Development and Validation of a Sensitive and Rapid Method to Determine Naratriptan in Human Plasma by LC-ESI–MS–MS: Application to a Bioequivalence Study

Manish Yadav[1,3], Chirag Patel[1], Mahendra Patel[1], Tulsidas Mishra[1], Girin A. Baxi[3], Puran Singhal[1], and **Pranav S. Shrivastav[2,*]**

[1]Bioanalytical Research Department, Veeda Clinical Research, Ahmedabad 3800015, India, [2]Chemistry Department, School of Sciences, Gujarat University, Ahmedabad 380009, India, and [3]Department of Chemistry, KSKV Kachchh University, Bhuj 370001, India

Abstract

A simple, sensitive, selective, and rapid high-performance liquid chromatography-tandem mass spectrometry method is developed and validated for the quantitation of naratriptan, using sumatriptan as internal standard (IS). The method included liquid–liquid extraction of naratriptan and IS with methyl-*tert*-butyl ether and dichloromethane mixture from 100 µL human plasma. The chromatographic separation is achieved on ACE C18 (50 mm × 2.1 mm, 5 µm) analytical column under isocratic conditions, using 0.1% acetic acid and acetonitrile (15:85, v/v) at a flow-rate of 0.4 mL/min. The precursor → product ion transitions for naratriptan (*m/z* 336.10 → 98.06) and IS (*m/z* 296.09 → 251.06) were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) and positive ion mode. The linearity of the method for naratriptan is determined in the range of 103–20690 pg/mL with the analysis time of 1.5 min. The method is fully validated according to USFDA guidelines. A systematic post-column infusion study is conducted for ion-suppression due to endogenous matrix components. The process efficiency of analyte (96%) and IS (93%) from spiked plasma samples was consistent and reproducible. The application of the method is demonstrated by a bioequivalence study of 2.5 mg naratriptan tablet formulation in 31 healthy volunteers under fasting conditions.

Introduction

Migraine is a paroxysmal disorder characterized by attacks of headache, nausea, vomiting, photophobia, and malaise. It is one of the most common form of disabling primary headache and affect nearly 12% of Caucasian population (1–3). The current approach to antimigraine therapy comprises potent serotonin 5-hydroxy-tryptamine (5-HT, 1B/1D) receptor agonist collectively termed triptans. They effect migraine relief by binding to these receptors in the brain, where they act to induce vasoconstriction of extracerebral blood vessels and also reduce neurogenic inflammation (4). Naratriptan is a novel second generation triptan antimigraine drug used to treat moderate to acute migraine cases. It is a selective 5-HT receptor agonist, with high affinity at the 5-HT (1B), 5-HT(1D) and 5-HT(1F) receptor subtypes (5,6). The probable sites of therapeutic action of naratriptan include cranial vasculature; the peripheral terminations of trigeminovascular sensory nerves; the first order synapses of the trigeminovascular sensory system; the descending pain control system; and the nuclei of the thalamus (7). It has very high oral bioavailability (63–74%) and higher lipophilicity compared to other triptan analogs and exhibits a distinct clinical therapeutic profile. The clinically recommended dose of naratriptan is 2.5 mg and has a plasma half life of 6 h (4,7–9).

Few methods are presented for the determination of naratriptan in biological matrices. Dulery et al. (10) have developed a liquid chromatographic-electrospray-mass spectrometric (LC-ESI–MS) assay for the determination of naratriptan, sumatriptan and MDL 74,721 in rabbit plasma (100 or 300 µL). The primary objective was to compare their pharmacokinetics after intravenous and oral administration of these three antimigraine compounds in rabbits. Vishwanathan et al. (11) have reported a rapid, sensitive, and selective method for the determination of antimigraine drugs rizatriptan, zolmitriptan, naratriptan, and sumatriptan in human serum by LC–ESI-MS–MS. The drugs were extracted by solid phase extraction on Oasis HLB cartridges employing 1.0 mL serum sample. The calibration curves were linear from 1–100 ng/mL and the chromatographic analysis required 5 min to separate all four compounds. The limit of detection (LOD) was 100 pg/mL for naratriptan based on the signal to noise ratio of 3. In the present study, a highly sensitive (103 pg/mL) and fully validated LC–ESI-MS–MS method has been developed and applied to a bioequivalence study in 31 healthy volunteers under fasting condition. The method is highly selective and rapid (1.5 min) to analyze naratriptan in 100 µL human plasma. Process efficiency, absolute and relative matrix effect and stability in spiked plasma samples are demonstrated at quality control levels.

Experimental

Chemicals and materials

Reference standards of naratriptan (99.3%) and sumatriptan (IS) (99.4%) were procured from Samex Overseas (Ahmedabad,

*Author to whom correspondence should be addressed: email pranav_shrivastav@yahoo.com.

India). High-performance (HP) LC grade acetonitrile, methyl-*tert*-butyl ether, dichloromethane, glacial acetic acid, and sodium hydroxide pellets were obtained from Merck Specialties Pvt. Ltd., (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system purchased from Millipore (Bangalore, India). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at –20°C until use.

LC conditions

A Waters Acquity LC (Milford, MA) was used for setting the reverse-phase liquid chromatographic conditions. The separation of naratriptan and sumatriptan was performed on an ACE C18 (50 mm × 2.1 mm, length × inner diameter) column (ACE, Aberdeen, Scotland), with 5 µm particle size. It was maintained at 35°C in a column oven with an alarm band of ± 5°C. The mobile phase consisted of 0.1% acetic acid and acetonitrile (15:85, v/v). For isocratic elution, the flow rate of the mobile phase was kept at 0.4 mL/min. The total chromatographic run time was 1.5 min. The autosampler temperature was maintained at 5°C.

MS conditions

Ionization and detection of naratriptan and IS was carried out on a Waters Quattro Premier XE Mass Spectrometer, equipped with ion spray interface and operating in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor protonated precursor → product ion transitions for naratriptan at *m/z* 336.10 → 98.06 and *m/z* 296.09 → 251.06 for IS (Figure 1A and 1B). The source dependent parameters and analyzer parameters kept for naratriptan and sumatriptan were, capillary voltage: 1.00 kV; extractor voltage: 3.00 V; RF lens: 0.0 V; source temperature: 120 ± 5°C; desolvation temperature: 400 ± 10°C; cone gas flow: 100 ± 10 L/h; desolvation gas flow: 800 ± 10 L/h. The optimum values for compound dependent parameters like cone potential and collision energy were set at 43.0 V and 24.0 eV for naratriptan; 28.0 V and 19.0 eV for IS, respectively. Quadrupole 1 and 3 were maintained at unit mass resolution. Dwell time was set at 200 ms for both naratriptan and sumatriptan (IS). Data collection, peak integration, and calculations were performed using Mass Lynx software version 4.1.

Standard stock, calibration standards, and quality control sample preparation

The standard stock solution of 1000 µg/mL naratriptan was prepared by dissolving an appropriate amount in water. Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with stock solution (2% of total volume of blank plasma). Calibration curve standards were made at 103, 207, 647, 1293, 2586, 5173, 10345, and 20690 pg/mL concentrations and quality control samples were prepared at four levels, viz. 18639 pg/mL (HQC, high quality control), 1789 pg/mL (MQC, medium quality control), 304 pg/mL (LQC, low quality control) and 106.5 pg/mL (LLOQ QC, lower limit of quantitation quality control). Stock solution (1000 µg/mL) of the internal standard (IS) was prepared by dissolving 10 mg of sumatriptan in 10 mL of water. An aliquot of 25 µL of this solution was further diluted to 50 mL in the same diluent to obtain a solution of 500 ng/mL. All the solutions (standard stock, calibration standards and quality control samples) were stored at 2–8°C until use.

Sample extraction protocol

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 100 µL of spiked plasma sample, 50 µL of IS was added and vortexed for 10 s. Subsequently, 50 µL of 0.1 N sodium hydroxide solution was added and vortexed for another 10 s. Further, 2.5 mL of methyl-*tert*-butyl ether and dichloromethane (80:20, v/v) was added and rotated on a cyclo-mixer for 10 min at 32 × *g*. Samples were then centrifuged at 3204 × *g* for 5 min at 10°C. After centrifugation, 2.0 mL of the supernatant organic layer was transferred to an evaporation tube. The supernatant was evaporated to dryness in a thermostatically controlled water bath maintained at 35 ± 5°C under a stream of nitrogen. After drying, the residue was reconstituted in 100 µL of mobile phase and 5 µL was used for injection into the chromatographic system.

Bioanalytical method validation

A thorough and complete method validation of naratriptan in human plasma was done following the USFDA guidelines (12).

System suitability test was performed by injecting six consecutive injections using aqueous standard mixture of naratriptan (1789 pg/mL) and IS (500 ng/mL) at the start of each batch during method validation. System performance was studied by injecting one extracted LLOQ sample with IS at the beginning of

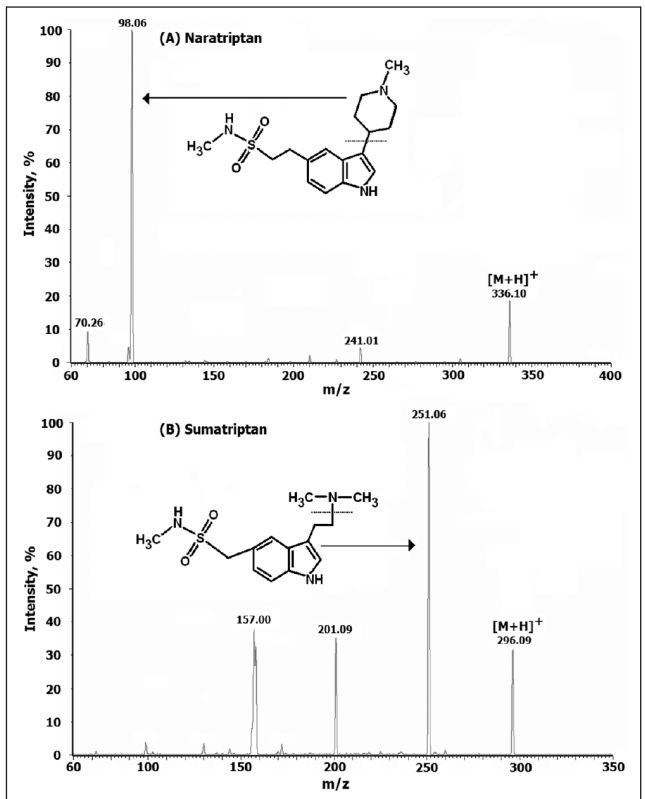

Figure 1. Product ion mass spectra of (A) naratriptan (*m/z* 336/10 → 98.06, scan range 60–400 amu) and (B) sumatriptan, IS (*m/z* 296.09 →251.06, scan range 60–350 amu) in positive ionization mode.

each analytical batch and before re-injecting any sample during method validation. A carry over experiment was performed to verify any carry over of analyte, which may reflect in subsequent runs. The design of the study comprised of the following sequence of injections: mobile phase solution → two samples of LLOQ → blank plasma → ULOQ sample → blank plasma → ULOQ sample → blank plasma.

The selectivity of the method towards endogenous plasma matrix components was assessed in nine different batches of plasma (K_3EDTA) which consisted of six normal control plasma, and one each of lipidemic, hemolytic, and heparinised human plasma. Check for interference due to commonly used medications (paracetamol, chlorpheniramine maleate, caffeine, acetylsalicylic acid, and ibuprofen) in human volunteers was done for ionization (ion suppression/enhancement), analytical recovery (precision and accuracy), and chromatographic interference (interference with MRM of drug and IS). Their stock solutions (100.0 µg/mL) were prepared by dissolving requisite amount in methanol. Further, working solutions (100.0 ng/mL) of each drug were prepared in the mobile phase, spiked in plasma, and analyzed under the same conditions at LQC and HQC levels. The MRM in the positive ionization mode were monitored at *m/z* 152/110 for paracetamol, *m/z* 275/230 for chlorpheniramine, and *m/z* 195/138 for caffeine. The response of acetylsalicylic acid (179/137) and ibuprofen (205/159) were insignificant in the positive mode as they give much higher response in the negative mode.

The linearity of the method was determined by analysis of five calibration curves containing eight non-zero concentrations. The area ratio response for naratriptan–IS obtained from multiple reaction monitoring was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression which was finalized during pre-method validation. A correlation coefficient (r^2) value > 0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was accepted as the LLOQ, if the analyte response was at least five times more than that of drug free (blank) extracted plasma. In addition, the analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with a precision (% CV) not greater than 20% and accuracy within 80–120%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ± 15%.

For determining the intra-batch accuracy and precision, replicate analysis of plasma samples of naratriptan was performed on the same day. The run consisted of a calibration curve and five replicates of LLOQ QC, LQC, MQC, and HQC samples. The inter-assay accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive validation days. The deviation at each concentration level from the nominal concentration was expected to be within ± 15% except LLOQ, for which it should be within ± 20%. Similarly, the mean accuracy should not deviate by ± 15% except for the LLOQ where it can be ± 20% of the nominal concentration.

Matrix ion suppression effects on the MRM LC–MS–MS sensitivity were evaluated by the post column analytes infusion experiment (13). A standard solution containing 100 ng/mL of naratriptan in water was infused post column via a "T" connector into the mobile phase at 10 µL/min employing in-built infusion pump. Aliquots of 5 µL of extracted control plasma were then injected into the column by the autosampler and MRM LC–MS–MS chromatograms were acquired for naratriptan. Any dip in the baseline upon injection of extracted blank plasma (without IS and analyte) would indicate ion suppression, while a peak at the retention time of naratriptan and IS indicates ion enhancement.

The relative recovery, matrix effect, and process efficiency were assessed as recommended by Matuszewski et al. (14). All three parameters were evaluated at HQC, MQC, and LQC levels in six replicates. Relative recovery (RE) was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC level. The recovery of IS was similarly estimated. Absolute matrix effect (ME) was assessed by comparing the mean area response of unextracted samples (spiked after extraction) with mean area of neat standard solutions (in mobile phase). The overall "process efficiency" (%PE) was calculated as (ME × RE)/100. Further, the effect of plasma matrix (relative matrix effect) on analyte quantitation was also checked in six different batches/lots of plasma. From each batch, six samples at LLOQ level were prepared (spiked after extraction) and checked for the % accuracy and precision (% CV) values. The deviation of the standards should not be more than ± 15% and at least 90% of the lots at each QC level should be within the aforementioned criteria.

All stability results were evaluated by measuring the area response (naratriptan–IS) of stability samples against freshly prepared comparison standards with identical concentration. Stock solutions of naratriptan and IS were checked for short term stability at room temperature and long term stability at 2–8°C. The solutions were considered stable if the deviation from nominal value was within ± 10.0%. Autosampler stability (wet extract), dry extract, bench top (at room temperature), and freeze-thaw stability were performed at LQC and HQC using three replicates at each level. Freeze-thaw stability was evaluated by successive cycles of freezing (at –20°C and –70°C) and thawing (without warming) at room temperature. Long term stability of spiked plasma samples stored at –20°C and –70°C was studied at both these levels. The samples were considered stable if the % accuracy of LQC and HQC were in the range of 85.00–115.00%.

To authenticate the ruggedness of the proposed method, it was performed on three precision and accuracy batches. The first batch was used for re-injection reproducibility; second batch was analyzed by two different analysts, and the third batch was studied on two different columns. Dilution integrity experiment was evaluated by diluting the stock solution prepared as spiked standard at 49265 pg/mL naratriptan concentration in the screened plasma. The precision and accuracy for dilution integrity standards at 1/5th (9853 pg/mL) and 1/10th (4926.5 pg/mL) dilution were determined by analyzing the samples against calibration curve standards.

Bioequivalence study design

The design of the study comprised "An open label, balanced, randomized, two treatment, two period, two sequence, single dose, crossover oral bioequivalence study of test formulation of

naratriptan dihydrochloride (2.5 mg tablets, Indian Healthcare Company) and a reference formulation (Amerge 2.5 mg tablets from GlaxoSmithKline, Philadelphia, PA) in 31 healthy adult human subjects under fasting conditions". Each subject was judged to be in good health through medical history, physical examination and routine laboratory tests. Written consent was taken from all the subjects after informing them about the objectives and possible risks involved in the study. An independent ethics committee constituted as per Indian Council of Medical Research (ICMR) approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA (15). The subjects were orally administered a single dose of test and reference formulation after recommended wash out period of 7 days with 240mL of drinking water. Blood samples were collected at 0.0 (pre-dose), 0.33, 0.67, 1.0, 1.33, 1.67, 2.0, 2.33, 2.67, 3.0, 3.33, 3.67, 4.0, 4.5, 5.0, 6.0, 8.0, 12.0, 18.0, 24.0, and 30.0 h after oral administration of test and reference formulation in labeled K3 EDTA-vacuettes. The maximum volume of blood withdrawn during the entire study did not exceed 250 mL for each subject, which included (other than for measurement) up to 10 mL for screening, about 10 mL for post study safety assessment (hematology and biochemical tests) and ~0.5 mL of heparinised blood was discarded prior to each sampling through venous cannula. A 5 mL blood sample was collected at each time point. Plasma was separated by centrifugation and kept frozen at –20°C till the completion of period and then transferred at –70°C until analysis. During the study, subjects had a standard diet and water intake was free. An incurred sample re-analysis (assay reproducibility test) was also conducted by computerized random selection of 20 subject samples. The selection criteria included samples which were near the C_{max} and the elimination phase in the pharmacokinetic profile of the drug. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the value should not be more than ± 20% (16).

Results and Discussion

Method development

To develop a rapid, rugged, and precise method, it was important to optimize the chromatographic and MS conditions, as well as to have an efficient and simple extraction procedure for naratriptan. The inherent selectivity of MS–MS detection was also expected to be beneficial in developing a selective and sensitive method. The present study was conducted using electrospray ionization (ESI) for LC–MS–MS analyses. As naratriptan and sumatriptan have secondary amine groups that may be protonated in solution under the experimental conditions, the intensity found was much higher in the positive mode compared to the negative mode. The Q1 MS full scan spectra was predominant with protonated precursor ions at *m/z* 336.10 and 296.09 for naratriptan and sumatriptan (IS) respectively. Protonation of naratriptan can occur at two different sites, one on the nitrogen of the sulfonamide side chain and the other on nitrogen of piperidine ring (11). The former gives rise to a product ion at *m/z* 241.01 due to alpha cleavage and the latter yields a major fragment at *m/z* 98.06, which undergoes a retro Diels-Alder reaction to form *m/z* 70 in the Q3 MS. The most stable and consistent product ion for sumatriptan was at *m/z* 251.06 due to the elimination of dimethyl amine group. The other peaks at *m/z* 201.09 and *m/z* 157 were assigned to the cleavage of CH_3NHSO_2H, followed by dimethyl amine group respectively from the protonated precursor ion. By suitable optimization of compound dependent and source dependent parameters, the most consistent and abundant product ions were obtained at *m/z* 98.06 and 251.06 for naratriptan and IS respectively. A dwell time of 100 ms for naratriptan and IS was adequate and no cross talk was observed between their MRMs.

In this effort to develop a simple and rapid method with adequate sensitivity, all three procedures [protein precipitation (PPT), liquid–liquid (LLE), and solid phase (SPE)] were tried during method development. Previous two reports have employed SPE for naratriptan and other triptans from rabbit plasma (10) and human serum (11). Thus, SPE was tried with MCX and HLB cartridges, which gave quantitative recovery with good peak shapes for both the drugs, but showed non linear behavior for highest calibration standard on calibration curve. Protein precipitation was then tested with solvents like acetonitrile and methanol in acidic/alkaline medium. However, specificity and peak shapes were significantly affected with frequent clogging of the column. Finally, reproducibility and recovery data supported liquid–liquid extraction to be used as extraction technique for naratriptan from human plasma. LLE was tested to isolate the drug from plasma using diethyl ether, methyl-*tert*-butyl ether, dichloromethane, *n*-hexane, and ethyl acetate individually as extraction solvents. The recovery obtained in all these solvents was inconsistent with some ion suppression (greater than 15% CV). Addition of strong base like sodium hydroxide helped in maintaining the analyte and IS in the neutral form for efficient extraction in methyl-*tert*-butyl ether and dichloromethane (80:20, v/v). Quantitative and precise recovery was obtained in this solvent system for both the drugs with minimum matrix interference.

For efficient chromatographic separation and peak shape, no. of trials were conducted by changing the mobile phase composition, pH, acid additives, injection volume (2–10 µL) and flow rate (0.2–0.6 mL/min). Dulery et al. (10) have used Nova-Pak C8/C18 (150 mm × 2 mm, 4 µm particle size) to separate naratriptan and sumatriptan under gradient conditions. In an another report, Vishwanathan and coworkers (11) have used an Alltech Solvent Miser Silica (150 mm × 2.1 mm, 5 µm particle size) column to separate naratriptan, sumatriptan, rizatriptan, and zolmitriptan in a chromatographic run time of 5 min. Isocratic elution of all four drugs was possible with 20 mM ammonium acetate (pH 2.7, adjusted with glacial acetic acid and formic acid)–methanol–acetonitrile (80:10:10, v/v/v) solvent system. To evaluate the analytical potential of different columns for fast chromatographic separation, four different reversed-phase columns were tested namely, Chromolith RP-18 (monolithic silica column, 100 × 4.6 mm, 5 µm, surface area 300 m^2/g, pore size 130 Å), Kromasil (50 and 100 × 4.6 mm, 5 µm, surface area 340 m^2/g, pore size 100 Å), Gemini C-18 (50 × 4.6 mm, 5 µm, surface area 375 m^2/g, pore size 110Å) and ACE C18 (50 × 4.6 / 2.1 mm,

5 µm, surface area 300 m^2/g, pore size 100 Å). Separation was tried using various combinations of methanol–acetonitrile, acidic buffers (acetic acid-ammonium acetate and formic acid-ammonium formate) and additives like formic acid and acetic acid on these columns to find the optimal mobile phase that produced the best sensitivity, efficiency, and peak shape. Though separation was possible in all the cases, superior peak shape and reproducibility was achieved on ACE C18 (50 × 2.1 mm, 5 µm) compared to other columns with 0.1% acetic acid and acetonitrile (15:85, v/v) as the mobile phase. The optimum injection volume was 5 µL, and the flow rate was 0.4 mL/min. Both the drugs were eluted in a run time of 1.5 min, with retention time of 1.04 and 0.91 min for naratriptan and sumatriptan (IS) respectively.

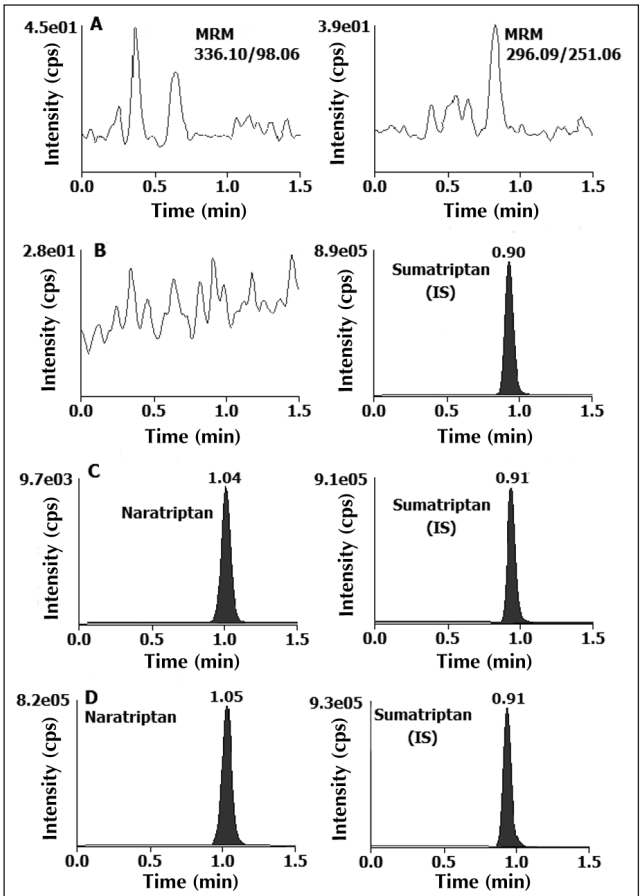

Figure 2. MRM ion-chromatograms of (A) extracted blank plasma (without IS and analyte), (B) blank plasma with sumatriptan (IS), (C) naratriptan at LLOQ (*m/z* 336/10 → 98.06) and IS, (D) real subject sample at 2.7 h after administration of 2.5 mg single dose of naratriptan.

Sumatriptan, which also belongs to the same class of antimigraine drugs was selected as the IS in the present study. Both the drugs have similar structures and properties and were easily extracted with methyl-*tert*-butyl ether and dichloromethane (80:20, v/v). There was no effect of sumatriptan on the recovery, sensitivity or ion suppression of naratriptan.

Assay performance and validation

Throughout the method validation, the % CV of system suitability test for naratriptan and IS was observed in the range of 0.00–0.50% and 0.62–1.67% for their retention time and the response respectively, which is not more than 2% as per the acceptance criteria. The signal to noise ratio for system performance was ≥ 200 for naratriptan and IS.

Carry-over evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. Negligible enhancement (< 0.1%) in the response was found in double blank after subsequent injection of highest calibration standard (aqueous and extracted) at the retention time of naratriptan and IS, respectively. Moreover, no ghost peaks appeared during the analysis of blank samples.

The calibration curves were linear over the concentration range of 103–20690 pg/mL. The best linear fit and least squares residuals for the calibration curve were achieved with a $1/x^2$ weighing factor, giving a mean linear regression equation $y = 0.0023x + 0.00013$ for the calibration curves, where y is the peak area ratio of the analyte to the IS and x the concentration of the analyte. The mean and standard deviation values for slope, intercept and correlation coefficient (r) observed was 0.00013 and 0.00001; 0.0023 and 0.00063; and 0.9992 and 0.0002, respectively. The lowest concentration (LLOQ) in the standard curve that can be measured with acceptable accuracy and precision was found to be 103 pg/mL in plasma at a signal-to-noise ratio (S/N) of ≥ 100.

The selectivity of the method was established with individual plasma samples for interfering matrix components. Representative MRM ion chromatograms in Figure 2 of extracted blank plasma (without IS and analyte), blank plasma fortified with IS (*m/z* 296.09 → 251.06), naratriptan at LLOQ (*m/z* 336.10 → 98.06) and an actual subject sample (after 2.7 h of dosage) demonstrates the selectivity of the method. The extraction procedure together with mass detection gave very good selectivity for the analysis of naratriptan and IS in the blank plasma. No endogenous interferences were found at the retention times of naratriptan (1.04 min) and IS (0.91 min) in the blank plasma. The area observed at the retention time of naratriptan at LLOQ and IS was less than 1%. None of the commonly used medications by human volunteers showed interfering signals at the retention time of naratriptan or IS.

The intra- and inter-batch precision and accuracy were established from validation runs performed at HQC, MQC, LQC, and LLOQ QC levels (Table I). The intra-batch precision ranged from 0.9 to 4.0% and the

Table I. Intra- and Inter-batch Precision and Accuracy for Naratriptan

		Intra-batch				Inter-batch			
QC ID	Nominal conc. (ng/mL)	*n**	Mean conc. observed (pg/mL)†	% CV	% Accuracy	*n**	Mean conc. observed (pg/mL)§	% CV‡	% Accuracy
HQC	18639	5	17622	0.9	94.5	25	17529	2.0	94.0
MQC	1789	5	1776	1.8	99.3	25	1750	3.9	97.8
LQC	304	5	305	4.0	100.3	25	290	4.8	95.4
LLOQ QC	106.5	5	112	2.4	105.2	25	111	6.1	104.2

* *n* = total number of observations.
† Mean of 5 replicates at each concentration.
‡ CV = coefficient of variance.
§ Mean of 5 replicates for five precision and accuracy batches

Table II. Absolute Matrix Effect, Relative Recovery, and Process Efficiency for Naratriptan

QC Level	A* (% CV†)	B‡ (% CV)	C§ (% CV)	% ME**		% RE††		% PE‡‡	
				Analyte	IS	Analyte	IS	Analyte	IS
LQC	2158 (0.8)	2115 (4.9)	2083 (2.8)	98.0	89.2	98.5	103.9	96.5	92.7
MQC	13013 (0.3)	12879 (0.8)	12558 (1.8)	98.9	99.2	97.5	93.8	96.4	93.1
HQC	131886 (2.6)	129621 (2.0)	125404 (4.4)	98.3	93.3	96.7	102.0	95.1	95.2

* Mean area ratio (analyte/IS) response of six replicate samples prepared in mobile phase (neat samples)
† Coefficient of variation
‡ Mean area ratio (analyte/IS) response of six replicate samples prepared by spiking in extracted blank plasma
§ Mean area ratio (analyte/internal standard) response of six replicate samples prepared by spiking before extraction
** B/A × 100 †† C/B × 100 ‡‡ C/A × 100 = (ME × RE)/100

Table III. Stability of Naratriptan under Different Conditions (n = 3)

Storage condition	Nominal Conc. (pg/mL)	Calculated conc. (pg/mL) Mean, stability samples ± SD	% Change*
Bench Top Stability; 6 h			
HQC	18639	17879 ± 305	–4.1
LQC	304	299 ± 14.6	–1.6
Wet Extract Stability; 22 h			
HQC	18639	17101 ± 359	–8.3
LQC	304	302 ± 2.1	–0.6
Dry Extract Stability; 24 h			
HQC	18639	17562 ± 116	–5.8
LQC	304	294 ± 9.0	–3.3
Freeze/Thaw Stability; 3 Cycles, –20°C			
HQC	18639	18851 ± 557	1.0
LQC	304	313 ± 26.6	2.9
Freeze/Thaw Stability; 3 Cycles, –70°C			
HQC	18639	18386 ±169	–1.4
LQC	304	309 ± 9.0	1.6
Long Term Matrix Stability; 82 days, –20°C			
HQC	18639	17465 ± 278	-6.3
LQC	304	291 ± 2.1	–4.3
Long Term Matrix Stability; 82 days, –70°C			
HQC	18639	17732 ± 260	–4.8
LQC	304	299 ± 2.0	–1.6

$$\text{\% Change} = \frac{\text{Mean stability samples} - \text{Mean comparison samples/Nominal conc.}}{\text{Mean comparison samples}} \times 100$$

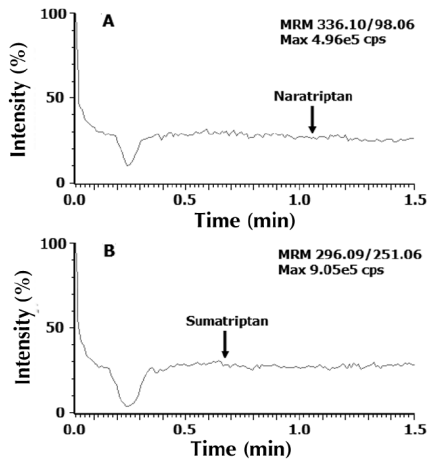

Figure 3. Post column analyte infusion experiment for (A) naratriptan and (B) sumatriptan, IS.

accuracy was within 94.5–105.2%. For the inter-batch experiments, the precision ranged from 2.0% to 6.1% and the accuracy was within 94.0–104.2%.

Matrix effect may be defined as a composite of some undesirable effects that originate from a biological matrix. Results of post-column infusion experiment in Figure 3 indicate no ion suppression or enhancement at the retention time of naratriptan and IS. However, considerable ion suppression (> 15%) was observed at 0.25 min, which did not affect the quantitation of naratriptan in subsequent measurements. The relative recovery, absolute matrix effect and process efficiency data for naratriptan and IS at LQC, MQC, and HQC levels is presented in Table II. The result for 'relative' matrix effect, which compares the precision (% CV) values between different lots (sources) of plasma (spiked after extraction) samples, was ≤ 2.2% which indicates absence of matrix effect. The average matrix factor value calculated as the response of post spiked sample/response of neat solutions in mobile phase at the LLOQ levels was 0.98, which indicates a minor suppression of 2%. The overall mean extraction efficiency for naratriptan and IS at the three quality control levels was 96% and 93.6%, respectively. The recovery for both drug and IS was consistent, precise, and reproducible.

The stability of naratriptan and IS in human plasma and stock solutions was examined under different storage conditions. Samples for short-term stability remained unchanged upto 6 h, and the drug and IS stock solutions were stable for 82 days at refrigerated temperature below 8°C. Naratriptan in control human plasma (bench top) at room temperature was stable at least for 6 h at 25°C and for minimum of three freeze and thaw cycles at –20°C and –70°C. Spiked plasma samples stored at –20°C and –70°C for long term stability experiment were found stable for a minimum period of 82 days. Dry extract stability of the spiked quality control samples stored at –20°C was determined up to 24 h. Autosampler (dry extract) stability of the spiked quality control samples maintained at 5°C was determined up to 22 h without significant drug loss. Different stability experiments in plasma at two QC levels, with the values for precision and percent change are shown in Table III.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration (above the upper limit of quantitation ULOQ), which may be encountered during real subject sample analysis. The precision for dilution integrity of 1/5 and 1/10th dilution were 3.1% and 1.2%, and the accuracy results were 103.3 and 94.3% respectively which is within the acceptance limit of 15% for precision (% CV) and 85.0–115.0% for accuracy.

Method ruggedness results for re-injection reproducibility ranged from 2.2 to 4.5% for precision and 92.8% to 103.6% for accuracy at LLOQ, LQC, MQC, and HQC levels. The precision (% CV) and accuracy values for two different columns ranged from 1.8% to 3.1% and 94.4% to 101.2%, respectively, at these quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 2.3–5.4% and 94.5–103.5% respectively.

Application to a pharmacokinetic/bioequivalence study

The validated method has been successfully used to quantify naratriptan concentration in the human plasma samples after the administration of a single 2.5 mg oral dose of naratriptan. Figure 4 shows the plasma concentration vs. time profile of naratriptan in human subjects under fasting condition. The method was sensitive enough to monitor the naratriptan plasma concentration up to 30 h. In all approximately 1500 samples including the calibration, QC, and volunteer samples were run and analyzed during a period of 5 days, and the precision and accuracy were well within the acceptable limits. The mean pharmacokinetic parameters obtained for the test and reference formulation are presented in Table IV. The 90% confidence interval of individual ratio geometric mean for test/reference was within 80–125% for AUC_{0-t}, AUC_{0-inf}, and C_{max}. The % change in the randomly selected subject samples for assay reproducibility (incurred sample re-analysis) was less than 8.0%. This authenticates the reproducibility and ruggedness of the proposed method. Further, there was no adverse event during the course of the study.

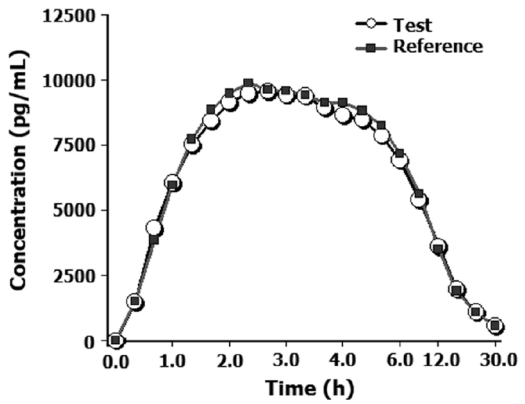

Figure 4. Mean plasma concentration-time profile of naratriptan after oral administration of 2.5 mg (test and reference) tablet formulation to 31 healthy volunteers.

Table IV. Mean Pharmacokinetic Parameters Following Oral Administration of 2.5 mg Tablet Formulation (Test and Reference) of Naratriptan in 31 Healthy Human Subjects

Parameter	Test Mean ± SD	Reference Mean ± SD
C_{max} (pg/mL)	10449 ± 2113	10342 ± 2605
T_{max} (h)	2.7 ± 1.8	2.8 ± 1.4
$t_{1/2}$ (h)	6.4 ± 0.8	6.5 ± 1.2
$AUC_{0-30\,h}$ (h.pg/mL)	101807 ± 21513	99493 ± 22217
AUC_{0-inf} (h.pg/mL)	106279 ± 23309	104422 ± 24509

C_{max} = maximum plasma concentration.
T_{max} = time point of maximum plasma concentration.
$t_{1/2}$ = half life of drug elimination during the terminal phase.
AUC_{0-t} = area under the plasma concentration-time curve from zero h to 30 h.
AUC_{0-inf} = area under the plasma concentration-time curve from zero h to infinity.

Conclusions

To summarize, the LC–ESI-MS–MS method for the quantitation of naratriptan in human plasma was developed and fully validated as per USFDA guidelines. The method offers significant advantages over those previously reported (10,11), in terms of lower sample requirement for processing (100 µL), simplicity of liquid–liquid extraction procedure, sensitivity (103 pg/mL) and overall analysis time. The efficiency of liquid–liquid extraction and a chromatographic run time of 1.5 min per sample make it an attractive procedure in high-throughput bioanalysis of naratriptan. Also, the on-column loading of sample at LLOQ level (0.52 pg/injection) was much lower compared to other reported procedures. The current method has shown acceptable precision, accuracy and adequate sensitivity for the quantitation of naratriptan in human plasma in a clinical study.

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