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METHOD DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATIONOF SULFAMETHOXAZOLE AND TRIMETHOPRIM ORAL SUSPENSION USP

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ABSTRACT
A brand-new, regulatory-based HPLC technique has been created and approved for use in the USP oral suspension assay of trimethoprim and sulfamethoxazole.Using waters X-bridge shield C-18, 4.6 mm x 100 mm, 3.5 µm, HPLC separation was accomplished buffer pH 5.5 and methanol (75:25) were used in the mobile phase proportion, with a flow rate of 1.2 ml/min. The 220 nm wave length, 20 µL injection volume, 50°C column temperature, and 25°C sampler temperature were used for the detection. Sulfamethoxazole and trimethoprim had retention times of roughly 2.2 and 2.9 minutes, respectively.Run time about 8 minutes. According to ICH criteria, the developed procedures have undergone validation. The technique demonstrated satisfactory results in terms of robustness, ruggedness, linearity, specificity, and precision. The USP approach and the suggested procedure were proven to be equal**.**

Key words: HPLC, Method validation, Method development, ICH, Oral Suspension

INTRODUCTION
A complex separation method called HPLC is utilized to isolate, elucidate, and quantify the components. [1-2]. The current study aims to develop a new in-house analytical approach to determine the the Sulfamethoxazole/Trimethoprim in Oral Suspension. Sulfamethoxazole chemically known as 4-Amino-N-(5-methylisoxazol-3-yl)-benzene sulfonamide. Trimethoprim chemically known as 5-(3,4,5-Trimethoxybenzyl)pyrimidine-2,4-diamine. A crucial stage in the synthesis of bacterial folate is inhibited by the bacteriostatic sulfonamide antibiotic sulfamethoxazole. It is typically administered in conjunction with trimethoprim, an inhibitor of dihydrofolate reductase that prevents dihydrofolic acid from being reduced to tetrahydrofolic acid. A prescription drug called trimethoprim is used to treat bacterial infection symptoms. One can use trimethoprim by itself or in combination with other drugs. Trimethoprim is an element of the antibiotic medication class. Co-trimoxazole belongs to the group of drugs known as sulfonamides and is a combination of trimethoprim and sulfamethoxazole. Sulfamethoxazole has the physical appearance of a white to pale yellow, crystalline powder that is sparingly soluble in ethanol, easily

soluble in acetone, and nearly insoluble in water. It dissolves in diluted acids and sodium hydroxide solutions. Trimethoprim is a white, crystalline powder with no smell. Trimethoprim is soluble in water, DMSO, dimethylacetamide, benzyl alcohol, propylene glycol, chloroform, methanol, ether and benzene. [3-5]A quick review of the literature revealed the absence of RP-HPLC Stability indicating techniques for trimethoprim and sulfa drugs analysis.(6-9]. A new RP-HPLC method for simultaneous determinations of trimethoprim and sulfamethoxazole has been attempted to be developed.[10-13] Many approaches based on biological studies have been published for the estimation of these medications both on their own and in combination with other medications in pharmaceutical dosage forms.[14-15]. In consideration of the literature review A new RP- HPLC with a regulatory basis An in-house technique for analysing trimethoprim and sulfamethoxazole in oral suspension has been developed.

MATERIALS AND METHODS

Drugs and Chemicals

Sulfamethoxazole and Trimethoprim RS, procured from sigma Aldrich. HPLC grade Monobasic potassium phosphate, Orthophosphoric acid, Sodium hydroxide, Methanol and Milli-Q water were obtained from Merck.

Instrumentation

An HPLC for waters (E-2695 – PDA 2996) that uses Empower -3 software to operate. Column used waters X-bridge shield C-18, which measures 4.6 mm by 100 mm and 3.5 µm. The mobile phase is degassed and the standard is dissolved using a PCI sonicator. The materials were weighed using an electronic balance made by Mettler Toledo. Met Rohm pH metres were used for all pH adjustments. The centrifuge used was a Remington. MobilePhase

Buffer preparation: Precisely measured 1.36 g of monobasic potassium phosphate into an appropriate receptacle. Pipetted 1.0 ml of triethylamine into the same container after adding roughly 1000 ml of water. After thoroughly mixing, sonicated to dissolve. After using diluted sodium hydroxide solution or orthophosphoric acid to adjust the pH to 5.5±0.05, the mixture is filtered through a 0.45µm membrane filter.

Mobile Phase preparation: Transferred 750 ml of buffer pH 5.5 and 250 ml of methanol intosuitable container. Mixed well and sonicated.

Diluent Preparation

Used mobile phase as diluent.

Standard Preparation

Preparation of Standard Stock solution for Sulfamethoxazole: After precisely weighing and transferring around 25 mg of Sulfamethoxazole RS into a 25 mL volumetric flask, 10 mL of methanol was added, and the mixture was sonicated to dissolve it. diluted with diluent to volume and thoroughly mixed. (Concentration of about 1000 µg/mL of Sulfamethoxazole).

Preparation of Standard Stock solution for Trimethoprim : Weighed accurately and transferred about 25 mg of Trimethoprim RS into a 100 mL volumetric flask, added about 10ml methanol and sonicated to dissolve. Diluted to volume with diluent and mixed well. (Concentration of about 250 µg/mL)

Preparation of Working Standard Preparation: Pipetted out 4.0 mL of stock standard solution into a 50 mL volumetric flask. Diluted to volume with diluent, Mixed well (Concentration of about 100 µg/mL of Sulfamethoxazole and 20 µg/ml of Trimethoprim).

Sample Preparation

Stock Sample Preparation: Measured the sample density using one bottle of oral suspension containing trimethoprim and sulfamethoxazole. Using the pipette that is attached to the 5 ml syringe, removed the 5 ml of suspension from the top, middle, and bottom of the sample. Accurately weighed, approximately 5.0 ml of the oral suspension of trimethoprim and sulfamethoxazole was transferred into a 100 ml volumetric flask, 35 mL of methanol was added, and the mixture was sonicated for 15 minutes with periodic shaking to dissolve the material.

Diluted with diluent to volume, thoroughly mixed. The sample solution was centrifuged for ten minutes. (Concentration of about 2000 µg/mL of Sulfamethoxazole and 400 µg/mL of Trimethoprim).

Preparation of working Sample Preparation: Pipetted out 5.0 mL supernatant sample stock solution into a 100 mL volumetric flask. Diluted to volume with diluent, Mix well. Filtered through 0.45µm nylon filter discarding first (4) ml of filtrate (Concentration of about 100 µg/mL of Sulfamethoxazole and 20 µg/mL of Trimethoprim).

Chromatographic conditions

Waters X Bridge shield C-18, Chromatographic separation was performed using 4.6 mm x 100 mm, 3.5 µm. The pH 5.5 buffer and methanol were combined to form the mobile phase 75:25), which had a 08-minute run period and a 1.2 ml/min flow rate. 220 nm is the detection wavelength. It was discovered that the retention times of trimethoprim and sulfamethoxazole were 2.9 and 2.2 minutes, respectively. Thermostatically controlled column oven set to 50°C, 20 µl injection volume, and a 25°C sampler temperature are maintained.

METHOD VALIDATION

The developed method was validated in terms of linearity, specificity, precision, accuracy, limit of detection, limit of quantitation, robustness and system suitability testing as per the ICH guidelines [16].

System Precision

System precision established by injecting standard solutions in five replicates. Relative standard deviation of peak area responses of Sulfamethoxazole and Trimethoprim for five replicate injections of the standard solution was calculated and reported.(Table 1)

Method Precision

Precision of the assay method was determined by injecting, six individual sample preparations of Sulfamethoxazole and Trimethoprim . The samples were prepared as per the method. Reported with Relative standard deviation for assay. (Table 1)

Method Accuracy

Accuracy study was performed by spiking the known amount of standard in different concentrations (40% to 160%) along with placebo. The Individual and average recovery was calculated and reported with Relative standard deviation. (Table 1)

Linearity and range

The HPLC system was calibrated for linearity by injecting different quantities of Sulfamethoxazole (100 µg/mL) and Trimethoprim (20 µg/mL), which ranged from 40% to
160% of the standard concentration. From 40% to 160%, the linearity graph was displayed. provided with a correlation coefficient report. (Table 1)

Ruggedness

Ruggedness of the method performed by analyst-2 on different day from method precision. Standard and sample solutions prepared and injected. Reported with Relative standard deviation for assay and system suitability parameters were calculated. (Table 1)

Solution stability

Solution stability study was performed at different days against the freshly prepared standard, and sample solution and the results were represented .The standard and sample solutions were prepared and injected. Replicate injections of the standard and sample solution were made at the following time intervals, Initial, 30-hrs, 52-hrs for standard and Initial, 29-hrs, 50-hrs for sample stored at 25°C temperature. The %difference in % assay from initial and time point for both standard and sample solution were generated. (Table 1)

Specificity

Specificity of the method can be studied in the presence of excipients, degradation products and impurities. Blank, standard and stressed sample solutions were prepared and injected into the

chromatographic system for identification and impurity interference with the respective API peaks.(Table 2)

Specificity by Forced Degradation:

Intentional drug product degradation is achieved through the use of forced degradationexperiments. These investigations evaluate an analytical method's capacity to measure a drug product and its breakdown products independently. Drug product is exposed to acid, base, oxidizing agent, heat and UV light degradation. The degraded samples were then analysed using the method to determine if there are interferences with the active. Thus, stability- indicating property was evaluated. (Table 2)

Robustness

Robustness of the method evaluated by varying chromatographic method parameters such as column temperature ± 5°C, Mobile phase pH± 0.2 and Flow rate ± 0.2 ml/min from the optimized conditions. Injected Standard solution into the chromatographic system. System suitability results were reported. (Table 3)

RESULTS AND DISCUSSION

Table 1 : Method Validation Results

Parameter	Sulfamethoxazole	Trimethoprim	AcceptanceCriteria
System precision (Five replicates)	% RSD - 0.0 USP Tailing – 1.0	%RSD - 0.1 USP Tailing – 1.2	% RSD NMT 2.0% USP Tailing NMT 2.0
Method precision (Average of six preparations)	% RSD = 0.8 %	% RSD = 1.0 %	% RSD NMT 3.0%
Method Accuracy	Accuracy 40% - 100.1 Accuracy 100% - 99.1 Accuracy 160% - 98.1	Accuracy 40% - 101.0 Accuracy 100% - 100.1 Accuracy 160% - 100.0	% Recovery 97.5% to 102.5%
Linearity and Range (Correlation co-efficient)	1.000	1.000	Corr.coefficient R^2 = NLT 0.999
Ruggedness (Average of six preparations)	% RSD - 0.6 USP Tailing – 1.1	%RSD – 0.3 USP Tailing – 1.0	% RSD NMT 3.0%
Solution stability (at 25°c) (Standard)	52 Hrs (Complies)		% Assay NMT 2.0 % from Initial
Solution stability (at 25°c) (Sample)	50 Hrs (Complies)		% Assay NMT 2.0 % from Initial

System precision parameters such % RSD of peak area responses and USP tailing factor found within limit. Method precision result shows repeatability of the method.% Recovery found within the acceptance range. Linear over the concentration range of 40% to 160% of assay concentration. Ruggedness study exhibited reproducibility of the developed method. Solution Stability data demonstrated the sample and standard solutions stable up to 50 hours and 52 hours respectively at 25°C. Above all the results reported in **Table 1.**

Table 2: Specificity Results

		Sulfamethoxazole		Trimethoprim	
S.no	Sample name	Purity angle	Purity Threshold	Purity angle	Purity Threshold
01	Acid	0.177	1.019	0.443	1.165

02	Base	0.164	1.013	0.430	1.159
03	$Kmno_4$	0.165	1.007	0.393	1.045
04	Heat	0.155	1.009	0.462	1.168
05	UV	0.128	1.005	0.465	1.127

In forced degradation study Sulfamethoxazole and Trimethoprim treated with different stress conditions such as Acid stress, base stress, peroxide stress, UV and Heat.In all conditions Purity threshold found more than the purity angle. It's highy showing the specificity of the method mentioned in **Table 2**.

Table 3 : Robustness Results

		Sulfamethoxazole			**Trimethoprim**		
Method Parameters		**Retention Time**	**%RSD**	**USP Tailing**	**Retention Time**	**%RSD**	**USP Tailing**
Normal Condition	Column Temp: 50°C Mobile phase pH: 5.5 Flow rate 1.2 mL/Min	2.261	0.0	1.0	2.901	0.1	1.2
Column Temp. Minus	45°C	2.285	0.1	0.9	3.039	0.1	1.1
Column Temp. Plus	55°C	1.993	0.0	0.9	2.89	0.1	1.1
Mobile phase pH Minus	5.3	2.270	0.3	0.9	2.484	0.3	1.1
Mobile phase pH Plus	5.7	1.942	0.1	1.1	3.088	0.1	1.1
Flow rate Minus	1.0 mL/Min	2.498	0.0	0.9	3.518	0.0	1.1
Flow rate Plus	1.4 mL/Min	1.863	0.1	0.9	2.567	0.1	1.1

Robustness study results showed the developed method not affected by the small changes. Method had undergone with Column compartment temperature change, mobile phase pH changes and flow rate changes Results showed in **Table 3.**

CONCLUSION

The current study used a newly developed in-house HPLC analytical method to estimate the USP 200 mg/40 mg of Sulfamethoxazole/Trimethoprim oral suspension per 5 millilitres.The method for the assay of sulfamethoxazole/trimethoprim oral suspension USP is determined by the aforementioned studies' findings, and it is found to be linear across the concentration range, specific, accurate, exact, robust, and robust. When kept at a temperature of 25°C, standard and sample solutions remain stable for up to 52 hours for the former and 50 hours for the latter. The internal method's results meet regulatory standards, ICH recommendations, and validation parameters.

CONFLICT OF INTEREST

None

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