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Sensitive method for the determination of phenytoin in plasma, and phenytoin and 5-(4-hydroxyphenyl)-5-phenylhydantoin in urine by high-performance liquid chromatography*

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Abstract: An isocratic high-performance liquid chromatographic method is described for the quantitative analysis of phenytoin (PHT) in plasma and both phenytoin and its main metabolite, 5-(4-hydroxyphenyl)-5-phenylhydantoin (p-HPPH) in urine. Following ethyl acetate (plasma) and Extrelut-1 (urine) extraction, samples are analysed by means of a reversed-phase column (Nova-Pak C_{18}), using a mobile phase consisting of methanol–water–tetrahydrofuran (40:60:4, v/v/v) with UV detection at 230 nm.

The chromatography is complete in 10 min and the results show good precision (RSD 1.23–4.49%) and sensitivity for a linear range of 0.4–4.0 μg ml^{-1} for PHT in plasma, 0.1–1.0 μg ml^{-1} for PHT in urine and 0.1–1.2 μg ml^{-1} for p-HPPH in urine. The results indicate the method to be suitable for pharmacokinetic studies.

Keywords: *High-performance liquid chromatography; phenytoin; 5-(4-hydroxyphenyl)-5-phenylhydantoin; plasma; urine.*

Introduction

Phenytoin (5,5-diphenylhydantoin; PHT) is one of the most efficacious and widely prescribed anticonvulsants for the treatment of epilepsy. Although PHT has a well defined therapeutic range, therapeutic concentrations are not easily achieved due to the fact that PHT is eliminated via a saturable metabolic process [1] obeying Michaelis–Menten kinetics. PHT exhibits non-linear pharmacokinetics and any small change in bioavailability may lead to a marked change in plasma levels [2]. Doluisio [3] classified phenytoin as a drug with 'high risk potential' with respect to bioavailability problems.

Methods for the assay of PHT and/or 5-(4-hydroxyphenyl)-5-phenylhydantoin (p-HPPH) in plasma and/or urine have been reviewed by Burke and Thénot [4].

More recently published methods [5–8] have not been validated for low concentrations of PHT and/or p-HPPH since they are intended for use in therapeutic drug monitoring (TDM) which requires high selectivity but not very low limits of detection. More sensitive methods have been reported [9–12] for use in other types of pharmacokinetics studies, including bioavailability determinations. Nevertheless, there is a need to improve these methods in terms of ease of sample handling and analysis time, while keeping their characteristics of linearity, sensitivity, accuracy and precision.

The purpose of the present study was to develop a rapid, simple and sensitive HPLC isocratic method for the quantitative determination of PHT in plasma and urine and its main metabolite p-HPPH in urine for use in a comparative study of the bioavailability after a single oral dose of two different PHT formulations.

Materials and Methods

Apparatus

A Merck–Hitachi liquid chromatograph equipped with a reversed-phase column, Nova-Pak C_{18} (150 × 3.9 mm i.d. particle size 4 μm) (Waters Assoc., Milford, MA, USA) was used. The solvent was delivered by means of a Merck–Hitachi pump, Model 6000 (Merck, Darmstadt, Germany) which was coupled to a

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Rheodyne injector, Model 7125 (Cotati, CA, USA) equipped with a 20-μl loop. A UV detector, Merck–Hitachi, Model 4200, operated at 230 nm, was used and peak-heights were measured by a Hewlett–Packard integrator, Model 3390 (Avondale, PA, USA) with a chart speed set at 0.20 cm min^{-1}.

Reagents and standards

Purified water (Milli-Q water purification system; Millipore, Bedford, MA, USA), HPLC-grade methanol and tetrahydrofuran (Romil Chemicals, Leics, England) were used throughout. Acetic acid, citric acid, chloroform, disodium hydrogen phosphate, ethyl acetate, methanol, sodium acetate were of analytical grade (Merck, Darmstadt, Germany). PHT and p-HPPH (Sigma Chemical, St Louis, MO, USA) were used as reference substances as 1 mg ml^{-1} in methanol (stock solution). Pentobarbital (PBT) was used as internal standard (Sigma Chemical, St Louis, MO, USA): 1 mg ml^{-1} in methanol (stock solution). Blank plasma and urine were obtained from healthy subjects undergoing no drug therapy.

Mobile phase for plasma and urine samples

For plasma, a mixture of water–methanol–tetrahydrofuran (60:40:4, v/v/v) at a flow rate of 1.2 ml min^{-1} was used. For urine, the eluent consisted of 0.2 M acetate buffer pH 4.0–methanol (60:40, v/v) at a flow rate of 1.0 ml min^{-1}. The mobile phase was prepared and degassed daily by passing through a 0.45-μm membrane filter (S-Pak filter, Millipore).

Sample preparation

Plasma. To plasma (0.5 ml) in a 16 × 125 mm screw-cap tube, with Teflon lining, was added methanol (50 μl) containing 2.5 μg of internal standard (PBT) and ethyl acetate (3 ml). After vortex mixing (30 s) and centrifugation (9000*g*) for 5 min at room temperature the organic phase was transferred to a second clean tube and the sample was completely dried under nitrogen.

The residue was reconstituted in 100 μl of mobile phase and was injected onto the HPLC system (10 μl).

Urine. Urine (0.5 ml) was applied to the Extrelut-1 column (Merck, Darmstadt, Germany) and methanol (20 μl) containing 1.0 μg of the internal standard (PBT) was added and allowed to stand for 1 min. A pH 6.0 buffer solution (0.5 ml) was then applied to the Extrelut-1 column and allowed to stand for 10 min. The drugs were eluted with chloroform (6 ml). The eluate was evaporated to dryness and the residue was washed with 0.5 ml of methanol, which was again evaporated to dryness. The extract was then reconstituted in 75 μl of mobile phase and injected onto the HPLC system (10 μl).

Results

Chromatography

Plasma. Figure 1 shows the chromatograms obtained for blank plasma (A) together with blank plasma spiked with PHT (0.8 μg ml^{-1}) and the internal standard [2.50 μg of PBT (B)] obtained by means of the described methodology.

Urine. Figure 2 shows the chromatograms obtained for blank urine (A) together with blank urine spiked with PHT and p-HPPH (1.0 μg ml^{-1}) and the internal standard [1.0 μg of PBT (B)] analysed under the same conditions.

Quantitation

Quantitation was based on peak-height ratios obtained for the drug and its metabolites using PBT as the internal standard.

Linearity

The linearity of the method was checked for PHT in plasma (0.4–4.0 μg ml^{-1}) and for PHT (0.1–1.0 μg ml^{-1}) together with p-HPPH (0.1–1.2 μg ml^{-1}) in urine. Peak-height ratios (reference to internal standard) and analyte concentrations were found to be linearly related over this range, see Table 1.

Between-day precision

Analysis of pooled plasma spiked with PHT (0.8, 1.0 and 2.0 μg ml^{-1}) gave relative standard deviations (RSD) which ranged between 1.88 and 4.49% (Table 2).

Analysis of pooled urine spiked with PHT and p-HPPH (0.8–1.0 μg ml^{-1}) gave RSDs which ranged between 1.53 and 2.62 and between 1.23 and 2.25, respectively, see Table 3.

Recovery

The mean relative recovery was calculated by comparing the concentrations obtained for

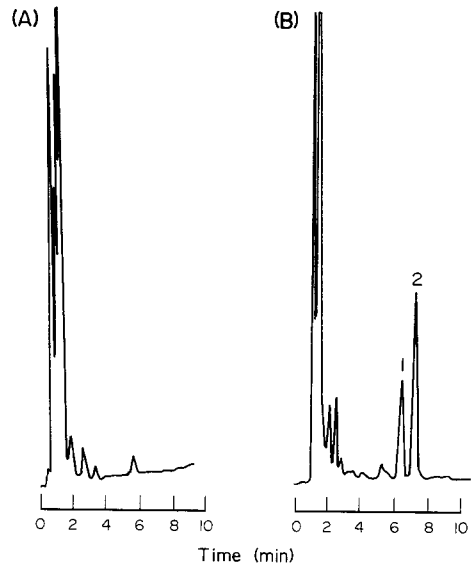

Figure 1
HPLC of PHT: 1, PHT; 2, PBT (internal standard). UV detection: 230 nm. Column: Nova-Pak C_{18} (4 μm). Mobile phase: methanol–water–tetrahydrofuran (40:60:4, v/v/v). A, blank extracted plasma; B, extracted spiked plasma with 0.800 μg ml^{-1} of PHT and 2.50 μg ml^{-1} of internal standard.

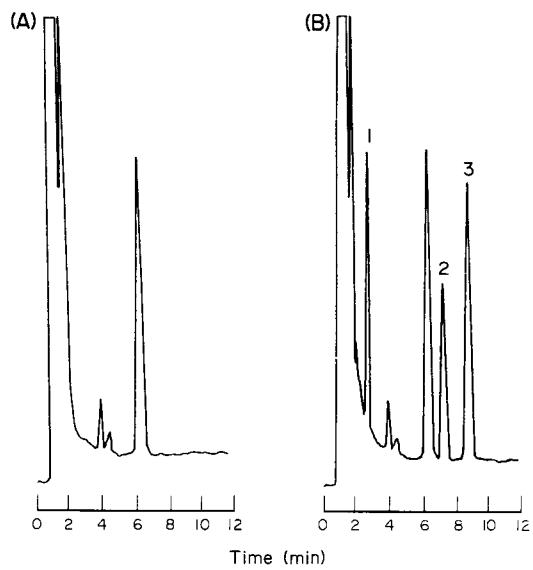

Figure 2
HPLC of PHT and its main metabolite, p-HPPH: 1, p-HPPH; 2, PHT; 3, PBT (internal standard). UV detection: 230 nm. Column: Nova-Pak C_{18} (4 μm). Mobile phase: 0.2 M acetate buffer pH = 4.0–methanol (60:40, v/v). A, blank extracted urine; B, extracted spiked urine with 1.00 μl ml^{-1} of PHT, p-HPPH and 2.50 μg ml^{-1} of internal standard.

Table 1
Results of linear regression analysis of calibration data

	Plasma	Urine	
	PHT	PHT	p-HPPH
Slope (b)	0.430	0.599	0.834
Intercept (a)	0.000*	0.000*	0.0413
Standard error of slope (S_b)	0.00213	0.00288	0.0105
Standard error of intercept (S_a)	0.0175	0.00605	0.0150
Range (μg ml^{-1})	0.400–4.00	0.100–1.00	0.100–1.20
Correlation coefficient (r)	0.999	0.999	0.999

*Forced through the origin.

Table 2
Between-day precision study of PHT in plasma*

	Nominal concentration (μg ml^{-1})	Measured value Mean (μg ml^{-1})	RSD (%)	n†
PHT	0.800	0.812	2.59	8
	1.00	1.10	4.49	12
	2.00	2.02	1.88	4

*Mean values of different spiked plasmas analysed on different days.
†Number of plasma samples analysed for each value. Experimental conditions as described in the text.

the drug-supplemented plasma and urine with the same nominal concentrations utilized for the calibration curve.

The mean relative recovery for PHT in plasma was 100% and ranged from 93.7 to 104% (Table 4) with a RSD of 3.74%.

In urine, the mean relative recovery for PHT and p-HPPH was 101 and 101% and ranged

Table 3
Between-day precision of PHT and p-HPPH in urine*

	Nominal concentration ($\mu g\ ml^{-1}$)	Measured value Mean ($\mu g\ ml^{-1}$)	RSD (%)	n†
PHT	0.800	0.796	1.53	4
	1.00	0.996	2.62	7
p-HPPH	0.800	0.802	2.25	4
	1.00	1.00	1.23	7

*Mean values of different urine samples analysed on different days.
†Number of urine samples analysed for each value. Experimental conditions as described in the text.

Table 4
Mean relative recovery of phenytoin from plasma

	Spiked value ($\mu g\ ml^{-1}$)	Recovered ($\mu g\ ml^{-1}$)	Mean relative recovery (%)	RSD (%)*
PHT	0.400	0.374	93.7	3.42
	0.800	0.830	104	2.77
	1.20	1.25	104	2.63
	1.60	1.62	101	0.711
	2.00	2.02	101	0.973
	3.00	2.94	97.8	0.840
	4.00	4.02	100	0.246
		Mean:	100	
		RSD (%):	3.74	

*Four replicate determinations for each concentration. Experimental conditions as described in the text.

Table 5
Mean relative recovery of PHT and p-HPPH from urine

	Spiked value ($\mu g\ ml^{-1}$)	Recovered ($\mu g\ ml^{-1}$)	Mean relative recovery (%)	RSD (%)*
PHT	0.100	0.101	101	5.84
	0.200	0.209	104	3.39
	0.400	0.403	101	3.52
	0.600	0.595	99.1	3.18
	0.800	0.800	100	1.77
	1.00	1.00	100	0.708
		Mean:	101	
		RSD (%):	3.10	
p-HPPH	0.100	0.108	108	5.48
	0.200	0.210	105	4.85
	0.400	0.386	96.6	1.31
	0.800	0.780	97.5	3.26
	1.00	1.00	100	2.03
	1.20	1.22	101	0.698
		Mean:	101	
		RSD (%):	4.88	

*Four replicate determinations for each concentration. Experimental conditions as described in the text.

from 99.1 to 104% and from 96.6 to 108% (Table 5), with RSDs of 3.10 and 4.88%, respectively.

Discussion and Conclusions

Different mobile phase compositions were evaluated in the present study for their ability to separate PHT, p-HPPH and PBT from interfering endogenous substances.

Methanol and water were used as the basis for the different mobile phases, but no good separation of the analytes was obtained by simply varying their proportions. Therefore, either a third solvent [isopropanol, tetrahydrofuran or acetate buffer (pH 4.8)] were added in at least six different combinations. As an example, methanol–water–tetrahydrofuran (55:45:10, v/v/v) did not achieve separation of p-HPPH (t_r = 3.91 min) from urine interferents whilst for methanol–water–pH 4.8 acetate buffer (50:50:15, v/v/v) p-HPPH and PHT gave retentions of 5.97 and 11.0 min, respectively, which were considered to be too large. No systematic method of optimization was followed, except for a trial and error procedure, following the general rules of solvent-dependent order of elution.

Isocratic elution was used for both plasma and urine analysis, and a good resolution was obtained for the three different drugs with the composition indicated. The optimum flow rate for the mobile phase was found to be 1.2 ml min^{-1} (plasma) and 1.0 ml min^{-1} (urine), resulting in analysis times of 8.50 and 10.0 min, respectively. The slightly smaller time for plasma was considered significant for use with a very large number of samples. Different detection wavelengths also were evaluated to achieve the maximum sensitivity towards PHT and p-HPPH (230 nm).

A simple extraction method with an organic solvent (ethyl acetate) was used which enabled interference of PHT and the internal standard by plasma components to be avoided. In urine, a biological fluid with more endogenous interferents, a liquid–liquid extraction method on Extrelut-1, especially adapted for small sample quantities, was applied successfully.

In the developed method small volumes of biological samples are used (500 µl of plasma and urine) which is an advantage in a bioavailability study, as a large number of blood samples have to be drawn from the same volunteer. This low sample volume did not

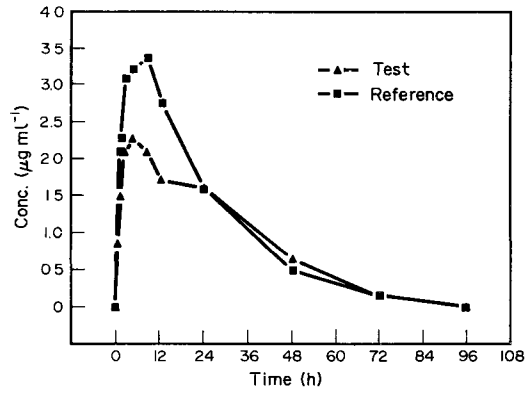

Figure 3
Concentration vs time curves of PHT in plasma of a healthy volunteer, who was given a single oral dose of 300 mg of PHT, in a bioavailability study.

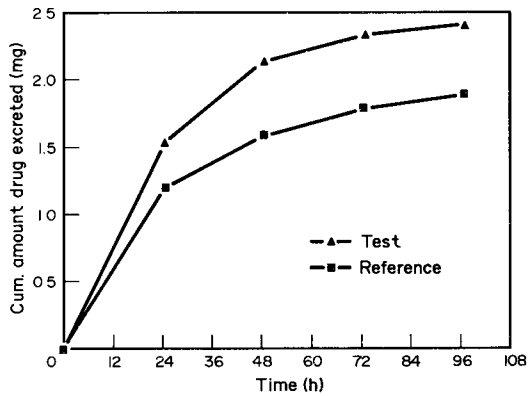

Figure 4
Cumulative amount of PHT excreted unchanged in urine of a healthy volunteer, who was given a single oral dose of 300 mg of PHT, in a bioavailability study.

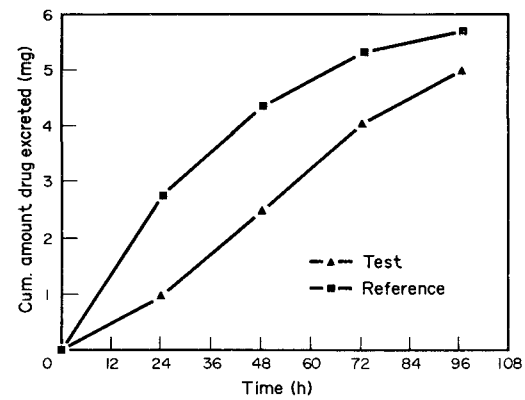

Figure 5
Cumulative amount of p-HPPH excreted in urine of a healthy volunteer, who was given a single oral dose of 300 mg of PHT, in a bioavailability study.

preclude the high sensitivity required to define clearly the elimination phase of PHT from plasma (Fig. 3) or to detect PHT and p-HPPH in urine over the collection period (Figs 4 and 5).

With regards to recovery, linearity and precision the method reported here shows excellent characteristics and is adequate for the determination of PHT and p-HPPH in plasma and urine in pharmacokinetic studies.

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