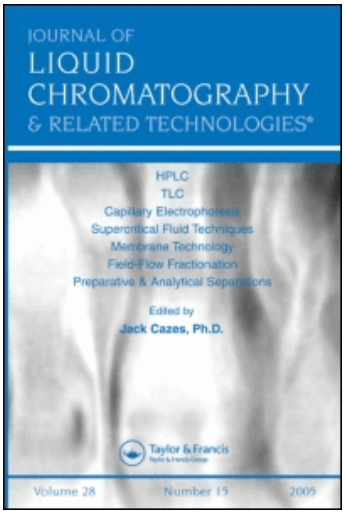

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MEASUREMENT OF PHENYTOIN, PHENOBARBITAL AND THEIR MAJOR METABOLITES IN SERUM, BRAIN TISSUE AND URINE

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ABSTRACT

A rapid HPLC method for the simultaneous determination of phenytoin, phenobarbital, 5-(p-hydroxyphenyl)-5-phenylhydantoin and p-hydroxyphenobarbital in serum, brain tissue and urine is described. The chromatographic separation is carried out using a Spherisorb 5 ODS column and monitoring at 195 nm. The mobile phase is a mixture of acetonitrile and phosphate buffer (28:72) with a flow rate of 2 ml/min. Serum and brain tissue homogenate samples are extracted with tert-butyl-methyl ether at low pH in the presence of an excess of ammonium sulfate. Glucuronide conjugates in urine samples are hydrolyzed by enzymatic cleavage with β-glucuronidase and then extracted with tert-butyl-methyl ether at low pH. Quantitation is based on peak-height ratio of analyte to internal standard (p-methylphenobarbital). The statistical analysis of the results demonstrate that the method is precise and accurate.

INTRODUCTION

Phenytoin (PHT) and phenobarbital (PB) are two of the most efficacious and widely prescribed anticonvulsants for the treatment of epilepsy. Phenytoin is extensively metabolized in the liver to 5-(p-hydroxyphenyl)-5-phenylhydantoin (p-HPPH), and between 60 to 70% of the administered dose is excreted in urine as a glucuronide conjugate of p-HPPH (1,2). Phenobarbital is also primarily metabolized to p-hydroxyphenobarbital (p-HPB) and partially conjugated with glucuronic acid, although, in this case a substantial fraction of the drug, about 30%, is excreted unchanged in the urine (3,4).

Despite the recently advocated single drug therapy for the chronic treatment of epilepsy (5,6), PHT and PB are two anticonvulsants frequently given in combination with success (8-11). The pharmacokinetic interactions between PHT and PB have been the subject of several repots (12-18), however, the results are still apparently contradictory and the anticonvulsant efficacy of the combination is not well understood.

Although a great number of papers and short communications dealing with the determination of PHT and PB have been published, no method has been evaluated for the simultaneous determination of both drugs and their major metabolites in the three pharmacologically most

important biological matrices (serum, brain tissue and urine).

In this paper we report an HPLC method for the simultaneous determination of PHT, PB, p-HPPH and p-HPB in small samples of serum, brain tissue and urine, which is a modification of two other previously published methods (19,20). p-Methylphenobarbital (p-MPB) is used as the internal standard for quantitation. The method is adequate for a systematic study in experimental animals of the potential metabolic interactions between both drugs and their repercusions on different brain areas, which is the site of action of these drugs.

MATERIALS AND METHODS

Reagents

Acetonitrile and methanol of HPLC grade were obtained from Fisher Scientific Co. (Fair Lawn, NJ 07410); tert-butyl-methyl ether for synthesis from Merck (Darmstadt, F.R.G.); PHT and PB from Bayer Laboratories (Barcelona, Spain); p-HPPH, p-HPB and p-MPB from Aldrich-Chemie (Steinheim, F.R.G.); ß-glucuronidase from Sigma Chemical Corp. (St. Louis, MO 63178). Water of HPLC grade was prepared with the Norganic water purification system (Millipore Corp., Bedford, MA 01730). All other chemicals were of reagent grade purchased from Merck.

Apparatus

The liquid chromatograph consisted of a Model 620 solvent delivery system equiped with a Model 7125 Rheodyne injector fitted with a 20 µl loop (Kontron AG, Zurich, Switzerland), an Uvikon Model 720LC UV-VIS variable wavelength detector (Kontron AG), a Model 3390A integrator (Hewlett-Packard, Avondale, PA 19311) and a Model 200 programmer (Kontron AG). The analyses were performed using a 250 mm x 4.6 mm i.d. column packed with Spherisorb 5 ODS (Kontron AG). The analytical column was protected with a 50 mm x 4.6 mm i.d. guard-column filled with Co:Pell ODS (Whatman Inc., Clifton, NJ 07014).

Standards

Stock solutions. These were prepared individually for PHT, PB, p-HPPH, p-HPB, and p-MPB containing 1 mg/ml of each compound in methanol.

Calibration standards for serum. These were prepared in serum of Wistar rats and in water at five different levels by diluting the appropriate volumes of each stock solution to obtain the following concentrations (µg/ml): p-HPB and p-HPPH 0.25, 1, 2, 4, 8; PB 5, 10, 20, 40, 60; PHT 1, 5, 10, 20, 40.

Calibration standards for brain tissue. These were prepared in brain tissue homogenates of Wistar rats and in water at five different levels containing the appro-

priate amounts at the following concentrations in brain tissue (μg/g): p-HPB and p-HPPH 0.25, 1, 2, 4, 8; PB 5, 10, 20, 40, 60; PHT 1, 5, 10, 20, 40.

Calibration standards for urine. These were prepared in urine of Wistar rats and in water at five different levels with the following concentrations (μg/ml): p-HPB 10, 20, 40, 60, 80; p-HPPH 25, 50, 100, 200, 400; PB 25, 50, 100, 150, 200; PHT 10, 20, 30, 40, 50.

Working internal standards. These were prepared daily in water to give concentrations of 15, 30 and 60 μg/ml of p-MPB.

Mobile Phase

The mobile phase was a mixture of acetonitrile and phosphate buffer pH 4.0 (28:72 by volume). The phosphate buffer was prepared by adding 150 μl of 1 mol/l potassium dihydrogen orthophosphate to 1000 ml of HPLC grade water and adjusting to pH 4.0 with 0.9 mol/l orthophosphoric acid. Before use, the mobile phase was filtered and degassed under reduced pressure through a 0.5 μm Millipore filter type HVLP04700 (Millipore Corp.).

Extraction Procedure for Serum

Two hundred μl of serum or calibration standard for serum together with 200 μl of working internal standard (15 μg/ml), and 2 drop of 3N HCl were dispensed into a

glass-stoppered centrifuge tube. After mixing, 2.5 ml of tert-butyl-methyl ether were added and the tube mixed vigorously by vortexing for 30 s. Then, using a calibrated spatula, an excess of crystalline ammonium sulfate was added and the tube vortex-mixed again. After centrifugation at 2800 xg for 5 min, the upper organic layer was transferred with the aid of a Pasteur pipette to a conical glass tube. Two ml of this organic phase were pipetted to a new conical glass tube and evaporated to dryness at 65 °C under a gentle stream of air.

The residue was then redissolved in 50 µl of a mixture of acetonitrile and water with the same volume relationship as the mobile phase and 20 µl of the solution injected into the chromatograph.

Extraction Procedure for Brain Tissue

Three hundred mg of brain tissue were weighed and homogenized with 1.5 ml of water in a glass pestle tube. One ml of the homogenate or calibration standard for brain tissue, 100 µl of working internal standard (30 µg/ml), and 3 drops of 6N HCl were added to a glass-stoppered centrifuge tube. After mixing, 5 ml of tert-butyl-methyl ether were added to the tube and mixed vigorously by vortexing for 30 s. Then, with a calibrated spatula an excess of crystalline ammonium sulfate was added and the tube vortex-mixed again. After

centrifugation at 2800 xg for 5 min, the organic upper layer was transferred to a glass conical tube. Four ml of this organic phase were evaporated to dryness at 65 °C under a gentle stream of air.

The residue was then redissolved in 50 µl of methanol and 20 µl of this solution injected into the chromatograph.

Extraction Procedure for Urine

Into a glass-stoppered centrifuge tube, 50 µl of urine or calibration standard for urine were dispensed and incubated at 37 °C in a water bath for 16 h with 250 µl of ß-glucuronidase, prepared according the indications of the product (Product No. 105-2000B). To the hydrolyzed urine, 100 µl of working internal standard (60 µg/ml) and 3 drops of 6N HCl were subsequently added. After mixing, 5 ml of tert-butyl-methyl ether were added and the tube mixed vigorously by vortexing for 1 min. Then the tube was centrifuged at 2800 xg for 5 min, and the organic upper layer was transferred to a glass conical tube. Four ml of this tert-butyl-methyl ether extract were finally transferred to a new glass conical tube and the tert-butyl-methyl ether removed by evaporation at 65 °C under a gentle stream of air.

The dry extract was then redissolved with 50 µl of a mixture of acetonitrile and water with the same volume

relationship as the mobile phase and 20 µl of the solution injected into the chromatograph.

Chromatographic Conditions

Chromatography was performed at room temperature using a flow rate for the mobile phase of 2 ml/min, with the detector at a wavelength of 195 nm, and a chart speed for the register system of 0.25 cm/min.

RESULTS

Chromatograms

A typical chromatogram of a standard mixture containing PHT, PB, p-HPPH and p-HPB is shown in Fig. 1. This standard mixture was periodically used to evaluate the resolving capacity of the chromatographic system. Under the chromatographic conditions used, all the assayed drugs were resolved. The relative retention times of the drugs and their metabolites relative to the internal standard were: p-HPB 0.32; p-HPPH 0.54; PB 0.61; p-MPB (int. stand.) 1.00; PHT 1.34.

Figs. 2A, 3A and 4A show chromatograms from extracts of serum, brain tissue and urine of Wistar rats treated with PHT and PB, respectively. Figs. 2B, 3B and 4B show typical chromatograms from extracts of blank serum, brain tissue and urine, respectively. As can be seen, there was no observed interference from normal , brain tissue or urine constituents.

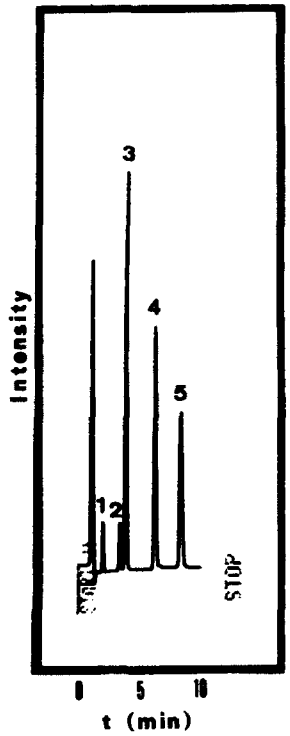

FIGURE 1. Typical chromatogram of a standard mixture containing (1) 3.2 µg/ml p-HPB, (2) 3.2 µg/ml p-HPPH, (3) 64 µg/ml PB, (4) 48 µg/ml p-MPB (int. stand.), and (5) 32 µg/ml PHT. Attenuation was 2^7.

Calibration and Linearity

Calibration equations were calculated for serum, brain tissue, urine and water from peak-height ratios versus concentration by linear regression analysis, with five determinations for each concentration. The results obtained exhibited good linearity (correlation coefficients ranged from 0.996 to 1.000). Table 1 shows the results obtained.

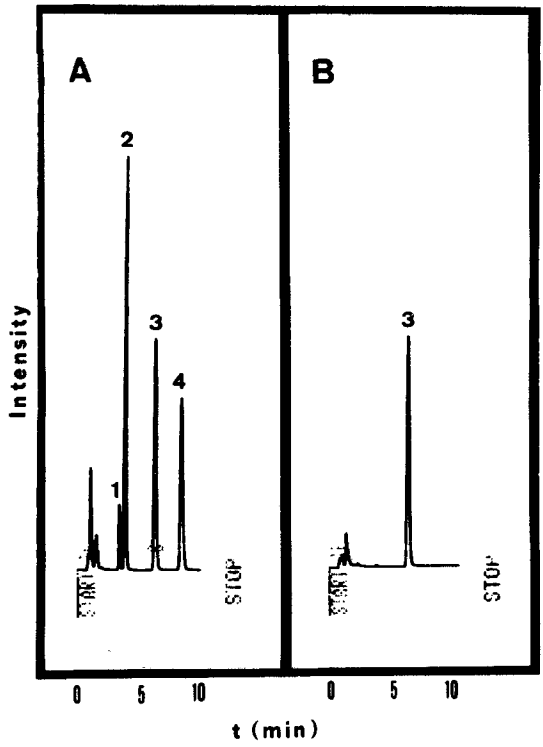

FIGURE 2. Chromatograms of:
A) a serum sample from a treated rat containing (1) 1.71 µg/ml p-HPPH, (2) 23.00 µg/ml PB, and (4) 13.74 ug/ml PHT;
B) a typical serum blank.

Peak 3 is the internal standard. Attenuation was 2^7.

In each case, the equations obtained from water standards were compared with those obtained from biological standards by means of the analysis of covariance. The results obtained showed no statistically significant differences ($p>0.05$), neither for the slope

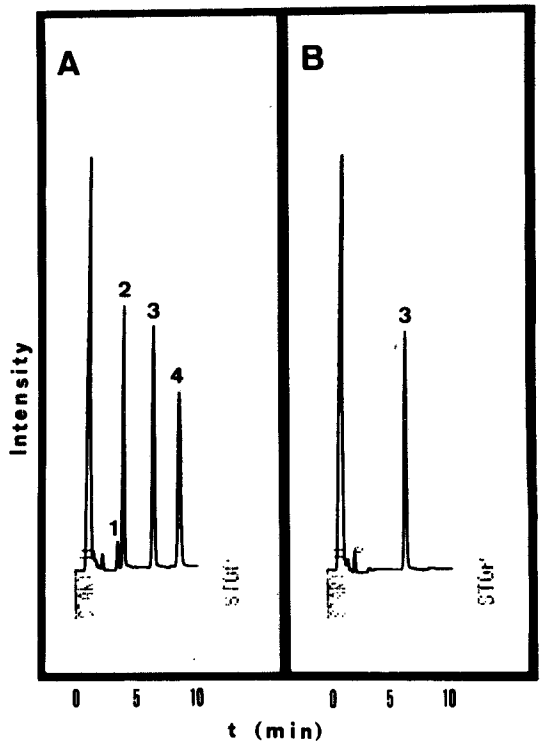

FIGURE 3. Chromatograms of:
A) a brain tissue homogenate sample from a treated rat containing (1) 0.64 µg/g p-HPPH, (2) 16.01 µg/g PB, and (4) 15.65-µg/g PHT;
B) a typical brain tissue homogenate blank.

Peak 3 is the internal standard. Attenuation was 2^7.

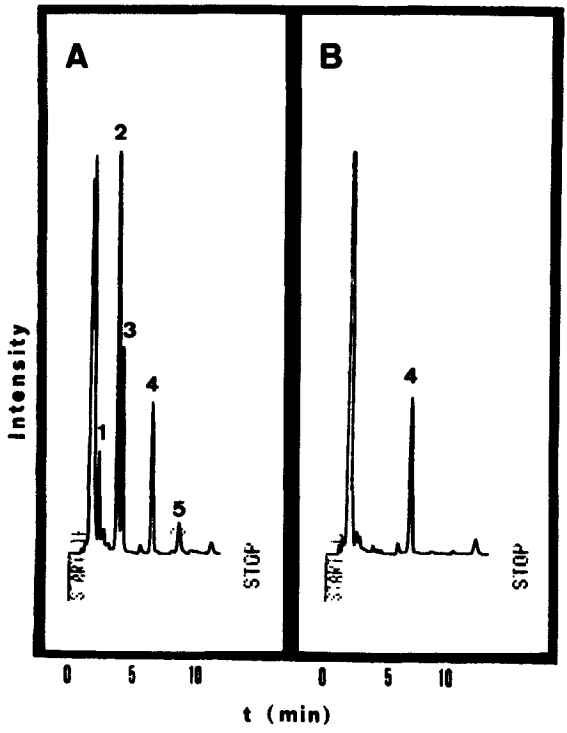

FIGURE 4. Chromatograms of:
A) an urine sample from a treated rat containing (1) 36.31 µg/ml p-HPB, (2) 228.08-µg/ml p-HPPH, (3) 161.00 µg/ml PB, and (5) 31.67 µg/ml PHT;
B) a typical urine blank.

Peak 4 is the internal standard. Attenuation was 2^9.

TABLE 1
Calibration Data and Statistical Comparison of the Calibration Curves Obtained from Biological and Water Standards

Compound	Parmater	Standard		Analysis of covariance
		Biological	Water	
Calibration for serum				
p-HPB	a	0.152	0.153	F(1,46)= 1.13ns
	b	0.005	0.002	F(1,47)= 0.97ns
	r	0.998	0.999	
p-HPPH	a	0.136	0.136	F(1,46)= 1.01ns
	b	0.007	0.003	F(1,47)= 1.79ns
	r	0.999	0.999	
PB	a	0.077	0.077	F(1,46)= 1.25ns
	b	0.022	0.009	F(1,47)= 2.38ns
	r	0.999	1.000	
PHT	a	0.048	0.049	F(1,46)= 2.17ns
	b	0.009	0.006	F(1,47)= 2.03ns
	r	0.999	0.999	
Calibration for brain tissue				
p-HPB	a	0.140	0.140	F(1,46)= 1.04ns
	b	0.007	0.005	F(1,47)= 1.59ns
	r	0.998	0.999	
p-HPPH	a	0.122	0.121	F(1,46)= 1.87ns
	b	0.005	0.001	F(1,47)= 0.92ns
	r	0.999	0.999	
PB	a	0.068	0.067	F(1,46)= 2.27ns
	b	0.013	0.009	F(1,47)= 2.00ns
	r	0.998	0.998	
PHT	a	0.045	0.046	F(1,46)= 3.13ns
	b	0.006	0.004	F(1,47)= 3.29ns
	r	0.999	0.999	
Calibration for urine				
p-HPB	a	0.016	0.016	F(1,46)= 0.68ns
	b	0.009	0.003	F(1,47)= 1.72ns
	r	0.997	0.998	
p-HPPH	a	0.012	0.012	F(1,46)= 0.82ns
	b	0.007	0.002	F(1,47)= 3.92ns
	r	0.999	0.999	
PB	a	0.008	0.008	F(1,46)= 1.44ns
	b	0.004	0.003	F(1,47)= 2.15ns
	r	0.996	0.998	
PHT	a	0.005	0.006	F(1,46)= 2.16ns
	b	0.005	0.002	F(1,47)= 1.20ns
	r	0.996	0.996	

a= slope; b= intercept; r= correlation coefficient; ns= not significant (p>0.05).

test nor for the intercept test (Table 1). On the basis of these results, calibration equations obtained from water standards can be used instead of biological standards for routine determinations.

Precision Studies

The precision of the method was determined by assaying samples of serum, brain tissue homogenates and urine containing known quantities of the drugs and their metabolites. Within-run precision was determined by analysing ten aliquots of each sample in one day. Run-to-run precision was calculated by analysing one aliquot of each remainder sample daily for a period of ten days. During this period of time the samples were stored at -20 °C in the dark. In both cases, the precision found, expressed as the coefficient of variation (CV), was less than 5% for all the assayed compounds. Precision data are given in Table 2.

Analytical Recovery

Recoveries were determined by comparing the peak-height ratios obtained from the extraction of samples of serum, brain tissue homogenates and urine with known amounts of the drugs and their metabolites after which p-MPB was added as an external standard, with peak-height ratios obtained by direct injection of the same amounts of drugs, their metabolites and p-MPB in meth-

TABLE 2
Precision and recovery for the Assay of Drugs Added to Serum, Brain Tissue Homogenates and Urine

		Precision				
		Within-run*		Run-to-run**		
Compound	Actual concn.	Mean concn.	CV (%)	Mean concn.	CV (%)	Recovery (%)
Serum						
p-HPB	0.25	0.254	3.6	0.251	3.4	92.4
	2.00	2.09	2.0	2.14	2.3	90.7
	8.00	8.16	1.1	8.07	1.4	87.2
p-HPPH	0.25	0.254	3.1	0.260	3.3	93.5
	2.00	2.01	1.7	2.10	1.3	91.0
	8.00	8.09	0.7	8.19	0.9	89.1
PB	5.00	5.04	2.3	5.07	2.5	95.5
	20.00	20.42	1.4	21.10	1.5	93.9
	60.00	60.53	0.7	61.08	0.9	91.0
PHT	1.00	1.07	2.6	1.02	3.0	96.5
	10.00	10.15	1.8	10.44	2.2	95.7
	40.00	40.41	1.1	40.82	1.4	93.8
Brain tissue homogenates						
p-HPB	0.25	0.257	4.4	0.264	4.5	91.1
	2.00	2.21	2.7	2.30	3.9	87.5
	8.00	8.19	1.1	8.11	1.4	84.9
p-HPPH	0.25	0.262	3.9	0.257	4.2	92.9
	2.00	2.05	2.1	2.00	2.8	90.1
	8.00	8.00	0.9	8.13	1.2	87.9
PB	5.00	5.12	3.2	5.17	3.5	94.6
	20.00	20.37	1.5	20.10	2.6	90.7
	60.00	60.81	1.0	61.03	1.2	89.1
PHT	1.00	1.04	2.6	1.09	3.1	95.8
	10.00	10.04	1.8	10.59	2.3	94.9
	40.00	41.11	0.9	40.61	1.1	90.3
Urine						
p-HPB	10.00	10.29	2.6	10.51	2.5	87.4
	40.00	40.12	1.1	40.19	1.4	82.1
	80.00	80.72	0.8	81.09	1.1	78.0
p-HPPH	25.00	25.27	1.0	25.32	1.3	87.5
	100.00	100.04	0.7	101.33	0.7	81.9
	400.00	402.06	0.3	403.88	0.3	77.8
PB	25.00	24.51	1.6	25.72	1.4	90.4
	100.00	101.39	0.5	100.97	0.6	89.3
	200.00	202.53	0.4	201.78	0.4	85.4
PHT	10.00	10.36	2.4	10.06	3.7	92.8
	30.00	30.52	1.5	30.89	1.5	91.4
	50.00	50.41	1.1	51.33	1.2	89.5

Concentrations are expressed in µg/ml for serum and urine, and in µg/g for brain tissue; *, ten samples assayed in one day; **, ten days with one sample assayed per day.

anol. As shown in Table 2, recoveries ranged from 77.8 to 96.5%.

DISCUSSION

The HPLC method reported is operationally simple and rapid. It involves a one-step extraction of small samples, yielding a simultaneous determination of PHT, PB, p-HPPH and p-HPB in serum, brain tissue and urine, using the same reagents and chromatographic conditions.

The extraction with an organic solvent at low pH in the presence of an excess of ammonium sulfate has proved to be an excellent technique for the extraction of antiepileptic drugs and for the precipitation of proteins and some lipids which may interfere with the analysis or contaminate the chromatographic system (19-21). We have chosen tert-butyl-methyl ether as an extraction solvent because of its excellent analytical recovery for both drugs and metabolites, as well as for its relatively low boiling point (55.2 °C). In addition, being the top layer, tert-butyl-methyl ether facilitates easy removal of the solvent after extraction. Other solvents tested were dichloromethane, dichloroethane, chloroform, diethyl ether, diisopropyl ether, and ethyl acetate; however, these solvents give a low recovery for the metabolites of the drugs. The low protein and lipid content of urine make it unnecessary to

use ammonium sulfate for the extraction of urine samples.

As reported elsewhere (20), we have found it necessary to redissolve in methanol the dry extract from brain tissue homogenates in order to achieve a greater recovery. In this way methanol redissolves some lipids of the extract together with some drugs trapped by them.

Since p-HPPH and p-HPB are known to be conjugated with glucuronic acid, the hydrolyzation of this glucuronides by enzymatic cleavage with β-glucuronidase seem to be the most appropriate technique (22), for which reason we incubated urine samples with β-glucuronidase during the night.

The efficacy of the extraction procedure together with the use of a guard-column maintains the performance of the analytical columns for a long period of time with relatively little maintenance. We usually purged the chromatographic system at the end of each day with water followed by acetonitrile, and the guard-column was refilled after each month of regular use.

We have used this method to carry out a systematic study of both acute and chronic pharmacokinetic interactions between PHT and PB (23,24). With it, we have confirmed the presence of p-HPPH in the brain after intraperitoneal administration of 30 mg/kg of PHT to Wistar rats. A peak level of 0.54 ± 0.065 μg/g was reach-

ed after 4 h from the injection, with a brain tissue ratio of 0.39±0.019. These results are in agreement with those reported by Chou and Levy (25). However, we were unable to confirm the presence of this metabolite in brain tissue after the maintenance of a serum level of 2.07±0.441 μg/ml of p-HPPH (concentration similar to that achieved with the administration of a dose of PHT) for 4 h. In all cases the number of animals used was ten. In common with other authors (26) we did not detect the presence of p-HPB in serum or brain tissue.

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