IJBPAS, January, 2018, 7(1): 38-48

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

SIMULTANEOUS DETERMINATION OF AMOXICILLIN AND RANITIDINE IN HUMAN PLASMA BY RP-HPLC METHOD

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Received 25th May 2017; Revised 26thJune 2017; Accepted 25th Oct. 2017; Available online 1st Jan. 2018

ABSTRACT

Gastro duodenal ulcer is a common disease caused by *Helicobacter pylori.* Amoxicillin and ranitidine are usually co prescribed drugs to treat such infections. An RP-HPLC method was developed and validated for the determination of amoxicillin and ranitidine in human plasma simultaneously. For this purpose, chromatographic system comprised of LC-10 AT VP pump and SPD-10 AVP UV/visible detector was used. The separation was best accomplished with mobile phase consisting of 0.02M phosphate buffer: acetonitrile (93:7 v/v, pH 3.0) from Hibar-Purospher star RP-18e (5 µm, 250 x 4.6 mm) column at ambient temperature. The flow rate was 1.0 ml/minute and separation was carried out at 230 nm. The method was found linear over the concentration range of 0.085µg/mL to 20µg/mL for amoxicillin and 0.078µg/ml to 20µg/ml for ranitidine with correlation coefficient of 0.999. The retention times of amoxicillin and ranitidine were about 8 and 11 minutes respectively. The proposed method is accurate, precise, reproducible and economical.

Keywords: Amoxicillin, Ranitidine, RP-HPLC, simultaneous, plasma

1. INTRODUCTION

Helicobacter pylori is the most common cause of gastrointestinal infections worldwide and its eradication significantly reduces the rates of the recurrence and complications [1]. Amoxicillin is a semi synthetic penicillin and commonly prescribed drug in penicillin class due to its good oral absorption. Ranitidine is a H_2 receptor blocker that inhibits production of gastric acid. It is the most commonly used drug to treat peptic ulcer and gastroesophageal reflux disease [2].

For the quantitative determination of amoxicillin trihydrate and ranitidine hydrochloride in plasma, several HPLC methods [3-11], HPLC with diode array detection[12], HPLC/MS [13], HPTLC [14], LC-MS/MS [15,16], UPLC with mass spectrometry methods [17] were presented in the literature. But all these methods were mostly discussed for amoxicillin alone, ranitidine alone or with other combination of drugs.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Amoxicillin trihydrate and ranitidine hydrochloride reference standards were gifted by Central Drug Laboratory, Karachi, Pakistan and Indus Pharma (Pvt) Limited respectively. Disodium hydrogen phosphate, acetonitrile HPLC grade, methanol analytical grade and *ortho* phosphoric acid were purchased from Merck, Darmstadt, Germany.

2.2. Instrumentation and chromatographic conditions

Shimadzu HPLC system equipped with LC-10 AT VP pump and SPD-10 A UV detector loaded with Shimadzu Class GC software was used. Purospher star RP-18e (5 µm, 250 × 4.6 mm) column fitted with a 100 µL loop. Mettler electronic balance and Millipore filtration assembly were also used. pH meter (370 pH meter, Jenway, Europe), analytical balance (Mettler Toledo B204-S, Switzerland), vortex mixer (Whirl Mixer, England), centrifuge (Hereues, Osterode, Germany) swinney membrane filter (Millipore, England), microlitre syringe (Hamilton, Switzerland), and sonicator (LC20H) were used.

2.3. Preparation of mobile phase

The mobile phase having 0.02M phosphate buffer: Acetonitrile (93:7) the pH was adjusted to 3.0 and the flow rate was established at 1.0 ml/minute. The mobile phase was degassed and filtered through 0.45µm filter. The performance was carried out at ambient temperature and detection was made at 230 nm.

2.4. Plasma drug analysis

Blood samples from the human volunteers were collected, plasma was separated and deproteinized by mixing plasma and acetonitrile in a ratio of 1:1, the mixture was vortexed for 5 minutes and centrifuged at 5000 rpm for 8 minutes. Supernatant was then filtered by 0.45 µ membrane filter.

2.5. Method development

The mobile phase which gives best separation was used. The method was optimized by change in composition, pH and flow rate of the mobile phase. Different mobile phases were considered which resulted in broad peaks and more retention time. The finest resolution and retention were achieved when 0.02M phosphate buffer: acetonitrile (93:7) was used and pH was adjusted to 3.0 with *ortho* phosphoric acid.

2.6. Method validation

In the present study, the proposed method was validated by using different parameters like linearity, specificity, accuracy, precision, limit of detection, limit of quantitation, freeze and thaw stability and long term stability of both drugs according to the ICH guidelines.

Specificity differentiates between the active and other substances present in the sample. Specificity was determined to ascertain the separation of amoxicillin and ranitidine with plasma constituents. It was assessed by injecting the active ingredients and plasma samples.

Linearity of the method was determined by making eleven dilutions of amoxicillin and ranitidine in plasma from 0.039µg/mL to 20µg/mL. All dilutions were filtered and injected into HPLC separately. The calibration curve was plotted between peak areas versus known concentrations.

Accuracy is the closeness of measured value to the actual value of analytes. It can be found out by repeat sample analysis that have known concentrations of drug. Accuracy can be measured by analyzing at least five aliquots for each concentration.

Precision describes the reproducibility of analytical method. For measurement of precision, five aliquots of each concentration were analyzed. Precision can be divided into intraday and interday. Intraday accuracy and precision is a measure of each accuracy and precision on same day while interday accuracy and precision is a measure of accuracy and precision on different days.

Recovery of an analyte is the response of detector that is obtained from an analyte's concentration when added and extracted from the biologic medium and compared to the response that is found from the actual concentration of the reference standard. It is

the extraction power of the method. The absolute analytical recovery was determined by comparing the peak area of amoxicillin and ranitidine in plasma to that in mobile phase. For both amoxicillin and ranitidine, five aliquots of three different concentrations of 20 µg/mL, 1.25 µg/mL and 0.1562µg/mL were analyzed separately and their percentage recoveries were determined. The relative analytical recovery was determined by comparing the peak area of five aliquots of three different concentrations high, medium and low of the drug (20, 1.25 and 0.1562µg/mL for amoxicillin and ranitidine) with the peak area of actual concentrations. Then percentage recoveries, mean, standard deviations and % CV were calculated.

The freeze and thaw stability of amoxicillin and ranitidine in plasma were determined by preparing fifteen samples each of low (0.085 µg/mL) and high (20µg/mL) concentration. All samples were stored at -20°C. After 24 hours, all samples were thawed; five samples were analyzed and compared with freshly prepared samples. Rests of the samples were re-frozen for next 24 hours. The same procedure was repeated for 2nd and 3rd cycle. Then mean, SD, % CV and percentage accuracy were calculated.

For long term stability, ten samples of the same above mentioned low and high concentrations of amoxicillin and ranitidine were prepared in plasma and stored at -20°C. To determine the long term stability out of ten, five samples were analyzed at the end of second week and the next five samples were analyzed at the end of fourth week and compared with freshly prepared samples. Statistical parameters i.e. mean, SD, % CV and percentage accuracy were determined.

3. RESULTS AND DISCUSSION

The aim of the present study was to develop and validate an HPLC method for the determination of amoxicillin and ranitidine in human plasma simultaneously.

3.1. Method development

For the determination of amoxicillin and ranitidine simultaneously in human plasma, different mobile phases with different compositions and ratios were tried out like methanol: water (30:70 v/v), phosphate buffer: methanol (90:10 v/v) and phosphate buffer: acetonitrile (93:7 v/v). It was found that methanol containing mobile phases resulted in broad peak with long tail while acetonitrile containing mobile phase give best peak with high resolution.

3.2. Method validation

In the present study, the method was validated according to the ICH guidelines by using different parameters like specificity, linearity, accuracy, precision, limit of

detection, limit of quantitation, freeze and thaw stability and long term stability of both drugs.

3.2.1. Linearity

The standard calibration curve for known concentrations of 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.1562 and 0.085 µg/mL of amoxicillin and 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.1562, 0.085 and 0.078 µg/mL of ranitidine were constructed for linearity, accuracy and precision in plasma and found to be linear with good correlation coefficient (R^2 = 0.999) (Table 1, Figure 1and 2).

3.2.2. Specificity

The chromatograms (Figure 3 and 4) show no interference of amoxicillin and ranitidine with the plasma.

3.2.3. Limit of Detection and Quantification

The lower limit of quantification for amoxicillin and ranitidine were found to be 0.085µg/mL and 0.078 µg/mL respectively and the limit of detection were 0.078µg/mL and 0.039µg/mL for amoxicillin and ranitidine sequentially.

3.2.4. Accuracy

There was no significant difference found between the amount of drug spiked in plasma and the amount recovered and results are presented in table 2.

3.2.5. Precision

Intraday and interday precision was determined by preparing and injecting concentrations of 20, 2.5, 0.3125 and 0.085µg/mL of amoxicillin and 10, 5, 0.6125 and 0.1562µg/mL of ranitidine. The mean, SD, precision and accuracy were determined and presented in table 3 and 4. The method was accurate and precise as the results were within acceptable limit.

3.2.6. Robustness

Robustness was analyzed by making deliberate changes in flow rate and pH of mobile phase and the values of %RSD were found less than 2%.

3.2.7. Freeze and thaw stability

Freeze and thaw stability of amoxicillin was determined by subjecting three freeze and thaw cycles to five samples of high concentration (20µg/mL) and five samples of low concentration (0.085µg/mL). Similarly for ranitidine, freeze and thaw stability was determined on same number of samples of high concentration (20µg/mL) and low concentration (0.078µg/mL). The mean, SD, %CV and % accuracy were calculated (Table 5).

3.2.8. Long term stability

The long term stability was determined on five samples of each high concentration (20µg/mL) and low concentration (0.085µg/mL) of amoxicillin and five

samples of each high concentration (20µg/mL) and low concentration (0.078µg/mL) of ranitidine. The mean, SD, %CV and % accuracy were determined for fresh samples of both drug and then after storage at -20°C at the end of 2nd and 4th week (Table 6).

Table 1: Linearity of amoxicillin and ranitidine in plasma

Drug	Regression Equation	R^2	LOD (µg/mL)	LOQ (µg/mL)
Amoxicillin	**y = 15641x - 185.4**	**0.999**	**0.078**	**0.085**
Ranitidine	**y = 42890x + 5964**	**0.999**	**0.039**	**0.078**

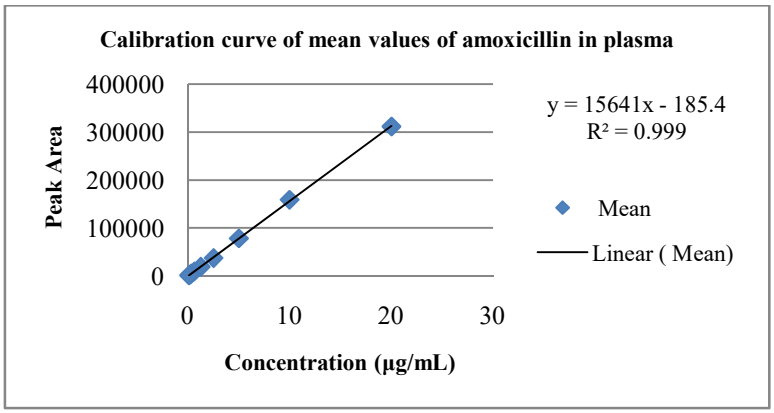

Figure 1: Linearity of amoxicillin in plasma

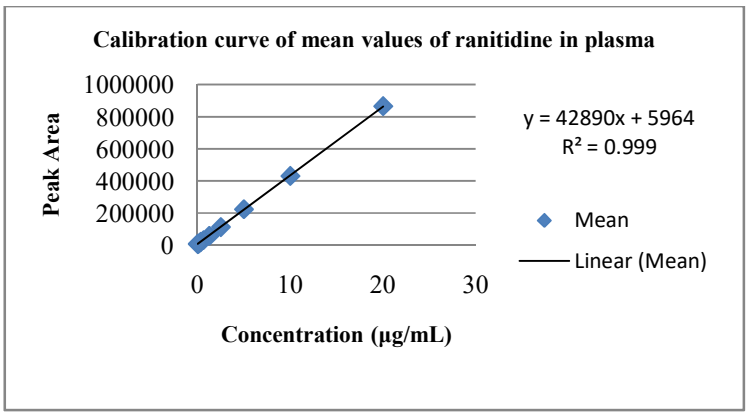

Figure 2: Linearity of ranitidine in plasma

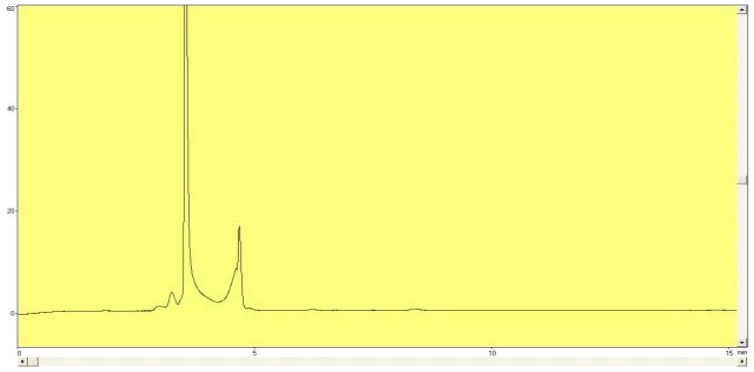

Figure 3: Chromatogram of blank plasma

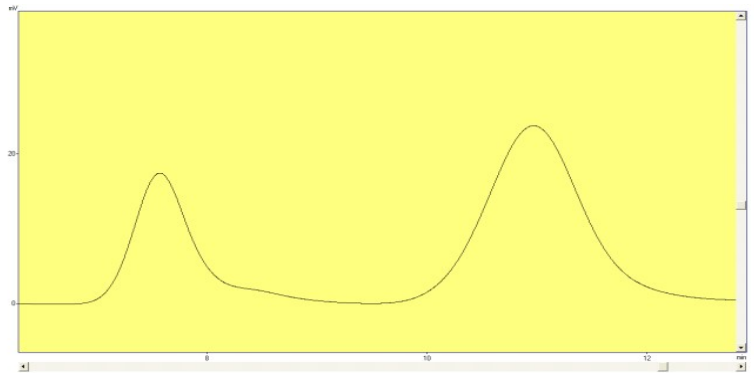

Figure 4: Chromatogram of amoxicillin and ranitidine in plasma

Table 2: Relative analytical recovery of amoxicillin and ranitidine

RELATIVE ANALYTICAL RECOVERY OF AMOXICILLIN AND RANITIDINE							
		Actual Concentrations (µg/mL)					
		20		1.25		0.1562	
		Measured conc. (µg/mL)	% Recovery	Measured conc. (µg/mL)	% Recovery	Measured conc. (µg/mL)	% Recovery
Amoxicillin	Mean (N=5)	19.669	98.343	1.2344	98.752	0.1514	96.927
	SD		1.350		0.797		1.328
	% CV		1.372		0.807		1.370
Ranitidine	Mean (N=5)	20.023	100.119	1.245	99.664	0.1558	99.744
	SD		0.134		0.537		0.252
	%CV		0.133		0.539		0.252

Table 3: Intraday accuracy and precision of amoxicillin and ranitidine in plasma

INTRADAY ACCURACY AND PRECISION OF AMOXICILLIN AND RANITIDINE IN PLASMA					
	Theoretical Conc.(µg/mL)	Mean (N=10)	SD	%CV	%Accuracy
Amoxicillin	20	19.732	0.341	1.728	98.659
	2.5	2.485	0.016	0.639	99.388
	0.3125	0.310	0.005	1.519	99.277
	0.085	0.083	0.001	1.807	97.294
Ranitidine	10	9.843	0.120	1.217	98.433
	5	4.892	0.089	1.819	97.844
	0.6125	0.6084	0.004	0.630	99.331
	0.1562	0.1550	0.001	0.736	99.232

Table 4: Interday accuracy and precision of amoxicillin and ranitidine in plasma

INTERDAY ACCURACY AND PRECISION OF AMOXICILLIN AND RANITIDINE IN PLASMA					
Amoxicillin	Day to day	Measured Conc.(µg/mL)			
		20	2.5	0.3125	0.085
	Mean (N=15)	19.774	2.493	0.302	0.083
	SD	0.336	0.020	0.016	0.001
	Precision	1.698	0.799	5.185	1.006
	% Accuracy	98.870	99.720	96.698	97.882
Ranitidine	Day to day	Measured Conc.(µg/mL)			
		10	5	0.6125	0.1562
	Mean (N=15)	9.712	4.845	0.610	0.155
	SD	0.030	0.042	0.002	0.0003
	Precision	0.313	0.864	0.303	0.217
	% Accuracy	97.122	96.892	99.533	99.065

Table 5: Freeze and thaw stability of amoxicillin and ranitidine

	FREEZE AND THAW STABILITY OF AMOXICILLIN AND RANITIDINE				
AMOXICILLIN		Low concentration 0.085 µg/mL			
		Fresh samples	FT cycle 1	FT cycle 2	FT cycle 3
	Mean (N=5)	0.0848	0.08452	0.0843	0.08382
	SD	0.0003	0.0003	0.00029	0.00019
	% CV	0.417	0.368	0.346	0.229
	% Accuracy	99.765	99.435	99.176	98.612
		High concentration 20 µg/mL			
		Fresh samples	FT cycle 1	FT cycle 2	FT cycle 3
	Mean (N=5)	19.935	19.843	19.715	19.569
	SD	0.186	0.196	0.179	0.169
	% CV	0.931	0.990	0.908	0.866
	% Accuracy	99.673	99.213	98.576	97.847
RANITIDINE		Low Conc. 0.078 µg/mL			
		Fresh samples	FT cycle1	FT cycle2	FT cycle3
	Mean (N=5)	0.0779	0.0778	0.0774	0.0770
	SD	0.00008	0.00009	0.0002	0.0004
	% CV	0.107	0.115	0.268	0.592
	% Accuracy	99.897	99.821	99.282	98.769
		High Conc. 20 µg/mL			
		Fresh samples	FT cycle1	FT cycle2	FT cycle3
	Mean (N=5)	19.925	19.891	19.721	19.576
	SD	0.096	0.100	0.150	0.232
	% CV	0.481	0.501	0.761	1.186
	% Accuracy	99.624	99.453	98.604	97.881

Table 6: Long term stability of amoxicillin and ranitidine in plasma

LONG TERM STABILITY OF AMOXICILLIN AND RANITIDINE IN PLASMA							
	Sample No.	Low Concentration			High Concentration		
		Fresh Samples	After 2 weeks at -20ºC	After 4 weeks at -20ºC	Fresh Samples	After 2 weeks at -20ºC	After 4 weeks at -20ºC
Amoxicillin	Mean (N=5)	0.0848	0.0837	0.0833	19.935	19.895	19.808
	SD	0.0004	0.0003	0.0002	0.186	0.087	0.090
	% CV	0.417	0.309	0.230	0.931	0.436	0.453
	% Accuracy	99.765	98.494	98.023	99.673	99.473	99.041
Ranitidine	Mean (N=5)	0.0779	0.0775	0.0772	19.925	19.846	19.685
	SD	0.00008	0.0002	0.0002	0.096	0.076	0.089
	% CV	0.107	0.282	0.322	0.481	0.384	0.454
	% Accuracy	99.897	99.410	99.076	99.624	99.232	98.424

4. CONCLUSION

This RP-HPLC method is suitable for the determination of amoxicillin and ranitidine simultaneously in human plasma. This proposed method is specific, less time consuming and can be used for pharmacokinetic studies of amoxicillin and ranitidine drugs.

ACKNOWLEDGEMENT

The authors are grateful to the Department of Pharmaceutics, Faculty of Pharmacy, University of Karachi for their continuous and generous support during the research work.

Authors are thankful to Indus Pharma (Pvt) Limited for providing Ranitidine standard and Central Drug Laboratory Karachi, Pakistan for providing samples of amoxicillin standard.

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