CHROMBIO. 234

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Simultaneous determination of phenytoin, phenobarbital and their para-hydroxylated metabolites in urine by reversed-phase high-performance liquid chromatography

RONALD WELLINGTON DYKEMAN and DONALD JOHN ECOBICHON*

Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec H3G 1Y6 (Canada)

Phenytoin (DPH) and phenobarbital (PB), alone or in combination, remain the drugs of choice in epilepsy characterized by general convulsive seizures [1]. Both DPH and PB are para-hydroxylated by hepatic smooth endoplasmic reticular enzymes to inactive metabolites which are subsequently conjugated with glucuronic acid and excreted in the urine. Of daily doses, approximately 60—70% of DPH and 25—80% of PB appear in the urine as glucuronidated 5-(p-hydroxyphenyl) 5-phenylhydantoin (HPPH) and 5 ethyl 5-(p-hydroxyphenyl) barbituric acid (pHPB) respectively [2,3]. Less than 4% of DPH and 30% of PB is excreted unchanged in the urine.

Urinary excretion data provide a non-invasive method of assessing the overall elimination constant for loss of drug from the body, as well as the rate constants for the production of metabolites [4]. Analytical systems applicable to the measurement of urinary DPH and PB should permit simultaneous quantitation of both unchanged drugs and metabolites. Gas—liquid chromatographic (GLC) techniques for the analysis of PB are characterized by poor reproducibility and non-linear calibration curves due to peak tailing as a result of irreversible or reversible adsorption to column packing. Attempts to circumvent these problems by derivatization have resulted in some improvement, though derivative formation may be incomplete and column degradation occurs with methylating agents [5]. The analysis of HPPH and pHPB by GLC has also been reported and could be subjected to the same criticism [6,7]. Radio-immunoassay methods have been utilized for DPH and PB determinations but recently pHPB has been shown to interfere substantially with the quantitation of PB in one

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*To whom correspondence should be addressed.

such system [8]. High-performance liquid chromatography (HPLC) circumvents many of the problems associated with GLC determinations, such as thermal degradation, prerequisite derivatization and laborious extractions. DPH, PB and HPPH have been successfully quantitated in urine by normal as well as reversed-phase methods [9—11]. The simultaneous determination of DPH, PB and HPPH in serum has recently been reported but PB and HPPH were poorly resolved [12]. This lack of resolution could invalidate quantitation of HPPH in urine where PB levels may greatly exceed HPPH and the degree of interference would depend on the amount of PB present. Residues of pHPB have not been evaluated in these or other HPLC systems and the simultaneous quantitation of pHPB, PB, HPPH and DPH by HPLC has not been reported. A reversed-phase HPLC technique has been developed which allows the simultaneous determination of pHPB, PH, HPPH and DPH in biological fluids by use of an inexpensive fixed wavelength liquid chromatograph.

MATERIALS AND METHODS

Apparatus

Chromatography was performed on a component system constructed from a Model 110 solvent-metering pump (Altex Scientific, Berkeley, Calif., U.S.A.), a Spectra Physics Model 3100 liquid chromatograph (Chromatronix, Berkeley, Calif., U.S.A.) equipped with a Model 230 dual channel UV absorbance detector and a 20-μl loop sample injection valve. The column, 300 × 3.9mm I.D., was packed with 10 μm μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.) The mobile phase was a 40:60 (v/v) mixture of methanol—NaH_2PO_4 (0.025 M) adjusted to pH 8. The column effluent was monitored at 0.01 and 0.04 a.u.f.s. at 254 nm and the flow-rate was 1.0 ml/min.

Reagents

Methanol and ethyl acetate, redistilled ACS reagent grade, were obtained from Caledon (Georgetown, Canada). All other chemicals were reagent grade.

Standards

Phenacetin (acetophenetidine), the internal standard, and phenytoin (sodium 5-5 diphenylhydantoin) were purchased from Sigma (St. Louis, Mo., U.S.A.). HPPH (5-(p-hydroxyphenyl) 5-phenylhydantoin) from Aldrich (Milwaukee, Wisc., U.S.A.) and phenobarbital from Allen Hanbury (Toronto, Canada). The pHPB (5-ethyl 5-(p-hydroxyphenyl) barbituric acid) was a generous gift from Hoffman-La Roche (Montreal, Canada). Stock solutions (10 mg/ml) of DPH, PB, HPPH and pHPB were prepared by dissolving each in 10 ml of methanol. Standard spiking solutions containing 100, 200, 400, 600 and 1000 μg/ml of PB, DPH, HPPH and pHPB were prepared by diluting aliquots of the stock solutions to 10 ml with methanol. A stock solution of phenacetin (4mg/ml), the internal standard, was prepared in methanol and diluted 1:10 for extractions. Calibration standards were prepared from 1:10 dilutions of the spiking solutions and 1:100 dilutions of the stock internal standard solution. These standard solutions were stable for at least 1 month at 4°.

Procedure

Aliquots of the total collection period urine volume were stored at $-20°$ or analyzed directly as follows. Samples (2ml) of urine and 2.0 ml of 12 M HCl were heated in loosely-capped round-bottomed centrifuge tubes for 120 min at 90°. After cooling to room temperature, 2.0 ml of 12 M NaOH were added and the acidic urine mixture adjusted to pH 7 with 20% NaOH. Following the addition of 0.2 ml of internal standard and 2.0 ml of 0.10 M phosphate buffer (pH 6.3), the parent drugs and metabolites were extracted with 10 ml of ethyl acetate on a serological shaker rotating at 40 rpm for 15 min. After centrifugation at 600 g for 10 min, the organic layer was transferred to a graduated conical drying tube. The ethyl acetate extraction was repeated and the combined organic levels evaporated to dryness under a stream of air in a Multi-Bloc heater at 50°. The extracted residue was redissolved in 2.0 ml of methanol and 20 μl was injected into the liquid chromatograph. The peak height ratios of pHPB:internal standard, PB:internal standard, HPPH:internal standard and DPH:internal standard were calculated.

Calibration and recovery

A standard curve was constructed by adding known amounts of pHPB (10—60 μg/ml) to drug-free urine. Extraction efficiency was determined by adding the internal standard to ethyl acetate layer residues from urine containing known amounts of pHPB, PB, HPPH and DPH. Efficiency was calculated by comparing the ratios of pHPB:internal standard added to ethyl acetate layer residues to ratios of pHPB:internal standard obtained from calibration solutions. Similarly, PB, HPPH and DPH extraction efficiencies were determined.

RESULTS AND DISCUSSION

Optimum separation was dependent upon achieving adequate retention of pHPB, minimizing the retention of DPH and resolving PB and HPPH. Retention times for pHPB, HPPH, and DPH were found to be mainly influenced by the methanol content of the eluent, while PB retention was more sensitive to changes in pH. From plots of retention time versus eluent methanol concentration, a 40:60 (v/v) mixture of methanol—NaH_2PO_4 (0.025 M) buffer was selected. The retention times for pHPB, PB, HPPH, S (phenacetin) and DPH were 5.3, 9.2, 11.4, 14.2 and 28.5 min respectively (Fig. 1A). Resolution of PB and HPPH was only achieved at pH 8 or above. Since silica dissolves at about pH 8, leading to column degradation with loss of the stationary liquid phase, and resolution and PB and HPPH are not sufficiently resolved below pH 8 maintenance of a pH of 8 is imperative. Using a potassium phosphate—acetonitrile eluent at pH 8, Soldin and Hill [13] reported the separation of parent anticonvulsants extracted from serum but not their metabolites. We have not found column deterioration at pH 8 during 3 months of continual use, results in agreement with Soldin and Hill [13]. The separation factor for PB and HPPH with the methanol—NaH_2PO_4 (0.025 M) eluent was found to be 1.35 compared to 1.14 previously reported for a 17:83 mixture of acetonitrile and water [12]. This difference may seem insignificant until the band size ratio of PB and HPPH at equal concentrations is considered. Under these conditions
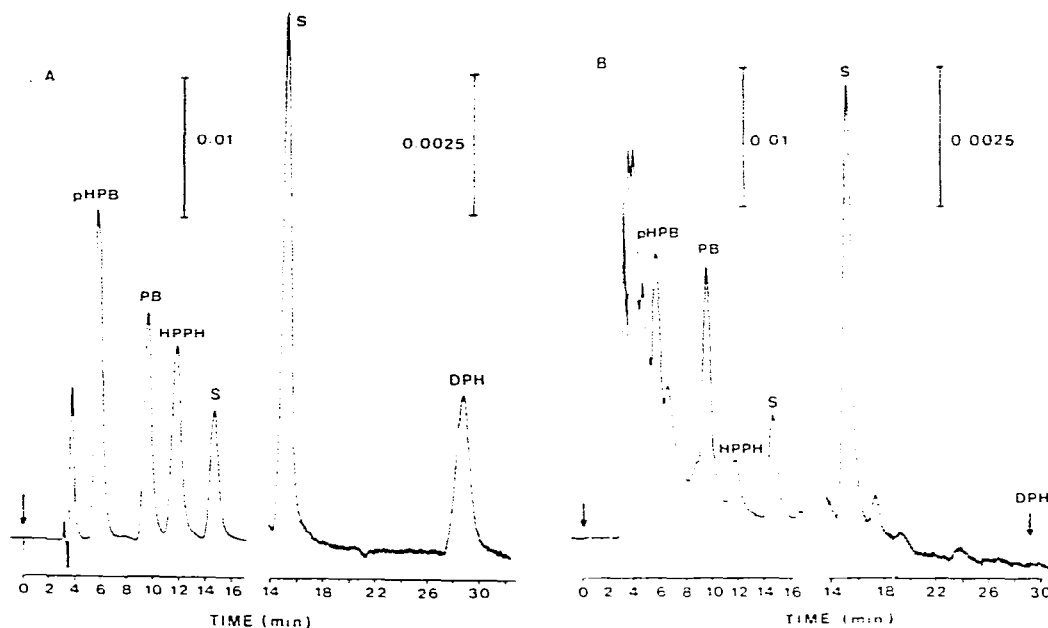
Fig. 1. High-performance liquid chromatogram of phenobarbital (PB), diphenylhydantoin (DPH) and the para-hydroxylated metabolites, hydroxyphenobarbital (pHPB) and 5-(p-hy-droxyphenyl)-5-phenylhydantoin (HPPH). The internal standard (S) was phenacetin. Trace A shows a typical chromatogram for the reference standard containing 40 µg/ml of pHPB, PB, HPPH and DPH measured at 0.04 or at 0.01 a.u.f.s. due to the low sensitivity of DPH. Trace B shows a chromatogram of a urine sample from a female patient containing, on analysis, 19 µg/ml pHPB, 59.5 µg/ml PB and 14.8 µg/ml HPPH. DPH was below the limit of detection at 0.01 a.u.f.s.

we found a band size ratio of 1:1—2:1 and a resolution of approx. 1.1—1.25 compared with 3:1—4:1 and an estimated resolution of 0.8—1.0 from the previous method [12]. Since a resolution of 1.0 is required for accurate peak height measurements, PB could interfere with the quantitation of serum HPPH in the acetonitrile—water system where HPPH levels are lower than PB as well as in urine where variable relative proportions of PB and HPPH may exist.

The response of the detector at 254 nm to DPH was much less than for pHPB, PB and HPPH but dual channel capability permits the simultaneous monitoring at 0.01 a.u.f.s. of DPH and at 0.04 a.u.f.s. of pHPB, PB and HPPH at levels found in clinical samples of serum and urine. The internal standard concentration of phenacetin (4 µg/ml of urine) was selected to retain detector response within scale at each of the detector settings, enabling peak height ratio quantitation of each of the compounds simultaneously. The detection limits for pHPB, PB, HPPH and DPH were 1.5 µg/ml, 2.0 µg/ml, 2.0 µg/ml and 5 µg/ml respectively. The sensitivity of the detector was not sufficient to measure free DPH in urine but would be suitable for determinations in serum. The 254 nm detection wavelength was selected because it is usually available in low cost detectors and is more selective than the 195 nm wavelength used in a previous study [12].

TABLE I

LINEAR REGRESSION PARAMETERS FOR STANDARD CALIBRATION CURVES

$n=20$.

Drug	Range (μg/ml)	Slope	Intercept	Correlation coeff.
pHPB	10— 60	0.067	-0.08	0.9987
PB	10—100	0.039	-0.03	0.9945
HPPH	10—100	0.036	-0.02	0.9995
DPH	10—100	0.007	0.00	0.9979

Standards in methanol were analyzed and from these data standard curves were constructed by plotting peak height ratio (drug:internal standard, y axis) versus drug concentration (μg/ml). Each point on the curve was the mean of at least 5 determinations. The relationships were linear for all compounds as summarized in Table I and standards prepared in urine gave similar results.

Extraction efficiencies of urine solutions containing pHPB, PB, HPPH and DPH were 90% (coefficient of variation, C.V., 3.5), 81% (C.V. 8.2), 83% (C.V. 3.8) and 102% (C.V. 6.0) respectively. Ethyl acetate solvent extraction of urine is convenient because of its relative density and volatility. Occasionally in samples of male urine, a small peak was observed to immediately precede the elution of PB, but no substantial interference was found.

A diol metabolite of DPH, 5-(3,4-dihydroxycyclohexa-1, 5-dienyl)5-phenyl-hydantoin occurs to the extent of about 10—20% in the urine of male volunteers under steady conditions of DPH [14]. Acid hydrolysis of the glucuronide of this diol has been shown to produce dehydration of the metabolite to give equal amounts of the 30H and 40H isomers of HPPH and the latter would interfere with the assay [15]. Procedures involving pre-extraction with isoamyl alcohol to remove this metabolite prior to hydrolysis have been described, but since pHPB and HPPH are known to be conjugated with glucuronic acid, enzymatic cleavage with β-glucuronidase would seem to be more appropriate [16]. Since our primary objective was to describe a chromatographic system capable of the simultaneous separation of both the parent drugs and their major metabolites, our extraction procedure did not include provision for removal of the diol metabolite.

Fig. 1B depicts a chromatogram of a patient's urine that contained, per ml, 19 μg of pHPB, 59.5 μg of PB and 14.8 μg of HPPH. DPH, if present, was below the detection limits of the assay. Day-to-day precision calculated from assays of a single sample containing 40 μg/ml of each compound on 10 consecutive days was for pHPB, PB, HPPH and DPH; 39.0 ± 1.18 μg/ml (C.V. 3.0), 41.5 ± 4.7 μg/ml (C.V. 11.4), 40.7 ± 1.39 μg/ml (C.V. 3.4) and 42.8 ± 0.48 μg/ml (C.V. 1.1) respectively.

The effects of concomitant administration of PB and DPH on drug metabolism are variable. Clinical studies have shown that PB increased plasma DPH levels by interfering with the formation of HPPH [17]. Conversely in other studies, DPH levels were found to rise following cessation of PB [18]. Investi-

gations utilizing the simultaneous quantitation of these compounds in urine and serum should permit evaluation of the factors affecting the dose-dependent kinetics of DPH, genetic and environmental contributions to hydroxylation, as well as the equivocal effects of simultaneous DPH and PB administration. The sensitivity of this method should permit investigation of factors affecting the hydroxylation of PB and DPH through the convenient and reproducible measurement of the parent drugs and their para-hydroxylated metabolites in biological fluids at levels encountered in clinical samples.

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