Development and Validation of HPLC Analytical Method for Quantitative Determination of Metronidazole in Human Plasma

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Summary: The objective of the present study was to develop a simple, rapid and sensitive reversed-phase high performance liquid chromatographic (RP-HPLC) analytical method with UV detection system for the quantitative determination of metronidazole in human plasma. The chromatographic separation was performed by using C18 RP column (250mm X 4.6mm, 5µm) as stationary phase and 0.01M potassium dihydrogen phosphate buffered at pH 3.0 and acetonitrile (83:17, v/v) as mobile phase at flow rate of 1.0 ml/min. The UV detection was carried out at 320nm. The method was validated as per the US FDA guideline for bioanalytical method validation and was found to be selective without interferences from mobile phase components, impurities and biological matrix. The method found to be linear over the concentration range of 0.2812µg/ml to 18.0µg/ml (r^2 = 0.9987) with adequate level of accuracy and precision. The samples were found to be stable under various recommended laboratory and storage conditions. Therefore, the method can be used with adequate level of confidence and assurance for bioavailability, bioequivalence and other pharmacokinetic studies of metronidazole in human.

Keywords: Metronidazole, RP-HPLC, Bioanalytical Method, UV detection, Validation by US FDA guideline, Fast Dissolving Tablets.

Introduction

Metronidazole (1-(β-hydroxyethyl)-2-methyl-5-nitroimidazole) is a nitroimidazole antibiotic which was initially used for the treatment of Trichomonas vaginalis but later on its efficacy and effectivity against the anaerobes and protozoans were successfully demonstrated [1]. Now a day it is widely used for the treatment of trichomoniasis, amoebiasis and giardiasis. [2] After oral administration of metronidazole 500mg; the peak plasma concentration achieved on average is about 10µg/mL in 1 h after administration. The oral bioavailability of the drug is about 100% with large volume distribution and protein binding of less than 20%. [3, 4]

Different analytical methods for the testing and quantitative determination of metronidazole in pharmaceutical formulations had been reported. [5-9] however the determination of metronidazole in biological fluid especially in plasma is mainly quantified by using high performance liquid chromatographic method [10-12] but in some cases the extraction method was complicated [13, 14] and in others they either used the complex mobile phase or mass spectroscopy as detection system [14-16] which is expensive and is not feasible for all laboratories.

In current study a simple, rapid and sensitive reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed for the quantitative determination of metronidazole in human plasma. In this study a simple UV- spectroscopic method has been used for the detection purpose which makes this method a cost effective and more feasible for even small scale laboratories. The method has been validated as per the US FDA guidance document "Bioanalytical Method Validation" (May, 2001) [17] and stability of samples have been determined on various laboratory conditions to which a biological sample may come across.

Molecular Formula of metronidazole $C_6H_9N_3O_3$ Chemical Structure of Metronidazole Fig. 1.

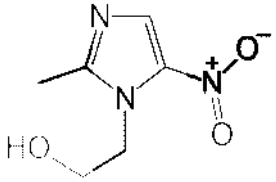

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Fig. 1: Chemical structure of metronidazole.

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Scope

The aim of the study was to develop and validate RP-HPLC method with UV detector for the quantitative determination of metronidazole in human plasma. It will provide a simple, rapid and sensitive method for the determination of peak plasma concentration, bioavailability, elimination half-life and other pharmacokinetic and bioequivalence studies of metronidazole.

Experimental

Chemicals

The chemicals used during this study includes analytical grade potassium dihydrogen phosphate from sigma Aldrich, analytical grade ortho-phosphoric acid from Merck, HPLC grade acetonitrile from Fischer Chemical while Metronidazole and drug free human plasma were obtained as gift sample from Sanofi-Aventis Pakistan and Liaquat National Hospital respectively.

Mobile Phase Preparation

0.01M potassium dihydrogen phosphate buffered at pH 3.0 with help of dilute phosphoric acid prepared then 0.01M phosphate buffer and acetonitrile are mixed together in a ratio of 83:17 (v/v), finally the resultant mixture is filtered through 0.45µm membrane filter.

Sample Preparation

Stock solution of Metronidazole (90µg/ml) was prepared by diluting the drug with mobile phase and then with human plasma. Mixture of 1ml sample solution and 1ml acetonitrile was vortexed for one min and centrifuged at 4,000rpm for 5 mins. The resultant samples were kept for drying and upon complete drying, reconstituted with 1ml mobile phase. Finally, the samples were run on HPLC after filtering them through 0.45µm filter.

Samples in mobile phase are prepared by dilution method from standard stock solution.

Chromatographic System

Shimadzu high pressure liquid chromatography (HPLC) prominence series model 20-A system with autosampler, built-in cooler, degassing unit and injection loop of 100µl and UV detector was used. The testing was conducted by using Isocratic reverse phase technique with Hibar® C18, 5µm (250mm X 4.6mm) HPLC column of Merck origin. The analysis of 25µl samples were carried out at a flow rate of 1.0ml/min with λmax of 320nm. The system also has Lab Solution software to control the analysis and for the data acquisition and integration.

Validation Parameters

System Suitability

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such [19]. The samples were run on system to check the system suitability before starting the validation.

Selectivity

To determine that the method is specific and selective for metronidazole following samples were run on HPLC as per FDA "Bioanalytical Method Validation" guidelines [17].

- 8 Samples of blank run prepared from 8 different sources.
- Sample of standard solution prepared in plasma.

Accuracy

The accuracy of method is developed by triplicate injection of samples having the concentration of 50% (4.5µg/ml), 100% (9µg/ml) and 150% (13.5µg/ml), prepared in plasma from stock solution by dilution method.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions [20]. The precision of the method was demonstrated at two different levels i.e. intra-batch and inter-batch precision.

Recovery

The absolute recovery of the method was determined by preparing the samples of 50%, 100% and 150% concentrations in both mobile phase and plasma. These samples were then analyzed on HPLC,

peak areas were recorded and recovery of the method determined by following formula,

Absolute Recovery =

$$\frac{\text{Area of Sample prepared in Plasm}}{\text{Area of sample prepared in mobile phase}} \text{ X } 100$$

Calibration Curve

In order to demonstrate the linearity and range and to construct a calibration curve, samples of 200%, 150%, 125%, 100%, 75%, 50%, 25%, 12.5 %,6.25% and 3.125%, concentration (18µg/ml to 0.28µg/ml) were prepared in human plasma from standard stock solution by dilution method and run on the HPLC.

Lower Limit of Quantification (LLOQ)

The limit of quantification of method was established by using "Signal to Noise Ratio (10:1)" method as described by the FDA bioanalytical method validation guideline and five samples of LLOQ concentrations were prepared by serial dilution method and run on the system.

Stability

The stability of the samples was demonstrated by using two concentrations i.e. 100% and 50% as defined in the FDA bioanalytical method validation guideline. The evaluation of stability was performed under the conditions described in the guidelines and results were calculated and compared with the initial results.

Freeze and Thaw Stability

In this study the samples were subjected to freezing for 24hrs at -20ºC and then they were thaw at room temperature until they are completely in a liquid state then again the cycle was repeated; totally the samples were subjected to three cycles of thawing and freezing and then after processing the samples were analyzed on HPLC. Stability was demonstrated by comparing the mean of resultant values with the mean value of initial results.

Short Term Temperature Stability

The three samples of 100% and 50% concentration were kept at room temperature for about 20 h and then analyzed at two occasions during this time period i.e. after 8 hrs and after 20 hrs. The stability was demonstrated by comparing the mean of resultant values with the mean value of initial results.

Long Term Stability

The long term stability was established by storing the three samples of 100% and 50% concentration at -20ºC and then analyzing them after 7, 14, 21 and 28 days. The stability was reported by comparing the mean of resultant values with the mean of initial results.

Stock Solution Stability

The prepared stock solution was kept for 24h and then samples were analyzed to establish the stock solution stability.

Post-Preparative Stability

This was also determined by keeping both the sample and standard vials after preparation i.e. processed samples to determine their stability for the time duration required for the validation activities.

Result and Discussion

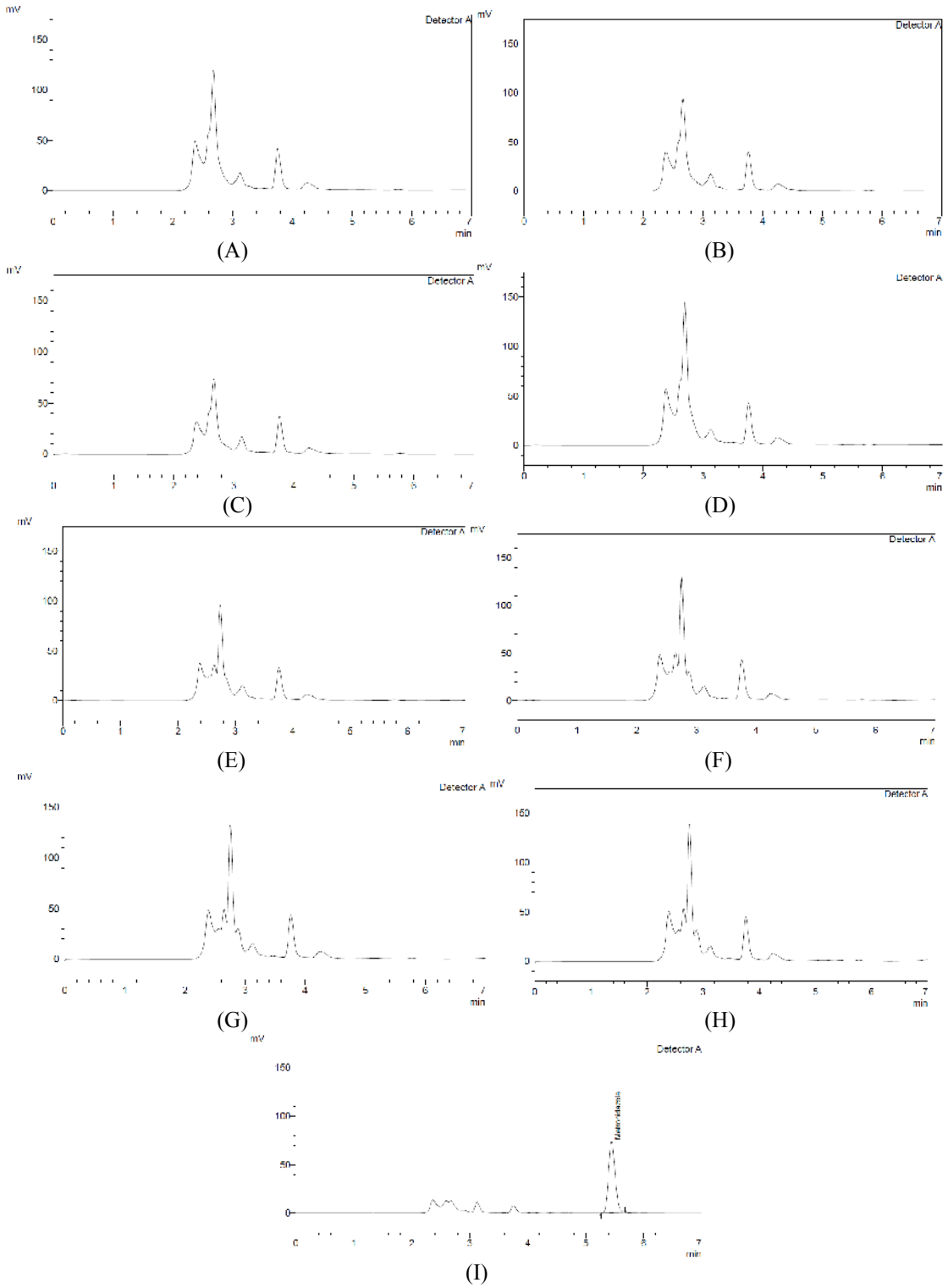
The bioanalytical method must be validated prior to use for pharmacokinetic and/or bioequivalence studies, because adequately validated methods are a necessity for approvable regulatory filings. The major advantage of method validation is that it builds a degree of assurance, not only for the developer but also to the user that the results obtained by the analysis are accurate and precise; secondly it makes the analysis inexpensive by eliminating the frustrating repetitions and results to better time management in the end. Since the minor changes in the analytical conditions like change of reagent supplier or grade, analytical setup are inevitable due to obvious reasons but the method validation process engrosses such conditions and pays for more than invested on the process.

Method Validation was achieved by performing the following parameters

System suitability was achieved by injecting six replicated injection of standard metronidazole solution. The %RSD of the peak areas of metronidazole were 0.01% and. The Mean theoretical plate count, based on USP tangent calculations (USP-2014) for metronidazole peak was 9574 (Table-1).

Table-1: System Suitability Parameters and Results Data.

Parameter	Value
Retention Time (mins)	5.402
Theoretical Plates	9574
Tailing Factor	1.168
Peak Area %RSD	0.01%



(A) – (H) Chromatogram of 8 different blank plasma samples with its impurities
(I) Chromatogram of spike amount of metronidazole in plasma

Fig. 2: Chromatograms of Specificity.

Specificity and Selectivity of the analytical method was demonstrated by injecting the blank samples of human plasma from eight different sources along with the sample of metronidazole in plasma. The resultant chromatograms of blank showed biological impurities up to 4.5 mins whereas the retention time of metronidazole in plasma was after 5.0 mins Fig. 2.

The *accuracy* of method was evaluated by measuring the percent recoveries, calculated by difference of measured value and nominal true value. The mean percent recovery of all four concentrations were found as 94.84% with standard deviation of ± 0.04 and %CV of 4.21% which is well within the acceptance limit of 85 - 115% as per the FDA US guideline for Bioanalytical method validation indicating that the method is accurate for the intended purpose (Table-2).

Intra-Batch Precision was established by injecting five samples of four different concentrations (0.2812 µg/ml – 13.50 µg/ml) including lower limit of quantification prepared in plasma. The %CV of five samples of each concentration (n = 5) was between 0.25 – 2.18 % that is less than 15% defined by the FDA US guideline for bioanalytical method validation. While the %CV of LLOQ concentration (0.2812µg/ml) was 6.39%, also within the acceptance limit of ≤ 20%. These results indicate that the method is precise with adequate level of replication. The overall mean recovery of drug samples in plasma was 95.58% ± 0.06 (Table -3)

Inter-Batch Precision of the method was evaluated by using different HPLC system, column and analysis was performed by different analyst on different days. The method adequately meets the acceptance criteria for inter-batch precision as the overall %CV is found to be 8.13% which is well less than 15% acceptance criteria. The %CV of 7.58% at LLOQ concentration is also within the acceptance limit of ≤ 20%. Hence the method has demonstrated the adequate level of inter-batch precision (Table-4).

Recovery of the method was estimated by injecting the samples of three different concentrations of metronidazole prepared in both mobile phase and plasma. The recovery of drug from plasma was found between 77.49 – 86.82% with mean value of 81.70% ± 4.08 which is within the acceptance limit of ≥ 70% (Table-5).

Calibration Curve was constructed by analyzing ten different samples in a concentration range of 0.2812 µg/ml - 18.0µg/ml including the LLOQ concentration in order to demonstrate the linearity of the method (Fig. 3). The method was found to be linear over a described concentration range with r^2 value of 0.9987.

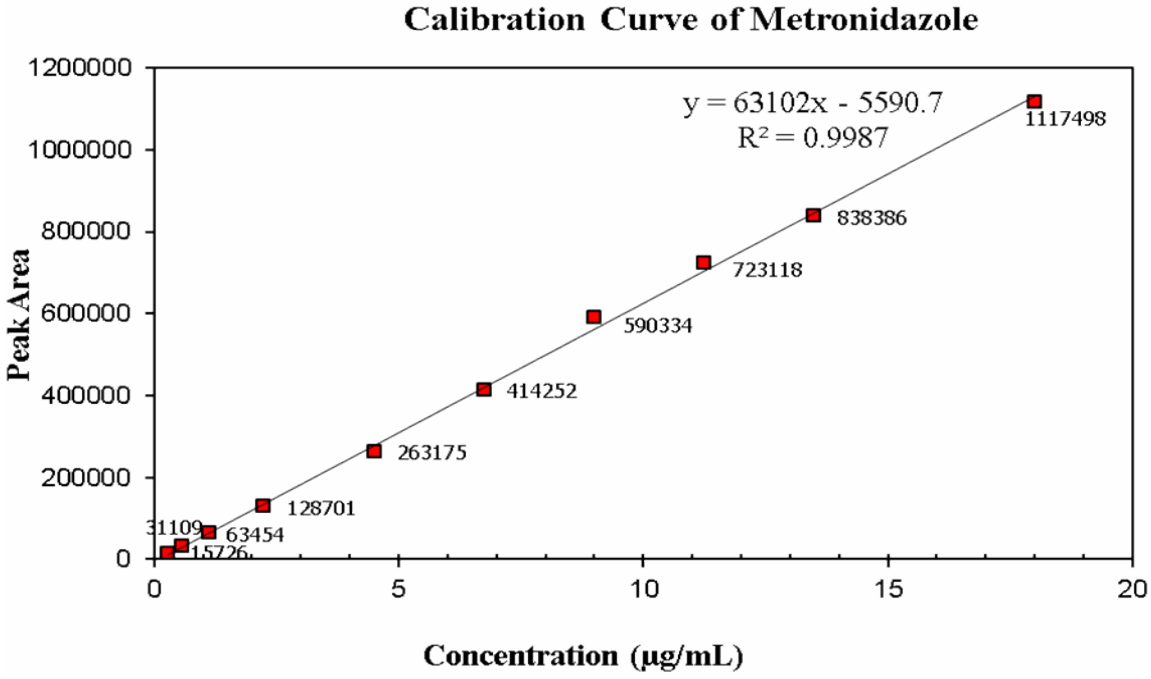


Fig. 3: Calibration Curve of Metronidazole.

Table-2: Accuracy Data of Metronidazole

Percent Concentration	Nominal True Value (µg/mL)	Measured Value (µg/mL)	% Result	Statistical Analysis	
		0.27	95.90	Mean	0.27
		0.27	96.43	SD	0.001
3.125	0.2812	0.27	97.07	%CV	0.53
		0.27	95.83	Mean	96
		0.27	96.07		
		4.03	89.49	Mean	4.02
		4.03	89.46	SD	0.004
50	4.50	4.02	89.33	%CV	0.09
		4.02	89.36	Mean	89
		4.02	89.30		
		9.00	100.05	Mean	9.00
		9.00	100.03	SD	0.002
100	9.00	9.00	100.05	%CV	0.03
		9.00	100.05	Mean	100
		9.01	100.10		
		12.64	93.64	Mean	12.64
		12.63	93.56	SD	0.01
150	13.50	12.64	93.66	%CV	0.05
		12.64	93.65	Mean	94
		12.65	93.67		
	Overall % Mean		94.84		
	Overall Standard Deviation		0.04		
	Overall % Coefficient of variation (CV)		4.21		

Table-3: Intra-Batch Precision Data of Metronidazole.

Percent Concentration	Nominal True Value (µg/mL)	Measured Value (µg/mL)	% Result	Statistical Analysis	
3.125	0.2812	0.26	93.43	Mean	0.28
		0.28	99.53	SD	0.018
		0.31	110.03	%CV	6.39
		0.27	96.33	Mean	100
		0.29	102.90		
50	4.50	4.02	89.30	Mean	4.02
		4.03	89.60	SD	0.01
		4.02	89.30	%CV	0.25
		4.01	89.04	Mean	89
		4.01	89.09		
100	9.00	9.03	100.29	Mean	9.02
		9.03	100.35	SD	0.197
		8.69	96.54	%CV	2.18
		9.17	101.93	Mean	100
		9.17	101.83		
150	13.50	12.64	93.64	Mean	12.48
		12.62	93.52	SD	0.16
		12.42	92.01	%CV	1.29
		12.46	92.30	Mean	92
		12.25	90.75		
	Overall % Mean		95.58		
	Overall Standard Deviation		0.06		
	Overall % Coefficient of variation (CV)		6.18		

Table-4: Inter-Batch Precision Data of Metronidazole.

Percent Concentration	Nominal True Value (µg/mL)	Measured Value (µg/mL)	% Result	Statistical Analysis	
3.125	0.2812	0.28	101.18	Mean	0.30
		0.31	111.48	SD	0.023
		0.28	99.52	%CV	7.58
		0.33	117.74	Mean	106
		0.28	100.92		
50	4.50	4.16	92.50	Mean	4.02
		4.03	89.56	SD	0.10
		3.89	86.46	%CV	2.40
		4.01	89.07	Mean	89
		4.01	89.21		
100	9.00	9.01	100.10	Mean	8.93
		9.15	101.72	SD	0.188
		8.82	98.05	%CV	2.10
		9.00	99.98	Mean	99
		8.67	96.31		
150	13.50	12.41	91.90	Mean	12.39
		12.64	93.60	SD	0.16
		12.23	90.63	%CV	1.31
		12.41	91.89	Mean	92
		12.25	90.72		
	Overall % Mean		96.63		
	Overall Standard Deviation		0.08		
	Overall % Coefficient of variation (CV)		8.13		

Table-5: Absolute Recovery Data of Metronidazole.

Percent Concentration	Nominal True Value (µg/mL)	Measured Value (µg/mL)	% Result	Statistical Analysis	
50	4.50	3.49	77.49	Mean	77.49
		3.49	77.54	SD	0.002
		3.48	77.43	%CV	0.32
100	9.00	7.80	86.71	Mean	86.78
		7.81	86.80	SD	0.006
		7.81	86.82	%CV	0.64
150	13.50	10.91	80.81	Mean	80.83
		10.91	80.83	SD	0.003
		10.92	80.85	%CV	0.33
Overall % Mean			81.70		
Overall Standard Deviation			4.08		
Overall % Coefficient of variation (CV)			4.99		

Table-6: Freeze and Thaw Stability Data.

Percent Concentration	Initial Concentration (µg/mL)	Conc. after Freeze and Thaw Cycles (µg/mL)	Percent Concentration	Initial Concentration (µg/mL)	Conc. after Freeze and Thaw Cycles (µg/mL)
50	4.02	4.00	100	8.69	9.02
	4.03	4.05		9.17	9.00
	4.02	4.03		9.17	9.05
Mean	4.02	4.03	Mean	9.01	9.02
SD	0.01	0.02	SD	0.278	0.025
%CV	0.16%	0.58	%CV	3.08%	0.28
Difference (Initial-Final)		-0.01	Difference (Initial-Final)		-0.01
% Degradation		0.15	% Degradation		0.13

Table-7: Short Term Stability Data.

Percent Conc.	Initial Conc. (µg/mL)	After 8 Hrs. (µg/mL)	After 20 Hrs. (µg/mL)	Percent Conc.	Initial Conc. (µg/mL)	After 8 Hrs. (µg/mL)	After 20 Hrs. (µg/mL)
50	4.02	4.02	4.03	100	8.69	9.03	9.01
	4.03	4.03	4.01		9.17	8.68	9.16
	4.02	4.02	4.03		9.17	9.16	9.02
Mean	4.023	4.02	4.027	Mean	9.01	8.96	9.06
SD	0.01	0.01	0.01	SD	0.278	0.25	0.08
%CV	0.16	0.18	0.31	%CV	3.08	2.74	0.90
Difference (Initial-Final)		-0.002	-0.004	Difference (Initial-Final)		0.05	-0.05
% Degradation		0.04	0.10	% Degradation		0.58	0.58

Table-8: Long Term Stability Data.

Percent Concentration	Initial Conc.(µg/mL)	After 7 Days (µg/mL)	After 14 Days (µg/mL)	After 21 Days (µg/mL)	After 28 Days (µg/mL)
50	4.01	3.97	4.13	4.06	4.13
	4.01	3.99	3.94	4.24	3.55
	4.16	4.14	4.15	3.61	3.95
Mean	4.06	4.03	4.075	3.97	3.88
SD	0.07	0.09	0.11	0.32	0.29
%CV	1.76%	2.31	2.78	8.13	7.58
Difference (Initial-Final)		0.029	-0.013	0.091	0.186
% Degradation		0.70	0.32	2.24	4.59
100	8.82	8.94	9.01	8.96	8.74
	9.15	9.02	9.04	9.10	8.62
	9.00	8.98	8.88	9.26	8.92
Mean	8.99	8.98	8.98	9.11	8.76
SD	0.14	0.04	0.29	0.15	0.15
%CV	1.50%	0.45	7.58	1.65	1.73
Difference (Initial-Final)		0.010	0.017	-0.115	0.234
% Degradation		0.24	0.41	2.82	5.77
Average % Degradation		0.47	0.05	0.29	5.18
Overall Average % Degradation			1.35		

The lower Limit of Quantification was established by using "Signal to Noise (10:1)" ratio method as described in the FDA US guideline for the bioanalytical method validation. The LLOQ concentration was found to be 0.2812µg/ml.

Freeze and Thaw Stability: The difference between initial concentration and after freeze and thaw cycles did not show any significant difference that indicates the samples were stable after three cycles of freezing and thawing (Table-6).

Short Term Temperature Stability: The difference between initial concentration and after 8h and 20 h did not show any practical difference thus

the samples were stable at room temperature for 20h (Table-7).

*Long Term Stability***:** The difference between initial concentration and after 7, 14 and 21 days did not show any significant difference while slight difference in concentration after 28th day was observed which is within the acceptance limit (≥ 15%) of accuracy and precision (Table-8). Thus overall results indicate that the samples were stable for 28 days at storage condition of -20ºC.

Stock Solution Stability: The result of stock solution analysis after 24 h was found to be 100.04% which showed that the stock solution was stable at room temperature for 24 h.

*Post Preparative Stability***:** There was no significant difference in the results of samples analyzed at the start of validation process and at the end of validation process which showed that the samples were stable during the whole validation process (Approx. 6 h).

Conclusion

The developed analytical method was validated as per the FDA US guideline on human plasma. The results of all the validation parameters were well within the acceptance limit defined by the FDA guideline. The method was validated by using plasma samples from eight different sources to demonstrate the robustness of the method over the physiological variation among the human population. Therefore, the method can be used with adequate level of confidence and assurance for bioavailability, bioequivalence and other pharmacokinetic studies of metronidazole in human.

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Declaration

We declare that this is an original research work and is not previously published or presented elsewhere in any language and is also not in consideration in any other journal simultaneously.

We would also like to declare that there are no conflicts of interest or financial interests between the authors or members of their immediate families

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