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DEVELOPMENT AND VALIDATION OF FAST REVERSED-PHASE HPLC METHOD FOR ANALYSIS OF ESOMEPRAZOLE IN RABBIT PLASMA

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Abstract: The present study focused to develop rapid, accurate and sensitive reversed-phase high pressure liquid chromatography method for the quantification of esomeprazole (ESO) magnesium in rabbit plasma. Chromatographic separation was achieved isocratically on a reversed-phase C_{18} column using simple mobile phase consisting of of methanol : acetonitrile: 0.05 M phosphate buffer, pH 7 adjusted with potassium hydroxide (45 : 10 : 45, v/v/v) at a flow rate of 1.0 mL/min and UV detection at 302 nm. The method was validated for system suitability, linearity, precision, accuracy, stability, robustness, LOD and LOQ. The described method stated good linearity over the range of 0.01 to 2.5 µg/mL (r = 0.999). The extraction recovery of esomeprazole was more than 95.3%. The method was precise with relative standard deviation < 1% with more than 90% accuracy and limit of quantification 0.0309 µg/mL. The freeze thaw stability studies indicated that the rabbit plasma samples containing esomeprazole could be stored in freezer at -20°C and handled under normal laboratory conditions without significant loss of drug. In conclusion, the developed method is simple, cost effective and reproducible, with improved sensitivity and running time of analysis.

Keywords: RP-HPLC, esomeprazole, rabbit plasma, method development, validation

Esomeprazole magnesium trihydrate (EMT), chemically described as bis (5-methoxy-2-[(S)-[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl] sulfinyl]-H-benzimidazol-1-yl) magnesium trihydrate (Fig. 1) is S-isomer of omeprazole, a proton pump inhibitor (1). Esomeprazole (ESO) irreversibly blocks the H^+/K^+-ATPase enzyme system of the gastric parietal cell, used for the treatment of peptic ulcer disease, gastroesophageal reflux disease, NSAIDS associated ulceration and Zollinger-Ellison syndrome (2). Several techniques such as spectroscopy (3, 4), liquid chromatography (5, 6), supercritical fluid chromatography (7), capillary electrophoresis (8), preparative chiral chromatography (1) and RP-HPLC (9-12) methods have been reported in the literature for the quantitative estimation of EMT in biological fluids and pharmaceuticals.

Capillary electrophoresis, preparative chiral chromatography, supercritical fluid chromatography involves a tedious extraction procedure involving too many steps, indicating need for the development of more efficient, sensitive and simple analytical technique. Compared to other analytical techniques described in the literature, HPLC would afford advantages in terms of improved resolution, speed, precision, sensitivity and accuracy of analysis. The present study, therefore focused to develop simple,

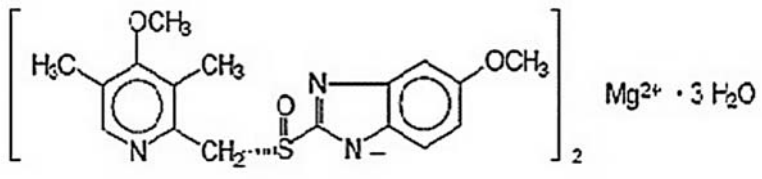

Figure 1. Structure of esomeprazole magnesium trihydrate

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sensitive and accurate HPLC method for the determination of esomeprazole in rabbit plasma followed by validation as per ICH guidelines.

EXPERIMENTAL

Chemicals

Esomeprazole magnesium trihydrate was a generous gift from Unison Chemicals Works, Lahore, Pakistan. HPLC grade methanol, acetonitrile and potassium dihydrogen phosphate were purchased from Sigma Aldrich (Germany). All chemicals and reagents were of analytical grade.

Instruments and chromatographic conditions

The HPLC analysis was carried out on Perkin Elmer HPLC system with UV-VIS detector (Perkin Elmer Series N3896) and computer running software Chromera (HPLC software version 3.4.0.5712) for data acquisition and processing. The chromatographic separation was performed on hypersil C18 column (250 × 4.6 mm with 5 µm particle size,

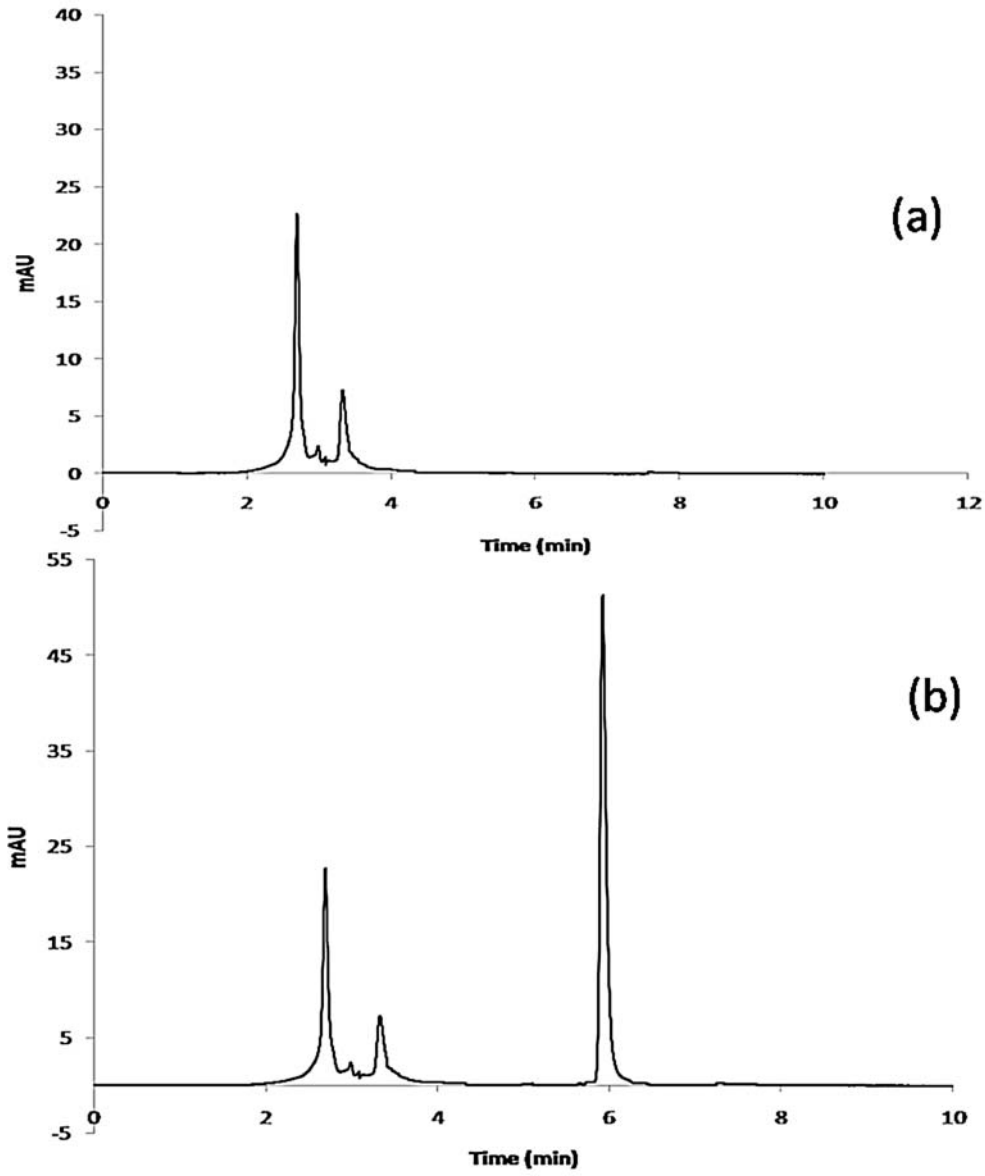

Figure 2: Representative chromatograms (a) drug free plasma (b) plasma spiked with esomeprazole

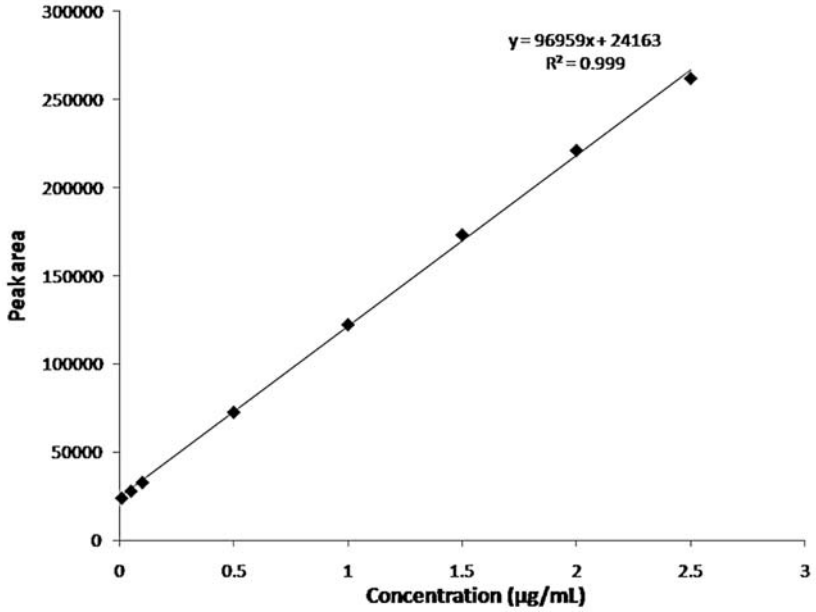

Figure 3. Representative calibration curve of esomeprazole in rabbit plasma

Table 1. Results of system suitability study.

	Retention time (min)	Peak area	Tailing factor
Mean (n = 6)	5.92	173806.7	1.77
SD	0.0251	513.16	
% RSD	0.424	0.295	

Supelco Analytical). The isocratic mobile phase consisted of methanol : acetonitrile : 0.05 M phosphate buffer (pH 7 adjusted with potassium hydroxide) in the ratio of 45 : 10 : 45 (v/v/v), and was pumped through the column at a constant flow rate of 1 mL/min with quantification achieved at 302 nm.

Sample preparation

Blood samples were collected from healthy rabbits in citrated tubes (Bolton Scientific Ltd.) and centrifuged at 3500 rpm for 15 min. The supernatant obtained was stored at -20^{o}C and used for method development and validation. The study was conducted with the approval of Ethical Committee for Utilization of Laboratory Animals, Bahauddin Zakariya University, Multan, Pakistan. Standard stock solution of esomeprazole (100 µg/mL) was prepared in mobile phase and further diluted with mobile phase to working solutions. Drug free blank plasma (100 µL) was spiked with working drug solutions to attain the desired concentration range. To 1 mL of the spiked plasma sample, 1 mL of methanol was added, vortexed for 20 min and centrifuged (Hermile Z 200-A) at 3000 rpm for 15 min. The supernatant was collected and 20 µL of the extracted drug solution was injected into HPLC system.

Selection and optimization of mobile phase

A combination of HPLC grade acetonitrile, methanol and 0.05 M phosphate buffer was selected for better analyte solubility and stability during study. To attain adequate resolution initially different solvent systems were tried with varied combinations for mobile phase optimization. In preliminary studies, methanol : acetonitrile : 0.05 M phosphate buffer (pH 7) in various proportions, like 30 : 10 :

60, 10 : 50 : 40, 70 : 30 : 10 were investigated. The mobile phase consisting of methanol : acetonitrile : 0.05 M phosphate (buffer pH 7) in volume ratio of 45 : 10 : 45 showed maximum separation, better peak resolution and sensitivity. The same was used in further studies.

Validation parameters of developed HPLC method

The method was validated in accordance with ICH guidelines (13). The parameters assessed were linearity, accuracy, precision, limit of detection, limit of quantification, robustness and freeze thaw stability of drug in plasma.

Linearity, limit of detection and limit of quantification

The linearity of the method was evaluated by repeatedly injecting (n = 3) different concentrations of the standard solution of the esomeprazole. Calibration curve was constructed by plotting sample peak area (mean) against concentration and regression equation was computed. The LOD and LOQ were calculated as:

$$LOD = \frac{3.3 \times S}{M} \qquad LOQ = \frac{10 \times S}{M}$$

where, S is the standard deviation of the peak area (n = 5) of sample and M is slope of the corresponding calibration curve.

Recovery, accuracy and precision

The extraction recovery values were calculated by comparing the peak area of extracted sample of drug to that of the unextracted pure drug solutions used for plasma spiking. Recovery experiments, intra-day precision, inter-day precision and accuracy were performed at low, medium and high concentrations (14). Accuracy was expressed as a percent-

Table 2. Extraction efficacy of analytical method.

Concentration (µg/mL)	Concentration found (mean ± SD)	Recovery %
2.5	2.406 ± 0.07	96.27
1	0.954 ± 0.01	95.4
0.05	0.047 ± 0.001	95.3

Table 3. Intra-day and inter-day precision and accuracy.

Conc. (µg/mL)	Intra-day (n = 6)			Inter-day (n = 6)		
	Conc. found (mean ± SD)	Accuracy (%)	RSD (%)	Conc. found (mean ± SD)	Accuracy (%)	RSD (%)
2.5	2.44 ± 0.079	97.6	3.25	2.43 ± 0.09	97.3	3.88
1	0.91 ± 0.025	91.3	2.75	0.91 ± 0.02	91.33	2.27
0.05	0.047 ± 0.002	94.6	4.39	0.04 ± 0.001	90.66	3.36

Table 4. Robustness by change in mobile phase composition.

Conc. (µg/mL)	Change in mobile phase composition			
	Methanol : acetonitrile : 0.5 M phosphate buffer (v/v/v)			
	(50 : 5 : 45)		(40 : 15 : 45)	
	Conc. found (µg/mL) Mean ± SD	RSD (%)	Conc. found (µg/mL) Mean ± SD	RSD (%)
2.5	2.46 ± 0.081	3.32	2.43 ± 0.04	1.70
1	0.93 ± 0.037	3.23	0.9 6 ± 0.02	2.60
0.05	0.049 ± 0.002	4.21	0.051 ± 0.002	3.92

Table 5. Robustness by change in flow rate.

Conc. (µg/mL)	Change in flow rate			
	1.2 mL/min		0.8 mL/min	
	Conc. found (µg/mL) Mean ± SD	RSD (%)	Conc. found (µg/mL) Mean ± SD	RSD (%)
2.5	2.45 ± 0.03	1.24	2.49 ± 0.04	1.61
1	0.95 ± 0.02	2.41	0.94 ± 0.03	3.39
0.05	0.047 ± 0 .001	3.22	0.045 ± 0.001	3.34

Table 6. Freeze thaw stability studies.

Freeze thaw cycle	Drug conc. spiked	Drug conc. found Mean ± SD	RSD %
Cycle 0	2.5	2.49 ± 0.056	2.28
	1	0.98 ± 0.015	1.55
	0.05	0.046 ± 0.001	3.29
Cycle 1	2.5	2.48 ± 0.025	1.01
	1	0.97 ± 0.02	2.58
	0.05	0.044 ± 0.002	4.66
Cycle 2	2.5	2.5 ± 0.03	1.44
	1	0.96 ± 0.01	1.58
	0.05	0.048 ± 0.002	2.12
Cycle 3	2.5	2.43 ± 0.025	1.03
	1	0.96 ± 0.026	2.75
	0.05	0.045 ± 0.001	2.54

age of the drug while precision was expressed as relative standard deviation (RSD).

Robustness and freeze thaw stability

The robustness of analytical method was determined by analysis of aliquots from homogeneous lots by differing physical parameter within the specified parameter of the assay i.e., by changing physical parameters like mobile phase ratio and flow rate. The freeze thaw stability was assessed by assaying six replicates of three dilution levels in rabbit plasma. The samples were frozen and thawed over 3 cycles. All samples were stored at -20^{o}C followed by unassisted thawing at room temperatures in dark to protect from photooxidation.

RESULTS AND DISCUSSION

The proposed analytical method for the estimation of esomeprazole by RP-HPLC method was optimized through a series of trials which have followed the aforementioned chromatographic conditions. In preliminary studies, to enhance the resolution and also to achieve acceptable retention time different mobile phase composition were tried. Finally the method was optimized by mobile phase composition of methanol : acetonitrile : 0.05 M phosphate buffer at pH 7 adjusted with potassium hydroxide in the ratio of 45 : 10 : 45, v/v/v. The extraction procedure was optimized to keep simple and minimum processing steps. The retention time of esomeprazole was found to be 5.92 min thus allowing the run time of 7 to 8 min. Figure 2 depicted the representative chromatograms of blank rabbit plasma and rabbit plasma spiked with esomeprazole.

Validation parameters

The chromatographic method should be able to separate the required analyte with good resolution, sensitivity, accuracy, precision and reproducibility. System suitability, an integral part of analytical procedures was performed before the initiation of validation and the results were within acceptable limits (Table 1).

Linearity, LOD and LOQ

The linearity of the assay method was validated over the concentration range of 0.01 to 2.5 µg/mL. The least square method was applied to calculate the linearity of the calibration graphs and the method found to be linear in the specified range with a correlation coefficient higher than 0.998 (Fig. 3), indicating a good linearity according to the ICH guidelines. The LOD and LOQ were established at 0.0102 µg/mL and 0.0309 µg/mL, respectively. The present method has improved sensitivity and retention time in comparison to methods described previously. In previous HPLC-UV study, retention time of 7.26 min with limit of detection 0.0507 µg/mL has been reported (9). Islam and coworkers employed HPLC method for the quantification of esomeprazole from human serum, the LOQ was 0.02 µg/mL; however, retention time was more than 10 min (16).

Extraction recovery

The recovery of esomeprazole after liquid-liquid extraction was found to be greater than 95% (Table 2), indicating that the extraction procedure has removed the interfering materials from plasma with good extraction recovery of esomeprazole. In previous studies, methanol, methyl t-butyl ether and a mixture of t-butyl methyl ether and dichloromethane have been utilized for omeprazole and esomeprazole extraction (9, 15, 16).

Accuracy and precision

The results of accuracy and precision of the method are presented in Table 3, respectively. The method showed more than 90% accuracy and was precise with % RSD in the range of 2.75-4.39% for intra-day and 2.27-3.88% for inter-day precision, respectively.

Robustness and freeze thaw stability studies

The robustness of the method was determined by small deliberate variations in mobile phase composition and flow rate. The method was robust to minor changes in mobile phase composition and flow rate with % RSD in the range of 1.70% to 4.21% (Table 4) and 1.24% to 3.34 % (Table 5), respectively. Table 6 represents the results of freeze thaw stability study. The method showed good stability with % RSD 1.01-4.66%. The results proved that there was no significant degradation in freeze thaw cycles (-20^{o}C to room temperature). Freeze thaw stability studies performed by other authors also showed minor changes in esomeprazole concentration (9).

CONCLUSION

In conclusion, a simple, rapid, sensitive and economical reverse phase HPLC method has been developed for the quantification of esomeprazole in rabbit plasma. The credibility of the proposed method has been established by validation as per ICH guidelines. The method employed more common instrumentation with UV detection, simpler extraction procedure and faster run time with no compromise on assay sensitivity. In comparison with the previously developed methods, the present method offers an undoubted advantage in term of overall analytical performance This assay method can be used in pharmacokinetic analysis and biodistribution studies of novel formulations of esomeprazole in rabbits.

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Conflict of interest

All authors declared that they have no conflict of interests.

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