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DETERMINATION OF BENOXAPROFEN [2-(4-CHLOROPHENYL)-α-METHYL-5-BENZOXAZOLEACETIC ACID, LRCL 3794] IN BIOLOGICAL FLUIDS

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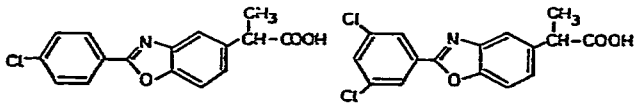
SUMMARY

Benoxaprofen, a novel anti-inflammatory compound, is efficiently (>95%) extracted from plasma and urine in the pH range 1 to 5 into either chloroform or ether. The compound is determined either by UV spectroscopy or by gas–liquid chromatography of the methyl ester (formed by reaction with diazomethane) on a column of 3% of silicone gum E-301 on DCMS-treated Chromosorb W, with detection by flame ionisation (limit 0.3 μg/ml) or electron capture (limit 0.01 μg/ml). For rapid routine use, the UV method (limit *ca.* 5 μg/ml) gives good agreement with the specific methods.

INTRODUCTION

Recent investigations in these laboratories have demonstrated that benoxaprofen [2-(4-chlorophenyl)-α-methyl-5-benzoxazoleacetic acid, LRCL 3794, structure I], possesses notable anti-inflammatory, analgesic and antipyretic properties in animal tests[1,2]. In order to study the absorption and excretion of this compound in animals and man, methods were required for determining benoxaprofen in plasma and urine. We have developed methods based on the UV spectrum of the free acid or the gas chromatographic properties of its methyl ester derivative. Gas chromatographic methods involving flame ionisation detection (GC–FID) and electron-capture detection (GC–ECD) are described.



I (benoxaprofen) II

MATERIALS

Apparatus

A Unicam SP 820 UV spectrophotometer fitted with an SP 250 scale-expansion unit and SP 21 recorder was used for the UV assay.

A Perkin-Elmer F11 gas chromatograph was fitted with a flame ionisation de-

tector and a glass column (3 ft. × 3 mm I.D.) packed with DCMS-treated Chromosorb W (80–100 mesh) containing a 3% loading of silicone gum E 301; the oven temperature was 210° and the injection-port temperature 230°. Nitrogen was used as carrier gas (flow-rate 25 ml/min).

A Hewlett-Packard gas chromatograph, Model 5710A, was fitted with an electron-capture detector and a glass column (3 ft. × 0.25 in. I.D.) packed with the identical stationary phase and support as used for flame ionisation detection; the column temperature was 220° and samples were injected on-column. The carrier gas was argon–methane (95:5) (flow-rate 45 ml/min).

Chemicals

Chloroform, diethyl ether, redistilled *n*-hexane (AnalaR grade); ethereal diazomethane; β-glucuronidase–aryl sulphatase solution from *Helix pomatia* (Boehringer Corporation Ltd.); 2-(4-chlorophenyl)-α-methyl-5-benzoxazoleacetic acid (benoxaprofen); 2-(3,5-dichlorophenyl)-α-methyl-5-benzoxazoleacetic acid (LRCL 4025, structure II); ^{14}C-labelled benoxaprofen, labelled at the 2-position of the benzoxazole ring (specific activity 0.32 mCi/g) and synthesised from ^{14}C-carboxy-4-chloroanilic acid in these laboratories by Mr. T. A. Hicks.

EXPERIMENTAL AND RESULTS

Extraction of benoxaprofen from plasma

The efficiency of extraction of benoxaprofen (pK_a between 3 and 4) from plasma was examined by using [^{14}C] benoxaprofen. At a concentration of 5 or 10 μg/ml in human plasma, extraction with chloroform at pH 1 removed 96.2 ± 0.7% (5 replicates) and 98.1 ± 0.4% (5 replicates) of the radioactivity, respectively, from the aqueous phase; when ether was used, the efficiency was similar. The extraction efficiency from rat plasma under these conditions was between 96.9 and 98.2% for the concentration range 1 to 100 μg/ml. The efficiency of this extraction procedure was used as a basis for both spectrophotometric and GC methods.

Spectrophotometric procedure for plasma

The UV spectrum of benoxaprofen in chloroform solution shows an absorption maximum at 309 nm ($\varepsilon = 36{,}480$) and a shoulder at 325 nm. Plasma (1 ml) was placed in a 10-ml stoppered tube containing 0.1 *M* Na_2HPO_4 (0.5 ml), 1 *M* HCL (0.3 ml) and chloroform (5 ml). The stoppered tube was shaken for 3 min and centrifuged at 1500 *g* for 5 min. The aqueous phase and protein precipitate were removed, and the absorbance of the chloroform phase was measured at 309 nm against chloroform on the SP 820 spectrophotometer (scale expansion × 5). Levels of benoxaprofen in plasma were determined by reference to a standard graph prepared from blank plasma samples containing suitable aliquots of a stock solution (200 μg/ml) of benoxaprofen in 0.1 *M* Na_2HPO_4,

GC procedures for plasma

The aim of these methods was to improve the specificity and sensitivity of the assay for benoxaprofen. The principle of the methods involved extraction of benoxaprofen and formation of the methyl ester (using ethereal diazomethane) for GC.

GC–FID method. Plasma (1 ml) was placed in a 10-ml stoppered tube, and a solution (20 μg/ml) of LRCL 4025 in 0.1 *M* Na_2HPO_4 (0.5 ml) was added as internal standard, followed by 1 *M* HCl (0.3 ml) and chloroform (5 ml). The stoppered tube was shaken for 3 min and centrifuged at 1500 *g* for 5 min. The aqueous phase and protein precipitate were removed, and the chloroform was transferred to a fresh 10-ml tube containing 0.1 *M* NaOH (2 ml). This tube was shaken for 2 min and centrifuged at 1500 *g* for 2 min, then the aqueous phase was transferred to a clean tube, made acid with 0.5 ml of 1 *M* HCl and extracted with chloroform (5 ml). After centrifugation and aspiration of the aqueous phase to waste, most of the chloroform was transferred to a clean 20-ml tube and evaporated to dryness under vacuum. The sides of the tube were washed down with methanol (0.5 ml), and diazomethane solution (0.5 ml) was added. The tube was lightly stoppered and left for 15 min in a fume cupboard. The stopper was then removed and the methanol was evaporated off under reduced pressure. The residue was briefly cooled in a mixture of solid carbon dioxide and ethanol, then dissolved in chloroform (50 μl), and aliquots (1 μl) of this solution were injected into the gas chromatograph. The retention times of benoxaprofen and internal standard were 3.6 and 5.1 min, respectively.

GC–ECD method. Plasma (0.5 ml) was added to a 10-ml stoppered tube. A solution (10 μg/ml) of LRCL 4025 in 0.1 *M* Na_2HPO_4 (0.1 ml) was added as internal standard, followed by 0.5 *M* HCl (0.5 ml) and diethyl ether (5 ml). The tube was stoppered, shaken for 3 min and centrifuged at 1500 *g* for 5 min. The ether was transferred to a clean 10-ml tube and evaporated to dryness under nitrogen at 50°, and the residue was esterified with diazomethane as described for the flame ionisation procedure. Redistilled hexane (50 μl) was then added, and 1 μl of the solution was injected into the Hewlett-Packard chromatograph. The retention times of benoxaprofen and internal standard were 3.4 and 5.4 min, respectively.

These three methods developed for assay of benoxaprofen in plasma were compared by assaying blank human plasma samples to which known amounts of the labelled compound had been added; the results (see Table I) demonstrated good compatibility between the methods. The UV analysis demonstrated reasonable precision in this experiment even at a level as low as 1.5 μg/ml. The speed of the assay (10 to 20 samples/h) makes UV analysis the method of choice when very good sensitivity is not required.

In another comparative experiment, a solution of [^{14}C]benoxaprofen in human plasma at a nominal concentration of 15.5 μg/ml was assayed by liquid scintillation

TABLE I

COMPARISON OF ASSAYS OF BENOXAPROFEN IN HUMAN PLASMA SPIKED WITH THE DRUG

Results are given as the mean (range) for three replicates. The ^{14}C level is the mean of two determinations.

Nominal content of drug (μg/ml)	*Content of drug found (μg/ml)*			
	UV method	*GC–FID*	*GC–ECD*	*^{14}C level*
1.5	1.4 (1.2–1.5)	1.2 (1.0–1.3)	1.7 (1.6–1.7)	1.4
5.0	4.8 (4.7–4.9)	4.6 (4.5–4.7)	5.1 (5.0–5.2)	4.5
10.0	9.8 (9.5–10.2)	9.8 (9.6–10.0)	9.7 (9.4–10.1)	9.1

counting to give a value of 15.02 ± 0.08 μg/ml (4 replicates). When assayed by GC–FID, the level obtained was 14.99 ± 0.09 μg/ml (4 replicates). This latter result was 96.7% of the nominal level and 99.8% of the ^{14}C-level by radioassay, and demonstrated a high degree of precision for the GC–FID method.

Omission of the alkaline-wash and back-extraction steps incorporated in the GC–FID procedure resulted in background peaks at a long retention time, but no difference in the overall result. A simple, single extraction was sufficient for the GC–ECD procedure, as the background was minimal with this detector.

Assay of benoxaprofen in urine

The GC methods have been used in assay of benoxaprofen in urine by procedures closely similar to those described for plasma. Benoxaprofen is excreted in animals and man either unchanged or as the glucuronide conjugate[3]. Free benoxaprofen was extracted at pH 5, which avoided co-extraction of large amounts of glucuronide, which might hydrolyse back to the parent drug. The extraction efficiency at 3 μg/ml of free benoxaprofen was greater than 95% in the pH range 1 to 5.

GC–FID method for free benoxaprofen. Urine (5 ml) was placed in a 40-ml stoppered tube containing a solution (20 μg/ml) of LRCL 4025 in 0.1 *M* Na_2HPO_4 (1 ml), and the pH was adjusted to 5 by dropwise addition of 5 *M* HCl. Chloroform (20 ml) was added, and the tube was stoppered and shaken for 2 min; the tube was then set aside for 5 min, and the aqueous phase was carefully removed with a Pasteur pipette and rejected. A portion (18 ml) of the chloroform phase was transferred to a 100-ml round-bottomed flask and evaporated to dryness under reduced pressure. The residue was dissolved in chloroform (2 ml) and transferred to a 20-ml stoppered tube. A second 2-ml portion of chloroform was used to wash out the flask and was also transferred to the stoppered tube. The solution was evaporated to dryness, and the derivatisation and GC procedures were carried out as described above for plasma.

GC–FID method for total benoxaprofen (free plus glucuronide). The sum of free and conjugated benoxaprofen in urine was assayed by using aliquots of fresh urine. The urine (1 ml) was diluted with 0.1 *M* acetic acid–sodium acetate buffer of pH 4.5 (5 ml) containing 15 μl of β-glucuronidase preparation (5.2 U/ml) in a 10-ml stoppered tube, and the mixture was incubated for 18 h at 37°. Internal-standard solution (1 ml, 20 μg of LRCL 4025) and concentrated HCl (0.25 ml) were then added, followed by chloroform (6 ml), and the mixture was shaken vigorously for 2 min. The tube was set aside for 5 min, the aqueous phase was aspirated off, 2 ml of 0.1 *M* NaOH were added to the chloroform layer, and the mixture was shaken for 2 min. After separation of the phases, the aqueous phase was transferred by Pasteur pipette to a clean 10-ml tube and acidified with 0.5 ml of 1 *M* HCl, and chloroform (5 ml) was added. The mixture was shaken for 2 min, and, after separation of the phases, the aqueous phase was discarded. Most of the chloroform phase was transferred to a clean tube and evaporated to dryness. The derivatisation and GC procedures were carried out as described above.

GC–ECD method for total benoxaprofen. An aliquot of urine (1 ml) was diluted with 1 ml of 0.1 *M* acetate buffer of pH 4.5 in a 10-ml tube, 15 μl of β-glucuronidase preparation were added, and the mixture was incubated at 37° for 18 h. After incubation, 0.5 ml of 1 *M* HCl was added, followed by internal-standard solution (0.1 ml, 2 μg of LRCL 4025) and ether (7 ml). The tube was stoppered, and the extraction,

derivatisation and assay procedures were carried out according to the GC–ECD method described for plasma.

DISCUSSION

The methods for plasma described here have been used in studies on animals and humans to demonstrate absorption of benoxaprofen[3–5]. Only unchanged benoxaprofen has been detected in the plasma of a number of species, including man[3]. The GC–FID method has been used routinely to determine plasma levels of benoxaprofen in human volunteers. A typical absorption pattern is shown in Fig. 1; after single oral doses of 40, 80 and 100 mg of benoxaprofen to a healthy male with a 1-week interval between doses, the compound was well absorbed, as demonstrated by the dose-related plasma levels in this subject.

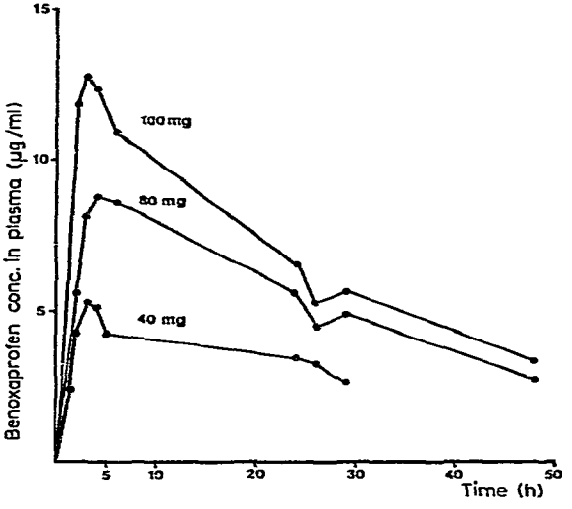


Fig. 1. Variation in plasma levels of benoxaprofen, with time, in a human subject given single oral doses of 40, 80 and 100 mg of the drug.

Similar studies in a larger number of volunteers have demonstrated that the compound has a long half-life (*ca.* 33 h) in human plasma[6]. This observation prompted development of a more sensitive assay based on the electron-capture properties of the chlorine atom in the methyl ester to permit accurate estimation of terminal-phase plasma levels below 1 μg/ml. With this method, accurate determination of the elimination rate and other pharmacokinetic parameters of the drug are possible. Peak heights obtained with the GC–ECD method have shown that the compound can be assayed accurately in plasma at levels as low as 0.01 μg/ml compared with *ca.* 0.3 μg/ml with the GC–FID assay. Excellent linearity in the ratio of peak height of benoxaprofen to that of the internal standard was achieved for the plasma-concentration range examined (0.01–20 μg/ml).

The GC methods described for assay of benoxaprofen in urine have been applied to samples collected from human volunteers after oral doses of the drug[5]. A

TABLE II

URINARY EXCRETION OF BENOXAPROFEN IN A HUMAN VOLUNTEER AFTER FIVE DOSES OF 50 mg GIVEN ONCE DAILY

Assay	*Concentration (μg/ml) in 24-h sample (n = 3; ± s.e.m.)*	
	GC–FID	*GC–ECD*
First	6.96 ± 0.03	7.35 ± 0.10
Second	7.31 ± 0.12	7.23 ± 0.08

comparison of the two GC methods for assay of total benoxaprofen (free and conjugated) was made on urine from a male volunteer who had received five daily oral doses of 50 mg of benoxaprofen. After incubation for 18 h at 37° with β-glucuronidase, duplicate samples of urine were assayed by both methods for total benoxaprofen; good agreement in recovery was achieved (see Table II).

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