

Method development and validation for naratriptan determination in human plasma by HPLC with tandem mass spectrometry detection, and its application to bioequivalence study

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The authors developed a simple, sensitive and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantification of naratriptan (NP) in human plasma using naratriptan-d3 (NPD3) as an internal standard (IS). Chromatographic separation was performed on a Zorbax SB-C18, 75 x 4.6 mm, 3.5 μ m column with an isocratic mobile phase composed of 0.1% formic acid : acetonitrile (50:50 v/v), at a flow-rate of 0.6 mL/min. NP and NPD3 were detected with proton adducts at m/z 336.5 \rightarrow 98.0 and 339.4 \rightarrow 101.0 in selected reaction monitoring (SRM) positive mode, respectively. The liquid-liquid extraction method was used to extract the NP and NPD3. This method was validated over a linear concentration range of 0.1-25.0 ng/mL with a correlation coefficient of (r^2) \geq 0.9998. The Intra-day and Interday precision was found to be 1.8 to 3.6%, and 2.3 to 2.6%, and accuracy to be 101.7- 104.2% and 101.8 to 102.9%, respectively. NP was found to be stable throughout freeze-thaw (three cycles), bench top and auto sampler stability studies. This method was successfully applied for the analysis of plasma samples following oral administration of NP (2.5 mg) in 31 healthy Indian male human volunteers under fasting conditions.

Uniterms: Liquid chromatography/quantitative analysis. Mass spectrometry/quantitative analysis. Naratriptan/determination/human plasma.

Os autores desenvolveram um método simples, sensível e específico de cromatografia líquida-espectrometria de massa-tandem (LC-MS/MS) para a quantificação de naratriptan (NP) em plasma humano empregando naratriptan-d3 (NPD3) como padrão interno de referência (IS). A separação cromatográfica foi realizada em coluna Zorbax SB-C18, 75 x 4,6 mm, 3,5 μ m com fase móvel isocrática composta por 0,1% ácido fórmico : acetonitrila (50:50 v/v) e taxa de fluxo de 0,6 mL/min. NP e NPD3 foram detectados com adutos de prótons a m/z 336.5 \rightarrow 98.0 e 339.4 \rightarrow 101.0 in em modo positivo do tipo monitoramento de reação selecionada (SRM), respectivamente. Extração líquido-líquido foi empregada para extrair NP e NPD3, sendo o método validado para uma faixa linear de concentração de 0,1-25,0 ng/mL resultando em coeficiente de correlação (r^2) \geq 0,9998. A variação intra e interdía observada para precisão foi de 1,8 a 3,6% e 2,3 a 2,6%, respectivamente; para exatidão a variação foi de 101,7 a 104,2% e 101,8 a 102,9%, respectivamente. O NP se mostrou estável frente a processos de congelamento-descongelamento (3 ciclos), e estudos de estabilidade de bancada e amostragem automática. O método desenvolvido foi aplicado com sucesso para a análise de amostras de plasma após a administração oral de 2,5 mg de NP em 31 voluntários humanos, de nacionalidade indiana, sexo masculino, sob condições aceleradas.

Unitermos: Cromatografia líquida/análise quantitativa. Espectrometria de massa/análise quantitativa. Naratriptan/determinação/plasma humano.

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INTRODUCTION

NP is chemically known as N-methyl-2-[3-(1-methylpiperidin-4-yl)-1H-indol-5-yl] ethane sulfonamide. The molecular formula is $C_{17}H_{25}N_3O_2S$, which corresponds to a molecular weight of 335.47. NP is a triptan drug used for the treatment of migraine headaches, and is a selective 5-hydroxytryptamine 1 receptor subtype agonist. It is well absorbed (74% oral bioavailability with peak plasma concentrations after 2-5 hours) having 28-31% protein binding, and is metabolized by a wide range of cytochrome P450 isoenzymes into a number of inactive metabolites, and has a 5-8 hour half life (Fuseau *et al.*, 1997).

Several techniques such as fluorometry and liquid chromatography (LC) methods have been reported in the literature for the quantitative estimation of NP in biological fluids (Vishwanathan *et al.*, 2000; Ptacek *et al.*, 2009; Dulery *et al.*, 1997; Gueorguieva *et al.*, 2005). Dulery *et al.* (1997) developed a method in rabbit plasma, using a liquid chromatographic-electrospray-mass spectrometric assay for the determination sumatriptan and naratriptan. To date, only a few methods have been developed in human plasma for quantification of NP (Vishwanathan *et al.*, 2000; Ptacek *et al.*, 2009; Dulery *et al.*, 1997; Gueorguieva *et al.*, 2005) by LC-MS/MS. Vishwanathan, Bartlett, Stewart (2000) developed a method for determination of antimigraine compounds rizatriptan, zolmitriptan, naratriptan and sumatriptan in human serum by liquid chromatography (LC MS/MS) with a solid phase extraction method, and observed good linearity between the concentration ranges of 1.0 - 100.0 ng/mL. However, the extraction method is expensive for large sample analysis.

The purpose of this investigation was to develop a simple, sensitive, selective and reproducible analytical method for the quantitative estimation of NP in a small volume of human plasma using deuterated internal standard. It is also envisaged that this method will be able to provide an efficient solution for pharmacokinetic, bioavailability or bioequivalence studies of NP.

MATERIALS AND METHODS

Materials

Naratriptan hydrochloride was obtained from APL Research, India, and naratriptan-d3 hydrochloride was obtained from Toronto Research Chemicals, Ontario, Canada. Analytical grade sodium carbonate, tertiary butyl methyl ether, formic acid and acetonitrile were purchased from SD fine chemical Ltd, Mumbai, India. Human plasma (K_2EDTA) was purchased from the Navjeevan blood bank,

Hyderabad, India. Double distilled water was used for all the experiments.

Instrumentation and detection

The HPLC system (1200 series, Agilent technologies, Waldbronn, Germany) was employed. A Mass spectrometer API 4000 triple quadrupole instrument (ABISCIEX, Toronto, Canada) was used. Turbo ion spray positive mode with unit resolution, SRM was used for detection. For NP, $[M+H]^+$ (m/z 336.5) was monitored as the precursor ion and a fragment at m/z 98.0 was chosen as the product ion. For NPD3, the $[M+H]^+$ (m/z 339.4) was monitored as the precursor ion and a fragment at m/z 101.0 was monitored as the product ion. Mass parameters were optimized as source temperature 600 °C, nebulizer gas 25 psi, heater gas 35 psi, curtain gas 20 psi, CAD gas 5 psi (nitrogen), ion spray voltage 5500 V, source flow rate 600 μ L/min without split, entrance potential 10 V, declustering potential 40 V, collision energy 16 V, and collision cell exit potential 6 V for both analyte and internal standard.

Chromatographic conditions

A Zorbax SB-C18, 75 \times 4.6 mm, 3.5 μ m, was selected as the analytical column. The mobile phase composition was 0.1% formic acid: acetonitrile (50:50 v/v). The flow rate of the mobile phase was set at 0.6 mL/min. The column temperature was set at 50 °C. NPD3 was used as the appropriate internal standard. The retention time of NP and NPD3 were found to be 1.2 ± 0.2 min, respectively with a total run-time of 2 min for each sample.

Preparation of standards and quality control (QC) samples

Standard stock solutions of NP (100.0 μ g/mL), and NPD3 (100.0 μ g/mL) were prepared in methanol. NPD3 dilution (250.0 ng/mL) was prepared in 50% acetonitrile from NPD3 standard stock solution. Standard and spiking solutions were stored under refrigerator conditions (2-8 °C) until analysis. Standard stock solutions were added to drug-free human plasma to obtain NP concentration levels of 0.1, 0.2, 0.5, 1.0, 5.0, 10.0, 15.0, 20.0 and 25.0 ng/mL for analytical standard, and of 0.1, 0.3, 7.5, 17.5 ng/mL for quality control standards, and stored in a freezer (-30 °C) until analysis. The aqueous standards were prepared in reconstitution solution of acetonitrile: 0.1% formic acid (50:50 v/v) and stored under refrigerator conditions (2-8 °C) for validation exercises until analysis.

Sample preparation

Liquid-liquid extraction was used to isolate NP and NPD3 from human plasma. A volume of 50 μL of NPD3 (30.0 ng/mL) and 250 μL of plasma sample (respective concentration) were added to polypropylene tubes, vortexed briefly and placed in an ice bath. This process was followed by addition of 250 μL of 0.5 N sodium carbonate, 2.5 mL of extraction solvent (tertiary butyl methyl ether) and vortexed for approximately 10 min. Further samples were centrifuged at 4000 rpm for 5 min at 4 °C. Supernatant from each sample was transferred into polypropylene tubes and evaporated to dryness under nitrogen at 40 °C. Finally, dried residue from each tube was reconstituted with 250 μL of reconstitution solution (acetonitrile: 0.1% formic acid (50:50 v/v) and vortexed briefly. Subsequently, 20 μL of sample from each tube was transferred into auto sampler vials and injected into the LC-MS/MS.

Recovery

The extraction recovery of NP and NPD3 from human plasma was determined by analyzing quality control samples. Recovery at three concentrations (0.3, 7.5 and 17.5 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 50% was considered adequate to attain the required sensitivity.

Limit of quantification (LOQ)

Peak response (peak area) was determined in blank plasma samples (six replicates from different plasma) and in spiked LOQ sample prepared from the same plasma. The peak area of blank samples should be no greater than 20% of the mean peak area of LOQ of NP, and no greater than 5% of NPD3. The precision and mean accuracy of the back calculated LOQ replicate concentrations must be ≤ 20 and ± 20 %, respectively.

Analytical curves, regression model

The analytical curves were constructed using values ranging from 0.1 to 25.0 ng/mL of NP in human plasma. Calibration curves were obtained by the weighted quadratic model with the log-transformed regression model. The ratio of NP peak area to NPD3 peak area was plotted against NP concentration in ng/mL. The suitability of the calibration curve was confirmed by back-calculating the concentrations of the calibration standards.

$$\text{Log } y = a \log (x^2) + b \log (x) + c$$

y = peak area ratio (PAR) of NP to NPD3

x = concentration (ng/mL) of NP in plasma.

Coefficients a , b and c , the coefficient of determination (r^2) and x was calculated using weighted 1 quadratic model with log-transformed regression analysis.

Calibration curve standards and quality control samples

Calibration curve standard samples and quality control samples were prepared in replicate ($n=6$) for analysis. The correlation coefficient (>0.9998) was obtained by using the quadratic model with the log-transformed regression model across the whole range of tested concentrations.

Precision and accuracy

Precision and accuracy for the back-calculated concentrations of the calibration points should be within $\pm 15\%$ of their nominal values. For LOQ however, precision and accuracy should be within $\pm 20\%$.

Stability (Freeze- thaw, Auto sampler, Bench top, Re-injection, Long term)

Low quality control and high quality control samples ($n=6$) were retrieved from the 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 NP in quality control samples was also evaluated by analysis after 100 days of storage at -30 °C. Autosampler stability was studied following a 101.5 h storage period in the autosampler tray. Bench-top stability was studied for a 25 h period. Re-injection stability was proved for one reinjection. Stability samples were processed and extracted along with the freshly spiked calibration curve standards. The precision and accuracy for the stability samples must be within 15 and $\pm 15\%$ of their nominal concentrations, respectively.

Matrix effect

The matrix effect was performed in order to determine the matrix effect (ion suppression/ ion enhancement) qualitatively on different lots of blank plasma (6 lots from K_2EDTA blank plasma), using triplicate analysis at low and high concentrations, which were extracted and injected as per method. The ion suppression matrix effect was evaluated by injecting reagent blank, different lots of blank

plasma (6 lots from K2 EDTA blank plasma) of extracted blank matrix while the aqueous standard was infused. No significant matrix effect was observed.

Column to column reproducibility

Column to column reproducibility was determined by injecting analytical standards, matrix blank and QC standards on different columns. The results showed good reproducibility.

Standard and stock solution stability

Standard stock solution stability at room temperature in white light under laboratory conditions and refrigerated conditions for NP and NPD3 was performed as part of the method development process. The results showed good stability for 57 days under refrigerated conditions.

Analysis of patient samples

The bio-analytical method described above was used to determine NP concentrations in plasma following oral administration in healthy human volunteers. These volunteers were contracted in APL Research Pvt. Ltd., Hyderabad, India... and each of the 31 healthy volunteers was administered a 2.5 mg dose (one 2.5 mg tablet) by the oral route with 240 mL of drinking water. The reference product Amerge tablets (GSK) 2.5 mg and test product NP tablet (Test tablet) 2.5 mg were used. The study protocol was approved by the IEC (Institutional Ethics Committee) as per ICMR guidelines (Indian Council of Medical Research). Blood samples were collected at pre-dose (0hr), 5 minutes prior to dosing, followed by further sample taken at 0.5, 1, 1.33, 1.67, 2, 2.33, 2.67, 3, 3.33, 3.67, 4, 4.5, 5, 6, 8, 10, 12, 14, 18, 24 and 30 hours. After dosing, 5 mL of blood was taken at each collection and placed in vacutainers containing K₂EDTA. Collections were taken for 44 (22 time points for test and reference, respectively) time points using centrifugation 3200 rpm at 10 °C for 10 min, and storage at -30 °C until sample analysis. Test and reference were administered separately to the same human volunteers under fasting conditions with proper wash-out periods as per the protocol approved by IEC.

Pharmacokinetics and statistical analysis

Pharmacokinetics parameters for the human plasma samples were calculated by a non-compartmental statistics model using WinNon-Lin5.0 software (Pharsight, USA). Plasma samples were taken for a period of 3 to 5 times

the terminal elimination half-life ($t_{1/2}$), considered the area under the concentration time curve (AUC) ratio higher than 80%, as per FDA guidelines. Plasma NP 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). K_e was estimated from the slope of the terminal exponential phase of the plasma of the NP concentration–time curve by 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 was assessed by analysis of variance (ANOVA) and by 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} .

RESULTS AND DISCUSSION

Method development and validation

LC-MS/MS is one of the most powerful analytical tools used in clinical pharmacokinetics given its selectivity, sensitivity and reproducibility. The goal of this work was to develop and validate a simple, sensitive and rapid assay method for quantitative determination of NP from plasma samples. A simple Liquid-liquid extraction technique was utilized for extraction of NP and NPD3 from the plasma samples. Chromatographic conditions, particularly the composition and nature of the mobile phase, were optimized through several earlier trials to achieve best resolution and to increase the signal of NP and NPD3. MS optimization was performed by direct infusion of solutions of both NP and NPD3 into the ESI source of the mass spectrometer. The critical parameters in the ESI source include the needle (ESI) voltage, which is directly related to the charged droplet formation and to the amount of gaseous ions formed. Capillary voltage was related to the gaseous ion guidance to the inside of the MS and is the last barrier between the atmospheric pressure and the high vacuum of the mass spectrometer. Other parameters, such as the nebulizer and heater gases, were optimized to obtain a better spray shape, resulting in improved ionization and droplet drying to form the protonated ionic NP and NPD3 molecules (Figures 1a, 1b). A CAD product ion spectrum for NP and NPD3 yielded high-abundance fragment ions of m/z 98 and m/z 101, respectively (Figures 2, 3). After the

SRM channels were tuned, the mobile phase was changed from an aqueous phase to a more organic phase with acid dopant to obtain a fast and selective LC method. Good

separation and elution were achieved using 0.1% formic acid: acetonitrile (50:50 v/v) as the mobile phase, at a flow-rate of 0.6 mL/min with a 10 μ L injection volume.

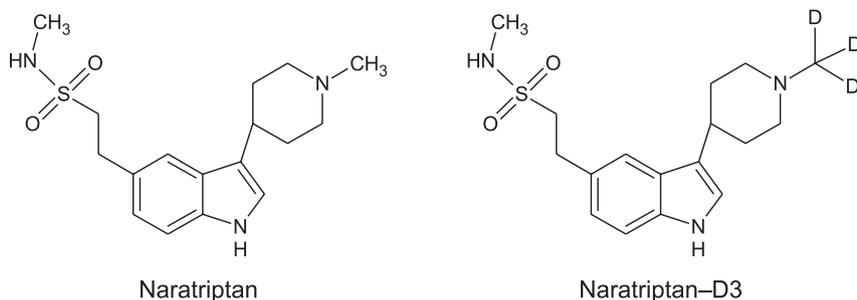


FIGURE 1 - Chemical structures of Naratriptan and Naratriptan -D3

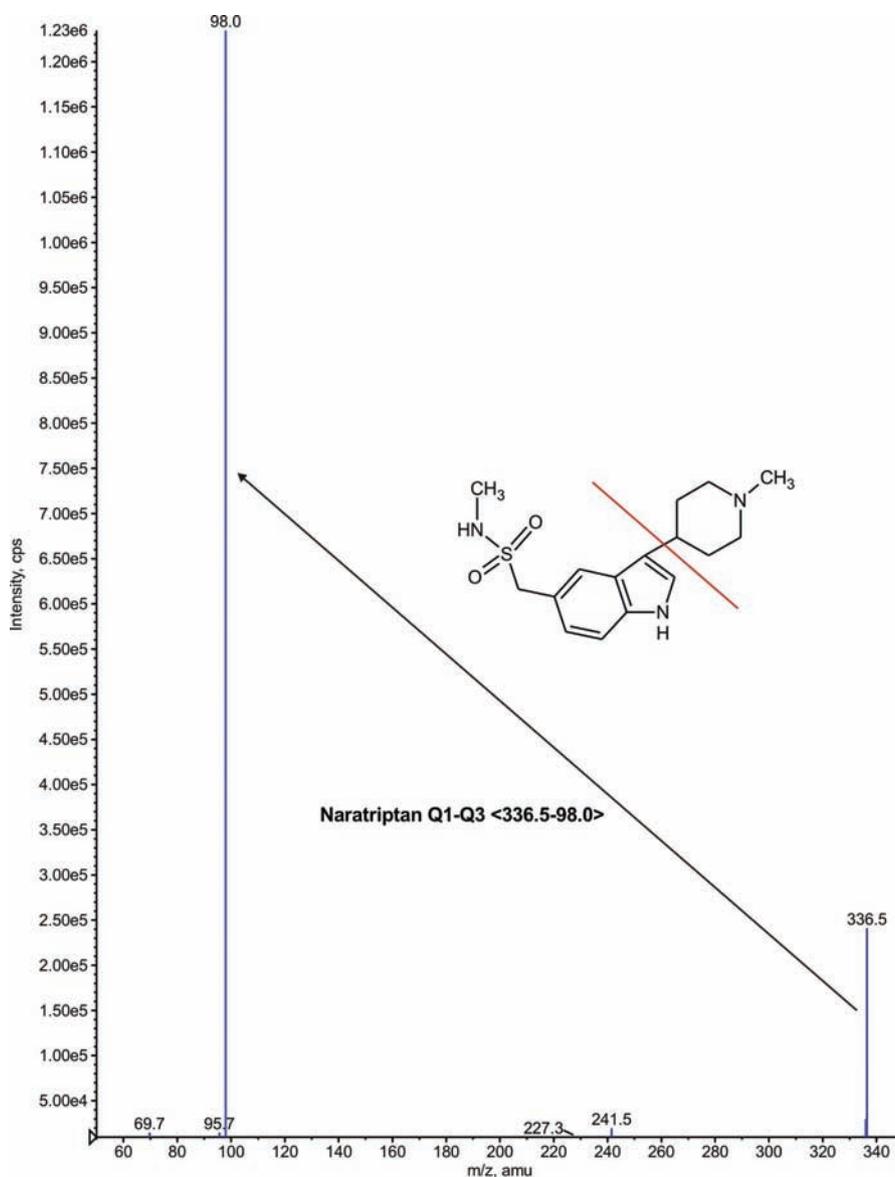


FIGURE 2 - Mass spectra of the Naratriptan Q1, Q3.

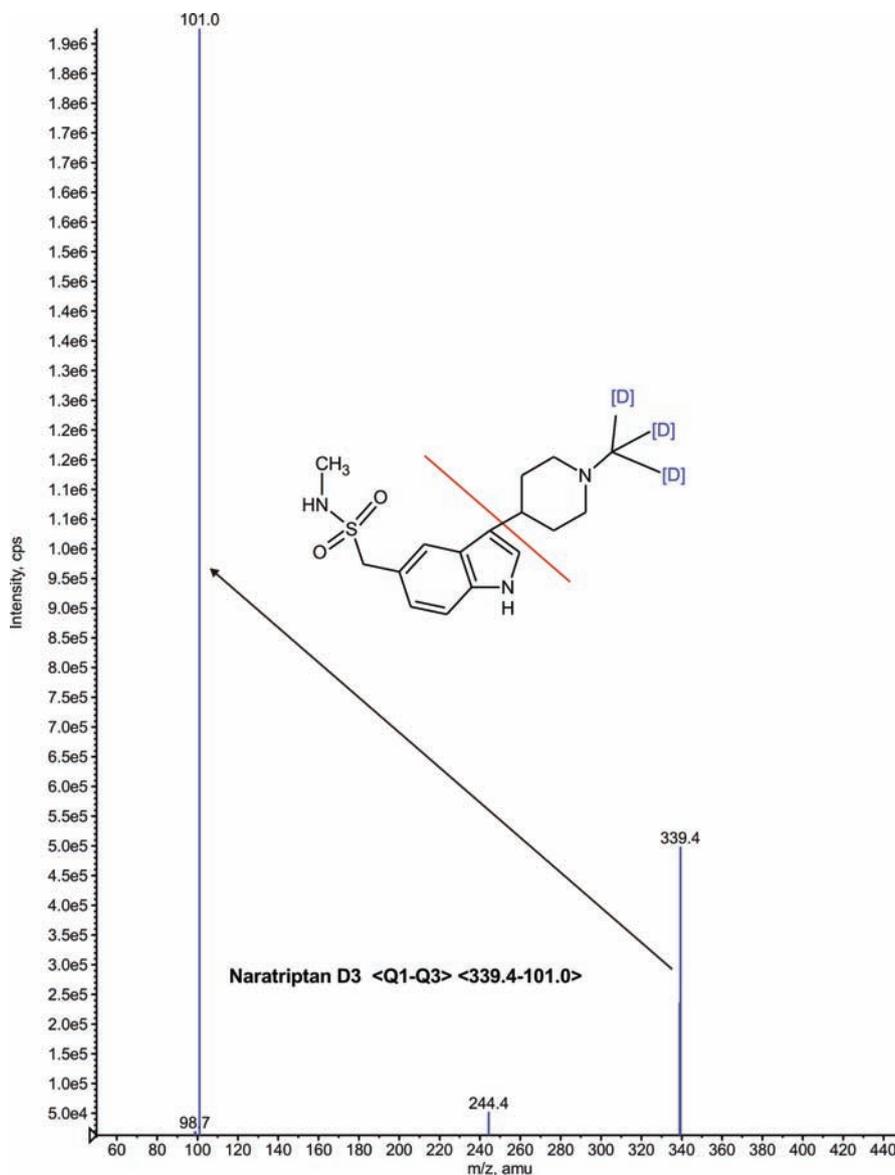


FIGURE 3 - Mass spectra of the Naratriptan -d3 Q1, Q3.

Selectivity

The analysis of NP and NPD3 using MRM function was highly selective with no interfering compounds (Figure 4A). Chromatograms obtained from plasma spiked with NPD3 (30.0 ng/mL) are shown in Figure 4B, and spiked with NP (0.1 ng/mL) and NPD3 (30.0 ng/mL) are shown in Figure 4C.

Matrix effect

To predict the variability of matrix effects in samples from individual subjects, the matrix effect was quantified by determining the matrix factor, which was calculated as follows.

$$\text{Matrix Factor} = \frac{\text{Peak response ratio in the presence of extracted matrix}}{\text{Peak response ratio in aqueous standards}}$$

Six lots of blank biological matrix were spiked, each in triplicate, with the aqueous standard at the mid QC level, and compared against aqueous standards of the same concentration in alternate injections. The overall precision of the matrix factor is expressed as coefficient of variation (% CV) and was determined to be $\leq 15\%$ for NP.

Linearity

Calibration curves were plotted as the peak area ra-

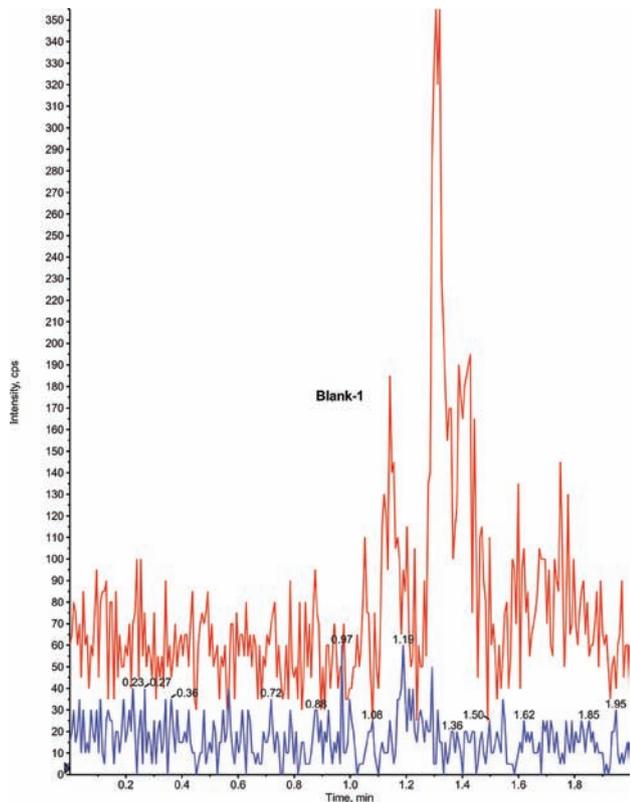


FIGURE 4A - SRM chromatograms of naratriptan and naratriptan-d3 in blank human plasma.

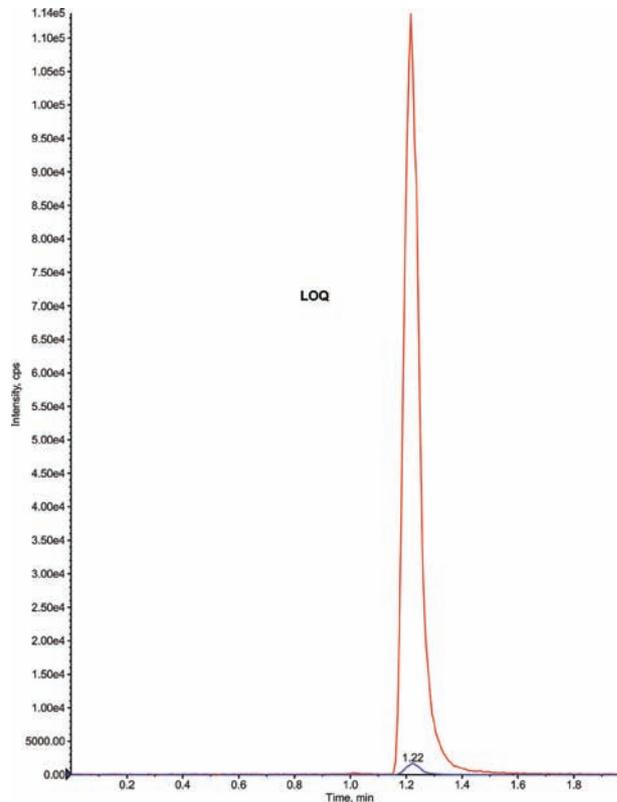


FIGURE 4C - spiked human plasma containing 0.1 ng/mL naratriptan and 30.0 ng/mL naratriptan-d3 [LLOQ].

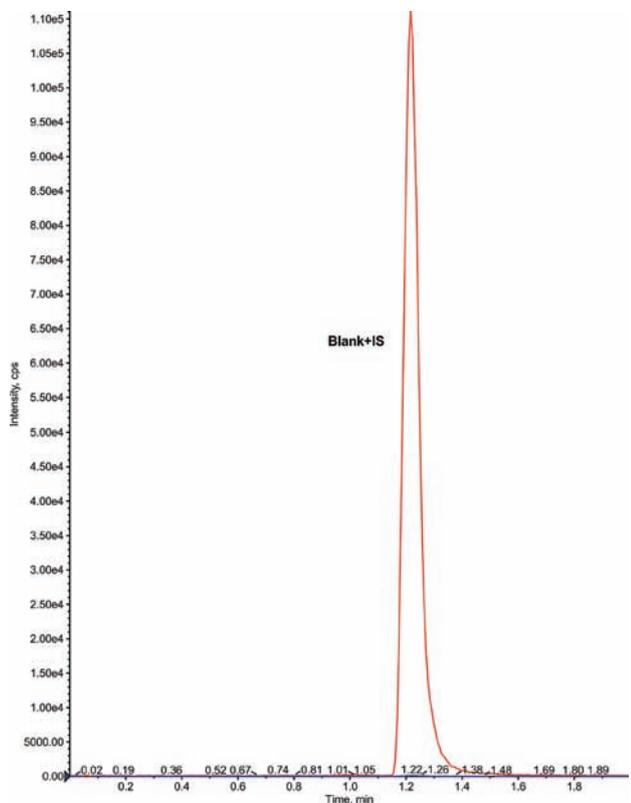


FIGURE 4B - Spiked human plasma containing 30.0 ng/mL naratriptan-d3 [Blank+IS].

tio (NP and NPD3) versus NP concentration. Calibration was found to be linear over the concentration range of 0.1 - 25.0 ng/mL. The R.S.D.s were less than 15%, and the accuracy ranged from 98.7 to 101.5%. The determination coefficients (r^2) were greater than 0.9998 for all curves (Table I).

TABLE I - Calibration curve details from one batch of the validation section

Spiked plasma concentration (ng/mL)	Concentration measured (mean±SD) (ng/mL)	R.S.D. ^a (% CV) ($n = 5$)	Accuracy %
0.1	0.1 ± 0.0	1.0	99.0
0.2	0.2 ± 0.0	3.0	101.5
0.5	0.4 ± 0.0	2.4	99.0
1.0	1.1 ± 0.1	1.3	100.7
5.0	5.1 ± 0.1	1.3	100.1
10.0	9.8 ± 0.1	1.3	98.7
15.0	15.1 ± 0.2	1.0	100.3
20.0	20.1 ± 0.3	1.3	100.4
25.0	25.1 ± 0.3	1.1	100.2

^a [Standard deviation/mean concentration measured] x 100.

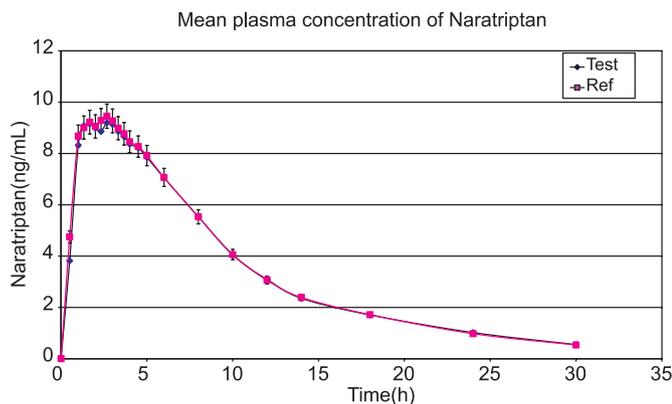


FIGURE 5 - Mean plasma concentrations of test vs. reference after a 2.5 mg dose (one 2.5 mg Tablet) single oral dose (31 healthy volunteers).

Precision and accuracy of quality control standards

The quantitative accuracy and precision of the assay was evaluated on an inter-assay basis, using prepared QC standards at three concentrations spanning the range of the analytical standard curve. Precision is expressed as % CV. Precision and accuracy for this method was controlled by calculating the intra and inter-batch variations at three concentrations (0.3, 7.5 and 17.5 ng/mL) of QC samples in six replicates. As shown in Table II, Intra and inter-day CV% ranged from 1.8 to 3.6 and from 2.3 to 2.6%, respectively, while Intra and inter-day accuracy ranged from 101.7 to 104.2% and from 101.0 to 102.9%, respectively, for NP. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

Stability (Freeze- thaw, Auto sampler, Room temperature, Long term)

Quantification of NP in plasma subjected to 3 freeze-thaw (-30 °C to room temperature) cycles verified the

stability of the analyte. No significant degradation of NP was observed even after a 101.5 h storage period in the autosampler tray, and the final concentrations of NP ranged from 97 to 104%. In addition, the long-term stability of NP in QC samples after 100 days of storage at -30 °C was also evaluated. The concentrations ranged from 99 to 106% (Table III). These results confirmed the stability of NP in human plasma for at least 100 days at -30 °C.

Recovery

The recovery following sample preparation using the Liquid-liquid extraction method with tertiary butyl methyl ether was calculated by comparing the peak area ratios of NP in plasma samples with the peak area ratios of solvent samples, and was estimated at control levels of NP. The recovery of NP, determined at three different concentrations 0.3, 7.5 and 17.5 ng/mL, were found to be 67.8, 70.5 and 71.7%, respectively. The overall average recovery of NP and NPD3 were found to be 70.02 and 70.49%.

Limit of detection (LOD)

The limit of detection was determined using aqueous solutions. For NP, 10 µL of 5 pg/mL solution was injected, yielding 0.05 pg/mL.

Application to biological samples

The above-validated method was used in the determination of NP in plasma samples for establishing the bioequivalence of a single 2.5 mg dose (one 2.5 mg tablet) in 31 healthy volunteers. Typical plasma concentrations versus time profiles are shown in Fig. 5. All the plasma concentrations of NP were within the standard curve region and remained above the 0.1 ng/mL LOQ for the entire sampling period. The observed maximum plasma concentration (C_{max}) for the standard and test were 94.5 ± 3.36 and 92.0 ± 3.33 ng/mL, respectively.

TABLE II - Precision and accuracy (analysis with spiked plasma samples at three different concentrations)

Spiked plasma concentration (ng/mL)	Within-run ($n=6$)			Between-run ($n=30$)		
	Concentration measured (ng/mL) (mean \pm SD.)	R.S.D. ^a (% CV)	Accuracy %	Concentration measured (ng/mL) (mean \pm SD.)	R.S.D. ^a (%CV)	Accuracy %
0.3	0.3 \pm 0.0	3.6	101.7	0.3 \pm 0.0	2.6	101.0
7.5	7.8 \pm 0.1	1.8	104.0	7.6 \pm 0.2	2.3	101.2
17.5	18.2 \pm 0.5	2.7	104.2	18.0 \pm 0.4	2.4	102.9

^a [Standard deviation/mean concentration measured] x 100.

TABLE III - Stability of the samples

Spiked plasma concentration (ng/mL)	Room temperature stability		Processed sample stability		Long-term stability		Freeze and thaw stability	
	25 hr		101.5 hr		100 days		Cycle 3 (48 hr)	
	Concentration measured (n=6) (ng/mL) (mean±SD)	R.S.D. ^a (n=6) (% CV)	Concentration measured (n=6) (ng/mL) (mean±SD)	R.S.D. ^a (n=6) (%CV)	Concentration measured (n=6) (ng/mL) (mean±SD)	R.S.D. ^a (n=6) (%CV)	Concentration measured (n=6) (ng/mL) (mean±SD)	R.S.D. ^a (n=6) (%CV)
0.3	0.3 ± 0.01	3.5	0.3 ± 0.0	3.54	0.3 ± 0.0	2.6	0.3 ± 0.0	3.01
17.5	17.9 ± 0.1	1.1	17.7 ± 0.4	2.10	17.8 ± 0.2	1.16	17.63 ± 0.2	0.93

^a[Standard deviation/mean concentration measured] x 100.

The corresponding time of maximum concentration (T_{max}) for reference and test were found to be 2.67 and 2.67 hr, respectively. The value of the area under the curve from time 0 to the last sampling time (AUC_{0-t}) for the standard and test were found to be 103.04 ± 2.86 and 102.08 ± 2.89 ng.hr/mL, respectively. The area under the curve from 0 to ∞ ($AUC_{0-\infty}$) was 108.48 ng.h/mL for the standard and 107.97 ng.hr/mL for the test. The elimination half-life ($t_{1/2}$) was 7.05 hr for the reference drug and 7.45 hr for the generic drug. In addition, the mean ratio of $AUC_{0-t}/AUC_{0-\infty}$ was higher than 90%, in accordance to the Food and Drug Administration Bioequivalence Guideline. The test/reference ratio (T/R) and 90% confidence intervals (90 CIs) for overall analysis fell within the previously stipulated range (80–125%). The T/R ratio and 90 CIs (in parenthesis) were 97.31% for C_{max} , 99.06% for AUC_{0-t} , and 99.53% for $AUC_{0-\infty}$. Therefore, it can be concluded that the two NP formulations (reference and test) analyzed are bioequivalent.

CONCLUSION

The proposed method offers significant advantages over previously reported methods in terms of simplicity, sensitivity, selectivity, ruggedness and reproducibility. The major advantage of this method is the use of a minimal volume of 250 μ L plasma sample that greatly facilitates blood sample collection. In addition, this method has shown a wider linearity range from 0.1 to 25.0 ng/mL. No side effects were observed during or after the study by the volunteers who participated. Limit of detection (LOD) was proved for 0.05 pg/mL. NP and NPD3 were eluted within 1.2 ± 0.2 min with a total run time of 2 min. This method was fully validated as per FDA guidelines and successfully applied in bioequivalence studies to evaluate the plasma concentrations of NP in healthy human volunteers.

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