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DETERMINATION OF THE ANTIHYPERTENSIVE DRUG 1-[2-ETHOXY-2-(3′-PYRIDYL)ETHYL]-4-(2′-METHOXYPHENYL)PIPERAZINE (IP/66) IN RAT AND HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ISOTOPE DILUTION MASS SPECTROMETRY

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SUMMARY

In connection with pharmacokinetic studies on the antihypertensive drug 1-[2-ethoxy-2-(3′-pyridyl)ethyl]-4-(2′-methoxyphenyl)piperazine (IP/66) (I), appropriate high-performance liquid chromatographic (HPLC) and gas chromatographic–mass spectrometric isotope dilution (GC–MS–ID) methods for its determination in rat and human plasma, respectively, were developed. In both techniques, deproteinized and basified plasma samples were extracted and purified by adsorption on an Extrelut-1 column, then the drug was eluted with dichloromethane. Quantitative HPLC analysis was performed on a C_8 reversed-phase column. The mobile phase was phosphate buffer (0.02 *M*, pH 2.8)–acetonitrile (65:35), with UV detection at 208 nm. The internal standard was 1-[2-butoxy-2-(3′-pyridyl)ethyl]-4-(2′-methoxyphenyl)piperazine, a homologue of I. The inter-assay coefficient of variation (C.V.) was 9.9% for a drug level of 2 μg/ml. Quantitative GC–MS–ID analysis was performed with a DB-17 fused-silica capillary column using the selected-ion monitoring technique. The deuterated form of I, 1-[2-ethoxy-2-(3′-pyridyl)ethyl]-4-2′-trideuteromethoxyphenyl)-piperazine, utilized as internal standard, was synthesized. The inter-assay C.V was 7.36% for a drug level of 1 ng/ml

INTRODUCTION

Selective α_1-adrenoceptor blocking agents are widely used in the treatment of various diseases characterized by overactivity of sympathetic nerves on smooth

muscle [1]. 1-[2-Ethoxy-2-(3′-pyridyl)ethyl]-4-(2′-methoxyphenyl)piperazine (IP/66) (I) was described as a potent α-adrenoceptor antagonist [2] and subsequent studies suggested a selectivity of this compound toward post- as compared with pre-junctional α-adrenoceptor-mediated responses [3]. Further, I has been described as possessing a remarkable stereospecificity in its α-adrenoceptor blocking property [4]. In view of this interesting pharmacodynamic profile, we decided to develop appropriate high-performance liquid chromatographic (HPLC) and gas chromatographic–mass spectrometric–isotope dilution (GC–MS–ID) [5] methods for the detection of I in rat and human plasma, respectively.

EXPERIMENTAL

HPLC method

Materials. I·HCl was synthesized in our laboratory as described previously [2], and the internal standard (I.S.), 1-[2-butoxy-2-(3′-pyridyl)ethyl]-4-(2′-methoxyphenyl)piperazine, was obtained in a similar manner. The purity of these products was checked to be at least 99%. All other reagents were of analytical-reagent grade and used without further purification. Diethyl ether, dichloromethane and acetonitrile (LiChrosolv) were obtained from Merck (Darmstadt, F.R.G.), 85% orthophosphoric acid, monobasic potassium phosphate and 70% perchloric acid were from Carlo Erba (Milan, Italy). Fresh deionized and distilled water was used throughout the experiments. Stock solutions of I·HCl (0.5 mg/ml) and I.S. (1 mg/ml) were prepared in 0.1 *M* hydrochloric acid. Storage of these solutions at 4 °C did not result in any detectable decomposition.

Chromatography. A Perkin-Elmer (Norwalk, CT, U.S.A.) Series 400 liquid chromatograph, equipped with an LC-85B variable-wavelength spectrophotometric detector set at 208 nm (sensitivity 0.16 a.u.f.s.) and an LCI-100 laboratory computing integrator, was used. Injections were made in a Model 7125 syringe-loading sample injector valve (Rheodyne, Cotati, CA, U.S.A.). A prepacked Perkin-Elmer C_8 reversed-phase column was used (25 cm × 4.6 mm I.D., particle size 10 μm). The mobile phase, constantly degassed with helium during the whole analytical procedure, was phosphate buffer (0.02 *M*, pH 2.8)–acetonitrile (65:35, v/v) and the flow-rate was 1.6 ml/min. All solutions were filtered through a Millipore (Molsheim, France) 0.2-μm filter and the assay was performed at room temperature.

Sample preparation. In a disposable glass centrifuge tube, 20 μl of 0.1 *M* hydrochloric acid (containing 4 μg of I.S.) were added to 1 ml of rat plasma. This mixture was vortex-mixed for 5 s, then placed for 30 min in a shaking water-bath (SW-20 C, Julabo Labortechnik) at 37 °C and 120 rpm. Plasma deproteinization was performed by addition of 40 μl of 70% perchloric acid and vortex-mixing for 5 s. Then a further purification was performed with 15 ml of diethyl ether by mechanical shaking for 10 min and centrifugation for 5 min (1000 *g*). This procedure was repeated twice and the organic layers were discarded, then 200 μl of 4 *M* sodium hydroxide solution were added to the residue. The solution was transferred dropwise into a dry Extrelut-1 column (Merck) allowed to stand for 30 min and then eluted with 15 ml of dichloromethane. The organic phase was evap-

orated under a stream of nitrogen at 60°C in a heating bath, the residue was dissolved in 50 μl of mobile phase and 10 μl of the solution were injected into the column.

Calibration graph and sample analysis. Standard samples were prepared by adding 20 μl of 0.1 *M* hydrochloric acid containing 1, 2, 4 and 5 μg of I·HCl and 4 μg of I.S. to 1 ml of blank rat plasma. A calibration graph was obtained by plotting the peak-area ratio (I/I.S.) against the concentration of I in standard samples. I.S. (4 μg) was added to plasma samples obtained from treated animals and the mixture was extracted. For each set of twenty plasma samples a new calibration graph was constructed. Concentrations of I·HCl in plasma samples were calculated from the regression curve by inverse prediction.

Preliminary studies indicated that in blank rat plasma spiked with I at concentrations ranging from 1 to 5 μg/ml and stored at −20°C no detectable drug decomposition occurred for at least three months.

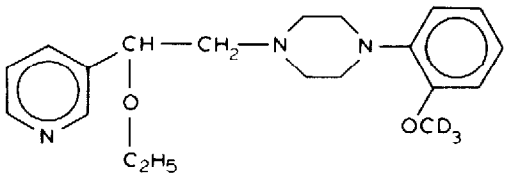
Detection of I in rat plasma. Male Wistar rats (Nossan, Correzzana, Milan, Italy) weighing 300–350 g were used. After overnight starvation, the rats were subdivided into six groups of five animals each and orally dosed with 25 mg/kg I·HCl (dissolved in water). After 0, 5, 10, 15, 30 and 60 min, blood samples (3–5 ml) were withdrawn by means of heart puncture, using heparinized syringes. Blood collection was carried out under light diethyl ether anaesthesia. The samples were placed in disposable tubes and rapidly centrifuged (2000 *g* for 10 min), then analysed according to the HPLC method described above.

GC–MS–ID method

Materials. I base and 1-[2-ethoxy-2-(3′-pyridyl)ethyl]-4-(2′-trideuteromethoxyphenyl)piperazine (I-d_3) were synthesized in our laboratories, the former according to ref. 2 and the latter as described below. All other reagents were as used for HPLC determinations. Stock solutions of I (10 μg/ml) and I-d_3 (50 μg/ml) were prepared in ethanol (LiChrosolv, Merck). Storage of these solutions at 4°C did not result in detectable decomposition.

Synthesis of the internal standard, 1-[2-ethoxy-2-(3′-pyridyl)ethyl]-4-(2′-trideuteromethoxyphenyl)piperazine (I-d_3). A solution of 1-[2-hydroxy-2-(3′-pyridyl)ethyl]-4-(2′-methoxyphenyl)piperazine [2] (3.13 g, 10 m*M*) in 48% hydrobromic acid was refluxed for 16 h and evaporated in vacuo. The crude residue was dissolved in water and the solution was made basic with 10% sodium hydroxide solution, extracted with diethyl ether and neutralized by the addition of dilute acetic acid to give 1-[2-hydroxy-2-(3′-pyridyl)ethyl]-4-(2′-hydroxyphenyl)piperazine (II) (1.7 g, 57%); m.p. 132°C (from ethyl acetate).

To a stirred solution of 150 mg (5.7 m*M*) of sodium hydride (55% in paraffin oil) in 12 ml of dimethyl sulphoxide (DMSO) were added in portions 1.7 g of II and then a solution of 1 g (7 m*M*) of iodomethane-d_3 in 2 ml of DMSO. The solution was stirred at room temperature for 4 h, poured into water and extracted with ethyl acetate. The organic phase was washed with 10% sodium hydroxide solution and water to neutrality and then extracted with dilute hydrochloric acid. The aqueous solution was made basic with 10% sodium hydroxide solution and the precipitate was filtered to give 1-[2-hydroxy-2-(3′-pyridyl)ethyl]-4-(2′-tri-



Isotopic distribution

$^2H_0$ = 1.06 %
$^2H_1$ = 9.61 %
$^2H_2$ = 3.28 %
$^2H_3$ = 87.0 %

Fig. 1. Structure of deuterium-labelled I and its isotopic distribution.

deuteromethoxyphenyl)piperazine (III) (1 g; 55%); m.p. 92°C (from diisopropyl ether). From 1 g of III I-d_3 (720 mg; 60%) (m.p. 183°C) was prepared according to the procedure described in ref. 2.

The isotopic distribution of the deuterated compound was checked by selected-ion monitoring (SIM) on 100 ng of I-d_3 using capillary GC–MS at $M/\Delta M$ 3000 resolution (10% valley definition) (Fig. 1).

Gas chromatography–mass spectrometry. A Pye 204 gas chromatograph (Pye Unicam, Cambridge, U.K.), equipped with a DB-17 fused-silica capillary column (15 m×0.32 mm I.D.) (J&W Scientific, Folsom, CA, U.S.A.) and directly coupled to a VG 7070 EQ mass spectrometer (VG Analytical, Manchester, U.K.), was used, and data were acquired using a PDP 8/A computer (Digital Equipment, Meynard, MA, U.S.A.). The sample (1 μl) was injected by a moving needle–solid inlet system [6]. The oven temperature and the injection port were set at 260 and 350°C, respectively. Helium was the carrier gas (inlet pressure 0.4 bar). The mass spectrometric resolution was 2500 $M/\Delta M$ (10% valley definition). The ion source and transfer line temperature was 250°C. The energy of the electron beam was 70 eV and the trap current 50 μA. Data were acquired by SIM at m/z 205.13 for I and m/z 208.15 for I-d_3.

Sample preparation. In a disposable glass centrifuge tube, 20 μl of a solution containing 15 ng of I.S. were added to 1 ml of human plasma. This mixture was vortex-mixed for 5 s, allowed to stand for 30 min to equilibrate in a shaking water-bath at 37°C and 120 rpm [5], and then treated as described above for the HPLC procedure to effect plasma deproteinization. The mixture was transferred into an Extrelut-1 pre-packed glass column (Merck), and after about 30 min it was eluted with 30 ml of diethyl ether. The eluate was discarded and 1 ml of 1 *M* sodium hydroxide solution was added at the top of the column. After about 15 min, elution was performed with 15 ml of dichloromethane. The organic phase was evaporated under a stream of nitrogen at 60°C in a heating bath, the residue was dissolved in 20 μl of methanol and 1-μl aliquots of the solution were injected into the GC–MS apparatus.

Calibration graph and sample analysis. To obtain a calibration graph, standard samples were prepared by adding 20 μl of methanol containing 1, 5, 15, 30 and 60 ng of I and 15 ng of I.S. to 1 ml of blank human plasma. The concentrations of I in plasma samples were calculated using the ID method. The calibration graph was constructed by plotting peak-area ratio (I/I-d_3) against I concentration in standard samples. I-d_3 (15 g) was added to plasma samples obtained from treated human subjects and the mixture was extracted. The concentrations of I in these plasma samples were calculated from the regression curve by inverse prediction.

Preliminary studies indicated that in blank human plasma spiked with I at concentrations ranging from 1 to 60 ng/ml and stored at −20°C no detectable drug decomposition occurred for at least three months.

Detection of I in human plasma. A tablet containing 5 mg of IP/66 hydrochloride was administered to a fasted healthy subject. Blood samples (3–5 ml) were collected 0, 0.5, 1, 2, 6 and 12 h after dosing. Plasma levels were measured according to the GC–MS–ID method described above.

RESULTS

High-performance liquid chromatography

Typical chromatograms are shown in Fig. 2. The retention times of I and I.S. were 5.3 and 10.8 min, respectively, for a total analysis time of 13 min. Maximum absorption was obtained at 208 nm. In spite of this short working wavelength, the baseline was stable and free from interferences (see Fig. 2).

Extraction efficiency. The percentage recovery of the extraction procedure for I·HCl and its I.S. was determined at three concentrations, ranging from 2 to 5 μg/ml. Peak areas obtained from extracted plasma samples were compared with those obtained by injection of standard solution. The mean recoveries [± coefficient of variation (C.V.)] of I·HCl and the I.S. were 82.8±10.2% ($n=6$) and 93.8±7.89% ($n=6$), respectively.

Accuracy and precision. The intra- and inter-assay precision were determined by performing three and six replicate analyses, respectively, on 1-ml rat plasma aliquots to which 2–4 μg of I·HCl and 4 μg of I.S. had been added. The concentrations in these samples were calculated from the regression curve by inverse prediction and are summarized in Table I. The mean (±C.V.) recovery of I·HCl

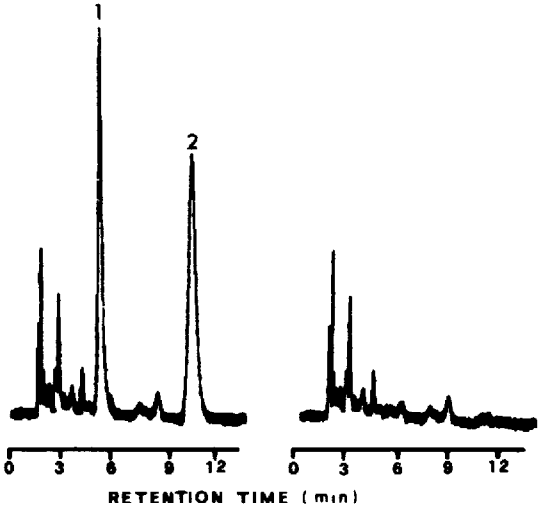


Fig. 2. Typical chromatograms of rat plasma collected 10 min after oral administration of 25 mg/kg I·HCl (left) or placebo (right). Peak 1 corresponds to a concentration of 2.2 μg/ml I and peak 2 to a concentration of 4 μg/ml internal standard.

TABLE I

PRECISION AND ACCURACY OF THE HPLC METHOD FOR THE DETERMINATION OF I

Samples of 1 ml of rat plasma were supplemented with 2 μg/ml (low concentration) and 4 μg/ml (high concentration) of I.

Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	n	Coefficient of variation (%)
Intra-assay			
2	2.0 ±0.19	3	9.5
4	4.01 ± 0.39	3	10.1
Inter-assay			
2	2.02 ± 0.2	6	9.9
4	4.16 ± 0.42	6	10.2

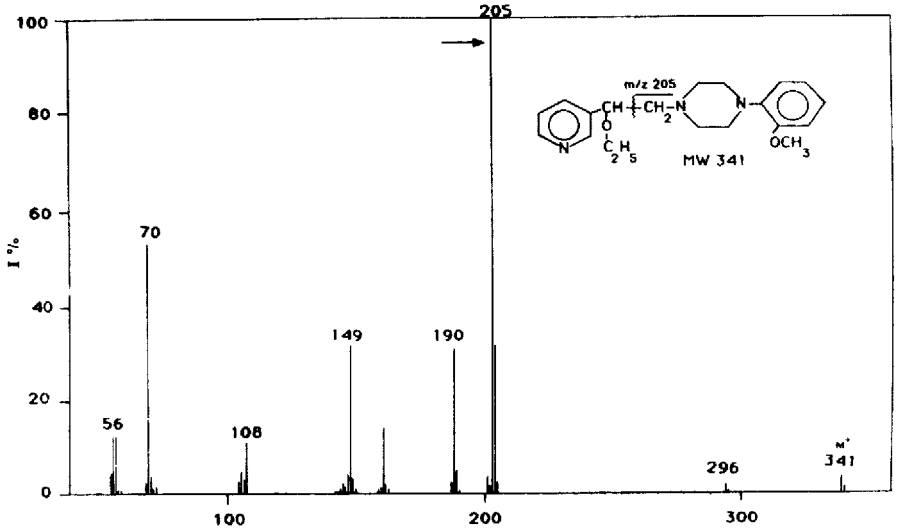


Fig. 3. Mass spectrum of I (electron impact, 70 eV).

from plasma was 102.67 ± 9.50% ($n=12$). There was no significant difference ($p<0.05$) in recovery over the whole concentration range.

Linearity. Good linearity was obtained for I·HCl over the range 1–5 μg/ml ($y=0.3334x+0.0289$, $r=0.9941$). The lowest detectable drug concentration was 1 μg/ml. At this concentration the signal-to-noise ratio is higher than 3.

Gas chromatography–mass spectrometry

Mass spectra and SIM of I and I-d_3. Figs. 3 and 4 show the mass spectra of I and I-d_3, respectively. As the molecular ions of I and I-d_3 (m/z 341 and 344, respectively) were of low abundance under the instrumental conditions used, the sensitivity of the assay was enhanced by chosing for SIM, the base peak ions at m/z 205 for I and m/z 208 for I-d_3. Fig. 5 shows a typical SIM report for human

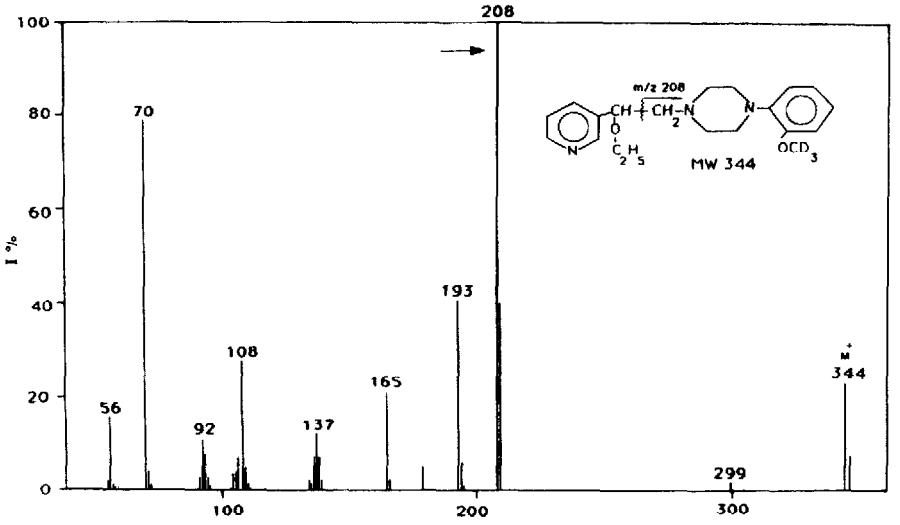


Fig. 4. Mass spectrum of I-d_3 (electron impact, 70 eV).

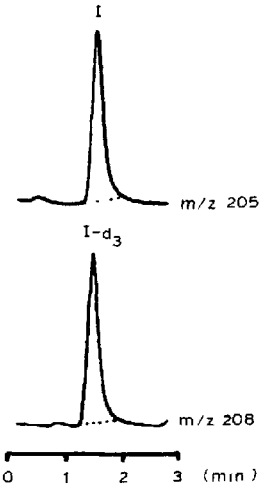


Fig. 5. Selected-ion monitoring of human plasma 60 min after oral administration of a tablet containing 5 mg of I. The upper peak corresponds to a concentration of 20 ng/ml I and the lower peak to a concentration of 15 ng/ml I-d_3.

plasma obtained 60 min after oral administration of a tablet containing 5 mg of I·HCl. The retention time of I and I-d_3 was 1.40 min.

Accuracy and precision. The intra- and inter-assay precision were determined by performing three or five replicate analyses, respectively, on 1-ml human plasma aliquots to which 1 and 30 ng of I and 15 ng of I-d_3 had been added. The concentrations in these samples were calculated from the regression curve by inverse prediction and are summarized in Table II. The mean ($\pm$C.V.) recovery of I was $101.01 \pm 5.50\%$ ($n=10$). There was no significant difference ($p<0.05$) in recovery over the whole concentration range.

TABLE II

PRECISION AND ACCURACY OF THE GC–MS–ID METHOD FOR THE DETERMINATION OF I

Samples of 1 ml of human plasma were supplemented with 1 ng/ml (low concentration) and 30 ng/ml (high concentration) of I.

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	*n*	Coefficient of variation (%)
Intra-assay			
1	1.01 ± 0.04	3	4.23
30	30.07 ± 1.18	3	3.91
Inter-assay			
1	1.01 ± 0.08	5	7.87
30	30.16 ± 0.85	5	2.81

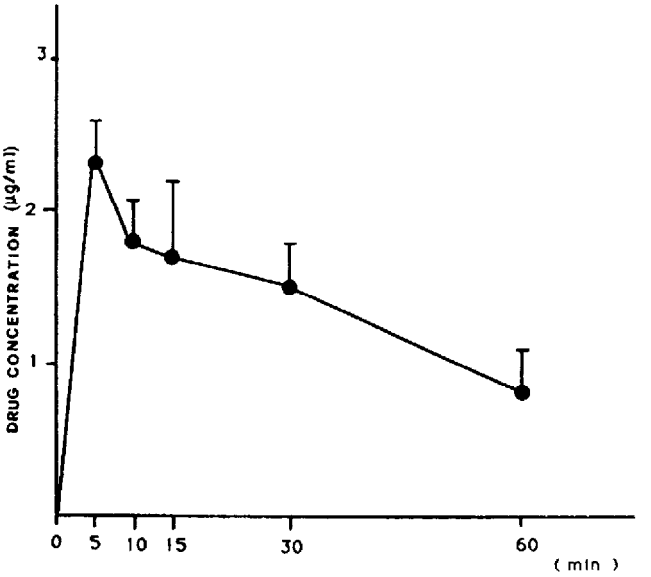


Fig. 6. Time dependence of I·HCl concentration in rat plasma after a single oral dose of 25 mg/kg I Each value is the mean ± S.E. for six animals.

Linearity. For human plasma samples good linearity was obtained for I over the range 1–60 ng/ml ($y = 0.0583x + 0.3688$, $r = 0.9935$). The method exhibits very good precision and accuracy at both low (1 ng/ml) and high (30 ng/ml) I concentrations (Table II), and therefore the amount of a possible interfering compound (which could be suggested by the large intercept) should be constant in each sample and unrelated to I concentration. The lowest detectable drug concentration was 1 ng/ml. At this concentration the signal-to-noise ratio is higher than 5.

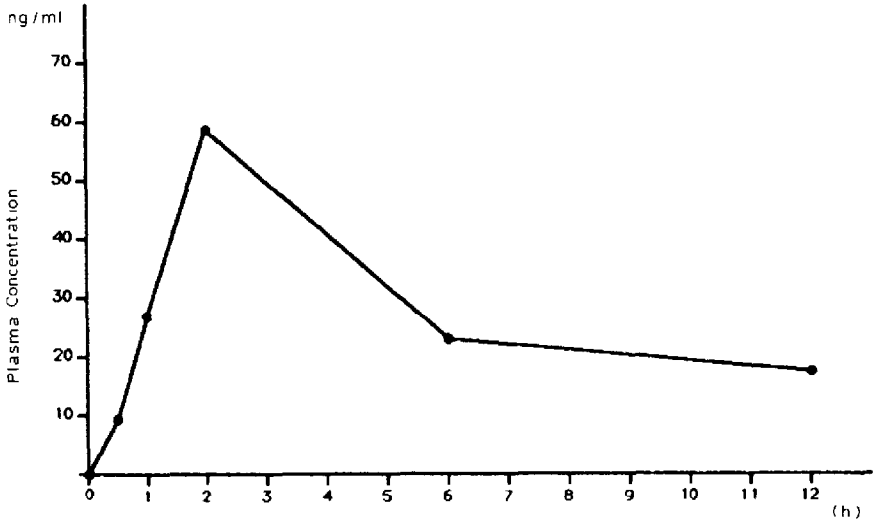


Fig. 7. Time dependence of I concentration in human plasma after a single oral dose of a tablet containing 5 mg of I.

Application of the method

Some results are presented for the application of the HPLC and GC–MS–ID method to the detection of I in rat and human plasma, respectively.

Rat plasma samples obtained as described above were analysed according to the HPLC method, and the results are shown in Fig. 6. The maximum drug concentration (17.4 ± 1.9 μg/ml) was detected after 5 min.

Human plasma samples obtained from a healthy subject treated as described under Experimental were analysed according to the GC–MS–ID method, and the results are shown in Fig. 7. The maximum concentration of I in plasma (58 ng/ml) was reached after 2 h.

DISCUSSION

The results indicate that both the HPLC and GC–MS–ID methods are precise, sensitive and reliable for detecting I in rat and human plasma. Although the range of drug sensitivity is obviously lower with the HPLC procedure, this method (which is less expensive and time-consuming than the GC–MS–ID method) could be usefully employed in animal pharmacokinetic studies, in which higher doses are administered and considerable amounts of the drug can be detected in organs and tissues. Further, the HPLC procedure might be further improved to obtain a stereospecific determination of biological concentrations of optical isomers of I. Indeed, a similar HPLC procedure has already been used for the resolution of the diastereoisomeric derivatives of 1-[2-hydroxy-2-(3′-pyridyl)ethyl]-4-(2′-methoxyphenyl)piperazine, a precursor of I [4].

In view of the low dosage of I to be used for therapeutic purposes in humans, the GC–MS–ID method is particularly relevant as it will permit plasma concentrations of I as low as 1 ng/ml to be determined. Hence the use of this procedure

may become mandatory in studies aimed at evaluating the absorption, distribution and excretion of I in humans.

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