BIOANALYTICAL METHOD FOR THE QUANTITATIVE ESTIMATION OF FROVATRIPTAN IN RABBIT PLASMA BY LC–MS/MS AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

S. Kumar Moola1,2, Bala Sekhara Reddy Challa3 and K.B. Chandrasekhar2

1ARPL Pvt. Ltd, Bangalore–560 099, India.
2Jawaharlal Nehru Technological University Anantapur, Anantapur–522001, India.
3Vagdevi College of Pharmacy, Gurazala–522 415, India.

ABSTRACT

Liquid chromatography–tandem mass spectrometry was used to develop a simple, sensitive and rapid method for the quantitative determination of frovatriptan in rabbit plasma using frovatriptan d3 as internal standard (IS). Chromatographic separation was performed on C18 column using a mobile phase composed of 5 mM ammonium formate (pH 3.0) and acetonitrile (60:40, v/v) at flow rate of 0.5 mL/min. Analyte and the IS were extracted form plasma by a simple liquid–liquid extraction procedure. Frovatriptan and frovatriptan d3 were detected with proton adducts at m/z 244.0→213.2 and 247.1→231.1 in multiple reaction monitoring (MRM) positive mode, respectively. The method was validated with the correlation coefficients of ($r^2$) ≥ 0.9963 over a linear concentration range of 5.00–12000.00 pg/mL. The method demonstrated intra and inter–day precision within 0.82 to 3.48% and 0.38 to 1.14% and accuracy within 97.23 to 100.32% and 97.79 to 100.91 % for frovatriptan. This method was successfully applied to a pharmacokinetic study in 6 rabbits.

Key words: Liquid chromatography, Mass spectrometry, Frovatriptan, Rabbit plasma, Pharmacokinetics.

INTRODUCTION

Migraine is a common headache disorder that disproportionately affects women. About half of all women affected by migraine, that they are more likely to experience migraine in association with menstrual periods [1,2]. Unlike other types of migraine, the pathophysiologic bases of menstrual migraine are related to neuroendocrine changes associated with the menstrual cycle, particularly the fluctuations of estrogen levels [3]. Management of menstrual migraines is challenging as the attacks are reported to be of longer duration and are more refractory to therapies [4]. When compared with sumatriptan, in vitro studies have shown that frovatriptan exhibits higher selectivity for the cerebral vasculature, with minimal effects on the coronary vasculature [5].

Frovatriptan belongs to the class triptans and was developed by Vernalis for the treatment of migraine headaches, in particular those associated with menstruation. Frovatriptan causes vasoconstriction of arteries and veins that supply blood to the head. Frovatriptan is believed to act on extracerebral, intracranial arteries and to inhibit excessive dilation of these vessels in migraine [6].

Mean maximum blood concentrations ($C_{max}$) in patients are achieved approximately 2–4 hours after administration of a single oral dose of frovatriptan succinate 2.5 mg. The absolute bioavailability of frovatriptan in healthy subjects is about 20% in males and 30% in females. Food has no significant effect on the bioavailability, but delays $t_{max}$ by one hour. Binding of frovatriptan to serum proteins is low (approximately 15%). The terminal elimination half–life of frovatriptan is approximately 26 hours [7-10].

Literature survey reveals that very few analytical methods have been reported for the determination of frovatriptan, including a high–performance liquid chromatography (HPLC) method [11] and a capillary zone electrophoretic method [12]. Currently, the goal of bio–analytical scientists is to develop reliable, rapid and efficient procedures for performing qualitative and quantitative analyses suitable for clinical and preclinical studies. Unfortunately, conventional HPLC methods must sacrifice either time or resolution. As a result, it is necessary to develop fast or ultra–fast methods such as LC–MS/MS without any loss of separation efficiency.
To date, no LC–MS/MS method has been reported for the determination of frovatriptan in any of the matrices. Thus the aim of this study was to develop a more specific, selective, sensitive and high-throughput LC–MS/MS method for quantitative determination of frovatriptan in rabbit plasma by LC–MS/MS with a small amount of sample volume. The application of this assay method to a pharmacokinetic study in healthy rabbits is described.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Frovatriptan succinate monohydrate (99.20%) obtained from TLC Pharmachem, whereas frovatriptan d3 hydrochloride (99.8%) obtained from USP. HPLC grade methanol was purchased from J.T. Baker (Phillipsburg, USA), while analytical grade ammonium formate and ammonium acetate was from S.D fine chemicals (Mumbai, India). Ammonium hydrogen carbonate, zinc sulfate solution, tert- butyl methyl ether (TBME) was purchased from Merck Speciality Limited (Mumbai, India). Ultra-pure water from Milli-Q water purification system (Millipore, Bedford, MA, USA) was used through the study. All other chemicals in this study were of analytical grade. Rabbits were obtained from Hyderabad Research Labs (Hyderabad, India).

**Instrumentation**

An HPLC system (1200 Series, Agilent Technologies, Germany) connected with triple quadrupole API–4000 mass spectrometer instrument (AB Sciex, Foster City, CA, USA). Data processing was performed with the Analyst 1.5.1 software package. Ionization was performed by electro spray ionization (ESI) source in positive mode with unit resolution.

**Detection**

Mass parameters were optimized to get the product ions of m/z: 213.2 and m/z: 231.1 from its respective precursor ions of frovatriptan [M+H]+ (m/z: 244.0) and frovatriptan d3 [M+H]+ (m/z: 247.1). The ionization soruce maintained at 550 °C and the ion spray voltage was set at 5500 V. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 35, 35, 32, and 5 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 65, 35, 10, 12 V for frovatriptan and 75, 35, 10, 12 V for the IS.

**Chromatographic conditions**

Chromatography was performed on Inertsil ODS C18, 150 × 4.6 mm, 5 μm analytical column at 40°C, with 5 mM ammonium formate (pH 3.0) and acetonitrile (60:40, v/v) as mobile phase at a flow rate of 0.5 mL/min. frovatriptan d3 was used as an internal standard in terms of chromatography and extractability. Analyte and the IS was eluted at 2.3 ± 0.3 min with 4 min total run time.

**Preparation of standards and quality control (QC) samples**

Two standard stock solutions of frovatriptan (0.1 mg/mL) were prepared in HPLC grade methanol for the preparation of calibration standards and quality control samples. From these stock solutions, appropriate dilutions were made using a 50:50, v/v mixture of acetonitrile and water as a diluent to produce working standard solutions of frovatriptan. The calibration curve (CC) standard samples of frovatriptan in rabbit blank plasma were prepared by spiking with an appropriate volumes of the working solutions giving the final concentrations of 5.00, 10.0, 50.00, 200.00, 500.00, 2400.00, 4800.00, 7200.00, 9600.00 and 12000.00 pg/mL. Similarly, quality control (QC) samples were also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 5.00 (lower limit of quantitation, LLOQ), 15.0 (low quality control, LQC), 6000 (medium quality control, MQC) and 9000 ng/mL (high quality control, HQC) as a single batch at each concentration. All the prepared plasma samples were stored at −30°C.

A 0.1 mg/mL of frovatriptan d3 stock solution was prepared by dissolving the compound in HPLC grade methanol. The working concentration of frovatriptan d3 (5.00 ng/mL) was prepared from the above stock solution using the diluent. Stock solutions of frovatriptan and frovatriptan d3 were found to be stable for 30 days at 2-8°C.

**Sample preparation**

Liquid–liquid extraction was used to isolate analyte and the IS from plasma. For this purpose, 50μL of the IS (5.00 ng/mL) and 100 μL of plasma sample (respective concentration) was added into labeled polypropylene tubes and vortexed briefly. To this, 100 μL of ammonium hydrogen carbonate and and 100 μL of zinc sulfate solution was added. After vortexing for 10 s, a 2.5–mL aliquot of the TBME was added using a Dispensette Organic pipette (GmbH, Wertheim, Germany). The sample was vortex for 10 min and then centrifuged for 4 min at 4000 rpm using a Heraeus Megafuse 3SR (Japan) centrifuge. The organic layer (2.0 mL) was transferred into a 10–mL glass test tube and evaporated at 40°C under a stream of nitrogen. The dried extract was reconstituted in 500 μL of the mobile phase and transferred into auto–injector vials. From these, a 20 μL aliquot was injected into the chromatographic system.

**Selectivity and specificity**

The selectivity of the method was determined by six different rabbit blank plasma samples, which were pretreated and analyzed to test the potential interferences of endogenous compounds co–eluting with analyte and the IS. Chromatographic peaks of analyte and the IS were identified based on their retention time and MRM
responses. The peak area of frovatriptan at the respective retention time in blank samples should not be more than 20% of the mean peak area of LOQ samples. Similarly, the peak area of frovatriptan d3 at the respective retention time in blank samples should not be more than 5% of the mean peak area of LOQ samples.

Recovery
The extraction recovery of frovatriptan and frovatriptan d3 from rabbit plasma was determined by analyzing quality control samples. Recovery at three concentrations (15.00, 6000.00, and 9000.00 pg/mL) was determined by comparing peak areas obtained from the plasma sample and the neat samples (aqueous samples).

Limit of detection (LOD) and limit of quantification (LOQ)
The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. LOD was determined using the signal–to–noise ratio (s/n) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples. The limit of quantitation (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOQ was found by analyzing a set of mobile phase and plasma standards with a known concentration of frovatriptan.

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

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

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

Calibration curve, precision and accuracy
The calibration curve was constructed using values ranging from 5.00 to 12000.00 pg/mL of frovatriptan in rabbit plasma. Calibration curve was obtained by quadratic model with weighted 1/x^2 regression analysis. Calibration curve standard samples and quality control samples were prepared in replicates (n=6) for analysis. Precision and accuracy for the back calculated concentrations of the calibration points, should be within ±15 and ± 15% of their nominal values. However, for LLOQ, the precision and accuracy should be within ± 20 and ± 20%.

Stability studies in plasma samples
LQC and HQC (n=6) samples were retrieved from a deep freezer after three freeze–thaw cycles and processed as per the procedure described earlier. Samples were stored at –30°C in three cycles of 24, 36 and 48 h. In addition, the long–term stability of frovatriptan in quality control samples was also evaluated by analysis after 65 days of storage at –30°C. Autosampler stability was studied following 72 h storage period in the autosampler tray and bench top stability was studied for 24 h period with control concentrations. Stability samples were processed and extracted along with the freshly spiked calibration curve standards. The precision and accuracy for the stability samples must be ≤ 15 and ± 15 %, respectively of their nominal concentrations.

Analysis of plasma samples
The bioanalytical method described above was used to determine frovatriptan concentrations in plasma following oral administration of frovatriptan tablet to healthy rabbits (n=6). New Zealand albino male rabbits with weight between 1.5-1.8 kg were selected for the study. The animals were kept in individual cages and maintained at 25 °C for 10 days prior to experiment. Standard diet and water ad libitum were given to them. All experiments have been performed according to guidelines of the Institutional Animal Ethics Committee, Bioneeds, Bangalore. Frovatriptan 0.23 mg/1.8 kg body weight was administered orally in a single dose of frovatriptan tablet. All studies were performed after keeping rabbits for overnight fasting. Blood samples (0.3 mL) were collected at predose and 0.25, 0.75, 1.5, 1.75, 2, 2.5, 3, 4, 6, 8, 12, 18 and 20 h of post–dose in heparinized Eppendorf tubes. These samples were centrifuged immediately at 3500 rpm and 4°C temperature for 10 min. Plasma samples were taken and stored at –30°C until assay. Pharmacokinetic parameters like peak plasma concentration (Cmax) time to reach peak plasma concentration (tmax), area under the (concentration–time) curve (AUC) and elimination half–life (t1/2) were calculated following non–compartment model of WinNonlin Version 5.1.

RESULTS AND DISCUSSION
Method development
LC–MS/MS has been used as one of the most powerful analytical tools in clinical pharmacokinetics for its selectivity, sensitivity and reproducibility. The goal of this work is to develop and validate a simple, sensitive, rapid and rugged assay method for the quantitative determination of frovatriptan from rabbit plasma samples. Chromatographic conditions, especially the composition and nature of the mobile phase, usage of different columns, different extraction methods such as solid phase, precipitation and liquid–liquid extraction methods were optimized through several trials to achieve the best resolution and increase the signal of frovatriptan and the IS.
The MS optimization was performed by direct infusion of solutions of both frovatriptan and the IS into the ESI source of the mass spectrometer separately. The critical parameters in the ESI source include the needle (ESI) voltage, source temperature and other parameters such as nebulizer gas, auxiliary gas, curtain gas and collision gas were optimized to obtain a better spray shape, resulting in better ionization of the protonated ionic frovatriptan and the IS molecules (Figure. 1A and 1B). Product ion spectrum for frovatriptan and the IS yielded high–abundance fragment ions of m/z 213.2 and m/z 231.1 (Figure, 2A and 2B). After mass spectrometer parameters optimized, chromatographic conditions such as mobile phase optimization, column optimization, extraction method optimization was performed to obtain a fast and selective LC method. A good separation and elution were achieved using 5 mM ammonium formate (pH 3.0) and acetonitrile (60:40, v/v) as the mobile phase, at a flow–rate of 0.5 mL/min and injection volume of 5 µL. Inertsil ODS C18 (50 mm × 4.6 mm, 5 µm) column gave good peak shape and response even at lowest concentration level for the analyte and the IS. The retention time of analyte and the IS obtained with the above optimized chromatographic conditions were low enough (2.3 and 2.3 min) allowing short run time of 4.0 min.

During method development different sample pre–treatment methods were investigated. Initially protein precipitation (PP) was tried using methanol and acetonitrile as precipitating agents resulted poor recoveries with some ion suppression and poor chromatography of the analyte. Thus, LLE was carried out using solvents like tert–butyl methyl ether, diethyl ether, ethyl acetate, hexane and dichloromethane alone and in combination under acidic/basic conditions. LLE is helpful in producing a spectroscopically clean sample and avoiding the introduction of non–volatile materials onto the column and MS system and also minimizing the experimental cost. Clean samples are essential for minimizing ion suppression and matrix effect on LC–MS/ MS. Among the different solvents checked alone and in combination for their suitability tert–butyl methyl ether was found to be optimal, which can produce a clean chromatogram for a blank sample and yields the highest recovery for the analyte from the plasma. Addition of ammonium hydrogen carbonate and zinc sulfate as extraction additives helped in achieving the highest recoveries for the analyte and the IS. The use of deuterated compounds as an IS is recommended for bioanalytical assays to increase assay precision and limit recovery variations. Hence frovatriptan stable labeled isotope frovatriptan d3 is selected for the present work and was found to be best in mimicking the analyte chromatography, extraction and ionization.

**Selectivity and specificity**

The analysis of frovatriptan and the IS using MRM function was highly selective with no interfering compounds (Fig. 3A). Chromatograms obtained from plasma spiked with frovatriptan (5.00 ng/mL) and the IS are shown in Fig. 3B.

**Limit of detection (LOD) and quantification (LOQ)**

The limit of detection was used to determine the instrument detection levels for frovatriptan even at low concentrations. An aliquot of 5 µL of a 0.5 pg/mL solution was injected and estimated LOD was 3.5 fg with S/N values ≥ 3–5. The limit of quantification for this method was proven as the lowest concentration of the calibration curve which was proven as 5.00 pg/mL.

**Matrix effect**

Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the mid QC level and compared with neat standards of same concentration in alternate injections. The overall precision of the matrix factor is 3.64 for frovatriptan. There was no ion– suppression and ion– enhancement effect observed due to analyte and the IS at respective retention time (Fig. 4).

**Linearity, precision and accuracy**

Four frovatriptan calibration curves were linear over the concentration range of 5.00–12000.00 pg/mL with a correlation coefficient (r²)≥0.99. Calibration curve details from one batch of the validation section are provided in the Table 1. The intra–day and inter–day precision and accuracy results in plasma QC samples are summarized in Table 2. The results revealed good precision and accuracy.

**Stability studies**

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

**Recovery**

The recovery of frovatriptan was determined at three different concentrations 15.00, 6000.00 and 9000.00 pg/mL, was found to be 99.37%, 90.40% and 98.82%, respectively. The overall average recovery of frovatriptan and the IS were found to be 96.19% and 92.95%, respectively.

**Application to real time plasma samples**

The above validated method was used in the determination of frovatriptan in plasma samples for establishing the pharmacokinetic of a single 0.23 mg/1.8 kg body weight in 6 healthy rabbits by oral route. Fig. 5 depicts the mean plasma concentration vs time profile of frovatriptan. All the plasma concentrations of frovatriptan were within the standard curve region and retained above the 5.00 pg/mL (LOQ) for the entire sampling period (Table-4).
### Table 1. Calibration curve details from one batch of the validation section

<table>
<thead>
<tr>
<th>Spiked concentration pg/mL</th>
<th>Mean ± SD</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.00</td>
<td>4.94 ± 0.13</td>
<td>2.65</td>
</tr>
<tr>
<td>10.00</td>
<td>9.89 ± 0.14</td>
<td>1.39</td>
</tr>
<tr>
<td>50.00</td>
<td>50.0 ± 1.59</td>
<td>3.19</td>
</tr>
<tr>
<td>200.00</td>
<td>203 ± 1.64</td>
<td>0.81</td>
</tr>
<tr>
<td>500.00</td>
<td>492 ± 6.52</td>
<td>1.33</td>
</tr>
<tr>
<td>2400.00</td>
<td>2398 ± 41.47</td>
<td>1.73</td>
</tr>
<tr>
<td>4800.00</td>
<td>4762 ± 61.81</td>
<td>1.30</td>
</tr>
<tr>
<td>7200.00</td>
<td>7200 ± 91.38</td>
<td>1.27</td>
</tr>
<tr>
<td>9600.00</td>
<td>9540 ± 157.32</td>
<td>1.65</td>
</tr>
<tr>
<td>12000.00</td>
<td>11980 ± 148.32</td>
<td>1.24</td>
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</table>

### Table 2. Intra-run and inter-run precision and accuracy

<table>
<thead>
<tr>
<th>Nominal concentration (pg/mL)</th>
<th>Intra-run n=6</th>
<th>Inter-run n=36</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D</td>
<td>%CV</td>
</tr>
<tr>
<td>5.00</td>
<td>5.02 ± 0.13</td>
<td>2.54</td>
</tr>
<tr>
<td>15.00</td>
<td>14.58 ± 0.51</td>
<td>3.48</td>
</tr>
<tr>
<td>6000.00</td>
<td>5838.00 ± 100.35</td>
<td>1.72</td>
</tr>
<tr>
<td>9000.00</td>
<td>8895.00 ± 73.22</td>
<td>0.82</td>
</tr>
</tbody>
</table>

### Table 3. Stability of the samples for frovatriptan

<table>
<thead>
<tr>
<th>Stability test</th>
<th>Plasma concentration (pg/mL)</th>
<th>Frovatriptan Concentration Measured(mean±SD) (n=6; pg/mL)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room temperature stability (24 h)</td>
<td>15.00</td>
<td>15.10 ± 0.33</td>
<td>2.17</td>
</tr>
<tr>
<td></td>
<td>9000.00</td>
<td>8905.36 ± 115.68</td>
<td>1.30</td>
</tr>
<tr>
<td>Autosampler stability (72 h)</td>
<td>15.00</td>
<td>15.31 ± 0.38</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td>9000.00</td>
<td>8919.64 ± 138.33</td>
<td>1.55</td>
</tr>
<tr>
<td>Freeze-thaw stability (3 cycles)</td>
<td>15.00</td>
<td>14.58 ± 0.28</td>
<td>1.91</td>
</tr>
<tr>
<td></td>
<td>9000.00</td>
<td>8823.21 ± 170.45</td>
<td>1.93</td>
</tr>
<tr>
<td>Long term stability (65 days)</td>
<td>15.00</td>
<td>15.34 ± 0.34</td>
<td>2.22</td>
</tr>
<tr>
<td></td>
<td>9000.00</td>
<td>8971.43 ± 135.47</td>
<td>1.51</td>
</tr>
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</table>

### Table 4. Pharmacokinetic parameters of frovatriptan in rabbit plasma

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC₀⁻t (pg · h/mL)</td>
<td>46403.81</td>
</tr>
<tr>
<td>Cmax (pg/ mL)</td>
<td>5192.17</td>
</tr>
<tr>
<td>AUC₀⁻∞ (pg · h/mL)</td>
<td>94342.34</td>
</tr>
<tr>
<td>Kel</td>
<td>0.03258</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>2.50</td>
</tr>
<tr>
<td>t₁/₂</td>
<td>21.28</td>
</tr>
<tr>
<td>ct</td>
<td>1561.83</td>
</tr>
<tr>
<td>Ct/kel</td>
<td>47938.53</td>
</tr>
</tbody>
</table>

AUC₀⁻∞: area under the curve extrapolated to infinity; 
AUC₀⁻t: area under the curve up to the last sampling time; 
Cmax: the maximum plasma concentration; 
Tmax: the time to reach peak concentration; 
Kel: the apparent elimination rate constant.
Figure 1A: Parent ion mass spectra of frovatriptan

Figure 1B: Parent ion mass spectra of frovatriptan-d3

Figure 2A: Product ion mass spectra of frovatriptan

Figure 2B: Product ion mass spectra of frovatriptan-d3

Figure 3A: Typical chromatogram of blank plasma sample

Figure 3B: Typical MRM chromatogram of LOQ sample along with the IS.
CONCLUSION

The LC–MS/MS assay reported here is simple, rapid, specific and sensitive for quantification of frovatriptan in rabbit plasma. To the best of our knowledge, this is the first LC–MS/MS report for the determination of frovatriptan in any of the biological matrices. The method described in this manuscript has been developed and validated over the concentration range of 5.00 – 12000.00 pg/mL in rabbit plasma. The intra–batch precision was less than 3.48% and accuracy ranged from 97.23% to 100.32 %. The inter–batch precision was less than 1.14% and accuracy ranged from 97.79% to 100.91%. The selectivity, sensitivity, precision and accuracy obtained with this method make it suitable for the purpose of the present study.

In conclusion, the method used in the present study is easy and fast to perform; it is also characterized with an adequate accuracy, precision, selectivity and stability. The simplicity of the method, and using rapid liquid–liquid extraction and sample turnover rate of 4.0 min per sample, make it an attractive procedure in high–throughput bioanalysis of frovatriptan.

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