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Reverse Phase High Performance Liquid Chromatography Method for the Simultaneous Estimation of Amoxicillin Trihydrate and Tinidazole in the Tablet Dosage Form

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Abstract: A simple, rapid and selective RP -HPLC method has been developed for determination of Amoxicillin trihydrate and Tinidazole in the pharmaceutical dosage form. The chromatographic separation was achieved with reverse phase phenomenex ® (Luna 5µ C18(2) 100A (250 × 4.60 mm) Column in isocratic mode and mobile phase containing 25mM Potassium dihydrogen phosphate (adjusted to pH 4.5 by Ortho-phosphoric acid) and Acetonitrile in the ratio of 40:60 % v/v was used. The flow rate was 1mL min^{-1} and effluent was monitored at 238 nm. Retention time was found to be 2.053 min for Amoxicillin trihydrate and 2.944 min for Tinidazole, respectively. Linear regression analysis data for the calibration plot showed that there was good linear relationship between response and concentration in the range of 5-50 µg/ml for both Amoxicillin trihydrate and Tinidazole, respectively. Percentage recoveries obtained for Amoxicillin trihydrate and Tinidazole were 100.87% and 99.52% respectively. The proposed method is precise, accurate, selective and rapid for the simultaneous determination of Amoxicillin trihydrate and Tinidazole in pharmaceutical dosage forms.

Key words: Amoxicillin trihydrate · Tinidazole · RP-HPLC Method · Validation

INTRODUCTION

Amoxicillin is chemically (2S.5R.6R)-6-{[(2R)-2-amino-2-(4-hydroxyphenyl)-acetyl] amino}-3, 3-dimethyl-7-oxo-4-thia-l-azabicyclo [3.2.0] heptane-2-carboxylic acid [1]. Amoxicillin is amino Penicillin with spectrum similar to that of Ampicillin [2]. Amoxicillin is a moderate-spectrum bacteriolytic β-lactum antibiotic used to treat bacterial infections caused by susceptible micro organisms. It is usually the drug of choice within the class because it is better absorbed following oral administration. Amoxicillin acts by inhibiting the synthesis of bacterial cell wall [3]. It is official drug in Indian Pharmacopoeia [4], British Pharmacopoeia [5]. The molecular formula is C16H19N3O5S and Molecular weight is 365.1 g/mol [6].

Tinidazole is chemically 1-(2- Ethyl Sulfonyl Ethyl) -2 - methyl - 5-nitroimidozole [7]. Tinidazole is a prodrug and antiprotozoal agent. The nitro group of tinidazole is reduced in Trichomonas by a ferredoxin-mediated electron transport system and used for Metronidazole-Resistant Vaginal Trichomoniasis [8]. The free nitro radical generated as a result of this reduction is believed to be responsible for the antiprotozoal activity. It is suggested

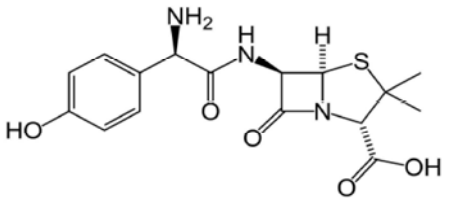

Fig 1: Chemical structure of Amoxicillin

that the toxic free radicals covalently bind to DNA, causing DNA damage and leading to cell death [9]. Tinidazole is the subject of monograph in each of the BP and the USP. British Pharmacopoeia [10] describes the non-aqueous titration method using perchloric acid as titrant and malachite green as indicator for the assay of tinidazole. The molecular formula is C8H13N3O4S and Molecular weight is 247.2 g/mol [11]. Both the drugs are used to treat Gastro intestinal infectious diseases and upper respiratory tract infections.

Literature survey revealed that a number of methods have been reported for estimation of Amoxicillin [12-20] and Tinidazole [21-27] individually or in combination with other drugs.Only one spectophotometric method [28]

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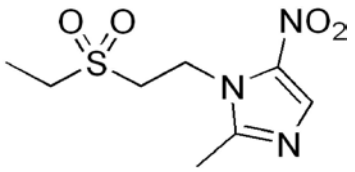

Fig. 2: Missing

and one LC estimation [29] method have been reported for the quantitative estimation of Amoxicillin trihydrate and tinidazole in pharmaceutical dosage form. Hence an attempt has been made to develop new HPLC method which is simple, rapid, reproducible and economical method for simultaneous estimation of Amoxicillin trihydrate and Tinidazole in tablet dosage form. This method has been successfully used for quality-control analysis of drugs and for other analytical purposes.

MATERIAL AND METHODS

Chemicals: Standard bulk drug samples of Amoxicillin and tinidazole were provided by Drugs India private Ltd (Hyderabad, India). Pharmaceutical dosage form used in this study was TINIMOX® labeled to contain 500 mg of Amoxicillin trihydrate and 300mg of Tinidazole (manufactured by Dr. Reddy's Laboratories, Hyderabad, India). Acetonitrile (HPLC grade) and ortho phosphoric acid was obtained from Merck Specialties Private limited, Mumbai, India. Water (HPLC grade) was obtained from milli-Q system; Hydrochloric acid (LR grade) was obtained from Ranbaxy Fine Chemicals Ltd, New Delhi, India. Sodium hydroxide (LR grade) and Potassium dihyrdrogen ortho phosphate purified were obtained from S.D. Fine Chemicals Ltd, Mumbai, India. Hydrogen Peroxide was obtained from Sabin Pharmaceuticals, Hyderabad, India.

Apparatus: HPLC method development and validation was done on a Shimadzu (Japan) Liquid chromatograph equipped with (LC-20 AD pump), LC-20A UV/Vis detector, (Rheodyne) 7725i injection with 20µl loop and LC-Solutions software. Stationary phase used was Luna 5µ C18 (2) 100A 250 × 4.60mm Column. Membrane filter of size 0.45µ (Rankem Nylon membranes, New Delhi, India) was used. All weighing were done on Shimadzu electronic balance, BL-220 H (Shimadzu Corporation, Japan).

Preparation of Standard Stock Solution: The standard stock solution 1mg/ml of Amoxicillin trihydrate and Tinidazole were prepared separately by dissolving 10 mg of each drug in 100 ml mixture of acetonitrile and water (60:40 v/v). From the standard stock solution, mixed standard solution was prepared to contain 100 µg/mL of Amoxicillin trihydrate and 100 µg/mL of Tinidazole.

Preparation of Mobile Phase: Acetonitrile : 25mM Potassium dihydrogen phosphate (adjusted to pH 4.5 by Ortho-phosphoric acid (10% aqueous) (60:40 v/v)was prepared, filtered through 0.45 µm membrane filter and sonicated on ultra sonic bath.

Preparation of Solutions for Calibration Curve: Standard Stock solution of Amoxicillin trihydrate and Tinidazole each were further diluted to get solutions of concentration ranging from 5-50 µg/ml.

Procedure for Sample Preparation/tablet Analysis: Sample Details: TINIMOX®
Label Claim: Each Tablet contains Amoxicillin Trihydrate equivalent to Amoxicillin 500mg and Tinidazole 300mg
Mfg. By: Dr.Reddy's Lab, Hyderabad

Twenty tablets were weighed and powder equivalent to 10 mg of Amoxicillin trihydrate was weighed accurately (0.2036g),to this 4mg of Tinidazole was added (standard addition method) and transferred to a 100ml volumetric flask and extracted with mixture of Acetonitrile and water (60:40 v/v). The combined extracts were filtered and transferred to a volumetric flask and the volume adjusted to 100 ml with Acetonitrile and water. From this solution, 3 ml was pipetted and transferred to 10 ml volumetric flask and made volume upto the mark with acetonitrile and water to get the concentration 30µg/ml of Amoxicillin trihydrate and 30 µg/ml of Tinidazole.

Dilutions for Precision Studies: Precision of the method was checked by system precision and repeatability (Intraday and Interday studies). In system precision 6 replicates of mixed standard (containing Amoxicillin trihydrate 30µg/ml and Tinidazole 25µg/ml) were used.

Dilutions for Recovery Studies: To study accuracy of the method, recovery studies were carried out by addition of standard drug solution to sample at 3 different levels, 50%, 100% and 150% of the test concentration (test concentration is 30µg/ml for Amoxicillin trihydrate and 30 µg/ml for Tinidazole).

Robustness Studies: Robustness of the method was determined by small, deliberate changes in flow rate, mobile phase ratio, Wavelength of detection and pH of

mobile phase. Flow rate was changed to 1 ± 0.05 ml/min. The mobile phase ratio was changed to ±2 units both acetonitrile and buffer, pH of mobile phase was changed to 4.5±0.2

LOD and LOQ Determination [30].

Limit of detection can be calculated by using following formula

$$LOD = 3.3\ \sigma/S$$

Limit of quantitation can be calculated based on standard deviation of the response and the slope.

$$LOQ = 10\ \sigma/S$$

Where
σ = Standard deviation of the response
S = Slope of the calibration curve

System Suitability Testing [30]: System suitability testing is used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. Parameters such as theoretical plates, tailing factor, resolution were determined and compared against the specifications.

RESULTS AND DISCUSSION

Once the HPLC method was developed, the method was validated in terms of parameters like linearity, precision, LOD, LOQ, recovery studies etc. The proposed HPLC method was validated as per ICH guidelines [31].

The solutions of Amoxicillin trihydrate and Tinidazole working standards were injected into the HPLC system and run in different solvent systems as mobile phases. Different mobile phases containing Acetonitrile, Buffers (Triethanolamine, phosphate) in different proportions were tried and finally Acetonitrile : 25mM potassium dihydrogen phosphate adjusted to pH4.5 with ortho phosphoric acid (60:40 v/v) was selected as an appropriate mobile phase which gave good resolution and acceptable peak parameters for both Amoxicillin trihydrate and Tinidazole. Representative chromatogram of mixed standard of Amoxicillin trihydrate and Tinidazole is shown in Figure 3.

From the standard stock solution further dilutions (Amoxicillin trihydrate 30 µg/ml and Tinidazole 30 µg/ml) were done using mobile phase and scanned over the

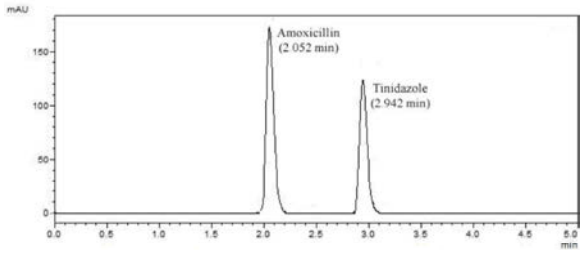

Fig. 3: Chromatogram of working standard mixture of Amoxicillin trihydrate and Tinidazole

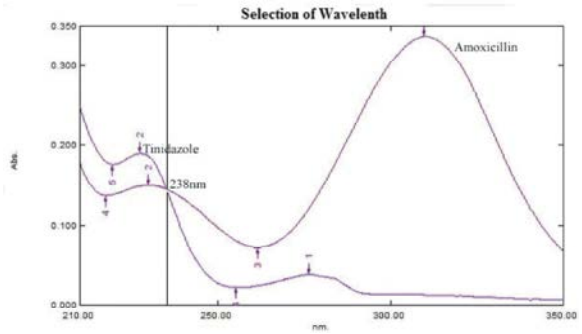

Fig. 4: Overlain of Amoxicillin trihydrate and Tinidazole standards

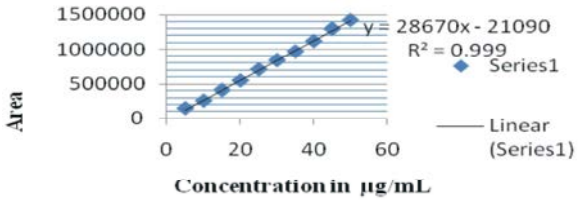

Fig 5: Calibration Curve for Amoxicillin trihydrate

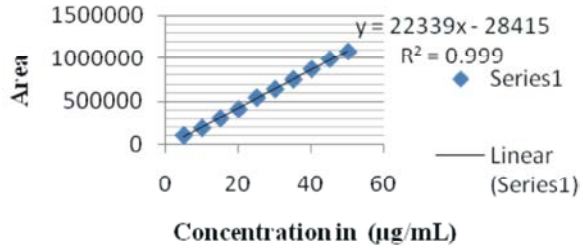

Fig 6: Calibration Curve for Tinidazole

range of 200-400 nm and the spectra were overlain. It was observed that at 238 nm both Amoxicillin trihydrate and Tinidazole showed considerable absorbance and therefore it was selected as detection wavelength. Overlain spectra of both drugs are shown in Fig.4.

Method Validation: The linear relationship was observed between the peak area and concentration over the range of 5-50µg/ml for Amoxicillin trihydrate and 5-50µg/ml for

Table 1: Optimized Chromatographic Conditions

Parameters	Method
Stationary phase (Column)	Phenomenex ® (Luna 5µ C18(2) 100A (250 × 4.60 mm) Column
Mobile phase	25mM Potassium dihydrogen phosphate (adjusted to pH 4.5 by Ortho-phosphoric acid) and Acetonitrile in the ratio of 40:60 % v/v
Flow rate	1 ml/min
Pressure	110 kgf
Run time	5 min
Column temperature(°c)	Ambient
Detection wavelength (nm)	238
Drugs Retention time (min)	
Amoxicillin trihydrate	2.052 ± 0.14
Tinidazole	2.942 ± 0.10

Table 2: System suitability parameters for Amoxicillin trihydrate

S.NO.	RT	Area	USP Plate count	USP Tailing
1	2.045	837040	3421.260	1.388
2	2.044	834617	3334.017	1.384
3	2.045	834524	3349.321	1.385
4	2.043	837656	3459.209	1.390
5	2.047	837562	3432.664	1.391
Mean		836280		
SD		1517		
% RSD		0.18		

Table 3: System suitability parameters for Tinidazole

S.NO.	RT	Area	USP Plate count	USP Tailing
1	2.935	615412	6567.838	1.404
2	2.937	612720	6800.499	1.401
3	2.939	615977	6490.672	1.398
4	2.942	618022	6545.286	1.401
5	2.933	613264	6527.171	1.401
Mean		615079		
SD		2146		
% RSD		0.34		

Table 4: Intraday Precision

	Concentration		Peak area	
S.NO	Amoxicillin trihydrate	Tinidazole	Amoxicillin trihydrate	Tinidazole
1	30	25	834512	538442
	30	25	837726	537768
	30	25	834052	538486
	30	25	829526	539018
	30	25	827483	534306
	30	25		
	837625	538648		
% RSD			0.53	0.32

Table 5: Interday Precision

	Concentration		Peak area	
S.NO	Amoxicillin trihydrate	Tinidazole	Amoxicillin trihydrate	Tinidazole
1	30	25	805746	515924
	30	25	807161	509796
	30	25	810133	515856
	30	25	806895	525417
	30	25	809121	525400
	30	25	807692	509974
% RSD			0.196	1.32

Table 6: Robustness Results for variations in flow rate

	Retention Time		Tailing factor	
Parameter	Amoxicillin trihydrate	Tinidazole	Amoxicillin trihydrate	Tinidazole
pH 4.3	2.045	2.938	1.41	1.471
pH 4.7	2.048	2.937	1.38	1.391

Table 7: Robustness Results for variations in pH.

	Retention Time		Tailing factor	
Parameter	Amoxicillin trihydrate	Tinidazole	Amoxicillin trihydrate	Tinidazole
pH 4.3	2.052	2.942	1.390	1.401
pH 4.7	2.047	2.937	1.384	1.401

Table 8: Robustness Results for variations in Mobile phase.

	Retention Time		Tailing factor	
Parameter	Amoxicillin trihydrate	Tinidazole	Amoxicillin trihydrate	Tinidazole
ACN : buffer (62 : 38)	2.045	2.935	1.388	1.404
ACN : buffer (58 : 42)	2.048	2.939	1.385	1.398

Table 8: Recovery Studies of Amoxicillin trihydrate and Tinidazole

Drug		50%	100 %	150%
Amoxicillin Trihydrate	Mean % recovery	98.62	99.54	101.85
	% RSD	1.5	0.53	1.2
Tinidazole	Mean % recovery	99.62	100.48	100.66
	% RSD	0.29	1.3	1.7

Table 9: Analysis of Formulation

	Amount(mg/tablet)			
Drug	Labelled	Estimated	% Label claim	% RSD
Amoxicillin trihydrate	500mg	498.16	99.63	0.42
Tinidazole	300 mg	302.02	100.67	0.65

coefficient, which was 0.999 for Amoxicillin trihydrate and 0.999 for Tinidazole. Correlation coefficient, y- intercept, slope of regression line are shown in Figure 5 and 6. Precision was carried out as system precision and repeatability as per ICH guidelines. It was determined at 3 concentration levels with 3 replicates at each level. For all three concentration levels % RSD obtained was less than 2 % for both the drugs. The results of precision are given in Table 4 and 5. Robustness studies were carried out after deliberate alterations of flow rate, mobile phase compositions and mobile phase pH. It was observed that the small changes in these operational parameters, did not lead to changes of retention times of peak of interest. Results of robustness studies are shown in table no.6 and 7.The proposed method was evaluated in the assay of Tablet formulation containing Amoxicillin trihydrate and Tinidazole. Five replicate determinations were carried out and % assay found was 98.60 -101.85 for Amoxicillin trihydrate and 99.60-100.60 % for Tinidazole, was shown in Table 8. Results of tablet analysis were shown in Table no.9.

CONCLUSION

The method described enables the quantification of Amoxicillin Trihydrate and Tinidazole in combined Tablet dosage form. The validation data demonstrate good precision and accuracy, which prove the reliability of the proposed method. Hence, this HPLC method can be used routinely for quantitative estimation of both components in solid oral dosage form.

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