top left**), a male dystrophic mouse treated with 10 mg/kg per day of tamoxifen for 15 months (TAM) (**top right**), a female dystrophic mouse (FEM) (**bottom right**), and a male wild-type mouse (wt) (**bottom left**). **B**: Higher magnification view of the marked area in **A**, showing the fibrotic scars stained in blue. **C**: The areas stained blue were quantified and expressed as the percentage of the total tissue surface. Sections collected 1.50 mm, 2.25 mm, and 3.00 mm from the apex were analyzed and the values were averaged for every mouse. The values represent means ± SEMs of 8 to 11 mice. Scale bars: 2 mm (**A**); 400 μm (**B**). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared with the Dys group; $^{\dagger}P \leq 0.05$, $^{\dagger\dagger\dagger}P \leq 0.001$ compared with the TAM group.

ratio was seven times lower in Dys mice than in wt mice (Figure 7E). TAM treatment elevated the ERβ2/ERβ1 ratio more than fourfold, bringing it close to that of wt mice (Figure 7E). The mRNAs encoding shorter ERβ variants (ERβ5, ERβ5A, and ERβ6), which are expressed in the ovaries, were not detected in the GAS muscles from any group (Figure 7F). Western blot analysis showed that ERα and ERβ1 proteins were, respectively, 4.3 times and 3.5 times more abundant in Dys muscles than in wt muscles (Figure 7, G and H), whereas ERβ2 was expressed at similar levels in both groups (Figure 7I). TAM did not modify ERα and ERβ1 protein expression but caused a fourfold accumulation of the ERβ2 isoform in dystrophic muscle (Figure 7I), resulting in complete normalization of the relative ERβ2/ERβ1 protein ratio (Figure 7J).

Levels of TAM and Its Metabolites in Plasma and Muscle

We determined the concentrations of the E and Z isomers of TAM and of three major TAM metabolites in the plasma and in the GAS muscle of the TAM group. Results and representative chromatographic profiles are shown in Table 4 and Supplemental Figures S5 and S6, respectively. TAM isomers were the major species, followed by 4-OH-TAM, 4-hydroxy-*N*-desmethyl-TAM (also known as endoxifen), and *N*-desmethyl-TAM isomers. The latter were below the limit of quantification of the assay for the plasma. The compounds were 9 to 20 times more abundant in the GAS muscle than in the plasma. The levels of TAM and its metabolites in the muscle and the plasma of the TAM-treated mice were in the low nanomolar range. Unexpectedly, these levels were up to two to three orders of magnitude lower than those found in the same tissues of patients with breast cancer under standard TAM treatment[41,42] or of normal mice and rats.[43] In addition, in our TAM-treated mice, the E and Z isomers were present in roughly similar quantities, which contrasts with humans treated for breast cancer whereby the E isomers are usually only present in trace amounts.[37,44] Of note, we analyzed the food pellets and ruled out a Z-to-E interconversion during the preparation and the storage of the modified chow.

Discussion

TAM, a first-generation SERM, administrated orally for 15 months at 10 mg/kg/day to *mdx*^{5Cv} mice caused remarkable muscular improvements: i) the ability of the mice to maintain their grip was increased, suggesting that the body musculature was able to develop more force; ii) the triceps surae, a large group of muscles in the leg, displayed a striking enhancement of contractile features; iii) the DIA, the most severely affected muscle in dystrophic mice, became bigger, contained more fibers, but less fibrotic deposits; and iv) the heart showed a significant reduction in the extent of

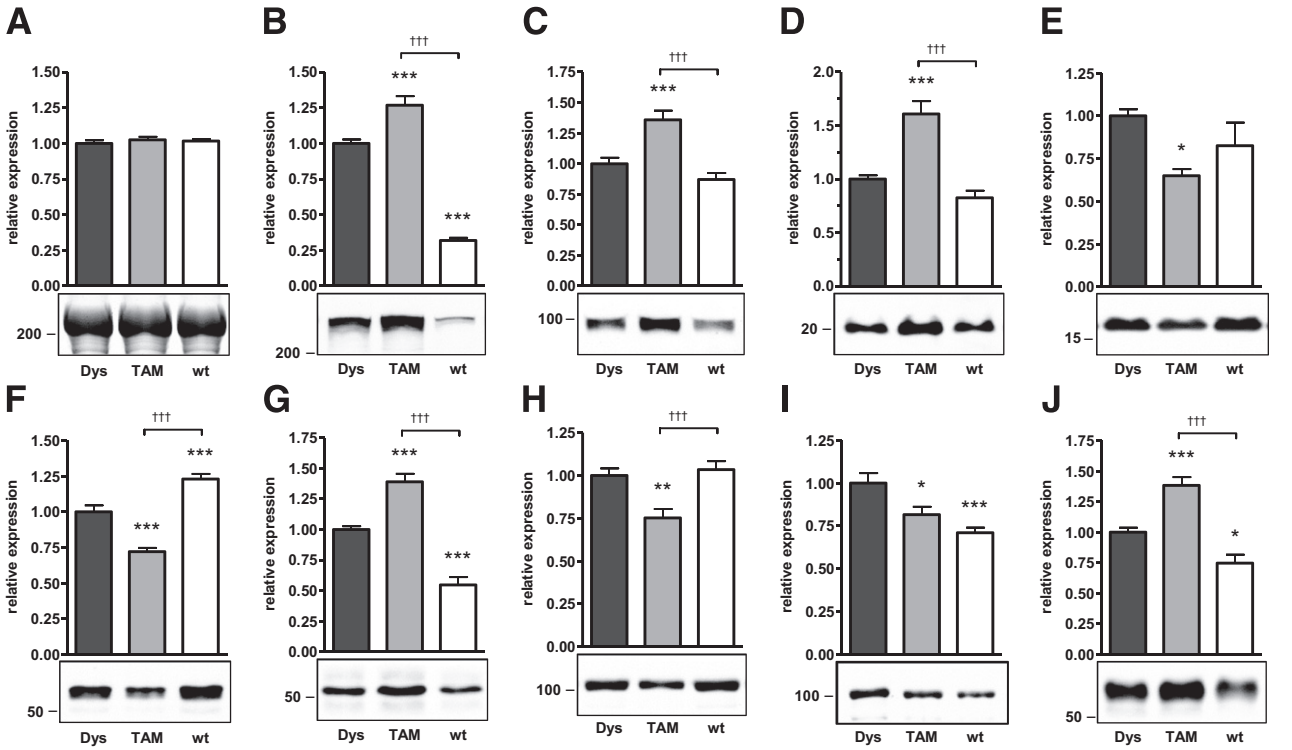

Figure 6 Effects of TAM treatment on the expression of muscle markers. Western blot analyses were performed on gastrocnemius extracts prepared from male dystrophic mice (Dys), male dystrophic mice treated with 10 mg/kg per day of tamoxifen for 15 months (TAM), and male wild-type mice (wt). **A**–**J**: The myosin heavy chains (**A**), shown by Coomassie Blue staining, were used for correcting the signals of the following muscle markers: utrophin (**B**), α7 integrin (**C**), αB-crystallin (**D**), parvalbumin (**E**), calsequestrin 1 (**F**), calsequestrin 2 (**G**), SERCA1 (**H**), SERCA2 (**I**), and calcineurin (**J**). The position of the molecular weight markers is indicated (in kDa). The values were normalized to the average value of the Dys group and represent the means ± SEMs of 11 mice per group. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ compared with the Dys group; $^{\dagger\dagger\dagger}P \leq 0.001$ compared with the TAM group.

fibrosis. To the best of our knowledge, this is the first report on the use of TAM on a model of muscular dystrophy.

Rationale for Using TAM

TAM and its active metabolites have been intensively studied for their ability to control survival, growth, and other functions of estrogen-dependent cell populations in the mammary glands, uterus, ovaries, and bones.[17–19,45] Apart from these effects, other actions, including prevention of oxidative stress,[20] protection against contraction-induced membrane damage,[14] modulation of calcium handling,[24–26] prevention of mitochondria-mediated cell death,[46] and inhibition of fibrosis[22,23,47] have been documented for TAM and its metabolites. These processes contribute to the pathogenic mechanisms at work in dystrophic muscle, and targeted interventions have been shown to improve the phenotype of dystrophic muscle to some extent.[7,31,33,35,48–51] Therefore, we reasoned that TAM should ameliorate the structure and the function of dystrophic muscles in mice. The findings described in the present report show that TAM remarkably ameliorated the function and the structure of murine dystrophic muscles.

All of the effects reported in the present study were obtained with tissue levels of TAM and its major metabolites much lower than those reported in prior studies on normal rodents.[43,52] In addition, we found that the E isomers accounted for an important part of the total TAM and metabolites. In humans, the unusual occurrence of high levels of the E isomers has been correlated with breast cancer resistance to TAM therapy and specific profiles of TAM-metabolizing cytochrome P450 enzymes.[53] More work is needed to clarify why the dystrophic mice display lower levels of TAM and its metabolites compared with normal mice[43] and high amounts of E isomers compared with humans.[37,44]

TAM Tolerability

As judged by the relative weight of selected organs and overall behavior, long-term administration of TAM to dystrophic mice was well tolerated. TAM significantly diminished the weight gain of the treated mice, which is likely because of the reduction of white fat. At the end of the treatment period, the TAM-treated males weighed the same as age-matched females.

Protective Actions of TAM on Muscle Function and Supporting Molecular Findings

Using several techniques, we have demonstrated that TAM ameliorates various force parameters of dystrophic muscle.

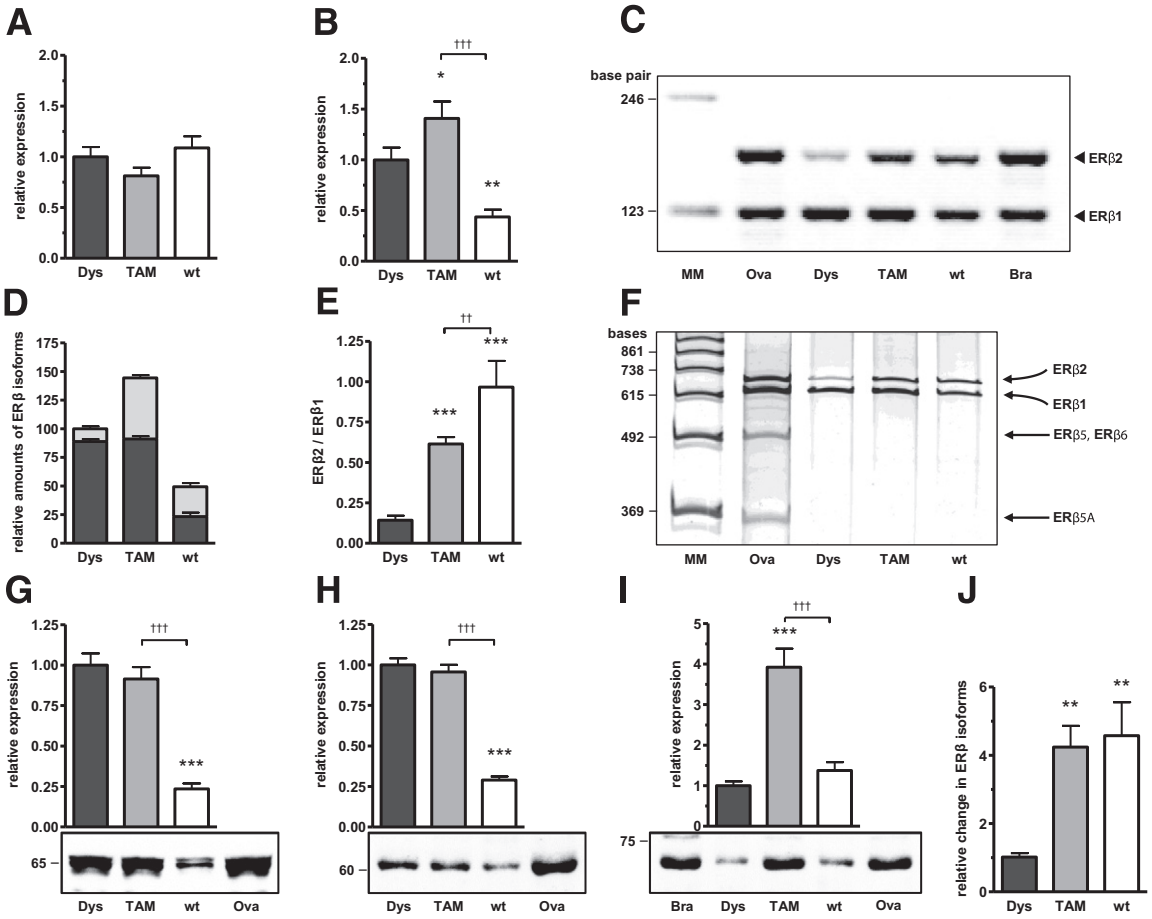

Figure 7 Effects of TAM treatment on the expression of estrogen receptors. Total protein and mRNA extracts were prepared from gastrocnemius muscles of male dystrophic mice (Dys), male dystrophic mice treated with 10 mg/kg per day of tamoxifen for 15 months (TAM), and male wild-type mice (wt). Brain (Bra) and ovary (Ova) extracts were included for comparison. The levels of ERα (**A**) and ERβ (**B**) mRNAs were determined by real-time qPCR. **C**—**E**: The relative abundance of the ERβ1 and ERβ2 isoforms were evaluated after nested PCR, followed by gel electrophoresis and densitometric analysis of the bands. **C**: Representative agarose gel, showing PCR amplification of the ERβ1 and ERβ2 isoforms. The molecular weight markers are shown (base pairs). **D**: Relative abundance of the ERβ1 and ERβ2 isoforms, normalized to the total ERβ content in the Dys group. **E**: ERβ2-to-ERβ1 mRNA ratio. **F**: Denaturing urea-polyacrylamide gel electrophoresis showed that gastrocnemius muscles did not express small ERβ isoforms (ERβ5, ERβ5A, ERβ6) that can be found in ovaries (**arrows**). The molecular weight markers are shown (bases). Western blot quantification of ERα (**G**), ERβ1 (**H**), and ERβ2 (**I**). The position of the molecular weight markers is indicated (in kDa). **J**: ERβ2-to-ERβ1 protein ratio normalized to the values of the Dys group. The data represent the means ± SEMs of 11 mice per group. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ compared with the Dys group; $^{\dagger\dagger}P \leq 0.01$, $^{\dagger\dagger\dagger}P \leq 0.001$ compared with the TAM group.

The wire test, like other hanging tests, is a rather stringent assay that challenges many muscles simultaneously, including those of the limbs as well as the trunk, abdominal, and back muscles.[40] The much-increased score at the wire test showed that TAM greatly improved overall muscle function of active dystrophic mice. This score could be affected by changes in force, fatigability, and possibly also balance. Therefore, we extended the evaluation of muscle function with the use *in situ* isometric contractions of the triceps surae, a large muscle group of the lower leg that is representative of most locomotor muscles. In agreement with the grid test findings, we found that the P_t and the P_o developed per unit of muscle cross section were much higher in the TAM-treated triceps than in triceps from untreated dystrophic mice. In addition, and as shown by longer TTP and $RT_{1/2}$ and smaller maximum rates of tension development and tension loss, TAM conferred much slower contraction kinetics to the triceps surae. The resistance of muscle to repeated tetanic contractions was also much higher in TAM-treated mice than in wt mice.

It has been established by others that the transient estrogen rise during the menstrual cycle correlates with enhanced muscle force,[12] and in certain paradigms estrogens conferred slower contraction and relaxation rates to the muscles,[12,54] involving either an alteration in calcium handling[54] or a decrease in type IIB fibers.[55]

TAM made dystrophic muscles even stronger than wt muscles, which one may find surprising. By contrast to what is expected with strategies aimed at re-introducing the missing dystrophin,[8] the mechanisms of action of active pharmacologic compounds do not necessarily involve the restoration of impaired signaling pathways and homeostatic

Table 4 Levels of TAM and its metabolites in plasma and gastrocnemius muscle

	Plasma		Gastrocnemius	
Compounds	ng/mL	nmol/L	ng/g	nmol/L
(Z)-tamoxifen	1.25 ± 0.30	3.35 ± 0.80	20.18 ± 4.58	54.32 ± 12.32
(E)-tamoxifen	1.75 ± 0.14	4.71 ± 0.38	16.01 ± 1.87	43.11 ± 5.02
(Z)-4-hydroxytamoxifen	1.22 ± 0.37	3.14 ± 0.97	13.31 ± 4.11	34.35 ± 10.60
(E)-4-hydroxytamoxifen	1.39 ± 0.11	3.59 ± 0.28	14.81 ± 2.06	38.23 ± 5.30
(Z)-*N*-desmethyl-tamoxifen	0.13 ± 0.03	0.37 ± 0.09	2.49 ± 0.50	6.96 ± 1.40
(E)-*N*-desmethyl-tamoxifen	0.30 ± 0.04	0.84 ± 0.10	3.69 ± 0.42	10.31 ± 1.16
(Z)-endoxifen	0.29 ± 0.05	0.78 ± 0.13	5.04 ± 0.83	13.49 ± 2.21
(E)-endoxifen	0.19 ± 0.02	0.51 ± 0.06	3.82 ± 0.49	10.22 ± 1.32

Values represent mean ± SEM of either 8 plasma or 11 muscles from TAM-treated mice. The plasma values for (Z) and (E)-*N*-desmethyl-tamoxifen are below the limit of quantification of the method and are shown for reference only.

balances back to normal levels. Moreover, it should be noted that the force developed by unexercised normal muscle does not represent an absolute upper limit that can in no condition be reached or exceeded. Instead, the force of normal muscles can be augmented by several conditions, including the use of doping substances and exercise that causes muscles to display an optimal redox balance and to ensure adaptation to the novel energy demand and structural requirements.[56–59] We suggest that TAM may have triggered and enhanced alternative pathways and compensatory mechanisms that could collectively ameliorate dystrophic muscle function and force output, possibly to levels above those of wt muscle.

Our findings of a slower rate of contraction and an enhanced resistance to fatigue in muscles from TAM-treated mice are of significance for the pathophysiology of muscular dystrophy. We established that the slower twitches resulted from a fast-to-slow fiber type shift and were accompanied by a molecular signature typical of slow-contracting muscles:

First, the slow-twitch phenotype is partly governed by the protein phosphatase calcineurin.[60] Our finding that TAM enhanced calcineurin expression in the GAS muscle suggests a fast-to-slow phenotype transition. This is in agreement with data showing that chronic activation of calcineurin in normal skeletal muscle promoted fast-to-slow fiber transition, increased endurance, improved resistance to fatigue, and enhanced mitochondrial oxidative function.[61,62] In support of a protective role for calcineurin in dystrophic muscle, reports indicate that inhibition of calcineurin activity by cyclosporin A aggravated the *mdx* phenotype, whereas constitutively active calcineurin protected *mdx* muscles from damage.[63]

Second, it is established that the fast-contracting type IIB fibers of both patients with DMD and *mdx* mice are more susceptible to damage than the slow-twitch type I fibers.[64,65] This might be because of higher antioxidant defense mechanisms and accumulation of utrophin, a dystrophin homologue, in slow compared with fast fibers.[66] The EDL, TA, and soleus muscles of the TAM-treated mice contained an increased number of type I fibers or of fast-twitch, fatigue-resistant type IIA fibers and, consequently, displayed an increased value of the (I + IIA)/IIB fiber ratio, which was restored to normal. This index was also normalized in the DIA, although this was achieved through a relative reduction of the type I and IIA fibers, which might result from a protection of the fragile type IIB fibers in that muscle.

Third, fiber type shift did not occur in the GAS muscle because the (I + IIA)/IIB fiber index was similar in all groups, which is in agreement with studies by others showing similar fiber type compositions in normal and dystrophic GAS muscle.[67] However, Western blot analyses showed changes in the levels of calcium handling proteins, again suggestive of a transition toward a slower phenotype. The GAS muscle from TAM-treated mice contained more of the slow type–specific protein calsequestrin-2 together with reduced levels of the fast type–specific proteins SERCA1, calsequestrin-1, and parvalbumin (reviewed in Berchtold et al[68] and Reggiani and Kronnie[69]). SERCA2 levels in dystrophic GAS muscle were also reduced by TAM close to normal amounts. Interestingly, SERCA2 was found to be overexpressed in the fast-twitch EDL muscle in *mdx* mice, likely as a compensatory mechanism.[70] We suggest that the TAM-induced reduction of SERCA2 in GAS muscle might result from an alleviation of the dystrophic symptoms.

We have also established that TAM treatment enhanced the accumulation of several structural proteins, such as the dystrophin homologue utrophin, α7 integrin, and αB-crystallin. When overexpressed in *mdx* mice, utrophin and α7 integrin have proven to be of therapeutic interest by acting as surrogates for the missing dystrophin.[32,71,72] αB-crystallin is a small heat shock protein that is much more abundant in slow-twitch than in fast-twitch muscles.[73] It acts as a chaperone for several myofibrillar proteins such as desmin, a muscle-specific intermediate filament that is critical for maintaining the integrity of the myofilaments, and for ensuring their proper anchoring to other binding partners.[73] Of note, mutations in either desmin or αB-crystallin result in a variety of muscular disorders.[74] In support of a protective role for TAM-induced accumulation

of structural proteins, recent studies reported that up to 20% of the force deficit in old *mdx* mice is due to altered myofilament architecture[75] and that reciprocally damaging contractions impair myofilament activity.[76] Taken individually, the overexpression level of every one of these structural proteins is likely too low to promote significant protection. We suggest that their simultaneous overexpression contributed to the TAM-induced increase of muscle force and to the recovery of membrane stability.

Altogether, the fiber type shifts, the increased levels of calcineurin, the accumulation of various structural proteins, and the alterations in calcium handling proteins suggest that TAM triggered complex transcriptional programs that protected the muscle, at least partly, via the acquisition of a slower and fatigue-resistant phenotype.[60,62]

Protective Actions of TAM on Overall Muscle Structure

Most muscles of the mdx^{5Cv} mouse undergo massive necrosis at ~3 to 5 weeks of age, followed by the formation of new myofibers retaining internal nuclei and displaying an important scattering of their diameter. From 8 to 10 weeks of age, the degeneration-regeneration cycles continue at a lower rate, and at ~1 year of age, as the self-repair capabilities of the muscle decline, connective tissue infiltration becomes prominent. In young dystrophic mice, centronucleated fibers are a reliable marker of the proportion of fibers that have disappeared due to prior necrosis.[31,34,51] In the present study, the interpretation of centronucleation is complicated by the long duration of treatment, during which the muscles likely underwent several cycles of necrosis-regeneration, and by the fact that the regenerated fibers are more vulnerable than the original ones.[77] However, it is likely that the decreased centronucleation in the soleus and the TA muscles is subsequent to prevention of necrosis and that the increased proportion of regenerated fibers in the EDL muscle and the DIA results from enhanced regeneration. This view is strongly supported by the relative weights of these muscles, the alteration of which parallels the centronucleation index. Whether these muscle-specific effects correlate with different expression profiles of the ERs and/or their nuclear cofactors in different muscles remains to be established. This possibility finds some support from earlier work to suggest higher ER levels in slow-twitch muscles from rabbit[78] as well as from recent findings from Feder et al[79] who found altered ER expression in EDL and quadriceps muscles of *mdx* mice (D. Feder, personal communication).

Normalization of myofiber size, reduction of the scattering of myofiber diameter, and decreased fibrosis are considered positive outcomes in the evaluation of therapeutic interventions in older dystrophic mice. Overall, several muscles from both the anterior and the posterior lower leg as well as the DIA showed a favorable evolution of one or more of these parameters with TAM. We believe that most of the musculature benefited similarly from TAM exposure, which is supported by the enhanced performance at the wire test.

Protective Actions of TAM on Diaphragm and Heart

TAM has been shown to prevent fibroblast activation, decrease collagen synthesis, and inhibit the release of transforming growth factor (TGF)-β, a major profibrotic mediator, in several conditions such as keloids, rhinophyma, Dupuytren disease, and retroperitoneal fibrosis.[22,23,47,80] Here, we demonstrate that TAM decreased the progression of fibrosis in the dystrophic heart and DIA. Furthermore, TAM showed additional protective effects on the DIA. It ameliorated the myofiber diameter, increased the proportion of regenerated fibers, and greatly enhanced the thickness of the muscle, which resulted mostly from an increase in the total number of myofibers. After TAM treatment, the net amount of tissue consisting of myofibers likely to contribute to the respiratory function was augmented by 72%. Collectively, these results suggest that TAM alleviated the muscular dystrophy in the DIA and strongly promoted the formation of new myofibers. In support of this, we found that the plasma level of TGF-β, a growth factor that controls muscle regeneration and fibrosis, was reduced (unpublished data). The DIA is the muscle of the dystrophic mouse that best mirrors the human condition.[81] Several pharmacologic interventions, such as immunosuppressors, green tea polyphenols, and blockers of TGF-β signaling pathways, reduced fibrosis in the DIA.[51,82,83] Other substances (reviewed in Judge et al[5]), such as halofuginone and deflazacort (but not prednisolone), were found efficacious for ameliorating cardiac function or reducing cardiac fibrosis. Together with losartan,[50] TAM appears to be one of the few compounds that reduces the development of fibrotic scars in both the DIA and the heart of dystrophic mice. This may be related to their common ability to reduce TGF-β. Improving respiratory and cardiac functions is a challenging issue for ameliorating the quality of life and increasing the life expectancy of patients with DMD.[4,5] This makes TAM particularly attractive as a therapeutic agent for treating muscular dystrophy.

Significance of ER Expression and Low Levels of TAM and Metabolites in Muscle

Natural estrogens and TAM are lipophilic compounds that accumulate in biological membranes, where they are thought to exert a variety of actions that involve neither ER nor transcription. In *in vitro* systems, short-term exposure to high concentrations of TAM were found to increase membrane fluidity,[84] to protect phospholipids from peroxidation[20] and to directly modulate the activity of ion channels and pumps.[24–26] Typically, these effects were seen with 1 to 20 μmol/L TAM in the extracellular fluid, which likely leads to much higher local concentrations in the membranes of the cultured cells. Several pharmacodynamic

studies on normal mice and rats reported that TAM and its metabolites reach concentrations in the low micromolar range in various tissues, including skeletal muscle.[43,52] Our findings show that the total concentration of TAM and its metabolites in the GAS muscle of dystrophic mice was ~200 nmol/L, which is likely insufficient for triggering physical actions on the membrane. Moreover, data from others suggest that direct membrane actions of TAM would not prevail *in vivo*. Koot and colleagues[14] reported that TAM-induced protection of rat skeletal muscles from damaging contractions was achieved after long-term treatment, whereas short-term (24 hours) treatment was ineffective. Therefore, we believe that the decreased CK value that we report here is the consequence of ER-dependent mechanisms that lead to myofiber stabilization rather than a direct effect on membrane fluidity or stability. In fact, we have recently demonstrated that doses of TAM as low as 0.1 mg/kg/day (ie, 100 times lower than the dose used in the present study) still produced significant improvements of most motor endpoints, lowered plasma CK levels, and reduced the number of Evans blue dye-permeable fibers and that TAM actions were antagonized by the ER blocker fulvestrant (O.M. Dorchies et al, manuscript in preparation), which provides strong support for receptor-mediated effects of TAM on dystrophic muscle.

Most of the effects of estrogens, TAM, and TAM metabolites result from their high-affinity binding to ERα and ERβ that are expressed in estrogen-responsive tissues of both males and females, including skeletal muscle.[10,11,28] Several ERβ isoforms exist. ERβ1 is considered as the physiologically active isoform, whereas ERβ2, a longer isoform with much reduced affinity for estrogens, would act in a dominant negative manner for the other ERs.[27,28] We report here, for the first time, that dystrophic muscle is enriched in both ERα and ERβ. This could well be the underlying reason for the unexpectedly high responsiveness of this tissue to TAM. Moreover, we found that the imbalance in the relative amounts of ERβ1 and ERβ2 tended to be normalized by TAM due to increased expression of ERβ2. This is particularly interesting in light of recent studies that demonstrate a role for ERβ in preventing both hypertrophy and fibrosis of the heart,[85,86] although these studies do not allow distinguishing the roles of different ERβ isoforms. Previous work by others have suggested that ER are expressed in various cell types within mammalian skeletal muscle, including endothelium, myoblasts, and myofibers.[10,11] In our hands, immunofluorescence labeling of mouse muscle tissues with the use of a large number of commercially available antibodies produced inconsistent staining patterns (data not shown). Consequently, more work is needed to unequivocally identify the cell type(s) that convey the increased ER expression in dystrophic skeletal muscles.

After binding their ligands, homodimers or heterodimers of ERα/ERβ regulate the transcription of target genes that bear palindromic estrogen-response elements in their promoter regions.[87] We have screened for the presence of estrogen-response elements in the upstream regions of the genes encoding several of the proteins whose expression was altered by TAM treatment. Although no complete estrogen-response element was found, these regions bear many estrogen-response element half-sites, which, in certain instances, may suffice to control the expression of estrogen target genes.[87] More experiments are needed to establish if TAM stimulated the expression of these proteins through increased transcription.

Tissue-specific estrogen sensitivity and response to TAM are essentially defined by the pattern of expression of ERα, ERβ, co-activators, and co-repressors. On binding to ERα or ERβ, TAM alters the set of co-regulators that are recruited, resulting in either proestrogenic or antiestrogenic effects in a tissue-specific manner.[28] Several of our findings suggest that TAM mimics estrogens on skeletal muscle. TAM increased the force and the resistance to fatigue and slowed the kinetics of contraction. Moreover, TAM-treated males weighed the same as age-matched females, most muscles from both groups had similar relative weights, and their plasma CK levels were similarly low. However, major differences remained between TAM-treated males and untreated females. As judged by the physical impulse scores determined from the wire hanging test and by the phasic and tetanic forces, the females were as weak as the untreated males, the female DIA accumulated much more adipose tissue, and both the female DIA and heart were not protected against fibrosis. Therefore, although our results indicate that TAM exerted protective effects on the overall musculature, this compound does not just "feminize" skeletal muscles of dystrophic mice nor does it fully mimic the natural estrogens. In fact, TAM binding to ER results in either proestrogenic or antiestrogenic actions, depending on the cell type,[19] which is characteristic of many SERMs, whereas natural estrogens elicit proestrogenic responses only. In addition, TAM and natural estrogens may modulate ER-independent pathways in a different manner,[24–26,88] resulting in distinct biological responses. Of note, it is likely that the levels of circulating estrogens were reduced in the relatively old females used in this study.

The issue of whether TAM is proestrogenic or antiestrogenic for dystrophic muscle is currently under investigation in our laboratory. This is complicated by the fact that several TAM metabolites exhibit a 30- to 100-fold higher affinity for ERs and display a stronger antiestrogenic activity than the parental drug and that the (E)-isomers display much lower antiestrogenic activity than the (Z)-isomers, at least as evaluated on breast cancer cells.[89] The use of other SERMs, such as raloxifene, whose biological activity does not depend on metabolites, might be useful for clarifying the roles of estrogen signaling in dystrophic muscle function. However, current work in our laboratory shows that raloxifene is much less efficacious than TAM on mdx^{5Cv} mice (O.M. Dorchies et al, manuscript in preparation).

Potential of TAM for DMD and Other Dystrophies

Over the past years, considerable efforts have been made toward therapies that replace or repair the defective dystrophin gene and permit the production of quasi-dystrophin.[8] However, technologic, cost, and safety issues obstruct the development of these approaches. In our view, the evaluation of known orally active small-molecular weight compounds with well-characterized pharmacodynamic and safety profiles presents significant advantages over other therapeutic avenues.[7] In particular, they might provide benefit to patients with DMD within a minimum period of time and are much less costly.

Our study suggests that TAM might be well-suited for this purpose. Besides its good safety profile in adults,[17–19,90] several studies report that it was also well tolerated when given for up to 48 months to 13-to 16-year-old prepubertal boys and for 12 months to girls as young as 3 years.[45,91,92] Importantly, in these studies, TAM did not alter the acquisition of male sexual traits. However, no data exist about the safety of TAM on growing boys as young as 5 to 7 years of age, at the time when the disease is diagnosed and treatment is likely to be initiated. This limitation should be taken into account if TAM is being evaluated on young patients with DMD.

Patients with DMD under usual steroid medications exhibit reduced growth and altered bone quality, which correlates with more frequent fractures. However, the reduction of the stature might participate in the therapeutic benefits of steroids (see Bianchi et al[93] and references within). By contrast, TAM prevents bone loss[17,94] and has been shown to increase the height of short boys by decreasing the rate of bone maturation.[92] At present, it is not known whether the foreseen action of TAM on stature might be a therapeutic issue for boy with DMD.

Our study shows that very low levels of TAM and TAM metabolites are sufficient to cause major therapeutic effects on the dystrophic mouse, which is encouraging in the perspective of a clinical application of our findings to patients with DMD. It is possible that therapeutic TAM concentrations might be reached with lower than standard TAM regimen, the safety of which has been established for more than 20 years. The specific benefits elicited by the E isomers of TAM metabolites, which were produced in substantial amounts in the dystrophic mice but are barely detected in humans,[37,44] deserve further examination. This could result in lower than expected benefits when extrapolating our results from mice to patients with DMD.

In conclusion, our preclinical evaluation of TAM in a mouse model of DMD showed promising improvements of skeletal and cardiac muscles. However, more investigations are required to establish the actions of TAM on further aspects of the dystrophic disease, such as the prevention of the initial muscle necrosis and the modulation of the inflammatory responses. Our further work will also aim at elucidating the molecular mechanisms that underlie the actions of TAM on dystrophic skeletal muscle, in particular with respect to the signaling pathways, the contributions of the ERs, and the specific activities of TAM metabolites.

Acknowledgments

We thank Colette Sauty-Defferard for animal care and excellent technical support, the other members of the laboratory for helpful discussions, and Prof. Randall Kramer (University of California, San Fransisco, CA) for the kind gift of anti-α7 integrin antibody. The BA-D5, SC-71, BF-35, and BF-F3 hybridoma developed by Prof. Stefano Schiaffino were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health & Human Development and maintained by The University of Iowa, Department of Biology (Iowa City, IA).

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2012.10.018*.

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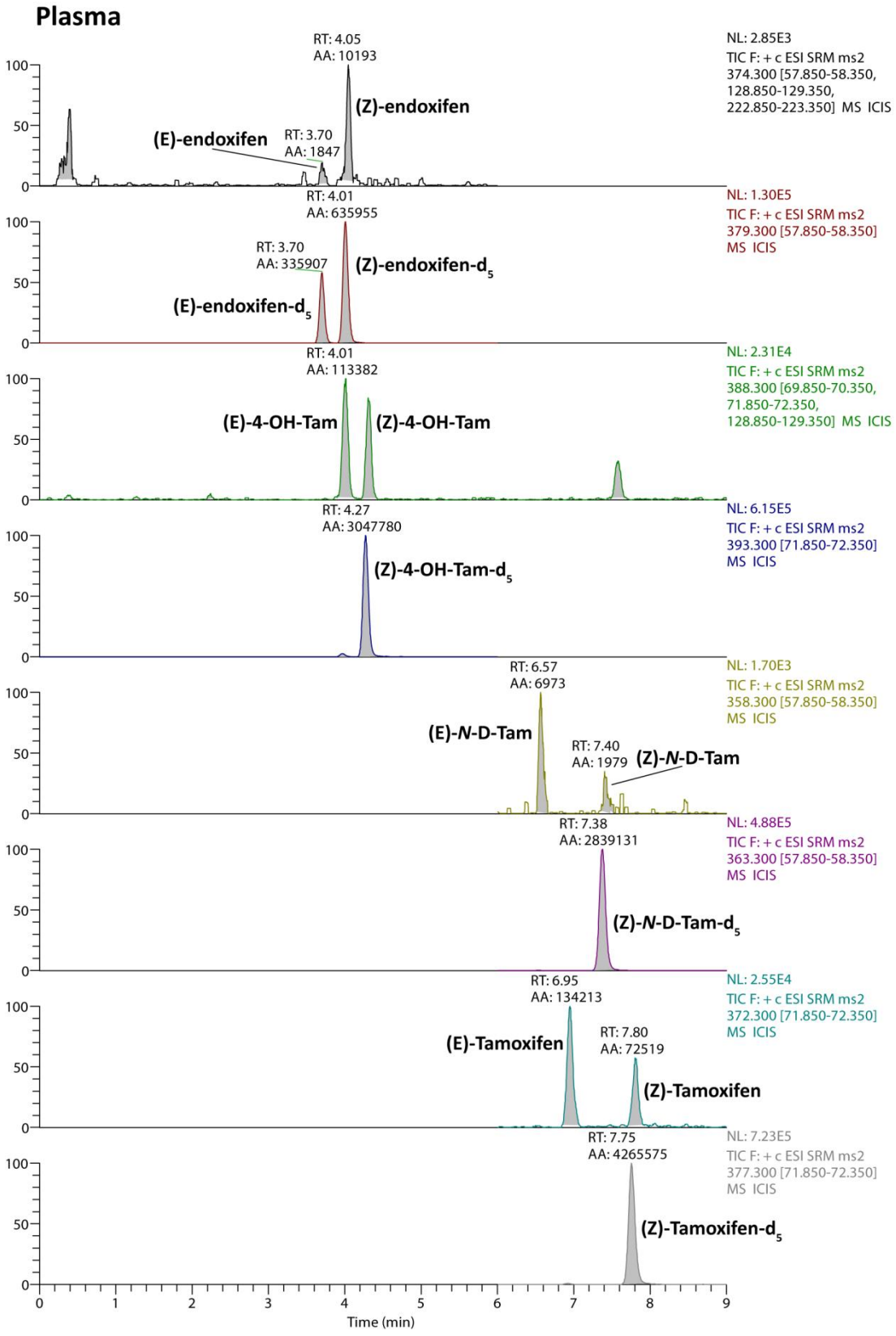
Supplemental Figure S5



Supplemental Figure S6