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Note

Determination of a new anti-inflammatory agent, 1-isobutyl-3,4-diphenylpyrazole-5-acetic acid, by high-performance liquid chromatography

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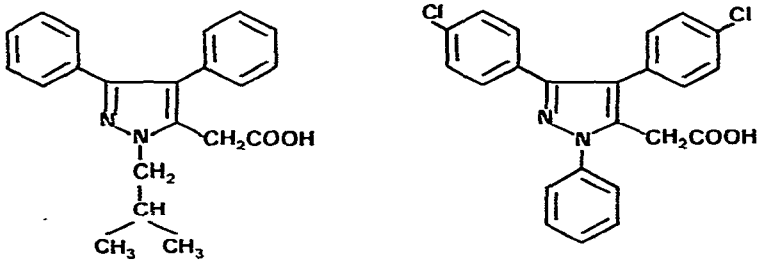
1-Isobutyl-3,4-diphenylpyrazole-5-acetic acid (LM 22070) (Fig. 1a) is a new drug which possesses promising analgesic, antipyretic, and anti-inflammatory properties. Its activity is either the same as (antipyretic action) or inferior to (analgesic and anti-inflammatory activity) that of indomethacin, but it is always markedly superior to that of phenylbutazone. Its ulcerogenic activity in the rat is three times weaker than that of indomethacin [1].

A rapid and sensitive method for the quantitation of LM 22070 in biological fluids was required to determine the pharmacokinetic parameters of this drug in humans. Therefore, a reversed-phase liquid chromatographic (RPLC) method based on ion-suppression chromatography and fluorescence detection was



a b

Fig. 1. (a) Chemical structure of LM 22070 (1-isobutyl-3,4-diphenylpyrazole-5-acetic acid). (b) Chemical structure of internal standard: 1-phenyl-3,4-di-*p*-chlorophenylpyrazole-5-acetic acid.

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

MATERIALS AND METHODS

Chemicals and reagents

LM 22070 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.

Stock solutions

LM 22070 stock solution (20 mg per 100 ml) and internal standard stock solution (1 mg per 100 ml) were prepared in methanol. A ten-fold dilution of these two solutions was performed before use.

Chromatography

High-performance liquid chromatographic analyses were performed on a Chromatem 38 liquid chromatograph equipped with an Altex pump and operated at ambient temperature (Touzart et Matignon, Paris, France). A Jobin-Yvon Model J.Y. 3 D spectrofluorescence detector (Jobin-Yvon, Longjumeau, Paris, France) was operated at 274 nm for excitation and 350 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.

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 efficiency of newly made columns was tested with a standard mixture (benzene—naphthalene—anthracene) using sodium nitrate as an unretained marker. The solvent system was methanol—water (80:20). Under these conditions, the column efficiency was 3700 theoretical plates for benzene, 4200 for naphthalene and 4700 for anthracene.

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 1.6 ml/min, generating a pressure of about 120 bars.

Extraction precedures

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 (0.1 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 LM 22070 excreted in urine, the conjugate 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 (0.1 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.

Calibration, repeatability and accuracy

Calibration curves were obtained by plotting peak area ratios (LM 22070/ internal standard) against drug levels from different pools of plasma containing concentrations ranging from 0.2 to 8 μg/ml.

Repeatability and accuracy of the assay were studied by measuring the LM 22070 concentration from various plasma samples spiked with 0.5, 2 and 6 μg/ml of the drug. The absolute analytical recovery from plasma was calculated by comparing the peak areas from plasma extracts containing 0.5, 2 and 6 μg/ml of LM 22070 to peak areas obtained by direct injections of pure standard solutions of the drug.

Calculations

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

Clinical study

A pharmacokinetic study of 1-isobutyl-3,4-diphenylpyrazole-5-acetic acid was performed on ten healthy male subjects. Each subject received one single intravenous dose containing 50 mg of the drug. Blood samples (5 ml) were collected before drug administration and 5, 15, 20, 60, 90, 120, 180, and 240 min after dosing. The plasma immediately harvested and frozen until analysis.

RESULTS AND DISCUSSION

The present RPLC method for the quantitative analysis of 1-isobutyl-3,4-diphenylpyrazole-5-acetic acid in plasma and urine involves a simple extraction of an acidified biological sample with diethyl ether, followed by ion suppression chromatography using a fluorescence detector.

Because of its lower polarity, ether is less likely to extract interfering compounds than many chlorinated solvents. So ether extracts of blank plasma were free of endogenous absorbing peaks able to interfere with the assay (Fig. 2A).

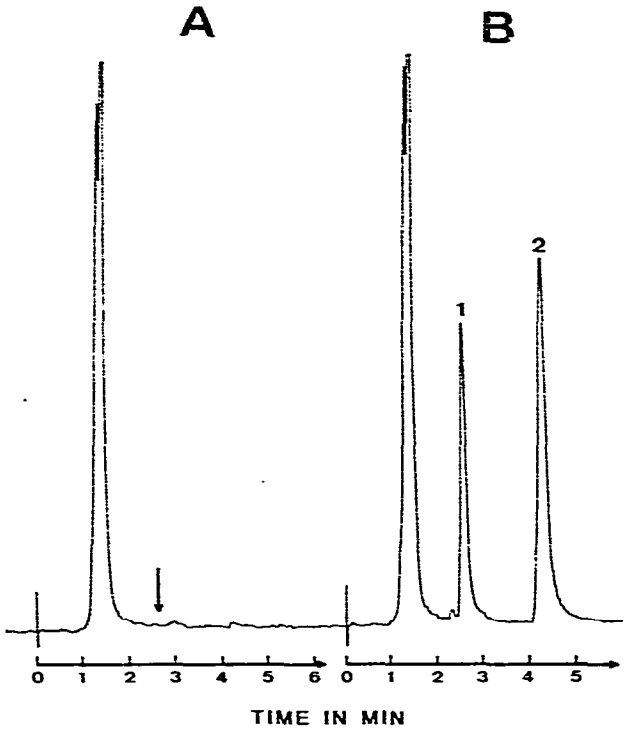

Fig. 2. (A) Chromatogram of a blank plasma. The arrow indicates the retention time of LM 22070. (B) Chromatogram of a plasma extract containing LM 22070 (1) 6 μg/ml and internal standard (2) 0.4 μg/ml.

The drug is a weak acid (pK_a = 5.5), so the buffered (pH 4.4) mobile phase suppresses the solute ionization and reduces peak tailing. In such conditions, good resolution and efficiency were obtained (R_s = 5.6, N = 2990 theorical plates for LM 22070).

Optical fluorescence response under the described chromatographic conditions was achieved by use of an excitation wavelength at 274 nm and an emission wavelength at 350 nm (Fig. 3). The excitation maximum of the internal standard was 273 nm and the emission maximum was 345 nm, under the same conditions.

Fig. 2B shows the chromatogram of an extract from 1 ml of human plasma containing LM 22070 (6 μg/ml) and internal standard (0.4 μg/ml). Under the described chromatographic conditions, the retention times of LM 22070 and internal standard were 2.6 min and 4.3 min, respectively. Fig. 4 shows the chromatogram of an urine extract (0.6 ml).

Because of the large variations in plasma levels during a 24-h kinetic study, it is often necessary to take variable volumes of biological sample for analysis. Thus it was found necessary to maintain a constant ratio of aqueous to organic phase during the initial extraction step, in order to obtain a reproducible recovery. The absolute analytical recovery of LM 22070 from plasma was 97.5% (Table I). It was quite comparable with the absolute analytical recovery of the internal standard (96%).

Estimates of repeatability and accuracy of the method are shown in Table II.

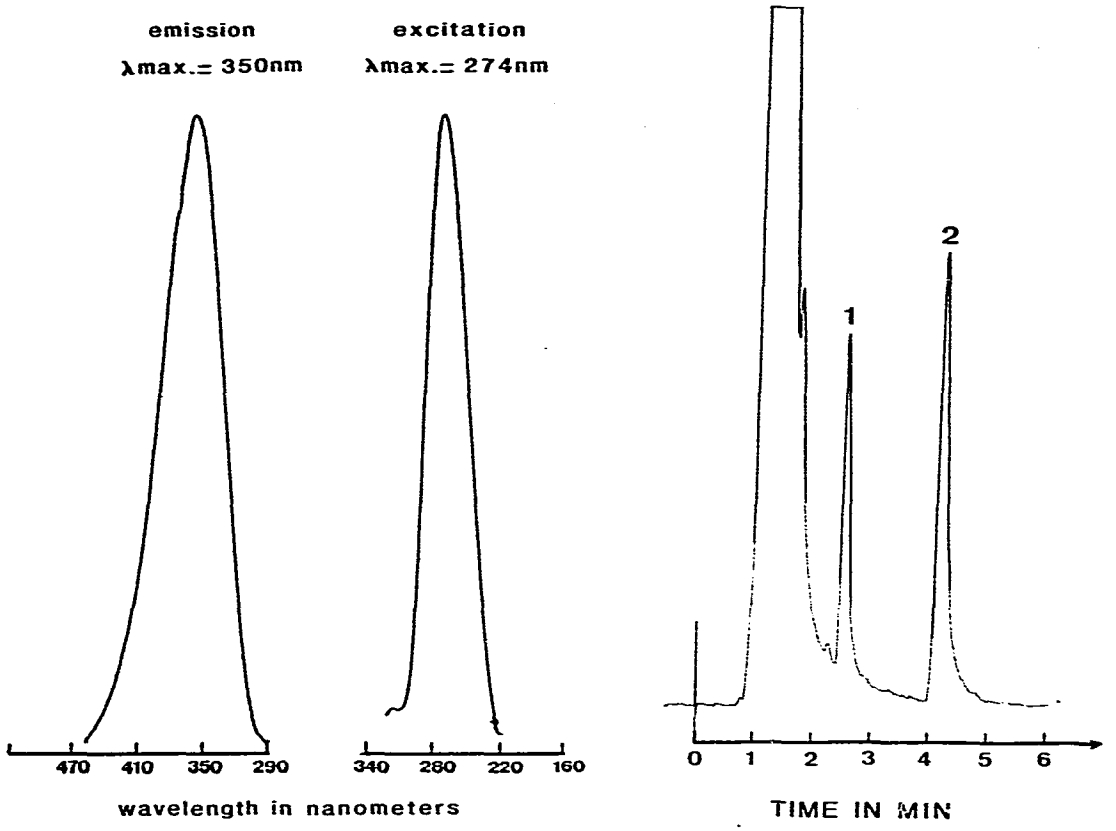

Fig. 3. Excitation and emission spectra of a 20 μg/ml solution of LM 22070 in acetonitrile—water—0.2 *M* phosphate buffer (pH 4.4) (65:15:20).

Fig. 4. Chromatogram of a 0.6-ml urine extract containing LM 22070 (1) 8.9 μg/ml and internal standard (2) 4 μg/ml.

TABLE I

ABSOLUTE ANALYTICAL RECOVERY OF LM 22070

Concentration (μg/ml)	*n*	Recovery (%)
0.5	3	97.5
2	4	96.5
6	4	99

Accuracy varied from 1.2 to 4.6%. It was still good at low concentrations and within the generally accepted limits for drug assays.

The results of calibration curves showed that a good linear fit was obtained in the therapeutic range of concentration (0.2—8 μg/ml) (slope 0.280; intercept 0.0238; $r = 0.999$).

TABLE II

REPEATABILITY AND ACCURACY

LM 22070 added (μg/ml)	*n*	LM 22070 measured (μg/ml)*	Mean error	Relative error (%)	CV** (%)
0.5	10	0.48 (0.022)	0.069	4	4.6
2	5	1.91 (0.051)	0.023	-4.5	2.6
6	5	5.72 (0.068)	0.030	4.6	1.2

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

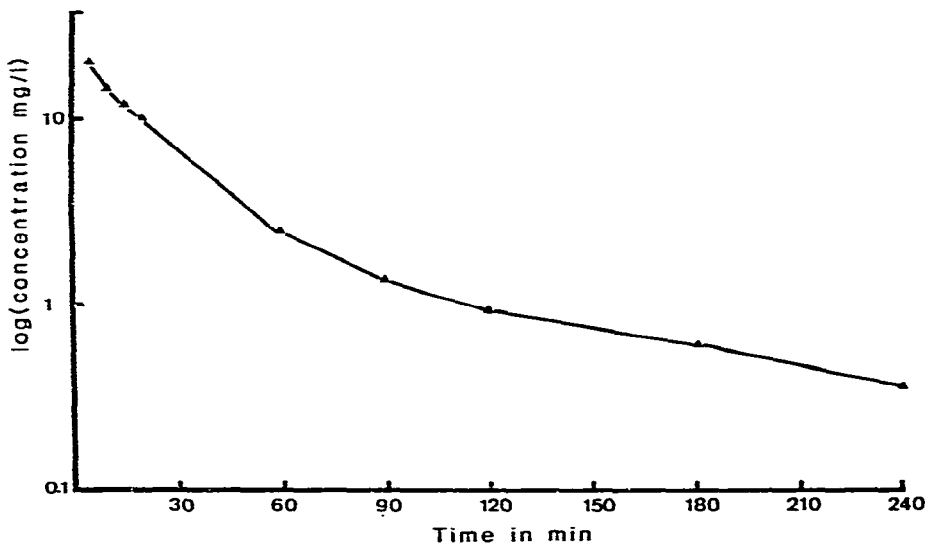

Fig. 5. Plasma concentration—time curve after a 50-mg intravenous injection of LM 22070.

Fig. 5 shows the plasma concentration—time curve after a 50-mg intravenous injection of LM 22070. Plasma levels varied from 21.5 μg/ml 5 min after administration to 0.36 μg/ml 4 h after dosing. The amount of LM 22070 excreted by the urine (free and conjugated) was 14.53 mg per 24 h (29.06%).

The detection limit is about 90 ng/ml in plasma and urine, both measured at a signal-to-noise ratio of 3:1.

The proposed RPLC method for the assay of 1-isobutyl-3,4-diphenylpyrazole-5-acetic acid is simple and sensitive enough to be used in pharmacokinetic and biopharmaceutic studies with this new drug.

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