Pharmaceutical Research, Vol. 7, No. 4, 1990

Report

Stereochemical Characterization of the Diastereomers of the Phenobarbital *N*-β-D-Glucose Conjugate Excreted in Human Urine

William H. Soine,[1,2] Phyllis J. Soine,[3] Suzanne E. Mongrain,[1] and Terry M. England[3]

Received July 31, 1989; accepted October 30, 1989

The absolute configuration of the *N*-β-D-glucoside metabolites of phenobarbital was determined by methylation of the diastereomers to make mephobarbital *N*-β-D-glucosides, followed by oxidative removal of glucose to give the optical isomers of mephobarbital. Following a single oral dose of phenobarbital to two male subjects, both phenobarbital *N*-β-D-glucosides were excreted in the urine. The absolute configuration (C-5 position) of the major phenobarbital *N*-β-D-glucoside excreted in the urine was the S form. A pronounced stereoselective formation and/or urinary excretion occurs for the *N*-glucoside conjugates of phenobarbital in humans.

KEY WORDS: 1-(β-D-glucopyranosyl)phenobarbital; diastereomers; absolute configuration; mephobarbital; product enantioselectivity; urine.

INTRODUCTION

It has been proposed that *N*-β-D-glucosylation is a general pathway for metabolism of barbiturate drugs. This is based on the isolation and identification of the *N*-β-D-glucose conjugates of amobarbital and phenobarbital (1–3). The coupling of D-glucose to N_1 of phenobarbital confers asymmetry at C_5 and generates two diastereomers (**1a** and **1b**). In man, amobarbital has been shown to form both *N*-β-D-glucoside diastereomers and a pronounced "product enantioselectivity" for one of the diastereomers was observed (4). Phenobarbital has been shown to form *N*-β-D-glucose conjugates in man (5–8); however, characterization of the diastereomers has not been reported. In this study, the absolute configuration for the C-5 substituents of the *N*-β-D-glucoside diastereomers of phenobarbital is determined and both diastereomers are identified as excretion products in human urine.

MATERIALS AND METHODS

Reagents and Chemicals

5(*S*)-5-Ethyl-1-(1-β-D-glucopyranosyl)-5-phenyl-2,4,6-(1H,3H,5H)pyrimidinetrione (**1a**) and 5(*R*)-5-ethyl-1-(1-β-D-glucopyranosyl)-5-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione (**1b**) were synthesized as previously described (9). In that report they were designated PBGA and PBGB, respectively. For each of the *N*-β-D-glucoside conjugates, greater than 98% of the peak area (198 nm) was associated with a single diastereomer unless otherwise noted. Phenobarbital was purchased from Merck and Co. Inc. (Rahway, NJ). *p*-Hydroxyphenobarbital and tris[3-(heptafluropropylhydroxymethylene)-*d*-camphorato], europium(III) derivative [Eu(hfc)$_3$], were purchased from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile (MeCN), ethylacetate (EtOAc), and monobasic and dibasic sodium phosphate were HPLC grade. All other chemicals were reagent grade.

Equipment

Melting points (uncorrected) were determined in an open capillary with a Thomas–Hoover Unimelt apparatus (Philadelphia, PA). IR spectra were determined with a Nicolet 5ZDX FT-IR (Madison, WI). ^{1}H-NMR spectra were obtained on a JOEL FX90Q spectrometer (Tokyo). Optical rotations were determined on a Perkin-Elmer 141 Polarimeter (Norwalk, CT). The thermospray mass spectra (LC/MS) were obtained with a Hewlett–Packard HP 5988A LC/MS system interfaced to a Hewlett–Packard HP1090 liquid chromatograph via a Hewlett–Packard thermospray interface.

HPLC Systems

The HPLC system for preparative purification of **1a** and **1b** from the urine extract utilized an Econosil C-18 semi-preparative reverse-phase column (250 × 9-mm i.d.; particle size, 10 μm; Alltech Associates) with a Perisorb RP-18 guard column (20 × 2-mm i.d.; particle size, 30–40 μm; Upchurch Scientific). The system used a 1.0-ml loop injector (Rheo-

[1] Department of Medicinal Chemistry, School of Pharmacy, Virginia Commonwealth University, Richmond, Virginia 23298-0581.
[2] To whom correspondence should be addressed.
[3] Bureau of Forensic Science, Division of Consolidated Laboratory Services, Commonwealth of Virginia, Richmond, Virginia 23219.

0724-8741/90/0400-0402$06.00/0

dyne, Inc.). The mobile phase was 15% (v/v) MeCN in 0.5 *M* ammonium acetate at a flow rate of 4.0 ml/min. The effluent was monitored at 254 nm.

The HPLC system used for detecting metabolites and monitoring the semipreparative purification of the metabolites utilized an Econosphere C-18 column (250 × 4.6-mm i.d.; particle size, 5 μm; Alltech Associates) with the guard column that was described previously. The mobile phase was 15% (v/v) MeCN in 0.025 *M* sodium phosphate buffer, pH 6.5. The flow rate was 1.4 ml/min and the effluent was monitored at 204 nm using a strip chart recorder. The same chromatographic conditions were used for UV characterization. Postcolumn ionization was accomplished by mixing a 0.2 *M* sodium borate buffer (pH 10.0) at a flow rate of 0.3 ml/min with a column eluate and passing the solution over a 50 × 2-mm column of 75-μm glass beads (10). The effluent was monitored with a diode array detector (2140 Rapid Spectral Detector, LKB-Produkter AB) from 190 to 300 nm. The retention time (t_R) was 22.2 min for **1a** and 25.0 min for **1b**. For both **1a** and **1b** the UV maximum was at 194 nm and, following postcolumn ionization, two UV maxima were observed at 198 and 238–242 nm.

The conditions used to obtain the thermospray mass spectra (LC/MS) have been previously described (11). The chromatography was performed with an Econosphere C-18 column using a mobile phase of 15% (v/v) MeCN in 0.1 *M* ammonium acetate buffer at a flow rate of 1.2 ml/min. The t_R was 15.2 min for **1a** and 16.6 min for **1b**. The chemical ionization mass spectrum exhibited a *m/z* 395 [21%, $(M+H)^+$] and 412 [100%, $(M+NH_4)^+$] for both **1a** and **1b**.

Conversion of 1a to *R*(−)-Mephobarbital

5(*S*)-5-Ethyl-1-(2,3,4,6-tetraacetyl-β-D-glucopyranosyl)-3-methyl-5-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione was prepared as previously described from 5(*S*)-5-ethyl-1-(2,3,4,6-tetraacetyl-β-D-glucopyranosyl)-5-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione (**1a** tetraacetate) by treatment with diazomethane (9). After hydrolysis of 95 mg (164 μmol) of the tetraacetyl intermediate in 9 ml of 10% H_2SO_4, 2.0 ml of 9.5 *M* H_2SO_4 containing 732 mg (2.45 mmol) of $Na_2Cr_2O_7$ was added. The reaction was heated at 93°C for 3 hr. The solution was cooled to room temperature, diluted with 10 ml of water, and extracted with CH_2Cl_2 (5 × 10 ml). The combined CH_2Cl_2 extracts were dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The white precipitate was recrystallized from methanol/water and dried under reduced pressure to yield 33 mg (132 μmol, 82% yield) of a white solid. m.p. 99–101°C (lit. 101°C) $[a]_D^{25} = -8.9°$ (lit. $[a]_D^{20} = -9.0°$) (12).

Conversion of 1b to (*S*/*R*)-(+)-Mephobarbital

(5*S*/5*R*:6/94)-5-Ethyl-1-(β-D-glucopyranosyl)-3-methyl-5-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione was prepared from 144 mg (364 μmol) of(5*S*/5*R*:6/94)-5-ethyl-1-(1-β-D-glycopyranosyl)-5-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione by treatment with diazomethane (9). The oil that was obtained was dissolved in 10 ml of 3.8 *M* H_2SO_4 containing 1.57 g (5.3 mmol) of $Na_2Cr_2O_7$ and heated at 83°C for 17 hr. The reaction was cooled to room temperature, diluted with 10 ml water, and extracted with CH_2Cl_2 (5 × 15 ml). The combined CH_2Cl_2 extracts were dried over anhydrous Na_2SO_4, concentrated, and purified by column chromatography. Eluting the column with $CHCl_3$:acetone (9:1, v:v), combining the fractions containing mephobarbital, and concentrating under reduced pressure gave 44 mg of a white solid. This material was recrystallized from methanol/water and dried under vacuum to yield 33 mg (132 μmol, 36% yield) of (*S*/*R*)-mephobarbital. The *N*-methyl resonances were at 5.09 (S isomer) and 4.98 ppm (R isomer) in the presence of a 0.26 molar ratio resulting from the incremental addition of $Eu(hfc)_3$ chiral shift reagent (13). The resolution of the *N*-methyl absorbance was not adequate for accurate quantitation of each isomer. m.p. 99–101°C (lit. 101°C) $[\alpha]_D^{20} = +7.9°$ (lit. $[\alpha]_D^{20} = +9.3°$) (12).

Partial Purification of 1a and 1b Conjugates from Urine

Total urine was collected for 60 hr from one male Caucasian (subject 1) and one male Oriental (subject 2) after taking a 90-mg oral dose of phenobarbital. The urine samples were refrigerated immediately upon collection and, within 48 hr, made acid to litmus with conc. HCl. Urine samples containing measurable quantities of phenobarbital *N*-β-D-glucosides were pooled. Purification of these samples was similar to the method used for the isolation of the amobarbital *N*-β-D-glucosides (4). Three liters of acidified urine, in which 1000 g of NaCl had been dissolved, was passed over a 450g column of XAD-4. The column was washed with 500 ml of 0.2 *N* HCl. The metabolite was eluted with 500 ml of methanol. The methanol eluate was evaporated to a thick oil under reduced pressure (water aspirator) followed by the addition of 15 ml of a saturated solution of $(NH_4)_2SO_4$. The extract was acidified with 10% H_2SO_4 until acid to litmus. This mixture was extracted for 42 hr with diethyl ether, then 28 hr with diisopropyl ether to remove phenobarbital and other lipophilic material. Each extract was checked by HPLC to verify that the phenobarbital *N*-glucosides were not present. The aqueous phase was extracted for 28 hr with EtOAc. The EtOAc fraction was dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. This material was partially purified by HPLC using the semipreparative column. A single fraction containing both **1a** and **1b** (10–18 min) was collected into 5–10 ml of glacial acetic acid. The sample was concentrated to a volume of 2 ml under reduced pressure. A portion of this material was characterized by HPLC-UV (both with and without postcolumn ionization) and LC/MS. The remaining fraction was rechromatographed using the same HPLC system. The eluate was collected in the presence of glacial acetic acid and the fractions containing **1a** and **1b** were kept separate. The samples were concentrated, and anhydrous Na_2SO_4 was added and extracted with EtOAc (4 × 2 ml). The EtOAc fraction was dried with anhydrous Na_2SO_4, then evaporated, and the residue was reconstituted with 1 ml of water and lyophilized. This procedure yielded 1–2 mg of a white solid, which cochromatographed with **1a**, and trace quantities of a yellow oil that cochromatographed with **1b**. The fraction corresponding to **1a** was further characteried by FT-IR, ^{1}H-NMR, and LC/MS. Insufficient material was present for characterization of **1b** by FT-IR and ^{1}H-NMR.

RESULTS

Determination of the Absolute Configuration of 1a and 1b

The conversion of **1a** tetraacetate to *R*(−)-mephobarbital is shown in Fig. 1. The glucose portion was oxidatively removed from the mephobarbital *N*-β-D-glucoside by treatment with sodium dichromate under strongly acidic conditions. *R*(−)-Mephobarbital was obtained in a reasonable yield and characterization for optical purity by melting point, optical rotation (12), and ^{1}H-NMR using a chiral shift reagent (13) indicated a single enantiomer. When the partially purified *N*-methyl homologue of **1b** (94% **1b** and 6% **1a**) was oxidized using the same conditions as described for *N*-methyl **1a**, only a 4% yield of mephobarbital was obtained. The oxidative removal of glucose from *N*-methyl **1b** required a longer reaction time at lower temperatures to obtain mephobarbital in an acceptable yield. The mephobarbital obtained from **1a** had a (−) rotation, indicating that it has an absolute configuration of R (14). The mephobarbital obtained from **1b** had a (+) rotation, consistent with an absolute configuration of S. The absolute configuration at C-5 of **1a** and **1b** is S and R, respectively.

Characterization of Biologically Derived 1a and 1b

The biologically derived **1a** was identical to synthetic **1a** by FT-IR, ^{1}H-NMR, LC/MS, and HPLC-UV (with and without postcolumn ionization). In the ^{1}H-NMR and FT-IR spectra, potassium acetate was observed as a contaminant at an approximately equimolar concentration. The spectrum of potassium acetate was subtracted from that of biologically derived **1a** to give a spectrum consistent with that of synthetic **1a**. The absolute configuration of the major phenobarbital *N*-β-D-glucoside excreted in urine by humans is S.

The quantity of biologically derived **1b** isolated in this study was insufficient to characterize by FT-IR or ^{1}H-NMR.

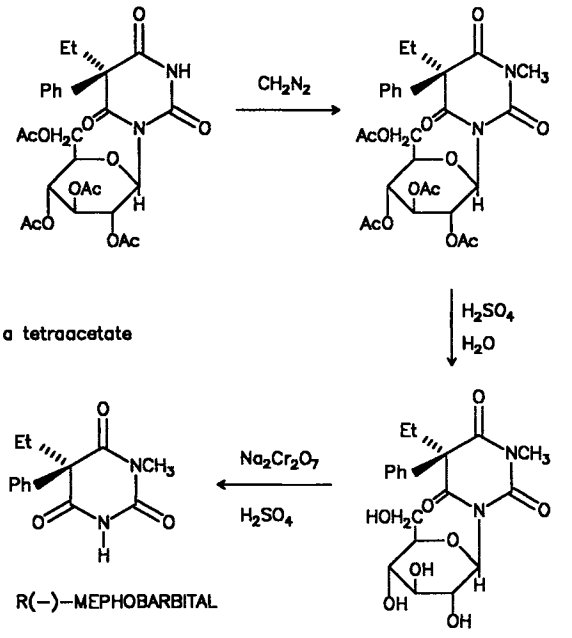

Fig. 1. Synthetic conversion of **1a** tetraacetate to *R*(−)-mephobarbital.

Using the initial LC/MS conditions (linear detection), only the molecular ion adduct plus ammonia $(M+NH_4)^+$ could be detected. Using single-ion monitoring (SIM) for both the molecular ion $(M+H)^+$ and the $(M+NH_4)^+$ ion, the chromatogram in Fig. 2 was obtained. The retention times and relative ion intensities were identical to those of synthetic **1b**. To confirm further the presence of **1b**, it was analyzed by HPLC-UV. Upon postcolumn ionization the UV maximum at 194 nm shifted to 198 nm, with a second maximum at 238–242 nm. These results are identical to those for synthetic **1b**. Following oral dosing with phenobarbital, **1b** (R configuration at C-5) is also excreted in the urine. The percentages of **1a** excreted by subjects 1 and 2 were approximately 5 and 3%, respectively. The percentages of **1b** excreted by subjects 1 and 2 were approximately 0.7 and 0.6%, respectively. The small percentage of phenobarbital *N*-glucosides recovered is probably due to the long half-life of phenobarbital and the short period of urine collection. Also, this study was initiated prior to recognition of the chemical instability of **1a** and **1b** (11) and some decomposition may have occurred prior to analysis.

DISCUSSION

Conjugation of a glycone to a barbiturate nitrogen may confer chirality to the aglycone and diastereomers would be formed. Spectroscopic methods are not currently available for predicting the absolute configuration associated with these chiral conjugates, especially the barbiturate *N*-β-D-glucoside conjugates. In contrast, the absolute configuration associated with *N*-alkyl barbiturates, especially compounds with medicinal applications, has been extensively studied. For the phenobarbital *N*-β-D-glucosides, the nitrogens were differentiated by methylation with diazomethane, after which removal of glucose to obtain mephobarbital was required. Cleavage of an *N*-linked barbiturate from glucose has been reported under strongly acidic conditions (7,15), however, attempts to hydrolyze *N*-methyl **1a** using 10% H_2SO_4 at 90°C for 24 hr or longer were unsuccessful. The acid stability of the *N*-methyl homologue **1a** was consistent with that observed for other barbiturate *N*-β-D-glucosides and hydantoin *N*-β-D-glucuronides (9,16).

The oxidative degradation of the glycoside ring was based on an analytical method developed for removing interfering substances when quantitating phenobarbital or mephobarbital in urine (17). Although this oxidative method was successful for these compounds, it is not a general method. If a C-5 substituent is a propyl or longer alkyl chain, significant oxidation will occur at the ultimate or penultimate (ω-1) carbon using these conditions (18). In the course of these studies, milder oxidizing conditions were evaluated [$KMnO_4/H_2SO_4$, Na*m*IO_4/H_2O or CH_2Cl_2, $Pb(OAc)_4$/HOAc or CF_3CO_2H or pyridine, Zn/HCl], however, no mephobarbital or extremely poor yields of mephobarbital were obtained.

Prior reports have shown that following dosing with phenobarbital, an *N*-β-D-glucoside(s) of phenobarbital was excreted in urine by humans. Early studies used methods that were not sufficiently sensitive to differentiate closely related diastereomers (2,3,7). Since **1b** was present at low concentrations, considerable problems were encountered

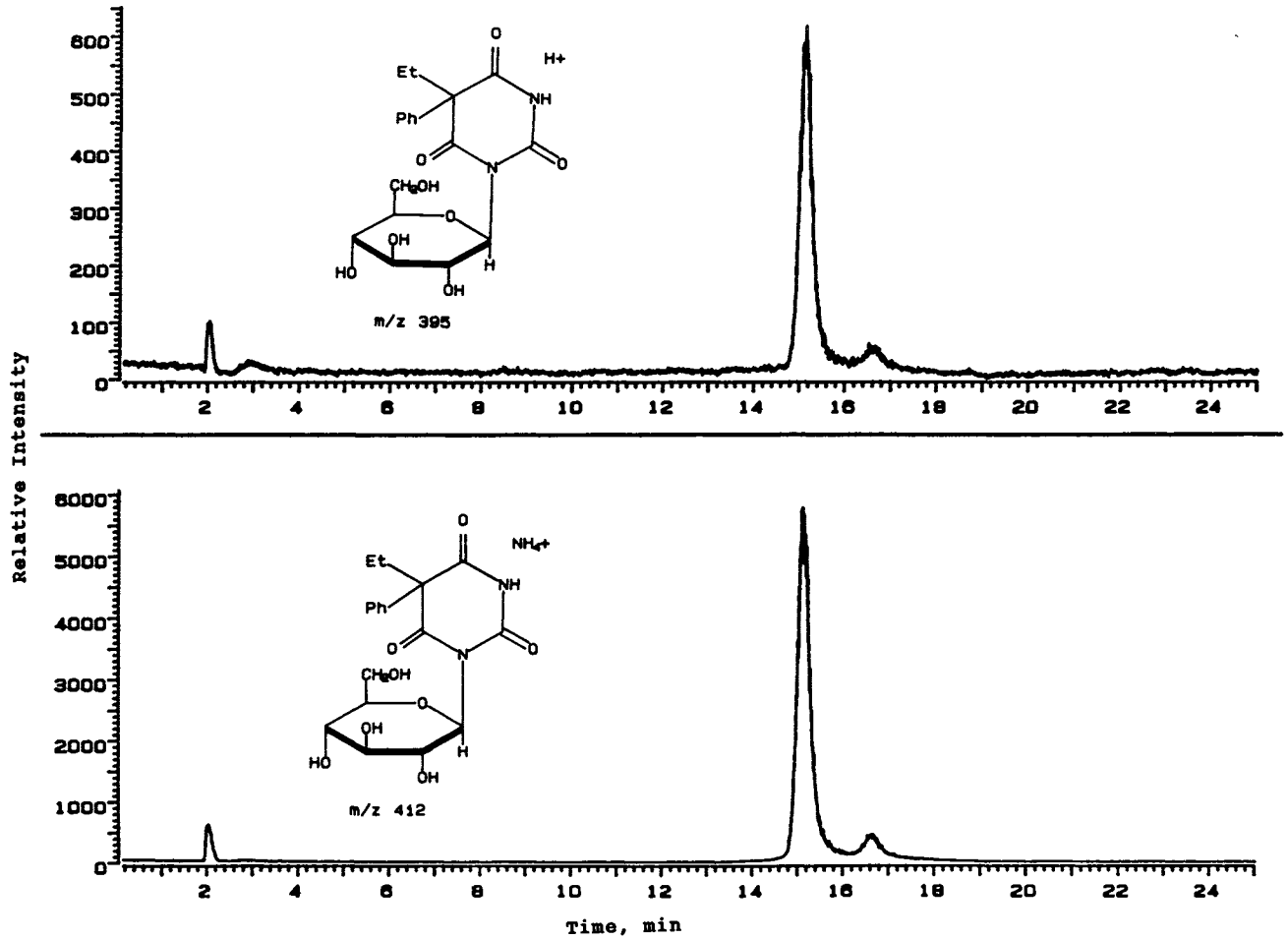

Fig. 2. A thermospray LC/MS tracing of the partially purified human urine extract in which the major ions of the phenobarbital *N*-β-D-glucosides, *m/z* 412 ($M+NH_4^+$) and *m/z* 395 ($M+H^+$), were monitored.

in confirming its presence in urine as a metabolite, possibly due to its chemical instability (11). Even isolating **1a** from urine in a form that was sufficiently pure and in adequate quantities for FT-IR and ^{1}H-NMR analysis proved very difficult. Characterization of **1b** was possible only using LC/MS and comparison of UV spectra at pH 6.5 and 10.0 during HPLC analysis. However, using spectrometrically independent UV and MS data, there is conclusive evidence that both diastereomers of phenobarbital *N*-β-D-glucoside are excreted in human urine following oral administration of phenobarbital.

Since the absolute configuration at the C-5 position of **1a** and **1b** is S and R, respectively, the major phenobarbital *N*-β-D-glucoside excreted in urine by humans has the S configuration. A product enantioselectivity has been previously observed for the urinary excretion of the amobarbital *N*-β-D-glucosides (4), but the absolute configuration has yet to be determined. It would be anticipated that the absolute configuration for the major amobarbital *N*-β-D-glucoside would be S, comparable to that obtained for the phenobarbital *N*-β-D-glucosides. In a closely related system, *N*-glucuronidation has been shown to be a major metabolic pathway for phenylethylhydantoin in man. However, the occurrence of stereoselective formation and/or excretion of the *N*-glucuronides in human urine has not been reported (19,20). In contrast, stereoselective formation, hydrolysis and/or urinary excretion of *O*-glucuronides of drug racemates by humans is frequently observed (21–27).

CONCLUSION

Although studies of the *N*-β-D-glucosylation pathway are very limited, it appears that product enantioselectivity for formation and/or excretion of the *N*-β-D-glucoside conjugates of phenobarbital and amobarbital is similar. It remains to be determined if the absolute configuration at C-5 of the barbiturate ring of the major diastereomer excreted in the urine is the same for both the phenobarbital *N*-β-D-glucosides and the amobarbital *N*-β-D-glucosides.

ACKNOWLEDGMENTS

This work was supported by grants from the Epilepsy Foundation of America and PHS Grant GM34507.

REFERENCES

1. B. K. Tang, W. Kalow, and A. A. Grey. *Res. Commun. Chem. Path. Pharm.* 21:45–53 (1978).
2. B. K. Tang, W. Kalow, and A. A. Grey. *Drug Metab. Disp.* 7:315–318 (1979).

3. W. H. Soine, V. O. Bhargava, and L. K. Garrettson. *Drug Metab. Disp.* 12:792–794 (1984).
4. W. H. Soine, P. J. Soine, B. W. Overton, and L. K. Garrettson. *Drug Metab. Disp.* 14:619–621 (1986).
5. D. Kadar, B. K. Tang, and A. W. Conn. *Can. Anaes. Soc. J.* 29:16–23 (1982).
6. V. O. Bhargava, W. H. Soine, and L. K. Garrettson. *J. Chromatogr.* 343:219–223 (1985).
7. B. K. Tang, B. Yilmaz, and W. Kalow. *Biomed. Mass Spectrom.* 11:462–465 (1984).
8. V. O. Bhargava and L. K. Garrettson. *Dev. Pharm. Ther.* 11:8–13 (1988).
9. W. H. Soine, P. J. Soine, T. M. England, B. W. Overton, and S. Merat. *Carbohydr. Res.* 193:105–113 (1989).
10. C. R. Clark and J.-L. Chan. *Anal. Chem.* 50:635–637 (1978).
11. F. B. Vest, W. H. Soine, R. B. Westkaemper, and P. J. Soine. *Pharm. Res.* 6:458–465 (1989).
12. J. Knabe and K. Philipson. *Arch. Pharm.* 299:231–242 (1966).
13. J. Avolio and R. Rothchild. *J. Magn. Res.* 58:328–333 (1984).
14. J. Knabe, H. Junginger, and W. Geismar. *Liebigs Ann. Chem.* 739:15–26 (1970).
15. B. K. Tang, T. Inaba, and W. Kalow. *Biomed. Mass Spectrom.* 4:73–76 (1977).
16. J. H. McGuire, T. C. Butler, and K. H. Dudley. *Drug Metab. Disp.* 10:595–598 (1982).
17. M. T. Bush and E. Helman. *Life Sci.* 4:1403–1409 (1965).
18. E. W. Maynert. *J. Biol. Chem.* 195:397–402 (1952).
19. R. K. Lynn, J. E. Bauer, W. P. Gordon, R. G. Smith, D. Griffin, R. M. Thompson, R. Jenkins, and N. Gerber. *Drug Metab. Disp.* 7:138–144 (1979).
20. P. J. Wedlund, W. S. Aslanian, E. Jacqz, C. B. McAllister, R. A. Branch, and G. R. Wilkinson. *J. Pharmacol. Exp. Ther.* 234:662–669 (1985).
21. B. Silber and S. Riegelman. *J. Pharmacol. Exp. Ther.* 215:643–648 (1980).
22. P. Seideman, O. Ericsson, K. Groningsson, and von C. Bahr. *Acta Pharm. Toxicol.* 49:200–204 (1981).
23. S. F. Sisenwine, C. O. Tio, F. V. Hadley, A. L. Liu, H. B. Kimmel, and H. W. Ruelius. *Drug Metab. Disp.* 10:605–608 (1982).
24. T. Walle, U. K. Walle, M. J. Wilson, T. C. Fagan, and T. E. Gaffney. *Br. J. Clin. Pharmacol.* 18:741–747 (1984).
25. E. Dumont, C. von Bahr, T. L. Perry, and L. Bertilsson. *Pharmacol. Toxicol.* 61:335–341 (1987).
26. M. El Mouelhi, H. W. Ruelius, C. Fenselau, and D. M. Dulik. *Drug Metab. Disp.* 15:767–772 (1987).
27. E. Nusser, K. Nill, and U. Breyer-Pfaff. *Drug Metab. Disp.* 16:509–511 (1988).