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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COMPARISON OF *IN VIVO* AND *IN VITRO* DRUG METABOLISM

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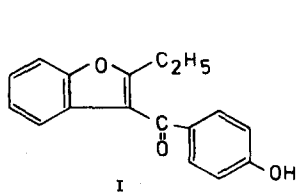
SUMMARY

Using 2-ethyl-3-(4-hydroxybenzoyl)benzofuran (EHBB) as an example, biotransformation in rabbits and rats and by rat hepatocyte suspensions was studied by high-performance liquid chromatography (HPLC) and mass spectrometry. The biotransformation of N-alkyl-substituted piperidines by rat hepatocytes gives valuable information about the pharmacodynamics of this series of compounds. It is demonstrated that a simple reversed-phase HPLC pre-column technique is much superior to the classical sample purification via extraction. The utility of hepatocytes for the investigation of drug metabolism is demonstrated.

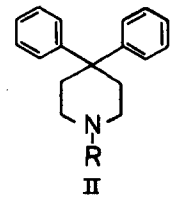
INTRODUCTION

For the registration of drugs, detailed information about the biotransformation and biological activity of their metabolites must be documented. In order to reduce at least partially the number of animal experiments and studies on humans we started *in vitro* investigations using hepatocyte suspensions[1–5].

2-Ethyl-3-(4-hydroxybenzoyl)benzofuran (EHBB) (I) and N-alkylated diphenylpiperidines (II) [R = H, CH_3, *n*-C_3H_7, $CH(CH_3)_2$, *n*-C_4H_9, $CH(CH_3)(C_2H_5)$, $CH_2CH(CH_3)_2$, $C(CH_3)_3$, $CH_2C(CH_3)_3$, $C(CH_3)_2(C_2H_5)$, *n*-C_6H_{13}, $CH_2C_6H_5$] were used as examples for demonstration of the utility of this *in vitro* model in drug research. EHBB is a drug used in the treatment of blood vessel diseases[6]. It also reduces blood serum levels of uric acid, triglycerides and cholesterol[6]. N-Alkylated diphenylpiperidines are used for the treatment of Parkinson's disease[7].



I



II

A drug and its metabolites are often separable by reversed-phase high-per-

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formance liquid chromatography (HPLC), but, proteins (*e.g.* from plasma, liquor cerebrospinalis or cell incubation media) and inorganic compounds have to be removed in time-consuming work-up procedures, which are the most inaccurate steps owing to the often unknown partition coefficients of the compounds being tested. If these by-products are not removed, the lifetime of the column is reduced considerably and overloading of the packing material with constituents of biological fluids causes a decrease in column efficiency. In biological samples, interesting compounds are often present at low concentrations and may be accompanied by large amounts of high-molecular-weight material (*e.g.*, proteins) or salts. Therefore, both enrichment of investigated compounds and sample clean-up have to be carried out prior to analysis.

Pre-columns have been used for enrichment procedures[8–11] and for sample clean-up[12–15]. Recently, a fully automated method was described for the analysis of drugs in biological fluids using pre-columns for simultaneous sample clean-up and enrichment[16]. Unfortunately, this method requires expensive equipment such as an autosampler and time-controlled pneumatic column switching valves. Such a modified HPLC system is especially suitable for routine analysis.

In this paper the advantages of a previously described[1] pre-column technique are demonstrated by comparison with the "classical" sample pre-treatment procedure.

EXPERIMENTAL

Chromatographic conditions

The separations were performed with a low-pressure gradient former (Model M 2500) and a constant-flow pump (Model M 600/200; Gynkotek, Munich, (F.R.G.). The syringe-loading sample injector (Model 7125; Rheodyne, Berkeley, CA, U.S.A.) was modified by replacing the sample loop by a pre-column as described in ref. 1. The pre-column (30 × 4.6 mm I.D.) was filled with Nucleosil 30 C_{18} (20–40 μm; Machery, Nagel & Co., Düren, F.R.G.). The analytical columns used were pre-filled with LiChrosorb RP-8 (particle size 5 μm; E. Merck, Darmstadt, F.R.G.) or Nucleosil 5 C_{18} (particle size 5 μm; Machery, Nagel & Co.). The dimensions of the columns were 100 × 4.6, 125 × 4 and 125 × 4.6 mm I.D. Detection was performed with variable-wavelength detectors (Spectro Monitor II; Laboratory Data Control, Riviera Beach, FL, U.S.A.; and LC 75; Perkin-Elmer, Überlingen, F.R.G.).

Mass spectrometer

Mass spectra were recorded with a VG Micromass MM 7070 F instrument, equipped with a data system (Vacuum Generators, Manchester, U.K.).

Reagents

EHBB was obtained from Sanol Schwarz (Monheim, F.R.G.). N-Alkyl-substituted diphenylpiperidines were supplied from the research laboratories of Byk Gulden (Konstanz, F.R.G.). All other reagents were of research grade, except acetonitrile, which was of the highest commercially available purity (LiChrosolv; Merck), and water, which was distilled twice before use. Recovery experiments from serum were carried out with standardized serum (Biotest, Frankfurt, F.R.G.). For

the cell preparations the following biochemicals were used: collagenase (Worthington; Seromed, Munich, F.R.G.), bovine serum albumin (Fraction V) (Sigma, St. Louis, MO, U.S.A.) and glucose (For Biochemical Use; Merck). The different media were prepared as follows: BS medium: 118 mmol NaCl, 2.5 mmol Na_2HPO_4, 2.0 mmol $NaH_2PO_4 \cdot 2H_2O$, 6.0 mmol KCl, 20.0 mmol $NaHCO_3$, 5.0 mmol glucose; perfusion medium: BS medium containing 0.5% (w/w) collagenase; cell preparation medium: BS medium containing 0.5% (w/w) fat-free bovine serum albumin; incubation medium: BS medium containing 0.5% (w/w) bovine serum albumin and 1.4 mmol $CaCl_2 \cdot 2H_2O$.

Sample preparation

Human serum. To 4 ml of standardized serum was added EHBB, resulting in concentrations of 5, 20 and 50 μg/ml of the drug. Then 1 ml of 1 *N* hydrochloric acid and 15 ml of water were added. The mixture was transferred to an Extrelut column (Merck) and EHBB was finally eluted with dichloromethane–isopropanol (9:1). The eluate was evaporated to dryness, dissolved in 1 ml of acetonitrile and 50 μl were used for HPLC separation.

Tissue samples. To tissue of known weight were added 30 ml of 0.05 *M* glycine buffer (pH 9.0) before homogenization. After centrifugation the supernatant was decanted, adjusted to pH 3 with 1 *N* hydrochloric acid and extracted twice with 60 ml of methylene chloride. After drying over sodium sulphate, 80 ml of the combined extracts were evaporated and dissolved in 1 ml of ethanol. These solutions were used for HPLC investigation.

Blood samples. Blood samples of defined quantity were adjusted to pH 3 with 1 *N* hydrochloric acid, extracted twice with 30 ml of methylene chloride and dried over sodium sulphate. Then 40 ml of the combined extracts were evaporated to dryness and the residue was dissolved in 1 ml of ethanol. These ethanolic solutions were investigated by HPLC.

Incubation by hepatocytes. The rat hepatocytes were prepared by recirculating collagenase perfusion of the isolated liver (20 min, *ca.* 20 ml/min, 37°C). The perfusion medium was gassed with carbogen (95% oxygen, 5% carbon dioxide) in an oxygenator. After the perfusion, the buffer was replaced with 50 ml of BS medium, prepared immediately before the addition. Then the liver was cut and the cells were suspended in 100 ml of cell preparation medium at 4°C. The suspension was shaken in an atmosphere of carbogen at 37°C for 10 min at a frequency of 4 Hz, filtered through a nylon gauze (75 μm) and finally cooled to 0°C for 15 min. The supernatant was decanted and incubation medium was added to a final volume of 60 ml to the suspension. Then 3 ml of the cell suspension were transferred into 50-ml erlenmeyer flasks, 10.5 ml of incubation medium were added, the mixture was shaken in an atmosphere of carbogen at 37°C for 15 min and 1.5 ml of an aqueous drug solution were added to the cell suspension. Samples were taken immediately before and after the drug solution was added and also after 15, 30, 60, 90 and 120 min. Carbogen gassing was repeated every 15 min. Suspensions of 1.2 ml were collected, cooled with liquid nitrogen and stored at −20°C before the analysis. The vitality of the cells was tested by the trypan blue reaction under a microscope.

TABLE I

RECOVERY OF EHBB FROM HUMAN SERUM, DETERMINED BY HPLC

Column, LiChrosorb RP-8 (5 μm, 100 × 4.6 mm I.D.); mobile phase, solvent A phosphoric acid (pH 3.0), solvent B acetonitrile, linear gradient elution from 0 to 25% B (2 min) to 55% B (2 to 25 min), to 100% B (25 to 25.5 min); flow-rate, 1 ml/min; temperature, ambient; injection, 50 μl; detection, absorption at 290 nm.

EHBB added (μg/ml)	*EHBB found ± standard deviation (n = 3) (μg/ml)*	*Recovery ± standard deviation (n = 3) (%)*
5	4.8 ± 0.15	96.0 ± 3.00
20	19.3 ± 0.66	96.5 ± 3.30
50	49.7 ± 1.89	99.4 ± 3.78
		Average: 97.3 ± 3.36

Animal experiments

Rabbits. Levels of EMBB were determined in blood from four rabbits (Neuseeländer) after intravenous injection of 2.5 mg/kg body weight of the drug. To investigate the accumulation of EHBB in different tissues, ten male rabbits were given 202 or 351 mg of the drug daily for 28 days. The animals were then killed and their blood, bile, gall bladder, vessels, brain, muscle tissues and livers stored at −20°C until taken for analysis.

Rats. The ductus choledochus of four male rats (anesthetized with dental gas) were dissected and connected to a PVC tube. After 30 min the rats were given 20 mg/kg body weight of EHBB (in 2 ml of water). The bile was collected and the amount determined every 30 min; 20 μl were used for the HPLC separation.

RESULTS

Determination of the recovery and detection limit of EHBB in biological fluids by HPLC

To determine the detection limit of EHBB in biological fluids the drug was added in different amounts to human serum and after extraction (see *Sample preparation*) the extracts were investigated by HPLC under the conditions given in Table I. Table I demonstrates that the method gives excellent recoveries (97.3 ± 3.36%). The detection limit of EHBB is 10 ng per gram of serum.

TABLE II

RABBIT SERUM LEVELS OF EHBB (mg/kg) AFTER INTRAVENOUS INJECTION OF 2.5 mg/kg BODY WEIGHT, DETERMINED BY HPLC

For chromatographic conditions, see Table I.

Time after administration (h)	*Animal 1*	*Animal 2*	*Animal 3*	*Animal 4*	*Mean ± standard deviation*
0.25	0.310	0.610	0.220	0.310	0.363 ± 0.17
1	0.052	0.027	0.030	0.077	0.047 ± 0.02
24	<0.010	<0.010	<0.010	<0.010	<0.010

TABLE III

HPLC DETERMINATION OF EHBB AND ITS METABOLITES IN RABBIT AORTA, GALL BLADDER, BRAIN, MUSCLE, LIVER AND BLOOD

For chromatographic conditions, see Fig. 1. Rabbits were given 202 mg/day of EHBB for 28 days.

Source	*Amount used (g)*	*Amount found (μg/g)*			
		EHBB	*Metabolite 1*	*Metabolite 2*	*Metabolite 3*
Aorta	0.6	0.3	–	–	–
Gall bladder	1.5	4.0	–	–	16.1
Brain	7.1	–	–	–	–
Muscle	30.35	–	–	–	–
Liver	30.3	0.2	–	–	0.1
Blood	2.9 ml	0.2	–	–	–

Determination of rabbit serum levels of EHBB by HPLC

EHBB (2.5 mg/kg body weight) was injected into rabbits intravenously. Blood samples (3 ml) were collected after 15 min, 1 and 24 h and after extraction (see *Sample preparation*) the extracts were investigated by HPLC. It follows from Table II that after 1 h the blood serum levels of EHBB had decreased drastically.

Determination of EHBB and its metabolites in different rabbit tissues

The mechanism of the biological effect of EHBB is still not known. To elucidate the mechanism of action of this drug, concentrations in different tissues and biological fluids from rabbits given orally 202 or 351 mg of EHBB daily for 28 days were determined. After extraction of the tissue and biological fluids (aorta, bile, gall bladder, brain, muscle, liver, blood) (see *Sample preparation*), the extracts were investigated by HPLC. Tables III and IV show that the drug is found in the aorta, gall bladder, liver and blood. In the gall bladder, liver and blood, three different metabolites can also be detected.

TABLE IV

HPLC DETERMINATION OF EHBB AND ITS METABOLITES IN RABBIT GALL BLADDER, BRAIN, MUSCLE, LIVER AND BLOOD

For chromatographic conditions, see Fig. 1. Rabbits were given 351 mg/day of EHBB for 28 days.

Source	*Amount used (g)*	*Amount found (μg/g)*			
		EHBB	*Metabolite 1*	*Metabolite 2*	*Metabolite 3*
Gall bladder	0.8	12.8	–	–	60.1
Brain	7.5	–	–	–	–
Muscle	28.0	–	–	–	–
Liver	31.6	0.1	–	–	–
Blood	3.2 ml	0.7	1.5	3.5	–

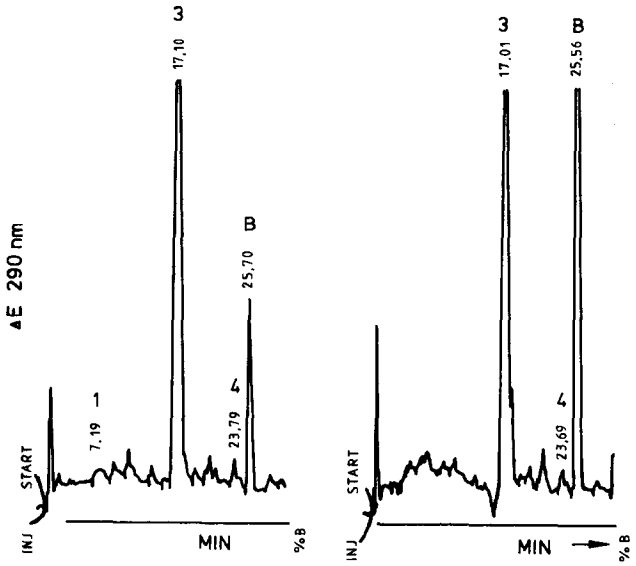

Fig. 1. HPLC traces of rabbit bile (left, direct injection; right, gall bladder homogenate). Rabbits were given 351 mg of EHBB for 28 days. Column, LiChrosorb RP-8 (5 μm, 125 × 4.6 mm I.D.); eluent, A, phosphoric acid (pH 2.7), B acetonitrile, linear gradient elution from 0 to 20% B (2 min), to 40% B (2 to 22 min), to 60% B (22 to 30 min); flow-rate, 1 ml/min; temperature, ambient; injection, 20 μl of bile or 20 μl of ethanolic extract; detection, absorption at 280 nm. Peaks: B = EHBB; 1, 3 and 4 = metabolites.

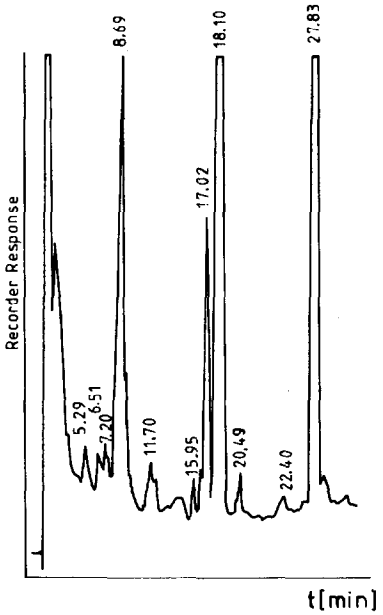

Fig. 2. HPLC trace of EHBB and its metabolites in rat bile, determined 4 h after feeding 20 mg/kg. Column, LiChrosorb RP-8 (5 μm, 125 × 4.6 mm I.D.); mobile phase, eluent A phosphoric acid (pH 2.7), eluent B acetonitrile, linear gradient elution from 0 to 20% B (2 min), to 40% B (2 to 22 min), to 60% B (22 to 30 min); flow-rate, 1 ml/min; temperature, ambient: injection, 20 μl. EHBB at 27.83 min, metabolites at 8.69, 17.02, 18.10 and 22.40 min.

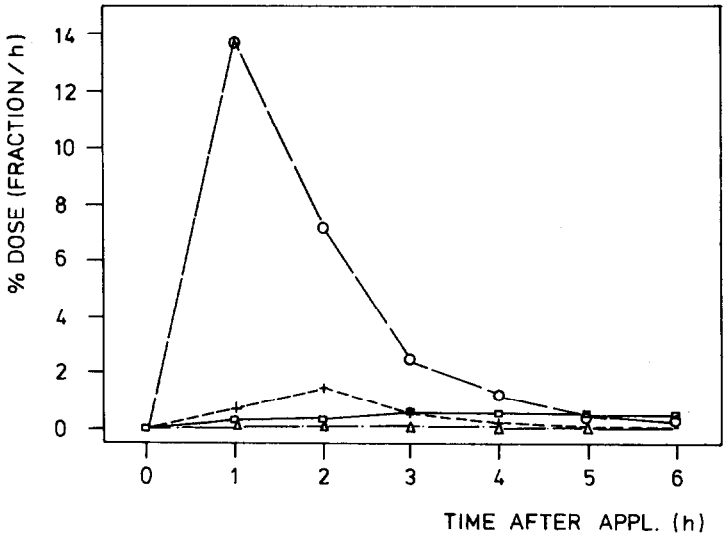

Fig. 3. Secretion of EHBB and its metabolites (% dose/h) in rat bile (bile flow from live animals). Animals were given 20 mg/kg of EHBB orally. Chromatographic conditions as in Fig. 1. □, EHBB; +, metabolite 1; △, metabolite 2; ○, metabolite 3.

To demonstrate the determination of EHBB and its metabolites, Fig. 1 shows an HPLC trace of rabbit bile and an extract of gall bladder homogenate.

Isolation of EHBB and its metabolites from rat bile by HPLC

As is demonstrated by Fig. 2, rat bile is an excellent source for the isolation and structural investigation of EHBB and its metabolites. After administration of 20

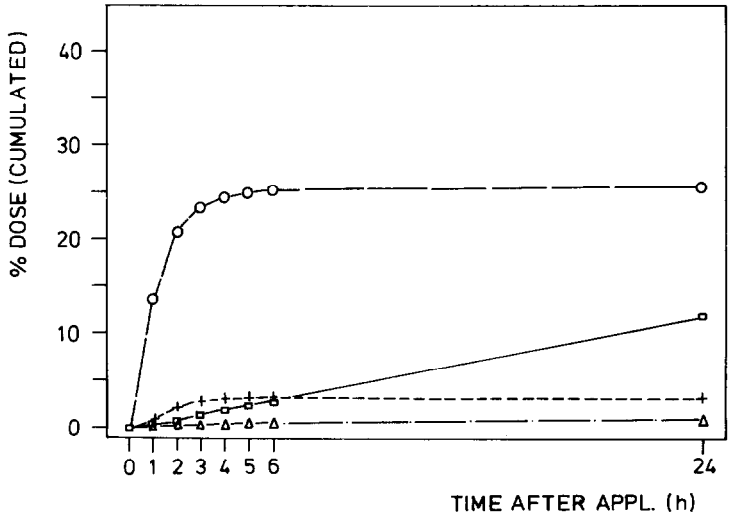

Fig. 4. Secretion of EHBB and its metabolites (% dose, cumulated) in rat bile (bile flow from live animals). Animals were given 20 mg/kg of EHBB orally. Chromatographic conditions as in Fig. 2. □, EHBB; +, metabolite 1; △, metabolite 2; ○, metabolite 3.

mg/kg body weight of EHBB, bile was taken from four live rats (see *Animal experiments*). Figs. 3 and 4 show the secretion of EHBB and its metabolites with the bile flow as a function of time and demonstrate that the bile secretion of EHBB and its metabolites increased rapidly up to 1 h after the administration of the drug, followed by a strong decrease. After 4–5 h the drug concentration in bile was almost zero. From Fig. 4 it follows that more than 40% of the administered drug (and metabolites) was secreted by the bile flow. The amount of the secreted drug and its metabolites in the bile flow is sufficient for structure determination by mass spectrometry.

Biotransformation of EHBB by rat hepatocyte suspensions; application of the pre-column enrichment technique

EHBB (100 μmol) was added to the hepatocyte suspension (see *Sample preparation*). After 60 min, 1 ml of supernatant was transferred into the pre-column for purification and enrichment[1] of EHBB and its metabolites and then separated by reversed-phase HPLC (Fig. 5). The amount of the separated material is sufficient for mass spectrometric investigations.

Biotransformation of N-alkyl-substituted 4,4-diphenylpiperidines by rat hepatocyte suspensions

For HPLC separation of the 4,4-diphenylpiperidines and their metabolites the samples were thawed, the cells destroyed by ultrasonics (5 min, ultrasonic apparatus) and finally centrifuged (2 min, Eppendorf).

A modification of our recently described "on-line" pre-column separation method for the determination of pharmaceuticals and their metabolites in biological fluids[1] allowed the direct use of the supernatant: 2 ml of water were injected on to

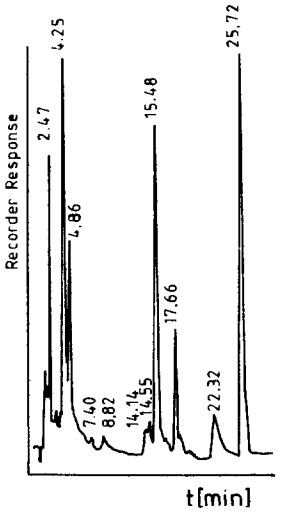

Fig. 5. HPLC trace of EHBB and its metabolites of an EHBB (100 μmol)-incubated (30 min) rat hepatocyte suspension. Weight (dry), 17.6 mg/ml; protein concentration, 5.1 mg/ml; number of cells, $2.7 \cdot 10^6$/ml; vitality (tested by microscope), 86%; pre-column, LiChroprep RP-8, 20–40 μm, 50 × 4.6 mm I.D.; injection, 1 ml of water, 1 ml of supernatant, 1 ml of water. Chromatographic conditions as in Fig. 2. EHBB at 25.75 min, metabolites at 7.40, 15.48, 17.66 and 22.32 min.

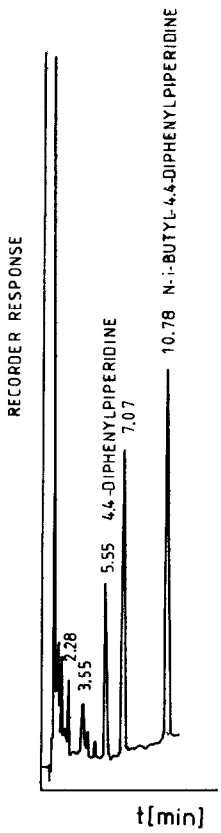

Fig. 6. HPLC trace of N-isobutyl-4,4-biphenylpiperidine and its metabolites in an N-isobutyl-4,4-diphenylpiperidine-incubated (10^{-4} mol, 30 min) rat hepatocyte suspension. Weight (dry), 17.6 mg/ml; protein concentration, 5.1 mg/ml; number of cells, $\sim 10^6$/ml; vitality (tested by microscope), 89%; pre-column, Nucleosil 30 C_{18}, 20–40 μm, 30 × 4.6 mm I.D.; injection, 2 ml of water, 100 μl of sample, 1 ml of water, 10 μl of perchloric acid (70%), 1 of ml water; analytical column, Nucleosil 5 C_{18} (Machery, Nagel & Co., 5–7 μm, 125 × 4 mm I.D.); mobile phase, eluent A 20 mmol sodium perchlorate (pH 2.5 adjusted with perchloric acid–acetonitrile (70:30, v/v), eluent B acetonitrile, linear gradient elution from 0% B to 1% B/min; flow-rate, 2 ml/min; temperature, ambient; detection, absorption at 220 nm.

the pre-column, followed by 100 μl of supernatant, 1 ml of water, 10 μl of perchloric acid (70%) and finally 1 ml of water. The pre-purified sample was than transferred to the analytical column by the back-flushing technique. By this method, for the starting materials and their metabolites up to 100 μl/l per compound could be determined. Fig. 6 shows a typical chromatogram for N-isobutyl-4,4-diphenylpiperidine and its metabolites.

Structural investigations of the metabolites of EHBB and N-alkyl-4,4-diphenylpiperidines

A comparison of Figs. 2 and 5 shows that in rat bile and the supernatant of rat hepatocytes the same metabolite peaks (1–4) are found after administration and incubation of EHBB. The mass spectrum of metabolite 4 (Fig. 7) is identical with that of an authentic sample of 3'-hydroxy-2-ethyl-3-(4-hydroxybenzoyl)benzofuran.

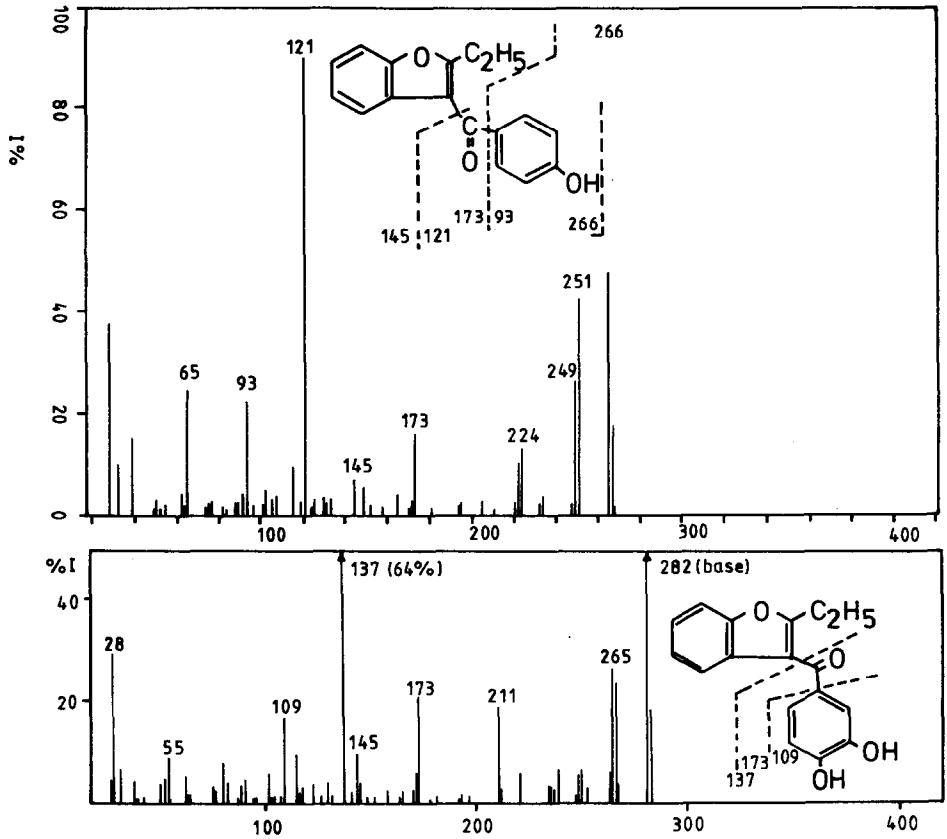

Fig. 7. Electron impact mass spectra (70 eV, direct inlet) of EHBB and EHBB metabolite 4, separated by HPLC. Chromatographic conditions as in Fig. 1.

The mass spectra of the HPLC-separated fractions (Figs. 2 and 5) of EHBB are also identical with those of synthetic samples. The mass spectra of metabolites 2 and 3 (Figs. 8 and 9) show also molecular ion peaks at *m/e* 282, which indicates that these metabolites are monohydroxylated EHBBs. The position of the hydroxyl group can be determined by nuclear magnetic resonance[17].

It follows from the mass spectrometric investigations of the HPLC-separated metabolites of the N-alkyl-substituted diphenylpiperidines that these compounds react mainly to give 4,4-diphenylpiperidine[17].

DISCUSSION

The results demonstrate that the proposed HPLC method is excellent for the determination of low concentrations of EHBB and N-alkyl-4,4-diphenylpiperidines in biological tissues. The method is highly suitable for pharmacokinetic studies of the drugs. It should be emphasized that relatively large amounts of EHBB can be detected in blood vessels. For the first time it was demonstrated that this drug is accumulated at its locus of action.

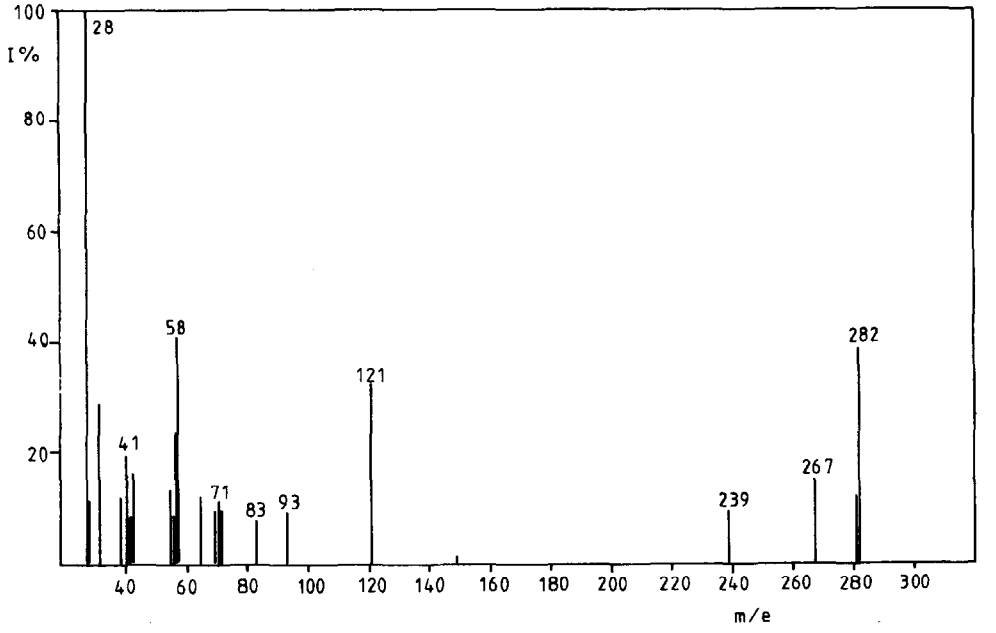

Fig. 8. Electron impact (70 eV, direct inlet) mass spectrum of EHBB metabolite 3, separated by HPLC. Chromatographic conditions as in Fig. 1.

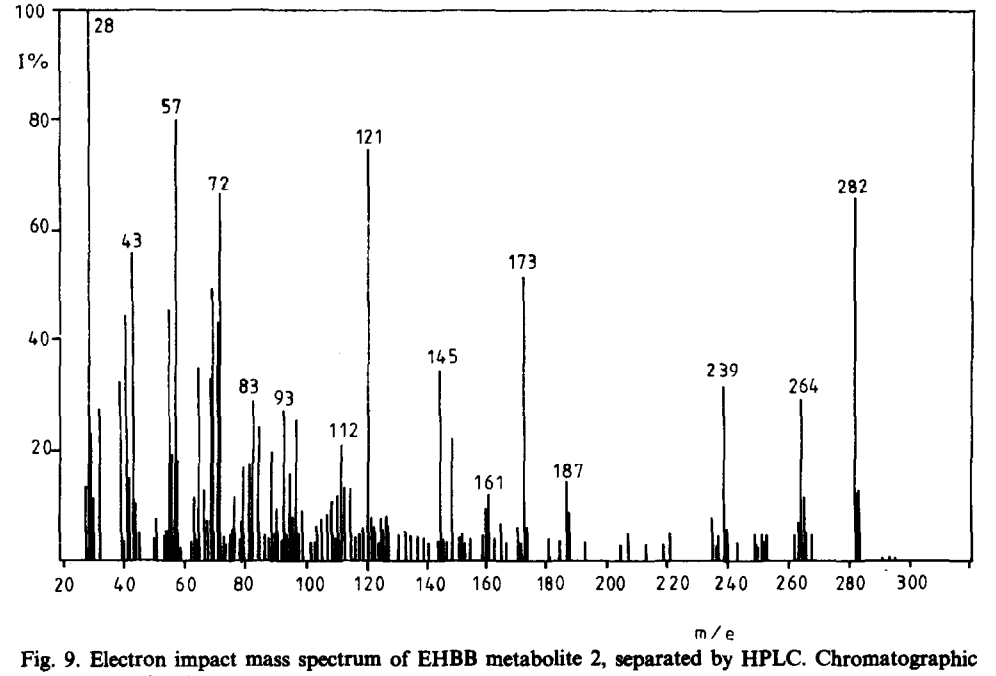

Fig. 9. Electron impact mass spectrum of EHBB metabolite 2, separated by HPLC. Chromatographic conditions as in Fig. 1.

Using this HPLC method we further succeeded in demonstrating that rat hepatocytes are exceptionally well suited for *in vitro* studies of the biotransformation of drugs. Although bile is a rich source for the isolation of the metabolites of EHBB in EHBB-administered animals, these metabolites can be isolated in a much simpler manner after incubation of hepatocyte suspensions. The "*in vitro* hepatocyte model" should in the future help to reduce the number of animal and human experiments necessary in drug research.

The metabolism (mainly dealkylation) of N-alkylated 4,4-diphenylpiperidines by rat hepatocyte suspensions is very dependent on the alkyl substituent. The low biotransformation rate for the *tert.*-butyl compound is striking; owing to its stability, the *tert.*-butyl compound shows the strongest biological activity. This valuable information can be obtained by the *in vitro* experiments described here, without treating live animals.

These investigations are also a further example demonstrating the utility of our recently developed pre-column sample clean-up and enrichment technique[1].

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