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REVERSED-PHASE CHROMATOGRAPHY OF FENFLUMIZOLE, A NEW POTENTIAL ANTI-INFLAMMATORY AGENT, AND ITS APPLICATION IN PHARMACOKINETIC STUDIES IN RAT, DOG AND MAN

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SUMMARY

Two high-performance liquid chromatographic methods for quantitation of fenflumizole in biological fluids are described. Fenflumizole is a new agent with demonstrable anti-inflammatory properties in laboratory rodents. The major difference between the two methods lies in the utilization of fluorescence detection of the column eluate in assay II as compared to the less selective ultraviolet—visible detection used in assay I. Assay II appeared suitable for monitoring metabolites in addition to the parent compound. The quantitation limit of plasma fenflumizole was below 10 ng/ml for assay I and approximately 100 pg/ml for assay II. The reproducibility within a plasma concentration range of 50—150 ng/ml was below 7% (standard deviation) in both assays. Fenflumizole was found to be subject to photodecomposition. The impact of this on the analytical performance was evaluated and precautionary measures were assessed. The reliability of the analytical methods is examined and their applicability in in vivo studies conducted in rat, dog, and man.

INTRODUCTION

Fenflumizole, 2-(2,4-difluorophenyl)-4,5-bis(4-methoxyphenyl)imidazole, is a potential anti-inflammatory agent of the non-steroidal type, structurally belonging to the series of substituted triarylimidazoles (Fig. 1) [1,2]. It has displayed analgesic and antipyretic properties similar to those of other non-steroidal anti-inflammatory drugs, but with fewer gastrointestinal side-effects [2].

This paper describes two rapid, accurate, and analytical high-performance liquid chromatographic (HPLC) procedures for the determination of fenflumizole in biological fluids. The validity of the methods is demonstrated by the determination of fenflumizole plasma levels after single administrations to rats, a beagle dog, and a human volunteer.

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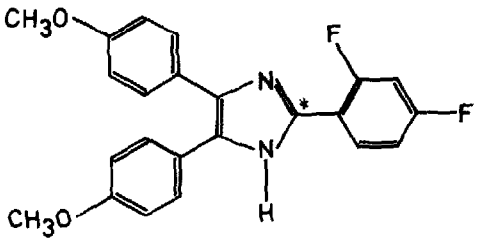

Fig. 1. Chemical structure of fenflumizole, a potential anti-inflammatory agent. Position of radioactive labelling at carbon-2 in the imidazole nucleus is shown by an asterisk.

EXPERIMENTAL

Reagents and materials

Fenflumizole was synthesized and supplied by the Synthesis Department of Dumex Ltd. [^{14}C]Fenflumizole was purchased from the Huntingdon Research Centre and was labelled at carbon-2 in the imidazole nucleus (Fig. 1). Acetonitrile was of HPLC grade, triethylamine of synthetic grade and all other chemicals were of analytical grade.

Instrumentation and analytical conditions

The liquid chromatograph consisted of a Waters Assoc. Model 6000 A constant flow pump, a Waters U6K injector, either a Waters Model 440 UV detector or a Kontron spectrofluorometer SFM-23 LC and a Hewlett-Packard Model 3380 integrator. The fluorometer was equipped with a slit width of 4 nm and a 150-W xenon lamp with a wavelength range of 200—800 nm. A Knauer stainless steel chromatographic column, 250 mm × 4 mm I.D. (Dr. Herbert Knauer, Taunus, F.R.G.), was packed with 5-μm Spherisorb ODS (Phase Separations, Queensferry, U.K.) using a slurry technique. A Beckman liquid scintillation counter LS 150 was used for determining extraction yields.

The mobile phase for the analysis of fenflumizole was a mixture of a 50 m*M* aqueous solution of triethylamine and acetonitrile (23:77). Chromatography was done at room temperature and the flow-rate was 1 ml/min. Under these conditions the capacity ratio of fenflumizole was 6.7 and the number of theoretical plates was calculated to be approximately 6000.

Analytical procedure for assay I

A 5-ml volume of the extraction medium was added to 1 ml of an aqueous sample (e.g. plasma, urine) and the mixture was rotated for 5 min. The extraction medium was a 1:1 mixture of *n*-hexane—dichloromethane. 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 residue was redissolved in 100 μl of the mobile phase, and an aliquot of 40 μl was injected onto the HPLC column. The absorbance at 280 nm (filter) of the eluate was monitored with a UV detector (Fig. 2).

Calibration standards within a range of 0—200 ng/ml were obtained by adding known amounts of fenflumizole to drug-free plasma. Ethanol was used

as solvent for the stock solution of fenflumizole. It was stored in the dark at 4°C when not in use because light-induced decomposition was an important factor (see below).

Analytical procedure for assay II

Owing to the expected presence of more polar metabolites in plasma and urine in relation to the parent compound, the extraction mixture was replaced by diethyl ether and UV detection was changed to fluorescence detection (Fig. 3). In all other respects the assays were similar. By recording the spectra, the excitation and emission maxima were found to be 316 nm and 410 nm. This was achieved by filling the flow cell with a standard solution of fenflumizole and scanning in situ.

Quantitation

A calibration curve was obtained by analysing 1-ml drug-free samples to which were added 0—200 ng of fenflumizole. A linear relationship between the detector response and the fenflumizole concentration was observed. From a computerized plot of the standard peak areas versus concentrations the actual concentrations of the samples were found. Regression coefficients and ordinate intercepts were obtainable by means of linear regression (method of least squares).

RESULTS AND DISCUSSION

Conditions for chromatography and extraction

Initially, an unbuffered mobile phase (methanol—water, 90:10) was used. However, continued use of this solvent failed to give reproducible retentions. A buffered solvent (acetonitrile—phosphate buffer, pH 3.0, 80:20) was therefore prepared. This helped the retention performance, but was inconvenient for routine use because it necessitated frequent regeneration of the chromatographic system, independent of the strength of the buffer. A 77:23 mixture of acetonitrile and 0.05 *M* triethylammonium acetate (pH 4.0) was finally chosen as a standard eluent. Separation with this solvent proved to be excellent and has subsequently been used in all analyses of biological samples.

The extraction procedure was closely linked to the choice of detection principle. When using absorbance at 280 nm (see Fig. 2) as detection method, fenflumizole was chromatographically separated from endogenous biological constituents after extraction with *n*-hexane—dichloromethane (1:1). However, it was necessary to consider the possibility that metabolites might occur in the effluents. With this in mind the technique was further improved by the use of a fluorimetric detector and a less restricting extraction with diethyl ether.

Figs. 2 and 3 show typical elution tracings from the alternative analytical methods. The UV detector leaves a poorly resolved crowding at the beginning of the chromatogram which does not fluoresce at the conditions specified.

The extraction recoveries were estimated by employing ^{14}C-labelled fenflumizole. For plasma the fenflumizole recoveries were found to be 84% in assay I and only 72% in assay II. The extractions from urine were fully

effective in both assays. These results were confirmed by liquid chromatography by ratioing the slopes of the standard curves of direct and processed standards. A time-dependent extraction yield was found for plasma when the tubes were rotated for less than 5 min.

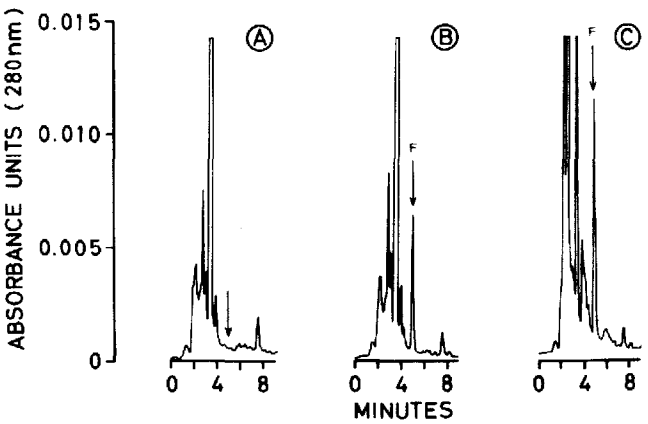

Fig. 2. HPLC UV traces of fenflumizole (F) in (A) blank plasma, (B) a plasma standard of 50 ng/ml, and (C) a plasma sample of approximately 100 ng/ml. Chart speed was 0.5 cm/min and injection volume 40 μl.

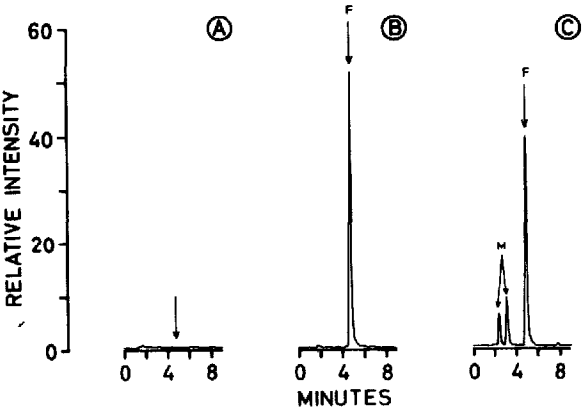

Fig. 3. HPLC fluorescence traces of fenflumizole (F) in (A) blank plasma, (B) a plasma standard of 50 ng/ml, and (C) a plasma sample of approximately 40 ng/ml; two metabolites (M) are visible. Chart speed was 0.5 cm/min and injection volume 40 μl.

Reliability and characteristics of the analytical methods

The analytical variation was evaluated by means of the precision and accuracy. The variation of the slope and the ordinate intercept of the standard curve and its deviation from linearity provided an estimate of the accuracy, and the precision was determined by including control samples in each analytical series (Table I). These were prepared by adding plasma to a known amount of fenflumizole. After mixing, volumes of 1 ml were stored at −20°C until analysis.

TABLE I

ESTIMATION OF ANALYTICAL VARIATION

Comparison of two analytical methods for determining fenflumizole concentrations (ng/ml) in plasma as indicated by ten consecutive analytical series over six weeks. Slope and ordinate intercepts were calculated according to the equation $Y = a + bX$ (linear regression). Analytical variation was estimated with the aid of quality control samples (pool I and pool II), which were assayed along with normal plasma samples. Figures given are means (± S.D.).

Parameter		UV detection (assay I)	Fluorimetric detection (assay II)
Ordinate intercept	*a*	2.74 (± 0.92)	−3.68 (± 1.26)
Slope	*b*	0.315 (± 0.05)	9.65 (± 0.79)
Correlation coefficient	*r*	0.997 (± 0.07)	0.999 (± 0.04)
Inter-assay analytical variation	Pool I	–	47.6 (± 3.3)
	Pool II	144.7 (± 9.7)	145.3 (± 8.1)

The comparative data in Table I show acceptable parameters for both methods. The fluorimetric detection method with its inherent high sensitivity demonstrates a correlation coefficient and a precision (defined as the reciprocal of the variance, $1/S.D.^2$) that are superior to those obtained with the UV detector. The reproducibility measured as the relative standard deviation is estimated to be below 7% in all assays.

In the assays described the quantitation limit was found to be below 10 ng/ml for assay I and approximately 100 pg/ml for assay II. The latter was limited primarily by the onset of a destructive photodecomposition within the flow cell caused by too intense excitation of the fluorophore.

Two other aspects were considered when judging the magnitude of laboratory error in the analytical method. First, storage of plasma pool samples at −20°C was found not to cause measurable changes of the fenflumizole content over at least eighteen months. Second, repetitive freeze—thaw cycles of plasma and urine up to three times left the sample unchanged with regard to nanogram-range concentrations of fenflumizole.

With regard to the plasma and serum analyses, fluorimetric detection in assay II did not require haemolysis-free samples, unlike the 280 nm absorbance measurement in assay I.

Photodecomposition

As with several other polyarylimidazoles, fenflumizole exhibits instability when exposed to certain wavelengths of light [1].

From the outset, daylight caused deterioration in stock solutions up to the mg/ml concentration range, and sunlight rapidly made this worse. As a rule the specimens were handled in subdued light or protected from light by the use of brownish-coloured or tinfoiled glass tubes. By applying this rule, the longevity of stock ethanol solutions of fenflumizole was found to be at least five days. Experiments on the photolytic properties of fenflumizole are in progress and will be reported.

Probe studies of plasma kinetics in the rat, beagle dog and normal man

The validity of the assays was assessed by determining plasma concentrations of fenflumizole in in vivo probe studies. Male Sprague-Dawley rats and a beagle dog were given single intravenous doses of 3 and 10 mg/kg body weight (Fig. 4), and a human male volunteer ingested fenflumizole in single doses of 0.1, 2 and 5 mg/kg body weight (Fig. 5). Although the determination of plasma fenflumizole was feasible with both assays, assay II appeared superior because the metabolite peaks were also of interest. A preliminary finding common to these studies was the rapid occurrence (within 1 h of administration) of two metabolite peaks both of higher polarity than the parent compound (Fig. 3). The terminal elimination half-lives (above 24 h) were estimated to be about 13 h for the rats, 37 h for the beagle dog and 55 h for the male human volunteer.

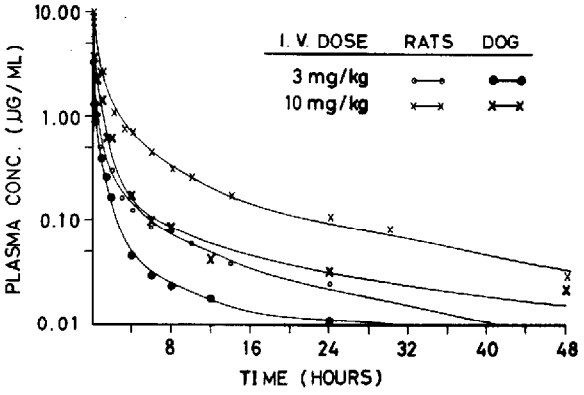

Fig. 4. Plasma concentration—time curves of fenflumizole for Sprague-Dawley rats and one beagle dog after the doses indicated. Each point on the curves for the rats is the mean of three animals, which were killed at this time.

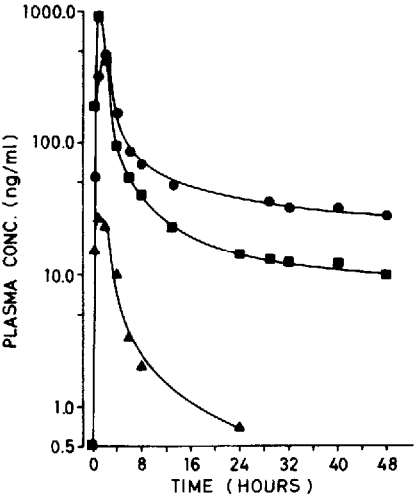

Fig. 5. Plasma concentration—time curves of fenflumizole for a male volunteer after ingestion of (▲) 0.1 mg/kg, (■) 2 mg/kg, and (●) 5 mg/kg.

CONCLUSIONS

Two assays have been developed to quantitate fenflumizole and metabolites in biological fluids. Fenflumizole is a potential non-steroidal anti-inflammatory agent, recently developed. The assays combine adequate sensitivity with appropriate precision and accuracy. The sample processing is limited to one extraction step. Precautions must be taken against light owing to photolability.

The validity of the analytical assays has been demonstrated in probe pharmacokinetic studies in rat, beagle dog and normal man. In forthcoming papers these applications will be discussed in detail.

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