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DETERMINATION OF ISOFEZOLAC IN BIOLOGICAL FLUIDS BY REVERSED-PHASE LIQUID COLUMN CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive, and specific reversed-phase high-performance liquid chromatography assay was developed for the determination of 1,3,4-triphenylpyrazole-5-acetic acid (isofezolac) in plasma and urine. The assay involves extraction into diethyl ether from plasma buffered at pH 4.4. The organic phase is evaporated and the residue, dissolved in the mobile phase [acetonitrile—water—0.2 *M* phosphate buffer (pH 3) (65 : 15 : 20)] is chromatographed at a flow-rate of 1.5 ml/min. The drug is detected by its UV absorption (detection limit 100 ng/ml) or its very intense fluorescence (detection limit 10 ng/ml). Absolute analytical recoveries for isofezolac varied from 92.9 to 100.4%. The accuracy is ca. 1%. Each separation requires about 6 min. This method was applied successfully to the determination of isofezolac in humans for pharmacokinetic studies.

INTRODUCTION

Isofezolac, 1,3,4-triphenylpyrazole-5-acetic acid (Fig. 1), is a new drug selected on the basis of its anti-inflammatory, analgesic and antipyretic properties. This new non-steroid anti-inflammatory drug appeared to be as active as indomethacin in the various tests performed. Its activity is always greater than that of phenylbutazone and its ulcerogenic activity in the rat is 2.5 times weaker than that of indomethacin [1].

In order to perform pharmacokinetic studies, we have developed a sensitive technique for the determination of isofezolac in biological fluids. The method consists of a reversed-phase high-performance liquid chromatographic (HPLC) separation. The drug is detected by its UV absorption or its very intense fluorescence.

1-Phenyl-3,4-di-*p*-chlorophenylpyrazole-5-acetic acid (Fig. 1) was used as internal standard.

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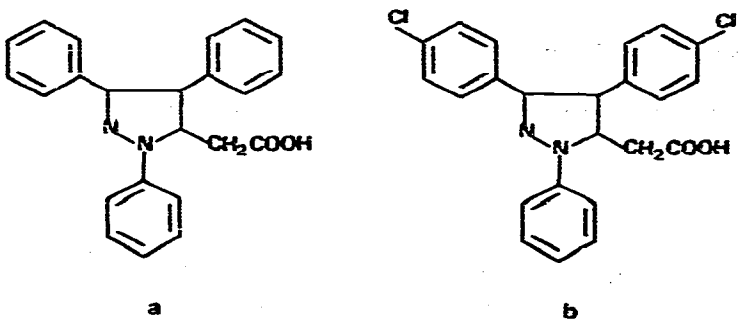

a b

Fig. 1. Chemical structures of isofezolac (a) and internal standard (b).

MATERIALS AND METHODS

Chemicals and reagents

Isofezolac and 1-phenyl-3,4-di-*p*-chlorophenylpyrazole-5-acetic acid (used as internal standard) were kindly supplied by Pharmuka (Gennevilliers, France).

All reagents were of analytical grade and used without further purification: diethyl ether (E. Merck, Darmstadt, G.F.R.), disodium citrate, 1 *N* sodium hydroxide, 0.1 *N* hydrochloric acid, orthophosphoric acid, potassium dihydrogen phosphate, acetonitrile and methanol (Prolabo, Paris, France), enzyme solution containing 100,000 units of β-glucuronidase per ml and 1,000,000 units of arylsulfatase per ml (I.B.F., Gennevilliers, France).

Buffer solutions

Citric acid—0.1 *M* disodium citrate buffer (pH 4.4) and 0.2 *M* phosphate buffer (pH 3) were stored at +4°C. For studies in which the pH of the eluent was varied, the pH was adjusted with a 10% solution of orthophosphoric acid or a 10% solution of sodium hydroxide in water. The phosphate concentration was maintained constant (0.02 moles/l) over the pH range investigated.

Stock solutions

Isofezolac stock solution (20 mg per 100 ml) and internal standard stock solution (20 mg per 100 ml) were prepared in methanol.

Apparatus

HPLC analyses were performed on a Chromatem 38 liquid chromatograph (Touzart et Matignon, Paris, France) operated at ambient temperature. A Jobin-Yvon Model J.Y. 3 D spectrofluorescence detector (Jobin-Yvon, Longjumeau, Paris, France) was operated at 273 nm for excitation and 335 nm for emission. The output of the detector was displayed on a recorder Model PE 1286 (Sefram, Paris, France) having a 1-V full-scale range. For UV detection, a Model 770 spectrophotometric detector (Spectra Physics, Santa Clara, CA, U.S.A.) was operated at 265 nm.

The column was 15 cm × 4.6 mm I.D. stainless steel, packed with LiChrosorb RP 8 (5 μm particle size; E. Merck) by a balanced density slurry technique [2]. A 20-μl loop injection valve Model 70-10 (Rheodyne, Berkeley, CA, U.S.A.) was used to introduce samples into the chromatographic system. The test of

the reversed-phase column was carried out with benzene, naphthalene and $NaNO_3$ as unretained compound. Under these conditions, the column efficiency was 3600 theoretical plates for benzene and 3150 for naphthalene.

Chromatographic conditions

The mobile phase, acetonitrile—water—0.2 *M* phosphate buffer (pH 3) (65:15:20), was filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) under negative pressure, and degassed by ultrasonic vibration. The column was preconditioned with mobile phase until a stable recorder output was obtained. The flow-rate was then adjusted to 1.5 ml/min, generating a pressure of about 105 bar.

Extraction procedures

Plasma. To 1 ml of plasma transferred into a 45-ml screw-capped centrifuge tube, were added 1 ml of citrate buffer, 100 μl of internal standard solution (4 mg per 100 ml) and 15 ml of diethyl ether. After agitation and centrifugation (5 min at 3000 *g*), the organic phase was carefully pipetted out into a 15-ml conical centrifuge tube and evaporated to dryness under a stream of nitrogen at 40°C. The sample residue was dissolved in various volumes (0.5—3 ml) of mobile phase depending on the expected drug concentration; 20-μl aliquots were injected into the chromatograph.

Urine. In order to determine the total amount of isofezolac excreted in urine, the conjugated drug must be first hydrolysed. The best conditions for complete hydrolysis consisted of incubating 0.1 ml of urine, 1 ml of citrate buffer (pH 4.4), 100 μl of internal standard dilution (4 mg per 100 ml) and 0.1 ml of the enzyme solution at 37°C for 16 h. The sample was then treated as before. The residue was dissolved with 1—2 ml of mobile phase and 20-μl aliquots were injected into the chromatograph.

Calculations

The calculations of plasma and urine concentrations were always made by the internal standard method using peak area ratios.

RESULTS AND DISCUSSION

Chromatographic conditions

Reversed-phase liquid chromatography using bonded hydrocarbon stationary phases has proved to be an extremely versatile and easily used analytical technique for the analysis of ionogenic compounds [3—5]. Since isofezolac and the internal standard are compounds with a carboxylic function they can be determined by such a method. In order to achieve the best chromatographic conditions, the composition and pH of the mobile phase were varied.

Mobile phases studied included various ratios (v/v) of acetonitrile—water—phosphate buffer; namely 50:30:20, 55:25:20, 60:20:20, 65:15:20 and 70:10:20. The capacity factor (k') and resolution (Rs) of the two compounds were affected at higher acetonitrile concentrations (Fig. 2). When the acetonitrile concentration exceeded 65%, a loss of resolution between isofezolac

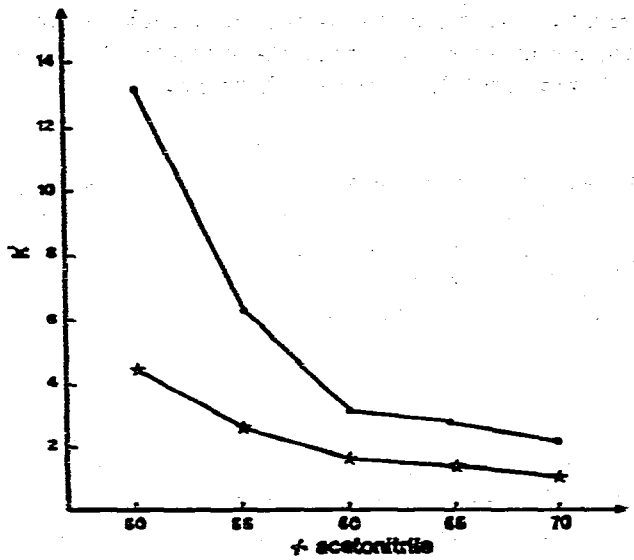

Fig. 2. Effect of the solvent acetonitrile content at pH 4 on capacity factors (k') of isofezolac (*) and internal standard (•).

and internal standard was observed. Moreover, the elution peak of isofezolac was near the peak of rapidly eluting plasma constituents.

At a lower acetonitrile concentration (50%) the resolution was very good (Rs = 11.2) but the elution time was more than 16 min. In order to optimize both resolution and elution time the amount of acetonitrile in the mobile phase was fixed at 65%.

The pH of the solvent system is the most critical factor influencing the reso-

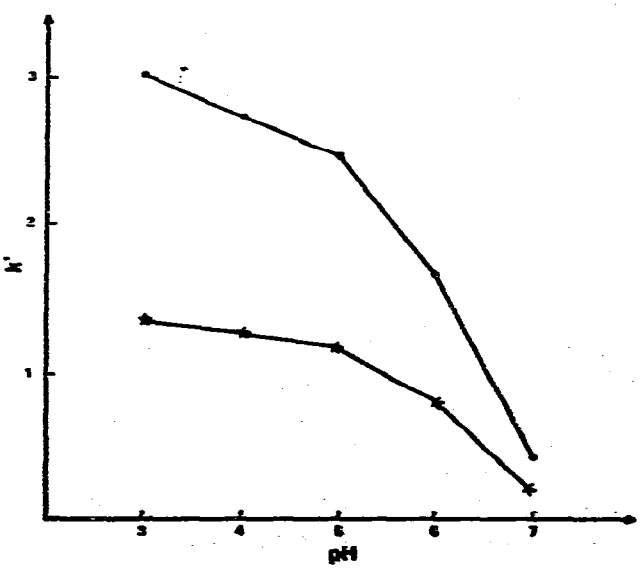

Fig. 3. Effect of variation of the solvent pH on capacity factors (k') of isofezolac (*) and internal standard (•). Mobile phase: acetonitrile—water—0.2 M phosphate buffer (65 : 15 : 20).

lution, as shown in Fig. 3. At pH > 5, the capacity factor decreased rapidly. Under these pH conditions, the two compounds are ionized and have a maximum affinity for the aqueous phase; thus they cannot be resolved. Excellent resolutions are achieved at pH < 5. The pH chosen for the separation of the compounds was pH 4. Resolution and column efficiency were very good (Rs = 7, N = 3090 theoretical plates for isofezolac). The total chromatographic time was less than 6 min. Moreover, at pH 4, the life of the column is very long.

Under the chromatographic conditions described, the retention times of isofezolac and internal standard were 2.7 and 4.5 min, respectively. Fig. 4B shows the chromatogram of an extract corresponding to 1 ml of human plasma containing 0.73 μg/ml isofezolac and 1 μg/ml internal standard. Fig. 4A shows the chromatogram of an extract of the same volume of a blank plasma from the same subject.

Fig. 5 shows the chromatogram of an extract corresponding to 0.1 ml of urine.

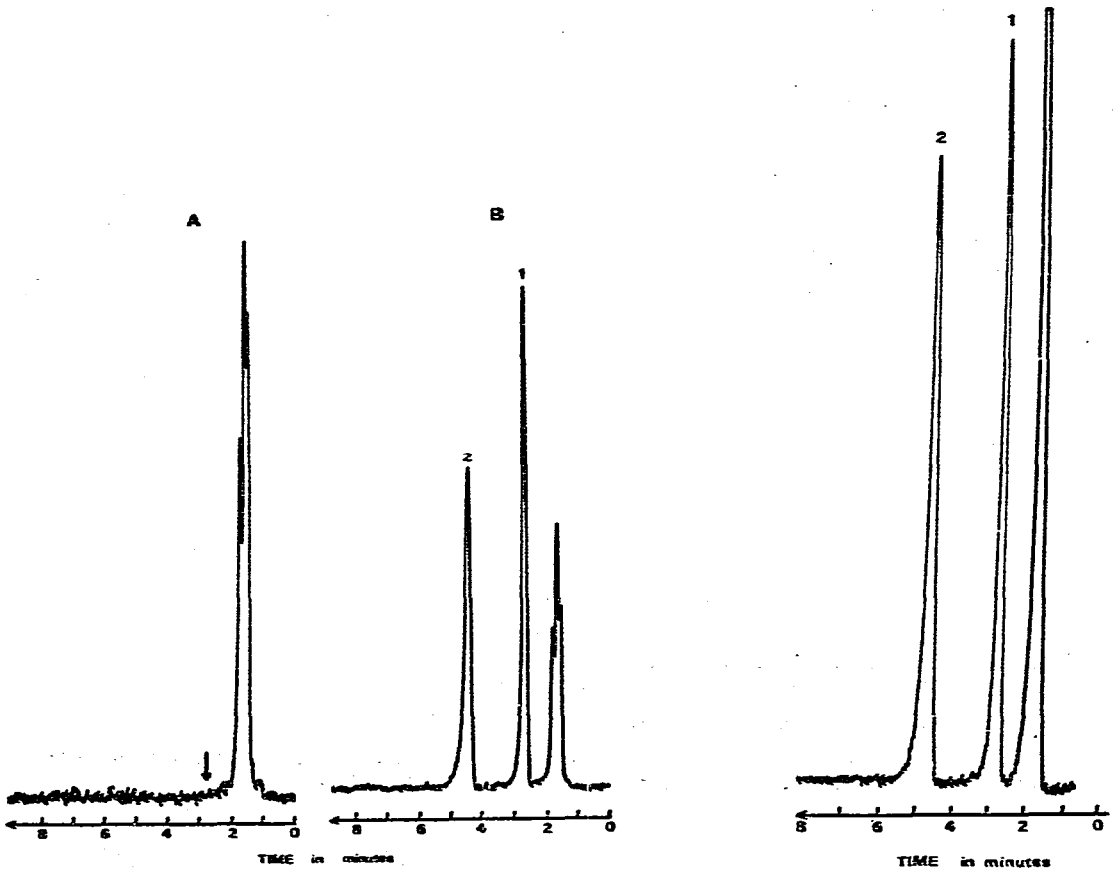

Fig. 4. Chromatograms of isofezolac in plasma. A, blank plasma sample (arrow indicates isofezolac elution position); B, plasma sample 2 h after a single oral dose of 50 mg. Isofezolac (peak 1) concentration = 0.73 μg/ml; internal standard (peak 2) concentration = 1 μg/ml.

Fig. 5. Chromatograms of isofezolac in urine (0—12 h collection). Isofezolac (peak 1) concentration = 16.53 μg/ml; internal standard (peak 2) concentration = 2 μg/ml.

Detection

Most of the pyrazole class of compounds exhibit very intense UV absorption and fluorescence. So monitoring of isofezolac plasma levels is possible with less than 1 ml of biological fluid, using either UV or fluorescence detectors.

Under the conditions of the assay (pH and solvents mixture) isofezolac and the internal standard give an absorption maximum at 265 nm (Fig. 6). Molar absorptivities are 18,600 for isofezolac and 21,800 for the internal standard. However, for pharmacokinetic studies, where a greater sensitivity is required, fluorescence detection must be preferred. Excitation and emission spectra are shown in Fig. 7. Isofezolac and the internal standard show the same excitation maximum at 273 nm. The emission maxima are 335 nm for isofezolac and 345 nm for the internal standard. In order to obtain greater sensitivity, the wavelengths chosen for the assay were 273 nm (excitation) and 335 nm (emission).

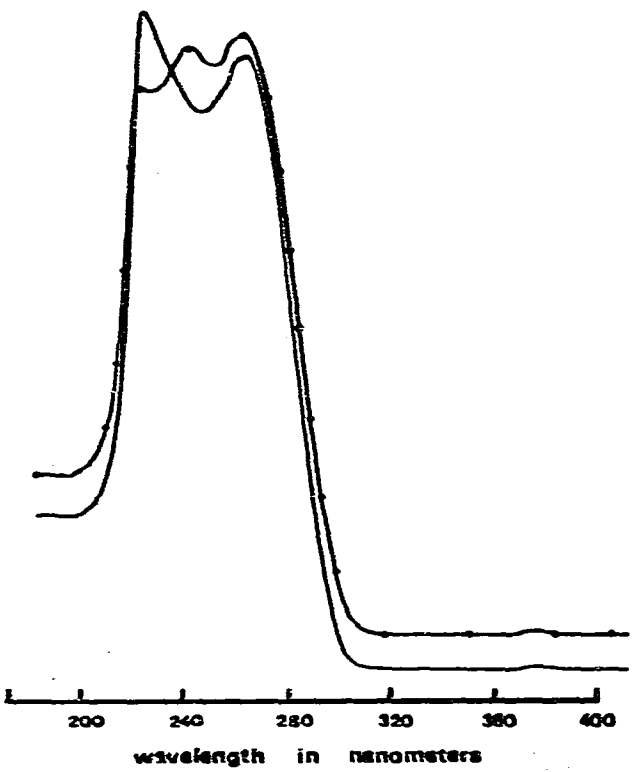

Fig. 6. UV absorption spectra of 2 μg/ml isofezolac (—) and internal standard (•—•—•) in acetonitrile—water—0.2 *M* phosphate buffer (pH 4) (65 : 15 : 20).

Repeatability and accuracy

Repeatability and accuracy of the assay were studied by measuring the concentrations of isofezolac in plasma samples spiked with isofezolac at concentrations of 0.2, 1 and 2 μg/ml. The results listed in Table I show that the repeatability and accuracy at lower concentrations are still very good and within the generally accepted limits for drug assays.

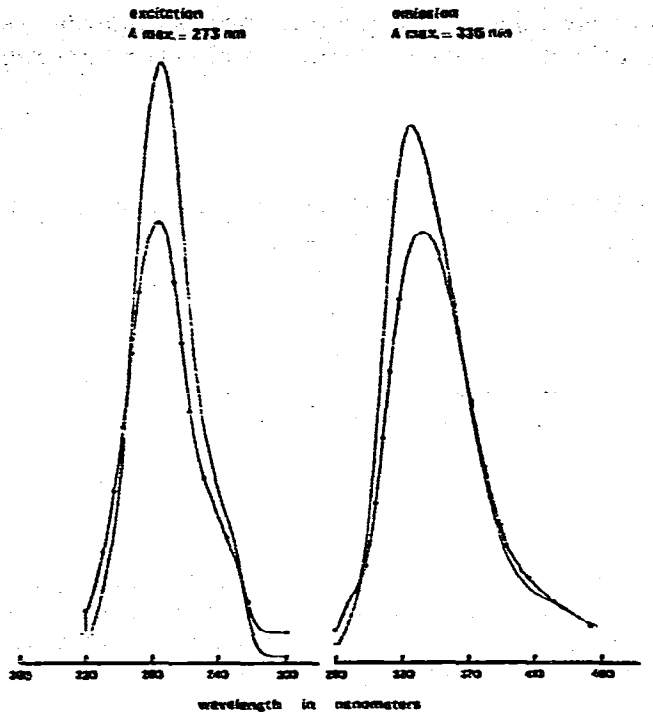

Fig. 7. Excitation and emission spectra of 200 μg/ml isofezolac (—) and internal standard (•—•—•) in acetonitrile—water—0.2 *M* phosphate buffer (pH 4) (65 : 15 : 20).

TABLE I

REPEATABILITY AND ACCURACY

Isofezolac added (μg/ml)	*n*	Isofezolac measured (μg/ml)*	Mean error	Relative error (%)	C.V.** (%)
0.5	5	0.203 (0.005)	0.002	1.5	2.34
1	5	0.994 (0.007)	0.003	0.6	0.66
2	5	1.986 (0.021)	0.009	0.7	1.04

*Standard deviation in parentheses.
**Coefficient of variation.

Recovery

The absolute analytical recovery from plasma of isofezolac and the internal standard was measured in the following way. The two compounds were added to plasma to achieve concentrations ranging between 0.2 and 2 μg/ml. The plasma samples were then assayed with the method described. Absolute analytical recovery was calculated by comparing the peak areas from plasma extracts

with the peak areas obtained by direct injection of the pure drug standards. As shown in Table II, the extraction efficiencies are quite comparable for the drug and internal standard.

TABLE II

ABSOLUTE ANALYTICAL RECOVERY OF ISOFEZOLAC AND INTERNAL STANDARD

Compound	Concentration (μg/ml)	Recovery (%)
Isofezolac	0.2	92.9
	1	92.9
	2	100.4
Internal standard	0.2	93.9
	1	94.1
	2	99.6

Linearity

The calibration curve was obtained by plotting the ratios of isofezolac peak area to that of internal standard, versus their respective concentrations in five different pools of plasma containing concentrations ranging from 0.2 to 2 μg/ml. The relation is linear over this range ($r = 0.9999$, slope = 0.7131, and intercept = 0.0063).

Detection limit

The described extraction procedure yields a relatively clean extract (Fig. 4A). Thus, isofezolac may be measured at the highest detector sensitivity. Under the conditions of this assay, the detection limit is about 10 ng/ml with fluorescence detector.

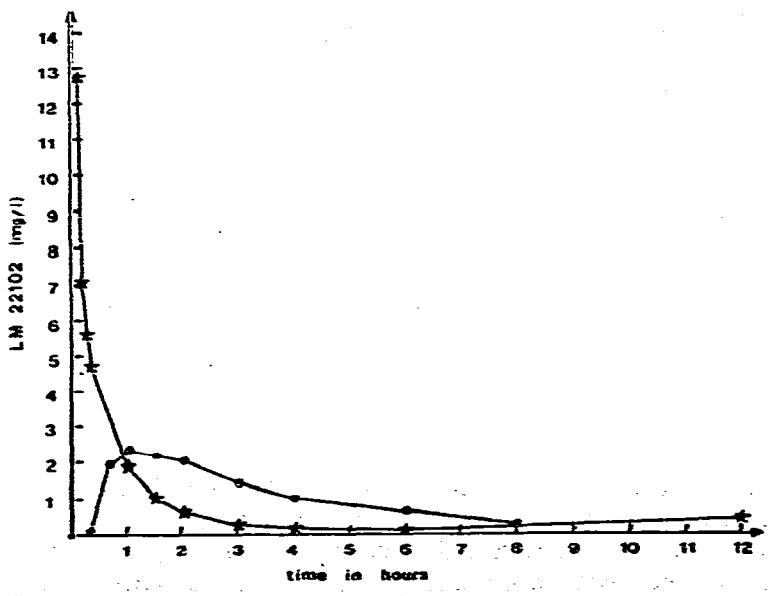

Fig. 8. Plasma decay of isofezolac after the administration of a single 50-mg dose of isofezolac. *, Intravenous administration; ●, oral administration.

Clinical studies

A complete profile of the plasma isofezolac levels of one subject from a clinical study is shown in Fig. 8. This subject received one single intravenous dose of 50 mg of isofezolac and a week later a single oral dose of 50 mg. The half-life of isofezolac is about 1 h and the analytical method is able to measure plasma concentrations for more than 24 h, which is sufficient to achieve correct pharmacokinetic studies.

The proposed method is simple, sensitive, and rapid for the determination of isofezolac in biological fluids during pharmacokinetic studies.

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