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Simultaneous determination of isbufylline and its major metabolites in rabbit blood and urine by reversed-phase high-performance liquid chromatography

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ABSTRACT

A sensitive high-performance liquid chromatographic assay for isbufylline and its major metabolites in rabbit blood and urine is described. After extraction, samples were eluted by a linear reversed-phase gradient. Specimens obtained after intravenous administration of isbufylline to rabbits were analysed to identify and subsequently quantify the potential metabolites. Using the ultraviolet absorption trace on the recorder as a reference, elution fractions were collected and analysed by mass spectrometry with the direct inlet system and gas chromatography–mass spectrometry after derivatization. Seven metabolites were identified and another five quantified. The method is specific, accurate, reproducibile and recommended for pharmacokinetic studies.

INTRODUCTION

Isbufylline (1,3-dimethyl-7-isobutylxanthine) is a compound with bronchodilator activity originating from research conducted on theophylline substitued at the 7-position with an isobutyl group. This chemical modification produces the pharmacological properties of this new compound: it has a potent anti-bronchospastic effect and lower excitatory action than theophylline on the central nervous and cardiac systems (reference bronchodilator drug) [1,2].

An assay has been previously reported for the quantification of plasma isbufylline alone [3]. Our method measures isbufylline and its metabolites. This

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chemical-analytical study was set up to perform pharmacokinetic and metabolic studies.

EXPERIMENTAL

Instruments and instrumental conditions

The assay was performed using a high-performance liquid chromatography (HPLC) Beckman System Gold constant-flow pump and a Model 166 UV detector set at 280 nm (San Ramon, CA, USA). The column was a reversed-phase 5-μm Nucleosil ODS (25 cm × 4.6 mm I.D.) (Chrompack, Middelburg, Netherlands). A Shimadzu C-R6A (Kyoto, Japan) integrator was also used.

Reagents

Isbufylline and its derivatives, 1-methyl-7-(2-hydroxy-2-methylpropyl)xanthine (MX/2/013, I), 1,3-dimethyl-7-(2-hydroxy-2-methylpropyl)xanthine (MX/2/004, II), 1-methyl-7-isobutylxanthine (MX/2/011, III), 1,3-dimethyl-7-(3-hydroxy-2-methylpropyl)xanthine (MX/2/017, IV), 3-methyl-7-isobutylxanthine (MX/2/007, V), 1,3-dimethyl-7-isobutyl uric acid (MX/2/006, VI), 3-methyl-7-(2-hydroxy-2-methylpropyl)xanthine (MX/2/014, VII) and 3-methyl-7-isobutyl uric acid (MX/2/012, VIII) were gifts from Malesci (Florence, Italy). 3-Isobutyl-1-methylxanthine (internal standard, I.S.) was obtained from Sigma (St. Louis, MO, USA). All solvents were of UV grade. Acetonitrile was purchased from Omnia Res. (Milan, Italy); the other solvents, N,O-bis(trimethylsilyl)-trifluoracetamide (BSTFA), trimethylchlorosilane and pyridine, were obtained from Pierce (Rockford, IL, USA). Solvent A was a 0.5% solution of acetic acid in deionized water from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Solvent B was 20% acetonitrile in solvent A. Solvents were filtered through a Millipore 0.40-μm filter.

Animal treatment

New Zealand male rabbits, HY/Cr, were purchased from Charles River (Calco, Italy) and were acclimatized to the research facilities, receiving standard laboratory chow and water *ad libitum*. The animals were housed separately in stainless-steel cages in rooms at controlled temperature (20°C), humidity (50%) and light cycle (12–12 h). Intravenous doses of isbufylline, 12 mg/kg in isotonic saline, were administered to five animals (3.0–3.5 kg weight). Blood samples (0.5–1.0 ml) were drawn from a vein (on the ear not used for intravenous injections) in heparinized syringes at different times after dosing. Urine was collected before and from 0 to 48 h after treatment. Samples were stored at −20°C until analysis.

Standard solutions

Stock solutions of compounds (100 μg/ml) in deionized water were prepared and stored at 4°C throughout the study. Stock standards were added to blank

pool of blood and urine to give final concentrations of 12.5 μg/ml. These solutions were further diluted ten-fold with the same blank pool of blood and urine used above, to provide the material for quality control. Each pool was divided into 1-ml samples and frozen at −20°C until analysis. The two sets of samples obtained were analysed over three months to obtain twenty replicates for each set.

Procedure

The concentrations of isbufylline and metabolites I, II and III in blood were quantified after extraction with 7 ml of chloroform–2-propanol (75:25, v/v), 1 ml of acetic acid (0.5%) plus 100 μl of 10 μg/ml I.S. for each 0.2–0.5 ml sample of blood. The mixture was shaken for 10 min, and after centrifugation (10 min at 1100 *g*, the organic layer was transferred and evaporated to dryness at 37°C under a stream of nitrogen. The dried residue (0.2 ml) was reconstituted in acetonitrile–0.25% acetic acid (10:90, v/v). The analysis was performed by linear gradient elution at room temperature; the concentration of acetonitrile in 0.5% acetic acid was increased linearly from 0 to 20% in 20 min. The flow-rate was 1.2 ml/min.

The extraction procedure for urine was slightly modified. After centrifugation at 700 *g* for 10 min and filtration through a 0.2-μm membrane filter type flowpore D (Flow Labs., Sartorius, Germany), urine samples (0.2 ml) were spiked with 3 μg of the I.S. and extracted with methylene chloride (5 ml) plus ammonium sulphate (1 g). The organic layer was evaporated to dryness under a gentle stream of nitrogen at 37°C. The solution used to reconstitute the dried residue (1 ml) was the same as that described for blood.

Identification of compounds

In order to identify the peaks potentially corresponding to the compounds in blood and urine matrices, HPLC elution fractions were collected, using the UV absorption trace on the recorder as a reference. Eluates were derivatized with 50 μl of BSTFA, 5 μl of trimethylchlorosilane and 50 μl of pyridine for 1 h at 60°C, and analysed by mass spectrometry.

Mass spectrometry

A VG 70-250 mass spectrometer (VG Analytical, Manchester, UK) was used in the electron-impact mode. Mass spectra were obtained with the direct inlet system (DIS) or by gas chromatography–mass spectrometry (GC–MS) after derivatization. The gas chromatograph was an HP 5890 and the column was CP Sil 8 CB (25 m × 0.32 mm I.D., film thickness 0.12 mm, Chrompack). Oven temperature was programmed at 80°C for 1 min, then at 10°C/min to 280°C. The ion source temperature was 250°C and the electron energy was 70 eV.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms obtained from various drug-free and spiked urine and blood samples, and samples taken after drug administration.

Peaks 2 and 9 (Fig. 1C) and 10 (Fig. 1F) were analysed with the DIS, and peaks 4 and 5 (Fig. 1C) by GC–MS.

The mass spectrum of peak 5 (Fig. 2A) is identical with that obtained from an

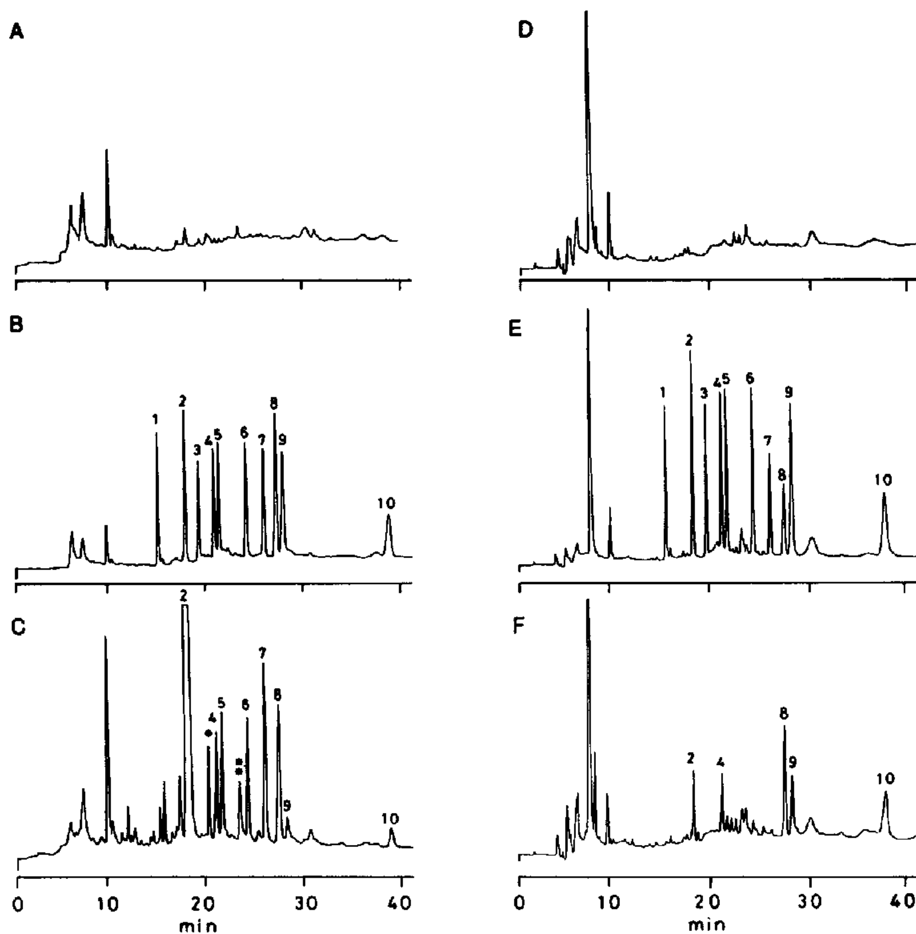

Fig. 1. Chromatograms of blood and urine extracts. (A) Blank urine; (B) blank urine spiked with 0.14 μg of test compounds and 0.21 μg of internal standard; (C) urine sample obtained after isbufylline administration, containing I (95.9 μg/ml), II (5.40 μg/ml), IV (6.10 μg/ml), V (10.00 μg/ml), VI (8.40 μg/ml), III (0.15 μg/ml) and isbufylline (0.30 μg/ml); (D) blank blood; (E) blank blood spiked with 0.20 μg of test compounds; (F) blood sample, obtained after isbufylline administration, containing I (2.56 μg/ml), II (2.26 μg/ml), III (2.71 μg/ml) and isbufylline (2.62 μg/ml). Peaks: 1 = VII; 2 = I; 3 = VIII; 4 = II; 5 = IV; 6 = V; 7 = VI; 8 = I.S.; 9 = III; 10 = isbufylline. Peaks indicated with asterisks were not characterized, but may be metabolites.

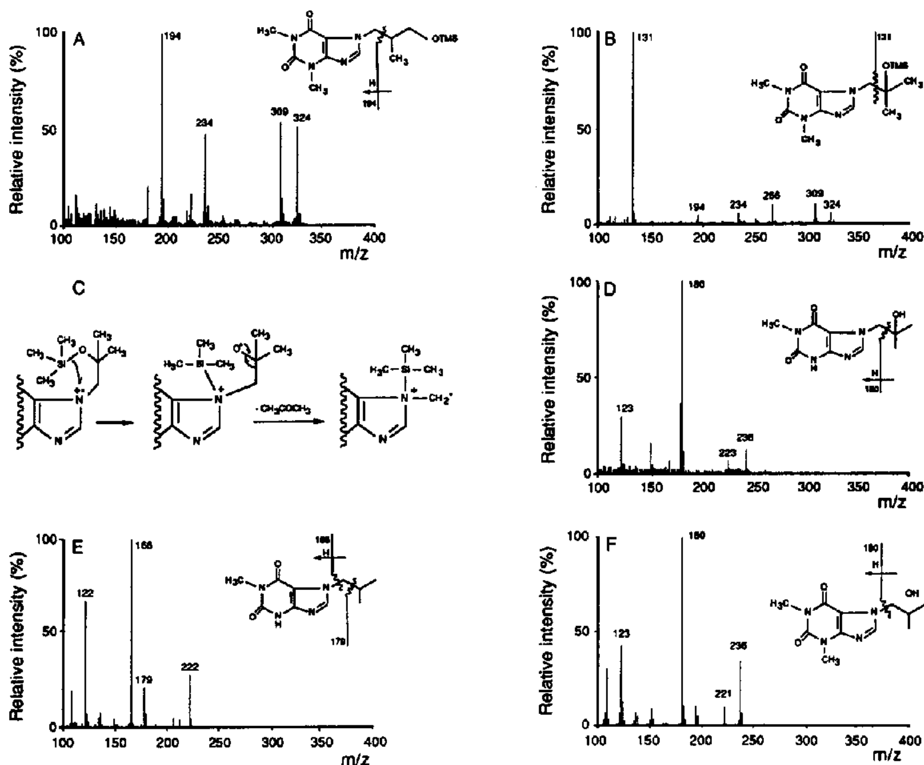

Fig. 2. Mass spectra of (A) peak 5 in Fig. 1C, (B) peak 4 in Fig. 1C, (D) peak 2 in Fig. 1C after derivatization, (E) peak 9 in Fig. 1F and (F) peak 10 in Fig. 1F. (C) Possible fragmentation pathway from ion *m/z* 324 to ion *m/z* 266.

authentic standard of IV, confirming the identification of this compound on the basis of the HPLC retention time. The ion at *m/z* 324 is the molecular ion. Loss of a methyl group gives the ion at *m/z* 309, and the elimination of trimethylsilanol produces the ion at *m/z* 234. The ion at *m/z* 194 is due to the fragmentation shown in Fig. 2A.

The mass spectrum of peak 4 (Fig. 2B) is identical with that of derivative II. It is quite similar to that of the previously examined metabolite, but there are some interesting differences. In particular, the ion at *m/z* 131 is due to the fragmentation shown in Fig. 2B, and is indicative of the hydroxylation site. The ion at *m/z* 266 is present only in this metabolite. It is probably given by loss of acetone (Fig. 2C). Similar results have been previously reported [4,5], where an analogous transfer of the trimethylsilyl residue is involved.

The mass spectrum of peak 2 (Fig. 2D) is identical with that obtained from an authentic standard of I, confirming the identification of this compound on the basis of its HPLC retention time. The ion at *m/z* 238 is the molecular ion. Loss of

TABLE I

PERFORMANCE DATA ON THE HPLC METHOD FOR THE DETERMINATION OF ISBUFYLLINE AND ITS METABOLITES IN BLOOD

Values are means ± S.D. (n = 10), with percentage C.V. values in parentheses. The intra-day precision and accuracy were considered at two concentrations (1.25 and 12.5 μg/ml).

Compound	Reproducibility[a]	Intra-day precision	Accuracy[b] (%)	Linearity[c]	Recovery[d] (%)	Detection limit (ng/ml)
Isbufylline	0.39 ± 0.02 (5.6)	12.96 ± 0.35 (2.7)	1.17 ± 1.63	$y = 0.37x - 0.015$; $r = 0.999$	81 ± 9.3	15
		0.98 ± 0.05 (5.7)	17.94 ± 7.09			
III	0.68 ± 0.03 (5.5)	13.07 ± 0.20 (1.5)	3.75 ± 1.30	$y = 0.68x - 0.075$; $r = 0.999$	103 ± 3.3	5
		1.19 ± 0.06 (5.2)	8.46 ± 6.12			
VI	0.66 ± 0.05 (7.5)	12.08 ± 0.30 (2.3)	4.03 ± 3.72	$y = 0.65x - 0.097$; $r = 0.997$	56 ± 4.5	10
		1.06 ± 0.04 (4.6)	16.53 ± 2.61			
V	1.08 ± 0.04 (3.7)	10.06 ± 0.23 (2.3)	18.50 ± 2.48	$y = 1.04x + 0.047$; $r = 0.990$	100 ± 2.3	5
		1.15 ± 0.02 (2.0)	11.04 ± 4.36			

IV	1.05 ± 0.07 (7.4)	3.33 ± 0.20 (1.5)	6.99 ± 3.97	$y = 1.11x - 0.095; r = 0.999$	100 ± 2.0	5
		1.25 ± 0.09 (7.6)	10.10 ± 9.53			
II	0.93 ± 0.06 (7.3)	12.98 ± 0.32 (2.4)	4.09 ± 3.07	$y = 0.95x + 0.007; r = 0.999$	104 ± 2.9	5
		1.18 ± 0.12 (10.0)	7.44 ± 5.24			
VIII	0.55 ± 0.02 (4.7)	11.87 ± 0.22 (1.8)	7.21 ± 2.38	$y = 0.51x + 0.007; r = 0.999$	67 ± 6.1	10
		1.06 ± 0.05 (5.4)	9.92 ± 3.47			
I	0.77 ± 0.01 (2.0)	13.75 ± 0.63 (4.5)	8.07 ± 3.72	$y = 0.79x + 0.060\ r = 0.999$	88 ± 8.7	<5
		1.28 ± 0.13 (10.1)	8.88 ± 5.61			
VII	1.02 ± 0.03 (2.7)	12.93 ± 1.57 (12.1)	7.06 ± 4.44	$y = 1.04x - 0.097; r = 0.999$	86 ± 7.6	5
		1.27 ± 0.07 (5.5)	4.71 ± 2.87			

[a] C.V. of the slopes of standard curves.
[b] Percentage error from the true value (1.25 and 12.5 μg/ml).
[c] One of the representative individual standard curves.
[d] Percentage of four replications at 0.5, 1, 5, 10 and 20 μg/ml.

TABLE II

PERFORMANCE DATA ON THE HPLC METHOD FOR THE DETERMINATION OF ISBUFYLLINE AND ITS METABOLITES IN URINE

Values are means ± S.D. (n = 10), with percentage C.V. values in parentheses. The intra-day precision and accuracy were considered at two concentrations (1.25 and 12.5 μg/ml).

Compound	Reproducibility[a]	Intra-day precision	Accuracy[b] (%)	Linearity[c]	Recovery[d] (%)	Detection limit (ng/ml)
Isbufylline	0.13 ± 0.01 (7.4)	12.74 ± 0.21 (1.6) 1.37 ± 0.05 (4.1)	3.81 ± 2.33 5.73 ± 3.80	$y = 0.13x + 0.002$ $r = 0.996$	98 ± 4.6	20
III	0.32 ± 0.01 (1.8)	12.22 ± 0.17 (1.4) 1.29 ± 0.04 (3.3)	2.85 ± 2.03 4.35 ± 2.37	$y = 0.33x - 0.002$; $r = 0.999$	95 ± 7.0	15
VI	0.34 ± 0.01 (1.4)	12.24 ± 0.24 (2.0) 1.25 ± 0.04 (3.2)	2.99 ± 1.72 4.30 ± 3.20	$y = 0.34x$; $r = 0.999$	88 ± 6.9	10
V	0.36 ± 0.01 (2.7)	12.03 ± 0.73 (6.1) 1.28 ± 0.02 (1.9)	5.26 ± 4.90 3.11 ± 2.17	$y = 0.37x + 0.002$; $r = 0.999$	101 ± 1.9	10
IV	0.35 ± 0.21 (5.9)	12.5 ± 0.15 (1.2) 1.30 ± 0.02 (1.3)	5.01 ± 2.12 7.52 ± 3.94	$y = 0.35x + 0.008$; $r = 0.998$	102 ± 4.9	10

II	0.34 ± 0.02 (5.0)	12.81 ± 0.22 (1.7)	3.12 ± 2.11	$y = 0.35x + 0.015$; $r = 0.997$	96 ± 5.5	10
		1.24 ± 0.04 (3.8)	4.46 ± 2.26			
VIII	0.31 ± 0.02 (6.7)	13.00 ± 0.72 (5.5)	11.71 ± 6.48	$y = 0.34x + 0.007$; $r = 0.997$	25 ± 4.2	15
		1.44 ± 0.05 (3.9)	13.42 ± 4.40			
I	0.48 ± 0.02 (4.6)	12.67 ± 0.06 (0.5)	2.80 ± 1.87	$y = 0.47x + 0.022$; $r = 0.999$	93 ± 5.1	5
		1.46 ± 0.10 (6.8)	8.8 ± 2.81			
VII	0.42 ± 0.01 (1.4)	11.58 ± 0.24 (2.1)	5.40 ± 2.80	$y = 0.43x - 0.005$; $r = 0.999$	98 ± 4.8	10
		1.26 ± 0.05 (4.5)	3.91 ± 2.17			

[a] C.V. of the slopes of standard curves.
[b] Percentage error from the true value (1.25 and 12.5 μg/ml).
[c] One of the representative individual standard curves.
[d] Percentage of four replications at 0.5, 1, 5, 10 and 20 μg/ml.

a methyl group gives the ion at *m/z* 223. The ion at *m/z* 180 is probably due to the fragmentation shown in Fig. 2D. Further loss of CH_3–N=C=O probably produces the ion at *m/z* 123.

The mass spectrum of peak 9 is identical with that of III (Fig. 2E). The molecular ion is at *m/z* 222. The ions at *m/z* 179 and 166 are probably due to progressive fragmentation of the aliphatic chain. The ion *m/z* 122 probably originates from the loss of CH_3–N=C=O from the ion at *m/z* 179.

The mass spectrum of the peak 10 is identical with that obtained from an authentic standard of the drug, confirming the identification of this compound on the basis of its HPLC retention time. The ion at *m/z* 236 is the molecular ion. Loss of a methyl group gives the ion at *m/z* 221. The ion at *m/z* 180 probably has the structure indicated in Fig. 2F. Further loss of CH_3–N=C=O probably produces the ion at *m/z* 123. These last two ions are similar to those described in Fig. 2D and E.

No interfering peaks were found in the HPLC chromatograms at retention times close to those of the compounds measured (capacity ratio $k'_{I} = 4.13$, $k'_{II} = 5.01$, $k'_{IV} = 5.12$, $k'_{V} = 5.93$, $k'_{VI} = 6.44$, $k'_{III} = 6.99$, $k'_{isbufylline} = 9.79$, $k'_{I.S.} = 6.82$). In Fig. 1C, there are two peaks that indicate two possible metabolites that have not been characterized, with capacity ratios of 4.69 and 5.80, respectively.

Reproducibilities were expressed as the coefficients of variation (C.V.) of the slopes of blood and urine standard curves. The day-to-day assay precision over three months was assessed by determining the C.V. at 1.25 μg/ml ($n = 10$) and 12.5 μg/ml ($n = 10$). The accuracy was calculated as the percentage error from the true value on processing samples containing 1.25 μg/ml ($n = 10$) and 12.5 μg/ml ($n = 10$). Calibration graphs were drawn daily in duplicate at 0.5, 1, 2, 4, 10 and 20 μg/ml; they passed through the origin and were linear for each compound, both for blood and urine. The recovery for isbufylline and its metabolites from blood and urine was calculated at 0.5, 1, 5, 10 and 20 μg/ml (four replications for each concentration) by comparing chromatographic peak areas of isbufylline and metabolites in blood and urine samples with peak areas obtained by direct injection of equal amounts of the compounds in solvent. The results are shown in Tables I and II for blood and urine, respectively. The analytical recovery and the detection limit at a signal-to-noise ratio of 3:1 are also reported in Table I and II for each compound, as are the precision and accuracy of the assay.

Although the time for analysis is longer than previous methods, data are obtained for both isbufylline and its metabolites, which is a significant improvement. Using the procedure described, the lifespan of the analytical column averaged 1000 h so the cost is contained. Although not reported here, similar performance was obtained when other sample matrices, such as plasma and red blood cells, were processed. An example of the potential use of the method is reported in Fig. 3, which summarizes the preliminary data of the kinetic and metabolic profile of isbufylline in rabbits after 12 mg/kg intravenous administration. No up to date data are available on the disposition profile of this com-

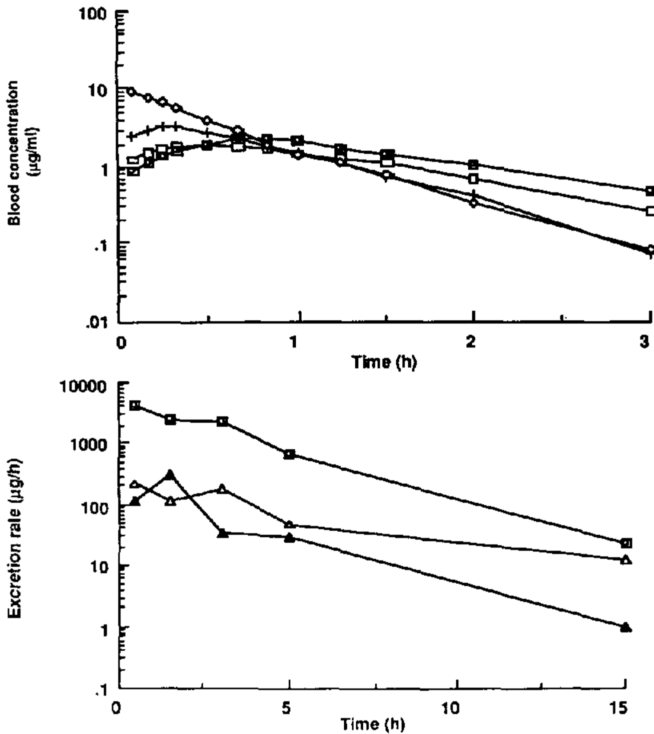

Fig. 3. Semilogarithmic plot *versus* time of mean blood concentrations of isbufylline (◇), I (⊞), II (□) and III (+) and the mean urinary excretion rates of I (⊞), IV (△), VI (▲), after intravenous injection of isbufylline (12 mg/kg) in rabbits.

pound and its metabolites, although they resemble those of theophylline [6]. The metabolites V and VI appear in the urine but are not detected in the blood, probably because their elimination is formation rate-limited during the decline phase.

The method described, routinely used in our laboratory, offers simplicity and requires only common instruments. Small samples are needed, and different matrices can be processed with the same performance to determine isbufylline and its metabolites.

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