Journal of Chromatography, 574 (1992) 319–325
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6185

Determination of the tricyclic compound adosupine and its three metabolites in plasma and brain of rat using high-performance liquid chromatography

V. D'Aranno*, A. Mancinelli☆ and S. Manzini

Department of Pharmacology, Menarini Ricerche Sud, Via Tito Speri 10, 00040 Pomezia, Rome (Italy)

(First received August 12th, 1991; revised manuscript received October 17th, 1991)

ABSTRACT

An analytical method for the detection in biological samples of the novel tricyclic compound adosupine (10-acetoamido-5-methyl-5,6-dihydro-11H-dibenzo[*b,e*]azepin-6,11-dione), which is capable of influencing various forms of urinary bladder hyperreflexia has been developed using high-performance liquid chromatography with UV detection. Liquid–liquid extraction was used to isolate the parent compound, three metabolites and an analogue (added as internal standard) from plasma and brain of rat. Adosupine was well separated from its three metabolites with 0.01 *M* disodium hydrogenphosphate–acetonitrile–methanol–nonylamine (59.986:38:2:0.014) at pH 4.5 as mobile phase using a C_{18} reversed-phase column. The standard curves were linear in the range 50–5000 ng/ml (or ng/g) for adosupine and metabolites in both plasma and brain. The between- and within-assay variations for high and low concentrations of the parent compound and the three metabolites were 8.2–14%. In the range 50–5000 ng/ml (or ng/g) the accuracy of the method was satisfactory, with the relative error always lower than 10%. Analytical recoveries of added adosupine and the three metabolites were higher than 82%. The method has been applied successfully, to investigate the pharmacokinetics of the drug and its distribution in the central nervous system of rats.

INTRODUCTION

Adosupine (10-acetoamido-5-methyl-5,6-dihydro-11*H*-dibenzo[*b,e*]azepin-6,11-dione) is a tricyclic compound which has been found to affect experimental urinary hyperreflexia in rats by acting at sites in the central nervous system (CNS), thus making it a potential candidate for clinical use in urinary incontinence [1]. Analytical methods to analyse compounds similar to adosupine, such as tricyclic antidepressants, are described in the literature [2]. Sensitive analytical methods use gas chromatography with nitrogen–phosphorus detection [3,4] or high-performance liquid chromatography (HPLC) with UV [5], fluorescence [6] or electrochemical detection [7,8].

In order to investigate the pharmacological activity of adosupine and to evaluate its pharmacokinetic profile during short and long-term studies in animals, we developed an analytical method to determine adosupine and three of its identified metabolites, termed M1, M2 and M3 (Fig. 1) in plasma and brain of rat, using HPLC with UV detection.

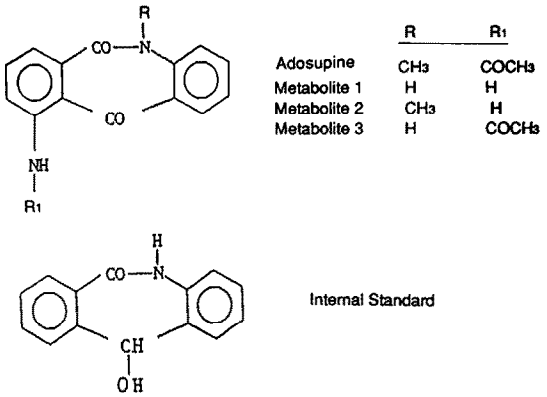
EXPERIMENTAL

Reagents and materials

Adosupine metabolite 1 (M1) (10-amino-5*H*-6,11-dihydro-dibenzo[*b,e*]azepin-6,11-dione), metabolite 2 (M2) (10-amino-5-methyl-5,6-dihydro-11*H*[*b,c*]azepin-6,11-dione), metabolite 3

☆ Present address: Sigma-Tau, Via Pontina Km 30.400-0040-Pomezia, Rome, Italy.

0378-4347/92/$05.00



	R	R_1
Adosupine	CH_3	$COCH_3$
Metabolite 1	H	H
Metabolite 2	CH_3	H
Metabolite 3	H	$COCH_3$

Fig. 1. Molecular structures of adosupine, metabolites M1, M2, and M3, and the internal standard.

(M3) (10-acetoamido-5*H*-6,11-dihydro-dibenzo-[*b*,*e*]azepin-6,11-dione), and the internal standard (I.S.) (11-hydroxy-5*H*-5,6-dihydro-dibenzo[*b*,*e*]-azepin-6-one) were synthesized by the Department of Chemistry, Menarini (Firenze, Italy).

Disodium hydrogenphosphate 12-hydrate, citric acid, hydrochloric acid, orthophosphoric acid, ethyl acetate, methanol, acetonitrile and benzene were purchased from Merck (Darmstadt, Germany), and nonylamine from Fluka (Buchs, Switzerland).

Solvents were of chromatographic grade. Water was bidistilled and filtered through a 0.4-μm Nucleopore filter.

Standards

Stock solutions were made by dissolving adosupine, M1, M2, M3 and I.S. in acetonitrile (1 mg/ml). They were stored at 4–6°C.

Working solutions of adosupine and the metabolites were prepared by appropriate dilution in acetonitrile of stock solutions to 5, 10, 20, 50, 100 and 250 μg/ml. The working solution of I.S. was prepared by diluting the stock solution with acetonitrile to 100 μg/ml. Stock and working solutions were prepared every 2 months.

Instrumentation and chromatographic conditions

The HPLC system consisted of an HPLC pump (Model 114, Beckman) equipped with an automatic sampler injector (Model Promis, Spark Holland), an RP-18 column (25 cm × 3.9 mm I.D., 5 μm particle size) (Brownlee Labs.) protected by an RP-18 precolumn (15 × 3.2 mm I.D., 7 μm) (Brownlee Labs.) and a UV detector (Model 165, Beckman) operated at a wavelength of 240 nm. A laboratory computing integrator (Model 3392, Hewlett-Packard) was used to calculate peak areas.

The mobile phase was 0.01 *M* disodium hydrogenphosphate–acetonitrile–methanol–nonylamine (59.986:38:2:0.014). The eluent was adjusted with 83% (w/v) of orthophosphoric acid to pH 4.5, and the flow-rate was 1.0 ml/min. The column and precolumn were heated to 38°C.

Sample preparation

Extraction from plasma. To 0.5–1 ml of plasma samples were added 1 ml of buffer (disodium hydrogenphosphate (0.2 *M*–citric acid (0.1 *M*) at pH 8, 40 μl of the working solution of I.S. (100 μg/ml) and 4 ml of ethyl acetate. The mixture was shaken for 20 min. After 15 min of centrifugation at 4270 *g* at 4°C, the organic phase was transferred to a conical glass tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The dry residue was reconstituted with 100 μl of acetonitrile, and 10 μl were injected into the HPLC column.

Extraction from brain. A half brain was homogenized in 2.5 ml of buffer (disodium hydrogenphosphate (0.2 *M*)–citric acid (0.1 *M*) at pH 8 containing 80 μl of I.S. working solution (100 μg/ml). After the addition of 6.5 ml of benzene, the samples were shaken for 30 min. After centrifugation (20 min at 4270 *g*, at 4°C), the organic phase was transferred to a glass tube containing 1 ml of 0.1 *M* HCl. After shaking and centrifugation, the organic phase was transferred to a conical glass tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The dry residue was reconstituted with 100 μl of acetonitrile, and 10 μl were injected into the HPLC column.

Recovery

The recoveries of adosupine and its metabo-

lites from plasma and brain were determined by adding known amounts of standards to drug-free samples in the range 0.1–5 μg, and comparing the peak areas obtained after extraction with those obtained after the injection of various concentrations of adosupine and related metabolites into the chromatographic column.

Calibration curves

Standard curves were constructed with 1 ml of plasma or a half brain (*ca.* 1 g) of untreated rat containing 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 μg of adosupine and its metabolites (M1, M2, M3). Samples were extracted as described above. The standard curves were defined by linear least-squares regression analysis. Concentrations of adosupine and its metabolites in plasma or brain samples were calculated by plotting the ratio of the peak area of adosupine or the metabolite to the peak area of I.S. on the appropriate standard curve.

Animal treatment and plasma and brain collection

Male rats were treated orally with 50 mg/kg of adosupine. Four animals for each time were sacrificed by decapitation at 5, 15, 30, 60, 90, 120, 180, 240, 300, 360, 420, 480, 540, and 600 min after administration. Blood samples were collected in plastic heparinized tubes and centrifuged, and the plasma was frozen until analysis. Brains were removed, dissected longitudinally and stored at −20°C until analysis.

For the distribution studies in the CNS, male rats were treated orally with 40 mg/kg of adosupine. Six animals for each time were sacrificed by decapitation at 60 and 180 min after administration, the brain and spinal cord were removed and dissected and various areas of the CNS were collected and stored at −20°C until analysis.

RESULTS AND DISCUSSION

Fig. 2a shows a representative chromatogram of the standards of adosupine and its three identified metabolites M1, M2 and M3; the I.S. peak is shown in Fig. 2c. They gave symmetrical and well separated peaks with retention times of 3.6, 4.2, 5, 6 and 7.8 min for M3, I.S., M1, adosupine and M2, respectively. To improve the separation of adosupine from M1 it was necessary to heat the chromatographic column at 38°C, because at room temperature these two peaks coeluted. For the extraction of adosupine and its metabolites from the brain, we had to use the hazardous solvent benzene because other solvents, such as hexane or ethyl acetate, gave unsatisfactory results.

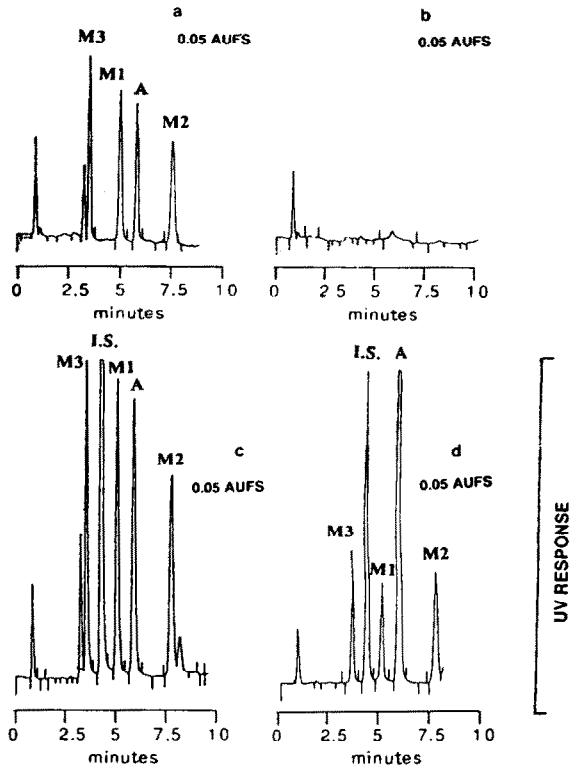


Fig. 2. Chromatograms of (a) 25 ng of standard of M3, M1, adosupine (A) and M2 with retention times of 3.6, 5, 6 and 7.8 min respectively; (b) plasma sample from an untreated rat; (c) plasma sample treated at the beginning of the extraction with 500 ng each of adosupine, M1, M2, and M3 and 4 μg of internal standard; (d) plasma sample from a rat orally treated with 50 mg/kg of adosupine and sacrificed 1 h after the administration. The concentrations of adosupine, M1, M2 and M3 are 3.98, 0.64, 1.1 and 0.79 μg/ml, respectively.

Blank plasma or brain samples were processed through the complete extraction procedure and examined by HPLC. At a signal-to-noise ratio of 3, there were no interfering compounds eluting at the retention times of adosupine, M1, M2, M3 and I.S. either in plasma and brain (Figs. 2b, 3a).

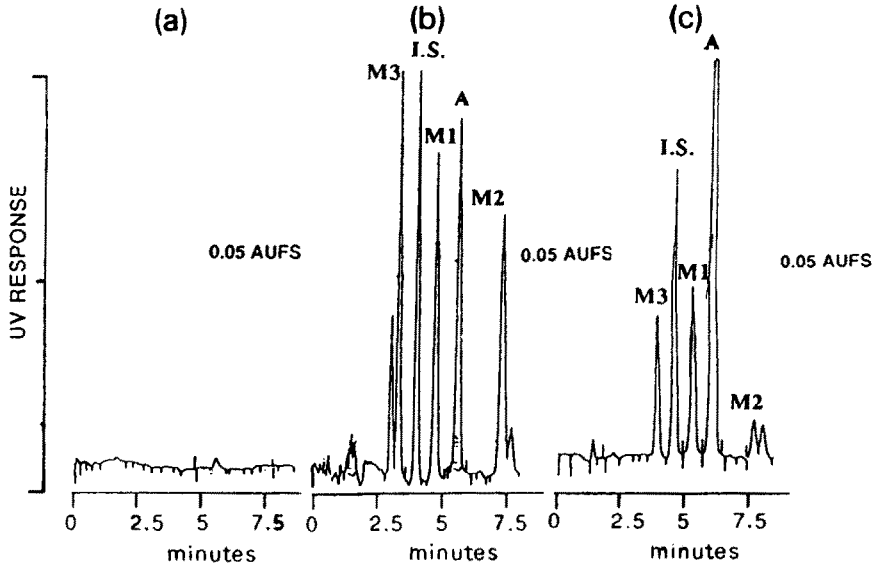


Fig. 3. Chromatograms of (a) brain sample from an untreated rat; (b) brain sample treated at the beginning of the extraction with 500 ng each of adosupine, M1, M2, and M3 and 8 μg of I.S.; (c) brain sample from a rat orally treated with 50 mg/kg of adosupine and sacrificed 1 h after the administration. The concentrations of adosupine, M1, M2 and M3 are 3.39, 0.9, 0.55 and 0.55 μg/g, respectively.

The detection limit was 50 ng/ml (ng/g) for adosupine, M1, M2 and M3 both in plasma and brain (Figs. 2c and 3b). The determination of linearity of the method in the range 50–5000 ng/ml (ng/g) for adosupine, M1, M2 and M3 produced coefficients of determination (r^2) of at least 0.999 for both plasma and brain samples.

The precision and the accuracy of the method were determined by adding known amounts (50–5000 ng/ml or ng/g) of adosupine and its three metabolites to drug-free plasma or brain, which was divided and stored at −20°C. Three samples were determined simultaneously (within) and seven on different days (between). The results are expressed by the coefficient of variation (C.V.) of the between- and within-assay (precision) (Tables I and II) and by the relative error (accuracy) (Tables III and IV).

TABLE I

BETWEEN- AND WITHIN-ASSAY VARIATIONS FOR THE DETERMINATION OF ADOSUPINE AND ITS METABOLITES IN RAT PLASMA

Values are C.V. %.

Concentration (ng/ml)	Adosupine		M1		M2		M3	
	Between	Within	Between	Within	Between	Within	Between	Within
50	8.1	7.3	8.1	7.7	8.2	7.8	4.1	7.0
100	6.7	9.4	6.1	9.7	7.2	9.1	3.6	8.4
250	5.1	9.2	5.1	6.7	7.1	11.0	2.7	7.9
500	4.3	5.4	3.1	3.8	6.7	7.8	3.0	7.9
1000	4.4	6.3	3.1	4.0	6.4	6.2	2.2	7.0
5000	3.2	1.8	3.1	3.0	6.3	2.2	1.7	3.1

TABLE II

BETWEEN- AND WITHIN-ASSAY VARIATIONS FOR THE DETERMINATION OF ADOSUPINE AND ITS METABOLITES IN RAT BRAIN

Values are C.V. %.

Concentration (ng/ml)	Adosupine		M1		M2		M3	
	Between	Within	Between	Within	Between	Within	Between	Within
50	7.1	11.0	6.1	6.3	8.2	11.0	4.8	12.5
100	6.7	7.5	5.2	14.4	7.7	12.3	4.8	14.0
250	5.4	10.0	5.3	11.0	6.6	10.0	3.7	10.0
500	5.1	10.8	4.7	4.9	6.5	8.7	3.6	9.5
1000	5.1	6.2	4.4	5.3	5.9	9.2	3.2	5.5
5000	4.3	2.0	4.0	2.5	5.0	3.6	3.6	2.7

TABLE III

ACCURACY FOR THE DETERMINATION OF ADOSUPINE AND ITS METABOLITES IN RAT PLASMA

Amount added (ng/ml)	Adosupine		M1		M2		M3	
	Found (ng/ml)	R.E.[a] (%)	Found (ng/ml)	R.E. (%)	Found (ng/ml)	R.E. (%)	Found (ng/ml)	R.E. (%)
50	46	−8	52	4	51	2	46	−8
100	93	−7	109	9	96	−4	102	2
250	250	0	263	5.2	256	2.4	250	0
500	465	7	530	6	500	0	483	−3.4
1000	1000	0	1001	1	1004	0.4	983	−1.7
5000	4850	−3	5001	0.2	5000	0	4900	−2

[a] Relative error.

TABLE IV

ACCURACY FOR THE DETERMINATION OF ADOSUPINE AND ITS METABOLITES IN RAT BRAIN

Amount added (ng/ml)	Adosupine		M1		M2		M3	
	Found (ng/ml)	R.E. (%)	Found (ng/ml)	R.E. (%)	Found (ng/ml)	R.E. (%)	Found (ng/ml)	R.E. (%)
50	45	−10	46	−8	45	−10	48	−4
100	94	−6	103	3	102	3	104	4
250	261	4	274	9.5	271	8.4	267	6.8
500	460	−8	469	6.2	523	4.6	579	3.8
1000	959	−4	984	−1.5	977	2.3	990	−1
5000	4900	−2	5000	0	5006	0.1	4970	−0.6

TABLE V

MEAN PERCENTAGE RECOVERY OF ADOSUPINE AND ITS METABOLITES FROM RAT PLASMA AND BRAIN

Values are mean ± S.D. of four determinations.

Amount (ng/g)	Adosupine		M1		M2		M3	
	Plasma	Brain	Plasma	Brain	Plasma	Brain	Plasma	Brain
100	102 ± 9	95 ± 7	103 ± 10	95 ± 8	105 ± 5	100 ± 6	98 ± 6	93 ± 6
250	98 ± 7	90 ± 5	100 ± 9	90 ± 5	102 ± 10	93 ± 8	99 ± 5	95 ± 6
500	96 ± 8	85 ± 7	97 ± 7	84 ± 6	99 ± 8	95 ± 6	94 ± 4	88 ± 8
1000	92 ± 7	88 ± 5	93 ± 5	87 ± 5	100 ± 11	94 ± 7	87 ± 7	89 ± 7
5000	92 ± 5	97 ± 5	93 ± 5	84 ± 6	103 ± 12	96 ± 7	82 ± 4	85 ± 4

The average recovery of the extraction procedure was 80–100% for adosupine and the metabolites (Table V). The recovery from plasma and brain of the internal standard was 95 ± 8% and 78 ± 9% (n = 4), respectively.

A decrease in the recovery from brain relative to that from plasma was observed for the more polar M1 and M3 (Table V), probably because the extraction from brain was executed with benzene, which required further washing with 0.1 *M* HCl after the first step of the extraction.

As an example of the application of this HPLC

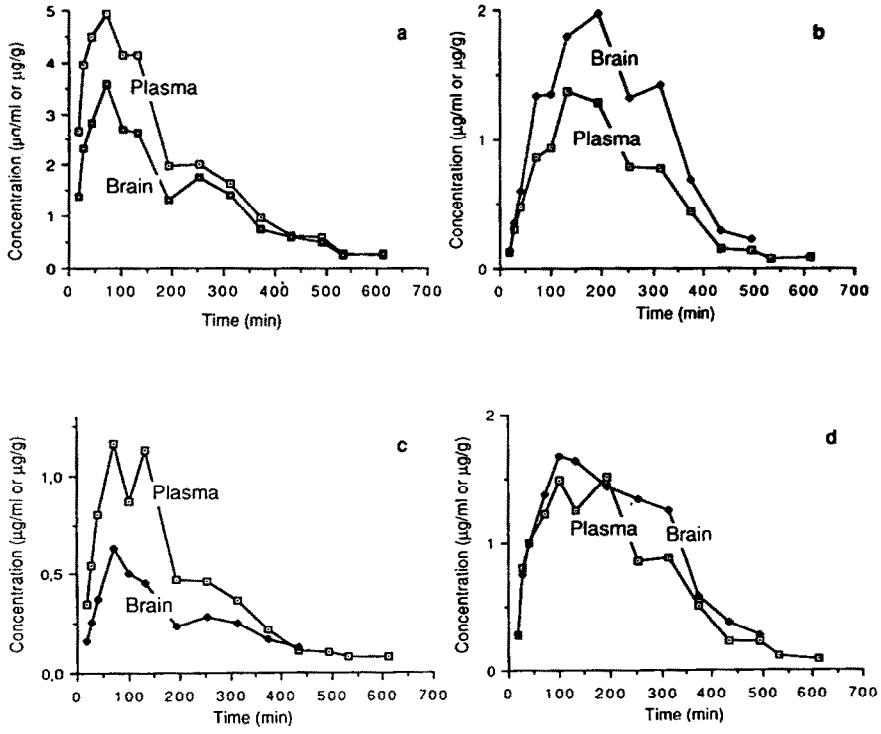


Fig. 4. Plasma and brain concentration–time profile of (a) adosupine, (b) M1, (c) M2 and (d) M3 following oral administration of 50 mg/kg of adosupine to rats. Each value is the mean of four determinations. For the sake of clarity, standard deviations are not shown; however, they never exceed 10% of the means.

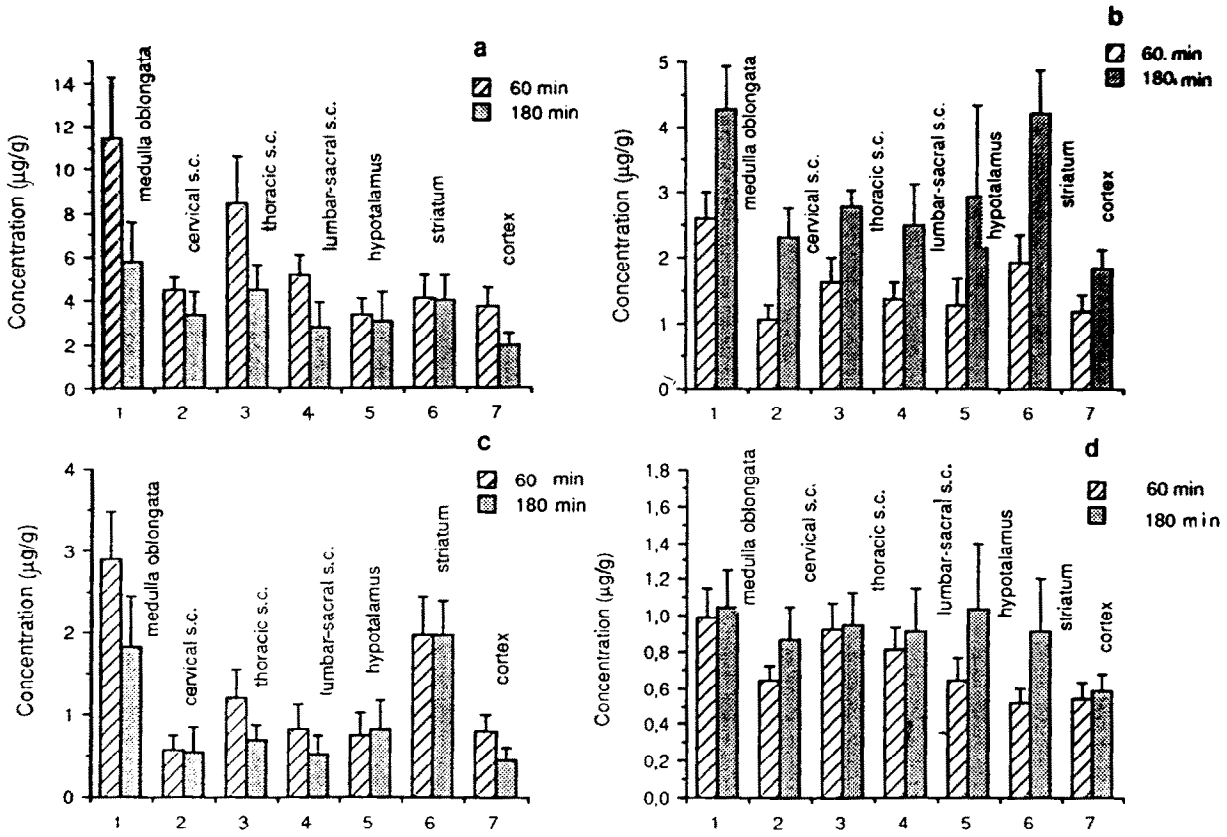


Fig. 5. Concentrations of (a) adosupine, (b) M1, (c) M2 and (d) M3 in different regions of the CNS of rats, 1 and 3 h after oral administration of adosupine (40 mg/kg). Columns represent means ± S.D. of six rats; s.c. = spinal cord.

method, concentrations of adosupine and its metabolites in plasma and brain are presented (Fig. 4a–d). Moreover, the method was successfully employed to determine the levels of adosupine and its metabolites in selected areas of the CNS (Fig. 5a–d).

CONCLUSIONS

The method described here is a simple, sensitive, and accurate assay for the determination of adosupine and the three metabolites M1, M2 and M3 in rat plasma and brain; it could be successfully used for pharmacokinetic and distribution studies.

ACKNOWLEDGEMENTS

The authors thank Drs. Carlo Mannucci and Andrea Perico of Menarini Pharmaceuticals, Florence (Italy) and Dr. Attilio E. G. Crea (Menarini Ricerche Sud, Pomezia, Italy) for their helpful technical advice. This study was supported by IMI grant No. 45054.

REFERENCES

1 *Eur. Pat.*, 0089322.
2 R. N. Gupta and G. Molnar, *Biopharm. Drug Dispos.*, 1 (1980) 259.
3 M. L. Constanzer and W. F. Bayne, *J. Chromatogr.*, 413 (1987) 277.
4 T. R. Norman and K. P. Maguire, *J. Chromatogr.*, 340 (1985) 173.
5 R. F. Suckow and T. B. Cooper, *J. Chromatogr.*, 230 (1982) 2.
6 P. Haefelfinger, *J. Chromatogr.*, 233 (1982) 269.
7 R. F. Suckow and T. B. Cooper, *J. Pharm. Sci.*, 70 (1981) 257.
8 A. L. Fischer, W. F. Bayne and D. G. Musson, *J. Pharm. Sci.*, 78 (1989) 971.