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RESEARCH ARTICLE

Simultaneous Quantification of Pantoprazole and Levosulpiride in Spiked Human Plasma Using High Performance Liquid Chromatography Tandem Mass Spectrometry

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Abstract: ***Background:*** Pantoprazole (PTZ) and Levosulpiride (LS) were proven as effective agents for the treatment of Gastro-Esophageal Reflux Disease (GERD). It is a complex motor disorder that results in regurgitation of the gastric contents into the lower esophagus with consequent symptoms such as heart burn, retrosternal pain, dysphagia and belching

Methods: A rapid, sensitive, selective and specific liquid chromatography- electro spray ionization tandem mass spectrometry (LC-MS/MS) method was developed for the simultaneous quantification of Pantoprazole (PTZ) and Levosulpiride (LS) in spiked Human Plasma. The method utilized SPE as sample preparation technique and the analysis was carried out on a HPLC system utilizing electro spray ionization as interface and triple quadrupole mass analyzer for quantification in MRM mode in positive mode. Iloperidone was used as internal standard (IS). Chromatographic separation was performed on a Phenomenex C-18 Column (4.6 mm x 50 mm, 5µ) with an isocratic elution mode utilizing a mobile phase composition of Solution containing a mixture of 70 volumes of acetonitrile: 30 volumes of methanol and 10mM ammonium formate (pH 4.0) at the ratio of 80:20 % v/v. The flow rate was maintained at 0.3 mL/min.

Results: PTZ, LS and IS were detected and quantified with proton adducts at m/z 383.37→200.00, m/z 341.42→112.15 and 426.48→261.00 respectively. The linearity and range was established by fortifying blank plasma samples in the concentration range of 3.5-2000 ng/mL for PTZ and 0.3-2400 ng/mL for LS. The correlation coefficient (r2) was found to be ≥ 0.993 for PTZ and (r2) ≥ 0.990 for LS. The lower limit of quantification for PTZ was 3.5 ng/mL and LS was 3.0 ng/mL. The intra and inter day precision and accuracy for PTZ and LS were within the limits fulfilling the international acceptance criteria. PTZ and LS were found to be stable throughout three freeze-thaw cycles, bench top and short term stability studies.

Conclusion: The proposed validated LC-MS/MS method offers a sensitive quantification of PTZ and LS in spiked human plasma and can be utilized for the quantification of PTZ and LS in real-time samples.

ARTICLE HISTORY

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1. INTRODUCTION

Pantoprazole, (PTZ), a substituted benzimidazole derivative is an irreversible proton pump inhibitor used for short treatment of acid related gastrointestinal disorders. It is chemically 6-(difluoromethoxy)-2-[(3, 4-dimethoxypyridin-2-yl) methanesulfinyl]-1H-1, 3-benzodiazole. PTZ acts by suppressing the final step in gastric acid production by covalently binding to the (H^+, K^+)-ATPase enzyme system at the secretory surface of the gastric parietal cell [1, 2].

Levosulpiride, (LS) an L-enantiomer of sulpiride is chemically N-[(1-ethylpyrrolidin-2-yl) methyl]-2-methoxy-5-sulfamoylbenzamide. It is a substituted benzamide atypical

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antipsychotic agent used to treat anxiety disorders, schizophrenia, depression and peptic ulcers [3, 4]. LS acts by blocking the presynaptic dopaminergic D_2 receptors [5].

PTZ and LS were proven as effective agents for the treatment of Gastro-Esophageal Reflux Disease (GERD) [6 &7]. It is a complex motor disorder that results in regurgitation of the gastric contents into the lower esophagus with consequent symptoms such as heart burn, retrosternal pain, dysphagia and belching [6]. The combination of PTZ and LS are available in the form of tablets and capsules in the market.

In Literature, there are few analytical methods available for the quantification of PTZ and LS. Various HPLC and liquid chromatography tandem mass spectrometry methods are available for the analysis of selected drug candidates either individually or in combination with other drugs in biological samples.

A validated LC-MS/MS method for the quantification of pantoprazole in human plasma and its application to bioequivalence study was reported by Challa and his coworkers [8]. Also, Yun Li and coworkers [9] reported an LC-MS/MS method for the quantification of pantoprazole in human plasma. The method was used for the quantification of the same during pharmacokinetic and bioequivalence study. Ling J and coworkers [10] reported an HPLC-MS method for the quantification of Pantoprazole in human plasma. An LC-MS/MS method for the quantification of Pantoprazole using Levosulphiride as internal standard in human plasma was reported by Osmair Peres and his coworkers [11]. Ramakrishna *et al* [12] reported a reverse phase (RP) HPLC method for the quantification of pantoprazole in human plasma. A modified reverse phase HPLC method for the analysis of Pantoprazole using Levosulphiride as internal standard was reported by Safwan Ashour and coworkers [13]. Kothapalli and coworkers [14] reported an RP HPLC method for the simultaneous quantification of Pantoprazole and Levosulpiride in degradation samples. Nicholas and coworkers [15] developed and improved ion pair liquid chromatography method for the quantification of sulphiride in plasma using fluorescence detection technique. A Hydrophilic Lipophilic Interaction Chromatography (HILIC) tandem MS/MS method for the quantification of Levosulphiride in human plasma was reported by Paek and Coworkers [16]. Also, Park *et al* and Prasad *et al* [17, 18] developed HPLC-MS/MS methods for the quantification of Levosulphiride in human plasma. In their work, they also discussed the application of the developed method for pharmacokinetic and bioequivalence studies. A simultaneous UPLC-MS/MS method for the quantification of rabeprazole and levosulphiride in human plasma has also been reported [19]. Another example of an LC-MS/MS method for a combination with PPI is reported by Rakesh Das and T K Pal [20] and the application of LC-MS/MS technique for monitoring pharmokinetics and drug interactions has been discussed in detail. To the best of our knowledge till now, there is no liquid chromatography-tandem-mass spectrometry (LC-MS/MS) method available for the simultaneous estimation of pantoprazole and levosulpiride in human plasma.

The aim of the present study is to develop a simple, selective and high throughput method for the simultaneous quantification of PTZ and LS in human plasma which employs SPE for sample preparation using LC-triple Quad MS/MS with electro spray ionization (ESI) as interface.

2. EXPERIMENTAL

2.1. Materials and Reagents

Working standards of pantoprazole (PTZ) were procured from Ranbaxy Laboratories (Mumbai, India) and Levosulpiride (LS) from INC Chem Laboratories (Hyderabad, India). Iloperidone (IS) was purchased from Sigma Aldrich (Hyderabad, India). LC-MS/MS grade Acetonitrile and Methanol were purchased from Sigma Aldrich (Mumbai, India). Ammonium Formate and formic acid of AR grade were purchased from Qualigens (Mumbai, India). Milli-Q water was prepared using a Milli-Q purification system (Millipore, Bedford, USA). Strata-C18-E 1 CC solid phase extraction cartridges were purchased from Phenomenex [Hyderabad, India],. Drug-free human plasma was procured from Government Hospital (Ooty, Tamilnadu).

2.2. LC-MS/MS Instrument and Conditions

Analysis was achieved using a ultra-fore LC system coupled with tandem quadrupole mass spectrometry (Shimadzu 8030, Tokyo Japan) equipped with electrospray ionization (ESI) interface, LC-20AD pump, SPD-M20 PDA detector, CTO-20AC column oven, CBM-20 alite controller and SIL-20AC auto sampler. Data acquisition was performed using LC Solutions. The parameters optimized for the analysis were as follows: Desolvation Line (DL) temperature 250^0C, Block Temperature 200^0C, Detector voltage 1.3kv, Nitrogen gas was used as Nebulizer gas (1.5L/min) and drying gas (15 L/min). Argon gas was used for collision induced dissociation (230kPa) experiments.

2.3. Chromatographic Conditions

Phenomenex C_{18} column (50 mm x 4.6 mm; 5 µm) was chosen as stationary phase. Mobile phase composed of a mixture of (acetonitrile and methanol 70:30, v/v) and 10mM ammonium formate (pH adjusted to 4.0 using formic acid) in the ratio of 80:20(v/v) at a flow rate of 0.3 mL/min. Injection volume was 10µL, the column temperature was set ambient. The total chromatographic run time was 4.00 min. The detection of PTZ, LS and IS was achieved in positive ion mode in MRM (Multiple Reaction Monitoring) with retention times approximately 2.08, 2.64 and 3.01 mins, respectively. The precursor to product ion transitions and collision energies for PTZ, LS and IS were m/z: 383.37→200.00, -15.0; 342.10→112.15,-29.0 and 427.20→261.00,-28.0, respectively.

2.4. Preparation of Calibration Standards and Quality Control Samples

Standard stock solutions of PTZ, LS and IS were prepared by dissolving 10 mg of each drug in 10 mL acetonitrile to give a final concentration of 1000 µg/mL. Various working standards ranging from 0.007 – 40 µg/mL of PTZ and 0.006 – 48 µg/mL of LS were prepared in mobile phase. From the above working standards ten calibration standards of PTZ and LS were prepared using blank plasma spiked at

different concentrations in the range of 3.5-2000 ng/mL and 3.0-2400 ng/mL, respectively. The stock solution of the IS was suitably diluted and spiked to the blank plasma to maintain a concentration of 250 ng/mL in each sample. The three quality control (QC) samples were similarly prepared at concentration of 10 ng/mL (LQC), 1000 ng/mL (MQC), 1800 ng/mL (HQC) for PTZ and 12 ng/mL (LQC), 1200 ng/mL (MQC), 2200 ng/mL (HQC) for LS. The Quality control samples were prepared as a single batch, stored frozen and the aliquots were thawed prior to stability studies.

2.5. Sample Preparation

Prior to sample preparation, the drug free plasma obtained from the Government Head Quarters Hospital, Ooty was processed and analyzed for the presence of interferences. These interference free samples were further used for spiking analysis. 0.1mL each of PTZ, LS and IS were spiked to 1.7 mL of plasma and transferred into 5 mL polypropylene tubes. The samples were vortexed for 2 minutes. From this, 1 mL was pipetted and subjected to SPE procedure using strata-C18-E 1CC cartridges on a positive pressure SPE unit. Before sample loading the SPE columns were sequentially conditioned with 1 mL of Acetonitrile. 1mL of spiked plasma was loaded to the SPE columns and were washed with 1mL of milli-Q water. Final elution from SPE columns was performed using 1 mL of acetonitrile. The eluents were collected in micro centrifuge tubes and were centrifuged at 4000 rpm for 15 minutes. From this, the supernatant liquid was taken and 10 µL aliquot was injected for further separation and quantification by LC-MS/MS.

2.6. Method Validation

Method validation was performed based on the industrial guidelines for the bio analytical method validation [21-24]. The present method was validated for sensitivity, selectivity, linearity, precision, accuracy, recovery, matrix effect and stability.

2.6.1. Selectivity

The selectivity of the method was measured by comparing the chromatograms of 6 different blank human plasma with corresponding spiked plasma at LLOQ which were pretreated and analyzed to test the potential interferences of endogenous compounds co-eluting with IS and analytes. Peak areas of endogenous compounds co-eluting with the analytes at their corresponding retention time should not be more than 20% peak area of LLOQ sample response. Endogenous compounds peak areas co-eluting with IS should not be more than 5% of the mean response of IS in LLOQ samples.

2.6.2. Linearity

Linearity was performed using the concentrations ranging from 3.5-2000 ng/mL for PTZ and 3.0 -2400 ng/mL for LS (n=6) in human plasma. Carry over of the sample after injection of the calibration standard of upper limit of quantification was performed by analyzing the blank plasma samples. Linearity was performed by a weighted least square ($1/x^2$) linear regression method. It involves the measurement of the ratio of peak analyte to the ratio of IS.

2.6.3. Matrix Effect

Matrix effect was used to evaluate ion suppression which was evaluated by comparing the peak areas of each analyte extracted from the blank matrix fortified with analyte to those of the respective analyte prepared at the same concentration with mobile phase. Experiments were performed at LQC and HQC in triplicates. Acceptable limit should be ≤15%.

2.6.4. Accuracy and Precision

Intra (within the day) and inter day (between the day) precision and accuracy were analyzed at four different concentration levels LLOQ, LQC, MQC and HQC for each analyte. Accuracy of the method was measured as % measured target concentration and precision was measured in terms of % CV in which the value should not exceed 15% for the nominal QC values and 20% for LLOQ.

2.6.5. Lower limit of Quantitation

A lower limit of quantitation was defined as the lowest concentration of analyte in the sample which can be quantified reliably with an acceptable accuracy and precision.

2.6.6. Stability

Stability was carried out to ensure that the storage conditions used without altering the concentration of analytes. Stability in plasma was measured in terms of freeze-thaw in which samples were frozen at -70°C in three cycles of 24, 36 and 48 h with one hour thaw after each interval, bench top study was studied for 8 h at room temperature and short term stability was studied for 6 h at room temperature. All comparisons were made against fresh calibration standards prepared in mobile phase. The samples were tested at LQC and HQC levels using 3 replicates. Samples were treated as stable if values were found in acceptable limits of accuracy and precision at (±15% CV) nominal value.

3. RESULTS AND DISCUSSION

3.1. Method Development and Optimization of Chromatographic Conditions

Liquid chromatography-tandem mass spectroscopy (LC-MS/MS) had led to major breakthrough in bio analytical method development because of its inherent speed, sensitivity and selectivity. The aim of this work was to develop and validate a sensitive, simple and rapid method for the simultaneous quantification of PTZ and LS in spiked human plasma. Mass spectrometer was operated in positive ion mode. The MRM transitions were selected as m/z 384.10→200.00; m/z 342.10→112.15; m/z 427.20→261.00 for PTZ, LS and internal standard (Fig. **2**). In the optimization of chromatographic conditions the use of mixture of organic solvents acetonitrile and methanol over single solvent in mobile phase had shown good response and symmetric peaks. Various buffers, such as ammonium formate, ammonium acetate and formic acid were used with altered flow rates from 0.3 mL/Min to 0.7 mL/min. Different mobile phase ratios like 90:10; 95:05; 85:15 were also tried. Different types of columns such as C_{18} and C_8 were used for the chromatographic separation. Finally, mobile phase composition of a mixture of organic solvents [acetonitrile and metha-

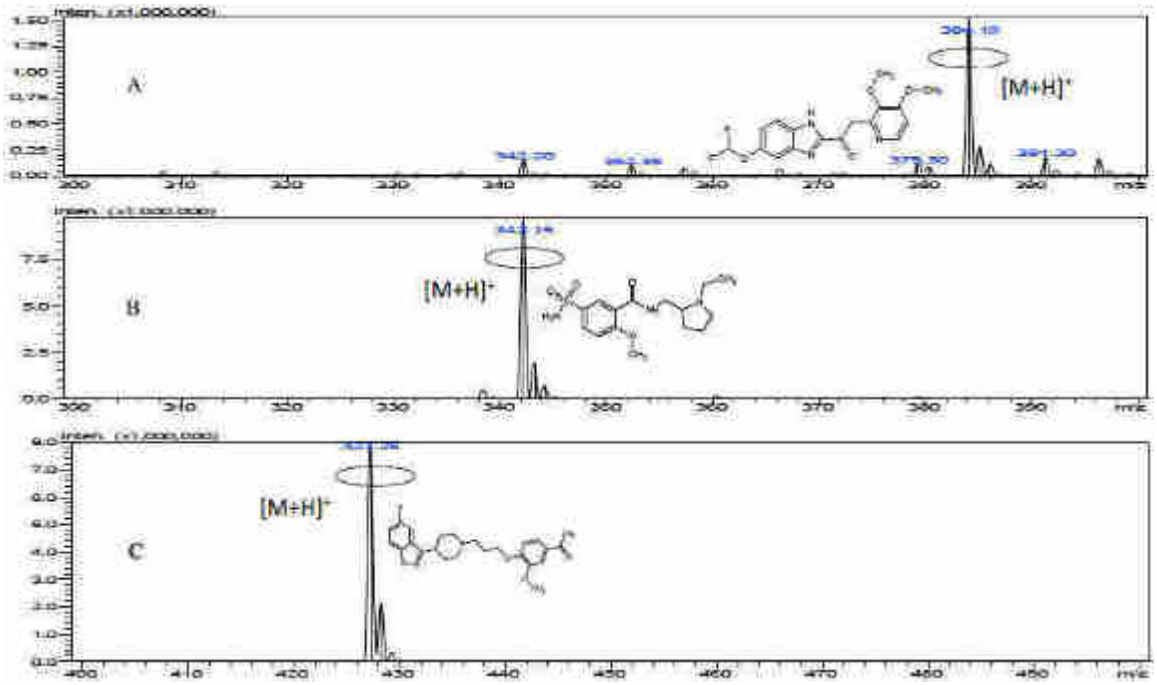

Fig. (1). MS spectra of (**A**) Pantoprazole, (**B**) Levosulpiride and (**C**) Iloperidone (IS) in Electro spray positive ionization mode.

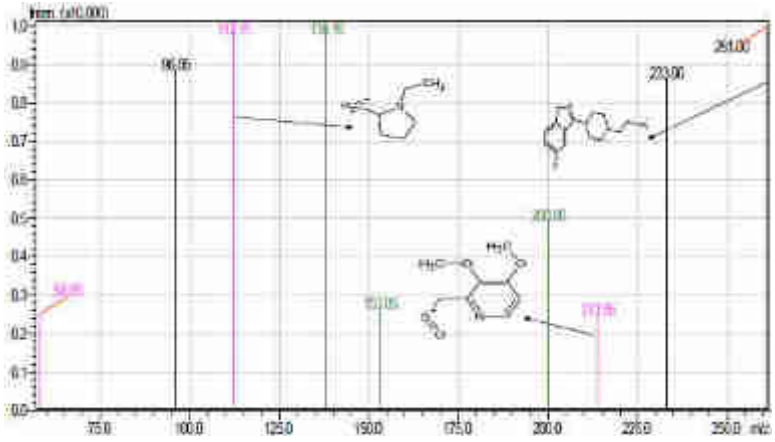

Fig. (2). Product ion scan of PTZ, LS and IS.

nol 70:30, v/v] and 10 mM Ammonium formate (pH adjusted to 4.0 using formic acid) in the ratio of 80:20 (% v/v) at a constant flow rate of 0.3 mL/min on a Phenomenex C_{18} Column (50 mm x 4.6 mm, 5 µm) achieved good resolution with high sensitivity of PTZ, LS and IS. The retention time of PTZ, LS and IS were 2.13, 2.77 and 3.17 mins, respectively (Fig. **2**). Several compounds were examined to find a suitable IS. The use of the structural analogs of PTZ viz Rabeprazole and Omeprazole coeluted with PTZ. Thus, based on solubility and antipsychotic class Iloperidone was selected as IS which was found to be compatible with analytes in chromatographic behavior.

3.2. Method Validation

3.2.1. Selectivity

No endogenous interferences were observed at the retention times of each analyte and the chromatograms are shown in the Fig. (**3**).

3.2.2. Linearity

Peak area ratio of PTZ and LS to the IS (y) versus the nominal concentration (x) of PTZ and LS were used to determine the linearity of each calibration curve. Concentrations ranging from 3.5-2000 ng/mL for PTZ and 3.0-2400 ng/mL for LS were found to be linear for all the analytes (Table **1**). The carry over in the blank plasma samples analyzed after the higher calibration standard were not observed. Correlation coefficients were found to be in the range of $0.993 < r^2 < 0.998$ for all the analytes.

3.2.3. Matrix Effect

Matrix effect was determined by the ratio of peak area of post spiked concentration to the peak area of neat concentrations. The precision of the matrix factor determined at LQC was found to be 0.81 and HQC was found to be 0.98, indicating that the matrix does not produce ion suppression effect during ionization of the drug candidates.

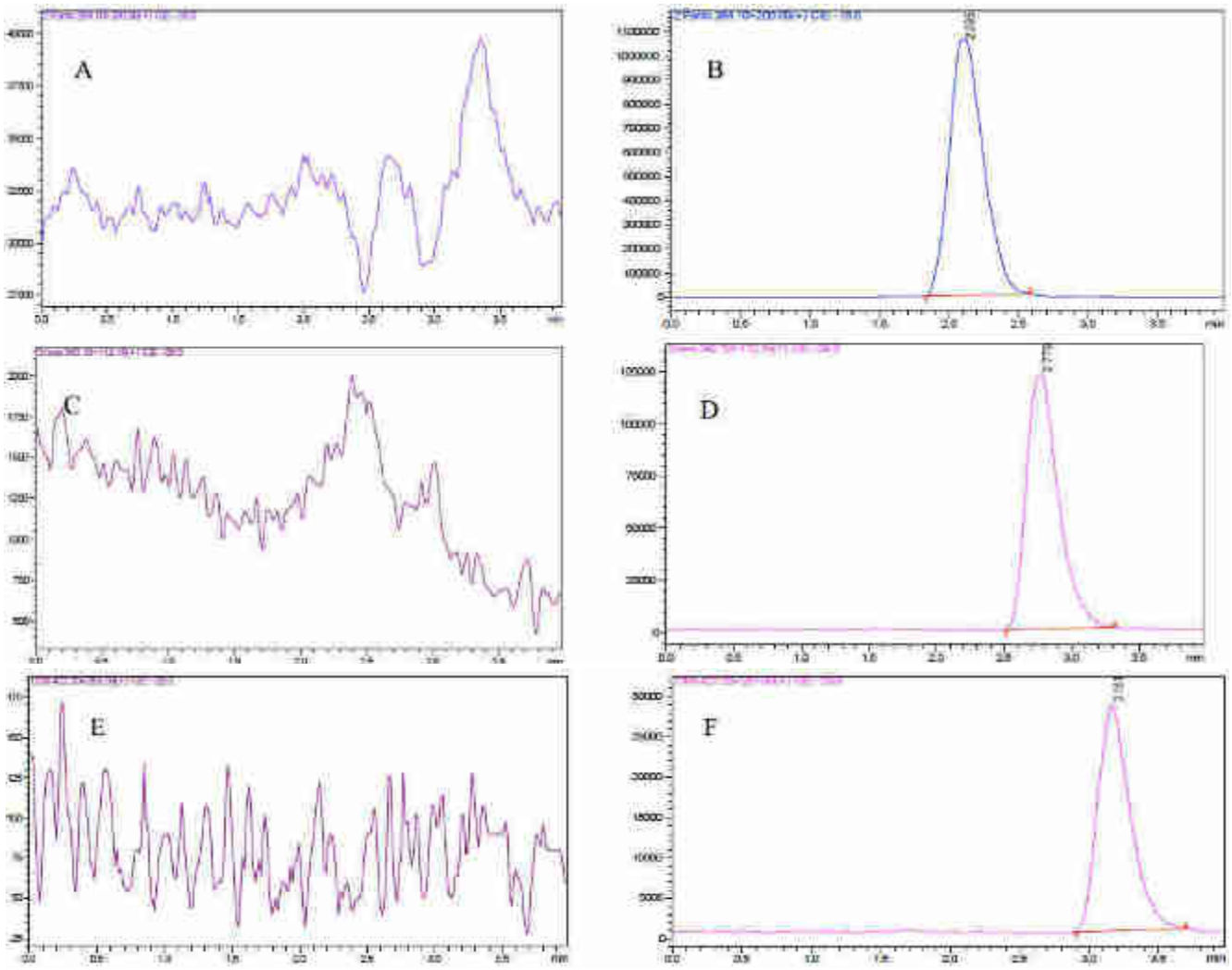

Fig. (3). MRM Chromatograms of (**A**) Blank Human Plasma at m/z 384 (**B**) human plasma spiked with Pantoprazole (2000 ng/mL), (**C**) Blank Human Plasma at m/z 342 for Levosulphiride, (**D**) human plasma spiked with Levosulpiride (2400 ng/mL), (**E**) Blank Human Plasma at m/z 427 and (**F**) human plasma spiked with Iloperidone (IS) (250 ng/mL).

Table 1. Summary of Calibration standards of PTZ and LS (n=6).

Concentration (ng/mL)	Pantoprazole			Concentration (ng/mL)	Levosulpiride		
	Mean (ng/mL) ± SD	CV (%)	Accuracy (%)		Mean (ng/mL) ± SD	CV (%)	Accuracy (%)
3.5	3.49 ± 0.0004	1.28	95.23	3.0	2.76 ± 0.0001	1.23	97.50
10	9.47 ±0.0053	4.16	98.18	12	11.10 ± 0.0019	2.32	97.38
100	105.53 ± 0.0042	3.26	101.61	120	119.34 ± 0.0006	0.47	99.18
400	397.25 ± 0.0073	1.49	100.69	400	397.83 ± 0.0023	0.56	98.87
700	700.24 ± 0.0090	1.04	100.96	800	806.68 ± 0.0104	1.28	99.38
1000	1002.59 ± 0.0151	1.22	99.47	1200	1199.14 ± 0.0101	0.84	99.78
1300	1288.87 ± 0.0137	0.86	98.04	1600	1615.92 ± 0.0122	0.75	100.48
1500	1491.58 ± 0.0065	0.35	99.91	2000	1974.36 ± 0.0214	1.08	99.15
1800	1816.77 ± 0.0234	1.04	101.51	2200	2196.83 ± 0.0114	0.51	99.56
2000	1986.19 ± 0.0035	0.14	99.27	2400	2402.97 ± 0.0315	1.31	101.62

Table 2. Recovery, and precision of PTZ and LS (n=6).

Analyte	QCs (ng/mL)	Mean Concentration Found (ng/mL) ± SD	Intra-day		Mean Concentration Found (ng/mL) ± SD	Inter-day	
			Accuracy (%)	Precision (% CV)		Accuracy (%)	Precision (% CV)
Pantoprazole	LLOQ(3.5)	3.37 ± 0.00005	96.28	1.57	3.36 ± .00008	96.19	2.51
	LQC(10)	9.50 ± 0.0100	95.00	4.21	9.03 ± 0.0016	90.33	5.17
	MQC(1000)	980.00 ± 0.0173	98.00	1.76	961.66 ± 0.023	96.16	2.40
	HQC(1800)	1760.00 ± 0.0520	97.77	2.95	1770.00 ± 0.02	98.33	1.12
Levosulpiride	LLOQ(3.0)	2.84 ± 0.00004	94.11	1.43	2.73 ± 0.00001	91.16	5.65
	LQC(12)	11.00 ± 0.004	91.66	9.09	10.05 ± 0.0076	87.50	5.21
	MQC(1200)	1150.66 ± 0.0390	95.88	3.38	1153.33 ± 0.0344	96.11	2.98
	HQC(2200)	2146.66 ± 0.0404	97.57	1.88	2165.00 ± 0.0308	98.40	1.42

Table 3. Summary of stability testing of PTZ and LS under various storage conditions (n=6).

Stability Test	Pantoprazole			Levosulpiride		
	QCs (ng/ml)	Mean(ng/ml) ± SD; Accuracy (% Nominal);	Precision (% CV)	QCs (ng/ml)	Mean (ng/ml) ± SD; Accuracy (% Nominal);	Precision (% CV)
Freeze thaw (3 cycles at -70 ± 2° C)	LQC (10)	9.40 ± 0.0003; 94.00	3.36	**LQC (12)**	11.6 ±0.0004;96.66	3.51
	HQC (1800)	1755 ± 0.0129; 97.5	0.73	**HQC (2200)**	2176 ± 0.0108; 98.92	0.49
Bench top (25 ° C for 8 h)	LQC (10)	9.52 ± 0.0003 ; 95.12	3.22	**LQC (12)**	11.5 ± 0.0003; 96.20	2.83
	HQC (1800)	1757 ± 0.0479; 97.63	2.72	**HQC (2200)**	2135 ± 0.0412; 97.04	1.93
Short term stability (25 °C for 6 h)	LQC (10)	9.73 ± 0.0002; 97.32	3.05	**LQC (12)**	11.67 ±0.0001; 97.25	1.23
	HQC (1800)	1775 ± 0.023; 98.61	1.34	**HQC (2200)**	2165 ±0.019; 98.40	0.88

3.2.4. Accuracy and Precision

Accuracy, intra-day and inter-day precision are summarized in Table **2**. All the values were found to be in acceptable limit for all the analytes.

3.2.5. Lower limit of Quantification

Lower limit of Quantification of pantoprazole and levosulpiride was found to be 3.5 ng/mL and 3.0 ng/mL, respectively which showed acceptable accuracy (Recovery – 80 - 120%) and Precision (% RSD < 20%) (Table **2**).

3.2.6. Stability Studies

Stability studies in matrix were performed for freeze-thaw (three cycles), bench top (25°C, 8h) and short term stability (25°C, 6h). No degradation of analytes were observed during the storage conditions. The stability studies were summarized in Table **3** which were found to be within the acceptance limits for all analytes.

CONCLUSION

A rapid, selective and sensitive SPE-LC-MS/MS method was successfully developed for the simultaneous quantification of PTZ and LS in human plasma with a chromatographic run time of 4.00 min. The analyte peaks were free from interferences in plasma. The method was validated as per USFDA and EMEA guidelines which showed high degree of precision and accuracy. The developed method can be used in clinical pharmacokinetics as well as in bioavailability/bioequivalence (BA/BE) studies for the simultaneous quantification of PTZ and LS.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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