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Note

Rapid method for simultaneous determination of phenobarbital, diphenylhydantoin and their main hydroxylated metabolites by nitrogen-selective gas chromatography

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Phenobarbital (PB) and diphenylhydantoin (DPH) remain the drugs of choice in epilepsy characterized by general convulsive seizures [1].

Some years ago a gas chromatographic method was developed and employed in this laboratory to assay PB and DPH plasma levels in epileptic patients [2]. The introduction of a nitrogen-selective detector to gas chromatography made this method simpler, quicker and highly precise for use in pharmacokinetic studies [3].

The principal metabolic route for both PB and DPH is the aromatic hydroxylation to *p*-hydroxy compounds, by the hepatic microsomal enzymes [4, 5], which are subsequently conjugated with glucuronic acid before being excreted into the urine [6, 7]. Urinary and also biliary excretion data provide precise information about the overall elimination constants for the loss of drug from the body as well as the rate constants for drug metabolism [8]. As the two drugs are very often administered together and as possible drug—drug interaction is expected in the level of urinary excretion and metabolism, the analytical systems applicable to the determination of PB and DPH in biological samples should permit simultaneous quantitation of both unchanged drugs and their metabolites.

To separate the two drugs from their *p*-hydroxylated metabolites several techniques have been published including spectrophotometry [4], paper chromatography [9], thin-layer chromatography [10], gas—liquid chromatography [11, 12] and liquid chromatography [13]. Most of the published methods present either problems of interference [4] or time consuming procedures [11—13]. In addition, amongst these methods only a few considered the importance of determining the two drugs and their hydroxylated metabolites in the same sample [13].

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In this report we describe a simple gas—liquid chromatographic method using nitrogen selective detection to assay PB, DPH, *p*-hydroxyphenobarbital (*p*-OHPB) and *p*-hydroxyphenylphenylhydantoin (*p*-HPPH), simultaneously in biological specimens, including urine and bile. The chromatographic analysis was realized in less than 10 min without the need for temperature programming.

MATERIALS AND METHODS

Instruments

A Packard Model 429 gas chromatograph equipped with nitrogen—phosphorus (N—P) selective detector and a 1.8 m × 2 mm I.D. spiral glass column packed with 3% OV-1 on 100—120 mesh Gas-Chrom Q, was used for the gas chromatographic analysis.

The column was silanized with 5% dimethylchlorosilane (Eastman-Kodak, Rochester, NY, U.S.A.) and conditioned overnight at 250°C before use. Column, injector and detector temperatures were fixed at 210, 240 and 250°C, respectively. Nitrogen was used as the carrier gas at a flow-rate of 60 ml/min. The chemical structures of the metabolites in the urine extract were confirmed by using an LKB 2091 mass spectrometer coupled to a gas chromatograph.

Reagents

p-Tolylphenylhydantoin (*p*-MPPH), *p*-OHPB and *p*-HPPH were purchased from Aldrich-Europe (Beerse, Belgium). DPH was obtained from Fluka (Buchs, Switzerland). Trimethylaniliniumhydroxide (TMPAH) was obtained from Eastman-Kodak. PB and all organic solvents of analytical grade were obtained from Merck (Darmstadt, G.F.R.).

A stock solution of the four tested compounds was prepared in pure methanol at a concentration of 1 mg/ml, the internal standard was made separately in solution at a concentration of 1 mg/ml. The two solutions were kept at +4°C when not in use. Prior to the assay, aliquots of the mother solution were diluted to the desired concentrations in blank samples.

Extraction procedure

To a 40-ml glass centrifuge tube, were added 0.1 ml of the internal standard (400 μg/ml), 0.5 ml of urine sample, 1.5 ml of phosphate buffer pH 6.8 and 10 ml of diethyl ether. After mixing for 10 min on a mechanical shaker and centrifuging at 1340 *g*, the diethyl ether extract was separated and filtered through filter paper. The filter paper was then washed with 3 ml of diethyl ether. The combined organic extract and washing were evaporated to dryness under vacuum in a water bath at 45°C. The dry residue was transferred into a small vial (1.5 ml) by dissolving it into 0.6 ml ethanol, twice. The ethanol was evaporated off on a sand bath at moderate temperature under a slight stream of nitrogen. The residue was then dissolved in 0.1 ml of 0.2 *M* TMPAH and 0.5—1 μl was injected into the chromatograph.

Conjugated metabolites

The conjugated metabolites were hydrolyzed with concentrated hydrochloric acid prior to the extraction. Urine (0.5 ml) was mixed with 0.5 ml 12 *N*

HCl in the centrifuge tube containing the internal standard and placed in a water bath at 90°C for 60 min. After cooling the mixture was carefully neutralized with 0.5 ml 12 *M* NaOH followed by the addition of 1.5 ml phosphate buffer pH 6.8. The sample was then processed as described above. This drastic hydrolysis liberated all the conjugated metabolites, either sulphates or glucuronides.

In order to determine both free and conjugated hydroxy metabolites the urine samples were analyzed before and after hydrolysis. The amounts of conjugated metabolites were then calculated from the difference between the total and the free compounds.

Calibration curves

Calibration curves were prepared by adding different amounts of the four compounds to drug-free urine; then the urine samples were assayed as described before. The peak height ratios of each compound to the internal standard were plotted against concentration in the range of 5—100 μg/ml.

RESULTS

Table I shows the within-run precision, at two different concentrations (n = 10) for each of the tested compounds. The coefficient of variation did not exceed 7.2% for *p*-OHPB and was less for the other three compounds.

TABLE I

WITHIN-RUN ESTIMATES OF THE PRECISION OF THE ANALYSIS OF PB, DPH, *p*-OHPB AND *p*-HPPH

Results are based on ten analyses for each of two concentrations of PB, DPH, *p*-OHPB and *p*-HPPH. The internal standard is *p*-tolylphenylhydantoin. Chromatography on a 1.8-m column containing 3% OV-1 on 100—120 mesh Gas-Chrom Q.

Drug	Concn.	Mean	± S.D.	C.V. (%)
PB	20	19.18	0.89	1.7
	40	37.90	0.91	
DPH	20	21.50	0.46	2.2
	40	40.50	1.10	2.8
p-OHPB	10	10.04	0.72	7.2
	40	38.80	2.20	5.8
p-HPPH	20	18.20	0.95	5.2
	40	42.40	1.10	2.5

Standard curves were constructed by plotting peak height ratios (drug/internal standard) versus drug concentrations (μg/ml). The instrument responses and the concentrations were linearly related for all compounds (Fig. 1) over a range of 5—100 μg/ml. The correlation coefficient of the linear regression approaches 0.99 for each of the four drugs.

Extraction efficiencies of urine samples containing known amounts of PB, DPH, *p*-OHPB and *p*-HPPH were 88, 97, 84 and 107% respectively.

Fig. 2 depicts chromatograms of rat urine (animal dosed with PB and DPH), before and after hydrolysis, compared to a chromatogram of the pure compounds in methanol. The retention times for PB, DPH, *p*-OHPB, internal stan-

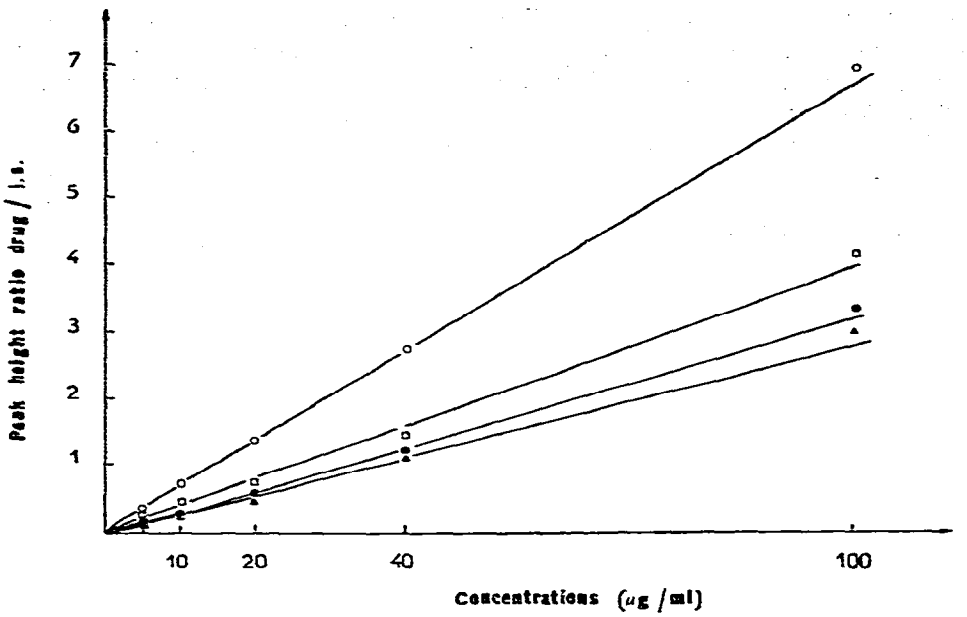

Fig. 1. Standard curves for determination of PB (○), *p*-OHPB (●), DPH, (□) and *p*-HPPH (▲). Internal standard, *p*-tolylphenylhydantoin.

dard and *p*-HPPH were 1, 3, 4, 6 and 9 min, respectively. No interference from urine components was observed and 100 μl of 0.2 *M* TMAH was enough to give complete methylation.

The quantification of the DPH in urine can be achieved by injecting a larger sample volume, as less than 5% of this drug is excreted in urine in an unchanged form.

The mass spectrum of the urine extract showed the formation of N,N,O-trimethyl derivatives of the two hydroxylated metabolites. The values of the more intense ions were 261 (100), 148 (83), 290 ($M^{+\cdot}$) (75) and 262 (50) for the *p*-OHPB-trimethyl derivative and 233 (100), 148 (53), 310 ($M^{+\cdot}$) (47) 224 (31), 118 (28) and 77 (27) for the *p*-HPPH-trimethyl derivative.

DISCUSSION

The proposed method possesses the important characteristics of precision, sensitivity and specificity over a wide range of PB, DPH, *p*-OHPB, and *p*-HPPH concentrations, in addition to extreme rapidity. The retention times for the four compounds and the internal standards are very satisfactory and the five peaks are very well separated.

The extraction procedure is efficient and using a pH of 6.8 to buffer the medium minimizes the extraction of the undesirable urine components. The use of the nitrogen-selective detector makes it possible to reduce sample size and to eliminate the sophisticated purification and clean-up steps. The limit of detection by the N—P detector is very satisfactory and superior to that reported for liquid chromatography [13]. The advantage in using such a detector is its relative insensitivity to hydrocarbons [14].

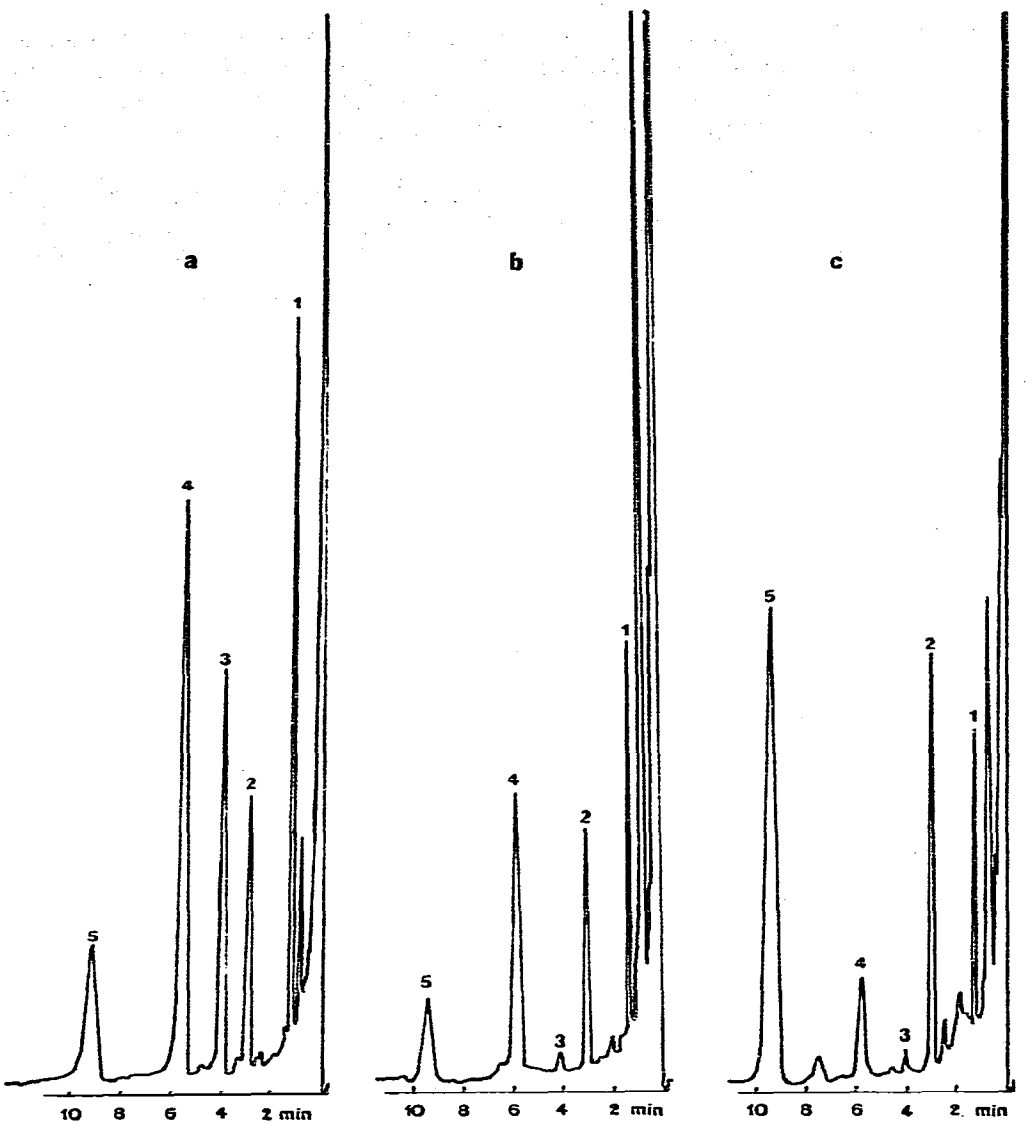

Fig. 2. Gas chromatograms of (a) pure solution of PB (1), *p*-OHPB (2), DPH (3), internal standard (4) and *p*-HPPH (5); (b) and (c) extracts of 0.25 and 0.5 ml of urine of rat pretreated with PB and DPH before and after hydrolysis respectively. Peaks as in a.

Most of the conjugated *p*-OHPB and *p*-HPPH excreted in the urine are in the form of glucuronides [12, 15], so acid hydrolysis can give a clear picture of the amount of glucuronides formed. But acid treatment of urine samples containing metabolites of PB and DPH in order to liberate the phenolic metabolites may transform the dihydrodiol metabolites into *m*- and *p*-hydroxy metabolites [16]. This can produce an error of about 10% in the case of *p*-HPPH. Therefore it might be preferable in precise metabolic studies to employ specific enzymatic cleavage. However with conjugated *p*-OHPB the values obtained with acid hydrolysis and β-glucuronidase treatment were very close

[12]. Diol metabolites can be removed from the medium by pre-extraction with isoamyl alcohol prior to hydrolysis [15], but since our aim was to describe a chromatographic method capable of the simultaneous separation of both parent drugs and their major metabolites, no attempts to remove the diol metabolites were made.

Investigations using the simultaneous quantitation of these compounds in urine and bile permitted the evaluation of the effects of the administration of one drug on the pharmacokinetics of the other [3]; in addition, more information about the factors affecting the *para*-hydroxylation in subjects under simultaneous DPH and PB treatment can be provided.

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