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ORIGINAL PAPER

Quantitative analysis of eletriptan in human plasma by HPLC-MS/MS and its application to pharmacokinetic study

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Abstract Authors developed a simple, sensitive, selective, rapid, rugged, and reproducible liquid chromatography–tandem mass spectrometry method for the quantification of eletriptan (EP) in human plasma using naratriptan (NP) as an internal standard (IS). Chromatographic separation was performed on Ascentis Express C18, 50×4.6 mm, 2.7 µm column. Mobile phase was composed of 0.1% formic acid: methanol (40:60 *v*/*v*), with 0.5 mL/min flow rate. Drug and IS were extracted by liquid–liquid extraction. EP and NP were detected with proton adducts at *m*/*z* 383.2→84.3 and 336.2→97.8 in multiple reaction monitoring (MRM) positive mode, respectively. The method was validated with the correlation coefficients of $(r^2) \geq 0.9963$ over a linear concentration range of 0.5–250.0 ng/mL. This method demonstrated intra- and inter-day precision within 1.4–9.2% and 4.4–5.5% and accuracy within 96.8–103% and 98.5–99.8% for EP. This method is successfully applied in the bioequivalence study of 24 human volunteers.

Keywords Liquid chromatography · Mass spectrometry · Eletriptan · Human plasma · Bioequivalence

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Abbreviations

CAD	Collisionally activated dissociation
EP	Eletriptan
HQC	High quality control
IS	Internal standard
LC-ESI-MS/MS	Liquid chromatography–electro spray ionization–tandem mass spectrometry
LLOQ	Lower limit of quantification
LOQ	Limit of quantification
LQC	Low quality control
MQC	Medium quality control
MRM	Multiple reaction monitoring
NP	Naratriptan

Introduction

Eletriptan is chemically designated as (*R*)-3-[(1-methyl-2-pyrrolidinyl) methyl]-5-[2-(phenylsulfonyl) ethyl]-1*H*-indole monohydrobromide. The empirical formula is $C_{22}H_{26}N_2O_2S{\cdot}HBr$ with molecular weight of 463.40. EP is a second generation triptan drug marketed and manufactured by Pfizer Inc used in the treatment of migraine. Specifically, it is a selective 5-hydroxytryptamine $1_B/1_D$ (5-HT_{1B}) receptor agonist that binds with high affinity to the 5-$HT_{[1B,\ 1D,\ 1F]}$ receptors has a modest affinity to the 5-$HT_{[1A,\ 1E,\ 2B,\ 7]}$ receptors and little to no affinity to the 5-$HT_{[2A,\ 2C,\ 3,\ 4,\ 5A,\ 6]}$ receptors. EP is well absorbed after oral administration with peak plasma levels occurring approximately 1.5 h after dosing to healthy subjects. The mean absolute bioavailability of eletriptan is approximately 50%. Plasma protein binding is moderate and approximately 85% [1–8].

The *N*-demethylated metabolite of EP is the only known active metabolite. This metabolite causes vasoconstriction similar to EP in animal models. Though the half-life of the

metabolite is estimated to be about 13 h, the plasma concentration of the *N*-demethylated metabolite is 10–20% of parent drug and is unlikely to contribute significantly to the overall effect of the parent compound. The terminal elimination half-life of EP is approximately 4 h. Following oral administration during a migraine attack, there was a reduction of approximately 30% in AUC and $T_{\max}$ was increased to 2.8 h. [9–11].

Literature survey reveals that Jocic et al. developed a study of forced degradation behavior of eletriptan hydrobromide by LC and LC-MS and development of stability-indicating method. In this, they described only about the degradation part of the drug [12]. Gillogly et al. developed pH-mediated acid stacking with reverse pressure for the analysis of cationic pharmaceuticals in capillary electrophoresis. This reverse-pressure method was used for detection and quantitation of several cationic pharmaceuticals that were prepared in Ringer's solution to simulate microdialysis sampling conditions [13]. Cooper et al. published the "Determination of eletriptan in plasma and saliva using automated sequential trace enrichment of dialysate and high-performance liquid chromatography". Using this technique, the procedure was observed to be specific and linear over the range 0.50–250 ng/ml [14] with long run time of 12 min. Recently, Suneetha and Lakshmana Rao developed a method RP-HPLC method for the estimation of eletriptan in pharmaceutical dosage forms in this method they quantified 200–1,000 μg/mL with long run time 10 min, limit of detection (LOD), limit of quantitation (LOQ) were 0.080 and 0.120 μg/mL, respectively [15]. There is no method reported for quantification of eletriptan by using LC-MS/MS in human plasma.

The main goal of the present study is to develop and validate the novel simple, sensitive, selective, rapid, rugged, and reproducible analytical method for quantitative determination of EP in human plasma by LC-MS/MS with a small amount of sample volume. The developed method would be applied in the bioequivalence study of different formulations in healthy human volunteers.

Materials and methods

Chemicals and reagents

Eletriptan hydrobromide was obtained from TLC Pharmachem while naratriptan hydrochloride was obtained from USP. HPLC-grade methanol purchased from JT Baker Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA. Formic acid was purchased from S.D. Fine Chemicals Mumbai. Sodium carbonate anhydrous, methyl *t*-butyl ether was purchased from Merck Specialty Private Limited, Worli, Mumbai. Ultra pure water from Milli-Q system (Millipore, Bedford, MA, USA) was used through the study. All other chemicals in this study were of analytical grade. Human plasma was obtained from Navjeevan blood blank, Hyderabad.

Instrumentation

HPLC system (1200 Series Agilent Technologies, Germany) connected with triple quadrupole mass spectrometer instrument (API 4000, Toronto, Canada). Data processing was performed with the Analyst 1.4.1 software package (SCIEX). Ionization was performed by electrospray positive mode with unit resolution.

Detection

Mass parameters were optimized to get the product ions of *m/z* 84.3, *m/z* 97.8 from its respective precursor ions of EP $[M+H]^+$ (*m/z*, 383.2) and NP $[M+H]^+$ (*m/z*, 336.2) with source temperature 500°C, ion spray voltage 5,500 V, heater gas, nebulizer gas at 35 psi each, curtain gas 20 psi, CAD gas 5 psi (all gas channels with nitrogen), source flow rate 500 μL/min without split, entrance potential 10 V, declustering potential 65 V for analyte and 75 V for internal standard, collision energy 35 V for both analyte and internal standard, collision cell exit potential 14 V for analyte and 16 V for internal standard.

Chromatographic conditions

Chromatography was performed on Ascentis Express C18, 50×4.6 mm, 2.7 μm analytical column at 40°C, with 0.1% formic acid: methanol (40:60 *v*/*v*) as mobile phase at a flow rate of 0.5 mL/min. NP was used as an internal standard in terms of chromatography and extractability. The drug and internal standard was eluted at 0.83±0.2 and 0.78±0.2 min with 2 min total run time.

Preparation of standards and quality control samples

Standard stock solutions of EP (50.0 μg/mL) and NP (50.0 μg/mL) was prepared in methanol. The internal standard spiking solution (500.0 ng/mL) was prepared in 50% methanol from NP standard stock solution (50.0 μg/mL). Standard stock solutions and internal standard spiking solutions were stored in refrigerator conditions (2–8°C) until analysis. Standard stock solution of EP was added to screened drug-free human plasma to obtain concentration levels of 0.5, 1.0, 5.0, 10.0, 50.0, 100.0, 150.0, 200.0 and 250.0 ng/mL for analytical standards and 0.5, 1.5, 75.0, 175.0 ng/mL for Quality control standards and stored in a −30°C freezer until analysis. Respective aqueous standards were prepared in reconstitution solution (methanol, 0.1%; formic acid, 40:60) and stored in refrigerator conditions 2–8°C until analysis.

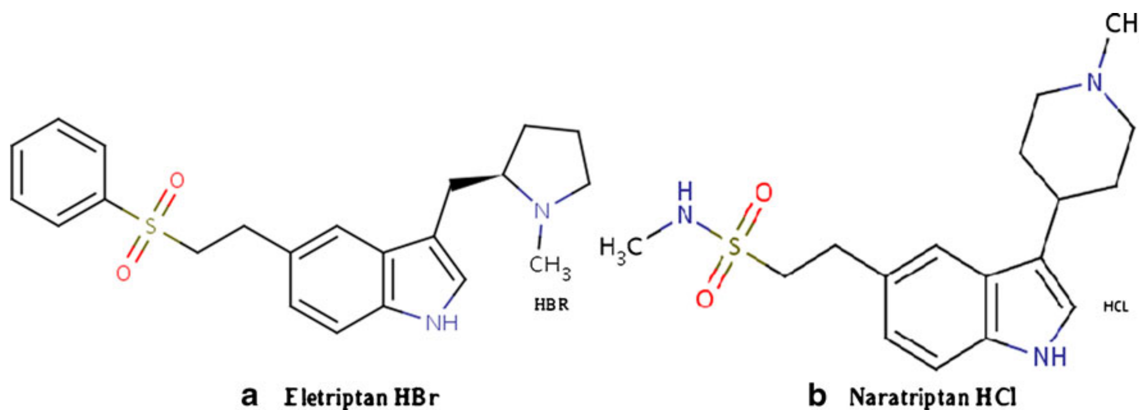

Fig. 1 Chemical structures of **a** eletriptan and **b** naratriptan HCl (IS)

Sample preparation

Liquid–liquid extraction was used to isolate drug and IS from human plasma. For this purpose, 50 μL of IS (500.0 ng/mL) and 100 μL of plasma sample (respective concentration) was added into labeled polypropylene tubes and vortexed briefly. Followed by 100 μL of 0.5 N sodium carbonate, 2.5 mL of extraction solvent (methyl tertiary butyl ether) were added and vortexed for 10 min. Then the samples were centrifuged at 4,000 rpm for 10 min at 20°C

Fig. 2 Mass spectra of elitriptan Q1➡elitriptan Q3

temperature. Subsequently, the supernatant from each sample was transferred into respective polypropylene tubes. After that, all the samples were kept for evaporation under nitrogen at 40°C. The dried residue was reconstituted with 500 μL of reconstitution solution and vortexed briefly. Finally, the extracted sample was transferred into auto sampler vials and injected into LC-MS/MS.

Selectivity and specificity

The selectivity of the method was determined by six different human blank plasma samples, which were pretreated and analyzed to test the potential interferences of endogenous compounds co-eluting with analyte and IS. Chromatographic peaks of analyte and IS were identified based on their retention times and multiple reaction monitoring (MRM) responses. The peak area of EP at the respective retention time in blank samples should not be more than 20% of the mean peak area of LOQ of EP. Similarly, the peak area of NP at the respective retention time in blank samples should not be more than 5% of the mean peak area of LOQ of NP.

Recovery

The extraction recovery of EP and NP from human plasma was determined by analyzing quality control samples. Recovery at three concentrations (1.5, 75.0, and 175.0 ng/mL) was determined by comparing peak areas obtained from the plasma sample and the standard solution spiked with the blank plasma residue. A recovery of more than 85% was considered adequate to obtain the required recovery.

Limit of detection and limit of quantification

The LOD is a parameter that provides the lowest concentration in a sample that can be detected from

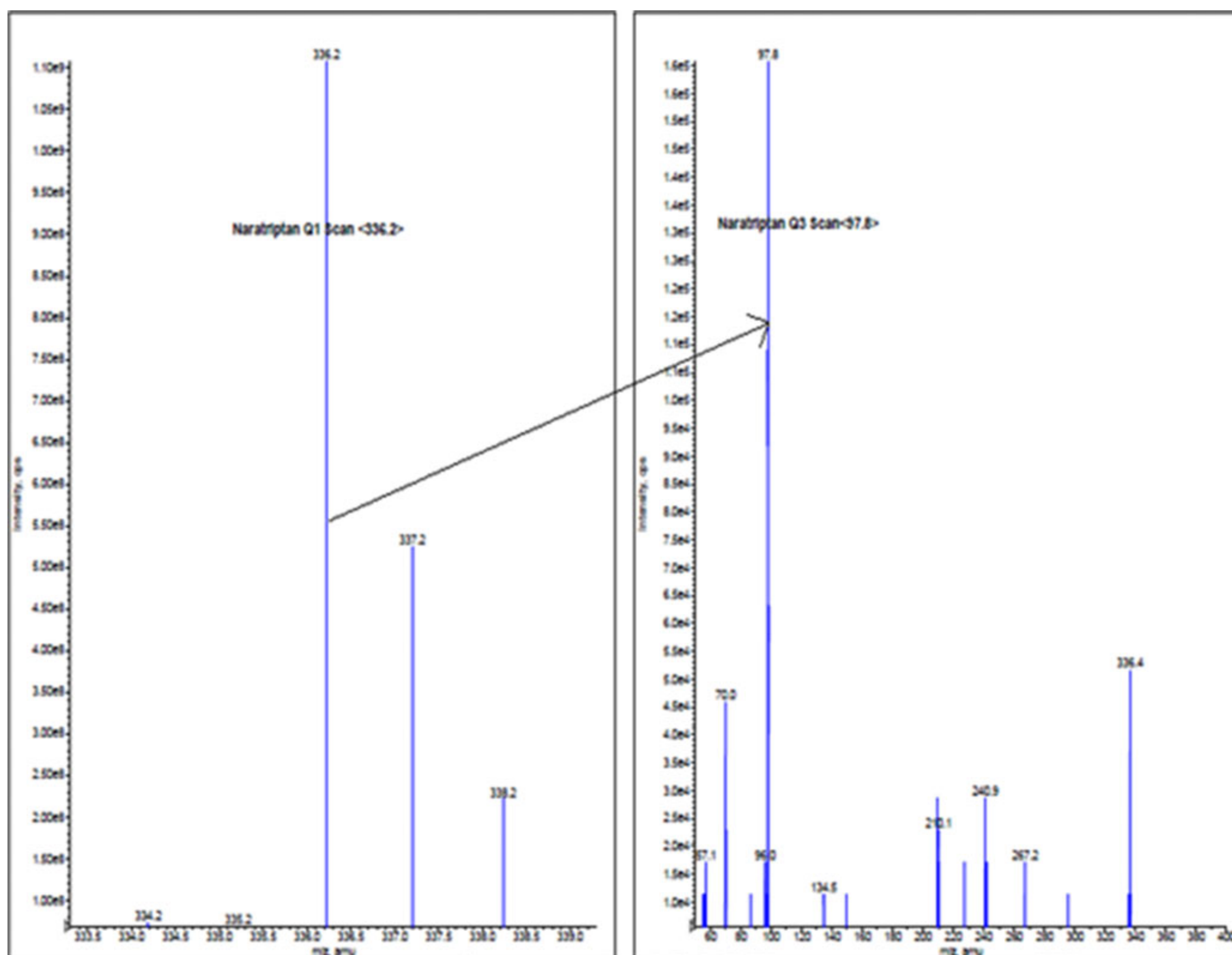

Fig. 3 Mass spectra of the naratriptan Q1➡naratriptan Q3

background noise but not quantitated. LOD was determined using the signal-to-noise ratio (S/N) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples.

The LOQ is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOQ was found by analyzing a set of mobile phase and plasma standards with a known concentration of EP.

Matrix effect

To predict the variability of matrix effects in samples from individual subjects, matrix effect was quantified by

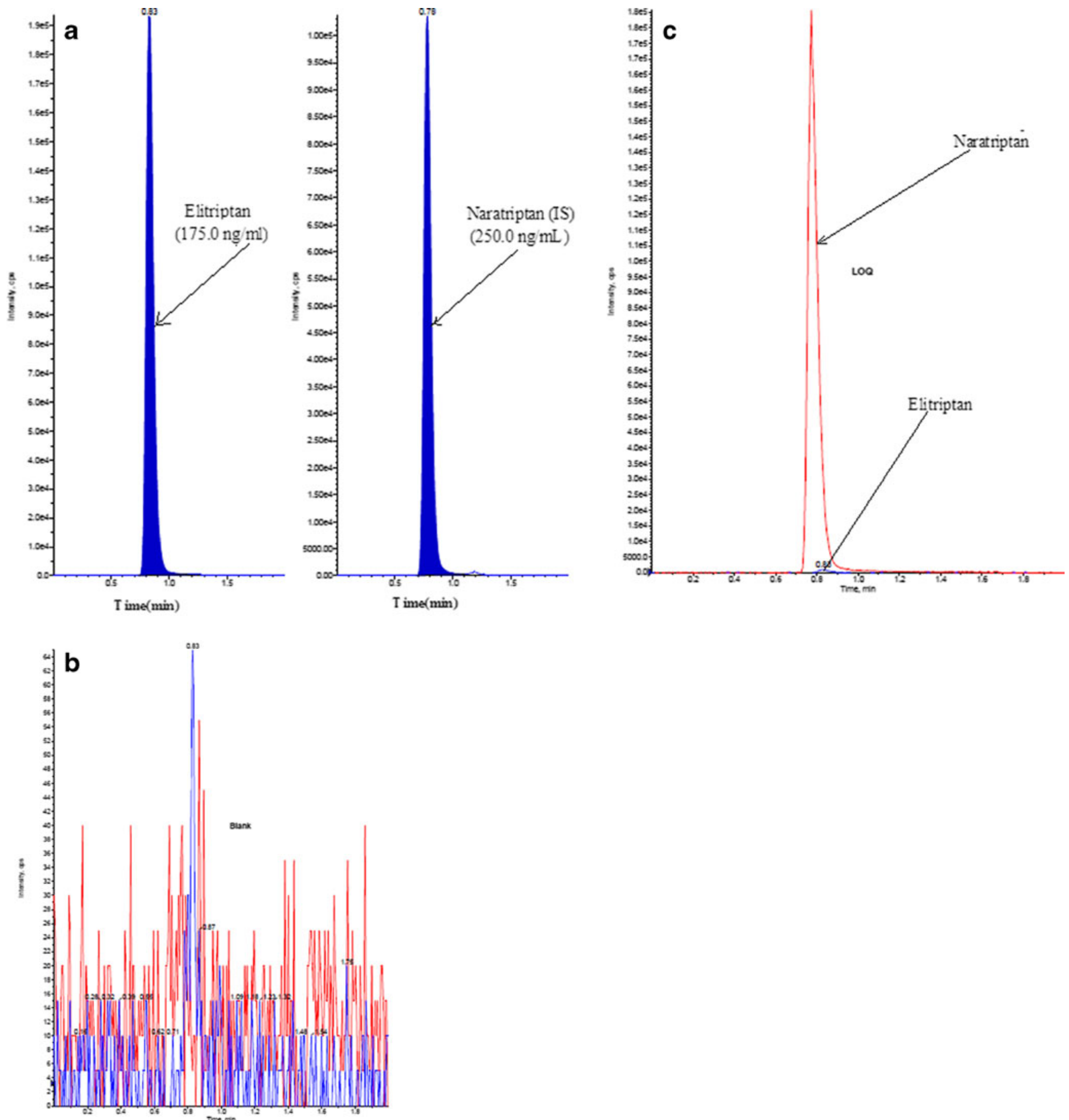

Fig. 4 **a** Chromatogram of spiked human plasma containing elitriptan (175.0 ng/ml) naratriptan (IS) (250.0 ng/ml). **b** MRM chromatograms of blank human plasma. **c** MRM chromatogram of spiked human plasma containing 0.5 ng/ml elitriptan and 250.0 ng/ml naratriptan (IS) (*LLOQ*)

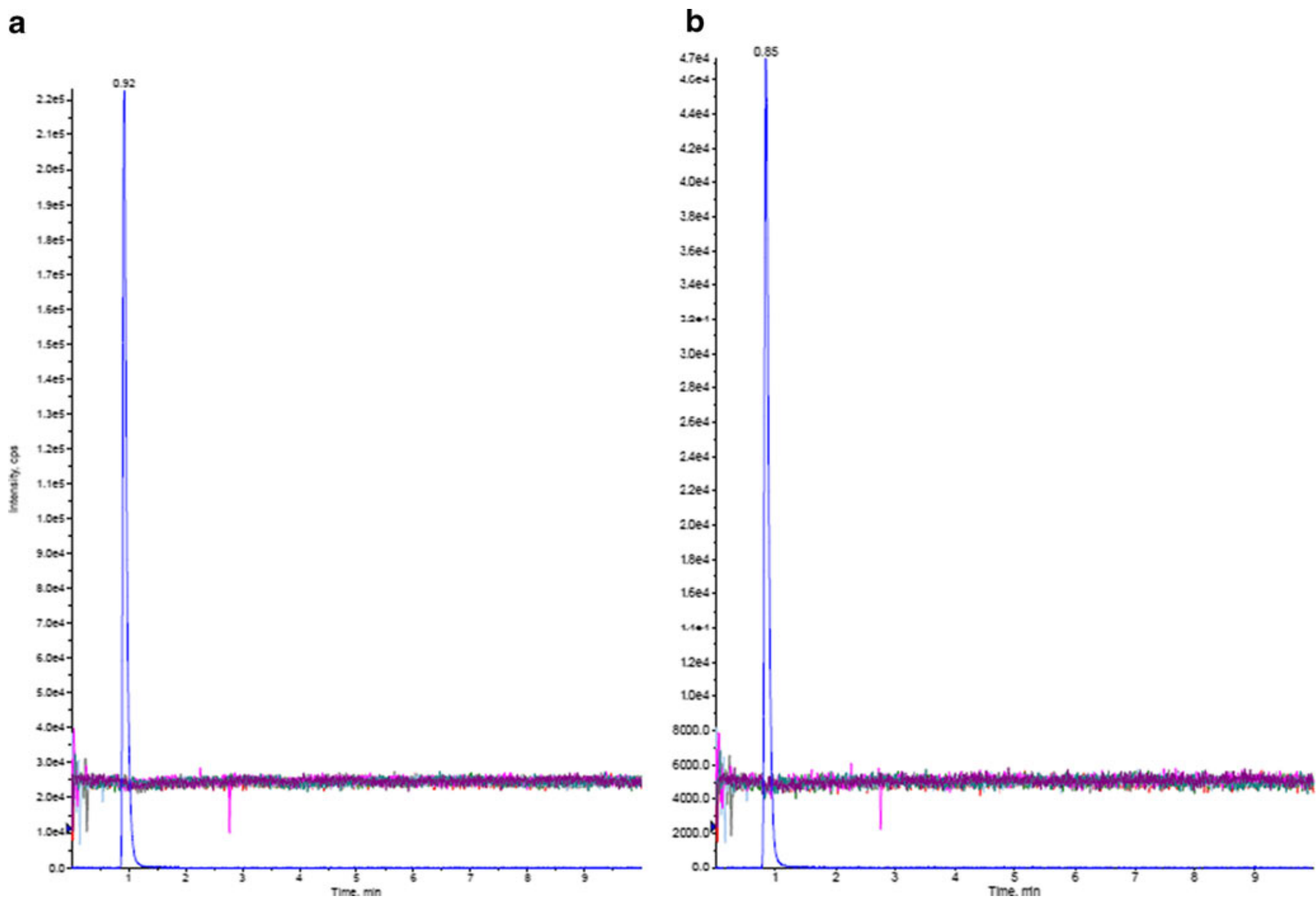

Fig. 5 **a** Matrix effect elitriptan using six different blank plasmas by infusion method. **b** Matrix effect naratriptan using six different blank plasmas by infusion method

determining the matrix factor, which was calculated as follows:

$$\text{Matrix factor} = \frac{\text{Peak response ratio in presence of extracted matrix(post extracted)}}{\text{Peak response ratio in aqueous standards}}$$

Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the medium quality control (MQC) level, and compared with aqueous standards of same concentration. The overall precision of the matrix factor is expressed as coefficient of variation (CV%) and CV% should be <15%.

Calibration curve, precision, and accuracy

The calibration curve was constructed using values ranging from 0.5 ng/mL to 250.0 ng/mL of EP in human plasma. Calibration curve was obtained by quadratic model with weighted $1/x^2$ regression analysis. The ratio of EP/NP peak area was plotted against the ratio of EP concentration in nanograms per milliliter. Calibration curve standard samples and quality control samples were prepared in replicates (n=6) for analysis. Precision and accuracy for the back calculated concentrations of the calibration points, should be within ≤15% and ±15% of their nominal values. However, for LLOQ, the precision and accuracy should be within ≤20% and ±20%.

Table 1 Calibration curve details

Spiked plasma concentration (ng/ml)	Concentration measured (ng/ml) (mean±S.D)	CV (%) (n=5)	Accuracy%
0.5	0.49±0.01	1.4	99.4
1.0	1.0±0.03	3.0	101.0
5.0	5.1±0.24	4.8	103.1
10.0	9.8±0.34	3.5	98.5
50.0	48.4±1.61	3.3	96.9
100.0	99.6±2.52	2.5	99.6
150.0	152.1±4.65	3.1	101.4
200.0	203.1±6.99	3.4	101.6
250.0	246.8±4.85	2.0	98.7

Table 2 Precision and accuracy (analysis with spiked plasma samples at three different concentrations)

Spiked plasma concentration (ng/ml)	Within-run			Between-run		
	Concentration measured (n=6; ng/ml; mean±S.D.)	CV (%)	Accuracy%	Concentration measured (n=30; ng/ml; mean±S.D.)	CV (%)	Accuracy%
1.5	1.48±0.07	5.3	98.8	1.492±0.08	5.5	99.5
75.0	77.25±5.99	7.8	103.0	74.814±3.47	4.6	99.8
175.0	169.42±10.70	6.3	96.8	172.397±7.56	4.4	98.5

Stability (freeze–thaw, auto sampler, bench top, long term) of EP in plasma

Low quality control (LQC) and high quality control (HQC) samples (n=6) were retrieved from a deep freezer after three freeze–thaw cycles according to the clinical protocol. Samples were stored at −30°C in three cycles of 24, 36, and 48 h. In addition, the long-term stability of EP in quality control samples was also evaluated by analysis after 45 days of storage at −30°C. Autosampler stability was studied following 54.5 h storage period in the autosampler tray with control concentrations. Bench top stability was studied for a 25-h period with control concentrations. Stability samples were processed and extracted along with the freshly spiked calibration curve standards. The precision and accuracy for the stability samples must be ≤15% and ±15% respectively of their nominal concentrations.

Analysis of patient samples

The bio analytical method described above was used to determine EP concentrations in plasma following oral administration healthy human volunteers. These volunteers/ subjects were contracted in APL Research Center Pvt. Ltd, Hyderabad, India and each subject was administered a 40-mg dose (one 40 mg tablet) in 24 healthy volunteers by oral administration with 240 mL of drinking water. The reference product Relpax Tablets (Pfizer, USA) 40 mg and Test product Eletriptan tablet (test tablet) 40 mg was used. Study protocol was approved by IEC (Institutional Ethics committee) as per ICMR (Indian council of medical research). Blood samples were collected as the pre-dose (0) h 5 min prior to dosing followed by further samples at 0.333, 0.667, 1.0, 1.25, 1.5, 1.75, 2.0, 2.333, 2.667, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 8.0, 10.0, 12.0, 16.0, 20.0, and 24.0 h. After dosing, 2.5 ml blood was collected each time in vaccutainers containing K_2EDTA. A total of 44 (22 time points for reference, 22 time points for test) time points were collected by using centrifugation 3,200 rpm, 10°C, 10 min and stored below −30°C until sample analysis. Test and reference eletriptan tablets were administered to same human volunteers under fed conditions separately with proper washing periods as per protocol (comparative, randomized, two-way crossover) approved by IEC.

Pharmacokinetics and statistical analysis

Pharmacokinetics parameters from the human plasma samples were calculated by a non-compartmental statistic model using WinNon-Lin5.0. software (Pharsight, USA). Blood samples were taken for a period of three to five times the terminal elimination half-life ($t_{1/2}$) and it was considered as the area under the concentration time curve (AUC) ratio higher than 80% as per FDA guidelines. Plasma EP concentration–time profiles were visually inspected, and $C_{\max}$ and $T_{\max}$ values were determined. The AUC_{0-t} was obtained by the trapezoidal method. $AUC_{0-\infty}$ was calculated up to the last measureable concentration and extrapolations were obtained using the last measureable concentration and the terminal elimination rate constant (K_e) it was estimated from the slope of the terminal exponential phase of the

Table 3 Stability of the samples

Spiked plasma concentration (ng/ml)	Room temperature stability		Processed sample stability		Long-term stability		Freeze and thaw stability	
	25 h		54.5 h		45 days		Cycle 3 (48 h)	
	Concentration measured (n=6; ng/ml; mean±S.D.)	CV (n=6; %)	Concentration measured (n=6; ng/ml; mean±S.D.)	CV (n=6; %)	Concentration measured (n=6; ng/ml; mean±S.D.)	CV (n=6; %)	Concentration measured (n=6; ng/ml; mean±S.D.)	CV (n=6; %)
1.5	1.49±0.12	8.6	1.57±0.15	9.9	1.470±0.02	2.0	1.57±0.07	4.9
175.0	178.28±6.4	3.6	182.38±18.95	10.4	165.354±6.77	4.1	180.060±7.08	3.9

plasma of the EP concentration–time curve (by means of the linear regression method). The terminal elimination half-life ($t_{1/2}$), was then calculated as 0.693/K_e. Regarding AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} bioequivalence were assessed by means of analysis of variance (ANOVA) and calculating the standard 90% confidence intervals (90% CIs) of the ratio's test/reference (logarithmically transformed data). The bioequivalence was considered when the ratio of averages of log-transformed data was within 80–125% for AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} [16–18].

Results and discussion

Method development

LC-MS/MS has been used as one of the most powerful analytical tools in clinical pharmacokinetics for its selectivity, sensitivity, and reproducibility. The goal of this work is to develop and validate a simple, sensitive, rapid, rugged, and reproducible assay method for the quantitative determination of EP from human plasma samples. Chromatographic conditions, especially the composition and nature of the mobile phase, usage of different columns, different extraction methods such as solid phase, precipitation, liquid–liquid extraction methods were optimized through several trials to achieve the best resolution and increase the signal of EP and NP. The MS optimization was performed by direct infusion of solutions of both EP and NP into the ESI source of the mass spectrometer. The critical parameters in the ESI source include the needle (ESI) voltage, capillary voltage, source temperature and other parameters such as nebulizer gas, heater gas and desolvation gases were optimized to obtain a better spray shape, resulting in better ionization of the protonated ionic EP and NP molecules (Fig. 1). Product ion spectrum for EP and NP yielded high-abundance fragment ions of m/z 84.3 and m/z 97.8respectively (Figs. 2 and 3). After mass spectrometer parameters optimized, chromatographic conditions such as mobile phase optimization, column optimization, extraction method optimization was performed to obtain a fast and selective LC method. A good separation and elution were achieved using 0.1% formic acid/methanol (40:60 v/v) as the mobile phase, at a flow rate of 0.5 mL/min and injection volume of 5 µL. Ascentis Express C18, 50×4.6 mm, 2.7 µm column, and liquid–liquid extraction method was optimized for the best chromatography. (Fig. 4a)

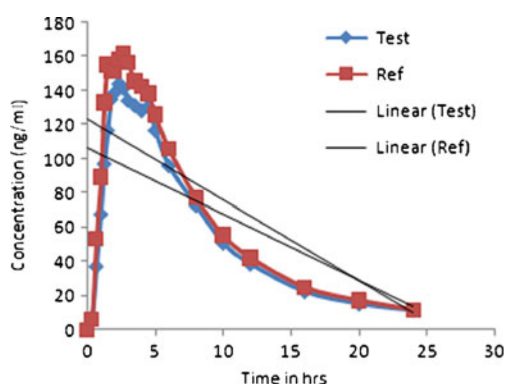

Fig. 6 Mean plasma concentrations test versus reference after a 40 mg dose (one 40 mg tablet) single oral dose (24 healthy volunteers)

Table 4 Mean pharmacokinetic parameters of elitriptan in 24 health volunteers after oral administration of 40 mg test and reference products

	Pharmacokinetic Parameter			
	C_{max} (ng/ml)	T_{max} (h)	AUC_{0-t} (ng. h/ml)	$AUC_{0-\infty}$ (ng. h/ml)
Test	144.05	2.333	1,320.85	1,424.58
Reference	161.57	2.667	1,335.54	1,434.84

$AUC_{0-\infty}$ area under the curve extrapolated to infinity, *AUC_{0-t}*: area under the curve up to the last sampling time, *C_{max}* the maximum plasma concentration, *T_{max}* the time to reach peak concentration

Method validation

The developed method was validated over a linear concentration range of 0.5–250.0 ng/ml. The validation parameters include selectivity and specificity, LOD, and LOQ, matrix effect, precision and accuracy, recovery, stability (freeze–thaw, auto sampler, bench top, long term) was evaluated under validation section.

Selectivity and specificity

The analysis of EP and NP using MRM function was highly selective with no interfering compounds (Fig. 4b).Chromatograms obtained from plasma spiked with EP (0.5 ng/mL) and NP (250.0 ng/mL) are shown in Fig. 4c.

Table 5 Pharmacokinetic test/reference of elitriptan after administration of 40 mg of test and reference, products in 24 healthy male volunteers

	Pharmacokinetic Parameter		
	C_{max} (ng/ml)	AUC_{0-t} (ng h/ml)	$AUC_{0-\infty}$ (ng h/ml)
Test/reference	89.16	98.90	99.28

Limits of detection and quantification

The limit of detection was used to determine the instrument detection levels for EP even at low concentrations. 5 μL of a 0.5 pg/mL solution was injected and estimated LOD was 2.5 fg with *S*/*N* values ≥3–5.

The limit of quantification for this method was proven as the lowest concentration of the calibration curve which was proven as 0.5 ng/ml.

Matrix effect

Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the MQC level, and compared with neat standards of same concentration in alternate injections. The overall precision of the matrix factor is 5.67 for eletriptan. There was no ion-suppression and ion-enhancement effect observed due to IS and analyte at respective retention time (Fig. 5a,b).

Precision and accuracy

Calibration curves were plotted as the peak area ratio (EP/NP) versus (EP) concentration.

Precision and accuracy of calibration curve standards, quality control standards represented in Tables 1 and 2.

Stability (freeze–thaw, auto sampler, bench top, long term)

Quantification of the EP in plasma subjected to three freeze–thaw cycles (−30°C to room temperature), autosampler, room temperature (Benchtop), long-term stability details were shown in Table 3.

Recovery

The recovery following the sample preparation using liquid–liquid extraction with methyl tertiary butyl ether was calculated by comparing the peak area of EP in plasma samples with the peak area of solvent samples and was estimated at control levels of EP. The recovery of EP determined at three different concentrations, 1.5, 75.0, and 175.0 ng/mL, were found as 95.43%, 91.95%, and 94.03%, respectively. The overall average recovery of EP and NP were found to be 93.81% and 87.18%, respectively.

Application to biological samples

The above validated method was used in the determination of EP in plasma samples for establishing the bioequivalence of a single 40 mg dose (one 40 mg tablet) in 24 healthy volunteers. Typical plasma concentration versus time profiles is shown in Fig. 6. All the plasma concentrations of EP were within the standard curve region and retained above the 0.5 ng/mL (LOQ) for the entire sampling period (Tables 4 and 5).

Conclusion

The method described in this manuscript has been developed and validated over the concentration range of 0.5–250.0 ng/mL in human plasma. The intra-batch precision was less than 7.8% and accuracy ranged from 96.8% to 103.0%. The inter-batch precision was less than 5.5% and accuracy ranged from 98.5% to 99.8%. The selectivity, sensitivity, precision, and accuracy obtained with this method make it suitable for the purpose of the present study. In conclusion, the method used in the present study is easy and fast to perform; it is also characterized with an adequate accuracy, precision, selectivity and stability. The simplicity of the method, and using rapid liquid–liquid extraction and sample turnover rate of 2.0 min per sample, make it an attractive procedure in high-throughput bioanalysis of eletriptan. The validated method was successfully applied in a bioequivalence study of two formulations (test and reference) by oral administration of 40 mg in 24 healthy human volunteers.

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