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Determination of a new antibronchospastic agent, MX2/120, in guinea pig plasma by high-performance liquid chromatography in a pharmacokinetic study

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Abstract

7-[(2,2-Dimethyl)propyl)]-1-methylxanthine (I, Lab code MX2/120) is a new potent antibronchospastic agent. A rapid and simple HPLC assay for I in guinea pig plasma has been developed. Compound I was extracted from plasma with dichloromethane by a solid-phase extraction procedure, after adding 1,3-dimethyl-7-pentylxanthine at a concentration of 5 μg/ml as the internal standard (I.S.). The extraction residue was redissolved in water–acetonitrile and chromatographed on a RP-18 reversed-phase column. The eluate was monitored by spectrophotometric detection at 280 nm. The method showed good linearity over the range 0.1–20 μg/ml ($r = 0.9998$) and is precise (C.V. × Student's t-test = 1.84%) and accurate (mean recovery ± limit of confidence = 100.25 ± 0.34). The HPLC assay was successfully applied to the determination of the pharmacokinetic profile of I after intravenous and oral administration in guinea pigs. The main pharmacokinetic parameters are presented.

1. Introduction

Several methylxanthine derivatives have been proposed as substitutes for theophylline in the treatment of obstructive respiratory diseases, and claimed to be free from central nervous and cardiovascular side-effects [1,2]. Among them, isbufylline (i.e. 1,3-dimethyl-7-isobutylxanthine [3]) was reported to show in guinea pigs remarkable and long-lasting antibronchospastic and antiinflammatory activity, coupled with minor side effects in comparison with theophylline [4].

Metabolism studies in humans suggested a rapid biotransformation involving the alkyl chains at positions 3 and 7 [5]. In order to protect the molecule from this metabolic degradation, a new series of compounds was synthesized [6]. 7-[(2,2-Dimethyl)propyl]-1-methylxanthine (MX2/120, I) was the most promising compound of the series in terms of bronchodilating activity, duration of action and lack of side effects [7].

This paper describes a high-performance liquid chromatographic (HPLC) method for the detec-

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tion of I in plasma samples, and its application to animal pharmacokinetic studies.

2. Experimental

2.1. Materials

7-[(2,2-Dimethyl)propyl]-1-methylxanthine (I) and the internal standard (I.S.) 1,3-dimethyl-7-pentylxanthine, were synthesized as previously described [6,8]. Both compounds were at least 99% HPLC pure. All other reagents were of analytical grade and used without further purification. Dichloromethane, acetonitrile and methanol (LiChrosolv) were obtained from Merck (Darmstadt, Germany) and orthophosphoric acid (85%), potassium dihydrogen phosphate and anhydrous sodium sulfate were obtained from Carlo Erba (Milan, Italy). Freshly deionized and distilled water was used throughout. Stock solutions of I (4 mg/ml) and I.S. (1 mg/ml) were prepared in water–acetonitrile (1:1, v/v). Storage of these solutions at 4°C for at least three months did not result in detectable decomposition.

2.2. Chromatography

A Perkin-Elmer (Norwalk, CT, USA) Series 400 liquid chromatograph equipped with a variable-wavelength 1050 Hewlett-Packard (Waldbron, Germany) spectrophotometric detector set at 280 nm and a LCI-100 Perkin-Elmer laboratory computing integrator were used. Injections were made with Model 7125 syringe-loading sample injector with a Rheodyne Valve (Cotati, CA, USA) and a 20-μl loop.

A prepacked RP-18 reversed-phase LiChroCart column was used (25 cm × 4 mm I.D., particle size 5 μm, LiChrospher, Merck) with a LiChrospher 100 RP-18 pre-column (4.0 × 4 mm I.D., particle size 5 μm). The mobile phase, constantly degassed with helium during the entire analytical procedure, was 0.02 *M* phosphate buffer (adjusted to pH 3.7 with 2 *M* H_3PO_4)–acetonitrile (65:35, v/v). The flow-rate was 1 ml/min. All solutions were filtered through a 0.2-μm filter (Millipore, Molsheim, France) and the assays were performed at room temperature.

2.3. Sample preparation

In a 10-ml glass centrifuge tube, 20 μl of a solution containing 5 μg of I.S. were added to 1 ml of guinea pig plasma. This mixture was vortex-mixed for 5 s, and then placed in a shaking vortex bath (SW-20 C Julabo Labortechnik, Seelbech, Germany) at 37°C and 120 rpm for 30 min. Drug extraction of plasma was performed as follows: a Sep-Pak cartridge rack from Waters, a SPE vacuum manifold and a Sep-Pak cartridge (360 mg of solid-phase C_{18} from Waters, Milford, MA, USA) were used. The sorbent was conditioned using 2 × 10 ml of methanol and 5 ml of water and was not allowed to dry at the end of the conditioning. A 1-ml volume of plasma, prepared as described above, was applied onto the cartridge and after absorption of the sorbent compounds the cartridge was washed with 10 ml of water. Finally the cartridge was dried under vacuum and compound I was eluted with 7 ml of dichloromethane. The solvent was dried with anhydrous Na_2SO_4 and evaporated under a gentle stream of nitrogen at 40°C. The residue was dissolved in 100 μl of water-acetonitrile (1:1, v/v) and a 20-μl aliquot was injected onto the liquid chromatograph.

2.4. Calibration curve and sample analysis

Standard samples were prepared by adding 20 μl of water–acetonitrile (1:1, v/v) containing 0.1, 0.5, 2, 5, 10, and 20 μg of I.S. to 1 ml of guinea pig blank plasma. The calibration curve was obtained by plotting the peak-area ratio of I/I.S. against the concentration of I in standard samples. Plasma samples obtained from treated animals were extracted after the addition of 5 μg of I.S. For each set of twenty plasma samples, a new standard curve was constructed. The concentrations of I in these plasma samples were estimated from the regression curve by interpolation.

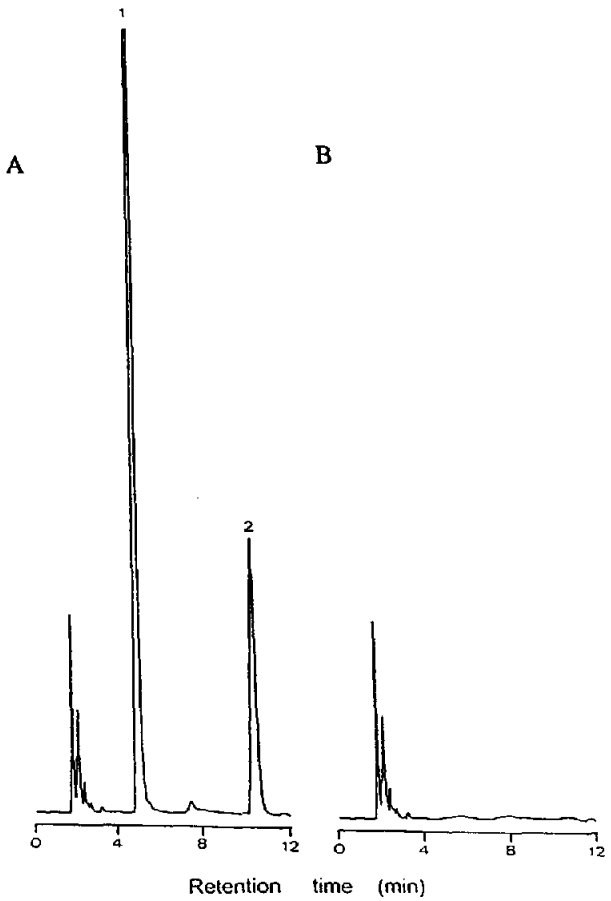

Fig. 1. Chromatograms of guinea pig plasma two hours after oral administration of 53 μmol/kg of I (A), and a blank plasma (B). Peaks: 1 = I (16 μg/ml) and 2 = I.S. (5 μg/ml).

3. Results

Typical chromatograms are shown in Fig. 1A. The retention times of I and I.S. were 4.9 and 10.4 min, respectively, and the total analysis time was 12 min. No interfering peaks were observed when blank plasma was extracted (Fig. 1B).

3.1. Extraction efficiency

The percentage recovery of the extraction procedure of I and I.S. was determined at four concentrations ranging from 0.1 to 20 μg/ml of plasma. A comparison of the peak area obtained from extracted plasma samples with those obtained by injection of a standard solution was made. The mean (± C.V.) recoveries of I and I.S. were 84.25 ± 7.92% ($n = 4$) and 90.50% ± 4.64 ($n = 8$), respectively.

3.2. Accuracy and precision

The intra- and inter-day precision of the assay was determined by carrying out four (intra) or six (inter) replicate analyses on 1-ml aliquots of guinea pig plasma spiked with 0.1, 10 and 20 μg of I and 5 μg of I.S. The concentrations of these samples were estimated from the regression curve and are summarized in Table 1.

The mean (± C.V.) recovery of the intra- and inter-day assays was 100.25 ± 0.90% ($n = 30$).

Table 1
Intra- and inter-day precision and accuracy for the determination of I in guinea pig plasma

Nominal concentration (μg/ml)	Mean concentration (μg/ml)	C.V. (%)	(Mean recovered) − (nominal concentration) difference (%)
Intra-day precision (n = 4)			
0.1	0.099	10.01	−1.0
10.0	9.9	1.91	−1.0
20.0	20.1	1.03	0.5
Inter-day precision (n = 6)			
0.1	0.098	10.03	−2.0
10.0	10.2	1.85	1.9
20.0	20.4	2.03	2.0

There was no significant difference ($p < 0.05$) in recovery over the concentration range studied. Thus the present method can be considered precise (C.V. × t = 1.84%) and accurate (mean recovery ± limits of confidence = 100.25 ± 0.34).

3.3. Linearity

Good linearity was obtained for I over the range 0.1–20 μg/ml ($y = 0.1868x - 0.0077$, $r = 0.9998$), where y is the peak-area ratio and x is the concentration of I in plasma, while r is the correlation coefficient.

The minimum quantitation limit (MQL) for I in plasma as estimated from regression analysis was 0.05 μg/ml.

3.4. Application of the method

To evaluate the applicability of the method, we measured the plasma level of I in a pharmacokinetic study following the intravenous and oral administration in guinea pigs of a dose of I (12.5 mg/kg) shown to exert an effect in acetylcholine- and histamine-induced bronchospasm in conscious guinea pigs [7]. Male Dunkin–Hartley guinea pigs (Rodentia, Torre Pallavicina, Bergamo, Italy) weighing 300–400 g were used in this study. All animals (136) were housed in stainless steel cages and fasted for 16 h prior to treatment. This study was approved by the Minister of Health of Italy.

3.5. Intravenous drug administration

The jugular vein was isolated and cannulated for intravenous drug administration in guinea pigs previously anaesthetized (i.p.) with a 10% solution of urethane (10 ml per kg bodyweight). Compound I was solubilized in glycofurol at a concentration of 12.5 mg/ml (53 μmol/ml), and administered in a volume of 1 ml/kg.

3.6. Oral drug administration

Compound I was suspended in an aqueous vehicle containing 0.9% NaCl, 0.4% Tween 80 and 0.4% carboxymethylcellulose at a concentration of 1.25 mg/ml (5.3 μmol/ml) in a volume of 5 ml/kg. The suspension was sonicated for 15 min in an ultrasonic bath before administration.

3.7. Sample collection

The carotid vein was isolated, cannulated and blood samples (ca. 2–4 ml) were drawn from guinea pigs anaesthetized with a 10% solution of urethane 30 min before blood sampling. Each sample was taken from an other animal and for each sampling time four animals were sacrificed. Blood samples were collected in PTFE tubes containing a solution of 30% potassium oxalate (2% of the total plasma volume), centrifuged at 314 *g* for 5 min and plasma samples were stored at −20°C until use. Blood samples were taken at 5, 15, 30, 45 min and 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 16, 20, 24, and 48 h.

3.8. Pharmacokinetic analysis

Pharmacokinetics parameters of I were estimated using a non-linear regression program (Easy Fit, by M. Vaccari and M. Rocchetti, Istituto Mario Negri, Milano). The parameters were calculated in the standard manner [9] from the biexponential (i.v.; Fig. 2A) or triexponential (p.o.; Fig. 2B) equation fitted to the mean drug concentration–time data. The C_{max} and the t_{max} were read from the experimentally observed data.

4. Discussion

The described HPLC assay is similar to those reported for the detection of xanthines in biological fluids, such as, for example, theophylline, caffeine, theobromine and derivatives [10]. It combines solid-phase extraction for sample clean-up with conventional reversed-phase HPLC for elution.

The pharmacokinetics of I was studied in plasma of male guinea pigs following intravenous and oral administration. The mean plasma concentrations and the pharmacokinetic parameters

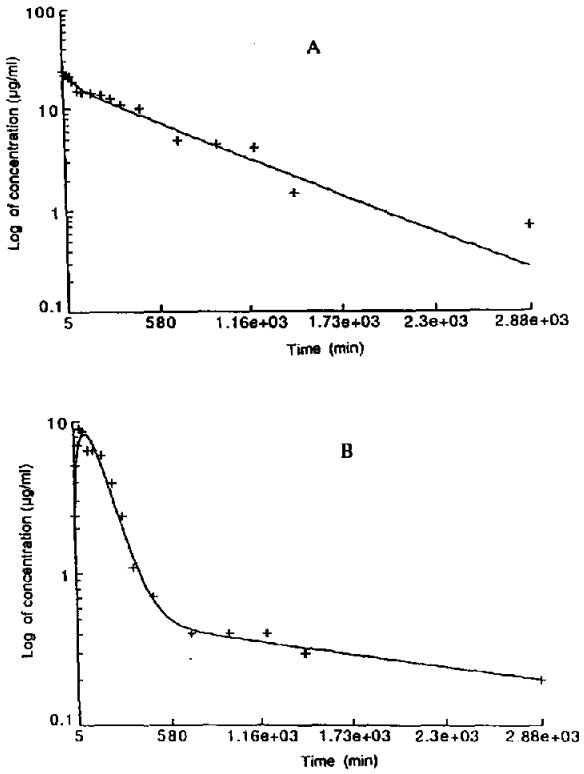

Fig. 2. Semilogarithmic plot of mean drug concentration–time data after intravenous (A) and oral (B) administration of I in guinea pigs.

Table 2
Plasma concentration of I in guinea pigs following intravenous and oral administration

Time (h)	Concentration (mean ± S.D.) (µg/ml)	
	Intravenous[a]	Oral[b]
0.08	23.7 ± 2.6	2.4 ± 0.70
0.25	22.1 ± 2.9	5.1 ± 1.60
0.50	22.0 ± 2.8	6.9 ± 2.40
0.75	21.4 ± 5.7	8.9 ± 2.30
1.00	18.9 ± 2.4	8.6 ± 0.80
1.50	14.9 ± 2.5	6.4 ± 0.60
2.00	14.5 ± 1.0	6.4 ± 1.20
3.00	14.2 ± 1.6	6.0 ± 0.10
4.00	14.0 ± 0.2	3.9 ± 0.90
5.00	12.6 ± 1.2	2.4 ± 0.50
6.00	11.1 ± 1.0	1.1 ± 0.30
8.00	10.1 ± 0.9	0.7 ± 0.30
12.00	5.0 ± 2.3	0.4 ± 0.12
16.00	4.5 ± 1.5	0.4 ± 0.05
20.00	4.1 ± 0.6	0.4 ± 0.07
24.00	1.5 ± 0.4	0.3 ± 0.15
48.00	0.7 ± 0.2	0.2 ± 0.01

[a] Intravenous administration: 12.5 mg/kg.
[b] Oral administration: 6.25 mg/kg.

after administration of I to guinea pigs (12.5 mg/kg i.v. or p.o.) are listed in Tables 2 and 3. After a single intravenous administration of 12.5 mg/kg of I the plasma concentration of I declined according to a biexponential profile. The pharmacokinetic parameters were calculated by using a biexponential fitting. The kinetics is characterized by a large volume of distribution (502 ml/kg), a low plasma clearance (57.00 ml h^{-1} kg^{-1}) and a long half-life of elimination (8.13 h). These data suggest that I was well distributed in the body tissue (in good agreement with the lipophilicity of the drug) and that the drug was slowly metabolized [11] and eliminated from the body. The AUC was 216.42 h mg ml^{-1}. A biexponential equation was used to fit the plasma concentration data obtained after oral administration of 12.5 mg/kg of I. The mean plasma concentration reached a C_{max} (8.9 µg/ml) at 0.75 h (t_{max}), thereafter the plasma concentration declined according to a biexponential profile. The half-life of absorption was 0.82 h. The apparent elimination half life was ca. 30 h. The AUC was 52.87 h µg ml^{-1}. The mean residence time (MRT) after i.v. administration was 11.42 h. The bioavailability (F) of I, esti-

Table 3
Pharmacokinetic parameters

Pharmacokinetic parameters	Intravenous	Oral
C_o (µg/ml)	24.89	
C_{max} (µg/ml)		8.90
t_{max} (h)		0.75
$t_{1/2\beta}$ (h)	8.13	34.83
$t_{1/2abs}$ (h)		0.82
$t_{1/2dis}$ (h)	0.56	
Vd (ml/kg)	505.00	
Cl (ml h^{-1} kg^{-1})	57.00	
AUC (h µg ml^{-1})	216.42	52.87
MRT (h)	11.42	25.43
F (%)		25.00

mated from mean ratio AUC p.o./AUC i.v. was ca. 25%. Comparison between $t_{1/2\beta}$ obtained after oral and intravenous administration (30 and 8.13 h., respectively), shows that the half-life of the compound strongly depends on the route of administration.

5. Conclusions

Our results indicate that the present HPLC method is precise, accurate, sensitive and reliable. Thus it can be used for plasma determinations of I in pharmacokinetic studies.

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