Original Article

Simultaneous RP-HPLC Estimation and Validation of Metronidazole, Furazolidone, and Dicyclomine in Capsule

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Abstract

For the first time, a new, simple, precise reversed-phase high-performance liquid chromatography method was developed for the simultaneous estimation of metronidazole, furazolidone, and dicyclomine hydrochloride in capsule dosage form. The method was performed with Thermo, C_8 (150 mm×4.6) column. The best separation was achieved by gradient elution with mobile phase of acetonitrile, water (40:60), and 20 mm phosphate buffer with 10% w/v sodium hydroxide (pH 7.5) with a detection wavelength of 215 nm. The separation was completed within 15 min of runtime. The retention time of metronidazole, furazolidone, and dicyclomine hydrochloride was found to be 1.79, 2.45, and 11.50 min, respectively. The proposed method was found to be linear. The method was statistically validated as per the ICH guidelines and shown to be simple, accurate, precise, linear, and reproducible in the range of 40.2–60, 40.2–60.4, and 3–5 µg/mL for metronidazole, furazolidone, and dicyclomine, respectively. For the first time, the developed method foretells the suitability of the method for the simultaneous estimation of three drugs in the commercially available dosage forms.

Keywords: *Dicyclomine hydrochloride, furazolidone, metronidazole, RP-HPLC, simultaneous estimation*

Introduction

Fumedil, a tripartite capsule, is commercially available with 10 mg of dicyclomine hydrochloride, 500 mg of metronidazole, and 50 mg of furazolidone. Dicyclomine hydrochloride[1,2] is chemically 2-(diethylamino)ethyl 1-cyclohexyl cyclohexane-1-carboxylate;hydrochloride. Metronidazole[3,4] is 2-(2-methyl-5-nitroimidazol-1-yl) ethanol, and furazolidone[5] is 3-[(E)-(5-nitrofuran-2-yl)methylidene amino]-1,3-oxazolidin-2-one [Figure 1]. The drug has a multitudinal usage and favorable profile in gastrointestinal tract (GIT) disorders and microbial infections such as bacteria and protozoa. Dicyclomine hydrochloride is a synthetic analog of acetylcholine endow with dual effect such as muscarinic blocker and local anesthetic effect.[6] As a consequence, it antagonizes muscarinic receptors on smooth muscle in the GIT, thereby preventing the actions of acetylcholine and reducing smooth muscle spasms in GIT, biliary, and urinary tract.[7-9] Metronidazole is a nitroimidazole antimicrobial that instigates DNA strand breaks, thereby inhibiting DNA synthesis and bacterial cell growth. Similarly, furazolidone is a nitrofuran antimicrobial agent used in the treatment of diarrhea or enteritis caused by bacteria or protozoan infections.[10-13] Furazolidone is also active in treating typhoid fever, cholera, and *Salmonella* infections. Its bactericidal activity is based on its interference with DNA replication and protein production. Furazolidone binds bacterial DNA which leads to the gradual inhibition of monoamine oxidase.[14,15]

The literature study shows good number of methods with single or dual combination of these drugs. Some of the determination methods include spectrophotometric determination,[16-18] UV[19] and reversed-phase high-performance liquid chromatography (HPLC),[20-25] quantitative analysis,[26] and electrochemical reduction using carbon electrode.[27]

However, literature reports revealed that there has been no precise reported HPLC/UV method for the analysis of three drug combinations. The aim of the present research study is to develop a HPLC method capable of separating and analyzing all three agents simultaneously in ACN/aqueous solution.[28,29] Our developed method was validated in accordance with the International Conference of Harmonization

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(ICH) Guidelines ICH Q2 (R1) and Association of Official Analytical Chemists[30-33] for specificity, linearity, limit of detection, limit of quantification, precision, accuracy, and stability.

Materials and Methods

Chemicals

Metranidazole, dicyclomine, and furazolidone were kindly supplied from ATOZ Pharmaceuticals Pvt. Ltd, Chennai. Fumedil capsules were procured from a local market. HPLC grade acetonitrile and potassium dihydrogen orthophosphate were obtained from Merck. Sodium hydroxide, methanol, and 0.45 µm disposable filter cartridge were obtained from Sigma. Milli-Q Water was prepared from Milli-Q® Integral 3 Water Purification System.

Instrumentation

HPLC measurements were carried out on a Waters e2695 Separations module coupled with 2998 PDA Detector. Analyte and internal standard (IS) were weighed using a Mettler Toledo XS104 analytical balance (Mettler-Toledo, LLC and Columbus, OH). UV absorbance spectrum was collected using a UV-Pharmaspec 1700 spectrophotometer (Shimadzu). pH was measured using a HI 2211 pH/ORP pH meter (Hanna). Ultrasonication and degassing of the solvent were done using a USC-100 Ultrasonicator (AMANM), and HSV-1 Vacuum Pump was used for the experiment. Glasswares and pipettes used were calibrated.

Analytical and chromatographic conditions

Chromatographic separation was achieved using Unisol C18 (250 mm×4.6 µm), Unisol C18 (150 mm×4.6 µm), and Thermo C8 (150 mm×4.6 µm) reversed-phase column using UV detection at 215 nm with a diode array detector. Initially, different mobile phases were tested with gradient elution [Table 1] to achieve efficient system suitability parameters and peak purity. Optimization of the mobile phase included several trials with various ratios of mobile phase and pumps. It was observed that peak tailing persisted till pH 7.0. So the mobile phase was set at a ratio of 40–60% and 70–30% in pumps A and B with a flow rate of 0–15 mL. At pH 7.5, the peak purity was better with optimal theoretical plates. The best peak separation was achieved with gradient elution. The separation was completed within 15 min of runtime. The retention time of metronidazole, furazolidone, and dicyclomine hydrochloride was found to be 1.79, 2.45 and 11.50 min, respectively [Figure 2].

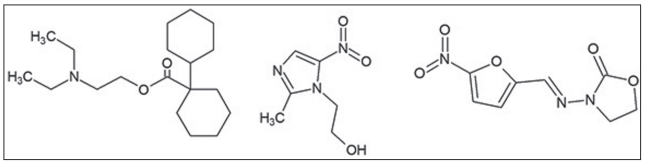

Figure 1: Structure of dicyclomine, metranidazole, and furazolidone

The mobile phase was prepared at the beginning of the day and filtered through a 0.45 µm disposable hydrophilic membrane filter cartridge. The mobile phase was pumped at gradient flow rates of 1, 1.5 and 2 mL/min, and the injection volume was 50 µL. The mixture of acetonitrile and water (40:60) was used as a diluent. The developed method was validated for specificity, linearity, limit of detection, limit of quantification, intra- and interday precision, accuracy, and stability based on the ICH guidelines.

Standard solution

In a 200 mL volumetric flask, 50 mg of furazolidone, 500 mg of metronidazole, and 10 mg of dicyclomine hydrochloride were weighed accurately and transferred. To this, 25 mL of mobile phase was added and sonicated for 10 min. When all the solids were dissolved, the compounds were diluted up to the mark with diluents.

Test preparation

Twenty capsules of Fumedil were weighed and the contents of the capsule were transferred. Empty shells were cleaned thoroughly and then weighed separately. The average net content of the capsule was by obtained subtracting this value from the weight of whole capsules. A quantity accurately equivalent to the net content was weighed and transferred into a 200 mL volumetric flask, and 25 mL of diluent was added to it. The contents were sonicated for 10 min, which ensured complete solubility. Then it was diluted up to the mark with diluents and filtered through a 0.45 µm filter.

Selection of wavelength

The solubility study was performed practically to confirm the solubility of metronidazole, furazolidone, and dicyclomine hydrochloride. Water, ethanol, and methanol were not suitable for all three components, whereas in acetonitrile all components were soluble. The peak resolution was significant in acetonitrile: water (40:60 % v/v), so it was chosen as a mobile phase for the development of the method.

A solution containing 10 µg/mL of metronidazole, furazolidone, and dicyclomine hydrochloride was prepared and used as a stock solution for the UV spectrum development. Peak maxima for metronidazole and furazolidone were

Table 1: Gradient flow table for optimized chromatographic conditions

Time (min)	Flow (mL)	A pump	B pump
0	1.0	40%	60%
4	1.0	40%	60%
6	1.5	70%	30%
7	2.0	70%	30%
12	2.0	70%	30%
15	2.0	40%	60%

Detection wavelength 215 nm, diluent: 50:50 (acetonitrile: water)

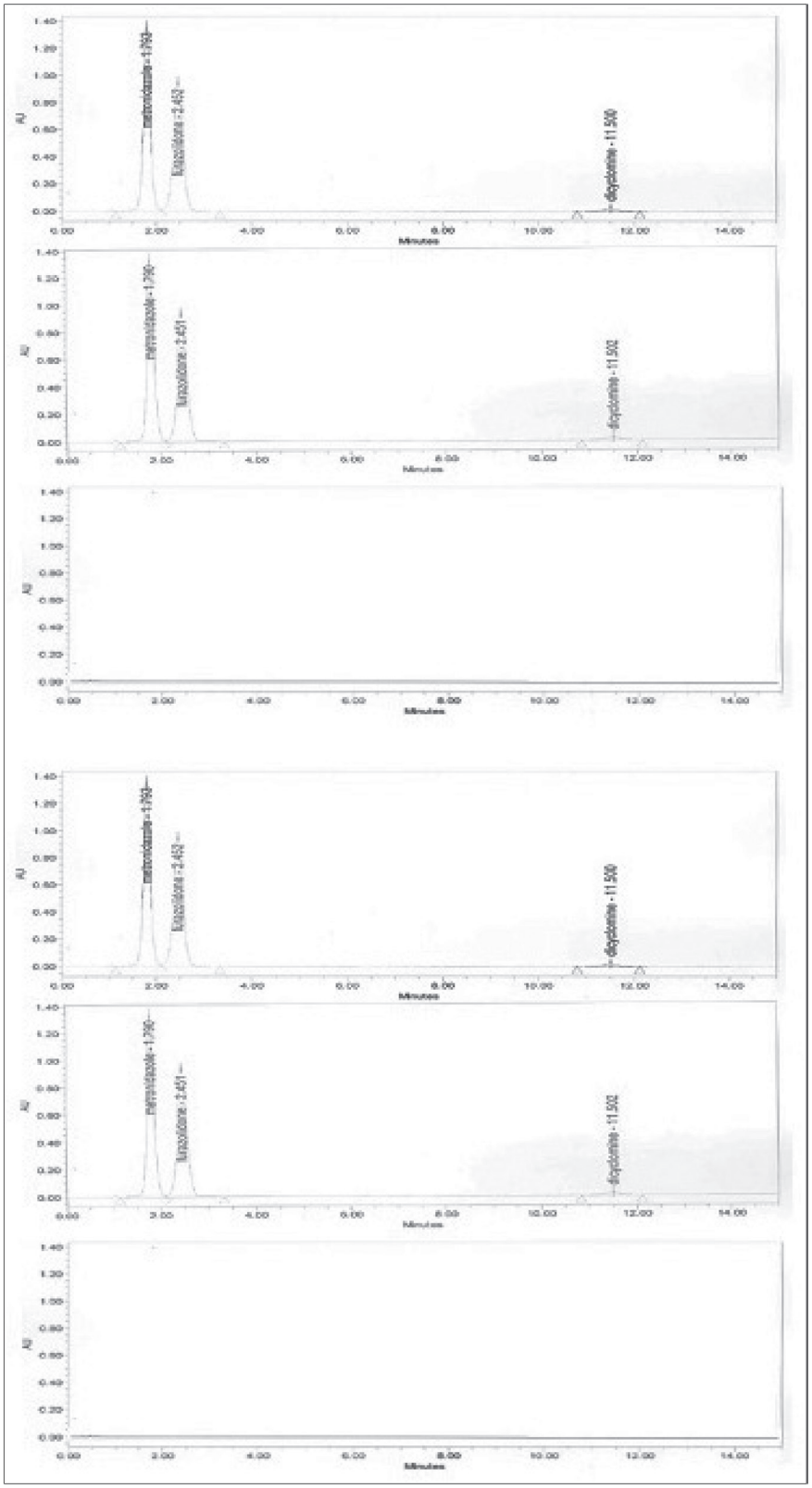

Figure 2: HPLC chromatograms. (a) Blank, standard solution of acetonitrile: water (40:60% v/v) and 20 mm phosphate buffer with 10% w/v sodium hydroxide (pH 7.5). (b) Spiked standards contain 2500 µg of metronidazole, 2500 µg of furazolidone, and 50 µg of dicyclomine in mobile phase. (c) Real sample extracted from the capsule acetonitrile: water (40:60% v/v)

observed at 317 and 364 nm. Because of no peak maxima for dicyclomine hydrochloride, the wavelength selection was a challenging task in this development work. Metronidazole and furazolidone have higher wavelength peak maxima, so the samples were scanned in the wavelength range of 190–400 nm. The middle wavelength of 215 nm was considered to be ideal for all. Furthermore, there was no impact on metronidazole and furazolidone responses, so the λ_{max} was set at 215.

Results

Method validation and optimized chromatographic techniques

The developed method was validated for the parameters such as linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), precision, specificity, and robustness, as recommended by the ICH and the Association of Official Analytical Chemists.

System suitability

System performance such as the number of theoretical plates (*N*), tailing factor, resolution (*R*), retention time (RT), and percentage relative standard deviation (%RSD) was determined for standard replicate injections of the selected drugs [Table 2].

By using the optimized condition, specificity, i.e., the ability of a method to discriminate between the analytes of interest and other components present in the sample, was performed with blank and placebo. It was shown that the blank and placebo do not interfere with the retention times of the selected drugs and hence the method is specific.

Accuracy

The accuracy of the method was confirmed by recovery analysis using a standard addition method. To the placebo, known quantities of pure drug solutions were spiked at three different concentrations ranging from 80%, 100%, to 120% levels [Table 3]. The percentage recovery of 100.12 ± 1.02% was observed for the spiked samples, regardless of the placebo addition.

Precision

The precision of the method was evaluated by intermediate precision where the interday, intraday, and analyst variations were assessed. The intraday precision was evaluated by analyzing six replicates of the calibration standards for the samples on the same day. In the meantime, the intraday precision was assessed by running calibration standards of the sample on three different days. Similarly, analyst-to-analyst precision was also performed by preparing and applying six different concentrations. The precision value was expressed as percentage RSD [Table 4].

Linearity

The linearity of the method was evaluated with several aliquots of the standard solutions of metronidazole, furazolidone, and dicyclomine at the 80–120% level. The analytical curve was

Table 2: System suitability results

Parameter	Drugs		
	Dicyclomine	Metronidazole	Furazolidone
Retention time (min)	11.50	1.79	2.45
Theoretical plates	10853	5948	8596
Tailing factor	1.09	0.93	0.84
Resolution	0.18	0.74	0.55
RSD of peak area	1.31	0.18	2.11
RSD of retention time	0.98	0.66	0.76

Table 3: Accuracy results of RP-HPLC method

Parameter	Amount taken (µg)	Amount recovered (µg)	% Recovery	Mean recovery	%RSD
Metronidazole					
80%	2.04	2.03	99.50	99.58	0.08
100%	2.52	2.51	99.60		
120%	3.00	2.99	99.66		
Furazolidone					
80%	0.204	0.204	100	99.75	0.22
100%	0.252	0.251	99.60		
120%	0.300	0.299	99.66		
Dicyclomine					
80%	0.04	0.04	100	99.94	0.10
100%	0.10	0.10	100		
120%	0.60	0.599	99.83		

prepared by plotting concentration versus corresponding mean peak area. The equation was obtained using the least-squares regression procedure.

The method was found to be linear for all three drugs (metranidazole, furazolidone, and dicyclomine) in the concentration ranges of 40.2–60, 40.2–60.4, and 3–5 µg/mL with the correlation coefficient of 0.99 [Figure 3].

Limit of detection and limit of quantification

Based on the standard deviation of the response and the slope, the detection limit and quantification were calculated. The detection limit was found to be 0.35, 0.39, and 0.37 µg/mL for metronidazole, furazolidone, and dicyclomine, respectively. The quantification limit was found to be 0.102, 0.103, and 0.105 µg/mL for metronidazole, furazolidone and dicyclomine, respectively.

Robustness and ruggedness

Robustness was tested by making small deliberate changes in the chromatographic conditions such as flow rate, pH, mobile phase composition, and column temperature at 100% concentration.

The ruggedness of the proposed analytical method was performed under different conditions such as different columns, analyst, instrument, and laboratory analysis of the same sample. It was observed that there were no significant changes in the resolution, so the method developed is robust and ruggedness was found to be good.

Discussion

Selection of λ_{max}

Because of no peak maxima for dicyclomine hydrochloride, the wavelength selection was a crucial point in this development work. Salfia and Fatima[19] reported λ_{max} of metronidazole at 340 nm using water as a solvent. From the solubility studies, it was observed that all three components were freely soluble in acetonitrile. The UV spectrum of metronidazole showed a λ_{max} at 317 nm and that of furazolidone showed λ_{max} at 364 nm. The simultaneous estimation of a solution containing 10 µg/mL of metronidazole, furazolidone, and dicyclomine hydrochloride was prepared and scanned in the UV region from 190 to 400 nm. Metronidazole and furazolidone had a higher wavelength peak maximum, and the middle wavelength of 215 nm was considered to be ideal for all. Furthermore, there was no impact on metronidazole and furazolidone responses, so the λ_{max} was set at 215 nm.

Method validation

After optimizing chromatographic conditions, system suitability and specificity were performed. The blank and placebo did not interfere with the retention times of the selected drugs, and also absence of overlapping peaks in

Table 4: Results of the precision study of dicyclomine, metronidazole, and furazolidone

Parameter	Results		
	Dicyclomine	Metronidazole	Furazolidone
Interday			
Mean %RSD of retention time	0.02	0.05	0.12
Mean %RSD of peak area	0.25	0.25	0.34
Mean % assay	100.22	100.03	99.93
Intraday			
Mean %RSD of retention time	0.04	0.07	0.16
Mean %RSD of peak area	0.18	0.72	0.64
Mean % assay	100.62	99.99	99.98
Analyst-to-analyst			
Mean %RSD of retention time	0.18	0.09	0.06
Mean % RSD of peak area	0.29	0.81	0.22
Mean % assay	99.98	99.52	100.04

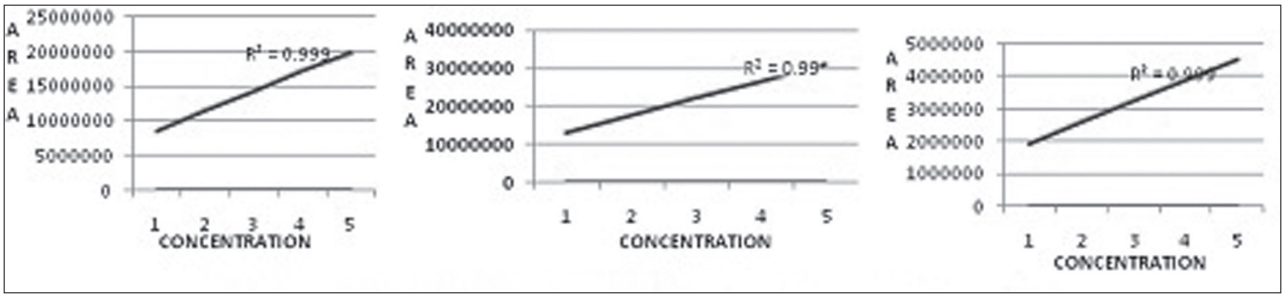

Figure 3: Linearity graph of metronidazole, dicyclomine, and furazolidone

the spectrum was observed. The capsule containing 500 mg of metronidazole, 50 mg of furazolidone, and 10 mg of dicyclomine was analyzed for the amount of label claim. The percentage label claim of the formulation was found to be in the ranges of 500.63–501.25 mg for metronidazole, 50.01–50.12 mg for furazolidone, and 10.00–10.06 mg for dicyclomine hydrochloride. The amount present in the formulation was in good agreement as per the guidelines with the label claim. Satish Kumar *et al*. reported the retention time of 3.440 min for metronidazole and 4.136 min for furazolidone, respectively, with the mobile phase of acetonitrile: methanol: phosphate buffer 10:40:50. But in the present study the retention time of metronidazole and furazolidone is only 1.79 and 2.45 min, which is considerably lower than the reported literature [Figure 2]. Moreover, the percentage RSD of RT and area and RSD of assay preparation were within acceptance limits of ICH guidelines. The linearity data showed an excellent correlation between the concentration of the drugs and the peak area. It also obeyed the limits as per the guidelines. The reported HPLC methods indicated some disadvantages such as too long runtime and also lengthy retention times of 8.1 and 4.2 min for metronidazole and furazolidone,[15] whereas the present research study showed very short retention time with the 14 min runtime. In addition, the recovery analysis studies in accuracy determination prove the supremacy of the proposed method. To the placebo, known quantities of stock solutions spiked at three different concentrations ranging from 80% to 120%. The recovery percentages were as follows: metronidazole min: 99.58%, max: 100.21%; furazolidone min: 99.75%, max: 100.12%; and dicyclomine hydrochloride min: 98.94%, max: 99.85%. The robustness of an analytical method was unaffected by deliberate change in the flow rate, pH, mobile phase composition and column temperature and was performed at 100% concentration. The ruggedness of the proposed analytical method was performed in different conditions such as different columns, analyst, instrument, and laboratories analysis of the same sample. It was observed that there were no changes in the parameters that the method developed is robust and rugged. As per the ICH guidelines, this ascertains that the developed method is unaffected by the variations in method parameters.

Based on the standard deviation of the response, the detection limit and quantification limit were calculated. The detection limit (LOD) was 0.35, 0.39, and 0.37 µg/mL and quantification limit (LOQ) was 0.102, 0.103, and 0.105 µg/mL for metronidazole, furazolidone, and dicyclomine, respectively.

Overall, the present developed method is the original firstborn method for the commercial validation of Fumedil capsules. Additionally, the work has exposed a simple mobile phase and stability for the sample throughout the validation. The retention time of the developed method for metranidazole, furazolidone, and dicyclomine was found to be better besides significant LOD and LOQ. It is obvious from the results that the proposed method can be applied readily for this drug combination with acceptable accuracy and precision. It was also noticed that there were no noticeable changes in the chromatogram when it was rugged and robust. This explores that the proposed method is well suitable for smaller sample size quantification and quantitation.

Conclusion

The developed and validated RP-HPLC with gradient flow was found to be specific, accurate, precise, reproducible, and time-saving. The above results showed that the method well agreed within the acceptance limits of guidelines. It will be virtually a valuable method for the commercial regular analysis and also for the quantification of metronidazole, furazolidone, and dicyclomine in capsule dosage form.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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