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Research Article

Procedures to Characterize *In Vivo* and *In Vitro* Enantioselective Glucuronidation Properly: Studies with Benoxaprofen Glucuronides[1]

Hildegard Spahn,[2,3] Seigo Iwakawa,[2] Emil T. Lin,[2] and Leslie Z. Benet[2,4]

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The diastereoisomeric glucuronic acid conjugates of *R/S*-benoxaprofen are the major benoxaprofen metabolites and are found in urine at high concentrations. The conjugates of *R*- and *S*-benoxaprofen can be separated directly on a C_{18} reversed-phase column using a mixture of acetonitrile and tetrabutylammonium hydroxide buffer, pH 2.5 (28:72, v/v), as the mobile phase. The *k'* values of *S*- and *R*-benoxaprofen glucuronides are 57.5 and 63.0, respectively. Diluted urine or deproteinized plasma samples were injected without further treatment. With fluorescence detection at 313/365 nm, quantifiable limits of 50 ng equiv./ml were found for the conjugates. The intra- and interday variability was below 12%. Utilizing this analytical procedure it is possible to characterize enantioselective glucuronidation both *in vivo* and *in vitro*. For *in vitro* procedures, apparent rates of formation and the *R/S* ratio may be substrate (benoxaprofen) and cosubstrate (UDPGA) dependent. Moreover, enantioselective cleavage of the formed benoxaprofen glucuronides by alkaline hydrolysis, hydrolytic enzymes, and acyl migration must be controlled for both *in vitro* and *in vivo* studies since *R*-benoxaprofen glucuronide is degraded faster than the *S*-diastereomer under certain conditions.

KEY WORDS: stereospecific assay; benoxaprofen; glucuronides; enantiomers; diastereoisomers.

INTRODUCTION

Formation of acyl glucuronides is a major metabolic pathway for several nonsteroidal antiinflammatory drugs (NSAIDs), especially for the 2-arylpropionic acids (1). For many of these compounds the percentage of drug excreted as glucuronide is very high and the renal clearance of unchanged drug is very low, approaching zero in some cases. For example, only small amounts of ketoprofen were recovered unchanged in urine (2), and both enantiomers of carprofen exhibited renal clearances of less than 1 ml/min in man (3).

In addition to common phase I and phase II metabolism, the chiral 2-arylpropionic acids may undergo stereoinversion, i.e., the more active *S*-(+)-enantiomer is preferentially formed from the *R*-(−)-enantiomer (4). As reviewed by Caldwell *et al.* (4) significant stereoinversion was found, for example, for ibuprofen, fenoprofen, and benoxaprofen. Although withdrawn from the market, the highly fluorescent and easily measured benoxaprofen [2-(*p*-chlorophenyl)-α-benzoxazoleacetic acid] is a useful model compound to investigate the major reactions that chiral 2-arylpropionic acids may undergo: formation of glucuronides, formation of isomeric conjugates and acyl migration, irreversible binding to proteins, and stereoinversion. We are particularly interested in developing sensitive, specific, and reliable analytical methods for the acyl glucuronide conjugates of NSAIDs such as benoxaprofen, zomepirac, indoprofen, and other less toxic members of this class of "profens," since we have hypothesized that the extent of measurable conjugate plasma concentrations and the facility of conjugates to acyl migrate and to bind irreversibly to proteins is correlated with the systemic toxicity observed for these compounds (5). For *in vitro* studies and animal studies on the enantioselective disposition of benoxaprofen, we describe here a method for the direct determination of the glucuronides. Conjugation with D-glucuronic acid *in vivo* forms diastereomeric glucuronides from 2-arylpropionic acid enantiomers, which can then be separated directly without hydrolysis and subsequent chiral derivatization.

Conjugation reactions of drug enantiomers with chiral coupling components resulting in diastereoisomers have been used for the determination of propranolol (5), oxazepam (6), and amobarbital (7) concentrations. Recently El Mouelhi *et al.* (8) presented a chromatogram of the separation of diastereomeric benoxaprofen glucuronides using a reversed-phase system with UV detection, although no information concerning the sensitivity, linearity, or reproducibility of the method was provided. All *in vitro* and *in vivo* benoxaprofen studies in the work of El Mouelhi *et al.* (8)

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[2] School of Pharmacy, Department of Pharmacy, University of California, San Francisco, California 94143.

[3] Pharmakologisches Institut für Naturwissenschaftler der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, Gebäude 75 A, D-6000 Frankfurt/M. 70, F.R.G.

[4] To whom correspondence should be addressed.

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were reported only as *S/R* glucuronide ratios without reference to specific concentrations.

The method described in the present article allows quantitation of *S*- and *R*-benoxaprofen glucuronide in urine and in plasma, even in low concentration ranges, since it is based on the fluorescence measurement of the highly fluorescent benoxaprofen. The method can also be used in enzyme kinetic studies with UDP-glucuronosyl-transferases, allowing the low formation rates to be quantitated without requiring the use of a fluorescent label such as 4-bromomethyl-7-methoxycoumarin (9). Furthermore, we address the questions of substrate (benoxaprofen) and cosubstrate (UDPGA) dependence of glucuronide formation rates and the steps necessary to control enzymatic and hydrolytic cleavage of the formed conjugates, which could lead to incorrect conclusions about formation processes, since the degradation can be stereoselective.

MATERIALS AND METHODS

Chemicals

R/S-Benoxaprofen was obtained from Eli Lilly (Bad Homburg, F.R.G.), before it was withdrawn from the market. *S*-(+)-Naproxen (enantiomeric purity *e*, 0.98) was provided by Grünenthal (Stolberg, F.R.G.). Ethylchloroformate, L-leucinamide, and triethylamine were purchased from Fluka (Buchs, Switzerland). Tetrabutylammonium hydrogen sulfate (TBA) was from Janssen Chimica (Beerse, Belgium). All solvents were analytical grade and from E. Merck (Darmstadt, F.R.G.). Benoxaprofen enantiomers were obtained following the method of Weber *et al.* (11) via the formation of diastereomeric amides with α-methylbenzylamine, separation by preparative high-performance liquid chromatography (HPLC), and subsequent cleavage (enantiomeric purities *e*: $R = 0.94$, $S = 0.91$).

Naproxen glucuronide can be used as the internal standard when determining benoxaprofen glucuronide directly. A naproxen glucuronide-containing solution was obtained by administering a commercial naproxen preparation [containing *S*-(+)-naproxen] to a healthy volunteer and collecting fractionated urine. The samples were immediately adjusted to pH 3 for stabilization. Urine samples containing a high concentration of naproxen glucuronide were used either without treatment or after extraction at pH 5 to remove unmetabolized drug. Purification of this glucuronide by extraction from urine at acidic pH using ethylacetate [according to Ray and Wade (12)] is also feasible. Three hours after oral naproxen administration a urine sample was obtained that contained 24 μg/ml naproxen equivalents as glucuronide. This sample was used without further purification. Desmethylnaproxen and its conjugate, which were also present in the sample, did not interfere in this assay. The stability of naproxen glucuronide under exaggerated assay conditions was tested by repetitive thawing and freezing (five times) of pH-stabilized samples and by keeping these samples at room temperature for 1 week. Repeated thawing and freezing did not affect the measured glucuronide concentration. Stabilized samples maintained at room temperature resulted in 5–6% loss over 1 week.

Equipment

HPLC analyses were performed using either a Knauer gradient system (HPLC programmer 50, HPLC pumps 64; Knauer, Berlin, F.R.G.) or a Beckman Model 110A solvent metering pump (Beckman Instruments, Inc., Berkeley, Calif.) with a Shimadzu RF 535 fluorescence monitor (Shimadzu Corp., Kyoto, Japan) (xenon lamp, excitation wavelength set at 313 nm, emission wavelength set at 365 nm) and either a Shimadzu C-R3A Chromatopac integrator or a 3392A integrator (Hewlett-Packard Co., Avondale, Pa.). A Beckman Ultrasphere HPLC column (Beckman Instruments, Berkeley, Calif.) was used as the stationary phase (0.46 × 25 cm, 5-μm particle size). The mobile phase contained 28% acetonitrile in 10 m*M* tetrabutylammonium hydroxide buffer, pH 2.5 (flow rate, 1.8 ml/min; temperature, ambient).

The diastereomeric benoxaprofen-L-leucinamide derivatives were separated using a Zorbax Sil column (DuPont, Wilmington, Del.) and a LichroCart 4-4 guard column (Merck, Darmstadt, F.R.G.).

Samples

Frozen urine samples, which had been acidified and diluted at the time of collection, were available from a previous study, in which a volunteer took 300 mg *R/S*-benoxaprofen orally and collected urine over 96 hr.

A 200-g White Wistar rat was administered 20 mg of *R/S*-benoxaprofen by gavage. Blood was drawn by retroorbital puncture after 2 hr. In addition, urine was collected at the same time. Urine was diluted 1:10 and acidified after collection and then immediately frozen. A further dilution (1:100) with mobile phase containing the internal standard naproxen glucuronide was prepared and the samples were centrifuged prior to chromatography.

Rat plasma was stabilized after collection by adding the same volume of pH 3 buffer. Plasma samples were assayed directly after pH adjustment: to each 0.05 ml of plasma sample, 0.05 ml of acetonitrile and 0.1 ml of mobile phase containing the internal standard naproxen glucuronide were added [final concentration of *S*-(+)-naproxen glucuronide, 0.5–1.0 μg equiv. naproxen/ml). The sample was then vortexed and centrifuged.

Preparation of *R*-(−)- and *S*-(+)-Benoxaprofen Glucuronide Reference Solutions Using Microsomal Preparations

Using the benoxaprofen enantiomers, the glucuronides of *R*-(−)-benoxaprofen and *S*-(+)-benoxaprofen were prepared separately in order to define the elution order in the given chromatographic system. The resulting reference solutions contained *R*- or *S*-benoxaprofen glucuronide, respectively, and were prepared with washed microsomes from livers of sheep or White Wistar rats as described in Ref. 13 with slight modifications. Briefly, 2 mg microsomal protein/ml, 50 m*M* Tris–HCl buffer, pH 7.4, 10 m*M* magnesium chloride, 0.04% Triton X-100, 10 m*M* UDPGA (in one experiment 2.5–15 m*M* UDPGA), 16 m*M* saccharic acid-1,4-lactone, 0.4 m*M* phenylmethylsulfonyl fluoride, and 0.05–3.2 m*M* substrate (added as a solution in methanol or dimethyl sulfoxide) were included in the incubation mixture.

The incubation time did not exceed 10 min. A 1:10 dilution in the mobile phase (detailed below) was used to determine the retention times of the diastereomeric glucuronides. A 50-μl aliquot of the incubation mixture was pipetted into 50 μl of acetonitrile. The mixture was diluted with 400 μl of mobile phase. After vortexing and centrifugation the supernatants can be directly injected onto the HPLC.

To prepare purified standard solutions from such incubations, the excess of substrate was extracted at pH 5.5 using dichloromethane/diethyl ether (1:4), protein was precipitated with acetonitrile, and the supernatant pH was stabilized.

Preliminary Enzyme Kinetic Studies with Sheep Liver Microsomes

Employing the procedure as described above, the influence of varying substrate concentrations on the total yield and the *S/R* composition of the diastereomeric products was investigated with sheep liver microsomes so as to estimate the substrate concentration range where the glucuronidation kinetics can be approximated by the Michaelis–Menten equation, i.e., at concentrations below those where substrate inhibition occurs (14).

In the second part of these studies, the stabilities of the products under incubation conditions (but without cosubstrate) were studied at one concentration of glucuronide over a 90-min period and the apparent first-order degradation rate constant was determined.

For these stability studies, benoxaprofen glucuronide was extracted from urine samples at pH 3 with ethyl acetate, purified by preparative HPLC, and again extracted with ethyl acetate. The resulting residue was dissolved in diluted phosphoric acid to yield a final pH of 4. A procedure for the biosynthesis, isolation, and purification of benoxaprofen glucuronide in semipreparative scale will be described elsewhere (15). Stability was tested with the mixture of diastereomeric glucuronides at final concentrations of 16.3 μ*M* *R*-glucuronide and 17.2 μ*M* *S*-glucuronide in the sheep liver microsome incubation medium (without UDPGA) at pH 7.4 and at pH 6.6 (a possible pH in inflamed tissue), with and without the addition of inhibitors of hydrolytic enzymes (phenylmethylsulfonyl fluoride and saccharic acid-1,4-lactone).

Chromatography

Aliquots (20 μl) were injected after centrifugation using the chromatographic system characterized above (see Equipment). Urine samples contained relatively small amounts of parent drug. When the resolution of the *R*- and *S*-glucuronides was performed in an isocratic system, the unchanged compound was barely detectable and did not lead to obvious baseline shifts. Thus the mobile phase listed above could be used without modification.

In plasma samples and samples from stability studies and studies with rat liver microsomes, however, high concentrations of unconjugated benoxaprofen were present. Here the use of a gradient system was necessary. After 75 min the composition of the mobile phase was changed to 80% acetonitrile within 5 min and maintained for a total period of 15 min in order to remove unconjugated benoxaprofen from the column.

Quantification of the Glucuronide Concentrations in Reference Solutions

As *S*-(+)- and *R*-(−)-benoxaprofen glucuronide were not available as pure reference compounds, solutions containing the glucuronides had to be used as the reference. Two urine samples with different compositions with respect to the *S/R* ratio were characterized and used as references at different dilutions, allowing a calibration curve to be established.

The characterization was performed via an indirect method, involving cleavage of the glucuronides and subsequent chiral derivatization. This procedure is based on the derivatization with L-leucinamide (16) as summarized in the following section.

Derivatization of R- *and* S-*Benoxaprofen with* L-*Leucinamide.* To 50 μl of a plasma or urine sample, 50 μl of 1 *M* sodium hydroxide solution was added and the conjugates were hydrolyzed at room temperature for 1 hr. Then 50 μl of 1 *M* hydrochloric acid and 0.5 ml of pH 5 buffer (citrate/sodium hydroxide), containing the internal standard naproxen (unconjugated) at a concentration of 10 μg/ml, were added. Benoxaprofen was then extracted with 2 ml of a 1:4 mixture of dichloromethane and diethyl ether, and the organic layer transferred into another tube. The organic layer was evaporated under nitrogen; 50 μl of toluene was added to remove traces of water and again evaporated. Then 100 μl of a solution of triethylamine (60 m*M*) in dried acetonitrile was added and, after 1 min, 50 μl of a 60 m*M* solution of ethylchloroformate in dried acetonitrile, followed 2 min later by 50 μl of a solution of L-leucinamide (1 *M*) and triethylamine (1 *M*). After 3 min the reaction was stopped with 0.2 ml of 0.25 *M* hydrochloric acid. The products were extracted with ethyl acetate. After evaporation of this solvent, the residue was dissolved in 200 ml of mobile phase, which consisted of a 50:1 (v/v) mixture of dichloromethane and methanol. An aliquot (5 μl) was injected onto a Zorbax Sil column (0.46 × 25 cm; particle size, 5 μm) equipped with a silica gel guard column (LichroCart 4-4, filled with LiChrosorb Si 60). The flow rate was 1 ml/min, and the temperature ambient, resulting in a medium pressure of 12.5 MPa.

To another 50 μl of the urine sample 100 μl of water and 0.5 ml of pH 5 buffer (containing the internal standard, unconjugated naproxen) were added, extracted with 2 ml of a 1:4 mixture of dichloromethane and diethyl ether, and treated as the hydrolyzed sample described in the preceding paragraph. Glucuronide concentrations were calculated by subtracting the amounts of unconjugated enantiomers from the total enantiomer concentrations. The capacity factors were 2.3 for naproxen and 3.82 for *S*- and 7.52 for *R*-benoxaprofen. The *R/S* separation and resolution factors were 1.97 and 10.25, respectively.

With this procedure based on the chemical chiral derivatization, intraday coefficients of variation of 4.1 and 5.3% were obtained for the derivatives of the *R*- and the *S*-enantiomers, respectively, at a plasma concentration of 8 μg/ml plasma (racemate). With urine samples, similar values

were obtained; the coefficients of variation were 5.1 and 4.7% at 25 μg/ml (racemate). The linearity of the calibration curve was proved up to a concentration of 60 μg/ml racemate when a 50-μl aliquot of an undiluted sample was used. The detection limit based on an injection volume of 5 μl was 50 ng/ml but can easily be improved by increasing the sample or the injection volume.

Stability Studies with Urine Samples

Concentrations of *R*- and *S*-benoxaprofen glucuronide in rat urine were determined under different sample treatment conditions.

(1) Urine was frozen after collection.
(2) The urine pH value was adjusted to 3, and the sample frozen prior to analysis.
(3) Urine was diluted, acidified, and analyzed immediately and again after freezing.

Furthermore, the stability of the diastereomeric glucuronides at 37°C was investigated in different buffers (pH 3, 5, 7, 7.4), as well as the stability in frozen samples, which were diluted and adjusted to pH 2.5 or 3. The glucuronide concentrations in the solutions were 1.15 μg/ml for the *S*-glucuronide and 0.85 μg/ml for the *R*-glucuronide.

Reproducibility, Linearity, and Sensitivity

Reproducibility studies were performed by analyzing on 3 different days, 24 samples each day, eight each of low [*R*, 0.42; *S*, 1.82 μg/ml (concentrations given as benoxaprofen equivalents)], medium (*R*, 3.84; *S*, 8.67 μg/ml), and high (*R*, 17.3; *S*, 29.5 μg/ml) concentrations of glucuronides. Concentrations and S/R ratios were determined. The calculations of variability were performed both with and without the internal standard *S*-(+)-naproxen glucuronide.

The linearity was checked by the injection of 20 different glucuronide amounts. The highest amount injected onto the column was 2.3 mg (*S*/*R* ratio, 1.7; detector on "low sensitivity"). Based on an injection volume of 20 μl and a sample dilution of 1:100, this would be equal to a concentration of 11.5 mg/ml. For the analysis of unknown samples, a five-point calibration curve was established [concentration range, 1–100 μg/ml; $y = 3.212\ (\pm 0.19)\,x + 0.0017\ (\pm 0.0002)$; r^2 ranged from 0.996 to 0.999].

The sensitivity of the method was determined by preparing dilutions of urine samples containing known concentrations (detector on "high sensitivity").

RESULTS

Under the conditions described a sufficient separation of the diastereoisomer glucuronides of benoxaprofen was achieved. The capacity factors (k') for the benoxaprofen glucuronides were 57.5 (*S*) and 63.0 (*R*), and the separation and resolution factors were 1.10 and 2.39, respectively. Figure 1 depicts an HPLC chromatogram of a human urine sample (which had been obtained previously, 0–4 hr after p.o. administration of racemic benoxaprofen). As benoxaprofen is highly fluorescent (10), even small concentrations of the glucuronides are easily detectable. The lower limit of determination based on a 1:10 dilution and a 20 μl injection volume

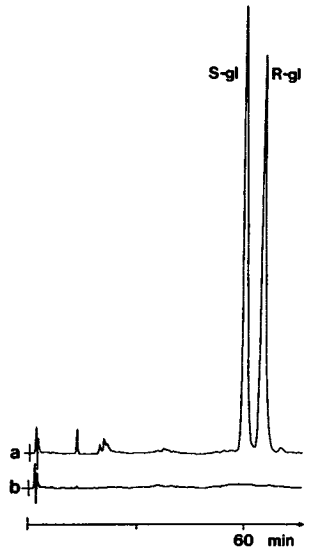

Fig. 1. HPLC resolution of *S*-(+)- and *R*-(−)- benoxaprofen glucuronide (S-gl, R-gl) in a human urine sample (0–4 hr after p.o. administration of 300 mg racemic benoxaprofen). (a) Concentrations—S-gl, 16.5 μg/ml; R-gl, 12.8 μg/ml; calculated as benoxaprofen equivalents. (b) Blank urine.

was 50 ng equiv./ml for each enantiomer (as glucuronide). Once quantification of the glucuronide concentrations in stabilized "standard" urine samples has been accomplished using the indirect method described above, the procedure for the direct determination is easy to perform and reproducible, even without an internal standard, as no extraction and derivatization procedures are required. (The k' for naproxen glucuronide as the internal standard was 11.8.)

Coefficients of variation (CV) were homogeneous over a wide concentration range, not exceeding 6% for intra- and 12% for interday variability during our studies. Intraday CVs are listed in Table I. Within the investigated range, the relationship between concentration and peak area was linear.

As acyl glucuronides are known to be unstable with respect to acyl migration, hydrolysis, or formation of microglobular bodies at physiologic pH (1,17,18), the pH of each urine sample had to be adjusted to 3. Since urine samples may contain high amounts of benoxaprofen glucuronides and the solubility limit of the metabolites might be exceeded upon cooling, the samples were diluted 1:10 and then frozen immediately after collection. Dilution prevents the formation of "microglobular bodies" (17), as does pH adjustment. When urine, containing benoxaprofen glucuronide, was not pH adjusted, glucuronide concentrations were significantly decreased. Further sample handling such as a second freez-

Table I. Intraday Coefficients of Variation (%; $N = 8$ for Each Concentration) Using Naproxen Glucuronide as Internal Standard

Concentration	*R*	*S*
Low (*R*, 0.42; *S*, 1.82 μg/ml)	6.0	4.8
Medium (*R*, 3.84; *S*, 8.67 μg/ml)	5.1	2.9
High (*R*, 17.3; *S*, 29.5 μg/ml)	2.5	2.2

ing and thawing of samples which had not been pH adjusted led to an almost complete disappearance of β-glucuronidase-cleavable peaks. However, two peaks susceptible to alkaline cleavage were observed, having a retention behavior almost similar to that of the diastereomeric glucuronides. The first peak eluted with the same retention time as *S*-benoxaprofen glucuronide, but it had a different peak shape and could be well identified. It was not completely separated (about 50%) from a second β-glucuronidase-resistant peak eluting between *R*- and *S*-glucuronide. In contrast, for samples adjusted to pH 3, the stability of the conjugates was markedly improved and the peaks remained cleavable during treatment with β-glucuronidase. The glucuronides could be detected, even after repetitive sample freezing and thawing, and additional peaks were avoided.

Measurements in Plasma and Urine

Comparison of the direct and the indirect methods (derivatization with L-leucinamide; Fig. 2) for 10 different urine samples (glucuronide concentrations between 0.5 and 35 μg/ml, calculated as *R*/*S*-benoxaprofen equivalents) indicated about 8% lower results for the direct method. The explanation for this difference results from traces of isomeric conjugates formed *in vivo* (18), which are not quantified by the direct procedure but are cleaved by alkaline hydrolysis.

Incubation at 37°C of the diastereomeric glucuronides in buffer solutions showed that the conjugate of the *R*-enantiomer disappeared more rapidly than the *S*-enantiomer at physiological pH. The apparent first-order rate constants characterizing the decrease in the glucuronide concentrations at different medium pH values (37°C) are given in Table IIA. In samples which were diluted, adjusted to pH 2.5 or 3, and frozen, no detectable decrease in the glucuronide concentrations was found over 3 months.

Two hours after p.o. administration to rats, the urine concentration of *S*-benoxaprofen glucuronide significantly exceeded that of the *R*-equivalents) (*S*/*R* ratio, 10). In rat plasma (after 2 hr) a similar difference in benoxaprofen glucuronides was found. *R*-Glucuronide could hardly be detected, whereas the concentration of the *S*-glucuronide was approximately 20 ng equiv./ml (determined using increased injection volumes).

In man, both the urinary excretion rate and the extent were also higher for the *S*-glucuronide (except during the first 2-hr interval after dosing), although the *S*/*R* ratio (~2) was much lower than that found in the rat. The cumulative excretion in urine within 96 hr is shown in Fig. 3.

Table II. Stability of *R*- and *S*-Benoxaprofen Glucuronide at Different pH Values: Apparent First-Order Decomposition Rate Constants (hr^{-1}) (A) in Buffer Solutions at 37°C and (B) in Incubation Media with Microsomes

pH	k (hr^{-1}) *R*	*S*
A		
3.0	0.030	0.027
5.0	0.064	0.062
7.0	0.277	0.165[a]
7.4	0.347	0.169[a]
B		
7.4 (with blocking hydrolytic enzymes)	0.964	0.914
6.6 (with blocking hydrolytic enzymes)	0.745	0.771
6.6 (without blocking hydrolytic enzymes)	3.03	1.92

[a] Additional peaks, which were not cleavable by β-glucuronidase, were observed.

Incubation Studies with Hepatic Microsomes

Incubation studies with sheep liver microsomes at pH 7.4 demonstrated that the *S*/*R* ratio of the glucuronide products is highly dependent upon the concentration of the substrate, both for incubation of racemic compound and incubation of the single enantiomers. The product *S*/*R* ratio was <1 for rat and >1 for sheep liver microsomes (approximately 0.4 with rat liver microsomes and 1.2 with sheep liver mi-

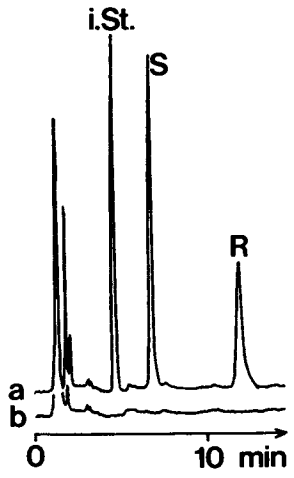

Fig. 2. Representative chromatograms showing (a) the separation of the diastereomeric L-leucinamide derivatives of benoxaprofen [extracted from a urine sample after the addition of unconjugated naproxen as internal standard (i.St.)] and (b) a blank urine sample.

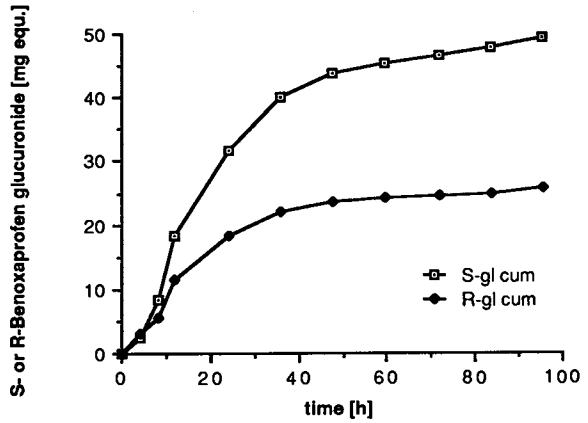

Fig. 3. Cumulative excretion of benoxaprofen glucuronides in human urine (after p.o. dosage of 300 mg of racemic compound).

crosomes at 0.5 m*M* racemic substrate) over a wide substrate concentration range. However, when the maximum formation rate was reached under our conditions (at 0.9 m*M* for sheep liver microsomes), the ratio became 1 and decreased for higher substrate concentrations together with a decrease in the total rate of formation. The absolute yield was—as can be expected—dependent upon the concentrations of UDPGA (Fig. 4). The dependency of the *S*/*R* ratio on the substrate concentration was similar for all cosubstrate concentrations, although the total yield was lower for lower UDPGA concentrations.

Product stability studies in microsome incubation media (without the addition of UDPGA) showed that, as in buffer solutions, the disappearance of glucuronides follows apparent first-order kinetics. There was no significant difference in the apparent decomposition rate constants between the *R*- and the *S*-glucuronides in the incubation medium at pH 7.4 or at pH 6.6 when inhibitors of hydrolytic enzymes were included. At pH 6.6, however, the *R*-glucuronide decomposed more rapidly, if no inhibitors of hydrolytic enzymes were added (Table IIB; $N = 3$ for all incubations). Decomposition in enzyme-blocked media at pH 7.4 was greater than that found in pH 7.4 buffer.

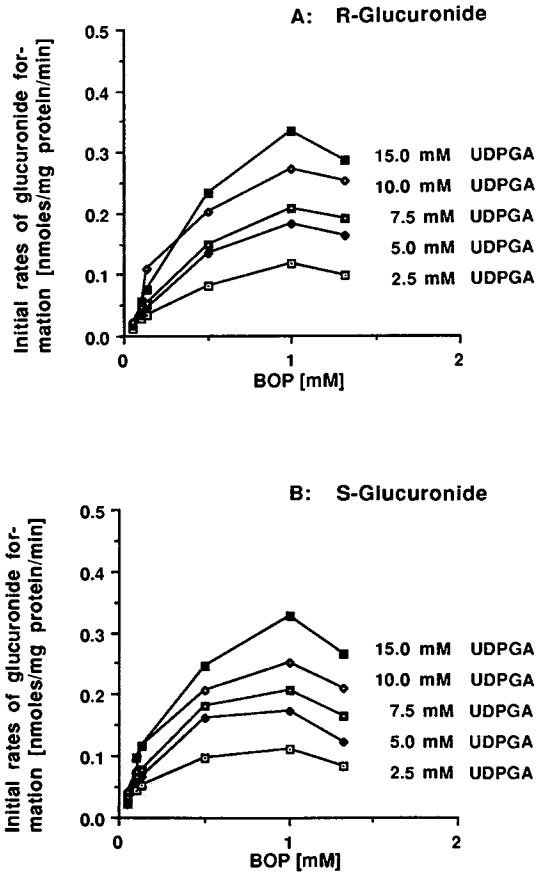

Fig. 4. The dependency of the initial rates of glucuronide formation with sheep liver microsomes as a function of the concentration of racemic substrate and the concentration of the cosubstrate UDPGA. (A) *R*-Benoxaprofen acyl glucuronide formation; (B) *S*-benoxaprofen acyl glucuronide formation.

DISCUSSION

In 1982 the occurrence of "microglobular bodies" in benoxaprofen urine samples and the influence of experimental conditions on their formation in clinical studies were discussed by Ridolfo *et al.* (17). These authors state that these structures "consist of a complex or mixture of two types of benoxaprofen conjugates of glucuronide—one a beta-glucuronide and the second compatible with a glucuronide structure that is not affected by beta-glucuronidase." We believe today that the second structure is caused in part by the formation of isomeric conjugates, which are known to be β-glucuronidase resistant (18). The findings in the present investigation with rat urine samples that are not pH stabilized are in accord with those of Ridolfo *et al.* (17): a sediment is formed upon cooling of the urine that can be cleaved by alkaline treatment, while only small amounts of glucuronides can be found in the supernatant. Furthermore, additional peaks occur which are not susceptible to β-glucuronidase but which can be cleaved by sodium hydroxide.

While the phenomenon of instability of acyl glucuronides and the formation of isomeric conjugates were previously described for several compounds (1), the appearance of glucuronide complexes (17) and a corresponding decrease in glucuronide concentration in the supernatant have been detected only with benoxaprofen. The different mechanisms by which the glucuronide concentrations can be affected require the use of a stabilization procedure. Hydrolysis, acyl migration, and sediment formation in urine can be prevented by acidification and dilution of the samples as described here. The analytical procedures presented give highly reproducible results.

The *in vitro* formation studies of benoxaprofen glucuronides from benoxaprofen enantiomers with racemic compound as well as with the single enantiomers using rat or sheep liver microsomes exhibited clear differences in the apparent rate of formation. The amount of conjugate formed from the *R*-enantiomer was significantly higher in rat liver microsomes and slightly lower in sheep microsomes in these *in vitro* experiments (substrate concentrations, 0.05–0.5 m*M*). This enantioselectivity in the glucuronidation of 2-arylpropionic acids was also found by El Mouelhi *et al.* (9), who described species-dependent enantioselective formation of the diastereomeric glucuronides of naproxen, ibuprofen, and benoxaprofen with immobilized UDPGT. The *S*/*R* ratios for the rate of formation of the glucuronides from benoxaprofen at pH 6 were 1.7, 1.2, and 0.5 with UDPGTs from rabbit, rhesus monkey, and human, respectively. In our studies with rat and sheep liver microsomes the *S*/*R* ratios of the apparent formation rates (pH 7.4) at substrate concentrations close to K_m were 0.4 and 1.2, respectively, for benoxaprofen glucuronides. We found a similar enantioselectivity of rat hepatic UDPGT for naproxen (13) and carprofen (unpublished data), the diastereomeric glucuronides of which can be separated on an octadecylsilane stationary phase in incubation mixtures as well as in plasma and urine samples (19).

The findings in our paper point out the complexities involved in attempting to characterize the enantioselective formation rates of acyl glucuronides and the pitfalls which El

Mouelhi *et al.* (9) possibly encountered in attempting to characterize species-dependent enantioselective glucuronidation by comparisons utilizing a single substrate concentration, a single UDPGA concentration, a single prolonged time point, and failure to control degradation of glucuronides back to parent aglycone. El Mouelhi *et al.* chose a pH of 6.0 for incubation, since hydrolysis and isomerization of the acyl glucuronide products are known to be decreased at acidic pH values (15), whereas we prefer to study the glucuronidation under more physiological conditions. Since the acidic pH is supposed to stabilize the product and thus cause less error, an interesting finding is the stereoselective instability of the benoxaprofen products at pH 6.6, if no inhibitors of hydrolytic enzymes are added (Table IIB). These experiments with and without inhibitors prove that the stereoselective decrease in the glucuronide concentration at pH 6.6 with microsomal protein is an enzymatic process, whereas the decomposition at pH 7.4 is rather a pH-dependent chemical instability. And in contrast to our findings in buffer solutions (Table IIA), there was no obvious stereoselectivity in the decomposition of the glucuronides at pH 7.4 when inhibitors of hydrolytic enzymes were added (Table IIB). These data suggest that true formation rates and *S/R* ratios cannot be obtained in a pH-"stabilized" microsomal incubation medium, unless enzyme inhibitors are also included.

It is obvious in examining Tables IIA and B that the rate of decomposition at pH 7.4 in incubation media with blocking hydrolytic enzymes is greater than that in pH 7.4 buffer and that stereospecificity in decomposition rate is not seen in the incubation media. These differences demonstrate the complexity of the system involved. We believe that the high decomposition rates in "blocked" media are due to the fact that the amount of blocking agents added was chosen so as to not affect glucuronide formation. Thus it is possible that we have not blocked all esterase cleaving ability. More detailed studies relating to the effect of blocking agents on inhibition of glucuronide formation vs inhibition of decomposition are ongoing in our laboratory.

With respect to *S/R* ratios in product formation, it should be obvious that if significant stereoselectivity in product decomposition occurs (which must be tested for each compound), then the concentrations in the medium and, of course, the *S/R* ratios at prolonged incubation times are highly dependent upon product loss, i.e., the *S/R* ratios may not necessarily reflect the ratios of the true formation rates. Using the initial apparent glucuronide formation rates and the first-order product decomposition rates determined above, the true formation rates and S/R ratios can be calculated. In such circumstances "stabilization" (pH adjustment and addition of inhibitors of hydrolytic enzymes) of the incubation system to minimize product loss is no longer necessary, as will be discussed in detail elsewhere.

Even if glucuronide decomposition was negligible, the *S/R* ratio should not be independent of the substrate concentration. As seen in Fig. 4, substrate inhibition occurs at benoxaprofen concentrations greater than 1 m*M* in sheep liver microsomes and the *S/R* ratio changes from a value greater than one to a value less than one. El Mouelhi *et al.* (9) performed all incubations at 3 m*M* UDPGA and reported that, under their experimental condition, "UDPGA concentration was not found to be a rate-limiting factor . . . ," a rather unusual finding for an enzyme with bisubstrate enzyme kinetic characteristics, as clearly shown in our work (Fig. 4), although the *S/R* ratio of the formed product was unaffected by UDPGA.

A third difficulty in interpreting the data of El Mouelhi *et al.* (9) is related to the probability that β-glucuronidase is present in their microsome preparation and should contribute to their final ratios, since they carried out incubations at pH 6 (for 6 hr), a pH where the hydrolytic activity of the enzyme is known to be considerable. It might also be suspected that at lower pH's there may be stereoselective degradation of the enzymatically formed β-1-acyl glucuronides as we have shown in Table II. Furthermore, at pH 6 acyl migration products will occur over prolonged incubation times. This discussion is not meant to imply that the finding of species-dependent enantioselective glucuronidation by El Mouelhi *et al.* (9) is incorrect. There are obvious differences in enzyme from different species as reported by El Mouelhi *et al.* (9), for experiments all carried out under the same conditions, and in the work reported here. However, the *S/R* ratios and the glucuronidation rate constants reported by El Mouelhi *et al.* (9) must be considered "apparent" values due to the complicating factors discussed above, which we have characterized here. That is, proper characterization of enantioselective glucuronidation requires investigation of substrate and cosubstrate dependence, studies of stereoselective degradation as a function of both pH and enzymatic processes, and an adequately validated specific and sensitive analytical method for diastereomeric glucuronides.

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