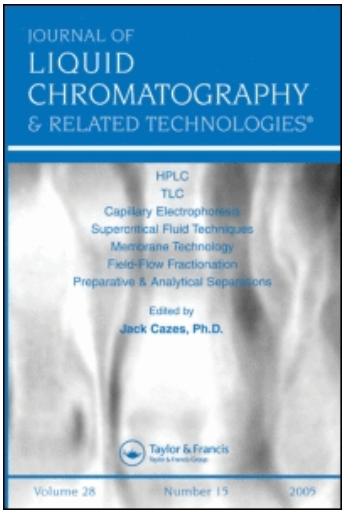

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MDL-035 IN THE PLASMA OF RATS, DOGS AND HUMANS.

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ABSTRACT

A sensitive and reproducible high performance liquid chromatographic method was set up for the assay of MDL-035, a new non-steroidal, nonacidic analgesic antiinflammatory agent, in the plasma of rats, dogs and humans. Plasma samples (0.5 ml) containing flurazepam as the internal standard, were diluted and extracted with ethyl ether. After centrifugation, the organic phase was taken to dryness, the residue was redissolved and injected into an RP-2 column. The elution was made in isocratic conditions using a CH_3CN/phosphate buffer solution as mobile phase. The UV detection was made at 320 nm. The method was validated for the concentration range from 0.05 to 10 µg/ml, and applied to pharmacokinetic studies. A typical plasma concentration-time profile in the rat after an oral administration is here presented.

INTRODUCTION

3-(1-methylethyl)-2-(4-methoxyphenyl)-3H-naphth-[1,2-d]-imidazole, MDL-035, is a new non-steroidal nonacidic analgesic antiinflammatory agent (1) currently being studied as anti-rheumatic agent.

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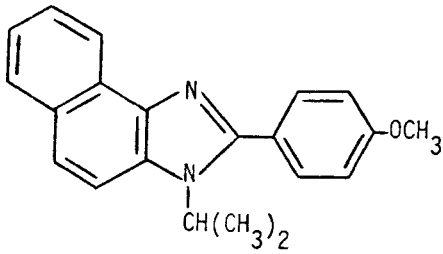

$C_{21}H_{20}N_2O$

M.W. 316.4

MDL-035, when tried in the pharmacological tests (1, 2), showed a high and prolonged activity. Remarkably, the compound resulted to be devoid of acute toxicity (LD_{50}= 15 g/kg, rat) and gastric lesivity (ED_{50} 1.05 g/kg, rat).

The aim of this work was to set up a sensitive and reproducible analytical method to determine the plasma levels of MDL-035 in preclinical and clinical pharmacokinetic studies and in toxicological investigations. The present HPLC method was validated for the concentration range from 0.05 to 10 µg/ml, providing for a sensitivity 8 times higher than that reported for the TLC method already available (3).

MATERIAL AND METHODS

The procedure involves the extraction with ethyl ether of the sample containing the internal standard. After evaporation of the organic solvent, the residue is redissolved and analyzed by HPLC using an RP-2 column as stationary phase and acetonitrile/phosphate buffer solution as eluant. The monitoring is made by UV detection at 320 nm.

Chemicals

MDL-035, Lepetit working standard of appropriate high purity.

Flurazepam, "Fabbrica Italiana Sintetici" (Milano, Italy), used as the internal standard.

Solvents and reagents high purity grade, Merck (Darmstadt, G.F.R.).

Distilled water, filtered through the Millipore Mille-Q system.

Plasma, from sprague-dawley rats, beagle dogs, and healthy volunteers.

Apparatus

The chromatographic determinations were made with a Waters Associates liquid chromatograph equipped with Model 6000A flow pumps, a Waters Model 620 solvent programmer, an LDC variable wavelength detector Spectromonitor III, a W.I.S.P. Mod. 710A automatic sampler, and a Tarkan 600 W+W recorder connected to a HP 3357 Data System, or a Hewlett-Packard Mod.3380A integrator for the quantitative determination of MDL-035 with reference to the internal standard. An RP-2 column, Brownlee Labs (Santa Clara, CA, USA), 25 cm x 4.6 mm, packed with 10 µ particles was used as stationary phase.

Standard solutions

Internal standard: 6 mg of flurazepam was dissolved in 10 ml of acetonitrile/2-propanol (1:1, v/v) solution.

MDL-035 and internal standard: 3 mg of MDL-035 and 6 mg of flurazepam were dissolved in 10 ml of acetonitrile/2-propanol (1:1 v/v) solution.

Extraction procedure

0.5 ml of rat, dog or human plasma was pipetted into a screw-cap tube containing 10 µl of the internal standard solution. The sample was then diluted with 0.5 ml of 1M Na_2HPO_4 and extracted with 10 ml of ethyl ether shaking for 10 min at 300 inversions per minute. After centrifugation at 2500 g for 5 min, 9 ml of the organic phase was transferred to a conical tube and taken to dryness at 37°C under a stream of nitrogen.

Chromatography

The residue was reconstituted with 20-25 µl of acetonitrile/2-propanol (1:1, v/v) with the aid of a Bransonic 12.

A suitable volume of this solution was finally put in the microvial of the HPLC automatic sampler. Ten microliters of the sample was injected into an RP-2 column and the isocratic elution was made at a flow-rate of 2 ml/min using a 45% B in A mixture as mobile phase, where A was a 0.05 M NaH_2PO_4/Na_2HPO_4 pH 7.8 aqueous solution and B was acetonitrile/water (9:1, v/v).

The UV detection of MDL-035 and the internal standard was made at 320 nm.

RESULTS AND DISCUSSION

Chromatographic separation

Figures 1-3 show that the method described affords a selective determination of MDL-035 and flurazepam in rat, dog and human plasma samples and a good chromatographic resolution of the peaks. The tracings are devoid of any peaks which could

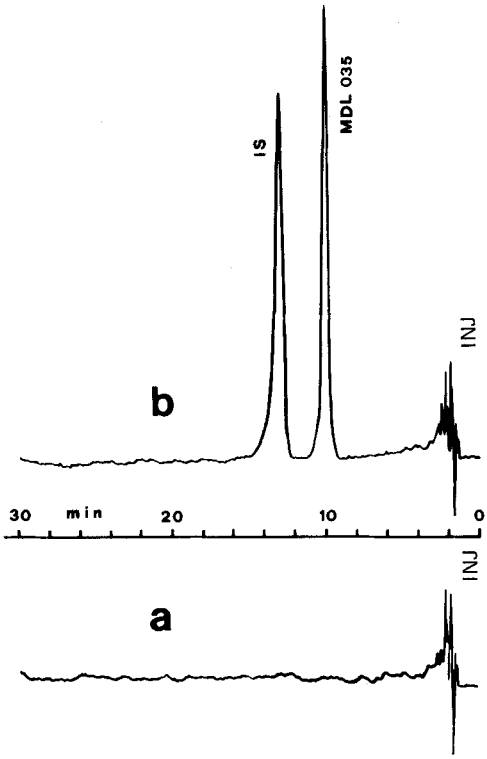

FIGURE 1 . Chromatograms of rat plasma samples (untreated animal)
a) no addition
b) plus MDL-035 (1 µg/ml) and the internal standard.

produce interference for the identification or the quantitative measurements of MDL-035 and the internal standard.

Validation of the method

In order to test the recovery, the precision (repeatability), the accuracy and the linearity of the method, plasma samples of untreated rats, dogs and humans were spiked with MDL-035 and flurazepam (internal standard) by adding known volumes of the standard solution (see Material and Methods).

Four concentration levels were established, based on the amounts of MDL-035 expected to be in the plasma: 0.05, 0.1, 1 and

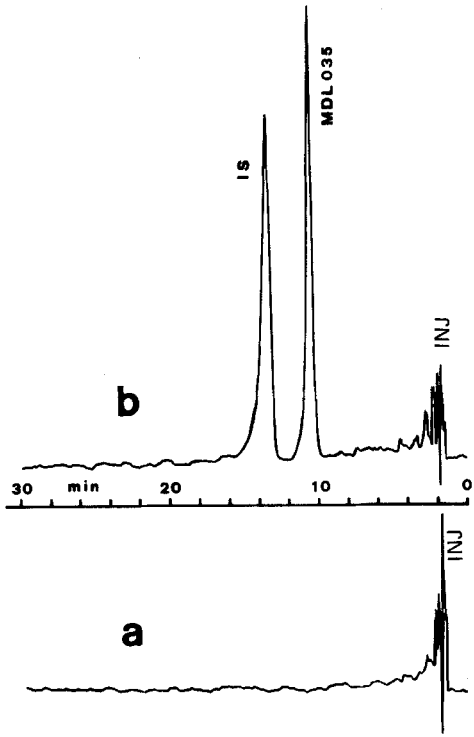

FIGURE 2. Chromatograms of dog plasma samples (untreated animal)
a) no addition
b) plus MDL-035 (1 µg/ml) and the internal standard.

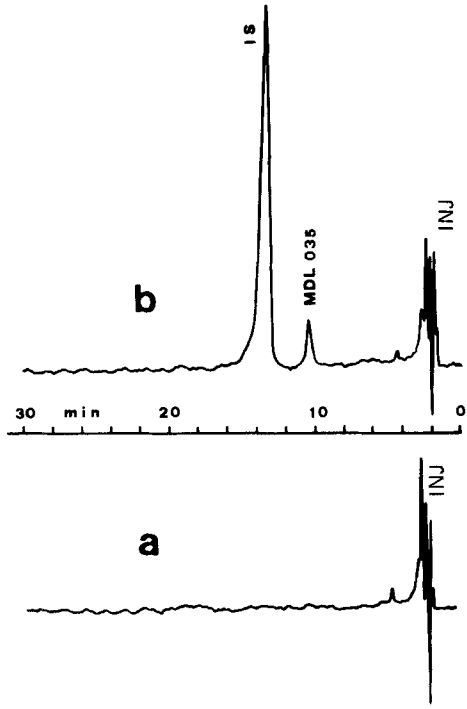

FIGURE 3. Chromatograms of human plasma samples (untreated subject)
a) no addition
b) plus MDL-035 (0.1 µg/ml) and the internal standard.

10 µg/ml . Five samples were prepared for each concentration level, extracted and analyzed as indicated above.

The average recovery of MDL-035 over the range tested was 106.7-117.7% (rat), 100.0-111.4% (dog), 101.9-106.7% (man), indicating that the extraction procedure yields a good recovery of both MDL-035 and flurazepam and the composition of plasma samples obtained from these three different species does not influence the recovery of the compounds (Table 1).

In Table 1 the mean recovery % (R %) and the coefficients of variation (C.V.%) of MDL-035 from rat, dog and human plasma samples are reported. C.V. = S.D./$\bar{X}$, where S.D. is the standard deviation and $\bar{X}$ is the mean of five analyses.

The precision of the method estimated by the coefficients of variation was satisfactory. The values of C.V.% calculated

TABLE 1

PLASMA CONCENTRATION (ng/ml)	RAT		DOG		MAN	
	R %	C.V.(%)	R %	C.V.(%)	R %	C.V.(%)
10000	108.4	6.0	111.4	2.7	101.9	1.4
1000	106.7	2.4	110.8	1.9	106.7	4.5
100	108.2	11.1	109.4	7.4	104.8	7.0
50	117.7	13.1	100.0	12.9	101.9	11.4

over the concentration levels tested ranged between 2.4-13.1 (rat), 1.9-12.9 (dog), 1,4-11.4 (man).

The accuracy was computed over the 20 points of each calibration curve and a mean recovery R of 110.3%, C.V. 9.5.% was found for rat plasma samples, R 108.3% C.V. 7.4% and R 103.7%, C.V. 6.9% for dog and human plasma samples, respectively.

The linear regression analysis provided for the equations:

$y = -0.0039 + 1.0848\ x \qquad r = 0.9979$ rat
$y = -0.0049 + 1.1146\ x \qquad r = 0.9996$ dog
$y = 0.0141 + 1.0183\ x \qquad r = 0.9999$ man

where y represents the amount of MDL-035 found, x the amount added and r the correlation coefficient.

The true value of the MDL-035 concentration in a plasma sample can be found by the equations above reported.

Operating wavelength

The operating wavelength was selected on the basis of the UV spectrum of MDL-035 in water-methanol 1:1 at different pH values. At pH 8.4 (next to the pH of the buffer used to make mobile phase) the UV spectrum exhibited 5 bands in the interval 220-350 nm, one of them with λ max 320 nm. At this operating wavelength the HPLC tracing resulted to be devoid of interferring peaks related to endogenous substances extracted from plasma.

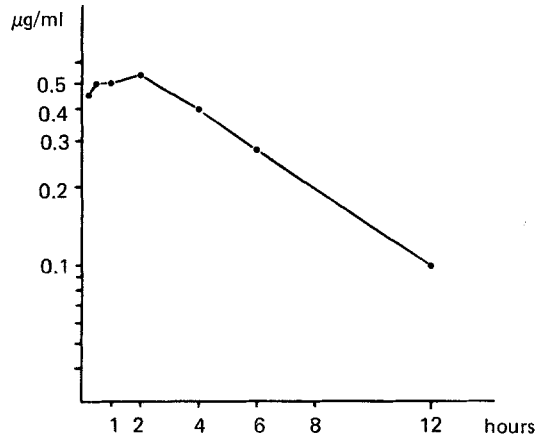

FIGURE 4. Mean plasma concentration-time curve of unchanged MDL-035 in rats treated with a 5 mg/kg oral dose.

Pharmacokinetic study

Some recent results of a pharmacokinetic study in the rat (4) are here reported. Peak plasma levels of unchanged MDL-035 in male rats were attained in 0.25-2 h (plateau) after the administration of a 5 mg/kg oral dose, reaching values of about 0.5 µg/ml. The compound was undetectable 24 h after treatment, while a mean concentration of 0.1 µg/ml was measured at the 12^{th}h (Fig.4). The elimination half life of the parent compound from plasma estimated after the 2^{nd}h, resulted to be about 4 h.

The method proved to be reliable and suitable for routine analysis.

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