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Note

Determination of 1,3-dimethyl-7-isobutylxanthine in guinea pig plasma by high-performance liquid chromatography

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The development of safer and selective theophylline derivatives free from central and cardiovascular side-effects is a major area of research [1,2]. Recently, in our laboratory, it has been discovered that 1,3-dimethyl-7-isobutylxanthine (TE/06, I) is two to five times more potent than theophylline as an antibronchospastic agent and also exhibits much lower central nervous and cardiac excitatory actions [3]. This paper describes a high-performance liquid chromatographic (HPLC) method for detection of I in plasma samples, that is suitable for use in animal and human pharmacokinetic studies.

EXPERIMENTAL

Materials

1,3-Dimethyl-7-isobutylxanthine (I) and the internal standard (I.S.), 1,3-dimethyl-7-isoamylxanthine, were synthesized as previously described [3]. These products were at least 99% pure. All other reagents were of analytical grade and used without further purification. Dichloromethane and acetonitrile (LiChrosolv) were obtained from Merck (Darmstadt, F.R.G.) and orthophosphoric acid, potassium phosphate monobasic and perchloric acid were from Carlo Erba (Milan, Italy). Freshly deionized and distilled water was used throughout. Stock solutions of I (2 mg/ml) and I.S. (5 mg/ml) were prepared in the HPLC mobile phase. Storage of these solutions at 4°C for at least two months did not result in detectable decomposition.

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Chromatography

We used a Perkin-Elmer (Norwalk, CT, U.S.A.) Series 400 liquid chromatograph equipped with a variable-wavelength LC-85B spectrophotometric detector set at 280 nm (sensitivity 0.32 a.u.f.s.) and an LCI-100 laboratory computing integrator. Injections were made with a Model 7125 syringe-loading sample injector with a Rheodyne valve (Cotati, CA, U.S.A.).

A prepacked RP-18 reversed-phase column was used (25 cm × 4 mm I.D., particle size 5 μm, Merck). The mobile phase, constantly degassed with helium during the whole analytical procedure, was 0.02 *M* phosphate buffer (pH 2.8)–acetonitrile (65:35, v/v). The flow-rate was 1.0 ml/min. All solutions were filtered through a 0.2-μm filter (Millipore, Molsheim, France) and the assays were performed at room temperature.

Sample preparation

In a 20-ml glass centrifuge tube, 20 μl of a solution containing 10 μg of I.S. were added to 1 ml of guinea pig plasma. This mixture was vortex-mixed for 5 s, and then placed for 30 min, in a shaking vortex bath (SW-20 C Julabo Labortechnik) at 37°C and 120 rpm. The plasma was deproteinized by adding 30 μl of 70% perchloric acid, followed by vortex-mixing for 5 s and addition of 100 μl of 4 *M* sodium hydroxide. The solution was extracted with 10 ml of dichloromethane by mechanical shaking for 10 min and centrifugation for 5 min (1000 *g*).

After removal of the aqueous phase by aspiration, the organic layer was transferred into a disposable 10-ml glass tube and evaporated under a gentle stream of nitrogen at 60°C on a heating bath. Finally, the residue was redissolved in 100 μl of acetonitrile–water (1:1, v/v) and 10 μl were injected into the column.

Calibration curve and analysis of samples

Standard samples were prepared by adding 20 μl of acetonitrile–water (1:1, v/v) containing 5, 10, 15 or 20 μg of I and 10 μg of I.S. to 1 ml of blank guinea pig 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 10 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.

RESULTS

Typical chromatograms are shown in Fig. 1. The retention times of I and I.S. were 5.7 and 9.2 min, respectively, and the total analysis time was 11 min.

Extraction efficiency

The percentage recovery of the extraction procedure for I and I.S. was determined at four concentrations ranging from 5 to 20 μg/ml of plasma. A comparison of the peak areas obtained from extracted plasma samples with those obtained

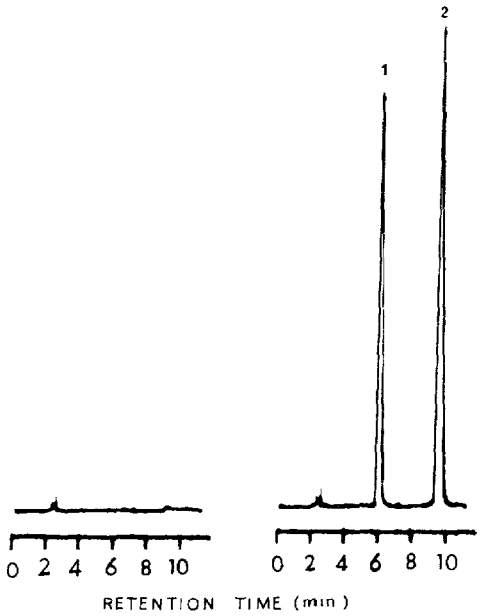

Fig. 1. Chromatograms of guinea pig plasma 60 min after oral administration of 25 mg/kg I (concentration 2.5 μg/l) (right) and a blank plasma (left). Peaks: 1 = I; 2 = I.S.

by injection of a standard solution was made. The mean (±C.V.) recoveries of I and I.S. were 69.89±4.33% ($n=8$) and 70.90±6% ($n=5$), respectively.

Accuracy and precision

The intra- and inter-day precision of the assay was determined by carrying out three (intra) or five (inter) replicate analysis on 1-ml aliquots of guinea pig plasma spiked with 5, 10, 15 and 20 μg of I and 10 μg of I.S. The concentrations of these samples were estimated from the regression curve and are summarized in Table I.

The mean (±C.V.) recovery of I was 99.31±6.78% ($n=20$). There was no significant difference ($p < 0.05$) in recovery over the concentration range studied.

Linearity

A good linearity was obtained for I over the range 5–20 μg/ml ($y=0.1087x + 0.0262, r = 0.9981$). The lowest detectable concentration was 2 μg/ml at a signal-to-noise ratio of ca. 3.

Application of the method

To evaluate the suitability of the method we measured the plasma level of I following administration of a dose (25 mg/kg per os) that exerts an effective antagonism toward acetylcholine- and histamine-induced bronchospasm in conscious guinea pigs [4]. Male albino guinea pigs (Pampaloni, Pisa, Italy) weighing 350–400 g were used. After overnight starvation, animals were orally dosed with 25 mg/kg I (dissolved in water). Blood samples (3–5 ml) were withdrawn after

TABLE I

INTRA- AND INTER-DAY PRECISION OF THE ASSAY

Nominal concentration (μg/ml)	Mean concentration (μg/ml)	Coefficient of variation (%)	Mean difference (%)
Intra-day precision (n=3)			
5	5.09	5.48	1.80
10	10.13	4.94	1.30
15	15.73	4.11	4.87
20	19.60	0.37	−2.00
Inter-day precision (n=5)			
5	4.94	8.28	−1.2
10	10.09	6.72	0.9
15	15.30	7.43	2
20	19 09	4.28	4.55

30 min by means of heart puncture, using heparinized syringe. Blood collection was carried out under light ether anaesthesia. Samples were than rapidly centrifuged at 2000 *g* for 10 min. Plasma samples were then analysed according to the HPLC method described. A plasma concentration of I of 14.0 ± 1.5 μg/ml ($n=8$) was found.

CONCLUSION

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

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