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Journal of Chromatography B, 673 (1995) 147–151

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Simultaneous determination of plasma phenytoin and its primary hydroxylated metabolites in carbon tetrachloride-intoxicated rats by high-performance liquid chromatography

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First received 30 January 1995; revised manuscript received 18 May 1995; accepted 8 June 1995

Abstract

We have developed a simple and sensitive method for the simultaneous determination of phenytoin (PHT), 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), and 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) in rat plasma by high-performance liquid chromatography. The three substances were separated on a reversed-phase column (5 μm TSK gel ODS-80TM, 250 mm × 4.6 mm I.D.) using acetonitrile–0.008 *M* NaH_2PO_4 (pH 6) (35:65, v/v) as a mobile phase at a flow-rate of 0.8 ml/min. Absorbance was monitored at 215 nm. The quantification limit was 50 ng/ml for each of PHT, *m*-HPPH and *p*-HPPH. The mean recoveries for DPH, *m*-HPPH and *p*-HPPH from plasma were 95.6 ± 3.6, 94.5 ± 4.2 and 98.6 ± 2.9%, respectively.

1. Introduction

Phenytoin (PHT) is one of the most widely used and extensively studied drugs for the treatment of epilepsy. It is metabolized by cytochrome P-450 (CYP) 2C9/10, producing various metabolites including 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), the major metabolite, and 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) [1,2].

Some reports have shown the simultaneous determination of PHT, *m*-HPPH and *p*-HPPH by high-performance liquid chromatography (HPLC) [3–7]. Sato et al. [4] determined the concentrations of PHT and HPPH (*m*-HPPH plus *p*-HPPH) in human urine, and Szabo et al. [5] analyzed both *m*-HPPH and *p*-HPPH and not PHT in human urine. Hsieh and Huang [6] developed an assay to determine the enantiomers of *p*-HPPH in biological fluids. In addition, Liu et al. [7] analyzed PHT and its metabolite (*p*-HPPH and *m*-HPPH), including three antiepileptic drugs and their principal metabolites, with photodiode-array detection in biological fluids. However, there are some problems with HPLC methods in terms of simplicity and sensitivity.

The purpose of this study was to develop a simple and sensitive HPLC method for the simultaneous measurement of PHT and its two metabolites in rat plasma, and to apply this method to a pharmacokinetic study in carbon tetrachloride (CCl_4)-treated rats.

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0378-4347/95/$09.50
SSDI 0378-4347(95)00250-2

2. Experimental

2.1. Reagents and standards

PHT sodium, *m*-HPPH, *p*-HPPH, 5-(4-methylphenyl)-5-phenylhydantoin (internal standard; MPPH) were purchased from Sigma (St. Louis, MO, USA). Other reagents and organic solvents were obtained from Wako Pure Chemical Industries (Osaka, Japan). Standard solutions of PHT, *m*-HPPH and *p*-HPPH (each 1 mg/ml) were prepared in the mobile phase, which remained stable for at least three months at 4°C. These solutions were then diluted as necessary to prepare the appropriate plasma standards for each drug and each assay (0.05, 0.1, 0.5, 1 and 5 μg/ml). Plasma was obtained from rats and MPPH (0.5 mg/ml) was also prepared in the mobile phase and stored at 4°C.

2.2. Analytical procedure

PHT and its metabolites (*m*-HPPH and *p*-HPPH) were determined according to a modification of the method of Dickinson et al. [3]. We added 50 μl of internal standard (MPPH; 10 μg/ml), 100 μl of 0.2 *M* hydrochloric acid and 3 ml of dichloromethane to 100 μl of serum in 15-ml culture tubes. After vortex-mixing for 2 min, the tubes were centrifuged at 1200 *g* for 5 min, and the aqueous phase was removed by aspiration. The organic phase was transferred into a clean conical tube and evaporated in a water bath at about 40°C under a gentle stream of nitrogen. The residue was dissolved in 100 μl of mobile phase, and 50 μl of the solution was injected into the HPLC apparatus.

2.3. Recovery

The recovery was calculated by comparing the peak heights of a series of PHT, *m*-HPPH and *p*-HPPH (0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 μg/ml) spiked samples after extraction from plasma, to the peak heights of a series of unextracted reference standard.

2.4. Chromatography

The apparatus (Model 114M, Beckman Instruments, San Ramon, CA, USA) was equipped with a variable-wavelength UV detector (Model 870-UV, Japan Spectroscopic Co., Tokyo, Japan), and separation was achieved using a C_{18} reversed-phase column (250 mm × 4.6 mm I.D., particle size 5 μm, TSKgel ODS-80TM, Tosoh, Japan). The mobile phase was composed of acetonitrile–0.008 *M* NaH_2PO_4 (pH 6) (35:65, v/v), and the flow-rate was 0.8 ml/min. The absorbance of the eluent was monitored at 215 nm. All instruments and the column were operated at ambient laboratory temperature (ca. 23°C).

2.5. Animal study

Male Sprague–Dawley (210–240 g) rats, 7–8 weeks of age, were obtained from CLEA Japan (Tokyo, Japan). The rats were kept in an air-conditioned room (25 ± 1°C, 50–60% humidity) with a 12-h light–dark cycle (8:00 a.m.–20:00 p.m.) and given free access to commercial rat chow (Oriental-MF, Tokyo, Japan) and water. Twenty hours before the experiment, the rats were cannulated in the right jugular vein under ether anaesthesia for drug administration and blood sampling. The polyethylene cannula was inserted and, to avoid removal by the rat during the experiment, pulled subcutaneously to the nape of the neck, thus allowing free movement of the animals during the experiment. The rats were fasted overnight in a metabolic cage in which they had free access to water, after which they were injected intravenously with 4 mg/kg of PHT. Normal control rats were given olive oil (2 ml/kg). CCl_4 was dissolved in olive oil and administered orally 24 h prior to the intravenous administration of PHT. Blood samples (about 0.2 ml) were obtained from the jugular vein at 0.25, 0.5, 1, 2, 4 and 6 h after intravenous administration of PHT.

2.6. Data analysis

For the pharmacokinetic parameters of PHT, the elimination half-life ($t_{1/2}$) was calculated as

$t_{1/2} = 0.693/K$, where K is the elimination rate constant as assessed by applying logarithmic regression analysis to the terminal part of the plasma concentration profile. The area under the plasma concentration–time profile (AUC) was calculated using the linear trapezoidal rule up to 2 h and extrapolated to infinity with k. The total body clearance (Cl) of the doses was determined as Cl = dose/AUC. The apparent volume of distribution (V_d) was calculated as $V_d = Cl/k$. Results are shown as means ± S.D. Statistical analysis was performed by the unpaired Student's t-test.

3. Results and discussion

Fig. 1 shows representative chromatograms obtained from rat plasma spiked with PHT, *m*-HPPH and *p*-HPPH (1 μg/ml) (Fig. 1, panel 1), blank rat plasma (panel 2) and a rat plasma sample obtained 2 h after intravenous administration of PHT (4 mg/kg) in control rats (panel 3). The results indicate that there was good separation among PHT, *m*-HPPH, *p*-HPPH and internal standard. The retention times of *p*-HPPH, *m*-HPPH and PHT were around 7.7, 8.5 and 16 min, respectively. No interfering peaks appeared when primidone (retention time: 4.3 min), ethosuximide (4.8 min), phenobarbital (8.8 min), carbamazepine (14.1 min), oxazolam (16.9 min), nitrazepam (19.9 min) or diazepam (53.1 min), which are usually administered to patients in combination with PHT, were added to plasma. Intra- and inter-assay variations in the results of analysis for PHT, *m*-HPPH and *p*-HPPH are indicated in Table 1; the coefficients of variation (C.V.) were less than 4% ($n = 10$). In the concentration range extracted in plasma samples of 0–5 μg/ml of PHT, *m*-HPPH and *p*-HPPH, the method showed good linearity (PHT: $r = 0.993$; *m*-HPPH: $r = 0.991$; *p*-HPPH: $r = 0.995$).

The buffer used in the present study, which was a slight modification of that reported by

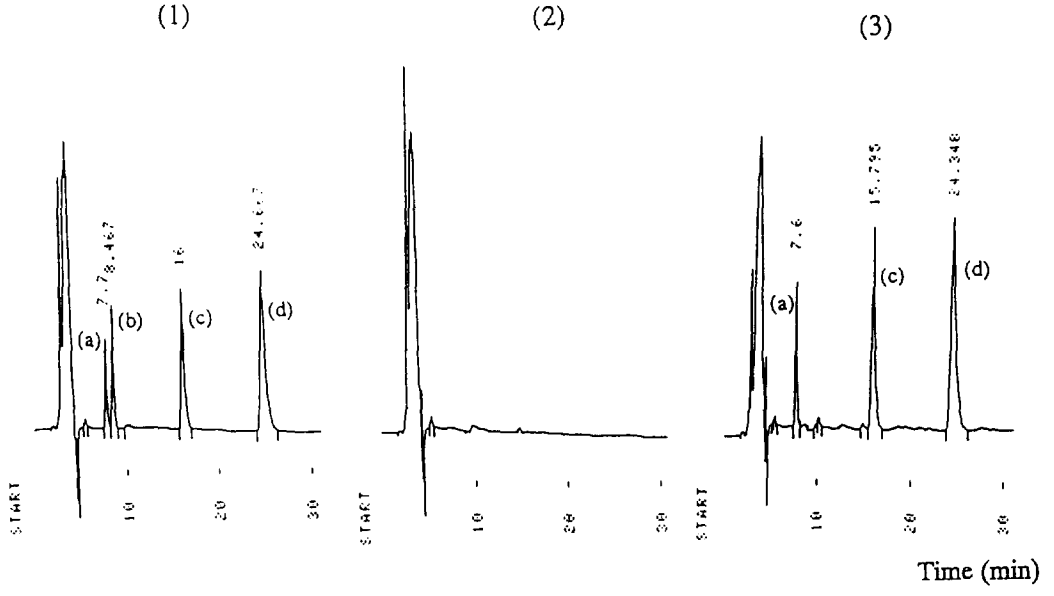

Fig. 1. Chromatograms of phenytoin and its primary hydroxylated metabolites. (1) Rat plasma spiked with PHT, *m*-HPPH and *p*-HPPH. The concentrations are 1 μg/ml for PHT, *m*-HPPH and *p*-HPPH. (2) Blank rat plasma. (3) Rat plasma obtained 2 h after intravenous administration of PHT (4 mg/kg) in control rats. Column, TSK gel ODS-8TM; mobile phase, acetonitrile–0.008 *M* NaH_2PO_4 (pH 6) (35:65, v/v); flow-rate, 0.8 ml/min; detection wavelength, 215 nm. In each case the injection volume was 50 μl. Peaks: a = *p*-HPPH = (5-(*p*-hydroxyphenyl)-5-phenylhydantoin), b = *m*-HPPH = (5-(*m*-hydroxyphenyl)-5-phenylhydantoin), c = PHT = phenytoin, d = internal standard = 5-(4-methylphenyl)-5-phenylhydantoin.

Table 1
Intra- and inter-assay variations in the simultaneous analysis of phenytoin and its primary hydroxylated metabolites in rat plasma

Concentration added (μg/ml)	PHT		*m*-HPPH		*p*-HPPH	
	Mean (μg/ml)	C.V. (%)	Mean (μg/ml)	C.V. (%)	Mean (μg/ml)	C.V. (%)
Intra-assay						
0.05	0.051	2.8	0.051	3.1	0.051	3.1
0.1	0.11	2.6	0.11	3.5	0.10	3.2
0.5	0.51	3.5	0.51	3.1	0.52	3.7
1	1.02	3.1	1.02	3.2	0.99	2.7
5	5.04	2.4	5.11	2.9	5.06	2.3
Inter-assay						
0.05	0.05	3.0	0.051	3.1	0.049	2.5
0.1	0.10	2.9	0.10	2.9	0.11	3.4
1	1.01	3.2	1.02	3.3	1.03	2.5
5	5.02	2.8	5.03	2.9	5.05	2.1

Each value represents the mean of ten experiments. The concentration was calculated on the basis of the peak-height ratios against the internal standard ($n = 10$). PHT: phenytoin; *m*-HPPH: 5-(*m*-hydroxyphenyl)-5-phenylhydantoin; *p*-HPPH: 5-(*p*-hydroxyphenyl)-5-phenylhydantoin.

Dickinson et al. [3], acetonitrile–water (4:6, v/v), was found to be superior as an HPLC buffer to methanol–0.02% aqueous ammonium acetate (1:1, v/v) [4], water–dioxane–tetrahydrofuran (80:15:5, v/v/v) [7], acetonitrile–35 m*M* monobasic ammonium phosphate (21:79, v/v) [6], and potassium phosphate buffer–acetonitrile–methanol (110:50:30, v/v/v) [7] used previously in terms of isolation and/or sensitivity.

Reversed-phase HPLC systems with octyl-[4] or octadecyl-silica (ODS-silica) [5,7–9] columns have been used in previous studies. In the present study, we used an ODS-silica column for HPLC analyses.

The sensitivities of the methods of Sato et al. [4], Szabo et al. [5] and Liu et al. [7] were much lower (about 5–25 times) than that of our method. There are no data in the literature regarding limits of quantification of PHT and its metabolites in rat plasma. The limits of quantification for PHT, *m*-HPPH and *p*-HPPH after extraction in our method using rat plasma were each 50 ng/ml. The mean recoveries for DPH, *m*-HPPH and *p*-HPPH added to rat plasma were 95.6 ± 3.6, 94.5 ± 4.2 and 98.6 ± 2.9% ($n = 10$), respectively.

The pharmacokinetic results of the animal experiments are shown in Table 2. The plasma concentration of PHT in olive-oil-treated rats was decreased according to the one-compartment model, and plasma *p*-HPPH levels reached a peak at around 0.5 h and then decreased gradually. However, *m*-HPPH, the minor metabolite, was not detected after administration of PHT (Fig. 2). The elimination of PHT in CCl_4-treated rats was delayed and *p*-HPPH production in the CCl_4-treated group occurred to a lesser extent than in the olive oil group.

In addition, the advantages of our HPLC

Table 2
Pharmacokinetic parameters following the intravenous administration of phenytoin in carbon tetrachloride-intoxicated rats

Treatment	$t_{1/2}$ (h)	V_d (ml/kg)	Cl (ml/min/kg)
Control	0.56 ± 0.11	530 ± 122	10.29 ± 0.98
CCl_4 treatment	2.67 ± 0.66[a]	486 ± 32	1.87 ± 0.42[a]

Control rats received olive oil (2 ml/kg). CCl_4 was administered orally at 0.5 ml/kg 24 h prior to the intravenous administration of PHT (4 mg/kg). $t_{1/2}$ = half life, V_d = apparent volume of distribution, Cl = total body clearance, $n = 5$.
[a] $p < 0.001$.

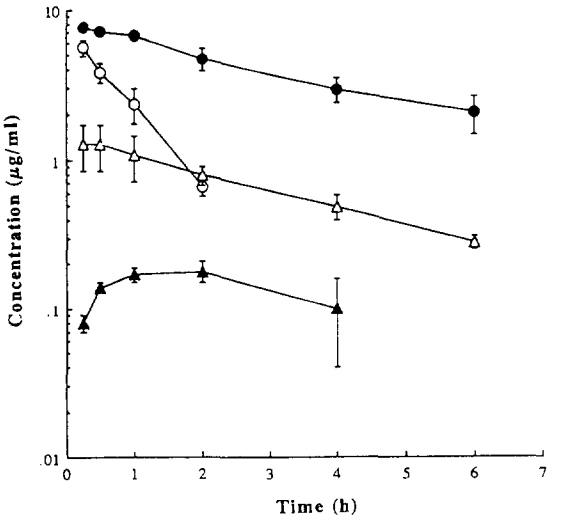

Fig. 2. Mean plasma concentration–time curves of phenytoin and its primary hydroxylated metabolite in carbon tetrachloride (0.5 ml/kg)-intoxicated rats: ○ = PHT (Control), ● = PHT (CCl_4-treated), △ = *p*-HPPH (Control), ▲ = *p*-HPPH (CCl_4-treated); mean ± S.D., $n = 5$.

procedure are that (1) no gradient elution is required, and (2) separation is achieved at room temperature. The findings obtained here suggest that this improved method is reliable in terms of simplicity and sensitivity for the simultaneous determination of PHT and its hydroxylated metabolites in rat plasma.

References

[1] M.E. Veronese, P.I. Mackenzie, C.J. Doecke, M.E. McManus, J.O. Miners and D.J. Birkett, Biochem. Biophys. Res. Commun., 175 (1991) 1112.
[2] M.E. Veronese, C.J. Doecke, P.I. Mackenzie, M.E. McManus, J.O. Miners, D.L.P. Rees, R. Gassen, U.A. Meyer and D.J. Birkett, Biochem. J., 289 (1993) 533.
[3] R.G. Dickinson, W.D. Hooper, M. Patterson, M.J. Eadie and B. Maguire, Ther. Drug Monit., 7 (1985) 283.
[4] A. Sato, K. Shimada, Y. Izumo and T. Sakaguchi, J. Chromatogr., 275 (1983) 97.
[5] G.K. Szabo, R.J. Pylilo, H. Davoudi and T.R. Browne, J. Chromatogr., 535 (1990) 279.
[6] C-Y. Hsieh and J. Huang, J. Chromatogr., 575 (1992) 109.
[7] H. Liu, M. Delgado, L.J. Forman, C.M. Eggers and J.L. Montoya, J. Chromatogr., 616 (1993) 105.