Original Article

Simultaneous Determination of Some 5-Nitroimidazole Derivatives and Ciprofloxacin by High Performance Liquid Chromatography

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ABSTRACT

Objective: An isocratic high performance liquid chromatographic method for simultaneous determination of Metronidazole, Tinidazole and Ciprofloxacin was developed. **Method:** Separation was achieved with a C_{18} (250x4.6 mm, 5 μm) column. Optimal chromatographic conditions were: mobile phase consisting of acetonitrile: 0.3% o-phosphoric acid modified with 0.1% triethylamine (20:80 v/v) at a flow rate of 0.7 mL min^{-1}, at 30°C and wavelength of 300 nm. **Results:** The retention times were 4.92 min for Metronidazole, 8.78 min for Tinidazole and 9.76 min for Ciprofloxacin. The responses were linear (R^2 = 0.9999) in the range of 12.5 – 100 μg mL^{-1}. The limits of detection were 0.012 μg mL^{-1} for Metronidazole and Tinidazole and 0.04 μg mL^{-1} for Ciprofloxacin. The limits of quantification were 0.125 μg mL^{-1} for Metronidazole and Tinidazole and 0.4 μg mL^{-1} for Ciprofloxacin. The recovery (%) was within the range of 99.57 – 100.3% for all analytes. **Conclusion:** The method developed can successfully be used for routine analysis and quality control of Metronidazole, Tinidazole and Ciprofloxacin.

Key words: Metronidazole, Tinidazole, Ciprofloxacin, RP-HPLC, Validation, Quality control.

INTRODUCTION

5-Nitroimidazole derivatives are antiprotozoal and antibacterial agents, the most common of which is Metronidazole (MET), chemically known as 2-(2-methyl-5-nitro-imidazol-1-yl) ethanol. It is widely used in clinical practice against a wide range of anaerobic microbes (from protozoa - *Giardia lamblia, Trichomononas vaginalis*, and *Entamoeba histolytica* to bacteria - *Helicobacter pylori, Clostridium difficile*, and *Bacteroides fragilis*).[1-2] Tinidazole (TIN), chemically known as 1-(2-ethylsulfonylethyl)-2-methyl-5-nitroimidazole is another representative of 5-nitroimidazoles, which has the same antimicrobial activity and a similar use as MET. In addition it has been used in regimens for the eradication of *Helicobacter pylori* in peptic ulcer disease.[3]

Ciprofloxacin (CIP) (1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(1-piperazinyl) - 3-quinolinecarboxylic acid) is a broad spectrum antibacterial agent of the fluoroquinolones group. It is active against bacteria causing respiratory, urinary tract, gastrointestinal and abdominal infections.[4] CIP has low toxicity and that's why it is has been widely used in practice. Despite its wide range action, CIP has decreased activity against anaerobic microorganisms. Therefore, a combination of CIP and an agent, active against anaerobes can be used to treat mixed aerobic / anaerobic infections.[5-6]

Combination of CIP and MET in a single dosage form is active against a broad spectrum of obligate anaerobic bacteria.[7] Combinations of CIP and TIN are also used, for example

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in treatment of chronic refractory pouchitis[8] and pelvic inflammatory disease.[9]

Various analytical techniques, such as high performance liquid chromatography,[10-11] thin layer chromatography,[11] spectral techniques (ultraviolet spectrophotometry,[12] spectrofluorimetry[13] and infrared spectroscopy[14]) and electrochemical techniques[15] have been used for determination of MET, TIN and CIP alone or in combination in different matrices.

The development of methods for simultaneous determination of the drugs used in the combination therapy of various diseases is an important analytical task, both in terms of quality control, as well as in therapeutic drug monitoring and drug safety. The presence of such methods would save time and resources for carrying out the analysis.

To the best of the autors knowledge there are no publications describing methods for simultaneous determination of MET, TIN and CIP, so the aim of this work was to develop a simple, sensitive, precise and accurate HPLC method for analysis of the above mentioned drugs.

MATERIALS AND METHODS

Chemicals and reagents

MET, TIN and CIP were purchased from Sigma-Aldrich (Germany) as standards. LC-grade acetonitrile was supplied from Merck (Germany). All other chemical reagents were of analytical grade.

Instrumentation and chromatographic conditions

Chromatographic separation was performed on modular HPLC system Shimadzu (Japan) equipped with LC-20AD quaternary pump with an auto sampler, Shimadzu DGU-20A$_5$ vacuum degasser and a Shimadzu SPD-20A UV/VIS detector. The data was recorded using Lab Solutions Software. A LiChrosorb C_{18} (250 mm x 4.6 mm, 5 µm) column was used as a stationary phase. Isocratic separation was performed with a mobile phase consisting of 20 volumes acetonitrile and 80 volumes 0.3% o-phosphoric acid (modified with 0.1% triethylamine) at a flow rate of 0.7 mL min^{-1}. The analysis was carried out at temperature of 30°C and injection volume was 20 µL. The UV detector was set at 300 nm.

Preparation of reference solutions

Accurately weighed amounts of MET, TIN and CIP were dissolved in solvent A (0.3% o-phosphoric acid solution: acetonitrile (80:20 v/v)) in a suitable volumetric flask, so the final concentration of working solutions to be 0.05 mg mL^{-1} for MET, TIN and CIP respectively.

Sample preparation

An amount equivalent to 250 mg of MET, TIN, CIP (after determination of the average mass of the tablets) was transferred into a suitable volumetric flask. A portion of solvent A was added and the sample was placed in an ultrasonic bath for 10 minutes. The volume was adjusted with solvent A and the sample was filtrated. An aliquot of the filtrate was suitably diluted to give a final concentration of 50 µg mL^{-1} of MET, TIN and CIP.

Validation procedure

The analytical method developed was validated in accordance to International Conference on Harmonization (ICH) guidelines.[16]

Selectivity

The ability of an analytical method to unequivocally assess the analyte in the presence of other components can be demonstrated by evaluating specificity. The specificity of the method was evaluated by assessing interference from excipients in the pharmaceutical dosage form prepared as a placebo solution.

Linearity

The linearity of the method was determined with five standard solutions in a concentration range of 12.5 to 100 µg mL-1 for MET, TIN and CIP. Calibration curves were constructed by peak areas against test substance concentrations. Each response was the average of three determinations.

Precision

The intraday precision was determined by six successive injections of sample at the 100% of claimed concentration of the tested substances MET, TIN and CIP (50 µg mL^{-1}). The inter day precision was determined by six replicate injections of sample in three different days at the same concentration levels and at the same experimental conditions.

Accuracy

The accuracy of the method was calculated by recovery studies. It is carried out by preparing the samples of 50%, 100% and 150% of target concentration. The samples were injected in triplicate for each concentration level. Recovery (*R* / %) and relative standard deviation (*RSD* / %), were calculated for each concentration level.

Detection (LOD) and quantification (LOQ) limits

The limit of detection (*LOD*) and limit of quantification (*LOQ*) were defined as the minimum concentration at which the analytes can be detected and quantified,

respectively. *LOD* and *LOQ* were determined by the signal-to-noise ratio.

Stability

The stability of working solutions was checked by re-injection of the samples at the day of experiment and after 24 h storage at room temperature in laboratory conditions.

Robustness

ICH defines the robustness of an analytical procedure as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. Robustness was determined by changing the flow rate (to 0.5 and 1 mL min^{-1}) and the concentration of acetonitrile in the mobile phase (to 18 and 22%).

RESULTS AND DISCUSSION

At the method development stage, an aqueous solutions, containing phosphoric acid and methanol or acetonitrile in different volumetric ratios were initially tried as mobile phases. The role of the organic modifiers such as triethylamine (0.1-3%) on retention behaviour of the analytes was also studied. Best results regarding resolution, peak shape and run time were achieved with mobile phase consisting of acetonitrile: 0.3 % phosphoric acid modified with 0.1% triethylamine (20:80 v/v). Thus, with avoiding the use of buffers, the mobile phase preparation was less time consuming and ensures trouble-free exploitation of HPLC system. Using this mobile phase, the total run time was around 11 min, which is comparable with published HPLC results for MET and CIP.[11] The respective retention times for MET, TIN and CIP were about 4, 8 and 9 min with good peak shapes, which are two-three times longer for MET and CIP when compare with UPLC.[10] But even at these optimal conditions the resolution between TIN and CIP was not satisfactory. Varying the column temperature (25°C-35°C) did not lead to significant changes in retention time and resolution regarding TIN and CIP. Changes in the flow rate were the next step in the chromatographic conditions optimization. Decreasing the flow rate from 1 mL min^{-1} to 0.7 mL min^{-1} gives the satisfactory resolution for peaks of TIN and CIP. The optimum wavelength for detection was set at 300 nm at which detector responses obtained were much better for all drugs. Thus, the selected optimal conditions for separation of MET, TIN and CIP were as follows: mobile phase consisting of acetonitrile:0.3 % phosphoric acid modified with 0.1% triethylamine (20:80 v/v) at a flow rate of 0.7 mL min^{-1}, at 30°C and optimal wavelength of 300 nm. The retention times were 4.92 min for MET, 8.78 min for TIN and 9.76 min for CIP.

Validation of the method

Selectivity

Experiments have shown that excipients do not interfere with the determination of the analytes, and that under optimal chromatographic conditions all compounds are completely separated from one another (Figure 1 and Figure 2). This indicated that the method is selective and can be used for identification and simultaneous quantification of MET, TIN and CIP.

The chromatographic parameters for all three drugs determined under optimal conditions were satisfactory and are presented in Table 1.

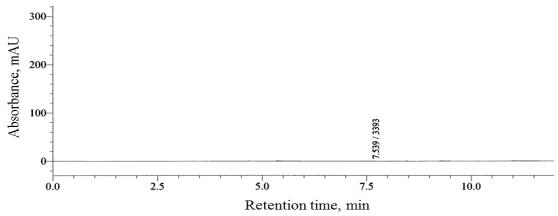

Figure 1: Chromatogram of placebo

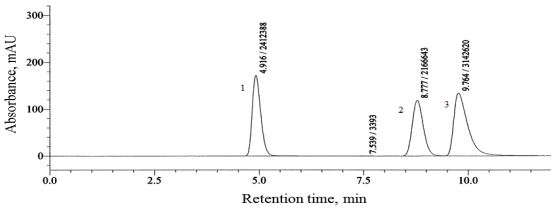

Figure 2: Typical chromatogram of MET, TIN and CIP 1-MET (retention time 4.92 time), 2-TIN (retention time 8.78 min); 3-CIP (retention time 9.76 min).

Table 1: Chromatographic data for HPLC method (system-suitability test).

Parameter	MET	TIN	CIP
Retention time, min	4.92	8.78	9.76
Tailing factor	1.34	1.22	1.98
Theoretical plates	2779	5249	4194
Resolution factor	3.20	2.35	1.80
Retention factor	1.21	1.57	1.85

Table 2: Validation data for the calibration plots.

Concentration level, %	Concentration µg mL^{-1}	MET Area[a]	TIN Area[a]	CIP Area[a]
25	12.5	611203	550043	787908
50	25.0	1232265	1105377	1589520
100	50.0	2416803	2166643	3157338
150	75.0	3657483	3238470	4775103
200	100.0	4855361	4315802	6357585
Slope	-	48496.7	43060.9	63615.2
Intercept	-	8546.1	12139.7	-5254.8
r^2	-	0.9999	0.9999	0.9999

[a]Average of three determinations

Table 3: Precision of the method.

Amount taken, mg tablet^{-1}	Amount found, mg tablet^{-1}		
	MET	TIN	CIP
250.0	249.1	248.9	249.4
	249.6	249.3	248.4
	250.3	249.6	249.2
	250.2	250.1	250.1
	249.2	249.4	249.6
	249.4	249.7	249.3
Mean	249.6	249.5	249.3
±SD	0.509	0.405	0.557
±RSD / %	0.204	0.162	0.224

Calibration and linearity

A series of 5 calibration solutions in a concentration range 12.5-100 µg mL^{-1} were prepared. An excellent correlation was observed between the peak areas and concentration of drugs of interest. Correlation coefficients were found to be 0.9999 for all three drugs. Calibration plot data - peak area (average from three determinations), slope, intercept, and correlation coefficient (*r2*) are listed in Table 2.

Precision

The values of *RSD* / % (Table 3) for MET, TIN and CIP were found to be in the range from 0.162 % to 0.224 % indicating good repeatability and reproducibility of the analytical procedure.

Accuracy

The accuracy of the method was calculated by recovery studies. It is carried out by preparing the samples of 50%, 100% and 150% of target concentration. Each concentration level was injected three times. The accuracy was expressed as the percentage of analytes recovered by the assay. The recovery *R* / % and *RSD* /% for all analytes were within the ranges of 99.57 – 100.3% and 0.174– 1.094% respectively. The results listed in Table 4 shows good accuracy and indicate that the proposed method is suitable for the quantitative determination of MET, TIN and CIP.

Limit of detection and limit of quantification

The limit of quantification (*LOQ*) and limit of detection (*LOD*) were evaluated based on signal-to-noise ratios by serial dilution of working reference solution. The *LOQs* for MET, TIN and CIP were found to be 0.125 µg mL^{-1}, 0.125 µg mL^{-1} and 0.4 µg mL^{-1} respectively;

Table 4: Recovery studies of MET, TIN and CIP.

Compound	Concentration level, %	Amount taken, µg mL^{-1}	Amount found, µg mL^{-1}	R / %	±SD[a] / %	RSD / %
MET	50	125	125.3	100.2	0.189	0.189
	100	250	250.6	100.3	0.173	0.174
	150	375	374.8	99.81	0.318	0.319
TIN	50	125	124.5	99.57	0.441	0.442
	100	250	249.1	99.65	0.507	0.509
	150	375	375.1	100.1	0.274	0.275
CIP	50	125	124.6	99.65	0.631	0.634
	100	250	249.9	99.97	0.556	0.557
	150	375	374.4	99.84	1.092	1.094

[a]Average of three determinations

the *LODs* – 0.012 μg mL^{-1}, 0.012 μg mL^{-1} and 0.04 μg mL^{-1} respectively.

Stability

It was found that the prepared samples are stable for 24 h, which was sufficient to perform and complete all measurements. No significant differences in analytes behavior was found.

Robustness

No significant differences were found in the retention time of MET, TIN and CIP when the composition and flow rate of the mobile phase were changed.

CONCLUSION

The validated RP-HPLC method developed here proved to be simple, specific, accurate, precise and sensitive. It can be used successfully for routine analysis and quality control of MET, TIN and CIP in pharmaceutical preparations. As prospect for future work, it would be interesting to track the performance of the method for determination of MET, TIN, and CIP in biological samples.

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CONFLICT OF INTEREST

The authors declare no conflic of interest.

ABBREVIATIONS

MET: Metronidazole, **TIN:** Tinidazole, **CIP:** Ciprofloxacin, **HPLC:** High Performance Liquid Chromatography, **UV:** ultraviolet, **ICH:** International Conference on Harmonization, **LOQ:** Limit of Quantitation, **LOD:** Limit of Detection, **RSD:** Relative Standard Deviation.

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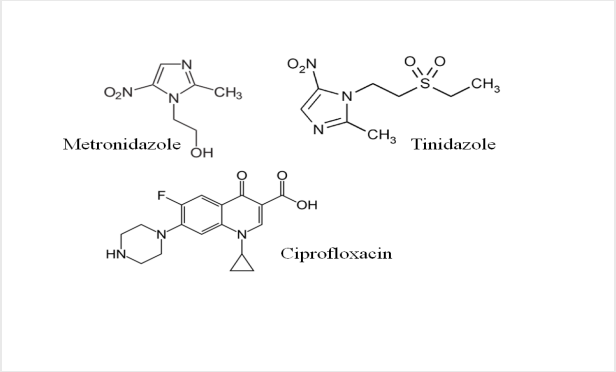
SUMMARY

- An isocratic high performance liquid chromatographic method for simultaneous determination of Metronidazole, Tinidazole and Ciprofloxacin was developed.
- A mixture of acetonitrile: 0.3% o-phosphoric acid modified with 0.1% triethylamine (20:80 v/v) was used as mobile phase.
- The retention times were 4.92 min for Metronidazole, 8.78 min for Tinidazole and 9.76 min for Ciprofloxacin.
- The developed method was validated as per ICH guidelines.
- The method developed can successfully be used for routine analysis and quality control of Metronidazole, Tinidazole and Ciprofloxacin in bulk and tablets.

PICTORIAL ABSTRACT



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