Journal of Chromatography, 306 (1984) 279—290
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1987

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF AMINEPTINE AND ITS MAIN METABOLITE IN HUMAN PLASMA

G. NICOT* and G. LACHATRE

Département de Pharmacologie Clinique, Centre Hospitalier Régional Universitaire, Hôpital Dupuytren, 87031 Limoges (France)

C. GONNET

Laboratoire de Chimie Analytique III (E.R.A. No. 474), Université Claude Bernard - Lyon I, 43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne (France)

J.-P. VALETTE, L. MERLE and Y. NOUAILLE

Département de Pharmacologie Clinique, Centre Hospitalier Régional Universitaire, Hôpital Dupuytren, 87031 Limoges (France)

and

N. BROMET

Département de Pharmacocinétique, Technologie Servier, 45007 Orléans (France)

(First received July 25th, 1983; revised manuscript received October 20th, 1983)

SUMMARY

An isocratic reversed-phase ion-pair liquid chromatographic method for the determination of amineptine and its main metabolite in plasma using an internal standard, is reported. The effects of stationary phase alkyl chain length and the concentration of alkyl sulfonate in the mobile phase were investigated. The drugs were extracted as ion pairs and the influence of various parameters on the extraction efficiency are discussed. Using a heptane—octanol—tetraheptylammonium bromide mixture (98 : 2 : 0.5, v/v/w) as extraction solvent, more than 60% of each drug was recovered with a very good selectivity. UV detection at 220 nm allowed drug determination down to 0.010 μg/ml. Linear standard curves up to 1.00 μg/ml were observed.

0378-4347/84/$03.00

INTRODUCTION

Amineptine* (dihydro-10,11-dibenzo[*a*,*d*]cycloheptenyl-5-amino)-7-heptanoic acid, used as an antidepressant [1, 2], is a molecule characterized by an amino acid side-chain attached to a tricyclic nucleus [3, 4]. Pharmacologically, amineptine appears to act on the dopaminergic system [5].

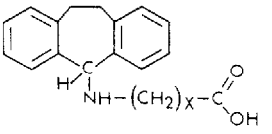
The biotransformation of this drug in man leads to various urinary metabolites which are not well known; in blood, a metabolite with a shortened amino acid side-chain, (dihydro-10,11-dibenzo[*a*,*d*]cycloheptenyl-5-amino)-7-pentanoic acid, has been identified [1].

Up to now, only one method for the determination of amineptine in plasma has been published [6]. This method was later applied to the estimation of amineptine and its main metabolite in human plasma [7]. This gas–liquid chromatographic (GLC) method requires costly equipment, including mass spectrometric detection and use of an external standard. A simple high-performance liquid chromatographic (HPLC) method for the plasma determination of amineptine and its main metabolite is reported here. The drugs are isolated from plasma by ion-pair extraction; the HPLC isocratic method is performed using an internal standard, a reversed-phase ion-pair system, and UV detection, and is found to be suitable for therapeutic determinations and pharmacokinetic investigations.

EXPERIMENTAL

Chemicals and reagents

Amineptine, its metabolite and internal standard, (dihydro-10,11-dibenzo-[*a*,*d*]cycloheptenyl-5-amino)-7-octanoic acid (Fig. 1), were provided by Servier Laboratories (Servier, Suresnes, France). Standard aqueous solutions of 10 μg/ml amineptine and its metabolite, and 10 μg/ml internal standard aqueous solutions were daily obtained from 1 mg/ml stock solutions. The latter were prepared by dissolving the compounds in methanol using an ultrasonic bath; the standard solutions were then stored at +4°C in brown glass flasks and were found to be stable for at least one month. Acetic acid (Rectapur grade), phosphoric acid and *n*-heptane (Normapur grade), and purified octanol were all obtained from Prolabo (Paris, France). Phosphate buffer (pH 7.0), acetonitrile and methanol



	X
amineptine	6
metabolite	4
int. standard	7

Fig. 1. Chemical structure of amineptine, its metabolite and internal standard.

*Trademark: Survector.

of LiChrosolv grade were purchased from Merck (Darmstadt, F.R.G.). The ion-pair reagents, tetraheptylammonium bromide (THABr) and heptanesulfonic acid sodium salt, were purchased from Eastman Kodak (Touzart et Matignon, Vitry-sur-Seine, France).

Chromatography

The chromatographic apparatus consisted of the following components: a Waters Model 6000 A pump (Waters, Paris, France), a Pye Unicam spectrophotometer (Pye Unicam, Paris, France) operated at 220 nm, and a Rheodyne 7125 injection valve (Touzart et Matignon, Vitry-sur-Seine, France) equipped with a 50-μl loop. The detector output was connected either to a Kontron W + W 610 recorder (W.W. Electronic Inc., Basel, Switzerland) or to an HP Model 3390 A integrator (Hewlett-Packard, Paris, France). A stainless-steel column (150 × 4.6 mm I.D.) was packed with a Nucleosil C_{18} (5 μm) stationary phase (Macherey-Nagel, Düren, F.R.G.), using a slurry packing technique [8] with some modifications to the solvents used: the slurry was made with *n*-butanol and the packing solvent was methanol.

The mobile phase consisted of acetonitrile—distilled water mixture (38 : 62, v/v). The aqueous phase contained 1.2 g/l heptanesulfonate and was adjusted to pH 3.0 with phosphoric acid. The mobile phase was filtered using a 0.45-μm Millipore filter and degassed in an ultrasonic bath. The separation was performed isocratically at room temperature.

Sample preparation and plasma extraction

Venous blood samples (5 ml) were collected into a 10-ml Vacutainer green-stoppered tubes (Becton-Dickinson, Missisauga, Canada) and centrifuged at 900 *g*. When the determination was not carried out immediately, the plasma was frozen at −20°C in plastic tubes; under these conditions, no degradation of drugs was noted after one month of storage.

For analysis, 2 ml of plasma were added to 1 ml of 0.05 *M* phosphate buffer (pH 7.0), 100 μl of 10 μg/ml internal standard solution and 10 ml of heptane—octanol—THABr (98 : 2 : 0.5, v/v/w) in 10-ml PTFE-lined screw-capped glass tubes. The tubes were shaken for 10 min on a Laboral oscillating agitator (Prolabo, Paris, France) and then centrifuged at 900 *g* for 10 min. An 8-ml volume of each upper organic phase was collected in a 10-ml conical base glass tube and 200 μl of a 0.17 *M* acetic acid—methanol (90 : 10, v/v) mixture were added. The tubes were capped and shaken on a Breda Scientific rotary agitator (Bioblock, Paris, France) for 5 min at 10 rpm; they were then centrifuged at 900 *g* for 5 min. The upper organic phase was discarded and 50 μl of acetic methanol phase were injected into the chromatograph.

Calibration curves and calculation

Plasma samples (2 ml) were spiked with increasing amounts of amineptine and its metabolite (final concentrations: 0.05, 0.10, 0.25, 0.50, 1.00 μg/ml of each drug in plasma) and with 100 μl of a 10 μg/ml internal standard solution. The samples were extracted as described above and standard curves were generated for each series of determinations by plotting peak height ratios (drug/internal standard) against known drug concentrations.

Plasma concentrations were interpolated from these standard curves. Accurate results in the range 0.01—1.00 μg/ml could also be alternatively obtained using a Hewlett-Packard integrator; in this case, calibration was obtained from a 0.50 μg/ml drug standard solution.

RESULTS AND DISCUSSION

Chromatographic system development

The influence of the counter-ion size on the retention of amineptine and its metabolite was studied. The effects of pentane-, hexane-, heptane- and octanesulfonate at 1.2 g/l in aqueous mobile phase were successively investigated. As

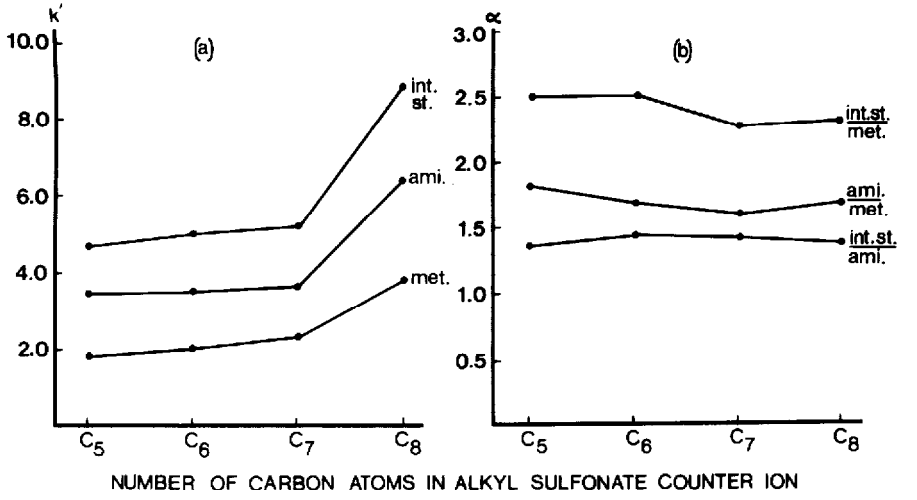


Fig. 2. Effect of the counter-ion size on the k' values (a) and selectivity factor values α (b) for amineptine, its metabolite and internal standard. Chromatographic conditions are as described in Experimental.

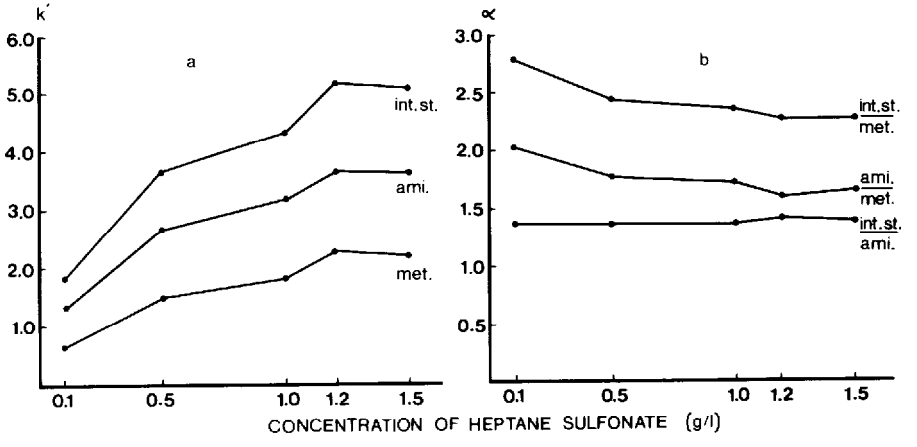


Fig. 3. Effect of the heptane sulfonate concentration on the k' values (a) and selectivity factor values α (b) for amineptine, its metabolite and internal standard. Chromatographic conditions are as described in Experimental.

can be seen from Fig. 2a, the k' values increase with increasing carbon number of the counter-ion alkyl chain, while selectivity factors α (Fig. 2b) show a relative constancy. Heptanesulfonate was chosen as counter-ion since in this condition the k' of the metabolite, the less retained peak, was large enough to be sufficiently resolved from the few endogenous compounds which eluted in less than 3.5 min.

Fig. 3a and b shows the effect of heptanesulfonate concentration in aqueous mobile phase on the capacity and selectivity factors. Concentrations from 0.1 to 1.5 g/l were successively used. According to the results obtained, a good compromise was obtained between retention time and selectivity factors by using 1.2 g/l heptane sulfonate in the aqueous mobile phase. Under these conditions, the metabolite elutes just after the solvent front; on the other hand, the resolution between metabolite and amineptine (6.7) and between amineptine and internal standard (5.4) is very good.

Extraction studies

The use of a polar solvent such as ethyl acetate in the extraction procedure showed that an important amount of endogenous compounds is co-extracted with the drugs of interest and these interfere with the detection of metabolite. It is well known that endogenous substances are co-extracted to a smaller extent by using non-polar solvents; unfortunately, the extraction recovery of polar compounds such as amineptine is very low under these conditions. Schill [9] studied the possibility of isolating drugs using an ion-pair extraction; recently, Smedes et al. [10] described an ion-pair extraction of catecholamines from plasma and urine. In the present study, a similar extraction procedure was used via the ion-pair formation in neutral medium between THABr (the cationic pairing ion) and the negatively charged carboxylate group of the drug. The back-extraction of drugs was then performed in an acetic acid—methanol mixture. The ionized acetate was used as competing ion in order to form an extractable ion-pair with THABr in the organic phase. Acceptable recovery and good selectivity were found using this procedure.

In order to determine the optimal conditions for drug extraction from human plasma, different parameters were investigated: the plasma pH, the nature and the concentration of the counter-ion, the nature of the organic phase, and the acetic acid concentration in the back-extraction step. All experiments were carried out with 2 ml of plasma spiked with the two drugs and the internal standard. The extraction conditions are given in the appropriate legends; throughout the set of experiments, the extraction efficiency was estimated by measuring drug peak heights.

The influence of plasma pH employing THABr as cationic counter-ion is illustrated in Fig. 4; the different pH values were obtained by adding NaOH or HCl. The relative extraction efficiency increases with increasing plasma pH up to pH 7—8 and then decreases. The poor extraction efficiency observed at pH values lower than 7.0 could be due to a simultaneous decrease of the ionized carboxylic group and protonation of the amine group leading to hydrophilic forms of the drugs. The ion-pair formation seems to be complete at pH values near 7—8, resulting from the ionization of the carboxylic group in this pH range. No hypothesis was established which could explain the decrease in extraction we observed for pH higher than 8.0.

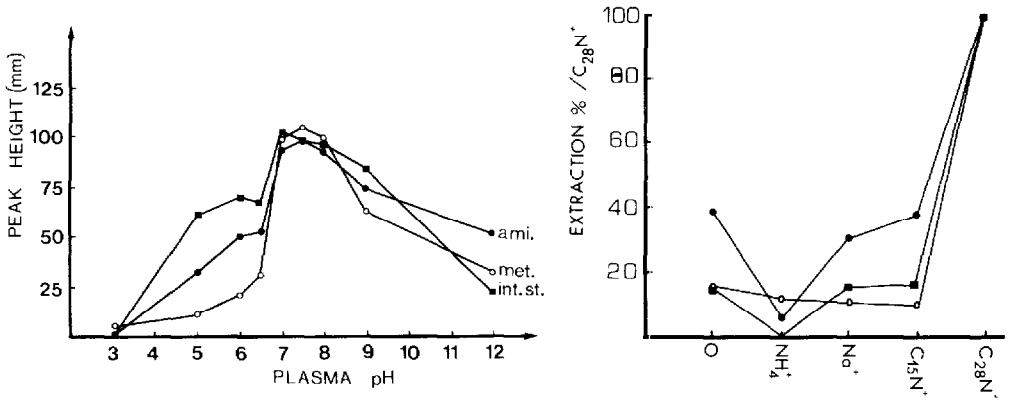


Fig. 4. Effect of the plasma pH on the extraction efficiency. Extraction conditions are as described in Experimental. ami. = amineptine; met. = metabolite; int. st. = internal standard.

Fig. 5. Effect of the counter-ion structure on the extraction efficiency. NH_4^+ = ammonium chloride, Na^+ = sodium sulfate, $C_{15}N^+$ = dodecyltrimethylammonium bromide, $C_{28}N^+$ = tetraheptylammonium bromide. Extraction conditions are as described in Experimental, except for NH_4^+, Na^+ and $C_{15}N^+$ which were dissolved in plasma. (▪), amineptine; (○), metabolite; (●), internal standard.

In order to determine the influence of the counter-ion structure, the peak heights of extracted drugs were estimated with various types of inorganic cations or alkyl quaternary ammonium ions. Due to their solubilities, THABr and dodecyltrimethylammonium bromide were respectively dissolved in the organic phase and in plasma; all these pairing ions were used at a concentration of 5 g/l in the organic phase. Fig. 5 shows that the extraction efficiency increases with the carbon number of the quaternary ammonium, while no improvement was noticed using inorganic cations (even for Na_2SO_4 concentrations up to 20 g/l). We thought, in agreement with previous reports [9, 10], that these results can be attributed to solvation effects in both phases; thus, it is possible that the attraction forces between inorganic cations and the aqueous phase are greater than those between ion pairs (formed by these inorganic cations and drugs) and organic phase. The reverse is true when inorganic cations are replaced by THABr.

It must be noted (Table I) that at pH 7.0, when 1 *M* NH_4Cl—NH_4OH buffer [10] was used, the extraction efficiency decreased compared to the results obtained with the phosphate buffer. This decrease is probably due to two competing interactions: interactions between carboxylic group (drug) and tetraheptylammonium ion, and interaction between carboxylic group (drug) and buffer ammonium ions. This decrease is more pronounced in the series metabolite > amineptine > internal standard and could be partly attributed to the more or less polar character of the ion pairs formed.

Extraction and back-extraction steps were investigated by varying the nature of extraction solvent. Solvent polarity, octanol percentage and THABr concentration in the organic phase were successively tested; the percentage of methanol in the aqueous phase and the molarity of the acetic acid solution used for the back-extraction were also studied. The effect of solvent polarity on the ex-

TABLE I

INFLUENCE OF THE NATURE OF THE BUFFER ON THE EXTRACTION EFFICIENCY

Buffer	Peak height (mm)					
	Metabolite $-(CH_2)_4-$		Amineptine		Internal standard	
NH_4Cl-NH_4OH (1 *M*, pH 7.0)	39		50		39	
	39	38*	44	48	35	38
	36		49		41	
Phosphate buffer (Merck, pH 7.0)	84		63		40	
	79	81	62	62	42	41
	80		61		41	

*Mean peak height.

traction yield was studied by using a different organic phase containing 2% of octanol and 1 g/l of THABr. Under these conditions, methylene chloride—octanol and methylene chloride—diethyl ether—octanol mixtures provide a poor extraction yield and should be avoided; a quantitative extraction was observed with toluene—octanol for the metabolite only. Nevertheless, it was found that extraction efficiency increased significantly for the three components when using hexane—octanol or heptane—octanol mixtures. As expected, it could be concluded that the ion pairs behave as lipophilic compounds and are much more soluble in non-polar solvents such as heptane or hexane.

As shown in Fig. 6, an increase in relative extraction efficiency was noticed for amineptine and particularly for its metabolite, with increasing octanol in heptane containing 1 g/l of THABr. Schill [9] described lipophilic alcohols such as octanol as having a moderate polarity and acting both as proton donor and acceptor. Thus, it could be assumed that octanol gave a high solvation for the amino acid side-chain of the studied compounds. In the case of internal standard, a slight increase in extraction efficiency was observed up to 1% octanol, then the extraction efficiency decreased again. The latter result could be attributed to the more non-polar character of this solute, due to the larger alkyl

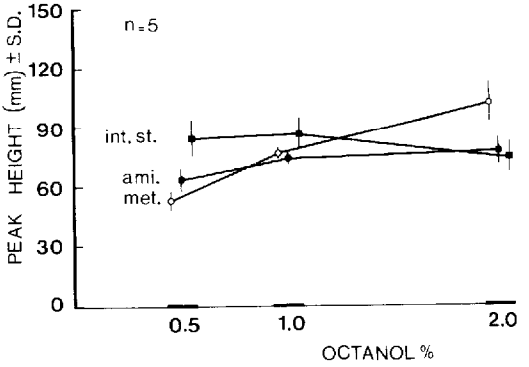


Fig. 6. Effect of the octanol percentage in heptane containing 1 g/l THABr, on the extraction efficiency. Experiments were carried out at plasma pH 7.0.

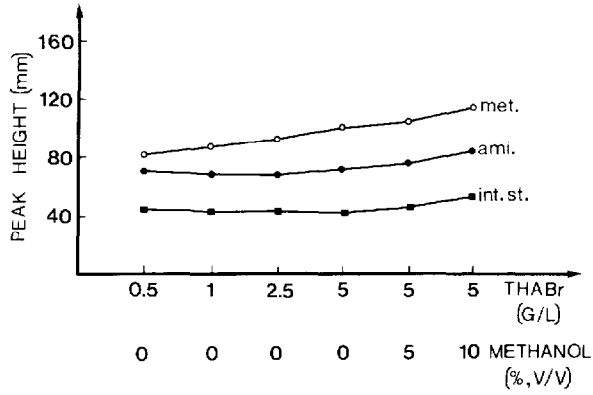


Fig. 7. Effects of the amount of the counter-ion THABr in the extraction phase and methanol percentage in the back-extraction phase, on the extraction efficiency. Other extraction conditions are as described in Experimental.

side-chain. Octanol concentrations higher than 2% were not used in order to avoid the co-extraction of interfering compounds.

The influence of the counter-ion concentration was studied by varying the amount of THABr in the heptane—octanol mixture (98 : 2, v/v) from 0.5 to 5 g/l. As shown in Fig. 7, the relative extraction efficiency was improved when the THABr concentration increased. This improvement was especially observed for amineptine and metabolite, and these findings are in agreement with results obtained by Smedes et al. [10] using an ion-pair extraction system for catecholamines; however, a real improvement was noted when methanol was added to the acetic acid solution used in the back-extraction. According to the limit of counter-ion solubility in organic phase and in order to suppress the co-extraction of undesirable endogenous anions, the following concentrations were chosen: 5 g THABr per litre of organic phase and 10% of methanol in the back-extraction solution.

Although an increase in recovery of amineptine and internal standard was noticed when extracting drugs with acetic acid solution from 0.17 *M* to 3.40 *M*, in these conditions a larger solvent front occurred and no improvement was noted for the metabolite recovery.

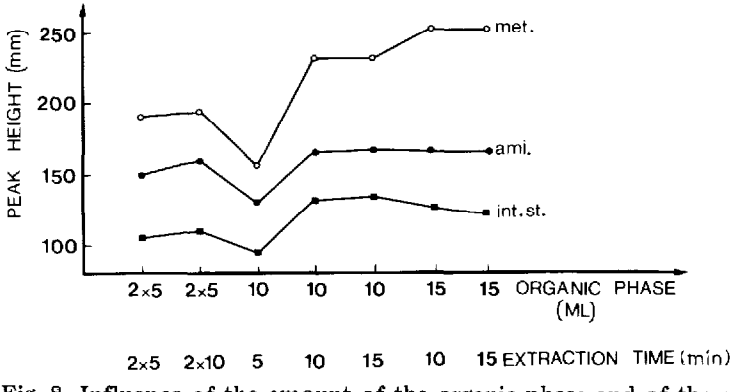


Fig. 8. Influence of the amount of the organic phase and of the extraction time, on the extraction efficiency. Plasma pH and extraction solvent are as described in Experimental.

In order to optimize the extraction procedure for 2-ml plasma samples, the volume of organic phase to be employed and the extraction time were investigated. Results are shown in Fig. 8. Solvent volumes less than 10 ml result in the formation of a precipitate which could adsorb the drugs and decrease the extraction percentage. A 10-min extraction time and a 10-ml volume of organic phase seemed to be in agreement with the quantitative and fast extraction requirements.

Linearity, sensitivity and selectivity

The standard curves were obtained by measuring the peak height ratios (drug/internal standard) on chromatograms obtained from drug-free plasma spiked with amineptine, its metabolite and internal standard. The linear curves were observed when plotting peak height ratios versus concentration (0.01, 0.05, 0.10, 0.50, 1.00 μg/ml of each drug). Each value was the mean of four measurements. The calibration curves for amineptine and its metabolite could be respectively expressed by the following equations: $Y = 0.0049\ X + 0.0228$ ($r = 0.999$), and $Y = 0.0085\ X - 0.0140$ ($r = 0.999$). The detection limit (signal-to-background ratio = 3) was at least 0.01 μg/ml for each drug.

TABLE II

SELECTIVITY OF THE HPLC SYSTEM

Drug (10 μg/ml)*	k'	Interference	Drugs (1 μg/ml)**	k'	Interference
Imipramine	6.09	—	Lorazepam	3.18***	+
Nortriptyline	6.27	—	Nitrazepam	3.36***	+
Maprotiline	6.45	—	Clorazepate	4.27***	+
Amitriptyline	7.00	—	Tianeptine	3.73	+
Protriptyline	7.55	—	Mianserin	3.73	+
Trimipramine	7.91	—	Desipramine	5.36	+
Desmethylclomipramine	9.18	—			
Clomipramine	11.00	—			
Meprobamate	ND	—			
Phenobarbital	1.18	—			
Valproic acid	3.00§	—			
Oxazepam	2.82	—			
Triazolam	1.36	—			
Bromazepam	1.55	—			
Diazepam	8.09	—			
Levomepromazine	7.18	—			

Metabolite (k' = 2.27), amineptine (k' = 3.64), internal standard (k' = 5.18)

*Non-extracted solution (50 μl injected).
**Extracted solution according to the amineptine assay procedure (50 μl injected).
***Peak height is no more than three times the level of the background noise.
§ Very poor signal at this concentration.

The method selectivity was studied and results are given in Table II. Among the drugs tested, tianeptine, mianserin and desipramine at therapeutic range interfere in the analysis described.

Reproducibility and recovery

Within-day reproducibility was determined by carrying out eight determinations from plasma samples spiked with 0.010, 0.250 and 1.000 μg/ml of amineptine and its metabolite. Day-to-day reproducibility was obtained by carrying out eight determinations over one month, no more than one assay per day, from plasma spiked with 0.250 μg/ml of each compound. Coefficients of variation (Table III) for within-day and day-to-day studies were respectively less than 11.93% and 8.15%.

Drug recovery was studied by adding a known amount of amineptine and its metabolite to a drug-free plasma sample at three different concentrations (0.10, 0.50 and 1.00 μg/ml). The organic phase was back-extracted with 200 μl of a 0.17 *M* acetic acid—methanol mixture containing an internal standard amount equivalent to a 100% extraction yield. For each concentration, five extractions were performed; means of peak height ratios were computed and compared to the mean of four height ratios of a drug and internal standard amount equivalent to a 100% extraction yield. Table IV shows that measured recoveries range from 62% to 73% for metabolite and from 64% to 73% for amineptine.

TABLE III

WITHIN-DAY AND DAY-TO-DAY REPRODUCIBILITY OF THE METHOD

Compound	Concentration (μg/ml)	Within-day (n = 8)		Day-to-day (n = 8)	
		S.D.	C.V.* (%)	S.D.	C.V. (%)
Metabolite	0.010	0.002	11.93		
	0.250	0.006	2.44	0.011	4.43
	1.000	0.021	2.04		
Amineptine	0.010	0.001	7.92		
	0.250	0.008	3.15	0.021	8.15
	1.000	0.019	1.88		

*Coefficient of variation.

TABLE IV

RECOVERY DATA FOR ASSAY OF AMINEPTINE AND ITS METABOLITE

Compound		Peak height after extraction (mm)					Mean of peak height without extraction (mm) (mean of four injections)	Percentage recovery (± 2 S.D.)
Name	Concentration (μg/ml)	1	2	3	4	5		
Metabolite —$(CH_2)_4$—	0.10	112.5	98.8	111	110	97.5	171	62 ± 8
	0.50	137.5	134	146	160	156	229	64 ± 10
	1.00	159	159	159	158	152.5	216	73 ± 2
Amineptine	0.10	63.8	57.5	66.3	62.5	67.5	100	64 ± 7
	0.50	82.5	80	89	99	96	133	67 ± 12
	1.00	94	93	93	91	89	126	73 ± 3

CLINICAL APPLICATIONS

The HPLC system described here was used for routine monitoring of plasma amineptine and its metabolite and for pharmacokinetic studies. Typical chromatograms of extracts from a blank sample, a spiked plasma and a patient plasma sample are shown in Fig. 9. No interfering peak originating from an endogenous compound was formed. Retention times were 4.0 min for metabolite, 6.0 min for amineptine and 8.0 min for internal standard. Samples can be injected at 10-min intervals. The analytical method described above has sufficient sensitivity for pharmacokinetic studies in human subjects. Using this method plasma kinetics were investigated in two adults suffering from depression who received a single 100-mg oral dose of amineptine (Survector). We obtained the following preliminary results: for one patient, peak concentrations of 378 ng/ml for amineptine and 532 ng/ml for metabolite were reached within 120 min and 180 min, respectively, after drug administration; for the other patient, peak concentrations of 1059 ng/ml for amineptine and 685 ng/ml for metabolite, were reached within 45 min and 60 min, respectively, after drug administration. In both cases, the drug could not be detected in blood, at 12 h and at 24 h for amineptine and for metabolite, respectively. Further studies on the pharmacokinetics in depressed and in uremic adults are in progress in our laboratory; the possible relationship between plasma concentration and effect of both amineptine and its metabolite is also being studied.

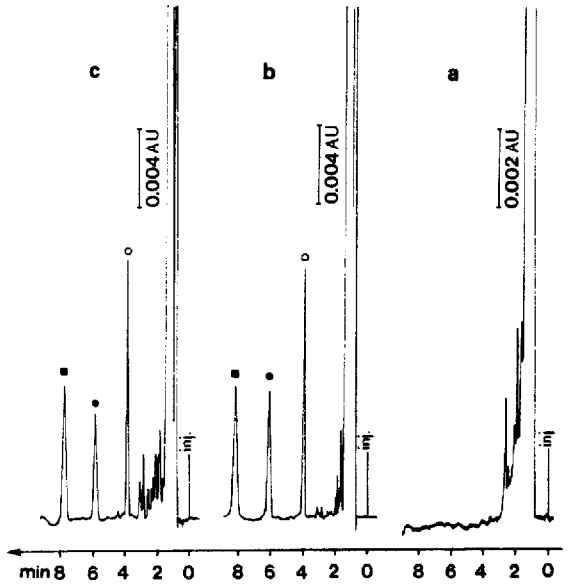


Fig. 9. Typical chromatograms of extracts from: (a) blank plasma; (b) plasma spiked with 250 ng/ml of metabolite (○), 250 ng/ml of amineptine (●) and 500 ng/ml of internal standard (■); (c) a patient plasma sample obtained 45 min after taking a 100-mg oral dose of amineptine, spiked with 500 ng/ml of internal standard (■) and containing 230 ng/ml of metabolite (○), 180 ng/ml of amineptine (●).

ACKNOWLEDGEMENTS

This work was supported in part by a grant from Inserm (CRL No. 825 020) and Servier Laboratories. The technical assistance of J. Mallon is appreciated.

REFERENCES

1 J.C. Poignant, L'Encephale, 5 (1979) 709.
2 M. Porot, C. Gerstner and M.A. François, Therapie, 35 (1979) 733.
3 C.E. Malen and J.C. Poignant, Experientia, 28 (1972) 811.
4 C.E. Malen, Psychol. Med., 11 (1979) 25.
5 R. Samanin, A. Jori, S. Bernasconi, E. Morpugo and S. Garattini, J. Pharm. Pharmacol., 29 (1977) 555.
6 C. Sbarra, P. Negrini and R. Fanelli, J. Chromatogr., 162 (1979) 31.
7 C. Sbarra, M.G. Castelli, A. Noseda and R. Fanelli, Eur. J. Drug Metab. Pharmacokin., 6 (1981) 123.
8 B. Coq, C. Gonnet and J.-L. Rocca, J. Chromatogr., 106 (1975) 249.
9 G. Schill, in J.A. Marinsky and J. Marcus (Editors), Ion Exchange and Solvent Extraction, Vol. 6, Marcel Dekker, New York, 1974, Ch. 1.
10 F. Smedes, J.C. Kraak and H. Poppe, J. Chromatogr., 231 (1982) 25.