Journal of Chromatography, 221 (1980) 75—84
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 648

RAPID QUANTITATIVE DETERMINATION OF UNDERIVATIZED CARBAMAZEPINE, PHENYTOIN, PHENOBARBITAL AND *p*-HYDROXYPHENOBARBITAL IN BIOLOGICAL FLUIDS BY PACKED COLUMN GAS CHROMATOGRAPHY

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(Received March 25th, 1980)

SUMMARY

A method is described for measuring, without derivatization, the concentrations of phenobarbital, *p*-hydroxyphenobarbital, carbamazepine and phenytoin in biological fluids of epileptic patients undergoing long-term therapy. This method uses, at an isothermal temperature, a special column packing (GP-2% SP-2510-DA on 100—120 mesh Supelcoport).

The lower limit of detection for all substances analyzed is 1 μg/ml of biological material. The recovery of the compounds is about 95%, the reproducibility of the method is good (coefficient of variation, 4.3%). The mass spectra confirm the identity of substances eluting from the column. There is no interference from other commonly used antiepileptic drugs or endogenous substances.

The method has the advantages of high specificity, sensitivity and rapidity and it appears to be suitable for the routine monitoring of blood and urine concentrations in patients receiving multi-drug therapy.

INTRODUCTION

Numerous methods are available for the quantitative measurement of carbamazepine (CBZ), phenytoin (PHT), phenobarbital (PB) and *p*-hydroxyphenobarbital (*p*OHPB) in biological fluids by gas—liquid chromatography (GLC) and high-performance liquid chromatography (HPLC). A general survey of the various analytical techniques employed in the quantitative analysis of antiepileptic drugs and their metabolites has appeared in a recent book [1]. More recently other methods utilizing GLC [2—9] and HPLC [2, 10—12] have been published. However, some problems are still controversial, or may only be

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solved with methods that are too complicated to be adopted for routine use, especially for the determination of CBZ and *p*OHPB.

CBZ is rather unstable at high temperatures and at an acid pH, and it degrades into iminostilbene in high and non-constant proportions [1]. To avoid this degradation many methods use the formation of several derivatives [1, 2, 4–8].

Methods for the determination of *p*OHPB by GLC are carried out via the formation of a derivative (frequently a methyl derivative) that requires thorough purification of the biological samples. These procedures are both complicated and time-consuming [13–15]. For these reasons, we have tried to identify and measure these products, possibly together with PB and PHT, without derivatization by using a column packing developed by Supelco (Bellefonte, PA, U.S.A.) GP-2% SP-2510-DA [16].

Other methods have been described previously for the determination of some anticonvulsant drugs in plasma utilizing this phase at a programmed temperature [3, 9]. During the preparation of our manuscript a brief report has been published [17] utilizing a mixture of GP-2% SP-2510-DA on 100–120 mesh Supelcoport and 3% OV-17 on 100–120 mesh Gas-Chrom Q in equal parts by weight, at an isothermal temperature.

Our paper describes a gas chromatographic method for the quantitative determination of PB, CBZ, PHT and *p*OHPB without derivatization utilizing columns packed with SP-2510-DA at an isothermal temperature. Moreover, the substances eluting from the columns were analyzed in a mass spectrometer to confirm the identity of the drugs.

MATERIALS AND METHODS

Reagents and standards

All chemicals were of analytical grade. PB, CBZ, PHT, *p*OHPB, 5-(*p*-methylphenyl) hydantoin (MPH) and 5-(*p*-methylphenyl) 5-phenylhydantoin (MPPH) were obtained from Aldrich Europe (Beerse, Belgium).

Apparatus

A Carlo Erba Fractovap 2351 gas chromatograph equipped with a dual flame-ionization detector and a Hewlett-Packard 3380A recorder–integrator were used. The silanized borosilicate glass columns were packed with GP-2% SP-2510-DA on 100–120 mesh Supelcoport (Supelco).

For the analysis of PB and CBZ, the column length was 150 cm, and for the analysis of PHT and *p*OHPB it was 50 cm.

The following flow-rates were used: hydrogen, 15 ml/min; air, 250 ml/min; carrier gas (nitrogen), 50 ml/min (CBZ, PB) and 80 ml/min (PHT, *p*OHPB). The temperature of the columns was 245°C; the temperature of the injectors was 275°C.

The columns were conditioned by the following procedure: 15 min at room temperature with a carrier gas flow-rate (nitrogen) of 50 ml/min; 16 h at 265°C with a nitrogen flow-rate of 50 ml/min. After conditioning, and when the gas chromatograph was not in use, a nitrogen flow-rate of 10 ml/min was maintained in the column, and the oven temperature was kept at 100°C. This step seems very useful in holding the characteristics of the column constant.

Extraction procedures

CBZ, PB, PHT. To 1 ml of plasma or urine were added 15 μg of MPH (as an internal marker for PB and CBZ) and/or 15 μg of MPPH (as an internal marker for PHT), 0.2 ml of 1 *N* HCl and 8 ml of chloroform. To determine the calibration curves, various amounts of PB (10—80 μg), CBZ (2—20 μg) and PHT (5—40 μg) were added.

The test-tubes were shaken mechanically for 20 min, then centrifuged and the supernatant was discarded. Six millilitres of the chloroform layer were transferred to a second test-tube and evaporated to dryness at 65°C. The residue was redissolved in 100 μl of chloroform, and 1—2 μl of this solution were injected into the gas chromatograph.

Free pOHPB. To 1 ml of urine, either 1 *N* HCl or 1 *N* NaOH was added to bring the pH to about 5; 0.5 *M* acetate buffer (pH 5) was added to bring the final volume to 5 ml. To determine the calibration curve, various amounts of *p*OHPB (5—40 μg) were added.

To the buffered mixture 10 μg of MPPH (as internal marker) and 15 ml of ethylacetate were added. The test-tubes were shaken for 15 min, then centrifuged and 14—15 ml of the organic phase were transferred to a second test-tube. The organic phase was evaporated to dryness at 65°C with a stream of nitrogen. The residue was redissolved in 100 μl of acetone and 1—2 μl of this solution were injected into the gas chromatograph.

Total pOHPB. One millilitre of urine was incubated with an equal volume of 37% HCl at 100°C for 30 min. After cooling 6 *N* NaOH was added to it to bring the pH to about 5; 0.5 *M* acetate buffer (pH 5) was added to bring the final volume to 5 ml. The buffered mixture was extracted and analyzed by the procedure described above.

The conjugated *p*OHPB was estimated by the difference between total and free *p*OHPB.

Recovery

Analytical recoveries of substances were established as follows. For standards we added known quantities of the substances to pooled drug-free plasma (PB, CBZ and PHT) or to drug-free urine (*p*OHPB). Aliquots (1 ml) of the plasma or urine were taken through the extraction procedure without an internal marker being added. After drying, the extracts were reconstituted in 100 μl of chloroform containing 15 μg of MPH and/or MPPH; 1—2 μl of these solutions were injected into the gas chromatograph.

A second series of standards was prepared simultaneously by extracting 1 ml of drug-free plasma or drug-free urine and, after drying, adding the substances and the marker at the same concentrations as noted above.

We compared the peak area ratios of the extracted standard to the ratios obtained from the standard to which the compounds were added after extraction. The analytical recoveries so measured were corrected to absolute recoveries by the calculated ratio between the volume of chloroform added and the volume of chloroform taken out and evaporated during the extraction procedure.

For the urine extracts, the absolute recoveries were considered to be identical to the analytical recoveries because the volume of the organic phase taken out and evaporated was the same as the volume added.

Reproducibility

For the reproducibility studies, 24 plasma samples and urine samples (1 ml) containing three different concentrations of every drug (PB: 5, 30, 80 μg; *p*OHPB: 2.5, 15, 40 μg; PHT: 2.5, 15, 40 μg; CBZ: 2, 6, 15 μg; 8 samples for concentration) were prepared. Samples were stored at −20°C until analysis. The analyses were performed about once every ten days.

Biological material

Five adult epileptic patients were studied for their rate of elimination of *p*OHPB. The patients had been receiving a long-term antiepileptic drug regimen including PB, for at least six months.

A blood sample was obtained from the patients before the morning administration of the drugs (7 a.m.). Urine had been collected over the 24 h previous to blood collection (from 7 a.m. to 7 a.m.).

Plasma samples for the determination of PB, CBZ and PHT were obtained after separation of the heparinized blood of the patients. All specimens were stored at 4°C until analysis.

Fig. 1. Gas chromatographic responses obtained with: (A) 5(*p*-methylphenyl)hydantoin (a), phenobarbital (b) and carbamazepine (c), after extraction of a calibrated sample; (B) extracted plasma of a patient receiving phenobarbital and carbamazepine; (C) 5-(*p*-methylphenyl) 5-phenylhydantoin (b) and phenytoin (a) after extraction of a calibrated sample; and (D) extracted plasma of a patient receiving phenytoin.

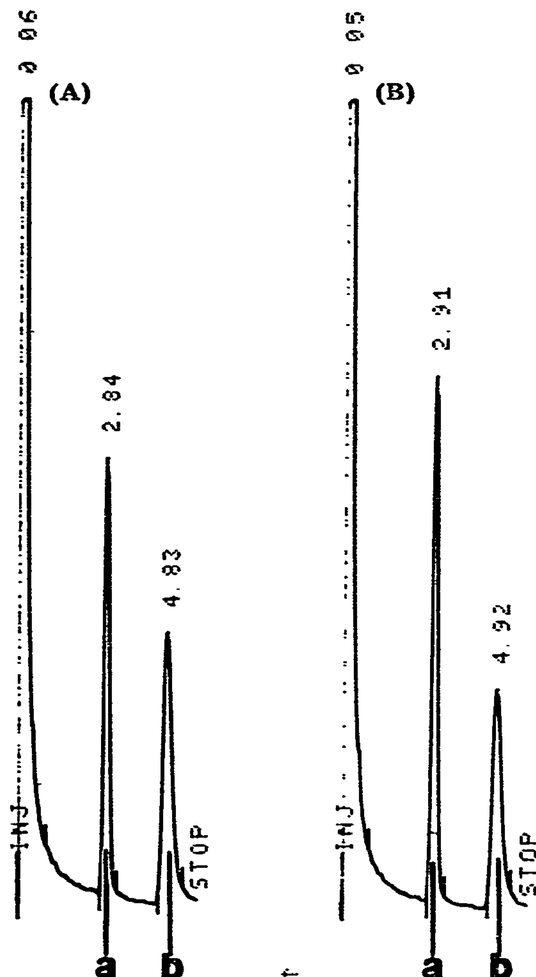


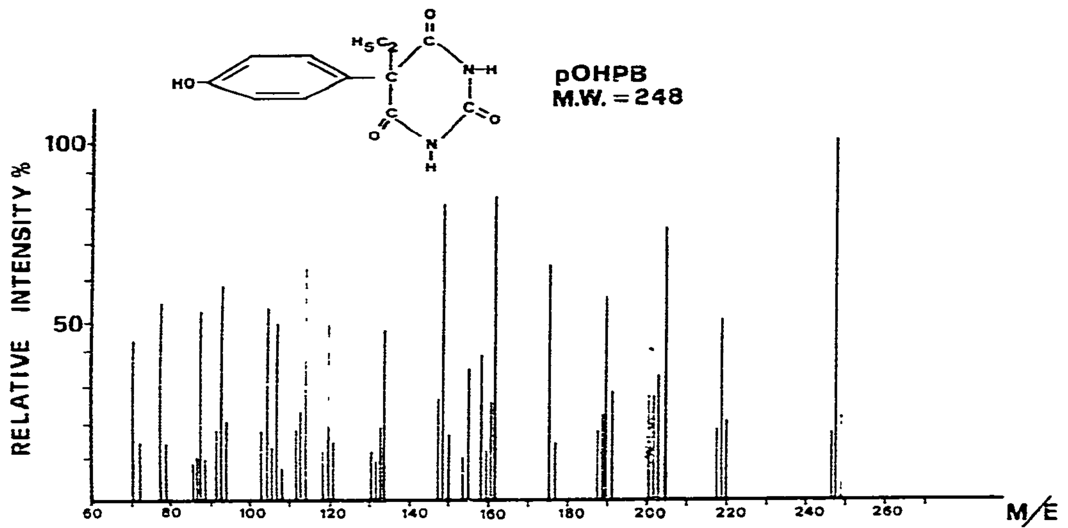
Fig. 2. Gas chromatographic responses obtained with: (A) 5(*p*-methylphenyl) 5-phenylhydantoin (a) and *p*-hydroxyphenobarbital (b), after extraction of a calibration sample; and (B) extracted urine after hydrolysis in a patient receiving phenobarbital.

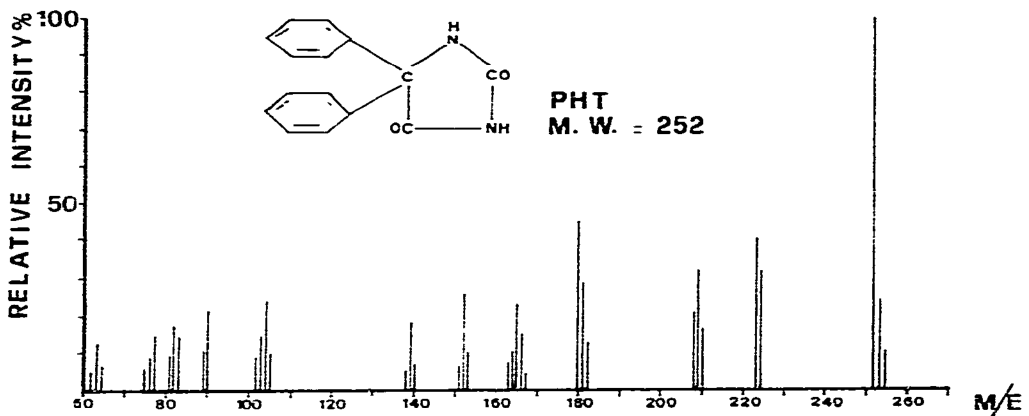
RESULTS AND DISCUSSION

Typical chromatograms of PB, CBZ and PHT obtained from the injection of internal standards (calibrators) and from the injection of plasma extracts of patients are shown in Fig. 1. In Fig. 2 chromatograms of *p*OHPB obtained from the injection of internal standards and from the injection of urine extracts are shown. There is no interference from endogenous plasma or urine substances and metabolites; retention times are short.

The concomitant administration of ethosuximide, valproic acid, primidone, 5-methylphenobarbital, diazepam, clonazepam and clobazam does not interfere with the analysis of CBZ, PHT, PB and *p*OHPB.

Identification of the compounds eluting from the column was carried out by means of combined gas chromatography—mass spectrometry (LKB—9000). Fig. 3 shows the mass spectra of PHT, CBZ, PB and *p*OHPB. After comparison





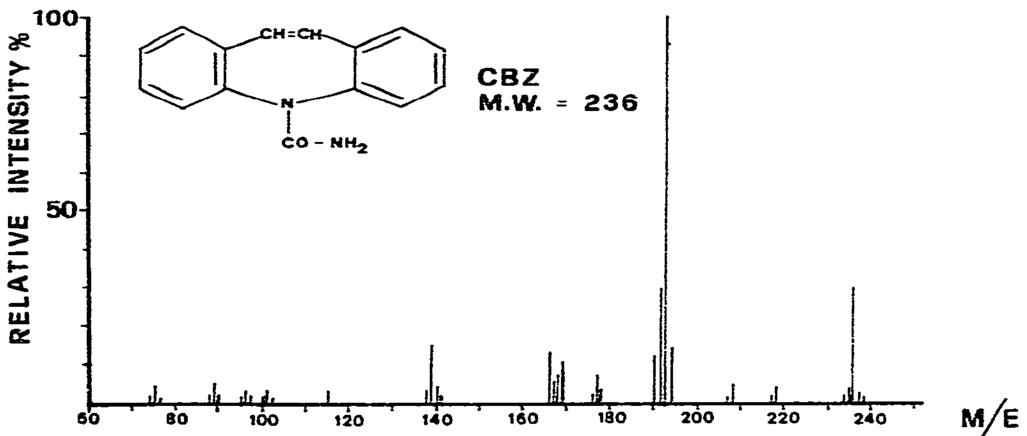


Fig. 3.

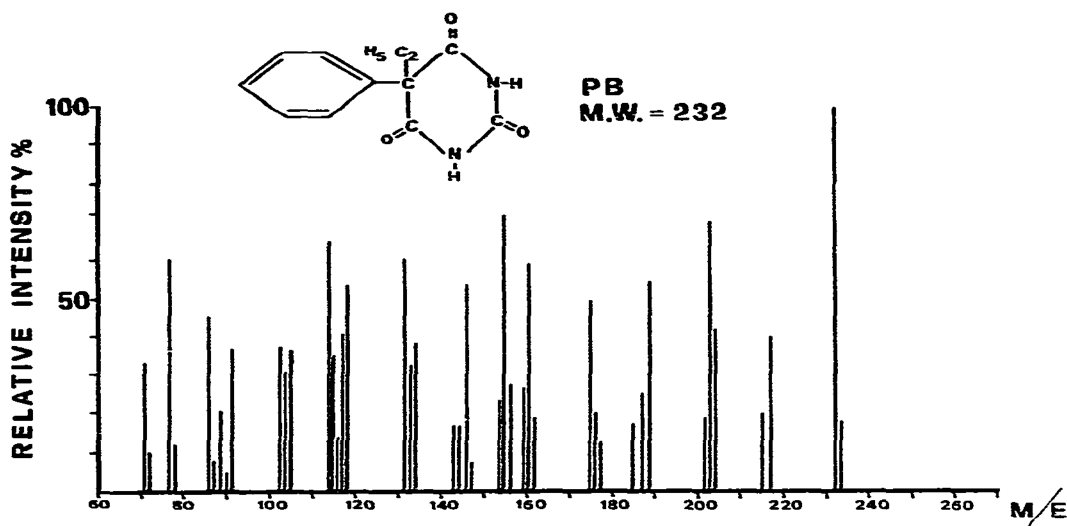


Fig. 3. Normalized electron-impact mass spectra of *p*OHPB, PHT, CBZ and PB obtained by applying a urine or plasma extract to the gas chromatograph—mass spectrometer.

of these mass spectra with the mass spectra of the pure reference substances obtained by direct inlet, it can be concluded that CBZ, PHT, PB and *p*OHPB leave the gas chromatographic column unchanged.

Therefore, in our procedure, these substances are being determined in intact form. The calibration curves of extracted substances are shown in Fig. 4. The linearity in the concentration ranges studied (2—20 μg/ml for CBZ; 5—40 μg/ml for PHT; 10—80 μg/ml for PB and 5—40 μg/ml for *p*OHPB) is very good for all the substances examined.

The minimal detectable amount using the described procedures is 1 μg/ml of plasma or urine for each substance. The recovery from human plasma (of PB,

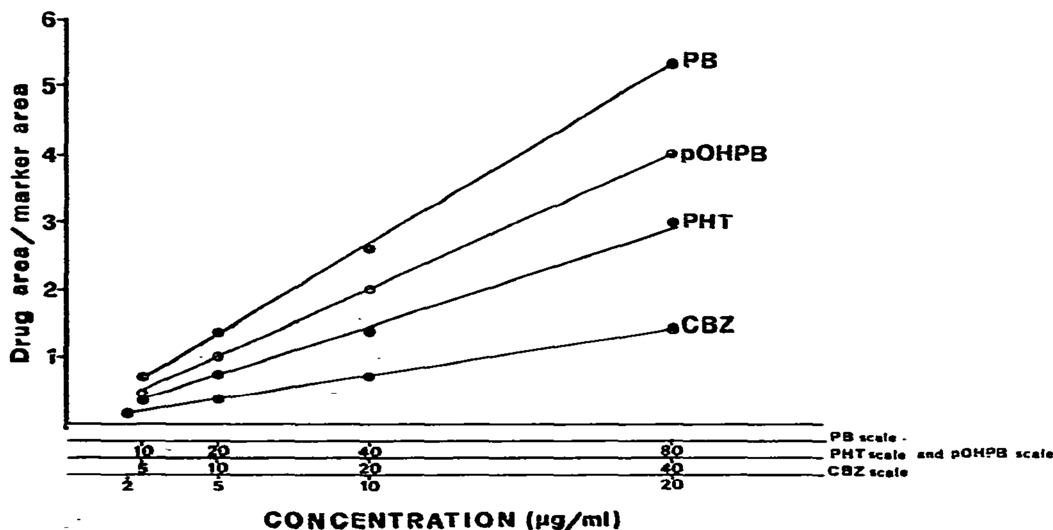


Fig. 4. Peak area ratio vs. concentration calibration curves for PB, *p*OHPB, PHT and CBZ. Marker for PB and CBZ is MPH; marker for PHT and *p*OHPB is MPPH.

PHT, CBZ) and from human urine (of *p*OHPB) is high and constant in the ranges examined, as reported in Table I. The PB standard curve derived from extracted urine is identical to that of the PB curve derived from extracted plasma. The day-to-day reproducibility of this procedure has given good results; data are shown in Table II.

On the column, CBZ undergoes a 5% degradation to iminostilbene at the various concentrations we analyzed over several months of working with different columns.

It is important for the stability of the columns to maintain a carrier gas flow-rate of about 10 ml/min at about 100°C when the columns are not in use. If this procedure is not followed, the peaks tail and the columns lose their sensitivity.

The excretion of PB and of free and conjugated *p*OHPB was studied in five epileptic patients; the results obtained are reported in Table III. Our results, similar to those obtained in other laboratories using different methods [13—15,

TABLE I

RECOVERY OF SUBSTANCES FROM HUMAN PLASMA (PB, CBZ AND PHT) AND FROM HUMAN URINE (*p*OHPB)

Amount added (μg/ml)		Amount found (μg/ml, mean ± S.D., n = 4)	Recovery (% ± S.D.)
PHT	5	4.8 ± 0.10	96.0 ± 2.00
	10	9.6 ± 0.19	96.0 ± 1.90
	20	19.4 ± 0.39	97.0 ± 1.95
	40	38.3 ± 0.95	95.7 ± 2.37
CBZ	2	1.9 ± 0.10	95.0 ± 5.00
	5	4.8 ± 0.10	96.0 ± 2.00
	10	9.6 ± 0.25	96.0 ± 2.50
	20	19.3 ± 0.47	96.5 ± 2.35
PB	10	9.7 ± 0.21	97.0 ± 2.10
	20	19.2 ± 0.30	96.0 ± 1.50
	40	38.4 ± 0.41	96.0 ± 1.02
	80	78.0 ± 0.91	97.5 ± 1.12
*p*OHPB	5	4.7 ± 0.22	94.0 ± 4.40
	10	8.9 ± 0.43	89.0 ± 4.30
	20	18.1 ± 0.41	90.5 ± 2.05
	40	35.3 ± 0.68	88.2 ± 1.70

TABLE II

REPRODUCIBILITY OF PHT, CBZ, PB ANALYSIS IN PLASMA SAMPLES AND OF *p*OHPB IN URINE SAMPLES

Drug	PHT			CBZ			PB			*p*OHPB		
Amount added (μg/ml)	2.5	15	40	2	6	15	5	30	80	2.5	15	40
Amount found (μg/ml)*	2.5	15.1	40.0	2.0	6.1	15.0	5.1	30.6	80.1	2.5	15.1	40.0
S.D.	0.14	0.45	0.13	0.13	0.23	0.55	0.23	1.12	1.60	0.29	0.61	1.17
C.V. (%)	5.57	2.99	2.62	6.50	3.70	3.67	4.52	3.66	2.00	11.44	4.08	2.94

*Mean of eight determinations.

TABLE III

PLASMA LEVELS AND RECOVERY OF PB DAILY DOSE AS PB AND *p*OHPB IN 24 HOURS

Urine collection was from five patients receiving long-term therapy. Percentage of daily PB dose is shown in parentheses.

Patients	Age (years)	Sex	Weight (kg)	PB daily oral dose (mg/kg)	Total daily drug regimen (mg/day)	PB plasma concentration (μg/ml)	PB urine amount (mg/24-h collection)	Free *p*OHPB urine amount (mg/24-h collection)	Conjugated *p*OHPB urine amount (mg/24-h collection)	Urine volume (ml)
C.F.	35	F	68	1.47	PHT = 350 PB = 100	24.6	14.2 (14.2)	3.2 (3.2)	7.5 (7.5)	1290
V.F.	20	F	60	2.50	PHT = 300 PB = 150	32.5	37.8 (25.2)	8.6 (5.7)	12.2 (8.1)	980
D.C.	35	F	55	1.82	CBZ = 1000 PB = 100	16.9	26.3 (26.3)	8.8 (8.8)	15.3 (15.3)	970
C.M.	45	F	56	2.23	PB = 125	18.3	24.7 (19.8)	5.1 (4.1)	11.3 (9.0)	980
V.P.	33	M	70	1.42	PB = 100	21.0	25.0 (25.0)	5.0 (5.0)	13.1 (13.1)	1016

18—22], confirm the relatively low elimination of PB either in intact form or as a *p*-hydroxy metabolite.

The possibility of measuring *p*OHPB by GLC without derivatization permits simplification of the extraction procedures. In fact, our method, in contrast to previously published papers, is based on simple solvent extraction from urine followed by sample concentration and direct injection onto the column. For these reasons our method is very simple and rapid; studies on PB metabolism can easily be performed in different situations (drug interactions, associated pathologies, etc.).

As described above, the gas chromatographic phase permits the determination of PB, PHT and CBZ without derivatization with good results for the linearity and reproducibility, which is an advantage especially for CBZ. Moreover, the gas chromatographic—mass spectometric analysis demonstrates that the substances are detected in intact form.

ACKNOWLEDGEMENT

The authors would like to thank Dr. M.P. Bertoni and Mr. L. Cappellini (from the Mario Negri Institute, Milan, Italy) for their kindness in performing the mass spectral analysis.

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