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Short communication

Simultaneous determination of primidone and its three major metabolites in rat urine by high-performance liquid chromatography using solid-phase extraction

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Abstract

A new high-performance liquid chromatographic method for simultaneous determination of primidone (PRM) and of its three major metabolites, phenobarbital (PB), *p*-hydroxyphenobarbital (*p*-HO-PB) and phenylethylmalonamide (PEMA), in rat urine, was developed. After acid hydrolysis, these compounds were extracted from urine by means of a Bond Elut Certify LRC column with good clean-up. The extracts were chromatographed on a C_{18} reversed-phase column using isocratic elution at 40°C, with UV detection at 227 nm. The limit of detection was 0.5 mg/ml for the four compounds. Good linearity ($r^2 > 0.99$) was observed within the calibration ranges studied: 37.4–299.3 μg/ml for PRM, 26.4–211.2 μg/ml for PB, 12.5–100.2 μg/ml for *p*-HO-PB and 12.1–97.0 μg/ml for PEMA. Repeatability was in the range 3.1–6.8%. This method constitutes a useful tool for studies on the influence of various parameters on primidone metabolism.

Keywords: Primidone; Phenobarbital; *p*-Hydroxyphenobarbital; Phenylethylmalonamide

1. Introduction

Primidone (PRM) is an antiepileptic drug. In animals and men, primidone is metabolized into two major pharmacologically active substances, phenylethylmalonamide (PEMA) and phenobarbital (PB).

Simultaneous determination of plasma concentrations of PRM and of its two metabolites, PEMA and PB, has been reported using gas or liquid chromatographic methods [1–4]. More recently, Moriyama et al. [5] described a rapid serum HPLC method, but it could not be directly applied to urine because many endogenous substances were not eliminated. Furthermore, we needed to determine, in addition to PEMA and PB, another metabolite present in urine, *p*-hydroxyphenobarbital (*p*-HO-PB) [6], in order to study the metabolism.

Liu et al. [7] reported a method for the determination of carbamazepine, phenytoin, PB, PRM, and their metabolites by HPLC with photodiode-array detection. Unfortunately, two of the metabolites, PEMA and *p*-HO-PB were not completely resolved and validation of the method was not carried out.

We have established a new validated HPLC

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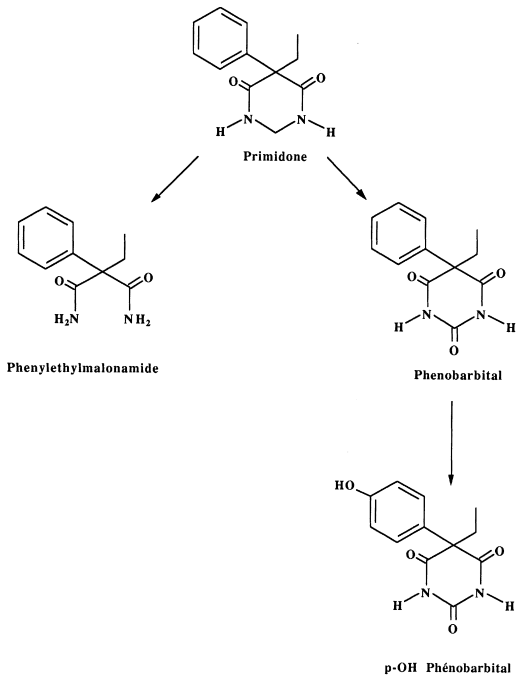

Fig. 1. Metabolic pathway of primidone.

method for the separation and quantitative determination of PRM and of its three metabolites, PEMA, PB and *p*-HO-PB in rat urine (Fig. 1: metabolic pathway of PRM).

2. Experimental

2.1. Chemicals

Primidone, phenobarbital and *p*-hydroxyphenobarbital were purchased from Sigma (St. Louis. MO, USA), R.P.R. (France) and Aldrich (Milwaukee, WI, USA), respectively.

Phenylethylmalonamide was synthesized according to the procedure described in [8].The identity of this compound was checked by ^{1}H NMR, EI-MS and carbon-hydrogen-nitrogen (CHN) microanalysis. The purity was tested by thin-layer chromatography (stationary phase: Kieselgel 60 ^{254}F, solvent: ethyl acetate).

All organic solvents were HPLC purity grade (Merck, Darmstadt, Germany). Other chemicals were of analytical grade (Prolabo, Paris, France).

2.2. Instruments and chromatographic conditions

The HPLC system consisted of an isocratic pump Beckman Model 110 A (Beckman, San Ramon, CA, USA), a sample injector with 20-μl loop (Rheodyne, Cotati, CA, USA), a variable wavelength UV detector (Beckman Model 166). The chromatography column Nucleosil 100-5 μm, C_{18}, 250×4.6 mm (Macherey-Nagel, Düren, Germany) was kept at a temperature of 40°C using a temperature programmable circulating water bath. The mobile phase was 0.01 *M* potassium phosphate buffer (pH 4.0)–methanol–acetonitrile (270:30:30, v/v/v) circulating at a flow-rate of 1.0 ml/min. It was degassed in an ultrasonic bath before use. The wavelength for detection was 227 nm. The data recording system consisted of an IBM personal computer PS/2 Model 55 SX with SYSTEM GOLD software (Beckman).

Bond Elut Certify LRC (Large Reservoir Capacity) columns, with a 130 mg capacity column bed were purchased from (Varian Les Ulis, France). The Certify extraction cartridge utilizes a packed bed consisting of a special, nonpolar C_8 sorbent and a strong cation exchanger (SCX), benzenesulfonic acid. A vacuum manifold capable of holding ten sample preparation columns (Vac-Elut, Varian) was used for simultaneous sample extractions.

2.3. Preparation of standard solutions and calibration

Stock solutions of standards were prepared by dissolving accurately weighed PRM, PB, *p*-HO-PB and PEMA in methanol in a volumetric flask. Urine standards were obtained by mixing known volumes of these stock solutions, methanol was evaporated and the residue was dissolved with a known volume of blank rat urine.

Urine standard curves were prepared at concentrations ranges of: 37.4–299.3 μg/ml for PRM, 26.4–211.2 μg/ml for PB, 12.5–100.2 μg/ml for *p*-HO-PB and 12.1–97.0 μg/ml for PEMA.

2.4. Clean-up procedure for urine samples

Bond Elut Certify LRC columns were activated prior to use by passage of 2.0 ml of methanol and 2.0 ml of 0.1 *M* acetate buffer (pH 4.0). A 0.5-ml

volume of centrifuged rat urine was transferred into a glass test tube, where 0.5 ml of 12 *M* HCl was added. The mixture was heated at 100°C for 2 h to perform the acid hydrolysis of the sulfo- and glucuro-conjugated compounds. After cooling, 0.45 ml of 12 *M* NaOH and 2.0 ml of 0.1 *M* acetate buffer (pH 4.0) were added to the acidic medium. The resulting mixture was adjusted to pH 4.0 and its volume to 50 ml, with distilled water. A 10.0-ml volume of the resulting solution was introduced into an activated Bond Elut Certify LRC column, using gentle suction (Vac-Elut). The column was washed with 1.0 ml of 0.1 *M* acetate buffer (pH 4.0) and dried under full vacuum for 10 min. The four compounds were eluted with 2.0 ml of hexane–ethyl acetate (95:5, v/v) and then with 4.0 ml of hexane–ethyl acetate (40:60, v/v). The organic phases were collected.

The aqueous phase was applied to the same Bond Elut column. These operations were repeated three times.

All organic phases were mixed and evaporated to dryness under vacuum. The residue was dissolved in 0.5 ml of mobile phase and a 20-ml aliquot was injected into the HPLC system as described above.

2.5. Study procedures and urine sample collection

Thirty female Sprague-Dawley rats (body weight ~300 g) received a daily oral dose of 20 mg/kg/body weight for 13 days via a cannula Carrieri (60-R). PRM was suspended in 0.212% carboxymethylcellulose CMC 12H. The animals had free access to food and water, and were placed in metabolic cages (Pajon, France).

Urine samples were collected daily at 1.00 p.m. during the thirteen days PRM administrative period, and for 3 days after the PRM administration period. All samples were pooled before analysis in order to collect larger amounts of the various metabolites and were stored at −20°C until analysed (total urine volume: 2700 ml).

2.6. Results of validation

The specificity of the method was checked by comparison of blank urine chromatograms with spiked urine chromatograms (Fig. 2).

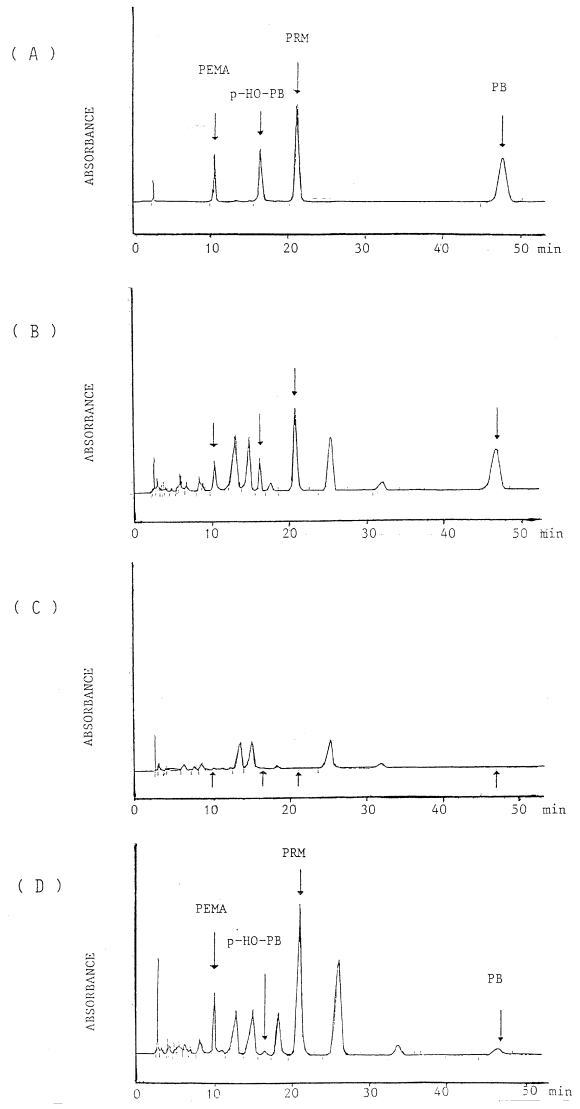

Fig. 2. Chromatograms of: (A) PRM and its metabolites in mobile phase, (B) blank rat urine spiked with PRM (24.77 μg/ml), PEMA (20.04 μg/ml), *p*-HO-PB (12.18 μg/ml) and PB (50.69 μg/ml); rat urine before (C) and after (D) primidone oral administration.

Known quantities of PRM, *p*-HO-PB, PB and PEMA, reflecting the compound ratio found in treated rat urine samples, were added in a blank urine sample and those solutions were subjected to the validation procedure.

Validation was performed according to the procedure described by Caporal-Gautier et al. [9]. Six

criteria were studied: repeatability, reproducibility, accuracy, linearity, limit of detection and limit of quantitation.

The stability of the four studied compounds was checked under sample preparation conditions and the mean value after storage at −20°C was within ±5%.

2.6.1. Extraction recovery

The extraction efficiency was estimated by measuring the peak areas of nonextracted standard solutions, and comparing these areas with the peak areas obtained from extraction of spiked urine samples at the same concentration (Table 1).

2.6.2. Calibration curves

Urine standard curves were prepared at concentrations ranges of: for PRM: 37.4, 74.8, 149.6, 224.5 and 299.3 μg/ml; for PB: 26.4, 52.8, 105.6, 158.4 and 211.2 μg/ml; for *p*-HO-PB: 12.5, 25.0, 50.0, 75.1 and 100.2 μg/ml; for PEMA: 12.1, 24.2, 48.5, 72.7 and 97.0 μg/ml and gave the regression line slope equations:

(PRM)	$y=-0.61722+7.4063x$	$r^2=0.999$
(PB)	$y=-0.00332+4.9579x$	$r^2=0.999$
(PEMA)	$y=-0.00212+9.6232x$	$r^2=0.999$
(*p*-HO-PB)	$y=-0.00605+5.6494x$	$r^2=0.999$

where y and x represent area and concentration, respectively. For each compound, five concentrations were studied ($n=3$ for each concentration).

2.6.3. Repeatability and reproducibility

The repeatability (intra-day precision) of the method for rat urine was evaluated by analyzing urine spiked with PEMA, *p*-HO-PB, PRM and PB at concentrations of 48.5, 50.l, 149.6 and 105.6 μg/ml respectively, in six replicates.

Reproducibility (inter-day precision) was studied for the same concentrations as those studied for repeatability on 3 different days.

Results corresponding to these two criteria were expressed by the relative standard deviation, calculated by the formula:

$$\text{R.S.D.\%} = S/X \times 100$$

where S is the standard deviation and X is the mean (Table 1).

2.6.4. Quantitation limit and detection limit

The experimental quantitations limits, defined as the lowest concentrations of PEMA, *p*-HO-PB, PRM and PB in a spiked urine sample which gives rise to a signal able to be quantified by the integrator (signal-to-noise ratio = 10), were found to be: 1.5 μg/ml for PEMA and *p*-HO-PB and 2 μg/ml for PRM and PB.

The detection limits for PEMA, *p*-HO-PB, PRM

Table 1
Study of the repeatability, reproducibility, accuracy, limit of quantification, limit of detection of rat urine spiked with PEMA, *p*-HO-PB, PRM and PB ($n=6$)

Compounds	Concentration (μg/ml)	Repeatability R.S.D. (%)	Reproducibility R.S.D. (%)	Accuracy (%)	Quantification limit (μg/ml)	Detection limit (μg/ml)
PEMA	12.2	8.8	9.9	59	1.5	0.5
	48.5	6.8	7.5	63		
	194.0	5.2	7.3	70		
p-HO-PB	12.5	10.3	14.3	56	1.5	0.5
	50.1	6.1	6.2	71		
	200.4	6.0	6.1	74		
PRM	37.4	6.9	10.1	97	2	0.5
	149.6	5.9	7.1	98		
	598.6	5.4	7.0	99		
PB	26.4	6.1	10.3	97	2	0.5
	105.6	4.8	7.3	100		
	422.4	3.2	8.3	97		

and PB were about 0.5 μg/ml (signal-to-noise ratio = 3) (Table 1).

3. Results and discussion

3.1. Optimization of the chromatographic system

Different proportions of methanol, acetonitrile and water containing 0.01 *M* phosphate buffer (pH 4.0) were tested as the mobile phase. The water–acetonitrile–methanol (270:30:30, v/v/v) ratio was chosen as the most optimum, with good resolution.

Peak area decreased with increasing flow-rate without affecting the resolution. A flow-rate of 1.0 ml/min was used.

A temperature of 40±0.2°C was used throughout the work in order to obtain symmetrical peaks.

Retention times of PEMA, *p*-HO-PB, PRM and PB were 10.6, 16.7, 21.1 and 47.6 min, respectively. The overall chromatographic run time was within ~50 min.

The detection wavelength was 227 nm at the maximal absorbance of primidone.

3.2. Extraction procedure

PRM was sparingly soluble in organic solvents and the best solubility was in ethyl acetate. The liquid–liquid extraction with ethyl acetate, was not suitable because of interference from endogenous substances. C_{18} cartridges (Waters) were also unsuccessful due to the interference from endogenous substances. We were able to separate PRM, PB, *p*-HO-PB and PEMA from the interfering endogenous compounds by using Bond Elut Certify LRC column for solid-phase extraction (SPE) under the specified conditions, resulting in a more selective analytical method for the determination of these four compounds in rat urine.

There are three general extraction mechanisms used in SPE: nonpolar, polar and ion-exchange. Selection is primarily based on the functional groups present in the analytes and the composition of the sample matrix.

Each sorbent within a given extraction mechanism exhibits unique retention properties and selectivity which may be quite specific for a given analyte. To achieve high recovery and good clean-up, it was necessary to use three extraction steps.

3.3. Application to the analysis of rat urine samples

The chromatograms are presented in Fig. 2: (A) PRM and its metabolites in mobile phase, (B) blank rat urine spiked with PRM (24.77 μg/ml), PEMA (20.04 μg/ml), *p*-HO-PB (12.18 μg/ml) and PRM (50.69 μg/ml); rat urine before (C) and after (D) PRM oral administration.

The total amounts found in 2700 ml of urine were 5.22 mmol (PRM + *p*-HO-PB + PB + PEMA). The total dose of PRM administered for the rats was 2.66 g or 12.2 mmol. These results showed that 42.78% of the PRM administered was eliminated in urine; 31.54% as PRM, 9.82% as PEMA, 0.83% as PB and 0.59% as *p*-HO-PB.

3.4. Discussion

The relatively low amount of recovered compounds in urine (42.78% of administered PRM) may be due to either excretion in faeces or diffusion in biological tissues.

Levels of PRM, PB, *p*-HO-PB and PEMA found in rat urine after an oral administration of primidone were the same before and after acid hydrolysis at 100°C. These results indicate that these four compounds were not conjugated in rat urine. This is contrary to the findings in humans: Paibir and Soine [10] found that the concentration of *p*-hydroxyphenobarbital present in human urine, after oral phenobarbital administration, was increased after acid hydrolysis and that it was possible that *N*-glucuronidation could be a method for elimination of the drugs by humans at a much higher or toxic dose.

4. Conclusion

This simple and efficient HPLC method allowed simultaneous separation and quantification of primidone and of its three metabolites in rat urine. The analytical assay method was successfully validated and it provides a useful tool for studies on the

influence of various parameters on primidone metabolism.

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