Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, No. 4, pp. 365–372, 1990
Printed in Great Britain

0731–7085/90 $3.00 + 0.00

LC determination of the diastereomers of 1-(β-D-glucopyranosyl)phenobarbital in human urine

WILLIAM H. SOINE,*§ PHYLLIS J. SOINE,† TERRY M. ENGLAND,† DEVIN F. WELTY‡ and JOHN H. WOOD‡

* *Department of Medicinal Chemistry, School of Pharmacy, Virginia Commonwealth University, Richmond, VA 23298-0581, USA*
† *Bureau of Forensic Science, Division of Consolidated Laboratory Services, Commonwealth of Virginia, Richmond, VA 23219, USA*
‡ *Department of Pharmacy and Pharmaceutics, School of Pharmacy, Virginia Commonwealth University, Richmond, VA 23298-0581, USA*

Abstract: The "product enantioselectivity" associated with the urinary excretion of the phenobarbital *N*-glucoside conjugates has not been determined previously. A liquid chromatography method using gradient elution was developed for quantifying both phenobarbital *N*-glucoside conjugates, phenobarbital, and *p*-hydroxyphenobarbital. Following a single oral dose of phenobarbital to male Caucasian and Oriental subjects, both phenobarbital *N*-glucoside conjugates were observed in the urine. In seven subjects, 3.3–10.6% of the phenobarbital dose was detected as a single phenobarbital *N*-glucoside (*S* configuration at the C-5 position of the barbiturate ring). The other phenobarbital *N*-glucoside diastereomer accounted for <1.5% of the phenobarbital dose. The urinary excretion of the major phenobarbital *N*-glucoside diastereomer paralleled the urinary excretion of phenobarbital and was comparable in both Caucasian and Oriental subjects. These results indicate a pronounced selectivity for the formation and/or urinary excretion of the phenobarbital *N*-glucosides.

Keywords: *Urine; reversed-phase chromatography; 1-(β-D-glucopyranosyl)phenobarbital, diastereomer, pharmacokinetics*

Introduction

It has become apparent that *N*-glucosylation is an important pathway for the metabolism and urinary excretion of phenobarbital in Man [1–6]. When glucose is coupled to phenobarbital, two diastereomers will be formed (Fig. 1); however, the differentiation of these diastereomers in urine after dosing with phenobarbital has not been extensively studied. Two analytical methods for the quantification of the phenobarbital *N*-D-glucosides have been previously reported [2, 5]. The first method was based on the methylation of the phenobarbital *N*-D-glucosides with diazomethane, followed by persilylation and analysis by gas chromato-

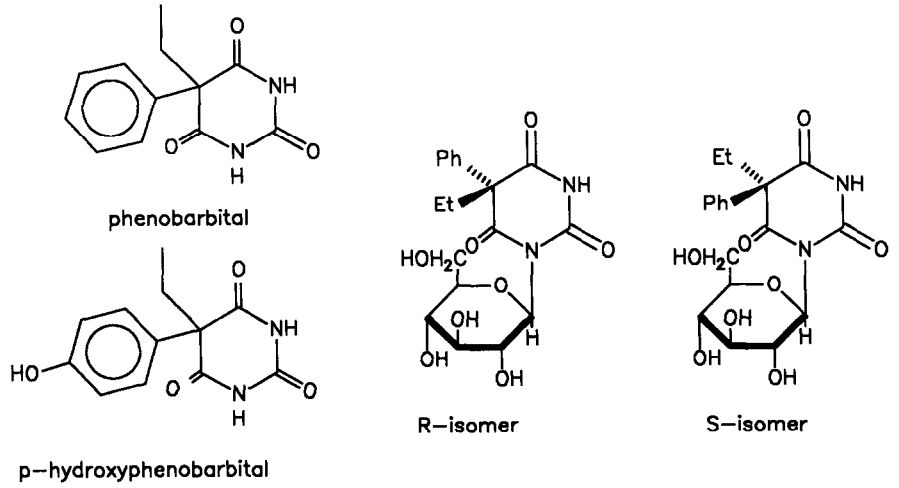

Figure 1
Structures of the major urinary excretion products of phenobarbital.

§ Author to whom correspondence should be addressed.

graphy–mass spectrometry (GC–MS) using chemical ionization (methane) detection [2]. The precision and sensitivity (0.1 ng ml^{-1}) of the assay were very good. The disadvantage of the GC–MS assay was that phenobarbital and *p*-hydroxyphenobarbital must be quantitated in a separate analysis and GC conditions have not yet been reported that are capable of separating the methylated/persilylated phenobarbital *N*-D-glucoside diastereomers.

A liquid chromatographic (LC) method was previously reported for the quantitation of phenobarbital *N*-D-glucosides; however, the problem of diastereomers was not addressed [5]. In later studies it was recognized that the LC method was differentiating the diastereomers [7], and that only a single diastereomer was being determined. The other diastereomer was eluting on the leading edge of the internal standard, making its identification and quantification unreliable [5]. This paper describes the initial results with a modified LC method that is capable of differentiating and quantitating the diastereomers of the phenobarbital *N*-glucose conjugates in urine. This LC method was then used for preliminary studies on the urinary excretion of these metabolites in Caucasian and Oriental subjects.

Experimental

Apparatus

A Model 1330 liquid chromatograph (Bio-Rad Laboratories, Richmond, CA) with binary gradient capability was used for this study. The pumps were interfaced to a WISP Model 712 autosampler (Waters Chromatography, Milford, MA), a Perisorb RP-18 guard column (30–40 μm, 20 × 2 mm, i.d.; Upchurch Scientific, Oak Harbor, WA), an Econosphere C_{18} column (5 μm, 250 × 4.6 mm, i.d.; Alltech Associates, Deerfield, IL), a Model 1306 variable wavelength UV detector (Bio-Rad Laboratories), and a Model 3393A digital integrator (Hewlett–Packard, Palo Alto, CA). Constant column temperature (Lauda K-2/R, Brinkman Instruments, Westbury, NY) was maintained using a circulating water jacket. The UV spectra were obtained on a DMS 100S UV–vis spectrometer (Sunnyvale, CA) at a slit width of 1.0 nm.

Materials

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**) had been synthesized previously [7, 8]. In those reports they were designated as PBGA and PBGB, respectively. For each of the phenobarbital *N*-β-D-glucose conjugates, >98% of the peak area (198 nm) was associated with a single diastereomer. Phenobarbital was purchased from Merck (Rahway, NJ), and *p*-hydroxyphenobarbital and methylphenylhydantoin (internal standard, IS) were purchased from Aldrich (Milwaukee, WI). Acetonitrile and ethylacetate were HPLC grade (Mallinckrodt Inc., St Louis, MO). The monobasic and dibasic sodium phosphate were HPLC grade (Fisher Scientific, Pittsburgh, PA), and the remaining chemicals were reagent grade.

Chromatographic conditions

The analyses required gradient elution. The eluents were: solvent A, acetonitrile–sodium phosphate buffer (pH 6.5, 25 mM), (10:90, v/v) solvent; B, acetonitrile–sodium phosphate buffer (pH 6.5, 25 mM), (25:75, v/v). The gradient used was 10 min of 100% A, linear programming over 28 min to 67% B, linear programming over 4 min to 100% B, re-equilibration over 2 min to 100% A. The injection volume was 20 μl, the flow-rate was 1.4 ml min^{-1}, and the eluate was monitored at 198 nm. The λ_{max} for the analytes when dissolved in 15% acetonitrile in 0.025 M sodium phosphate buffer, pH 6.5, were 196 nm for **1a** and **1b**, 199 nm for phenobarbital and 197–198 nm for *p*-hydroxyphenobarbital. The analysis was carried out at 25.0°C. The mobile phase was degassed by purging with helium.

Sample standards

Individual stock solutions (4 mM) of phenobarbital, *p*-hydroxyphenobarbital, **1a**, **1b** and internal standard (methylphenylhydantoin, 5.0 mM) were prepared in methanol and stored at −20°C. When stored under these conditions, no decomposition of the stock solutions was detectable after 12 months of storage. Standard solutions 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 acidified reconstituting solution [H_3PO_4 (1 M)–acetonitrile–

water (1:12:87, v/v/v)] or citric acid acidified urine (urine samples collected over citric acid).

Sample preparation

To prepare the sample for analysis, 5.0 μM (950 μg or 100 μl) of a 1:100 dilution of internal standard in methanol were transferred to a screwcap test tube (100 mm × 13 mm), evaporated to dryness and reconstituted with 200 or 400 μl of urine. To each test tube was added anhydrous Na_2SO_4 (app. 150 mg) and 20 μl of 1 M H_3PO_4 followed by addition of 3.0 ml (Pipetman, Rainin Inst. Co., Woburn, MA) of ethyl acetate. The test tube was capped and placed on a rotary mixer at 25 rpm for 30 min. Then 0.5 ml of a saturated solution of $(NH_4)_2SO_4$ was added and the test tube was recapped and placed on the rotary mixer for an additional 10 min. After centrifugation at 500*g* for 10 min, the ethyl acetate layer was transferred and dried with approx. 150 mg of anhydrous Na_2SO_4 for 30 min. A 2.0-ml aliquot (Pipetman) of the ethyl acetate layer was transferred to a test tube (76 × 12 mm), evaporated to dryness in a vortex evaporator (Haake Buchler Instruments, Saddle Brook, NJ) at 27°C under reduced pressure using a water aspirator. The residue was reconstituted with 200 μl of acidified reconstituting solution. To insure dissolution of the analytes the sample was left for 4 h at room temperature or overnight in the refrigerator before transfer to the LC vials for analysis [9].

Collection of urine samples for analytical study

Three male non-smoking Caucasian subjects and four non-smoking Oriental subjects received a 90-mg oral dose of phenobarbital (3 × 30 mg tablet, Eli Lilly, Indianapolis, IN) just prior to bedtime. Total urine was collected for approximately 55 h followed by collection of morning urines for 5 additional days. Morning-urine samples consisted of urine collected between recorded urination prior to bedtime and initial urination at the beginning of the day. Since **1a** and **1b** are unstable under neutral and basic conditions [10], the subjects collected the urine in the presence of citric acid (final pH of the urine ranged from 2.3 to 2.6). The urine samples were refrigerated immediately upon collection, frozen within 48 h, and kept at −20°C until analysed.

Analysis of urinary excretion of analytes

The percentage of dose of phenobarbital, **1a**, **1b** and *p*-hydroxyphenobarbital in Table 1 was calculated by measuring the concentration of analyte in each urine sample. Duplicate samples were prepared and analysed for each data point. Complete absorption was assumed [11]. The parameter estimates for the urinary excretion rate and ordinate intercepts for each compound were determined by linear-regression analysis for correlated data [12] using interactive matrix language (IML) of the statistical analysis system (SAS) [13]. The pooled data shown in Fig. 2 were obtained by mathe-

Table 1
Urinary excretion of phenobarbital and metabolites

Subject No.	R*	Last sample (h)	PBOH†	% Dose detected as **1a**	**1b**	PB‡	Total % dose detected
2	C	152.0	1.7	7.7	0.1	12.9	22.4
3	C	152.5	1.7	8.3	0.5	15.6	26.1
4	C	151.4	3.4	3.3	ND	9.0	15.7
1	O	152.9	2.5	10.6	1.5	11.3	25.9
5	O	152.0	2.5	4.1	0.9	8.6	16.1
6	O	152.2	1.9	6.1	0.5	6.1	14.6
7	O	175.7	1.7	5.2	0.1	6.6	13.6
AVG§	C		2.3	6.4	0.2	12.5	21.4
SD	C		1.0	2.7	0.3	3.3	5.3
AVG	O		2.2	6.5	0.8	8.2	17.6
SD	O		0.4	2.9	0.6	2.4	5.7

*R = race, C = Caucasian, O = Oriental.
†PBOH = *p*-hydroxyphenobarbital.
‡PB = phenobarbital.
§AVG = average, SD = standard deviation.

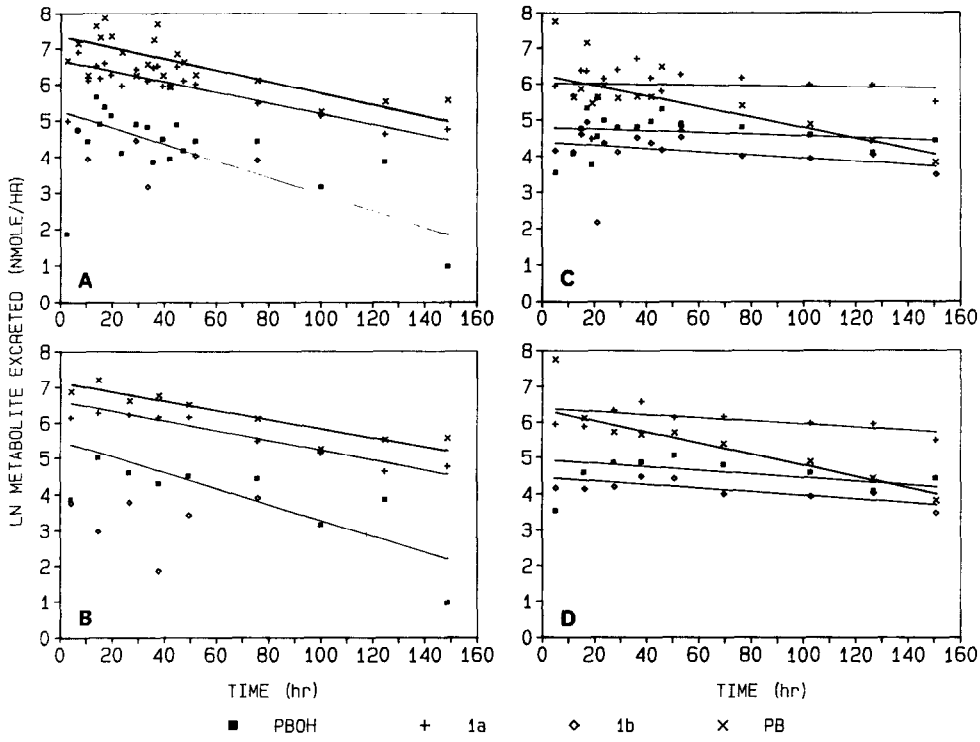

Figure 2
Plots of the urinary excretion profiles in a Caucasian and Oriental subject: (A) subject No. 3, unpooled data (see Table 3); (B) subject No. 3, pooled data (K_{PB} = 0.0131 h^{-1}, *Y*-INT = 7.14, r^2 = 0.827, K_{1a} = 0.0139, *Y*-INT = 6.61, r^2 = 0.944, K_{PBOH} = 0.0223, *Y*-INT = 5.47, r^2 = 0.703); (C) subject No. 1, unpooled data (see Table 3); (D) subject No. 1, pooled data (K_{PB} = 0.0156, *Y*-INT = 6.35, r^2 = 0.967, K_{1a} = 0.0045, *Y*-INT = 6.39, r^2 = 0.456, K_{1b} = 0.0050, *Y*-INT = 4.45, r^2 = 0.616, K_{PBOH} = 0.0043, *Y*-INT = 4.98, r^2 = 0.487).

matically pooling the data into collection periods which ranged from 9.3 to 14.7 h, depending on the frequency of urination. The calculations started with the second urine sample obtained the morning after taking the drug.

Results and Discussion

Analytical methodology and assay validation

A chromatogram of *p*-hydroxyphenobarbital, **1a**, **1b** and phenobarbital dissolved in the acidified reconstitution solution is shown in Fig. 3(a). A chromatogram of an extract of blank urine with internal standard is shown in Fig. 3(b), and a chromatogram of *p*-hydroxyphenobarbital, **1a**, **1b** and phenobarbital (10 μmol ml^{-1} of each analyte) extracted from blank urine is shown in Fig. 3(c). The calibration curve was prepared using standards extracted from blank urine. The concentration of analytes in urine were calculated from peak–area ratio of analyte to internal standard versus concentration of the standards (1, 2, 4, 10, 20 and 40 μM) using linear regression with zero intercept. The slopes (± standard error) were 0.0977 ± 0.0010 for *p*-hydroxyphenobarbital, 0.0646 ± 0.0012 for **1a**, 0.0606 ± 0.0011 for **1b**, and 0.0578 ± 0.0010 for phenobarbital with correlation coefficients (r^2) of 0.99 or better (n = 16). *p*-Hydroxyphenobarbital was not detected at concentrations below 0.5 μm and phenobarbital, **1a** and **1b** were not detected below 1.0 μm following extraction of 200 μl of urine. These analytes could be detected at 0.25 and 0.5 μM, respectively, when 400 μl of urine was extracted. A chromatogram of a urine sample from an Oriental subject (No. 7) following oral administration of phenobarbital is shown in Fig. 3(d).

In two separate runs of 60 analyses the reproducibility (SD) of the retention times for all analytes was ±0.04 min. In the blank urines no significant interfering absorbances were observed; however, a small peak was occasionally observed in certain individuals that eluted at 28.33–28.56 min. This resulted in a shoulder on the trailing edge of **1a** and required careful monitoring of the integration parameters.

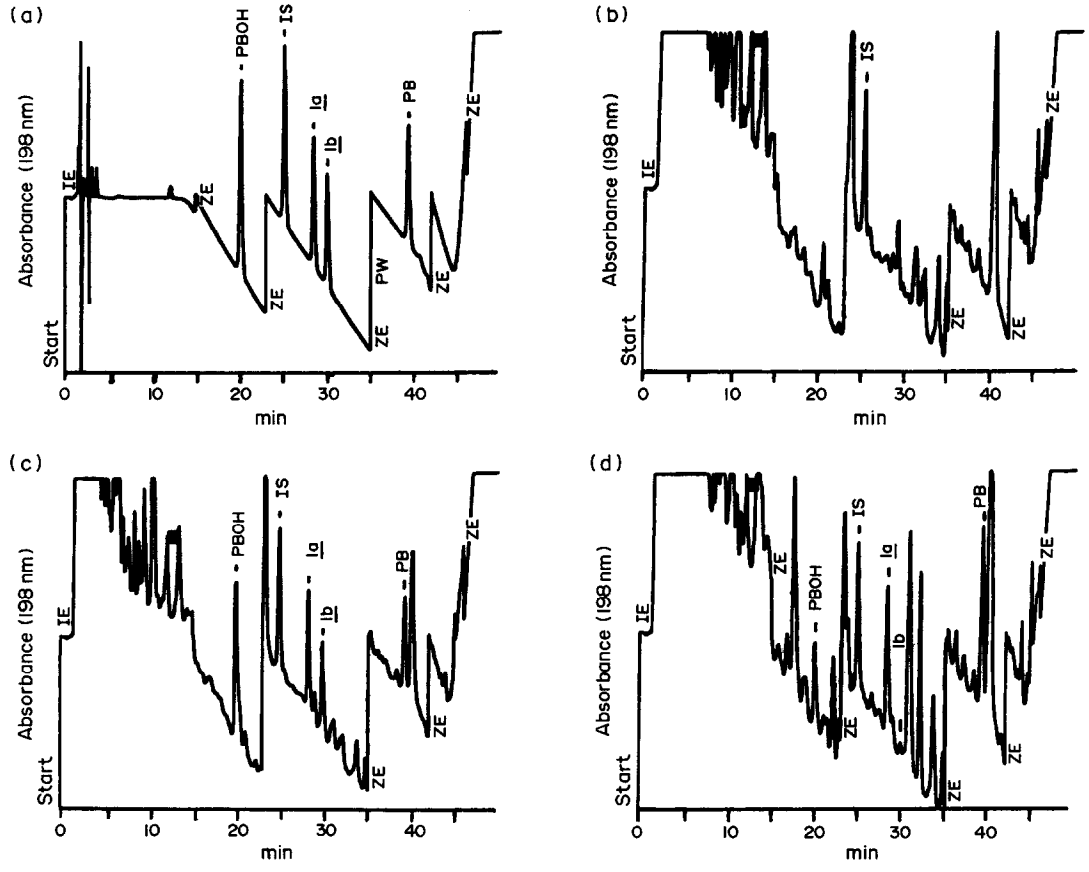

Figure 3
Chromatograms of (a) the 10 μM standards of phenobarbital and metabolites dissolved in acidified reconstituting solution (*p*-hydroxyphenobarbital, 10.0 μM, 20.19 min; **1a**, 10.0 μM, 28.47 min; **1b**, 10.3 μM, 30.02 min; phenobarbital, 10.0 μM, 39.14 min); (b) a 400-μl blank urine that was acidified with citric acid (contains internal standard, 25.15 min); (c) the 10 μM standards of phenobarbital and metabolites extracted from 200 μl of urine; (d) urine obtained 59 h after subject No. 7 had taken a 90-mg oral dose of phenobarbital (an extract of 400 μl of urine; *p*-hydroxyphenobarbital, 4.7 μM; **1a**, 14.0 μM; **1b**, 0.7 μM; phenobarbital, 14.8 μM.

Recovery of *p*-hydroxyphenobarbital, **1a**, **1b** and phenobarbital from urine was determined by comparison of triplicate analyses of standards prepared in blank urine at 40, 20, 10 and 4 μM with equivalent quantities of compounds dissolved in the reconstituting solution. By comparison of peak areas the recovery from urine was 96.4 ± 2.0% of *p*-hydroxyphenobarbital, 100.5 ± 4.0% of **1a**, and 98.9 ± 2.6% for **1b**. The apparent recovery of phenobarbital decreased linearly from approximately 84 to 52%. This was probably due to interference by the strongly absorbing peak at 39.9 min (t_R phenobarbital = 39.1 min). The apparent recovery of internal standard from urine was 118.2 ± 3.2%. No interfering absorbances which co-eluted with the internal standard were detected in blank urines. Neither were there any absorbances associated with decomposition of the standards that could contribute to the enhanced recovery of internal standard. The use of this internal standard for numerous urine samples from different individuals gave a consistent peak area and was very reproducible.

The within-run precision was evaluated by analysing urine samples containing either 20 μM (n = 6) or 4 μM (n = 6) of all of the standards. At the high concentration the relative standard deviation was 1.7% for *p*-hydroxyphenobarbital, 2.4% for **1a**, 1.8% for **1b** and 4.5% for phenobarbital and at the low concentration it was 0.9, 3.6, 1.4 and 1.9%, respectively.

Modification of the initial LC method was necessary to obtain better resolution of **1b** from the internal standard as well as better resolution of *p*-hydroxyphenobarbital and phenobarbital from naturally occurring interfering compounds in urine. In addition, the

mobile phase was acidified to pH 6.5 to minimize decomposition. Changes were also incorporated into the urine collection and extraction procedure so that all aqueous solutions were strongly acidic to minimize ring opening and decomposition [10]. The extraction procedure was improved by adding anhydrous sodium sulphate at the first step so that recovery was almost quantitative for **1a** and **1b**. The major limitation of the assay was that **1a** and **1b** could only be detected above approximately 0.5 μM. Following a single 90 mg dose of phenobarbital, the concentration of the phenobarbital *N*-D-glucosides was approaching or below the lower limit of detection for the *N*-glucoside conjugates at the end of 6 days. Although post-column ionization has been proposed as an improved method for detecting barbiturates [14], the extinction coefficient of the diastereomers at 240 nm following post-column ionization was approximately 25% that at the shorter wave length. Current studies are underway to determine if derivatives of **1a** and **1b** could still be resolved and also provide increased sensitivity for detection, especially for **1b**.

Application to urinary excretion of phenobarbital and metabolites

Metabolite **1a** was detected in over 98% of the samples analysed. Metabolite **1b** was detected in 21 out of 28 samples when the concentration of **1a** was ≥9.0 μm. In some samples no **1b** was detected when the concentration of **1a** ranged up to 19.3 μM. Metabolite **1b** was detected in 19 out of 79 samples when the concentration of **1a** ranged from 9.0 to 0.5 μM. The highest concentration of **1b** detected was 4.4 μM, and the average concentrations each day, *when detected*, are shown in Table 2. In subject No. 1, shown in Fig. 2, the ratio of **1a/1b** was 6.8 ± 1.5 throughout the period of urine collection. The ratio of **1a/1b**, in all samples where both diastereomers were detected (n = 40 urine samples), was 7.1 ± 4.8 (range 0.4–31.0).

The fraction excreted as phenobarbital *N*-D-glucosides was less than the fraction excreted as phenobarbital. This is consistent with the study in which these metabolites were quantitated using ^{14}C-phenobarbital [1] or GC–MS [2]. For the Oriental subjects the fraction of dose excreted as **1a** approached that excreted as phenobarbital, whereas, for the Caucasian subjects the fraction of the dose excreted as **1a** was approximately one-half that excreted as phenobarbital. Generally, the concentration of **1a** was higher in the urine of the Oriental subjects than in the Caucasians, but overall no difference in the fraction excreted was observed (Table 1). Also, **1b** was detected more frequently in the urine of the Orientals than the Caucasians.

The fraction of the dose quantified as free *p*-hydroxyphenobarbital was usually less than that excreted as the *N*-D-glucosides. Since the samples were acidified immediately upon collection, hydrolysis of the *p*-hydroxyphenobarbital *O*-glucuronide could have occurred prior to analysis. Whether or not free *p*-hydroxyphenobarbital and/or some *p*-hydroxyphenobarbital *O*-glucuronide was quantified in some samples cannot be stated unambiguously for this study. Therefore, the quantitative results reported in Tables 1 and 3 may be subject to this additional error and further statistical comparisons were not done. In a previous report, 7–10% of an oral dose of phenobarbital was excreted as free *p*-hydroxyphenobarbital in the urine after 8 days, and the

Table 2
Concentrations of **1a** and **1b** detected in the urine of the Caucasian and Oriental subjects

Time (h)	**1a** Mean ± SD	Range	**1b*** Mean ± SD	Range
0–8	7.5 ± 4.6	14.9–1.9	1.7 ± 0.6	2.3–1.0
8–32	6.0 ± 3.5	14.7–1.5	1.4 ± 0.7	3.2–0.5
32–59	8.1 ± 7.7	40.9–1.2	1.4 ± 0.7	3.0–0.7
80	8.7 ± 7.5	25.0–1.6	2.1 ± 1.4	4.4–0.8
104	8.9 ± 6.2	19.3–3.3	1.2 ± 0.6	1.9–0.7
128	6.4 ± 5.0	14.7–ND†	1.9	
152	4.8 ± 4.6	15.7–1.8	1.5 ± 0.8	2.3–0.8
176	3.4 ± 2.5	8.6–1.0	1.2 ± 0.8	2.6–0.4

*When detected.
†ND = Not detected.

Table 3
Parameters derived for urinary excretion of phenobarbital and metabolites in Caucasian and Oriental male subjects using unpooled data*

Subject No.	R	Analyte	n†	K (h^{-1})	SE of K	Y-INT	SE of Y-INT	r^2
2	C	PBOH	19	0.0080	0.0065	4.52	0.42	0.080
		1a	19	0.0043	0.0019	5.98	0.13	0.220
		1b		—	—	—	—	—
		PB	19	0.0082	0.0029	6.85	0.19	0.320
3	C	PBOH	12	0.0302	0.0031	6.20	0.16	0.930
		1a	12	0.0254	0.0054	5.96	0.27	0.760
		1b		—	—	—	—	—
		PB	9	0.0351	0.0041	7.17	0.21	0.910
4	C	PBOH	12	0.0063	0.0016	5.40	0.11	0.600
		1a	12	0.0100	0.0020	5.53	0.14	0.730
		1b		—	—	—	—	—
		PB	12	0.0031	0.0026	6.11	0.19	0.130
1	O	PBOH	15	0.0024	0.0030	4.80	0.19	0.050
		1a	15	0.0011	0.0037	6.03	0.23	0.010
		1b	14	0.0036	0.0045	4.28	0.67	0.049
		PB	15	0.0158	0.0034	6.72	0.21	0.630
5	O	PBOH	13	0.0087	0.0021	5.26	0.15	0.610
		1a	13	0.0210	0.0093	6.05	0.65	0.320
		1b	12‡	0.0019	0.0023	3.88	0.33	0.061
		PB	13	0.0067	0.0021	6.34	0.15	0.480
6	O	PBOH	11	0.0034	0.0036	4.67	0.27	0.090
		1a	11	0.0070	0.0053	5.95	0.40	0.160
		1b	—	—	—	—	—	
		PB	11	0.0082	0.0014	6.10	0.11	0.790
7	O	PBOH	11	0.0027	0.0021	4.29	0.19	0.150
		1a	11	0.0034	0.0029	5.45	0.26	0.140
		1b	—	—	—	—	—	
		PB	11	0.0062	0.0028	5.84	0.25	0.350
Summary for unpooled data								
	C	PBOH		0.0115	3.1E-05	5.06	0.29	
		1a		0.0097	3.3E-05	6.07	0.19	
		PB		0.0089	3.3E-05	6.78	0.13	
	O	PBOH		0.0044	2.4E-05	4.76	0.23	
		1a		0.0081	2.5E-05	5.87	0.15	
		PB		0.0092	2.5E-05	6.14	0.10	
Summary for pooled data								
	C	PBOH		0.0124	1.9E-05	5.19	0.07	
		1a		0.0096	2.6E-04	6.05	0.07	
		PB		0.0075	2.0E-05	6.65	0.06	
	O	PBOH		0.0052	1.4E-05	5.19	0.07	
		1a		0.0071	1.9E-04	5.99	0.06	
		PB		0.0093	1.5E-05	6.17	0.05	

*Abbreviations are the same as Table 1.
†n = number of observations.
‡Positive slope.

ratio of free to conjugated *p*-hydroxyphenobarbital was approximately 1:2 [15].

In Fig. 2 are representative urinary excretion curves for *p*-hydroxyphenobarbital, **1a**, **1b** and phenobarbital using natural collection periods or mathematically pooled samples. The results for the linear regression for the unpooled data to estimate a K for phenobarbital, **1a**, **1b** (when it accounted for >1% of the dose) and *p*-hydroxyphenobarbital for each individual are listed in Table 3. In spite of the large fluctuations observed for the urinary excretion of the analytes, the graphical representations of the data using the unpooled data indicated that the linear model selected was adequate to describe the data.

The urinary excretion of **1a** would be expected to parallel the excretion of phenobarbital provided its formation is considerably slower than its urinary excretion. Therefore, it was of interest to determine if parallel excretion of phenobarbital and **1a** was occurring. For each individual, upon comparison of the apparent K values obtained for the metabolites

with the K obtained for phenobarbital, parallel elimination was observed ($P > 0.90$). Although the statistical tests [12] indicated that the urinary excretion of phenobarbital and **1a** were parallel, by collecting only overnight urines for the later time points, this relationship is not always readily apparent (see Fig. 2). In future studies total urine collections should be obtained. These results are consistent with the report by Tang *et al.* [2] who indicate that the urinary excretion of the phenobarbital *N*-glucoside(s) paralleled the elimination of phenobarbital. In a structurally similar compound, amobarbital, the urinary excretion of the amobarbital *N*-D-glucoside(s) and 3′-hydroxyamobarbital was first order and paralleled the decline of the serum concentration of amobarbital [16].

In conclusion, this LC method described here is capable of differentiating the phenobarbital *N*-glucosides. The method requires a minimum of sample preparation and was suitable for these initial studies on the urinary excretion of phenobarbital and its major metabolites. In the subjects studied, it appears that *N*-glucosylation of phenobarbital is a quantitatively important pathway for the excretion of phenobarbital and a pronounced product enantioselectivity occurs for the formation and/or excretion of these metabolites.

Acknowledgements — The authors would like to thank Vernon M. Chinchilli for his help in the statistical analysis. This work was supported by grants from the Epilepsy Foundation of America and Public Health Service Grant GM34507.

References

[1] B.K. Tang, W. Kalow and A.A. Grey, *Drug Metab. Disp.* **7**, 315–318 (1979).
[2] B.K. Tang, B. Yilmaz and W. Kalow, *Biomed. Mass Spectrom.* **11**, 462–465 (1984).
[3] D. Kadar, B.K. Tang and A.W. Conn, *Can. Anaes. Soc. J.* **29**, 16–23 (1982).
[4] W.H. Soine, V.O. Bhargava and L.K. Garrettson, *Drug Metab. Disp.* **12**, 792–794 (1984).
[5] V.O. Bhargava, W.H. Soine and L.K. Garrettson, *J. Chromatogr.* **343**, 219–223 (1985).
[6] V.O. Bhargava and L.K. Garrettson, *Dev. Pharm. Ther.* **11**, 8–13 (1988).
[7] W.H. Soine, P.J. Soine, T.M. England, B.W. Overton and S. Merat, *Carbohydr. Res.* **193**, 105–113 (1989).
[8] R.M. Graham, S.M. Mongrain, W.H. Soine and P.J. Soine, *Va. J. Sci.* **39**, 167 (1988).
[9] P.J. Soine and W.H. Soine, *J. Chromatogr.* **422**, 309–314 (1987).
[10] F.B. Vest, W.H. Soine, R.B. Westkaemper and P.J. Soine, *Pharm. Res.* **6**, 458–465 (1989).
[11] T.R. Browne, J.E. Evans, G.K. Szabo, B.A. Evans and D.J. Greenblatt, *J. Clin. Pharmacol.* **25**, 51–58 (1985).
[12] E.F. Vonesh and R.L. Carter, *Biometrics* **43**, 617–628 (1987).
[13] SAS Institute Inc., *SAS User's Guide: Basics, Version 5 Edition; SAS User's Guide: Statistics, Version 5 Edition; SAS/GRAPH User's Guide, Version 5 Edition*. SAS Circle, Cary, NC (1985).
[14] C.R. Clark and J.-L. Chan, *Anal. Chem.* **50**, 635–637 (1978).
[15] L.O. Boreus, B. Jalling and N. Kallberg, *Acta Paediatr. Scand.* **67**, 193–200 (1978).
[16] B.K. Tang, W. Kalow, L. Endrenyi and F.-Y. Chan, *Eur. J. Clin. Pharmacol.* **22**, 229–233 (1982).

[*Received for review 2 August 1989; revised version received 5 January 1990*]