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Note

Gas chromatographic determination of 3-(4-biphenylylcarbonyl)propionic acid (Fenbufen) and two metabolites in human plasma

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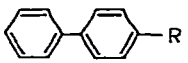
Fenbufen**, 3-(4-biphenylylcarbonyl)propionic acid (I) is a new non-steroidal anti-inflammatory agent[1]. Human pharmacokinetic studies required the development of a method suitable for the determination of I and of its two major metabolites, γ-hydroxy-4-biphenylbutyric acid (II) and 4-biphenylacetic acid (III), in biological fluids.



I : R = $-CO-CH_2-CH_2-COOH$

II : R = $-CHOH-CH_2-CH_2-COOH$

III : R = $-CH_2-COOH$

A spectrophotometric method with thin-layer chromatographic (TLC) separation of the three compounds has been applied in animal studies. As its sensitivity was 1 μg/ml, this method did not permit an accurate measurement of plasma concentrations in man after the administration of a single therapeutic dose of 600 mg of Fenbufen.

In this work, a gas chromatographic (GC) method is described that is suitable for bioavailability studies in man. It involves three steps: double extraction, derivatization and TLC separation of compounds in two groups before GC. Such a preliminary separation was required for two reasons: (i) plasma concentrations of II are 5–10 times greater than those of I and III, and (ii) under the GC conditions chosen, the retention time of II was not sufficiently different from that of Fenbufen to permit a direct analysis. (Benzoyl-3-phenyl)-2-propionic acid was used as an internal standard (I.S.). It was added to the plasma and, after derivatization and TLC, withdrawn with I and III, whereas its methyl ester was added to II just before injection into the gas chromatograph.

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EXPERIMENTAL

Reagents

Fenbufen and its two metabolites were obtained from Lederle Laboratories, Pearl River, N.Y., U.S.A.

Acetone, dichloromethane, *n*-hexane and methanol (reagent grade, Carlo Erba, Milan, Italy) were distilled before use.

Diazomethane was prepared when required in a gaseous form by mixing N-nitroso-4-toluenesulphonamide (Fluka, Buchs, Switzerland) with a half-saturated solution of potassium hydroxide in methanol at room temperature[2–4].

Thin-layer chromatograms were developed at room temperature in Shandon tanks on plastic sheets (20 × 20 cm) pre-coated with F 1500 LS 254 silica gel (Schleicher & Schüll, Dassel, G.F.R.). Before use, these plates were washed by ascending migration in acetone. *n*-Hexane–acetone (85:15) was used as the solvent system and renewed before each migration (100 ml).

Preparation of standard solutions

Stock solutions were prepared by dissolving 1 mg/ml of I, II, III and the internal standard in acetone. No evidence of decomposition was observed after storage for 6 months at 4°.

Working solutions were prepared by diluting the stock solutions in acetone to obtain various concentrations between 1 and 25 μg/ml for I and III, between 5 and 125 μg/ml for II and between 5 and 10 μg/ml for the internal standard.

Standard graphs were prepared after adding 0.2 ml of each of these diluted solutions to 1 ml of plasma.

The internal standard in its methyl ester form was obtained by methylation of acetone dilutions derived from the stock solution and containing 50 and 100 μg/ml of the acid form of the internal standard. Volumes of 50 μl of these solutions were added to each sample just before injection into the gas chromatograph.

Gas chromatographic conditions

A Hewlett-Packard, Model 5710 A dual-column gas chromatograph with flame-ionization detectors was used. The two glass columns (2 m × 2 mm I.D.) containing 3% OV-17 W/HP (80–100 mesh) operated in a differential mode. The packed columns were conditioned at 300° for 4 h with no carrier gas flow, and then at 275° for 16 h with a carrier gas flow-rate of 10 ml/min.

The following gas chromatographic conditions were used: detector temperature, 300°; injector temperature, 250°; nitrogen flow-rate, 30 ml/min for the working column and 40 ml/min for the reference column; amplifier range, 10; electrometer attenuation setting, ×2; recorder chart speed, 6 mm/min. For the detector, a hydrogen flow-rate of 30 ml/min and an air flow-rate of 300 ml/min were used.

The oven was programmed as follows: (i) for separation of compounds I, III and the internal standard: initial temperature 205°, isothermal for 4 min, then increased from 205° to 255° at 4°/min and cleared for 4 min at 290°; (ii) for separation of compound II and the internal standard: initial temperature 225°, isothermal for 4 min, then increased from 225° to 260° at 4°/min.

Procedure

A 1-ml volume of plasma, 0.2 ml of internal standard solution, 1 ml of 1 *N* hydrochloric acid and 20 ml of dichloromethane were introduced in a 30-ml glass-stoppered test-tube. The mixture was shaken for 5 min on a rotating mixer (60 rpm) and was centrifuged at 2500 rpm for 5 min. An 18-ml volume of the organic layer was transferred into a 50-ml test-tube. To the residue, 10 ml of dichloromethane were added and the extraction procedure was repeated; 10 ml of the organic layer were withdrawn and added to the first 18 ml. The solvent was evaporated to dryness by passing a gentle stream of nitrogen into the test-tube in a 35° water-bath. The dry residue was dissolved in two consecutive volumes of 2.5 ml of acetone and the solution was transferred into a 10-ml conical test-tube.

Methylation was performed directly by passing a stream of diazomethane into the acetone solution of the plasma extract until the occurrence of a yellow coloration, which indicates the presence of an excess of reagent. The solvent was evaporated under conditions described previously. The methylated extract was dissolved in 100 μl of acetone and the solution spotted quantitatively on the silica gel plates; the test-tube was rinsed with 50 μl of acetone and spotted at the same place. The TLC plates were fully developed with *n*-hexane–acetone (85:15) in unequilibrated tanks and subsequently air-dried at room temperature. The positions of the spots for compounds I, II, III and the internal standard were located on the plates using a longwave UV lamp (254 nm). The spots corresponding to compounds I, III and the internal standard were scraped off and transferred together into a 15-ml test-tube; the spot of compound II was scraped off separately and introduced into another 15-ml test-tube. A 5-ml volume of acetone was added to each tube, which was shaken (Vortex) for 5 sec and centrifuged at 1200 *g* for 3 min. A 4.5-ml volume of the supernatant was transferred into a 10-ml conical test-tube and evaporated under the conditions already described. A volume of 20–50 μl of acetone was added to the extract of I, III and the internal standard, whereas 50–100 μl of internal standard solution were added to II. Volumes of 1–3 μl were injected in the GC column.

RESULTS AND DISCUSSION

The R_F values of the compounds studied in their methyl ester form, with full ascending migration (20 cm), are shown in Table I. γ-Hydroxy-4-biphenylbutyric acid (II) is distinguished well from the other three derivatives, which permits a good separation. When using the long wave UV lamp, the limit of detection was 0.5 μg.

As shown in Figs. 1 and 2, a good separation was obtained. The retention

TABLE I

R_F VALUES OF THE METHYL ESTERS OF I, II, III AND THE INTERNAL STANDARD (I.S.)

Compound	R_F
I	0.24
II	0.12
III	0.41
I.S.	0.30

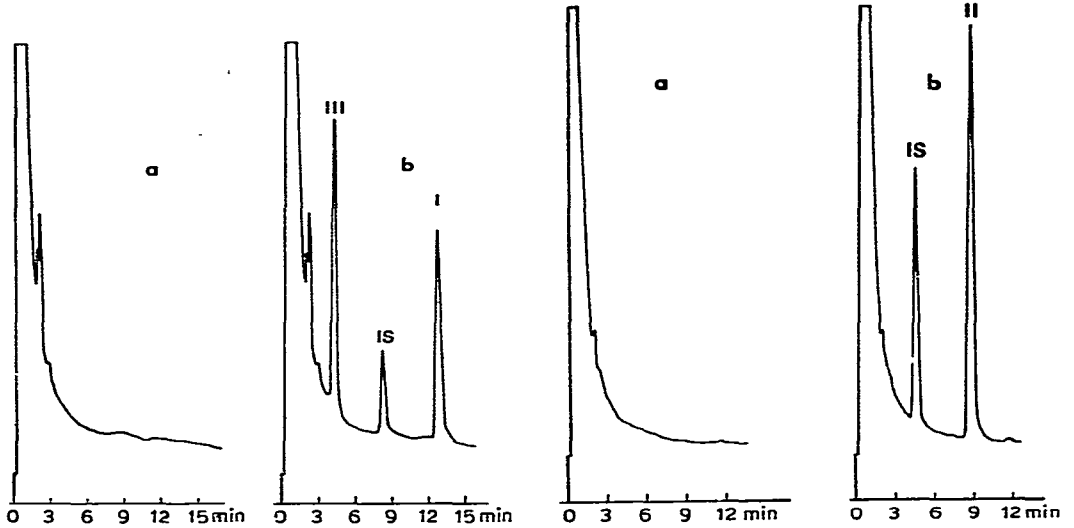

Fig. 1. (a) Chromatogram of an extract of 1 ml of control plasma. (b) Chromatogram of an extract of 1 ml of plasma containing 1 μg of I, 1 μg of III and 0.5 μg of internal standard.

Fig. 2. (a) Chromatogram of an extract of 1 ml of control plasma. (b) Chromatogram of an extract of 1 ml of plasma containing 10 μg of II and 2.5 μg of internal standard.

times of I, III and the internal standard (Fig. 1b) were 12.4, 4.3 and 8.4 min, respectively, and those of II and the internal standard (Fig. 2b) were 8.8 and 4.6 min, respectively.

Typical standard graphs are shown in Fig. 3 for compounds I, II and III. They were obtained by using plasma samples spiked with I, II and III with concentrations varying from 0.2 to 25 μg/ml and the ratios of compound to internal standard from 0.2 to 6. For the three compounds studied, good linearity between the ratio of the peak areas and the corresponding plasma concentrations was found. The precision of the method was 5.8% for Fenbufen, 8.4% for II and 7.0% for III. The limit of detection was 50 ng/ml for I and 100 ng/ml for II and III.

The recovery and the inter-assay reproductibility of the method were checked by analysing 13 different plasma samples spiked with various concentrations of the three derivatives. The results are shown in Table II.

Interferences from other non-steroidal anti-inflammatory and analgesic agents (indomethacin, aminopyrine, acetophenetidin, acetylsalicylic acid, propoxyphene), glucocorticoids (prednisone), sedatives and hypnotics (phenobarbitone, oxazepam, trichlorourethane), antibiotics (benzylpenicillin), antitussives (codeine) and central nervous system stimulants (caffeine) which could possibly be associated with Fenbufen were carefully checked. Only caffeine, at concentrations greater than 0.5 μg/ml, was found to interfere in the determination of 4-biphenylacetic acid (partial overlapping of the GC peaks).

In conclusion, the method described was found to be suitable for pharmacokinetic studies of Fenbufen after the administration of a single therapeutic dose, even in patients receiving other anti-inflammatory and analgesic agents.

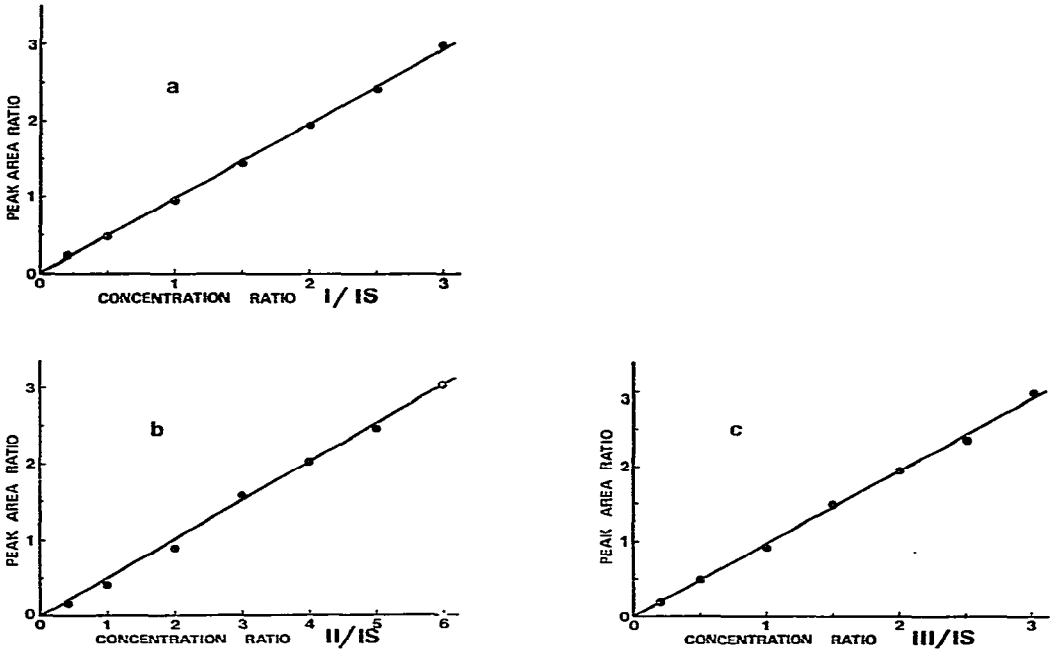

Fig. 3. Typical standard graphs for I (a), II (b) and III (c).

TABLE II

RECOVERY OF ADDED FENBUFEN AND ITS METABOLITES FROM PLASMA

Compound	*Added (μg/ml)*	*Found (μg/ml)*	*Recovery % ± S.D.)*	*Number of trials*
I	2	1.94	97 ± 2.4	8
	1	0.98	98 ± 5.2	5
II	10	9.81	98 ± 3.6	8
	5	4.88	98 ± 1.4	5
III	2	2.00	100 ± 3.7	8
	1	1.00	100 ± 6.1	5

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