High-performance liquid chromatographic analysis of phenobarbital and phenobarbital metabolites in human urine

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Abstract

A HPLC assay using UV detection and post-column alkalinization was developed to quantify possible urinary excretion products of phenobarbital in human urine. After filtration the urine was injected directly onto the HPLC column for analysis of phenobarbital, *p*-hydroxyphenobarbital, phenobarbital N-glucosides and phenobarbital N-glucuronides. The accuracy and precision of the assay were within $\pm 15\%$ and the limit of detection (LOD) was 1 μM, suitable for pharmacokinetic studies. Phenobarbital was administered orally to five male subjects and urine was collected for a period of 96–108 h. Phenobarbital, *p*-hydroxyphenobarbital, and phenobarbital N-glucosides were detected and quantified in the urine of all five subjects. The phenobarbital N-glucuronides were not detected in the urine. This assay provides a rapid method with improved selectivity to analyze urine for phenobarbital and its metabolites.

Keywords: Phenobarbital; *p*-Hydroxyphenobarbital; Phenobarbital N-glucosides; Phenobarbital N-glucuronides

1. Introduction

Mice have been shown to excrete both phenobarbital N-glucosides and N-glucuronides in their urine after being dosed with phenobarbital. The phenobarbital N-glucuronides accounted for a higher percentage of the dose than the phenobarbital N-glucosides, i.e., $7.8 \pm 2.3\%$ and $1.6 \pm 0.6\%$, respectively [1]. In addition, a structurally related drug, phenytoin, has been shown to be excreted in human urine as a N-glucuronide conjugate [2]. To determine if humans excrete phenobarbital N-glucuronides as urinary biotransformation products of phenobarbital,

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an analytical method was needed for their detection and quantification.

Glucuronide conjugates are often detected and quantified after hydrolysis of the conjugate using β-glucuronidase or acid [1,3–6]. The barbiturate or imide N-glycosides may not be amenable to the enzymatic deconjugation, since 5-ethyl-5-phenylhydantoin N-glucuronides have been shown to be resistant to β-glucuronidase hydrolysis [7] and the hydantoin and barbiturate N-glycosides do not undergo hydrolysis under strongly acidic conditions [1,2,8–10]. The synthetic standards of phenobarbital and its metabolites, including phenobarbital N-glucuronides, were used to develop a HPLC method for the detection and quantification of phenobarbital N-
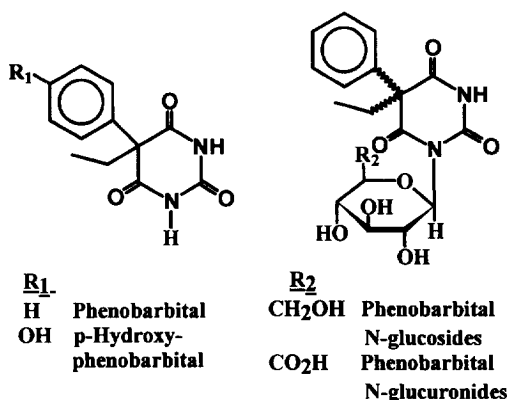
Fig. 1. Structures of phenobarbital and its metabolites.

glycosides, p-hydroxyphenobarbital and phenobarbital in human urine (structures shown in Fig. 1). It was observed that phenobarbital N-glucuronides were not excreted in the urine as significant metabolites of phenobarbital.

2. Experimental

2.1. Materials

All solvents and buffers were HPLC grade from Fisher Scientific (Fair Lawn, NJ, USA). Sodium phenobarbital (5-ethyl-5-phenyl-2,4,6-(1H,3H,5H)-pyrimidinetrione), USP, was from Penick Chemical Division (New York, USA). p-Hydroxypheno-barbital.H_2O (5-ethyl-5-(p-hydroxyphenyl)-2,4,6-(1H,3H,5H)-pyrimidinetrione) was purchased from Sigma (Milwaukee, WI, USA). The (5R)- and (5S)-5-ethyl-1-(β-D-glucopyranosyl)-5-phenyl-2,4,6-(1H,3H,5H)pyrimidinetrione ((5S)- and (5R)-PbG), and (5R)- and (5S) - 5 - ethyl - 1 - (β - D - gluco - pyranosyluronate) - 5 - phenyl - 2,4,6 - (1H,3H,5H) - pyrimidinetrione ((5S)- and (5R)- PbGA) were synthesized as previously reported [8,9].

2.2. Analytical HPLC

HPLC analyses were performed on a Beckman System Gold Liquid Chromatograph (San Ramon, CA, USA) consisting of three Beckman 110B solvent

delivery modules, a Beckman 406 analog interface Module, a programmable Beckman Model 507 autosampler (with a 100-μl loop) and a Beckman 168 diode array detector module. Chromatographic separation was achieved on a C_{18} reversed-phase column (Econosphere, 250×4.6 mm I.D., particle size 5 μm, Alltech, Deerfield, IL, USA) with a pellicular ODS guard column (20×2 mm I.D., particle size 37–53 μm, Whatman, Fairfield, NJ, USA). The HPLC column was jacketed with circulating water maintained at a temperature of 25.0±0.1°C using a Brinkman mgw Lauda RMS20 temperature-programmable circulating water bath. Elution of the analytes was isocratic with 12% (v/v) acetonitrile–H_3PO_4 (pH 2.2–2.3; 0.025 M)(12:88, v/v) for 50 min. After each analysis the mobile phase was changed to acetonitrile–water (40:60, v/v) over a period of 1 min and the column was washed for 20 min. Equilibration for 20 min with the initial mobile phase was done before the next injection. Post-column alkalinization was achieved by the insertion of a mixing chamber (50×4.6 mm I.D., Supelco, Belle-fonte, PA, USA) filled with acid washed silanized glass beads (particle size 75 μm, Supelco) after the column outlet. A 0.05 M borate buffer (pH 12.7–12.9) was introduced at a flow-rate of 0.3 ml/min (Beckman 110A pump) via a Swagelok tee inserted in the line between the analytical column and the mixing column (final apparent pH 9.5–10.0). A back-pressure regulator (500 psi, Upchurch Scientific, Oak Harbor, WA, USA) was installed as a pulse dampner between the outlet from the pump and the Swagelok tee just prior to the mixing chamber. The wavelength of detection was 240 nm (bandwidth of 10 nm). The diode array detector was programmed to scan from 198 to 298 nm during selected time periods at 4-nm intervals.

2.3. Dosing of subjects and collection of urine

Five healthy non-smoking male volunteers were recruited to participate in the study with the approval of the Committee on the Conduct of Human Research, Virginia Commonwealth University, Richmond, VA, USA. The subjects were Caucasian ($n=3$) and South Asian ($n=2$) with ages ranging from 25 to 48 years. The subjects had not received any barbitu-

rate or any 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 for 24 h before the study and for the entire period of urine collection.

Each subject received an oral dose of 100 mg sodium phenobarbital USP (tablets, Eli Lilly, Indianapolis, IN, USA). The drug was taken at night just prior to bedtime. A blank urine sample was collected just prior to dosing. Complete urine samples were collected in glass containers containing approximately 10 g of citric acid (final pH of 2.5–3.0) [10]. The volume of urine was measured. The urine voided every 12 h (range 9–14 h) was pooled and stored at 4°C until analysis. Urine was collected for 96–108 h following dosing.

Each urine specimen was filtered through a Gelman HPLC disk filter (pore size 45 μm). The filtered urine was injected directly onto the column. The injection volume was 25 μl and all samples were analyzed in duplicate. Blank urine containing the synthetic standards of p-hydroxyphenobarbital, (5S)- and (5R)-PbG, (5S)- and (5R)-PbGA and phenobarbital were run after every sixth sample to monitor any shift in retention times.

A urine sample from subject 1 (pooled urine sample from 0–65 h) was analyzed after acid hydrolysis. A 1-ml volume of filtered urine was transferred to a screw-cap test-tube, 200 μl of 21% H_3PO_4 was added, the tube was capped, and the mixture was heated at 95°C for 2 h. The sample was analyzed as previously described.

3. Results

The HPLC assay was developed for the simultaneous detection and quantification of p-hydroxyphenobarbital, (5S)- and (5R)-PbG, (5S)- and (5R)-PbGA, and phenobarbital in urine. After injection of the urine directly onto the column and monitoring the column effluent at 200 nm, absorbances were observed in the blank urine that interfered with the detection and quantification of the phenobarbital N-glycosides. After post-column alkalinization of the column effluent to an apparent pH of 10 and analysis of the blank urine at 240 nm, the chromatogram

showed minimal interferences in the urine after 21 min. When post-column alkalinization 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. Fig. 2 shows the chromatogram for a blank urine sample and a blank urine sample containing the synthetic standards of the analytes (the periodic pulsing has been subtracted from the chromatogram). No standard was available for the O-glucuronide of p-hydroxyphenobarbital. To determine if the conjugate could interfere with the assay, urine from subject 1 was analyzed after acid hydrolysis [1,11]. The concentration of p-hydroxyphenobarbital increased from 4.1 μM to 8.6 μM, while the concentration of the rest of the analytes remained the same.
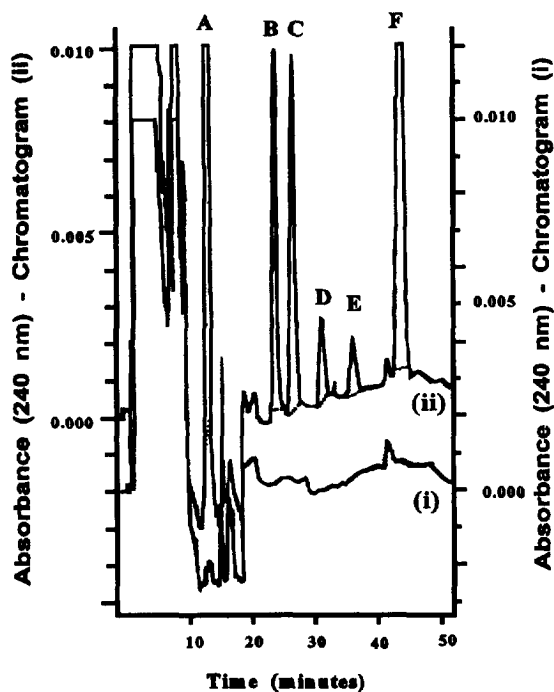
The linearity, precision and accuracy for the

Fig. 2. HPLC–UV chromatogram of (i) blank urine and (ii) blank urine containing synthetic standards of the analytes: A, p-hydroxyphenobarbital (13.5 min, 11.0 μM); B, (5S)-PbG (24.3 min, 8.2 μM); C, (5R)-PbG (27.2 min, 8.0 μM); D, (5S)-PbGA (31.9 min, 2.5 μM); E, (5R)-PbGA (36.9 min, 2.0 μM); F, phenobarbital (44.2 min, 48.0 μM).

analytes were determined in the urine from subject 1 using peak heights. For the measurement of noise, only the random baseline noise was considered. The standard curve was linear ($r^2 \geq 0.975$) for p-hydroxy-phenobarbital (0.6–200.0 μM), (5S)- and (5R)-PbG (2.0–50.0 μM), (5S)- and (5R)-PbGA (2.0–10.0 μM), and phenobarbital (3.2–80.0 μM). The precision (% C.V.) and the accuracy (% S.E.) were determined for each analyte by six consecutive analyses at three or more concentrations. The results are shown in Table 1. The limit of identification (LOI) for (5S)-PbG, (5R)-PbG, (5S)-PbGA and (5R)-PbGA was calculated as the concentration for which the ratio of signal to noise was six [12]. It was 2.0 μM for each conjugate. The limit of detection (LOD), determined as the lowest concentration of the analyte for which the signal to noise ratio was three [12] was 1.0 μM for the N-glycoside conjugates. When (5R)-PbG was present slightly below the LOI of the assay [1.9 μM shown in Fig. 3(i)], the on-line UV scans obtained at the apex of the peaks for the metabolite and the synthetic (5R)-PbG (con-

centration 8.0 μM) were normalized at 240 nm. The metabolite was quantitatively measured only if the correlation coefficient (r^2) for the normalization of the UV scans was greater than or equal to 0.98.

p-Hydroxyphenobarbital, (5S)- and (5R)-PbG, and phenobarbital could be detected in the urine of all five subjects. No UV absorbances corresponding to the retention times of (5S)- and (5R)-PbGA were observed in any of the five subjects. The amount of each analyte quantified in subjects 1–5 is shown in Table 2.

4. Discussion

A review by Tang on the glucosidation of drugs indicates that very few drugs have been evaluated as candidates for glucosylation in mammals [13]. To understand the role of glucosylation in the metabolism of drugs, it is important to determine the relative importance of other complementary pathways, such as glucuronidation. Since phenobarbital

Table 1
Precision and accuracy for the quantitation of phenobarbital and its metabolites in human urine

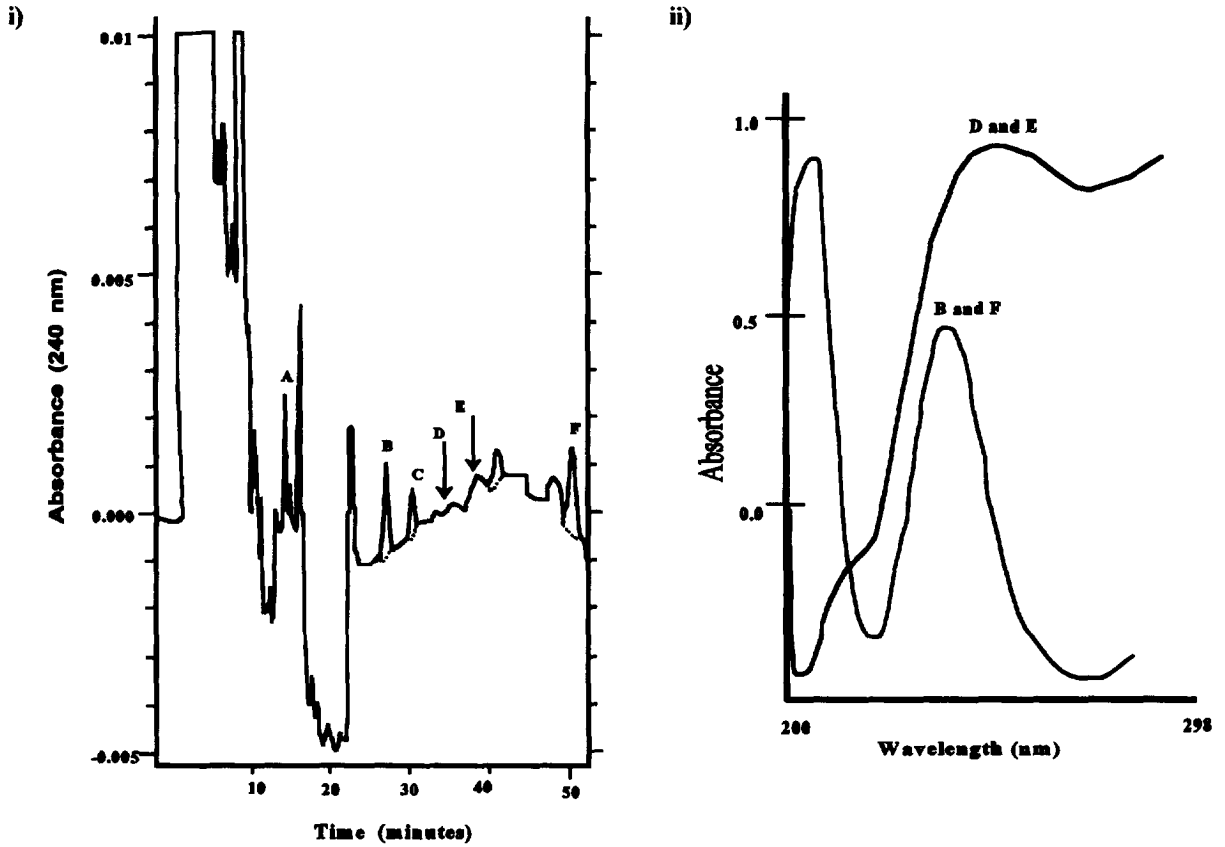| Analytes	Concentration (μM)	Precision (% C.V.)	Accuracy (%)
p-Hydroxy-phenobarbital	0.6	2.55	6.71
	55.0	1.38	3.00
	165.0	1.25	3.20
	275.0	2.55	5.33
(5S)-PbG	2.0	5.78	5.47
	10.0	3.15	3.85
	30.0	1.05	3.53
	50.0	0.72	3.41
(5R)-PbG	2.0	7.63	10.69
	10.0	3.92	3.43
	30.0	2.35	3.45
	50.0	1.98	1.47
(5S)-PbGA	2.0	5.35	5.94
	6.0	2.37	5.32
	10.0	1.43	5.94
(5R)-PbGA	2.0	10.06	7.69
	6.0	4.84	8.66
	10.0	3.76	9.62
Phenobarbital	3.2	5.59	4.59
	16.0	3.10	15.27
	48.0	0.94	10.84
	80.0	0.93	10.77

Fig. 3. (i) Chromatogram of urine from subject 1 after administration of phenobarbital (56–65 h): A=*p*-hydroxyphenobarbital (14.5 min, 1.5 μM); B=(5*S*)-PbG (27.3 min, 4.2 μM); C=(5*R*)-PbG (30.8 min, 1.9 μM); D=retention time of the synthetic (5*S*)-PbGA (34.1 min); E=retention time of the synthetic (5*R*)-PbGA (38.8 min); F=phenobarbital (50.7 min, 7.1 μM). (ii) Normalized on-line UV scans obtained at the apex of peaks B and F and at the retention times of (5*S*)-PbGA (peak D) and (5*R*)-PbGA (peak E).

Table 2
Quantitation of phenobarbital and its metabolites from human urine

Subject	Race	Total percent of the dose						
		PbOH[a]	(5*S*)-PbG	(5*R*)-PbG	(5*S*)-PbGA	(5*R*)-PbGA	Pb[b]	Total
1	A[c]	2.63	6.26	1.30	ND[e]	ND	5.67	15.9
2	A	3.35	7.02	1.50	ND	ND	7.56	19.4
3	C[d]	3.08	14.65	1.89	ND	ND	6.61	26.2
4	C	3.06	7.10	1.66	ND	ND	5.59	17.4
5	C	4.05	4.20	0.60	ND	ND	7.41	16.3
Average		3.27	6.15	1.27	NA[f]	NA	6.56	19.0
Standard deviation		0.52	3.98	0.49	NA	NA	0.93	4.2

[a] PbOH=*p*-Hydroxyphenobarbital; [b] Pb=Phenobarbital; [c] A=Asian; [d] C=Caucasian; [e] ND=Not detected; [f] NA=Not applicable.

had been shown to form N-glucuronide conjugates in mice, it was anticipated that N-glucuronidation may be important in the biotransformation of phenobarbital by humans. Initial studies to preparatively isolate and identify phenobarbital N-glucuronides from a subject's urine did not give definitive results (experimental details not included). The failure to detect the phenobarbital N-glucuronides could have been due to (a) loss of analyte as a result of sample manipulation, or (b) the individual being tested was deficient in being able to form the phenobarbital N-glucuronides or was excreting it at very low levels (although the subject was known to excrete the phenobarbital N-glucosides from prior studies). This analytical method was developed so that individual urine samples could be readily screened for the conjugates with minimal loss prior to analysis. Prior studies have been done at 198 nm (pH 6.5) [3] or 220 nm (pH 5) [4], and required extraction with extensive sample preparation. Initial attempts to inject the urine sample directly and analyze the chromatogram at 200 nm revealed the presence of numerous interferences. The interferences were minimized by using a post-column alkalinization method developed by Clark et al. [14] for the improved detection of barbiturates by HPLC. Alkalinizing the column effluent and monitoring it at 240 nm provided a considerable improvement over prior HPLC methods [3,4,11]. The LOD for the prior methods and this method was comparable. The accuracy and precision of the assay were within the 15% acceptance criteria proposed by the 'Joint Conference on Validation and Control of Bioanalytical Methods' for the analytical methods used in bioavailability, bioequivalence and pharmacokinetic studies [15]. This assay provides a rapid method for the analysis of phenobarbital and its metabolites with improved selectivity.

The urine samples from five subjects (three Caucasians, two Asians) were analyzed after receiving an oral dose of phenobarbital. The phenobarbital N-glucosides had exhibited facile hydrolysis of the barbiturate ring under neutral to basic conditions (1–6 ring opening) [10]. Since it was anticipated that the phenobarbital N-glucuronides would exhibit comparable hydrolytic activity, immediate acidification of the urine minimized any possible decomposition of the N-glycoside conjugates prior to analysis.

The half life of phenobarbital is 99 ± 18 h [16], so urine was collected for approximately one half-life. The percentage of the dose identified as p-hydroxy-phenobarbital, (5S)- and (5R)-PbG, and phenobarbital (Table 2) is comparable to the amount reported in related urinary excretion studies of phenobarbital [3–6]. Since (5R)-PbG could be quantified when it accounted for only 0.6% of the dose, this suggests that if phenobarbital N-glucuronide was present, it could have been detected at a comparable percentage. The dose of phenobarbital given to the subjects is in the range of a typical daily therapeutic dose (100 mg, approximately 1.5 mg/kg for these subjects). The dose used in the mouse study was 40 mg/kg, approximately a 25-fold increase in dose. In general, the metabolism and clearance of most drugs is dependant on the concentration of the drug. When comparing glucuronidation and glucosylation of pranoprofen in mice, it was observed that the glucoside conjugate was primarily formed when the drug concentration was low and that glucuronidation became quantitatively more important at higher drug concentrations [17]. It is possible that phenobarbital N-glucuronidation could be a method for elimination of phenobarbital by humans at a much higher or toxic dose.

This study shows that imide N-glucuronidation is of negligible importance in the urinary biodisposition of phenobarbital at low doses in humans. In vitro studies of barbiturate metabolism using enzyme preparations, in conjunction with in vivo studies would be beneficial in understanding the importance of N-glucosylation with respect to the other oxidative and conjugative pathways for the metabolic biodisposition of the barbiturates and structurally related drugs.

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