Validated Analytical method for the simultaneous estimation of Sulfamethoxazole and Pyrimethamine in Bulk and Combined Tablet Dosage Form by UHPLC-PDA

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ABSTRACT

A simple, accurate, precise, rapid, selective and reproducible RP-UPLC method was developed for simultaneous estimation of Sulfamethoxazole (SMX) and Pyrimethamine (PYM) in the pharmaceutical dosage form. Chromatographic separation was carried out using Acquity UPLC ($BEHC_{18}$1.7µm, 2.1x50 mm)column and mobile phase consists of phosphate buffer: acetonitrile (60:40 V/V ; pH 6.8). The flow rate was 0.5 mL/min and detection was set at 220 nm in UV detector. Retention time of SMX and PYM were 1.61 min and 3.02 min respectively. The method shows good linearity over the concentration range of 100-600 µg/mL SMX and 5-30 µg/mL PYM. The correlation coefficients for the calibration curve of sulfamethoxazole and pyrimethamine was found to be 0.999 and 0.999 respectively. The developed method was validated according to ICH guidelines.

Keywords: RP-UPLC, Sulfamethoxazole (SMX), Pyrimethamine (PYM), Validation.

INTRODUCTION

Ultra Performance Liquid Chromatography (UPLC) is a relatively new modern technique which gives an direction for liquid chromatography and it is applicable for particle having less than 2µm in diameter to acquire better resolution ,speed and sensitivity as compared with High-Performance Liquid Chromatography (HPLC). It uses fine particles and saves time and reduces solvent consumption. The UPLC system reduces analysis time up to nine time comparing to the conventional system using 5µmparticle packed analytical columns. In UPLC the separation is performed under tremendous pressures (up to 100 MPa is possible), but it has no negative impact on analytical column as well as other components of chromatographic system. Separation efficiency remains maintained and also it is even improved more[1-4].

Malaria is an infectious disease commonly found in the tropical countries. Eukaryotic plasmodium parasites (mainly *Plasmodium falciparum* and *Plasmodium vivax*) are the root cause of this deadly disease. The mode of transmission includes the bite of anopheles mosquitoes (Mojab, 2012; Dua *et al*., 1998) [5]

Sulfamethoxazole is an antibacterial drug which has been used to the treatment of various bacterial infections in humans and other species. It is the sulfonamide drug most commonly used by combination with trimethoprim for the treatment of urinary tract infections or with pyrimethamine for the treatment of Chloroquine-resistant Plasmodium falciparum malaria. Sulfamethoxazole abbreviated as SMX is 4-Amino-N-(5-methyl-3-isoxazolyl) benzenesulfonamide; N1-(5-Methylisoxazol-3-yl) sulfanilamide, Molecular formula $C_{10}H_{11}N_3O_3S$ and structuralformula shown in figure 1. It is slightly soluble in water (0.5 g/L) and benzene, slightly soluble in chloroform, diethylether, Isopropanol and soluble in ethanol and methanol, Melting point is 167 Cº(Gennaro, 1995, Rudy et al., 1973 and Budavari, 2000).[6]

Pyrimethamine (PYM), an antimalarial drug, works by blocking the biosynthesis of pyrimidines and purines, which plays an important role in DNA synthesis and cell multiplication. This is achieved by inhibiting the dihydrofolate reductase of plasmodia. It is chemically 5-(4-Chloro phenyl)-6-ethylpyrimidine-2,4-diyl diamine and structural formula shown in figure 1.(Meena and Sandhya, 2013).[7]

The literature study reveals that there are numerous analytical methods re-ported for quantification of SMX and PYM. The study includes UV spectrophotometry[18-16],HPLC[17-27], LC-MS/MS[28], and HPTLC [29].However,no methods were reported by UPLC till now.

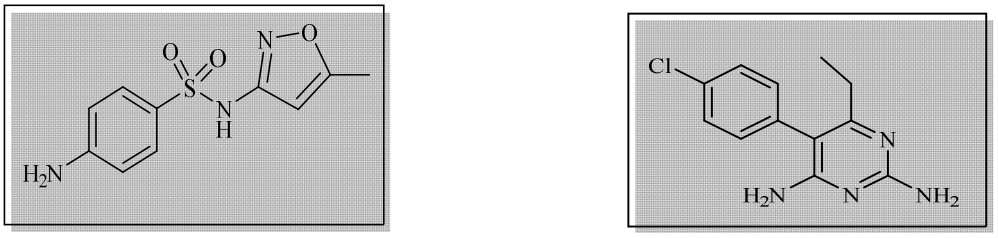

Fig 1: Structure of Sulfamethoxazole and Pyrimethamine

MATERIALS AND METHODS

Chemicals and reagents

Reference standard of SMX and PYM gift sample provided from Synthiya research lab private limited, Puducherry. Tablet used for analysis, P-KALFIN (label claim: 500mg of sulfamethoxazole and 25mg of Pyremethamine) were purchased from the local pharmacy in Chennai. HPLC grade acetonitrile and water were purchased from Merck. potassium dihydrogen phosphate & ortho-phosphoric acid were procured from Ranken.

Instrumentation

Chromatography was performed on UPLC Agilent technology-1200 infinity series with high speed auto sampler containing PDA detector. The chromatographic separation was achieved by using Acquity UPLC ($BEHC_{18}$ 1.7µm, 2.1x50 mm) column. Data acquisition and integration were performed using open lab CHEMSTATION software.

Chromatographic conditions

The chromatographic separation was achieved using Acquity UPLC ($BEHC_{18}$ 1.7µm, 2.1x50 mm)column with isocratic elution of mobile phase consists of mixture of phosphate buffer at pH 6.8 and acetonitrile (60:40 V/V) at a flow rate of 0.5 mL/min. The volume of sample solution injected was 20 µL and the total run time was 5mins. UV detection was done at 220 nm. The eluent was monitored using UV detector at a wavelength of 220 nm.

Preparation of buffer

10mM phosphate buffer was prepared by dissolving 1.36g of potassium dihydrogen orthophosphate in 1000 mL distilled water. Then pH was adjusted to 6.8 with ortho phosphoric acid and solution was filtered through 0.45µ nylon filter.

Preparation of mobile phase

Mobile phase was prepared by mixing 10mM phosphate buffer:Acetonitrile in the ratio of 60:40 and filtered through 0.45µnylonfilter.

Preparation of standard solution

Standard stock preparation

Standard stock solution of SMX and PYM were prepared separately in mobile phase of concentration 2000µg/ml and 1000µg/ml respectively. Working standard solution was prepared

by mixing 10 ml of SMX and 1 ml of PYM standard stock solution in 50 ml volumetric flask, diluted with the mobile phase to the mark.

Sample preparation

Transfer 60.15 mg of P-KALFIN formulation into a 100ml standard flask. About 50ml of mobile phase was added to this standard flask and sonicated in an ultrasonic bath for 15 min and then volume make up with same. The solution was filtered through 0.45µm nylon syringe filter.

RESULTS AND DISCUSSION

Method development

Variety of mobile phase was investigated in the development of a RP-UPLC method for the simultaneous estimation of Sulfamethoxazole (SMX) and Pyrimethamine (PYM). The system suitability was appropriate using Acquity UPLC ($BEHC_{18}$ 1.7µm, 2.1x50 mm)column with isocratic elution of mobile phase consists of mixture of phosphate buffer at pH 6.8 and acetonitrile (60:40 V/V) which results in the retention time of SMX and PYM were 1.61 min and 3.02 min respectively.

Method validation

The optimized method was validated as per ICH Q2 (R1)[31]and the following parameters were considered: system suitability, accuracy, precision, robustness, specificity, linearity, LOD and LOQ.

System suitability

System suitability was performed by six replicate injection of standard solution with the concentration of 20µg/mL of MF and 400µg/mL of MN was injected. The parameters like retention time, theoretical plate, resolution and peak area are shown in theTable1and Figure2.

Specificity

Specificity is the ability to check clearly the analyte in the presence of components which may expect to be present. Typically, these might include impurities, degradants and matrix. There was no interference from excipient and other component with the drug peak. So, the developed method has been found to be specific (Figure 2).

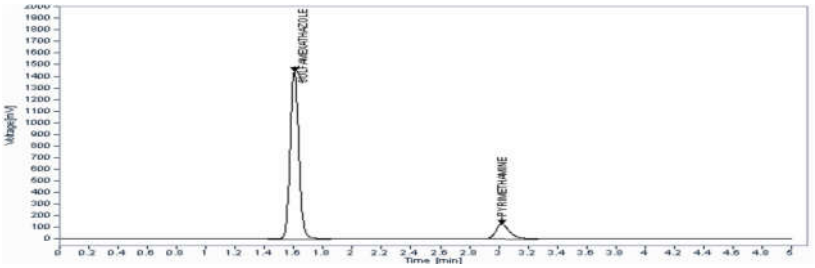

Figure2.UPLC chromatogram of Sulfamethoxazole and Pyrimethamine

Table 1: System suitability parameters for Sulfamethoxazole and Pyrimethamine

Name	**Retention time**	**Peak area** ± SD	**Theoretical plates** ±SD	**Asymmetry** ± SD
Sulfamethoxazole	1.61	6023.448	3571.58	0.918
Sulfamethoxazole	1.60	5880.107	3640.04	0.929
Sulfamethoxazole	1.60	5873.124	3603.65	0.922
Sulfamethoxazole	1.60	5905.951	3620.60	0.913
Sulfamethoxazole	1.59	5890.538	3568.71	0.916
Pyrimethamine	3.02	824.266	5165.11	0.699
Pyrimethamine	3.01	829.389	5359.78	0.732
Pyrimethamine	3.03	827.304	5303.10	0.737
Pyrimethamine	3.00	837.734	5185.19	0.742
Pyrimethamine	2.99	827.035	5315.52	0.736

Linearity

The linearity of the method was performed by preparing the concentration range of 5-30µg/mL and 100.-600µg/mL for SMX, PYM, from standard stock solution. Calibration curves were constructed by plotting concentration versus area of SMX and PYM.

Table 2. Regression characteristics determined by the proposed method

Parameters	**Sulfamethoxazole**	**Pyrimethamine**
Linearity range (µg/ml)	5-30	100-600
Slope	14.325	42.813
Intercept (a)	263.37	1.253
Correlation coefficient (r^2)	0.999	0.999
Regression equation: A = a + bc, where A is the absorbance, a is the intercept, b is the slope and c is the concentration.		

Accuracy

The accuracy was calculated by the analysis of tablet and standard at low,medium and high con centration level.The accuracy was estimated from three replicate injections and calculated as the µg/mL drug recovered from the drug matrix.The method is found to be accurate and results are summarized in table 3 & 4.

Table 3: Accuracy table of Sulfamethoxazole

Drug	Accuracy level	Amount of drug (µg/mL)	Quantity added (mg)	Recovered (µg/mL) ± SD (n=3)	% Accuracy ± SD (n=3)
Sulfamethoxaole	50%	400	5	4.925	99.92
	100%	400	10	9.836	99.78
	150%	400	15	14.604	98.78

SD: Standard deviation, n = 3

Table 4: Accuracy table of Pyrimethamine.

Drug	Accuracy level	Amount of drug (µg/mL)	Quantity added (mg)	Recovered (µg/mL) ± SD (n=3)	% Accuracy ± SD (n=3)
Pyrimethamine	50%	20	0.25	0.257	100.22
	100%	20	0.50	0.506	98.69
	150%	20	0.75	0.769	100.04

SD: Standard deviation, n = 3

Precision

The precision of the proposed assay method was assessed by analyzing standard solution of 400 µg/mL of SMX and 20 µg/mL of PYM for six times and calculate the % RSD. The precision of test method results are displayed in Table 5

Table 5.Precision Study of Sulfamethoxazole

S.No	System precision	Method precision	Intermediate precision
1	5760.461	6125.437	6167.931
2	5743.031	6074.125	6170.465
3	5756.569	6073.124	6252.388
4	5778.487	6134.846	6246.909
5	5793.133	6092.334	6246.232
Mean	5766.336	6099.973	6216.785
SD	19.613	28.774	43.52
%RSD	0.34	0.47171	0.70

Table 6.Precision Study of Pyrimethamine

S.No	System precision	Method precision	Intermediate precision
1	755.544	826.64	830.051
2	754.110	818.18	849.053
3	758.130	810.46	849.356
4	746.049	816.46	852.302
5	749.233	829.44	840.003
Mean	752.613	820.32	844.153
SD	4.893	7.0648	9.13
%RSD	0.65	0.927	1.08

Robustness

The robustness of a method was analysed by changing experimental, chromatographic condition. Altering in flow rate (0.6±1% mL/min), changes in column oven temperature (40±5 °C), Changes in mobile phase buffer pH (3.5±0.2),changes in mobile phase composition and changes in wavelength allowable limits from actual chromatographic condition. It was noted that there was norecognizable change in mean RT and RSD and parameters fell within the limitof ≤ 2. The theoretical plate, tailing factor, resolution was found to be good of SMX and PYM. This method is robust with variability condition.

Solution stability

Stability of sample solution was confirmed by storing it at ambient temperatur e for 15hrs.The assay of Sulfamethoxazole and Pyrimethamine were analysed. It was found that percentage labeled amount of Sulfamethoxazole at 5,10 and 15 hours were 100.79, 100.54 and 100.06 respectively; Percentage labeled amount of Pyrimethamine at 5, 10 and15 hours was 100.87, 100.62,and 100.05respectively.

CONCLUSION

The major supremacy of the UPLC method is significant saving in run time. Based on the study reports of the present research work, it is obvious that the developed method also had a very short noticeable reduction in the total runtime. In addition, it is a very simple and a novel method in the midst of commercial applicability. The current developed method offers a lot of advantages over the others like speedy acquisition of results, remarkable savings in operational cost and short, sharp retention time with good resolution. Moreover ,the results of the validation studies indicated that the developed RP-UPLC method is simple, accurate and robust.The validated data by ICH guidelines also confirms the effectiveness of the developed method for quantitative analysis of SMX and PYM in bulk and pharmaceutical dosage form.

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