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VALIDATION STUDY OF THE HPLC ASSAY OF SOME ANTIAMOEBIC AGENTS

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ABSTRACT

A simple reversed phase HPLC method has been developed for the assay of tinidazole (TZ) and metronidazole (MZ) in their pure form and in formulations. Methanol, water and acetic acid in the ratio 65 : 33 : 2 was used as mobile phase with flow rate of 1 mL per min. LiChrospher C18-5 µm (25 x 0.46 cm) column was used and UV detection was made at 268 nm. The internal standard method using sparfloxacin (SP) as the internal standard is used. The linear dynamic range was found to be 0.08 - 9.5 µg mL^{1} and 0.07 - 8.5 µg mL^{-1} for metronidazole and tinidazole, respectively. The proposed method is successively employed for the determination of these drugs in tablets and injections. The excipients present in the formulations did not interfere with the assay procedure. Proposed method was found to be simple, sensitive, reproducible, rugged and accurate. Analytical parameters were calculated and full statistical evaluation is included.

Key words: RP-HPLC, Tinidazole, Metronidazole, Sparfloxacin, Pharmaceutical formulations.

INTRODUCTION

5-Nitroimidazoles such as tinidazole and metronidazole are extensively used as antiamoebic drugs. They are used in the treatment of trichomoniasis and giardiasis. Metronidazole is the drug of choice for the treatment of dracunculus infestations.[1] Official methods of Indian Pharmacopoeia[2] and British Pharmacopoeia[3] describes non-aqueous perchloric acid titration and United States Pharmacopoeia[4] describes HPLC methods for the assay of these drugs. Other analytical methods reported for the assay of tinidazole and metronidazole include GLC[5,6], spectrophotometry[7-11], volumetry[12] and gravimetry[13]. Few HPLC methods are also reported for the assay of these drugs. Some of these are found to be less sensitive[14-17], involves narrow detection limits[18,19] and most of these methods use mobile phases which are critical of pH.

In continuation of our HPLC work[20] in developing new methods for the assay of medicinal agents, we have successfully developed a sensitive RP-HPLC method for the assay of tinidazole and metronidazole. Present method is simple, precise and rugged. Also, method uses simple mobile phase without the need of buffer, involves no complex procedure to prepare sample solutions, and offers better sensitivity than the reported methods. Also, present assay procedure employs internal standard method with sparfloxacin as an internal standard. It is interesting to note that, sparfloxacin, itself an important anti-bacterial drug, can effectively be used as internal standard for the analysis of tinidazole and metronidazole.

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EXPERIMENTAL

Instrumentation and reagents

A Merck-Hitachi 7000 series low pressure gradient HPLC system equipped with a L7100 gradient pump, L7612 solvent degasser, a Rheodyne model 7125 injection valve with a fixed loop of 20 μL and Merck-Hitachi L7400 multiwavelength UV-Vis detector was used. The analyte peaks were resolved on a LiChrospher C18-5 μm (25 x 0.46 cm) column. A computer based Winchrom chromatographic software conditions was used for data integration.

Tinidazole and metronidazole were obtained as gift samples from Sarabhai Pharmaceuticals, India and sparfloxacin was obtained as gift sample from Sun Pharmaceuticals, Bombay, India. Methanol and water used for mobile phase were HPLC grade obtained from E.Merck India limited. Acetic acid (1 : 1) used was analytical reagent grade. Triply distilled water was employed for all other purposes.

Preparation of standard solutions

Standard stock solutions of 1 mg mL^{-1} of tinidazole, metronidazole and sparfloxacin were prepared using a mixture of methanol and water (1 : 1 v/v) in separate volumetric flasks. From this standard stock solution, mixed standard (working standard) solutions were prepared by suitable dilution with the mobile phase to contain 0.01-12 μg mL^{-1} of tinidazole and metronidazole and 10 μg mL^{-1} of sparfloxacin as internal standard in different 10 mL volumetric flasks. Before subjected to analysis all the working standard solutions were filtered through 0.45 μ filter and degassed.

Preparation of sample solutions

For tablets: Five tablets were weighed accurately and powdered well. The fine powder equivalent to 100 mg of tinidazole and metronidazole was dissolved in 100 mL volumetric flask using methanol and water (1 : 1 v/v). From this sample stock solution mixed solutions of working range were prepared by further dilution with the mobile phase to contain 0.01-12 μg mL^{-1} of tinidazole and metronidazole and 10 μg mL^{-1} of sparfloxacin as internal standard in different 10 mL volumetric flasks. Before subjected to analysis all the working standard solutions were filtered through 0.45 μ filter and degassed.

For injections: Suitable volume of the injections was diluted appropriately with the methanol and water (1 : 1 v/v) to contain 1 μg mL^{-1} of tinidazole or metronidazole. From this sample stock solution, mixed solutions of working range were prepared by further dilution with the mobile phase to contain 0.01-12 μg mL^{-1} of tinidazole and metronidazole and 10 μg mL^{-1} of sparfloxacin as internal standard in different 10 mL volumetric flasks. Before subjected to analysis all the working standard solutions were filtered through 0.45 μ filter and degassed.

Assay procedure

The chromatographic conditions followed for the assay of tinidazole and metronidazole are given below.

Mobile phase	:	Methanol : water : acetic acid (65 : 33 : 2 v/v)
Column	:	LiChrospher C18-5 μm (25 x 0.46 cm)
Flow rate	:	1 mL per min
Detection	:	UV set at 268 nm
Injection volume	:	20 μL
Run time	:	12 min.

With the above chromatographic conditions, 20 μL of the standard and sample solutions (twelve different concentrations in the range containing 0.01-12 μg mL^{-1} of tinidazole and metronidazole and 10 μg mL^{-1} of sparfloxacin as internal standard) were injected and the chromatograms were recorded. The system suitability factors for this chromatogram are given in Table 1. The retention times for metronidazole, tinidazole and sparfloxacin were found to be 3.14, 5.32 and 9.68 min, respectively. Concentration of the drug (sample) was calculated by calculating the response factor (peak area ratio of the standard peak and internal standard peak) of the standard solutions and sample solutions.

RESULTS AND DISCUSSION

Optimization of the variables

Optimum conditions, which are necessary for the quantitative analysis of the drug with maximum sensitivity, were established by a number of preliminary experiments. Optimum conditions were fixed by varying one parameter at a time by fixing other parameters constant and observing its effect on the response factor and also on the peak resolution. Effect of wavelength on the response factor and on the peak resolution was observed over the wavelength range 240-280 nm. 268 nm ± 2 unit was found to be optimal. Similarly effect of composition of the mobile phase was studied by changing the composition of the water, methanol and acetic acid. The optimum ratio of methanol : water : acetic acid was found to be 65 : 33 : 2 v/v with ± 2 mL of methanol and water. Effect of flow rate was observed by varying the flow rate from 0.8-1.5 mL min^{-1}. Lower flow rates lead to increase in resolution time and high flow rates found to considerable increase in the pressure. Therefore, 1 mL min^{-1} was found to be optimal for all measurements. Also, for internal standard, we tested different compounds and found that sparfloxacin was the best. Under these optimum conditions we have observed good resolution between sample peaks and internal stand peak with maximum sensitivity.

Validation studies

Linearity and range of the method was done by analyzing twelve different concentrations (n = 5) of the mixed standard solutions containing 0.01-12 μg mL^{-1} of tinidazole and metronidazole and 10 μg mL^{-1} of sparfloxacin under the chromatographic conditions mentioned above. The response factor of the standard solutions was calculated. The calibration curve was plotted using response factor v/s concentration of the standard solutions. Calibration curve was found to be linear over the concentration range 0.08-9.5 μg mL^{-1} and 0.07-8.5 μg mL^{-1} for metronidazole and tinidazole respectively. The data were analyzed by linear regression least squares fit method. The calibration graph shows negligible intercept and is described by the calibration equation, $y = a + bx$, where y is the peak area, ‘b’ is the slope, ‘a’ is the intercept and ‘x’ is the concentration of the analyte. To calculate the limit of quantitation (LOQ) and limit of detection (LOD) signal to noise ratio 10 : 1 and 3 : 1 respectively were used. Linear regression least squares fit data are given in Table 2.

The validity of the method for the analysis of metronidazole and tinidazole in its pure form and in its formulations was examined by analyzing various available formulations using the proposed procedure. To study the accuracy and to check the interference from the excipients used in the formulations, recovery studies were carried out by standard addition method. To the pre-analyzed formulations, known amounts of the analyte at three different concentration levels were added and assayed. The results obtained are given in Table 3. The recoveries were above 100% in most of the cases. The lower values of the RSD of the assay indicate the method is precise and accurate. Also, the results depicted that the present method is useful for

bulk drug analysis as well as commercial pharmaceutical formulations. Precision of the method was demonstrated by repeatability studies. This was done by injecting consequently the standard solution ten times and passing through the assay procedure. The lower values of RSD indicate the method is precise.

Chromatographic parameters were not affected significantly with the slight changes in the chromatographic conditions like composition of the mobile phase and flow rate (1 mL per min ± 0.1). Assay procedure described in the proposed method was repeated with different C18 columns and no significant change in chromatographic parameters was observed. Also, three different persons (analysts) carried out analyses and no considerable changes were noticed in the chromatographic parameters. All this shows that the proposed chromatographic procedure adopted in the method is rugged.

Further, to study the specificity of the method, analysis has been performed in the presence of excipients and other additives such as glucose, dextrose, lactose, starch, sodium alginate, talc, magnesium alginate, magnesium stearate and ascorbic acid. Under the chromatographic conditions employed recovery studies were carried out. To a known amount of drug (metronidazole 10 μg mL^{-1}) excipients in different concentrations were added and analysed. Results of the recovery analysis are presented in the Table 4. Excipients up to concentration mentioned in the Table do not interfere in the assay. Also, recoveries in most of the cases are found to be 100% and lower values of the RSD indicate the good precision of the method.

Table 1: System suitability parameters for the HPLC assay of metronidazole and tinidazole

Parameters	MZ	TZ	SP
Retention time t_R in min	3.14	5.32	9.68
Relative retention time RRT in min	-----	1.79	3.92
Capacity factor k	1.12	2.59	5.54
Selectivity factor α	-----	2.31	2.13
Resolution R	-----	6.05	11.32
Number of theoretical plates N*	1440.00	5765.00	10735.00
Height equivalent to theoretical plates (HETP) h, in cm	0.0173	0.0043	0.0023

*Calculated as $N = 5.54 (t_R/W_{0.5})^2$

Table 2: Linear regression least squares fit data for the estimation of tinidazole and metronidazole

Parameters	TZ	MZ
Linear dynamic range (μg mL^{-1})	0.07 - 8.5	0.08 - 9.5
Slope (m)	0.1104	0.1089
Intercept (b)	0.0087	0.0054
Standard deviation of slope (Sm)	0.0022	0.0018
Standard deviation of intercept (Sb)	0.0091	0.0074
Correlation coefficient (R)	0.9991	0.9994
Limit of quantitation (LOQ, μg mL^{-1})	0.07	0.08
Limit of detection (LOD, μg mL^{-1})	0.021	0.024

Table 3: Recovery study for spiked concentrations of drugs to the preanalyzed dosage forms

Formulations	Label claim mg per tablet	Amount added in mg	Recovery (% mean ± RSD)*	
			Proposed method	Official method [IP]
Tinidazole				
(i) Tiniba[a]	300	0	99.48 ± 0.66	98.97 ± 1.07
		25	101.03 ± 0.64	98.74 ± 0.87
		50	99.21 ± 0.40	101.16 ± 0.80
		100	101.12 ± 0.55	102.07 ± 1.07
(ii) Fasagyl[b]	500	0	100.85 ± 0.35	98.12 ± 0.85
		25	99.03 ± 0.25	99.21 ± 1.24
		50	99.66 ± 0.32	101.41 ± 0.57
		100	101.18 ± 0.59	101.56 ± 1.15
Metronidazole				
(i) Metrogyl[c]	400	0	100.24 ± 1.14	101.12 ± 1.01
		25	100.29 ± 0.64	99.08 ± 0.89
		50	101.12 ± 0.92	98.46 ± 0.59
		100	100.58 ± 0.32	101.18 ± 1.02
(ii) Aristogyl[d]	200	0	99.21 ± 0.82	101.01 ± 1.20
		25	101.29 ± 0.52	102.11 ± 0.48
		50	100.82 ± 0.43	101.21 ± 0.42
		100	100.18 ± 1.10	100.59 ± 0.82
Metronidazole Injection				
Metrogyl[e]	500 mg/5 mL	0	101.11 ± 0.43	102.08 ± 1.21
		25	99.63 ± 0.81	101.04 ± 0.55
		50	101.14 ± 0.62	101.58 ± 1.02
		100	100.48 ± 1.21	102.04 ± 0.72

*Average of four determinations

Marketed by, [a]Zydus Cadila Healthcare limited, [b]Pfizer Ltd. (India), [c & e]Unique J.B. Chemicals and Pharm Ltd limited and [d]Aristo Pharm Pvt Ltd.

Table 4: Determination of metronidazole[a] in the presence of excipients

Excipients	Amount added in mg	% Recovery of MZ ± % RSD[b]
Glucose	10	98.9 ± 0.82
Starch	08	101.2 ± 0.65
Lactose	12	100.2 ± 0.48
Talc	08	98.95 ± 0.58
Dextrose	14	100.4 ± 0.67
Stearic acid	11	99.3 ± 0.88
Sodium alginate	07	100.2 ± 0.75
Magnesium alginate	08	101.04 ± 0.90
Magnesium stearate	10	100.3 ± 0.85

[a]5 mg mL-1 of metronidazole was taken Average of six replicate analysis

CONCLUSION

The results of the proposed HPLC method showed that the data are consistent with the label claim of the formulations. The calibration curve showed linear response over the range of concentration used in the assay procedure. The lower value of RSD for the reproducibility studies and recovery studies show that the method is precise and accurate. Further, there is no interference of the excipients used in the formulations. Since sparfloxacin is an important antibacterial drug, present method can be applied reversibly for the assay of sparfloxacin using tinidazole or metronidazole as internal standard. The present RP-HPLC method uses simple mobile phase methanol, water and acetic acid without the need of buffer and all other reported HPLC procedures are critical of the pH of the mobile phase used, involves no complex procedure to prepare sample solutions, offers better sensitivity than some of the reported methods. Thus, the developed HPLC method is simple, accurate, precise and rugged. Hence, this method can be adopted for the quality control of tinidazole and metronidazole in bulk as well as in formulations.

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