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Development of a RP-UPLC method for the simultaneous analysis of Secnidazole, Fluconazole, and Azithromycin: Application in pharmaceuticals and human serum

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Abstract : A novel approach was carried out to develop and validate a rapid and selective analytical method by using Reverse Phase Ultra Performance Liquid Chromatographic (RP-UPLC) technique for the simultaneous separation and analysis of secnidazole, fluconazole, and azithromycin in raw materials, their pharmaceutical dosage forms and human serum. The developed analytical UPLC method is superior in technology to conventional HPLC with respect to speed, resolution, solvent consumption and cost of analysis. Elution time for the separation was 10 min in reverse phase mode and ultra violet detection was carried out at 210 nm. Efficient separation was achieved on BEH C18 sub-2-µm UPLC column using 0.002M Na_2HPO_4 and acetonitrile as organic solvent in a gradient program. Benzophenone was used as internal standard. Resolutions between secnidazole, fluconazole, and azithromycin were found to be more than 4.5. The active pharmaceutical ingredient was extracted from tablets using methanol and acetonitrile (50:50 v/v) as diluent. The calibration graphs were linear for secnidazole, fluconazole, benzophenone and azithromycin. The method showed excellent recoveries for all drugs in bulk and formulated products. The test solution was found to be stable in diluent for 72 h when stored in the refrigerator between 2 to 8 ^{o}C. The developed UPLC method was validated that meets the requirements laid down by the International Conference on Harmonization (ICH) guidelines with respect to linearity, accuracy, precision, specificity and robustness, and has been successfully applied to pharmaceutical formulations because no chromatographic interferences from the tablet excipients are found. To the best of our knowledge, a validated reverse phase analytical method for the simultaneous separation and quantification of secnidazole, fluconazole, and azithromycin by using UPLC technique disclosed in this investigation was not published elsewhere.

Keywords: Column liquid chromatography, Ultra Performance Liquid Chromatography(UPLC),Secnidazole , Fluconazole ,Azithromycin ,Validation and Simultaneous quantification.

Introduction

Ultra Performance Liquid Chromatography (UPLC) system is an innovative product that brought revolution in high performance liquid chromatography by outperforming conventional HPLC. UPLC decreases sample run times up to a factor of 10, uses up to 95 percent less solvent and significantly improves productivity in the lab. The sub-2-µm hybrid particle chemistry, which offers significant benefits over today's HPLC systems equipped with standard 5-µm particle chemistries. UPLC achieves the speed by using novel sub two-micron particles that accelerate chromatographic run times and also double peak capacity or resolution.

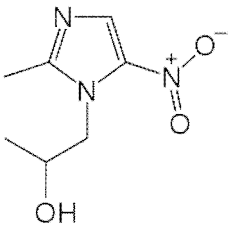
UPLC was designed as a total system to leverage both ultra-high pressure and small particle separation attributes that result in uniquely superior performance. With significant improvements in resolution, sensitivity and speed that can be achieved for chromatographic separations by minimizing the band spreading contributions of both the instrument and the column.

UPLC system will eliminate significant time and cost per sample from analytical process while improving the quality of results, the system allows chromatographers to work at higher efficiencies with a much wider range of linear velocities, flow rates, and backpressures. UPLC Photodiode Array (PDA) Detector detects and quantifies lower concentrations of sample analyte, trace impurities at levels to 0.004% with maximum sensitivity and compares spectra across wavelengths and broad concentration ranges. It is easy to identify components that are difficult to detect by conventional HPLC-based methods. The present study was conducted to analyze and quantify secnidazole, fluconazole, and azithromycin in pharmaceutical formulations and human serum by using RP-UPLC technique is described in this investigation.

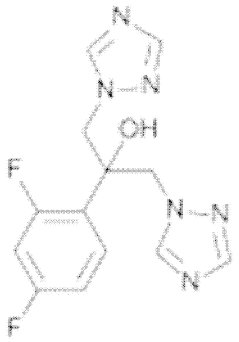
Secnidazole (SNZ; CAS 3366-95-8) is a nitroimidazole drug [1], an orally active antiamebic, antiprotozoal drug used for the treatment of infection [2]. In an infection, the infecting organism seeks to utilize the host's resources to multiply. The injecting organism, or pathogen, interferes with the normal functioning of the host and can lead to chronic wounds, gangrene, loss of an infected limb, and even death. SNZ has the chemical name (1-(2-methyl-5-nitro-1*H*-imidazol-1-yl) propan-2-ol). Figure 1 shows the chemical structure of SNZ. Its molecular formula is $C_7H_{11}N_3O_3$, and its molecular weight is 185.18. It is available as a tablet and usually 2 grams single dosed with food. Fluconazole (FCZ; CAS 86386-73-4) is a triazole group drug [3], an orally active used in the treatment and precention of superficial and systemic fungal infections. Like other imidazole and triazole class antifungals, fluconazole inhibits the fungal cytochrome P450 enzyme 14α-demethylase. This inhibition prevents the conversion of lanosterol to ergosterol, an essential component of the fungal cytoplasmic membrane, and subsequent accumulation of 14α-methyl sterols. Fluconazole is primarily fungi static. FCZ has the chemical name (2-(2,4-difluorophenyl)-1,3-*bis*(1*H*-1,2,4-triazol-1-yl)propan-2-ol). Figure 1 shows the chemical structure of FCZ. Its molecular formula is $C_{13}H_{12}F_2N_6O$, and its molecular weight is 306.27. It is available as a tablet and usually dosed once daily after breakfast. Azithromycin (AZTM; CAS 83905-01-5) is the first clinically developed antibiotic in a new subclass of the macrolides, is one of the world's best-selling antibiotics, and is derived from erythromycin. The azalides, characterized by the expansion of the 14-membered aglycone ring of erythromycin with endocyclic ionizable nitrogen [4, 5]. AZTM has the chemical name (2*R*,3*S*,4*R*,5*R*,8*R*,10*R*,11*R*,12*S*,13*S*, 14*R*)-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-15-oxo-11-{[3,4,6-trideoxy-3-(dimethyl --amino)-β-D-*xylo*-hexopyranosyl]oxy}-1-oxa-6-azacyclopentadec-13-yl 2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranoside). Figure 1 shows the chemical structure of AZTM. It differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring, thus making the lactone ring 15-membered onto which two sugar moieties are linked. The insertion of a methyl-substituted nitrogen on the lactone ring at position 9a of the large macrolactone ring produces an enhanced spectrum and potency against bacteria compared with other macrolides and erythromycin [6, 7] and superior stability in acidic environment [8]. An aminosugar, *d*-desosamine, is attached through a β-glycosidic bond to the C5 position of the lactone ring. A neutral sugar, *l*-cladinose is attached via a α-glycosidic linkage to the C3 position of the lactone [9]. Its molecular formula is $C_{38}H_{72}N_2O_{12}$, and its molecular weight is 748.98. It is reported to exist extensively as dihydrate [10], which is white crystalline powder with a molecular formula of $C_{38}H_{72}N_2O_{12}\cdot 2H_2O$ and a molecular weight of 785.0 [11]. AZTM does not interact with the hepatic cytochrome P450 system and is not associated with the pharmacokinetic drug interactions seen with erythromycin and other macrolides [12]. It is available as a tablet and usually dosed 1 hour before or 2 hours after the meal. The combination kit with azithromycin, secnidazole and fluconazole was more effective with better symptomatic relief and less recurrence rate and may be routinely recommended in

all cases of lower genital infection and syndromic management of pelvic inflammatory disease in a cost effective, safe and effective strategy [13, 14].

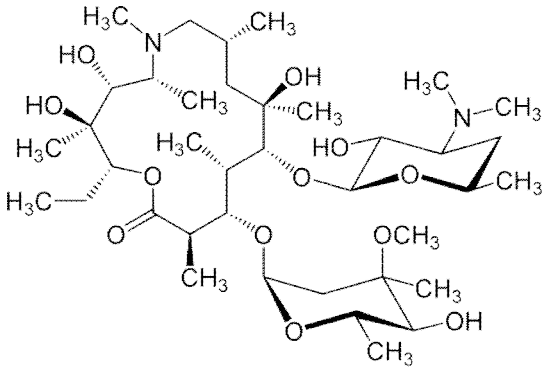
A few analytical methods have been reported for the quantification of secnidazole [15, 16], fluconazole [17], and azithromycin [18, 19] individually. A review of the literature did not reveal the presence of simultaneous method applied to the separation and quantification of above three drugs. It was felt necessary to develop a suitable RPLC method for the separation and quantification of three drugs by one method. We present the development and validation of a RP-UPLC assay using UV detector for measurement and allowed determination of each analyte without the need for development of separate and distinct methods for each analyte.



Secnidazole



Fluconazole



Azithromycin

Fig. 1. Chemical structures of three drugs

Experimental

Chemicals

Bulk sample of fluconazole was obtained from Auctus Pharma (Hyderabad, India). Secnidazole and azithromycin were obtained from Alchem Laboratories Ltd. (Mumbai, India). Commercially available FAS-3 combikit manufacture by Hetero Healthcare Ltd and ZOCON-AS combikit manufacture by FDC Limited was purchased from local pharmacy each containing one tablet of fluconazole (150 mg), two tablets of secnidazole (1 gm each), and one tablet of azithromycin (1 gm) as individual dosage forms in a blister pack. HPLC grade acetonitrile, methanol was obtained from Apchem, Ashonuj Chem Pvt. Ltd (Navi Mumbai, India). Na_2HPO_4, and NaOH were obtained from Merck (Darmstadt, Germany), Benzophenone was obtained from Alfa Aesar, Lancaster. High purity water was obtained from Millipore Milli-Q Plus water purification system. The chromatographic separation and quantification was carried out on a Waters Acquity UPLC with PDA Detector with a separation module. Empower software (Waters) was used for data handling installed on a Pentium computer (Lenovo). Acquity BEH-Shield RP18 and BEH-C18 columns were purchased from Waters.

Equipment

The LC system used for method development and method validation consisted of a Waters Acquity UPLC with PDA Detector with a separation module. Empower software (Waters) was used for data handling installed on a Pentium computer (Lenovo). Acquity BEH-Shield RP18 and BEH-C18 columns were purchased from Waters.

Chromatographic Conditions

The analysis was carried out on Acquity BEH-Shield RP18 column (100 X 2.1 mm, 1.7µm). The mobile phase composition was 0.002M Na_2HPO_4 (pH adjusted to 10.0 with 3% aq. NaOH solution) and acetonitrile as organic solvent with a linear time gradient program [Time/% acetonitrile: 0/5, 1.5/5, 3/30, 5/90, 8/90, 9/5, 10/5] at a flow rate of 0.30 mL min^{-1}, 40 °C column temperature and detection was monitored at 210 nm with analyses run time of 10 min. The injection volume was 0.5 µL, acetonitrile and methanol (50:50, *v/v*) was used as sample diluent.

Preparation of Solutions

Preparation of Standard Solution

A mixture of secnidazole, fluconazole, azithromycin, and benzophenone 800 µg mL^{-1}, 400 µg mL^{-1}, 10,000 µg mL^{-1}, and 200 µg mL^{-1} were prepared respectively by dissolving the appropriate amount in acetonitrile

and methanol (50:50 v/v) and filled to the mark with diluent.

Preparation of Sample Solutions
Twenty tablets were weighed to determine the average tablet weight and powdered in a mortar. Powder equivalent to 40.0 mg fluconazole, 80.0 mg of secnidazole, 1000.0 mg of azithromycin, and 20.0 mg of benzophenone was transferred into a 100 mL volumetric flask. About 50 mL diluent was added and kept on a rotary shaker for 20 min to disperse the material completely followed by sonication for 10 min, cooled to room temperature, make up to mark with diluent and mixed well. About 10 mL of sample solution was centrifuged for 15 min at 2,500 rpm. The supernatant was collected and filtered through a 0.45 µm Nylon-66 membrane filter.

Serum drug sample solution
Blood samples were collected from volunteers and then centrifuged at 3000 rpm for 15 min. The supernatant obtained was stored at -20°C. After thawing, serum was deprotonated by acetonitrile and spiked with working solutions to produce desired concentrations of above three drugs. A 0.5 µL volume of each sample was injected and chromatographed under the above conditions.

Method Validation

Method validation was performed according to ICH guidelines with respect to precision, linearity, accuracy, specificity and robustness [20].

Precision
The precision of the developed method was evaluated by six replicate injections of the above standard mixture. The RSD of three drugs and benzophenone was calculated for peak areas, USP tailing factor and plate count. The intermediate precision of the method was also evaluated on a different column dimension.

Linearity
Stock solutions of secnidazole, fluconazole, azithromycin, and benzophenone 1600 µg mL^{-1}, 800 µg mL^{-1}, 20,000 µg mL^{-1}, and 400 µg mL^{-1} were prepared respectively by dissolving the appropriate amount in acetonitrile and methanol (50:50 v/v) and diluted to the required concentrations. The solutions were prepared at five concentration levels ranging from 25% to 120% of the target concentration. The correlation coefficients, slopes and *Y*-intercepts of the calibration curve were determined.

Accuracy
Standard addition and recovery experiments were conducted to determine accuracy of the method for the quantification of three drugs and benzophenone. The study was carried out at 80%, 100% and 120% for three replicate injections of each concentration of the analyte followed by calculation of the percentage recovery.

Solution Stability and Mobile Phase Stability
Solution stability of three drugs solution along with benzophenone in a tightly capped volumetric flask for 72 h when stored in a refrigerator between 2 to 8 °C temperature was studied. The contents of three drugs were determined in 8 h intervals. Mobile phase stability was assessed over a period of 72 h by injecting the freshly prepared sample solutions in 8 h interval as well. The contents of three drugs were determined in the test solutions.

Specificity
The specificity of the analytical method was checked in different conditions of acid hydrolysis (0.1N HCl), base hydrolysis (0.1N NaOH) and peroxide treatment (0.3% H_2O_2). Aliquot quantities of mixture of three drugs were weighed in different volumetric flasks and added 3.0 mL of 0.1N HCl, 3.0 mL of 0.1N NaOH, and 1.0 mL of 0.3% H_2O_2 respectively and diluted to 75 mL with diluent. These solutions were refluxed at 80 °C for 8 h, cooled to room temperature, made up to 100 mL with diluent and analyzed by UPLC.

Robustness
The experimental conditions were deliberately altered and resolution between secnidazole, fluconazole, benzophenone and azithromycin was evaluated. The impact of flow rate and temperature changes on resolution, tailing factor and plate count was studied by the alteration of ± 0.05 mL min^{-1}. Column temperatures were also changed to 35 and 45 °C instead of 40 °C. The mobile phase composition and injection volume was kept constant. The effect of different column dimension was also studied.

Results and Discussion

The primary target in developing this UPLC method was to achieve simultaneous determination of these drugs in pharmaceutical formulations and also in human serum under common conditions that are applicable for routine quality control, research and development of these drugs in ordinary laboratories. The development of UPLC methods for the determination of drugs has received more attention recently because of their speed, resolution, sensitivity, and cost effectiveness which is important in the quality control of drugs and drug products.

This work was intended to develop a precise, reliable, and speedy method, based on reverse-phase UPLC separation combined with UV detection for simultaneous drug assay in raw material, bulk drug samples, dosage formulations and, especially in human serum.

UPLC Method Development and Optimization

Development of a rugged and suitable UPLC method for the separation of secnidazole, fluconazole, and azithromycin required a number of trials to be carried out using different mobile phase compositions. As part of the preliminary work, separation was attempted using BEH C18 (100 X 2.1 mm, 1.7 µm) column with 0.01M ammonium acetate buffer and acetonitrile in a different gradient programs at a flow rate of 0.3 mL^{-1}, the sensitivity of secnidazole and fluconazole was less with unsymmetrical peak shapes and azithromycin was not even detected at a common UV maxima of 210 nm. Separation between secnidazole and fluconazole was only 0.2 min in a total run time of 10 min. Trials were also done at acidic pH 3.5 as there was no improvement in sensitivity of azithromycin but there was considerable improvement for secnidazole and fluconazole. Benzophenone peak shape and sensitivity was good in all above trials. It was found in the literature that azithromycin has two pKa values at 8.1 and 8.9. Attempts were made with different basic pH in a range of $\pm$2.0 pKa. Triethylamine (TEA) was used to adjust the buffer pH to 10.0 which has shown dramatic improvement in sensitivity of azithromycin, but more noise in chromatographic base line due to lower common UV at 210 nm. Several mobile phase compositions were also employed but no success in separation and sensitivity. Trials were made to adjust the buffer pH to 10.0 with alternative reagents and the suitable one found was 3% aq. NaOH. To improve the peak shapes of all three drugs alternative buffer chosen was 0.002M Na_2HPO_4, pH adjusted to 10.0 with 3% aq. NaOH after a number of trials. To improve the resolution, attempts were made with different percentages of acetonitrile and methanol in the mobile phase. Trials were also done at different flow rates and different temperatures to optimize the peak shape, sensitivity, tailing factor and resolution for all three drugs and benzophenone. The choice of C18 columns in UPLC is less except BEH-C18 and BEH-Shield RP18 in reverse phase conditions.

The typical retention times of secnidazole, fluconazole, benzophenone and azithromycin were 3.7, 4.1, 5.6, and 6.1 min respectively with a total chromatographic run time of 10 min. The resolution (R_s) between secnidazole and fluconazole was ~4.8, fluconazole and benzophenone was ~19.0, and benzophenone and azithromycin was ~5.7. The system suitability test results are summarized in Table 1.

Potential interferences from excipients (dibasic calcium phosphate anhydrous, pregelatinized starch, croscarmellose sodium, lactose, magnesium stearate, microcrystalline cellulose, povidone, hypromellose, titanium dioxide, sodium lauryl sulfate, triacetin and Ponceau 4R) were also investigated. No interferences from excipients were observed. Analyses was performed for different batches of pharmaceutical dosage forms of two manufacturers (each $n = 3$). In all batches the contents of secnidazole, fluconazole, and azithromycin were well within the limits of $\geq$ 90.0% and $\leq$ 110.0% (w/w). The Assay results for dosage forms are summarized in Table 2.

Method Validation

Precision

The area RSD values for secnidazole, fluconazole, benzophenone, and azithromycin were found to be within 2.0% confirming a suitable precision of the method. The method was observed to be rugged because of the fact that the variation of the previously described various parameters did not impact on the results.

Linearity

The correlation coefficient obtained was greater than 0.99 for all products. Slope and *Y*-Intercept values were 14750 and 65791 for secnidazole, 9631 and 47218 for fluconazole, 15676 and 75228 for benzophenone, and 15551 and 92418 for azithromycin. Linearity was determined for five concentrations of each three replicate injections. The RSD values of the slope and *Y*-intercept values were 0.9 and 7.7% for secnidazole, 0.7 and 5.3% for fluconazole, 0.7 and 7.1% for benzophenone and 0.4 and 1.8% for azithromycin which confirmed the linear relationship between peak areas and concentrations. The Linearity test results are summarized in Table 1.

Table 1. System suitability, Precision, Linearity, Accuracy, LOD, LOQ and Robustness of the proposed method

Parameter	SNZ		FCZ		AZTM	
System suitability	Bulk		Bulk		Bulk	
R_t	3.7		4.1		6.2	
R_s	-		7.8		11.1	
N	53669		162676		270009	
USP Tailing	1.8		1.4		1.4	
Capacity factor	2.7		3.1		5.2	
Precision % RSD (n = 6)	0.8		0.6		0.4	
Linearity (n = 3)	Formulation	Serum	Formulation	Serum	Formulation	Serum
r	0.995	0.999	0.996	0.999	0.992	0.999
Slope	14749.7	14763.2	9631.3	9622.1	15551.0	15534.0
% RSD for slope	0.9	1.2	0.7	1.1	0.4	0.8
Intercept	65790.7	65783	47218.0	47214	92417.7	92420.1
% RSD for Intercept	7.7	8.1	5.3	6.2	1.8	2.2
Accuracy (% recovery)						
80% (n = 3)	99.3	99.0	100.9	99.8	96.6	96.8
100% (n = 3)	98.5	98.2	100.8	100.1	97.6	98.4
120% (n = 3)	97.1	97.6	100.6	98.6	97.5	99.2
LOD (μgmL^{-1})	0.0026	0.0029	0.0019	0.0021	0.0091	0.0098
LOQ(μgmL^{-1})	0.0072	0.0080	0.0051	0.0068	0.0226	0.0295
Robustness	(R_t)		(R_t , R_s)		(R_t , R_s)	
Different flow 0.25 mL min^{-1}	4.0		4.3, 7.7		6.4, 9.2	
Different flow 0.35 mL min^{-1}	3.3		3.8, 10.8		5.8, 10.5	
Different Temp. 35 °C	3.7		4.1, 9.3		6.0, 8.3	
Different Temp. 45 °C	3.6		4.0, 9.9		6.0, 9.0	
Different Column: Acquity BEH-C18 (50 X 2.1mm, 1.7 µm)						
Different flow 0.25 mL min^{-1}	3.3		3.7, 5.3		5.9, 6.9	
Different flow 0.35 mL min^{-1}	2.9		3.4, 9.4		5.6, 13.0	
Different Temp. 35 °C	3.0		3.5, 9.3		5.7, 10.6	
Different Temp. 45 °C	2.9		3.5, 7.5		5.6, 9.1	
Different pH						
Different pH 10.2	3.7		4.1, 9.7		6.2, 7.4	
Different pH 9.8	3.5		3.9, 8.7		5.9, 7.2	

n: number of determinations, R_t : retention time, R_s : USP resolution, N : number of theoretical plates, r : correlation coefficient
SNZ: Secnidazole, FCZ: Fluconazole, AZTM: Azithromycin

Table 2: Assay results for tablet dosage form by the proposed method

Formulation	Amount of drug taken (mg)			Amount of drug found (mg)			% Amount found ($n^a = 3$) ± SD^b		
	SNZ	**FCZ**	**AZTM**	**SNZ**	**FCZ**	**AZTM**	**SNZ**	**FCZ**	**AZTM**
Manufacturer-1	81.87	48.73	1032.9	82.00	48.79	1010.48	100.15± 1.04	100.12± 0.68	97.83± 1.30
Manufacturer-2	79.94	40.32	1000.0	78.15	39.46	981.7	97.77± 0.32	97.86± 0.76	98.17± 1.18

[a]n is number of determinations, [b]SD is standard deviation

Table 3: Assay results for serum samples by the proposed method

Formulation	Amount of drug taken (mg)			Amount of drug found (mg)			% Amount found ($n^a = 3$) ± SD^b		
	SNZ	**FCZ**	**AZTM**	**SNZ**	**FCZ**	**AZTM**	**SNZ**	**FCZ**	**AZTM**
Manufacturer-1	80.62	48.70	1030.1	81.10	48.90	1001.5	100.60± 1.12	100.41± 0.86	97.22± 1.53
Manufacturer-2	79.20	40.18	1002.6	77.93	39.61	968.7	98.40± 0.63	98.58± 0.98	96.62± 1.34

[a]n is number of determinations, [b]SD is standard deviation

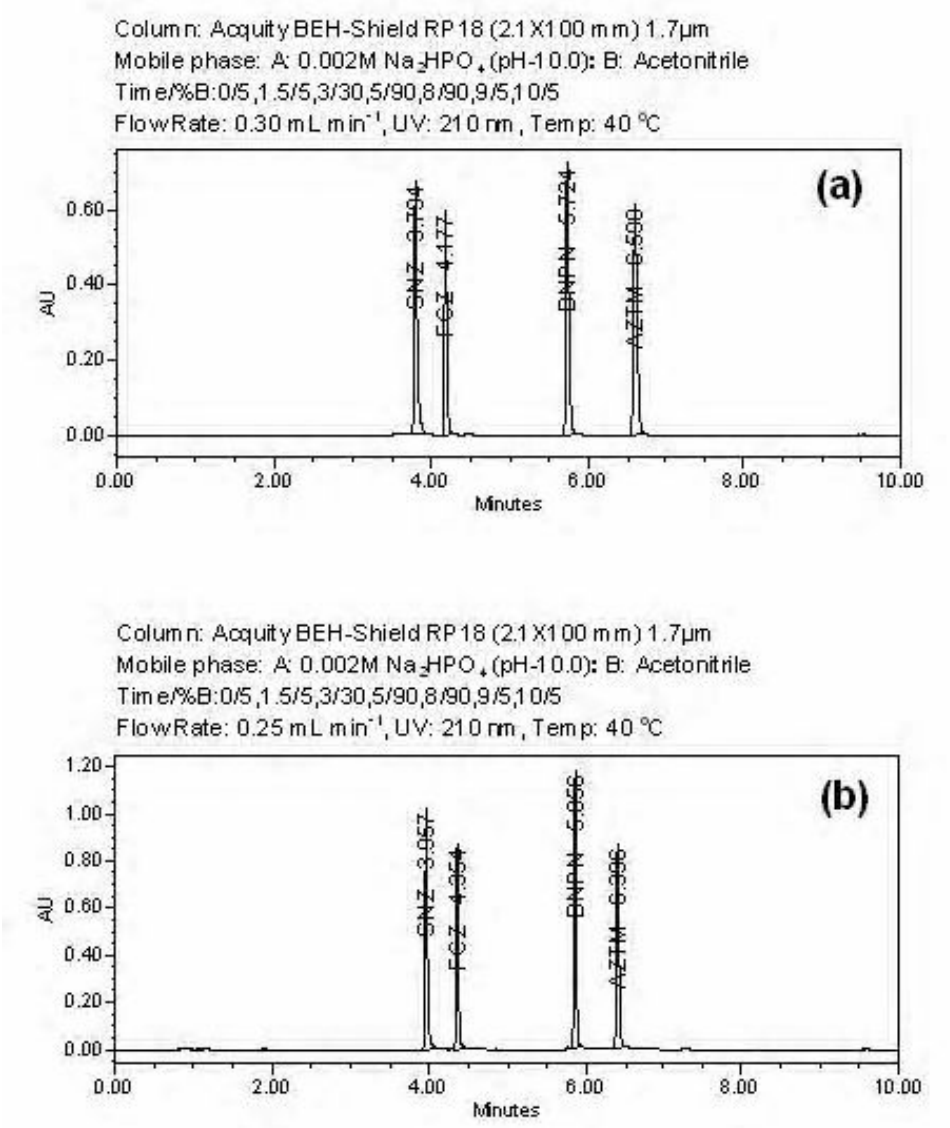


Fig. 2. Representative chromatograms of secnidazole, fluconazole, benzophenone and azithromycin

Accuracy

The percentage recovery obtained for secnidazole, fluconazole, and azithromycin ranged from 95.0% and 105.0% in bulk drugs and in pharmaceutical dosage forms, respectively. The Accuracy test results are summarized in Table 1.

Solution Stability and Mobile phase Stability

No significant changes were observed in the content of secnidazole, fluconazole, benzophenone, and azithromycin during solution and mobile phase stability experiments. The stability data confirmed that sample solutions were stable up to 72 h when stored in a refrigerator between 2 to 8 °C temperature.

Specificity

Degradation was not found in acid hydrolysis and peroxide treatment samples, but found 31% and 25% of degradation in base hydrolysis for bulk drugs and dosage forms. Degradants that are formed have base line chromatographic resolution with main components and peak purity flags passes for all three bulk drugs and dosage forms. Hence the method is specific.

Detection and quantification limit

The analytical sensitivity of the method was anticipated from the signal to noise ratio 3:1 for LOD and 10:1 for LOQ. The minimum limits at which the analytes could be readily detected and quantified for three drugs were summarized in Table 1.

Robustness
Deliberate changes in chromatographic conditions (flow rate, column, and column temperature) resulted in R_s values >5.0 between secnidazole, fluconazole, benzophenone and azithromycin illustrating a good robustness of the method. The Robustness test results are summarized in Table 1.

***Serum drug analysis*:**
The availability of all three drugs from human serum was determined by the stated chromatographic conditions. Blood samples of healthy volunteers (age group between 23-26 years, non-smokers, and not taking any other medicines) were collected. Multiple blood samples were collected in evacuated glass tubes through an internal cannula placed in the forearm veins or directly from a vein. The blood was then slightly shaken and centrifuged at 3000 rpm for 15 min and the plasma was separated. The obtained plasma was processed as mentioned above and stored at -20°C for analysis. The assay results for serum samples are summarized in Table 3.

Conclusion

An accurate and reliable Reverse Phase UPLC method with UV detection was described for the separation and simultaneous quantification of secnidazole, fluconazole, and azithromycin on Waters Acquity UPLC. The buffer choice and pH 10.0 with 3% aq. NaOH solution were found to be crucial in the separation of these drugs. The method was fully validated and applied successfully to measure the three drugs in serum samples. A total chromatographic run time of 10 min allows application in routine and quality control analyses of combination kit product consisting of secnidazole, fluconazole and azithromycin dosage forms.

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