Journal of Chromatography, 343 (1985) 219—223
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2662

Note

High-performance liquid chromatographic analysis of 1-(β-D-glucopyranosyl)phenobarbital in urine

VIJAY O. BHARGAVA, WILLIAM H. SOINE* and LORNE K. GARRETTSON

Departments of Pharmacy/Pharmaceutics and Medicinal Chemistry, School of Pharmacy, and Department of Pediatrics, School of Medicine, Virginia Commonwealth University, Richmond, VA 23298 (U.S.A.)

(First received December 20th, 1984; revised manuscript received April 15th, 1985)

Although phenobarbital (PB) has been in use as a hypnotic and anticonvulsant since 1912, the quantitative disposition of this drug in man has not been fully established [1]. PB had been shown to be excreted almost exclusively in the urine as PB, *p*-hydroxyphenobarbital (HPB) and the O-glucuronide of HPB [2]; however, these studies had accounted for only 35—50% of the PB dose (urine was collected for five to nine half-lives) [3, 4]. Recently, Tang et al. [5] proposed that 1-(β-D-glucopyranosyl)phenobarbital (PNG), shown in Fig. 1, was a quantitatively significant urinary metabolite of PB in humans. When this additional metabolite was quantitated, 87% of a single dose of radiolabelled PB could be accounted for in the urine after 4.5 half-lives. Additional studies have confirmed the occurrence of PNG as a metabolite of PB and suggest it may account for a significant percentage of the urinary excretion of PB in some humans [6, 7].

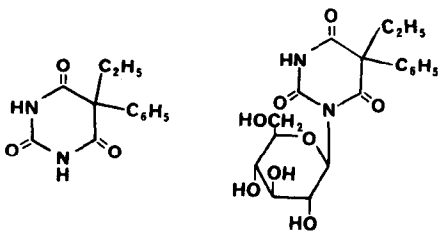

Fig. 1. Structures of phenobarbital and 1-(β-D-glucopyranosyl)phenobarbital.

0378-4347/85/$03.30

To faciliate further study of the excretion of this newly recognized and novel metabolite, an isocratic high-performance liquid chromatographic (HPLC) method was developed for the detection and quantification of PNG, PB and HPB in urine. This method is described in this report.

EXPERIMENTAL

Phenobarbital was purchased from Mallinckrodt (St. Louis, MO, U.S.A.). *p*-Hydroxyphenobarbital and 5-methyl-5-phenylhydantoin, the internal standard (I.S.), were purchased from Aldrich (Milwaukee, WI, U.S.A.). 1-(β-D-Glucopyranosyl)phenobarbital was synthesized as previously described [7]. Acetonitrile, monobasic and dibasic sodium phosphate were HPLC grade and purchased from Fischer Scientific (Pittsburgh, PA, U.S.A.). All other chemicals were reagent grade. The β-glucuronidase/sulfatase, derived from *Helix aspersa*, Type HA-4, was obtained from Sigma (St. Louis, MO, U.S.A.).

The liquid chromatograph (Model 6000A, Waters Assoc.) was equipped with a variable-wavelength detector (Model HM/HPLC Holochrome, Gilson) and separation was achieved using a C_{18} reversed-phase column (250 × 4.6 mm I.D., particle size 5 μm, Ultrasphere ODS, Beckman) with a C_{18} guard column (32 × 4 mm I.D., particle size 7—50 μm, Waters Assoc.). The mobile phase was a mixture of acetonitrile—0.025 *M* sodium phosphate buffer, pH 7.0 (15:85). The volume of sample injected was 20 μl (Model 7125, Rheodyne). The separation was carried out at ambient temperature at a flow-rate of 1.4 ml/min and chart-speed of 0.25 cm/min. The effluent was monitored at 204 nm with a sensitivity of 0.05 a.u.f.s.

Individual stock solutions of PB, HPB, PNG and I.S. were prepared in methanol at a concentration of 1 mg/ml and stored at 4° C. The solutions were stable for at least one month under these conditions. Standards were prepared in urine by transferring known amounts of the stock solution to a 10-ml volumetric flask, evaporating the methanol under a stream of nitrogen and dissolving the residue in blank urine.

To prepare the sample for analysis, 4.0 μg (20 μl of a 1:5 dilution of I.S. stock solution) of I.S. were added to 200 μl of urine in a 10-ml screw-capped test-tube followed by addition of 1.0 ml of saturated ammonium sulphate, and 2.0 ml of ethyl acetate. The solution was extracted using a rotary mixer at 25 rpm for 30 min. After centrifugation at 500 *g* for 5 min the organic layer was transferred to a conical drying tube. The ethyl acetate was evaporated to dryness in a vortex evaporator (Buchler Instruments) at a pressure of 30 mmHg (no heat). The residue was reconstituted in 200 μl methanol and a 20-μl aliquot was injected onto the column.

For analysis of the O-glucuronide of HPB, 200 μl of urine were added to a screw-capped test-tube containing 4.0 μg of I.S. and 300 μl of 0.05 *M* sodium acetate buffer, pH 4.7 containing 500 U of β-glucuronidase/sulfatase. The solution was incubated for 24 h at 37—38° C. After the incubation, 1.0 ml of saturated ammonium sulfate and 2.0 ml of ethyl acetate were added and the sample was extracted and analyzed as previously described.

Urine specimens were obtained from eight patients diagnosed as having epilepsy and receiving a chronic regimen of PB. These patients were receiving

no other anticonvulsant medications. The urine samples were stored frozen at −20°C until analyzed.

RESULTS

Blank urine and PB and metabolites dissolved in water were put through the analysis procedure and their chromatograms are shown in Fig. 2B and A, respectively. The retention times of HPB, PNG, IS and PB were 7.0, 9.4, 11.5 and 21.6 min, respectively. The chromatogram of the urine of patient No. 1 is shown in Fig. 2C.

Recovery of PB and metabolites was determined by triplicate analysis of standards prepared in blank urine: PB (200 and 50 μg/ml), HPB (120 and 30 μg/ml) and PNG (120 and 30 μg/ml). The recovery was determined relative to the I.S. by direct injection of equivalent quantities of compound dissolved in deionized water. The mean values were 100.6 ± 0.1% for PB, 96.8 ± 0.22% for HPB and 81.1 ± 4.1% for PNG.

The within-run precision was evaluated by analyzing urine samples (n = 8) spiked with 80 μg/ml PB and 60 μg/ml of both HPB and PNG. The coefficient of variation was 5.0% for PB, 5.8% for HPB and 4.7% for PNG. The reproducibility observed with the same standard on five consecutive days gave coefficients of variation of 5.5% for PB, 3.0% for HPB and 5.6% for PNG.

A standard calibration curve was obtained by extraction of compounds from blank urine and plotting peak height ratios of drug or metabolite to internal standard as a function of drug or metabolite concentrations. In concentration ranges 5—200 μg/ml (22—862 nmol/ml) PB, 2—120 μg/ml (8—484 nmol/ml) HPB, and 2—120 μg/ml (5—305 nmol/ml) PNG, the y intercept was 0.05, 0.06, and 0.02, respectively, with a correlation coefficient of 0.997 or better. The lower limit of detection was 1.0 μg/ml HPB, PNG and PB.

To determine if PNG was stable to conditions necessary for quantitation of the glucuronide of HPB, duplicate samples of a PNG standard were analyzed

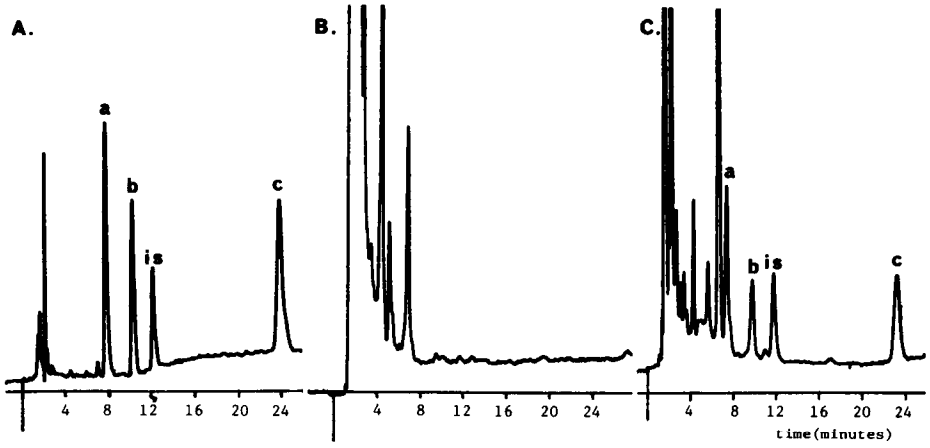

Fig. 2. Chromatograms of: A, HPB (a), PNG (b), internal standard (is) and PB (c) dissolved in water and carried through the analysis procedure; B, blank urine extract; and C, patient on PB therapy (HPB = 6.5 μg/ml, PNG = 37.6 μg/ml, PB = 15.0 μg/ml).

before and after a 24-h hydrolysis with β-glucuronidase/sulfatase. The average values obtained were 95.8 μg/ml before hydrolysis and 95.4 μg/ml after hydrolysis.

DISCUSSION

Kadar et al. [6] had previously reported a mass spectrometric method for quantification of PNG in urine. Their method required derivatization of PNG to the tetraacetate prior to analysis and use of the N-glucoside of amobarbital as an internal standard. Due to the limited availability of their internal standard and the desire to measure the metabolite without derivatization, reversed-phase HPLC was evaluated as an alternative method. By modifying the previously reported HPLC methods for quantification of PB and HPB [8—10], separation of PB, HPB, PNG and I.S. was found to be optimal between pH 7.0 and 8.0 when the mobile phase contained 15% acetonitrile. Upon analysis of a blank urine extract with the detector at 254 nm, a strongly absorbing substance was observed to co-chromatograph with HPB; however, at 204 nm this interference was not observed (Fig. 2B). In addition, at 204 nm the sensitivity was enhanced three- to five-fold. Previous assays in which PB, HPB and the O-glucuronide of HPB were quantitated, β-glucuronidase and/or acid hydrolysis at high temperatures were used to hydrolyze the O-glucuronide of HPB prior to analysis. In this assay β-glucuronidase/sulfatase was used and the observed percentage of HPB and its O-glucuronide conjugate was consistent with a previous study [5]. An average of 56% (range 31—87%) of the HPB excreted, was as the O-glucuronide (Table I). It should be noted that Tang et al. [11] had reported that the structurally related N-glucoside of amobarbital was stable

TABLE I

PHENOBARBITAL AND METABOLITE CONCENTRATIONS IN RANDOMLY SAMPLED HUMAN URINE SPECIMENS BEFORE AND AFTER HYDROLYSIS WITH β-GLUCURONIDASE

Patient	Age (years)	Sex	Dose (mg/day)	Concentration, μg/ml (nmol/ml)					
				Before β-glucuronidase			After β-glucuronidase		
				PB	PNG	HPB	PB	PNG	HPB
1	70	F	90	15.0 (64.7)	37.6 (95.4)	6.5 (25.7)	15.2 (65.4)	38.1 (96.7)	20.6 (82.9)
2	22	F	120	26.0 (112.2)	8.7 (22.0)	23.4 (94.3)	25.5 (109.9)	9.0 (22.9)	41.6 (167.6)
3	22	F	60	8.7 (37.3)	2.7 (6.8)	5.3 (21.2)	8.8 (37.9)	2.7 (6.8)	9.7 (37.2)
4	22	M	100	17.3 (74.5)	N.D.* —	4.7 (10.1)	16.7 (72.0)	N.D. —	7.1 (28.5)
5	42	M	60	11.4 (48.9)	5.0 (15.1)	2.8 (11.4)	12.0 (51.7)	5.9 (14.9)	5.7 (23.4)
6	15	F	60	7.2 (30.8)	10.6 (26.8)	12.4 (50.0)	7.5 (32.1)	10.1 (25.6)	24.7 (99.8)
7	26	M	90	19.5 (83.9)	3.1 (7.9)	5.1 (20.7)	19.3 (83.2)	3.2 (8.0)	19.3 (77.8)
8	15	M	90	47.8 (206.0)	6.3 (15.9)	25.7 (107.7)	49.0 (211.2)	6.4 (16.2)	30.6 (112.1)

*N.D. = Not detected.

during β-glucuronidase incubation. It was observed that PNG was also stable under these conditions as seen in the control experiment and in Table I, in which the concentration of PNG in the patient's urine did not change after enzymatic hydrolysis.

Randomly collected urine samples from eight patients being treated with PB were analyzed for PNG. PNG was detected in all but one patient and the ratio of PNG to PB or HPB was highly variable. This variability was similar to that reported by Kalow et al. [12] for formation of the N-glucoside of amobarbital.

This analytical method has been developed, tested and used in the detection and quantification of PNG, PB and HPB in the urine of subjects receiving only PB. Possible interference in this assay due to other anticonvulsant drugs has yet to be assessed.

It is hoped that this analytical procedure will facilitate further investigation into the N-glucoside pathway and its possible impact on genetic [12] and age-dependent [13, 14] variability observed in PB elimination.

ACKNOWLEDGEMENT

This work was supported in part by a grant from the VCU Grants-in-Aid Program for Faculty.

REFERENCES

1 M.P. Whyte and A.S. Dekaban, Drug Metab. Dispos., 5 (1976) 63.
2 T.C. Butler, J. Pharmacol. Exp. Ther., 116 (1956) 326.
3 A.J. Ravn, M. Lunding and O. Secher, Acta Pharmacol. Toxicol., 27 (1969) 1983.
4 P. Lous, Acta Pharmacol. Toxicol., 10 (1954) 166.
5 B.K. Tang, K. Kalow and A.A. Grey, Drug Metab. Dispos., 7 (1979) 315.
6 D. Kadar, B.K. Tang and A.W. Conn, Can. Anaesth. Soc. J., 29 (1982) 16.
7 W.H. Soine, V.O. Bhargava and L.K. Garrettson, Drug Metab. Dispos., 12 (1984) 792.
8 R.W. Dykeman and D.J. Ecobichon, J. Chromatogr., 162 (1979) 104.
9 M.W. Anders and J.P. LaTorre, Anal. Chem., 42 (1970) 1430.
10 H.E. Kunze, W.D. Hooper and M.J. Eadie, Ther. Drug Monitor., 3 (1981) 34.
11 B.K. Tang, T. Inaba and W. Kalow, Drug Metab. Dipos., 3 (1975) 479.
12 W. Kalow, D. Kadar, T. Inaba and B.K. Tang, Clin. Pharmacol. Ther., 21 (1977) 530.
13 B. Jalling, Develop. Med. Child Neurol., 16 (1970) 781.
14 L.K. Garrettson and P.G. Dayton, Clin. Pharmacol. Ther., 11 (1970) 674.