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ION-PAIR EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TIANEPTINE AND ITS METABOLITES IN HUMAN PLASMA, URINE AND TISSUES

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SUMMARY

An isocratic reversed-phase ion-pair liquid chromatographic method for the determination of tianeptine and its two main metabolites in plasma, urine and tissues, using an internal standard, is reported. The influence of two stationary phases on the retention of the drugs was studied. The drugs were extracted as ion pairs, using a heptane—octanol—tetraheptylammonium bromide mixture (98:2:0.5, v/v/w) as extraction solvent. This extraction procedure yielded plasma drug recoveries of > 60% and allowed UV detection at 220 nm without interference from endogenous components of plasma, urine or tissues. Linear standard curves up to 1.00 μg/ml and drug determination down to 0.01 μg/ml were

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observed. This method has been successfully applied to the analysis of human plasma and urine samples and of encephales from tianeptine-dosed rats.

INTRODUCTION

Tianeptine, the sodium salt of (3-chloro-6-methyl-5,5-dioxo-6,11-dihydro-dibenzo[*c,f*][1,2] thiazepin-11-yl)-7-aminoheptanoic acid, used as an antidepressant [1, 2], is a molecule characterized by an amino acid side-chain attached to a tricyclic nucleus. From the pharmacological standpoint, tianeptine appears to act on the serotoninergic system [3].

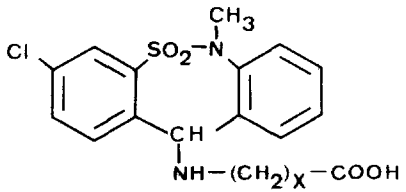
The biotransformation of this drug in man leads to various urinary metabolites [4], but at present, all of these metabolites have not been isolated to confirm their identity. Nevertheless, two compounds are known to be 2-acid and 4-acid homologues of tianeptine; these compounds, which are produced as a result of beta-oxidation of the heptanoic acid side-chain [5], are found in human plasma and are major urine components.

Up to now, only gas chromatographic (GC) methods for the determination of tianeptine in biological fluids have been published [6]. A simple high-performance liquid chromatographic (HPLC) method for plasma, urine and tissue determination of tianeptine and its two main metabolites is reported here. The drugs are isolated from biological fluids and tissues by ion-pair extraction. The HPLC method, which is performed using an internal standard, a reversed-phase ion-pair system and UV detection, is found to be suitable for therapeutic determinations and pharmacokinetic investigations.

EXPERIMENTAL

Chemicals and reagents

Tianeptine, its metabolites (Fig. 1) and the internal standard (dihydro-10,11-dibenzo[*a,d*]cycloheptenyl-5-amino)-7-octanoic acid were provided by Servier Labs. (Servier, Suresnes, France). A 10-μg/ml aqueous solution of tianeptine and each of its metabolites, and a 10-μg/ml internal standard aqueous solution were obtained daily from a 1-mg/ml stock solution. The stock solution was prepared by dissolving the compounds in methanol, using an ultrasonic bath;



	X
metabolite II	2
metabolite I	4
Tianeptine	6

Fig. 1. Chemical structure of tianeptine and its two main metabolites.

the standard solution was then stored at +4°C in brown glass flasks and was found to be stable for at least one month. Acetic acid (Rectapur grade), phosphoric acid (Normapur grade), *n*-heptane (Normapur grade) and purified octanol were all obtained from Prolabo (Paris, France). Phosphate buffer (0.05 *M*, pH 7.0), acetonitrile and methanol of LiChrosolv grade were purchased from Merck (Darmstadt, F.R.G.). Phosphate buffer (0.5 *M*, pH 7.0) was prepared by mixing 0.5 *M* KH_2PO_4 and K_2HPO_4 aqueous solutions. The ion-pair reagents, tetraheptylammonium bromide (THABr) and pentanesulphonic 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 6000A pump (Waters Assoc., Paris, France), a Shimadzu spectrophotometer operated at 220 nm and a Rheodyne 7125 injection valve (Touzart et Matignon) equipped with a 50-μl loop. The detector output was connected either to a Kontron W + W 610 recorder or to a Delsi Enica 10 integrator (Delsi, Paris, France). A stainless-steel column (150 × 4.6 mm I.D.) was packed with Hypersil ODS (5 μm) stationary phase (Shandon, Runcorn, U.K.) using a slurry packing technique [7], 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 an acetonitrile–distilled water mixture (45:55, v/v). The aqueous phase contained 2.7 g/l pentanesulphonate 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 flow-rate was 1.3 ml/min and the separation was performed isocratically at room temperature.

Sample collections

Venous blood samples (5 ml) were collected into 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 was noted after six months of storage. The volume of human urine samples was recorded after collection and urine was kept frozen in plastic flasks until the day of analysis. Brains of rats treated with tianeptine were taken immediately after they were killed and wrapped in a piece of preweighed aluminium foil; the samples were weighed and stored at −20°C until they could be extracted.

Extraction from biological samples

Extraction from plasma. 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 25-ml PTFE-lined screw-capped glass tubes. The tubes were shaken for 10 min on a Laboral oscillating agitator (Prolabo) 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

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 the acetic acid—methanol phase were injected into the chromatograph.

Extraction from urine. After urine was centrifuged at 900 *g* to remove particulates, extraction was processed similarly to the plasma extraction, except that 1 ml of 0.5 *M* phosphate buffer (pH 7.0) was added to each sample instead of 0.05 *M* phosphate buffer.

Extraction from tissues. Brain extracts were prepared for chromatographic analysis by the following procedure: a 1-g amount of brain tissue was placed into a 25-ml glass tube. To each sample of tissues, 2 ml of 0.05 *M* phosphate buffer (pH 7.0) were added. The samples were homogenized using an ultra Turrax (Janke and Kunkel, Stauffen, F.R.G.) for 30 s. After vortex mixing for 15 s and then centrifugation, the resultant clear supernatant was transferred into a 25-ml PTFE-lined screw-capped glass tube. Then, extraction was carried out as described for plasma.

Calibration curves and calculation

The standard calibration curves were obtained for each series of determinations by analysis of blank plasma, urine or brain tissue samples to which known quantities of drugs were added with a constant quantity of internal standard. These calibration curves were generated by plotting peak-height ratios (drug/internal standard) against known drug concentrations. The quantities of tianeptine and its metabolites used for the establishment of the standard curves were of the same magnitude as those found in biological samples (our study): from 0.01 to 1.00 μg/ml in plasma and urine and from 0.01 to 1.00 μg/g in brain tissue.

Plasma, urine or brain tissue concentrations of tianeptine and its metabolites were interpolated from these standard curves. Accurate results could also be obtained alternatively using an integrator; in this case, calibration was obtained from a 0.25-μg/ml or a 0.25-μg/g standard drug concentration.

RESULTS AND DISCUSSION

Chromatographic separation

Using a chromatographic system with a Nucleosil C_{18} 5-μm stationary phase, already employed for determination of a drug [8] whose structure appears to be similar to that of tianeptine, we noted a discrete endogenous compound, which elutes with the same retention time as metabolite II of tianeptine (Fig. 2, left panel). In order to resolve this problem, we modified the mobile phase through the alkyl chain-length or concentration of the counter-ion. Unfortunately, the mobile phase employed was unable, in any case, to separate the endogenous compound and metabolite II. A good separation was achieved using a Hypersil ODS 5 μm stationary phase, with the mobile phase previously described. In this latter system, only the retention time of the metabolite II eluting peak was delayed; the endogenous compound eluted near the front of the solvent (Fig. 2, right panel). Although no hypothesis was established that

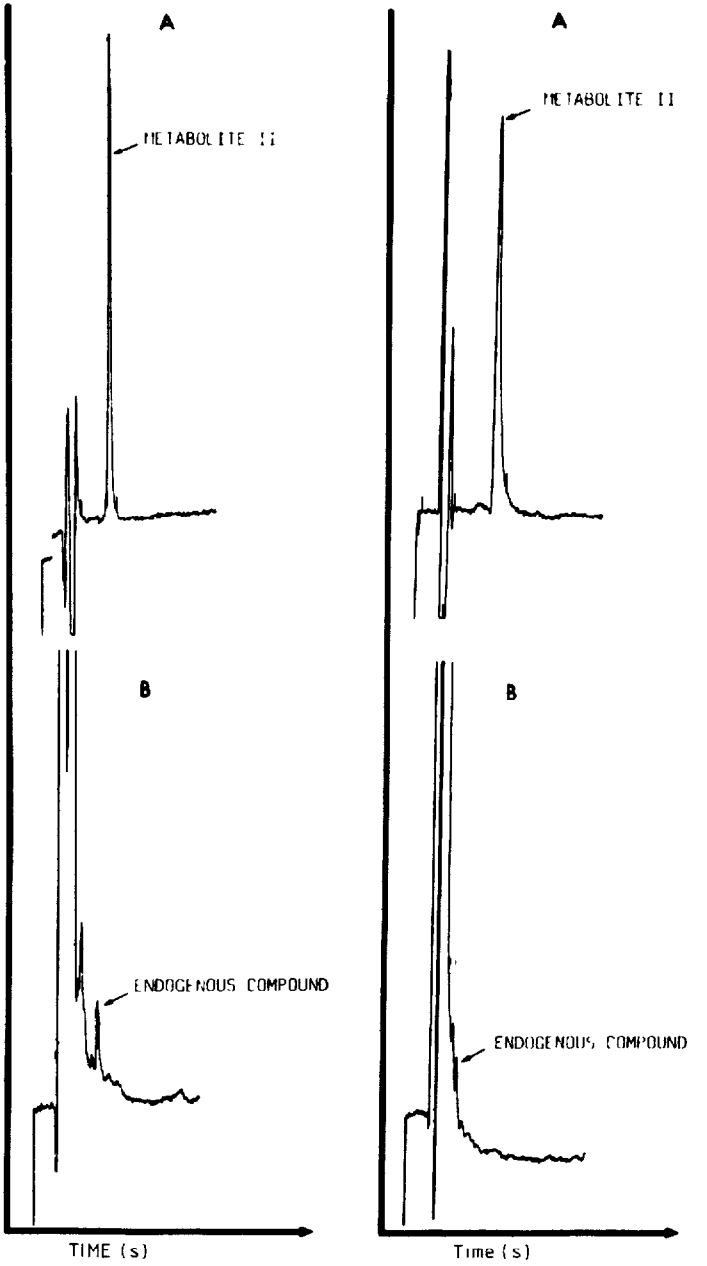


Fig. 2. Typical chromatograms of metabolite II aqueous solutions (A) and blank plasma extracts (B). Chromatographic conditions: column Nucleosil C_{18}, 5 μm (150 × 4.6 mm I.D.) (left panel); column Hypersil ODS, 5 μm (150 × 4.6 mm I.D.) (right panel). In both cases: mobile phase consisted of acetonitrile—distilled water containing 2.7 g/l pentane sulphonate at pH 3.0 (45:55, v/v); flow-rate, 1.3 ml/min; detector at 220 nm.

could explain the differences exhibited by these two stationary phases, such variations in retention and selectivity from one commercial packing to another have already been observed, especially when analysing more-polar solutes [9].

The fairly important accessibility of the residual silanols [10] or the differences in geometric structure of silice [11] could explain the behaviour of these two commercial bonded phases.

Extraction studies

In order to resolve the co-extraction of endogenous substances using a polar solvent, Nicot et al. [8] recently described an ion-pair extraction of amineptine

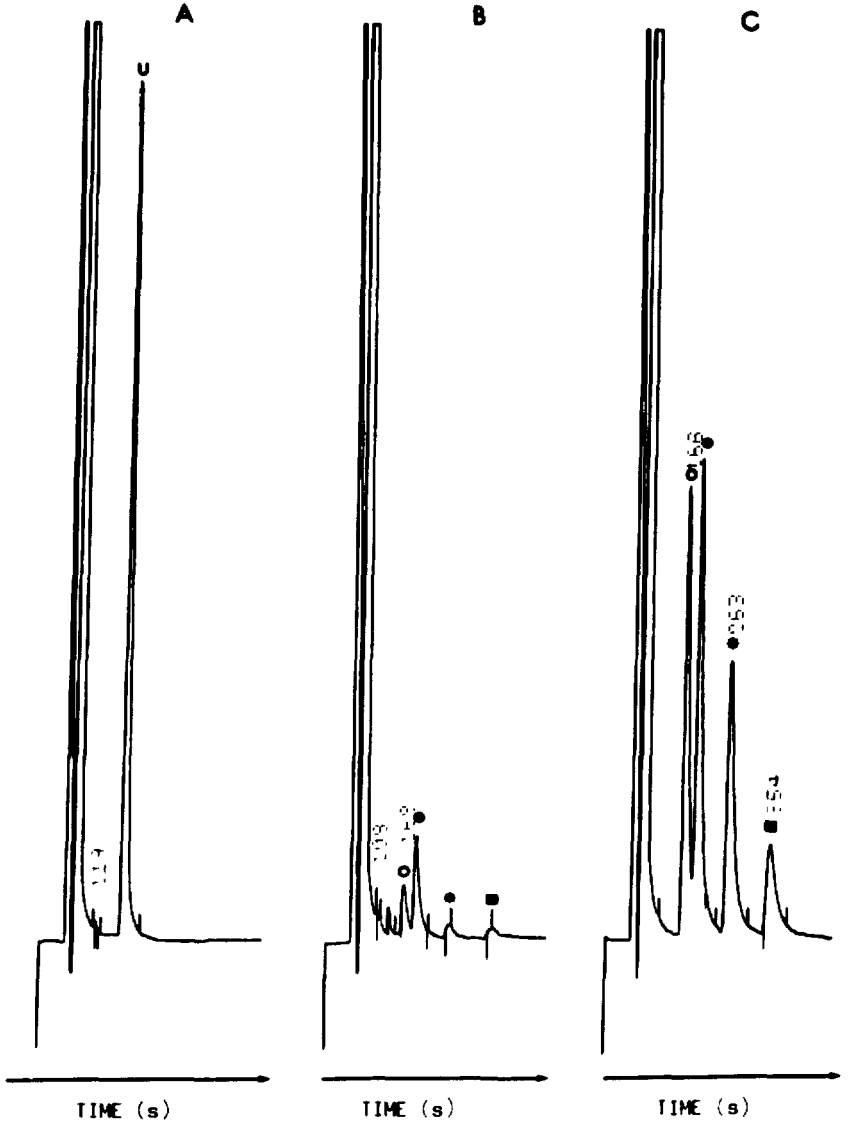


Fig. 3. Typical chromatograms of extracts from a plasma spiked with 500 ng/ml metabolite II (○), 500 ng/ml metabolite I (●), 500 ng/ml tianeptine (*) and 500 ng/ml internal standard (■): Extraction phase· (A) methylene chloride—octanol—THABr (98:2·0.5), (B) toluene—octanol—THABr (98:2:0.5); (C) heptane—octanol—THABr (98:2 0.5). Other extraction conditions are as described in Experimental and in all cases, 50 µl of extract were injected. (U = unknown compound in methylene chloride.)

from plasma via the ion-pair formation in neutral medium between THABr (the cationic pairing ion) and the negatively charged carboxyl group of the drug. In the present study, we used a similar extraction procedure.

Several solvent mixtures (methylene chloride—octanol, toluene—octanol, heptane—octanol) containing 5 g/l THABr were tested to identify the solvent for optimum recovery of tianeptine and its metabolites from human plasma. Only heptane—octanol appeared to be a practical extraction solvent for these drugs (Fig. 3).

Linearity, sensitivity and selectivity

Linear standard curves were observed when plotting peak-height ratios versus plasma concentrations (0.01, 0.05, 0.10, 0.25, 0.50, 1.00 μg/ml of each drug). Each value was the mean of four measurements. The calibration curves for tianeptine, metabolite I and metabolite II could be expressed by the following equations, respectively: $y = 3.27x - 0.057$ ($r = 0.998$), $y = 4.26x - 0.076$ ($r = 0.999$) and $y = 4.60x - 0.138$ ($r = 0.997$). The detection limit (signal-to-noise ratio = 3) was at least 0.01 μg/ml for each drug compound. The selectivity of the method was studied and the results are given in Table I. Among the drugs tested at therapeutic range, amineptine and diazepam interfere with tianeptine, clobazam interferes with metabolite I and chlorazepate, flunitrazepam, desmethyldiazepam and triazolam interfere with metabolite II.

Reproducibility and recovery

Within-day reproducibility was determined by carrying out eight deter-

TABLE I

SELECTIVITY OF HPLC SYSTEM

k' = Capacity factor; ND = not detected.

Drug (10 μg/ml)*	k'	Inter-ferences	Drug (1 μg/ml)*	k'	Interferences with
Bromazepam	1.59	–	Clorazepate	3.41	Metabolite II
Oxazepam	2.00	—	Desmethyldiazepam	3.43	Metabolite II
Nitrazepam	2.18	—	Flunitrazepam	3.43	Metabolite II
Lorazepam	2.22	—	Triazolam	3.56	Metabolite II
Clonazepam	2.59	—	Clobazam	4 07	Metabolite I
Medazepam	ND	—	Diazepam	5.93	Tianeptine
Tetrazepam	ND	—	Amineptine	6.28	Tianeptine
Clomipramine	ND	—			
Desipramine	ND	—			
Desmethylclomipramine	ND	—			
Imipramine	ND	–			
Nortriptyline	ND	—			
Protriptyline	ND	–			
Trimipramine	ND	—			
Meprobamate	ND	—			

Metabolite II (k' = 3.10), metabolite I (k' = 4.07), tianeptine (k' = 6.28), internal standard (k' = 9.0)

*Extracted aqueous solution according to the tianeptine assay procedure (50 μl injected).

TABLE II

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

Compound	Added concentration (μg/ml or μg/g)	Within-day (n = 8)						Day-to-day (n = 8)	
		Plasma measured concentration (μg/ml)		Urine measured concentration (μg/ml)		Brain tissue measured concentration (μg/g)		Plasma measured concentration (μg/ml)	
		Mean ± S.D.	C.V.* (%)	Mean ± S.D.	C.V.* (%)	Mean ± S.D.	C.V.* (%)	Mean ± S.D.	C.V.* (%)
Metabolite II	0.010	0.012 ± 0.001	13.41	0.010 ± 0.002	25.60	0.012 ± 0.003	26.58	0.010 ± 0.003	34.90
	0.050	0.045 ± 0.004	10.17	—	—	—	—	0.049 ± 0.006	12.09
	0.250	0.255 ± 0.009	3.86	0.244 ± 0.015	6.33	0.227 ± 0.020	9.14	0.245 ± 0.016	6.57
	0.500	—	—	0.514 ± 0.020	3.97	0.502 ± 0.025	5.00	—	-
	1.000	1.015 ± 0.052	5.21	1.065 ± 0.028	2.68	0.894 ± 0.046	5.19	0.992 ± 0.082	8.33
Metabolite I	0.010	0.017 ± 0.002	15.27	0.010 ± 0.002	17.79	0.011 ± 0.001	10.52	0.014 ± 0.005	35.82
	0.050	0.049 ± 0.003	7.08	—	—	—	—	0.052 ± 0.005	10.75
	0.250	0.254 ± 0.009	3.92	0.256 ± 0.001	5.54	0.248 ± 0.013	5.38	0.245 ± 0.016	6.70
	0.500	—	—	0.511 ± 0.019	3.78	0.523 ± 0.011	2.27	—	—
	1.000	0.994 ± 0.055	5.54	1.071 ± 0.020	1.90	1.149 ± 0.100	8.77	1.018 ± 0.090	8.87
Tianeptine	0.010	0.011 ± 0.002	18.26	0.014 ± 0.007	28.09	0.008 ± 0.002	24.15	0.012 ± 0.003	26.59
	0.050	0.051 ± 0.002	4.84	—	-	—	—	0.052 ± 0.006	11.97
	0.250	0.253 ± 0.012	4.96	0.248 ± 0.010	4.19	0.248 ± 0.012	4.89	0.246 ± 0.018	7.69
	0.500	—	—	0.516 ± 0.019	3.69	0.531 ± 0.018	3.41	—	-
	1.000	1.022 ± 0.055	5.40	1.048 ± 0.032	3.09	1.213 ± 0.042	3.52	1.000 ± 0.082	8.20

*Coefficient of variation.

TABLE III

ABSOLUTE RECOVERY FOR ASSAY OF TIANEPTINE AND ITS METABOLITES

Compound	Concentration (μg/ml)	n	Percentage recovery (mean ± S.D.)	
			Plasma	Brain tissue
Metabolite II	0.050	5	65 ± 5	—
	0.100	4	—	18.5 ± 1.9
	0.250	5	59 ± 1	—
	0.500	5	—	15.0 ± 0.6
	1.000	5	59 ± 4	16.0 ± 1.2
Metabolite I	0.050	5	73 ± 5	—
	0.100	4	—	65.6 ± 3.4
	0.250	5	63 ± 1.5	—
	0.500	5	—	49.6 ± 0.9
	1.000	5	64 ± 4.5	47.0 ± 1.5
Tianeptine	0.050	5	69 ± 4.5	—
	0.100	4	—	36.4 ± 5.0
	0.250	5	64 ± 2	—
	0.500	5	—	32.3 ± 1.1
	1.000	5	64 ± 3	32.6 ± 1.3

minations from plasma, urine and brain tissue samples containing tianeptine and its metabolites at concentrations in the range of 0.01—1.00 μg/ml (or 0.01—1.00 μg/g). Day-to-day reproducibility was obtained from plasmas spiked with 0.01, 0.05, 0.25 and 1.00 μg/ml of each compound drug, by carrying out eight determinations over fifteen days and no more than one assay per day for each concentration. The data presented in Table II indicate that within-day and day-to-day coefficients of variation for metabolite I, metabolite II and tianeptine were generally $< 12\%$ except for the concentrations near the limit of detection; in this latter case, coefficients of variation were $< 36\%$, 29% and 27% for plasma, urine and brain tissue, respectively.

Absolute drug recovery was studied by adding known amounts of tianeptine and its metabolites to drug-free plasma and brain tissue samples at different concentrations. The solvent 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, four or five extractions were performed; means of peak-height ratios of drug and internal standard were computed and compared to the mean of four peak-height ratios obtained after injection of standard solutions. The results in Table III illustrate the consistency of metabolites and tianeptine recoveries in plasma over the concentration range of interest. On the other hand, a variation of extraction recoveries was noted between metabolite I, metabolite II and tianeptine in brain tissues; the poor recovery of metabolite II was not explained.

CLINICAL APPLICATIONS

The HPLC system described here was used for routine monitoring of plasma

tianeptine and its metabolites and for pharmacokinetic studies.

Typical chromatograms of extracts from blank human plasma, spiked human plasma, patient plasma and spiked human urine samples are shown in Fig. 4. No interfering peak originating from an endogenous compound was formed. Retention times were ca. 2.8 min for metabolite II, 3.3 min for metabolite I, 4.5 min for tianeptine and 6.0 min for internal standard. Sample can be injected at 10-min intervals.

By using this method, we investigated the plasma kinetics of tianeptine and its metabolites in twelve uraemic subjects ranging in age from 35 to 66

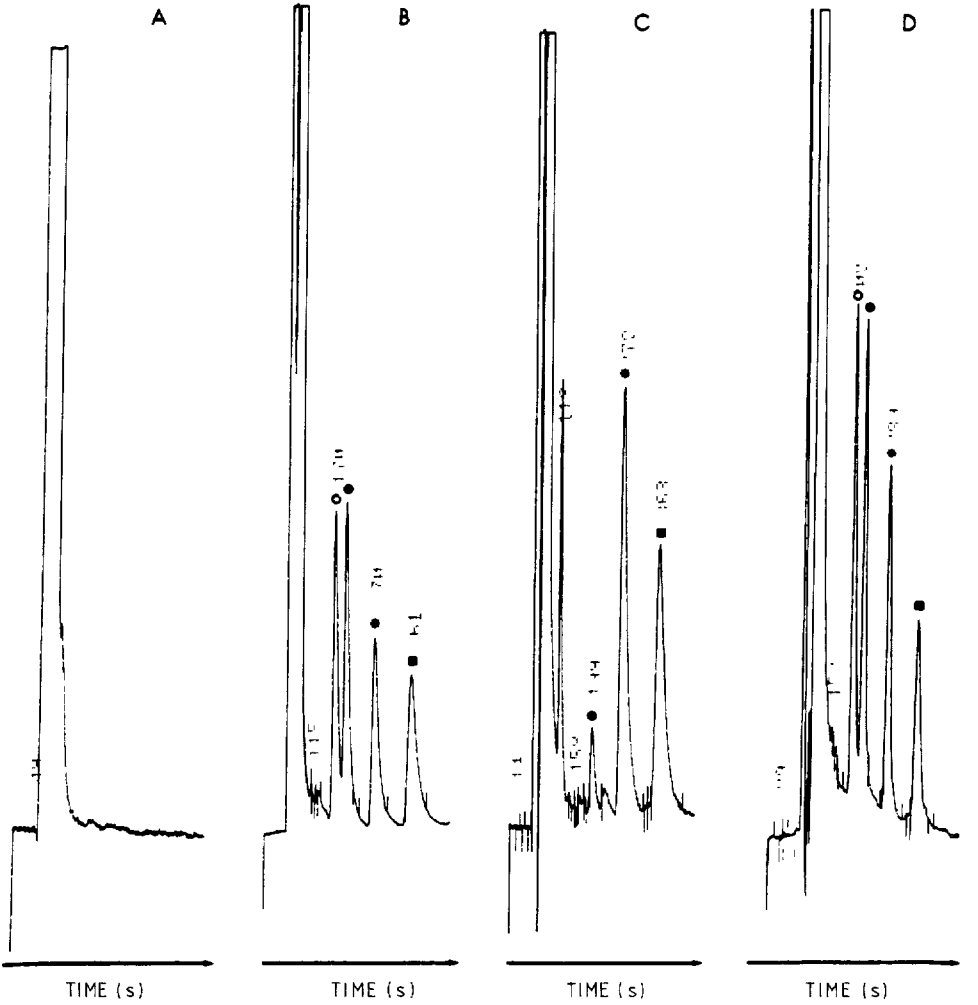


Fig. 4. Typical chromatograms of extracts from: (A) blank plasma; (B) plasma spiked with 250 ng/ml metabolite II (○), 250 ng/ml metabolite I (●), 250 ng/ml tianeptine (∗), 500 ng/ml internal standard (■); (C) a patient plasma sample obtained 75 min after taking a 12.5-mg oral dose of tianeptine, spiked with 500 ng/ml internal standard (■) and containing 37 ng/ml metabolite I (●) and 324 ng/ml tianeptine (∗) (metabolite II is < 10 ng/ml); (D) a human urine sample spiked with 500 ng/ml internal standard (■), 250 ng/ml metabolite II (○), 250 ng/ml metabolite I (●) and 250 ng/ml tianeptine (∗).

years. These subjects received a single 12.5-mg oral dose of tianeptine. The drug seems to be rapidly adsorbed into the circulation, reading peak plasma levels of 222 (132—316) ng/ml for unchanged drug, 65 (35—88) ng/ml for metabolite I, and < 58 ng/ml for metabolite II, respectively, 1.9 h, 3.5 h and 2.9 h after dosing. The practical effectiveness of the assay was also demonstrated by assaying urine specimens obtained from one of these twelve subjects over a 24-h period. Table IV shows that the urinary major component is metabolite I; comparison of extracts from these urines before and after hydrolysis with β-glucuronidase at 37°C for 12 h shows no significant differences, indicating

TABLE IV

URINARY EXCRETION OF TIANEPTINE AND ITS METABOLITES

Sample time (h)	Amount excreted (μg)*		
	Metabolite II	Metabolite I	Tianeptine
0— 6	30.4	70.4	—
6—12	—	38.4	—
12—24	—	64.0	—
24—48	—	57.6	—
Total	30.4	230.4	—

*Amount excreted in urine after oral administration of a 12.5-mg tablet of tianeptine to a 35-year-old woman.

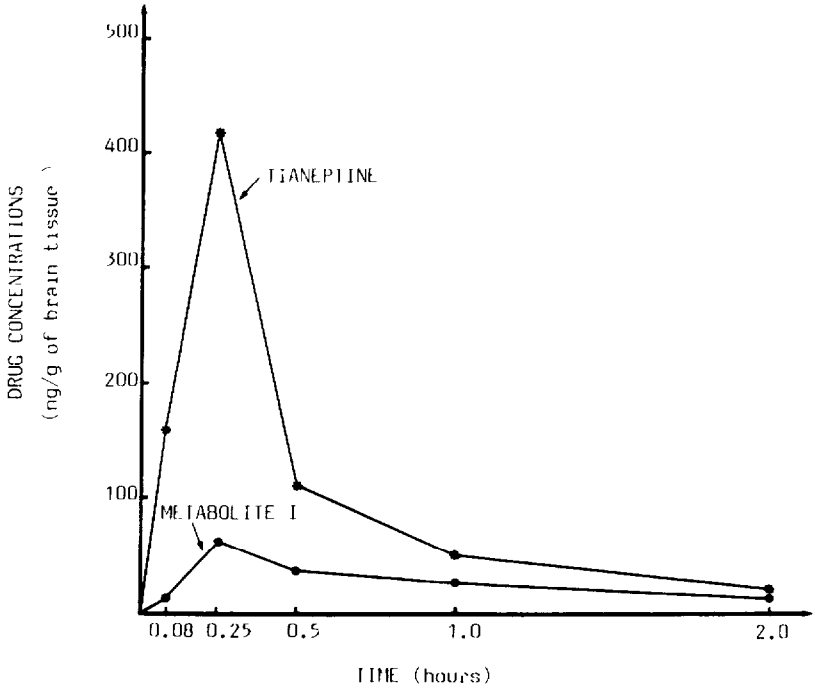


Fig. 5. Brain tissue concentrations of tianeptine (*) and metabolite I (•) in rats, following an intraperitoneal dose of 10 mg/kg. Each point represents the mean of five experiments.

that conjugation was not an important metabolic pathway as previously reported by Taylor et al. [5].

This system has been applied successfully to study the kinetic behaviour of tianeptine in rat brain tissue following the intraperitoneal administration of 10 mg of tianeptine per kg of body weight. Fig. 5 shows that peak brain concentrations of tianeptine and metabolite I were obtained in 15 min, falling rapidly to < 20 ng/ml within 2 h.

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