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Development and Validation of a New RP-HPLC method for the simultaneous estimation of Sulfamethoxazole sodium and Trimethoprim in finished tablet dosage form

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A B S T R A C T

The main aim and objective of the recent research work was development of a new, precise, rapid, accurate RP-HPLC method for the simultaneous Estimation of Sulfamethoxazole sodium and Trimethoprim in finished tablet dosage form. After optimization the good chromatographic separation was achieved by Isocratic and gradient mode with a mixture of Ammonium Acetate buffer pH 5.8 Adjusted with 1ml of Orthophosphoricacid, ACN: Buffer (400:600) v/v as the mobile phase with Octadecylsilan Rp Aqueous-AR-5 (250 x 4.6 mm, 5 µm), column as stationary phase at flow rate of 1.0 mL/min and detection wavelength of 210 nm. The Retention time of Sulfamethoxazole sodium and Trimethoprim was found to be 3.170 & 4.377 min. The linearity of this method was found in the concentration range of 50-150 µg/mL. The correlation coefficient R^2 value is found to be 0.999&0.995. The LOD for this method was found to be 0.0003µg/mL. The LOQ for this method was found to be 0.0009 µg/mL. This method was found to be good percentage recovery about 99.77% indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard with the sample so, the method specifically determines the analyte in the sample without interference from excipients of formulation. The method was extensively validated according to ICH guidelines for Linearity, Range, Accuracy, Precision, Specificity and Robustness.

Keywords: UV spectrophotometer, Sulfamethoxazole sodium and Trimethoprim, RP-HPLC

A R T I C L E I N F O

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1. Introduction

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Often a time lag exists from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs [1, 2].

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization (ICH) guidelines (Q2A and Q2B) [3,4]. Method development is a continuous process that progresses in parallel with the evolution of the drug product. The goal and purpose of the method should reflect the phase of drug development. During early drug development, the methods may focus on API behavior. They should be suitable to support preclinical safety evaluations, pre-formulation studies, and prototype product stability studies. As drug development progresses, the analytical methods are refined and expanded, based on increased API and drug product knowledge. The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines. Scouting experiments are frequently performed during method development to establish the performance limits of the method, prior to formal validation experiments. These may include forced degradation studies, which are an integral part of development of a stability-indicating method. API is typically subjected to degradation by acid, base, peroxide, heat, and light. This allows for a determination of the capability of the method to separate and quantify degradation products, while providing insight into the main mechanisms of degradation. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients [5].

In method development, an attempt to select the best chromatographic conditions like the best column, the best mobile phase, the detection wavelength etc. to be used for routine analysis of any drug is done. For the method development by HPLC method some information about the sample is very essential i.e. number of components present in the sample, pKa values of different components, UV-Visible Spectra of each analyte, solubility in different solvents, concentration range of each component, nature of sample etc. Prior to method development there must be some technical information i.e. chromatography method selection according to the sample properties, the sample when analyzed with HPLC, the condition where all compounds elute in a reasonable time, optimization of HPLC method with regard to analysis time, resolution, selectivity and sensitivity [1].

2. Experimental

Instruments and chemicals: The following instruments were used to carry out the research work: UV-Visible Spectrophotometer (Analytical Technologies Ltd), HPLC (SHIMADZU)gradient, HPLC(SHIMADZU) Autosampler, Ultra sonicator (Citizen, Digital Ultrasonic Cleaner), pH meter (Elico), Electronic balance (Shimadzu), Syringe (Hamilton), HPLC Column (Octadecylsilane Rp Aqueous-AR-5 (250 x 4.6 mm , 5µm). All the chemicals which were used such as Ammonium Acetate, Acetonitrile, Water, Ethanol, Orthophosphoric acid, Tetrahydrofurol, Potassium dihydrogen phosphate, Ammonium dihydrogen phosphate, Sodiu, dihydrogen phosphate Sodium hydroxide, Chloroform, Ether (95%) were AR grade. Drugs such as Sulfamethoxazole sodium and Trimethoprim bulk drugs. Gift samples were obtained from Natco Pharma Ltd, HYD and Sulfamethoxazole sodium and Trimethoprim dosage form were obtained from local pharmacy.

Method of validation [6-13]

Preparation of standard solution sulphamethoxazole sodium & trimethoprim

Weigh and transfer **800** mg of Sulphamethaxazole sodium working standard and 160 mg of Trimethoprim working standard into 100 mL volumetric flask, add 60 mL of diluent and sonicate to dissolve and dilute to volume with diluent. Transfer 10 mL of standard stock solution into 100 mL volumetric flask and dilute to volume with diluent.

Precision

Standard stock solution preparation:

Weigh and transfer 800 mg of Sulphamethaxazole sodium working standard and 160 mg of Trimethoprim working standard into 100 mL volumetric flask, add 60 mL of diluent and sonicate to dissolve and dilute to volume with diluent.

Standard preparation

Transfer 10 mL of standard stock solution into 100 mL volumetric flask and dilute to volume with diluent.

Sample Preparation:

Finely grind pre weighed 20 tablets. Transfer grinded Sample quantitatively equivalent to 800 mg of Sulphamethaxazole sodium and 160 mg of Trimethoprim in to 100 mL volumetric flask add 60 mL of diluent,

sonicate to dissolve for 10 minutes and dilute to volume with diluent. Further filter the solution through 0.45µ filter paper. Dilute 10 ml of filtrate to 100 ml with mobile phase.

System precision

Finely grind pre weighed 20 tablets. Transfer grinded Sample quantitatively equivalent to 800 mg of Sulphamethaxazole sodium and 160 mg of Trimethoprim in to 100 mL volumetric flask add 60 mL of diluent, sonicate to dissolve for 10 minutes and dilute to volume with diluent. Further filter the solution through 0.45µ filter paper. Dilute 10 ml of filtrate to 100 ml with mobile phase. The peak areas were noted down and %RSD were calculated.

Method precision: Finely grind pre weighed 20 tablets. Transfer grinded Sample quantitatively equivalent to 800 mg of Sulphamethaxazole sodium and 160 mg of Trimethoprim in to 100 mL volumetric flask add 60 mL of diluent, sonicate to dissolve for 10 minutes and dilute to volume with diluent. Further filter the solution through 0.45µ filter paper. Dilute 10 ml of filtrate to 100 ml with mobile phase. Peak areas were noted down .Average, Standard deviation, %RSD were calculated

Accuracy

Accuracy is a measure of the closeness of test results obtained by a method to the true value. Accuracy indicates the deviation between the mean value found and the true value. It is determined by applying the method to samples to which known amounts of analyte have been added. It should be analyzed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay.

Standard stock solution preparation

Weigh and transfer 100 mg of Sulphamethaxazole sodium working standard and 50 mg of Trimethoprim working standard into 100 mL volumetric flask, add 60 mL of diluent and sonicate to dissolve and dilute to volume with diluent.

Standard preparation

Transfer 10 mL of standard stock solution into 100 mL volumetric flask and dilute to volume with diluent.

Preparation of 100% Solution

Finely grind pre weighed 20 tablets. Transfer grinded Sample quantitatively equivalent to 100 mg of Sulphamethaxazole sodium and 50 mg of Trimethoprim in to 100 mL volumetric flask add 60 mL of diluent, sonicate to dissolve for 10 minutes and dilute to volume with diluent. Further filter the solution through 0.45µ filter paper. Dilute 10 ml of filtrate to 100 ml with mobile phase.

Preparation of 120% Solution

Finely grind pre weighed 20 tablets. Transfer grinded Sample quantitatively equivalent to 120 mg of Sulphamethaxazole sodium and 60 mg of Trimethoprim in to 100 mL volumetric flask add 60 mL of diluent, sonicate to dissolve for 10 minutes and dilute to volume with diluent. Further filter the solution through 0.45µ filter paper. Dilute 10 ml of filtrate to 100 ml with mobile phase.

Preparation of 140% Solution

Finely grind pre weighed 20 tablets. Transfer grinded Sample quantitatively equivalent to 140 mg of Sulphamethaxazole sodium and 70 mg of Trimethoprim in to 100 mL volumetric flask add 60 mL of diluent, sonicate to dissolve for 10 minutes and dilute to volume with diluent. Further filter the solution through 0.45µ filter paper. Dilute 10 ml of filtrate to 100 ml with mobile phase.

Procedure

Inject each concentration in to the chromatographic system and measure the peak area. .Plot the graph of peak area on y axis versus concentration on x axis. calculate the correlation coefficient .

3. Results and Discussion

Determination of working wavelength (λmax)

In simultaneous estimation of two drugs isobestic wavelength is used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are interconvertible. So this wavelength is used in simultaneous estimation to estimate both drugs accurately. The wavelength of maximum absorption (λ max) of the solution of the drugs in mobile phase were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400nm against mobile phase as blank. The absorption curve shows characteristic absorption maxima at 209nm for Sulfamthoxazole sodium. Thus 209nm was selected as detector wavelength for the HPLC chromatographic method.

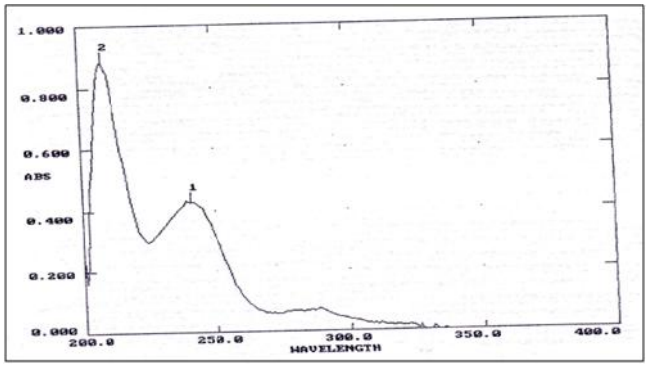

Figure 1: UV-VIS Spectrum of Sulfamethoxazole sodium and Trimthoprim.

Method development for Assay:

Trial -1

Chromatographic conditions

Column : Zodiac Rp Aqueous-AR-5 (250 x 4.6 mm, 5 µm)
Elution mode : Gradient.
Mobile phase : phosphate buffer pH 5.8: ACN [40:60]
Flow rate : 1.0 mL /min
Detection wavelength : 210 nm
Injection volume : 20 µL
Run time : 6 min

Observation

The two peaks are not well resolved. Resolution was found to be less than 2 . So this trial is not considered.

Trial -2

Chromatographic conditions

Column : Develosil Rp Aqueous-AR-5 (250 x 4.6 mm, 5 µm)

Elution mode : Isocratic
Mobile phase : ACN:Methanol: Buffer pH 5 (200:400:400)
Flow rate : 1.0 mL/min
Detection wavelength : 210 nm
Injection volume : 20 µL
Run time : 6 min
Mixed standard solution is used for recording chromatogram.

Observation

It was observed that theoretical plates are very low. So this trial was not considered.

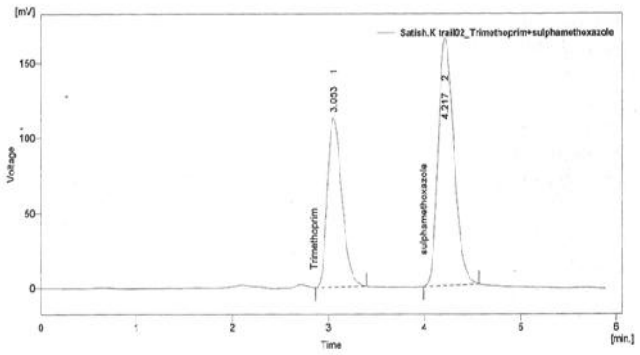

Figure 2: Chromatogram of Trial 2

Table 2: Results for Trial 2

Result Table (Uncal - Satish.K trail02_Trimethoprim+sulphamethoxazole)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	3.053	1187.405	112.209	38.7
2	4.217	1878.850	164.257	61.3
	Total	3066.255	276.466	100.0

Column Performance Table (From 50% - Satish.K trail02_Trimethoprim+sulphamethoxazole)

	Reten. Time	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	3.053	0.167	1.629	1859	37187	-
2	4.217	0.183	1.415	2931	58613	3.912

Trial -3

Chromatographic conditions
Column: Develosil Rp Aqueous-AR-5 (150 x 4.6 mm , 5 µm)
Elution mode:Isocratic
Mobile phase: Ammonium Acetate buffer pH4.5: Acetonitrile (985:15)
Flow rate : 1.5 mL/min
Detection wavelength : 244 nm
Injection volume : 20 µL
Run time : 10 min
Mixed standard solution is used for recording chromatogram.

Observation

The Sulphamethaxazole sodium and Trimethoprim peak was observed at 3.410 min with peak area 2001568, theoretical plates 3396 and tailing factor 1.51. The Theoritical plates, tailing factor and resolution was found to be within limits. So this trail was considered and validated according to ICH guidelines.

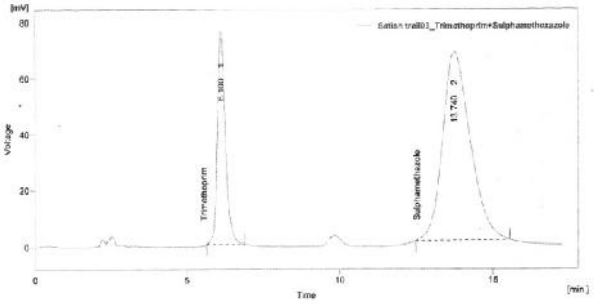

Figure 3: Chromatogram of Trail 3

Table 1: Results for Trial 3

Result Table (Uncal - Satish trail03_Trimethoprim+Sulphamethoxazole)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.100	1392.386	75.935	24.8
2	13.740	4212.724	67.162	75.2
	Total	5605.110	143.098	100.0

Column Performance Table (From 50% - Satish trail03_Trimethoprim+Sulphamethoxazole)

	Reten. Time	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	6.100	0.273	1.492	2759	27592	-
2	13.740	0.957	1.491	1143	11428	7.310

Table 2: Optimized Trial

Mobile phase	**Potassium di hydrogen phosphate buffer pH 5.8:ACN (600:400)**
Column	Octadecylilan C18 (250 x 4.6 mm , 5 µm)
Flow rate	1.0 mL/min
Column temperature	Room temperature(20-25^{o}C)
Sample temperature	Room temperature(20-25^{o}C)
Wavelength	210 nm
Injection volume	20 µL
Run time	6 min
Retention time	4.377 min for Sulphamethaxazole sodium and Trimethoprim

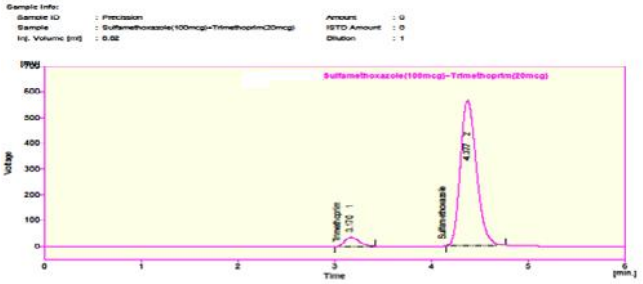

Figure 4: Chromatogram of Sulphamethaxazole sodium and Trimethoprim

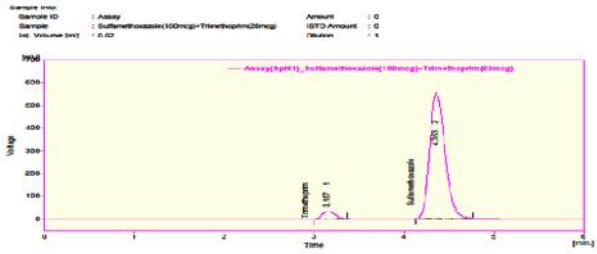

Figure 5: Chromatogram of Sulphamethaxazole sodium and Trimethoprim.

Table 3: Results for Sulphamethaxazole sodium and Trimethoprim.

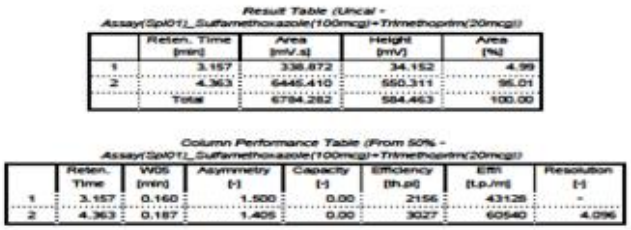

Table 4: Results of assay

Drug	Label claim(mg)	Amount found(mg)	% Assay
Sulphamethaxazole sodium and Trimethoprim	240	239.94	99.9

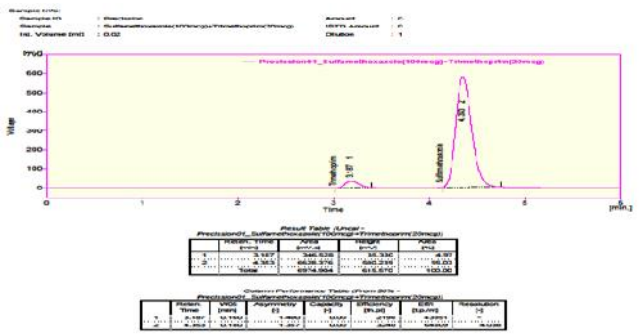

Figure 6: List of Chromatograms for system precision

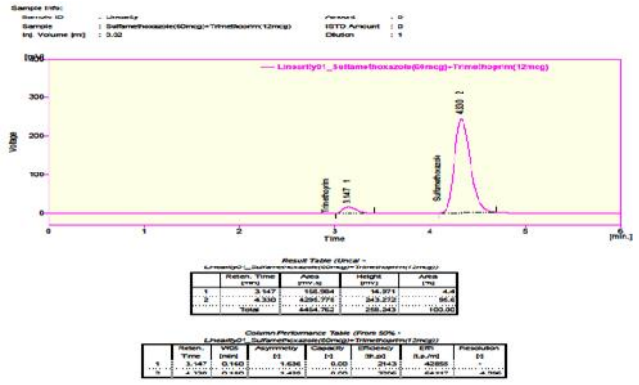

Figure 7: List of Chromatogram of linearity for preparation 1

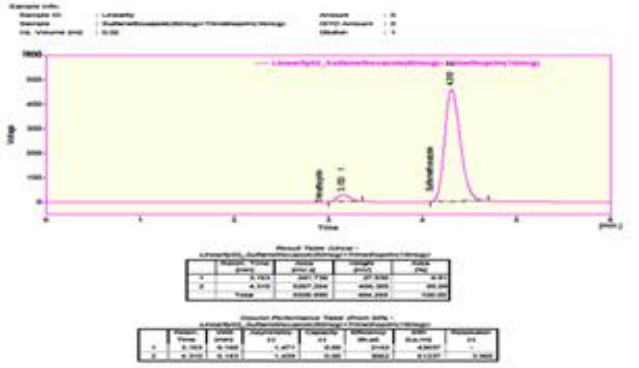

Fig 8: Chromatogram of linearity for preparation 2

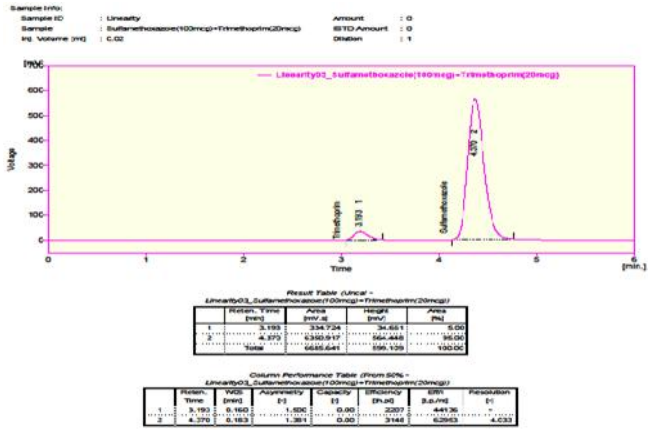

Fig 9: Chromatogram of linearity for preparation 3

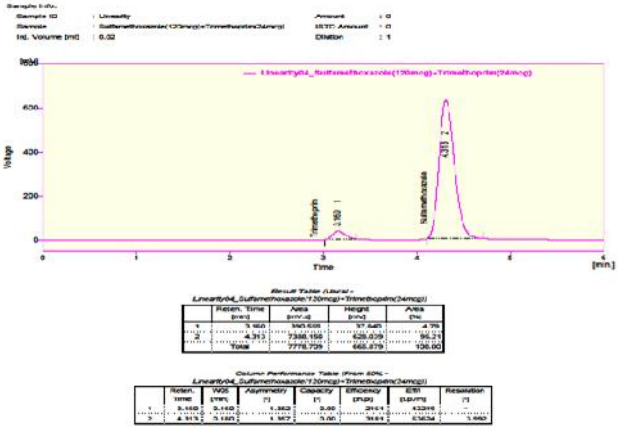

Fig 10: Chromatogram of linearity for preparation 4

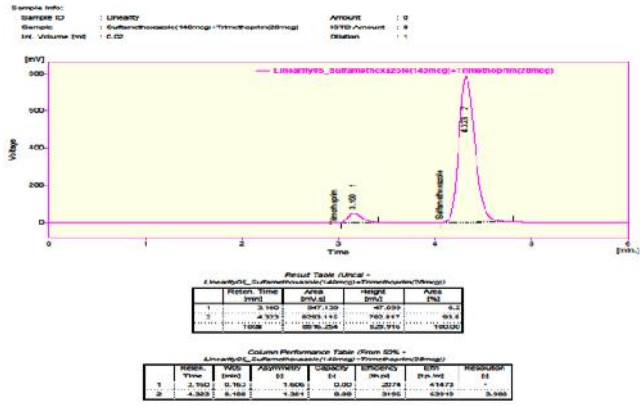

Figure 11: Chromatogram of linearity for preparation 5

A graph was plotted against the concentrations of the solutions and the peak areas. The correlation coefficient R2 was determined and was found to be 0.998.

Table 5: Linearity data of Sulfamethoxazole sodium

S.No	Concentration (µg/mL)	Area
1	60	4295.778
2	80	5267.254
3	100	6350.917
4	120	7388.150
5	140	8293.115

Table 6: Linearity data of Trimethoprim.

S. No	Concentration (µg/mL)	Area
1	60	168.984
2	80	241.736
3	100	334.917
4	120	390.559
5	140	480.139

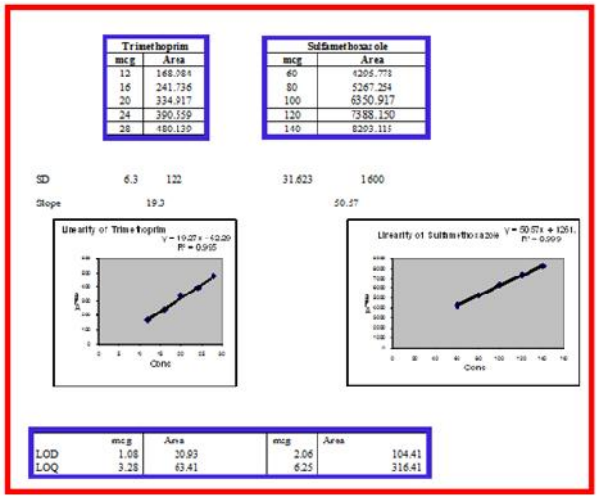

Figure 12: Graph for Linearity data of Sulphamethaxazole sodium and Trimethoprim

Table 7: Observation for linearity

S.No	Parameter	Sulphamethaxazole sodium & Trimethorim
1	Correlation coefficient	0.999&0.995
2	Slope	50.57&19.3
3	Intercept	5526

Observation

The correlation coefficient for linear curve obtained between concentration vs. Area for standard preparations of Sulphamethaxazole sodium and Trimethorim was found to be 0.998 respectively.

Specificity

The standard solution 100 µg/mL of Sulphamethaxazole sodium and Trimethorim was injected and the chromatogram was recorded for. The sample solution 100 µg/mL of Sulphamethaxazole sodium and Trimethorim was injected and the chromatogram was recorded.

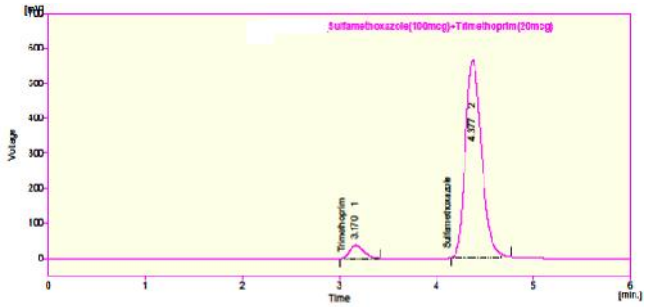

Table 13: Results for standard.

Figure 8: Chromatogram of standard

Name	RT	Area	TP	TF
Sulphamethaxazole sodium Trimethoprim	4.379& 3.190	6810& 177	3109& 1855	1.22

Table 9: Results for formulation.

Name	RT	Area	TP	TF
Sulphamethaxazole sodium and Trimethoprim	4.377& 3.170	6810& 197	3231& 1955	1.18

Observation: It was observed that diluent or excipient peaks do not interfere with analyte peak.

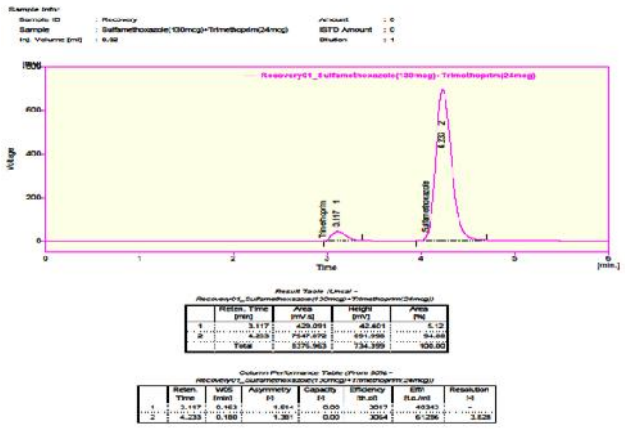

Figure 13: Chromatogram of 50% recovery

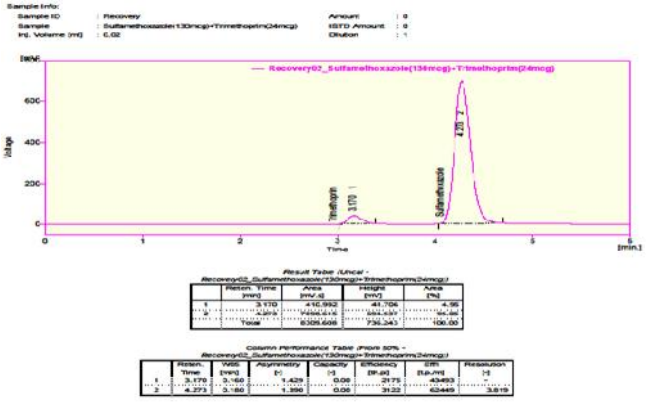

Figure 14: Chromatogram of 100% Recovery.

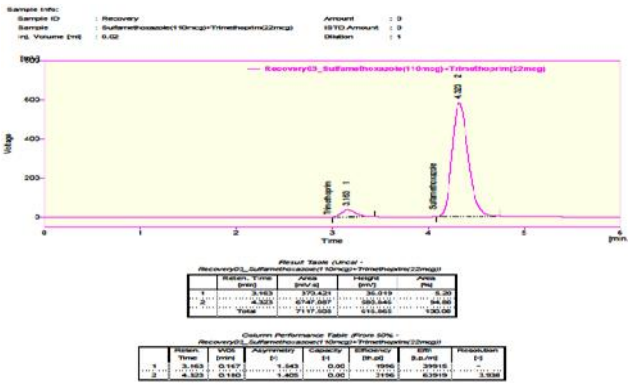

Figure 15: Chromatogram of 150% Recovery

Table 10: Results for Recovery of Sulphamethaxazole sodium and Trimethorim.

Conc	Amount present (µg/mL)	Amount added (µg/mL)	Amount found (µg/mL)*	Percent Recovery *	% Mean Recovery
50%	90	10	89.56	89.13	99.77
100%	110	10	108.02	106.02	
150%	130	10	127.27	126.18	

*Mean of three observations

4. Conclusion

A new precise, accurate, rapid method has been developed for the simultaneous estimation of Sulfamethoxazole sodium and Trimethoprim in pharmaceutical dosage form by RP-HPLC. The optimum wavelength for the determination of Sulfamethoxazole sodium &Trimethoprim was selected at 210nm. Various trials were performed with different mobile phases in different ratios, but Ammonium Acetate buffer pH 5.8: ACN: Buffer (600:400) was selected as good peak symmetry. The Retention time of Sulfamethoxazole sodium and Trimethoprim was found to be 6 min. The different analytical performance parameters such as linearity, precision, accuracy, and specificity, LOD, LOQ were determined according to International Conference on Harmonization ICH Q2B guidelines. The calibration curves were obtained by plotting peak area versus the concentration over the range of 50-150 µg/mL. From linearity the correlation coefficient R^2 value was found to be 0.998. The proposed HPLC method was also validated for system suitability, system precision and method precision. The % RSD in the peak area of drug was found to be less than 2%. The number of theoretical plates was found to be more than 2000, which indicates efficient performance of the column. The LOD for this method was found to be 0.0003µg/mL. The LOQ for this method was found to be 0.0009 µg/mL, indicates the sensitivity of the method. The percentage of recovery of was found to be 99.77 shows that the proposed method is highly accurate. Hence the proposed method is highly sensitive, precise and accurate and it successfully applied for the quantification of API content in the commercial formulations of Sulfamethoxazole sodium & Trimethoprim. in Educational institutions and Quality control laboratories.

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