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QUANTITATIVE ANALYSIS OF MINAPRINE AND SOME OF ITS METABOLITES WITH APPLICATION TO KINETIC STUDIES IN RATS

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SUMMARY

A simple and rapid high-performance liquid chromatographic method is described for the quantitative analysis of the psychotropic drug minaprine and three of its metabolites (M_1, M_3 and M_{11}), including one as yet undetected metabolite (M_{11}) known as a monoamine oxidase type A inhibitor *in vitro*. After selective extraction all four compounds were separated on a reversed-phase μBondapak C_{18} column using sodium acetate (0.03 *M*)–acetonitrile–methanol (88:7:5) (pH 3.3) as the mobile phase. The eluted compounds were detected with a UV detector at 254 nm. The sensitivity of the method is 0.02 μg per millilitre of body fluid or per gram of tissue for M_1 and M_{11} and 0.05 μg for minaprine and M_3.

The method has been applied successfully to the determination of minaprine and the metabolites in plasma and brain and is compared here with an gas–liquid chromatographic method with an electron-capture detector previously developed for the detection of minaprine and M_{11}. M_{11} was identified in rat urine by gas chromatography–mass spectrometry.

INTRODUCTION

Minaprine (morpholinoethylamino-3-methyl-4-phenyl-6-pyridazine dihydrochloride) belongs to a series of novel psychotropic agents[1] and has been reported to possess antidepressant and psychostimulant properties[2,3]. Biochemical studies have shown that it induces a marked increase of striatal acetylcholine[4] and affects serotonergic (Mandell, personal communication) and dopaminergic[5] metabolism, probably through a transient inhibition of monoamine oxidase (MAO) activity.

A detailed study of the metabolic profile of minaprine in man and animals has recently appeared[6]. ^{14}C-labelled minaprine and thin-layer chromatography were used to separate minaprine from its metabolites, which were then identified by mass spectrometry (MS). These include the main metabolite, 4-hydroxyminaprine (M_3), the N-dealkylated derivative, amino-3-methyl-4-phenyl-6-pyridazine (M_1), and other metabolites arising from oxidation and/or cleavage of the morpholino ring. Phar-

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macological studies indicate that some of these metabolites are not biologically active, while M_1 and M_3 retain much of the parent compound's pharmacological and biochemical effects (Biziere, personal communication).

As part of a study to assess the role of minaprine metabolites in the parent drug's effects, we developed the present high-performance liquid chromatographic (HPLC) procedure for quantitation of minaprine and its metabolites M_1 and M_3 in biological samples. The procedure includes quantitation of aminoethylamino-3-methyl-4-phenyl-6-pyridazine (M_{11}), a possible but as yet undetected metabolite which shows *in vitro* the ability to reduce MAO A activity (substrate serotonin) in the rat hypothalamus (Biziere, personal communication).

The performance of this technique is compared with that of a gas–liquid chromatographic procedure with an electron-capture detector (GLC–ECD), previously developed in our laboratory (unpublished work). Preliminary data are reported on the concentrations of minaprine and its metabolites (see Fig. 1 for structures) in the plasma and brain of minaprine-treated rats.

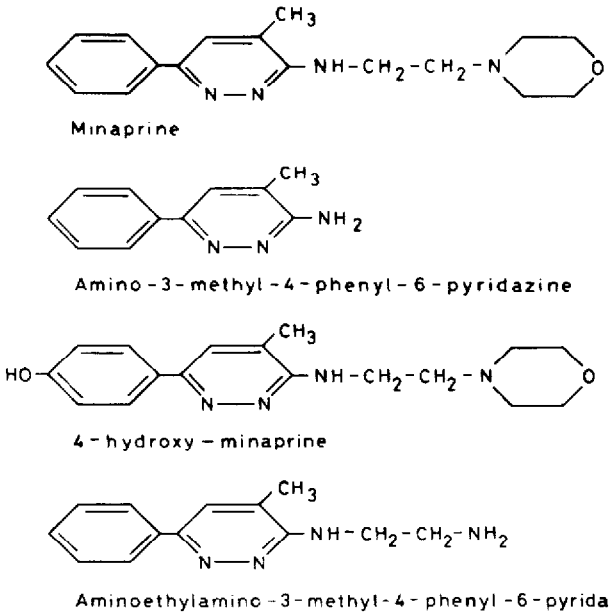


Fig. 1. Structures of minaprine, amino-3-methyl-4-phenyl-6-pyridazine (M_1), 4-hydroxyminaprine (M_3) and aminoethylamino-3-methyl-4-phenyl-6-pyridazine (M_{11}).

EXPERIMENTAL

Chemicals

Minaprine, M_1, M_3, M_{11} and 1-(6-chloro-2-pyridinyl)piperazine (CM 57191; internal standard for HPLC) were supplied by Clin-Midy (Montpellier, France). Quipazine maleate (internal standard for GC–ECD) was supplied by Miles Laboratory (Elkhart, IN, U.S.A.), pentafluoropropionic anhydride by Pierce (Rockford, IL,

U.S.A.), methanol and chloroform (HPLC grade) from E. Merck (Darmstadt, G.F.R.) and acetonitrile (HPLC grade) from Koch-Light (Colnbrook, Great Britain). Other reagents were ethyl acetate (Pestenal grade), acetone, hexane and formic acid (Carlo Erba, Milan, Italy).

Apparatus

HPLC was carried out on a Waters system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model U6K universal liquid injector, a Model 6000 A solvent delivery system and a reversed-phase column (μBondapak C_{18}, 30 cm × 3.9 mm I.D.) at room temperature. The mobile phase was sodium acetate buffer (0.03 *M*)–acetonitrile–methanol (88:7:5) (pH 3.3) at a flow-rate of 1.5 ml/min. Compounds eluted from the column were detected with a Model 440 UV monitor at 254 nm.

A Carlo Erba GC instrument was used, coupled with an electron-capture detector and a glass column (2 m × 3 mm I.D.) packed with 80–100-mesh Supelcoport with 3% OV-17 as the stationary phase. The column, detector and injector port temperatures were maintained at 260, 275 and 275°C, respectively. The carrier gas was nitrogen at a flow-rate of 30 ml/min.

Gas chromatography–mass spectrometry (GC–MS) was performed on an LKB 2091 instrument equipped with an LKB 2130 computer system for data acquisition and calculation. The chromatograph was operated under the conditions described above and mass spectra were collected in the electron-impact mode at 70 eV.

Animals

Male CD-COBS rats (Charles River, Como, Italy), average weight 200 g, were used. They were dosed with minaprine hydrochloride (15 mg/kg i.p. or 50 mg/kg p.o.) and heparinized blood, urine and brains were collected at pre-set times after each dose.

Extraction procedure

HPLC. To 0.25–2.0 ml of heparin-treated plasma 25 μl of a methanolic solution of CM 57191 (10 μg/ml), 0.1 *M* sodium hydroxide solution and 0.1 *M* phosphate buffer (pH 9) were added to a final volume of 2 ml. The samples were then extracted with 5 ml of chloroform–acetonitrile (9:1) by shaking for 30 min on an automatic shaker. After centrifugation the organic phase was separated and evaporated to dryness. The residues were dissolved in 0.1 ml of acetate buffer (pH 2) and washed with 1 ml of *n*-hexane. A volume of 25 μl of the buffer phase was injected into the HPLC column. Brains (*ca.* 1 g) were homogenized in 0.1 *M* hydrochloric acid (4 ml/g). After centrifugation the supernatant was made alkaline with 1 *M* sodium hydroxide solution and 0.1 *M* phosphate buffer, extracted with 10 ml of chloroform–acetonitrile, then processed as described for plasma.

GLC-ECD. To 0.25–2.0 ml of heparin-treated plasma were added 25 μl of a methanolic solution of quipazine (4 μg/ml) and the samples were extracted at pH 9 with 10 ml of ethyl acetate. After centrifugation the organic phase was evaporated to dryness and the residue was dissolved in 0.5 ml of benzene. Then 100 μl of an ethyl acetate solution of pentofluoropropionic anhydride (50%, v/v) were added and the samples were heated at 60°C for 30 min. After the reaction the samples were

washed with water (1 ml) and 5% aqueous ammonia (1 ml) and 1–3 μl were injected into the GC column.

Brains were homogenized (6 ml/g) in cold acetone–1 *M* formic acid (85:15) and centrifuged. The supernatant was shaken twice with *n*-heptane–chloroform (4:1), the organic phase was discarded and the aqueous phase was used for drug extraction as described for plasma.

Internal standard calibration graph

Drug-free plasma, urine and brain samples containing known amounts (20–500 ng) of the compounds under investigation were analysed concurrently with each set of unknown samples. Calibration graphs were constructed by plotting the ratio of the peak area of the compounds to that of the internal standard and comparing the amounts of the compounds added.

Percentage recoveries were calculated by comparing the peak-area ratios of the drug after plasma, urine and brain extraction with the peak-area ratios obtained by direct injection of their standard solutions.

RESULTS AND DISCUSSION

GLC-ECD method

The pentafluoropropionyl derivatives of minaprine, M_{11} and quipazine (internal standard) gave well separated symmetrical peaks, with retention times of 11.6, 3.3 and 4.6 min, respectively. Under the experimental conditions used the ratios of the peak area of minaprine and M_{11} were linear in the range 0.06–0.4 ng per injection. The detection limit for both the parent compound and its metabolite was 0.06 ng per injection (3 μl). Measurement of the pentafluoropropionyl derivative of the metabolites M_1 and M_3 did not yield optimal sensitivity and reproducibility, probably because they were readily absorbed into the stationary phase of the column packing and/or were unstable under the experimental conditions.

Extraction of minaprine and M_{11} from biological samples with ethyl acetate, benzene and chloroform was investigated. All of these solvents proved suitable for minaprine extraction, but ethyl acetate and chloroform gave more consistent recovery of the metabolite. Ethyl acetate (Pestanal grade) was chosen because it gave a clearer chromatogram than chloroform. Within the range 0.01–0.2 μg the mean recovery of minaprine from control urine and plasma was 93.0 ± 5.2% and from brain homogenates 94.0 ± 5.6%. Recovery of M_{11} from body fluids was 71.0 ± 5.3% and from brain homogenates 70.0 ± 9.6%.

Specificity of the analysis was confirmed when unknown urine, plasma and brain samples of rats given minaprine were analysed by GC–MS. The mass spectra were identical with those obtained after injection of minaprine or M_{11} pentafluoropropionate. The metabolite, however, was identified only in trace amounts in rat urine. Fig. 2 shows the mass spectra of minaprine (A) and M_{11} (B) pentafluoropropionate. The minaprine mass spectrum contains the molecular peak (*m/e* 444) and a fragment at *m/e* 325 ($-C_2F_5$), and also some ions generated from the morpholine moiety: *m/e* 100, 113 (base peak) and 114. Other fragments are at *m/e* 119 ($C_2F_5^+$), 169, 212, 332 (loss of the alkyl group of the secondary amide with transfer of 2H as illustrated in Scheme 1), 344 and 358.

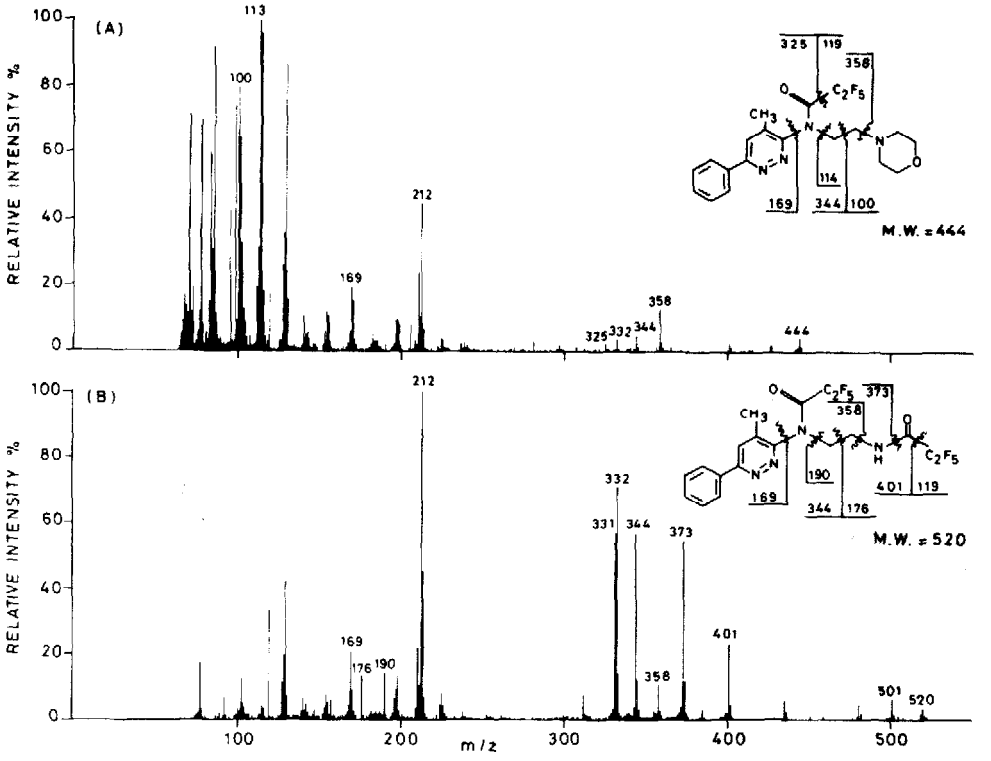
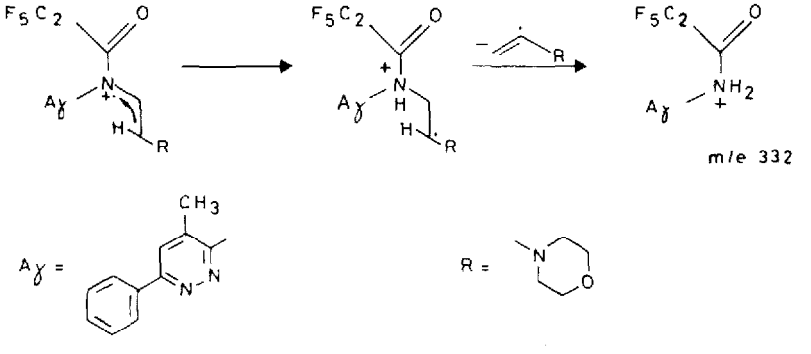
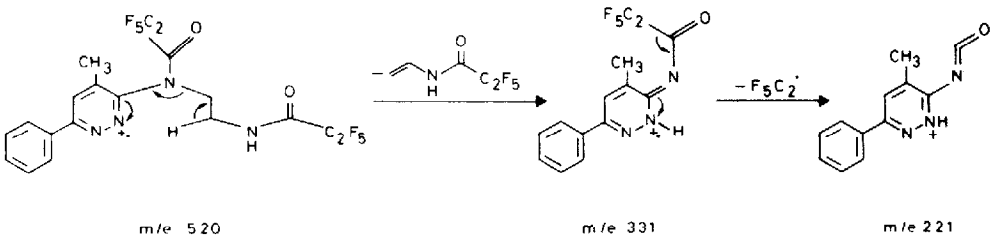


Fig. 2. Mass spectra of pentafluoropropionate minaprine and M_{11}.

The mass spectrum of the metabolite shows the molecular peak (*m/e* 520) and other peaks due to progressive fragmentation of the side-chain: *m/e* 501 (–F), 401, 373, 358, 344, 332, 331, 212 (base peak) and 169. The ion at *m/e* 331 could be formed through a McLafferty rearrangement (see Scheme 2). Cleavage of the chain with retention of the charge on the aliphatic amide moiety leads to ions at *m/e* 176 and 190. The mass spectra of the O-TMS derivatives of M_3 and M_1 have been reported by Davi *et al.*[6].



Scheme 1.



Scheme 2.

HPLC method

The major reported metabolite of minaprine is 4-hydroxyminaprine (M_3)[6]. As reported above, it does not chromatograph well on a GC column or else it is unstable under the experimental conditions suitable for minaprine quantitation. In order to detect M_3, in addition to any other metabolites, in plasma and brain we developed an HPLC procedure for the simultaneous determination of minaprine and its metabolites. Using sodium acetate buffer (0.03 *M*)–acetonitrile–methanol (88:7:5), minaprine, M_3, M_1 and M_{11} were readily separated. At a flow-rate of 1.5 ml/min and pH 3.3, the retention times were 5.4, 7.8, 10.1, 14.5 and 16.9 min for M_3, M_{11}, CM 57191 (internal standard), M_1, and minaprine, respectively. Examples of chromatograms of extracts from (A) drug-free plasma, (B) plasma of rats treated with minaprine (15 mg/kg i.p.) and (C) plasma spiked with minaprine and its metabolites are reported in Fig. 3. In chromatogram B a peak (M_x) is evident with a retention time of 9.2 min. This peak also appeared on injecting brain extracts and may represent a metabolite of minaprine. Studies are now in progress to identify this metabolite.

The chloroform–acetonitrile (9:1) mixture was an ideal extraction solvent be-

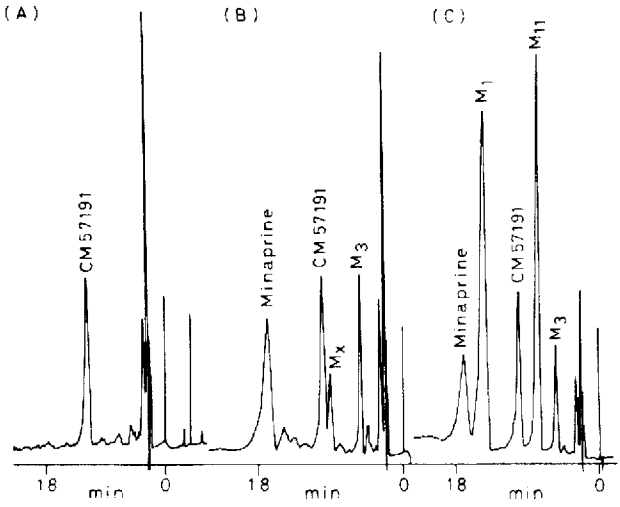


Fig. 3. HPLC traces of extracts from (A) drug-free plasma, (B) plasma of rat treated intraperitoneally with minaprine dihydrochloride (15 mg/kg) and (C) plasma spiked with minaprine, M_1, M_3 and M_{11}.

cause it extracted only a few impurities and no interfering substances from biological samples, yielding at the same time consistent recoveries of minaprine and its metabolites. Under these experimental conditions all four compounds were extracted reproducibly with mean recoveries of 80.0 ± 8, 82.5 ± 9.0, 92.1 ± 4.6 and 88.2 ± 7.7% from control plasma and 71.3 ± 3.4, 77.1 ± 9.1, 88.4 ± 3.4 and 85.5 ± 1.0% from brain homogenates for M_3, M_{11}, M_1, and minaprine, respectively. The calibration graphs were linear in the range of 0.02–0.2 μg for M_1 and M_{11} and 0.05–0.5 μg for M_3 and minaprine. The sensitivity limit of the method was 0.02 μg per millilitre of plasma or gram of brain for M_1 and M_{11} and 0.05 μg for M_3 and minaprine.

Comparison of HPLC and GLC-ECD

Plasma samples from rats treated intraperitoneally with minaprine dihydrochloride (15 mg/kg) were first analysed by GLC-ECD and then by HPLC. For this comparison only minaprine was considered, as M_{11} was not present in rat plasma and M_1 and M_3 could not be determined with our GLC method. The results are illustrated in Fig. 4. Statistical analysis of HPLC (x) and GLC-ECD (y) gave a slope of 0.9902 and a correlation coefficient (r) of 0.998. The good correlation found between the two techniques indicates that both methods are reliable for minaprine quantitation. GLC-ECD is more sensitive than HPLC but with the former technique minaprine needs to be derivatized before GLC analysis. Thus, for routine analysis

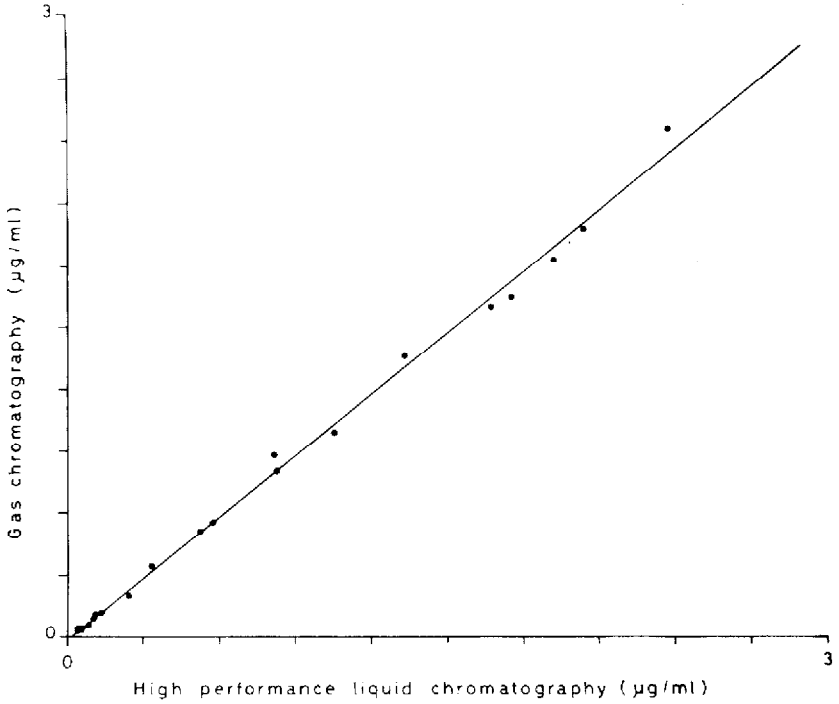


Fig. 4. Determination of minaprine in rat plasma by GC-ECD (y) and HPLC (x). $r = 20$; $y = 0.9902x - 0.00917$; $r = 0.998$.

and/or kinetic studies HPLC is easier and faster for quantitation of minaprine and its metabolites.

Animal studies

The HPLC procedure provided an opportunity for investigating the kinetic profile of minaprine and its metabolites in rat plasma and brain. Fig. 5 shows the curve of plasma concentration *versus* time obtained from rats injected intraperitoneally with minaprine dihydrochloride (15 mg/kg). At this dose minaprine was rapidly absorbed into the bloodstream of the rat. It reached plasma peak levels between 1 and 5 min but rapidly declined thereafter with an elimination half-life ($T_{\frac{1}{2}}$ of 22 min. The 4-hydroxy derivative (M_3) was detected in plasma 5 min after injection of the parent drug, rising to a plasma peak after 15 min. From the peak this metabolite disappeared from plasma ($T_{\frac{1}{2}}$ = 67 min) more slowly than the parent drug. The plasma area under the curve (AUC) ratio of M_3 (87.75 μg/ml · min) to minaprine (52.22 μg/ml · min) was 1.7, indicating that this is a quantitatively significant metabolite of minaprine in rat plasma.

The two urinary metabolites M_{11} (present results) and M_1 (Davi *et al.*[6]) of minaprine were not found in rat plasma within 8 h of minaprine injection.

Minaprine rapidly enters the brain from the bloodstream. Peak brain concentrations were reached after 5 min (see Table I) with levels about double those in plasma, and declined thereafter with a $T_{\frac{1}{2}}$ of 15 min. Like the peak concentrations, the brain AUC of minaprine (99.7 μg/g · min) was about twice the plasma AUC. Only traces of the 4-hydroxy derivative were found in rat brain and even these were only detectable at 60 min. As in plasma, M_1 and M_{11} were undetectable in rat brain within the sensitivity of the method. For M_{11}, which *in vitro* is a competitive inhibitor of MAO type A, this may be related to rapid oxidative deamination of the compound

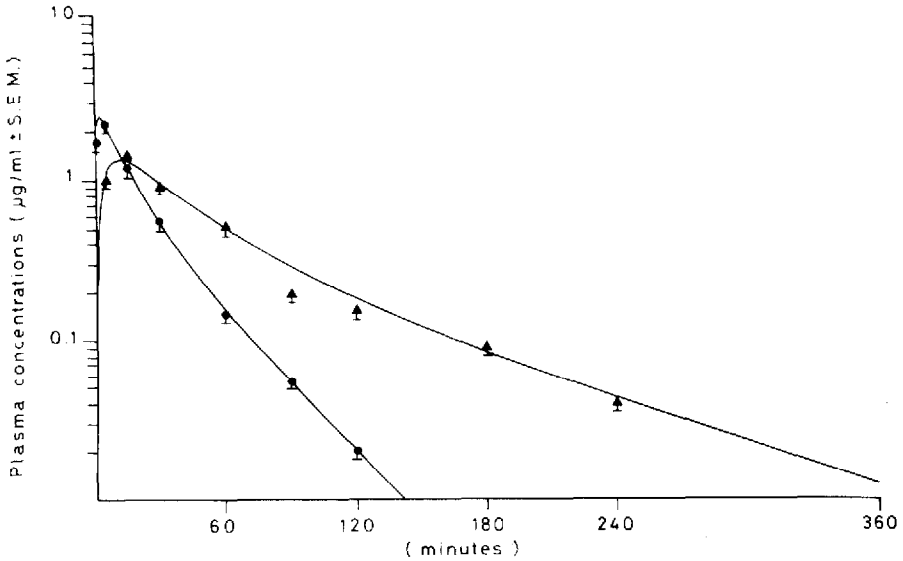


Fig. 5. Plasma concentration *versus* time curves for minaprine and 4-hydroxyminaprine in rats treated intraperitoneally with minaprine dihydrochloride (15 mg/kg).

TABLE I

BRAIN CONCENTRATIONS OF MINAPRINE AND ITS ACTIVE METABOLITES AFTER INTRAPERITONEAL INJECTION OF MINAPRINE DIHYDROCHLORIDE (15 mg/kg)

Each point is the mean ± standard error of the mean (S.E.M.) for four rats. $T_{\frac{1}{2}}$ = half-life; AUC = area under the curve.

Time after minaprine injection (min)	*Brain concentration (μg/g ± S.E.M.)*			
	Minaprine	M_3	M_1	M_{11}
1	0.70 ± 0.09	<0.05	<0.02	<0.02
5	4.84 ± 0.49	<0.05		
15	2.57 ± 0.24	<0.05		
30	1.04 ± 0.04	<0.05		
60	0.20 ± 0.04	0.18 ± 0.03		
90	0.05 ± 0.01	<0.05		
120	0.03 ± 0.01*			
240	<0.01*			
$T_{\frac{1}{2}}$ (min)	15	N.D.**	N.D.	N.D.
AUC (μg/ml · min)	99.7	N.D.	N.D.	N.D.

* Obtained by GLC-ECD analysis.
** N.D. = not determinable.

to the corresponding acid. Experiments in which rats were pre-treated with pargyline (100 mg/kg i.p. before oral 50 mg/kg minaprine dihydrochloride) showed, however, that M_{11} does not accumulate in rat brain even after blocking of MAO.

CONCLUSION

GLC-ECD and HPLC have been used for the quantitation of minaprine in plasma and tissues. Correlation studies showed that both techniques give comparable results for its quantitation. For kinetic studies, however, HPLC has a distinct advantage over GLC because the former can quantitate simultaneously the parent drug, its main metabolite and other possible metabolites in plasma and/or tissues. The 4-hydroxy derivative of the drug was present in rat brain only in trace amounts. The metabolites M_1 and M_{11} were not seen in plasma and brain, although they were present in rat urine. All three metabolites investigated therefore cannot play any role in the pharmacological and biochemical effects of minaprine in the animal species considered. This does not exclude the possibility that other as yet unidentified minaprine metabolites may enter the brain.

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