Development and validation of a high throughput UPLC–MS/MS method for simultaneous quantification of esomeprazole, rabeprazole and levosulpiride in human plasma ★

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A B S T R A C T

A high throughput ultra pressure liquid chromatography–mass spectrometry (UPLC–MS/MS) method with good sensitivity and selectivity has been developed and validated for simultaneous quantification of esomeprazole, rabeprazole and levosulpiride in human plasma using lansoprazole as internal standard (IS). The extraction method based on liquid–liquid extraction technique was used to extract the analytes and IS from 50 μL of human plasma using methyl tert-butyl ether:ethyl acetate (80:20, v/v), which offers a high recovery. Chromatographic separation of analytes and IS was achieved on a Hypersil gold C18 column using gradient mobile phase consisting of 2 mM ammonium formate/acetoneitrile. The flow rate was set at 0.5 mL/min to elute all the analytes and IS within 1.00 min runtime. Detection of target compounds was performed on a triple quadrupole mass spectrometer by multiple reaction monitoring (MRM) mode via positive electrospray ionization (ESI). Method validation results demonstrated that the developed method has good precision and accuracy over the concentration ranges of 0.1–2000 ng/mL for each analyte. Stability of compounds was established in a battery of stability studies, i.e., bench top, autosampler, dry extract and long-term storage stability as well as freeze-thaw cycles. The validated method has been successfully applied to analyze human plasma samples for application in pharmacokinetic studies.

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1. Introduction

Esomeprazole is chemically (S)-5-methoxy-2-[(4-methoxy-3,5-dimethylpyrroldin-2-yl) methylsulfinyl]-3H-benzimidazole (Fig. 1). Esomeprazole (S-isomer of omeprazole), the first single optical isomer proton pump inhibitor, generally provides better acid control than current racemic proton pump inhibitors and has a better pharmacokinetic/pharmacodynamic profile relative to racemic product omeprazole [1]. Esomeprazole is a potent inhibitor of gastric acid secretion and accumulates in the acidic compartment of the parietal cells where the molecule is transformed to its active sulfonamide form [2]. It has been well proven as an effective agent in the treatment of gastroesophageal reflux disease (GERD), functional dyspepsia, nonsteroidal antiinflammatory drugs-induced gastrointestinal symptoms, ulcers, Helicobacter pylori infection and Zollinger–Ellison syndrome [3–6].

Rabeprazole is chemically 2-[(4-(3-methoxypropoxy)-3-methylpyrroldin-2-yl) methyl sulfinyl]-1H-benzo[d]imidazole (Fig. 1), a new proton pump inhibitor, belongs to a class of substituted benzimidazole molecules [7]. Rabeprazole covalently binds and inactivates the gastric parietal cell H + /K + -ATPase, the proton pump responsible for the terminal step in gastric acid secretion [8]. It is clinically effective in the treatment of acid related diseases such as gastric ulcers, duodenal ulcers, GERD, functional dyspepsia and peptic ulcer disease, in association with Helicobacter pylori eradication [9].

Levosulpiride is chemically N-[(2S)-1-ethylpyrrolidin-2-yl] methyl]-2-methoxy-5-sulfamoyl benzamide (Fig. 1). Levosulpiride is a levo enantiomer of sulpiride and a benzamide derivative, indicated as an antipsychotic, antidepressant, antiemetic and anti-dyspeptic drug, as well as a drug for the treatment of somatoform disorders [10]. Levosulpiride is an antidopaminergic gastrointestinal prokinetic agent and its effect is mediated through the blockade of enteric (neuronal and muscular) inhibitory D2 receptors [11]. It has been shown to increase lower esophageal sphincter pressure, accelerate gastric emptying, enhance gallbladder emptying, and improve symptoms in functional dyspepsia and diabetic gastroparesis [12].

The combined usage of proton pump inhibitors (esomeprazole and rabeprazole) and prokinetics (levosulpiride) shows more...
beneficial effects than monotherapy in the treatment of functional dyspepsia [13]. Delayed gastric emptying, Helicobacter pylori infection, abnormal duodenojejunal motility, and central nervous system dysfunction are major causes of functional dyspepsia; hence, these patients are subjected to combination therapy with proton pump inhibitors and prokinetics to get effective treatment [14]. So there is a need for the method to estimate the plasma concentrations of esomeprazole, rabeprazole and levosulpiride simultaneously.

As per literature, several liquid chromatography tandem mass spectrometry (LC–MS/MS) methods are reported for the estimation of esomeprazole [15–17], rabeprazole [18–22] and levosulpiride [23–25] individually in biological samples. Till date, there is no ultra pressure liquid chromatography–mass spectrometry (UPLC–MS/MS) method available for the estimation of esomeprazole, rabeprazole and levosulpiride simultaneously in human plasma. The reported methods have major disadvantages including lacked sensitivity and a longer runtime. The method proposed by Gopinath et al. [17] for determination of esomeprazole in human plasma is sensitive but it has used expensive solid phase extraction technique for sample preparation. Other methods reported by Hultman et al. [15] for determination of esomeprazole in human, rat and dog plasma and Mogili et al. [16] for quantification of esomeprazole in human plasma requires long chromatographic run which is not useful for analyzing more samples per day. The method developed by Hishinuma et al. [19] for the quantification of rabeprazole in human serum is sensitive with lower limit of quantification of 0.25 ng/mL, but the method needs long chromatographic run time. Another method reported by Huang et al. [20] for the quantification of rabeprazole in human plasma, has utilizes very small amount of plasma for sample preparation, but this method also requires longer chromatographic run time which is not suitable for high throughput sample analysis. Phapale et al. [23] reported the LC–MS/MS method for quantification of levosulpiride in human plasma, but it has long chromatographic runtime compared to the current proposed UPLC method.

The present study describes a simple, selective and sensitive method, which employs liquid–liquid extraction technique for sample preparation and liquid chromatography with electrospray ionization (ESI)–tandem mass spectrometry for simultaneous quantitation of esomeprazole, rabeprazole and levosulpiride in human plasma. The proposed method has significant advantages over earlier reported methods like simultaneous quantitation of three analytes, shorter run time (1.00 min), wider linearity range with a more sensitivity (lower limit of quantification:0.1 ng/mL), and simple reproducible extraction. The present method has been validated as per the current US Food and Drug Administration (USFDA) guidelines [26]. The application of this assay in a clinical pharmacokinetic study following oral administration of esomeprazole, rabeprazole and levosulpiride is described.

2. Experimental

2.1. Materials and reagents

Reference standards of esomeprazole magnesium hydrate, rabeprazole sodium, levosulpiride and lansoprazole (internal standard, IS) were purchased from Sigma-Aldrich (Hyderabad, India). HPLC grade ammonium formate was procured from Thermo Fisher Scientific (Mumbai, India). HPLC grade methanol and acetonitrile were procured from J.T Baker (Phillipsburg, USA). Milli-Q water (18.2 mΩ and TOC ≤ 50 ppb) generated using a Milli-Q purification system (Millipore, Bangalore, India) was used throughout the study. Methyl tert-butyl ether and ethyl acetate were purchased from Rankem (Hyderabad, India). Drug-free human plasma was procured from King George Hospital (Visakhapatnam, India).

2.2. Chromatographic and mass spectrometric conditions

Waters Acquity UPLC system (Waters corporation, Milford, USA), consisting of binary solvent manager, sample manager and column manager, was used for solvent and sample delivery. Mobile phase A consisted of 2 mM ammonium formate in Milli-Q water and mobile phase B was acetonitrile. The analytes and IS were separated by the following gradient (minutes, % mobile phase B): 0.01; 10; 0.20; 10; 0.50, 90; 0.70, 90; 0.80, 10; 1.00, 10, and delivered with a flow rate of 0.5 mL/min on a Hypersil Gold C18 column (50 mm × 3.0 mm, 1.9 μm; Thermo scientific, India) maintained at 40 °C. The sample manager was maintained at 5 °C and injection volume was 2 μL. The total chromatographic run time was 1.00 min. The analytes and IS were detected using a Waters XEVO triple quadruple mass spectrometer (Waters corporation, Milford, USA) equipped with 2 spray source. The quantifications of the analytes and IS were achieved by operating the mass spectrometer in positive ion ESI with multiple reaction monitoring (MRM) mode. Nitrogen gas was used as both
cone gas and desolvation gas with a flow rate of 833.33 mL/min and 13,333.33 mL/min, respectively. The source dependent parameters, i.e., capillary voltage, extractor voltage, source temperature and desolvation temperature, were set at 3.50 kV, 3 V, 150 °C and 500 °C, respectively. The precursor to product ion transitions along with the cone voltage and collision energy for each analyte and IS were as follows: m/z 346.1 → 198.1, 21 V, 14 eV for esomeprazole; m/z 360.1 → 242.1, 20 V, 12 eV for rabeprazole; m/z 342.2 → 112.2, 15 V, 16 eV for levosulpiride; m/z 370.2 → 252.1, 20 V, 20 eV for IS with a dwell time 100 ms. Data acquisition and calculations were performed using Masslynx software, version 4.0.

2.3. Preparation of calibration standards and quality control samples

Individual standard stock solutions of esomeprazole, rabeprazole and levosulpiride were prepared by dissolving requisite amounts in methanol to obtain the final drug concentration of 1 mg/mL, respectively. A series of combined working stock solutions with concentrations in the range of 0.1–2000 ng/mL were prepared by serial dilutions with methanol:water (70:30, v/v). Calibration standards were prepared by spiking (2% of total plasma volume) in blank human plasma with combined working stock solutions. A nine-point calibration curve standards were made at 0.1, 0.2, 1.0, 5.0, 50.0, 500.0, 1000.0, 1600.0 and 2000.0 ng/mL concentrations, respectively. The quality control (QC) samples were similarly prepared at concentration of 1600 ng/mL (high quality control, HQC), 1000 ng/mL (middle quality control, MQC), 1.0 ng/mL (low quality control, LQC) and 0.1 ng/mL (lower limit of quantification quality control, LLOQ QC) with blank human plasma by separate weighting of standards. Stock solution (1 mg/mL) of IS was prepared by dissolving appropriate amount in methanol, and its working stock solution (250 ng/mL) was prepared by diluting the stock solution in methanol.

Fig. 3. Typical multiple reaction monitoring mode chromatograms of analytes: (A) esomeprazole, (B) rabeprazole and (C) levosulpiride (left panel) in human blank plasma and IS (right panel).
Sample preparation

A simple liquid–liquid extraction method was developed for extraction of the analytes and IS from human plasma. Prior to analysis, all frozen subject samples, calibration standards, and QC samples were thawed at ambient temperature. Then, 5 µL of IS working stock solution was added into each 1.5 mL eppendorf tube except for blank plasma. 50 µL of standards, QCs, subject samples, and blank plasma were transferred into eppendorf tubes. After vortexing for 30 s, 50 µL of Milli-Q water was added to each tube and vortexed to mix. The analytes and IS were extracted with 1 mL of methyl tert-butyl ether:ethyl acetate (80:20, v/v) by vortexing.

Fig. 4. Typical multiple reaction monitoring mode chromatograms of human plasma spiked with (A) esomeprazole, (B) rabeprazole and (C) levosulpiride at LLOQ level (left panel) and IS (right panel).

| Concentration added (ng/mL) | Esomeprazole (n=5) | Rabeprazole (n=5) | Levosulpiride (n=5) |
|-----------------------------|--------------------|-------------------|---------------------|
|                             | Mean (ng/mL)       | CV (%)            | Accuracy (%)        | Mean (ng/mL)       | CV (%)            | Accuracy (%)        |
| 0.100                       | 0.102              | 10.05             | 101.80              | 0.096              | 7.48              | 96.00               |
| 0.200                       | 0.198              | 6.26              | 99.10               | 0.202              | 5.53              | 101.00              |
| 1.000                       | 1.016              | 8.60              | 101.58              | 0.983              | 8.36              | 98.26               |
| 5.000                       | 5.030              | 2.98              | 100.59              | 5.112              | 3.95              | 102.24              |
| 50.000                      | 50.545             | 3.33              | 101.09              | 51.735             | 2.64              | 103.47              |
| 500.000                     | 503.012            | 2.74              | 100.60              | 511.713            | 2.18              | 102.34              |
| 1000.000                    | 1013.954           | 2.65              | 101.40              | 1000.592           | 3.25              | 100.06              |
| 1600.000                    | 1616.992           | 2.75              | 101.96              | 1571.033           | 5.16              | 98.19               |
| 2000.000                    | 2028.137           | 2.95              | 101.41              | 2078.221           | 3.31              | 103.91              |

Table 1

Precision and accuracy data for back-calculated concentrations of calibration standards.
for 10 min and followed by centrifugation at 5000 g for 10 min. The organic layer was transferred into a clean test tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen in the Turbo vap evaporator (Caliper life sciences, USA). The dried extract was reconstituted with 0.2 mL of mobile phase and 2 μL of aliquot was injected into the UPLC–MS/MS system for analysis.

2.5. Method validation

The bioanalytical method was thoroughly validated to meet the acceptance criteria of draft industrial guidance for the bioanalytical method validation [26]. The method was validated for selectivity, linearity, precision and accuracy, recovery, matrix effect, dilution integrity and stability. The selectivity was evaluated by comparing the chromatograms of six different blank human plasma with corresponding spiked plasma at LLOQ QC level. Peak areas of endogenous compounds co-eluting with the analytes should be less than 20% of the peak area of LLOQ response, and peak areas of endogenous compounds co-eluting with IS should be less than 5% of the mean response of IS in LLOQ samples. Linearity

| Table 2 | Intra- and inter-day accuracy and precision of esomeprazole, rabeprazole and levosulpiride. |
|---|---|
| Analyte | Level (ng/mL) | Intra-day (n=6) | Inter-day (n=30) |
| | | Mean (ng/mL) | CV (%) | Accuracy (%) | Mean (ng/mL) | CV (%) | Accuracy (%) |
| Esomeprazole | LLOQ (0.100) | 0.100 | 9.96 | 99.83 | 0.101 | 9.57 | 100.93 |
| | LQC (1.000) | 1.31 | 11.45 | 102.90 | 1.070 | 5.90 | 106.95 |
| | MQC (1000.000) | 1029.001 | 7.10 | 101.50 | 1023.426 | 4.93 | 102.34 |
| | HQC (1600.000) | 1623.924 | 2.59 | 101.32 | 1641.746 | 3.66 | 102.61 |
| Rabeprazole | LLOQ (0.100) | 0.098 | 10.48 | 97.67 | 0.099 | 11.19 | 99.00 |
| | LQC (1.000) | 0.992 | 7.78 | 99.15 | 1.012 | 7.69 | 101.19 |
| | MQC (1000.000) | 976.600 | 8.03 | 97.66 | 999.933 | 5.68 | 99.99 |
| | HQC (1600.000) | 1674.207 | 6.49 | 104.64 | 1667.869 | 4.80 | 104.24 |
| Levosulpiride | LLOQ (0.100) | 0.096 | 8.91 | 95.67 | 0.098 | 9.87 | 98.40 |
| | LQC (1.000) | 1.008 | 7.85 | 100.75 | 1.003 | 5.23 | 100.28 |
| | MQC (1000.000) | 996.342 | 3.31 | 99.63 | 1008.885 | 3.59 | 100.89 |
| | HQC (1600.000) | 1621.134 | 2.15 | 101.32 | 1622.351 | 2.42 | 101.40 |

| Table 3 | Mean overall recoveries of the analytes and IS. |
|---|---|
| Analytes | Sample concentration (ng/mL) | Response unextracted (Mean ± CV, %) | Response extracted (Mean ± CV, %) | Recovery (%) | Overall recovery (Mean ± CV, %) |
| | | | | | |
| Esomeprazole | 1 | 4866 ± 1.52 | 4399 ± 2.42 | 92.26 | 91.68 ± 1.22 |
| | 1000 | 4,564,899 ± 1.50 | 4,211,683 ± 1.50 | 92.26 | 91.68 ± 1.22 |
| | 1600 | 7,251,748 ± 0.44 | 6,699,120 ± 0.42 | 92.38 | 91.76 ± 1.26 |
| Rabeprazole | 1 | 3916 ± 1.29 | 3575 ± 1.23 | 92.26 | 91.76 ± 1.26 |
| | 1000 | 3,917,516 ± 1.07 | 3,415,019 ± 1.30 | 87.17 | 86.96 ± 1.26 |
| | 1600 | 6,242,951 ± 0.54 | 5,520,253 ± 0.36 | 88.42 | 88.42 ± 1.26 |
| Levosulpiride | 1 | 7895 ± 0.75 | 7895 ± 0.75 | 99.45 | 99.45 ± 0.75 |
| | 1000 | 9,131,233 ± 0.33 | 8,364,505 ± 0.84 | 91.60 | 91.60 ± 0.84 |
| | 1600 | 14,171,522 ± 0.89 | 12,543,580 ± 3.44 | 88.51 | 88.51 ± 3.44 |
| IS | 250 | 2,190,808 ± 3.64 | 1,887,323 ± 1.60 | 86.15 | 86.15 ± 3.64 |

| Table 4 | Stability data of esomeprazole, rabeprazole and levosulpiride in human plasma. |
|---|---|
| Condition | Esomeprazole (n=6) | Rabeprazole (n=6) | Levosulpiride (n=6) |
| | Mean (ng/mL) | CV (%) | Change (%) | Mean (ng/mL) | CV (%) | Change (%) | Mean (ng/mL) | CV (%) | Change (%) |
| Bench top (27 °C, 8 h) | | | | | | | | | |
| LQC | 1.045 | 6.22 | 2.85 | 1.036 | 5.04 | 2.85 | 1.025 | 3.92 | 3.92 |
| HQC | 1.039 | 8.59 | 2.25 | 1.039 | 8.56 | 1.36 | 1.024 | 4.15 | 4.15 |
| Auto sampler (4 °C, 24 h) | | | | | | | | | |
| LQC | 1.039 | 8.59 | 2.25 | 1.039 | 8.56 | 1.36 | 1.024 | 4.15 | 4.15 |
| HQC | 1.039 | 8.59 | 2.25 | 1.039 | 8.56 | 1.36 | 1.024 | 4.15 | 4.15 |
| Dry extract (4 °C, 24 h) | | | | | | | | | |
| LQC | 1.039 | 8.59 | 2.25 | 1.039 | 8.56 | 1.36 | 1.024 | 4.15 | 4.15 |
| HQC | 1.039 | 8.59 | 2.25 | 1.039 | 8.56 | 1.36 | 1.024 | 4.15 | 4.15 |
| Freeze–thaw (– 80 °C, After 4 cycles) | | | | | | | | | |
| LQC | 1.052 | 8.25 | 3.54 | 1.052 | 8.25 | 3.54 | 1.052 | 8.25 | 8.25 |
| HQC | 1.052 | 8.25 | 3.54 | 1.052 | 8.25 | 3.54 | 1.052 | 8.25 | 8.25 |
| Long term (– 80 °C, 60 days) | | | | | | | | | |
| LQC | 1.061 | 6.08 | 4.45 | 1.061 | 6.08 | 4.45 | 1.061 | 6.08 | 6.08 |
| HQC | 1.061 | 6.08 | 4.45 | 1.061 | 6.08 | 4.45 | 1.061 | 6.08 | 6.08 |

for 10 min and followed by centrifugation at 5000 g for 10 min. The organic layer was transferred into a clean test tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen in the Turbo vap evaporator (Caliper life sciences, USA). The dried extract was reconstituted with 0.2 mL of mobile phase and 2 μL of aliquot was injected into the UPLC–MS/MS system for analysis.

2.5. Method validation

The bioanalytical method was thoroughly validated to meet the acceptance criteria of draft industrial guidance for the bioanalytical method validation [26]. The method was validated for selectivity, linearity, precision and accuracy, recovery, matrix effect, dilution integrity and stability. The selectivity was evaluated by comparing the chromatograms of six different blank human plasma with corresponding spiked plasma at LLOQ QC level. Peak areas of endogenous compounds co-eluting with the analytes should be less than 20% of the peak area of LLOQ response, and peak areas of endogenous compounds co-eluting with IS should be less than 5% of the mean response of IS in LLOQ samples. Linearity
was tested for each analyte in the concentration range of 0.1–2000 ng/mL. For the determination of linearity, five standard calibration curves, containing at least nine non-zero standards, were constructed by a weighted least squares ($1/x^2$) linear regression method through the measurement of the peak area ratio of analyte to IS. In addition, blank and blank + IS samples were analyzed to confirm the absence of direct interferences; blank and blank + IS data were not included to construct calibration curves. Intra-day precision and accuracy were determined by analyzing six replicates of each QC (LLOQ, LQC, MQC and HQC) sample of two different batches on the same day. Inter-day precision and accuracy were determined by analyzing six replicates of each QC (LLOQ, LQC, MQC and HQC) sample of five different batches. The acceptance criteria included accuracy within ±15% deviation from the nominal values, except for the LLOQ, where it should be ±20% and precision of ±15% coefficient of variance (%CV), except for LLOQ, where it should be ±20%. The extraction recovery of each analyte was estimated at three different QC levels (six replicates of each LQC, MQC and HQC) by comparing the peak area response of extracted analytes with unextracted analytes (reconstitution solution spiked with the analytes) that represent 100% recovery. Similarly, recovery of IS was estimated by comparing the mean peak area of extracted QC samples ($n=18$) with mean peak area of unextracted QC samples. Recovery of the analytes and IS should be precise and reproducible at all QC levels. The matrix effect was evaluated by comparing the peak areas obtained from each analyte in post-extraction blank plasma samples with those of the respective analyte dissolved at the same concentration in reconstitution solution (mobile phase). The matrix effect was determined at MQC level using six replicates at each level for each analyte, and IS was determined at a single concentration of 250 ng/mL. Dilution integrity was performed to extend the upper concentration limits with acceptable precision and accuracy. Six replicates of each analyte at concentration two times of the upper concentration limits with acceptable precision and accuracy. Six replicates of each analyte at concentration two times of the upper concentration limit were prepared and diluted to 2- and 4-fold with blank plasma. These samples were processed and analyzed. Stability tests were conducted to evaluate the analyte stability in stock solutions and plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2–8 °C) was determined by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench-top stability (8 h), processed sample stability (autosampler stability for 24 h), dry extract stability (8 h), freeze–thaw stability (four cycles) and long-term stability (60 days) were tested at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15%) and precision (±15% CV).

### Pharmacokinetic study

The pharmacokinetic study was carried out in healthy male volunteers ($n=6$). Blood samples were collected in K2-EDTA vacutainer collection tubes (BD, Franklin, NJ, USA) following oral administration of esomeprazole (40 mg), rabeprazole (20 mg) and levosulpiride (75 mg) at pre-dose and 0.25, 0.50, 1.00, 1.50, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 16 and 24 h. The tubes were centrifuged (Thermo Scientific, Germany) at 3200 rpm for 10 min and then the plasma was collected. The collected plasma samples were stored at −80 °C till use. This study was carried out as per the approval and guidelines of the local ethical committee. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. Along with the clinical samples, the QC samples

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**Table 5** Pharmacokinetic data of esomeprazole, rabeprazole and levosulpiride ($n=6$, Mean ± SD).

| Parameters       | Esomeprazole (40 mg) | Rabeprazole (20 mg) | Levosulpiride (75 mg) |
|------------------|-----------------------|---------------------|-----------------------|
| $C_{\text{max}}$ (ng/mL) | 1560.229 ± 68.82 | 670.440 ± 18.10 | 171689 ± 6.71 |
| $T_{\text{max}}$ (h)    | 3.50 ± 0.55  | 3.33 ± 0.52   | 4.33 ± 0.52   |
| $T_{1/2}$ (h)        | 2.32 ± 0.27  | 3.46 ± 0.44   | 2.33 ± 0.32   |
| $\text{AUC}_{0\rightarrow t}$ (ng h/mL) | 8181.486 ± 460.55 | 1749.192 ± 98.88 | 1014206 ± 48.87 |
| $\text{AUC}_{0\rightarrow \infty}$ (ng h/mL) | 8191.343 ± 457.40 | 1753.775 ± 99.88 | 1015472 ± 48.34 |

$C_{\text{max}}$, the maximum plasma concentration; $T_{\text{max}}$, the time to reach $C_{\text{max}}$; $T_{1/2}$, elimination half-life; $\text{AUC}_{0\rightarrow t}$, the area under the plasma concentration-time curve from zero to the last sampling time; $\text{AUC}_{0\rightarrow \infty}$, the area under the plasma concentration-time curve from time zero to infinity.
at low, middle and high concentration levels were also assayed in triplicate. Plasma concentration vs time profiles of esomeprazole, rabeprazole and levosulpiride was analyzed by non-compartmental method using WinNonlin Version 5.3 (Pharsight Corporation, CA, USA).

3. Results and discussion

3.1. Method development and optimization

3.1.1. Optimization of the mass spectrometric conditions

Mass spectrometric conditions were optimized so as to achieve the maximum stable response of the precursor ions and the major product ions of the analytes. Mass spectrometer was operated in positive ion mode to get a good response. The inherent selectivity of MRM mode for quantification of the analytes was expected to be beneficial in developing a selective and sensitive method. All analytes showed the singly charged protonated ions [M+H]+ as the prominent ion in the full scan of Q1 spectrum and was used as the precursor ion to obtain Q3 product ion spectra. The cone voltage and collision energy were optimized to get highest intensity for precursor ion and product ion, respectively. The mass transition ion pair was selected as m/z 346.1→198.1 for esomeprazole, m/z 360.1→242.1 for rabeprazole and m/z 342.2→112.2 for levosulpiride. The product ion mass spectra for each analyte are presented in Fig. 2.

3.1.2. Optimization of the chromatographic conditions

Chromatographic conditions, especially the composition of mobile phase and column, were optimized in order to achieve a good chromatographic resolution and symmetric analyte peak shapes within a short run time. The feasibility of various mixtures of solvents such as methanol and acetonitrile with different

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**Fig. 6.** Typical multiple reaction monitoring mode chromatograms of the analytes: (A) esomeprazole, (B) rabeprazole and (C) levosulpiride (left panel) in human volunteer sample and IS (right panel).
buffers such as ammonium formate, formic acid solution with altered flow rates on different types of columns such as C18 and C8 were tested for complete chromatographic resolution of the analytes and IS from interfering biological matrix. Finally, the gradient mobile phase system consisting of 2 mM ammonium formate–acetonitrile mixture (minutes, % mobile phase B): 0.01, 10; 0.20, 10; 0.50, 90; 0.70, 90; 0.80, 10; 1.00, 10, delivered with a 0.5 mL/min flow rate on a Hypersil gold C18 column achieved the good chromatographic separation of the analytes and IS with desired response. The retention time of esomeprazole, rabeprazole, levo-
sulpiride and IS were 0.60, 0.54, 0.73 and 0.56 min, respectively. Several compounds were investigated to find a suitable IS and finally, lansoprazole was found to be compatible with targeted analytes in terms of extraction efficiency, chromatographic behavior and ionization yield.

3.2. Method validation

3.2.1. Selectivity

Representative chromatograms obtained from blank plasma sample and blank plasma spiked with LLOQ standard of each analyte and IS are presented in Figs. 3 and 4. There were no significant endogenous interferences observed in the respective MRM channel at the retention time of each analyte and IS in blank plasma sample.

3.2.2. Linearity

Linearity of each calibration curve was determined by plotting the peak area ratio of analyte to IS (y) versus the nominal concentration (x) of the calibration points, and fitted to the y = mx + c using a regression factor (1/x^2). The nine-point calibration curve was found to be linear over the concentration range of 0.1–2000 ng/mL for all the analytes. Correlation coefficients were in the range 0.980 < r^2 < 0.995 for all the analytes. The accuracy values ranged from 96.00% to 103.91%, while the precision (%CV) summarized in Table 2. The intra-day accuracy ranged between 95.67% and 111.45% with a precision of 1.31%–10.48%; the inter-day accuracy between 98.40% and 106.95% with a precision of 2.42%–11.19%.

3.2.4. Recovery

The extraction recoveries of all the analytes and IS were good and reproducible. The mean overall recoveries (with the precision) of all the analytes are summarized in Table 3.

3.2.5. Matrix effect

There was no effect of endogenous contribution from blank plasma in the measurement of all the analytes and IS. The average matrix factor values (matrix factor = peak area of post-spiked concentrations/peak area of neat concentrations) ranged from 0.897 to 0.996, while the precision (%CV) values ranged from 1.08% to 2.36% for all the analytes.

3.2.6. Dilution integrity

The upper concentration limit was extended to 4000 ng/mL for all the analytes by a half and quarter dilution with screened human blank plasma. The mean back-calculated concentrations for a half and quarter dilution samples were within 85%–115% of nominal value, while precision values ranged from 1.24% to 5.14% for all the analytes.

3.2.7. Stability studies

Analysis of stock solution stability was performed at the concentration of 2000 ng/mL. After storage for 24 days at 2–8 °C and at room temperature for 6 h, more than 97% of esomeprazole, rabeprazole and levosulpiride remained unchanged. The results of bench-top stability (8 h), processed sample stability (auto sampler stability for 24 h), dry extract stability (8 h), freeze–thaw stability (four cycles) and long-term stability (60 days) are summarized in Table 4 and found to be within the acceptance limit.

3.3. Application of the method in pharmacokinetic study

The established UPLC–MS/MS method was successfully applied to the determination of esomeprazole, rabeprazole and levosulpiride concentrations in human plasma samples collected from healthy volunteers (n=6). The mean plasma concentrations vs time profiles of esomeprazole, rabeprazole and levosulpiride are shown in Fig. 5. In addition, the pharmacokinetic parameters are presented in Table 5. The maximum concentration (Cmax) in human plasma (1560.229 ng/mL for esomeprazole, 670.440 ng/mL for rabeprazole and 171689 ng/mL for levosulpiride) was attained at 3.50 h, 3.33 h and 8191.343 ng h/mL for rabeprazole 1749.192 ng h/mL and 1753.775 ng h/mL and for levosulpiride 1014.206 ng h/mL and 1015.472 ng h/mL. These values were in close proximity when compared with earlier reported values [15–22]. Fig. 6 represents the chromatograms of three analytes after oral administration.

4. Conclusion

In summary, a selective, sensitive and rapid UPLC–MS/MS method for simultaneous quantification of esomeprazole, rabeprazole and levosulpiride in human plasma was developed and fully validated as per USFDA guidelines. Till date, there has been no reported UPLC–MS/MS method for simultaneous quantification of esomeprazole, rabeprazole and levosulpiride in any biological matrix. This method offers significant advantages in terms of wide range of linearity, good recovery, rapid extraction and short run time. Moreover, this method provides superior sensitivity with the LLOQ as low as 0.1 ng/mL for each analyte. Finally, the simplicity of sample preparation and the short chromatographic runtime give the method capability for high sample throughput. From the results of all the validation parameters, we can conclude that the present method is useful for pharmacokinetic/bioequivalence studies with desired precision and accuracy.

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References

[1] L.J. Scott, C.J. Dunn, G. Mallarkey, et al., Esomeprazole: a review of its use in the management of acid-related disorders in the US, Drugs 62 (2002) 1091–1118.
[2] T. Andersson, M.H. Alin, G. Hasselgren, et al., Pharmacokinetic studies with esomeprazole, the (s)-isomer of omeprazole, Clin. Pharmacokinet. 40 (2001) 411–426.
[3] J.E. Richter, P.J. Kahrlas, J. Johanson, et al., Efficacy and safety of esomeprazole compared with omeprazole in GERD patients with erosive esophagitis: a randomized controlled trial, Am. J. Gastroenterol. 96 (2001) 656–665.
[4] T. Lind, L. Rydberg, A. Kyléback, et al., Esomeprazole provides improved acid control vs. omeprazole in patients with symptoms of gastro-oesophageal reflux.
reflux disease, Aliment. Pharmacol. Ther. 14 (2000) 861–867.
[5] N.B. Vakil, R. Shaker, D.A. Johnson, et al., The new proton pump inhibitor esomeprazole is effective as a maintenance therapy in GERD patients with healed erosive esophagitis. A 6-month, randomized, double blind, placebo-controlled study of efficacy and safety, Aliment. Pharmacol. Ther. 15 (2001) 926–935.
[6] K.R. Devault, J.F. Johanson, D.A. Johnson, et al., Maintenance of healed erosive esophagitis: a randomized six-month comparison of esomeprazole twenty milligrams with lansoprazole fifteen milligrams, Clin. Gastroenterol. Hepatol. 4 (2006) 852–859.
[7] C.P.M. Dekkers, J.A. Beker, B. Thjodleifsson, et al., Comparison of rabeprazole 20 mg versus omeprazole 20 mg in the treatment of active duodenal ulcer: a European multicentre study, Aliment. Pharmacol. Ther. 13 (1999) 179–186.
[8] F. Pace, S. Pallotta, S. Casalini, et al., The effect of L-sulpiride on lower gastrointestinal disorders, Part I: functional dyspepsia, Neurogastroenterol. Motil. 18 (2006) 346–353.
[9] S. Gopinath, R.S. Kumar, M.B. Shankar, et al., Development and validation of a sensitive and high-throughput LC-MS/MS method for the simultaneous determination of esomeprazole and naproxen in human plasma, Biomed. Chromatogr. 27 (2013) 894–899.
[10] F. Sabbatini, G. Petrelli, S.L. Manna, et al., The effect of L-sulpiride on lower gastrointestinal disorders, Part I: functional dyspepsia, Neurogastroenterol. Motil. 39 (1999) 927–933.
[11] F. Rossi, A. Forgione, Pharmacotoxicological aspects of levosulpiride, Pharmacol. Res. 31 (1995) 81–94.
[12] V. Andresen, M. Camilleri, Challenges in drug development for functional gastrointestinal disorders, Part I: functional dyspepsia, Neurogastroenterol. Motil. 18 (2006) 346–353.
[13] S.D. Aros, F. Cremonini, N.J. Talley, Treatment of functional dyspepsia, Curr. Treat. Options Gastroenterol. 7 (2004) 121–131.
[14] N.J. Talley, M.D. Silverstein, L. Agreus, et al., AGA technical review: determination of esomeprazole and rabeprazole concentrations in human serum by liquid chromatography/tandem mass spectrometry, J. Chromatogr. 350 (2007) 345–353.
[15] I. Hultman, H. Stenhoff, M. Liljeblad, Determination of esomeprazole and its two main metabolites in human, rat and dog plasma by liquid chromatography with tandem mass spectrometry, J. Chromatogr. B 848 (2007) 317–322.
[16] R. Mogili, K. Kanala, C.K. Bannoith, et al., Quantification of esomeprazole in human plasma by liquid chromatography tandem mass spectrometry and its application to bioequivalence study, Der. Pharm. Lett. 3 (2011) 138–145.
[17] T. Hishinuma, K. Suzuki, H. Yamaguchi, et al., Simple quantification of lansoprazole and rabeprazole concentrations in human serum by liquid chromatography/tandem mass spectrometry, J. Chromatogr. 39 (1999) 927–933.
[18] J. Hwang, Y. Xu, S. Gao, et al., Development of a liquid chromatography/tandem mass spectrometry assay for the quantification of rabeprazole in human plasma, Rapid Commun. Mass. 19 (2005) 2321–2324.
[19] Y. Zhang, X. Chen, Q. Gu, et al., Quantification of rabeprazole in human plasma by liquid chromatography–tandem mass spectrometry, Anal. Chim. Acta 523 (2004) 171–175.
[20] L. Yu, H. Deng, B. Xiang, Liquid chromatography–tandem mass spectrometry for the quantification of rabeprazole in human plasma and its application to pharmacokinetic study, Arzneimittelforschung 60 (2010) 262–266.
[21] P.B. Phapale, H.W. Lee, M.S. Lim, et al., Liquid chromatography–tandem mass spectrometry quantification of levosulpride in human plasma and its application to bioequivalence study, J. Chromatogr. B 878 (2010) 2280–2285.
[22] Y. Wang, Z. Wang, J. Wang, et al., Determination of rabeprazole in rat plasma by gradient elution LC-ESI-MS and its application, Lat. Am. J. Pharm. 32 (2013) 208–213.
[23] R. Mogili, K. Kanala, C.K. Bannoith, et al., Quantification of esomeprazole and its two main metabolites in human plasma by liquid chromatography tandem mass spectrometry and its application to bioequivalence study, Der. Pharm. Lett. 3 (2011) 138–145.
[24] S. Gopinath, R.S. Kumar, M.B. Shankar, et al., Development and validation of a sensitive and high-throughput LC-MS/MS method for the simultaneous determination of esomeprazole and naproxen in human plasma, Biomed. Chromatogr. 27 (2013) 894–899.
[25] Y. Wang, Z. Wang, J. Wang, et al., Determination of rabeprazole in rat plasma by gradient elution LC-ESI-MS and its application, Lat. Am. J. Pharm. 32 (2013) 208–213.