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Simultaneous Determination of Metformin, Cimetidine, Famotidine, and Ranitidine in Human Serum and Dosage Formulations Using HPLC with UV Detection

M. Saeed Arayne[1], Najma Sultana[2], M. Hashim Zuberi[1], and **Farhan Ahmed Siddiqui[1]**

[1]Department of Chemistry, University of Karachi, Karachi – 75270 and [2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Karachi, Karachi – 75270

Abstract

A new, simple, and reliable reversed-phase high-performance liquid chromatographic method has been developed and validated for the simultaneous determination of metformin (Metf), cimetidine (Cimt), famotidine (Famt), and ranitidine (Rant) in their synthetic mixtures and tablet formulations. These drugs were separated on a Purospher Star RP18 endcapped (250 mm × 4.6 mm i.d.) column packed with 5-µm particles. The mobile phase, optimized through an experimental design, consisted of methanol–water–triethylamine (20:80:0.05), whose pH was adjusted to 3.0 with phosphoric acid (85%) pumped at a flow rate of 1.0 mL/min. UV detection was performed at 229 nm. The method was validated in the sample concentration range of 5–25 µg/mL for all the drugs, where it demonstrated good linearity with *r* = 0.9998, 0.9979, 0.9997, and 0.9987 (*n* = 6), respectively. For independent 100% level samples, the intra-day and inter-day precision was in the range i.e. < 2.0 for all the drugs. The method demonstrated robustness, resisting to small deliberate changes in pH, flow rate, and composition (organic:aqueous ratio) of the mobile phase. The limit of detection values were 0.071, 0.116, 0.134, and 0.110 µg/mL, while the limit of quantitation were 0.217, 0.352, 0.405, and 0.368 µg/mL for Metf, Cimt, Famt, and Rant, respectively. The applicability of the method was demonstrated by determining the drug content in pharmaceutical formulations, where it exhibited good performance.

Introduction

Metformin (Metf) is prescribed for the treatment of type II diabetes mellitus, presently the most commonly used antidiabetic drug. It lowers the blood glucose concentration without causing hypoglycemia. Proposed mechanisms of action include decreased glucose production in the liver, decreased intestinal absorption of glucose, increased glucose uptake from the blood into the tissues, and decreased insulin requirements for glucose disposal. It is absorbed slowly from the small intestine and does not undergo hepatic metabolism.

H2-receptor antagonist (H2RA) is classified in those drugs which are used to block the action of histamine on parietal cells in the stomach and to decrease the acid production by these cells (1). Three H2RAs are widely available and used in our community. These are cimetidine (Cimt), famotidine (Famt), and ranitidine (Rant); their chemical structures are given in Figure 1.

These drugs compete with histamine for H2 receptors and block gastric acid secretion and some cardiovascular effects of histamine. In literature, Lee et al. (2) and Girardin et al. (3) reports H2RA interferes with the absorption of many drugs when given concurrently, like Metf and glibenclamide. Therefore, there was a need to develop an effective and suitable liquid chromatographic (LC) method for therapeutic drug monitoring and pharmacokinetic studies conducted in humans. Several high-performance liquid chromatographic (HPLC) methods have been reported for the quantitation of Metf (4–9). Determination

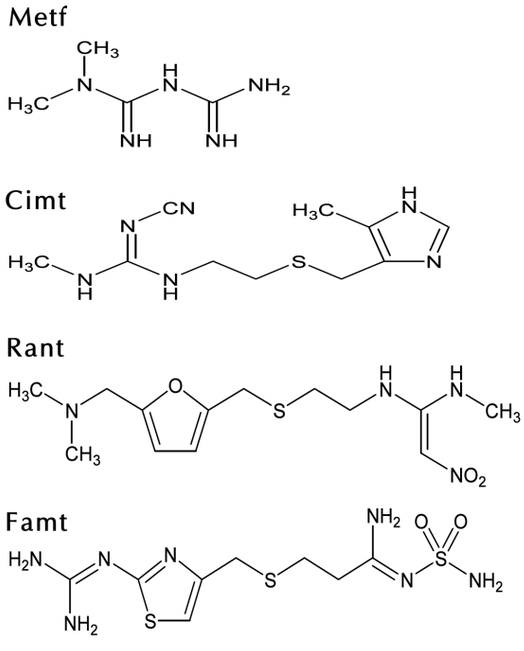

Figure 1. Chemical structures of Metf and H2-receptor antagonists.

*Author to whom correspondence should be addressed: Email: msarayne@gmail.com.

of Metf in human plasma by HPLC was reported by Amini et al. (10), but intra- and inter-day coefficient of variation (CV) and percent error values of the assay method were all less than 8.3%. HPLC–UV determination of Metf in human plasma for application in pharmacokinetics and bioequivalence studies was reported by Prta et al. (11). Mistri et al. (12) reported a liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for simultaneous determination of antidiabetic drugs Metf and glyburide in human plasma, but inter-batch and intra-batch CV across four validation runs was less than 8%. The accuracy was within ±8%, but in newly developed method, the recovery and CV values are far better than it. Column HPLC method for the simultaneous determination of Metf in a pharmaceutical preparation was reported by Ali et al. (13), but this method was applied only in pharmaceutical formulation, so our work has an advantage on it.

Several HPLC methods have been reported for these H2RA (14–22); Helali et al. (23) reported a stability indicating method for Famt in pharmaceuticals using a porous graphitic carbon column. Determination of Famt in low-volume human plasma by normal-phase LC–MS–MS was reported by Zhong et al. (24). Gschwend et al. (25) reported the pharmacokinetics and bioequivalence study of Rant in healthy male subjects. Determination of Rant in rat plasma by HPLC was reported by Li et al. (26), but percent relative standard deviation (%RSD) of these methods were much greater than newly developed work.

A method for determination of Cimt in human plasma and urine by LC was reported by Iqbal et al. (27), but CV of this method was up to 4.2%, which was much greater than our work, but there was not any single HPLC method reported for the simultaneous determination of these co-administered drugs in active and dosage forms; so there was a need for a method for the simultaneous analysis of these drugs.

Experimental

Materials and Methods

Chemicals and reagent

Metf hydrochloride (Neodipar 250 mg), Cimt (Ulcerax 400 mg), Rant (Nulcer 150 mg), and Famt (Hiler 20 mg) reference standard were kindly supplied by Sonaphy Aventis Limited (Karachi, Pakistan), Sami Pharmaceuticals (Karachi, Pakistan), Bosch Pharmaceuticals (Karachi, Pakistan), and Getz Pharma Pakistan (Karachi, Pakistan), respectively, and tablets were purchased from a local market. Analytical-grade phosphoric acid (85% pure), TEA (triethylamine), and HPLC-grade methanol were purchased from Merck (Darmstadt, Germany). Water was twice distilled and deionized by Stedec CSW-300 (Lahore, Pakistan). Drug-free human serum was obtained from the National Institute of Cardiovascular Disease in Karachi, Pakistan (NICVD).

Instrumentation and chromatographic condition

The liquid chromatographic system consisted of Shimadzu model LC-10 AT VP pump, Rheodyne manual injector fitted with a 20-µL loop, a Shimadzu model SPD-20AV variable wavelength UV-vis detector (Kyoto, Japan). The chromatographic system was integrated via Shimadzu model CBM-102 Communication Bus Module. Analysis was performed on a Purospher Star RP18 end-capped (5 µm) analytical reversed-phase column.

The mobile phase consisted of methanol–water–triethylamine (20:80:0.05), whose pH was adjusted to 3.0 with phosphoric acid (85%). Prior to delivering into the system, it was filtered through 0.45-µm filter and degassed using an ultrasonic bath. The analysis was carried out under isocratic conditions using a flow rate of 1.0 mL/min at room temperature. The samples were introduced by injector with a 20-µL sample loop. Chromatograms were recorded at 229 nm using a detector SPD-20AV Shimadzu UV visible. Class GC-10 software was used for data requisition.

Method validation

Wavelength selection

In addition, the UV spectra of individual drugs were recorded in the wavelength range from 200 to 400 nm and compared. The 229 nm isobestic point was considered satisfactory, permitting the detection of all drugs with adequate sensitivity.

Standard solution preparations

Stock solutions (100 µg/mL) of Metf and H2RA (Cimt, Famt, and Rant) were prepared daily by dissolving 10 mg of pure drug active of each drugs in 100 mL of 20% methanol in water (v/v). The stocks solutions were sequentially diluted to give working solution at concentrations in the range 5.0–25.0 µg/mL with 20% methanol (diluent) for preparation of calibration curves.

Assay procedure for dosage forms

Individual tablets were pulverized using a mortar and pestle and completely transferred to a 100-mL conical flask. The volume was adjusted with 20% methanol, and the flask was mechanically shaken for 5 min. Stocks solutions were sequentially diluted to give working solution at concentrations in the range of 5.0–25.0 µg/mL. The samples were filtered through a 0.45-mm membrane filter, and the amount of Metf and H_2RA per tablet was calculated from the related linear regression equations.

Serum drug analysis

The availability of Metf, Cimt, Famt, and Rant from pooled human serum was determined by the stated chromatographic conditions. Blood samples of ten healthy volunteers were collected. Volunteers (age range 22–25 years) were non-smokers, not involved in any strenuous activity, and not taking any other medication. Multiple blood samples (10 mL) were collected in evacuated glass tubes through an indwelling cannula placed in the forearm veins or directly from vein. The blood was then slightly shaken and centrifuged at 10,000 rpm for 10 min, and the plasma separated.

To 1.0 mL of plasma, 10.0 mL of acetonitrile was added, the mixture was vortexed for 1 min and than centrifuged for 10 min at 10,000 rpm. Supernatant was filtered through 0.45-µm pore size membrane filter. Serum thus obtained was mixed in ratio of 1:1 with drug solutions to produce desired drug concentration in serum. These solutions were stored at –14°C pending analysis.

Result and Discussion

Development of optimum mobile phase

In order to develop an reversed-phase HPLC method initially, different ratios of methanol and water was tried for simultaneous estimation of Metf and H_2RA (Cimt, Famt, and Rant). The best separation was obtained in mobile phase composition of methanol, water, and TEA (triethylamine) in the ratio of 20:80:0.05 (v/v). Flow rate selection was based on peak parameters (height, asymmetry, tailing), baseline drift, run-time, and ease of preparation of mobile phase. Individual drug solutions were injected into the column at the concentration of 100 µg/mL, and elution pattern and resolution parameters were studied as a function of pH. It was observed that all H_2RA are pH-sensitive, and good separation was achieved when pH was maintained at 3.0 (± 1).

For validation of analytical methods, the guidelines of the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (28) and FDA (29) have recommended the accomplishment of accuracy tests, precision, specificity, linearity, and robustness of the method.

Specificity and robustness

The mobile phase, methanol–water–triethylamine (20:80:0.05) in various proportions, was investigated after several trials. This system is quite robust. Other ODS columns have been tested with minimal effect on the resolution of the analytes. A Purospher Star RP_{18} (250 mm × 4.6 mm) column is recommended because it demonstrated ruggedness and reproducibility in this assay. Typical chromatograms of Metf and H_2RA (Cimt, Famt, and Rant) are shown with reference drug (Figure 2) in human serum (Figure 3) and blank serum (Figure 4) at a flow rate of 1.0 mL/min. The optimum wavelength for detection was 229.0 nm at which much better detector response for each drug was obtained. The retention times for the investigated drugs were found to be Metf 2.64 min, Cimt 4.76 min, Famt 6.81 min, and Rant 8.12 min.

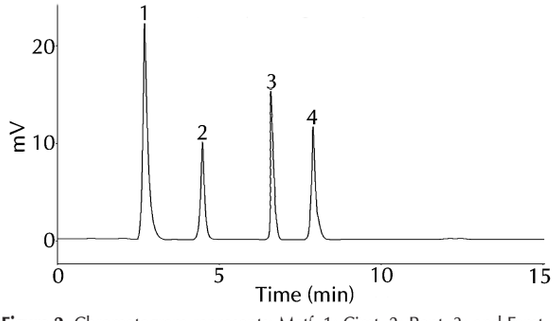

Figure 2. Chromatogram represents: Metf, 1; Cimt, 2; Rant, 3; and Famt, 4 in dosage formulation.

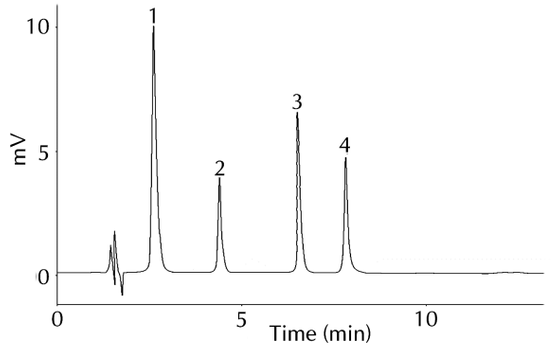

Figure 3. Chromatogram of: Metf, 1; Cimt, 2; Rant, 3; and Famt, 4 in the presence of human serum.

Linearity and calibration

The linearity of the method was determined by injection of Metf and H_2RA standard solutions at five concentration levels in the range of 5.0–25.0 µg/mL in raw material and in human serum as shown in Table I. No significant changes in the concentration of Metf and H_2RA were observed during three

Table I. Linearity, Accuracy, and Precision in Reference Drug and in Human Serum by Proposed Method

	Raw Material				Human Serum			
	Inj. conc.	Found conc.	% Recovery	% RSD	Inj. conc.	Found conc.	% Recovery	% RSD
Metf								
	5.00	4.99	99.80	1.33	5.00	4.92	98.40	1.05
	10.00	10.15	101.50	1.05	10.00	9.84	98.40	0.97
	15.00	14.84	98.93	0.74	15.00	15.20	101.33	0.35
	20.00	20.14	100.70	0.86	20.00	20.08	100.40	1.25
	25.00	24.95	99.80	0.93	25.00	25.11	100.44	1.74
Cemt								
	5.00	4.94	98.80	0.58	5.00	4.88	97.60	1.87
	10.00	9.88	98.80	0.64	10.00	10.14	101.40	0.44
	15.00	15.20	101.33	1.04	15.00	14.93	99.53	0.57
	20.00	20.08	100.40	1.74	20.00	20.34	101.70	0.99
	25.00	24.87	99.48	0.55	25.00	24.97	99.88	1.22
Famt								
	5.00	5.07	101.40	1.22	5.00	4.92	98.40	1.38
	10.00	10.12	101.20	1.07	10.00	10.07	100.70	1.47
	15.00	15.03	100.20	1.38	15.00	14.97	99.80	1.01
	20.00	19.82	99.10	0.36	20.00	20.22	101.10	0.45
	25.00	24.65	98.60	0.94	25.00	24.91	99.64	1.35
Rant								
	5.00	5.07	101.40	0.97	5.00	4.95	99.00	0.84
	10.00	9.85	98.50	0.48	10.00	9.94	99.40	1.41
	15.00	14.95	99.67	1.32	15.00	15.22	101.47	1.49
	20.00	20.18	100.90	1.05	20.00	19.78	98.90	1.05
	25.00	25.33	101.32	1.11	25.00	24.69	98.76	0.55

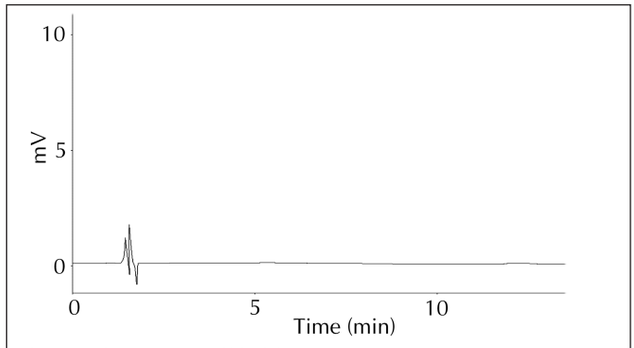

Figure 4. Chromatogram of blank human serum.

days (Table II). Least-square regression calibration curves were constructed by plotting peak areas of Metf and H_2RA as a function of the drug concentration in the standard working solution. The calibration curves could be represented by the following regression equations:

y (Metf) = 12471x + 1710.3 (r = 0.9998, n = 5)
y (Cimt) = 15640x + 19078 (r = 0.9979, n = 5)
y (Famt) = 7158.5x + 1453.3 (r = 0.9997, n = 5)
y (Rant) = 14300x – 3187 (r = 0.9987, n = 5)

where x is the concentration of Metf and H_2RA in µg/mL and y is the peak-area. Repeatability is given as inter- and intra-day precision, and accuracy was evaluated by analyzing three different concentrations of Metf and H_2RA.

Accuracy and precision

The accuracy of an analytical method is defined as the similarity of the results obtained by the analytical method to the true value and the precision as the degree of that similarity (28,29). Accuracy of the method was performed by spiking drugs with placebo: starch 10, lactose 40, talc 2, and magnesium stearate 1. A 1:1 blend of drugs and placebo was prepared (Table III). Five concentrations range of 8–12 µg/mL were assayed in one day to determine intra-day precision and accuracy. In addition, analyses of five samples of five concentrations on three consecutive days were used to determine inter-day precision and accuracy, as shown in Table III.

Recovery studies

To keep an additional check on the accuracy of the developed assay method and to study the interference of formulation additives, analytical recovery experiments were performed by adding known amounts of pure drug to the pre-analyzed samples of commercial dosage forms. The percent analytical recovery values calculated by comparing concentration obtained from the spiked samples with actual added concentrations are also listed, as shown in Table IV. The percent recovery of the added pure drug was calculated as follows:

$$\%\text{Recovery} = [(Cv - Cu)/Ca] \times 100$$

where Cv was the total drug concentration measured after standard addition, Cu was drug concentration in the formulation, and Ca was drug concentration added to formulation. Precision and accuracy with all the drugs were performed separately in presence of excipients and formulations.

LOD and LOQ

LOD and LOQ were estimated in accordance with the baseline noise. LOD was obtained as the sample concentration that causes a peak three times as high as the baseline noise level, and the LOQ was calculated as being ten times as high as the baseline noise level (28,29). Using the parameters mentioned earlier, LOD and LOQ were estimated to be 0.071, 0.116, 0.134, 0.110 µg/mL, and

Table II. Inter-day and Intra-day Precision*

Drug conc. (µg/mL)	Metf (CV)		Cimt (CV)		Rant (CV)		Famt (CV)	
	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intera-day
5	0.17	0.18	0.51	0.07	0.13	0.2	0.07	0.42
8	1.31	0.23	0.06	0.88	0.52	0.17	0.48	0.57
10	0.63	0.28	0.28	0.01	0.69	0.3	1.31	0.37
15	0.25	0.8	0.1	0.03	0.13	0.1	0.94	0.56
20	0.83	0.64	0.5	0.26	0.34	0.11	0.16	0.53
25	1.13	0.39	0.3	0.31	0.6	0.12	0.96	0.31

* n = 6.

Table III. Precision and Accuracy of the Method in Presence of Excipients

Analyte	Spiked conc. (µg/mL)*	Measured conc. (µg/mL)	% Recovery	% RSD
Metf	8	7.93	99.13	1.07
	10	10.12	101.2	1.37
	12	12.07	100.6	2.58
Cimt	8	8.19	102.4	0.51
	10	9.97	100.9	1.85
	12	12.11	99.7	1.65
Famt	8	7.89	100.5	1.13
	10	9.83	98.6	1.72
	12	11.94	99.5	0.62
Rant	8	8.05	100.6	1.44
	10	10.21	102.1	1.27
	12	12.02	100.2	1.18

* n = 5.

Table IV. Accuracy and Precision of Method in Pharmaceutical Formulations by Standard Addition Method

Conc. of drug in formulations (µg/mL)	Conc. of drug added (µg/mL)	Total conc. of drug found (µg/mL)	% Analytical recovery	CV
Metf				
5	3.6	8.69	102.5	0.16
5	4	9.12	102.5	0.12
5	4.4	9.52	102.72	0.07
Cimt				
8	3.6	11.62	100.2	0.29
8	4	11.89	99.13	0.13
8	4.4	12.2	98.6	0.2
Rant				
5	3.6	8.53	99.16	0.06
5	4	8.87	98.55	0.02
5	4.4	9.42	100.2	0.11
Famt				
10	3.6	13.91	102.6	0.12
10	4	14.4	102.9	0.18
10	4.4	14.6	101.38	0.07

0.217, 0.352, 0.405, 0.368 µg/mL for Metf, Cimt, Famt, and Rant, respectively. The LOD and LOQ were calculated according to ICH guideline as:

$$LOD = 3.3\sigma/S \text{ and } LOQ = 10\sigma/S$$

where σ is the standard deviation of the lowest standard concentration, and S is the slope of the standard curve.

Recovery from serum

In order to evaluate the applicability of the proposed method in the perspective of serum, serum samples were spiked with all these drugs at the aforementioned concentration levels and assayed in triplicate. The blood samples were treated in accordance with the protocol described earlier, and the obtained recoveries and coefficient of variation are illustrated in Table I. Statistical analysis showed that no considerable differences exist among the mean recoveries of both the drugs in serum samples.

Recoveries of different concentrations of all the drugs from serum were calculated by dividing the integrated peak area by the respective nominal drug concentration for calibration and serum samples and expressed as the amount (expressed in %) (Table I).

Conclusion

An HPLC method for simultaneous determination of Metf and H_2RA (Cimt, Famt, and Rant) for in vitro analysis have not been reported. The presented method in addition to its novelty for determination of four ingredients at single wavelength is sufficiently rapid, simple, and sensitive as well as precise and accurate, which complies with ICH guidelines for accuracy, precision, and stability for standards and QC samples. The assay of the two active ingredients was not interfered by the excipients in the products also in human serum. The linearity, accuracy, precision, LOD, LOQ, and specificity were established. In addition to the analysis of these drugs, this rapid and reproducible analytical method is suitable for dissolution studies and could also be used for pharmacokinetic studies conducted in humans.

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