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Research Article

DEVELOPMENT AND VALIDATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR BIOANALYTICAL APPLICATION WITH RANITIDINE HCL

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ABSTRACT

A simple, rapid and specific bioanalytical procedure was developed and validated for the quantification of Ranitidine HCl (RHCl) in human plasma by High-Performance Liquid Chromatography (HPLC) with UV detection. The chromatographic separation was achieved in a Cyano Column (250mm x4.6mm;5µm). The mobile phase was a mixture of 10mM phosphate buffer and acetonitrile (50:50, v/v) at a flow rate of 1.0 mL min^{-1}. The UV detection was set at 320 nm and 0rnidazole was used as an internal standard (IS). An original pretreatment procedure of plasma sample was developed, based on liquid-liquid extraction with dichloromethane. The method was validated over the concentration range of 50 ng mL^{-1} to 2000 ng mL^{-1} for Ranitidine HCl in human plasma with correlation coefficient of 0.9985.The limit of detection(LOD) was 5 ng mL^{-1}. In stability tests, RHCl in human plasma was stable during storage and assay procedure. The method was very rapid, simple, sensitive and economical, and was successfully applied to human pharmacokinetic study.

Keywords: Ornidazole, HPLC, Validation, Plasma, Pharmacokinetic study, Ranitidine HCl (RHCl).

INTRODUCTION

RanitidineHCl(CASNO.71130-06-8),N-(2-[(5-(dimethylamino-methyl)furan-2-yl)methylthio]ethyl)- N-methyl- 2-nitroethene- 1,1-diamine hydrochloride (Fig 1), is a histamine H_2-receptor antagonist that inhibits stomach acid production [1-2]. RHCl is indicated in active and maintenance therapies of duodenal ulcer. It is also used in treatment of active, benign gastric ulcer, pathological hypersecretory conditions, and gastro-esophageal reflux disease. Serum concentrations of 36-94 ng/ml of ranitidine have been shown to inhibit 50% of stimulated gastric acid secretion [3]. High performance liquid chromatography (HPLC) method has been extensively used for the analysis of ranitidine from biological fluids (plasma or urine) due to high sensitivity. It is necessary to validate the analytical method for the determination of RHCl in its pharmacokinetic study. Keeping this fact in mind, we have developed and validated a method for the quantitation of RHCl.

Literature survey reveals that very few analytical methods have been reported for the quantification of RHCl in human plasma. Quantitation using HPLC either by UV or fluorescence detection technique was performed [4-5]. Rustum et al [6] developed a method for analysis of RHCl in plasma and urine by using UV-detection. In the method of Boutagy et al [7] simultaneous analysis of ranitidine and cimetidine in human plasma by HPLC was performed. Khedr et al [8] established a method for determination of RHCl in rabbit plasma by HPLC with fluorescence detection. All these methods are unsatisfactory for pharmacokinetic studies because of complicated sample preparation and high cost technique. It is necessary to develop less expensive and simple method. An ideal method should be simple, rapid separation capability, sensitive and specific.

Hence the main objective of this work was to develop a simple, sensitive, rapid and reliable HPLC method for the quantification of RHCl in human plasma. The developed method was validated according to FDA guidelines and applied to the analysis of RHCl in human plasma using ornidazole as internal standard (IS).

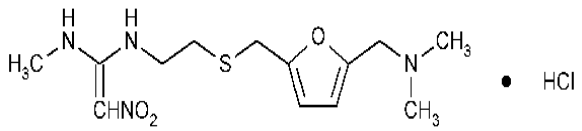

Fig. 1: Structure of Ranitidine HCl

MATERIALS AND METHODS

RHCl was obtained from Albert David limited (Mfg.), Kolkata, India) and Ornidazole from Akums Drugs & Pharmaceuticals Limited (Delhi, India).Dichloromethane(analytical reagent grade) and acetonitrile(LC grade) were purchased from Merck Privated limited (Mumbai, India). All aqueous solutions including the buffer for mobile phase were prepared with HPLC-grade water (resistivity of 18.2 M Ω.cm) generated from Milli-Q gradient water purification system of Millipore (Elix 3, Milli-Q A10 Academic).The blank human plasma with EDTA-K_3 anticoagulant was collected from Clinical Pharmacological Unit (CPU) of Bioequivalence Study Centre, Jadavpur University, Kolkata, India.

Instrumentation and chromatographic conditions

The HPLC apparatus (Knauer, Germany) adjusted with HPLC pump (Knauer 1000), Rheodyne injector (D-14163 Berlin), UV detector (Knauer 2500) and EZChrom (version 3.1.6) software. Reverse phase -HPLC analysis was performed isocratically at room temperature using a cyano, 250 X 4.6mm, 5µ particle size stainless steel column. A mixture of 10mM dipotassium hydrogen phosphate buffer (PH-4.8) and acetonitrile in the ratio of 50:50 (v/v) was used as mobile phase. The mobile phase was filtered through 0.45 µm membrane filter. The eluent was monitored with a UV detector set at 320 nm at a flow rate of 1.0 mL min^{-1} and a sample size of 50 µL was injected through the Rheodyne injector.

Preparation of stock and working solutions

Stock solutions of RHCl and Ornidazole (IS) were prepared at concentration of 10µg mL^{-1} in water and methanol respectively. Both the stock solutions were stored at -20^0 C in polypropylene vials until they were used for working solutions by adding appropriate volume of mobile phase. Working solutions of different concentrations were prepared from above-mentioned stock solution afresh before to use.

Preparation of calibration standards and quality control (QC) samples

Eight different concentrations (50, 100, 250, 500, 750, 1000, 1500 and 2000 ng mL^{-1}) in plasma were prepared by adding required volume of working solution of analyte to blank plasma. Three different QC samples LQC (150 ng mL^{-1}), MQC (1200 ng mL^{-1}) and HQC (1600 ng mL^{-1}) were prepared similarly. For internal standard, the final concentration in plasma was 1000ng mL^{-1}.The plasma samples were subjected to the sample preparation procedure and injected into the HPLC system. Plasma calibration curve was

prepared by taking area ratio of analyte to internal standard as Y-axis and concentration of analyte (ng mL^{-1}) as X-axis. Six replicates of each concentrations of calibration curve were prepared.

Sample preparation

Liquid –liquid extraction procedure was used for the extraction of the RHCl from the plasma. For calibration standards and quality control samples, an aliquot quantity of 0.45 mL plasma sample was taken in a 10 mL stopper test tube and 0.05 mL internal standard was added and mixed. To it 4.5 mL of Dichloromethane was added and mixed for 15 min followed by centrifugation at 5000 rpm for 20 min.The organic layer was separated, transferred to a separated test tube and evaporated to dryness under the stream of N_2 at 40^0 C.The residue was reconstituted in 200 µL of mobile phase, filter through 0.22 µm membrane filter and 50 µL was injected into the HPLC system.

Method validation

The method was validated according with to currently accepted principles of the US Food and Drug Administration (FDA) industry guidance for validation of bioanalytical methods [9]. The validation parameters addressed were linearity, precision, accuracy, specificity and stability.

Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in presence of other components in the sample. The specificity of the method was evaluated by screening six different sources of blank plasma. These lots were spiked with known concentration of analytes along with IS at low, medium and high concentrations. The spiked samples were analyzed after extraction to confirm lack of interference and absence of lot to lot variation.

Sensitivity

For determination of sensitivity, the lowest concentration-standard used to produce the calibration plot was regarded as the LLOQ. Analysis at theLLOQ should meet two criteria:

- The response at the LLOQ should be five times the response of the blank.
- The response at the LLOQ should be identifiable, discrete, and reproducible with precision of 20% and accuracy of 80–120%.

Six samples containing 0.05 µg mL^{-1} RHCl regarded as the LLOQ, were investigated. Reproducibility and precision were determined.

Accuracy and precision

Inter- and intra-assay precision and accuracy were determined by analyzing six replicates at four different concentrations (LLOQ, LQC, MQC and HQC) levels in three different days. The comparison was made between the obtained values and the experimental values. Precision was expressed as percentage of relative standard deviation (% R.S.D.). The mean value of accuracy should be within 15% of actual value except at LLOQ, where it should not deviate by more than 20%. The precision at each concentration level should not exceed 15% of R.S.D. except for the LLOQ where it should not deviate by more than 20%. Should not exceed 20% of Extraction Recovery. The extraction recovery of the analytes from the plasma was evaluated by comparing the mean detector responses of three replicates of processed QC samples at low, medium and high concentrations to the detector responses of standard solutions of same concentration, where as the recovery of the IS was determined at a single concentration of 1000ng mL^{-1}. Recovery of an analyte need not be 100 %, but the extent of recovery of analyte and the IS should be consistent, precise and reproducible.

Stability

The stability of RHCl in plasma was evaluated with four studies; freeze-thaw, ambient, short-term and long-term stability study. Six replicates of two QC samples at concentration of 1500 ng mL^{-1} (LQC), and 1600 ng mL^{-1} (HQC) was prepared and then subsequent HPLC analysis was carried out.

The freeze-thaw stability study was carried out by comparing the QC samples that had been frozen and thawed three times, then extracted and analyzed. For ambient stability study the QC samples were kept at room temperature for 24 h, then extracted and analyzed. The short-term, long-term stability study was carried out with plasma samples spiked with RHCl, which were stored at -20^0 C for 1 and 3 months respectively, then extracted and analyzed.

RESULTS AND DISCUSSION

Linearity

The calibration curves were found to be linear over a range from 50 to 2000 ng mL^{-1} of the peak-area ratio of RHCl to internal standard versus concentration (Fig-2).The average correlation coefficients obtained was 0.9985. The calibration equation shows an excellent linearity with average slope 0.001 and intercept -0.00317.The limit of detection (LOD) and lower limit of quantification (LLOQ) were 5 ng mL^{-1} and 50 ng mL^{-1}, respectively. Table 1 shows all the back calculated values with excellent accuracy and precision.

Specificity

Representative chromatograms of blank plasma, blank plasma spiked with RHCl and IS, and volunteer plasma sample after administration of an oral dose of 300 mg RHCl are shown in Fig 3. The analyte was separated well from IS under the described chromatographic conditions. Total run time of the chromatogram was 10 min and the retention time of drug and IS were about 8.76 and 3.60 min, respectively. No interfering peaks at these times were found in the chromatogram obtained from blank plasma.

Accuracy and precision

The intra- and inter-day precision (% R.S.D.) values of RHCl for various concentrations ranged from 4.395% to 2.0611% and 5.139% to 1.875% respectively. At the same concentrations, the percentage of accuracy was in the range of 98.25% to 99.73% and 99.33% to 99.88% respectively. Both accuracy and precision were in the acceptable range for bioanalytical purpose. The precision and accuracy data for both intra and inter day assays of three QC samples are presented in Table 2. The assay method demonstrated high degree of accuracy and precision.

Table 1: Summary of calibration standards

Conc. added (ng mL^{-1})	Mean conc. found (ng mL^{-1})	S.D.	R.S.D. (%)	Accuracy (%)	n
50 (LLOQ)	48.35	18.61	18.4476	96.71	6
100	97.39	7.364	9.6750	97.39	6
250	258.77	13.70	5.294	103.50	6
500	507.55	11.49	2.263	101.51	6
750	729.67	8.24	1.12	97.28	6
1000	989.39	12.83	1.296	98.93	6
1500	1529.77	16.17	1.057	101.98	6
2000	1984.39	24.28	1.22	99.21	6

S.D. = Standard deviation; R.S.D. (%) (Relative standard deviation) = [(S.D./Mean) X 100]; Accuracy (%) = [(Mean cons. found / Conc. added) X 100]; n = number of replicates.

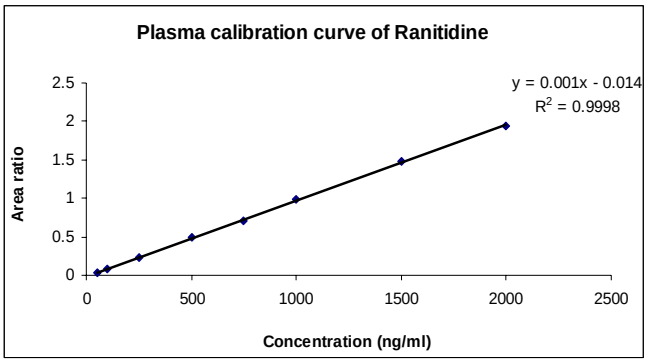

Fig. 2: Plasma calibration curve of Ranitidine.

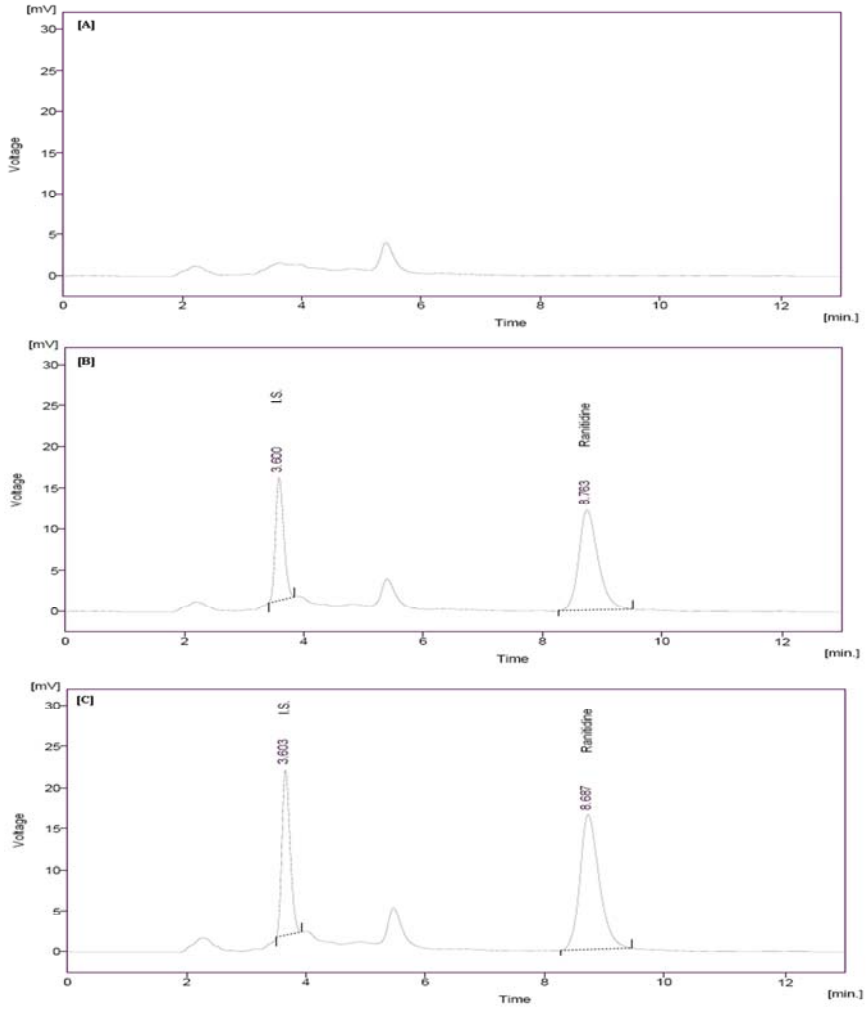

Fig. 3: [A] Blank plasma; [B] Blank plasma spiked with raw drugs; [C] Human plasma containing Ranitidine HCl and IS.

Extraction recovery

The recoveries (mean) of RHCl at low-(150 ng mL^{-1}), medium-(1200 ng mL^{-1}) and high-(1600 ng mL^{-1}) quality control samples was 89.23%, 90.18% and 85.27%, respectively. The recovery (mean) of IS was 91.24% at the concentration used in the assay (Table 3).

Stability

The predicted concentrations for each analyte at LQC and HQC samples deviated within ± 15% of the nominal concentrations in a batter of stability tests viz., three freeze/thaw cycles, bench-top (24 h), at -20° C for at least for 1 month and at -20° C for at least 3 month (Table 4). The results were found to be within the assay variability limits during the entire process.

Table 2: Assessment of accuracy and precision from quality-control sample

QC Sample (ng mL^{-1})	Mean (ng mL^{-1})	S.D.	R.S.D. (%)	Accuracy (%)
Intra Day (n=12)				
50	49.135	2.15989	4.3958	98.25
150	148.5958	9.769243	6.574372	99.06389
1200	1201.81	18.82584	1.566457	100.1508
1600	1595.816	32.89165	2.061117	99.73852
Inter Day (n=24)				
50	49.26694	2.255257	5.13913	99.33884
150	145.4019	11.04327	7.594992	96.93463
1200	1193.686	24.32474	2.037784	99.47382
1600	1598.233	29.97912	1.875767	99.88956

S.D. = Standard deviation; R.S.D. (%) (Relative standard deviation) = [(S.D./Mean) X 100]; Accuracy (%) = [(Mean / Conc. Added) X 100]; n = number of replicates.

Table 3: Extraction recovery of analytes (n = 6)

Analyte	QC Sample (ng mL^{-1})	Extraction recovery (%)	R.S.D (%)
RHCl	150	89.23	4.27
	1200	90.18	3.41
	1600	85.27	1.23
IS	1000	91.24	2.07

R.S.D. (%) (Relative standard deviation) = [(Standard deviation /Mean) X 100]; n = number of replicates.

Table 4: Stability of analyte at different conditions (n = 6) *

Storage condition	QC sample (ng mL^{-1})	Mean (ng mL^{-1}) ±S.D.	R.S.D. (%)	Accuracy (%)
3 Freeze/thaw cycle	150	149.78±2.58	1.72	99.85
	1600	1586.37±10.58	0.66	99.14
24 h Ambient	150	147.12± 4.91	3.34	98.08
	1600	1582.96±10.52	0.66	98.93
1 month frozen (-20 ^{0}C)	150	144.71±3.70	2.56	96.47
	1600	1580.60±13.95	0.88	98.78
3 month frozen (-20 ^{0}C)	150	143.72±3.39	2.36	95.81
	1600	1551.09±8.31	0.53	96.64

* The data presented in this table are the percentage of measured value vs. theoretical value.

S.D. = Standard deviation; R.S.D. (%) (Relative standard deviation) = [(S.D./.Mean) X 100]; Accuracy = [(Mean / Conc. Added) X 100] ; n = number of replicates.

Application in clinical pharmacokinetics

The HPLC method was successfully applied to the pharmacokinetic study of RHCl in healthy human volunteers. Twelve normal, healthy, Indian male volunteers aged between volunteers aged 21 – 27 years were admitted in CPU (Clinical Pharmacological Unit) of Bioequivalence Study Centre (Dept. of Pharm Tech., Jadavpur University, Kolkata, India) to take part in the study.

The study was designed as a randomized, single-dose, two-period, and two-sequence crossover study under fasting conditions with a one–week wash out period. The study was approved by Drugs Control General of India (DCGI), New Delhi and Institutional Ethical Committee (IEC) of Jadavpur University, Kolkata, India.

Each volunteer was given either single dose of test product containing 300 mg of RHCl tablet (Formulated in our lab) or reference preparation tablets (Ultac, Cipla Ltd., India). A total of 15 blood samples were collected at 0 h (before drug administration) and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0 & 24.0 (after drug administration) in the test tubes containing EDTA at each time point. Breakfast, lunch and dinner were provided after 3 h, 6 h, and 13 h respectively after drug ingestion.

Collected blood samples were centrifuged immediately; plasma was separated and stored frozen at -20^0 C with appropriate labeling of subject code number, study date, and collection time, till the date of analysis. This study was done according to the U.S. Food and Drug Administration (FDA) [10] and European Agency for the Evaluation of Medicinal Products (EMEA) [11].

Evaluation of pharmacokinetics parameters

The method described above was successfully applied to a pharmacokinetic study of RHCl in human plasma. The maximum plasma concentration (C_{max}) ranged from 1260.4±0.378 ng mL^{-1} at the time of 4.15± 0.41h (t_{max}) and the elimination half-life ($t_{1/2}$) ranged from 3.10±0.796 h.The representative chromatograms of a plasma sample of the pharmacokinetic study are shown in Fig.3(C). All the pharmacokinetics parameters were similar between the reference and test products.

CONCLUSION

In summary, the method we have developed and validated is sensitive, specific, and reproducible. A simple and convenient extraction procedure makes this method more feasible for the bio-analysis of RHCl. Sample preparation is very simple, involving simple liquid-liquid extraction followed by separation of supernatant, evaporation and reconstitution of the dry extract with mobile phase before injection onto the HPLC.

In addition, as the run time is less, the method offers high-throughput quantitation.The method was validated in accordance with FDA regulations and the results obtained were within acceptance criteria. The method proved simpler, easier, and rapid, especially for screening purposes, than previously reported bioanalytical methods. The applicability of this method has been demonstrated by successfully analyzing the plasma samples of the clinical study.

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