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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF FENFLUMIZOLE AND ITS DEMETHYL METABOLITES IN BIOLOGICAL SAMPLES

CARSTEN MIDSKOV

Biological Department, A/S Dumex (Dumex Ltd.), 37, Prags Boulevard, DK 2300 Copenhagen S (Denmark)

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SUMMARY

This paper describes sensitive, selective and precise methods for the assay of fenflumizole and its chromatographically verified demethyl and didemethyl metabolites in whole blood, isolated red blood cells, plasma, saliva, urine and tissue (skin and fat) from human subjects. Also conjugates of the two metabolites with glucuronic acid and sulphate were assayable. The compounds were quantitated by means of reversed-phase liquid chromatography after diethyl ether extraction, followed by fluorescence and/or electrochemical detection. The assay using fluorescence detection is quantitative down to ca. 150 pg/ml; with electrochemistry this limit was ca. 600 pg/ml and included the demethyl metabolites only. Proteinaceous materials show an extraction yield of 70–75%, whereas analytes in sample materials without proteins show yields of better than 95%. The precision at concentration levels of ca. 50 ng/ml for the parent compound and ca. 5 ng/ml for the metabolites is at most 6% (relative standard deviation) with both detection modes. The analytical procedures developed were applied after both single and repetitive administration of fenflumizole. The administration of ^{14}C-labelled fenflumizole in the single-dose study revealed the presence in plasma and urine of as yet unknown metabolites. The in vivo retention time of ^{14}C activity was substantially greater in the blood cells than in plasma. Measurements of ^{14}C activity in excreta demonstrated that excretion via the faeces is the preferred route.

INTRODUCTION

Fenflumizole, 2-(2,4-difluorophenyl)-4,5-bis(4-methoxyphenyl)imidazole (FFZ), is a new imidazole derivative (Fig. 1). It has been found to be an orally effective anti-inflammatory analgesic and antipyretic in animal models [1]. Though FFZ also possesses anti-aggregatory and anti-bronchoconstrictory activities and has been shown to have probably beneficial effects on ischaemic heart disease, its intended use is primarily in the treatment of rheumatoid arthritis and allied disorders. To date two analytical methods have been reported for deter-

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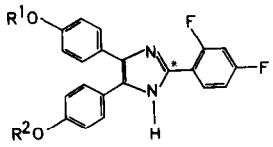

COMPOUND NO.	R^1	R^2
1.	CH_3	CH_3
2.	H	CH_3
3.	H	H
4.	Glu	CH_3
5.	Glu	Glu
6.	Sulphate	CH_3
7.	Sulphate	Sulphate

Fig. 1. Chemical structures of fenflumizole (FFZ, No. 1) and its known metabolites (Nos. 2–7). An asterisk at C-2 in the imidazole nucleus indicates the position of ^{14}C labelling. Compound No. 2 is monodemethyl fenflumizole (MD-FFZ), No. 4 its glucuronide and No. 6 its sulphoconjugate. Compound No. 3 is didemethyl fenflumizole (DD-FFZ), No. 5 its glucuronide and No. 7 its sulphoconjugate.

mination of intact FFZ [2]. Both used reversed-phase liquid chromatography (LC). In one case the column effluent passed through a UV detector (assay I), in the other through a fluorescence detector (assay II). As described previously, assay I [2] was found to be unsatisfactory for metabolic and pharmacokinetic studies in terms of sensitivity and chromatographic resolution, but for plasma these criteria were apparently fulfilled by fluorescence detection (assay II). However, in metabolic and pharmacokinetic studies the ability to quantify metabolite levels in other materials is also mandatory.

This paper describes a methodology for the quantitation of FFZ and its two demethyl metabolites with glucuronic acid and sulphate conjugates by serial fluorescence and electrochemical detection of effluents from reversed-phase LC. The practical application is demonstrated in two pilot studies by the analysis of whole blood, isolated red blood cells, plasma, saliva, urine and tissue (skin and fat) from human subjects. Radioactivity measurements are also included to demonstrate the presence of as yet unknown metabolites.

EXPERIMENTAL

Chemicals, reagents and utensils

FFZ and its two metabolites monodemethyl (MD-FFZ) and didemethyl fenflumizole (DD-FFZ) (Fig. 1) were synthesized and supplied by the Synthesis Department of Dumex. ^{14}C-Labelled FFZ was purchased from the Huntingdon Research Centre and synthesized as described by Scott and Hawkins [3]; the specific activity was 15.3 mCi/g (566 MBq/g). Acetonitrile and tetrahydrofuran were of HPLC grade, triethylamine of synthetic grade and all other chemicals were of analytical grade. β-Glucuronidase (EC 3.2.1.31) and phenolsulphatase (EC 3.1.6.1.) were purchased from Sigma (St. Louis, MO, U.S.A.) and subtilisin-A (EC 3.4.21.14) from Novo Industri (Bagsvaerd, Denmark). The liquid scintillation solution (Dynagel®) was from Baker Chemicals (Deventer, The Netherlands). Skin, fat, whole blood, erythrocytes (red blood cells), plasma, urine,

saliva and faeces were obtained from male adult volunteers and immediately placed in a deep-freezer at −20°C.

The excision of tissue specimens was performed with a scalpel under local lidocaine anaesthesia. For the isolation of red blood cells, polypropylene tubes from TecNunc (Roskilde, Denmark) coated with potassium EDTA were used.

To collect saliva the volunteers were requested to chew parafilm in order to stimulate the salivary flow. The saliva was collected as a mixture from the diverse glands by asking the subjects to spit into glass vials.

Faeces was collected in plastic containers and immediately covered by a tight plastic lid.

Instrumental and analytical parameters

The LC system consisted of a Perkin-Elmer Series 10 pump (Beaconsfield, U.K.), a Kontron Spectrofluorometer SFM 23 (Zürich, Switzerland), a Perkin-Elmer LC 4B amperometric detector and a Merck/Hitachi Model 833A Chromato data processor (Tokyo, Japan). Samples were injected with a Kontron MSI 660 autosampler.

The ^{14}C radioactivity of whole blood, plasma, urine and faeces was measured using a Beckman 150 liquid scintillation counter. Quench correction was made by the external standard method. For combustion of faeces and whole blood a biological materials oxidizer was used (B 306 Tri Carb from Packard, Downers Grove, IL, U.S.A.).

The fluorometer had a slit width of 4 nm and a 150-W xenon lamp with a wavelength range of 200–800 nm.

The electrochemical detector had a glassy carbon electrode and an Ag/AgCl reference electrode, and was operated in the oxidative mode at 0.525 V applied potential.

The column, packing material and standard mobile phase that were used in assay A (see below) were as described previously [2]. In both assays B and C, mobile phases I and II were used (see below). Mobile phase I was acetonitrile–50 m*M* triethylamine buffer (pH 4.0)–tetrahydrofuran (40:60:20, v/v/v). Mobile phase II consisted of the same components in the ratio 30:70:10 (v/v/v).

Chemical analyses of samples

Because of their (slight) photolability, FFZ, MD-FFZ and DD-FFZ solutions were prepared in brown glass vessles and handled in subdued light [4].

Assay A: simultaneous determination of FFZ, MD-FFZ and DD-FFZ in serum, plasma, whole blood, isolated red blood cells and tissue. The previously reported analytical method proved to be excellent for serum or plasma (assay II in ref. 2). Briefly, following diethyl ether extraction from serum or plasma, the evaporated residue was dissolved and chromatographed with standard mobile phase at ambient temperature and passed through the fluorescence detector [2]. Whole blood, red blood cells and tissue were processed as follows prior to extraction.

(i) Preparation of whole blood. The samples of whole blood were subjected to three freeze–thaw cycles for complete lysis of the cells before extraction as for serum.

(ii) Preparation of isolated red blood cells. Shortly after collection into polypropylene tubes coated with EDTA, 4 ml of stabilized blood were centrifuged at 2200 *g* and buffy coat, leucocytes and plasma discarded. A volume of distilled water equal to that of the remaining cell suspension was added and this suspension was cooled to −20°C to lyse the red blood cells. The haemolysate was extracted the same way as serum.

(iii) Preparation of tissue. About 200 mg (dry weight) of tissue, e.g. adipose tissue or skin (stratum corneum epidermis), were added to 500 µl of a proteolytic enzyme solution, prepared by dissolving 200 mg of subtilisin-A in 100 ml of 0.05 *M* sodium hydroxide–borax buffer at pH 9.3. The tissue was then incubated at 60°C in a water-bath. After 40 min, the proteolytic digestion was stopped by cooling to room temperature. From this point the procedure outlined for serum was followed.

Assay B: determination of FFZ, MD-FFZ and DD-FFZ in urine and saliva. To 5 ml of urine or saliva were added 5 ml of diethyl ether in a 10-ml glass tube. The tubes were capped and mixed for 5 min on a rotating shaker at 60 rpm. After centrifugation, 4 ml of the organic supernatant were transferred to a clean tube and dried under a stream of nitrogen gas at ambient temperature. The aqueous phase was left for hydrolysis of conjugates of MD-FFZ and DD-FFZ (see assay C). The organic residue was redissolved in 100 µl of mobile phase I. After agitation on a Vortex mixer, free FFZ was quantified by injection of 40 µl onto the column, which was maintained at 50°C. To determine free MD-FFZ and DD-FFZ, 40 µl of the remainder were injected after changing to mobile phase II and raising the temperature to 55°C. Both column effluents were passed first through a fluorescence detector and then, if possible, through an electrochemical detector.

Assay C: determination of sulphate and glucuronide conjugates of MD-FFZ and DD-FFZ in urine. A 2-ml sample of the aqueous phase resulting from the diethyl ether extraction carried out in assay B was subjected to two enzymic hydrolyses. To each of two tubes (tube 1 and tube 2) was added 1 ml of the aqueous phase mentioned in assay B. After evaporation of residual diethyl ether under a nitrogen stream, 50 µl of ethanol were added. Hydrolysis of the glucuronide conjugates was carried out by adding to tube 1 150 µl of acetic acid–sodium acetate buffer (0.2 *M*, pH 5.0) and 250 µl of freshly prepared aqueous solution of 2500 Fishman U of β-glucuronidase. Following incubation of the mixture for 1.5 h at 37°C in a water-bath, 500 µl of disodium hydrogen phosphate–sodium dihydrogen phosphate buffer (0.1 *M*, pH 7.5) were added, and the tubes were allowed to cool in the air at room temperature. The freed metabolites were extracted by the addition of 5 ml of diethyl ether. After rotation for 5 min at 60 rpm, the tubes were centrifuged and 4 ml of the organic layer were removed for evaporation. The residues were redissolved and treated as described in assay B for determination of free MD-FFZ and DD-FFZ.

The sulphate conjugates of MD-FFZ and DD-FFZ were hydrolysed by the addition to tube 2 of 2 ml of Tris–HCl buffer solution (pH 7.2) (Sigma, St. Louis, MO, U.S.A.) of 1 I.U. of phenolsulphatase. The hydrolysis was carried out similarly to that for the glucuronide conjugates except that the incubation period was extended to 5 h. To stop the reaction the tubes were removed from the water-bath

and, after cooling in air at room temperature, 5 ml of diethyl ether were added. The subsequent procedure was identical with that described for deglucuronidated MD-FFZ and DD-FFZ (assay B).

Radioactivity counting of samples

Plasma and urinary activities were measured without further processing using 4 ml of scintillation cocktail to 0.5 ml of sample. Faeces and whole blood were combusted, although faeces were minced and freeze-dried before combustion. Samples were 50 mg of faeces and 250 μl of whole blood, and to both were added 4 ml of scintillation cocktail.

Quantitation by chemical methods

All calibration solutions of the compounds were ethanolic. Stock solutions of 100 μg/ml were diluted shortly before use to standard solutions containing 10 ng/ml FFZ, MD-FFZ and DD-FFZ (assay A) and to 1 μg/ml MD-FFZ and DD-FFZ (assays B and C). Linear calibration curves (linear regression by least-squares method) were obtained for each analyte from the peak heights. The calibration curves of the three compounds were based on the analysis of 1–5 ml drug-free samples, which were added to evaporated ethanolic standard solutions of 0–200 ng of each compound. For preparation of tissue standards, 200 mg of the respective drug-free tissue were added to 0–200 ng of each compound in ethanol and evaporated.

Samples containing glucuronides and sulphate conjugates of MD-FFZ and DD-FFZ were quantitated after deconjugation by means of standards of unconjugated compounds (0–1000 ng/ml).

Quantitation by radioactivity measurements

Concentrations of ^{14}C radioactivity were determined using fixed, quenched standards. The degree of quenching for each sample was calculated from standard quench curves showing counting efficiency versus external standard ratio. Typical results for the latter were: faeces, 0.17–0.21; whole blood, 0.16–0.22; urine and plasma, 0.23–0.26. All counting efficiencies were in the range 70–75%.

RESULTS AND DISCUSSION

Chromatograms from plasma and urine extracts are shown in Figs. 2 and 3.

Reliability and features of the assays

The lower quantitation limit for the pertinent compounds in routine work was 100–200 pg with fluorescence detection and 400–800 pg with electrochemical detection (ED) at a signal-to-noise ratio of 3. With ED the detection sensitivity of DD-FFZ was about twice as good as that of MD-FFZ. Standard curves in assays A, B and C were all linear over a concentration range from 100 pg to 200 ng per ml of sample. The coefficients of determination of linear calibration graphs taken for both detection methods were, from ten consecutive analytical series, at least

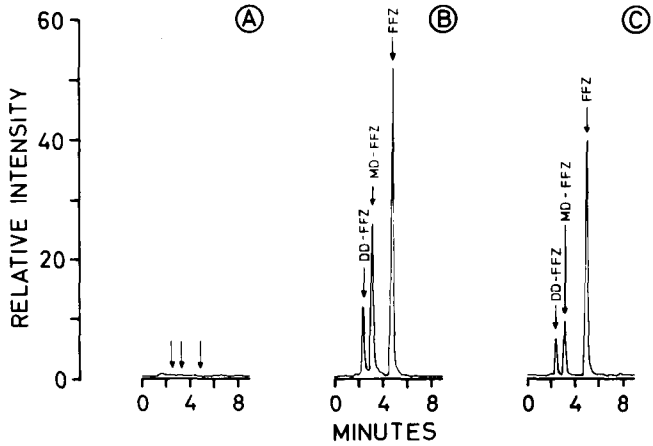

Fig. 2. Plasma HPLC fluorescence profiles (relative intensity) of FFZ and the metabolites MD-FFZ and DD-FFZ (assay A). (A) Blank standard; (B) standard; (C) human plasma collected after an intravenous dose of FFZ (7.9 mg). In the non-blank standard the DD-FFZ peak represents 25 ng/ml, the MD-FFZ peak 52 ng/ml and the FFZ peak 107 ng/ml. The corresponding figures for plasma sample are 15, 21 and 86 ng/ml.

0.992 ± 0.004 [mean $\pm$ standard error of the mean (S.E.M.)]; fluorescence detection was normally better.

The inter-assay precision was evaluated for the parent compound and each metabolite by assaying pooled serum and urine samples. Ten 1-ml samples of each pool, containing ca. 50 ng of FFZ and 5 ng of MD-FFZ and DD-FFZ, were analysed over six weeks. The precision at the concentrations studied amounted to relative standad deviations of less than 6% with both detection methods. The accuracy of the chromatographic assays was determined by adding known amounts of FFZ, MD-FFZ and DD-FFZ to drug-free plasma and urine and analysing the samples as described. The actual concentrations in serum and urine were fixed at ca. 50 ng/ml FFZ and 5 ng/ml MD-FFZ and DD-FFZ. The accuracy for ten assays with fluorescence detection averaged 2.5% (S.E.M. 0.8%) for both serum and urine. Accuracies evaluated with the same samples in the same runs but with ED were somewhat higher: ten serum samples had a mean deviation of -4.1% (S.E.M. 3.8%) and the figures for the urine samples were -2.5% and 2.0%.

The absolute extractive recoveries of FFZ and the two demethylation products were determined by spiking 1-ml blank samples to fixed concentrations of 50 ng (FFZ) and 5 ng (MD-FFZ and DD-FFZ). The samples were then submitted to the extraction procedure described above. The peak heights of these compounds were related to those recorded when the same concentrations of unextracted analytes were subjected to chromatography. Extraction yields appeared better than 95% in those cases where the sample material was without proteins, i.e. urine and saliva. However, with proteinaceous materials such as serum/plasma, whole blood and tissue, the recovery was 70–75%.

With the experimental processes described in assays A, B and C no interferences occurred with the following ten commonly prescribed antiphlogistics: apazone, benzydamine, fenbufen, ibuprofen, indomethacin, naproxen,

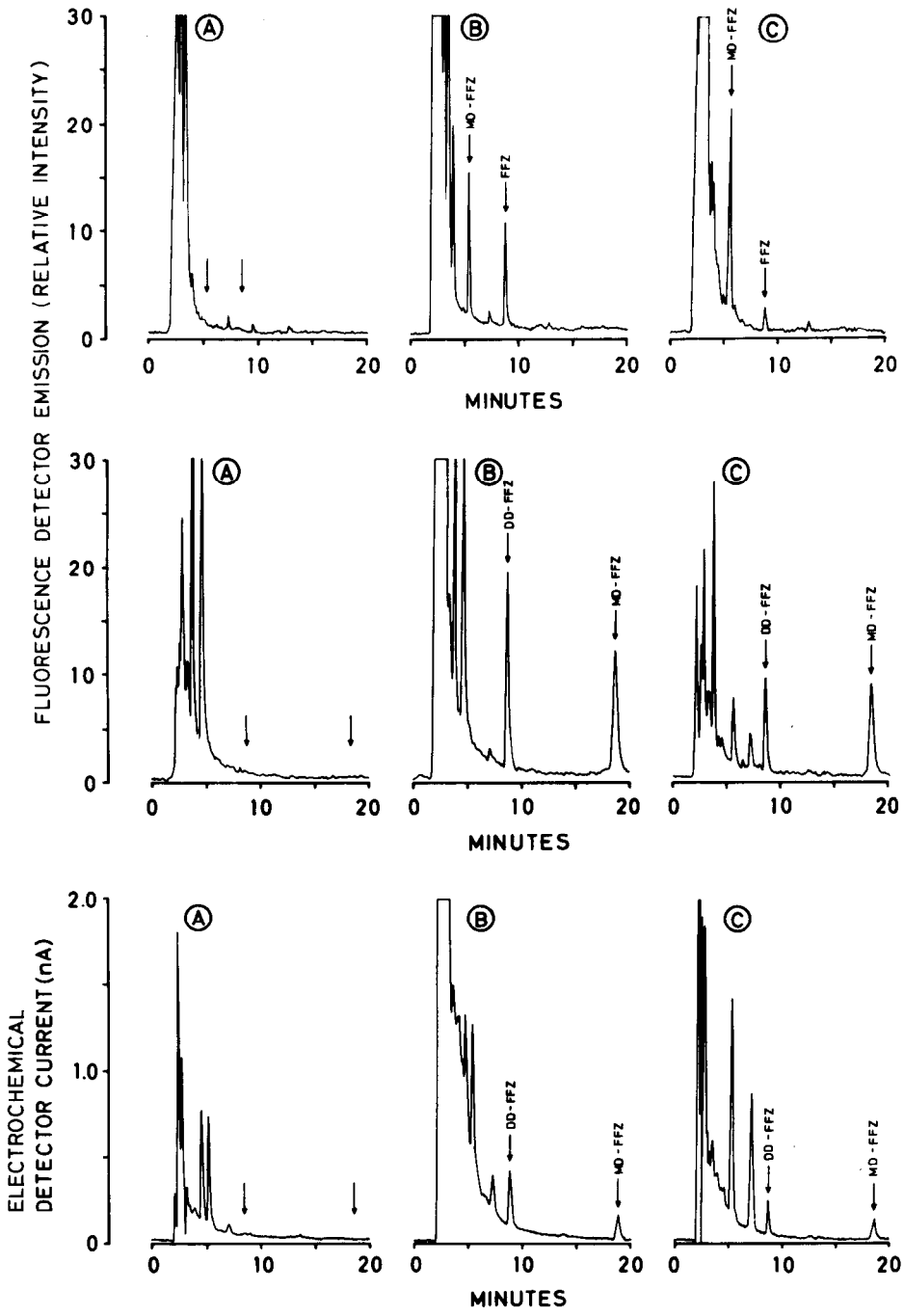

Fig. 3. Urinary HPLC fluorescence and electrochemical profiles of FFZ and the metabolites MD-FFZ and DD-FFZ (assay B). The upper panel shows traces obtained with mobile phase I, and the central and lower panels show those obtained with mobile phase II. Each panel shows the following human urine traces: (A) blank standard; (B) standard; (C) urine collected after an intravenous dose of FFZ (7.9 mg). Each of the peaks in the non-blank standards represents: upper panel, 3.1 ng/ml; central and lower panels, 4.2 ng/ml. Peaks from the urine samples represent: upper panel, 4.3 ng/ml MD-FFZ and 0.6 ng/ml FFZ; central and lower panels, 3.1 ng/ml MD-FFZ and 1.8 ng/ml DD-FFZ.

phenylbutazone, piroxicam, salicylic acid acetate (aspirin) and tolfenamic acid. Also, the tranquillizer diazepam, the antimalarial chloroquine diphosphate, the analgesics acetaminophen and propoxyphene hydrochloride and the uricosuric allopurinol were tested and found not to interfere. The antiphlogistic benoxaprofen was found to interfere totally with FFZ when fluorescence detection was used, but this presents no problem as the sale of this drug has been suspended by the manufacturer since 1982.

All the radioactivity measurements were carried out in triplicate. Intra-assay deviations were less than 5% of the mean. The standard Poisson error was fixed to 2%, except for no-activity cases.

In the search for a simultaneous quantitation of FFZ, MD-FFZ and DD-FFZ, the organic modifiers of choice were acetonitrile (assay A) and acetonitrile–tetrahydrofuran (assays B and C). Isocratic elution was preferred to gradient elution because the former is considered to be more stable and reliable.

The chromatographic identification of MD-FFZ and DD-FFZ was performed by obtaining virtually superimposable excitation and emission spectra of synthesized reference substances and trapped column effluent volumes of the respective two peaks. Moreover, adjacent excitation and emission maxima for all three compounds were observed at the wavelengths $\lambda_{ex}/\lambda_{em} = 326/410$, which were then used as the common detection conditions. Previously, the optimum detection parameters for FFZ were reported as $\lambda_{ex}/\lambda_{em} = 316/410$ [2]. However, the maximum sensitivity for the metabolites was not met using these conditions.

Sulphoconjugation and glucuronidation of MD-FFZ and DD-FFZ were examined by hydrolysis experiments. Storage at $-20\,^{\circ}C$ for six months did not cause any premature hydrolysis of these urinary conjugates.

The quantitation was carried out without authentic standards as conjugated drug metabolites were not available. Fig. 4 shows the sulphoconjugates to be less easily hydrolysed than the glucuronides; the maximum yields of the former are attained after incubation for 5 h, whereas those of the latter are reached after 1.5 h. No increase of the free components was observed if additional enzyme was added or if the incubation time was prolonged.

Tissue samples containing FFZ, MD-FFZ and DD-FFZ were subjected to proteolysis prior to extraction to make quantitation of the analytes more precise. Tissue samples treated with subtilisin-A did not contain compounds which, after diethyl ether extraction, interfered when fluorescence detection was used.

Studies in humans

Blood and plasma profiles from the single-dose study show the retention time of ^{14}C activity to be higher in erythrocytes than in plasma (Fig. 5). Furthermore, it appears that FFZ is metabolized to a greater extent than is accounted for by MD-FFZ and DD-FFZ formation (Fig. 5). Unfortunately, the use of ED does not seem to add extra information to that already obtained by fluorescence detection. The excretion profiles demonstrate that the biliary excretion is preferred to renal excretion (Fig. 6). A more detailed outline has been published [5].

The study of tissues and red blood cells demonstrates that the former accumulate substantial amounts of FFZ during repetitive dosing and that FFZ binds

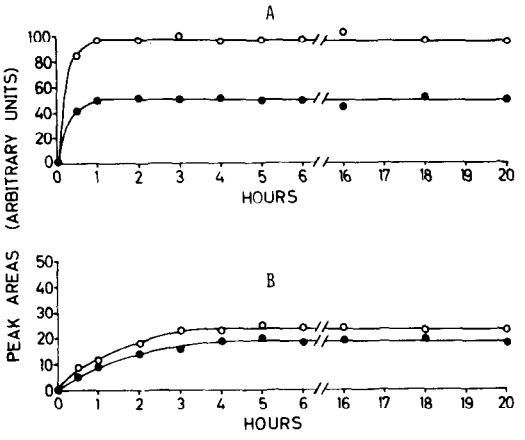

Fig. 4. Effect of incubation time on the enzymic hydrolysis of conjugates of MD-FFZ (●) and DD-FFZ (○) in urine. (A) Glucuronides; (B) sulphoconjugates.

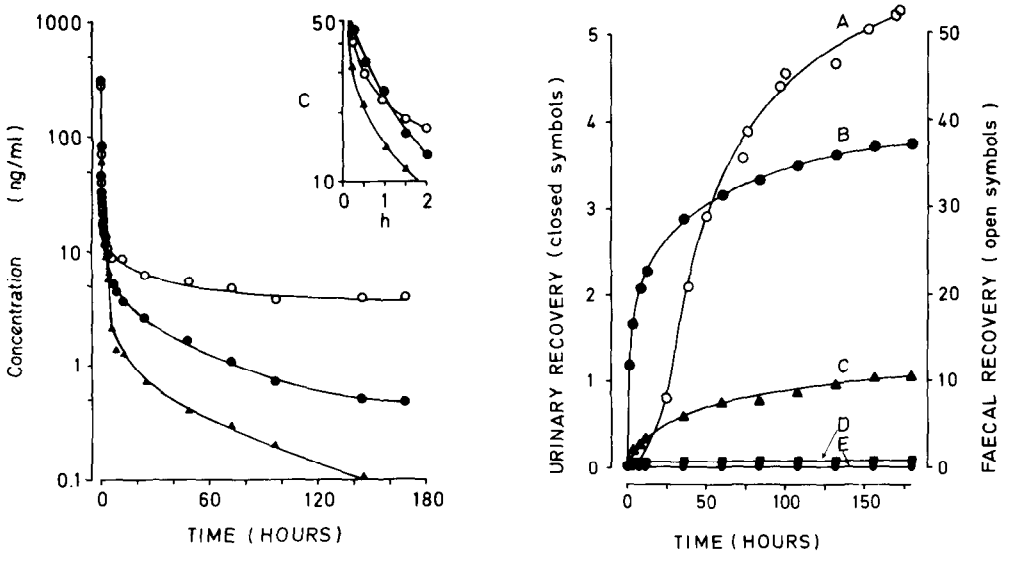

Fig. 5. Concentration-time relationships of ^{14}C activity in whole blood (○) and plasma (●) and of the total of FFZ, MD-FFZ and DD-FFZ in plasma (▲) for a male subject following an intravenous injection of 7.9 mg of [^{14}C]FFZ. The insert details the first 2 h. Activity was converted into concentration units of FFZ.

Fig. 6. Cumulative excretion in faeces and urine of ^{14}C activity (curves A and B) and of compounds identified in urine (curves C, D and E) following an intravenous injection of 7.9 mg of [^{14}C]FFZ. Curve A, ^{14}C activity in faeces; curve B, ^{14}C activity in urine; curve C, glucuronides of MD-FFZ and DD-FFZ in urine; curve D, sulphoconjugates of MD-FFZ and DD-FFZ in urine; curve E, sum of FFZ, DD-FFZ and MD-FFZ as free compounds in urine.

strongly to the blood cells. The tissues contained FFZ in concentrations of 50 μg/g (fat) and 5 μg/g (skin). The FFZ concentration in saliva represents ca. 10% of that in plasma, whereas the red blood cells contain ca. four times that in plasma. The binding to the red blood cells seems to persist throughout the lifetime of the cells. No further metabolites in skin and adipose tissue or blood cells are recognized.

CONCLUSION

Precise, accurate and sensitive methodologies and assays were developed and applied to quantify the extent of FFZ metabolization and to determine the concentrations of the parent compound and two metabolites with conjugates in several biological fluids, tissues and excreta. The ready practicability of the developed procedures was shown to be a useful means for studying the pharmacokinetics and bioavailability of FFZ and its monodemethyl and didemethyl metabolites [5].

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