Analysis of Methadone and its Primary Metabolite in Meconium

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Abstract

Methods for analysis of methadone and its principal metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) in meconium, based on fluorescence polarization immunoassay (FPIA) and high-performance liquid chromatography (HPLC) and diode array detection were developed. Meconium and urine samples of 16 neonates from 15 methadone-using mothers were analyzed. Because of the lower detection limit and the possibility of coanalyzing EDDP, meconium analysis with HPLC for detecting methadone use is very much preferable to FPIA. Identical results were obtained with HPLC analysis for both matrices: methadone or EDDP or both could be detected in the urine and meconium samples from 15 children. The amount of EDDP in meconium was much higher than the amount of methadone (ratio, 9.6). EDDP only was detected in eight of the meconium samples. A positive correlation was found between the methadone dose of the mothers and the methadone concentration in meconium, but not with the EDDP concentration in meconium.

Introduction

Intrauterine exposure to drugs of abuse is associated with high incidence of perinatal complications (1). Meconium has been reported to be a more suitable specimen than urine for detecting exposure to drugs of abuse (2). The following potential advantages of meconium were mentioned: meconium provides a longer history of drug exposure than urine, contains higher drug concentrations, and is collected more easily. In literature, assays of methadone in meconium based on enzyme multiplied immunoassay technique (EMIT) (3), radioimmunoassay (4), and gas chromatography–mass spectrometry (GC–MS) (3) have been described. No high-performance liquid chromatographic (HPLC) assay has been developed until now. In this article, we describe techniques for analysis of methadone with fluorescence polarization immunoassay (FPIA) and analysis of methadone and its principal metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), with HPLC and diode array detection. Meconium samples of 16 neonates from 15 methadone-using mothers were investigated with these methods.

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Experimental

Materials

The TDX/FLX methadone FPIA assay, which is a semiquantitative reagent system for the detection of methadone in human urine samples, was obtained from Abbott (Amstelveen, The Netherlands).

Methadone was obtained from the OPG (Utrecht, The Netherlands), and EDDP-perchlorate, was from Sigma Chemical (St. Louis, MO). Clomipramine was a gift from Ciba-Geigy (Arnhem, The Netherlands). Extrelut 20 columns were obtained from Merck (Amsterdam, The Netherlands)

All other chemicals were obtained from Merck.

Borax buffer solution (pH 9.0) was prepared by dissolving 5 g sodium borate in 100 mL distilled water and adjusting pH with 4M HCl.

The extraction fluid consisted of dichloromethane–isopropanol (80:20).

Apparatus

FPIA. This technique was performed with a TDX/FLX from Abbott.

HPLC. HPLC was performed with a M45 pump, a 996 photodiode array detector, and Millennium software (Waters, Etten-Leur, The Netherlands). A reversed-phase C_{18} stationary phase (Lichrospher 100 RP-18 endcapped, 119 × 4.6 mm, 5 µm, catalog #214408, Merck) was also used.

The mobile phase (flow rate, 0.6 mL/min) consisted of a mixture of 530 mL distilled water, 146 µL triethylamine, and 350 µL phosphoric acid brought to a pH of 3.3 with 10% KOH and 470 mL acetonitrile. This system is commonly used for toxicological screening in The Netherlands (5). Methadone and EDDP concentrations were measured at 204 nm.

Table I. Self-Reported Methadone Doses of Mothers of Neonates

Baby no.	Dose (mg)	Baby no.	Dose (mg)
1	25	9	110
2	90	10	110
3	45	11	60
4	60	12	?
5	40	13	15
6	17,5	14	10
7	55	15	15
8	50	16	15

Identification took place by matching UV spectra of eluting peaks with UV spectra in the library of the system and UV spectra of the standards.

GC–MS. The analyses were performed with a Varian 3400 series GLC (Varian, Houten, The Netherlands) interfaced with the Finnigan Matt Incos XL-mass selective detector (Finnigan, Veenendaal, The Netherlands) and a Varian 1075 autosampler. The column was a J&W Scientific DB-5 MS (30 m × 0.255 mm, 0.22-µm film thickness) (Interscience, Breda, The Netherlands). Helium was used as carrier gas at a flow rate of 1.2 mL/min. The injection port temperature was 280°C, and the detection port temperature was 300°C. The oven temperature was programmed from 80°C (0 min) to 280°C at 10°C/min and held at 280°C for 2 min. The GC–MS was operated in the scan mode (50–350 amu) and the selected ion monitoring (SIM) mode with the following ions (m/z) monitored for each component at their

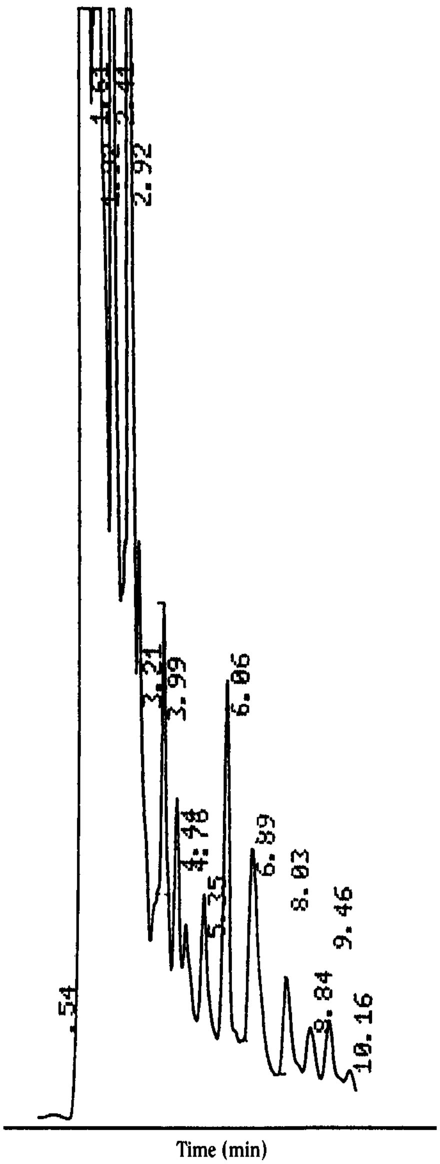

Figure 1. HPLC chromatogram of a spiked meconium sample. EDDP (1500 ng/g), 6.89 min and methadone (920 ng/g), 8.03 min.

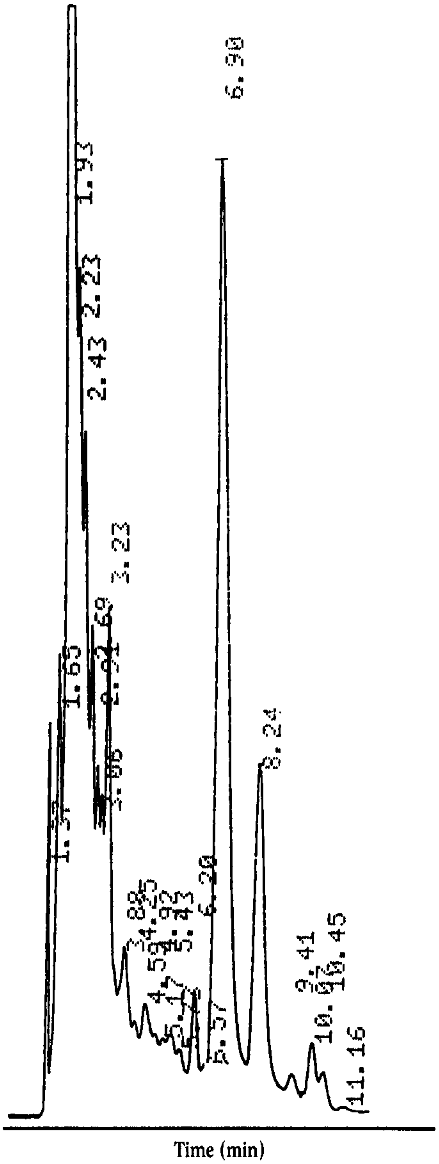

Figure 2. HPLC chromatogram of sample taken from Baby 8 on day 0. EDDP (2728 ng/g), 6.9 min and methadone (8637 ng/g), 8.24 min.

respective retention times (Rt): methadone, 72, Rt, 15.4 min and EDDP, 275–278, Rt, 16.5 min. Identification took place by comparing the MS scan with the system library.

Method

Immunoassay meconium. Water (5 mL) and one drop of 0.5M HCl were added to 500 mg meconium. The mixture was consecutively mixed on a vortex mixer (1 min), mixed ultrasonically (5 min), vortex mixed again, and centrifuged at 2683 × *g* (15 min). The TDX/FLX methadone assay was performed on the supernatant according to the Abbott manual.

HPLC assay meconium. Water (10 mL) and two drops of 0.5M HCl were added to 500 mg meconium. The extraction procedure was the same as described previously. The watery extract was transferred quantitatively to another vessel and brought to a volume of 20.0 mL with borax buffer solution (pH 9.0).

This solution was added to an Extrelut column (Merck). After 10 min, 40 mL of a mixture of dichloromethane and isopropanol (80:20) was added to the column. The organic extract was collected and evaporated on a water bath of 40°C under a stream of nitrogen. Then the extract was redissolved in 0.1 mL mobile phase. An aliquot (20 µL) was injected into the HPLC.

HLPC assay urine. Borax solution (0.3 mL) and 50 µL internal standard solution were added to 1.0 mL urine and extracted with 5 mL heptane–isoamylalcohol (98.5:1.5). The organic extract was evaporated as described previously. The residue was redissolved in 0.2 mL mobile phase. A 20-µL aliquot was injected into the HPLC.

Validation

Fortified meconium and urine specimens were prepared by adding the appropiate amount of standard material. No internal standard was used for the HPLC assay in meconium. Clomipramine was used as an internal standard for the HPLC assay in urine. Standard solutions were 4.6 mg methadone in 100 mL water, 10 mg methadone in 100 mL water, and 2.5 mg EDDP in 50 mL methanol. Internal standard solution was clomipramine, 2 mg in 100 mL methanol–water (1:1). Methadone standard solution (4.6 mg/100 mL) was added in amounts of 5, 10, 15, 20, and 40 µL to blank 500 mg meconium samples, resulting in 460, 920, 1380, 1840, and 3680 ng methadone/g meconium, respectively. Also, EDDP standard solution was added in concentrations of 10, 15, 20, 40, and 60 µL, corresponding with 1000, 1500, 2000, 4000, and 6000 ng EDDP/g meconium. Methadone standard solution (10 mg/100 mL) was added in amounts of 25, 50, 75, and 100 µL to 1 mL urine, resulting in 2500, 5000, 7500, and 10,000 ng/mL methadone/mL urine, respectively. Also, 50, 100, 200, and 300 µL EDDP standard solution were added corresponding with 2500, 5000, 10,000, and 15,000 ng/mL urine.

Standard lines were prepared and recovery and coefficients of variation were determined with these spiked meconium and urine samples. The detection limit of the HPLC assay was defined as five times the noise level. The detection limit of the FPIA assay was determined by analyzing blank meconium samples from 19 neonates. The detection limit was calculated from the mean polarization minus five times the standard deviation.

Table II. Methadone and EDDP in Neonatal Urine*

Baby no.	Days after birth (methadone and EDDP in urine)							
	0	1	2	3	4	5	6	7
1(F)†		521						
1(H)‡		982						
		4476						
2(F)	820	347	nd§	nd				
3(F)	1145							
4(F)	1188		774	403				
5(F)		408	607					
6(F)		nd	nd					
6(H)		297						
		833						
7(F)		1654	380					
8(F)	1634	813	nd	nd				
8(H)	1733		207					
	7199		1856					
9(F)			2216					
10(F)		>4000						
11(F)		1257						
12(F)	383	nd	nd	nd				
13(F)		nd	nd	nd			nd	
13(H)			nd				nd	
			nd				nd	
14(F)				nd	nd			nd
14(H)				nd	92			
				157	263			
15(F)		420	572	nd	nd	nd	nd	
15(H)			862			nd		
			1306			187		
16(F)	nd		nd	nd				
16(H)	nd		nd					
	159		131					

* The EDDP results are underlined.
† F = Fluorescence polarization immunoassay.
‡ H = High-performance liquid chromatography.
§ nd = Not detected.

Patients

Meconium was collected from neonates (n = 16, including one set of twins) of drug-dependent mothers (n = 15). All the mothers were on methadone maintenance during pregnancy. Methadone was detected several times during the second and the third trimesters in urine samples from 12 mothers. No urine analysis had been performed with the other three mothers. The dose range of methadone (dose known from 14 mothers of 15 of the children) was 10–110 mg/daily

Table III. Methadone and EDDP in Neonatal Meconium*

Baby no.	Days after birth (methadone and EDDP ng/g in meconium)							
	0	1	2	3	4	5	6	7
1(F)†		1272						
1(H)‡		1272						
		21305						
2(F)	2938				1648		nd§	
2(H)	3179				930		356	
	9800				8327		9004	
3(F)	1944	nd	nd					
3(H)	1543	660	nd					
	16898	11098	5537					
4(F)	2132	1969		nd	nd			
4(H)	2286	2306		388	247			
	405	1714		2409	3156			
5(F)		nd			nd			
5(H)		694			nd			
		7178			512			
6(F)			nd	nd				
6(H)			324	nd				
			2562	582				
7(F)	3985	3250	nd					
7(H)	2701	1186	nd					
	16835	9139	nd					
8(F)	3322	3832	nd	nd				
8(H)	2728	2041	nd	nd				
	8637	16141	41455	6665				
9(F)			5509	5351	3769			
9(H)			7721	6083	2081			
			37382	40976	22009			
10(F)			8951	3296	2559			
10(H)			10222	4314	648			
			60656	74336	21975			
11(F)		nd	nd					
11(H)		1463	nd					
		3935	2954					
12(F)	nd	nd		nd	nd	nd		nd
12(H)	nd	248						
	nd	5015						
13(F)		nd	nd	nd				
13(H)		nd	nd					
		nd	nd					
14(F)	nd	nd		nd				nd
14(H)		388						
		592						
15(F)				nd	nd	nd	nd	nd
15(H)					nd			nd
					2733			nd
16(F)	nd	nd	nd		nd			
16(H)	nd				127			
	2134				153			

* The EDDP results are underlined.
† F = Fluorescence polarization immunoassay.
‡ H = High-performance liquid chromatography.
§ nd = Not detected.

(mean, 48 mg) (Table I). These doses were self-reported doses. During prenatal care, information about the dose was asked of the mothers on several occasions.

When possible, the stool samples, which were obtained directly from the diapers, were pooled for each infant and run by day for 3 and sometimes 7 days after birth. Urine of the neonates was collected in a collection bag to prevent cross contamination of meconium. All samples were stored at −20°C until analysis.

Results

Analytical method

As the lower limits of detection, we found 1264 ng/g meconium (methadone) for the FPIA screening and 99 ng/g meconium (methadone) and 113 ng/g meconium (EDDP) for the HPLC assay. According to the Abbott assay manual, there is no crossreactivity of EDDP with the FPIA; 100,000 ng/mL EDDP in urine is below the detection level.

We found 76 ng/mL (methadone) and 127 ng/mL (EDDP) in urine as the lower limits of detection for the HPLC assay. The HPLC assay was found to be linear for methadone in meconium from 460 to 3680 ng/g (correlation coefficient, 0.9997) and for EDDP from 1000 to 6000 ng/g (correlation coefficient, 0.9962). The HPLC assay in urine was found to be linear from 2500 to 100,000 ng/mL for methadone (correlation coefficient, 0.9983) and from 2500 to 15,000 ng/mL for EDDP (correlation coefficient, 0.9970). The within-run coefficient of variation of the HPLC assay in meconium was 4.9% (methadone, 2000 ng/g, $n = 4$) and 4.3% (EDDP, 2000 ng/g, $n = 5$).

The within-run coefficients of variation of the HPLC assay in urine were 5.6% for methadone (2500 ng/mL) and 2.4% for EDDP (2500 ng/mL) ($n = 4$). The recoveries for these concentrations were 72.6 ± 3.5% (mean plus or minus standard deviation, $n = 4$) and 82.5 ± 1.1% ($n = 4$) for methadone in meconium and urine, respectively, and 61.9 ± 2.7% ($n = 5$) and 46.7 ± 1.1% ($n = 4$) for EDDP in meconium and urine, respectively. The recovery of methadone (1700 ng/g) by the TDX assay was 63.7 ± 6.1% (mean plus or minus standard deviation, $n = 12$). Amphetamine, caffeine, benzoylecgonine, cocaine, codeine, and morphine did not interfere with the assay. Typical chromatograms are shown in Figures 1 and 2. During our investigation, a slight shift in the retention time of methadone was observed. This was caused by aging of the column and slight differences in mobile phase composition.

Patient results

The results of urine and meconium analysis are shown in Tables II and III.

Urine analysis. Positive urine samples were found with FPIA in 12 children. The four children with negative urine samples were also analyzed with HPLC. Three children who were negative by FPIA proved positive by HPLC. Two were positive for methadone and EDDP, and the third was positive for EDDP only. Concentrations found in two of these three children were just above the detection level.

Meconium analysis. Meconium samples of eight children were found positive with FPIA. HPLC analysis revealed that 15 children were positive for drugs: 14 children were positive for methadone and EDDP, and one child was negative for methadone but positive for EDDP. The mother of the one child with both negative urine and meconium samples was on a low 15 mg/day methadone dose and had not used any methadone the last month before birth. With HPLC, concentrations of methadone up to 10 µg/g and of EDDP up to 74 µg/g were measured in meconium. Much higher concentrations of EDDP than methadone were measured in meconium. The ratio of EDDP to methadone, which was measured with HPLC, in all the samples that contained both methadone and EDDP was 9.6 ± 7.5 (mean plus or minus standard deviation, $n = 32$).

Only EDDP was measured in eight samples. We found a positive correlation between the methadone doses of the mothers and the methadone concentrations in meconium measured in the first meconium sample of each child ($r = 0.903$, $p < 0.001$). No correlation was found between the methadone dose and the EDDP concentration in meconium.

Because we wanted to be sure about the presence of EDDP and methadone in meconium, four meconium samples from different children (baby 2, day 0; baby 3, day 2; baby 5, day 1; and baby 8, day 2) were also qualitatively analyzed with GC–MS.

We could confirm the analytical results of FPIA and HPLC in all samples. The presence of methadone and EDDP in the SIM mode and of EDDP in the scan mode was detected. Methadone could not be detected in the scan mode because of interference from coeluting substances.

Discussion

The main metabolic reaction of methadone is *N*-demethylation resulting in a substance that cyclizes to form the major metabolite EDDP. In subjects on methadone maintenance, up to approximately 33% is excreted as unchanged drug in urine and up to about 43% as EDDP. The ratio of EDDP to unchanged methadone is usually higher in the urine of patients on methadone maintenance treatment than in isolated dose cases.

Urinary excretion of methadone is increased in acid urine. Up to 30% of a dose may be eliminated in the feces. EDDP has also been detected in feces (6,7). Nevertheless, essential information about the formation and disposition of EDDP is still lacking (8).

In our investigation, we found EDDP concentrations that were much higher than methadone in meconium. The ratio of EDDP to methadone in meconium was higher than reported in urine (7). No methadone could be measured at all in some meconium and urine samples, whereas a considerable amount of EDDP was found. We cannot determine if the EDDP in meconium was the result of metabolism of the mother or the fetus or both. Although we could not find any report about EDDP in literature, placental transfer of EDDP seems very likely, just as is the case for nearly any other drug (9). However, fetal metabolism by *N*-demethylation has been described (10).

Screening of methadone use by the immunoassay of meconium is hampered by a very high detection limit in comparison with HPLC. Moreover, FPIA has no crossreactivity with EDDP. These facts could be the explanation for the few positive neonates in the other studies about methadone in meconium. A screen based on immunoassay was used in these studies (3,4).

To our knowledge, few reports on correlation between dose and concentrations of drugs in meconium in humans existed until now. Nicotine metabolites were detected in meconium at concentrations proportional to the degree of maternal active and passive smoking (11). A correlation has been reported between concentrations of cocaine and morphine in meconium and the amount of drug used by the mother in pregnancy (12). Inverse correlations have been found between the concentration of the metabolite of cocaine benzoylecgonine in meconium and the weight, the length, and the head circumference of the neonate (13). The methadone concentration in meconium is a potentially useful tool to get information on the compliance of the mother.

Conclusion

HPLC analysis of methadone and EDDP in meconium is the method of choice over FPIA because of a much lower detection limit and the ability to coanalyze EDDP.

In our population of 16 children from 15 mothers who were known methadone users during pregnancy, comparable results were obtained both by HPLC analysis of meconium samples and urine samples.

A positive correlation was found between the methadone dose and the methadone concentration in meconium.

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