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High-performance liquid chromatographic evaluation of Med 15 and its metabolites Med 5 and tolmetin in rat plasma

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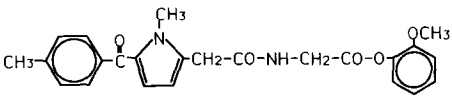
ABSTRACT

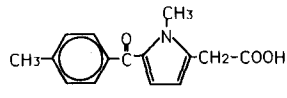
A simple and reliable high-performance liquid chromatographic method is described for the quantitative analysis of the new non-steroidal anti-inflammatory agent Med 15 and its metabolites Med 5 and tolmetin in rat plasma. After selective extraction the three analytes and an internal standard (*p*-phenylphenol) were separated on a reversed-phase Ultrasphere 5 μm column using potassium dihydrogenphosphate (0.05 *M*)–acetonitrile (52:48) (pH 4.7) as the mobile phase. The analytes were detected at 313 nm; the sensitivity of the method proved to be 0.05 μg/ml for all three compounds. The method has been applied to investigate Med 15 pharmacokinetics in rats.

INTRODUCTION

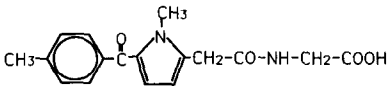
Med 15 is a new non-steroidal anti-inflammatory agent (NSAIA) in which tolmetin is linked to glycine through an amide bond, and the glycine moiety is in turn linked to guaiacol via an ester bond (Fig. 1).



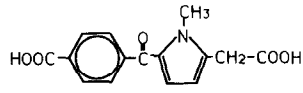
MED 15



TOLMETIN



MED 5



MCPA

Fig. 1. Molecular structures of Med 15, Med 5, tolmetin and MCPA.

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Oral administration of acidic NSAIAs produces typical gastrointestinal side-effects, mainly in the gastric mucosa [1–3]. Many attemps have been made to develop NSAIAs with an improved gastrointestinal tolerance. Esterification of acidic NSAIAs suppresses gastrotoxicity without impairing anti-inflammatory activity [4]. In a previous pharmacological investigation, Med 15 showed sustained anti-inflammatory activity with a particularly good gastrointestinal tolerability [5,6]. This paper describes a reversed-phase high-performance liquid chromatographic (HPLC) method with UV detection for simultaneous determination of Med 15 and its hydrolysis products, namely Med 5 and tolmetin (Fig. 1) using *p*-phenylphenol as internal standard.

EXPERIMENTAL

Chemicals and reagents

Med 15 and Med 5 were supplied by Sigma-Tau (Pomezia, Rome, Italy), and tolmetin and *p*-phenylphenol (internal standard, I.S.) were obtained from Sigma (St. Louis, MO, USA). All other chemicals used were of reagent grade; water was purified with a Milli-Q reagent-grade system. Standard solutions of Med 15, Med 5, tolmetin and the I.S. were prepared in acetonitrile and stored at 4°C.

Animals and treatment

Fasted male Sprague–Dawley rats (Charles River, Como, Italy) (average weight 200 g) were administered a single 82 mg/kg oral dose of Med 15 in a 0.5% carboxymethylcellulose sodium salt suspension. At various times after administration the animals (four per group) were sacrified. Heparinized blood samples were immediately centrifuged and the resulting plasma was separated and stored at −20°C until analysis. Animals were fed 3 h after drug administration.

Chromatographic conditions

The chromatographic system consisted of a Model L-6200 pump (Merck-Hitachi, Darmstadt, Germany), a Model 7125 sample injector (Rheodyne, Cotati, CA, USA) equipped with a 20-μl loop, and a Model L-4200 variable-wavelength UV detector (Merck-Hitachi) operated at 313 nm.

Separation was carried out on a 150 mm × 4.6 mm I.D. Ultrasphere ODS 5 μm column (Beckman, San Ramon, CA, USA) with a guard column (40 mm × 4.6 mm I.D. Perisorb RP-18, 30–40 μm particle size) at room temperature. A Merck-Hitachi D-2500 integrator was used to record chromatograms and calculate the peak heights of the analytes.

The isocratic mobile phase consisted of a 48:52 (v/v) mixture of acetonitrile and potassium dihydrogenphosphate (0.05 *M*) adjusted with phosphoric acid 8.5% (v/v) to a final pH of 4.7. A gradient flow-rate was used to eliminate long retention time of MED 15. It was 0.5 ml/min during the first 5 min, then increased to 0.7 ml/min and after 10 min increased to 1.0 ml/min; Fig. 2 shows the detailed profile of this flow gradient.

An unknown peak present in plasma samples of rats treated with Med 15 (Fig. 2D, MCPA) was identified with a Carlo Erba HRGC 5160 gas chromatograph (Carlo Erba, Milan, Italy) connected to a Finnigan MAT 8222 mass spectrometer with

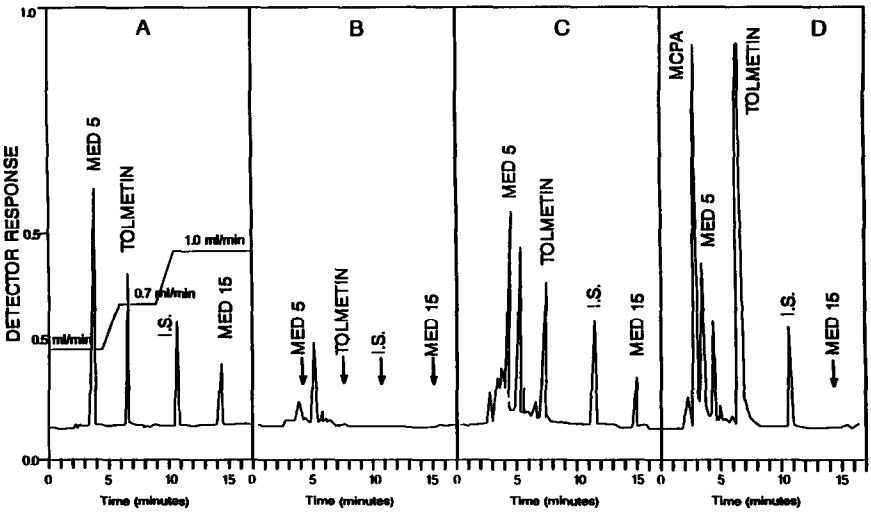

Fig. 2. Typical HPLC chromatograms of (A) analytes as authentic standards, (B) drug-free extracted plasma, (C) analytes added to drug-free plasma, (D) 1 h plasma sample of a rat treated orally with Med 15.

double-focus geometry, equipped with both electron impact and chemical ionization modes (Finnigan MAT, San José, CA, USA). Gas chromatographic analysis was performed with a HP 101 column (15m × 0.32 mm I.D.) (Hewlett-Packard, Palo Alto, CA, USA). The column temperature was programmed from 60°C to 150°C at 25°C/min, and from 150°C to 280°C at 15°C/min, then maintained at 280°C for 5 min. The helium (carrier gas) pressure was 0.25 kg/cm^2.

Extraction procedure

A 1-ml volume of rat plasma was acidified (90 μl of 1.0 *M* HCl), and 20 μl of the I.S. solution (1.0 mg/ml) were added and vortex-mixed for 10 s. The samples were extracted with 6 ml of benzene–*tert.*-butyl alcohol (90:10, v/v) by shaking for 20 min on an automatic shaker. After centrifugation (2000 *g* for 15 min), the organic layer was transferred to a conical tube and evaporated to dryness at 40°C, under a gentle stream of air. The residue was reconstituted with 0.2 ml of mobile phase, sonicated for few seconds and injected into the HPLC column.

Recovery and calibration curves

Percentage recoveries of Med 15, Med 5 and tolmetin from plasma were assessed by comparing the peak-height ratio (drug to I.S.) obtained after extracting known amounts of analyte (0.05–2 μg/ml range) with that obtained when identical amounts of the working standard were dispensed without extraction. Calibration curves were constructed by adding known amounts (0.05–2 μg/ml) of Med 15, Med 5 and tolmetin, to plasma of untreated animals and processing the samples as decribed above. Values of the peak-height ratio (drug to I.S.) for Med 15, Med 5 and tolmetin were plotted in calibration graphs, and they were used to calculate the drug concentration in the unknown sample.

RESULTS AND DISCUSSION

The major difficulty in the analysis of Med 15 and its metabolites is due to their amphoteric properties, which make it difficult to extract them from aqueous media. Extraction from moderately acidic rat plasma at the carefully selected pH of 3.5 provided a good recovery for all the analytes tested: on average, 80.7, 82.6, 99.3 and 90% for Med 15, Med 5, tolmetin and the I.S., respectively. The interassay reproducibility proved to be 5.1% for Med 15, 7.5% for Med 5, and 3.88% for tolmetin (Table I), and linearity was observed for all analytes in the range 0.05–2 μg/ml plasma; higher concentrations were diluted with phosphate buffer (pH 7.4).

The use of acidified plasma, moreover, completely prevents the hydrolysis of Med 15. Extraction recovery tests were carried out by adding Med 15 and the other analytes to previously acidified plasma samples; in this situation no hydrolysis of Med

TABLE I

RECOVERY OF MED 15, MED 5 AND TOLMETIN FROM RAT PLASMA

Each value is the mean (±S.D.) of five determinations.

Compound	Concentration (ng/ml)	Recovery (%)	Coefficient of variation (C.V.) (%)
Med 15	50	80.2(±5.0)	6.2
	100	81.0(±2.7)	3.3
	250	79.0(±5.6)	7.1
	500	88.0(±1.7)	1.9
	1000	80.8(±2.7)	3.3
	2000	75.4(±7.1)	9.4
Mean		80.7	
S.D.		4.11	
C.V.		5.1%	
Med 5	50	71.2(±8.1)	11.5
	100	85.0(±7.3)	8.6
	250	80.0(±9.0)	11.2
	500	86.0(±4.9)	5.7
	1000	88.4(±8.1)	9.2
	2000	85.0(±6.9)	8.1
Mean		82.6	
S.D.		6.20	
C.V.		7.5%	
Tolmetin	50	99(±2.0)	2.0
	100	102(±9.3)	9.1
	250	92(±3.0)	3.3
	500	99(±2.4)	2.4
	1000	102(±5.2)	5.1
	2000	102(±3.7)	3.6
Mean		99.3	
S.D.		3.88	
C.V.		3.90%	

15 occurred. When Med 15 (0.1–5 μg/ml) was incubated at 37°C in fresh rat plasma without acidification, its disappearance was rapid and complete. Med 5 showed very different behaviour when incubated under these conditions. It is very stable in plasma, with a half-life longer than 5 h. The hydrolysis of Med 15 in plasma is enzyme-mediated, since in aqueous solution it proved to be stable for up to 5 h at both 21 and 37°C. The enzyme instability of esterified NSAIAs has been widely documented in the literature [7–10].

Typical chromatograms from (A) standard solutions (concentrations as in point C), (B) drug-free plasma extract, (C) plasma spiked with analytes examined (0.25 μg/ml) and the I.S. (15 μg/ml) and (D) a plasma sample from a rat treated with Med 15 (82 mg/kg) are shown in Fig. 2. There were no interfering peaks from the drug-free plasma samples at retention times of Med 15, Med 5, tolmetin and the I.S. Selectivity was also tested by injecting various NSAIAs, namely sulindac, naproxen, indomethacin, flufenamic acid, ibuprofen and acetylsalicylic acid. None of these interfered with the peaks of Med 15, its metabolites and the I.S.

Chromatogram D in Fig. 2 shows an unknown peak with a retention time of 3.0 min. According to Sumner *et al.* [11] it was identified by gas chromatography–mass spectrometry as MCPA, the major metabolite of tolmetin (Fig. 3) [12]. MCPA fraction peaks eluted from HPLC system were pooled, evaporated, methylated with CH_2N_2 and identified by gas chromatography–mass spectrometry as 5-(*p*-carboxybenzoyl)-1-methylpyrrole-2-acetic acid dimethyl ester.

The HPLC method provided an opportunity for investigating the kinetic profile of Med 15 in rat plasma. Fig. 4 shows the curve of plasma concentration *versus* time obtained from rats treated orally with Med 15 (82 mg/kg). Plasma analysis showed undetectable parent drug at all times tested, as a results of a marked presystemic

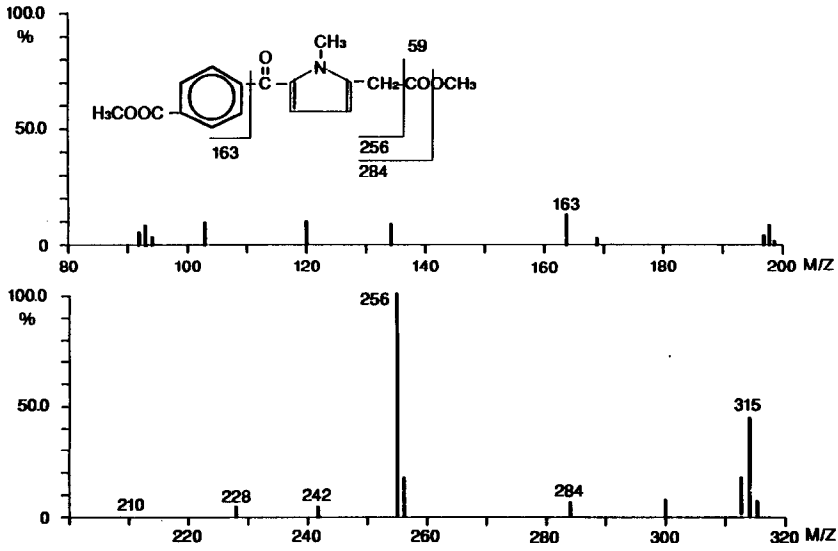

Fig. 3. Mass spectrum of MCPA as its dimethyl ester obtained in electron impact ionization mode (70 eV).

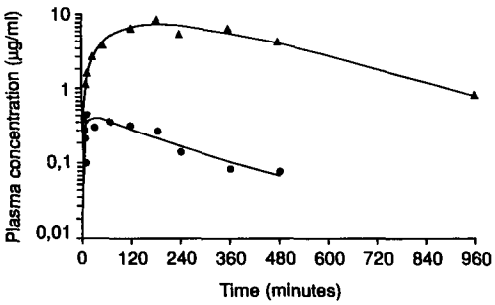

Fig. 4. Plasma concentration–time curves of Med 5 (●) and tolmetin (▲) after oral administration of Med 15 to rats (82 mg/kg), obtained with a computer-assisted process according to the open one-compartment model for oral administration. Each point represents the mean of four findings.

enzyme hydrolysis. A single kinetic curve is suitable for obtaining kinetic results of only qualitative character. The half-life is *ca.* 180 min for both Med 5 and tolmetin and the kinetic behaviour is considered to be monophasic.

CONCLUSION

A sensitive and specific HPLC assay has been developed for the simultaneous analysis of Med 15 and its metabolites. The lowest detectable concentration (0.05 µg/ml) and the good validation allow this method to be used in pharmacokinetic and bioavailability studies in humans.

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