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Challenges in the simultaneous quantitation of sumatriptan and naproxen in human plasma: Application to a bioequivalence study

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ABSTRACT

An ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method has been developed for the simultaneous determination of sumatriptan and naproxen in human plasma using naratriptan and indomethacin as the internal standards (ISs). The plasma samples were prepared by solid phase extraction on Phenomenex Strata-X cartridges using 100 μL human plasma sample. Chromatography was carried out on Waters Acquity UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm) analytical column under isocratic conditions using a mobile phase consisting of methanol–acetonitrile–4.0 mM ammonium acetate (70:10:20, v/v/v). The precursor → product ion transition for both the analytes and ISs was monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring and positive ionization mode. The method was validated over a wide dynamic concentration range of 0.050–100 ng/mL for sumatriptan and 0.050–100 μg/mL for naproxen. Matrix effect was assessed by post-column analyte infusion and the extraction recovery was >95.0% across four quality control levels for both the analytes. Stability was evaluated under different conditions including bench top, processed sample, freeze and thaw and long term. The method was applied to support a bioequivalence study of 85 mg sumatriptan + 500 mg naproxen sodium fixed dose formulation in 28 healthy Indian subjects. Assay reproducibility was demonstrated by reanalysis of 123 incurred samples.

1. Introduction

Migraine is a common syndrome that affects a sizeable portion of the world's population and its frequency of occurrence is about three times more in women than men [1,2]. Acute migraine is defined as spontaneous, short, recurring, moderate-to-severe attacks of unilateral throbbing headache, associated with nausea, vomiting, anorexia, phonophobia and photophobia [3]. Triptans, including sumatriptan, are a group of tryptamine-based drugs used in the acute treatment of migraine headaches. They work in the early stages of migraine by acting as serotonin receptors (5-HT) 1B and 1D. Triptans block vasoconstriction and transmission of signals to the trigeminal nucleus and thus prevent peripheral sensitization [4]. Sumatriptan [SUM,1-[3-(2-dimethylaminoethyl)-1H-indol-5-yl]-*N*-methyl-methanesulfonamide] was the first antimigraine agent approved by US FDA in 1991 for the treatment and management of acute migraine cases. SUM is available commercially in oral and subcutaneous forms, as a nasal spray. It is rapidly absorbed after oral or subcutaneous administration and is mainly distributed in tissues. It has poor oral bioavailability (14%), primarily due to extensive hepatic first-pass metabolism and partly due to incomplete absorption. The volume of distribution of SUM is 2.4 L/kg and is 10–21% protein bound [4,5].

Naproxen [NAP, (+)-(*S*)-2-(6-methoxynaphthalen-2-yl) propanoic acid] is a non-steroidal anti-inflammatory drug (NSAID) possessing analgesic, anti-inflammatory and antipyretic properties by decreasing the formation of prostaglandin precursors [6]. It works for a variety of pain and inflammatory syndromes, including migraine. In the treatment of migraine, it helps to relieve the pain through the analgesic property while the anti-inflammatory effect decreases the neurogenic inflammation in the trigeminal ganglion [4]. NAP is highly protein bound (~99%), mainly to albumin with a volume distribution of 0.16 L/kg. It is rapidly and completely absorbed from the gastrointestinal tract with an in vivo bioavailability of 95%. It is metabolized in the liver and its metabolites are eliminated primarily in the urine (~95%), with a half life of up to 12 h. Due to the complimentary mode of action of SUM and NAP, the combined use of these drugs can offer more favorable clinical benefits than either drug alone in acute migraine therapy. The fixed dose combination tablet TREXIMET® from GlaxoSmithKline, which contains 85 mg sumatriptan (as succinate

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salt), and 500 mg naproxen (as sodium salt) has shown greater efficacy compared to their individual use due to a synergistic effect [7,8].

Several methods are reported for the determination of SUM in biological matrices by HPLC with fluorescence [9,10], electrochemical [11–13], ultraviolet detection [14,15] and LC–MS/MS [16–22]. In majority of these published methods, the sensitivity was ≥1 ng/mL [9–12,16,20,22] with high volume of biological fluid (≥0.5 mL) for sample processing [9–13,16,17,19–21]. Tan et al. [22] have developed an evaporation free solid phase extraction (SPE)-LC–MS/MS method for rapid (3.0 min) determination of SUM in human plasma. A critical comparison of different analytical methods for identification and determination of several triptans including SUM has been reviewed by Saka [23]. Like SUM, there are numerous methods for the determination of NAP in biological fluids by a variety of analytical techniques like capillary electrophoresis [24], flow injection chemiluminescence [25], room temperature liquid phosphorimetry [26], electrokinetic capillary chromatography [27], fluorescence spectrometry [28–32], GC–MS [33], HPLC with chemiluminescence [34], fluorescence [33,35–38] and UV detection [33,36,39–46] and LC–MS/MS [46–48]. NAP has been determined in human plasma and dried blood spots by LC–MS/MS for pharmacokinetic comparison in the concentration range of 0.5–100 μg/mL [47]. Recently, a sensitive LC–MS/MS method (0.1 μg/mL) has been described for the determination of NAP in human plasma and gives a comprehensive account of various published methods for quantification NAP from different matrices [48]. Simultaneous determination of SUM and NAP in human plasma has been a subject of very few reports. Berges et al. [49] have studied the pharmacokinetics and tolerability of fixed-dose combination tablet of SUM and NAP (85/500 mg) in healthy volunteers. They used two different extraction procedures (SPE for SUM and protein precipitation for NAP) for sample preparation using 100 and 50 μL plasma respectively, followed by LC–MS/MS analysis. The linear range was validated from 0.1 to 100 ng/mL for SUM and 0.1 to 100 μg/mL for NAP. To the best of our knowledge there are no reports on the use of UPLC–MS/MS for their simultaneous determination in human plasma. UPLC with 1.7 μm particle size has significantly improved resolution, with reduced run time and improved sensitivity for the analyses of many compound types.

Thus, in the present work a highly sensitive, selective and rapid UPLC–MS/MS method has been developed and fully validated as per the US FDA guidelines for simultaneous measurement of SUM and NAP in subject samples. The method offers a wide dynamic concentration range, small turnaround time for analysis and utilizes only 100 μL human plasma for sample processing using solid phase extraction. Interference due to matrix was ascertained by post column infusion technique and was successfully applied to a bioequivalence study of fixed dose combination of SUM and NAP (80 + 500 mg) tablet formulation in 28 healthy Indian male subjects under fasting. The reproducibility in the measurement of study data has been demonstrated by reanalysis of incurred samples.

2. Experimental

2.1. Chemicals and materials

Reference standard of sumatriptan (SUM, 99.90%), naproxen (NAP, 99.78%), naratriptan (NARA, IS for SUM, 99.93%) and indomethacin (INDO, IS for NAP, 99.82%) were procured from Nosch Labs Pvt. Limited (Hyderabad, India), Divis Laboratories Limited (Hyderabad, India), Vivan Life Sciences Pvt. Limited (Mumbai, India) and Lotus International Pvt. Limited (Mumbai, India) respectively. HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Baker, S.A.de C.V. (Estado de Mexico, Mexico). Bio ultra grade ammonium acetate was purchased from Sigma–Aldrich (St. Louis, MO, USA) and *ortho*-phosphoric acid (*o*-PA, 88%) was from Merck Specialties Pvt. Ltd. (Mumbai, India). Solid phase extraction cartridges Phenomenex Strata™-X (30 mg, 1 cc) were obtained from Phenomenex India (Hyderabad, India). Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at −20 °C until use.

2.2. Liquid chromatographic and mass spectrometric conditions

A Waters Acquity UPLC system (MA, USA) was used for setting the reverse-phase liquid chromatographic conditions. The analysis of both the analytes and their ISs was performed on a Waters Acquity UPLC type BEH C18 (50 mm × 2.1 mm, 1.7 μm) analytical column and maintained at 30 °C in a column oven. The mobile phase consisted of methanol–acetonitrile–4.0 mM ammonium acetate (70:10:20, v/v/v) and was delivered at a flow rate of 0.25 mL/min. The sample manager temperature was maintained at 5 °C and the pressure of the system was 5800 psi. Ionization and detection of the analytes and ISs was carried out on a Waters Quattro Premier XE (USA) triple quadrupole mass spectrometer, equipped with electrospray ionization and operating in positive ionization mode. The source dependent parameters maintained for SUM, NAP and ISs were, cone gas flow: 100 ± 10 L/h; desolvation gas flow: 800 L/h; capillary voltage: 2.0 kV, source temperature: 110 °C; desolvation temperature: 400 °C; extractor volts: 5.0 V. The pressure of argon used as collision activation dissociation gas was 0.141 Pa. The optimum values for compound dependent parameters like cone voltage and collision energy were set at 30 V and 21 eV for SUM, 35 and 17 eV for NAP, 41 V and 25 eV for NARA and 28 and 24 eV for INDO respectively. Quadrupoles 1 and 3 were maintained at unit mass resolution and the dwell time was set at 100 ms. MassLynx software version 4.1 was used to control all parameters of UPLC and MS.

2.3. Preparation of standard stock, calibration standards and quality control samples

The standard stock solutions of SUM (200 μg/mL) and NAP (10.0 mg/mL) were prepared by dissolving their requisite amounts in methanol. Further, an intermediate solutions of SUM (20.0 μg/mL and 0.50 μg/mL) and NAP (5.0 mg/mL and 1.0 mg/mL) were prepared in methanol:water (50:50, v/v) respectively. Calibration standards and quality control (QC) samples were prepared by spiking (2% of total plasma volume) blank plasma with working solutions prepared from intermediate solutions for both the analytes. Calibration curve standards were made at the following concentrations 0.05, 0.10, 0.30, 1.00, 2.00, 4.00, 10.0, 20.0, 50.0, and 100 ng/mL for sumatriptan and for identical values in μg/mL for NAP respectively. The quality control samples were prepared at five levels, viz. 80.0 ng/mL (HQC, high quality control), 40.0/2.40 ng/mL (MQC-1/2, medium quality control), 0.15 ng/mL (LQC, low quality control) and 0.05 ng/mL (LLOQ QC, lower limit of quantification quality control) for SUM and for identical values in μg/mL for NAP respectively. Separate stock solutions (200 μg/mL) of the internal standards were prepared by dissolving 2.0 mg of reference standards in 10.0 mL of methanol. Their combined working solution (0.200 μg/mL for NARA and 1.000 μg/mL INDO) was prepared from their stock solutions in methanol:water (50:50, v/v). All the solutions (standard stock, calibration standards and quality control samples) were stored at 2–8 °C until use.

2.4. Protocol for sample preparation

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, 40 μL of internal standard was added and vortexed for 10 s. Further, 100 μL of 3% (v/v) *o*-PA was added and vortexed for another 10 s. Samples were then centrifuged at 13,148 × *g* for 5 min at 10 °C and thereafter loaded on Phenomenex Strata-X (30 mg, 1 cc) cartridges, after conditioning with 1 mL methanol followed by 1 mL of water. Washing of samples was done with 1 mL 4.0 mM ammonium acetate in water followed by 1 mL of 5% methanol in water. Subsequently, the cartridges were dried for 1 min by applying nitrogen (1.72×10^5 Pa) at 2.4 L/min flow rate. Elution of analytes and ISs was done using 300 μL of mobile phase into pre-labeled vials, briefly vortexed for 15 s and 10 μL was used for injection in the chromatographic system.

2.5. Procedures for method validation

The method validation was performed as per the USFDA guidelines [50]. System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of analytes and ISs at the start of each batch during method validation. System performance was studied by injecting one extracted blank (without analytes and ISs) and one extracted LLOQ sample with ISs at the beginning of each analytical batch and before re-injecting any sample during method validation. The carryover effect of the auto-sampler was evaluated by sequentially injecting extracted blank plasma → ULOQ sample → extracted blank plasma → LLOQ sample → extracted blank plasma at the start and end of each batch.

The selectivity of the method toward endogenous plasma matrix components was assessed in eight lots (6 normal of Na-heparin plasma, 1 hemolyzed, and 1 lipemic) of blank human plasma. The selectivity of the method toward commonly used medications by human volunteers was also ascertained. This included paracetamol, chlorpheniramine maleate, diclofenac, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (100 μg/mL) were prepared by dissolving requisite amount in methanol. Further, working solutions (1.0 μg/mL) were prepared in the mobile phase, spiked in plasma and analyzed under the same conditions at LQC and HQC levels in triplicate to check for any possible interference at the retention time of analytes and ISs. The cross talk of MRM for analytes and ISs was checked using highest standard on calibration curve and working solution of ISs.

The linearity of the method was determined by analysis of five linearity curves containing ten non-zero concentrations. The area ratio response for analyte/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. The lowest standard on the calibration curve was accepted as LLOQ, if the analyte response was at least ten times more than that of drug free (blank) extracted plasma.

For determining the intra-day accuracy and precision, replicate analysis of plasma samples was performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ, LQC, MQC-1/2 and HQC samples. The inter-day accuracy and precision were assessed by analysis of five precision and accuracy batches on three consecutive validation days. The precision (% CV) at each concentration level from the nominal concentration should not be greater than 15%. Similarly, the mean accuracy should be within 85–115%, except for the LLOQ where it can be 80–120% of the nominal concentration.

Ion suppression/enhancement effects on the MRM UPLC–MS/MS sensitivity were evaluated by post column analyte infusion experiment [51]. A standard solution containing SUM and NAP (at MQC level) and ISs was infused post column into the mobile phase at 10 μL/min employing infusion pump. Aliquots of 10 μL of extracted control plasma were then injected into the column by the autosampler and MRM UPLC–MS/MS chromatogram was acquired for the analytes and ISs. The relative recovery, process efficiency and matrix effect were assessed as reported previously [52]. All three parameters were evaluated at HQC, MQC-1/2 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. The overall 'process efficiency' (% PE) was calculated as (ME × RE)/100. To evaluate the relative matrix effect, calibration lines from eight plasma lots (including hemolyzed and lipemic) were constructed and the precision (% CV) values for slopes were calculated. For a method to be practically free from relative matrix effect the % CV should not exceed 3–4% [53].

All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against freshly prepared comparison standards with identical concentration. Stock solutions of analytes and ISs 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, bench top (at room temperature), and freeze–thaw stability were performed at LQC and HQC using six replicates at each level. Long term stability of spiked plasma samples stored at −20 °C and −70 °C was also studied at both these levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15.0%.

To authenticate the ruggedness of the proposed method, it was done on two precision and accuracy batches. The first batch was analyzed on two different columns (same make but different batch no.), while the second batch was analyzed by two different analysts who were not part of method validation. Dilution integrity experiment was conducted by diluting the stock solution prepared as spiked standard at 2 times ULOQ concentration (200 ng/mL) for sumatriptan and for identical value in μg/mL for NAP respectively with screened blank human plasma. The precision and accuracy for dilution integrity standards at 1/5th and 1/10th dilutions for SUM and NAP were determined by analyzing the samples against freshly prepared calibration curve standards.

2.6. Bioequivalence study design and incurred sample reanalysis

The bioequivalence study was conducted with a single fixed dose of a test (85 mg sumatriptan + 500 mg naproxen sodium tablets from a Generic Company) and a reference (TREXIMET®, 85 mg sumatriptan (as succinate) + 500 mg naproxen sodium tablets) (GlaxoSmithKline, Research Triangle Park, NC 27709) formulation to 28 healthy adult Indian 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. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization, E6 Good Clinical Practice [54]. The subjects were orally administered a single dose of test and reference formulations after recommended wash out period of 7 days with 200 mL of water. Blood samples were collected at

0.00 (pre-dose), 0.33, 0.67, 1.00, 1.33, 1.67, 2.00, 2.50, 3.00, 3.50, 4.00, 4.33, 4.67, 5.00, 5.33, 5.67, 6.00, 6.50, 7.00, 8.00, 10.0, 12.0, 24.0, 48.0, 72.0 and 96.0 h after oral administration of test and reference formulation. Plasma was separated by centrifugation and kept frozen at −70 °C until analysis. During study, subjects had a standard diet while water intake was unmonitored. The pharmacokinetic parameters of sumatriptan and naproxen were estimated by non-compartmental model using WinNonlin software version 5.2.1 (Pharsight Corporation, Sunnyvale, CA, USA).

An incurred sample re-analysis (assay reproducibility test) was also conducted by random selection of 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 values should not be more than ±20% [55].

3. Results and discussion

3.1. Method development

Mass spectrometry: As both the analytes have a very different structure and ionization behavior, the tuning of mass parameters was done using ESI in positive as well as negative ionizations modes. SUM and its internal standard, NARA have been studied previously in positive ionization mode as they have secondary amino groups which can be readily protonated [56]. Although, NAP and INDO can be easily ionized in the negative ionization mode due to the presence of carboxylic acid group, it was observed that both were readily ionized in the negative as well positive ionization modes [57]. Moreover, the peak intensities were comparable in both the modes and hence all four analytes were ionized under positive ionization mode without polarity switch. The Q1 MS full scan mass spectra contained protonated precursor $[M+H]^+$ ions at *m*/*z* 296.2, 231.1, 336.2 and 358.1 for SUM, NAP, NARA and INDO respectively. The most abundant and consistent product ions in Q3 MS spectra were observed at *m*/*z* 58.1, 185.1, 98.1 and 139.1 for both the analytes and ISs respectively as shown in Fig. 1a–d. The product ion fragment at *m*/*z* 58.1 for SUM corresponded to dimethyl amine group, while the fragment at *m*/*z* 185.1 for NAP was formed due to elimination of carboxylic acid group from the precursor ion. Similarly, the stable fragments for NARA and INDO at *m*/*z* 98.1 and 139.1 can be attributed to *N*-methyl piperidine and *p*-chloro benzoyl substructure respectively from their precursor ions. The source dependent and compound dependent parameters were suitably optimized to obtain a consistent and sufficient response for both the analytes. A dwell time of 100 ms was adequate for the analytes and ISs and no cross talk was observed between their MRMs.

Plasma extraction: As both the drugs have significant difference in drug–plasma binding, physico-chemical properties (pK_a of 9.63 and 4.15 for SUM and NAP respectively), calibration range and their dose strength used in combination therapy, it was difficult to optimize a single extraction procedure for both the analytes. Several methods have used either liquid–liquid extraction (LLE) [9,11,21] or solid phase extraction (SPE) [13,16–19,22] for the extraction of SUM (as a single analyte) from human plasma. However, protein precipitation (PP) has been successfully established for sample clean-up of NAP from human plasma [32,47,48]. Berges et al. [49] have reported a separate SPE procedure for extraction of SUM and a protein precipitation protocol with acetonitrile for NAP for their simultaneous quantification from human plasma. In our effort to have a simple, efficient and a single process for sample preparation of both the analytes, several trials were carried out with all three extraction procedures namely PP, LLE and SPE. Acetonitrile and methanol were tested initially as protein precipitants; however, the recovery was neither consistent nor quantitative for SUM (~25–30%) at different QC levels. Further, LLE was initiated using ethyl acetate, dichloromethane and methyl *tert*-butyl ether, alone and in combination under acidic (formic/acetic acid), alkaline ($NaOH/Na_2CO_3$) and neutral conditions. Significant improvement in the recovery (75–90%) was observed for SUM in almost all the solvent systems and extremely poor for NAP under alkaline conditions. Extraction in presence of acidic additives gave much better result (70–80%), nevertheless the recovery was inconsistent at LLOQ level for both the analytes. Thus, SPE was tried on Phenomenex Strata™-X (30 mg, 1 cc), which has a reversed phase functionalized polymeric sorbent that gives strong retention of neutral, acidic, or basic compounds. Addition of 3% (v/v) *o*-PA to break the drug–protein binding (especially for NAP) gave a consistent and quantitative recovery (95–97%) for both the analytes and ISs at all QC levels.

Chromatography: Chromatographic separation of the analytes was initiated under isocratic conditions to obtain adequate response, sharp peak shape and a short analysis time on Waters Acquity UPLC type BEH C18 (50 mm × 2.1 mm, 1.7 μm) analytical column. To find the best eluting solvent system, various combinations of methanol/acetonitrile along with buffers (ammonium formate/formic acid, ammonium acetate/acetic acid) having different ionic strengths (1–10 mM) in the pH range of 4.0–6.5 and volume ratios were tested. The mobile phase consisting of methanol–acetonitrile–4.0 mM ammonium acetate (70:10:20, v/v/v), pH 4.8 adjusted with acetic acid was found most suitable for baseline separation of analytes. The capacity factors, which describe the rate at which the analytes migrate through the column were 1.77 and 1.02 for SUM and NAP respectively, based on the dead time of 0.45 min. The selectivity factor (α) of the column for the chromatographic separation of the analytes was 1.73. The number of theoretical plates obtained for SUM, NAP, NARA and INDO were 2500, 1095, 2130 and 1444, respectively, and the resolution factor between the analytes was no less than 3.24. Also, the reproducibility of retention times for the analytes, expressed as % CV was ≤0.6% for 105 injections on the same column. Ideally, a deuterated analog should be preferred as an internal standard, however, use of NARA and INDO as internal standards worked well in maintaining the ionization efficiency of the analytes and overall performance of the method. Moreover, there was no drug–drug interaction between the analytes and ISs (especially between naproxen and indomethacin), which was checked at LQC and HQC levels. Also, there was practically no effect of ISs on analyte recovery, sensitivity or ion suppression. Representative MRM ion chromatograms in Figs. 2 and 3 of (a) blank plasma spiked with ISs, (b) SUM and NAP at LLOQ and (c) a real subject sample at C_{max} demonstrate the selectivity of the method to differentiate and quantify the analytes from endogenous components in the plasma matrix or other components in the sample. Moreover, there was no interference at the retention times of analytes and ISs.

3.2. System suitability, system performance and auto-sampler carryover

The precision (% CV) of system suitability test was observed in the range of 0.12–0.32% for the retention time and 0.91–1.02% for the area response for both the analytes and ISs. The signal to noise ratio for system performance was ≥30 for both the analytes. 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. There was practically negligible carry-over (≤0.09%) in extracted double blank plasma (without analyte and IS) after subsequent injection of highest calibration standard (aqueous and extracted) at the retention time of analytes and ISs.

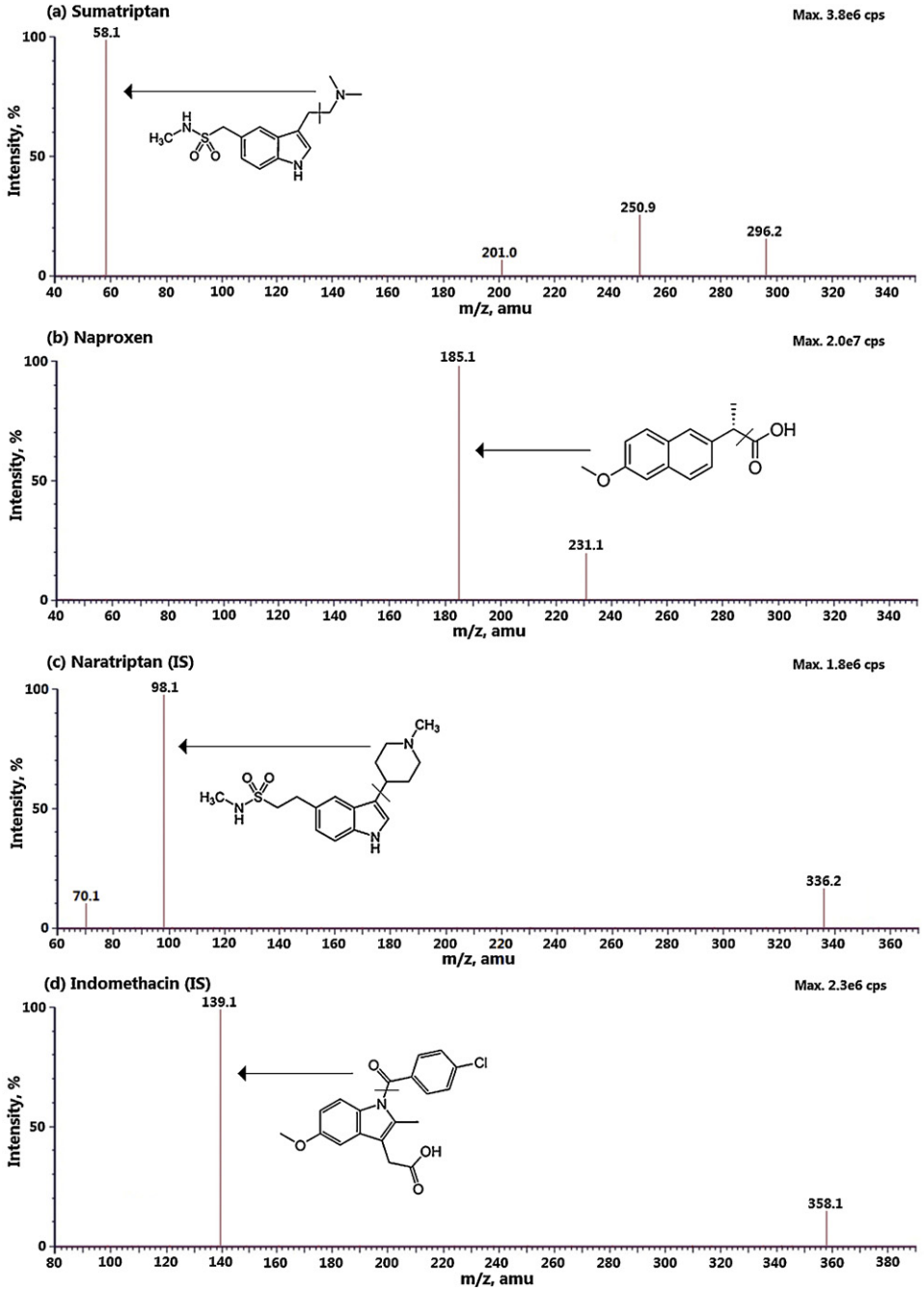

Fig. 1. Product ion mass spectra of (a) sumatriptan (m/z 296.2 → 58.1, scan range 40–350 amu), (b) naproxen (m/z 231.1 → 185.1, scan range 40–350 amu), (c) naratriptan, IS (m/z 336.2 → 98.1, scan range 60–370 amu) and (d) indomethacin, IS (m/z 358.1 → 139.1, scan range 80–390 amu) in positive ionization mode.

3.3. Linearity, lower limit of quantification and accuracy and precision

The five calibration curves were linear over the concentration range of 0.05–100 ng/mL for SUM and 0.05–100 μg/mL for NAP, with a correlation coefficient (r^2) ≥ 0.9996 for both the analytes. The mean linear equations obtained were $y = (0.045519 \pm 0.000344)x - (0.000010 \pm 0.000031)$ and $y = (0.085455 \pm 0.000946)x + (0.000004 \pm 0.000066)$ for SUM and NAP respectively. The calculated standard deviation value for slope, intercept and correlation coefficient were 0.000344, 0.000031, 0.0002 and 0.000946, 0.000066 and 0.0001 for SUM and NAP respectively. The accuracy and precision (% CV) observed for the calibration curve standards ranged from 98.2 to 100.9% and 0.56 to 2.20% for SUM and 97.9 to 101.6% and 0.80 to 1.75% for NAP respectively. The lowest concentration (LLOQ) in the standard curve that can be measured with acceptable accuracy and precision was 0.050 ng/mL and 0.050 μg/mL for SUM and NAP respectively in plasma at a signal-to-noise ratio (S/N) of ≥30. The LOD values were 0.017 ng/mL for SUM and 0.017 μg/mL for NAP at S/N ≥ 10. The intra-batch and inter-batch precision and accuracy were established from validation runs performed at five QC levels (Table 1). The intra-batch precision (% CV) ranged from 0.68 to 2.44 and the accuracy was within 97.0 to 103.4% for both the analytes. Similarly, for the inter-batch experiments, the precision varied from 0.36 to 3.12 and the accuracy was within 98.3 to 102.0%.

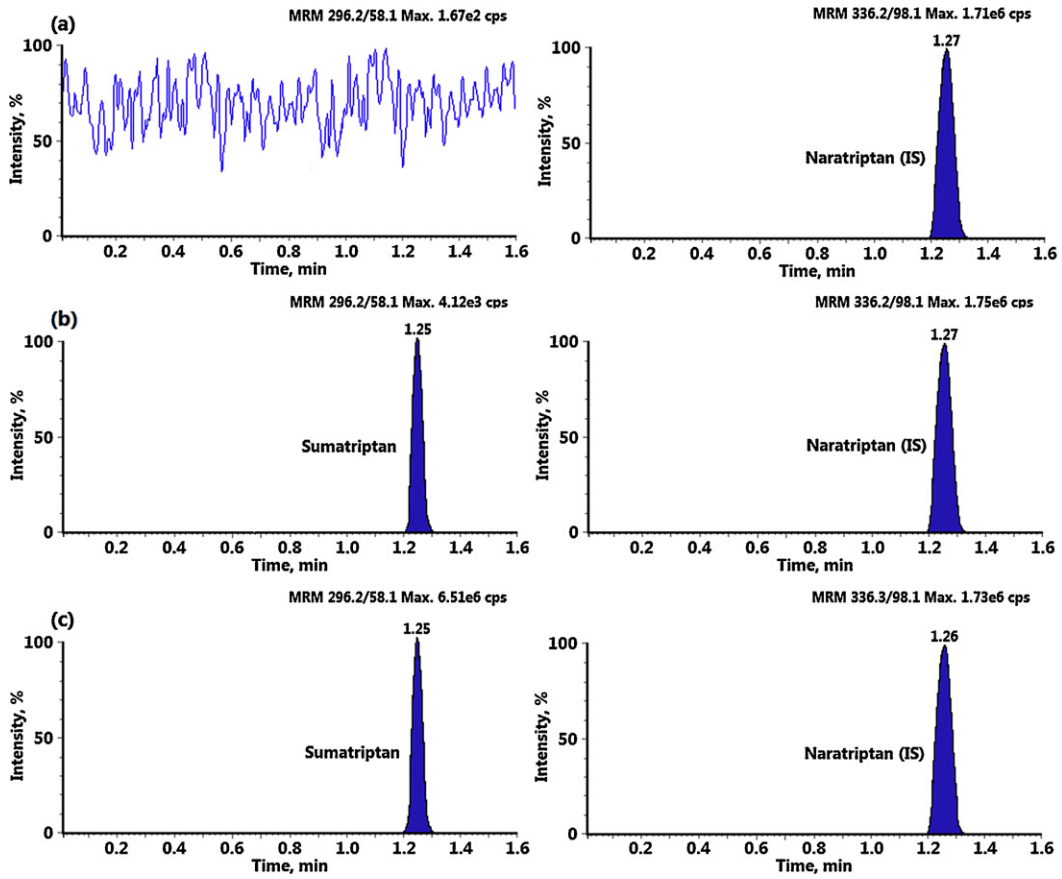

Fig. 2. MRM ion-chromatograms of (a) blank plasma with naratriptan, IS, (b) sumatriptan at LLOQ (m/z 296.2 → 58.1) and naratriptan, (c) sumatriptan in subject sample at C_{max} after oral administration of 85 mg sumatriptan + 500 mg naproxen sodium fixed dose formulation.

3.4. Matrix effect, ion suppression and extraction recovery

Matrix effect is responsible for suppression or enhancement in the measurement of analyte signal due to endogenous or exogenous components present in biological fluids. Matrix effect can directly impact the accuracy, precision, ruggedness and the overall reliability of a validated method. Post-column analyte infusion technique gives a qualitative indication (suppression or enhancement) due to the presence of matrix [51]. Chromatograms in Fig. 4a–d show negligible ion suppression or enhancement at the retention time of analytes and ISs as evident from the flat baseline. The concept of relative matrix effect as recommended by Matuszewski et al. [53] gives a comparison of matrix effect values between different lots of biofluids. The coefficient of variation (% CV) of the slopes of calibration lines for relative matrix effect in eight different plasma lots was ≤3.35 for both the analytes (Table 2).

The relative recovery, absolute matrix effect and process efficiency data at different QC levels are presented in Table 3. The relative recovery of the analyte is the 'true recovery', which is unaffected by the matrix as it is calculated by comparing the area ratio response (analyte/IS) of extracted (spiked before extraction) and unextracted (spiked after extraction) samples. The recovery across quality control levels was within 95–98% for both the analytes and ISs.

Table 1
Intra-batch and inter-batch precision and accuracy for sumatriptan and naproxen.

QC ID	Nominal conc.	Intra-batch				Inter-batch			
		n	Mean conc. observed[a]	% CV	% accuracy	*n*	Mean conc. observed[b]	% CV	% accuracy
Sumatriptan (ng/mL)									
HQC	80.0	6	81.8	1.59	102.3	30	78.8	2.04	98.5
MQC-1	40.0	6	41.0	2.44	102.5	30	39.5	2.93	98.8
MQC-2	2.40	6	2.43	1.01	101.3	30	2.43	3.12	101.3
LQC	0.150	6	0.152	2.05	101.2	30	0.151	1.37	100.7
LLOQ QC	0.050	6	0.051	0.74	102.0	30	0.051	0.36	102.0
Naproxen (μg/mL)									
HQC	80.0	6	82.7	1.55	103.4	30	80.2	1.06	100.3
MQC-1	40.0	6	38.8	2.03	97.0	30	39.3	1.66	98.3
MQC-2	2.40	6	2.40	0.68	100.0	30	2.39	2.63	99.6
LQC	0.150	6	0.151	1.81	100.7	30	0.148	2.61	98.7
LLOQ QC	0.050	6	0.049	1.95	98.0	30	0.051	2.23	102.0

CV: coefficient of variation; *n*: total number of observations.
[a] Mean of 6 replicates at each concentration.
[b] Mean of 6 replicates for five precision and accuracy batches.

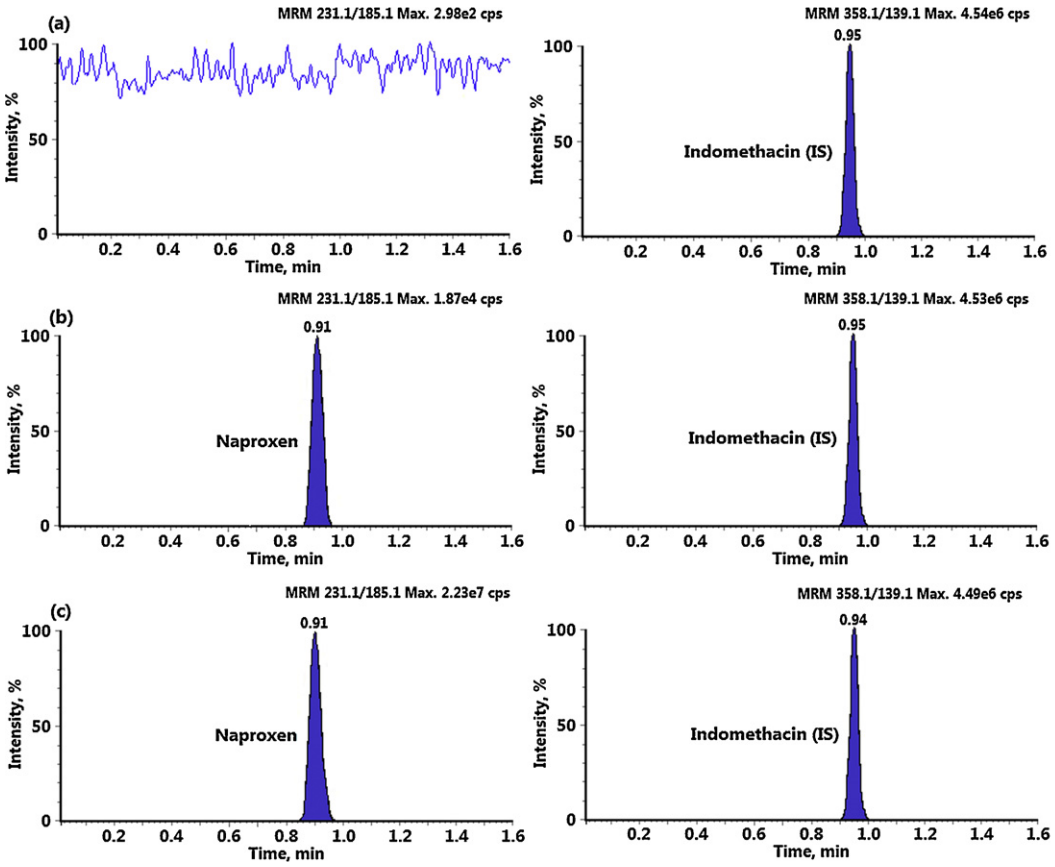

Fig. 3. MRM ion-chromatograms of (a) blank plasma with indomethacin, IS, (b) naproxen at LLOQ (m/z 231.1 → 185.1) and indomethacin, (c) naproxen in subject sample at C_{max} after oral administration of 85 mg sumatriptan + 500 mg naproxen sodium fixed dose formulation.

3.5. Stability results, method ruggedness and dilution reliability

The stability of analytes and ISs in human plasma and stock solutions was examined under different storage conditions. Stock solutions for short term stability were stable at room temperature up to 26 h and between 2 and 8 °C for a minimum period of 25 days for long term stability of both the analytes and ISs. Analytes in control human plasma (bench top) at room temperature were stable for at least 11 h at 25 °C and for minimum of five freeze and thaw cycles. Autosampler stability of the spiked quality control samples was determined up to 78 h. Wet extract stability of the spiked quality control samples was determined up to 59 h without significant loss of the analytes. Long term stability of the spiked quality control samples was unaffected up to 148 days. The detailed results for stability experiments are presented in Table 4.

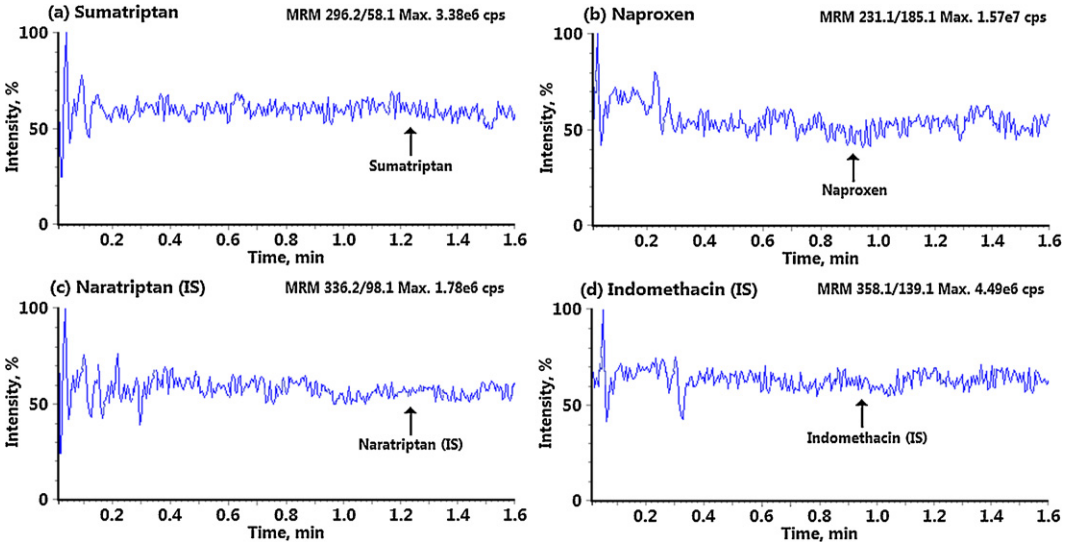

Fig. 4. Post column analyte infusion MRM LC–MS/MS chromatograms for (a) sumatriptan, (b) naproxen, (c) naratriptan and (d) indomethacin.

Table 2
Relative matrix effect in eight different lots of human plasma for sumatriptan and naproxen.

Plasma lot	Slope of calibration curve	
	Sumatriptan	Naproxen
Lot-1	0.043125	0.083859
Lot-2	0.043585	0.085254
Lot-3	0.041254	0.084265
Lot-4	0.043585	0.089655
Lot-5	0.043554	0.085426
Lot-6 (heparinized)	0.042154	0.084585
Lot-7 (hemolyzed)	0.045585	0.085654
Lot-8 (lipemic)	0.041254	0.089585
Mean	0.043012	0.086035
±SD	0.001439	0.002293
% CV	3.35	2.67

SD: standard deviation; CV: coefficient of variation.

The precision (% CV) and accuracy values for two different columns for method ruggedness ranged from 1.8 to 3.6% and 98.5 to 103.4% respectively at all five QC levels. For the experiment with different analysts, the results for precision and accuracy were within 1.9–3.5% and 97.6–103.8% respectively at these levels. For dilution reliability experiment the precision and accuracy values for 1/5th and 1/10th dilution ranged from 0.7 to 1.3% and 97.2 to 103.2% for both SUM and NAP respectively.

3.6. Application of the method in healthy human subjects and incurred sample results

The validated method was successfully applied for the assay of SUM and NAP in healthy Indian male subjects. Fig. 5 shows the plasma concentration vs. time profile for SUM and NAP under fasting condition. Table 5 summarizes the mean pharmacokinetic parameters after oral administration of combination tablet of 85 mg SUM/500 mg NAP sodium test and reference formulation. About 2268 samples including the calibration and QC samples along with subject samples were analyzed during a period of 6 days and the precision and accuracy for calibration and QC samples were well within the acceptable limits. SUM and NAP combined formulation shares a very similar pharmacologic profile with the individual components, however, its pharmacokinetics is markedly different. The rapid absorption of SUM and the delayed release of NAP

Table 3
Absolute matrix effect, relative recovery and process efficiency for sumatriptan and naproxen.

Analyte	*A* (% CV)	*B* (% CV)	*C* (% CV)	Absolute matrix effect, % ME [B/A × 100]	Relative recovery, % RE [C/B × 100]	Process efficiency, % PE [C/A × 100]
LQC						
Sumatriptan	1169 (0.57)	1183 (2.73)	1154 (0.77)	101.2 (97.9)[a]	97.5 (98.8)[a]	98.7 (96.7)[a]
Naproxen	5360 (1.57)	5425 (1.39)	5257 (1.58)	101.2 (98.2)[b]	96.9 (98.4)[b]	98.0 (96.6)[b]
MQC-2						
Sumatriptan	18,796 (1.11)	18,924 (0.28)	18,395 (1.89)	100.7 (99.0)[a]	97.2 (98.4)[a]	97.9 (97.4)[a]
Naproxen	86,135 (0.38)	88,658 (0.83)	84,658 (0.09)	102.9 (97.6)[b]	95.5 (97.2)[b]	98.3 (94.9)[b]
MQC-1						
Sumatriptan	312,205 (0.87)	317,854 (2.13)	305,485 (1.27)	101.8 (99.8)[a]	96.1 (97.0)[a]	97.8 (96.8)[a]
Naproxen	1,436,856 (2.41)	1,475,475 (0.47)	1,402,548 (0.98)	102.7 (97.5)[b]	95.1 (98.8)[b]	97.6 (96.3)[b]
HQC						
Sumatriptan	702,549 (1.73)	716,569 (0.45)	692,547 (0.60)	102.0 (98.2)[a]	96. 6 (98.7)[a]	98.6 (96.9)[a]
Naproxen	2,876,584 (0.70)	2,925,458 (1.82)	2,823,665 (0.28)	101.7 (97.6)[b]	96.5 (98.7)[b]	98.2 (96.3)[b]

CV: coefficient of variation; *A*: mean area response of six replicate samples prepared in mobile phase (neat samples); *B*: mean area response of six replicate samples prepared by spiking in extracted blank plasma; *C*: mean area response of six replicate samples prepared by spiking before extraction.
[a] Values for internal standard, naratriptan.
[b] Values for internal standard, indomethacin.

Table 4
Stability of sumatriptan and naproxen under various conditions ($n = 6$).

Storage conditions	Nominal concentration	Sumatriptan		Naproxen	
		Mean stability sample (ng/mL) ± SD	% change	Mean stability sample (μg/mL) ± SD	% change
Bench top stability (room temperature, 11 h)					
HQC	80.0	80.3 ± 1.6103	0.37	80.9 ± 0.5310	1.13
LQC	0.150	0.145 ± 0.0028	−3.33	0.154 ± 0.0023	2.67
Freeze and thaw stability in plasma at −20 °C					
HQC	80.0	80.7 ± 0.9965	0.86	79.5 ± 1.5434	−0.63
LQC	0.150	0.155 ± 0.0034	3.33	0.157 ± 0.0015	4.67
Freeze and thaw stability in plasma at −70 °C					
HQC	80.0	80.8 ± 0.5805	1.00	81.2 ± 0.8701	1.50
LQC	0.150	0.151 ± 0.0057	0.67	0.147 ± 0.0044	−0.30
Autosampler stability (4 °C, 78 h)					
HQC	80.0	82.3 ± 1.5723	2.86	79.6 ± 1.0368	−0.50
LQC	0.150	0.147 ± 0.0026	−0.30	0.151 ± 0026	0.67
Wet extract stability (2–8 °C, 59 h)					
HQC	80.0	79.5 ± 2.1881	−0.63	80.5 ± 1.2462	0.63
LQC	0.150	0.147 ± 0.0014	−0.30	0.148 ± .0017	−1.33
Long term stability in plasma at −20 °C					
HQC	80.0	80.8 ± 1.3509	1.00	78.9 ± 1.4967	−1.38
LQC	0.150	0.150 ± 0.0048	0.00	0.154 ± 0.0027	2.67
Long term stability in plasma at −70 °C					
HQC	80.0	79.9 ± 1.7556	−0.13	81.4 ± 1.6634	1.75
LQC	0.150	0.145 ± 0.0029	−3.33	0.154 ± .0034	2.67

SD: standard deviation; n: number of replicates at each level.
% change $= \frac{\text{mean stability samples} - \text{mean comparison samples}}{\text{mean comparison samples}} \times 100$.

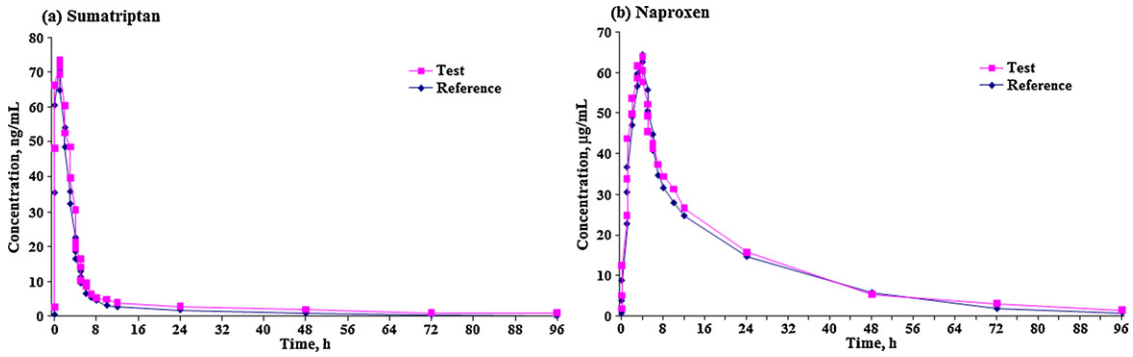

Fig. 5. Mean plasma concentration–time profile of sumatriptan and naproxen after oral administration of test (85 mg sumatriptan + 500 mg naproxen sodium tablets from a Generic Company) and a reference (TREXIMET®, 85 mg sumatriptan (as succinate) + 500 mg naproxen sodium tablets) (GlaxoSmithKline, Research Triangle Park, NC 27709) formulation to 28 healthy volunteers.

Table 5
Mean pharmacokinetic parameters following oral administration of 85 mg sumatriptan and 500 mg naproxen sodium test and reference formulation to 28 healthy Indian subjects under fasting.

Parameter	Sumatriptan		Naproxen	
	Test Mean ± SD	Reference Mean ± SD	Test Mean ± SD	Reference Mean ± SD
C_{max} (ng/mL)/(µg/mL)	78.04 ± 6.50	76.91 ± 7.65	60.36 ± 3.12	61.54 ± 2.45
T_{max} (h)	0.88 ± 0.14	1.00 ± 0.17	3.98 ± 0.35	4.12 ± 0.23
$t_{1/2}$ (h)	2.24 ± 0.22	2.32 ± 0.24	14.07 ± 0.83	13.82 ± 0.96
AUC_{0-96} (h ng/mL)/(h µg/mL)	196 ± 12	201 ± 8	953 ± 48	982 ± 59
AUC_{0-inf} (h ng/mL)/(h µg/mL)	204 ± 14	208 ± 11	1103 ± 65	1132 ± 84
K_{el} (1/h)	0.339 ± 0.029	0.327 ± 0.043	0.049 ± 0.002	0.050 ± 0.003

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 0 h to 96 h; AUC_{0-inf}: area under the plasma concentration–time curve from 0 h to infinity; K_{el}: elimination rate constant; SD: standard deviation.

sodium from the fixed dose contribute toward its therapeutic gain over monotherapy with either component. The C_{max} for SUM and NAP occurs at approximately 1 h (range 0.3–4.0 h) and 5 h (range 0.3–12 h) respectively after oral administration of this fixed dose formulation. The mean C_{max} values for SUM following oral administration of TREXIMET are similar to that of SUM when given as 100 mg alone (IMITREX®, sumatriptan succinate) [7]. Haberer et al. [8] have conducted several studies in healthy volunteers for this combination tablet. The C_{max}, T_{max}, AUC_{0-inf} and $t_{1/2}$ values for both the components obtained in the present work were comparable with their study. The mean T_{max} and $t_{1/2}$ values obtained for both the drugs in the present study (SUM ~1.0 h and ~2.3 h; NAP ~4.0 h and 14 h respectively) were well within the range reported by Haberer et al. [8] from different studies conducted under fasted conditions. Further, the values for C_{max}, AUC_{0-inf} and $t_{1/2}$ were very similar to the work of Niazi et al. [58] for 500 mg NAP under fasting with identical no. of subjects. No statistically significant differences were found between the two formulations in any parameter. The ratios of mean log-transformed parameters (C_{max}, AUC_{0-t}, and AUC_{0-inf}) and their 90% CIs were all within the defined bioequivalence range of 80–125%. These observations confirm the bioequivalence of the test sample with the reference product in terms of rate and extent of absorption. The % change for assay reproducibility in 123 incurred samples was within ±13% for both the analytes. This authenticates the reproducibility of the proposed method.

4. Conclusion

The developed UPLC–MS/MS method for the quantitation of SUM and NAP in human plasma was fully validated as per USFDA guidelines. The proposed method has a much higher sensitivity for both the analytes compared to all other methods presented either as a single analyte or in combination in different biological matrices. The method offers significant advantages over those previously reported, in terms of lower sample requirement for analysis, simplicity of extraction procedure and overall analysis time. The efficiency of solid-phase extraction and chromatographic run time of 1.6 min per sample renders the method useful in high-throughput bioanalysis. Absence of matrix interference is effectively shown by post-column infusion and by the precision (% CV) values for the calculated slopes of calibration curves. The validated method showed acceptable data for all the validation parameters, with adequate sensitivity and selectivity for their simultaneous quantification in a clinical setting. Further, incurred sample reanalysis of 123 samples reaffirms the reproducibility of the proposed method.

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