Journal of Chromatography, 496 (1989) 478–484
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4964

Note

Determination of muzolimine in plasma and urine by high-performance liquid chromatography

M A OSMAN*, L K DUNNING, V P BHAVNAGRI and L K CHENG

Drug Metabolism Department, A H Robins Company, 1211 Sherwood Avenue, Richmond, VA 23220 (U S A)

(First received May 31st, 1989, revised manuscript received July 13th, 1989)

Muzolimine, 5-amino-2-[1-(3,4-dichlorophenyl)ethyl]-2,4-dihydro-3H-pyrazol-3-one (Fig 1), is a potent 'high-ceiling' diuretic [1–3]. It produces a distinct diuretic effect even in cases of far advanced renal insufficiency [4]. The only reported method to assay the drug from biological fluids has been a thin-layer densitometric determination method [5,6].

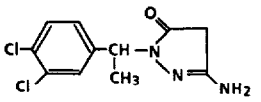
In order to support preclinical and clinical pharmacokinetic studies, a selective high-performance liquid chromatographic (HPLC) method for muzolimine in plasma and urine was developed The method was successfully applied to animal studies. In this method, cysteine was added to plasma and urine to stabilize muzolimine as described in previous methods

EXPERIMENTAL

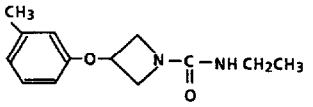
Reagents

Acetonitrile, monobasic sodium phosphate and phosphoric acid were all supplied by Baker (Phillipsburg, NJ, U.S A) Hexane and methylene chloride (analytical-reagent grade) were supplied by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Muzolimine was provided by Bayer (Wuppertal, F R G), and internal standards (Fig. 1) were synthesized in house. L-Cysteine was purchased from Sigma (St. Louis, MO, U.S.A.).

Muzolimine



Internal Standard for Plasma



Internal Standard for Urine

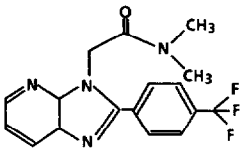


Fig 1 Chemical structures of muzolimine and the internal standards

High-performance liquid chromatography

The HPLC system consisted of a solvent delivery system (Waters Model 510; Waters Assoc., Milford, MA, U.S.A.), an autosampler (Waters Model 710B) and a spectrophotometric detector (Waters Model 490).

The chromatographic separation was achieved on a 10-μm, 30 cm$\times$3 9 mm I.D C_{18} reversed-phase μBondapak column (Waters) for plasma and a 5-μm, 15 cm$\times$3 9 mm I.D. reversed-phase phenyl Nova Pak column (Waters) for urine. The column was kept at ambient temperature (approximately 22°C). The mobile phase used for plasma was acetonitrile–0.05 *M* phosphate buffer pH 4.2 (45 55, v/v) and for urine acetonitrile–0.05 *M* phosphate buffer pH 4.2 (28 72, v/v), and it was delivered at a flow-rate of 1.5 ml/min. The compounds eluted were detected by a spectrophotometric detector at 272 nm. The output signal generated by the spectrophotometric detector was processed by a computer automated laboratory system (Computer Inquiry Systems, Walwick, NJ, U.S.A. and Hewlett-Packard Computer Model 1000, Palo Alto, CA, U.S.A.). The chromatographic tracings were recorded on a 10-mV chart recorder (Hewlett-Packard).

Standard solutions

Stock solutions, corresponding to 200 μg/ml in methanol, were prepared for muzolimine and the internal standard. Standard solutions for muzolimine were prepared at concentrations ranging from 0.05 to 10 μg/ml for plasma and from

0.25 to 10 μg/ml for urine by spiking drug-free plasma or urine with the appropriate concentrations of the drug. Cysteine was added to drug-free plasma and urine at 4 mg/ml to keep muzolimine from degrading. Plasma and urine standard samples were immediately frozen after preparation and kept at −20°C until assayed. The stock solutions of the internal standards were diluted in water to 200 μg/ml before being used to spike plasma or urine samples

Extraction procedure

A 1-ml volume of urine or 0.5 ml of plasma of the standard or unknown sample was transferred into a 16 mm × 125 mm culture tube containing 0.1 ml of 200 μg/ml internal standard solution A 5-ml mixture of hexane–methylene chloride (6·4, v/v) was added to each tube The solutions were mixed vigorously on a vortex-mixer for 30 s and centrifuged at 550 *g* for 5 min. The organic phase was transferred to a clean 15-ml centrifuge tube and evaporated to dryness with a stream of nitrogen at 60°C, and the residue was reconstituted in 0.2 ml of mobile phase. A 100-μl volume of the solution was then injected into the HPLC system.

Precision, reproducibility and accuracy studies

To test the precision and reproducibility of this method, six standard curves for muzolimine in plasma and urine were run on consecutive working days. The ranges of the curves were 0–10 μg/ml. The coefficient of variation of the peak-height ratio was determined at each concentration level of the standard curve. The slope, intercept and the correlation coefficient of the daily standard curves were also calculated

The accuracy of the method was determined by assaying thirty randomized samples for plasma and urine prepared with various concentrations of muzolimine.

Pilot study in dog

A pilot study was conducted in dogs to evaluate the analytical method. After an overnight fast, a male beagle dog was dosed with 1 mg/kg muzolimine dissolved in water. Blood and urine samples were collected over 6 h after dosing. Plasma was separated from the red blood cells immediately and transferred to clean tubes containing cysteine (4 mg/ml) within 3 min after blood collection The samples were immediately frozen and analyzed for muzolimine within two weeks. The volume of urine samples was measured, and cysteine was added (4 mg/ml). Then the samples were immediately frozen and kept at −20°C

RESULTS AND DISCUSSION

Typical chromatograms for drug-free plasma and urine are shown in Figs. 2 and 3, respectively. Both chromatograms showed no interfering peaks at the retention times of muzolimine and the internal standard.

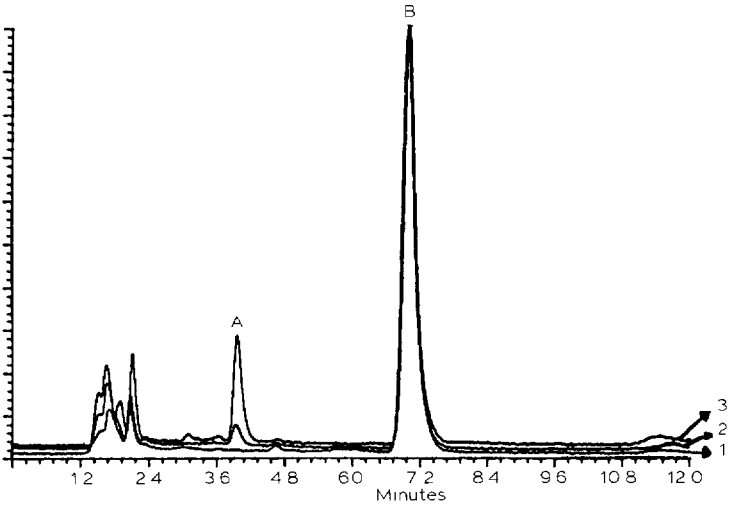


Fig 2 Multiple plot of three chromatograms obtained from extracts of (1) drug-free plasma spiked with 0 μg/ml muzolimine and 2 μg internal standard, (2) drug-free plasma spiked with 0 1 μg/ml muzolimine and 2 μg internal standard and (3) plasma sample collected from a dog at 0 17 h after oral dosing with 1 mg/kg muzolimine Peaks A = muzolimine (0 45 μg/ml), B = internal standard (2 μg)

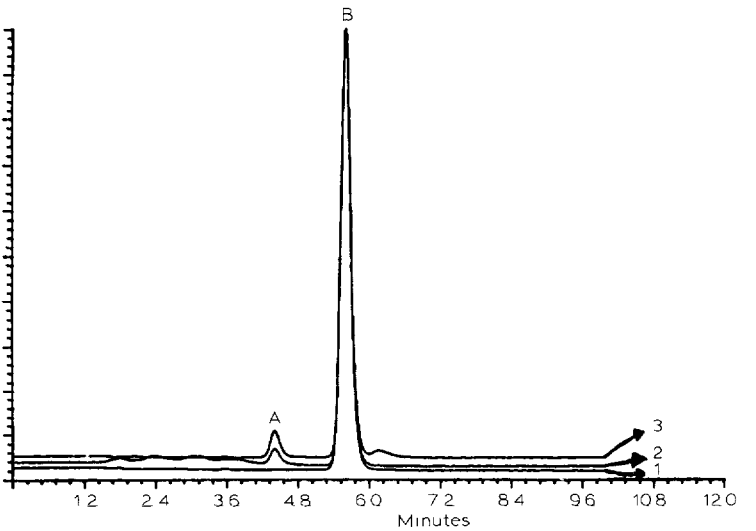


Fig 3 Multiple plot of three chromatograms obtained from extracts of (1) drug-free urine spiked with 0 μg/ml muzolimine and 2 μg internal standard, (2) drug-free urine spiked with 0 25 μg/ml muzolimine and 2 μg internal standard and (3) urine sample collected from a dog after oral dosing with 1 mg/kg muzolimine Peaks A = muzolimine (0 45 μg/ml), B = internal standard (2 μg)

The results of the six consecutive standard curves for muzolimine were linear between 0 and 10 μg/ml with a slope for plasma of 0.795 ± 0 04 and for urine of 0 139 ± 0.01. Good reproducibility of the method was reflected by the correlation coefficient values, 0.9987 ± 0 002 (mean ± S D) for the plasma standard curve and 0 9981 ± 0.0013 for the urine standard curve. The intercept values of the regression lines were always negligible. The below quantifiable limit for plasma is 0.05 μg/ml and for urine it is 0.25 μg/ml. The peak height of the curves for muzolimine showed a coefficient of variation that was below 10% in the concentration range 0–10 μg/ml. The extraction efficiency of muzolimine by the procedure used in this method was 84%

Tables I and II show the recovery values for the thirty spiked plasma and urine samples. The percentage muzolimine found was generally within 10% of the theoretical concentration added.

This method was employed in the analysis of plasma and urine samples obtained from a dog study Figs 2 and 3 show extracts of plasma and urine sam-

TABLE I

DETERMINATION OF UNKNOWN AMOUNTS OF MUZOLIMINE ADDED TO PLASMA

Concentration added (μg/ml)	*n*	Concentration found (mean ± S D) (μg/ml)	Coefficient of variation (%)	Found (%)
0 00	4	BQL[a]	-	-
0 064	6	0 06 ± 0 014	23 6	93 8
0 13	4	0 14 ± 0 19	13 2	107 7
1 28	6	1 19 ± 0 083	7 0	93 3
5 10	4	4 89 ± 0 110	2 3	95 8
10 2	6	10 2 ± 0 816	8 1	100 0

[a]BQL = Below quantifiable limit, i e concentration < 0 05 μg/ml

TABLE II

DETERMINATION OF UNKNOWN AMOUNTS OF MUZOLIMINE ADDED TO URINE

Concentration added (μg/ml)	*n*	Concentration found (mean ± S D) (μg/ml)	Coefficient of variation (%)	Found (%)
0 00	5	BQL[a]	-	-
0 32	5	0 34 ± 0 5	14 3	106 3
1 60	5	1 57 ± 0 18	11 8	97 9
3 20	5	3 28 ± 0 15	4 5	102 3
6 40	5	6 27 ± 0 31	4 9	98 0
8 00	5	7 82 ± 0 25	3 2	97 8

[a]BQL = Below quantifiable limit, i e concentration < 0 25 μg/ml

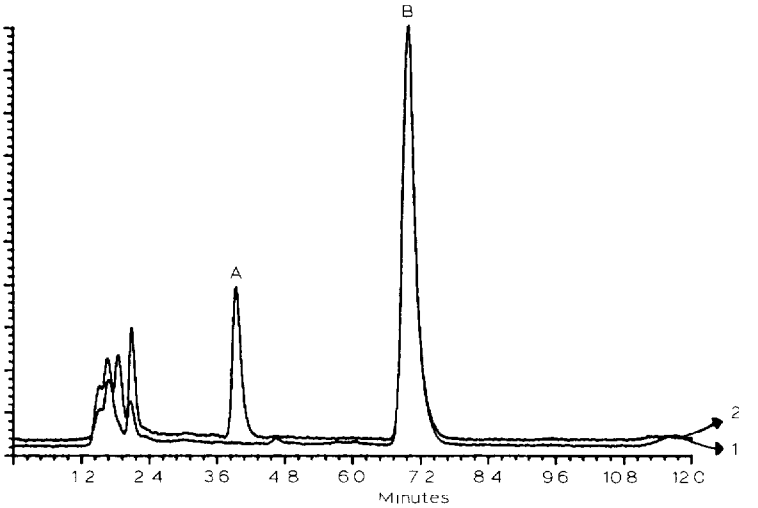


Fig 4 Multiple plot of two chromatograms obtained from extracts of (1) plasma sample collected before dosing a healthy volunteer with a 40-mg capsule of muzolimine (A = 0 μg/ml muzolimine, B = 2 μg internal standard) and (2) plasma sample collected at 2 h after dosing (A = 0 6 μg/ml muzolimine, B = 2 μg internal standard)

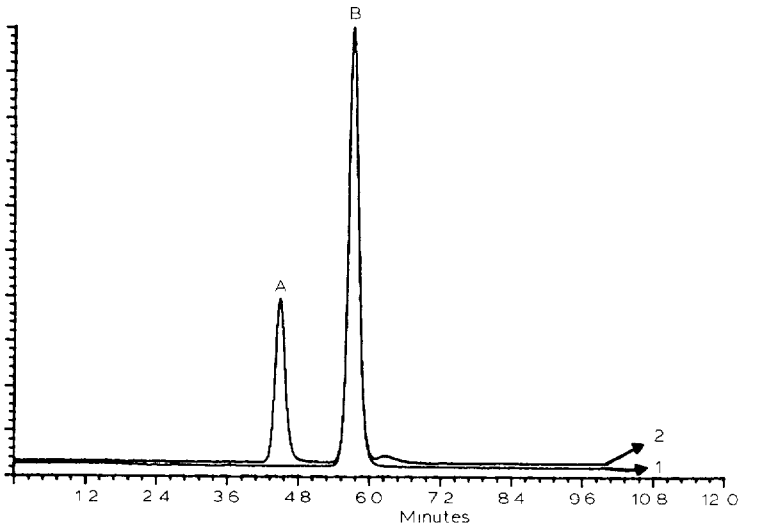


Fig 5 Multiple plot of two chromatograms obtained from extracts of (1) urine sample collected before dosing a healthy volunteer with a 40-mg capsule of muzolimine (A = 0 μg/ml muzolimine, B = 2 μg internal standard) and (2) urine sample collected after dosing (A = 2 83 μg/ml muzolimine, B = 2 μg internal standard)

ples obtained from the dog. Figs 4 and 5 show chromatograms of plasma and urine extracts of samples obtained from healthy human volunteers dosed with 40 mg of muzolimine. Fig. 6 shows a drug concentration–time profile for mu-

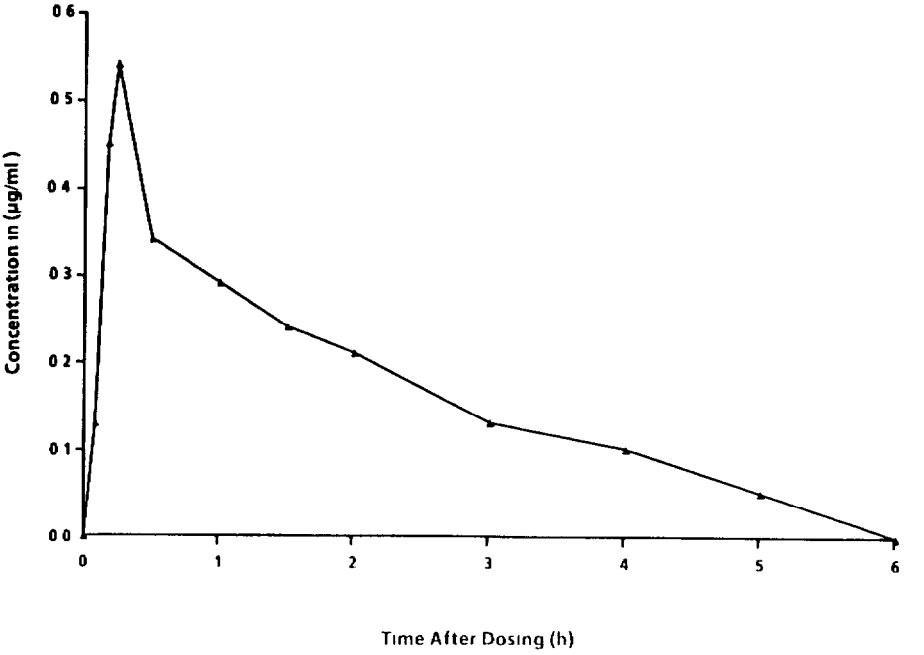


Fig 6 Plasma concentration–time curve of muzolimine after a single oral dose of 1 mg/kg muzolimine in dog

zolimine in one of the dogs used in the study. This method has a linear range of 0–10 μg/ml which was intended to cover all the expected sample concentrations generated from studies on low, high and multiple doses of muzolimine in animal and human studies.

CONCLUSIONS

A sensitive and selective HPLC method was developed for the determination of muzolimine in plasma and urine. The concentration–response curve was linear from 0 to 10 μg/ml. The method has a high degree of precision and accuracy It was utilized successfully in the analysis of plasma and urine samples obtained from animal and human bioavailability studies.

REFERENCES

1 D Loew, Curr Med Res Opin , 4 (1976) 455
2 D Loew, W Ritter and J Dycka, Eur J Clin Pharmacol , 12 (1977) 341
3 E Moller, H Horstmann, K Meng and D Loew, Experientia, 33 (1977) 382
4 P Schmidt, D Loew, J Dycka, H Kopsa, P Balcke, J Zazornik and E Deutsch, Eur J Clin Pharmacol , 20 (1981) 23
5 W Ritter, J Chromatogr , 142 (1977) 431
6 W Ritter, Methodol Surv Biochem Anal , 12 (1983) 231