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REACTION OF 1-O-ACYL GLUCURONIDES WITH 4-(p-NITROBENZYL)PYRIDINE

RICHARD B. VAN BREEMEN AND CATHERINE C. FENSELAU

Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine

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ABSTRACT:

In this investigation, 31 1-O-acyl glucuronides were synthesized and 25 of these were shown to react with 4-(p-nitrobenzyl)pyridine (NBP), a standard chemical nucleophil, on thin layer chromatography plates. A quantitative NBP assay was developed based on existing methods, and the rates of reaction of three acyl glucuronides, clofibric 1-O-acyl glucuronide, indomethacin 1-O-acyl glucuronide, and flufenamic 1-O-acyl glucuronide, were determined. These rates ranged from 0.436 min^{-1} to 1.08 min^{-1}. Chlorambucil, a powerful alkylating agent, reacted with NBP 127 times faster than the most reactive of the three glucuronides assayed. The half-lives of these three 1-O-acyl glucuronides, determined at pH 2.0, 4.0, 6.0, 7.4, and 10.0 in aqueous phosphate solution, ranged from greater than 1000 hr at pH 2 to less than 1 min at pH 10.0. Determination of the rates of reaction of 1-O-acyl glucuronides with NBP and the rates of hydrolysis as a function of pH further characterize these compounds as activated phase II metabolites.

Glucuronidation, the predominant mammalian phase II reaction, is generally considered by biochemists, pharmacologists, and toxicologists to be a metabolic pathway for detoxification of xenobiotic and endogenous compounds (1). Many compounds containing carboxylic acid functional groups are metabolized to acyl-linked or 1-*O*-acyl glucuronides in which glucuronic acid is conjugated through an acetal linkage to the carboxyl group of the aglycon. We have previously shown that 1-*O*-acyl glucuronides are subject to nucleophilic substitution reactions in which glucuronic acid serves as a good leaving group. The attacking nucleophil can be a small molecule like methanol (2) or ethanethiol (3), which transacylates 1-*O*-acyl glucuronides to form the methyl ester or thiol ester derivatives of the aglycon, respectively. Alternatively, the nucleophil can be a group on a biopolymer such as an amino acid side chain on serum albumin (4, 5). Transacylation of a 1-*O*-acyl glucuronide by a nucelophilic group on serum albumin results in the covalent binding of the carboxylic acid aglycon to the protein (4, 5). Although reaction of albumin with 1-*O*-acyl glucuronides is biologically significant, it is markedly influenced by specific reversible binding phenomena, and various nucleophilic centers appear to be involved (4, 5).

In this investigation the reactivities of 1-*O*-acyl glucuronides will be further characterized, so that they may be compared with those of more familiar electrophilic and cytotoxic agents, and so that the range of electrophilicities within this class may be determined. Possible correlation of relative chemical reactivities of these glucuronides with such properties as the relative pK_a values of the aglycons will also be considered. For these studies, a standard chemical nucleophil must be used. Reaction of a compound with NBP[1] is frequently used to indicate whether or not a compound is an alkylating agent, and the rate of this reaction can be measured to indicate the alkylating strength of the compound (6–16). NBP is the most commonly used nucleophil for the determination of the relative reactivity of electrophils. Furthermore, alkylation of NBP has been correlated with toxicity and mutagenicity of electrophilic agents (13–16).

The first quantitative application of NBP alkylation was made in 1955 by Epstein *et al.* (6), who measured the blue, alkylated NBP product spectrophotometrically. In 1961, Friedman and Boger (7) modified the procedure to stabilize the alkylated product and improve sensitivity. Since then, numerous modifications of either Epstein's method in nonaqueous solvents or Friedman's method in aqueous buffer have been published (8–11). These modifications have, in general, attempted to improve reproducibility and make the NBP assay a faster and more routine analysis.

Although highly reactive alkylating agents like nitrogen mustards and epoxides are usually reacted with NBP, we investigated the reaction of 1-*O*-acyl glucuronides, a group of acylating agents, with NBP. The nucleophilic pyridine nitrogen of NBP reacts with the electrophilic acyl carbon of the glucuronide. Glucuronic acid is displaced, and NBP is acylated to form a pyridinium salt (fig. 1.) Treatment of the pyridinium intermediate with base (pH $\geq$ 12) results in the loss of an acidic, benzylic proton from NBP and formation of an intensely blue product with an absorbance maximum of 540–560 nm (6, 7).

Initially, we examined the reaction on TLC plates of 31 1-*O*-acyl glucuronides with NBP and then developed a quantitative NBP assay to measure the relative reactivity of each of three acyl glucuronides, clofibric 1-*O*-acyl glucuronide, indomethacin 1-*O*-acyl glucuronide, and flufenamic 1-*O*-acyl glucuronide. The stability of each of these three glucuronides was also determined in aqueous solution at five different pH values.

Materials and Methods

Flufenamic acid (5.34 Ci/mol) and clofibric acid (3.87 Ci/mol) labeled with ^{14}C on the carboxylic acid groups were purchased from Research Products International (Mount Prospect, IL) and [2-^{14}C]indomethacin (7.95 Ci/mol) was purchased from New England Nuclear (Boston, MA). Radiolabeled compounds were pure by TLC and at least 95% pure by reversed phase HPLC with UV detection or scintillation counting (see methods described below). Unlabeled flufenamic acid, indomethacin,

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[1] Abbreviations used are: NBP, 4-(*p*-nitrobenzyl)pyridine; TLC, thin layer chromatography.

Send reprint requests to: Dr. Richard B. van Breemen, Department of Pharmacology, Johns Hopkins University School of Medicine, 727 North Wolfe Street, Baltimore, MD 21205.

4-(p-NITROBENZYL)PYRIDINE (NBP) REACTION SCHEME

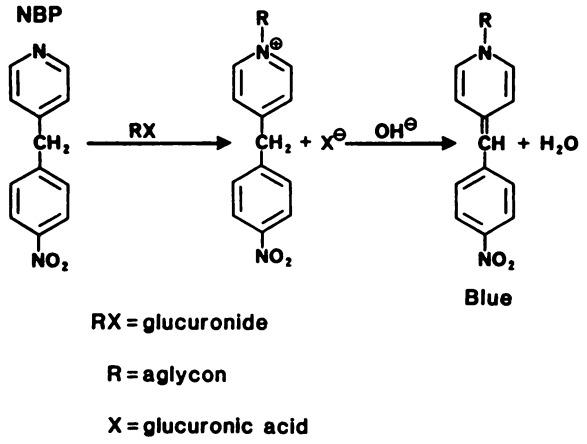

FIG. 1. *Reaction scheme for the acylation of NBP by 1-O-acyl glucuronides.*

UDP-glucuronic acid, furosemide, ketoprofen, and sorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Clofibric acid and valproic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). The following were gifts from the respective companies: etodolac, furobufen, and prodolic acid from Ayerst Laboratories (New York, NY); benoxaprofen from Eli Lilly Co. (Indianapolis, IN); nafenopin and pirprofen from Ciba-Geigy Pharmaceuticals Division (Summit, NY); flurbiprofen from The Upjohn Company (Kalamazoo, MI); diflunisal from Merck Sharpe & Dohme (Rahway, NJ); tolmetin and zomepirac from McNeil Pharmaceutical (Spring House, PA); gemfibrozil, meclofenamic acid, and mefenamic acid from Warner-Lambert Co. (Ann Arbor, MI); ciprofibrate and oxarbazole from Sterling-Winthrop Research Institute (Rensselaer, NY); bumetanide and carprofen from Hoffmann-LaRoche Inc. (Nutley, NJ); naproxen, tiopinac, and tixanox from Syntex Laboratories, Inc. (Palo Alto, CA); isoxepac and piretanide from Hoechst-Roussel Pharmaceuticals, Inc. (Somerville, NJ); and fenbufen and triflocin from Lederle Laboratories (Pearl River, NY).

Synthesis and Purification of 1-*O*-Acyl Glucuronides. 1-*O*-acyl glucuronides, both ^{14}C-labeled and unlabeled, were synthesized *in vitro* using the microsomal enzyme, UDP-glucuronyltransferase, which had been partially purified from rabbit liver and immobilized on cyanogen bromide-activated Sepharose beads according to published procedures (17). UDP-glucuronyltransferase activity for the conjugation of the standard substrate, *p*-nitrophenol, was 8.8 ± 1.5 nmol·min^{-1}·mg^{-1} protein (95% confidence limit, n = 12) and 45 ± 6 nmol·min^{-1}·ml^{-1} beads (95% confidence limit, n = 12). A typical incubation with this enzyme preparation contained UDP-glucuronic acid (80 mg, 130 μmol); carboxylic acid substrate (10 mg, ~34 μmol, which was typically the limit of solubility); 20 ml of 0.01 M phosphate buffer, pH 6.0; and 40 ml of beads containing immobilized UDP-glucuronyltransferase. This reaction mixture was gently rocked in a 60-ml bottle at room temperature for 12–15 hr. Next, the beads were removed by filtration and washed with 20 ml of distilled water. The combined filtrate was acidified to pH 2.0 with aqueous HCl, and precipitated aglycons were removed with a 0.45 μm Nylon-66 filter (Rainin Instrument Co., Woburn, MA).

Prepacked octadecylsilane cartridges (Waters Associates, Milford, MA) were used to purify the acyl glucuronides based on the method of Pallante *et al.* (18). The cartridge was rinsed with 2 ml of distilled water and then 1.5 ml of diethyl ether (J. T. Baker, Phillipsburg, NJ). The glucuronide was eluted with 4 ml of acetonitrile (Burdick and Jackson, Muskegon, MI), and the solvent was removed under a stream of nitrogen.

These procedures produced 100 μg–1 mg of each of 31 glucuronides. Parallel incubations provided 2–4 mg of the 1-*O*-acyl glucuronides of flufenamic acid, indomethacin, and clofibric acid.

Quantitation of Glucuronides by HPLC. Because rate studies of the reaction of NBP with 1-*O*-acyl glucuronides required known amounts of each reactant, glucuronides synthesized above had to be quantitated. Standard curves for the 1-*O*-acyl glucuronides of flufenamic acid, indomethacin, and clofibric acid were constructed by HPLC with UV detection using ^{14}C-labeled glucuronides of known specific activity. Solutions of each acyl glucuronide to be quantitated were prepared in 400 μl of tetrahydrofuran (freshly distilled under nitrogen from lithium aluminum hydride so that no peroxides were present by the starch-iodide test) and 50 μl of distilled water. Gradient HPLC was used to analyze the glucuronides. All solvents were HPLC grade (Burdick and Jackson) and were filtered and degassed before use. Over 10 min, a gradient was run from 40–60% acetonitrile for flufenamic glucuronide, 30–55% for indomethacin glucuronide, and 25–50% for clofibric glucuronide. The cosolvent was distilled water, which contained 1% (v/v) glacial acetic acid. The variable wavelength UV detector was set at 280 nm for flufenamic glucuronide, 254 nm for indomethacin glucuronide, and 278 nm for clofibric glucuronide. Each glucuronide solution was assayed three times and the concentration was determined from the standard curve. The standardized glucuronide solutions were used in the NBP reactions described below.

Reaction of NBP with 1-*O*-Acyl Glucuronides. As a qualitative test for the reaction of acyl glucuronides with NBP, each of the 31 synthetic glucuronides and their corresponding aglycons were spotted on silica gel TLC plates (Analtech, Newark, DE; 20 × 20 cm, 250 μm thickness) developed to 18 cm in a solvent system of benzene:butanol:water:methanol (1:2:1:1.25; v/v/v/v). After air drying, plates were visualized with NBP spray reagent as described by Sladek (19) except that the plates were heated for 1 hr at 125°C.

Next, a quantitative NBP assay was developed based on procedures published by Kawazoe *et al.* (8), Barbin *et al.* (9), and Phillips *et al.* (10). Reactions were performed under argon in 100-μl amber-colored reaction vials with Teflon-lined screw caps (Pierce Chemical Co., Rockford, IL). One vial was used per time point. Each vial contained 25 μl of potassium phosphate aqueous buffer, 0.067 M, pH 6.0; 50 μl of 2% NBP (w/v) in ethylene glycol (J. T. Baker Chemical Co.), and 25 μl of acyl glucuronide standard solution. Three concentrations of each glucuronide were used, ranging from 0.932–1.55 mM in the reaction mixture. Vials were vortexed for 1 min, equilibrated to 60°C in a dark oven for 2 min, and then incubated in the oven for 0, 20, 40, or 60 min. Vials were then frozen in a dry ice-acetone bath. After thawing for 15 min at room temperature, 100 μl of triethylamine (Aldrich Chemical Co.) in acetone (1:1, v/v) was added to each vial under nitrogen in order to develop the blue color of acylated NBP. The absorbance at 560 nm was read 1 min later in a 100-μl Ultramicro cuvette on a Guilford (Oberlin, OH) spectrophotometer.

Control experiments were carried out as described above in triplicate, in which either aglycon or glucuronic acid, 1.55 mM, was substituted for glucuronide. Peroxide-containing tetrahydrofuran was also tested as a reactant with NBP.

Reaction of NBP with Chlorambucil. Vials containing NBP solution and phosphate buffer were preheated to 60°C in the oven; then, 25 μl of a standard solution of chlorambucil in tetrahydrofuran was added. Three concentrations were used to make the reaction mixture 0.932, 1.24 or 1.55 mM in chlorambucil. After vortexing for 15 sec, vials were incubated either 0, 2, 4, or 6 min before being frozen in a dry ice-acetone bath. Alkylated NBP was analyzed as described above.

Stability of 1-*O*-Acyl Glucuronides in the NBP Assay. In order to evaluate the stability of the 1-*O*-acyl glucuronides of flufenamic acid, indomethacin, and clofibric acid under conditions of the NBP assay, reaction mixtures containing each 1-*O*-acyl glucuronide but no NBP were prepared as described above. Aliquots of each mixture were analyzed by HPLC before incubation and after incubation for 60 min at 60°C.

Stability of 1-*O*-Acyl Glucuronides in Aqueous Solution. Solutions containing either flufenamic glucuronide, indomethacin glucuronide, or clofibric glucuronide at approximately 100 nM were prepared in 0.1 M phosphate aqueous solution at pH 2.0, 4.0, 6.0, 7.4, and 10.0 (pH adjusted with aqueous HCl or KOH) and were incubated in a 37°C water bath for up to 12 hr. Aliquots were removed and analyzed by HPLC

every 4 hr except for pH 7.4 and pH 10 solutions, which were analyzed hourly and every minute, respectively. All measurements were made in triplicate.

Measurement of Aglycon pK_a. The pK_a values of clofibric acid, flufenamic acid, and indomethacin were measured by potentiometric titration (20). A mixed solvent system consisting of methanol:water, 1:1 (v/v), was used because of the limited solubility of these compounds in water.

Results

Each of the 31 synthetic 1-*O*-acyl glucuronides was further purified by TLC and derivatized on the silica gel plate with an NBP spray reagent. Upon alkalinization with 4% KOH aqueous spray solution, 25 of the glucuronide spots turned blue, indicating acylated NBP. The blue color faded rapidly in the presence of air. This reaction was dependent only upon the presence of a sufficient quantity of glucuronide (approximately ≥50 μg). Insufficient quantity and/or low reactivity of the acyl glucuronides resulted in no detectable NBP reaction for the glucuronides of carprofen, fenbufen, oxarbazole, sorbic acid, tixanox, and triflocin. The 25 other 1-*O*-acyl glucuronides gave a positive reaction with NBP on TLC plates. These were the glucuronides of benoxaprofen, bumetanide, ciprofibrate, clofibric acid, diflunisal, etodolac, flufenamic acid, flurbiprofen, furobufen, furosemide, gemfibrozil, indomethacin, isoxepac, ketoprofen, meclofenamic acid, mefenamic acid, nafenopin, piretanide, pirprofen, prodolic acid, naproxen, tiopinac, tolmetin, valproic acid, and zomepirac.

Having demonstrated that 1-*O*-acyl glucuronides react with NBP, a quantitative assay was developed based on existing methods. Published procedures had to be modified because of the limited glucuronide quantities (2–4 mg), their low reactivity and instability, and the instability of the acylated NBP reaction products. The slightly acidic aqueous phosphate buffer, pH 6, used by Kawazoe *et al.* (8), was selected to minimize hydrolysis and rearrangement of the acyl glucuronides, which can occur rapidly under alkaline conditions (21). The maximum reaction temperature, 60°C, recommended by Kawazoe *et al.* (8) was chosen to increase the slow reaction rate. Exclusion of air and light was found to be essential, otherwise the acylated NBP product was too unstable to give reproducible reaction rate measurements. The NBP-indomethacin reaction product was particularly unstable, even after alkalinization. Any exposure to air resulted in its immediate decomposition with loss of blue color. Because of the small (μl) scale of the reactions, triethylamine in acetone was used for alkalinization (6) instead of addition of aqueous potassium hydroxide followed by extraction of derivatized NBP with organic solvent (8). Finally, a new solvent system was devised incorporating ethylene glycol, tetrahydrofuran, and aqueous phosphate buffer, which could solubilize the polar glucuronides, the non-polar NBP, and the ionic potassium phosphate buffer even after addition of triethylamine and acetone.

The decomposition of flufenamic, indomethacin, and clofibric 1-*O*-acyl glucuronides during the 1-hr incubation at 60°C in the NBP solvent system was measured using HPLC. Indomethacin 1-*O*-acyl glucuronide was found to be the least stable, decomposing 6.6 ± 1.6%. Clofibric and flufenamic 1-*O*-acyl glucuronides were considerably more stable, decomposing 1.3 ± 0.1% and 2.6 ± 0.1%, respectively. Decomposition of these glucuronides was therefore determined to be a minor side reaction in this NBP assay.

Another group of control assays demonstrated that none of the unconjugated drugs nor glucuronic acid reacted with NBP under the assay conditions. Although tetrahydrofuran was distilled from lithium aluminum hydride immediately before use in order to remove peroxides, NBP incubation with peroxide-contaminated tetrahydrofuran gave no colored product with NBP.

Known quantities of the glucuronides of flufenamic acid, indomethacin, and clofibric acid, and chlorambucil, a nitrogen mustard, were reacted with excess NBP, and the absorbances at 560 nm were plotted *vs.* reaction time. Fig. 2 shows the reaction of NBP with 1.55 mM clofibric, flufenamic, or indomethacin 1-*O*-acyl glucuronide. Fig. 3 shows alkylation of NBP by three concentrations of chlorambucil. Since absorbance increased linearly with reaction time, this indicated that initial reaction rates had been measured. The slopes of these lines were determined by linear regression analysis and were used to calculate the pseudo-first order reaction rate constants for each glucuronide and for the reference alkylating agent, chlorambucil (table 1). Because partial degradation of the acylated (or alkylated) NBP probably occurred during incubation, each measured rate was actually the difference between formation and degradation of this product. Furthermore, these rates were relative measurements, since the extinction coefficients for acylated NBP were not determined. It was assumed that the extinction coefficients of the three acylated NBP products (NBP-clofibrate, NBP-indomethacin, and NBP-flufenamate) and alkylated NBP were identical.

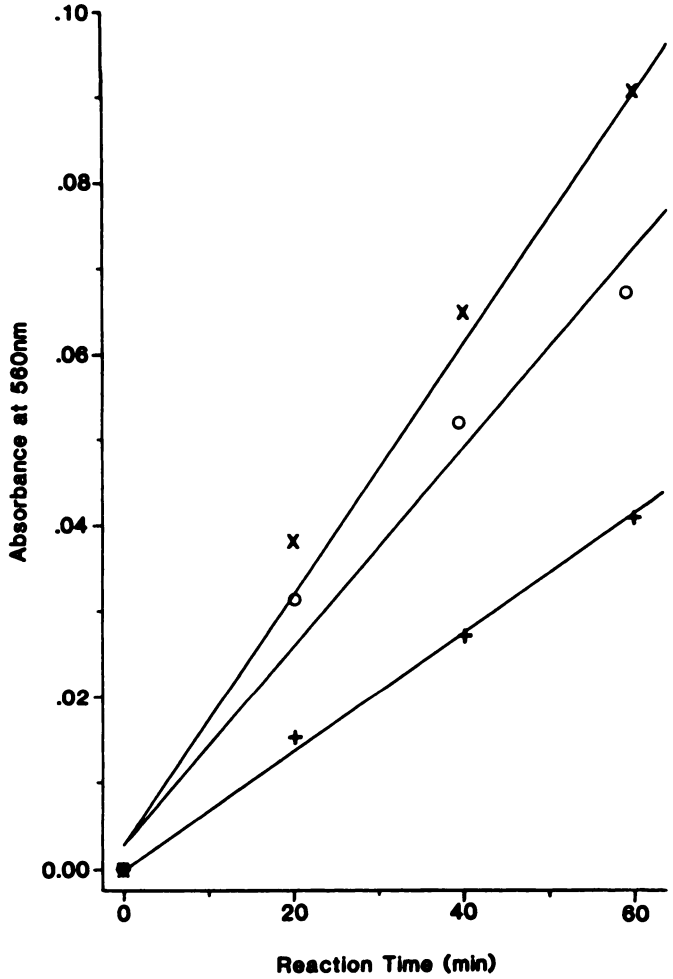

FIG. 2. *Reaction rates of NBP with indomethacin (○), clofibric (×), or flufenamic (+) 1-O-acyl glucuronide, 1.55 mM.*

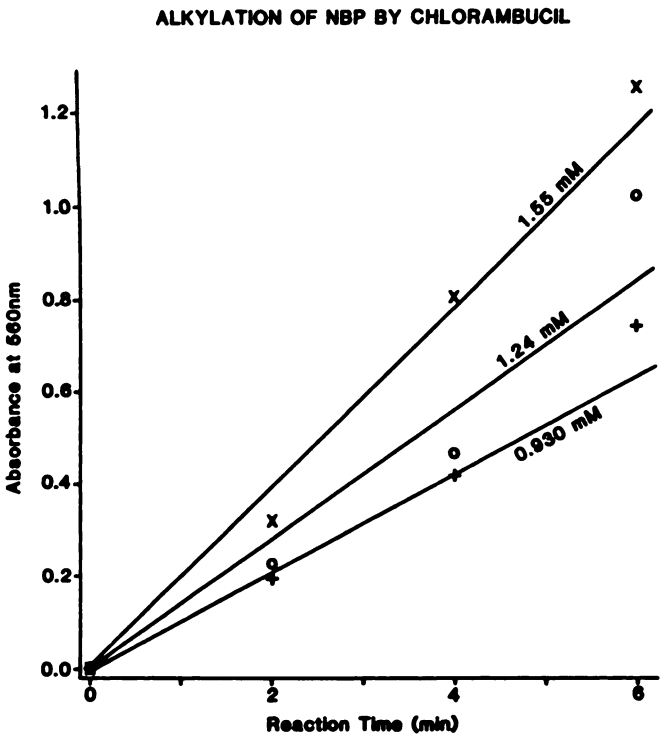

FIG. 3. *Rates of alkylation of NBP by chlorambucil.*

TABLE 1

Relative rate constants for alkylation of NBP[a]

	Rate Constant
	min^{-1}
Flufenamic 1-*O*-acyl glucuronide	0.436 ± 0.135
Indomethacin 1-*O*-acyl glucuronide	0.709 ± 0.081
Clofibric 1-*O*-acyl glucuronide	1.08 ± 0.23
Chlorambucil	137 ± 6

[a] Relative standard deviations were calculated for ≥3 rate determinations.

Clofibric 1-*O*-acyl glucuronide, the most reactive of the glucuronides in table 1, reacted 2.5 times faster with NBP than did flufenamic 1-*O*-acyl glucuronide, which was the least reactive. Chlorambucil, a powerful alkylating agent, reacted 127 times faster with NBP than did clofibric 1-*O*-acyl glucuronide. Although they are electrophilic and reactive metabolites, acyl glucuronides were found to be considerably weaker electrophils than nitrogen mustards.

Stability of 1-*O*-Acyl Glucuronides in Aqueous Solution. Disappearance of the 1-*O*-acyl glucuronides of flufenamic acid, indomethacin, or clofibric acid in aqueous phosphate solution at pH 2.0, 4.0, 6.0, 7.4, or 10.0 was measured by HPLC with UV detection. Gradient reverse phase HPLC was used to separate each 1-*O*-acyl glucuronide from its decomposition products, which included isomers from intramolecular acyl migration and the hydrolysis products, aglycon and glucuronic acid. For each assay, the logarithm of the peak height of the 1-*O*-acyl glucuronide was plotted *vs.* incubation time, and the slope of this line and its standard deviation were determined by linear regression analysis. These slopes were used to calculate the half-lives of the glucuronides in aqueous solution at each pH value (table 2). Because some of the slopes were near zero, the extrapolated half-lives are extremely long compared to the experimental 12-hr incubation time. Therefore, standard deviations are presented in table 2 only for half-lives which are less than 150 hr.

TABLE 2

Half-lives of 1-O-acyl glucuronides in aqueous solution at 37°C

pH	Flufenamic Glucuronide	Indomethacin Glucuronide	Clofibric Glucuronide
2.0	1109 hr	71.1 ± 39 hr	124 ± 53 hr
4.0	495 hr	146 ± 20.3 hr	49.5 ± 7.0 hr
6.0	70.7 ± 2.9 hr	21.0 ± 1.4 hr	48.4 ± 10.7 hr
7.4	6.96 ± 0.02 hr	1.41 ± 0.01 hr	7.26 ± 0.61 hr
10.0	4.8 ± 0.1 min	<1 min	<2 min

The half-lives of the three 1-*O*-acyl glucuronides measured here represent the sum of hydrolysis and intramolecular acyl migration. Both of these reactions are consequences of the electrophilicity of the glycosidic acyl bond. Rapid hydrolysis of all three glucuronides occurred during alkaline incubations as indicated by the disappearance of the 1-*O*-acyl glucuronide HPLC peak and corresponding increase in the aglycon peak. At pH 2 and 4, only slow hydrolysis was observed. Clofibric and flufenamic glucuronides exhibited their greatest stability at pH 2, whereas indomethacin glucuronide was most stable at pH 4.

Intramolecular acyl migration was suggested by the appearance of new peaks eluting near the 1-*O*-acyl glucuronides in these same incubations. Acyl migration was significant at pH 6, 7.4, and 10 for indomethacin 1-*O*-acyl glucuronide, which was generally the most susceptible of the three glucuronides to both hydrolysis and acyl migration. Rearrangement reactions occurred only at pH 7.4 and 10 for flufenamic and clofibric glucuronides. Flufenamic 1-*O*-acyl glucuronide was the most stable of the three glucuronides at all pH values except pH 7.4.

Aglycon pK_a Values. Potentiometric titration in methanol:water, 1:1 (v/v), of indomethacin, flufenamic acid, and clofibric acid was undertaken to confirm the relative order of the literature pK_a values using a single method and apparatus. The uncorrected pK_a values were 4.4, 4.0, and 3.5 compared to the literature values of 4.5 (22), 3.9 (23), and 3.0 (24) for indomethacin, flufenamic acid, and clofibric acid, respectively. The significance of these values is discussed below.

Discussion

The reaction of NBP with 1-*O*-acyl glucuronides in this simple, chemical system is further evidence of the electrophilicity of these conjugated metabolites. Measurement of the rates of NBP acylation permits evaluation of the relative reactivities of different 1-*O*-acyl glucuronides, and these may be compared with familiar, highly electrophilic alkylating agents such as nitrogen mustards. Although this assay is cumbersome in that two reaction steps are required, and it is affected by light and air, it is extremely sensitive and permits measurement over a wide range of reaction rates.

Based on their NBP reactivities, 1-*O*-acyl glucuronides are weak electrophils compared to the nitrogen mustards. The relative rates of NBP acylation for the three 1-*O*-acyl glucuronides tested ranged from 0.436 to 1.08 min^{-1}. A potentially wider range of reaction rates might be expected, if a larger set of drugs were studied.

A number of factors can influence the reactivity of the acyl carbon of the glycosidic linkage of 1-*O*-acyl glucuronides toward nucleophilic substitution. Since the glucuronic acid portions of these conjugates are identical, the aglycon determines the relative reactivity of the acyl linkage for each glucuronide (25). When

unconjugated, the structure of the aglycon also determines the acidity of its carboxylic hydrogen. Transacylation at pH 6.0 probably proceeds by nucleophilic attack on the carboxyl group carbon atom with displacement of a glucuronic acid anion (3, 4). The influence on this reaction of electron density at the carbonyl carbon should be the inverse of its influence on conjugate base formation for the free acid as characterized by pK_a values. However, steric factors might be equally important in determining reactivity of acyl glucuronides toward nucleophilic substitution reactions.

Flufenamic glucuronide, with an aglycon pK_a value between those of clofibric acid and indomethacin, was less reactive toward NBP than were the other two glucuronides. Therefore, no correlation was found between aglycon pK_a values alone and reaction rates of acyl glucuronides with NBP. Similarly, no correlation was found between aglycon pK_a and glucuronide stability in aqueous solution, since flufenamic glucuronide was the most stable glucuronide over a range of pH values. This lack of correlation reflects, among other things, the importance of seric factors in determining the reactivity of acyl glucuronides with nucleophils.

A qualitative correlation exists between reaction with NBP and glucuronide stability, however. The most stable of the glucuronides, flufenamic glucuronide, was also the least reactive compound toward NBP. Indomethacin and clofibric glucuronides, which were much less stable than flufenamic glucuronide in aqueous solution at most pH values, were also much more reactive toward NBP.

Because 1-*O*-acyl glucuronides are at least weakly electrophilic, acylation of macromolecules might occur *in vivo*. Cytotoxicity and immunogenicity have been proposed as some possible consequences of chronic exposure (4). Physiological effects due to 1-*O*-acyl glucuronides should be dependent upon their concentration and distribution *in vivo* and the length of exposure. Although glucuronides are typically excreted rapidly from the body, exposure may be prolonged when the parent drugs are administered chronically over many years. This is often the case for anti-inflammatory (*i.e.* benoxaprofen and indomethacin) and hypolipidemic agents (*i.e.* clofibrate). Among the three drugs whose metabolites are studied here, only clofibrate has been judged to have unacceptable toxicities as a result of chronic exposure (26).

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