ORIGINAL ARTICLE

Optimization of a micro-high-performance liquid chromatography method for determination of metronidazole benzoate in their standard powder and in dosage pharmaceuticals

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HIGHLIGHTS

- A new method of estimating MET in pharmaceuticals.
- Use of HPLC-UV technology for LC100 in the estimation of MET.
- Study the structural synthesis of MET in the neutral, acidic and base.
- Studying the relative stability of MET during the experimental estimation process.
- Perform different applications for the purpose of validating the chromatographic method in the estimation of MET.

Abstract

Context: In this manuscript, a high-performance liquid chromatography (HPLC) method for the determination of metronidazole in pharmaceuticals was described and developed. **Methods:** The reversed-phase HPLC (RP-HPLC) method was developed and the results obtained to determine the form of metronidazole. Chromatographic analysis was performed in HPLC-ultraviolet (HPLC-UV) system with Ion Pac column; Arcus EP-C18; 5 µm, 4.6 mm× 250 mm, with acetonitrile: triethylamine 30:70 (v/v)+0.5 M potassium dihydrogen orthophosphate buffer at pH 4.5 as mobile phase, at a flow rate of 1.0 ml/min. UV detection in HPLC system was performed at 310 nm. **Results:** The method was validated for accuracy, precision, specificity, linearity, and sensitivity. The retention time for the metronidazole was 9.9 min. Calibration plots were linear over the concentration ranges 1–5 µg/L for the metronidazole. The limit of detection was 0.115 µg/ml and the limit of quantitation was 0.437 µg/ml. The accuracy of the proposed method was determined by recovery studies and found to be from 93.3% to 100%. **Conclusion:** Commercial tablet formulation was successfully analyzed using the developed HPLC-UV method that has been validated; accuracy, precision, and specificity were found to be within the acceptable limits. Moreover, results obtained by the suggested methods showed no significant difference between the results obtained from the suggested method.

Key words: Detection limit, metronidazole drug, micro-high-performance liquid chromatography, quantification limit, statistical analysis

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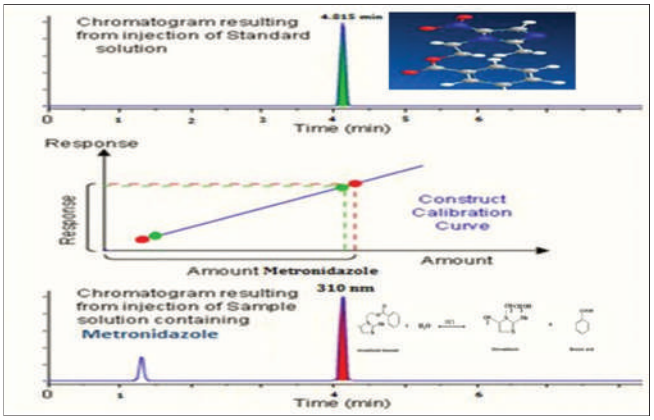
Graphical Abstract



INTRODUCTION

Metronidazole benzoate (MET) is used as an antiprotozoal, chemical name AUPIC: 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl) ethyl benzoate ($C_{13}H_{13}N_3O_4$, Mol. Wt. 275.27), MET is a white crystalline powder or light yellow odorless, almost soluble in dichloromethane, chloroform, and soluble acetone in ethanol, almost insoluble in water. Its melting point is 99–102, is a benzoic acid derivative used as antiamebic, antiprotozoal, and antibacterial [Figure 1].[1-6]

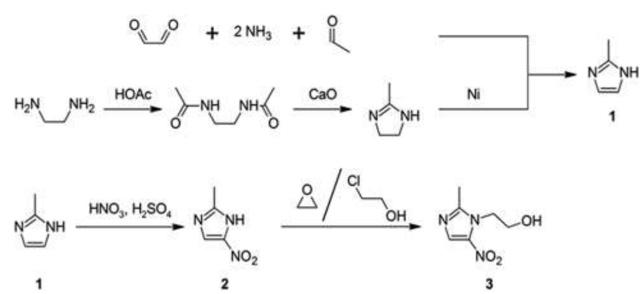
The medical properties of metronidazole are antimicrobial and antimicrobial agents. Nitroimidazole derivatives are also used in the treatment of anaerobic bacterial infection. The drug is converted into anaerobic bacteria by the enzyme pyroxene and verdoxine oxidase. The nitro group in metronidazole is chemically reduced by ferredoxin metabolism or by the associated ferredoxin group. Therefore, the new product is responsible for destroying the structure of the DNA spiral chains, thereby inhibiting the synthesis of DNA in microbial and bacterial organisms.[7-10]

A number of different analytical methods can be used to test and identify MET in pharmaceuticals, especially the high performance liquid chromatography method, which is considered to be a modern and advanced method. Quantification of metronidazole is measured in pharmaceuticals using high-performance liquid chromatography (HPLC).[11-14]

In this study, reversed-phase HPLC (RP-HPLC) was developed using ultraviolet detector, a simple, fast, and sensitive method for quantitative determination of metronidazole in pharmaceuticals. Stability of samples was determined in different laboratory conditions. It is very important to develop an appropriate analytical method to estimate the content of metronidazole in its pharmaceutical forms. In the HPLC method, aneleunt solution consisting of a mixture of solvents such as acetonitrile, methanol, and potassium hydrogen phosphate is used. A chromatographic separation column (Ion Pac Arcus EP-C18; 5 µm, 4.5 mm × 250 mm) is selected with a qualitative and quantitative estimate of this type of pharmaceutical and appropriate separation conditions are applied. This method was validated in accordance with the Food and Drug Administration Guidance Document, entitled "Dynamic Verification Method" (May 2001).[15-20] The RP-HPLC method was also validated in accordance with the International Council for Harmonisation (IHC) guidelines.

Metronidazole Synthesis

Metronidazole is produced by imidazole synthesis or ethylenediamine and acetic acid, followed by treatment with lime, then nickel as in the steps of the following equation.[21,22]



The objective of the study

The objective of the study was to develop and verify the RP-HPLC method with an ultraviolet (UV) detector for quantitative determination of MET in pharmaceuticals.

EXPERIMENTAL

Instrumentation

LC-100 series S-HPLC features fully automatic digital computer control. Its electronic circuit design, internal mechanical structure design, processing technology, functions of cinematography workstation, and the technical criteria make it leading instruments with excellent stability and reliability. The LC100-type HPLC-UV consists of a double-beam optical spectrometer (Angstrom Advanced Inc., USA), model UV-100 PC with 1 cm path length quartz cell is used and it is connected to IBM compatible computer. The software was UVPC personal spectroscopy software version Matlab, R2003b was used for the proposed chemometric methods, the partial least squares (PLS) were performed with PLS_Toolbox for use with Matlab R2003b, VP pumps, and variable wavelength programmable UV detector. Peak areas were integrated using an Angstrom Advanced Inc. LC solution software program. The chromatographic separation and quantification were performed on Ion Pac column; Arcus EP-C18 (250 mm × 4.6 mm; particle size 5 µm) analytical column maintained at room temperature. The mobile phase, drug standard solutions, and tablet sample solutions were filtered through a millipore membrane filter before injection into the HPLC system.[23-28]

CHEMICALS AND REAGENTS

Pure Standard

Standard MET with claimed purity of 98%, according to manufacturer certificate and was kindly donated by AARTI Drug Industries Pharma, India, for medical devices and pharmaceuticals.

Market Sample

Flagyl Espagne-France® tablets batch No. 75014-Paris France were labeled to contain 500 mg MET per tablet were manufactured by Sanofi Pharma for Pharmaceuticals and Medical Appliances Flagyl-France, the other drug METROSULE-500 tablets batch No. MBO/12120686 were manufactured by Limited Ajanta.

Configure the Samples for Measurement

- HPLC grade (Sigma-Aldrich® Chemie GmbH, Germany) solutions
- Stock standard solutions of MET were prepared in acetonitrile: triethylamine 30:70 (v/v) + 0.5 M potassium dihydrogen orthophosphate buffer at pH 4.5 to prepare concentration of 1 mg/ml from MET.[29-32]
- Working standard solutions of MET was prepared in acetonitrile: triethylamine 30:70 (v/v) + 0.5 M potassium dihydrogen orthophosphate buffer at pH 4.5 to prepare the concentration of 1.0, 2.0, 3.0, 4.0, and 5.0 µg/ml.

Table 1: Parameters of RP-HPLC method

Mobile phase	Acetonitrile: triethylamine 30:70 (v/v)+0.5 M potassium dihydrogen orthophosphate buffer at pH 4.5
Flow rate	1.0 mL/min
Detection wavelength	310 nm
Column temperature	Room temperature
Injection volume	20 µL
Run time	15 min

Sample Updating

To perform model updating, the optimized PLS calibration set was augmented with different samples of Flagyl Espagne-France® tablets containing known amounts from standard MET and METROSULE-500 tablets were manufactured by Limited Ajanta. One known concentration to three unknown concentrations of samples containing different concentrations of each was added purpose for done the initial calibration and the predictive ability of the updated sample was checked using external validation samples, then calculate the perform sample updating for each component using the developed method RP-HPLC with three concentrations of the added updating samples.[33-36]

PROCEDURE

Standard Drug Solution

The mobile phase was used as solvent for the preparation of standard solutions. Standard stock solution of metronidazole (500 µg/mL) was prepared by dissolving an accurately weighed amount of metronidazole (50 mg) in 100 mL of mobile phase in 250 mL volumetric flask. The flask was then made up to the mark with mobile phase. The stock solution was diluted aptly with mobile phase to prepare the working standard solutions of metronidazole (1, 2, 3, 4, and 5 µg/mL).

Chromatographic Conditions

Table 1 shows the values of the basic parameters obtained using the reverse-phase chromatography system (RP-HPLC).

RESULTS

The Calibration Curve

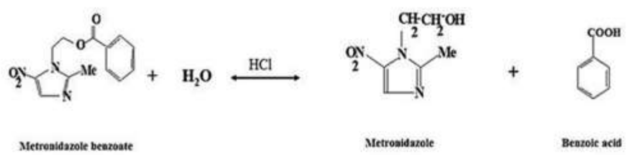
Calibration curves of the proposed method were prepared over concentration range of 1–5 µg/ml for MET. Solution was prepared in triplicate and 20 µl of each solution was injected onto the column. The peaks were determined at 310 nm. The calibration curve of metronidazole was constructed by plotting the peak area versus concentration.

Stress Degradation Studies

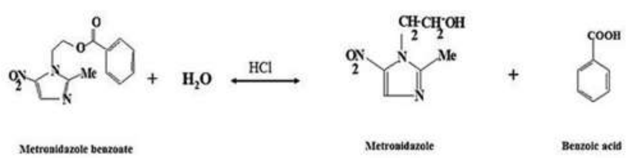
Stress degradation studies were carried out using different ICH prescribed stress conditions such as acidic, basic, oxidative, thermal, and photolytic stresses.[37-39]

Acid Degradation

About 60 mg from tablet powder of metronidazole was taken in 100 ml volumetric flask. 5 ml of 0.1 N HCl was added to the flask and kept at 70–80°C reflux condition for 2–3 h. After completion of the stress, the solution was neutralized using 0.1 N NaOH and completed up to the mark with mobile phase. Hydrolysis of MET may be hydrochloric acid.



One such reaction is hydrolysis, “splitting with water.” Hydrolysis of esters is stimulated by any acid or base [Figure 2].



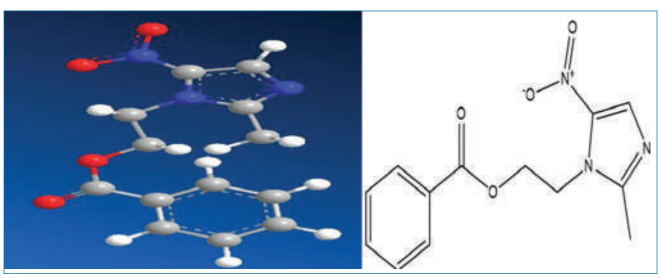
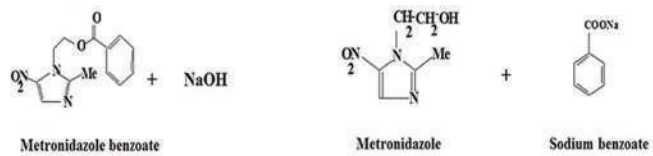


Figure 1: Structure of the metronidazole benzoate

The base degradation

When using base such as NaOH or potassium hydroxide to suppress ester, products of carboxylic salt, and alcohol. 60 mg from tablet powder of metronidazole was taken in 100 ml volumetric flask. 5 ml of 0.1 N NaOH was added in the flask and kept at 70–80°C reflux condition for 2–3 h. After completion of the stress, the solution was neutralized using 0.1 N HCl and completed up to the mark with mobile phase [Figure 3].



Oxidative degradation

About 60 mg from tablet powder of MET and 5 ml of 20% H_2O_2 were added in 100 ml volumetric flask. The flask was kept at 70–80°C reflux condition for 2–3 h. After completion of the stress, the flask was completed up to the mark with mobile phase [Figure 4].

Table 2: Characteristic absorption bands in the infrared spectra of metronidazole and its crud

Functional groups	MET	MET-Crud
ν(O-H bound)	3710.70	-
ν(O-H associated)	3220.85 3100.79	3220.52 3100.88
ν(C=O)	2342.02	-
ν(C=C) ν(C=N)	-	-
ν(N=O)	1535.59 1368.90	1535.71 1368.91
ν(C=C)	1265.53	1265.55
ν(C-N)	1187.45	1187.51
ν(C-O)	1074.51	1074.47
o(C-H)	825.72	825.72
M-OH	-	-

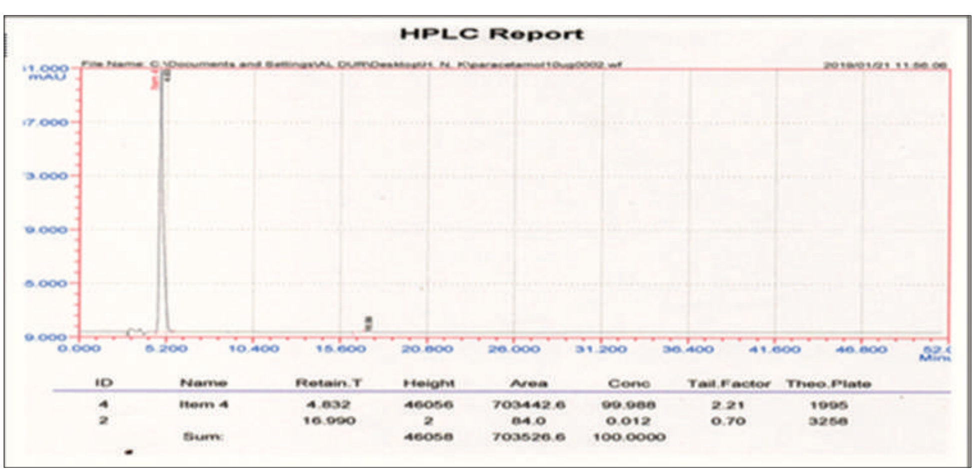


Figure 2: Chromatogram of acid degradation

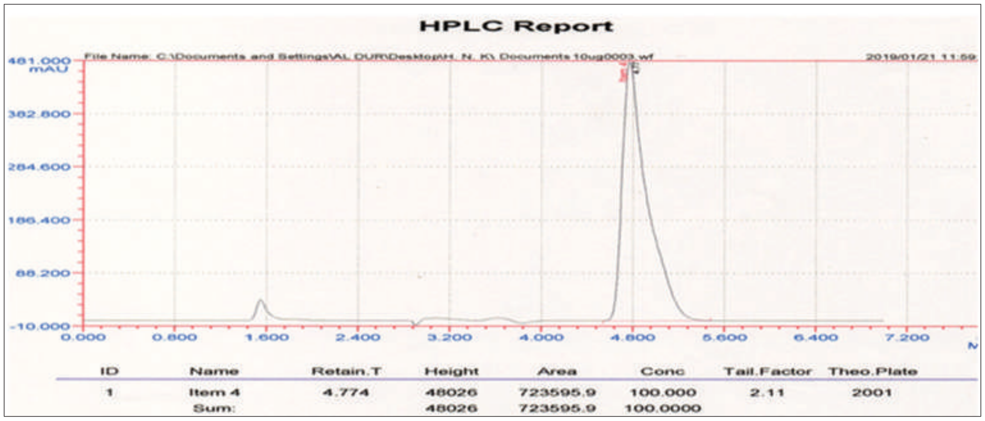


Figure 3: Chromatogram of base degradation

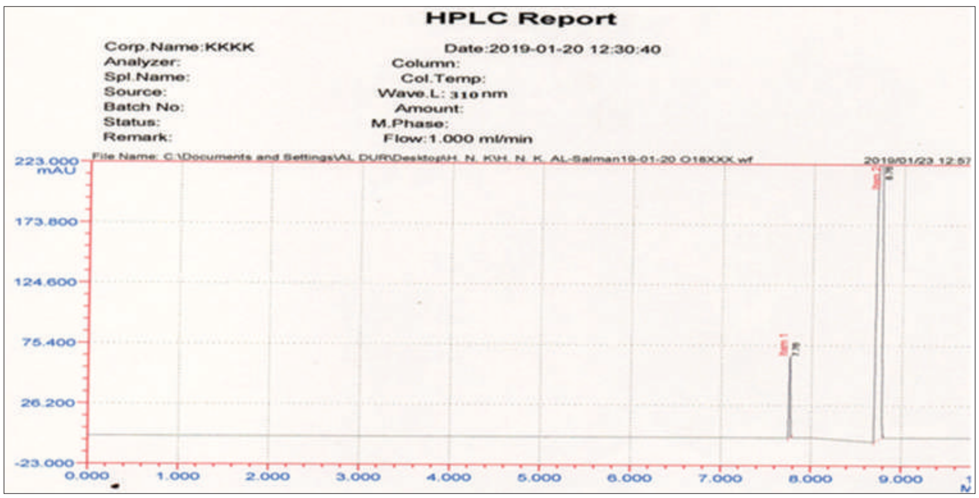


Figure 4: Chromatogram of oxidative degradation

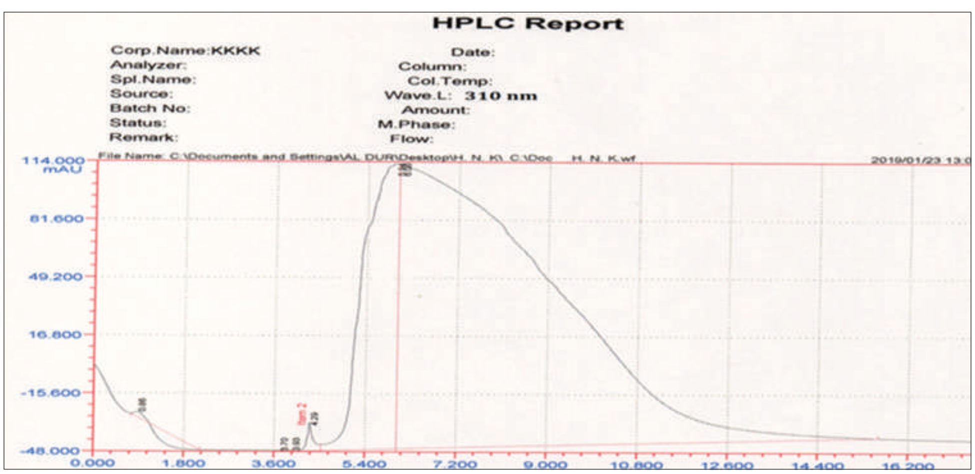
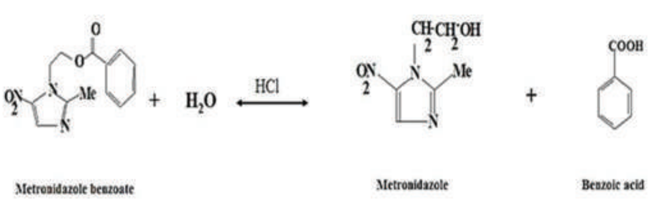


Figure 5: Chromatogram of photolytic degradation



Photolytic degradation

For photolytic degradation study, 60 mg from tablet powder of metronidazole benzoate was transferred into a glass Petri dish and placed in the direct sunlight for 2–3 h. After completion of the stress, the tablet powder was transferred

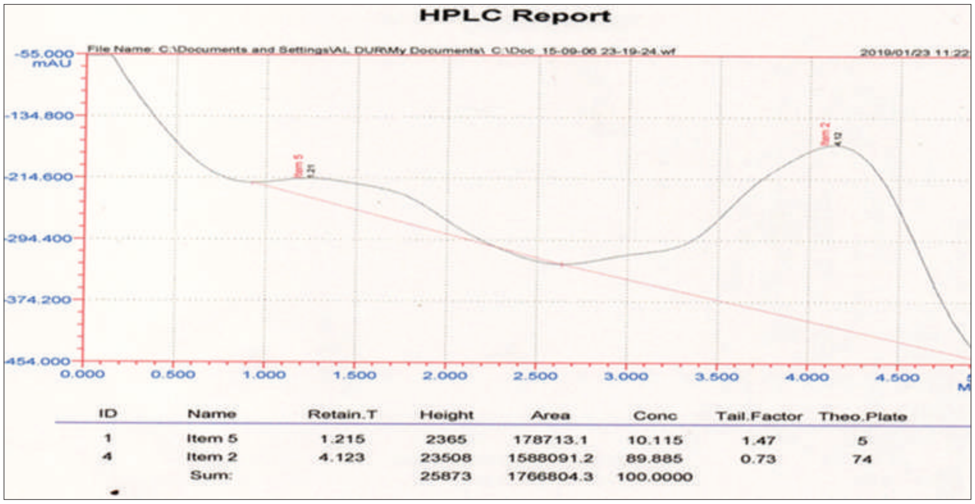


Figure 6: Chromatogram of thermal degradation

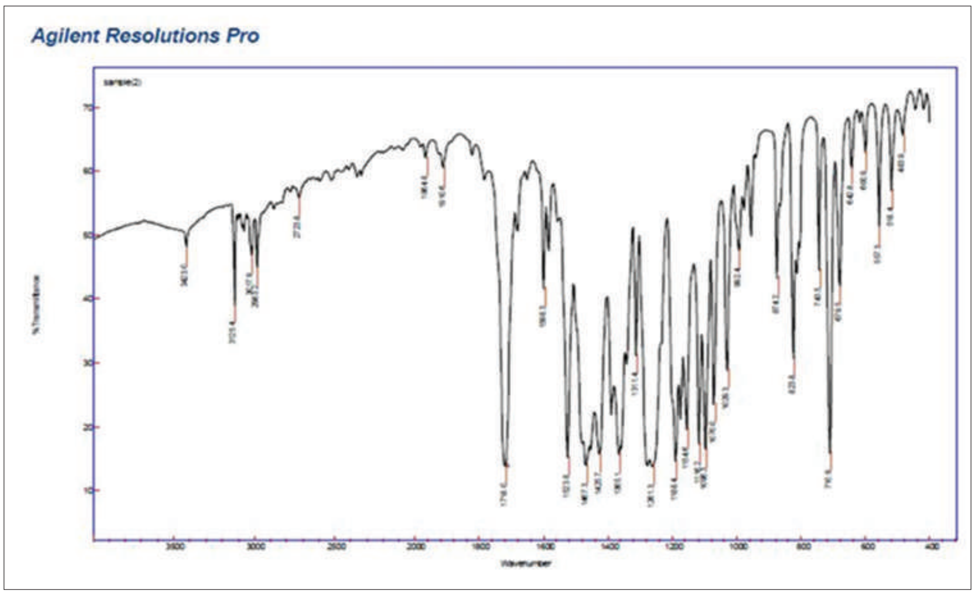


Figure 7: IR spectrum of metronidazole

to a 100 ml volumetric flask and made up to the mark with mobile phase. The infrared spectrum of the solution is then analyzed. The process of decomposition in this way leads to the partial disintegration of the metronidazole compound and the uncontrolled interference with pharmaceutical additives and this is evident in Figure 5, where the peaks of HPLC-UV appear irregular and sometimes overlapping.

Thermal degradation

For this, 60 mg from tablet powder of MET was taken in glass Petri dish and placed in hot air oven at 105°C for 2–3 h. After specified time, the tablet powder was transferred to a 100 ml volumetric flask and made up to the mark with mobile phase. Increasing the temperature of the metronidazole solution >100°C indicates that it is difficult to control the synthetic structure of the metronidazole and thus obtain complete thermal dissolution of the compound, this is shown in Figure 6.

Table 3: System suitability

Parameters	Value of metronidazole	Recommended limits
Retention time	4.815 (%RSD 0.532)	RSD ≈
Peak area	682,935.4 (%RSD 0.447)	RSD ≤ 2
USP plate count	1990	≥ 2000–2500
USP tailing factor	2.14	≤ 2–2.5
Resolution	1.5 min	≥ 3

INFRARED SPECTRUM OF METRONIDAZOLE

For Pure Metronidazole Powder

In the infrared spectrum of metronidazole [Figure 7, Table 2], stretching vibrations of –OH-associated group

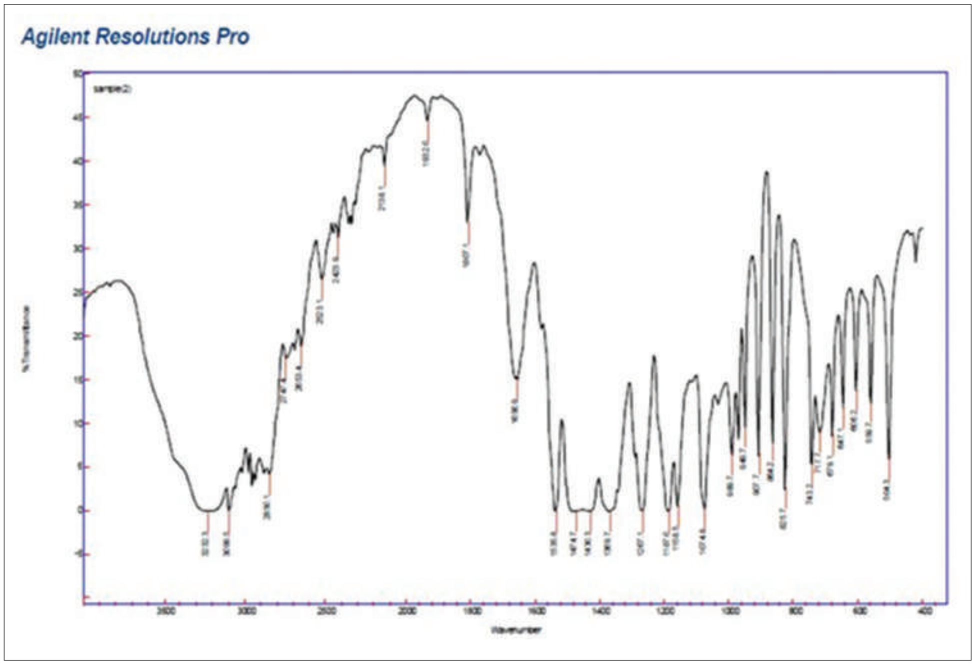


Figure 8: IR spectrum of metronidazole and its crud

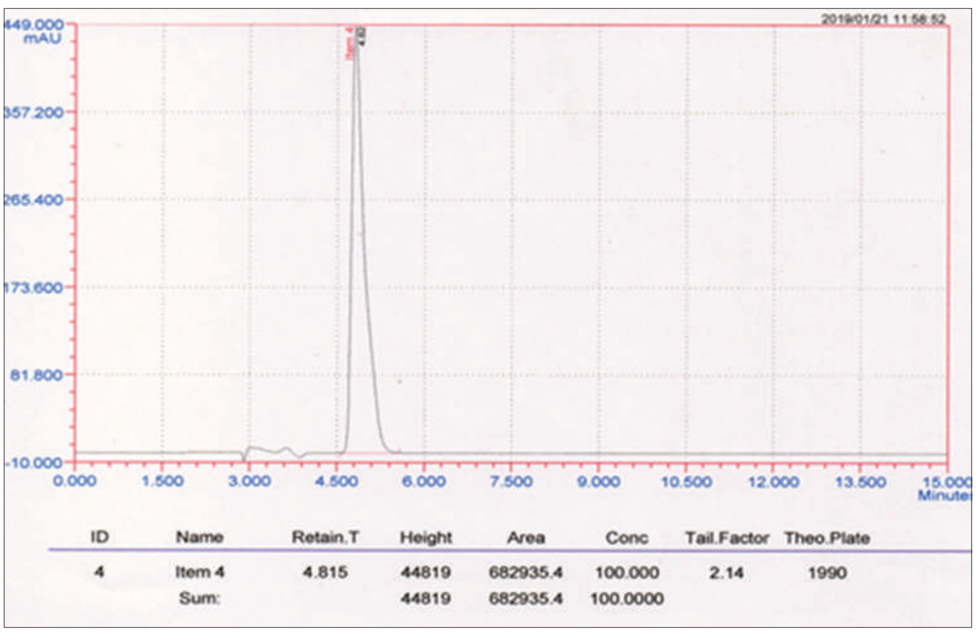


Figure 9: Chromatogram obtained after method optimization

were indicated by two absorption bands at 3220.85 cm^{-1} and 3100.79 cm^{-1}. Bonds –C=C– and –C=N– of imidazole cycle are characterized by fluctuations in the frequency range from 1700 cm^{-1} to 1500 cm^{-1}, but in the spectrum of pure metronidazole, they are pronounced not strongly.[41]

Metronidazole and its Crud

The characteristic absorption bands at 1535.59 cm^{-1} and 1368.90 cm^{-1} characterize stretching vibrations of nitro group, as is evidenced by the data in comparative, Table 2, the changes in significant fluctuations do not occur, so we can conclude that it is not involved in the formation of the bonds with the other materials in crud. The characteristic absorption bands at 1265.53 cm^{-1} and 1187.45 cm^{-1}, which correspond to –C=C– and –C=N– bonds in the structure of

Table 4: Results of method robustness

Parameter	Metronidazole (3 μg/mL)		
	Found (μg/mL)	%Recovery	%RSD
Analyst	3.0	100.0	0.301
Column	2.9	96.66	0.184
System	2.8	93.33	0.115

metronidazole, based on the data in Table 2, are stored in the test samples [Figure 8]

DISCUSSION OF THE RESULTS

The Optimization of HPLC conditions

The chromatographic conditions were developed to separate all the degradation products from the peaks of metronidazole. During the process of HPLC method optimization, several trials were taken using Ion Pac Arcus EP-C18; 5 µm, 4.5 mm × 250 mm, with the use of suitable organic phase, acetonitrile: triethylamine 30:70 (v/v) + 0.5 M potassium dihydrogen orthophosphate buffer at pH 4.5 and 1 ml/min flow rate. The wavelength was monitored at 310 nm. The retention time for metronidazole was 4.815 min. Good peak shape was observed of the new analytical method [Figure 9].

The System Suitability

Studies were carried out for the purpose of adapting the HPLC-UV system. The standard metronidazole (3 µg/mL) was used through three replicas of the same concentration that was replicated using the optimal method. Table 3 shows the system suitability. These results meet the requirements of separation method and metronidazole estimates in various pharmaceuticals.

The Validation of Method and Assay

In accordance with ICH guidelines, the new chromatographic method HPLC-UV and parameters such as specificity, linearity range and sensitivity, regression, precision, accuracy, and rigidity were used to validate the method used.[40] To assess the method validity, the effect experimental conditions on the peak areas of the analytes were examined. The validity of the method was checked at concentration of 3 µg/mL for metronidazole. Table 4 summarized all the results. The results revealed that the peak areas for the drugs were unaffected small changes in flow rate, composition of mobile phase, temperature, and detection wavelength, indicating significant validity of the method.

The Specificity[41]

The specificity of the proposed method was studied using the study of forced degradation. The analysis was performed to ensure that the proposed method was able to separate metronidazole from the potential degradation products generated during the study of forced degradation. Studies were performed using acid, base, oxidation, photolysis, and heat for the tablet sample at a concentration of 3 µg/ml of metronidazole. Table 5 shows the results of forced decomposition. Chromatograms shapes are shown in Figures 2-6. The highest percentage of deterioration occurred under the alkaline conditions of the drug. The lowest percentage of the degradation of metronidazole occurred in the case of thermal and in the case of photosynthesis. One peak degradation was observed in decomposition products.

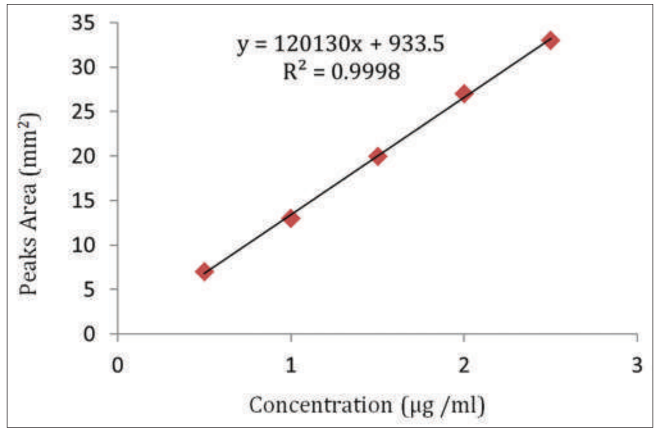


Figure 10: Linearity of the calibration curve

Table 5: Results of forced degradation studies

Type of degradation	Metronidazole (60 µg/mL)	
	%Recovery	%Degradation
Undegraded	100.02	0.000
Acid	98.459	1.541
Base	94.372	5.628
Oxidative	95.179	4.821
Photolytic	98.114	1.886
Thermal	98.882	1.118

Table 6: Regression statistics of the proposed method

R^2	0.9998
Standard error	0.3830
Standard error estimate	0.3651
Intercept	120130X
Slope	933.5
LLOD (µg/ml)	0.115
LLOQ (µg/ml)	0.437

Table 7: Summarized results of accuracy

Claimed conc. (µg mL–1)	Found conc. (µg mL–1)	Recovery±RSD
1	1	100±0.378
2	2	100±0.317
3	2.84	94.6±0.322
3.0 µg mL-1 for drugs (Flagyl Espagne-France® tablets)	2.98	99.3±0.359

Table 8: Results of precision studies

Claimed conc. (µg mL-1)	Intraday		Interday	
	Found (µg mL-1)	Recovery±RSD%	Found (µg/ml)	Recovery±RSD%
1	1	100±0.378	1	100±0.333
2	2	100±0.317	2.1	110±0.300
3	2.84	94.6±0.322	2.75	91.6±0.399
4	3.98	99.5±0.398	4	100±0.320
5	4.9	98.0±0.359	4.87	97.4±0.387
3.0 µg/ml drug (Flagyl Espagne-France® tablets)	2.98	99.3±0.359	2.9	96.6±0.338

Table 9: Assay of metronidazole in tablets

Analyte	Labeled claim (mg)	Found (mg)	Mean (mg)	%Recovery	%RSD
Negazole tablets	900	850	880	99.3	±0.359
	900	900			
	900	890			
METROSULE-500	650	650	651.66	102.5	±0.167
	650	645			
	650	660			

Other degradation products due to stress do not interfere with the detection of metronidazole, so the method can be considered as an indicator of stability.

The Linearity Range and Sensitivity[42,43]

Under the optimum experimental conditions, a linear relationship was established by plotting the peaks areas for drug against the drug concentration (µg/mL). The concentration range was found to be 1 µg/mL–5 µg/mL for metronidazole. The linear regression analysis of the data gave from the following equations:

$y = 120130x + 933.5$ ($R^2 = 0.9998$) for metronidazole

On the assumption that: y = peak area, x= concentration of the drug (µg/mL), and R^2 = Regression coefficient. The high values of regression coefficients with small intercept indicate the good linearity of the calibration curve that shows in Figure 10.

The Regression[44]

The sensitivity of the proposed method was assessed by calculating limit of quantitation (LLOQ) and limit of detection (LLOD). The LOD and LLOQ were calculated as follows:

LLOQ=10 × SD/S; LLOD= 3.3 × SD/S

Where, SD = standard deviation of the drug response and S = Slope of the calibration curve. LLOD values were found to be 0.115 µg/ml while LLOQ values were found to be 0.437 µg/ml. These values demonstrate the satisfactory sensitivity of the proposed method for the analysis of selected drug. Table 6 shows the results of regression statistics of the proposed method.

The Accuracy[45]

For the pre-analysis tablet sample solutions, a known amount of standard solution was added at three different levels, 10%, 20%, and 30%. The solutions were reanalyzed by the proposed method. The percentage recovery was between 98% and 100% with percentage RSD<0.4%. The results indicate good accuracy of the method. The selectivity of the method was demonstrated by the non-interference of the excipients with the analysis of the analytes. The results are summarized in Table 7.

The Precision[46]

The precision was established by analyzing metronidazole at a concentration of 3 µg/ml. The system precision was tested by applying the developed method for the determination of metronidazole in the pure standard metronidazole for three successive times ($n = 3$). The method precision was tested by repeated analysis of metronidazole in tablet sample for three successive times ($n = 3$). The results are summarized in Table 8. The percentage RSD values for system precision and method precision were ≤0.4%, indicating that the proposed method has good precision in the analysis of metronidazole.

THE APPLICATIONS OF METHOD

The analytical method of metronidazole and METROSULE-500 was assessed by examining commercially available tablets (Negazole tablets, Gulf Pharmaceutical Industries Limited, U.A.E., that claiming to contain 500 mg of metronidazole). The percentage of metronidazole was found where the values were 99.3 ± 0.359%, while the ratio of metronidazole in METROSULE-500 (Limited Ajanta) was found where the values were 102.5 ± 0.167%. This result indicating the values of percentage recovery and RSD% that the proposed method was accurate and precision in metronidazole analysis in dosages forms. Table 9 summarized the applications results.

CONCLUSION

This work described HPLC system (LC100 Angstrom advanced) equipped with a UV detector for metronidazole determination in two commercial pharmaceutical drugs. This developed method considered as simple, inexpensive and needs only a very small volume of the sample as well as used it is an ultraviolet detector makes this system very specific due to one peak in the chromatogram. In this application, there is no need for high sensitivity since the pharmaceutical drugs have a very low concentration. The method was validated as per the HPLC-UV guidelines and the developed method obeys Beer's law over the concentration range of 1.0–5.0 µg/mL for drugs.

Based on the results, this study divulges with important analytical method used to determine the presence of metronidazole in the dosage form. The developed and validated stability-indicating HPLC-UV method for the quantification of metronidazole is simple, accurate, precise, sensitive, specific, rugged, and robust. The proposed method can, thus, be applied for routine analysis of metronidazole in tablet dosage form.

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AUTHORS' CONTRIBUTIONS

This research was done individually in the laboratories of the College of Pharmacy, University of Basrah. This research was completed over a period of 3 months with serious and continuous work, and therefore, excellent results were obtained in finding an easy and sensitive method to estimate the metronidazole.

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