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Development and validation of a Sensitive bioanalytical method for the quantitative estimation of Pantoprazole in human plasma samples by LC–MS/MS: Application to bioequivalence study

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ABSTRACT

The present study aims at developing a simple, sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the quantification of pantoprazole sodium (PS) in human plasma using pantoprazole D3 (PSD3) as internal standard (IS). Chromatographic separation was performed on Zorbax SB-C18, 4.6 mm × 75 mm, 3.5 μm, 80 Å column with an isocratic mobile phase composed of 10 mM ammonium acetate (pH 7.10): acetonitrile (30:70, v/v), pumped at 0.6 mL/min. PS and PSD3 were detected with proton adducts at m/z 384.2 → 200.1 and 387.1 → 203.1 in multiple reaction monitoring (MRM) positive mode, respectively. Precipitation method was employed in the extraction of PS and PSD3 from the biological matrix. This method was validated over a linear concentration range of 10.00–3000.00 ng/mL with correlation coefficient (r) ≥ 0.9997. Intra- and inter-day precision of PS were found to be within the range of 1.13–1.54 and 1.76–2.86, respectively. Both analytes were stable throughout freeze/thaw cycles, bench top and postoperative stability studies. This method was successfully utilized in the analysis of blood samples following oral administration of PS (40 mg) in healthy human volunteers.

1. Introduction

Pantoprazole sodium (PS) is a substituted benzimidazole, sodium 5-(difluromethoxy)-2-[[(3,4-dimethoxy-2-pyridinyl)methyl]sulfinyl]-1*H*-benzimidazole sesquihydrate with a molecular weight of 426.52 Da (Fig. 1) [1]. It belongs to a class of proton pump inhibitors (PPIs) and is indicated for the treatment of ulceration in the esophagus caused by gastroesophageal reflux disease. PS is an acid-activated prodrug which is capable of reacting with free thiol (–SH) groups present on the ATPase enzyme. The covalent binding of PPIs to H^+/K^+-ATPase irreversibly inhibits hydrogen ion transport [2–4]. Following intravenous or oral administration, PS is extensively metabolized in the liver via cytochrome P450 system. It has an absolute bioavailability of ~77% with a peak plasma concentration (C_{max}) and $t_{1/2}$ of 2.5 μg/mL and 1.1 h, respectively. It is extensively metabolized in the liver by phase I and phase II metabolism [5].

Several techniques such as titrimetry, spectrophotometry and liquid chromatography (LC) methods have been reported in literature for the quantitative estimation of PS in pharmaceutical and biological fluids. Titrimetry and spectrophotometry suffers from disadvantages such as narrow linearity range, low sensitivity and lack of selectivity. Moreover, spectrophotometry involves a tedious extraction procedure involving too many steps. This may result in inaccurate and imprecise results [6]. Patel et al. [7] quantified the pantaprazole, rabeprazole, Esomeprazole, Domperidone and Itopride in pharmaceutical products by RP-HPLC. Cass et al. [8] quantified the PS enantiomers in human plasma using a multidimensional high-performance liquid chromatography (HPLC) with an UV detector at 285 nm. They observed a good linearity between the concentration ranges of 0.20–1.5 μg/mL.

Abbreviations: LC–MS/MS, liquid chromatography–tandem mass spectrometry; MRM, multiple reaction monitoring; PS, pantoprazole sodium; PSD3, pantoprazole D3; PPIs, proton pump inhibitors; LLOQ, lower limit of quantification; LOQ, Limit of Quantification; LQC, Lower Quality control; MQC, Middle Quality control; HQC, High Quality control.

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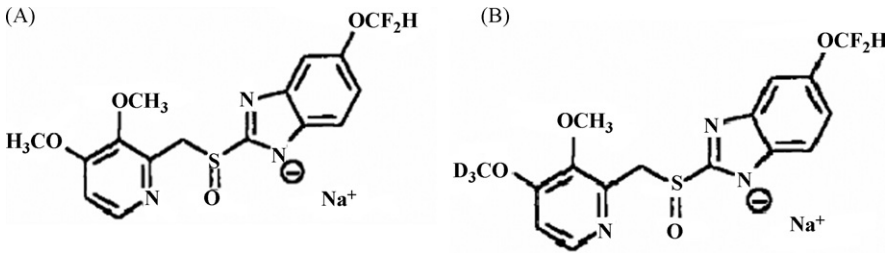

Fig. 1. Chemical structures of (A) pantoprazole sodium and (B) pantoprazole sodium D3 (IS).

However, the run time of each sample was ~28 min which is very tedious and time consuming. Nowadays, LC–MS/MS has gained importance for the quantitative estimation of drugs in various biological matrices including plasma, serum, urine, and ocular fluids, due to its high sensitivity, selectivity and reproducibility [9,10]. LC–MS/MS is an analytical tool which combines the physical separation capabilities of liquid chromatography and analytical capabilities of mass spectrometry. Generally, its application is oriented towards the specific detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture) [5].

In the present study, we have reported a high sensitive, selective and reproducible analytical method for the determination of PS in plasma samples utilizing liquid chromatography coupled to electrospray (ES) tandem mass spectrometry. Deuterated compound pantoprazole D3 (PSD3) was used as an internal standard. We have developed and validated the method as per the FDA guidelines over a concentration range of 10.00–3000.00 ng/mL using a simple precipitation technique for extraction of drug and internal standard. This method was successfully employed in the analysis of human plasma samples following oral administration of PS (40 mg) in healthy human volunteers [12].

2. Experimental

2.1. Materials and reagents

Pantoprazole Sodium (PS) was purchased from Apotex Inc, Canada and Pantoprazole D3 (PSD3) (internal standard, IS) was obtained from Synfine Research, Canada. Formic acid was purchased from Merck (Darmstadt, Germany). HPLC grade methanol and acetonitrile were purchased from Jt. Baker Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA. Ammonium acetate (reagent grade) was purchased from Merck Limited, Worli, Mumbai. Ammonia solution (25%, w/w) was purchased from Merck Speciality private limited, Worli, Mumbai. Human plasma was obtained from Biological Speciality Corporation, USA. Ultrapure water from Milli-Q-system (Millipore, Bedford, MA, USA) was used through the study. All other chemicals in this study were of analytical grade.

2.2. Instrumentation and chromatographic conditions

An aliquot of the plasma extract (5 μL) was injected into Zorbax SB-C18, 4.6 mm × 75 mm, 3.5 μm 80 Å column. Separation and elution were achieved using 10 mM ammonium acetate (pH 7.10): acetonitrile (30:70, v/v) as the mobile phase, at a flow-rate of 0.6 mL/min. The 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany) is equipped with G1312A binary pump, G1322A degasser, G1367A autosampler, G1330B thermostat, G1316A thermostated column compartment and G1323B control module. The column was placed at room temperature (25–30 °C) and run time between injections was 2.5 min. Mass spectrometric detection was performed using Applied Bio system/MDS SCIEX LC system API 4000 model (Toronto, Canada). A triple quadrupole mass spectrometer, equipped with an electrospray source interface in positive ionization mode was used for detection. Data processing was performed using Analyst 1.4.1 software package (SCIEX). The source temperature was maintained at 400 °C and the ion source consisted of gas channels, nebulizer gas 35 psi, heater gas/desolvation gas 45 psi, curtain gas 25 psi and collisionally activated dissociation (CAD) gas 4 psi (nitrogen). The electrospray source was operated in the positive ionization mode (ES+) at 5500 V. Quantitative determination was performed in multiple reaction monitoring (MRM) scan mode using the following transitions m/z 384.2 → 200.1 and m/z 387.1 → 203.2 for quantification of PS and PSD3, respectively.

2.3. Preparation of standards and quality control (QC) samples

Stock solution of PS (1000 μg/mL) was prepared in methanol. From this 400.00 μg/mL dilution was prepared in 25% methanol containing 3.5% ammonium hydroxide. Further dilutions (6000.00, 4000.00 and 200.00 ng/mL) were made in plasma. Aliquots of 6000.00, 4000.00 and 200.00 ng/mL were used to spike blank human plasma in order to obtain calibration curve standards of 10.00, 20.00, 100.00, 300.00, 600.00, 1200.00, 1800.00, 2400.00, and 3000.00 ng/mL Dilution integrity sample (4500.00 ng/mL) in plasma was prepared from 6000.00 ng/mL sample. Three levels of QCs at concentrations of 30.00, 900.00, and 2100.00 ng/mL (low, medium and high) were prepared by using the same blank plasma. According to the clinical protocol, spiked calibration curve standards and QCs were stored at. −10 to −30 °C. Stock solutions of PSD3 (100.00 μg/mL) were prepared in methanol. PSD3 was further diluted to 500.00 ng/mL using 40% acetonitrile in 10 mM ammonium acetate (pH 7.10).

2.4. Sample preparation

A sample volume of 100 μL was taken into polypropylene eppendroff tubes (ETs), and mixed with 100 μL of internal standard (500.00 ng/mL). Protein precipitation was carried out by addition of 0.8 mL of acetonitrile and vortexed for ~3 min. ETs were centrifuged at 4000 rpm for 10 min at 10 °C. Further, 200 μL of supernatant was transferred into labeled polypropylene tubes vials containing 200 μL of 10 mM ammonium acetate at pH 7.10 and vortexed. Sample volume of 5 μL was injected into the HPLC system connected to mass spectrometer.

2.5. Recovery

Recovery of PS was evaluated by comparing the mean peak area of six extracted low, medium and high quality control samples (30.00, 900.00, and 2100.00) to mean peak area of six extracted-spiking drug free-plasma samples with the same amount of low, medium and high PS quality control samples. Similarly Recovery of PSD3 was evaluated by comparing the mean peak area of six

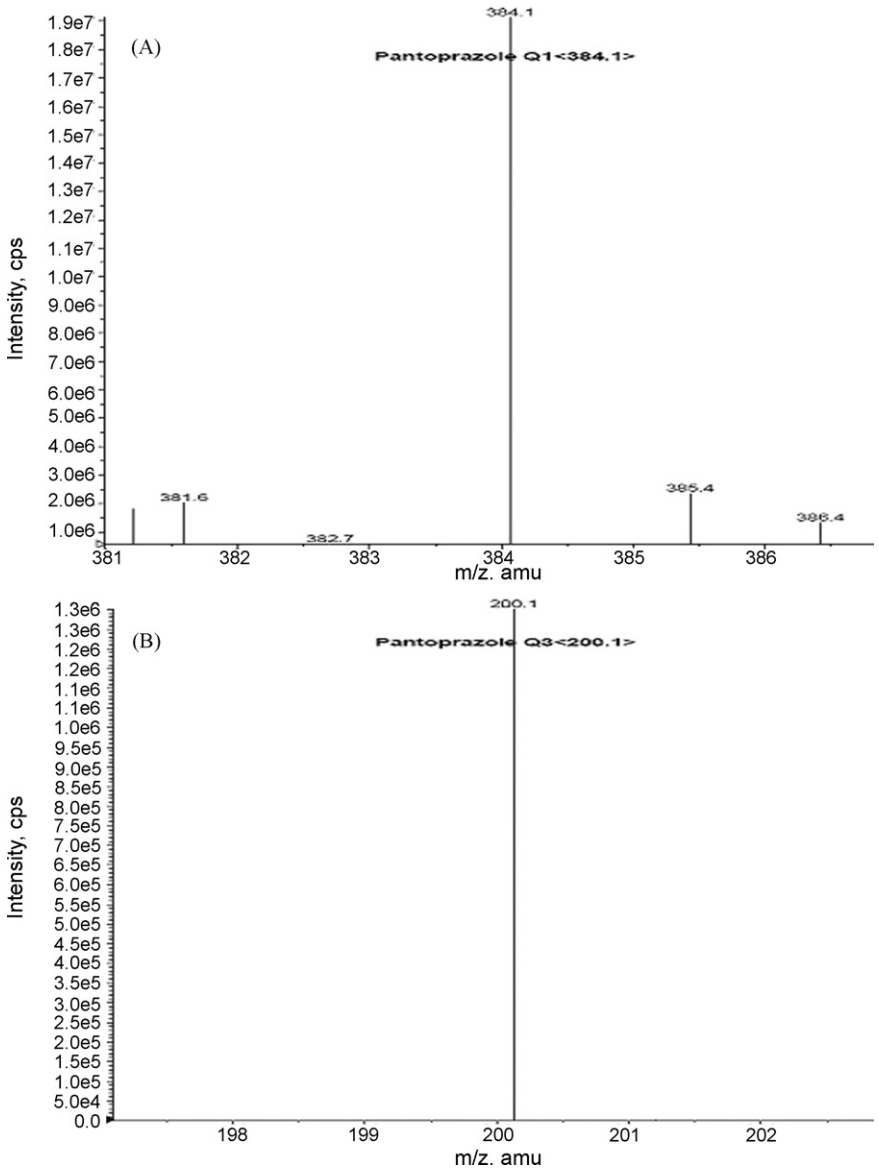

Fig. 2. Mass spectra of (A) pantoprazole Q1 scan and (B) fragmentation of pantoprazole.

extracted quality control samples to mean peak area of PSD3 in samples prepared by spiking extracted drug free-plasma samples with the same amount of PSD3.

2.6. Selectivity

The selectivity of the method was determined by, blank human plasma samples from six different healthy volunteers were pre-treated and analyzed to test the potential interferences of endogenous compounds co-eluting with analyte and IS. Chromatographic peaks of analytes and IS were identified on the basis of their retention times and MRM Responses. The peak area of PS at respective RT in blank samples should not be more than 20% of mean peak area of LOQ of PS. Similarly peak area of PSD3 at respective RT in blank sample should not be more than 5% of mean peak area of LOQ of PSD3.

2.7. Limit of quantification (LOQ)

Limit of quantification was estimated in accordance with baseline noise method. The LOQ was estimated at a signal-to-noise ratio (S/N) of 10. LOQ was experimentally performed by six injections of PS at LOQ concentration.

2.8. Analytical curves

The analytical curves of PS were constructed in the concentrations ranging from 10.00 to 3000.00 ng/mL in human plasma. Calibration curves were obtained by weighted linear regression (weighing factor: $1/x^2$). The ratio of PS peak area to PSD3 peak area was plotted against the ratio of PS concentration to that of PSD3 concentration in ng/mL. The fitness of calibration curve was confirmed by back-calculating the concentrations of calibration standards.

2.9. Calibration curve standards, regression model, precision, and accuracy batches

Calibration curve standard samples were prepared in duplicates and QC samples were prepared in replicates ($n = 6$) for analysis. Correlation coefficient (>0.9997) was obtained by using simple linear regression model in whole range of tested concentrations. Accu-

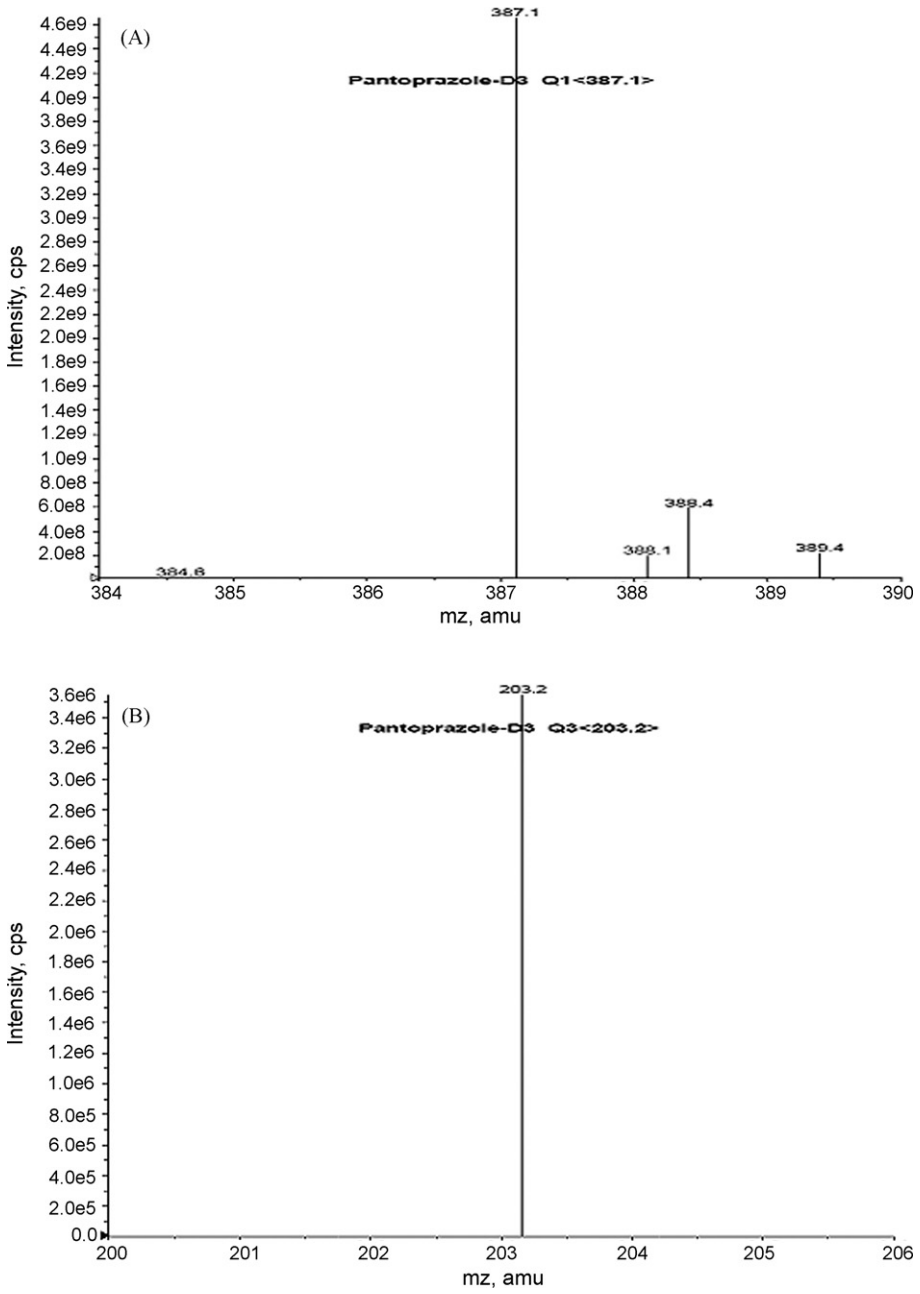

Fig. 3. Mass spectra of (A) pantoprazole D3 Q1 scan and (B) fragmentation of pantoprazole D3.

racy and precision for the back calculated concentrations of the calibration points, should be within ±15% of their nominal values. However, for LLOQ the precision and accuracy should be within ±20%.

2.10. Stability

Low QC and high QC samples ($n = 6$) were retrieved from deep freezer after three freeze/thaw cycles according to the clinical protocols. Samples were stored at −10 to −30 °C in three cycles of 24, 36 and 48 h**.** In addition, the long-term stability of PS in QC samples was also evaluated after 55 days of storage at −10 to −30 °C. Autosampler stability at refrigerated temperature was studied following 55.5 hr storage period in the autosampler tray. Bench top stability was studied for 48 h period. Stability samples were processed and extracted along with the freshly spiked calibration curve standards. The precision and accuracy for the stability samples must be within 15 and ±15%, respectively, of their nominal concentrations.

2.11. Matrix effect

The matrix effect due to plasma matrix was used to evaluate ion suppression/enhancement in a signal when comparing the absolute response of QC samples after pretreatment (precipitation with acetonitrile) with the absolute response of reconstitution samples extracted blank plasma sample spiking with analyte. Experiments were performed at low, high quality control levels in triplicate. The acceptable precision (%CV) should be ≤15%.

2.12. Analysis of patient samples

The above described bioanalytical method was used to determine plasma concentrations of PS following oral administration

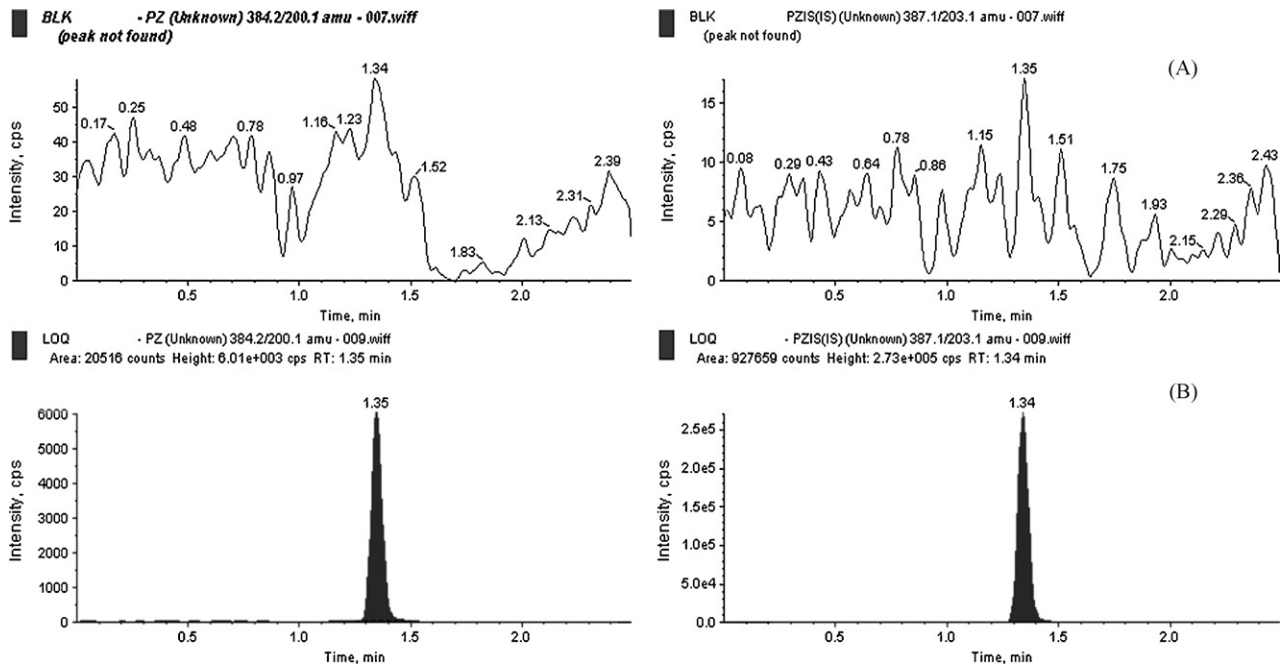

Fig. 4. Chromatograms of pantoprazole and pantoprazole D3 (IS) in human plasma: (A) blank human plasma and (B) spiked human plasma containing 10.00 ng/mL pantoprazole and 500.00 ng/mL pantoprazole D3.

in healthy human volunteers. The volunteers were contracted in (Apotex Research Pvt. Ltd, Bangalore, India), and each subject was administered 40 mg dose (one 40 mg tablet) in 29 healthy volunteers.

2.13. Pharmacokinetics and statistical analysis

Pharmacokinetics parameters from the human plasma samples were calculated by a non-compartmental statistics model using WinNon-Lin5.0 software (Pharsight, USA). Blood samples were taken for a period of 3–5 times the terminal elimination half-life ($t_{1/2}$). As per FDA guidelines, the ratio of area under concentration time curves (AUC) for test and reference in the range of 80–125% were considered to be bioequivalent. Plasma PS concentration–time profiles were visually inspected and C_{max} and T_{max} values were determined. The AUC_{0-t} was obtained by trapezoidal method. $AUC_{0-\infty}$ was calculated up to the last measurable concentration and extrapolations were obtained using the last measurable concentration and the terminal elimination rate constant (K_e). The slope of the terminal exponential phase obtained from plasma of PS concentration–time curve by means of the linear regression method was utilized for calculation of K_e. The terminal elimination half-life ($t_{1/2}$) was then calculated as $0.693/K_e$. Regarding AUC_{0-t} and C_{max} bioequivalence was assessed by means of analysis of variance (ANOVA) and calculating the standard 90% confidence intervals (90% CIs) of the ratios test/reference (logarithmically transformed data). Bioequivalence was considered when the ratio of averages of log transformed data was within 80–125% for AUC_{0-t} and C_{max} [10].

3. Results and discussion

3.1. Method development

LC–MS/MS has been used as one of the most powerful analytical tool in clinical pharmacokinetics for its selectivity, sensitivity and reproducibility. The goal of this work was to develop and validate a simple, rapid and sensitive assay method for the quantitative determination of PS from plasma samples. A simple extraction technique was utilized in the extraction of PS and PSD3 from the plasma samples. Chromatographic conditions, especially the composition and nature of the mobile phase, were optimized through several trials to achieve best resolution and increase the signal of PS and PSD3. The MS optimization was performed by direct infusion of solutions of both PS and PSD3 (IS) into the ESI source of the mass spectrometer. The vital parameters in the ESI source consisting of the needle (ESI) voltage, is directly related to charged droplet formation and the amount of gaseous ions formed [11]. Other parameters, such as the nebulizer and the desolvation gases were optimized to obtain a better spray shape, resulting in better ionization and droplet drying to form, in our case, the protonated ionic PS and PSD3 (IS) molecules (Fig. 1). A CAD product ion spectrum for PS and PSD3 yielded high-abundance fragment ions of m/z 200.1 and m/z 203.2, respectively (Figs. 2 and 3). After the MRM conditions were optimized and the mobile phase was changed from an aqueous phase to a more organic phase. A good separation and elution was achieved using 10 mM ammonium acetate (pH 7.10): acetonitrile (30:70, v/v) as the mobile phase, at a flow-rate of 0.6 mL/min and injection volume of 5 μL.

3.2. Selectivity and specificity

The selectivity of the method assessed by comparing chromatograms of a blank plasma (Fig. 4A), Spiked plasma (Fig. 4B). The retention times were 1.35 and 1.34 min for PS and PSD3, respectively (Fig. 4B). As shown in the figure, there were no significant endogenous peaks. That could interfere with the PS and PSD3. The results indicate that the method exhibited both good specificity and selectivity.

3.3. Limit of quantification (LOQ)

The LOQ signal-to-noise (S/N) values found for six injections of PS at LOQ concentration was 97.43. It can be concluded that the sensitivity is more for this method.

Table 1
Calibration curve details of the validation section.

Spiked plasma concentration (ng/mL)	Concentration measured (mean) (ng/mL)	Precision (%CV) or RSD[a] ($n = 5$)	Accuracy %
10.00	10.00	0.00	100.00
20.00	20.00	0.50	100.00
100.00	98.10	1.28	98.10
300.00	303.00	1.73	101.00
600.00	604.56	2.54	100.76
1200.00	1202.76	1.85	100.23
1800.00	1806.04	1.30	100.34
2400.00	2393.00	2.07	99.70
3000.00	2991.84	1.04	99.73

[a] [Standard deviation/mean concentration measured] × 100.

Table 2
Precision and accuracy (analysis with spiked plasma samples at three different concentrations).

Spiked plasma concentration (ng/mL)	Within-run ($n = 6$)			Between-run ($n = 30$)		
	Concentration measured (ng/mL) (mean ± S.D.)	Precision (%CV) or RSD[a]	Accuracy %	Concentration measured (ng/mL) (mean ± S.D.)	Precision (%CV) or RSD[a]	Accuracy %
30.00	27.33 ± 0.45	1.53	90.99	27.89 ± 0.80	2.86	92.95
900.00	859.73 ± 13.21	1.54	95.53	862.24 ± 14.36	1.67	95.80
2100.00	1955.55 ± 22.07	1.13	93.10	1982.36 ± 34.90	1.76	94.40

[a] [Standard deviation/mean concentration measured] × 100.

3.4. Matrix effect

The ion suppression/enhancement in the signal were found %CV 1.88 at low QC level and %CV 0.51 at high QC level for PS, indicating that the matrix effect on the ionization of analytes is not obvious under these conditions.

3.5. Linearity, precision and accuracy

Calibration curves were plotted as the peak area ratio (PS/PSD3) versus PS concentration. Calibration was found to be linear over the concentration range of 10.00–3000.00 ng/mL. The precision was less than 2.54% and the accuracy of the mean of measured concentrations ranged from 98.10 to 101.00%. The correlation coefficient (r^2) was greater than 0.9997 for all curves (Table 1). Precision and accuracy for this method were controlled by calculating the intra- and inter-batch variations at three concentrations (30.00, 900.00 and 2100.00 ng/mL) of QC samples in six replicates. As shown in Table 2, the intra-batch precision and accuracy were between 1.13 and 1.54 and 90.99 and 95.53%. Similarly the inter-batch precision and accuracy were between 1.67 and 2.86 and 98.10 and 100.76%. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

3.6. Stability

PS in plasma was subjected to three freeze/thaw (−10 to −30 °C to room temperature) cycles. The obtained accuracy of PS was between 99.0 and 104.0% of the theoretical values. No significant degradation of the PS was observed even after 55.5 h storage period in the autosampler tray and the final concentrations of PS was between 97.0 and 101.0% of the theoretical values. In addition, the long-term stability of PS in QC samples after 55 days of storage at −10 to −30 °C and room temperature stability for 48 h was also evaluated. The concentrations ranged from 98.0 to 102.0% for long-term stability and 98.0 to 101.0% for autosampler stability studies. These results confirmed the stability of PS in human plasma for at least 55 days at −10 to −30 °C (Table 3).

3.7. Recovery

The extraction recoveries of PS was determined at three different concentrations 30.00, 900.00 and 2100.00 ng/mL, were found to be 98.17 ± 10.078, 97.52 ± 6.846 and 98.33 ± 8.007%, respectively. The overall average recoveries of PS and PSD3 were found to be 98.01 ± 7.915 and 109.57 ± 14.177%. Recoveries of the analyte and IS were high and were consistent precise and reproducible.

Table 3
Stability of pantaprazole in human plasma samples.

Spiked plasma concentration (ng/mL)	Concentration measured ($n = 6$) (ng/mL) (mean ± S.D.)	Precision (%CV) (or) RSD[a] ($n = 6$) (%)	Accuracy (%)
Bench top stability at room temperature for 48 h			
30.00	29.91 ± 0.88	2.95	99.70
2100.00	2004.48 ± 10.05	0.50	95.45
Auto sampler stability at refrigerated condition for 55.5 h			
30.00	29.66 ± 0.40	1.36	98.87
2100.00	1986.48 ± 27.16	1.37	94.59
Long-term stability at −10 to −30 °C for 55 days			
30.00	29.52 ± 0.56	1.89	98.40
2100.00	2004.45 ± 30.43	1.52	95.45
Freeze/thaw stability for 3 cycles (−10 to −30 °C)			
30.00	28.83 ± 0.33	1.15	96.10
2100.00	1958.51 ± 27.29	1.39	93.26

[a] [Standard deviation/mean concentration measured] × 100.

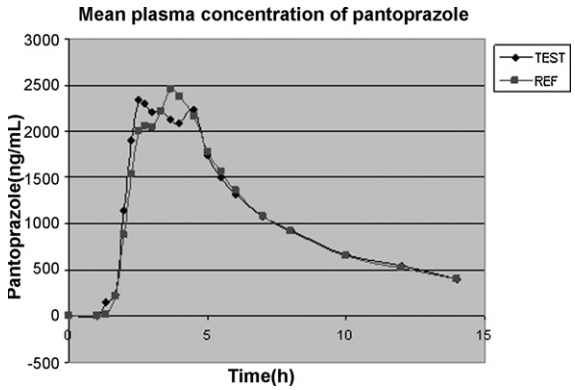

Fig. 5. Mean plasma concentrations of test vs. reference after a 40 mg dose (one 40 mg tablet) single oral dose (29 healthy volunteers).

3.8. Application to biological samples

The above validated method was used in the determination of PS in plasma samples for establishing the bioequivalence of a single 40 mg dose (one 40 mg tablet) in 29 healthy volunteers. Typical plasma concentration versus time profiles is shown in Fig. 5. All the plasma concentrations of PS were in the standard curve region and remained above LOQ (10 ng/mL) for the entire sampling period. The maximum plasma concentration (C_{max}) for the test and the reference were found to be 2333.40 and 2457.92 ng/mL, respectively. The corresponding time of maximum concentration (T_{max}) for the test and the reference were found to be 2.50 and 3.66 h, respectively. The value of AUC from t_0 to t_{last} (AUC_{0-t}) for the standard and test were found to be 13859.27 and 14097.54 ng h/mL, respectively. And the area under the curve from 0 to ∞ ($AUC_{0-\infty}$) was 16557.31 ng h/mL for the standard and 16946.94 ng h/mL for the test. The elimination half-life ($t_{1/2}$) was 4.66 h for the reference drug and 4.95 h for generic drug. In addition, the mean ratio of $AUC_{0-t}/AUC_{0-\infty}$ was higher than 90% following the FDA Bioequivalence Guidelines [8]. The ratio of test/reference (T/R) and 90% confidence intervals (90 CIs) for overall analysis was in the range 80–125%. The ratio T/R and 90 CIs (in parenthesis) were 94.93% for C_{max}, 101.72% for AUC_{0-t} and 102.35% for $AUC_{0-\infty}$. Therefore, it can be concluded that the two pantoprazole formulations (reference and test) analyzed are bioequivalent in terms of rate and extent of absorption.

4. Conclusion

In this article we have reported the use of LC–MS/MS for the accurate, precise and reliable measurement of PS concentrations in human plasma after oral administration of 40 mg to healthy volunteers. The method described here is fast, robust, and sensitive. Each sample requires less than 3 min of analysis time. The assay method is also highly specific due to the inherent selectivity of tandem mass spectrometry and has significant advantages over other techniques previously described for measuring pantoprazole in biological fluids. The sensitivity of the assay is sufficient to follow accurately the pharmacokinetics of PS following oral administration.

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