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LC–ESI-MS/MS determination of in vivo metabolites of almotriptan in rat plasma, urine and feces: Application to pharmacokinetics

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ABSTRACT

A highly sensitive and specific liquid chromatography–electrospray ionization tandem mass spectrometric (LC–ESI-MS/MS) method for investigating the in vivo metabolites of almotriptan in rat plasma, feces and urine was developed. Chromatographic separation was achieved on a Lichrospher RP-18 column (250 mm × 4.6 mm, 5 μm), using 20 mM ammonium acetate (pH 3.5) and acetonitrile (60:40, v/v) as a mobile phase at 25 °C. MS/MS detection was performed by positive ion electrospray ionization using target ions at *m/z* 336 $[M+H]^+$, *m/z* 368 and *m/z* 282 $[M+H]^+$ for almotriptan and its two metabolites, respectively. Two metabolites viz., γ-aminobutyric acid and sulfonamide were detected in plasma as well as feces after 24 h of oral administration of almotriptan, while only γ-aminobutyric acid was found in urine. The method was sensitive with a lower limit of quantification of 1.43 ng/mL and linear over the range of 1.43–5000 ng/mL in plasma. The method was validated and successfully applied to a pharmacokinetic study of almotriptan in rat plasma using sumatriptan as an internal standard. The peak plasma concentration (C_{max}) after 0.3 h of 5 mg/kg oral dose of almotriptan was determined to be 69.85 ng/mL.

1. Introduction

Almotriptan,3-[2-dimethylaminoethyl]-5[1-pyrrolidiylsulfonyl]-1H-indole is a novel selective serotonin 5-$HT_{1B/1D}$ receptor agonist used in the treatment of acute migraine [1]. It is chemically related to sumatriptan, selective for cranial, as opposed to peripheral vasculature and shown potent affinity for 5-$HT_{1B/1D}$ receptors in the central nervous system [2]. The pharmacodynamic profiles of almotriptan were extensively investigated using in vitro and in vivo animal models [3]. In humans, it has shown a rapid onset of action with sustained efficacy in most patients [4–6]. It exhibits less spasmogenic effect on cardiac arteries and therefore an improved vascular profile compared to sumatriptan [7]. Almotriptan shows one of the highest absolute bioavailabilities of the 5-$HT_{1B/1D}$ receptor agonists used for symptomatic relief of migraine [8]. It is well absorbed and mainly excreted in an unchanged form in urine [9]. However, the triptan class of anti-migraines generally metabolize to varying extends by monoamine oxidase A [10,11]. Salva et al. examined the urinary profiles following oral administration of almotriptan to healthy volunteers and showed two major phase I metabolites corresponding to carboxylic acids formed by oxidation and opening of pyrrolidine ring. However, the plasma level concentrations of these metabolites were found to be low compared with those of the unchanged compound [12]. Thus it is of great importance to develop highly sensitive and specific methods to detect and determine the metabolites of almotriptan in biological matrices.

A thorough literature search has revealed that only a few bioanalytical methods were reported for determination of almotriptan and its metabolites in human plasma. The LC with UV methods were found to be less sensitive to study the metabolites in plasma, urine and feces [13–19]. Aubets et al. have studied the disposition and metabolism of almotriptan in rats, dogs and monkeys [20]. However, the method was not only tedious and time consuming but also not validated for stability of almotriptan in plasma samples. Thus, a simple and rapid method for accurate determination of almotriptan in biological matrices was needed. Rapid as well as efficient methods to address the issues related to drug metabolites are of great importance. To the best of authors' knowledge, no validated LC–MS method for determination of in vivo metabolites and pharmacokinetics of almotriptan in rats was reported in literature.

The present manuscript describes, for the first time, a simple, rapid, specific, sensitive LC–MS method to determine almotriptan and its metabolites in plasma, urine and feces. The method was successfully applied to study pharmacokinetics of almotriptan following an oral administration of 5 mg/kg drug to rats.

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2. Experimental

2.1. Chemicals and reagents

All the reagents were of analytical-grade unless stated otherwise. Glass-distilled and deionized water (Nanopure, Barnsted, USA), HPLC grade acetonitrile and methanol (Qualigens Fine-chem., Mumbai, India), ammonium acetate, formic and trifluoroacetic acids (S.D. Fine chem. Mumbai, India) were used. Almotriptan and sumatriptan (IS) obtained as gift samples (Optimus Pharma Pvt. Ltd., Hyderabad, India) were used. Control plasma, urine and feces used in the present study were collected from Wistar rats (Pharmacology Division, Indian Institute of Chemical Technology, Hyderabad, India) and stored at −20 °C until the time for use. Nylon syringe filters (0.22 μm) were obtained from (Millipore, Mumbai, India). Centrifuge model 2-16P (Sigma, Zurich, Switzerland) was used. Heparin coated capillaries (Sangius Counting, Nümbrecht, Germany) and blood collection tubes (Sarstedt, Leicester, UK) were used.

2.2. Animals

Six Wistar rats (200 ± 20 g) housed under standard conditions with ad libitum access to water and standard laboratory diet were used throughout the experiments. After a single dose by oral administration of 5 mg/kg almotriptan to healthy Wistar rats ($n = 6$), blood samples (0.4 mL) were collected at regular time intervals for studying pharmacokinetics. Blood, urine and feces were collected into the processed test tubes at 24 h post dose for investigating the metabolites. Plasma was separated by centrifugation at 4000 × g for 20 min and stored frozen at −20 °C. Samples were thawed and allowed to reach room temperature and the concentration of almotriptan was determined from the calibration curve on the same day. Statistical analysis was performed using Microsoft Excel 2000 while pharmacokinetic software 'Ramkin' based on non-compartment model was used to calculate the peak plasma concentration (C_{max}), the time to C_{max} (t_{max}), elimination half-life ($t_{1/2}$) and AUC from 0 to infinity ($AUC_{0-\infty}$), AUC from 0 to time (AUC_{0-t}), mean residence time (MRT) and clearance (CL).

2.3. Liquid chromatography–mass spectrometry (LC–MS)

An Agilent 1200 series HPLC instrument (Agilent Technologies, USA) coupled with quadrupole time-of-flight (Q-TOF) mass spectrometer (Q-TOF LC/MS 6510 series classic G6510A, Agilent Technologies, USA) equipped with an ESI source was used. The chromatographic separation was achieved on a Lichrospher C_{18} (250 × 4.6 mm i.d.; particle size 5 μm) analytical column at 25 °C. The mobile phase containing 20 mM ammonium acetate (pH adjusted to 3.5 with trifluoroacetic acid) and acetonitrile (60:40, v/v) at a flow rate of 1.0 mL/min was used. The run time was 17.0 min. Even though last peak eluted at 9 min run time chromatogram was extended up to 17 min in order to ascertain that no other were formed. The data acquisition was under the control of Mass Hunter workstation software. The typical operating source conditions for MS scan in positive ESI mode were optimized; the ionization voltage 80 V; the capillary voltage 3000–3500 V; the skimmer at, 60 V; nitrogen was used as the drying (300 °C; 9 L/min) and nebulizer (45 psi) gas. For collision-induced dissociation (CID) experiments, keeping MS^1 static, the precursor ion of interest was selected using the quadruple analyzer and the product ions were analyzed using a time-of-flight (TOF) analyzer. Ultra high pure nitrogen was used as collision gas, and the pressure in the collision cell was maintained at 18 Torr. All the spectra were an average of 20–25 scans recorded under identical experimental conditions.

2.4. Preparation of stock, working, calibration and quality control standards

Stock solutions of 1 mg/mL almotriptan and sumatriptan (IS) were prepared separately in methanol and stored at 5 °C. The stock solution of almotriptan was diluted quantitatively with water to give working standards of 300, 500, 700, 1000, 2000, 4000, 5000, 9000, 10,000 and 50,000 ng/mL. The stock solution of sumatriptan was also diluted further to prepare working standards of 500, 4000, 9000 and 10,000 ng/mL. Quality control samples of 50, 400, 900 ng/mL almotriptan and sumatriptan were prepared by taking 100 μL of the respective working standard, adding 200 μL blank rat plasma and 700 μL of acetonitrile. Calibration standards 30, 50, 70, 100, 200, 500, 1000, 5000 ng/mL of almotriptan were prepared by taking 100 μL of 300, 500, 700, 1000, 2000, 5000, 10,000 and 50,000 ng/mL working standards respectively, adding 100 μL of 500 ng/mL of IS, 200 μL blank rat plasma and 600 μL of acetonitrile. The calibration standard 30 ng/mL of amotriptan was further diluted to 1.43 ng/mL and used for lower limit of quantification. A reference standard containing both 1000 ng/mL of almotriptan and sumatriptan was prepared by taking 100 μL each of 10,000 ng/mL drug and IS, adding 200 μL blank rat plasma and 600 μL of acetonitrile.

2.5. Protein precipitation procedure

The Quality control samples, Calibration standards and reference standard containing the working standard, blank rat plasma and acetonitrile were vortexed and centrifuged at 4000 × g for 20 min to precipitate proteins and to form clean supernatant liquids. The 10 μL of the obtained clean supernatant was directly injected onto the LC–MS system and same procedure was used for pharmacokinetic and metabolite studies.

2.6. Urine and feces sample preparation

One volume of urine was diluted with four volumes of 0.1 M ammonium acetate buffer (pH 7), filtered through a 0.22 μm nylon membrane and directly injected 10 μL onto the LC–MS system for analysis. Feces samples of 1 g quantity were extracted with 1 mL mixture of acetonitrile, methanol, water (60:20:20, v/v) by vortexing and centrifuging at 4000 × g for 20 min to obtain a clean supernatant. The clean supernatant was filtered through a 0.22 μm nylon filter and injected 10 μL directly onto the LC–MS system for analysis.

3. Results and discussion

3.1. Liquid chromatography–mass spectrometry (LC–MS)

The LC–MS method provided a highly selective method for determination of almotriptan. The chemical structures of almotriptan and IS are shown in Fig. 1. The analytes were easily protonated and generated positive product ions. They were identified at m/z 296 for IS, and 336 for almotriptan. The mass spectra and the proposed fragmentation patterns of almotriptan and IS are given in Table 1 and Fig. 2. The LC–MS chromatograms of blank plasma, feces and urine are shown in Fig. 3. Almotriptan and IS spiked plasma samples and extracted plasma sample drawn after oral administration are shown in Fig. 4, respectively. The retention times for almotriptan and the IS were determined to be 5.34 and 3.18 min, respectively.

While method optimization, different mobile phase compositions were tried to achieve good resolution and symmetric peak shapes for almotriptan as well as sumatriptan. The mobile phases containing acetonitrile/water and methanol/water binary solvent

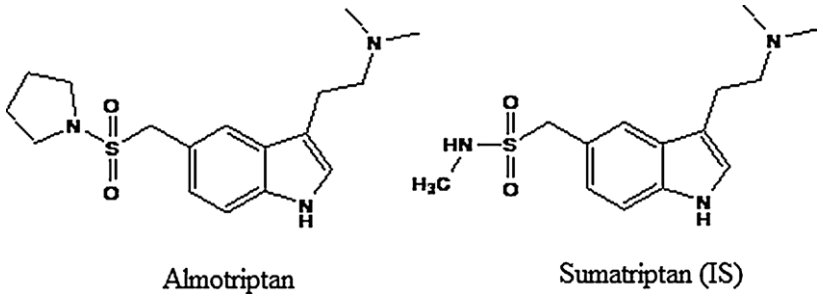

Fig. 1. Chemical structures of almotriptan and sumatriptan (IS).

Table 1
ESI–LC/MS full-scan and CID product ion spectral data of almotriptan and sumatriptan (IS).

Compound	Collision energy (V)	Mass to charge ratio	
		Precursor ion (*m*/*z*)	Product ions (*m*/*z*)
Almotriptan	80	336.21	291.11, 201.13, 158.09
Sumatriptan (IS)	80	296.14	251.08, 201.13, 158.09

MS conditions: positive ESI mode; the drying gas temperature 300 °C; capillary voltage 3000–3500 V.

systems using different buffers such as ammonium acetate and formate were tried. As a result, good separation, peak shape and resolution were obtained on a Lichrospher RP-18 column (250 × 4.6 mm, 5 μm) by using 20 mM ammonium acetate (pH adjusted to 3.5 with trifluoroacetic acid) and acetonitrile (60:40 v/v) as mobile phase in an isocratic mode at 25 °C at a flow rate of 1.0 mL/min. The run time was 17.0 min.

3.2. In vivo metabolites

3.2.1. Metabolites in plasma and feces

The full scan mass spectrum of free fraction of plasma and feces after administration of almotriptan was compared with those of blank (i) plasma, (ii) feces and (iii) almotriptan standard to find out the possible metabolites in plasma and feces. The compounds were analyzed by LC–MS/MS. The task of metabolite identification has been greatly facilitated by recent developments in high resolution LC/MS technology [time of-flight (TOF) and Fourier transform (FT) mass spectrometers], which allow determination of molecular formulae and product ion formulae with minimal uncertainty [21–24]. The parent drug and its two metabolites were found in plasma and feces after 24 h administration of almotriptan. The parent ion, its γ-aminobutyric acid metabolite (M1) and sulfonamide metabolite (M2) in plasma and feces were found after administration of almotriptan. Their retention times were 5.34, 3.41 and 8.46 min respectively (Fig. 5). After 24 h, the measured concentrations from of drug, metabolites M1 and M2 were 12, 35,

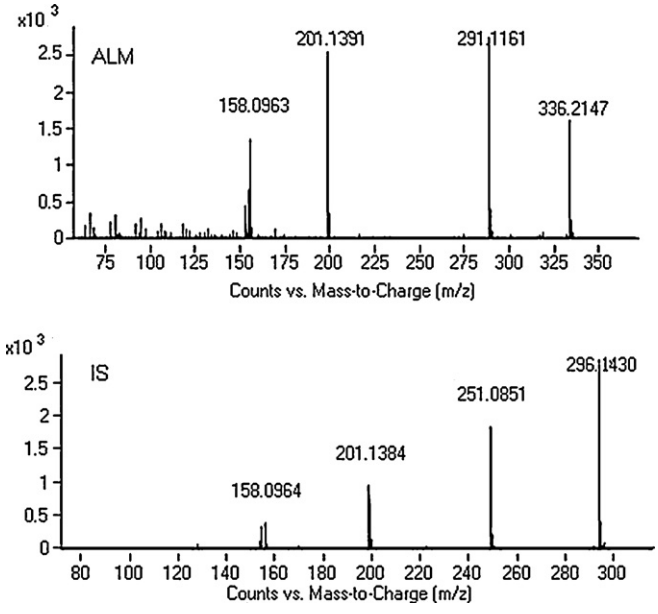

Fig. 2. Product ion mass spectra of almotriptan and sumatriptan (IS) (MS conditions: positive ESI mode; the drying gas temperature 300 °C; capillary voltage 3000–3500 V).

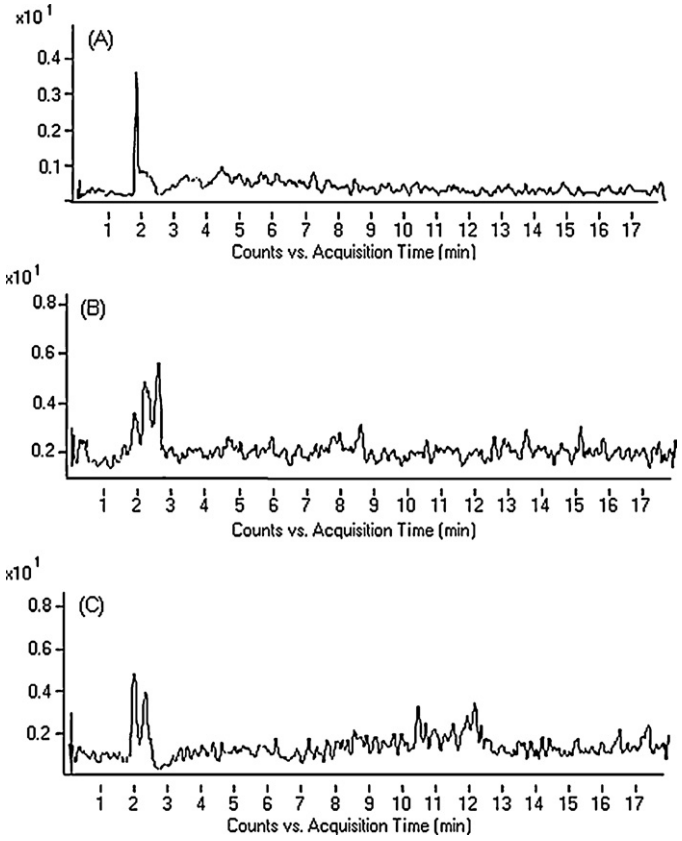

Fig. 3. LC–MS chromatograms of blank rat (A) plasma, (B) feces and (C) urine.

Table 2
HRMS data of M1 and M2 metabolites of almotriptan.

Molecular/fragment ion	Observed mass *m/z* (amu)		Theoretical mass *m/z* (amu)		Error (ppm)		Molecular formula	
	M1	M2	M1	M2	M1	M2	M1	M2
$[M+H]^+$	368.1635	282.1281	368.1639	282.1271	2.68	−4.96	$C_{17}H_{26}N_3O_4S$	$C_{13}H_{20}N_3O_2S$
Fragment 1	323.1067	254.0956	323.1060	254.0958	−2.81	1.16	$C_{15}H_{19}N_2O_4S$	$C_{11}H_{16}N_3O_2S$
Fragment 2	201.1381	190.1334	201.1386	190.1339	3.21	2.26	$C_{13}H_{17}N_2$	$C_{11}H_{16}N_3$
Fragment 3	158.0961	172.0419	158.0964	172.0427	1.16	−4.01	$C_{11}H_{12}N$	$C_7H_{10}NSO_2$
Fragment 4	81.0016	135.0913	81.0005	135.0917	−4.98	2.66	CH_5O_2S	$C_8H_{11}N_2$
Fragment 5	–	94.0659	–	94.0651	–	−3.01	–	C_6H_8N

9.8 ng/mL and 7.2, 18, 5.4 ng/mL in plasma and feces respectively. Mass defect filter (MDF) approach was used for screening and structural confirmation of metabolites. The identities of the detected metabolites were determined based on the empirical formulae of their protonated molecules and the interpretation of their product ion spectra. The protonated molecular ion and its fragment ions for M1 and M2 were at *m/z* 368, 323, 201, 158, 81 and 282, 254, 190, 172, 135, and 94 respectively, (Table 2 and Fig. 6). The metabolic pathway of almotriptan and fragmentation pathways are shown in Figs. 7 and 8 respectively.

3.2.2. Metabolites in urine

The full scan mass spectrum of free fraction of urine after administration of almotriptan was compared with those of blank urine samples and almotriptan solution to find out the possible metabolites in urine. The compounds were analyzed by LC–MS/MS. The parent drug and γ-aminobutyric acid metabolite (M1) in urine (mixed 0–24 h) were found after administration of almotriptan. Their retention times 5.34 and 3.41 min are shown in Fig. 5. After 24 h, the measured concentrations of drug and metabolite M1 in urine were 8.7 and 33.0 ng/mL respectively. The detected metabolite was identified based on the empirical formulae of the protonated molecule and interpretation of the product ion spectra. The molecular and fragment ions of M1 were at *m/z* 368, 323, 201, 158 and 81.

3.3. Validation

Validation according to the FDA guidelines [25] was performed for the assay of almotriptan in rat plasma.

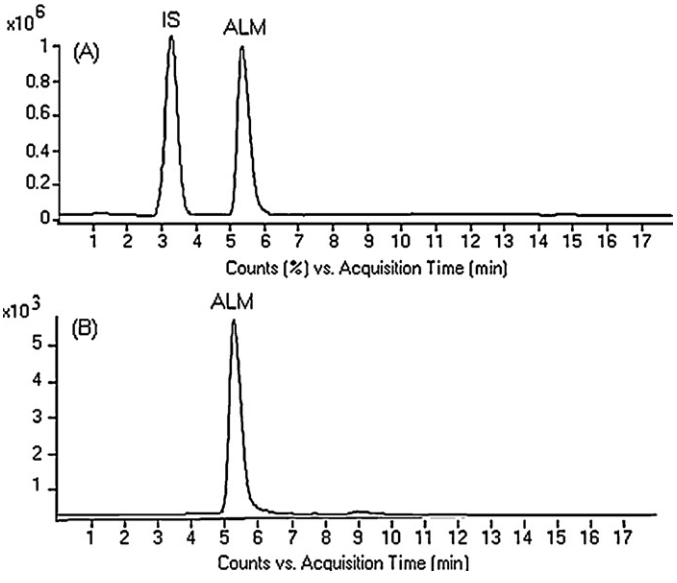

Fig. 4. LC–MS chromatograms of rat plasma (A) spiked with 1000 ng/mL of almotriptan and IS and (B) after 0.3 h following a 5 mg/kg oral dose of almotriptan.

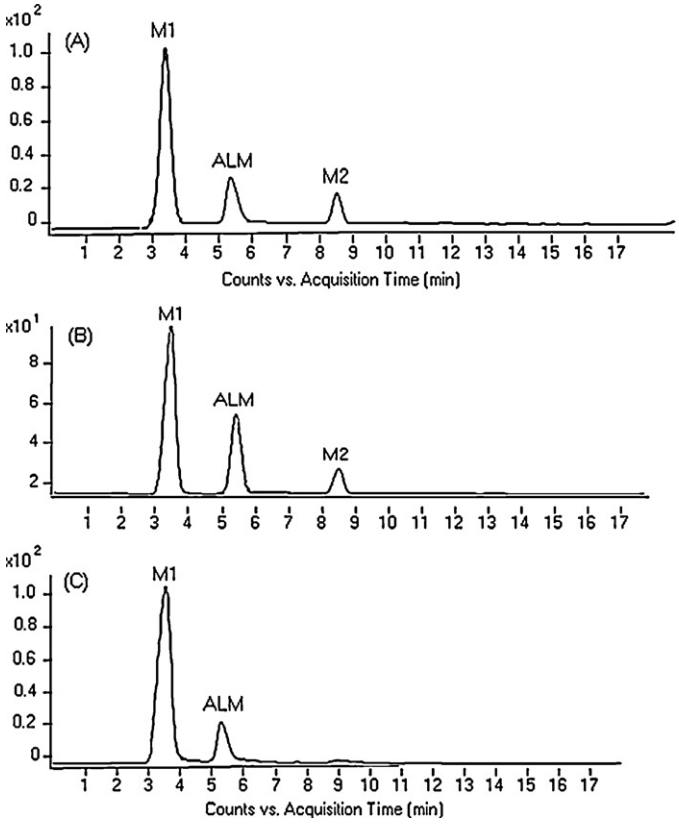

Fig. 5. LC–MS chromatograms of rat (A) plasma, (B) feces and (C) urine samples after 24 h oral dose of 5 mg/kg almotriptan.

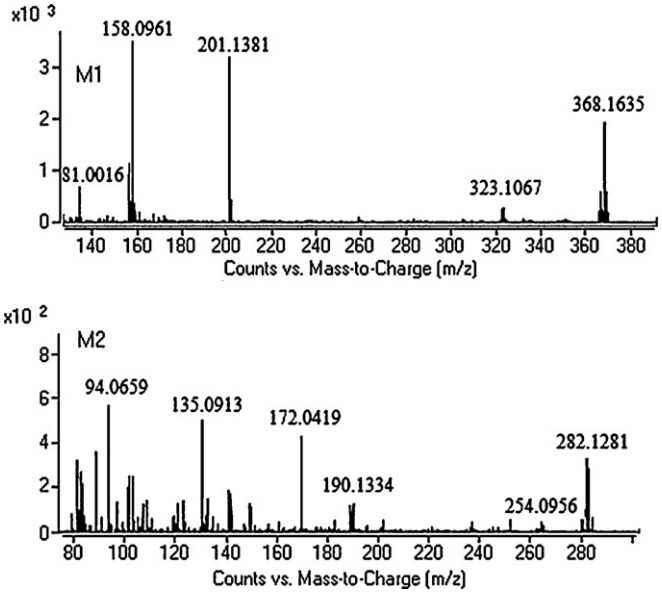

Fig. 6. HRMS spectra of almotriptan metabolites M1 and M2.

Table 3
Calibration curves, LOD and LOQ for almotriptan in rat plasma, feces and urine.

Matrix	Regression equation	r^2	$S_{y,x}$	LOD (ng/mL)	LOQ (ng/mL)
Plasma	$y = 21{,}113x - 10{,}192$	0.9998	8.58	0.42	1.43
Feces	–	–	–	0.52	1.72
Urine	–	–	–	0.46	1.53

r^2: correlation coefficient; $S_{y,x}$: standard error of estimate.

Table 4
Precision and accuracy data.

Added concentration (ng/mL)	Intraday									Inter day		
	Day 1			Day 2			Day 3			EC	RSD (%)	R.E. (%)
	EC	RSD (%)	R.E. (%)	EC	RSD (%)	R.E. (%)	EC	RSD (%)	R.E. (%)			
1.43	1.41	2.13	1.3	1.40	2.87	2.0	1.42	2.58	1.4	1.41	4.12	0.7
50	52	3.45	4.0	52	2.96	4.0	53.5	2.73	6.0	52.5	4.81	5.0
400	383	3.14	4.2	385	1.89	3.7	388	3.42	3.0	385	5.91	3.7
900	912	2.47	1.3	925	3.14	2.7	935	3.12	3.8	924	3.95	2.6

EC: experimental concentration; RSD: relative standard deviation; R.E.: relative error ($n = 6$).

Table 5
Recovery data.

Compound	Added concentration (ng/mL)	Recovery (%)	RSD (%)
Almotriptan	50	91.6	3.89
	400	90.9	3.76
	900	90.7	2.76
Sumatriptan (IS)	50	93.8	3.19
	400	94.6	3.48
	900	92.8	4.72

$n = 6$.

3.3.1. Specificity

No significant interference from endogenous substances was observed in the chromatograms of drug-free plasma, urine and feces from six different rats at the retention times of the analyte and IS. Representative LC–MS chromatograms of blank plasma, urine, feces and spiked plasma samples are shown in Fig. 3.

3.3.2. Linearity of calibration curves and limit of quantification (LOQ)

The linearity of the method was evaluated by calibration curves ($n = 6$) in the range of 1.43–5000 ng/mL. Calibration curves were constructed by plotting peak area ratios of almotriptan to IS against concentration with a weight of $1/x^2$. The calibration curves were constructed and found to be linear having correlations coefficient $r^2 > 0.9998$. The results are given in Table 3. The limit of detection (LOD) and quantification (LOQ) were determined as the concentration with a signal-to-noise (S/N) ratio of 3 and 10, respectively. The LOD and LOQ limits in plasma, feces and urine are recorded in Table 3.

3.3.3. Accuracy and precision

Six replicates ($n = 6$) of the LOQ (1.43 ng/mL) and three QC samples (50 ng/mL (LQ), 400 ng/mL (MQ) and 900 ng/mL (HQ)) were used to determine the accuracy and precision. Six replicates of each LOQ and QC concentrations were analyzed every day to determine the intra-day accuracy and precision. This process was repeated over 3 days in order to determine the inter-day accuracy and precision. Precision was calculated and expressed as percent relative standard deviation within a single run (intra-day) and among different runs (inter-day). The accuracy was calculated as % bias, i.e. the deviation between theoretical and experimental concentration. Both accuracy and precision were well within acceptable limits. Intra- and inter-day accuracy and precision of quality control data are given in Table 4.

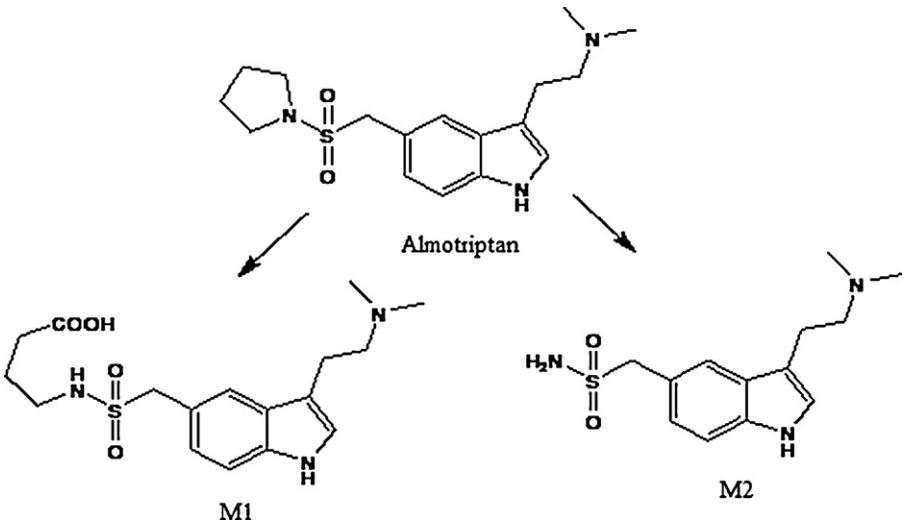

Fig. 7. Metabolic pathway of the almotriptan.

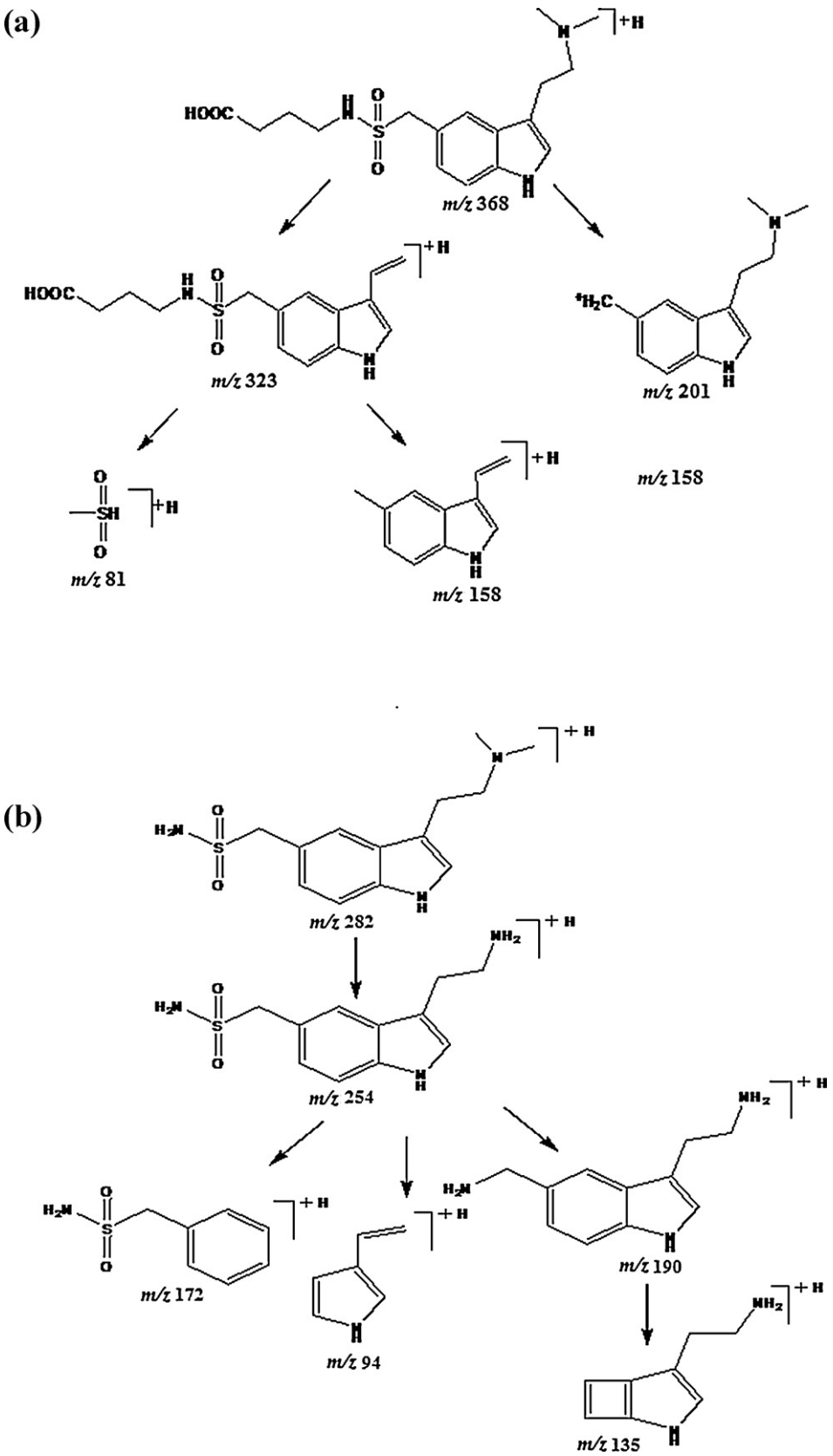

Fig. 8. (A) Mass spectral fragmentation of metabolite (M1) (for conditions see Section 2.3). (B) Mass spectral fragmentation of metabolite (M2) (for conditions see Section 2.3).

Table 6
Stability data.

Storage conditions	Added conc. (ng/mL)	Calculated conc. (ng/mL) (mean ± S.D.)	R.E. (%)
Rat plasma at room temperature for 6 h	50	51.12 ± 2.15	2.2
	400	413.00 ± 3.26	3.2
	900	878.00 ± 7.12	2.4
Processed samples at 10 °C for 4 h	50	51.41 ± 3.25	2.8
	400	414.00 ± 8.12	3.5
	900	862.00 ± 11.00	4.2
Rat plasma after three freeze/thaw cycles	50	48.08 ± 3.12	3.8
	400	423.00 ± 6.89	5.7
	900	921.00 ± 9.74	2.3
Rat plasma for 30 days at −20 °C	50	52.00 ± 6.25	4.0
	400	429.00 ± 8.05	7.2
	900	952.00 ± 14.00	5.7

R.E.: relative error ($n = 6$).

3.3.4. Extraction recovery and matrix effect

The extraction recovery of the analytes, by protein precipitation procedure was determined by comparing the peak areas of extracted plasma from the QC samples ($n = 6$) with those obtained from the direct injection of the standard solutions without any preparation at same concentrations. The recovery of almotriptan and IS was determined at three concentration levels of QC at low, medium and high concentrations are given in Table 5.

To evaluate the matrix effect, the chromatographic peak areas of each analyte from the spike-after-extraction samples at low (LQ), medium (MQ) and high (HQ) concentration levels were compared to those for the clean standard solutions at the same concentrations. Matrix effects on recovery of blank plasma samples spiked after the sample preparation with 50, 400 and 900 ng/mL of almotriptan were found to be within the acceptable limits (95–105%). Similar evaluation was performed on IS and no significant peak area differences were observed. Thus, ion suppression or enhancement due to plasma matrix was found to be negligible using the present method.

3.3.5. Stability

The stability of almotriptan was performed under all storage conditions at three QC concentration levels (50, 400 and 900 ng/mL) in six replicates. The freeze/thaw stability test was performed after three complete freeze/thaw cycles (−20 to 25 °C). The long-term stability was assessed after storage of QC samples at −20 °C for 30 days. For the short-term stability, QC samples were kept at ambient temperature (25 °C) for 6 h and then analyzed. Stability of processed samples was assessed by re-injection of extracted QC samples after 4 h conservation in autosampler (set at 10 °C). The stock solutions of almotriptan stored at −20 °C for 1 month were compared with freshly prepared stock solutions. The results are given in Table 6. It could be seen from Table 6 that the results are well within the acceptance limits.

3.4. Pharmacokinetic study

The developed method was applied to a pharmacokinetic study of almotriptan in plasma samples. Six male Wistar rats (200 ± 20 g) were collected from Pharmacology Division, Indian Institute of Chemical Technology, Hyderabad, India. Before orally administering a single dose of almotriptan (5 mg/kg) the rats were fasted for 12 h but with access to water and were further fasted for 2 h after administration. Blood samples of 0.4 mL were collected in heparin containing tubes from the epicanthic veins of rats by capillary tube at 0, 0.1, 0.3, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0 and 8.0 h post-dose. The blood samples were immediately centrifuged at 4000 × g for 20 min at room temperature. The plasma samples were stored at −20 °C until analysis. The mean plasma concentration–time curves of almotriptan were shown in Fig. 9. The pharmacokinetic parameters, the peak plasma concentration (C_{max}), the time to C_{max} (t_{max}), elimination half-life ($t_{1/2}$), the AUC from 0 to infinity ($AUC_{0-\infty}$), The AUC from 0 to time (AUC_{0-t}), mean residence time (MRT) and clearance (CL) were calculated for each subject by the 'Ramkin' software (Drug and Statistics, Mathematical Pharmacology Professional Committee of China, Shanghai, China). The pharmacokinetic parameters of almotriptan are given in Table 7.

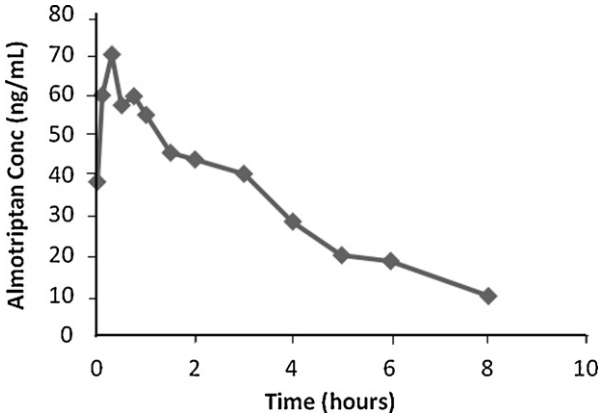

Fig. 9. Concentration vs. time profile over 8 h of almotriptan in rat plasma after oral administration of a dose of 5 mg/kg ($n = 6$).

Table 7
Pharmacokinetic parameters.

Parameter	Value
t_{max} (h)	0.3
C_{max} (ng/mL)	69.85
$t_{1/2}$ (h)	2.31
$AUC_{0-\infty}$ (ng/mL/h)	287.93
AUC_{0-t} (ng/mL/h)	255.03
MRT (h)	3.8
CL (mL/h/kg)	18,279.01

C_{max} (ng/mL): maximum plasma concentration; t_{max}: time to C_{max}; AUC: area under plasma concentration–time curve; $t_{1/2}$: half-life; MRT: mean residence time; CL: clearance.

4. Conclusions

A highly sensitive and specific liquid chromatography/tandem mass spectrometric (LC–ESI-MS/MS) method for investigating the in vivo metabolites and pharmacokinetics of almotriptan in rats was developed. The metabolites were identified by LC–MS/MS and their structures confirmed by infusion of the M1 and M2 samples in

electrospray ionization high resolution tandem mass spectrometry (ESI-HR-MS/MS). The method showed excellent sensitivity, linearity, precision, accuracy and was successfully applied to evaluate the pharmacokinetics of almotriptan in rats.

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