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Bioanalytical Method Development and Validation of Estimation of Nimorazole by RP-HPLC in Human Plasma

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Abstract : Nimorazole is an antiprotozoal medicine used to treat infections caused by protozoa in the stomach, intestines, or genital areas. The main principle of this study was to develop RP-HPLC technique for the quantitative determination of Nimorazole in human plasma. The separation was accomplished by the isocratic method by using column C18 (Thermosil ODS), detection wavelength was 294 nm. The analyte was extracted in acetonitrile by liquid-liquid extraction. Acetonitrile: water in the ratio 30:70 was used as mobile phase for estimation of the drug in human plasma with a flow rate of 0.8 mL/min at a detection wavelength of 294 nm. Retention time was found to be 7.933 ± 0.23 min.The developed method was found to be linear over the concentration range of 60-360 µg/mL, with a correlation coefficient of 0.9991. The LOD and LOQ were found to be 10 µg/mL and 40 µg/mL, respectively. The method ensure for Precision and % RSD was found to be less than 2 % and the mean % recovery was found to be 99.58%. This method was effectively and favourably applied to the plasma samples and it seems to be appropriate tool for regular therapeutic drug monitoring of anti-infective drugs.
Keywords: Bioanalytical method, RP-HPLC, Nimorazole.

Introduction

Bioanalytical method and validation are applying to build up a quantitative analytical method which can be associated for the biochemical process. It is applied for quantitative evaluation of drug and metabolites, clinical pharmacokinetics, new drug development, research process and therapeutic drug monitoring[1]. Nimorazole (NMZ) is chemically 4-[2-(5-nitroimidazol-1-yl)ethyl] morpholine (figure 1). Nimorazole is a nitroimidazolewith anti-infective drug against infections caused by protozoa in the stomach, intestines, or genital areas and is used to treat certain infections of the gum. It is also being studied in the treatment of cancer[2-3].A systematic literature evaluation shows that a number of methods available for estimation of Nimorazole in both single or in blend with other combinations in different dosage form and

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physiological fluids using various analytical techniques such as spectrophotometric methods[4-6], separation techniques, including HPLC[7-14], LC–MS[15] and HPTLC[16]have been also reported. There is no method reported for determination of Nimorazolein human plasma by RP-HPLC.Hence the present study describes the development and validation of a specific, fast, precise and robust RP-HPLC bioanalytical approach for Nimorazole in human plasma.

Materials and Methods

Chemicals and reagents

The active pharmaceutical ingredient Nimorazole was supplied as gift sample from Lupin's Pharmaceuticals Inc. Aurangabad, India. Nimorazol tablet was purchased from the local pharmacy, Pune, which contains Nimorazole 500 mg. All required chemicals, reagents, methanol, acetonitrile were of HPLC grade and purchased from Merck Chemicals, Mumbai, India.

Chromatographic conditions

Jasco HPLC system equipped with a pump (model Jasco PU 2080), Intelligent LC pump with sampler programmed at 20 µL injection capacity, UV/ VIS detector operated at a wavelength of 294 nm and JascoBorwin chromatography data system software was used (version 1.5, LC-Net II/ADC). 30: 70 % of acetonitrile: water used as mobile phase and flow rate of 0.8 mL/min. Separation was done by using Thermosil C18 (250 mm × 4.6 mm, 5 µm) column. Before estimation, using sonicator both mobile phase and sample solutions were degassed and filtered through 0.2-mm filter paper.

Preparation of stock solution and extraction procedure

Nimorazole was prepared by accurately weighing 10 mg into a 10 mL volumetric flask. The drug was dissolved in methanol and the solution was diluted to volume. The stock solution was further diluted with methanol to obtain a solution of NMZ 10 µg/mL.

Plasma preparation is done by using liquid-liquid extraction method. A 50 µL drug free plasma sample was transferred to eppendrof tube. A suitable amount of standard NMZ solutions and 200 µL acetonitrile used as extracting solvent were added. The mixture was centrifuge for 15 min at 15000 rpm. Subsequent aliquots of 60-360 µg/mL concentrations were prepared by diluting with 100 µL of acetonitrile and clear supernatant liquid of each concentration were injected for analysis and chromatogram were taken.

Assay of marketed formulation

For analysis of marketed formulation, twenty tablets of NMZ (500 mg) were finely powdered in a mortar and mixed thoroughly. Amount of the powder equivalent to one tablet content was accurately weighed, transferred into 50 mL volumetric flask and diluted with methanol. The sample solution was then filtered. After suitable dilution, the drug concentration of NMZ was determined by HPLC.

Validation of the method[17-20]

Validation was carried out for the various parameters such as Linearity, LOD and LOQ, Precision, Robustness, Accuracy etc.

Linearity

To evaluate the linearity and range of the developed method, six different standard concentrations (60-360µg/mL) of NMZ were prepared. Replicates of each concentration were independently prepared and injected into HPLC system. The peak area for each concentration was recorded and then plotted against the corresponding concentration to obtain the calibration graph and regression analysis was evaluated for NMZ.

Limit of detection and Limit of quantification

For determining the detection and quantification limits,injecting gradually low concentrations of the standardsolutions using the developed RP-HPLC method.The LODis the smallest concentration of the analyte

that gives a measurable response and LOQ is the smallest concentration of the analyte, which gives response that canbe accurately quantified.

Precision

Precision of the method was carried out by using three replicate injections of standardsolution of NMZ **(**120, 240, 360 µg/mL)was determined each dayof 3 consecutive days for Intraday and Interday precision studies and percentage RSD were calculated.

Robustness of the method

For assessment of robustness method, some experimental conditions were slightly changed such as wavelength, ratio of mobile phase and flow rate.The solution containing NMZ was injected under the varied conditions and change in the responses of NMZ was noted.

Accuracy

Accuracy was analysed by the recovery of added standard addition method, at 80, 100 and 120% level.The experiment was conducted in triplicate. Percentage recovery and relative standard deviation were calculated.

Specificity:

The specificity of the method was determining by analysing drug standard and sample. The identities of the chromatographic peak for NMZ from the sample were confirmed by comparing the t_R with those of standard.

Results and Discussion:

Bioanalytical RP-HPLC method was developed for estimation of NMZ in human plasma.Different mobile phases in varied proportions were tried.From a mixture acetonitrile: water 30: 70 v/v gave symmetric peak and good resolution for NMZ. The retention time of NMZ was found to be7.933 minute (figure 2).The method was validated as per ICH guidelines.

Linearity

Linearity of the method was determined at six concentrations levels ranging from 60-360 µg/mL. The Standard plots were constructed between concentrations versus peak area. The linear regression equations were Y = 37080x - 13706 (r^2 = 0.999) for NMZ. The plots obtained from linear regression are given in figure 3

Limit of detection and limit of quantification

The results of the LOD and LOQ were found to be 10 µg/mL and 40 µg/mL respectively for NMZ.

Precision

The precision of the method were determined by intra-day and inter-day precision. The % RSDvalues obtained were less than 2 % for intra-day and inter-day variation**,** respectively, which indicates that developed method is preciseat the given concentration(Table 1).

Robustness

Robustness of the method was checked for NMZ to remains unaffected by deliberately varying the method parameters. The relative standard deviation of peak areas was found to be less than 2 % indicate the robustness of the method as shown in Table 2.

Accuracy

Accuracy of the method was evaluated at three levels of the specified concentration in triplicate and the percent recovery was calculated. The results of the accuracy are shown in Table 3.

Assay of marketed formulation

Nimorazol tablets were analysed as a commercial formulation containing 500 mg of NMZ, Each sample was analyzed in triplicate.The mean recovery measured was 99.83 %.

Conclusion

The proposed bioanalytical RP-HPLC method for determination of Nimorazole in human plasma is rapid, accurate, precise and sensitive hence it can be useful for routine analysis as well asbio-equivalence studies, in preclinical and clinical pharmacokinetic studies.

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Table 1: Precision study of Nimorazole (n=3)

Conc. (µg/mL)	Intra-day precision			Inter-day precision		
	Measured area	**SD**	**(%) RSD**	**Measured area**	**SD**	**(%) RSD**
120	4193760	20075.98	0.48	4302121	16799.12	0.39
240	8596559	67315.46	0.78	8622630	29608.99	0.34
360	14553664	52618.15	0.36	13117862	125702.8	0.96

Table 2: Robustness study of NMZ

Conditions	t_R(min.)	Avg. Area	% RSD
Mobile phase ± 1.0mL			
ACN :Water (31:69 v/v)	7.97	714931.6	0.72
ACN :Water (30:70 v/v)	**7.93**	**1374032**	**1.76**
ACN :Water (29:71 v/v)	7.69	2317041	1.37
Wavelength ±2nm			
292	7.97	722597.2	0.14
294	**7.93**	**1204032**	**0.20**
296	7.05	2110375	0.53
Flow Rate ± 0.2min.			
0.6	7.98	1203717	0.19
0.8	**7.93**	**1305214**	**0.26**
1.0	7.01	1305720	0.26

Table 3: Accuracy study of NMZ

Label claim	Amt added (%)	Total Amt (mg)	Recovered Amt (mg)	% Recovery	Mean % Recovery ± SD
NMZ (500 mg)	80	900	897.0	99.67	99.58 ± 0.3384
	100	1000	998.7	99.87	
	120	1100	1091.3	99.21	

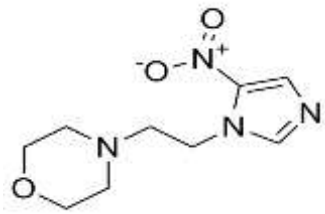

Figure 1: Chemical structure of Nimorazole

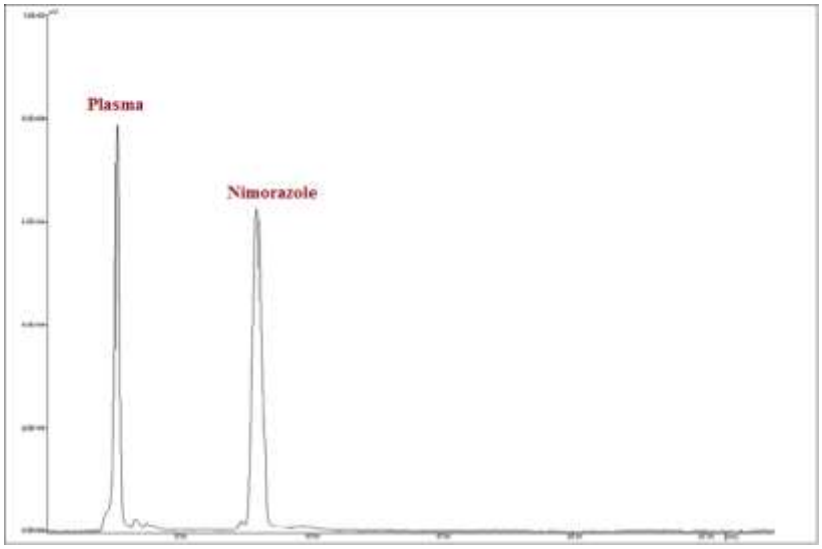

Figure 2: Chromatogram of Nimorazole with Plasma

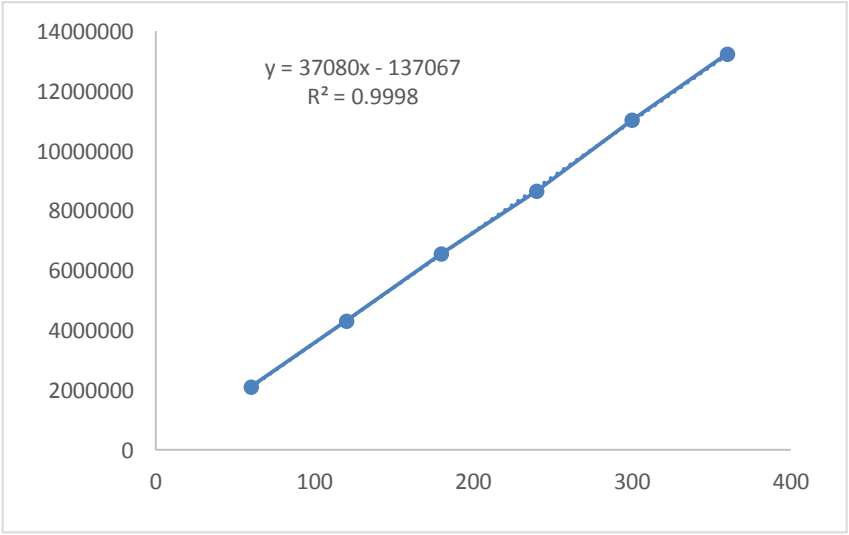

Figure 3: Calibration curve of Nimorazole in human plasma

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