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HPLC analysis for amobarbital *N*-glycosides in urine

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Abstract

A study was undertaken to determine if humans excrete both amobarbital *N*-glucuronides and *N*-glucosides in urine after an oral dose of amobarbital. Amobarbital *N*-glucuronides were synthesized and characterized. A reverse phase LC method using post-column pH adjustment and UV detection at 240 nm was developed and used for the quantification of the amobarbital *N*-glucosides and *N*-glucuronides in human urine. Amobarbital was administered orally to seven male subjects and the total urine was collected for a period of 48–53 h after dosing. After filtration, the urine was injected directly onto the HPLC column to analyze for the presence of metabolites. The previously identified (5*S*)-amobarbital *N*-glucoside was detected in all seven subjects. The (5*R*)-amobarbital *N*-glucoside was detected at lower concentrations in only four of the subjects. At the levels at which amobarbital *N*-glucosides were detected, there was no evidence for the formation and excretion of the amobarbital *N*-glucuronides. Amobarbital *N*-glucuronidation is not a quantitatively significant pathway for the biodisposition of amobarbital in humans. © 1997 Elsevier Science B.V.

Keywords: Amobarbital *N*-glucoside; Amobarbital *N*-glucuronide; Human metabolism; Amobarbital metabolism

1. Introduction

The identification of a limited number of glucoside conjugates of chemicals by mammals suggests that glucosylation is a minor pathway for their elimination [1,2]. Often the compounds forming β-glucosides are shown to preferentially form β-glucuronides, for example, *p*-nitrophenol, pranoprofen, and phenobarbital in mice [2–4], and bile acids in humans [5]. Some compounds form exclusively β-glucoside conjugates, such as pantothenic acid in dogs [6], 3-(4-pyrimidinyl)-5-(4-pyridyl)-1,2,4-triazole in rat, dog and monkey [7] and specific sulfonamides in humans [8]. To show that a drug is excreted exclusively as a glucoside is difficult since often glucuronide conjugates are not available as standards to prove their absence [8]. At this time there are no guidelines that allow prediction of whether a compound will be eliminated as glucoside or glucuronide conjugates. Each compound must be evaluated experimentally in the specific species of interest to determine the preferential route of excretion.

The barbiturates are widely used clinically, yet in humans the metabolic biodisposition of this class of drugs has not been completely elucidated. The major Phase I metabolites of most 5,5-disub-

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stituted barbiturates in humans result from oxidation of the substituents at the C-5 position of the barbiturate ring [9]. The major Phase II metabolites of the parent barbiturates result from conjugation of the ring nitrogen with glucose [2,10–12]. In humans, the formation of the *N*-glucoside conjugates of amobarbital (*I*) and phenobarbital can be quantitatively as important as the oxidative pathway in their biodisposition [2,10,12,13]. However, there still remains a significant portion of a barbiturate dose that has not been identified (up to 50% of the dose depending on the barbiturate and species).

The elimination of amobarbital in humans is reported to be $\geq 95\%$ by metabolism, with excretion of the metabolites almost exclusively via urine [14]. Approximately 66–94% of an oral dose of amobarbital can be accounted for in humans, with the amobarbital *N*-glucoside conjugates accounting for 5–38% of the dose [2,10]. Some of the variability associated with the formation or excretion of amobarbital *N*-glucosides may be due to a genetically controlled recessive trait. There also appears to be an ethnic difference in the biodisposition of amobarbital independant of the apparent genetic deficiency [2]. Amobarbital appears to be one of those compounds that form β-glucoside conjugates, but not β-glucuronide conjugates, since no *N*-glucuronides of amobarbital have been reported. That amobarbital *N*-glucuronides have not been identified as metabolites of amobarbital may be because the conjugates are not formed and excreted or they may not have been previously detected depending on the method of analysis (LC, enzymatic assays, GC/MS) [10,14–16]. The hypothesis that *N*-glucuronidation could be an important metabolic pathway for amobarbital was supported by reports of *N*-glucuronidation of phenobarbital in mice [4], of 5-ethyl-5-phenyl hydantoin in dogs [17] and of 5,5-diphenylhydantoin in rats and man [18]. This study was performed to determine if amobarbital *N*-glucuronide(s) are significant metabolic disposition products of amobarbital in humans. The amobarbital *N*-glucuronides were synthesized and an HPLC method was developed for the detection and quantification of amobarbital *N*-glycosides in human urine. It was observed that amobarbital *N*-glucuronides were not excreted in urine as significant metabolites of amobarbital.

2. Experimental

2.1. Chemistry

Dichloroethane and dichloromethane were dried over CaH_2 and distilled from P_2O_5 over nitrogen. Amobarbital and glucurono-3,6-lactone were purchased from Sigma (St. Louis, MO). Amobarbital *N*-glucoside ((5*R*)- and (5*S*)-AMG) was synthesized by the method of Soine et al. [19]. 3′-Hydroxyamobarbital was available from a previous study [20]. Celite 521 and trimethylsilyl trifluoromethanesulfonate were purchased from Aldrich (Atlanta, GA). Acetonitrile was Mallinckrodt HPLC grade (Scientific Products, McGaw Park, IL). All other reagents were analytical grade unless otherwise specified.

Melting points were determined on a Thomas Hoover Unimelt apparatus (Philadelphia, PA), in an open capillary and are uncorrected. Reactions were routinely monitored by TLC using silica gel GHLF plates (250 μm, 2.5 × 10 cm) (Analtech Inc., Newark, DE). Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA). Infra-red spectra were recorded on a Nicolet 5ZDX FT-IR spectrometer (Madison, WI). ^{1}H- and ^{13}C-NMR spectra were obtained on a 300 MHZ General Electric QE-300 FT-NMR spectrometer (Fremont, CA). Proton and carbon assignments are based on COSY and HETCOR experiments. Chemical shifts are in parts per million (δ) relative to tetramethylsilane in deuterochloroform ($CDCl_3$) or the HOD absorbance in deuterium oxide. Coupling constants are expressed in Hz.

LC analyses were conducted on a Beckman System Gold Liquid Chromatograph (San Ramon, CA), with a C_{18} reverse phase column (250 × 4.6 mm i.d., particle size 5 μm, Econosil, Alltech Associates, Deerfield, IL) and a Pellicular C_{18} guard column (20 × 2 mm i.d., particle size 37–53 μm, Whatman). The system consisted of a Programmable Model 507 Autosampler, an

Analog Interface Module 406, a UV/Vis Diode Array Programmable Detector 168, and a 110 B Solvent Delivery Module. The mobile phase flow rate was 1.4 ml min^{-1}. The column temperature was maintained at 25°C using an Alltech water jacket and a Brinkmann Instruments water circulator (Westbury, NY).

2.1.1. 5-Ethyl-5-(3′-methylbutyl)-1-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate)-2,4,6-(1H,3H,5H)pyrimidinetrione; II(A,B)

Methyl-1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranosyluronate was synthesized using a minor modification of the procedure of Bollenback et al. [21]. The methyl-1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranosyluronate (17.3 g, 45.9 mmol) was dissolved in freshly distilled dry methylene chloride (80 ml) under a nitrogen atmosphere. Freshly distilled persilylated amobarbital (15.6 g, 42.1 mmol) [19], was added to the solution with the exclusion of moisture. The reaction flask was placed in an ice bath followed by dropwise addition of trimethylsilyl trifluoromethanesulfonate (4.3 ml, 22.1 mmol). The reaction mixture was stirred and slowly allowed to warm to room temperature. The reaction was monitored by TLC every hour and after 3 h the reaction appeared to be complete (diethyl ether, $Rf_{II(A,B)} = 0.72$, $Rf_I = 0.81$). The reaction mixture was diluted with 80 ml of methylene chloride and carefully poured into a beaker containing 100 ml of water and crushed ice. The resulting mixture was stirred vigorously for 15 min, neutralized with saturated solution of $NaHCO_3$, and the emulsion formed was filtered over a layer of sand-Celite and the organic phase was separated and dried over anhydrous Na_2SO_4. The methylene chloride layer was filtered and evaporated under reduced pressure (72 mm Hg) at 40°C to give a sticky yellow material. The residue was treated with anhydrous ether to precipitate a mixture of amobarbital methyl-tri-*O*-acetyl-β-D-glucuronate diastereomers, II(A,B) (9.1 g, 16.8 mmol, 40% yield). The precipitate was recrystallized from methanol m.p. 169–170°C. ^{1}H-NMR ($CDCl_3$): δ 0.75 (*t*, $J = 8.1$, 3H, H_3-8), 0.85 (*t*, $J = 8.1$, 6H, CH_3-12), 0.9–1.0 (m, 2H, CH_2-10), 1.4 (m, 1H, H-11), 1.90–2.10 (m, 13H, $3COCH_3$ and $2H_2$-7), 3.68 (s, 3H, OCH_3), 4.18 (br.d, $J = 12.2$ Hz, 1H, H-5′), 5.0–5.1 (*t*, $J = 10.2$ Hz, 1H, H-2′), 5.1–5.3 (m, 2H, H-4′, 3′), 5.72 (*d*, $J = 10$, 1H, H-1′), 8.73 (s, 1H, NH), 8.87 (minor s, NH). IR (KBr): cm^{-1} 1766 (strong), 1730 (s), 1711 (s), 1703 (s), 1249 (s), 1229 (s). ^{13}C-NMR ($CDCl_3$) δ 9.6 (C-8), 20.9 (C(=O)$\underline{C}H_3$), 22.6 (C-12), 28.5 (C-11), 33.8, 33.9, 34.07 (C-7, C-9, C-10), 53.3 (OCH_3), 58.9 (C-5), 69.3 (C-2′), 70.6 (C-3′), 72.3 (C-4′, C-5′), 81.8 (C-1′), 149.0 (C-2), 166.9 (C-6′), 169.0, 170.0 (C4, C6, $\underline{C}(=O)CH_3$). The sample was analyzed by HPLC in a mobile phase of acetonitrile-H_2O (40:60, v/v). Two chromatographic peaks were observed at 15.3 and 15.7 min, corresponding to II(A) and II(B), respectively.

2.1.2. 5-Ethyl-5-(3′-methylbutyl)-1-(methyl-β-D-glucopyranosyluronate)-2,4,6-(1H,3H,5H)-pyrimidinetrione; III(A,B)

Compound II(A,B) (5 g, 9.22 mmol) was suspended in 45 ml of dry MeOH and 1 ml of concentrated H_2SO_4 and heated to 70°C. The reaction was complete in 5 h (TLC, diethyl ether, $Rf_{III(A,B)}$ 0.15; HPLC, mobile phase of acetonitrile-H_3PO_4 (pH 2.25; 0.025 M (25:75, v/v), $t_{RIII(A)}$ 12.0 min, $t_{RIII(B)}$ 13.9 min). The mixture was cooled and concentrated under reduced pressure, and extracted with methylene chloride (9×15 ml), followed by ethyl acetate (10×15 ml). The ethyl acetate fractions were combined and evaporated under reduced pressure to give 1.48 g (3.55 mmol, 39% yield) of the product III(A,B); mp 87–88°C. ^{1}H-NMR ($CDCl_3$): δ 0.75 (*t*, $J = 8.1$, 3H, CH_3-8), 0.85 (*t*, $J = 8.1$, 6H, CH_3-12), 0.9–1.0 (m, 2H, CH_2-10), 1.3–1.4 (m, 1H, CH-11), 1.78–2.05 (m, 4H, $2CH_2$-7), 3.68–3.71 (m, 2H, H-3′, 4′), 4.12–4.24 (m, 1H, H-5′), 5.74 (*d*, $J = 10.2$, 1H, H-1′), 8.73 (s, 1H, NH). IR (KBr): cm^{-1} 1757 (s), 1731 (s), 1258 (s). ^{13}C-NMR ($CDCl_3$) δ 9.9 (C-8), 22.6 (C-12), 28.5 (C-11), 33.8, 33.9, 34.1 (C-7, C-9, C-10), 53.3 (OCH_3), 58.9 (C-5), 69.3 (C-2′), 71.7 (C-3′), 72.3 (C-4′, C-5′), 82.3 (C-1′), 149.6 (C-2), 170.1 (C-6′), 172.3, 173.6 (C-4, C-6). Analysis calculated for $C_{18}H_{28}N_2O_9 \cdot 1H_2O$:C, 49.75; H, 6.96; N, 6.45. Found: C, 49.86; H, 6.92; N, 6.46.

2.1.3. 5-Ethyl-1-(β-D-glucopyranosyluronate)-5-(3'-methylbutyl)-2,4,6-(1,3H,5) pyrimidinetrione; IV(A,B)

Compound II(A,B) (3 g, 5.53 mmol) was suspended in 50 ml of 10% HCl and the reaction mixture was stirred at reflux. After 10 h the reaction appeared to be complete based on disappearance of II(A,B) and III(A,B) (TLC, diethyl ether, Rf IV(A,B) = 0.04; HPLC mobile phase of acetonitrile-H_3PO_4 (pH 2.25; 0.025 M) (25:75, v/v), $t_{RIV(A)}$ 6.47 and $t_{RIV(B)}$ 7.14 min). Amobarbital (t_{RI} 13.8 min) and traces (0.7%) of III(A,B) were detected. The reaction mixture was cooled, concentrated under reduced pressure to a volume of 10 ml and extracted with methylene chloride (5 × 15 ml) and ethyl acetate (6 × 20 ml). The ethyl acetate fractions were pooled, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to give IV(A,B) (1.34 g, 3.33 mmol, 60% yield), mp 123–124°C. ^{1}H-NMR (D_2O): δ 0.81 (*t*, $J = 8.1$, 3H, CH_3-8), 0.86 (*t*, $J = 8.1$, 6H, CH_3-12), 0.98–1.08 (m, 2H, CH_2-10), 1.38–1.46 (m, 1H, CH-11), 1.87–2.05 (m, 4H, $2CH_2$-7), 3.59 (*t*, $J = 10.2$, 1H, H-3'), 3.69 (*t*, $J = 10.1$, 1H, H-4'), 4.02–4.11 (m, 1H, H-5'), 4.92–5.02 (br.t., 1H, H-2'), 5.58 (*d*, $J = 10.2$, 1H, H-1'), 5.67 (*d*, $J = 10.2$, 1H, H-1'). IR (KBr): cm^{-1} 3450–3270, 1724 (s), 1707 (s). ^{13}C-NMR (D_2O) δ 9.2 (C-8), 22.17 (C-12), 28.12 (C-11), 33.32, 33.96, 36.47 (C-7, C-9, C-10), 59.07 (C-5), 69.08 (C-2'), 71.54 (C-3'), 78.33, 78.36 (C-4', C-5'), 82.09 (C-1'), 150.1 (C-2), 173.13, 174.05 (C-4, C-6), 180.02 (C-6'). λ_{max} 202 nm, pH 2.25 ($\epsilon = 1.65 \times 10^4$ L·cm^{-1} × mol^{-1}), λ_{max} 240 nm, pH 10.3 ($\epsilon = 8.28 \times 10^3$ l·cm^{-1}·mol^{-1}). Analysis calculated for $C_{17}H_{26}N_2O_9 \cdot 1H_2O$: C, 48.57; H, 6.71; N, 6.66. Found: C, 48.95; H, 6.78; N, 6.08.

2.2. Biological methods

2.2.1. Materials

Phosphoric acid, sodium hydroxide and sodium phosphate, monobasic, were of HPLC grade from Fisher Scientific (Pittsburg, PA). Sodium borate, decahydrate, was purchased from J.T. Baker (Philipsburg, NJ). Acetonitrile was Mallinckrodt HPLC grade (Scientific Products, McGaw Park, IL). Citric acid, analytical grade, was purchased from Sigma Chemicals (St. Louis, MO). All other reagents and solvents were analytical grade unless otherwise specified. Urine samples were filtered through 0.45 μm Acrodisc LC 13 filter (Gelman Sciences, Ann Arbor, MI).

2.2.2. Chromatographic conditions

The chromatographic analyses were conducted on a Beckman System Gold Liquid Chromatograph described under 'Synthetic Chemistry'. The injection volume was 25 μl. The mobile phase was acetonitrile-H_3PO_4 (pH 2.25; 0.025 M) (14:86, v/v), at a flow rate of 1.4 ml min^{-1}. pH adjustment was achieved by the insertion of a mixing column filled with glass beads (50 × 2 mm i.d., bead size 75 μm) after the column effluent. A 0.1 N borate buffer, pH 10.3, was introduced at a flow rate of 0.3 ml min^{-1} (Beckman 110A pump) via a Swagelok tee inserted in the line between the analytical column and the mixing column. The column effluent was monitored at 240 nm with a band-width of 4 nm. The UV scans of the diastereomers of amobarbital *N*-glucoside and amobarbital *N*-glucuronide were obtained in the region between 210 and 250 nm using the photodiode array detector. Stock solutions of amobarbital *N*-glucuronide (1.19 mM) and amobarbital *N*-glucoside (1.73 mM) were prepared in acetonitrile-H_3PO_4 (pH 2.25; 0.025 M) (30:70, v/v) and were stored refrigerated. During analysis, standards (25 nmol ml^{-1}) of (5*R*)- and (5*S*)-AMG and IV(A,B) were run after every sixth sample to monitor any shift in retention times. The acidified urine samples were filtered through a 0.45 μm filter immediately before analysis. All samples were run in duplicate. Accuracy of the assay was evaluated by determining the percent standard errors (% S.E.) of estimation for (5*R*)-, (5*S*)-AMG, IV(A), and IV(B). The lower limit of quantitation (LOQ) was based on the criteria that the mean experimental concentration should be within 15% (20% at the LOQ) of the theoretical concentration and the % CV should not exceed 15% (20% at the LOQ) [22,23]. The limit of detection (LOD) was based on the baseline noise determined as S_B, the standard deviation, using blank urines ($n = 9$). Using the equation for LOD at 99.86% confidence interval (LOD = 3 × S_B/sensitivity), LOD was estimated [22,23].

2.2.3. Urine collection

Seven healthy male volunteers were recruited to participate in the study with approval of the Committee on the Conduct of Human Research, VCU. The subjects were both Caucasian ($n = 3$) and Asian ($n = 4$) with ages ranging from 24 to 48 years (mean 28.4 years). A medical history was obtained and laboratory screening tests (free and conjugated bilirubin, δ-glutamyltransferase) were obtained prior to the study and were within normal limits. The subjects had received no barbiturate or imide drug for a minimum of 30 days before the study. They were instructed not to take any medication for 3 days prior to dosing and during the study. Alcohol consumption was prohibited 24 h before the study and for the remainder of the urine collection period.

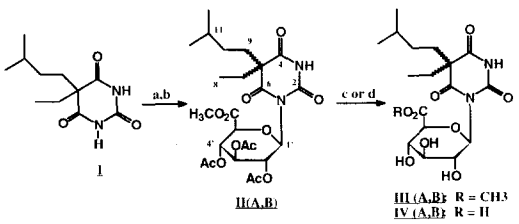
Each subject received an oral dose of 100 mg amobarbital sodium, sterile, U.S.P. (Eli Lilly and Co., Indianapolis, IN) packed *secundem artem* in a gelatin capsule. Subject no. 1 also received an oral dose of 200 mg amobarbital sodium, pulvules (Eli Lilly and Co, Indianapolis, IN). The drug was taken just prior to retiring for the night. Blank urine samples were collected prior to drug administration. Complete urine samples were collected in the presence of approximately 10 g of citric acid, to a resultant pH of 2.5–3.0. These samples were collected in approximately 12 h fractions and for a period of 48–53 h from the time of dosing. The volume of each sample was measured and 15 ml of each sample was separately frozen at −20°C until analysis.

3. Results

The amobarbital methyl triacetyl *N*-glucuronates were synthesized as a mixture of diastereomers (see Scheme 1) under condensation conditions developed by Vorbrüggen and Krolikiewicz [24]. Selective hydrolysis of II(A,B) in anhydrous methanol in the presence of conc. H_2SO_4 gave amobarbital methyl *N*-glucuronates (III(A,B)), or complete hydrolysis of II(A,B) with 10% HCl at 80°C gave amobarbital *N*-glucuronides (IV(A,B)).

Subject no. 1 was initially administered sodium amobarbital (200 mg). Attempts to isolate and detect IV(A,B) from the 0–24 and 24–48 h urine using solid phase extraction (XAD-2, Whatman C_{18} (Partisil ODS-5 and Partisil*ODS), Partisil C_8 columns, and Whatman strong anion exchange (SAX) columns) and liquid-liquid extraction [19] were unsuccessful.

The assay was developed for the simultaneous detection of (5*R*)- and (5*S*)-AMG and IV(A,B) by evaluating the effect of different concentrations of acetonitrile/buffer at different column temperatures. When using acetonitrile-H_3PO_4 (pH 2.25; 0.025 M) (14:86, v/v) at 25°C, the diastereomers of (5*R*)-and (5*S*)-AMG elutes at 31.9 ± 0.1 and 36.5 ± 0.1 min ($R_s = 1.4$) and IV(A,B) elutes at 34.1 ± 0.1 and 39.1 ± 0.1 min ($R_s = 2.6$). The amobarbital *N*-glucosides are also well resolved from the amobarbital *N*-glucuronides ($R_s = 1.4$ for (5*R*)-AMG and IV(A); $R_s = 1.6$ for (5*S*)-AMG and IV(B), see Fig. 1a). Sample preparation consisted of filtration to remove precipitates from the urine, and injection of the urine directly on to the column. When monitoring the column effluent at a wavelength of 202 nm, absorbances were observed in the blank urine that interfered with the detection and quantification of (5*R*)- and (5*S*)-AMG and IV(A,B). With post-column pH adjustment of the column effluent to an apparent pH of 10–10.3, analysis of blank urine at 240 nm resulted in fewer endogenous interferences from urine (see Fig. 1b). A small peak was occasionally observed in the urine of some individuals as a shoulder just prior to the elution of (5*S*)-AMG



Scheme 1. Synthesis of amobarbital *N*-glucuronides. (a) HMDS, TMSCl; (b) $CF_3SO_3Si(CH_3)_3$, Methyl-1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranosyluronate; (c) CH_3OH, H_2SO_4 for $R = CH_3$; (d) 10% HCl for $R = H$.

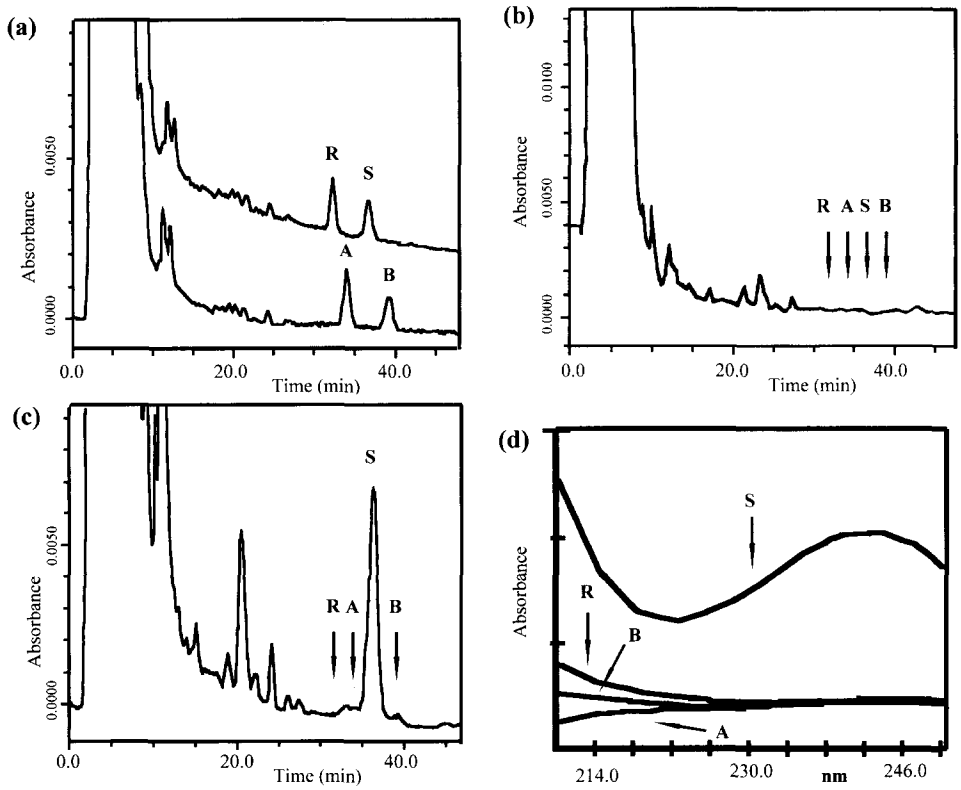


Fig. 1. (a) Chromatograms of the standards of (5*R*)-AMG (R), (5*S*)-AMG (S), IV(A) (A), and IV(B) (B) at 10 nmol ml^{-1} dissolved in urine acidified with citric acid; (b) Chromatogram of blank urine acidified with citric acid; (c) Chromatogram of Subject no. 7 urine obtained from 12.2 to 23.2 h after dosing with 100 mg sodium amobarbital ((5*S*)-AMG-38.3 nmol ml^{-1}, (5*R*)-AMG, IV(A) and IV(B) not detected); (d) UV scans of chromatographic peaks corresponding to retention times of (5*R*)-, (5*S*)-AMG, IV(A) and IV(B) shown in chromatogram (c). (The periodic pulsing due to post-column pH adjustment has been subtracted from the chromatogram).

(0.4–0.5 min, $R_s = 0.3$). When post-column pH adjustment was used, a periodic pulsing (due to the post-column pump) was observed in the chromatogram. At maximum sensitivity, the periodic nature of the pulse (every 27 s) could be readily differentiated from the random noise. In Fig. 1, the periodic pulsing has been subtracted from the chromatogram.

The accuracy and precision of the analytical method was determined by analyzing for (5*R*)- and (5*S*)-AMG and IV(A,B) dissolved in blank urine samples from each subject ($n = 7$). For measurement of noise, only the random baseline noise was considered. The percent coefficient of variation (%CV) for (5*R*)- and (5*S*)-AMG was 1.5 and 4.5% at 40 nmol ml^{-1}, 5.9 and 7.0% at 25 nmol ml^{-1}, and 16.5 and 11.0% at 3 nmol ml^{-1}, respectively. The % CV for IV(A) and IV(B) was 6.0 and 4.9% at 40 nmol ml^{-1}, 9.3 and 13.0% at 25 nmol ml^{-1}, and 14.0 and 17.7% at 3 nmol ml^{-1}. Accuracy of the assay (%S.E.) for (5*R*)- and (5*S*)-AMG was 2.1 and 2.3% at 40 nmol ml^{-1}, 5.2 and 5.9% at 25 nmol ml^{-1} and 8.3 and 9.5% at 3 nmol ml^{-1}, respectively. The %S.E. for IV(A) and IV(B) was 3.4 and 4.0% at 40 nmol ml^{-1}, 6.3 and 7.2% at 25 nmol ml^{-1}, and 8.9 and 10.1% at 3 nmol ml^{-1}. The lower limit of quantitation (LOQ) was determined to be 3 nmol ml^{-1}.

Calibration standards were prepared in blank urine for (5*R*)- and (5*S*)- AMG at concentrations of 3.3, 6.5, 13.0, 26.0, 52.0, and 104.0 nmol ml^{-1}, and for IV(A) and IV(B) at concentrations of 3.1,

Table 1
Urinary percentages of amobarbital metabolites excreted by humans

Subject no.	Ethnic origin	Time (h)	(5*S*)-AMG[a]	(5*R*)-AMG[a]	AMGR A	AMGR B
1	C[b]	0–23.2	13.9	ND[d]	ND	ND
		23.2–38.7	6.9	ND	ND	ND
		38.7–48.0	2.1	ND	ND	ND
2	C	0–22.8	12.2	0.7	ND	ND
		22.8–39.5	6.5	ND	ND	ND
		39.5–46.3	<LOQ[e]	ND	ND	ND
3	C	0–23.0	12.7	1.2	ND	ND
		23.0–42.3	8.9	ND	ND	ND
		42.3–50.3	<LOQ	ND	ND	ND
4	A[c]	0–24.7	12.6	ND	ND	ND
		24.7–48.0	6.6	ND	ND	ND
5	A	0–24.0	13.1	ND	ND	ND
		24.0–48.0	3.3	ND	ND	ND
6	A	0–22.2	9.0	0.7	ND	ND
		22.2–45.2	8.2	ND	ND	ND
		45.2–52.7	2.5	ND	ND	ND
7	A	0–23.2	12.8	1.3	ND	ND
		23.2–48.1	6.9	ND	ND	ND
Mean		0–48.0	19.8	0.9	ND	ND
S.D.		0–48.0	2.1	0.3	ND	ND

[a]Percentage of 100 mg dose.
[b]Caucasian.
[c]Asian.
[d]Not detected.
[e]<LOQ, below limit of quantitation.

6.3, 12.5, 25.0, 50.0 and 100.0 nmol ml^{-1}. Calibration curves were constructed for each analyte by plotting chromatographic peak height versus the nominal concentration of the calibration standards and fitting curves to the data using linear regression. For each glycoside analyzed, the standard response was linear with a $r^2 \geq 0.996$. The limit of detection (LOD) was estimated to be 1.1 and 1.5 nmol ml^{-1} for (5*R*)- and (5*S*)-AMG and 1.3 and 2.3 nmol ml^{-1} for IV(A) and IV(B), respectively [23,24]. (5*R*)-AMG, (5*S*)-AMG, IV(A) and IV(B) could be differentiated from the urinary interferences present in blank urine when present at 1 nmol ml^{-1} by visual evaluation of the UV spectrum collected at the retention time of the analytes.

The urine samples for all of the individuals displayed a chromatographic peak for (5*S*)-AMG at 36.5 ± 0.1 min with a λ_{max} at 240 nm. A representative chromatogram and UV is shown in Fig. 1c and 1d. In four of the seven individuals, (5*R*)-AMG was detected at 31.9 ± 0.1 min with a λ_{max} at 240 nm. Chromatographic peaks at 34.1 ± 0.1 and 39.1 ± 0.1 were observed in some samples, but the UV spectrum of the peaks did not exhibit a λ_{max} at 240 nm. The presence of IV(A) or IV(B) could not be confirmed as they were not detected in any of the urine samples (see Table 1).

4. Discussion

In our studies to understand the role of glucosylation in metabolism of xenobiotics, it is important to determine the relative importance of other complementary pathways, such as glucuronida-

tion. Intuitively, if amobarbital is a substrate for glucosylation, it has potential to be a substrate for glucuronidation, since the mechanism by which β-glucosides are formed is believed to be an SN_2 displacement reaction similar to that proposed for glucuronidation [6,7,25]. Glucuronides are readily formed for most lipophilic nucleophiles [25]. Amobarbital is lipophilic (log $P = 2.07$) and a sufficient ratio of the lactim $\leftrightarrows$ lactam tautomers exists making the nitrogen sufficiently nucleophilic that conjugation with the nitrogen has been shown to occur [10,14]. Since it was anticipated that amobarbital would form *N*-glucuronide conjugates, the study was designed to isolate and characterize the amobarbital *N*-glucuronides, then develop an assay to quantify the importance of the pathway in the biodisposition of amobarbital. The specific synthetic targets were the diastereomers of III(A,B) and IV(A,B). If IV(A,B) was detected, its conversion to III(A,B) was intended to aid in isolation and provide additional evidence for the presence of the glucuronyl moiety.

The spectroscopic characterization and acid stabilty of IV(A,B) was consistent with that reported for structurally related 5,5-disubstituted barbiturate *N*-glucuronides and *N*-glucosides [10,26,27]. Since the amobarbital *N*-glucosides had exhibited facile hydrolysis of the barbiturate ring under neutral or weakly alkaline conditions (1–6 ring opening) [26], the acid stability of the amobarbital *N*-glucuronides allowed acidification of the urine with citric acid to minimize any decomposition of the *N*-glycoside conjugates prior to analysis. Based on the relative chromatographic retention times of the amobarbital *N*-glucuronides, the diastereomers were termed A and B, with A representing the early eluting diastereomer, and B representing the late eluting diastereomer. The absolute stereochemistry of the individual diastereomers of IV(A,B) was not determined.

The approach was to isolate IV(A,B) by concentrating the urine and extraction of the metabolite(s) using solid phase extraction methods or continuous liquid-liquid extraction. These procedures were successful when isolating the synthetic standards of IV(A,B) dissolved in urine at a concentration of 10 nmol ml^{-1}. However, after processing the urine from subject no. 1 after dosing with 200 mg of sodium amobarbital, the presence of IV(A,B) could not be confirmed, although compounds with similar retention times to IV(A,B) were observed.

The failure to detect IV(A,B) could have been due to: (1) it being below our limit of detection and was escaping detection as a result of extensive sample manipulation, or (2) the single individual being tested was deficient in being able to form IV(A,B) or was excreting it at very low levels (although this individual was known to excrete (5*S*)-AMG from prior studies). To evaluate these possibilities an LC method was developed in which the individual urine samples could be readily screened for IV(A,B) with minimal loss prior to analysis. The analytical method developed was to directly inject the urine sample on the LC. Analysis of the urine at 200 nm revealed the presence of numerous compounds which closely eluted with IV(A,B). The interferences observed in the chromatogram were minimized by using a post-column pH adjustment method developed by Clark et al. [28] for the improved detection of barbiturates by LC. By monitoring the column effluent at a wavelength of 240 nm, the detection of the amobarbital *N*-glycosides at concentrations previously observed for (5*R*)- and (5*S*)-AMG was possible. The lower limit of quantitation, using post-column pH adjustment, was identified to be 3 nmol ml^{-1} for both (5*R*)- and (5*S*)-AMG, IV(A) and IV(B). Analysis of the UV scans of the chromatographic peaks allowed for identification and differentiation of the *N*-glucosides and *N*-glucuronides from the background interferences at levels of 1 nmol ml^{-1}. The other metabolite of amobarbital, 3′-hydroxyamobarbital [29], eluted prior to the amobarbital *N*-glycosides and did not interfere with the assay. No other chromatographic peaks were observed in the chromatogram that were associated with the dosing of amobarbital.

The observation of a racial difference in the metabolism of amobarbital [2,30] required that both Asians and Caucasians be included in this study. The urine samples from seven individuals (3 Caucasians, 4 Asians) were analyzed. The half life of amobarbital in males is reported to be

22.7 ± 1.6 h [29]. Since 20.7 ± 2.4% of the dose was accounted for as the amobarbital *N*-glucosides after approximately two half lifes, these results are consistent with comparable studies [2,10,14]. A 'product enantioselectivity' was observed for the metabolism of amobarbital with (5*S*)-AMG being formed almost exclusively. No evidence was obtained for the formation or excretion of IV(A,B) in either the Asian or Caucasian subjects. The inability to detect IV(A,B) in the urine does not rule out its formation, however, it does show that *N*-glucuronidation is of negligible importance in the biodisposition of amobarbital. In vitro studies of barbiturate metabolism using liver enzyme preparations, in conjunction with in vivo studies would be beneficial in understanding the importance of *N*-glycosylation with respect to the other oxidative and conjugative pathways for the metabolic biodisposition of the barbiturates.

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