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Development and Validation of RP-HPLC Method for Quantitative Analysis of Sulfamethoxazole and Trimethoprim in Liquid Suspension: A Comparative Study with Compendial Method

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ABSTRACT

Co-trimoxazole is a combination of trimethoprim or sulfamethoxazole. It is used to treat common infectious diseases, including the lung disorders, urinary disorders, and gastrointestinal infections. The current study was performed to develop a new RP-HPLC technique. The main purpose was to analyze SMX (Sulfamethoxazole) and TMP (Trimethoprim) in a liquid medium of 60mL. The analyses of SMX and TMP were performed on RP-HPLC with a C18 column (25 cm × 4.6 mm) packed with 5 µm ODS, L1 stationary phase, while the mobile phase consisted of methanol and water with a ratio of 6:4. The pH of the system was adjusted to 2.6 by using dilute phosphoric acid. The injection volume was 20µL having a flow rate of 1 mL/minute and column temperature of 40ºC. The analysis of all chromatograms was performed at a single wavelength of 254 nm. The validation of the method was determined for range, precision, linearity, specificity, accuracy, and system suitability. This method was found to be more environmentally friendly with respect to the other compendial methods, which are used for the TMP and SMX analysis.

Keywords: co-trimoxazole, chromatogram, RP-HPLC, sulfamethoxazole, trimethoprim

1. INTRODUCTION

Sulfamethoxazole (SMX) is an antibiotic drug that belongs to sulfanilamides [1]. It is utilized to address a range of health conditions that are generated within the body [2, 3]. These antibiotics can be provided to the body either through the mouth or by penetration through the injection [4]. They are quickly absorbed by the body and eliminated through the kidneys [5]. It is 4-amino–*N*-(5-methel-1,2-oxazol-3-yl)benzene-1-

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sulfonamide. It is insoluble in water but easily soluble in acetone and sparingly soluble in alcohol [5, 6]. Trimethoprim (TMP) is a known biological agent, which inhibits bacterial activity. It is used to treat bacterial infections of the respiratory and urinary systems [7]. It is 2,4-diamino-5-(3'4'5'-trimethoxy benzyl)pyrimidine. These are white and cream colour substances [8]. The antibiotic mechanism of TMP is enhanced by sulfonamides [9]. They are used to cure bacterial infections [10]. The phenomenon of supra-additive takes place by sulfonamides addition. This is also the most common addition of multiple combinations for two antibiotic medicines of sulphamides. They have synergic effects and there is a 5:1 ratio of trimethoprim and sulfamethoxazole, respectively [11]. Their combination is known as co-trimoxazole, which is mainly used to treat lung disorders like pneumonia, Coccidiosis, diarrhea, and gastroenteritis. They are also used for hyper-alimentation in the form of an aqueous solution. Their injections can cure bacterial infections in cattle and horses [12, 13].

Previous research has described that there are many analytical tools to determine SMX and TMP; either in individual form or in combination form [14]. A number of HPLC (high-pressure liquid chromatography) methods have been used for the estimation of TMP and SMX in pharmaceutical or biological samples [15]. Reverse phase high-pressure liquid chromatography (RP-HPLC) is the most used HPLC having 65-90%, respectively [16]. Reasonably, its extensive use is because of its features, which are unity and ease of its use for handling those substances, which have very diverse polarity [17, 18]. Compendial methods are also used to know the quality level of various medicinal products. These methods are not validated [18, 19].

In this research study, the RP-HPLC method was developed and its validity was checked for the identification of SMX and TMP as compared to the compendial methods.

2. MATERIALS AND METHODS

2.1. Reagent and Chemicals

Trimethoprims and sulfamethoxazole were bought from Shandong Rongyuan Pharmaceutical Company Limited, China and Andhra Organics Limited, India, respectively. The distilled water of 0.01 µS/cm conductivity was prepared in a laboratory. The source of methanol and orthophosphoric acid was Merck, Germany.

2.2. Instrumentation

The analyses were done by using the instruments including, Sonicator (Korea 60ºC), aluminumfoil (China 0.2 mm), hot plate (China 400ºC), analytical balance (Sartorius, Germany Min 0.0001g: Max 320 g), column (Merck Germany, C_{18}), nylon filters (Sartorius, Germany 0.45µ), USA pH 0-14, pH metre (Jenco), vacuum pump (Japan20psi), pH meter (Jenco 6173), Shimadzu LC-20AT Series with a dual pump, Dynamica, HALO DB-20 UV/Visible spectrophotometer, and 254 nm wavelength was used for analysis.

2.3. Collection of Samples

The standard molecules of SMX and TMP were taken from Andhra Organics Ltd. in India and Shandong Rongyuan Pharmaceutical Co., Ltd. in China with high purity levels of 99.44% and 99.66%, respectively, on an anhydrous basis.

2.4. Mobile Phase Preparation

The mobile phase was prepared by adding 600 mL of methanol in 400 mL of distilled water and adjusting the volume to the desired amount. The pH level of the solution was maintained at 2.6 level by adding a small amount of dilute phosphoric acid.

2.5. Standard Preparation

200 mg of sulfamethoxazole and 40 mg of trimethoprim were put into a flask of 100 mL. The mixture was diluted using methanol. A separate 50 mL volumetric flask was used to transfer 10 mL of this solution for further analysis.

2.6. Sample Preparation

To prepare the samples, 200 mg of SMX and 40 mg of TMP were added to a flask of 100 mL. From this solution, 10 mL was transferred to a 50 mL flask and the resulting solution was filtered. The concentration of TMP in the filtered solution was found to be 0.08 mg/mL and the concentration of SMX was found to be 0.4 mg/L.

2.7. Chromatographic System Configuration

The column used for analysis was 25 cm in length and 4.6 mm in diameter, it was also loaded with an ODS (octadecylsilyl) stationary phase

with a particle size of 5 µm, specifically the L1 type. The detector used in the analysis had a wavelength of 254 nm. The column was maintained at a temperature of 40°C and the flow rate of the mobile phase was set at 1 mL/minute. A sample injection volume of 20 µL was used for the analysis.

2.8. Method Validation

The developed method was validated through the following characteristics:

2.9. Linearity

Linearity is a measure of the relationship between the concentration of an analyte and the corresponding response of a measurement method. The coefficient of determination (r^2) of the regression line is used to determine the linearity of the method. For quantitative analysis, a value of r^2 greater than 0.99 generally denotes a strong linear connection between the analyte concentration and the observed response, which is considered acceptable. This indicates that the test results obtained by the technique will fall within a predictable range that is directly proportional to the analyte concentration that is being measured.

2.10. Specificity

The ability to dissociate analyte components in the presence of other components like matrix components is known as specificity [17]. If the method remained unaffected in the presence of impurities and exponents, it means that the method has specificity.

2.11. Accuracy

Accuracy is a measure of how closely an analytical method can determine the true value of a sample. An accurate method gave us an accurate value under different measurements. The accuracy of the method was shown because no divergence occurred from the true value. Accuracy reflects the degree to which an analytical method provides reliable and correct results that are free from significant errors or biases.

2.12. Precision

If the procedure is repeated multiple times for the samplings of a homogeneous sample and the results give closed values to each other, the analytical method is said to have precision.

3. RESULTS AND DISCUSSION

It is mandatory to analyze the drugs before their utilization. Analyzing data can provide both qualitative and quantitative information, which is crucially significant. Understanding the therapeutic mechanism of drugs is a vital aspect that cannot be overlooked. A number of methods are being used for simultaneous analysis of trimethoprim and sulfamethoxazole. FT-IR, amperometry, HPTLC, UV spectroscopy, and spectrometry were used for SMX and TMP analysis (see Table 1).

Table 1. Assay Calculations

Trimethoprim				Sulfamethoxazole			
Peak area of STD	Peak area of Sample			Peak area of STD		Peak area of sample	
70503891	7729721			28393293		28691409	
6940642	7688064			28513675		29049886	
Average = 7708893				Average = 28870648			
Average	6997267			Average		28453484	
S.D	80079.14			S.D		85122.93	
RSD	1.144			RSD		0.299	
Calculations of peak area of STD and sample							
Trimethoprim	7708893	37	10	100	25	5	99.67	100
	6997267	100	25	5	10	40	100	
	101.57%							
Sulfamethoxazole	28870648	200.4	10	100	25	5	99.80	100
	28453484	100	25	5	10	200	100	
	101.47%							

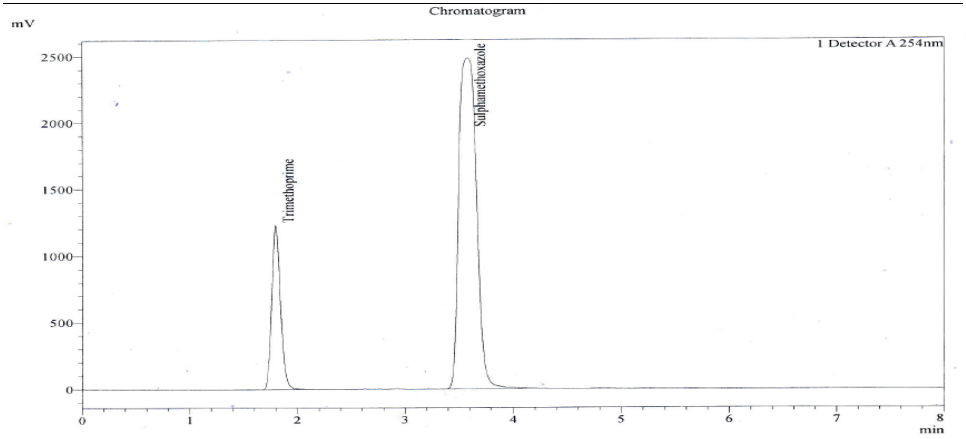

Figure 1. Chromatogram of TMP-SMZ Standard 1

Pharmaceutical industries specifically use RP-HPLC for conducting their analysis. The results obtained from the analysis of SMX and TMP are as follows (Figures 1 to 4; Tables 1-5).

Table 2. TMP-SMZ Standard 1 at 254 nm

Peak	Name	Area	Concentration	Tailing factor	Height	No. of theoretical plate
1	TMP	6940642	1.000	1.188	1229461	1969
2	SMZ	28393293	1.000	1.136	2483206	2819
Total		35333936			3712667	

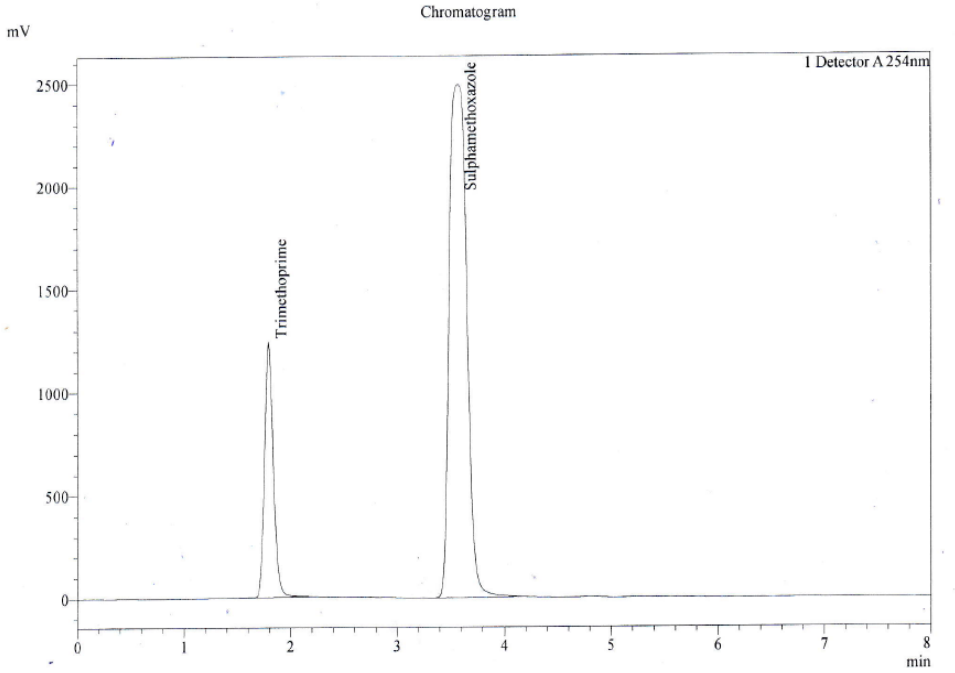

Figure 2. Chromatogram of TMP-SMZ Standard 2

Table 3. TMP-SMZ Standard 2 at 254 nm

Peak	Name	Tailing factor	Area	Height	Concentration	Number of theoretical plate
1	TMP	1.198	7053891	1233870	1.008	1948
2	SMZ	1.138	28513675	2488856	1.002	2797
Total			35567567	3722726		

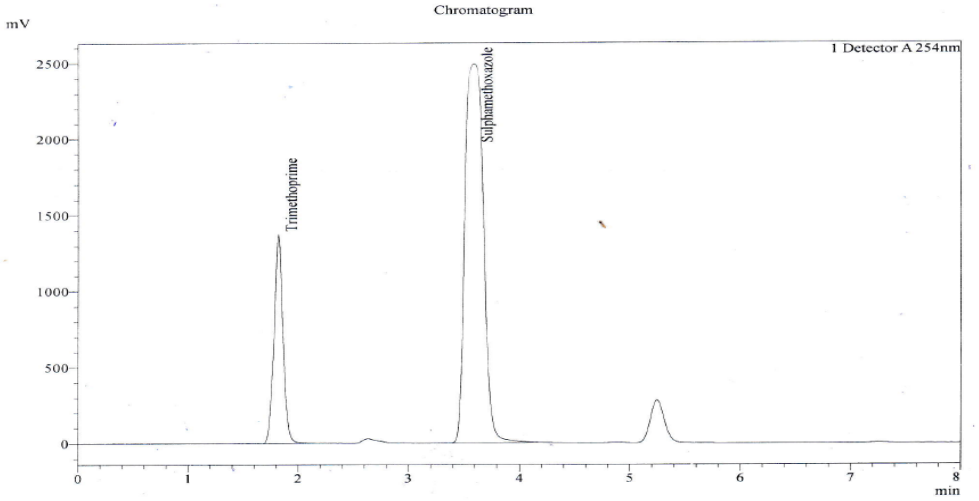

Figure 3. Chromatogram of TMP-SMZ Test Sample 1

Table 4. TMP-SMZ Test Sample 1 at 254 nm

Peak #	Name	Height	Area	Number of theoretical plate	Concentration	Tailing factor
1	TMP	1372780	7688064	2038	1.099	1.075
2	SMZ	2489631	2904986	2747	1.021	1.141
Total		3862411	3673790			

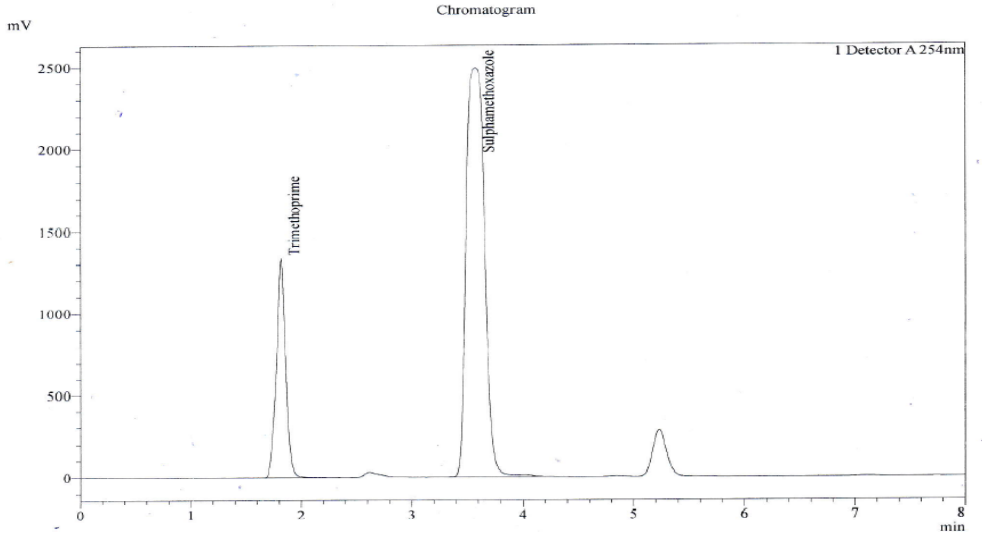

Figure 4. Chromatogram of TMP-SMZ Test Sample 2

Table 5. Results of TMP-SMZ Test Sample 2 at 254 nm

Peak	Name	Concentration	Area	Number of theoretical plate	Height	Tailing factor
1	TMP	1.105	7729721	1867	1333312	1.010
2	SMZ	1.008	28691409	2763	2488096	1.128
Total			36421130		3821408	

3.1. Method validation

3.1.1. Linearity. The method will show linearity if the results of the test are directly proportional to the quantity of the drug [18]. The percent concentrations of 80, 90, 100, 110, and 120 were injected, giving the following linearity results. Table 6 and Figure 5 & 6 show the results for the linearity.

Table 6. Linearity Values

Sr.#	Sample absorbance area		Solution volume (mL)	Conc. (mcg/mL)	% of drug
	Trimethoprim	Sulfamethoxazole			
1	2422329	16193821	25.6	4.0	80
2	2471764	16524307	28.8	4.5	90
3	2522209	16861538	32.0	5.0	100
4	2568653	17198768	35.2	5.5	110
5	2624106	17512744	38.4	6.0	120

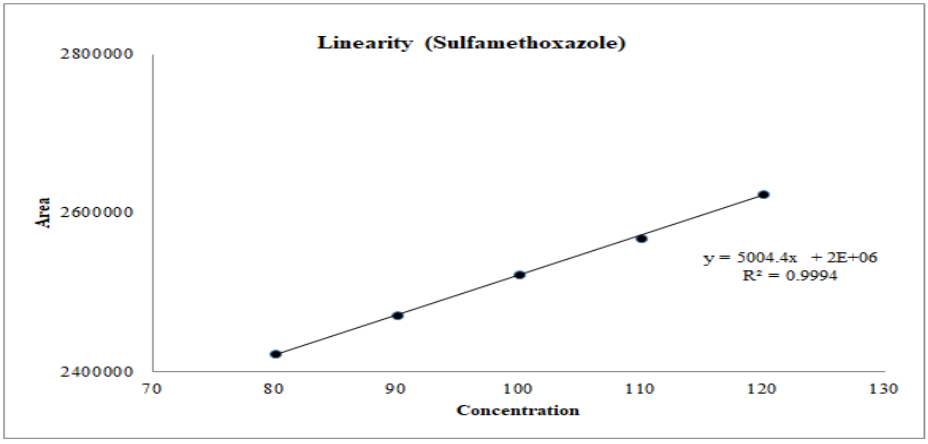

Figure 5. Linearity (Sulfamethoxazole)

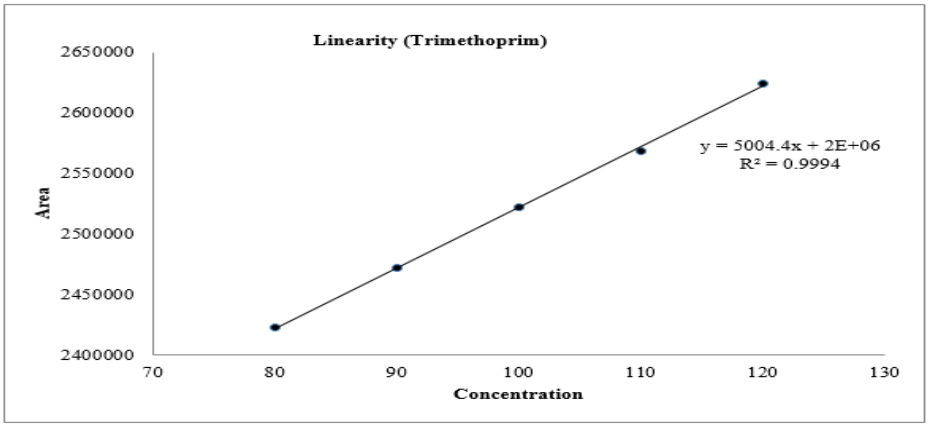

Figure 6. Linearity (Trimethoprim)

3.1.2. Specificity. Specificity is the ability of a solution in which components of the matrix dissolve the analyte. In the case of standard and placebo, the following results were obtained (see Table 7).

Table 7. Specificity

Sample	Standard samples (TMP/ SMX)	Placebo
Chromatogram (Peak Area / Spectrum)	Positive	Negative

3.1.3. Accuracy. Various trials were carried out to confirm the accuracy of this newly developed approach and the results are presented in Tables 8 and 9.

3.1.4. Sulfamethoxazole Results. Absorbance area of 80% reference: 16184214

Absorbance area of 100% reference: 16853525

Absorbance area of 120% reference: 17532744

Table 8. Accuracy Results for Sulfamethoxazole Samples

In placebo percentage of active	120%		100%		80%	
Absorbance area of test solution	17524535	17545738	16870344	16880168	16205637	16190974

Label claim (%)	99.95%	100.04%	100.10%	100.16%	100.13%	100.07%
Deviation from calculated results	0.05%	0.07%	0.10%	0.16%	0.13%	0.04%
Average recovery	100.01%		100.13%		100.08%	

Table 9. Accuracy Results for Trimethoprim Samples

TMP Content added in placebo	120%		100%		80%	
Absorbance area of test solution	2624684	2628955	2524891	2528142	2419311	2421047
Label claim (%)	99.98%	100.14%	100.07%	100.20%	99.96%	100.03%
Deviation from calculated results	0.02%	0.14%	0.07%	0.20%	0.04%	0.03%
Average Recovery	100.06%		100.13%		99.99%	

3.1.5. Precision. If there is a degree of closeness between the individual test findings, the procedure has precision. The following results were obtained for instrument precision (see Table 10).

Table 10. Instrument precision

Sr. No.	Area of Internal Standard (Sulfamethoxazole)	Areas of standards (Trimethoprim)
1.	16852548	2499147
2.	16850018	2498046
3.	16861538	2522209
4.	16841827	2546895
5.	16791588	2506789
RSD	0.1645%	0.8137%
S.D	27692.63	20461.58
Mean	16839503.8	2514617.2

3.2. Method Precision

It was expressed as RSD and found out by:

3.2.1. Repeatability. The assay was performed on separate samples in 3 replicate sets (see Tables 11 and 12).

3.2.1.1. Sulfamethoxazole Results. Absorbance area of the reference: 16835314

Concentration of reference: 32 µg/ml

Wavelength: 254 nm

3.2.1.2. Trimethoprim Results. Absorbance area of the reference: 2519356

Concentration of reference: 32 µg/ml

Wavelength: 254 nm

Table 11. Repeatability of Sulfamethoxazole Samples

Samples		Repeatability (Sulfamethoxazole)			
		Absorbance Area	Analyte concentration (µg/ml)	%results	Deviation from calculated results
I	1	16794517	32	99.76	0.24%
	2	16840138	32	100.03	0.03%
	3	16841974	32	100.04	0.04%
II	4	16863183	32	100.17	0.17%
	5	16874201	32	100.23	0.23%
	6	16806357	32	99.83	0.17%

SD = 0.1849; Average = 100.01%; RSD = 0.1849%

Table 12. Repeatability of Trimethoprim Samples

Samples		Repeatability (Trimethoprim)			
		Analyte concentration (µg/ml)	% results	Area of absorbance	Deviation from calculated results
1I	1	32	100.04	2520302	0.04%
	2	32	99.96	2518346	0.04%
	3	32	100.44	2530556	0.44%

Samples		Repeatability (Trimethoprim)			
		Analyte concentration (µg/ml)	% results	Area of absorbance	Deviation from calculated results
III	4	32	100.41	2529654	0.41%
	5	32	99.91	2517178	0.09%
	6	32	100.2	02524356	0.20%

Average = 100.16%; SD = 0.2280; RSD = 0.2277%

3.2.2. Reproducibility. The term reproducibility describes how effectively an analytical process may be used by many analysts working in the same laboratory. The following results were obtained when the assay was performed on three distinct samples as indicated in (see Tables 13 and 14).

3.2.2.1. Sulfamethoxazole Results. Absorbance area of the reference: 16813456

Concentration of reference: 32 µg/ml

Wavelength: 254 nm

3.2.2.2. Trimethoprim Results. Absorbance area of the reference: 2516209

Concentration of reference: 32 µg/ml

Wavelength: 254 nm

Table 13. Reproducibility of Sulfamethoxazole Samples

Samples	Reproducibility			
	Analyte concentration (µg/ml)	% results	Absorbance area	Variation from theoretical results (%)
1	32	99.97	16808945	0.03
2	32	100.12	16832952	0.12
3	32	100.10	16829435	0.10

SD = 0.0771; RSD = 0.0770%; Mean = 100.06%

Table 14. Reproducibility of Trimethoprim Samples

Samples	Reproducibility			
	Analyte concentration (µg/ml)	% results	Absorbance area	Fluctuation from theoretical results (%)
1	32	100.49	2528516	0.49
2	32	100.11	2519063	0.11
3	32	100.23	2521964	0.23

Mean = 100.28%; SD = 0.1924; RSD = 0.1919%

The RP-HPLC method developed in this research study was found to be the most effective method among all, which covers all the analytical techniques [15–17]. It was also observed that this technique was better than compendial methods since it is free of pollution, which showed that it is environmentally friendly. The mobile phase is methanol: water (6:4). The pH of the mobile phase was maintained at 2.6 level by using dilute phosphoric acid. The combined determination of Sulfamethoxazole and Trimethoprim requires this pollution-free analysis.

4. CONCLUSION

The current study concluded that the newly developed RP-HPLC method was cost-effective and convenient to use. It also fulfilled all the validation parameters like linearity, specificity, accuracy, and precision. The primary significance of this method lies in its environmentally friendly nature and suitability for our environment. Further studies can be used for the determination of impurities in samples using the LC-MS technique and future researchers can further establish their research by using this study results.

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