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Use of normal-phase microcolumn high-performance liquid chromatography for the study of hydrolytic stability, metabolic profiling and pharmacokinetics of an antiepileptic drug, benzonal

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Abstract: A normal-phase microcolumn HPLC method is proposed for the quantitative determination of the most frequently used antiepileptic drugs and some compounds developed recently. The main advantage of this method, in comparison with other micro-scale HPLC techniques for antiepileptic drugs, is that it is used under isocratic conditions at room temperature (22°C). The hydrolytic stability of benzonal (BZ) has been studied using this method together with mass spectrometry (MS), and IR, UV and NMR spectroscopy. Three pathways of hydrolytic degradation were established in alkaline conditions, whereas in an acid medium only one of these routes was followed. An investigation of the metabolic profiling of BZ in guinea-pigs showed that the drug undergoes fast hydrolysis in the intestinal tract forming phenobarbitone (PB) and benzoic acid (BA). Only PB was detected in brain tissue and it is probably responsible for the whole therapeutic effect. The pharmacokinetic parameters of this metabolite (PB) were determined in guinea-pigs, and their identity with the pharmacokinetic parameters of PB as the parent drug was confirmed.

Keywords: *Normal-phase microcolumn HPLC; antiepileptic drugs; hydrolytic stability; metabolism; pharmacokinetics; benzonal.*

Introduction

Development of simple microcolumn HPLC methods for the quantitative determination of drugs in biological media is important because such methods save considerable time and materials and are highly sensitive [1–6]. Although these methods have attracted much interest [2–6], no simple microcolumn isocratic method has been described for the quantitative determination of antiepileptic drugs. In many cases, the absence of a reliable quantitative method for new antiepileptic drugs in biological media is the cause of unsatisfactory investigations of their pharmacokinetic characteristics. For example, several new compounds exhibiting antiepileptic properties were discovered among barbituric acid and cyclic urea derivatives during the last decades in the USSR. Two of these substances, benzonal (BZ) and benzobamyl (BZB) have been included in the State Pharmacopoeia of the USSR for use in the treatment of epilepsy; two others, halonal (HL) and halodiph (HP), have been recommended recently for this purpose. Many investigations have been carried out for evaluation of their antiepileptic effectiveness [7, 8]. Separation of the enantiomers of some of these drugs was also reported [9], but in all these studies little attention was paid to their metabolism and pharmacokinetics [10].

The aims of the present work were to develop a simple, isocratic, microcolumn, normal-phase HPLC method for the quantification of antiepileptic drugs in biological media and to demonstrate its use in studies on the stability, metabolism and pharmacokinetics of BZ.

Experimental

Equipment and materials

Chromatographic analyses were carried out with a Milikhrom (Nauchpribor, USSR) microcolumn liquid chromatograph equipped with a diode matrix-type UV detector set in the wavelength range 190–360 nm. The stainless steel columns (62 × 2 mm i.d.) were packed

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with 5-μm Silasorb-600 (Chemapol, Czechoslovakia). Mass spectra were recorded with a Chrom-Mass system Ribermag R10-10B (France). IR spectra were obtained with an IR-75 spectrophotometer and UV spectra with a UV–vis spectrophotometer (both Carl Zeiss, Germany). NMR spectra were obtained with a FX-90 instrument (Jeol, Japan). Elemental analysis was carried out using an automotive C, H and N analyser. Tissue samples were homogenized by means of an homogenizer MPW-324 (Poland). Halonal (HL), benzonal (BZ), phenobarbitone (PB) and halodiph (HP) were kindly supplied by the Drug Design Laboratory of Tomsk Polytechnic (Tomsk, USSR). Ethosuximide (ETS) and puphemid (PPH) were gifts from the Armenian Institute of Fine Organic Synthesis (Erevan, USSR). Other drugs were obtained by extraction with acetone, ether or chloroform of commercial pharmaceuticals. Hexane, isopropanol, acetone, ether and chloroform were of analytical reagent grade and were used without further purification.

Sample preparation

Serum samples. 150 μl of 0.25 M hydrochloric acid, 100 μl of an internal standard solution and 1 ml of chloroform were added to 100 μl of the serum sample in a 10-ml screw-capped centrifuge tube and mixed for 3 min. The extraction tubes were centrifuged at room temperature for 10 min at 3000*g*. 1 ml of the organic layer was transferred to a conical glass tube and evaporated under a stream of nitrogen at 35°C. The residue was reconstituted in 30 μl of eluent and 3–5 μl were injected into the chromatograph.

Tissue samples were ground and then mixed with isotonic saline solution (1 ml solution to 0.5 g of tissue materials) and homogenized for 10 min. 200 mg of the homogenate was analysed in the same way as the blood serum samples.

Chromatographic conditions

The mobile phase was hexane–isopropanol–chloroform (70:8:22, v/v) with a flow rate of 100 μl min^{-1}. Detection of eluates was carried out at 230 nm. Chromatographic analysis was performed at ambient temperature. Some substances for further analysis were prepared chromatographically by separation under the proposed conditions with repeated runs of the analytical method.

Quantification

Quantification was carried out using the peak-height method. Phenazepam (PZ) (4 mg l^{-1} in chloroform) was used as an internal standard. Calibration graphs were obtained using various amounts of the stock solutions and the same amount of the internal standard. The intra-assay and inter-assay variations were determined by analysing 10 serum samples spiked with the drugs. The detection limits of each drug were determined in donor blood samples containing known amounts of drug. The recovery of each drug from blood serum samples was determined versus standard solutions injected into the chromatograph.

Interference

Fifteen drug-free serum samples were extracted and analysed for possible interference by endogenous constituents. The retention times of other antiepileptic drugs were also determined in order to avoid interference.

Mass spectrometry (MS) conditions

The eluent was collected after the chromatographic separation. The mobile phase was evaporated and the dry residue was reconstituted in diethyl ether. This solution was applied to the tungsten emitter and thus directly introduced into the ion source of the mass spectrometer. EI–MS spectra were recorded at 70 eV and 453 K.

Study of hydrolytic stability of BZ

The hydrolytic stability of BZ was studied in aqueous solution (0.2%, w/v) in a glass thermostatic reactor cell at different temperatures (273–333 K) and pH (3–12). Samples of the reaction mixture were analysed at various times by the proposed chromatographic method. The reaction products were also prepared chromatographically and their chemical composition and structure were established.

Study of gastrointestinal metabolism of BZ

The gastrointestinal metabolism of BZ was experimentally investigated in guinea-pigs (n = 30). A 100 mg kg^{-1} dose of BZ was orally administered and various subgroups of animals were decapitated at 1-, 3-, 7- and 12-h intervals. The parent drug and its metabolites were extracted from: various parts of the gastrointestinal tract; blood serum; and tissue homogenates of liver, kidneys and brain. The sep-

aration of the parent drug and its metabolites from each other and from other endogenous substances was accomplished using the proposed HPLC method. Identification of the isolated substances was carried out in the same way as that used to identify the hydrolysis products of BZ.

Pharmacokinetic study of BZ metabolite

A pharmacokinetic study of PB as the parent drug and PB as the metabolite of BZ was carried out in guinea-pigs. One group of animals (n = 10) was treated orally with PB and the other (n = 10) with an equivalent dose of BZ. The concentration of PB in blood serum was determined and the main single-dose pharmacokinetic parameters were calculated on the basis of a one-compartment linear model using the following equations [11]:

$$c = c_{max} \cdot \exp(-k_{el} \cdot t); \quad (1)$$

$$k_{el} = \frac{\ln 2}{t_{1/2}}; \quad (2)$$

$$\frac{CL}{f} = \frac{D}{AUC}; \quad (3)$$

$$AUC_{(0-\infty)} = AUC_{(0-t)} + \frac{c_t \cdot t_{1/2}}{0.693}; \quad (4)$$

$$\frac{V}{f} = \frac{D \cdot t_{1/2}}{0.693 \cdot AUC}; \quad (5)$$

where c_{max} is the peak concentration of PB in blood serum, c is the drug concentration at time t, k_{el} is the rate constant of elimination, $t_{1/2}$ is the half-life of elimination, CL is the body clearance of the drug, D is the single dose, f is the bioavailability (for PB it is considered to be equal to 1), and AUC is the area under curve. A 2-week chronic pharmacokinetic study of BZ and PB was also carried out using a 40 mg kg^{-1} dosage of BZ and equivalent dosage of PB twice daily. CL, V and the steady-state concentration (c_{SS}) of these drugs were estimated using the equations:

$$CL = \frac{D}{c_{SS} \cdot \tau}; \quad (6)$$

$$V = \frac{D}{\tau \cdot c_{SS} \cdot k_{el}}; \quad (7)$$

$$c_{SS} = \frac{D}{CL \cdot \tau}; \quad (8)$$

where τ is the dosage interval.

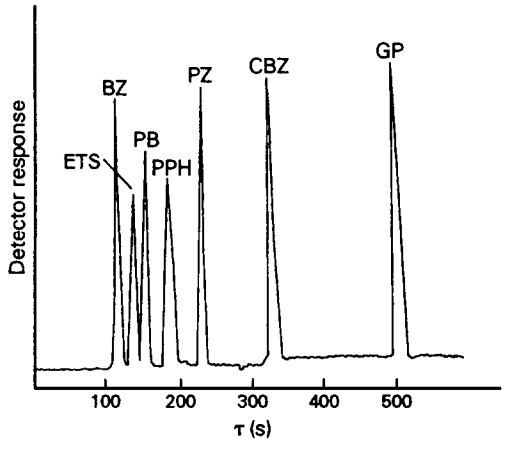

Figure 1
Chromatogram of the donor serum sample spiked with BZ, PPH, PB, CBZ, HP and PZ.

Results and Discussion

The following drugs were separated under the proposed conditions: BZ+PPH+PB+carbamazepine(CBZ)+HP using PZ as the internal standard (Fig. 1). The products of the hydrolytic degradation of BZ in acidic (Fig. 2b)

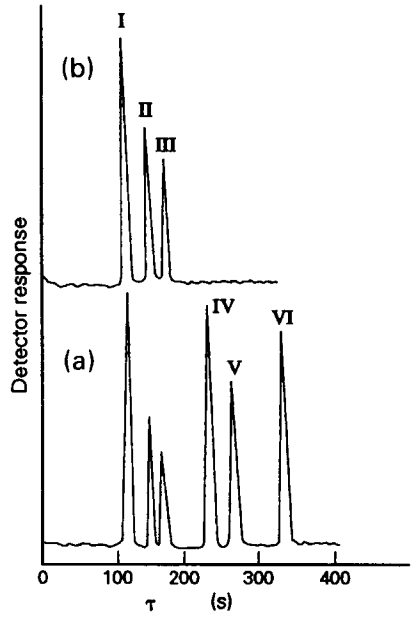

Figure 2
Chromatograms of the products of hydrolytic degradation of BZ in alkaline conditions (a): I = benzonal, II = phenobarbitone, III = benzoic acid; IV = benzoylurea; V = ethylphenyl malonic acid; VI = benzoylcarbamidoethylphenyl malonic acid; and acidic conditions (b): I = benzonal; II = phenobarbitone; III = benzoic acid.

Table 1
Analytical characteristics of microcolumn HPLC method proposed

Drug	Capacity factor (K')	Detection limit ($\mu g\ ml^{-1}$)	Linear range ($\mu g\ ml^{-1}$)	Recovery (%) ±SD	Concentration ($\mu g\ ml^{-1}$)	Reproducibility			
						Intra-assay mean ± SD ($\mu g\ ml^{-1}$)		Inter-assay mean ± SD ($\mu g\ ml^{-1}$)	
Halonal (HN)	0.16	0.1	0.1–50	97 ± 2.0	5	5.0	0.14	5.1	0.17
Benzonal (BZ)	0.16	0.1	0.1–60	95 ± 1.8	5	5.1	0.15	5.1	0.20
Ethosuximide (ETS)	0.42	0.1	0.1–20	90 ± 2.1	10	9.9	0.20	10.0	0.22
Phenobarbitone (PB)	0.53	0.1	0.01–50	94 ± 2.5	5	4.9	0.16	5.0	0.18
Phenytoin (PHT)	0.53	0.1	0.1–50	97 ± 2.0	10	10.0	0.24	10.1	0.27
Diazepam (DZ)	0.53	0.01	0.01–20	94 ± 2.0	0.02	0.02	0.08	0.019	0.01
Benzobamyl (BZB)	0.74	0.1	0.1–60	98 ± 2.1	5	5.0	0.15	5.0	0.18
Puphemid (PPH)	0.89	0.1	0.1–80	96 ± 2.4	10	10.1	0.21	10.0	0.23
Thiopentone sodium (TP)	1.05	0.1	0.1–50	95 ± 1.7	6	5.9	0.18	6.0	0.21
Methylphenylhydantoin (MPH)	1.11	0.1	0.1–50	98 ± 1.5	6	6.0	0.15	6.0	0.16
Phenazepam (PZ)	1.52	0.01	0.01–10	97 ± 2.4	0.02	0.02	0.009	0.021	0.01
Oxazepam (OZ)	1.68	0.01	0.01–10	98 ± 2.0	0.02	0.02	0.008	0.02	0.001
Carbamazepine (CBZ)	2.32	0.1	0.1–20	99 ± 1.5	2	2.0	0.12	2.1	0.15
Clonazepam (CZ)	2.32	0.01	0.01–10	97 ± 2.1	0.02	0.019	0.009	0.02	0.012
Clozepide (CLZ)	2.95	0.05	0.05–10	95 ± 2.1	0.1	0.011	0.09	0.12	0.012
Halodiph (HP)	4.05	0.1	0.1–40	99 ± 1.8	2	2.0	0.1	2.0	0.1
Diazepoxide (DP)	5.53	0.1	0.1–35	95 ± 2.3	5	5.1	0.14	5.0	0.17

and alkaline (Fig. 2a) media were also separated under the same conditions. Regression lines were obtained by plotting the peak-height ratio of each drug to that of the internal standard against the concentration in the spiked plasma samples. The linearity of the method was studied for each drug in spiked plasma samples; 20 serum samples were

Table 2
Elemental analysis of compounds corresponding to peaks I–VI (Fig. 2)

	Carbon		Hydrogen		Nitrogen		Oxygen	
Peak	theor. (%)	exp. (%)	theor. (%)	exp. (%)	theor. (%)	exp. (%)	theor. (%)	exp. (%)
I	67,85	68,01	4,76	4,60	8,33	8,52	19,05	18,78
II	62,06	62,07	5,17	5,17	12,06	12,97	20,77	20,69
III	68,85	68,22	4,91	4,71	—	—	26,22	27,58
IV	61,16	63,46	6,06	5,77	—	0,34	32,32	30,74
V	58,50	58,52	4,87	4,26	17,07	17,83	19,50	20,10
VI	66,27	65,02	5,23	5,09	8,13	8,97	23,25	22,23

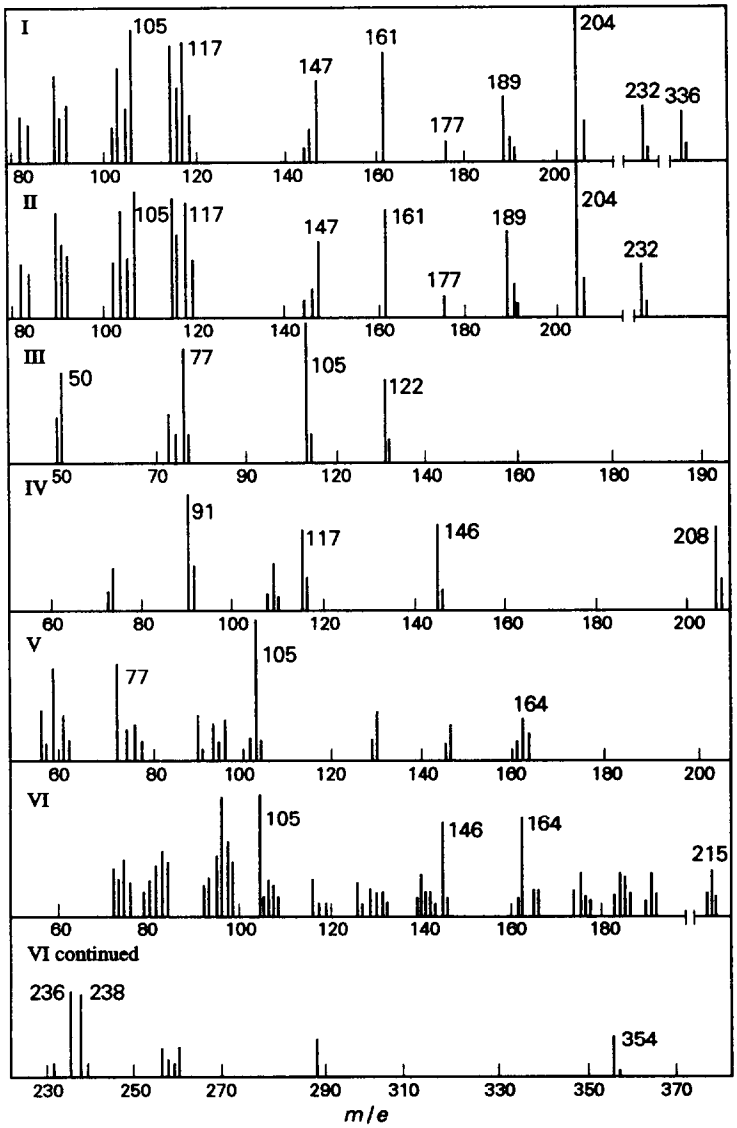

Figure 3
MS spectra of the products of hydrolytic degradation of BZ in alkaline conditions (pH 11).

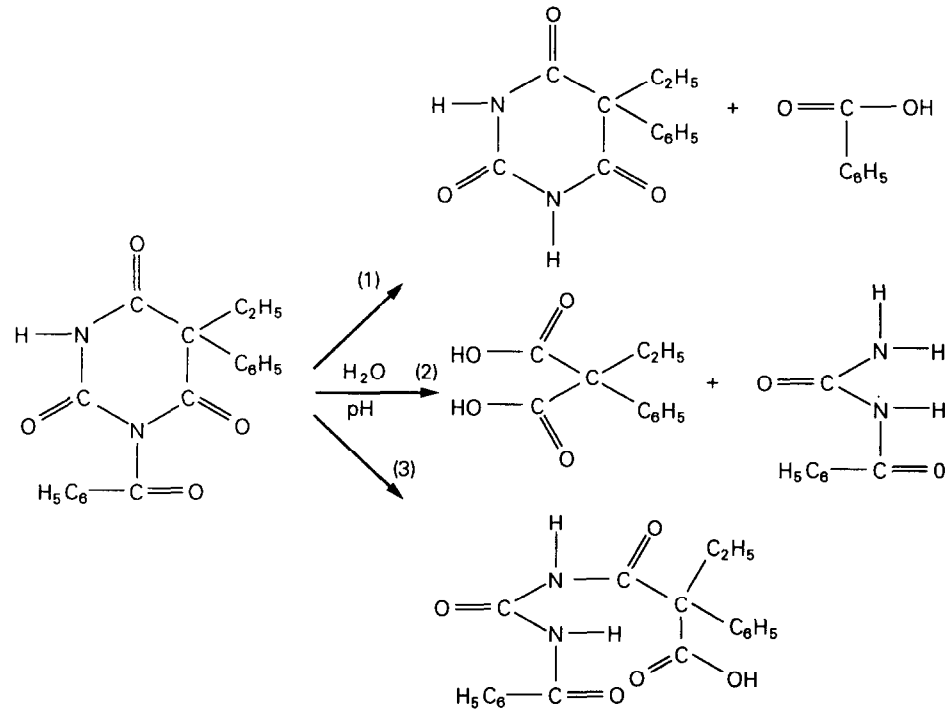

Figure 4
Scheme for the hydrolysis of BZ in alkaline conditions.

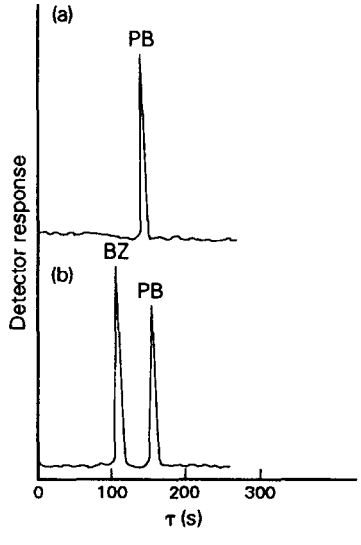

Figure 5
Chromatogram (a) of the serum of an epileptic patient receiving oral clinical doses of BZ (10 mg kg^{-1} daily).

analysed in the concentration ranges given in Table 1.

The analytical recoveries of each drug were measured at seven different concentrations in the linearity range. The precision within each assay and from day to day was established at concentrations within the therapeutic ranges

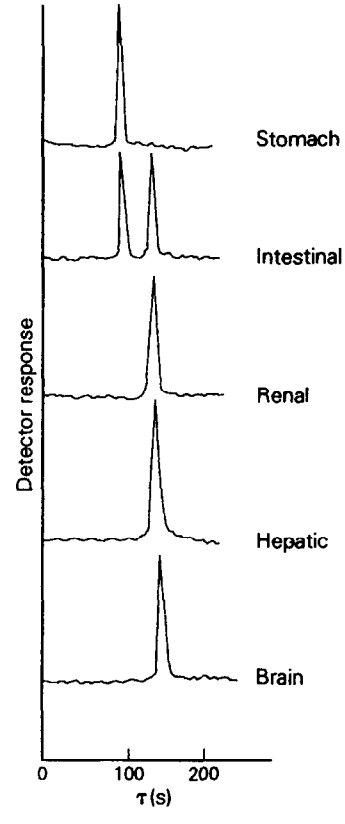

Figure 6
Chromatograms of extracts of the stomach and intestinal contents, of renal, hepatic and brain tissue homogenates and of the blood serum of guinea-pigs, which had received a single dose (60 mg kg^{-1}) of BZ.

for each drug during 2 weeks. Serum samples of 10 patients were analysed; no interference was encountered between these drugs and endogenous compounds in serum. Other antiepileptic drugs, which might be extracted in the same way were also tested for possible interference and it was established that: BZ interferes with HL; phenytoin (PHT) and diazepam (DZ) interfere with PB; and clonazepam (CZ) interferes with CBZ. Other commonly used drugs did not interfere with the analysis (Table 1).

The quantitative hydrolytic degradation study of BZ in acidic conditions (pH = 6.0, T = 311 K, $t_{react.}$ = 1.5 h) showed that two compounds and unreacted BZ may be observed in reaction products (Fig. 2b); in contrast five new substances can be detected in alkaline conditions (pH 11.0; T = 311 K; $t_{react.}$ = 1.5 h). Elemental analysis (Table 2) and MS spectra (Fig. 3) together with the chromatographic retention times of these substances showed that the compounds (chromatographic peaks I–VI in Fig. 2) may be identified as follows: (I), BZ; (II), PB; (III), benzoic acid; (IV), benzoylurea; (V), ethylphenyl malonic acid and (VI), benzoylcarbamido ethylphenyl malonic acid.

Detection of these substances in the hydrolytic reaction mixture leads to postulation of a scheme of alkaline hydrolysis of BZ (Fig. 4). Only pathway (1) occurs in acidic conditions.

A further step in the study was to investigate the intestinal metabolism of BZ. This study was stimulated by the fact that BZ could not be detected in the blood serum of epileptic patients receiving oral clinical doses of this drug (Fig. 5). Chromatographic analysis of ventricular and intestinal contents, of hepatic, renal and brain tissue homogenates and of blood serum (Fig. 6) shows that the metabolism of BZ does not take place in the stomach and starts only in the small intestine. The parent drug is not absorbed into the blood

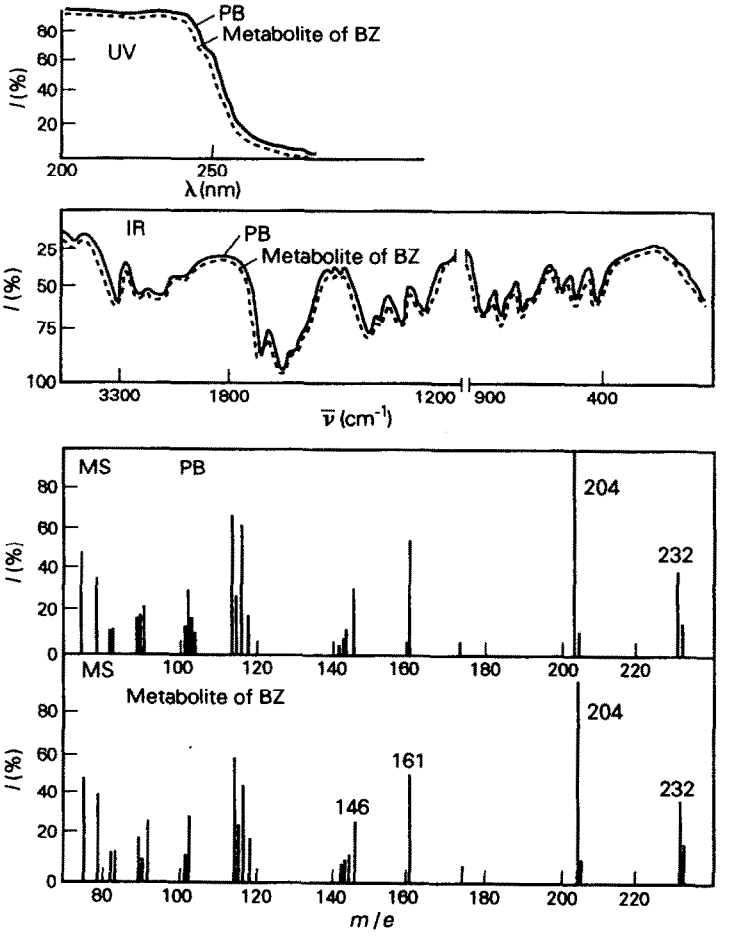

Figure 7
Spectra of PB and of the metabolite of BZ.

Table 3
Pharmacokinetic parameters of benzonal (BZ) and phenobarbitone (PB) in guinea-pigs receiving BZ and PB

Drug	Weight of animals (g)	Dose of drug (mg kg^{-1})		Maximum concentration (μg ml^{-1})	t_{max} (h)	$t_{½}$ (h)	k_{el} 10^{-2} (h^{-1})	AUC (μg ml^{-1} h)	CL (ml h^{-1} kg^{-1})		V_d (ml kg^{-1})		c_{ss} (μg ml^{-1})	
		Single	Multiple						Single dosing	Multiple dosing	Single dosing	Multiple dosing	Calculated	Measured
BZ	470	177	40	110	4.5	11.3	6.1	1332	108	127	1740	2100	22	18
BP	525	144	28	88	5.0	13.6	5.1	1062	134	139	2660	2790	17	16

serum and only its metabolite was detected in the biological media tested. Elemental and spectral analyses (Fig. 7) of the main metabolic product showed the metabolite to be PB, which has been widely used in antiepileptic therapy since 1912. In this case PB might be produced as a result of the hydrolytic degradation of BZ following the scheme in Fig. 4. Further clinical studies carried out in healthy volunteers (single dose) and in epileptic patients (undergoing long-term treatment) confirmed the results obtained in guinea-pigs. Thus, on the basis of these studies it may be concluded that the antiepileptic drug BZ acts as a prodrug substance rather than as the original parent drug.

In order to establish if there are any pharmacokinetic differences between the parent drug PB and the PB formed in intestinal tract as a metabolite of BZ, a thorough and comparative pharmacokinetic study of these drugs was conducted. The values of the main pharmacokinetic parameters given in Table 2 and the shape of c–t curves after single and multiple doses of BZ and PB showed no marked differences between the pharmacokinetic properties of these compounds.

Good correlation of the values of c_{SS} predicted on the basis of the single-dose study of these drugs with the actual c_{SS} measured during the chronic dosing showed the adequacy of the chosen pharmacokinetic model and the reliability of the estimated parameters.

Thus, on the basis of this study, it may be concluded that in metabolic and pharmacokinetic properties, BZ cannot be distinguished from those of the well-known drug PB, from which it is synthesized.

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