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Simultaneous determination of ranitidine and metronidazole in human plasma using high performance liquid chromatography with diode array detection

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Abstract

The development and validation of a simple method for the simultaneous determination of ranitidine and metronidazole in human plasma is described. Plasma samples (250 μL) were deproteinized by precipitation with 60% perchloric acid, centrifuged and the supernatant directly injected into the HPLC. Separation was achieved in isocratic mode with a Shimpak C_{18} column and a mobile phase consisting of 10 mM potassium dihydrogen phosphate pH 3.5:acetonitrile (90:10, v/v) with UV detection at 315 nm. The method showed good selectivity and sensitivity. Good and consistent recovery for metronidazole and ranitidine was obtained: 96.22 ± 3.52 and $95.00 \pm 4.50\%$ for ranitidine (25–1000 ng/mL) and metronidazole (60–10,000 ng/mL), respectively ($n = 3$). With this one-step sample preparation method, both ranitidine and metronidazole could be quantified simultaneously in human plasma with good precision (R.S.D. < 15%) and accuracy (bias values below 15%). The limit of quantification for ranitidine and metronidazole were 20 and 40 ng/mL plasma, respectively.
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Keywords: Ranitidine; Metronidazole; Reversed-phase chromatography; Validation; Plasma

1. Introduction

Ranitidine and metronidazole have been successfully used in combination therapy with antibiotics for the treatment of gastric *Helicobacter pylori* infections [1]. Ranitidine and metronidazole have low plasma protein binding (<20%) and their effective plasma concentrations are 100 ng/mL and 6 μg/mL, respectively [2].

Extraction and clean-up of drugs from biological fluids are usually the first and most difficult step in bioanalysis due to the need to selectively remove interferents such as proteins without significant analyte loss. Traditional extraction methods have relied upon protein precipitation agents, such as tungstic and perchloric acid to remove proteins prior to further sample processing [3]. Perchloric acid has been used in the quantification of ranitidine, metronidazole and other drugs from plasma and other biomatrices, such as liver, kidney and muscle [4–8]. Extraction of ranitidine from biological fluids is usually performed using liquid–liquid extraction with organic solvents such as dichloromethane [9,10], while metronidazole is usually extracted following precipitation with methanol [11–13]. Solid phase extraction is also used for quantification of ranitidine [14–16] and metronidazole [17,18]. Recently, a method based on direct injection of plasma samples following precipitation with perchloric acid for the quantification of ranitidine was described [5]. When analyzing samples from patients treated with ranitidine and metronidazole in combination, it is desirable to have an analytical method that can quantify both analytes in only one method. This paper describes the development and validation of a method for the simultaneous quantification of ranitidine and metronidazole in human plasma. Different

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extraction methods were evaluated for the extraction of ranitidine from plasma, including dichloromethane extraction following plasma alkalinization, ethanol/sucrose precipitation followed by dichloromethane extraction and perchloric acid precipitation followed by direct injection of the supernatant. However, the perchloric acid precipitation method was chosen due to its simplicity, lack of volume transfer steps and the good and consistent recovery achieved for both metronidazole and ranitidine, which together eliminated the need for the use of an internal standard.

2. Experimental

2.1. Chemicals

Ranitidine hydrochloride USP (from Ashish, India) and metronidazole hydrochloride USP (from Ashish, India) were used throughout the study. Dichloromethane, sodium hydroxide, perchloric acid, phosphoric acid, potassium dihydrogen phosphate and HPLC-grade acetronitrile were purchased from Merck, Darmstadt, Germany. Water was purified with a reverse osmosis system coupled to an ion exchange unit (Gehaka, Brazil).

2.2. Sample preparation

Human plasma was obtained from the hemotherapy center of João Pessoa (João Pessoa, Paraíba, Brazil) and collected after approval from the research ethics committee. The plasma was kept in freezer at −20 °C.

Ranitidine hydrochloride and metronidazole hydrochloride solutions were prepared in water (5 and 50 mg/mL stock solutions, respectively). For the validation studies, plasma samples (250 μL) were spiked with ranitidine and metronidazole stock solutions keeping a 50:1 plasma to stock solution ratio. Ranitidine was extracted from plasma using three different methods to evaluate the best absolute recovery. Metronidazole was extracted from plasma using only the perchloric acid method described below.

2.2.1. Sodium hydroxide precipitation

A previously described method was used [9]. Briefly, plasma samples (100 μL) were spiked with ranitidine to obtain a concentration of 100 or 1000 ng/mL. A 50 μL volume of 1 M NaOH was added to the sample and the analyte extracted with 1.0 mL of dichloromethane. The samples were vortexed and centrifuged (9000 rpm, 6 min). The aqueous layer was discharged and 100 μL of phosphoric acid added to the organic layer prior to evaporation to dryness under a stream of nitrogen. The residue was reconstituted in 100 μL of mobile phase.

2.2.2. Ethanol–sucrose precipitation

To 250 μL samples of plasma spiked with 100 or 1000 ng/mL of ranitidine, 250 μL of a solution of ethanol: sucrose 40% (90:10, v/v) was added. Samples were then vortexed, and extracted with 1000 μL of dichloromethane followed by vortexing and centrifugation at 3000 rpm for 15 min. The aqueous layer was discharged and the organic layer evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 250 μL of mobile phase.

2.2.3. Perchloric acid precipitation

Precipitaion with perchloric acid was carried out according to a previously published method [5]. Samples of spiked plasma (250 μL) were transferred to 1.5 mL ependorff tubes and the protein precipitated with 20 μL of 60% perchloric acid. The ependorff tubes were vortexed and then centrifuged for 6 min at 9000 rpm. The supernatant was collected, transferred to autosampler vials and directly injected into the HPLC.

2.3. Chromatographic system

The HPLC system consisted of an LC-10AD pump, a column oven (model CTO-10AS), a diode array detector (SPD-M 10A), an autosampler SIL-10AD, and a controller module SCL-10A (all from Shimadzu) coupled to a personal computer running the software Shimadzu Class VP for data acquisition.

The mobile phase consisted of potassium dihydrogen phosphate (pH 3.5; 0.01 M):acetonitrile (90:10, v/v). The phosphate buffer pH was adjusted with phosphoric acid 4%.

Separation was achieved at 25 °C using a Shimpak C_{18} column (300 mm × 4.6 mm i.d.; 5 μm) fitted with a Phenomenex Security guard C_{18} column (4.0 mm × 3.0 mm i.d.). The flow rate was set at 1.0 mL/min and a discrete channel on the diode-array detector configured to acquire data at 315 nm. Samples were introduced using the autosampler and the injection volume was 40 μL.

2.4. Method validation

2.4.1. Recovery

Recovery was assessed by spiking plasma samples with both ranitidine (from 30 to 1000 ng/mL) and metronidazole (from 60 to 10,000 ng/mL). The samples were then prepared as described in Section 2.2.3 and the absolute recovery calculated by comparing the response of ranitidine or metronidazole in the extracted samples, to those obtained for freshly prepared solutions of each standard in deionized water.

2.4.2. Selectivity

Selectivity of ranitidine and metronidazole from plasma interferents was investigated by using three batches of normal plasma from the hemotherapy center of João Pessoa and six plasma batches (four normal, one lipemic and one hemolyzed plasma) from the outpatient center of the University Hospital of the Federal University of Paraíba.

2.4.3. Precision, accuracy and linearity

Intra- and inter-day precision and accuracy were evaluated for both ranitidine and metronidazole. Ranitidine precision and accuracy was evaluated by spiking plasma samples with ranitidine at three different concentrations defined as quality control (QC) samples at 100, 400 and 800 ng/mL, while metronidazole QC's were spiked at 300, 2000 and 8000 ng/mL. Three samples at each concentration were prepared daily by taking 250 μL aliquots from a 1000 μL volume of spiked plasma, and the analyses repeated for 3 consecutive days. Calibration curves were prepared daily using the range of 20–1000 ng/mL for ranitidine and 40–10,000 ng/mL for metronidazole (external calibration).

2.4.4. Limit of detection and limit of quantification

The limit of quantification was determined as the lowest concentration of ranitidine or metronidazole, which could be quantified with a value of bias below 20% and a signal to noise ratio of at least 5. The limit of detection was considered as the concentration of each analyte producing a signal to noise ratio of 3 [19].

2.4.5. Stability studies

The stability of ranitidine and metronidazole in plasma samples extracted with perchloric acid precipitation and stored in the autosampler rack (25 °C) was evaluated for 120 h. We also evaluated the stability of ranitidine and metronidazole stock solutions stored at room temperature (20 °C) as well as the stability of ranitidine and metronidazole in plasma samples stored in the refrigerator (2 °C) for 10 days. The stability was evaluated by comparing the responses of ranitidine and metronidazole in stored samples with those of a freshly prepared sample at the same concentration level. An upper and lower limit of 10% of the original concentration was established as acceptance criteria.

2.4.6. Ruggedness

Ruggedness of the method was evaluated by monitoring the number of theoretical plates in relation to the injection number and by evaluating the performance of the method when using two batches of the same chromatographic column.

3. Results and discussion

3.1. Recovery

The recovery of ranitidine by different extraction procedures was evaluated (Fig. 1). Plasma extraction with dichloromethane following alkalinization with 1 M NaOH presented the lowest recovery of all three methods tested for ranitidine (average recoveries of 41.90 and 46.70% for 100 and 1000 ng/mL, respectively). An increase in column backpressure and precolumn degradation was also noticed with the NaOH/dichloromethane method.

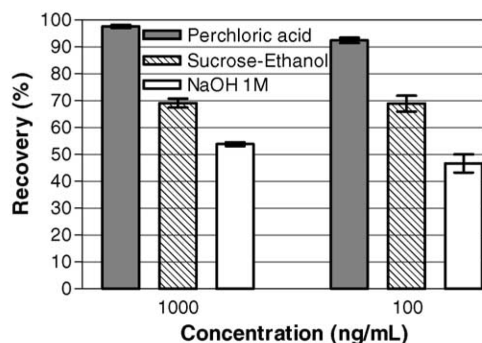

Fig. 1. Ranitidine recovery using three different extraction methods. Values are mean ± S.D. of three replicate experiments.

The ethanol–sucrose method showed intermediate recovery values for ranitidine between those obtained for the NaOH/dichloromethane and the perchloric acid method (Fig. 1). The average recovery for ranitidine with this method was 68.90 and 69.10% for 100 and 1000 ng/mL, respectively. One disadvantage of the method is the possibility of emulsion formation if the proportion of plasma to ethanol/sucrose solution is not ideal. Also, the long time required to separate and evaporate the organic layer is a drawback of the method, although automated liquid handling systems can overcome this limitation.

The best recovery values for ranitidine were obtained with perchloric acid precipitation (Figs. 1 and 2). Apart from higher absolute recovery values, the method does not require an evaporation step, the main limitation of the other methods tested. Thus, approximately 40 samples can be prepared in 1 h using the perchloric acid precipitation, a reasonable throughput for pharmacokinetics/bioequivalence studies.

Perchloric acid precipitation was chosen due to simplicity, speed and good reproducible average recovery values: 96.22 ± 3.52% ($n = 3$) and 95.00 ± 4.50% ($n = 3$) for ranitidine and metronidazole, respectively (Fig. 2).

Direct injection of plasma samples after precipitation of proteins is reported to have disadvantages such as low recoveries due to drug co-precipitation and unavoidable dilution of the sample [3] as well as increased column backpressure. In our study, precipitation of proteins with 60% perchloric acid gave the highest recoveries of all three methods tested for ranitidine. However, we found that the centrifugation rate following precipitation is a key parameter to be controlled, with centrifugation rates of 4000 rpm and lower producing extracts that resulted in peak tailing after only 25 injections due to incomplete precipitation of the protein into the pellet at the bottom of the ependorff tube.

3.2. Selectivity and chromatography

Fig. 3 shows chromatograms of plasma spiked with 800 ng/mL of ranitidine and 300 ng/mL of metronidazole as well as a blank plasma sample. There was good chromatographic resolution ($k = 2.2$ and 3.4 for ranitidine and metron-

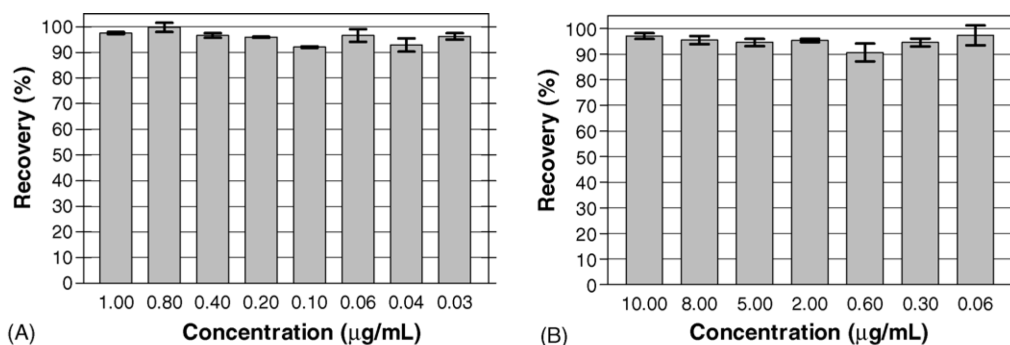

Fig. 2. Ranitidine (A) and metronidazole (B) recovery using perchloric acid 60%. Each bar represents the mean ± S.D. of three replicates.

idazole, respectively) between the analytes and plasma interferents. The detection at a relatively high wavelength (315 nm) contributed to increase the selectivity of the method. Selectivity was further demonstrated by performing peak purity analysis, which revealed similarity indexes of 0.9998 ± 0.0368 and 0.9997 ± 0.0358 for ranitidine and metronidazole, respectively ($n = 5$).

Ranitidine is usually chromatographed in reversed-phase mode using acetonitrile as organic modifier and an ion-pairing reagent to reduce its interaction with uncapped silanol groups in the stationary phase leading to tailing [5,10]. In our work, by using a C_{18} Shimpak Column (Shimadzu), there was no need for the use of ion-pairing reagents and the calculated tailing factor for ranitidine and metronidazole was 1.35 and 1.50, respectively. These results were reproduced with a different batch of the same Shimpak C_{18} column.

Under these analytical conditions, ranitidine and metronidazole also presented selectivity for six plasma samples tested (four normal plasma, a lipemic plasma and a hemolyzed plasma) from the outpatient centre of University Hospital at the Federal University of Paraíba.

3.3. Precision, accuracy and linearity

Tables 1 and 2 shows intra- and inter-day values of accuracy and precision for the determination of ranitidine and metronidazole, respectively. Ranitidine and metronidazole presented inter- and intra-day precision, and accuracy with R.S.D. values below 15%, except at the LOQ.

The method presented good intra-day (R.S.D. = 10.8%) and inter-day (R.S.D. = 6.8%) precision values in low concentrations for determination of ranitidine, while metronidazole presented good intra-day precision (R.S.D. = 8.2%) and inter-day precision (R.S.D. = 9.4%).

Ranitidine and metronidazole demonstrated good accuracy, with estimated values deviating from nominal values by a maximum of 4.9 and 7.2%, respectively. Ranitidine showed bias values lower than 5.6% with an LOQ of 20 ng/mL, while metronidazole presented an LOQ at 40 ng/mL (Table 2) and a bias value of 7.2% at LOQ.

Linearity was demonstrated for ranitidine (20–1000 ng/mL) and metronidazole (40–10,000 ng/mL). Both analytes gave linear responses in the concentration range tested: Ranitidine ($Y = 78.14 \pm 0.24X - 64.68 \pm 110.50$, $r = 1.000$, $n = 3$)

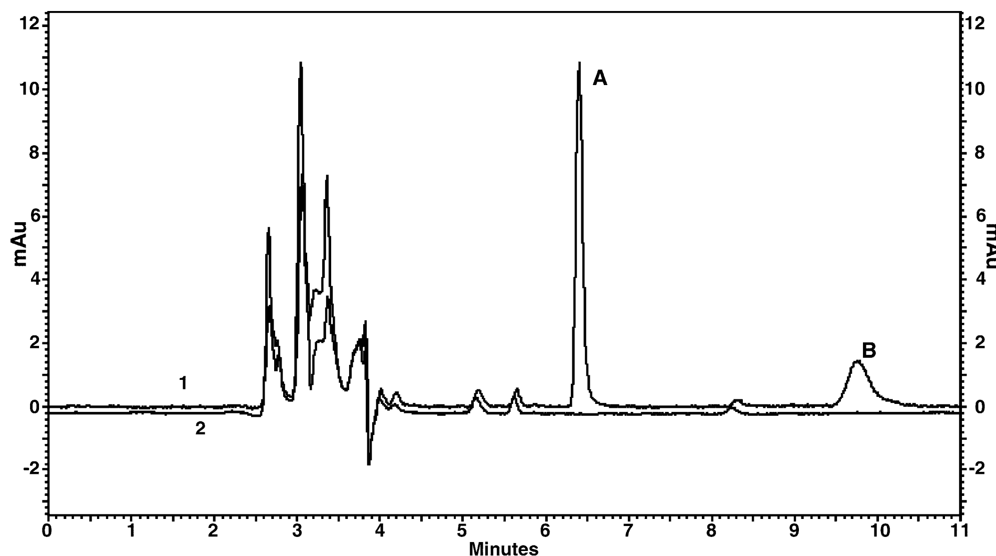

Fig. 3. (1) Chromatogram of plasma spiked with ranitidine (A) at 800 ng/mL and metronidazole (B) at 300 ng/mL, (2) chromatogram of human blank plasma.

Table 1
Inter- and intra-day precision and accuracy for ranitidine

Concentration (ng/mL)	Precision (average ± S.D.)				Accuracy	
	Intra-day		Inter-day		(%)[a]	Bias (%)
1000	1018.07 ± 7.5	979.6 ± 46.9	1010.4 ± 22.2	1002.7 ± 31.1	100.3	0.3
QC_H	800.6 ± 18.6	801.4 ± 12.1	806.1 ± 2.2	802.7 ± 11.2	100.3	0.3
600	592.6 ± 28.9	592.8 ± 10.6	599.3 ± 3.1	594.9 ± 15.9	99.2	−0.8
QC_M	401.0 ± 4.5	399.5 ± 2.4	394.1 ± 2.6	399.0 ± 6.7	99.8	−0.2
200	208.4 ± 11.2	197.7 ± 3.4	200.4 ± 3.7	202.2 ± 7.8	101.1	1.1
QC_L	103.4 ± 2.7	99.3 ± 2.1	98.0 ± 0.3	100.3 ± 2.9	100.3	0.3
60	59.4 ± 2.3	57.0 ± 2.2	58.2 ± 0.8	58.2 ± 2.3	97.0	−3.0
40	41.6 ± 0.4	39.7 ± 0.7	39.2 ± 0.8	40.2 ± 1.3	100.4	0.4
25	27.3 ± 1.4	25.9 ± 0.4	25.5 ± 0.9	26.2 ± 1.1	104.9	4.9
20	21.4 ± 2.3	20.2 ± 1.1	20.4 ± 0.1	20.7 ± 1.4	103.3	3.3
10	15.7 ± 0.9	10.8 ± 1.3	12.8 ± 1.4	13.1 ± 2.4	131.0	31.0

QC_H = quality control high, 800 ng/mL; QC_M = quality control medium, 400 ng/mL; QC_L = quality control low, 100 ng/mL. Each value is expressed as mean ± S.D. of three determinations.
[a] % of nominal concentration = (average measured value/nominal value) × 100. Bias % = [(measured value − true value)/true value] × 100.

and metronidazole ($Y = 105.88 \pm 0.05X - 1055.52 \pm 220.8$, $r = 1.000$, $n = 3$).

There was no need for an internal standard, mainly due to the consistent recovery of ranitidine and metronidazole and the absence of solvent transfer steps during sample preparation. The data showed a good correlation between the concentration and the response for both analytes in plasma during the inter-day analysis.

Fig. 4 shows plots of the standardized residuals plotted against the concentration values for ranitidine and metronidazole quantified using either external calibration (plots I and III, respectively) or internal standard calibration with metronidazole as internal standard of ranitidine (plot II) or ranitidine as internal standard of metronidazole (plot IV). These graphs show the absence of no-random patterns along the concentration axis, showing that the external calibration used is adequate and that the homocedasticity was not violated. Also, there was no value of residue situated more than three standard deviations from zero, showing the absence of outliers.

3.4. Stability studies

Ranitidine and metronidazole did not show evidence of significant degradation in the stability studies. When stored in plasma at 2 °C or in stock solutions at room temperature both ranitidine and metronidazole remained stable at all concentrations tested, with the larger variations at 3.7 and 8.3% in plasma (Table 3) and 9.9 and 1.5% in stock solution (Table 4) for ranitidine and metronidazole, respectively. When stored in plasma at 2 °C the chromatographic peaks of ranitidine and metronidazole did not suffer any interference from the matrix components during the storage time (8 days).

Fig. 5 shows the stability of ranitidine and metronidazole in extracted samples stored in the autosampler at 25 °C. Ranitidine and metronidazole showed limited stability after extraction, being stable in the autosampler rack for a period of only 24 h. After this time the presence of matrix interferents that coeluted with the analytes was observed (Fig. 5). For this reason, we limited the total analysis time to 3 h and stored the

Table 2
Inter- and intra-day precision and accuracy for metronidazole

Concentration (ng/mL)	Precision (average ± S.D.)				Accuracy	
	Intra-day		Inter-day		(%)[a]	Bias %
10000.0	9914.3 ± 91.0	9901.2 ± 363.0	10194.2 ± 149.3	10003.3 ± 247.2	100.0	0.0
QC_H	8016.2 ± 230.3	8096.9 ± 56.2	8154.6 ± 44.3	8089.2 ± 134.8	101.1	1.1
5000.0	4956.8 ± 232.0	5000.1 ± 72.3	5028.4 ± 14.8	4995.1 ± 125.7	99.9	−0.1
QC_M	1998.8 ± 39.8	2019.3 ± 2.6	1941.6 ± 54.9	1986.6 ± 48.7	99.3	−0.7
600.0	634.5 ± 23.2	589.8 ± 10.0	586.6 ± 7.1	603.6 ± 26.7	100.6	0.6
QC_L	302.8 ± 12.1	309.8 ± 10.2	294.3 ± 11.9	302.3 ± 12.0	100.8	0.8
100.0	97.3 ± 3.4	99.3 ± 8.2	92.1 ± 5.6	96.2 ± 6.1	96.2	−3.8
60.0	59.2 ± 3.5	55.1 ± 3.9	66.1 ± 2.7	60.2 ± 5.7	100.3	0.3
40.0	39.1 ± 0.9	47.0 ± 2.1	42.6 ± 2.1	42.9 ± 3.8	107.2	7.2
30.0	37.2 ± 1.4	39.1 ± 3.6	37.9 ± 2.1	38.1 ± 2.4	127.0	27.0
20.0	42.0 ± 3.7	40.4 ± 5.3	30.1 ± 1.4	37.5 ± 6.5	187.5	87.5

QC_H = quality control high, 8000 ng/mL; QC_M = quality control medium, 2000 ng/mL; QC_L = quality control low, 300 ng/mL. Each value is expressed as mean ± S.D. of three determinations.
[a] % of nominal concentration = (average measured value/nominal value) × 100. Bias % = [(measured value − true value)/true value] × 100.

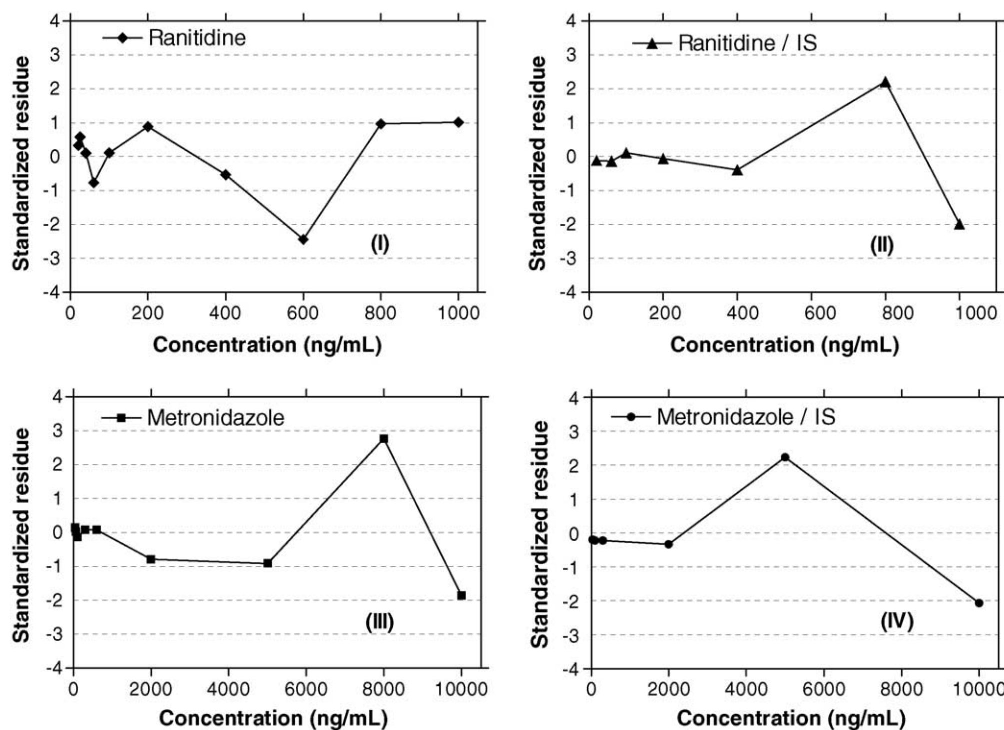

Fig. 4. Standardized residue analysis for ranitidine and metronidazole quantified with external (plots I and III, respectively) or internal standard calibration (plots II and IV, respectively). The concentrations of internal standards were 400 ng/mL for ranitidine as IS of metronidazole and 300 ng/mL for metronidazole as IS of ranitidine.

Table 3
Stability studies of ranitidine and metronidazole in spiked plasma at 2 °C

Time (days)	Concentration (%)					
	Metronidazole			Ranitidine		
	8000 ng/mL	2000 ng/mL	300 ng/mL	800 ng/mL	400 ng/mL	100 ng/mL
0	100.0 ± 0.3	99.5 ± 0.6	105.1 ± 1.2	98.8 ± 0.4	98.1 ± 0.3	91.8 ± 5.2
1	100.6 ± 0.2	99.3 ± 1.2	106.7 ± 2.3	102.3 ± 0.6	100.3 ± 0.2	86.1 ± 1.4
6	102.1 ± 0.2	96.4 ± 1.6	101.5 ± 1.5	97.8 ± 0.9	100.7 ± 1.9	88.6 ± 6.7
8	101.6 ± 1.3	97.4 ± 0.7	96.8 ± 1.5	100.2 ± 2.7	101.8 ± 2.7	94.2 ± 2.4

Each value is expressed as mean ± S.D. of three determinations.

Table 4
Stability studies of ranitidine and metronidazole in stock solution at 25 °C

Time (h)	Remaining (%)					
	Metronidazole			Ranitidine		
	8000 ng/mL	2000 ng/mL	300 ng/mL	800 ng/mL	400 ng/mL	100 ng/mL
0	98.2 ± 0.6	98.5 ± 0.1	104.9 ± 2.5	94.9 ± 0.4	98.3 ± 1.4	98.4 ± 3.9
3	98.5 ± 0.0	98.7 ± 0.4	106.5 ± 0.8	92.4 ± 2.2	97.0 ± 0.5	109.1 ± 6.6
6	98.6 ± 0.2	98.7 ± 0.0	100.8 ± 0.1	93.5 ± 0.9	91.5 ± 2.6	98.4 ± 1.4
12	98.4 ± 0.2	98.9 ± 0.5	100.4 ± 1.6	93.2 ± 1.1	100.8 ± 1.2	109.1 ± 1.2
24	93.7 ± 0.0	98.3 ± 0.7	100.4 ± 0.3	98.4 ± 2.5	109.7 ± 1.8	108.9 ± 3.0
48	104.2 ± 0.2	99.2 ± 0.9	109.8 ± 2.0	102.5 ± 0.5	97.4 ± 1.7	102.1 ± 2.5
72	103.7 ± 0.1	102.0 ± 0.5	109.5 ± 0.5	108.9 ± 0.5	97.9 ± 1.0	98.4 ± 0.3
192	97.9 ± 0.2	98.6 ± 0.1	106.4 ± 0.7	–	–	–
240	–	–	–	104.8 ± 1.2	100.1 ± 0.9	99.1 ± 0.6

Each value is expressed as mean ± S.D. of three determinations.

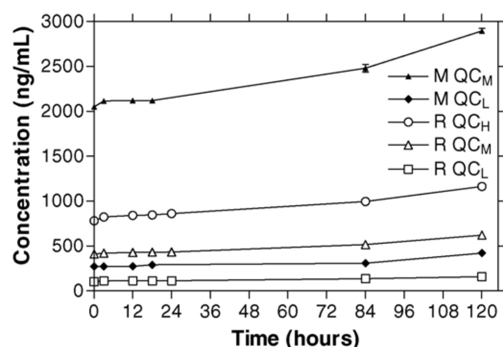

Fig. 5. Stability studies for extracted samples of ranitidine and metronidazole stored in autosampler at 25 °C. MQC_M = metronidazole, 2000 ng/mL; MQC_L = metronidazole, 300 ng/mL; RQC_H = ranitidine, 800 ng/mL; RQC_M = ranitidine, 400 ng/mL; RQC_L = ranitidine, 100 ng/mL.

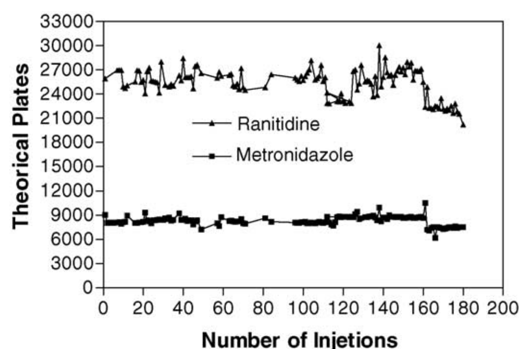

Fig. 6. Theoretical plates vs. number of injection for ranitidine and metronidazole.

samples at 20 °C after preparation and until placing them in the autosampler for analysis.

3.5. Ruggedness

Ruggedness of the method was evaluated by monitoring the performance of the column as a function of the number of injections and also by comparing the peak areas of ranitidine and metronidazole when analysed with C_{18} Shimpak columns from two different batches. A Student's *t*-test revealed that no significant differences existed between the areas of ranitidine ($P = 0.352$) or metronidazole ($P = 0.753$) when plasma extracts were chromatographed on the two columns. There was a decrease of approximately 20% in the number of theoretical plates calculated in relation to the ranitidine peak after 160 injections (Fig. 6), and peak broadening and tailing was detected after 200 injections. Metronidazole peak shape was less affected. The peak broadening and tailing was reversed after changing the pre-column cartridge and washing the column with a solution of acetonitrile:trifluoracetic acid 0.1% (80:20, v/v).

4. Conclusion

The main advantage of the method described here is its simplicity. The good and consistent recovery with the perchloric acid precipitation, and the lack of elaborate and time-consuming sample preparation procedures eliminated the need of an internal standard without compromising accuracy and precision. Critical points must be evaluated during the validation of bioanalytical methods to assure the quality of chromatogram and consequently the results. The method is particularly suited for monitoring plasma levels of ranitidine and metronidazole when used in combination therapy.

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