African Journal of Pharmacy and Pharmacology Vol. 6(2), pp. 98-107, 15 January, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.436
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Validated high performance liquid chromatography (HPLC) method for the determination of sumatriptan in rabbit plasma: Application to pharmacokinetic study

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Accepted 8 September, 2011

A new, sensitive and specific isocratic reverse phase-high performance liquid chromatography (RP-HPLC) method with fluorescence detection was developed and validated for the determination of sumatriptan in rabbit plasma using sulpiride as an internal standard (IS). Sumatriptan was extracted from plasma by a liquid-liquid extraction with a mixture of tert-butyl methyl ether, dichloromethane and ethyl acetate (2:2:3, v/v). Chromatographic separation of the analyte and internal standard was achieved on a Phenomenex C4 (250 × 4.6 mm, 5 µm) analytical column maintained at 40°C. The mobile phase was composed of 25 mM ammonium acetate (pH 6.5) and acetonitrile (85:15, v/v), pumped isocratically at a flow rate of 0.9 ml/min. Column eluent was monitored at excitation and emission wavelengths of 225 and 350 nm. The calibration curve was linear over a concentration range of 1 to 300 ng/ml (r^2 = 0.9999) with a limit of quantification, 1 ng/ml. The intra-day and inter-day precision and accuracy were between 2.24 and 4.28% and -1.10 and 2.86%, respectively. The mean recoveries of sumatriptan and sulpiride were 89.92 and 91.03%, respectively. Sumatriptan containing plasma samples were stable at -20°C for 14 days. The validated method was successfully applied for pharmacokinetic study after a single oral administration of sumatriptan (50 mg) to rabbits.

Key words: Sumatriptan, rabbit plasma, reverse phase-high performance liquid chromatography (RP-HPLC), liquid-liquid extraction, pharmacokinetics.

INTRODUCTION

Migraine is a chronic, episodic and neurological disorder characterized by unilateral headache often associated with nausea and/or vomiting which usually begins in childhood, adolescence or early adult life (Dulery et al., 1997). Sumatriptan succinate is a basic compound (pKa 9.63) and chemically known as {3-[2-(dimethylamino)ethyl]-N-methyl-1H-indole-5-methanesulphonamide succinate} (Figure 1a). It is a white to off-white powder which is readily soluble in water and in saline and the partition coefficient of the sumatriptan base in n-octanol/water ($P_{o/w}$) is 0.65. It is a highly selective 5-hydroxy-tryptamine-1 receptor agonist and proved to be a novel and effective in acute treatment for migraine headache. The clinical routes of administration are oral, subcutaneous and intranasal, with the absolute bioavailabilities of approximately 14, 96 and 15%, respectively (Balaguer-Fernandez et al., 2008; Ravi et al., 2009). It has been shown to have a low to moderate oral bioavailabilities in laboratory animals, such as rabbits (23%), rats (37%) and dogs (58%). The lower bioavailabilities are primarily due to pre-systemic first-pass metabolism and partly due to incomplete absorption (Barrow et al., 1997; Dixon et al., 1993).

Several analytical methods have been developed previously for the determination of sumatriptan in biological fluids, including high performance liquid chromatography (HPLC) with ultraviolet (UV) detection (Majithiya et al., 2006), HPLC with fluorescence detection (Ge et al., 2004), HPLC with coulometric detection

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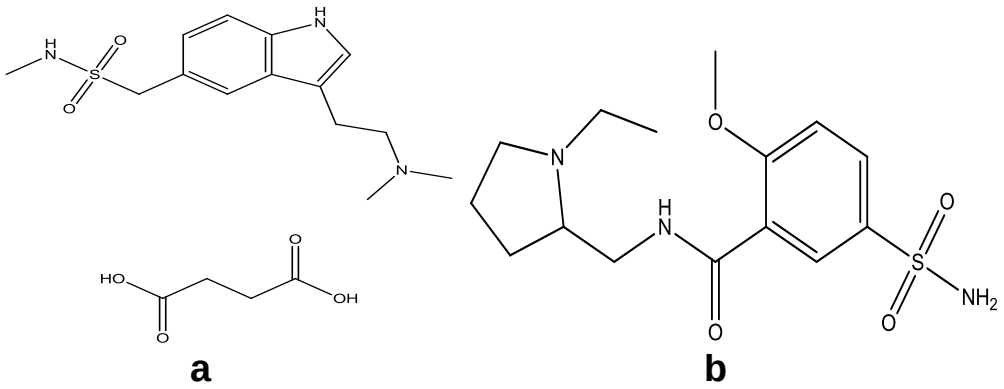

Figure 1. Chemical structures of (a) sumatriptan succinate and (b) sulpiride (IS).

(Andrew et al., 1993; Dunne and Andrew, 1996; Franklin et al., 1996) and HPLC with mass spectrometric detection (Biddlecombe et al., 2001; Boulton et al., 2003; Cheng et al., 1998; Dulery et al., 1997; McLoughlin et al., 1996; Vishwanathan et al., 2000). The drawbacks of the reported HPLC-UV method were low sensitivity (3 ng/ml) and longer run time of analysis (25 min). HPLC with coulometric and mass spectrometric detection methods involved extraction of sumatriptan from biological fluids using liquid-liquid extraction or solid phase extraction technique. In all the methods, 1 ml plasma/serum samples were used to achieve 1 ng/ml of limit of quantification (LOQ) except the method reported by Biddlecombe et al. (2001) in which 0.1 ml of plasma was used to achieve 0.1 ng/ml LOQ. Although, mass spectrometry provides excellent sensitivity and specificity with short analysis time but might not be ubiquitously applicable in laboratories due to cost implications.

Till date, only one method was available in literature for the determination of sumatriptan in plasma using HPLC with fluorescence detection (Ge et al., 2004). Although, the reported method had 1 ng/ml sensitivity with 0.5 ml plasma volume, our study could not adopt the method due to lack of specificity. Therefore, a new, sensitive and specific HPLC method with fluorescence detection was developed and validated for the determination of sumatriptan in rabbit plasma.

EXPERIMENTAL

Chemicals and reagents

Sumatriptan succinate was purchased from Nosch Labs (Hyderabad, India). Suminat® tablets were purchased from GlaxoSmithKline (Middlesex, UK). Sulpiride was purchased from Shanghai PI Chemicals (Shanghai, China). Methanol and acetonitrile (HPLC grade) were purchased from J.T. Baker (Phillipsburg, USA). Tert-butyl methyl ether (TBME) was purchased from Acros Organics (New Jersey, USA). Dichloromethane (DCM) and sodium hydroxide was purchased from R&M Chemicals (Essex, UK). Ethyl acetate (EA) was purchased from Lab Scan (Bangkok, Thailand). Ammonium acetate was purchased from Nacalai Tesque (Kyoto, Japan). Blank rabbit plasma was collected from marginal ear vein of several New Zealand rabbits and was stored at -20°C until further use.

Instrumentation and chromatographic conditions

The HPLC system consisted of a Shimadzu chromatographic system (Kyoto, Japan) equipped with an LC-20AD solvent delivery pump, RF-10AXL fluorescence detector, SIL-20AHT autosampler, CTO-10AS VP column oven and LC Solution chromato software. The analysis was performed on a reversed-phase C4 analytical column (Phenomenex, 250 × 4.6 mm i.d., 5 µm particle size) protected by a C4 guard column (Phenomenex Kromasil, 10 × 4 mm i.d., 5 µm particle size). The mobile phase consisted of a mixture of 25 mM ammonium acetate (pH 6.5) and acetonitrile (85:15, v/v) and was delivered at a flow rate of 0.9 ml/min.

Fluorescence detection was performed with excitation wavelength 225 nm and emission wavelength 350 nm. The column oven temperature was maintained at 40°C. The injection volume was 50 µl.

Preparation of stock solutions, standards and quality control samples

A stock solution of sumatriptan at a concentration of 1 mg/ml was prepared in methanol. The concentration was expressed as the amount of sumatriptan in the base form. The stock solution of sumatriptan was subsequently diluted in the same solvent to obtain working standard solutions in the range of 0.02 to 6 µg/ml. The stock solution of sulpiride (Figure 1b), as an internal standard (IS) was prepared at a concentration of 1 mg/ml in methanol and was further diluted with the same solvent to give a working concentration of 1 µg/ml. The working standard solutions of sumatriptan (25 µl) were added to 475 µl blank rabbit plasma to produce final concentrations of 1 to 300 ng/ml for sumatriptan. Quality control (QC) samples were prepared at three concentration levels of 5 ng/ml (low), 150 ng/ml (medium) and 250 ng/ml (high). For each solution, IS was added at a constant level of 20 µl of 1 µg/ml stock solution. All solutions were stored under refrigeration at 4°C prior to usage.

Extraction procedure

To 0.5 ml aliquot of plasma, 20 µl of 1 µg/m l of IS, 0.5 ml of 1 M

sodium hydroxide and 7 ml mixture of TBME, DCM and EA (2:2:3, v/v) as an extraction solvent were added. The mixture was vortexed for 2 min and centrifuged at 4000 rpm for 15 min. The supernatant was transferred to reacti-vial and evaporated to dryness at 50°C under a gentle stream of nitrogen gas. The residue was reconstituted with 0.2 ml of 10% v/v methanol and then the samples were transferred to autosampler vials. An aliquot of 50 µl was injected into the HPLC system for analysis.

Bioanalytical method validation

The method was validated according to USFDA guidance for bioanalytical method validation (USFDA, 2001) for specificity, linearity, sensitivity, accuracy, precision, recovery and stability.

Specificity

Specificity is described as the ability of a method to discriminate the analyte from all potentially interfering substances. The specificity of the method was investigated by comparing the chromatograms of blank plasma obtained from six rabbits with that of plasma samples spiked with sumatriptan and IS.

Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample. To evaluate the linearity of the method, five calibration plots at seven concentration levels consisting of 1, 10, 25, 50, 100, 200 and 300 ng/ml were determined in rabbit plasma. The linearity of each calibration curve was determined by plotting the peak area ratio of sumatriptan to IS of plasma standards versus the nominal concentration using linear regression analysis. The lowest concentration on the calibration curve that can be reproducibly quantified with acceptable precision and accuracy (± 20%) was considered as a limit of quantification (LOQ) of the assay.

Intra-day and inter-day precision and accuracy

Intra-day and inter-day precision and accuracy were evaluated by analyzing QC samples at low, medium and high concentrations of 5, 150 and 250 ng/ml. For the intra-day variation, sets of five replicates were analyzed on the same day and for the inter-day validation, five replicates of three concentration levels were analyzed on three different days. To be acceptable, the measures should be within ±15% at all concentrations.

Extraction recovery

The recovery of sumatriptan at three QC levels (5, 150 and 250 ng/ml) was determined by comparing the peak area of extracted QC samples with the peak area obtained from direct injections of a standard solution containing the same concentration of sumatriptan. Five replicates were prepared at each concentration level.

Stability studies

Stability experiments were performed with low, medium and high QC samples to evaluate the sumatriptan stability under different conditions. Experiments were performed in triplicate to determine stability of bench top (6 h) and autosampler (24 h) samples at room temperature (25 ± 2°C), freeze thaw stability (three cycles at -20°C and room temperature) and short term stability at -20°C for 14 days.

Dilution integrity

Dilution integrity experiment was performed due to the probability of encountering samples with concentrations above the upper limit of quantitation (ULOQ) in the pharmacokinetic study. Hence, it is necessary to dilute the study samples with drug free plasma to bring them within the calibration range. To determine the effect of dilution on the integrity of samples, a concentration of 600 and 1200 ng/ml was diluted with drug free rabbit plasma at one and four fold dilution, respectively to obtain sumatriptan concentration of 300 ng/ml. The samples were prepared in three replicates. The samples were analyzed and the obtained concentrations were compared with theoretical values.

Pharmacokinetic study in rabbits

Six healthy male New Zealand rabbits (2.8 to 3.4 kg) were used for the study. The study was conducted in accordance with Animal Ethical Guidelines for investigations in laboratory animal and the study protocol was approved by the Animal Ethics Committee of Universiti Sains Malaysia. After an initial period of acclimatization for 1 week to laboratory conditions, the rabbits were randomly divided into two groups of three each. All the rabbits were fasted for 12 h with *ad libitum* access to water prior to the experiment. One group received reference product (Suminat® conventional tablets), whereas the other group received test product (orally disintegrating tablets prepared in our research laboratory). A dose of 50 mg sumatriptan was administered to the each rabbit of both groups. The excipients present in reference product were lactose, microcrystalline cellulose, croscarmellose sodium, magnesium stearate and Opadry YS-1-1441-G whereas the test product were Eudragit EPO, microcrystalline cellulose, Kollidon CL-SF, calcium silicate, ammonium bicarbonate, aspartame, pineapple flavor, magnesium stearate and aerosil. The tablets were administered at the back of the pharynx using a gastric intubation tube (made of silicone rubber) with one tablet set on the tip of tube and immediately 5 ml of water was administered through the tube to facilitate swallowing of the tablet and to prevent it from sticking to the rabbit's throat. Animal had access to food 4 h after dose administration. About 2 ml of blood sample was withdrawn from marginal ear vein into heparinized eppendorf tubes at time intervals of 0 (pre-dose), 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12 and 16 h post administration. According to U.S. Food and Drug Administration (FDA) and European Agency for the Evaluation of Medicinal Products (EMEA) regulations, the sampling schedule should be planned to provide a reliable estimation of the extent of absorption (CPMP, 2001; US FDA, 2003). This can be achieved if AUC_{0-t} is at least 80% of $AUC_{0-\infty}$. Usually the sampling time should extend to at least three terminal elimination half-lives of the active ingredient. Time periods between sampling should not exceed one terminal half-life (Nation and Sansom, 1994). From the pharmacokinetic data reported in the existing literature, it was found that the half-life ($t_{1/2}$) of sumatriptan is approximately 1 to 2 h (Humphrey et al., 1991). Hence, in the present study, the samples were collected up to 16 h after drug administration as to cover a minimum of three half lives of the sumatriptan. The time interval between the sample collections was also maintained not to exceed more than one terminal half life, until it covers the three $t_{1/2}$ of sumatriptan. The plasma was separated by centrifugation at 4000 rpm for 15 min and was stored at -20°C until analysis. After a wash out period of one week, the animals were crossed-over and received the alternate product. According to the standard for bioequivalence tests (USFDA, 2003), the wash-out period should be at least 5 times the half-life of the active ingredient after

administration. The half-life of sumatriptan is approximately 1 to 2 h, and thus 7 days was enough for sumatriptan to be eliminated completely from the body even when individual variations are taken into consideration.

The pharmacokinetic parameters, namely, maximum plasma concentration (C_{max}) and time to reach maximum plasma concentration (T_{max}) were obtained directly from the data. The area under the plasma concentration-time curve from zero to infinity ($AUC_{0-\infty}$) was calculated by adding the area from time zero to the last sampling time (AUC_{0-t}) and the area from the last sampling time to infinity ($AUC_{t-\infty}$). The former was calculated using the trapezoidal formula and the latter by dividing the last measurable plasma drug concentration with the terminal elimination rate constant (K_e). The value of K_e was calculated using the least-squares regression analysis of the terminal portion of the log plasma concentration versus time curve. The elimination half life ($t_{1/2}$) was calculated by dividing 0.693/Ke.

Statistical analysis

The results are reported as mean ± standard deviation. An analysis of variance (ANOVA) was performed on the pharmacokinetic parameters, $AUC_{0-\infty}$, C_{max}, $t_{1/2}$ and K_e which distinguishes the effects due to subjects, periods and treatment (Wagner, 1975). The 'P' value was calculated from the obtained 'F' value using GraphPad Prism, version 5.02 (GraphPad Prism software, San Diego, CA). A parametric approach was used to compare the bioequivalence of the pharmacokinetic characteristics between the reference and test products. In this approach both reference and test groups should preferably have equal variance prior to analysis. In a statistical perspective, logarithmic transformation of $AUC_{0-\infty}$ and C_{max} values make the distribution appear more symmetric, closer to the normal distribution and achieves a relatively homogeneous variance between the groups (Zhu et al., 2009). Therefore, $AUC_{0-\infty}$ and C_{max} were logarithmically transformed before statistical analysis. The T_{max} values were analyzed using Wilcoxon Signed Rank test for paired samples. A statistical significant difference was considered at $P < 0.05$.

RESULTS AND DISCUSSION

Several variables of the HPLC method with respect to their effect on the separation of sumatriptan and IS from the matrix were investigated. In extensive preliminary experiments, parameters such as choice of analytical column, composition of the mobile phase, organic modifier, pH and molarity of buffer salt in addition to mobile phase flow rate and column temperature were optimized in order to provide a good performance of assay for the determination of sumatriptan in rabbit plasma.

Method development and optimization

The selection of wavelength is a prerequisite for the determination of a drug with adequate sensitivity and without interference from the endogenous compounds present in biological fluids. A Luminescence spectrophotometer (LS45 Luminescence spectrometer, PerkinElmer Instruments) scan in the range of 200 to 600 nm showed detection of sumatriptan with maximum sensitivity at the excitation and emission wavelengths of 225 and 350 nm. These wavelengths were selected for the quantification of sumatriptan in rabbit plasma.

Several reversed-phase analytical columns, such as, C18, C8, C4 and CN (in the order of increasing polarity of the stationary phase) were tested with the mobile phase composition of 50 mM ammonium acetate (pH 6.5) and acetonitrile (85:15, v/v) at a flow rate of 0.8 ml/min for the separation of sumatriptan and IS from the endogenous compounds present in rabbit plasma. Initial separation studies were performed with C18 (Phenomenex, 250 × 4.6 mm, 5 µm) column. For organic polar molecules, the sample retention decreases with increase in the length of the bonded phases. In contrast, sumatriptan was eluted at longer retention time from the C18 (Thermo-Hypersil 250 × 4.6 mm, 5 µm). The late elution could be due to the relatively lower organic content (15% acetonitrile) in the mobile phase which resulted in an increase in the affinity of the drug to the stationary phase (Sankalia et al., 2007). Moreover, the resolution between sumatriptan and IS was poor and also the chromatographic response for both analytes was low. In general, the polar compounds should have longer retention in C4 as compared to C8 due to less hydrophobic nature. However, sumatriptan and IS did not show any difference in the elution time from C8 (Phenomenex, 250 × 4.6 mm, 5 µm) and C4 (Phenomenex-Kromosil, 250 × 4.6 mm, 5 µm). The analytes were eluted with good chromatographic response from both the columns but good resolution was observed between sumatriptan and IS from C4 as compared to C8. Cyano (CN, Phenomenex 250 × 4.6 mm, 5 µm) chromatographic column is used for polar basic compounds in both reversed and normal phase modes. Sumatriptan and IS had a longer elution in CN as compared to the other columns (C18, C8 and C4). Moreover, chromatographic response was poor for both analytes and the peak shape of sumatriptan was also not optimal. Based on these findings, the analytical column C4 was found to be the most suitable for the determination of sumatriptan in plasma.

The amount of organic modifier present in the mobile phase influences analytes which are retained predominantly by adsorption onto the stationary phase. In the preparation of the mobile phase, several combinations of buffer and organic modifier at the ratios of 85:15, 80:20, 70:30 and 60:40 (v/v) were tested using C4 as an analytical column. Variations in the mobile phase lead to considerable changes in the chromatographic parameters. An increase in the content of the organic modifier resulted in a decrease in the retention time of the analytes.

At the ratio of 85:15 (v/v), both the analytes were eluted with good chromatographic response and optimum resolution. The peak shape of the analytes was also found to be symmetrical. At the ratios of 80:20 and 75:25 (v/v), satisfactory resolution was not achieved between

sumatriptan and IS, although the retention time was decreased without any effect on analytes response. Further increase in the content of the organic modifier to 60:40 (v/v) resulted in coelution of IS with endogenous and solvent front peaks. Hence, the ratio of mobile phase at 85:15 (v/v) was selected for further experiments.

When experiments were performed with methanol instead of acetonitrile as the organic modifier in the mobile phase, late elution of sumatriptan and IS with peak tailing and also increase in the column pressure were observed. Moreover, a significant interference was observed at the retention time of IS from endogenous compounds present in plasma. Hence, the experiments were carried out with acetonitrile as an organic modifier.

The selection of buffer pH mainly depends on the pKa of the analyte. For the basic compounds, pH needs to be selected approximately 2.5 pH units below the pKa. The pKa value for the sumatriptan and sulpiride is 9.63 and 9.12, respectively. The pH effect of the mobile phase was studied in the range of 3.5 to 7.5. The chromatographic response of both analytes was increased with increase of pH from 3.5 to 6.5. Further increase in pH to 7.5 resulted in a decrease of the chromatographic response of IS. A shoulder peak at earlier eluting portion of IS was observed at lower pH and gradually resolved while increasing the pH of mobile phase. The shoulder peak and IS was well resolved at the pH of 6.5. Moreover, the retention time of both analytes were gradually increased while increasing the pH from 3.5 to 7.5. Therefore, the pH value of 6.5 was considered to be optimal as it gave a good resolution between sumatriptan and IS and provided good compromise between retention time, chromatographic response and peak shape.

The buffer molarity was tested at 25, 50 and 75 mM. There were no significant changes in the retention time, chromatographic response and peak symmetry with the change in buffer molarity. Thus, a buffer molarity of 25 mM was selected for further analysis. The effect of flow rate at 0.7, 0.8 and 0.9 ml/min with optimized mobile phase composition was studied. The retention time of both analytes were decreased when the flow rate was increased from 0.7 to 0.9 ml/min. When the flow rate increased from 0.7 to 0.8 ml/min there was a decrease in peak width of both analytes but at flow rates of 0.8 and 0.9 ml/min, there was no significant decrease in the peak width. Hence, a flow rate of 0.9 ml/min was selected as the optimum flow rate since it yielded good peak shapes with reasonable retention time and without endogenous peak interference at the retention time of both analytes.

The effect of column temperature at 25°C (room temperature) and at 30 and 40°C (elevated temperatures) was studied. When the column temperature was increased from 25 to 40°C, there was a decrease in retention time and increase in chromatographic response of both analytes. There was no significant effect on the peak width of IS, but an increase in oven temperature caused a decrease in the peak width of sumatriptan. Thus, 40°C was selected as the optimum oven temperature with optimized chromatographic conditions.

After several trials, the mobile phase consisted of a mixture of 25 mM ammonium acetate (pH 6.5) and acetonitrile (85:15, v/v) was finally adopted at a flow rate of 0.9 ml/min. The described chromatographic conditions achieved satisfactory resolution and symmetrical peak shapes for IS and sumatriptan with the retention time of 7.13 and 8.73 min, respectively. No interference from the endogenous compounds present in plasma was observed at the retention time of both analytes.

Choice of internal standard

Several substances were tested for the selection of internal standard (IS). Sulpiride was selected as the most suitable IS, because the plasma samples showed no interference at its retention time and the peak was also well resolved from the sumatriptan. Moreover, it is a stable compound and does not exist endogenously in the plasma. In addition, a significant advantage of this IS was its elution time which was shorter than that of sumatriptan.

Sample preparation

At an initial study, sumatriptan was extracted with protein precipitation technique (PPT) due to the advantages of shorter processing time, consumption of less organic solvent, fewer steps and good clean up of plasma samples. Protein precipitation with perchloric acid (PCA), trichloroacetic acid (TCA), acetonitrile, methanol and a mixture of acetonitrile/PCA and acetonitrile/TCA has been investigated. Extraction of plasma samples using all the aforementioned solvents (except methanol) showed no interference at the retention times of sumatriptan and IS. However, the results from these investigations showed relatively poor recovery (40 to 60%) for both analytes. Protein precipitation with methanol did not successfully remove all the protein present in the plasma, hence resulted in an incomplete precipitation. Thus, liquid-liquid extraction (LLE) was selected for further experiments.

In LLE, initially sumatriptan was extracted with TBME (7 ml) alone and the samples produced interference at the retention time of sumatriptan and had a poor recovery about 30%. When a mixture of TBME and DCM at the ratio of 5:2 was used as an extraction solvent, they produced a small interference at the retention time of sumatriptan but recovery was improved to 50.12%. No interference was observed at the retention time of sumatriptan when blank plasma samples extracted with a ratio of 4:3, and also recovery was improved to 73.95%. However, further increase in DCM amount in an extraction solvent mixture caused precipitation of the

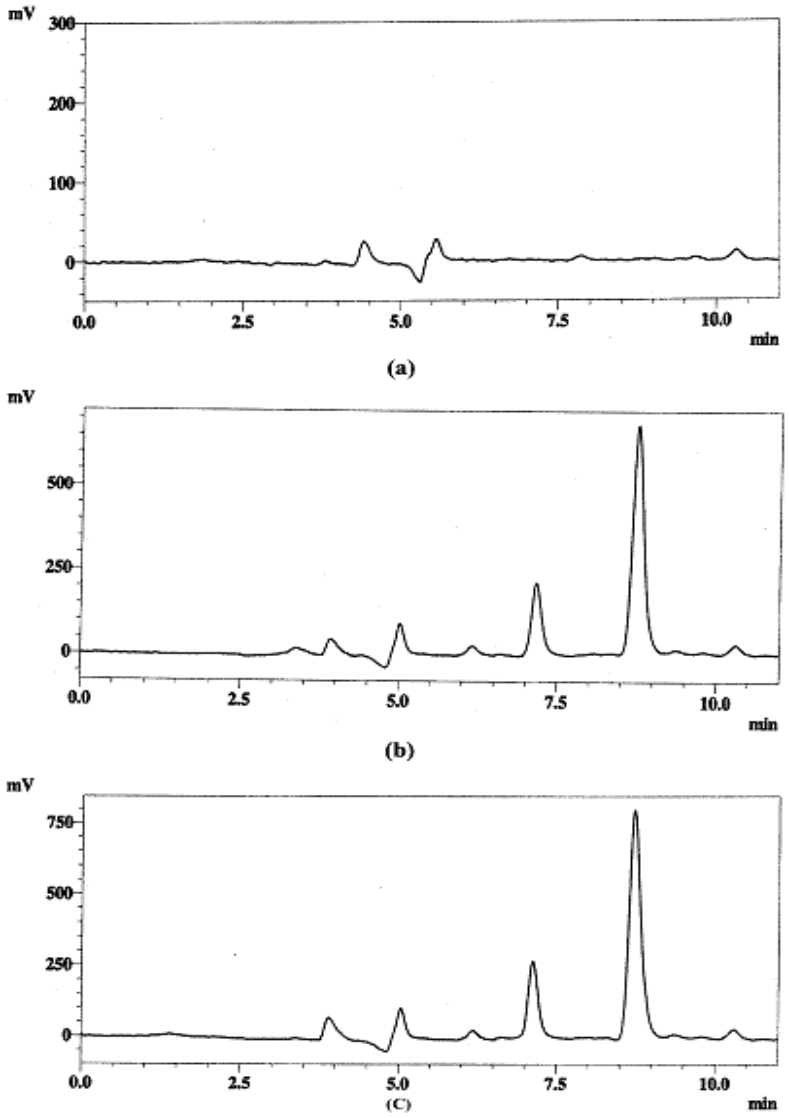

Figure 2. Representative HPLC chromatograms: (a) blank rabbit plasma, (b) rabbit plasma spiked with IS (7.13 min) and 200 ng/ml sumatriptan (8.73 min) and, (c) rabbit plasma collected at 1.5 h after oral administration of sumatriptan.

samples. Improvement in the recovery was observed when EA was added to the mixture of TBME and DCM. After several trials with different ratios of TBME, DCM and EA as an extraction solvent, it was found that the ratio of 2:2:3 achieved an efficient recovery for sumatriptan (90.13%) and IS (91.38%) without any interference from endogenous compounds at the retention time of both analytes.

Bioanalytical method validation

Specificity

The chromatograms of blank plasma, plasma spiked with sumatriptan and plasma obtained after 1.5 h oral administration are depicted in Figure 2. Figure 2a shows that blank rabbit plasma had no interference from endogenous substances at the retention times of the analyte and IS. Figure 2b indicated a good resolution between analyte and IS under the optimized chromatography conditions. The retention time was 7.13 min for IS and 8.73 min for sumatriptan with a precision of 0.22 and 0.18%. The developed method was therefore found to be selective for sumatriptan in the presence of endogenous matrix components.

Linearity

The calibration curve exhibited an excellent linearity over

Table 1. Summary of the calibration curve results for sumatriptan. Mean ± SD, n = 5.

Concentration added (ng/ml)	Concentration found (ng/ml)	Precision RSD (%)	Accuracy RE (%)
1	1.02 ± 0.06	5.77	2.43
10	9.95 ± 0.36	3.62	-0.51
25	25.67 ± 1.06	4.14	2.69
50	51.02 ± 2.42	4.74	2.04
100	98.48 ± 1.94	1.97	-1.52
200	198.82 ± 4.96	2.50	-0.59
300	301.13 ± 6.62	2.20	0.38

Table 2. Experimental values of mean concentration, %RSD and %RE presented for validation parameters of sumatriptan.

Study	Concentration added ng/ml)	Concentration found (ng/ml)	%RSD	%RE
Intra-day[a]	5	4.94 ± 0.11	2.25	-1.10
	150	152.05 ± 5.30	3.49	1.37
	250	253.83 ± 8.28	3.26	1.53
Inter-day[b]	5	5.14 ± 0.22	4.28	2.86
	150	150.24 ± 4.11	2.74	0.16
	250	252.76 ± 5.67	2.24	1.10
Bench top[c]	5	4.93 ± 0.05	1.02	-1.36
	150	147.73 ± 2.12	1.43	-1.51
	250	248.87 ± 1.01	0.40	-0.45
Freeze and Thaw[d]	5	4.89 ± 0.09	1.89	-2.19
	150	148.10 ± 3.70	2.50	-1.27
	250	243.61 ± 4.09	1.68	-2.56
Autosampler[e]	5	4.84 ± 0.08	1.67	-3.23
	150	145.91 ± 1.51	1.04	-2.72
	250	247.37 ± 1.42	0.57	-1.05
Short term[f]	5	4.79 ± 0.06	1.18	-4.11
	150	143.05 ± 3.47	2.42	-4.64
	250	241.58 ± 4.93	2.04	-3.37

[a]Intra-day accuracy and precision was determined with 5 replicates for each concentration. [b]Inter-day accuracy and precision was determined with 15 replicates (day 1, n = 5; day 2, n = 5; day 3, n = 5) for each concentration. [c]After 6 h at room temperature (25 ± 2°C), n = 3. [d]After 3 freeze and thaw cycles at -20°C, n = 3. [e]After 24 h at room temperature (25 ± 2°C), n = 3. [f]14 days at 4°C, n = 3.

the concentration range of 1 to 300 ng/ml of sumatriptan. The mean linear regression equation from five calibration plots was y = 0.0173 (±0.0004) × -0.0022 (±0.0002) with a correlation coefficient of 0.9999 (±0.0001). The results indicated that there was no significant inter-day variability of slopes and intercepts over the optimized concentration range. The present method had an LOQ of 1 ng/ml with an accuracy of 2.43% and precision of 5.77% (n = 5). The linearity results are presented in Table 1.

Intra-day and inter-day precision and accuracy

The intra-day accuracy (relative error (%RE)) ranged between -1.10 and 1.53% with a precision (relative standard deviation (%RSD)) of 2.25 to 3.49%. The inter-day accuracy ranged between 0.16 and 2.86% with a precision of 2.24 to 4.28%. All the results for precision and accuracy were within the acceptable limits (±15%). The results are as shown in Table 2.

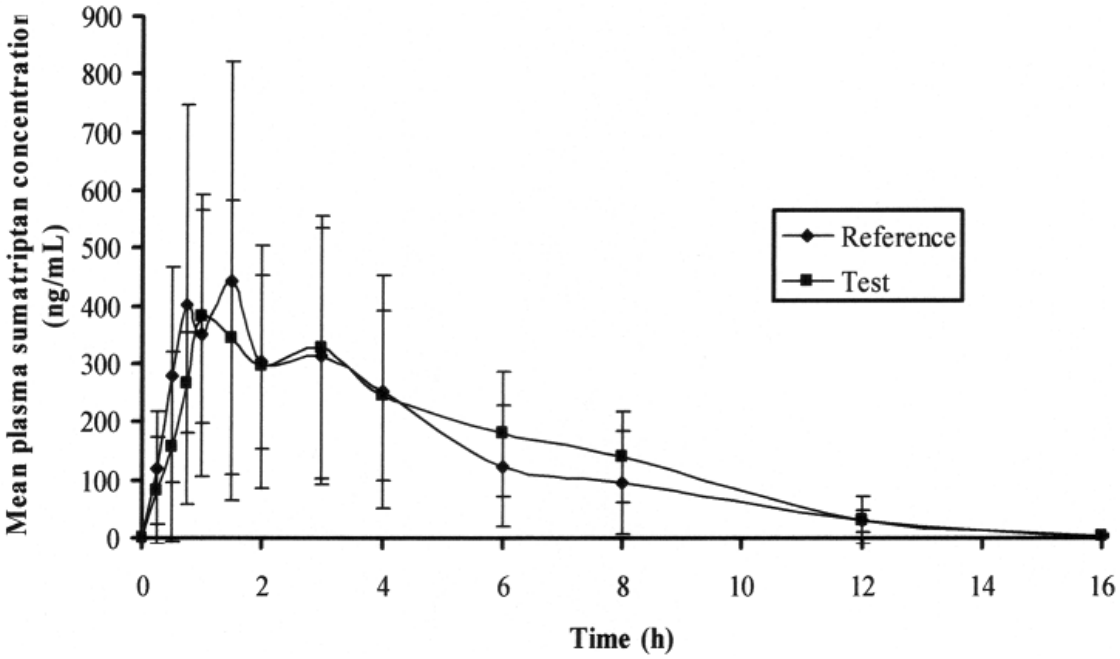

Figure 3. Mean plasma concentration-time profile in six rabbits obtained after a single oral administration (50 mg) of reference and test formulations of sumatriptan.

Extraction recovery

The mean extraction recoveries of sumatriptan at concentrations of 5, 150 and 250 ng/ml were 90.65, 88.68 and 90.42% with a precision of 2.62, 2.25 and 1.76%, respectively. The mean extraction recovery of IS was 91.03%. The extraction recovery of the analytes was shown to be consistent and reproducible.

Stability studies

Sumatriptan was found to be stable in the blank rabbit plasma for 6 h at bench top, and 24 h in an autosampler at room temperature, at repeated freeze-thaw cycles (three cycles) and at -20°C for 14 days. The stability results showed that the accuracy was in the range of -4.64 to -0.45 with a precision of 0.40 to 2.50. The values obtained were within the acceptable limits of precision and accuracy (±15%). The stability results are summarized in Table 2.

Dilution integrity

The experiments performed with one fold dilution of 600 ng/ml sumatriptan, produced mean concentration of 297.11 ng/ml with a precision and accuracy of 1.22 and -0.96%, respectively. In addition, four fold dilution of 1200 ng/ml sumatriptan produced mean concentration of 295.59 ng/ml with a precision and accuracy of 0.73 and -1.47%, respectively. The resulted precision and accuracy values were well within the acceptable limits of ±15% (US FDA, 2001).

Pharmacokinetic study in rabbits

The oral pharmacokinetics of reference and test formulations was compared in terms of rate (C_{max} and T_{max}) and extent (AUC_{0-t} and $AUC_{0-\infty}$) of absorption. The plasma concentration time profiles following oral administration of reference and test are depicted in Figure 3 and it is indicative for the suitability of the current method for pharmacokinetic studies of sumatriptan in rabbit plasma. From the data it was observed that individual plasma profiles were highly variable with many rabbits displaying double peaks while few of them displayed multiple peaks. The probable reason for multiple peaks observed after oral administration of sumatriptan may be due to alteration in the gastric motility in the gut by 5-HT_1 like agonists (sumatriptan) (Sifrim et al., 1999; Vingerhagen et al., 2000). The other probable reason may be presence of multiple absorption sites for sumatriptan in the gastrointestinal tract of rabbits (Dulery et al., 1997). As mentioned earlier, many of the rabbits exhibited double peaks of sumatriptan plasma concentrations. The probable reason for the appearance of double peaks may be as a result of the presence of two compartment absorption phases with only one disposition phase (Christensen et al., 2003; Fowler et al., 1991). Other researchers also reported the wide inter-

Table 3. Pharmacokinetic parameters of sumatriptan after a single oral administration (50 mg) of reference (Suminat®) and test formulations to rabbits.

Parameter	Reference	Test
AUC_{0-t} (h.ng/ml)	2135.87 ± 1515.89	2227.44 ± 1204.11
$AUC_{t-\infty}$ (h.ng/ml)	11.55 ± 13.63	4.92 ± 3.67
$AUC_{0-\infty}$ (h.ng/ml)	2147.42 ± 1529.06	2232.35 ± 1203.24
C_{max} (ng/ml)	523.32 ± 346.17	510.00 ± 222.57
T_{max} (h)	1.42 ± 0.96	1.54 ± 0.84
$t_{1/2}$ (h)	1.70 ± 0.23	1.39 ± 0.26
K_e (1/h)	0.4128 ± 0.056	0.5124 ± 0.082

Mean ± SD, n=6.

subject variability in plasma concentrations of sumatriptan after oral administration of conventional and fast disintegrating tablets (Carpay et al., 2004; Dahlof, 2001; Ferrari et al., 2008; Fowler et al., 1989; Lacey et al., 1995). The absorption profile results showed that the concentrations of drug in plasma increased rapidly, then fluctuated and reached maximum in all the rabbits at approximately 0.5 to 3.0 h for both preparations. Thereafter, drug concentrations in plasma declined gradually over a period of 16 h.

The pharmacokinetic parameters results are shown in Table 3 and the data demonstrates a wide inter-subject variability between rabbits after oral administration of sumatriptan. Sumatriptan was rapidly absorbed after oral administration of both preparations and most rabbits had maximum plasma concentrations in the range of 0.5 to 1.5 h. The individual T_{max} values varied between 0.5 to 3 h for both preparations. The coefficient of variation (%CV) values for T_{max} was more than 50%, which suggested that the inter-subject variability of sumatriptan after oral administration. The C_{max} values were varied between 216.25 and 1045.11 ng/ml for both preparations after a single oral administration of sumatriptan. The probable reason for variability in C_{max} values between subjects may be due to the inter-subject variability in plasma concentrations (Hiremath, 2000). The resulted higher %CV values (>40%) also represent the inter-subject variability of sumatriptan after oral administration. High inter-subject variability might be due to variability of gastric emptying and small intestine transit time (Rani et al., 1996). The resulted $t_{1/2}$ values demonstrated that a washout period of 1 week was sufficient due to the fact that no plasma sample showed any sumatriptan levels at zero hours of blood collection in the phase 2 experiment. The $AUC_{0-\infty}$ values in individual rabbits varied between 909.79 and 4736.62 ng.h/ml for both preparations. The $AUC_{t-\infty}$ values calculated were found to be less than 1% of the $AUC_{0-\infty}$. It indicates that the sample collection duration was sufficient for calculating at least 80% of $AUC_{0-\infty}$ and provided a reliable estimation of extent of absorption. When compared statistically, there was no significant difference ($P > 0.05$) between the pharmacokinetic variables of two formulations.Thus, reference and test formulations were bioequivalent in their rate and extent of absorption.

Conclusions

A new, sensitive and specific isocratic HPLC method was developed and validated for the determination of sumatriptan in rabbit plasma. The validated method showed satisfactory data for all the validation parameters tested. The previous reported fluorescence method had similar limit of quantitation (1 ng/ml) but lack of specificity restrict its pharmacokinetic application. Hence, the developed method could be an alternative to the reported method for the analysis of sumatriptan in plasma samples. The method was validated for sumatriptan concentration in the range of 1 to 300 ng/ml. This range is suitable for measuring sumatriptan in plasma samples after an oral administration of 50 mg tablet in pharmacokinetic study to rabbits. This study demonstrates inter-subject variability between rabbits associated with double and multiple peaks of plasma concentrations after a single oral administration of sumatriptan. The results of pharmacokinetic variables were comparable between the two formulations. Thus, reference and test formulations are bioequivalent in their rate and extent of absorption.

ACKNOWLEDGEMENT

The authors would like to thank Universiti Sains Malaysia for providing research grant (304/PFarmasi/638093) to support this work. The author (Ravi Sheshala) gratefully acknowledges Universiti Sains Malaysia, Penang, Malaysia for the fellowship provided.

REFERENCES

Andrew PD, Birch HL, Phillpot DA (1993). Determination of sumatriptan succinate in plasma and urine by high-performance liquid chromatography with electrochemical detection. J. Pharm. Sci., 82: 73-76.

Balaguer-Fernandez C, Femenia-Font A, Rio-Sancho SD, Merino V, Lopez-Castellano A (2008). Sumatriptan succinate transdermal delivery systerms for the treatment of migraine. J. Pharm. Sci., 97: 2102-2109.

Barrow A, Dixon CM, Saynor DA, Perren MJ, Stowe R, Smith I (1997). The absorption, pharmacodynamics, metabolism and excretion of ^{14}C-sumatriptan following intranasal administration to beagle dog. Biopharm. Drug Dispos., 18: 443-458.

Biddlecombe RA, Benevides C, Pleasance S (2001). A clinical trial on a plate? The potential of 384-well format solid phase extraction for high-throughput bioanalysis using liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass. Spectr., 15: 33-40.

Boulton DW, Duncan GF, Vachharajani NN (2003). Validation and application of high-performance liquid chromatography/tandem mass spectroscopy assay for sumatriptan in human plasma. Biomed. Chromatogr., 17: 48-52.

Carpay J, Schoenen J, Ahmad F, Kinrade F, Boswell D (2004). Efficacy and tolerability of sumatriptan tablets in a fast-disintegrating, rapid-release formulation for the acute treatment of migraine: results of a multicenter, randomized, placebo-controlled study. Clin. Ther., 26: 214-223.

Cheng KN, Redrup MJ, Barrow A, Williams PN (1998). Validation of a liquid chromatographic tandem mass spectrometric method for the determination of sumatriptan in human biological fluids. J. Pharm. Biomed. Anal., 17: 399-408.

Christensen ML, Mottern RK, Jabbour JT, Fuseau E (2003). Pharmacokinetics of sumatriptan nasal spray in adolescents. J. Clin. Pharmacol., 43: 721-726.

Committee for Proprietary Medicinal Products (CPMP) (2001). Note for Guidance on Investigation of Bioavailability and Bioequivalence, London, UK. Available at: http://www.egagenerics.com/doc/emea_bioequiv-1401-98.pdf.

Dahlof CG (2001). Sumatriptan: Pharmacological basis and clinical results. Curr. Med. Res. Opin., 17: S35-S45.

Dixon CM, Saynor DA, Andrew PD, Oxford J, Bradbury A, Tarbit MH (1993). Disposition of sumatriptan in laboratory animals and humans. Drug Metab. Dispos., 21: 761-768.

Dulery BD, Petty MA, Schoun J, David M, Huebert ND (1997). A method using a liquid chromatographic-electrospray-mass spectrometric assay for the determination of antimigraine compounds: preliminary pharmacokinetics of MDL 74,721, sumatriptan and naratriptan in rabbit. J. Pharm. Biomed. Anal., 15: 1009-1020.

Dunne M, Andrew P (1996). Fully automated assay for the determination of sumatriptan in human serum using solid-phase extraction and high-performance liquid chromatography with electrochemical detection. J. Pharm. Biomed. Anal., 14: 721-726.

Ferrari A, Pinetti D, Bertolini A, Coccia C, Sternieri E (2008). Interindividual variability of oral sumatriptan pharmacokinetics and of clinical response in migraine patients. Eur. J. Clin. Pharmacol., 64: 489-495.

Fowler PA, Lacey LF, Thomas M, Keene, ON, Tanner RJ, Barber NS (1991). The clinical pharmacology, pharmacokinetics and metabolism of sumatriptan. Eur. Neurol., 31: 291-294.

Fowler PA, Thomas M, Lacey LF, Andrew P, Dallas FA (1989). Early studies with the novel 5-HT1-like agonist GR43175 in healthy volunteers. Cephalalgia, 9: 57–62.

Franklin M, Odontiadis J, Clement EM (1996). Determination of sumatriptan succinate in human plasma by high-performance liquid chromatography with coulometric detection and utilization of solid-phase extraction. J. Chromatogr. B., 681: 416-420.

Ge Z, Tessier E, Neirinck L, Zhu Z (2004). High performance liquid chromatographic method for the determination of sumatriptan with fluorescence detection in human plasma. J. Chromatogr. B., 806: 299-303.

Hiremath SRR (2000). Textbook of Biopharmaceutics and Pharmacokinetics. Prism Books: Bangalore, India, pp. 32-34.

Humphrey PPA, Feniuk W, Marriott AS, Tanner RJN, Jackson MR, Tucker ML (1991). Preclinical studies on the anti-migraine drug, sumatriptan. Eur. Neurol., 31: 282-290.

Lacey LF, Hussey EK, Fowler PA (1995). Single dose pharmacokinetics of sumatriptan in healthy volunteers. Eur. J. Clin. Pharmacol., 47: 543-548.

Majithiya RJ, Majithiya JB, Umrethia ML, Ghosh PK, Murthy RSR (2006). HPLC method for the determination of sumatriptan in plasma and brain tissue. Ars. Pharm., 47: 199-210.

McLoughlin DA, Olah TV, Ellis JD, Gilbert JD, Halpin RA (1996). Quantitation of the 5HT1D agonists MK-462 and sumatriptan in plasma by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. A., 726: 115-124.

Nation RL, Sansom LN (1994). Bioequivalence requirements for generic products. Pharmacol. Ther., 62: 41-55.

Rani PU, Naidu MUR, Kumar TR, Shobha JC, Vijay T, Sekhar KR, Sekhar EC (1996). A bioequivalence study of two brands of sumatriptan tablets. Curr. Ther. Res., 57: 589-598.

Ravi S, Darwis Y, Khan N (2009). Development and validation of an RP-HPLC-UV method for analysis of sumatriptan succinate in pharmaceutical dosage forms. Acta Chromatogr., 21: 421-432.

Sankalia JM, Sankalia MG, Sutariya VB, Mashru RC (2007). Nateglinide quantification in rabbit plasma by HPLC: Optimization and application to pharmacokinetic study. J. Pharm. Biomed. Anal., 44: 196-204.

Sifrim D, Holloway RH, Tack J, Zelter A, Missotten T, Coulie B, Janssens J (1999). Effect of sumatriptan, a $5HT_1$ agonist, on the frequency of transient lower esophageal sphincter relaxation and gastroesophageal reflux in healthy subjects. Am. J. Gastroenterol., 94: 3158-3164.

USFDA (2001). Guidance for Industry: Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Rockville, MD. Available at: www.fda.gov/cder/guidance/index.htm.

USFDA (2003). Guidance for Industry: Bioavailability and bioequivalence studies for orally administered drug products-General considerations. US Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Rockville, MD. Available at: www.fda.gov/cder/guidance/index.htm.

Vingerhagen S, Hauksen T, Gilja OH, Berstad A (2000). Influence of $5HT_1$ receptor agonist on gastric accommodation and initial transpyloric flow in healthy subjects. Neurogastroent. Motil., 12: 95-101.

Vishwanathan K, Bartlett MG, Stewart JT (2000). Determination of antimigraine compounds rizatriptan, zolmitriptan, naratriptan and sumatriptan in human serum by liquid chromatography electrospray tandem mass spectrometry. Rapid Commun. Mass Spectr., 14: 168-172.

Wagner JG (1975). Statistics: Fundamentals of clinical pharmacokinetics. Drug Intelligence Publications: Hamilton, USA, pp. 285-305.

Zhu H, Zhou H, Seitz K (2009). Bioavailability and Bioequivalence. In: Qiu Y, Chen Y and Zhang GGZ (eds) Developing Solid Oral Dosage Forms: Pharmaceutical Theory and Practice, Academic Press, USA, pp. 341-364.