Section A-Research paper
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METHOD DEVELOPMENT AND VALIDATION OF HPLC-UV METHOD FOR ESTIMATION OF ORNIDAZOLE IN HUMAN PLASMA

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Abstract

An efficient, Sensitive and rapid high-Performance liquid Chromatographic Method with UV detection has been developed for the quantitation of antibacterial medications i.e. ornidazole from human plasma. The chromatographic separation was carried out on RP C_{18}(Waters) column (250×4.6 mm, 10mcm) using isocratic mobile phase, Ammonium Acetate (pH 4(pH 4.0,20 mM): acetonitrile (70:30 % v/v) pumped at a flow rate of 1ml/min. The analyte ornidazole and internal standard tinidazole were monitored at 318 nm. The method is specific and linear over the range of 0.025- 3.00 µg/ml with intraday precision of 1.9169% CV and interday precision of 1.1093% CV. Ornidazole was found stable in plasma during sample processing (autosampler) and 30 days of storage in a freezer. This validated method can be applied for bioavailability/ bioequivalence studies.

Keywords: Ornidazole, nitroimidazoles, bioanalytical and Reverse Phase Liquid Chromatography.

INTRODUCTION

Ornidazole (Fig.1), α-(chloromethyl)-2-methyl-5-nitro-1H-imidazole-1-ethanol-1-(3-chloro-2-hydroxy propyl)-2-methyl-5-nitroimidazole.[1,2] is antibacterial medications which inhibit the growth of both anaerobic bacteria and certain anaerobic protozoa, such as Trichomonas, vaginalis, *Entamoeba histolytica* and *Giardia lamblia.* It is chemically known as 1-chloro-3-(2-

methyl-5-nitro-1*H*-imidazol-1-yl)propan-2-ol [3]. Ornidazole is official in United States Pharmacopoeia and Indian Pharmacopoeia[4-5]. The Ornidazole activity is mediated predominantly by the reduction of the nitro group to a more reactive amine that attacks microbial DNA, bringing about loss of helical structure of DNA and subsequent DNA breakage thus inhibiting the further synthesis and ultimately disrupting transcription and cell death[6].

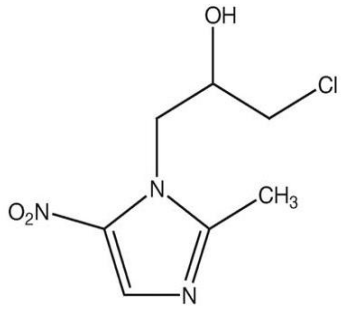

Fig. 1: Structure of Ornidazole

Several analytical methods that have already been reported for the estimation of OND in bulk and in the formulation as well as drugs combination are UV-Spectrophotometry, voltammetry , RP-HPLC and HPTLC[7-20] . Also, researchers have developed bioanalytical methods for the estimation of ornidazole in different biological matrices[21-30]. Few studies have sought to determine its metabolites. Heizmann et al. reported an HPLC-UV method to quantify ornidazole and two of its metabolites (6-hydroxy ornidazole and 2´,3´-dihydrodiol ornidazole) in biological fluids[31].

Although the literature survey reveals that there are analytical methods for the determination of Ornidazole in biological matrices. But these methods suffer from drawbacks such as long retention time and cumbersome extraction procedures. Hence an attempt has been made to develop a simple, specific and Sensitive HPLC method for the estimation of Ornidazole from human plasma using UV detection.

MATERIAL AND METHOD (EXPERIMENTAL)

Reagent and Solution

The working Standard of ornidazole and Tinidazole (Internal Standard) was procured from Endoc Pharma. Acetonitrile, Methanol and Ethyl Acetate were Merck HPLC grade(India). Deionized and ultrapure water used in all experiments was obtained from Milli-Q System (Millipore, USA). Ammonium Acetate used for the preparation of buffer solution was of AR (Analytical Reagent) grade (Qualligen, India).

Selection of wavelength

Standard solutions of both drugs were scanned in a UV spectrophotometer between 200 nm and 400 nm on spectrum mode, using methanol as a reference solvent. The drugs were identified by UV detector λmax at 318 nm.

Method development

Preparation of Standard solution and internal standard solution :
10 mg of working standard of Ornidazole was accurately weighed and dissolved in 10 ml Methanol to give a stock solution of 1mg/ml (Solution A). 1 ml of Solution A was diluted to 10 ml to get a solution of 100 µg/ml (Solution B). Further, 1ml of Solution B was diluted to 10 ml to get a solution of 10 µg/ml(Solution C). Further dilutions were made in the mobile phase. Similarly, dilutions were made for tinidazole which was used as an internal standard (I.S.).

Instrumentation

Jasco PU 1580 Intelligent pump was used with Jasco UV 1575 Intelligent UV detector. Integration and data analysis were carried out using Borwin Chromatographic software (Integrator, Jasco Japan). The UV- Vis detector was set at a wavelength of 318 nm. Waters RP-C18 column (250×4.6 mm, 10 mcm) was used for chromatographic separation.

Plasma Extraction Method

To 0.5ml of a standard solution of ornidazole was spiked in 0.5 ml plasma sample. In the resulting solution 50 µl of I.S. (internal standard –- Tinidazole, 20 µg/ml)was added, vortexed for 1 min. To this 0.5ml of 0.1 N HCl was added was kept for vortexing for 5 min. The drug was then extracted using 5ml of Ethyl acetate by vortexing for 10 mins. The organic layer was separated and evaporated to dryness under a stream of Nitrogen. The residue was reconstituted with the mobile phase and 100 µl was injected.
After the development of the extraction procedure, appropriate chromatographic conditions were optimized which are given in table 1.

Table 1: Optimized Chromatographic Conditions

HPLC Pump	Jasco PU 1580 Intelligent HPLC Pump
Detector	Jasco FP 1575 Intelligent UV-Vis Detector
Integrator	Borwin Chromatography Software
Column	Waters RP-C18 column
Wavelength	318 nm
Injection Loop	100 µl
Mobile Phase	0.02 M Ammonium Acetate: 300 ml of acetonitrile (700: 300v/v) adjusted to pH 4.1 for final mobile phase.
Flow rate	1ml/min

Once the chromatographic conditions were set, validation of the analytical method was done for Standard Solution.

Determination of Extraction efficiency

The extraction efficiency of ornidazole from the biological matrix was determined by % recovery. The recovery study was carried out by using LQC , MQC and HQC standards and % recovery was calculated by comparing the analytical results for extracted samples at with unextracted standards that represent 100% recovery.

Bioanalytical Method validation:

The developed bioanalytical method was validated in terms of specificity, matrix effect, linearity, precision, accuracy, limit of detection, limit of quantification and stability studies[32] .

Specificity and matrix effect

The specificity of the method was ascertained by analyzing the standard ornidazole extracted from plasma and compared with a chromatogram of blank plasma. To check the matrix effect, LQC (0.075 µg/ml) and HQC(2.5 µg/ml) were spiked in three different blank plasma and analyzed in triplicates using the optimized HPLC method.

Linearity, LOD & LOQ:

The Linearity Range of the Standard solution was found to be 0.0250µg/ml, 0.050µg/ml, 0.100 µg/ml, 0.2500µg/ml, 0.500µg/ml, 1.000µg/ml, 2.000µg/ml and 3.000µg/ml.

A calibration curve was constructed and the correlation coefficient was calculated. LOD and LOQ were calculated using the formula LOD=3.3(SD/S) and LOQ=10(SD/S), respectively based on the standard deviation (SD) of the response and slope(S) of the calibration curve.

Precision and Accuracy studies:

Precision was calculated by Intraday and Interday studies. Both precisions were determined at three different QC levels (low, medium, high) viz. 0.0750µg/ml, 1.00 µg/ml and 2.5 µg/ml. Six samples of each concentration were prepared to study Intraday and Inter-day precision and the results are presented as %RSD. The accuracy of the method was proved by finding the ratio of the mean of back-calculated QC concentration against the actual concentration.

Stability:

The stability of ornidazole reference standard in human plasma was determined at different storage conditions viz. Benchtop stability, post-preparative stability and freeze-thaw stability (three cycles) at LQC (0.075 µg/ml) , MQC (1 µg/ml and HQC level (2.5 µg/ml). Benchtop stability was evaluated for three different concentrations i.e. high (HQC), medium (MQC), low (LQC) concentrations of the drug. For benchtop stability, six replicate quality control samples corresponding to HQC, MQC & LQC were placed at room temperature for 8 hours and then analyzed. Stability was calculated by comparing the results with fresh samples.

Autosampler stability was evaluated by extracting and keeping the HQC, MQC and LQC samples (in a replicate of six each) in the autosampler for 24 hours. These samples were then analyzed and compared for their stability with fresh samples.

Dry Extract stability was evaluated by analyzing dry extracts of HQC, MQC and LQC samples (in a replicate of six each) which were extracted and kept in the refrigerator for 24 hours at 20°c and then analyzed. Stability was calculated by comparing the results with fresh samples.

For freeze-thaw stability Eighteen aliquots, each of HQC, MQC and LQC were stored at -20°c for 24 hours. Eighteen samples (6 each of HQC, MQC and LQC) were withdrawn and thawed at room temperature. When completely thawed, the samples were refrozen for 24 hours under the same conditions (FT3 Cycle). In the next cycle, an additional set of 6 HQC, MQC and LQC each was withdrawn along with the previous set and allowed to thaw. After thawing both the sets were replaced back (FT2 cycle). After 24 hrs additional set of 6 HQC, MQC and LQC each (FT1 Cycle) were withdrawn along with FT3 and FT2 sets and allowed to thaw. These samples were then extracted and analyzed. Stability was calculated by comparing the results with fresh samples.

For long-term stability, Eighteen aliquots each of HQC, MQC and LQC were stored at -20°c. Eighteen samples (6 each of HQC, MQC and LQC) were withdrawn and analyzed after 10 days, 20 days and 30 days of storage. Stability was calculated by comparing the results with fresh samples.

System Suitability Testing

System suitability testing for Quality control samples of Ornidazole was carried out by injecting 6 replicates of higher limit of Quantification (HLOQ) I.E. 3.000 µg/ml.

Result and discussion

Ornidazole is soluble in methanol, ether, ethanol and chloroform. The calibrator solutions were prepared in methanol. Sample preparation is a crucial step in bioanalytical method development. Liquid-liquid extraction was used for sample preparation of plasma samples. Ornidazole is an acid having a pKa 2.4; hence it was extracted from an aqueous medium at low pH into an organic solvent. Various solvents like methanol, ethyl acetate, methyl *tert*-butyl ether, dichloromethane, chloroform and acetone were used. Methanol when tried gave the low molecular weight plasma protein impurities, since water soluble impurities also get extracted. Ethyl acetate, dichloromethane, n-hexane and heptane were also tried. Best recovery was obtained between the range of 86.19% - 79.98% using ethyl acetate as extracting solvent, whereas use of dichloromethane, Tert methyl butyl ether and n-hexane gave low recovery (45.00% - 50.00%) interference in final eluted samples and inconsistent results.

Optimization of the HPLC method

Since Ornidazole is strong acidic in nature (ionizes in basic medium) so reverse – phase chromatography is the best choice. The efficiency of two different reverse–phase column C_{18} and C_8 was evaluated. C_{18} column was preferred for the separation of the drug because C_8 column was giving decreased retention of a drug (2.5 min) which resulted in the merging of plasma impurities peaks and drug peaks. Even resolution between the drug peak and internal standard (Tinidazole) peak could not be achieved. However the above-mentioned problems were resolved

using C_{18} column, there was no other interfering peak around the retention time of Ornidazole. Sufficient resolution between the drug (Ornidazole) and internal standard (Tinidazole) peak was also obtained.

Various mobile phases containing water and acetonitrile in different compositions were tried but desired resolution could not be achieved between solvent peaks, drug peaks and plasma impurities. Good resolution between the solvent peaks, drug and internal standard which are resolved properly from plasma impurities were obtained using water: Acetonitrile (70:30% v/v), But the peak was broad and showed tailing (> 2) and the response was not reproducible. Hence water in the mobile phase was replaced with 20 mM Ammonium Acetate buffer which resulted in the improvement of peak shape i.e. sharp peak with reduced tailing and fronting. This is because NH_3 ion gets adsorbed and blocks the silanol groups of the stationary phase. The retention time of Ornidazole was affected by the pH values of the systems. pKa value of Ornidazole is 2.4 so in the acidic pH probability of the drug remaining in unionized form is more which in turn has an effect on peak shape and retention time, therefore considering the pka value (2.4), pH of ammonium acetate buffer was adjusted to 4.0. Thus mobile phase 20 mM Ammonium Acetate (pH 4.0): Acetonitrile (70:30v/v) resulted in improved sensitivity and selectivity and also gave reproducible results.

With the aim of the optimization of mobile phase pH (2.0, 4.0, 5.0), the remaining two factors were kept constant, i.e. mobile phase composition (20 mM Ammonium Acetate buffer: Acetonitrile 70:30v/v) and flow rate of 1 ml/min. Since pKa of Ornidazole is 2.4 so in the acidic pH probability of the drug remaining in unionized form is more which in turn has an effect on peak shape and retention time. As pH increased towards basic, % of the ionized drug increased (5.0) resulting in decreased retention time for Ornidazole because of less interaction between the drug and C18 column under ionized conditions[33-34]. At lower pH (2.0) the Ornidazole eluted out faster with the mobile phase, however, due to unionized species, the drug has a tendency to stick with the stationary phase too. This results in tailing and hence an increase in asymmetric value at lower pH. At pH value (4.0), ionized species are not portioned with the stationary phase and hence give a symmetric peak. The resolution was poor at pH 2.0, 4.0, 5.0 with using Tinidazole as the internal standard but highest at pH 4.0(Highest plate number and good peak symmetry). Thus, the best chromatographic separation was achieved at pH 4.0 with improved peak shape, tremendous decrease in tailing and reproducible response, and hence was considered to be optimum.

The absorption maximum was found to be 318 nm during the UV scan. During the Liquid – Liquid extraction, the plasma was acidified which kept the drug unionized and lead to good recovery from the plasma matrix, as Ornidazole is strong acid having pKa 2.4.

Bioanalytical Method validation

The developed bioanalytical method was validated in terms of specificity, matrix effect, linearity, precision, accuracy, limit of detection, limit of quantification and stability studies11-13.

The method was found to be highly specific showing no interference from human blank plasma at the retention time of Ornidazole and Tinidazole(IS), which were 6.8 (±0.1) and 5.2 (± 0.1) minutes respectively. The chromatogram did not show any significant noise or any matrix effect. The Linearity Range of the ornidazole standard solution was found to be 0.0250µg/ml to 3.000µg/ml with a correlation coefficient of 0.99 which is presented in fig 2. The results are presented in table 2 and 3. A representative chromatogram of the plasma sample spiked with 2µg/ml of Ornidazole is presented in fig 3.

Fig. 2: Calibration curve for standard Ornidazole solution for bioanalytical method

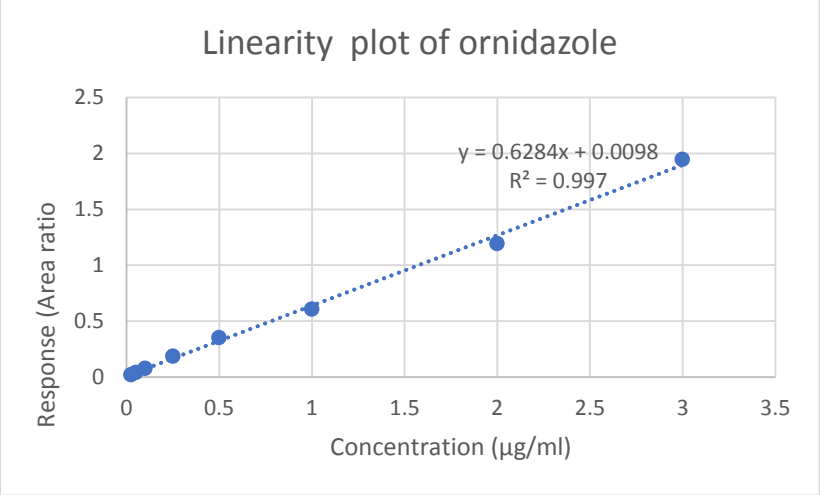

Fig. 3: Chromatogram of human plasma sample spiked with 2 µg/ml of Ornidazole and Internal Standard Tinidazole

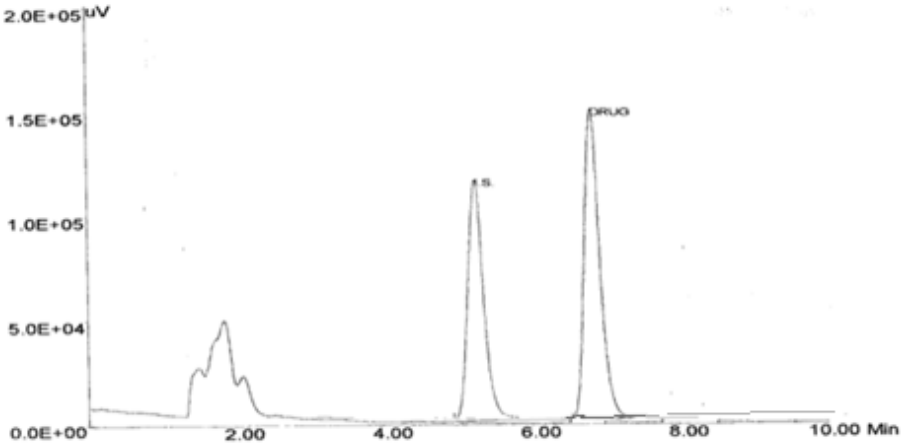

Table 2: Calibration data for the Analysis of Ornidazole in Quality control samples.

Conc. (µg/ml)	AREA RATIO (Area of analyte/ Area of I.S.)						±S.D.	%CV
	Linearity 1	Linearity 2	Linearity 3	Linearity 4	Linearity 5	MEAN AREA		
0.025	0.0215	0.0214	0.0212	0.0219	0.0220	**0.0216**	**0.0004**	**1.6390**
0.050	0.0412	0.0403	0.0406	0.0418	0.0420	**0.0412**	**0.0007**	**1.7637**
0.100	0.0793	0.0790	0.0791	0.0788	0.0795	**0.0791**	**0.0003**	**0.3696**
0.250	0.1877	0.1868	0.1850	0.1848	0.1858	**0.1860**	**0.0012**	**0.6478**
0.500	0.3417	0.3381	0.3437	0.3686	0.3763	**0.3537**	**0.0175**	**4.9416**
1.000	0.6004	0.6000	0.5985	0.6149	0.6215	**0.6071**	**0.0104**	**1.7207**
2.000	1.1999	1.2054	1.1968	1.1880	1.1881	**1.1956**	**0.0076**	**0.6338**
3.000	1.9370	1.9463	1.9499	1.9458	1.9521	**1.9462**	**0.0058**	**0.2964**

Table 3: Calibration data for the Analysis of Ornidazole in Quality control sample

Sr.No.	Slope	(Regression Value)
Linearity 1	0.0006	0.9992
Linearity 2	0.0006	0.9989
Linearity 3	0.0006	0.999
Linearity 4	0.0007	0.9989
Linearity 5	0.0007	0.999
Mean	**0.00064**	**0.999**
± S.D.	**0.0001**	**0.00012**
% C.V	**0.0108**	**0.0123**

Limit of detection (LOD) was found to be 0.0080µg/ml and Limit of Quantitation (LOQ) was found to be 0.0250µg/ml.

Precision and Accuracy studies:

The % CV for intra-day precision was found to be between 1.9169%- 0.6072% and for inter-day precision was found to be between 1.1093% to 0.4630%. The results are presented in table 4 and 5.

Table 4: Precision and Accuracy Data of Ornidazole in Quality control samples (Intraday)

Concentration of Standard solution			
QC ID	**LQC**	**MQC**	**HQC**
Actual Conc. (in µg/ml)	**0.0750µg/ml**	**1.00µg/ml**	**2.50µg/ml**
1	0.0757	1.0316	2.4744
2	0.0752	0.9955	2.5217
3	0.0750	1.0352	2.5133
4	0.0749	1.0302	2.4919
5	0.0742	1.0013	2.4964
6	0.0754	0.9794	2.5237
7	0.0745	1.0038	2.5049
8	0.0748	0.9916	2.4906
9	0.0755	0.9978	2.4842
10	0.0746	0.9981	2.5147
11	0.0746	0.9829	2.4752
12	0.0755	0.9866	2.5215
MEAN	**0.0750**	**1.0028**	**2.5011**
SD	**0.0005**	**0.0192**	**0.0181**
% CV	**0.6072**	**1.9169**	**0.7221**
%ACCURACY	**100.0004**	**100.2824**	**100.0417**

Table 5: Precision and Accuracy Data of Ornidazole in Quality control samples (Interday)

Concentration of Quality control samples			
QC SET ID	**LQC**	**MQC**	**HQC**
Actual Conc. (in µg/ml)	**0.075 µg/ml**	**1.00 µg/ml**	**2.50 µg/ml**
1	0.0757	1.0316	2.4744
2	0.0752	0.9955	2.5217
3	0.0750	1.0352	2.5133
4	0.0749	1.0302	2.4919
5	0.0742	1.0013	2.4964
6	0.0754	0.9794	2.5237
7	0.0745	1.0038	2.5049
8	0.0748	0.9916	2.4906
9	0.0755	0.9978	2.4842
10	0.0746	0.9981	2.5147
11	0.0746	0.9829	2.4752
12	0.0755	0.9866	2.5215
13	0.0750	1.0016	2.4936
14	0.0752	1.0053	2.5083
15	0.0751	0.9969	2.5084
16	0.0745	1.0004	2.4912

17	0.0748	0.9983	2.5029
18	0.0754	1.0022	2.5086
19	0.0751	1.0022	2.5049
20	0.0756	1.0006	2.4952
21	0.0746	0.9942	2.5059
22	0.0748	0.9955	2.4873
23	0.0759	1.0016	2.5040
24	0.0750	1.0027	2.5107
25	0.0754	1.0007	2.4922
26	0.0756	1.0029	2.5051
27	0.0745	0.9983	2.4959
28	0.0746	0.9999	2.4963
29	0.0754	1.0026	2.5027
30	0.0751	0.9980	2.5056
31	0.0757	1.0006	2.4949
32	0.0750	0.9951	2.5111
33	0.0746	0.9988	2.4966
34	0.0742	1.0046	2.5097
35	0.0756	0.9982	2.4959
36	0.0752	1.0006	2.5015
MEAN	**0.0751**	**1.0010**	**2.5011**
±S.D.	**0.0004**	**0.0111**	**0.0116**
% C.V	**0.5895**	**1.1093**	**0.4630**
% ACCURACY	**100.0768**	**100.0993**	**100.0456**

The stability of the analyte was evaluated using Quality control samples at LQC, MQC and HQC for bench-top stability, autosampler, dry Extract stability and three repeated freeze-thaw cycles.

The Benchtop stability revealed that the unprocessed samples remain stable for 8 hours at room temperature as the content of genistein was found to be in the range of 99.84 to 99.98 % w/w.

The post-preparative study indicated solution remains stable for 4 hours after processing of samples as the content of genistein was found to be in the range of 99.19 to 99.90% w/w. The dry sample stability study also confirmed the stability of ornidazole. The content of ornidazole was in the range of 99.97 to 99.96 % w/w, after the completion of the third freeze-thaw cycle, which falls in the acceptable range.

The long-term stability of Ornidazole in frozen plasma (-20^0) was determined after 10, 20 and 30 days, wherein it was found stable. All the values of validation parameters were in the acceptable range. System suitability testing for Quality control samples of Ornidazole was carried out by injecting 6 replicates of higher limit of Quantification (HLOQ) I.E. 3.000 µg/ml. The results are tabulated in table 6.

Table 6: System suitability data for Quality control samples of Ornidazole

Sr.No.	Area of HLOQ (3.000µg/ml)	Resolution	Tailing factor	Theoretical plates
1	2556834.35	5.933	1.22	5038.7
2	2518359.48	5.931	1.28	5052.37
3	2537285.49	5.935	1.26	5011.37
4	251606.65	5.927	1.25	5043.47
6	2524839.49	5.929	1.25	5036.56
MEAN	**2524185.0920**	**5.9310**	**1.2520**	**5036.4940**
±S.D.	**17181.5106**	**0.0032**	**0.0217**	**15.3036**
% CV	**0.5796**	**0.0804**	**1.7316**	**0.3039**

CONCLUSIONS

The study demonstrates that the developed validated bioanalytical HPLC method is simple, precise, accurate, and comparable to the existing HPLC methods. The optimized validated method has advantages like easy sample preparation and small sample size. Hence Validated HPLC bioanalytical method is highly suitable for pharmacokinetic studies and bioanalysis.

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