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Note

High-performance liquid chromatographic determination of the diastereomers of 1-(β-D-glucopyranosyl) amobarbital in urine

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Amobarbital, a commonly used barbiturate, has been shown to undergo a novel conjugation with glucose during its metabolism in humans [1–3]. Since glucose is being coupled to a barbiturate with two different substituents at the 5-position, these conjugates will exist as two diastereomers, as shown in Fig. 1. A previous study suggests that one of these diastereomers appears to be formed and/or excreted almost exclusively [4]. To facilitate further study of this novel metabolite(s), an isocratic high-performance liquid chromatographic (HPLC) method was developed for detection and quantification of the diastereomers of the N-glucose conjugate of amobarbital in urine. This method is described in this report.

EXPERIMENTAL

Amobarbital was purchased from Mallinckrodt (St. Louis, MO, U.S.A.). 3′-Hydroxyamobarbital was synthesized by the method of Maynert [5] and repeated recrystallizations gave a white crystalline material with a melting point of 185–186°C (lit. 187–188°C). Analysis of this sample with the HPLC method described in this paper indicated that at least five other compounds were present in small quantities; however, the crystalline material was approximately 82% of the desired compound based on total peak areas. The identity of the synthetic 3′-hydroxyamobarbital was confirmed by gas chromatography–electron-impact mass spectrometry (GC–EIMS) of the persilylated compound (m/z 458). No further purification was carried out on this metabolite. The 1-(β-D-glucopyranosyl) amobarbitals were synthesized and purified as previously described [4]. AMO

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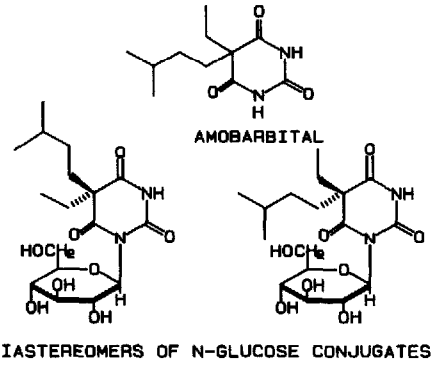

Fig. 1. Structures of amobarbital and the diastereomers of 1-(β-D-glucopyranosyl)amobarbital.

A refers to the early eluting conjugate (14.2 min) and AMO B refers to the later eluting conjugate (15.9 min) when using the following analytical system. For each of the N-glucose conjugates, greater than 98% of the peak area was associated with a single diastereomer. Butalbital was purchased from Sigma (St. Louis, MO, U.S.A.), and α-methyl-α-phenylsuccinimide was purchased from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile and monobasic and dibasic sodium phosphate (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). All other chemicals were reagent grade.

The liquid chromatograph (Model 1330, Bio-Rad Labs.) was equipped with a variable-wavelength detector (Model 1306, Bio-Rad Labs.), and separation was achieved using a C_{18} reversed-phase column (250×4.6 mm I.D. cartridge, particle size 5 μm, Econosphere C_{18}, Alltech Assoc.) with a C_{18} guard column (20×2 mm I.D., particle size 30–40 μm, Perisorb RP-18, Upchurch Scientific). The mobile phase was a solution of 20% (v/v) acetonitrile in 0.025 *M* sodium phosphate buffer, pH 6.5. The flow-rate was 1.4 ml/min, and the effluent was monitored at 198 nm with a digital integrator (Model 3393A integrator, Hewlett-Packard). The volume of sample injected was 20 μl (WISP Model 712, Waters Assoc.). The analysis was carried out at 25.0°C, and constant temperature was maintained using a circulating water jacket (Lauda K-2/R, Brinkmann).

Individual stock solutions (4 m*M*) of amobarbital, AMO A, AMO B, α-methyl-α-phenylsuccinimide and butalbital (9.0 m*M*) were prepared in methanol and stored at −20°C. The solutions were stable for at least four months under these conditions. Standards were prepared by dilution of known amounts of stock solution with methanol, transferring known amounts of this solution to a screw-cap test tube, evaporating the methanol under a stream of nitrogen and dissolving the residue in mobile phase, water or urine.

To prepare the sample for analysis, 5.0 nmol (1.1 μg or 100 μl) of a 1:200 dilution of butalbital (internal standard) in methanol were transferred to a screw-cap test tube (100 mm×13 mm), evaporated to dryness and reconstituted with 200 or 400 μl of urine. To the urine sample were added 1.0 ml of a saturated solution of ammonium sulfate and 3.0 ml (Pipetman, Rainen) of ethyl acetate. The solution was extracted using a rotary mixer at 25 rpm for 30 min. After centrifugation at 500 *g* for 10 min, the ethyl acetate layer was transferred and

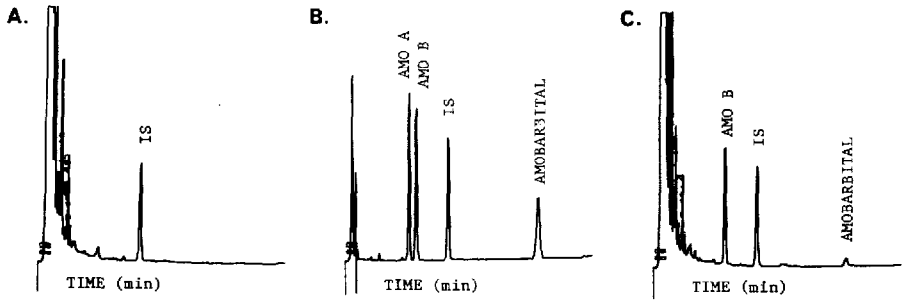

Fig. 2. Chromatograms of (A) blank urine extract with butalbital as internal standard (IS), (B) AMO A (14.2 min), AMO B (15.9 min), IS (22.8 min) and amobarbital (42.7 min) dissolved in mobile phase and (C) urine obtained 8.8 h after taking a 200-mg dose of sodium amobarbital (AMO A, not detected; AMO B, 31.5 nmol/ml, amobarbital, 6.8 nmol/ml).

dried with approximately 90 mg of anhydrous sodium sulfate, after which 2.0 ml (Pipetman) of the ethyl acetate were transferred to a test tube (76 mm × 12 mm) and evaporated to dryness in a vortex evaporator (Buchler Instruments) at 30 mmHg and 27°C. The residue was reconstituted with 200 μl of mobile phase, and the sample was allowed to stand overnight in the refrigerator before transfer to the vials for analysis. If the sample was transferred immediately, incomplete dissolution of all analytes in the sample was observed.

Seven individuals received a dose of 100 or 200 mg of sodium amobarbital prior to going to bed. Blank urines were obtained from each individual just prior to taking the drug. The urine samples analyzed were collected each morning for the following two days. The urine samples were stored at −20°C until analyzed. These individuals were receiving no other medication.

RESULTS

A chromatogram of an extract of blank urine with butalbital added as internal standard is shown in Fig. 2A, and Fig. 2B is the chromatogram of amobarbital, AMO A, AMO B and butalbital dissolved in mobile phase. In the blank urine no interfering absorbances were observed; however, peaks were often observed at 4.1, 13.4, 19.4 and 40.1 min. Compounds evaluated as potential internal standards were barbituric acid (1.6 min), ethylphenylmalonamide (4.6 min), phenylhydantoin (5.2 min), phenobarbital (15.1 min), butabarbital (17.8 min), α-methyl-α-phenylsuccinimide (19.4 min), butethal (20.6 min) and glutethimide (54.4 min). In one series of experiments the column temperature was increased to 35.0°C, and the retention times of AMO A, AMO B, butalbital and amobarbital were 12.9, 14.2, 19.4 and 35.6 min, respectively.

Recovery of AMO A, AMO B and amobarbital was determined by triplicate analysis of standards prepared in blank urine at 142, 61 and 6 nmol/ml. The recovery was determined relative to butalbital by direct injection of equivalent quantities of compounds dissolved in mobile phase. The recovery of internal standard from urine was 95.9 ± 1.7%. At all three concentrations the mean recovery values were 100.3 ± 2.0% for AMO B and 98.7 ± 2.0% for amobarbital. At the two

TABLE I

CONCENTRATION OF N-GLUCOSIDE METABOLITES OF AMOBARBITAL IN HUMAN URINE

Subject No.	Dose (mg)	Time (h)	Concentration (nmol/ml)		
			AMO A	AMO B	Amobarbital
1	200	7.6	0	32.7	0
		32.1	0	21.8	0
2	200	10.4	0	31.7	4.1
		31.8	0	26.7	0
3	100	7.8	0	10.4	0
		34.0	0	29.5	0
4	200	8.8	0	31.5	6.8
		31.4	2.2	15.4	1.7
5	200	8.0	1.9	56.4	0
		33.3	0	18.0	0
6	200	7.5	0	21.4	4.3
		30.0	0	20.6	4.3
7	100	12.0	0	22.0	0
		30.0	0	23.1	0

higher concentrations the recovery for AMO A was $97.2 \pm 1.4\%$ but at 6 nmol/ml the recovery was $83.9 \pm 0.8\%$.

The within-run precision was evaluated by analyzing urine samples spiked with either 6 nmol/ml ($n=9$) or 30 nmol/ml ($n=11$) of AMO A, AMO B and amobarbital. At the high concentration the relative standard deviation was 3.0% for AMO A, 2.8% for AMO B and 13.9% for amobarbital and at the low concentration it was 3.6, 3.3 and 24.5%, respectively.

A standard calibration curve was obtained by extraction of compounds from blank urine and plotting peak-area ratios of drug or metabolite to internal standard as a function of drug or metabolite concentrations. Keeping the internal standard concentration constant at 25 nmol/ml, at concentration ranges of 3–140 nmol/ml for AMO A and AMO B and of 5–120 nmol/ml for amobarbital, the y-intercepts were 0.3 (slope = 0.98), 2.1 (1.01) and −0.3 (0.97), respectively, with correlation coefficients (r^2) of 0.997 or better. The lower limit of detection for AMO A and AMO B was 2 nmol/ml and 3 nmol/ml for amobarbital.

Table I shows the urine concentrations of AMO A, AMO B and amobarbital observed in individuals approximately 8 and 32 h after receiving a dose of 100 or 200 mg of sodium amobarbital.

DISCUSSION

Prior assays for quantitation of the urinary excretion of amobarbital and 3′-hydroxyamobarbital utilized GC [6] or GC–MS [7]. An MS method reported by Tang et al. [8] for simultaneous quantification of amobarbital, 3′-hydroxyamobarbital and 1-(β-D-glucopyranosyl)amobarbital in urine had the disadvantage

that it was not capable of differentiating the possible diastereomers formed and required derivatization of the glucoside conjugate to the tetraacetate prior to analysis. During the development of this assay the HPLC conditions previously used for quantitation of 1-(β-D-glucopyranosyl)phenobarbital [9] were found to be effective for the separation of the diastereomers of 1-(β-D-glucopyranosyl)amobarbital. However, the time needed for each analysis was decreased by increasing the acetonitrile concentration to 20%. Decreasing the buffer pH to 6.5 was intended to prolong column life. Using these assay conditions 3′-hydroxyamobarbital had a retention time which was much shorter than that for AMO A and AMO B, but it corresponded to a strongly absorbing substance in the blank urine. Analysis of additional blank urines indicated that the intensity of this absorbance was highly variable in different individuals and at different times of the day making this assay inappropriate for either detection or quantitation of 3′-hydroxyamobarbital. During routine analysis of samples some samples were turbid when reconstituted. To minimize the occurrence of turbid samples the ethyl acetate fraction was dried with anhydrous sodium sulfate. Of the various compounds tested as internal standard, only butalbital did not co-chromatograph with any of the compounds of interest or with any other substances in the urines. In an attempt to decrease the time of analysis by increasing the column temperature to 35.0°C, the weakly absorbing substances at 13.5 and 40.1 min were no longer resolved from AMO A and amobarbital. No additional efforts were made to optimize conditions at this higher temperature, even though it may be possible to decrease analysis time significantly.

This assay is useful for differentiating the diastereomers and for detection and quantification of AMO B. It can be seen in Table I that one diastereomer, AMO B, was produced almost exclusively at levels which enabled reliable quantitation. Both AMO A and amobarbital were not detected or were present near the limits of detection for the assay described. The low concentration of AMO A is consistent with that observed in the isolation of these metabolites for characterization [4], and the low levels of amobarbital in urine are consistent with that observed in comparable studies [7].

It would appear that there is a pronounced "product enantioselectivity" [10] during the metabolism/excretion of amobarbital. This almost exclusive formation of AMO B indicates that the previous mass spectral method used for simultaneously studying both 3′-hydroxylation and N-glucosylation of amobarbital would give comparable quantitative information as this HPLC method on the excretion of amobarbital via the N-glucosylation pathway. The primary advantage of this HPLC method is that it can differentiate the diastereomers of the N-glucosides. This product enantioselectivity may be important for a better understanding of this novel type of metabolism in humans.

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