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A method using a liquid chromatographic-electrospray-mass spectrometric assay for the determination of antimigraine compounds: preliminary pharmacokinetics of MDL 74,721, sumatriptan and naratriptan, in rabbit

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Abstract

MDL 74,721 (I), sumatriptan(II) and naratriptan(III) are new 5-HT_1-like agonists that have potential as a novel treatment for migraine. Liquid chromatographic-electrospray-mass spectrometric (LC-ESI-MS) assays have been developed to compare the pharmacokinetics of these three antimigraine compounds. The concentration of each parent drug was determined using a solid-phase extraction method and LC-ESI-MS analysis demonstrating the high sensitivity and specificity of the methods down to subnanogram levels in rabbit plasma samples. Pharmacokinetic parameters evaluated after administration of single intravenous and oral doses were very similar and the ANOVA analysis did not show any statistically significant differences for $t_{1/2}$, C_{max}, V or AUC (normalised). The pharmacokinetic parameters showed short $t_{1/2}$ (range 1.14–1.9 h) either after intravenous (iv) or oral (po) administration and high total body clearance (CL) after the po dose both probably due to extensive and rapid metabolism of the parent drugs as suggested by the low values for bioavailability (range 13.4–22.8%). © 1997 Elsevier Science B.V.

Keywords: Pharmacokinetics; 5-HT_1-agonists; Electrospray; Mass spectrometry; High-performance liquid chromatography; Solid-phase extraction

1. Introduction

Migraine is a common disorder characterized by unilateral headache often accompanied by nausea and/or vomiting which usually begins in childhood, adolescence or early adult life. One of the first theories on migraine was described by Graham and Wolff [1] suggesting that the pathogenesis of the disease is primarily of vascular origin, the headache being associated with a marked and prolonged phase of cranial vasodilatation.

The role of neurotransmitters, particularly 5-hydroxytryptamine (5-HT), as a central mediator of a migraine attack has received much attention [2–5]. The characteristics of 5-HT receptors that mediate contraction of blood vessels have been studied for several years [6,7].

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MDL 74,721 (I), with a high affinity for the 5-HT_{1A} receptor, is a member of the family of compounds (including sumatriptan [8–10] and naratriptan) that acts as a 5-HT_1 receptor agonist. These compounds have been identified as potential antimigraine drugs.

Liquid chromatography is widely used for the quantitative determination of pharmaceutical compounds with UV, fluorescence or electrochemical detection. More recently, mass spectrometry (MS) has been introduced as a highly sensitive and specific detector for HPLC analyses. During the past 10 years several approches have tried to interface LC to MS, including thermospray [11,12], particle beam [13,14] and recently electrospray (ES) interfaces [15,16]. For the determination of pharmaceutical compounds for preclinical and clinical studies, LC-MS with atmospheric pressure ionization (API), is rapidly becoming a powerful analytical tool [17,18].

The present paper describes the analytical method and the pharmacokinetics of MDL 74,721, sumatriptan and naratriptan in the rabbit after intravenous and oral administration and compares the pharmacokinetic parameters of these three antimigraine compounds.

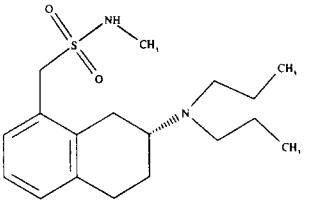
2. Materials and methods

2.1. Reference compounds and reagents

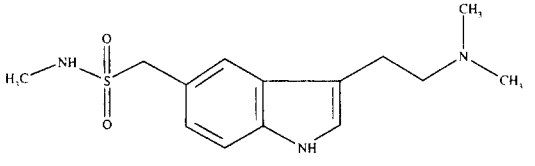
MDL 74,721G (I) (1-naphthalenemethanesulfonamide,7-(dipropylamino)-5,6,7,8-tetrahydro-*N*-methyl,(R),(Z)-2-butenedioate (1:1), Sumatriptan(II) (3-[2-(dimethylamino)ethyl]-*N*-methyl-1H-indole-5-methanesulfonamide hemi(butanedioate)), naratriptan(III) *N*-methyl-3-(1-methyl-4-piperidinyl)-1H-indole-5-ethanesulphonamide and MDL 74,967P (IV) (1-naphtalenemethanesulfonamide,7-(dipropylamino)-5,6,7,8-tetrahydro-*N*-phenyl-methyl-(S)-ethanedioate (1:1) were synthetized at the Marion Merrell Strasbourg Center.

Compound IV was selected as the internal standard of I and I was selected as the internal standard of compounds II and III. Chemical structures of the compounds are shown in Fig. 1.

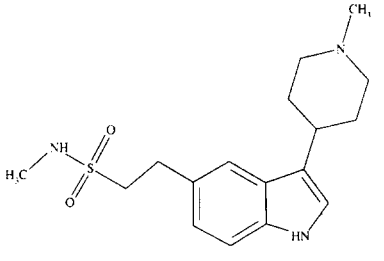
Methanol (Uvasol) was purified by a Millipore MilliQ system and had a resistance greater than 10 MΩ cm^{-1}.



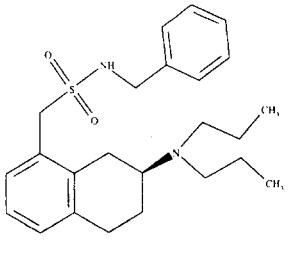
I (MDL 74721)



II (Sumatriptan)



III (Naratriptan)



IV (MDL 74967)

Fig. 1. Chemical structures of I (MDL 74,721), II (sumatriptan) and III (naratriptan) and IV (MDL 74,967).

Table 1
Details of body weights, doses and dose solutions administered

	Drug	Body weight (kg)	Mode of administration	Dose (mg kg^{-1})	Volume (ml kg^{-1})
1		3.6	iv	0.5	0.5
2		3.2	iv	0.5	0.5
3		2.2	iv	0.5	0.5
	I				
1		3.7	po	2.5	0.5
2		3.2	po	2.5	0.5
3		2.1	po	2.5	0.5
4		2.6	iv	0.25	0.5
5		2.6	iv	0.25	0.5
6		2.4	iv	0.25	0.5
	II				
4		2.7	po	1.25	0.5
5		2.6	po	1.25	0.5
6		2.4	po	1.25	0.5
7		2.4	iv	0.25	0.5
8		2.5	iv	0.25	0.5
9		2.4	iv	0.25	0.5
	III				
7		2.5	po	1.25	0.5
8		2.6	po	1.25	0.5
9		2.4	po	1.25	0.5

2.2. Drug administration

For each compound under study, three male New Zealand SPF (specific pathogen free) rabbits were supplied by Elevage scientifique du Domaine des Dombes (Romans, France). The animals were subjected to an acclimatization period of 2 weeks under controlled temperature (21 ± 1°C) and humidity (55 ± 10%) conditions. The animals were fasted 12 h before drug administration but had free access to drinking water. I was administered as an intravenous (iv) dose of 0.5 mg kg^{-1} and as an oral (po) dose of 2.5 mg kg^{-1}. Sumatriptan and naratriptan were administered as single doses of 0.25 mg kg^{-1} (iv) and 1.25 mg kg^{-1} (po). A one week wash-out separated iv and po administrations. Details of body weights and dose solutions are given in Table 1.

2.3. Plasma sample collection

An indwelling arterial catheter was inserted under local anesthesia in the central artery of a rabbit ear for plasma sample collection and a cannula was inserted in the lateral vein of the opposite ear prior to iv administration. A 2 ml blood sample was taken as a baseline for the measurement of the blank plasma concentrations. For both iv and po administration, blood samples were taken at the following times: basal, 1, 5, 15, 30, 45 min and 1, 1.5, 2, 3, 4, 5, 6, 7 and 8 h after drug administration.

Blood samples (1–2 ml depending on the time point) for I, II and III determination were drawn into tubes containing heparin (50 IU ml^{-1} blood). The plasma was separated in a refrigerated centrifuge, frozen and stored at −20°C until analyzed.

2.4. Standard and sample preparation

Stock solutions of I, II, III and IV at 1 mg ml^{-1} of free base were prepared in acetonitrile/water (50:50, v/v) and stored at 4°C. For each assay, working solutions were prepared daily by dilution of the corresponding stock solution to yield 0.01, 0.1 and 1 ng ml^{-1} for I, II and III and 1 ng ml^{-1} for the corresponding IS.

For each calibration curve, duplicate samples of rabbit plasma (500 μl) were spiked with I, II or III at various concentrations: 0, 0.5, 1, 2, 5, 10, 20, 50, 100 ng ml^{-1}. The concentration of IS was set at 40 ng ml^{-1}.

Quality-control plasma samples were prepared at 1 and 10 ng for the within-day reproducibility. The concentration of IS was set at 40 ng ml^{-1}. Five plasma samples were extracted and analysed for each concentration.

Plasma samples (100 or 300 μl), spiked with 20 ng of IS were deproteinised by adding 800 μl of methanol. After stirring on a vortex mixer, the samples were centrifuged 10 min at 15 000 rpm in an Eppendorf centrifuge. Then the supernatant was transfered into a 10 ml Benchmate tube and evaporated to dryness under a stream of nitrogen at 40°C. The sample was reconstituted in 1 ml of a mixture of water/methanol (90:10, v/v).

Sample preparation was developed using a Benchmate robot (Zymark, Paris, France). Disposable extraction columns (3 ml, Lichrolut C18-Select B, for the extraction of I) were purchased from Merck (Darmstadt, Germany) and (3 ml, CN, for the extraction of II and III) Baker (Deventer, The Netherlands).

Columns were conditionned with 3 ml of methanol and then washed with 3 ml of distilled water. One ml of sample was loaded onto the cartridge and the column was washed with 5 ml distilled water and 3 ml methanol/water (20:80, v/v). The compounds were eluted in 2 ml acetonitrile containing 10% of a 1 N HCl solution. The extract was evaporated to dryness under a stream of nitrogen at 40°C and the residue was reconstituted in 300 μl of the mobile phase A. Thirty μl of this extract were injected on the LC-MS system.

2.5. Liquid chromatography-mass spectrometry

The HPLC system used was an HP1050 quarternary solvent delivery pump with an HP1050 autosampler (Hewlett Packard, Les Ulis, France).

For the analysis of I and III, separation was achieved using a 150 mm × 2 mm i.d. Nova-Pak C8 column (4 μm particle size) (Waters, Molsheim, France) and for the analysis of II, separation was achieved using a 150 mm × 2 mm i.d. Nova-Pak C18 column (4 μm particle size) (Waters, Molsheim, France).

Solvent A, 90% 20 mM ammonium acetate with 10% acetonitrile and solvent B, 80% acetonitrile with 20% 20 mM ammonium acetate were used. The initial gradient conditions consisted of 80% of solvent A and 20% of solvent B at a flow rate of 0.5 ml min^{-1}. The final conditions were achieved in 20 min with 20% solvent A and 80% solvent B. The column was reequilibrated at initial conditions for 10 min before the next injection. Column temperature was maintained at 35°C.

The mass spectrometer employed was a single stage quadrupole (SSQ-700) or a triple stage quadrupole (TSQ-7000) manufactured by Finnigan MAT (San Jose, CA, USA), fitted with an atmospheric pressure ionization (API) interface. The ion source was operated in the electrospray (ESI) mode and in the positive ion mode. HPLC solvent entered the ESI source through a 100 μm i.d. fused silica capillary. A potential of 4.8 kV was applied to the needle electrode, producing charged liquid droplets at atmospheric pressure in a nitrogen sheath gas set at a pressure of 40 psi. The auxilliary gas was set at 15 ml min^{-1}. A potential of + 5.8 V was applied to the capillary and + 44.6 V to the tube lens. The temperature of the heated capillary was set at 230°C.

Quantification of I, II and III were performed by focusing the mass spectrometer in the selected ion monitoring mode on the quasimolecular ion of each compound (m/z 339.1 for I, m/z 296.1 for II and m/z 336.1 for III). The ESI-LC-MS was automated by icl procedures which pilot the HP1050 autosampler, the HP1050 LC-pump and all the ESI-MS parameters. Peak areas for all components were integrated using CHRO software on SSQ 700 or TSQ 7000 and peak area

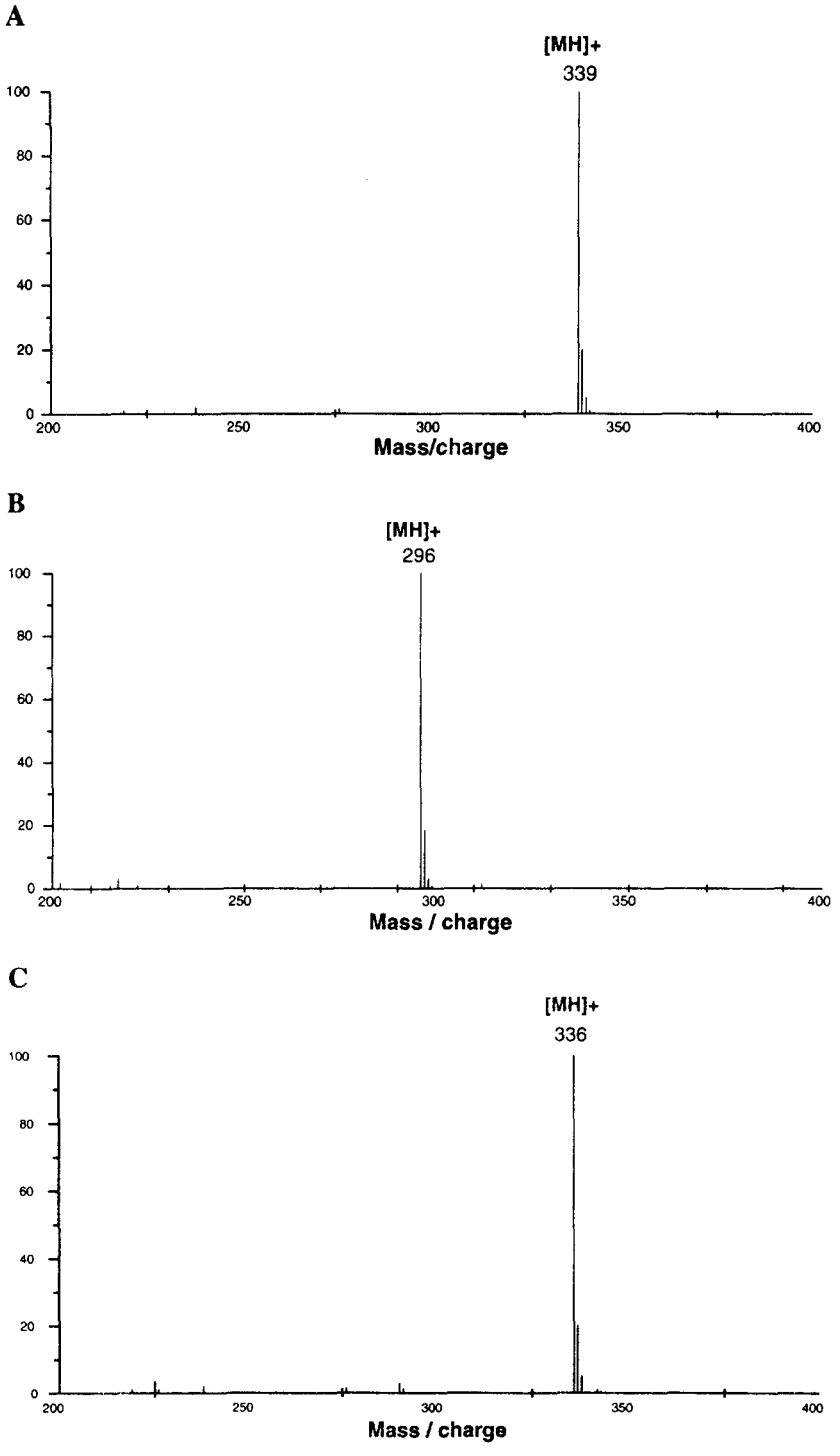


Fig. 2. Electrospray mass spectrum of (A) I, (B) II and (C) III.

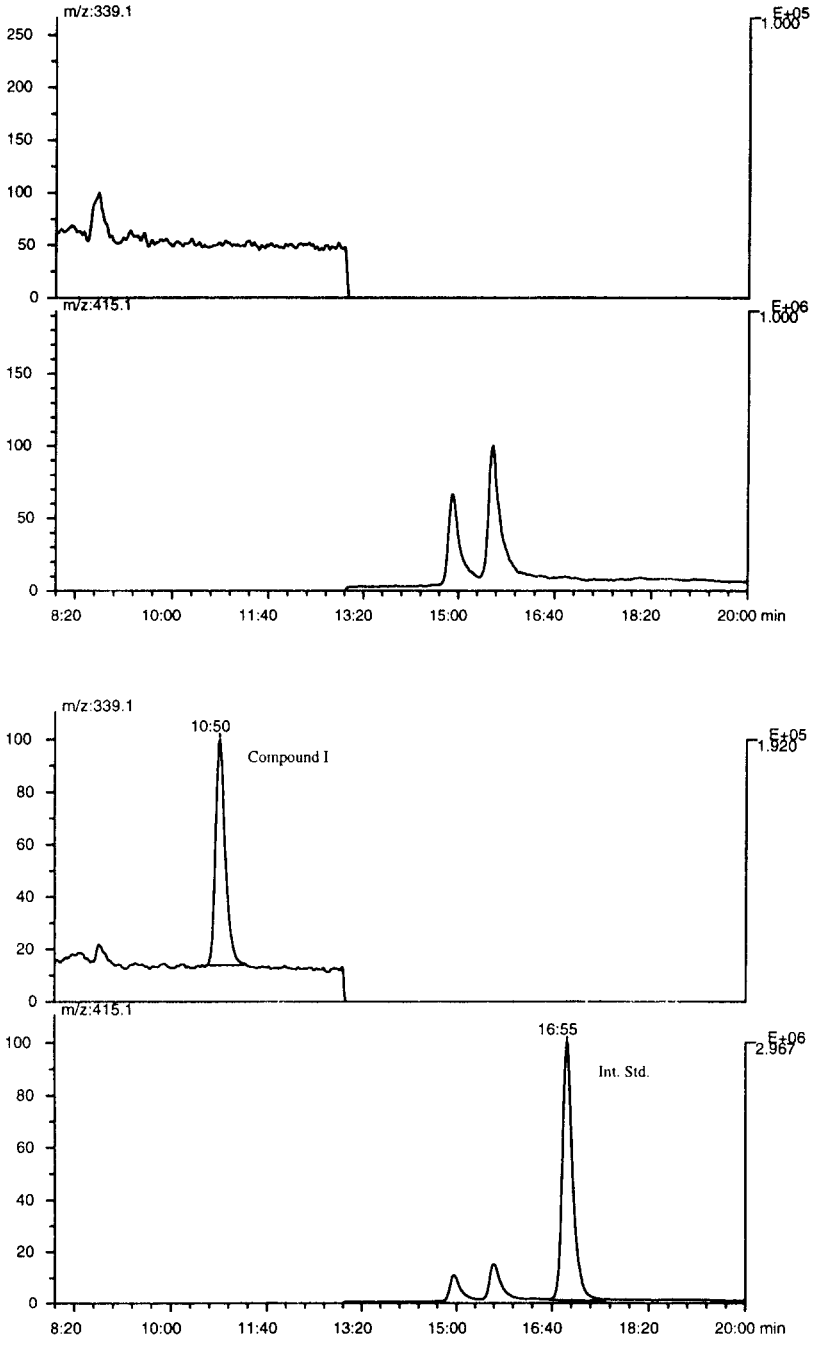


Fig. 3. Selected ion monitoring traces obtained from a control plasma sample (top) and spiked with 0.5 ng of I and 10 ng of IS (bottom).

ratios (area of drug/area of IS) were plotted versus concentration of each compound using unweighted linear regression.

2.6. Pharmacokinetic calculations

A multicompartimental model was fitted to the plasma concentration-time courses of I, II and III following intravenous and oral administration using the weighted least squares algorithm [12] of the SIPHAR pharmacokinetic package (Simed, Paris, France). The area under the plasma concentration-time curve (AUC) was calculated using the trapezoidal rule. The apparent volume of distribution (V), the total body clearance (CL) and the terminal half-life ($t_{1/2}$) were obtained from fitted data using standard equations. Maximun plasma concentration (C_{max}) and time of the C_{max} (t_{max}) are observed values. The $t_{1/2}$ was expressed as the mean ± S.D. of individual $t_{1/2}$.

The absolute bioavailability (F) after oral administration was calculated from the following equation:

$$F\% = \frac{AUC_{po}}{AUC_{iv}} \times \frac{dose_{iv}}{dose_{po}} \times 100$$

The pharmacokinetic parameters were analysed by one-way analysis of variance (ANOVA) on the statistical package of the SIPHAR software. When an effect was observed, the pairwise test was applied to identify significant differences.

3. Results

3.1. LC-MS analysis

Mass spectra of I, II, III and IV are shown in Fig. 2. Each compound presents a very simple mass spectrum with a major ion corresponding to $[M + H]^+$. Quantitative determination of I, II and III were performed by focusing the mass spectrometer in the selected ion monitoring mode on the quasi-molecular ion $[M + H]^+$ of each molecule; m/z 339.1 for I, m/z 296.1 for II and m/z 336.1 for III.

Chromatograms of extracts of plasma containing 0 and 0.5 ng ml^{-1} of I are shown in Fig. 3. Identical results were found for II and III. There were no interferences with the determination of each parent drug and their internal standard from either endogenous plasma components or potential metabolites.

The solid–liquid extraction (C18 cartridges for I and CN cartridges for II and III) yielded good recoveries and reproducibilities of the automated method. Good adsorption of the analytes from aqueous solutions and quantitative elution when using acetonitrile containing HCl were seen. The washing of columns by a mixture of methanol/water allowed us to eliminate many of the endogenous compounds in plasma samples leading to clean extracts. The percentage recovery calculated using peak areas from unextracted and extracted standards were 76.3 ± 7.4% for I, 78.1 ± 8.2% for II and 81.3 ± 6.7% for III. The calibration curves obtained with spiked plasma samples showed good linearity for I, II and III in the concentration range 0.25–50 ng 500 μl^{-1} with correlation coefficients ranging from 0.9992 to 0.9998.

Within-day reproducibility was determined by extraction of five sets of standards, each set of standards consisting of rabbit plasma containing I, II or III at concentration of 1 and 10 ng 500 μl^{-1}. Data are summarised in Table 2. The relative errors expressed in percent (RE%) represent the accuracy of the analytical method and the coefficient of variation expressed in percent (CV%) represent the precision. At 1 ng 500 μl^{-1}, CVs were below 10.6% and at 10 ng 500 μl^{-1} they were below 6.2%. All concentrations were within ± 10% of the theoretical concentration.

3.2. Pharmacokinetics of MDL 74 721 (I)

The mean plasma concentration-time course of I after a single intravenous dose of 0.5 mg kg^{-1} and a single oral dose of 2.5 mg kg^{-1} are shown in Fig. 4A. The calculated pharmacokinetic parameters are summarized in Table 3.

Following the intravenous dose of 0.5 mg kg^{-1} I given as a bolus, plasma concentration-time curves showed typical bicompartmental characteristics with $t_{1/2}$ ranging from 1.24 to 1.58 h and AUCs ranging from 108.1 to 234.7 ng h ml^{-1}. After iv administration, V and CL ranged from 4.9 to 8.3 l kg^{-1} and from 35.0 to 76.6 ml min^{-1} kg^{-1}, respectively.

Table 2
Within-day accuracy (RE%) and precision (CV%) of the LC-ESI-MS method

Compound	Added concentration (ng 500 μl^{-1})	Plasma concentration (mean ± S.D.) ($n = 5$) (ng 500 μl^{-1})	CV (%)	RE (%)
I	1	0.94 ± 0.10	10.6	−6
	10	9.98 ± 0.62	6.2	−0.2
II	1	0.90 ± 0.06	6.6	−10
	10	9.80 ± 0.38	3.8	−2
III	1	1.03 ± 0.10	9.7	+3
	10	10.27 ± 0.63	6.1	+2.7

Following the oral administration of 2.5 mg kg^{-1} of I, plasma concentration-time curves showed typical multicompartmental characteristics (Fig. 4A). In comparison with iv administration, similar values were calculated for $t_{1/2}$ with a range of 1.24–1.83 h. For the AUCs, the range was 81.9–279.0 ng h ml^{-1}. Larger variations were observed on C_{max} and t_{max} with ranges of 49.0–201.5 ng ml^{-1} and 5–30 min, respectively. The calculated V and CL showed larger values compared to those of the iv administration with ranges of 22.0–55.1 l kg^{-1} for V and 161.7–508.3 ml min^{-1} kg^{-1} for CL. The mean (± S.D.) F calculated from AUC after po and iv administration was shown to be 22.8 ± 21.9% with values ranging from 6.9 to 47.9%.

3.3. Pharmacokinetics of sumatriptan(II)

The mean plasma concentration-time course of II after a single intravenous dose of 0.25 mg kg^{-1} and a single oral dose of 1.25 mg kg^{-1} are shown in Fig. 4B. The calculated pharmacokinetic parameters are summarized in Table 3.

Following the intravenous dose of 0.25 mg kg^{-1} II given as a bolus, plasma concentration-time curves also showed typical bicompartimental characteristics with $t_{1/2}$ ranging from 1.83 to 2.06 h and AUCs ranging from 85.2 to 262.8 ng h ml^{-1}. After iv administration, V and CL values were very close to those of I and ranged from 2.3 to 6.9 l kg^{-1} for V and from 15.0 to 41.6 ml min^{-1} kg^{-1} for CL.

After the oral administration of 1.25 mg kg^{-1} of II, plasma concentration-time curves showed characteristic multiple peaks leading to a wide inter-subject variability. The tmax of the first peak ranged from 1 to 5 min. Due to experimental problems with rabbit 4, data were available only for rabbits 5 and 6. For these two animals, C_{max} values were 96.5 and 277.4 ng ml^{-1}. $t_{1/2}$ values were 1.2 and 1.5 h and AUC values 122.1 and 188.8 ng h ml^{-1}. The calculated V and CL showed larger values compared with those of the iv administration with a range of 14.0–18.3 l kg^{-1} for V and 110.0–170.0 ml min^{-1} kg^{-1} for CL. The values for F were 9.3 and 24.5%.

3.4. Pharmacokinetics of naratriptan(III)

The mean plasma concentration-time course of III after a single intravenous dose of 0.25 mg kg^{-1} and a single oral dose of 1.25 mg kg^{-1} are shown in Fig. 4C. The calculated pharmacokinetic parameters are summarized in Table 3.

After the intravenous dose of 0.25 mg kg^{-1} of III, plasma concentration-time curves also presented typical bicompartmental characteristics with $t_{1/2}$ ranging from 1.07 to 1.17 h and AUCs ranging from 160.8 to 177.3 ng h ml^{-1}. V and CL values showed small variation after iv administration with a range of 2.4–2.6 l kg^{-1} for V and of 25.0–25.9 ml min^{-1} kg^{-1} for CL.

Following the oral administration of 1.25 mg kg^{-1} of III, plasma concentration-time curves showed as for I typical multicompartmental characteristics. $t_{1/2}$ values ranged from 1.22 to 2.30 h

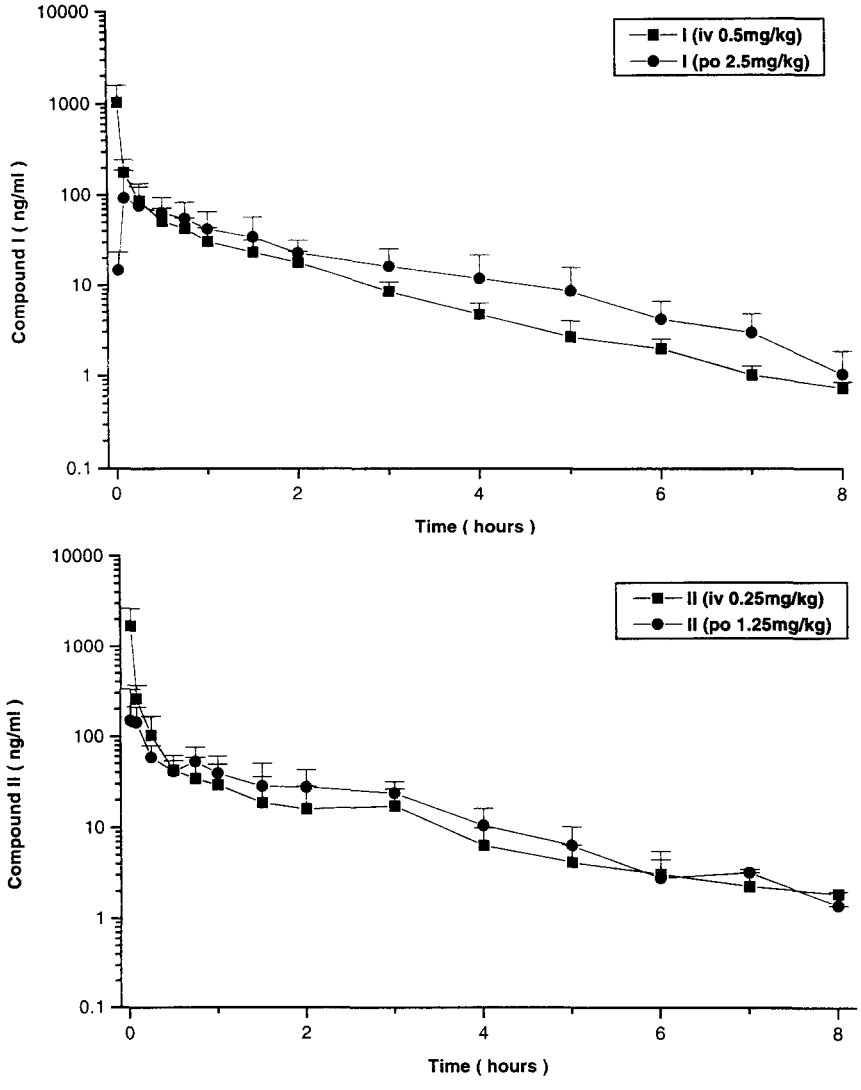


Fig. 4.

and AUC values from 73.1 to 185.5 ng h ml^{-1}. The t_{max} of III ranged from 1 to 30 min and C_{max} ranged from 42.7 to 94.8 ng ml^{-1}. As for I and II after oral administration, V and CL presented high values ranging from 14.9 to 50.2 l kg^{-1} for V and from 111.7 to 283.3 ml min^{-1} kg^{-1} for CL. The F values ranged from 9.2 to 20.9%.

4. Discussion

The method which has been developed to quantify I, II and III in rabbit plasma samples by automated solid-phase extraction and an electrospray-LC-MS technique, demonstrates specificity, sensitivity and reproducibility. The use of the new

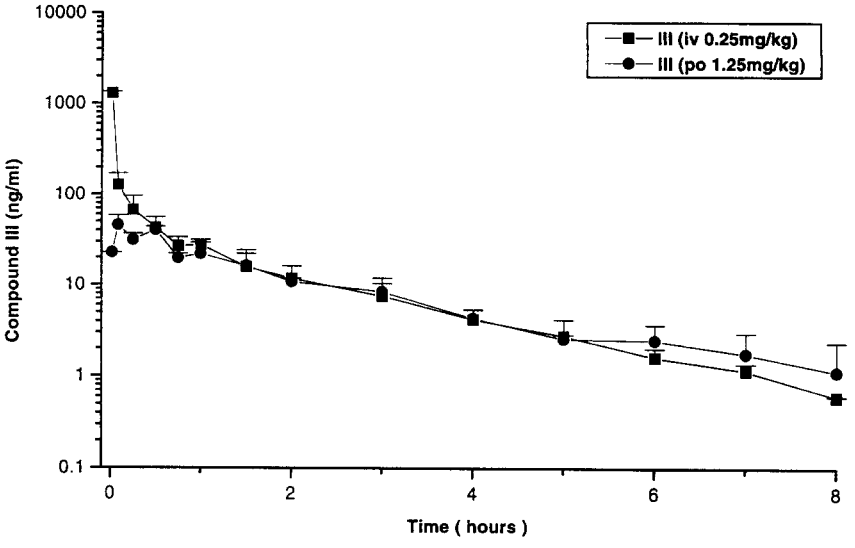


Fig. 4. (A) Mean (± S.D.) plasma concentration-time curves of I after an iv dose of 0.5 mg kg^{-1} and an oral dose of 2.5 mg kg^{-1} to rabbit ($n = 3$). (B) Mean (± S.D.) plasma concentration-time curves of II after an iv dose of 0.25 mg kg^{-1} and an oral dose of 1.25 mg kg^{-1} to rabbit ($n = 3$). (C) Mean (± S.D.) plasma concentration-time curves of III after an iv dose of 0.25 mg kg^{-1} and an oral dose of 1.25 mg kg^{-1} to rabbit ($n = 3$).

API source interface from Finnigan has shown that it is possible to get a sensitive, reproducible and stable signal over a 1 or 2 months period without any cleaning of the ion source. The sensitivity of the method was comparable with those described for sumatriptan [19,20], and allowed us to extract only a few hundred microliters of plasma samples to determine accurately I, II and III. The range of concentrations covers realistic concentrations as found in the pharmacokinetic study in the rabbit.

The second objective of this preliminary study was to determine and compare the pharmacokinetic parameters of I, II and III. After the iv

Table 3
Pharmacokinetic parameters after iv and po administration (mean ± S.D.) ($n = 3$)

Compound	AUC (ng h ml^{-1})	$t_{1/2}$ (h)	CL_{tot} (ml min^{-1} kg^{-1})	aVd (l kg^{-1})
PK values after iv administration				
I	172.5 ± 63.3	1.43 ± 0.17	53.3 ± 21.3	6.4 ± 1.7
II	191.5 ± 93.8	1.93 ± 0.12	23.9 ± 15.4	3.8 ± 2.6
III	166.6 ± 9.3	1.14 ± 0.06	25.3 ± 0.5	2.5 ± 0.1

Single iv dose of 0.5 mg kg^{-1} for I and 0.25 mg kg^{-1} for II and III

Compound	C_{max} (ng ml^{-1})	t_{max} (min)	AUC (ng h ml^{-1})	$t_{1/2}$ (h)	CL_{tot} (ml min^{-1} kg^{-1})	aVd (l kg^{-1})	F (%)
PK values after po administration							
I	103.4 ± 85.1	16.7 ± 12.6	153 ± 93.3	1.55 ± 0.29	339 ± 173.5	43.9 ± 18.9	22.8 ± 21.9
II*	186.9	3	155.5	1.35	140	16.2	16.9
III	73.9 ± 28.5	15.3 ± 14.5	113.9 ± 62.1	1.70 ± 0.60	215.6 ± 91.3	31.7 ± 17.7	13.4 ± 6.5

Single po dose of 2.5 mg kg^{-1} for I and 1.25 mg kg^{-1} for II and III.

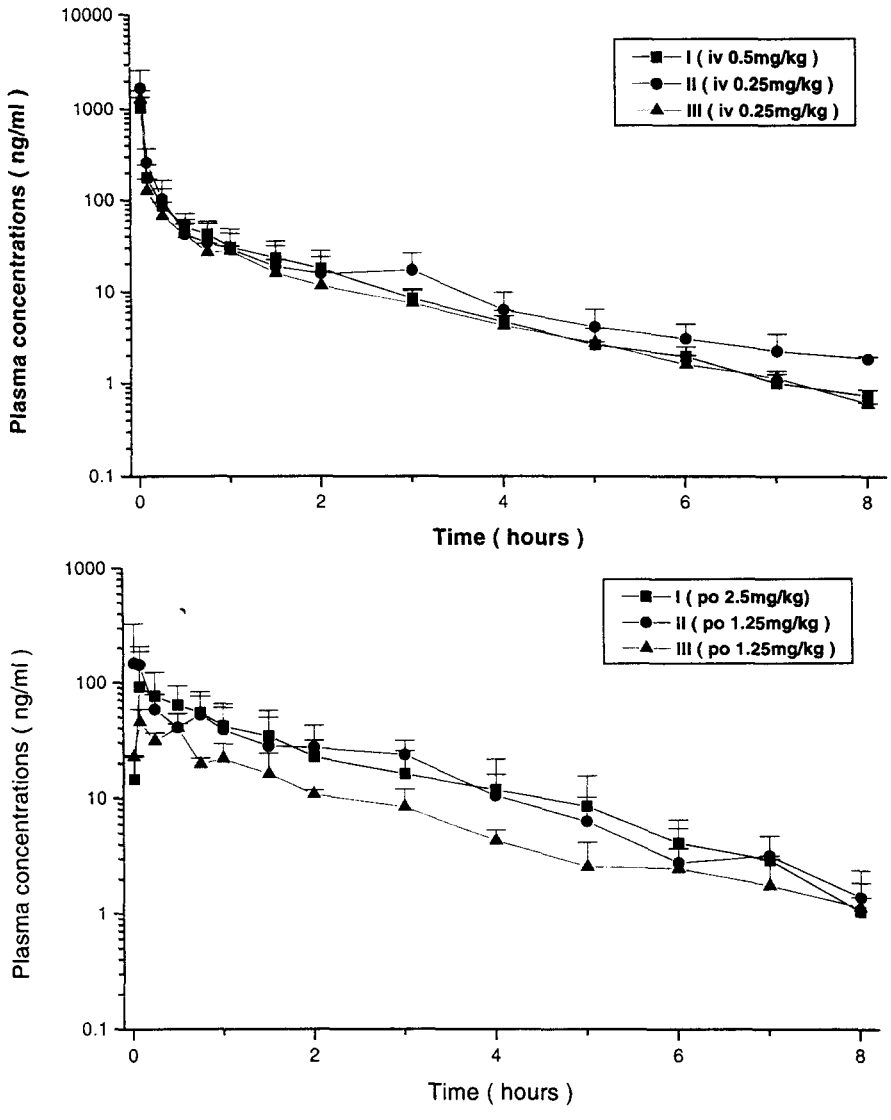


Fig. 5. (A) Comparison of mean (± S.D.) plasma concentration-time curves after a single iv dose of I, II and III to rabbit. (B) Comparison of mean (± S.D.) plasma concentration-time curves after a single po dose of I, II and III to rabbit.

administration, the plasma concentration-time curves were very similar for I, II and III as shown in Fig. 5A and indicated low interindividual variation. The $t_{1/2}$, CL and V for I, II and III were very similar too. The ANOVA analysis did not show any statistically significant differences. For the comparison of the AUCs, they were normalised to the same iv dose of 0.5 mg kg^{-1}. After normalisation, no statistically significant differences were found.

After the oral administration, plasma concentration-time curves of I, II and III (Fig. 5B) showed larger interindividual variation than after the iv dose. There was a rapid absorption of parent drug with a mean t_{max} between 1 and 25 min. There were no statistically significant differences. The wide interindividual variability for II was associated with the presence of multiple plasma concentration peaks suggesting multiple absorption sites (Fig. 5B) as described in animals

[21,22] and in man [23]. For $t_{1/2}$, V and normalised AUC, there were no statistically significant differences. After normalisation of C_{max} values to a po dose of 2.5 mg kg^{-1}, the ANOVA analysis revealed no statistically significant differences.

The quite large increase of CL and V values after oral administration were probably due to extensive metabolism of parent drugs as described for II in animals [21,22]. The same phenomenon could also explain the large CL and V of I and III after oral administration.

The pharmacokinetic parameters of II after iv administration in the present study compare favorably to those reported by Dixon et al. [21] although our CL values (23.9 ± 15.4 ml min^{-1} kg^{-1}) were lower than those reported (47 ± 7 ml min^{-1} kg^{-1}). The oral bioavailability in the present study was 16.9% for II compared with 23% reported by Dixon et al. [21] for the rabbit. On the other hand, the bioavailability of III was determined to be 13.4 ± 6.5% in the rabbit while Perren et al. [24] have reported oral bioavailability of 71% and 95% in the rat and dog, respectively. This may reflect an interspecies difference since the bioavailability of II has been shown to vary widely between different species (man = 14%, dog = 58%) [21]. Studies with [^{14}C]sumatriptan showed that parent drug was well absorbed (≈ 90%) [22]. The low values of F also suggest extensive first-pass metabolism of parent drugs.

5. Conclusion

The present paper describes a rapid and accurate method using liquid chromatographic-mass spectrometric assay for the analysis of drug levels in small quantities of plasma obtained from small laboratory animals. The sensitive technique should permit the determination of pharmacokinetic parameters from individual animals and a rapid comparison of the pharmacokinetics of competitive compounds at an early step of development.

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