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Note

Quantitation of 1-(β-D-glucopyranosyl)phenobarbital in plasma and bile

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Phenobarbital (PB) is frequently used as a sedative, a hypnotic and an anticonvulsant. It is also one of the most commonly recommended drugs for seizures in the neonate [1]. Studies in adults [2] and neonates [3] have shown that 1-(β-D-glucopyranosyl)phenobarbital (PNG) is a quantitatively significant metabolite of PB.

Studies to identify an animal model to investigate the disposition of xenobiotics via the N-glucosidation pathway have suggested that glucosidation may not be a significant metabolic pathway in several of the commonly used animal species [4,5]. However, the excretion of the glucoside metabolite via the biliary route in these animals has not been investigated. Biliary excretion of this metabolite may be possible owing to its large molecular mass. Several high-performance liquid chromatographic (HPLC) methods measuring PB and *p*-hydroxyphenobarbital (HPB) [6–8] have been reported. Recently we described an HPLC procedure to measure PNG in addition to the above compounds [9]. Since all of these procedures are applicable only for urine samples, pharmacokinetics and disposition of PB, particularly via the N-glucosidation pathway in adults and neonates, have not been completely elucidated. A recent assay quantitating PB, HPB and PNG in urine by gas chromatography–chemical-ionization mass spectrometry [10] may be applicable to plasma and bile. However, this assay requires administration of radiolabelled drug and the use of a mass spectrometer, which may not be easily available. In view of these factors, we describe here the development of a rapid and sensitive method for the detection of PB and its major metabolites in plasma and bile, which may be used when working with neonatal and pediatric populations or with small laboratory animals (e.g. rats).

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EXPERIMENTAL

Materials

PB was purchased from Mallinckrodt (St. Louis, MO, U.S.A.). HPB and 5-methyl-5-phenylhydantoin, the internal standard (I.S.), were purchased from Aldrich (Milwaukee, WI, U.S.A.). PNG was synthesized as previously described [2]. Acetonitrile, monobasic and dibasic sodium phosphate were HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). All other chemicals were reagent grade.

Instrumentation

The liquid chromatograph (Model M-45, Waters Assoc.) was equipped with a variable-wavelength UV detector (Model 481, Waters Assoc.) and separation was achieved on 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 acetonitrile–0.025 *M* sodium phosphate buffer, pH 7.0 (15:85). Samples were injected into a Rheodyne injector (Model 7125). The separation was carried out at ambient temperature at a flow-rate of 1.4 ml/min and a chart speed of 0.25 cm/min. The effluent was monitored at 254 and 260 nm for plasma and bile, respectively.

Analytical procedures

Plasma. To prepare samples for analysis, 10.0 μg (50 μl of a 1:5 dilution of a 1 mg/ml methanol solution) of I.S. was placed in a microcentrifuge tube and evaporated to dryness under a stream of nitrogen. Then 50 μl of plasma was added to the tube, followed by 100 μl of ice-cold acetonitrile. The mixture was vortex-mixed for 5 s and centrifuged at 13 000 *g* for 2 min to pellet the precipitated proteins. A 20-μl aliquot of supernatant was injected into the chromatographic system.

Bile. To prepare the sample for analysis, 20.0 μg (100 μl of a 1:5 dilution of a 1 mg/ml methanol solution) of I.S. was placed in a 10-ml tube and evaporated to dryness under a stream of nitrogen. Then 200 μl of bile was added to the tube, followed by 1.0 ml of saturated ammonium sulphate. The solution was extracted twice with 1 ml of ethyl acetate. The samples were mechanically shaken for 15 min and then centrifuged at 500 *g* for 5 min. The organic layers were pooled in a conical drying-tube and evaporated to dryness in a vortex–evaporator (Buchler Instruments) at a pressure of 30 mmHg (no heat). The residue was reconstituted in 200 μl of methanol, and a 20-μl aliquot was injected onto the column.

Calibration curves

Calibration curves were constructed by using blank plasma or bile to which increasing amounts of PB and its metabolites were added to yield final concentrations in the ranges 5–200 μg/ml for plasma and 5–80 μg/ml for bile. Plasma and bile standards stored at −20°C were found to be stable for a period of up to six weeks. Peak-height ratios of PB and its metabolites to the I.S. were used to

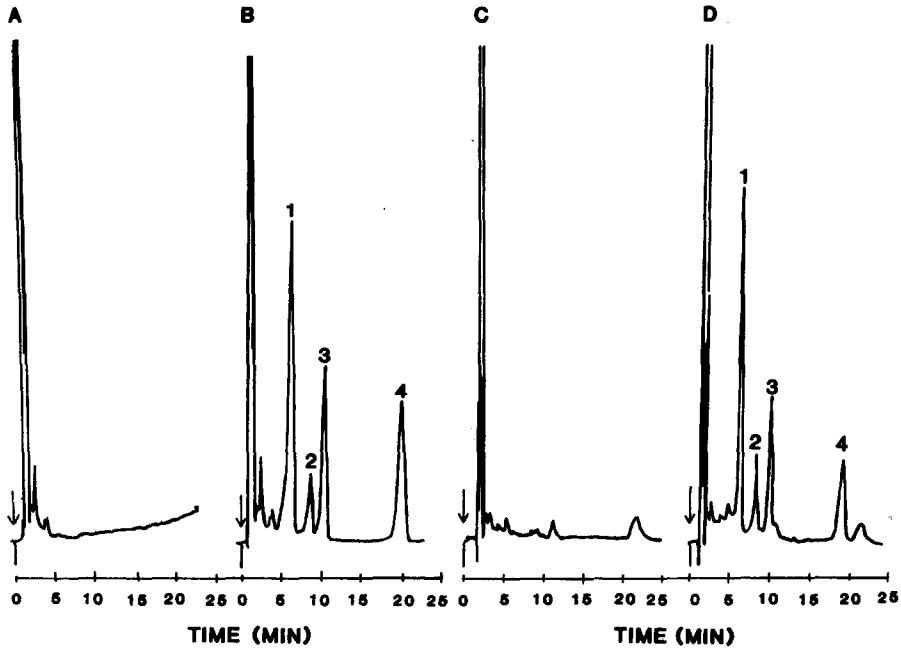

Fig. 1. Chromatograms of (A) blank plasma, (B) plasma spiked with HPB (1), PNG (2), internal standard (3) and PB (4), (C) blank bile extract and (D) bile extracted after spiking with HPB (1), PNG (2), internal standard (3) and PB (4). Concentration of PB and metabolites was 10 μg/ml and detector setting was 0.04 a.u.f.s.

construct the standard curve. All standard curves were calculated by least-squares regression analysis of peak-height ratios versus drug concentration.

RESULTS AND DISCUSSION

Using the chromatographic conditions described, PB, HPB, PNG and the I.S. yielded sharp, well resolved peaks with no interference from endogenous compounds at 19.06, 6.36, 8.3 and 10.13 min, respectively. Representative chromatograms of human plasma and rat bile before and after spiking with PB and its metabolites are shown in Fig. 1.

The standard curves based on peak-height ratios of the drug and its metabolites to the I.S. were all linear and highly reproducible. In concentration range of 5–200 μg/ml PB, HPB and PNG in plasma, the y-intercepts were −0.006, 0.017 and 0.007 with slopes of 0.013, 0.027 and 0.006, respectively. The standard curves for bile were constructed within the concentration range of 5–80 μg/ml PB, HPB and PNG. The y-intercepts obtained were −0.04, 0.028 and −0.021 with slopes of 0.06, 0.17 and 0.05 for PB, HPB and PNG, respectively. The correlation coefficients for all standard curves were 0.993 or better. The least amount that could be quantitated was 1.0 μg/ml of HPB, PNG and PB.

The precision of the assay was evaluated by analysing spiked plasma and bile samples at two different concentrations ($n=8$). The concentrations used were

10 μg/ml and 40 μg/ml PB, HPB and PNG. The within-day coefficient of variation (C.V.) was 2.72% for PB, 3.13% for HPB and 5.83% for PNG. The between-day C.V., calculated by comparing the same standards on five consecutive days, was 5.5% for PB, 3.78% for HPB and 6.2% for PNG.

Analytical recovery studies, in which spiked bile samples were compared with aqueous standards over a concentration range of 5–80 μg/ml, showed average recoveries of 98.3, 93.2 and 80.4% for PB, HPB and PNG, respectively.

A wavelength of 254 nm was chosen to monitor the plasma levels so that the assay would be adaptable to a fixed-wavelength detector. No interferences from endogenous substances in plasma were observed at this wavelength. Several wavelengths were evaluated to quantitate PB and its metabolites in bile. The optimum wavelength was found to be 260 nm with no interferences from endogenous substances in the bile. A flow-rate of 1.4 ml/min was found to be optimal for both separations.

PNG has been identified as a quantitatively significant metabolite of PB in adults [2,11] as well as in neonates [3]. Other barbiturate glucosides have also been identified [12]. These studies have only examined metabolite levels in urine. To study the disposition of PB completely it is necessary to quantitate levels of PB and its metabolites in both plasma and urine. Individual differences in the capacity to N-glucosidate phenobarbital may help explain the genetic [13] and age-dependent [14,15] variability observed in PB elimination. Animal species capable of barbiturate metabolism via N-glucosidation have been studied. In vivo studies conducted in dogs, mice, hamsters, guinea-pigs and albino rats have failed to reveal any amobarbital-N-glucoside in urine, except trace amounts in albino rats [4]. A recent study has indicated that N-glucosidation occurs to some extent in cats [5]. This study reports a discrepancy between in vivo urinary data and in vitro estimates of N-glucosidation capacity observed in cats. There is a possibility that, owing to the large molecular mass of the glucoside metabolite, it may be excreted in the bile and this may explain the discrepancies in the data.

In summary, the present method enables the quantitation of PB and its two major metabolites, HPB and PNG, in plasma and bile. Levels observed in previous studies [9,16] indicate that the sensitivity of this assay is adequate for the measurement of unchanged PB in plasma. Metabolite levels in plasma have not been followed to the author's best knowledge. The total concentration of [^{14}C]phenobarbital and/or its metabolites in bile has been shown to be ten-fold higher than in plasma [17], although individual metabolite levels in bile have not been reported. The small volume of biological fluid required for this method (50 μl plasma and 200 μl bile) is especially important when dealing with neonates or small animals (e.g. rats), where samples are difficult to obtain and volume is limited. Consequently, the procedures should permit more detailed pharmacokinetic and drug disposition studies of phenobarbital-N-glucoside.

REFERENCES

1 C.T. Lombroso, Pediatrics, 53 (1974) 536.
2 W.H. Soine, V.O. Bhargava and L.K. Garrettson, Drug Metab. Dispos., 12 (1984) 792.

3 V.O. Bhargava and L.K. Garrettson, Acad. Pharm. Sci., 15 (1985) 147.
4 B.K. Tang, A.A. Grey, P.A.J. Reilly and W. Kalow, Can. J. Physiol. Pharmacol., 58 (1980) 1167.
5 C.C. Giovanna, J. Malle, D. Kadar, B.K. Tang and W. Kalow, Can. J. Physiol. Pharmacol., 63 (1984) 1263.
6 R.W. Dykeman and D.J. Ecobichon, J. Chromatogr., 162 (1979) 104.
7 M.W. Anders and J.P. LaTorre, Anal. Chem., 42 (1970) 1430.
8 H.E. Kunze, W.D. Hooper and M.J. Eadie, Ther. Drug Monit., 3 (1981) 34.
9 V.O. Bhargava, W.H. Soine and L.K. Garrettson, J. Chromatogr., 343 (1985) 219.
10 B.K. Tang, B. Yilmaz and W. Kalow, Biomed. Mass Spectrom., 11 (1984) 462.
11 B.K. Tang, W. Kalow and A.A. Grey, Drug Metab. Dispos., 7 (1979) 315.
12 B.K. Tang, W. Kalow and A.A. Grey, Res. Commun. Chem. Pathol. Pharmacol., 21 (1979) 45.
13 W. Kalow, D. Kadar, T. Inaba and B.K. Tang, Clin. Pharmacol. Ther., 21 (1977) 530.
14 B. Jalling, Dev. Med. Child Nerol., 16 (1970) 781.
15 L.K. Garrettson and P.G. Dayton, Clin. Pharmacol. Ther., 11 (1970) 674.
16 W. Pitlick, M. Painter and C. Pippenger, Clin. Pharmacol. Ther., 23 (1978) 346.
17 C.D. Klaassen, Br. J. Pharmacol., 43 (1971) 161.