A Simple RP–HPLC Method for the Determination of Omeprazole in Human Serum and Urine: Validation and Application in Pharmacokinetic Study

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ABSTRACT: A Simple RP–HPLC method with UV detection has been validated to determine omeprazole concentrations in human serum and urine samples. The mobile phase consisted of a mixture of potassium dihydrogen phosphate buffer (pH 7.2 ± 0.05; 0.2 M) and acetonitrile (70:30, v/v), pumped at a flow rate of 1.0 ml/min through the C-8 column at room temperature. Peaks were monitored by UV absorbance at 302 nm at a sensitivity of 0.0001. The developed method was selective and linear for omeprazole concentrations ranging between 5 to 1000ng/ml for serum samples and 1 to 100µg/ml for urine samples. The recovery of omeprazole ranged from 95.68 to 99% and 95.54 to 99.8% for the serum and urine samples respectively. The limit of quantitation (LOQ) of omeprazole was 5 ng/ml. The intraday accuracy ranged from 93.54 to 104.38% and 100.55 to 103.48% for the serum and urine respectively. The interday accuracy varied from 97.61 to 113.95% and 97.42 to 109.97% for the serum and urine respectively. For the LOQ, good values of precision (6.03 and 10.13% for intraday and interday, respectively) were also obtained. Acceptable results were obtained during stability study. This method proved to be simple, accurate and precise for pharmacokinetic and bioequivalence studies of omeprazole.

Key words: Omeprazole; RP-HPLC; Method validation.

INTRODUCTION

Chemically omeprazole, (5-methoxy-2{[(4-methoxy-3, 5-dimethyl-2-pyridinyl) methyl] sulphinyl}-1H-benzimidazole), is a substituted benzimidazole with a powerful and selective inhibiting activity on gastric acid secretion.[1–3] It is more effective than ranitidine and cimetidine in the treatment of gastric ulcer.[4,5] Omeprazole suppresses gastric acid secretion by specific inhibition of the H^+/K^+ ATPase enzyme system at the secretory surface of the gastric parietal cell.[6,7] Omeprazole formulations must be administered in the form of enteric preparations to prevent degradation in acidic medium.[8–10] Absorption of omeprazole is rapid, with peak plasma levels occurring within 0.5 to 3.5 h. Omeprazole is extensively metabolized by the liver cytochrome P450 (CYP) enzyme system, being characterized by an elimination half-life lower than 1 h.[2,11] The main metabolites present in serum are hydroxyomeprazole and omeprazole sulphone.[12]

Several liquid chromatographic methods have been described in the literature to determine omeprazole and its metabolites in biological fluids.[13–20]

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Those methods were based on liquid–liquid, off-line solid-phase extraction or on-line solid-phase extractions which were very laborious when large number of samples were required in biological experiments like bioequivalence study. That's why an attempt was made to validate a simple and rapid method to analyze omeprazole in human serum and urine samples as well as to apply this method in pharmacokinetic study.

MATERIALS AND METHODS

Chemicals and reagents. The omeprazole standard and the internal standard pantoprazole were provided by Aristopharma Ltd., Dhaka, Bangladesh. HPLC grade acetonitrile and methanol, potassium dihydrogen phosphate, potassium hydroxide and sodium carbonate were procured (Merck, Germany).

Instrumentation. A Shimadzu (Japan) HPLC system consisting of a SCL-10Avp system controller and two LC-8A pumps were used. Ultraviolet detection was achieved with a SPD-10Avp UV-VIS detector (Shimadzu, Japan). The drug analysis data were acquired and processed using LC solution (Version 1.03 SP3, Shimadzu, Japan) software running under Windows XP on a Pentium PC.

Chromatographic conditions. The mobile phase used was a mixture of potassium dihydrogen phosphate buffer (pH 7.2 ± 0.05; 0.2 M) and acetonitrile (70:30, v/v), pumped at a flow rate of 1.0 ml/min through the column (XTerra C8; 5µ , 4.6 X 250 mm, Waters, USA) at room temperature. The mobile phase was degassed prior to use under vacuum by filtration through a 0.2µ nylon membrane. Concentrations were measured at 302 nm by UV detector at a sensitivity of 0.0001.

Preparation of calibration standards for serum and urine sample assay. Stock solution of omeprazole was prepared having a concentration of 250 µg/ml in methanol: 0.1M sodium carbonate aqueous solution mixture (5:95, v/v). The stock solution was further diluted with diluent (0.1M sodium carbonate aqueous solution) to prepare the working standard solution having a concentration of 10 µg/ml. Pantoprazole (internal standard) stock solution (250 µg/ml) was prepared in methanol:0.1M sodium carbonate aqueous solution mixture (5:95, v/v), from which a working standard solution of 10 µg/ml in diluent was prepared. All the solutions were prepared before analysis protected from direct light.

Omeprazole calibration standards for serum were obtained by adding required amount of the working solution and 20 µl of pantoprazole (internal standard) solution (10 µg/ml) to drug free serum to achieve the omeprazole concentrations of 1000, 500, 200, 100, 50, 20, 10, 5 ng/ml. Then they were vortexed for about 2 minutes for proper mixing and liquid-liquid extraction was performed by adding 2 ml methanol in 1 ml of each of the standards solutions. After vortex mixing they were centrifuged at 10,000 rpm for 5 minutes and the supernatants were collected.

Calibration standards for urine samples were prepared by adding required amount of omeprazole working solution and 100 µl of pantoprazole solution (250 µg/ml) to drug free urine to achieve the omeprazole concentrations of 100, 50, 20, 10, 5, 2, 1 µg/ml. Then they were vortexed for about 2 minutes for proper mixing. All these standards were stored at -30^0C until further analysis and analyzed by the HPLC method for the construction of calibration curves and method validation. In all cases the injection volume was 20 µl.

Preparation of QC samples. A series of quality control serum samples were prepared by spiking blank serum with 20 µl of Pantoprazole (internal standard) solution (10 µg/ml) and required amount of omeprazole to yield the final serum samples of 10, 100, 1000 ng/ml of omeprazole. These samples were then vortexed for 2 minutes and omeprazole was extracted according to method described in the previous section.

Similarly A series of control urine samples were prepared by spiking blank urine with 100 µl of Pantoprazole (internal standard) solution (250 µg/ml) and required amount of omeprazole to yield the final urine samples of 1, 10, 100 µg/ml of omeprazole and kept at −80°C until further analysis as quality control sample. All the samples were protected from light

throughout the experiment. These QC samples were used for method validation.

Validation. Calibration curves were constructed by using ratio of peak area of omeprazole to pantoprazole (internal standard) against the concentration of omeprazole and used to determine the omeprazole concentrations in unknown samples. The following parameters were determined for the validation of the analytical method developed for the determination of omeprazole in human serum and urine: specificity, selectivity, linearity, range, precision, accuracy, limit of detection, limit of quantitation, recovery, stability and ruggedness.[21-23]

Application of the method. This validated method had been applied to measure omeprazole concentration in serum and urine samples to evaluate the bioavailability of single dose of omeprazole 20 mg capsule (Omep-20; manufactured by Aristopharma Ltd., Dhaka, Bangladesh) in 24 healthy volunteers. The medication was administered under fasting conditions with 250 ml of water. Blood samples were collected at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6.5, 8, 10, 12 and 24 h after drug administration. After each blood sampling, serum was separated by centrifugation at 3000 rpm for 15 min and stored at –80°C until further assay. Serum samples were prepared by liquid-liquid extraction method. Urine samples were also collected in the time intervals of 0, 0–2, 2–4, 4–8, 8–12, 12–24, 24–36 and 36–48 h post dosing. The samples were kept at -30^0C in a marked tube until further analysis. Urine samples were prepared by adding the internal standard followed by vortexing for proper mixing. All the samples were analyzed by the described HPLC method.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions. A number of stationary and mobile phases were checked to determine the optimum separation and the highest analytical sensitivity for omeprazole and such sensitivity were given by the slope of calibration curves obtained for the assayed conditions. The optimal condition, with which the best result was obtained, has been reported in the experimental section. The mobile phase was buffered at a pH of 7.2 to ensure the stability of omeprazole since it rapidly degraded in acidic environment.[3] A higher pH was avoided because of the risk of degradation of the silica-based column. The mean retention times for omeprazole and the internal standard were 8.8 min and 11.2 min respectively. The analysis time was set at 15 min. This time assured the elution of omeprazole and pantoprazole without any interference. Baseline resolution of the substances was achieved under the chromatographic conditions of the study. The suitability of the chromatographic system was checked daily before analysis by evaluating the tailing factor, the resolution and the system repeatability of three injections of a solution of omeprazole (100ng/ml) and internal standard in mobile phase which demonstrated good performance of the chromatographic system throughout the study.

Selectivity. Drug-free human serum and urine samples from six different sources were analyzed to ensure selectivity of the method.[24] These chromatograms were free of interferences at the retention times of omeprazole and the internal standard (Figure 1A to D and 2A to D). The chromatograms for serum and urine samples are shown in Figure 1E and 2E respectively after oral administration of 20 mg of omeprazole capsule.

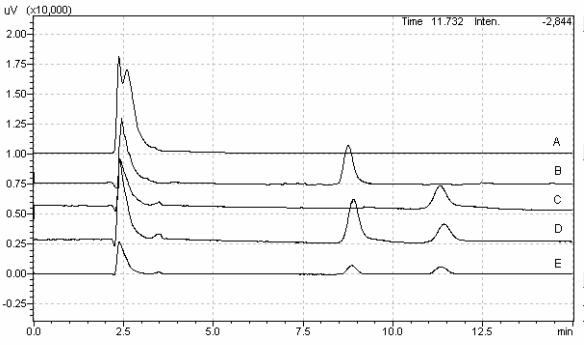

Figure 1. Representative chromatograms obtained from A) drug-free serum sample B) serum spiked with 500 ng/ml of omeprazole C) serum spiked with internal standard (pantoprazole, 200 ng/ml) D) calibration standard for serum sample spiked with 500 ng/ml of omeprazole and internal standard (pantoprazole, 200 ng/ml) E) extracted serum sample from a volunteer 2.0 h after a 20 mg oral dose of omeprazole.

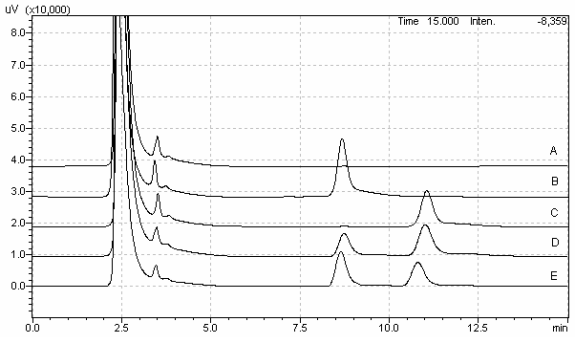

Fig. 2. Representative chromatograms obtained from A) drug-free urine sample B) urine spiked with 50 µg/ml of omeprazole C) urine spiked with internal standard (pantoprazole, 25 µg/ml) D) calibration standard for urine sample spiked with 50 µg/ml of omeprazole and internal standard (pantoprazole, 25 µg/ml) E) processed urine sample from a volunteer 4.5 h after a 20 mg oral dose of omeprazole.

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Sensitivity, Linearity and Range. According to IUPAC as cited in Roger Causon a method is said to be sensitive if small changes in concentration cause large changes in the response function.[25] Sensitivity can be expressed as the slope of the linear regression calibration curve, and it is measured at the same time with the linearity test. The sensitivity attainable with an analytical method depends on the nature of the analyte and the detection technique employed.[26]

The linearity of the assay method was validated for serum (eight standards) and urine (seven standards) samples. The slope and the intercept of the calibration graphs were calculated through least squares by weighing linear regression of omeprazole to internal standard peak-area ratio. The concentration of omeprazole was studied over the range of 5 to 1000 ng/ml in serum and 1 to 100 µg/ml in urine. Data were fitted to the equation $y=mx + b$, where y is the peak area ratio, x is the drug concentration, m and b are the slope and y-axis intercept of the calibration curve, respectively. The calibration curve was found to be linear over the specified range. To construct a calibration curve in bioanalysis, a linear regression analysis is generally used. This analysis assumes univariant regression, implying that the residuals are minimized around the dependent variable (or response) and that the independent variable (or concentration) is errorless. Other assumptions involved are the independence and normal distribution of residuals as well as their homocedasticity (equal variances). Homocedasticity was not observed in our data, as usually occurs in bioanalysis.[27-29] The mean regression parameters are given in Table 1. The mean (± S.D.) of the slope and intercept of the serum standards were 0.0057 (±0.0003) and 0.0951 (±0.0315), respectively. As for the urine standards, the mean (±S.D.) of the slope and intercept were respectively 0.151 (±0.0233) and −0.158 (± 0.101). The coefficient of determination was greater than 0.991 on all calibration curves in serum and urine.

Limit of Quantitation (LOQ). The lower limit of quantification can be defined as the lowest concentration on the calibration curve with acceptable precision and accuracy (CV<20%).[21] The lower limit of quantitation (LOQ) of omeprazole was found to be 5ng/ml (precision 6.03% and 10.13% for intraday and interday, respectively).

Precision and accuracy. The precision of the method was assessed by determining the intra-day and inter-day CV for different concentrations and are mentioned in the Table 2 and 3. The values obtained were in all cases lower than 14.14% for both serum and urine samples. The accuracy of the method for both serum and urine was determined using standard addition method and the results are given in Table 4. The precision and accuracy of the method was found to be well within the limits considered as acceptable.[24] Hence the results indicate that the method is precise, accurate and reliable.

Recovery. The method of extraction of omeprazole and pantoprazole was evaluated for efficiency and the results are shown in the Table 5. The recovery of omeprazole was measured for all QC samples and the average recovery was found to be 97.57% and 98.17% from serum and urine samples respectively. In case of internal standard, mean recovery of 98.1% and 99.6% were obtained for serum and urine samples. The method showed good efficiency in terms of recovery.

Stability. The stability of the analytes in human serum and urine under different temperature and timing conditions were evaluated for different storage conditions. The results are depicted in the Table 6.

Table 1. Regression data for the standard curve (n=6) of omeprazole in human serum and urine

		Correlation coefficient (r^2)	Slope	Intercept
Serum	Mean	0.9918	0.0057	0.0951
	S.D.	0.0071	0.0003	0.0315
	R.S.D.	0.7141	5.5007	33.0701
	Standard error	0.0032	0.0001	0.0141
Urine	Mean	0.9958	0.1509	- 0.158
	S.D.	0.0036	0.0233	0.101
	R.S.D.	0.3613	15.465	- 63.52
	Standard error	0.0018	0.0117	0.050

Table 2. Intra-day and Inter-day precision and accuracy of omeprazole in serum (n=6)

Concentration (ng/ml)	Concentration found (ng/ml) Mean ± SD	Precision CV (%)	Accuracy (%)
Intra-day			
10	9.35 ± 0.7	7.61	93.54
100	104.39 ± 9.2	8.77	104.38
1000	955.61 ± 68.5	7.17	95.56
Inter-day			
10	11.39 ± 1.6	14.14	113.95
100	108.28 ± 10.1	9.37	108.28
1000	976.09 ± 39.9	4.09	97.61

Table 3. Intra-day and Inter-day precision and accuracy of omeprazole in urine (n=6)

Concentration (µg/ml)	Concentration found (µg/ml) Mean ± SD	Precision CV (%)	Accuracy (%)
Intra-day			
1	1.03 ± 0.1	2.86	103.48
10	10.19 ± 1.1	11.15	101.91
100	100.55 ± 1.3	1.33	100.55
Inter-day			
1	1.09 ± 0.1	6.32	109.97
10	10.47 ± 0.7	6.36	104.69
100	97.42 ± 9.5	9.7	97.42

Table 4. Accuracy of the method for the determination of omeprazole in serum and urine (n=6)

For serum samples				
Concentration of omeprazole (ng/ml)			Omeprazole found (ng/ml) (Mean ± SD) (n=6)	% Accuracy (Mean ± SD) (n=6)
Initial quantity (a)	Quantity of standard added (b)	Total quantity (a+b)		
50	0	50	49.36 ± 4.1	98.72 ± 8.2
50	50	100	104.49 ± 11.2	104.49 ± 11.2
50	150	200	199.05 ± 15.4	99.52 ± 7.7
For urine samples				
Concentration of omeprazole (µg/ml)			Omeprazole found (µg/ml) (Mean ± SD) (n=6)	% Accuracy (Mean ± SD) (n=6)
Initial quantity (a)	Quantity of standard added (b)	Total quantity (a+b)		
5	5	10	10.91 ± 1.1	109.05 ± 10.5
5	15	20	20.33 ± 1.7	101.63 ± 8.7
5	45	50	49.78 ± 0.9	99.55 ± 1.9

Table 5. Extraction efficiency of the bioanalytical method (n=6).

		Recovery (%) [Mean ± SD]	
		Omeprazole	Pantoprazole (I.S.)
Serum concentration (ng/ml)	10	95.7 ± 1.8	
	100	98.0 ± 2.3	
	1000	99.0 ± 5.7	
	200	–	98.1 ± 7.9
Urine concentration (µg/ml)	1	99.8 ± 0.73	
	10	99.2 ± 2.7	
	100	95.5 ± 4.6	
	25	–	99.6 ± 2.9

Table 6. Stability of omeprazole in serum and urine QC samples in different storage conditions (n=6).

		Recovery % (Mean ± SD)		
	Concentration	6 hr at ambient temp	3 freeze thaw cycle	1 month storage at -80^0 C
Serum (ng/ml)	10	94.37 ± 1.6	91.29 ± 0.8	88.39 ± 2.9
	100	97.10 ± 3.8	98.02 ± 4.8	88.02 ± 1.7
	1000	93.69 ± 2.8	95.23 ± 3.6	91.73 ± 2.6
Urine (µg/ml)	1	99.31 ± 2.1	91.01 ± 1.1	91.09 ± 3.4
	10	95.23 ± 7.2	89.24 ± 1.3	89.89 ± 0.9
	100	97.02 ± 3.1	98.02 ± 6.5	93.67 ± 6.3

For short term stability determination, stored serum and urine aliquots were kept at ambient temperature for 6 hr exceeding that expected to be encountered during the routine sample preparation. Samples were pretreated and analyzed as above mentioned. The mean recoveries of the low, medium and high QC levels ranged from 93.69 to 97.1% for serum samples and 95.23 to 99.31% for urine samples. These results indicated reliable stability behavior under the experimental condition of the regular runs.

The stability of omeprazole in serum and urine samples over three cycles of freeze (-80^0C) and thawing (room temperature) were assessed. The mean recoveries of the low, medium and high QC levels (10, 100 & 1000 ng/ml for serum and 1, 10, 100 µg/ml for urine) ranged from 91.29 to 98.02 % for serum samples and from 89.24 to 98.02 % for urine samples, which indicate that the analyte is stable over the storage condition. The stability was also satisfactory after one month storage at -80^0C.

Ruggedness. Peak shape and resolution of omeprazole from other peaks in the matrix remained visually acceptable throughout the assay. The limit of quantitation demonstrated a reproducible response readily distinguishable from the noise level. The method was applied in three pharmacokinetic studies to evaluate the ruggedness of the method. The method was used for a period of 6 months using 5 analytical columns, two apparatus, four analysts and several batches of chemical reagents. It was found that the method does not change with time or study conditions.

Application of the method. The analytical method had been applied successfully to the analysis of samples from several pharmacokinetic studies. In a study with 24 volunteers, serum and urine samples together with calibration standards and quality control samples were assayed for omeprazole content. Figure 3 and 4 illustrate mean serum concentrations of omeprazole versus time profile curve and mean percentage of cumulative excretion of omeprazole over 48 hours respectively following single oral dose of 20 mg of omeprazole capsule.

A few HPLC-UV, LC-MS/MS, and GC-MS methods have been reported in different literatures.[30,31] Some of these methods use complicated extraction instruments, long and tedious extraction procedures, and large amounts of solvents or biological fluids for extraction while other methods have a long turnaround time during analysis. To minimize these limitations, the present

investigation provides a rapid, selective and sensitive HPLC-UV method that has short and simple extraction procedure, consume small amounts of solvent and biological fluid for extraction with a short turnaround time.

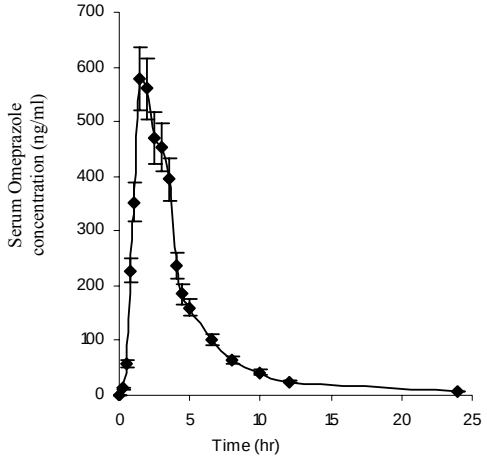

Figure 3. Mean (±S.D.) serum omeprazole concentration versus time curve following oral administration of single dose 20-mg omeprazole capsule (n=12)

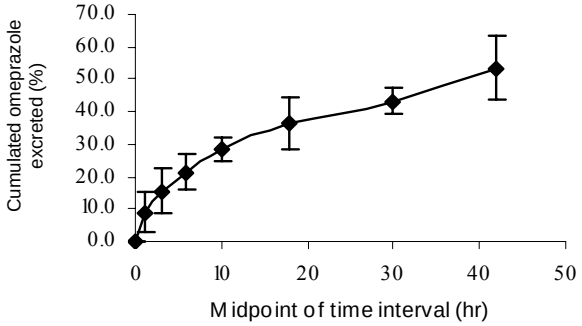

Figure 4. Mean (±S.D.) cumulative percent of omeprazole excreted versus midpoint of time interval curve following oral administration of single dose 20-mg omeprazole capsule (n=12)

The present HPLC method fulfils the acceptance criteria generally established for bioanalytical assays when applied in pharmaceutical analysis. The validation data also indicates that the method is able to quantify the drug at minor quantity like 5 ng/ml in biological samples with good precision and accuracy that can be applied for the quantization of omeprazole in biological samples. However, the HPLC method validation should be useful for monitoring serum and urinary drug concentrations and development of pharmacokinetic studies. In the explored range the method was accurate, precise, and selective enough to allow the analysis of omeprazole in human serum and urine samples after single oral administration of 20 mg of omeprazole capsule. The internal standards pantoprazole selected as structural analogues of omeprazole was allowed to compensate the signal suppression effect and reduce inaccuracy problems. In comparison with the previously developed methods, the present method offers an undoubted advantage in term of overall analytical performance.

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