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Research Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF RANITIDINE FROM PLASMA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Rapid and simple bioanalytical high performance liquid chromatographic (HPLC) method for the determination of Ranitidine using Nizatidine as an internal standard was developed and validated as per regulatory requirements. Sample preparation was accomplished through solid phase extraction and chromatographic separation on a reverse phase column. The mobile phase consists of mixture of phosphate buffer and methanol at a flow rate of 1 ml/min. The wavelength used for the detection of ranitidine was 315nm with a total run time of 8 minutes. The retention times of ranitidine and nizatidine were found to be 6 and 4.5 minutes respectively. The method was developed and tested for the linearity range of 10 ng/ml to 1000 ng/ml. The method was validated for accuracy, precision, linearity, recovery and stability in compliance to international regulatory guidelines

Keywords: Ranitidine, HPLC, Validation, Plasma.

INTRODUCTION

Ranitidine, N, N-dimethyl-5-[2-(1-methylamino-2 nitrovinylamino) ethylthiomethyl]-furfuylamine, is a H2 receptor antagonist commonly used in the treatment of duodenal and gastric ulceration. Ranitidine can be found in many pharmaceutical forms such as tablets, injectable solutions and oral liquids[1, 2]. Peptic ulcer is a common disease worldwide, with an estimated 10% of the population effected. Research has shown that the infection of Helicobacter pylori is a key factor in the occurrence and reoccurrence of peptic ulcer. Helicobacter pylori is a gram negative and microaerophilic organism which can wreck the mucosa, disturb the secretion of gastric acid and induce inflammation [3]. The most prominent effects of H2-receptor antagonists are on basal acid secretion; less profound but still significant is suppression of stimulated (feeding, gastrin, hypoglycaemia, or vagal stimulation) acid production [4]. Ranitidine is highly effective in the treatment of duodenal and stomach ulcers and Zollingen-Ellison syndrome. Ranitidine has been shown to be five to eight times more potent as an inhibitor of gastric secretion than other histamine H2 receptor antagonists [5].

High performance liquid chromatography has already become one of the most widely applied analytical separation techniques because of its superior performance and reliability, especially in the pharmaceutical, environmental, forensic, clinical, food and flavour sciences[6].

Validation of the bioanalytical method is the primary requirement for a method which is part of the pharmacokinetic study and/or bioequivalence study. Guidelines for the bioanalytical method validation have been available since 2001 by USFDA[7] and recently the EMEA has also released the guidelines for the same[8]. Many concept papers [9] and other literature [10] have been published at various forums for the increased requirement of bioanalytical method validation.

Some of the bioanalytical methods have been reported for the estimation of ranitidine from the pharmaceutical preparations [1, 11, 12, 13, 14, 15], and biological matrices by various techniques involving HPLC [2, 3, 4, 5, 16, 17]. An attempt was made to develop the bioanalytical method for the estimation of ranitidine from plasma using nizatidine as internal standard and to validate as per international regulatory guidelines. Weighting factors are used during the regression calculations [18] and the suitable regression factor was evaluated.

EXPERIMENTAL

Chemicals

Ranitidine was gift sample from Cipla Pharmaceuticals, India, and Solid Phase Extraction Cartridges were of waters. Plasma was obtained from commercial supplier. All the other chemicals were of analytical grade.

Preparation of solutions

Potassium phosphate buffer was prepared by dissolving approximately 1.75 gm of potassium phosphate in 1000 ml of water and the pH was adjusted to 7.0 with ortho phosphoric acid. 20% methanol in phosphate buffer was prepared by mixing 20 ml of methanol and 80 ml of phosphate buffer. 5% ammonia solution in methanol was prepared by mixing 5 ml of ammonia with 95 ml of methanol. 10% methanol was prepared by mixing 10 ml of methanol and 90 ml of water. Mobile phase was prepared by mixing phosphate buffer and methanol in the ratio of 65:35.

Preparation of standards

Ranitidine and nizatidine stock solutions were prepared at a concentration of 0.5 mg/ml by dissolving in methanol and the stock solutions were stored in the refrigerator. Spiking solutions of ranitidine for the preparation of calibration standards and quality control samples were prepared in 10% methanol and spiked in to the plasma at the ratio of 1:50. The calibration curve from 10.0 to 1000.5 ng/ml was generated using eight calibration standards at the concentrations of 10.0 ng/ml (STD 1), 20.1 ng/ml (STD 2), 50.1 ng/ml (STD 3), 150.1 ng/ml (STD 4), 250.2 ng/ml (STD 5), 500.5 ng/ml (STD 6), 750.6 ng/ml (STD 7) and 1000.5 ng/ml (STD8). The Quality Control samples were prepared at the concentrations of 10.3 ng/ml (LLOQQC), 29.9 ng/ml (LQC), 450.2 ng/ml (MQC) and 805.8 ng/ml (HQC). The bulk spiked calibration standards and quality control samples were stored in the freezer. Internal standard dilution was prepared by adding 0.15 ml of internal standard stock solution in to a 10ml volumetric flask and made up the volume with 10% methanol.

Sample preparation and extraction

Ranitidine from the plasma was extracted using solid phase extraction technique. Aliquot 0.4 ml of plasma into the extraction tubes and added 50µl of internal standard dilution and vortexed to mix the contents. 1 ml of 1N hydrochloric acid was then added and vortexed to mix the contents. SPE cartridges were conditioned with 1 ml of methanol and water and on to the conditioned cartridges to load the above sample and washed the cartridges with 1 ml of methanol. Finally the cartridges were eluted with 1 ml of 5% ammonia in methanol solution and evaporated the elution solution at approximately 50°C till the tubes were dried. The dried tubes were then reconstituted with 0.3 ml of 20% methanol in phosphate buffer before injection into HPLC column.

Chromatographic conditions

Waters HPLC (2695) with the C18 column (150x4.6mm, 5µm) (YMC Pack Pro-C18) was used and the wavelength used for the detection was 315nm. The other method conditions included were the column oven temperature of 40°C, flow rate of 1 ml/min, run time of 8 minutes and injection volume of 30µl.

Validation

The method performance was evaluated for selectivity, accuracy, precision, linearity, and robustness, stability during various stress conditions including bench top stability, freeze thaw stability, auto sampler stability, stability of stock solutions etc. and dilution integrity and recovery.

Selectivity

Selectivity was evaluated by extracting different blank plasma samples. The absence of interfering peaks at the same retention time of analytes or internal standard was considered as evidence for selectivity.

Linearity

Calibration curves were constructed using linear regression (with weighting of $1/x^2$) within the range of 10-1000 ng/ml of ranitidine.

Recovery

Recovery of analyte was evaluated by comparing the response of ranitidine in three quality control samples (LQC, MQC and HQC) with the response of ranitidine in equivalent aqueous solutions. Recovery of internal standard was calculated in the same manner at the MQC level.

Precision and accuracy

For precision and accuracy studies, samples were prepared at four concentration levels, limit of quantification (LOQQC), low (LQC), medium (MQC) and high (HQC) quality controls. Corresponding to 10, 30, 450 and 800 ng/ml respectively with six replicates each. Precision and accuracy was evaluated at inter and intra batch.

Dilution Integrity

Dilution integrity was evaluated by diluting the sample having the concentration of approx. 1600 ng/ml (approx. two times of HQC) with 1/5 and 1/10 dilutions and quantified against the calibration curve to evaluate the ability to dilute the pharmacokinetic samples.

Stability studies

The stability of the ranitidine in solutions and plasma samples was also evaluated during method validation. Ranitidine stability was evaluated using two concentration levels (low

and high quality control, corresponding to 30 and 800 ng/ml respectively). The stability of ranitidine was also evaluated in post-extracted samples kept in the auto sampler at 10 °C for 60 hours, as well as in plasma samples kept at freezer and after being stressed to 3 freeze-thawing cycles (24 hours each cycle). All samples described above were compared to freshly prepared ranitidine samples at the same concentration level.

RESULTS AND DISCUSSION

Chromatographic optimization

HPLC linked with photodiode array ultraviolet detector (DAD) has proven to be an important tool in the identification of compounds[19]. During our work the UV detector was used for the selection of the best wavelength (315 nm) to maximize the signal of compounds and minimize the signal of plasma components (interferents).

The chromatography conditions, especially the composition of mobile phase were optimized through several trials to achieve good resolution and symmetric peak shape for the analyte (ranitidine) and internal standard. The composition of the mobile phase was optimized by varying the percentage and pH of potassium phosphate buffer and percentages and type of organic component (Methanol or Acetonitrile). Finally 10 mM potassium phosphate buffer (pH 7.2): Methanol (65:35), chosen as the final mobile phase since it provided the best separation, with higher sensitivity and selectivity for the UV signal of analyte and internal standard.

Sample preparation

The solid phase extraction procedure was considered as the suitable extraction procedure using waters HLB cartridges after evaluating the acceptability of other extraction techniques like precipitation, liquid- liquid extraction. The extracted eluent was dried and reconstituted to get cleaner extract since the cleaner extract increases the column life. The Quality control samples were prepared at the concentrations specified in the bioanalytical method validation guidelines. The LOQQC was prepared at approximately same concentration of lowest calibration standard. The LQC was prepared at the concentration less than three times of lowest calibration standard. MQC concentration was prepared at approximately 45% of the highest calibration standard. HQC concentration was prepared at the concentration of approximately 80% of the highest calibration standard.

Selectivity

The described method used reversed-phase HPLC for separation of ranitidine from nizatidine (internal standard) and was shown to be selective for the analyte and its internal standard (retention times for ranitidine and nizatidine were 6 and 4.5 minutes respectively). No interfering peaks were observed with the same retention time of the analyte when plasma samples from different volunteers were analysed. Figures 1, 2 and 3 represent the chromatogram of blank plasma sample and lowest calibration standard and highest calibration standards respectively.

Linearity

Linearity was demonstrated from 10.0-1000.5 ng/ml. Table 1 show data from calibration curves analysed for the evaluation of precision and accuracy during different days. The calibration curve includes 8 calibration standards which are distributed throughout the calibration range. Correlation coef ficient was considered for the evaluation of goodness fit. The average correlation coefficient was found to be 0.9896 with goodness of fit.

Table 1: Precision and accuracy of Calibration standards:

Batch	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	Regression Coefficient (r value)
	Concentration (ng/ml)								
	10.0	**20.1**	**50.1**	**150.1**	**250.2**	**500.5**	**750.6**	**1000.5**	
Precision and Accuracy Batch-1	10.1	19.9	48.0	154.1	240.2	510.2	780.2	1080.8	0.9914
Precision and Accuracy Batch-2	10.7	20.6	52.1	165.2	238.2	520.7	680.2	980.4	0.9846
Precision and Accuracy Batch-3	9.8	21.2	51.9	160.2	259.5	474.6	729.8	1095.3	0.9927
Average	10.20	20.57	50.67	159.83	245.97	501.83	730.07	1052.17	0.9896
Standard Deviation	0.458	0.651	2.312	5.559	11.763	24.162	50.001	62.573	
%CV	4.5	3.2	4.6	3.5	4.8	4.8	6.8	5.9	

Precision and accuracy

Precision and accuracy was evaluated by analysing 3 precision and accuracy batches. Each precision and accuracy batch consists of calibration curve and six replicates of LOQQC, LQC, MQC and HQC. Precision and accuracy was evaluated both inter and intra batches. The intraday and inter day precision and accuracy of the method for each ranitidine concentration level (10.3, 29.9, 450.2 and 805.8 ng/ml) are presented in Table 2. The mean accuracy for each concentration level ranged from 99.6 to 104.9 and the mean precision for each concentration level ranged from 5.2 to 10.8.

Recovery

The recovery was evaluated by comparing response of extracted and unextracted samples. Extracted samples include six replicates of extracted LQC, MQC and HQC samples. Unextracted samples included the aqueous solutions equivalent to extracted samples. Internal standard recovery was evaluated in the same manner at MQC level. The average recovery for ranitidine in plasma was ranged from 86 to 88% for the low, medium and high quality control samples respectively with an average of 87.3%. The average recovery of the internal standard was 89.9%.

Stability studies

Stability studies were performed to evaluate the stability of ranitidine both in aqueous solution and in plasma after exposing to various stress conditions. The stability studies performed include stock solution stability of ranitidine and nizatidine in stock solution, stock dilution stability of ranitidine in dilutions, bench top stability in plasma, free thaw stability in plasma, long term storage stability in plasma, and auto sampler stability of processed samples. All stability evaluations were performed as per international regulatory guidelines.

Ranitidine and nizatidine stock solutions (1 mg/ml) remained stable when stored at refrigerator conditions for 7 days including the storage at room temperature for 8 hours. Ranitidine was stable in plasma samples when stored at room temperature for 18 hours. Ranitidine was found to be stable for 3 freeze and thaw cycles. Ranitidine was stable and did not show any degradation when stored in the freezer for 85 days. Ranitidine in the processed samples was stable for 60 hours when stored in the auto sampler at 10°C.

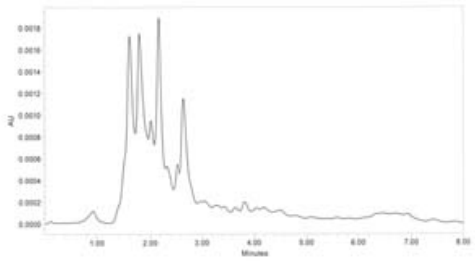

Fig. 1: Representative chromatogram of blank plasma sample

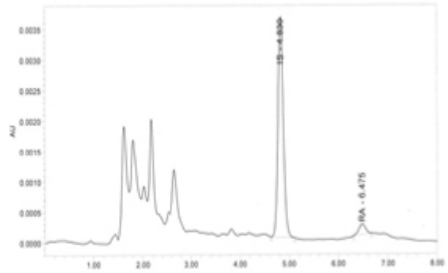

Fig. 2: Representative chromatogram of lowest calibration standard (IS-Internal Standard and RA-Ranitidine)

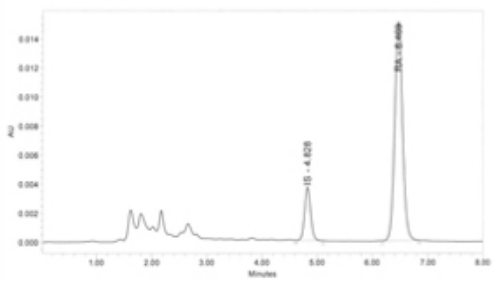

Fig. 3: Representative chromatogram of highest calibration standard (IS-Internal Standard and RA-Ranitidine)

Table 2: Between and within batch precision and accuracy of QC samples:

Batch-1	**LOQQC**	**LQC**	**MQC**	**HQC**
Nominal Concentration	**10.3**	**29.9**	**450.2**	**805.8**
Precision and Accuracy Batch-1	9.8	30.6	431.4	764.5
	9.7	32.9	425.8	789.9
	10.2	31.3	444.9	732.4
	9.5	33.2	478.9	756.3
	11.0	31.7	489.5	843.6
	10.9	29.2	467.3	834.8
Average	10.20	31.26	455.43	789.61
Standard Deviation	0.583	1.482	23.966	41.296
% Nominal	99.0	104.5	101.2	98.0
%CV	5.7	4.7	5.3	5.2
Precision and Accuracy Batch-2	12.3	32.6	445.6	778.2
	11.3	35.4	411.5	821.6
	10.4	26.4	489.6	831.5
	9.6	28.0	513.5	821.3
	11.4	31.7	487.5	777.9
	9.3	33.4	431.8	745.9
	10.3	29.3	472.7	885.8
Average	10.72	31.25	463.25	796.07
Standard Deviation	1.155	3.405	39.462	33.794
% Nominal	104.0	104.5	102.9	98.8
%CV	10.8	10.9	8.5	4.2
Precision and Accuracy Batch-3	14.1	31.7	514.3	886.9
	9.9	32.1	513.6	872.9
	10.8	31.4	468.9	779.9
	10.9	34.4	444.9	774.9
	11.2	28.7	513.0	813.9
	9.8	31.5	542.9	810.4
	9.4	32.9	469.8	875.9
Average	11.12	31.63	499.60	823.15
Standard Deviation	1.566	1.820	35.780	46.874
% Nominal	107.9	105.8	111.0	102.2
%CV	14.1	5.8	7.2	5.7
Global Precision and Accuracy				
Average	10.65	31.37	471.85	802.24
Standard Deviation	1.148	2.215	36.957	41.430
% Nominal	103.4	104.9	104.8	99.6
%CV	10.8	7.1	7.8	5.2

CONCLUSION

We described here the development of a new, selective, precise and accurate method for the quantification of ranitidine in human plasma using HPLC with UV detection and solid phase sample extraction technique which is suitable for application to a pharmacokinetic, bioequivalence and drug interaction studies for the estimation of ranitidine from plasma. The limit of quantification of the method was set to 10 ng/ml considering the dosage of ranitidine administered and it is determined not only by detection technique but also by the effective clean-up of sample and thus improving the signal to noise ratio. The method reported here uses a simple and effective extraction technique with good and reproducible recovery.

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