LIQUID CHROMATOGRAPHIC TECHNIQUE FOR THE SIMULTANEOUS DETERMINATION OF SULPHAMETHOXAZOLE AND TRIMETHOPRIM IN PHARMACEUTICAL FORMULATIONS

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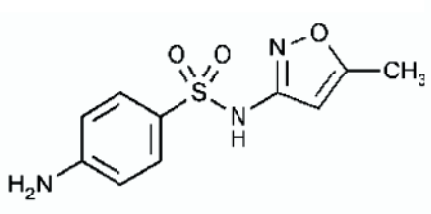
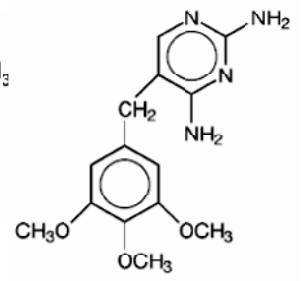
ABSTRACT
This paper describes the development and validation of a simple, specific, precise, and accurate Liquid Chromatographic method for the simultaneous determination of sulphamethoxazole and trimethoprim in pharmaceutical dosage forms. The chromatographic resolution was achieved with 50mM sodium phosphate buffer and acetonitrile (85:15) on a reversed phase column - Octyldecylsilane C18 column (100 x 4.6 mm, i.d., 5µm) - at ambient temperature. The flow rate through the column was 1ml/min and the UV detection was at 260nm using Agilent HPLC 1100LC System. The mean retention times for trimethoprim and sulphamethoxazole were 2.998 and 6.205 minutes respectively. Calibration curves were rectilinear over the ranges 5–80 mg/L (trimethoprim) and 25–400 mg/L (sulphamethoxazole). The RSD was less than 2.61% and percentage recovery was between91.93% - 103.98% with respect to precision and accuracy. The method has been used to analyze brands of cotrimoxazole tablets. The percentage content of sulphamethoxazole and trimethoprim were found to be comparable with BP 2002 requirement. This method, which has a fair run time of 6 minutes, is cost effective for routine analytical work and for quality control and product monitoring.

Keywords: Sulphamethoxazole, Trimethoprim, HPLC, Pharmaceutical formulations

INTRODUCTION
Sulphamethoxazole [A] belongs to the sulphonamide group of antibiotics with intermediate-acting antimicrobial property (Brayfield, 2011) while trimethoprim [B] is a drug substance used as an antimicrobial agent.



[A]



[B]

The combination of sulphamethoxazole and trimethoprim is effective in the treatment of a variety of infections including pneumonia, prostatitis, urinary tract infections and some non-tuberculous mycobacterial infections. Sulphamethoxazole inhibits bacterial synthesis of dihydrofolic acid by competing with p-aminobenzoic acid. Trimethoprim blocks the production of tetrahydrofolic acid by binding to and reversibly inhibiting the required enzyme, dihydrofolatereductase (Givianrad and Mohagheghian, 2012).

There is need to ascertain the chemical and biological equivalence of antibiotics due to global health problem posed by antibiotic and multi-drug resistance (Kasim *et al*., 2012). Therefore, sensitive, accurate, and precise analytical methods are continually made available for the direct measurement of drugs in given sample matrices. Such measurements are generally validated so that accurate information is generated for pharmacokinetic and clinical monitoring (Harmita *et al*., 2012)

High performance liquid chromatographic (HPLC) analysis is a general approach for the determination of sulphamethoxazole (SMX) and trimethoprim (TMP) since it provides adequate sensitivity and precision for monitoring therapeutic steady state concentration (Behzadian *et al.*, 1998). Several works have been done on the determination of sulphamethoxazole and trimethoprim either independently or simultaneously in combined dosage forms or in environmental and biological samples(*Kasim et al.,* 2012*; Ghanem and Abu-Lafi*, 2013; *Gonzalez et al.,* 2015).The physicochemical and *in-vitro* bioavailability equivalence of six brands of co-trimoxazole tablets marketed in Tigray, Ethiopiahas been reported (*Haliu et al.*, 2011). The highest and the lowest TMP contents of the tablets evaluated using HPLC were 99.93 and 98.59% while the highest and the lowest SMX contents were101.25 and 100.01% respectively.

A reversed phase-liquid chromatography for the simultaneous analysis of trimethoprim, sulphamethoxazole, and acetylsulphamethoxazole in small amount of blood has been reported (Renn*et al*., 1999). The mobile phase consisted of acetonitrile and phosphate buffer (20/80) but a low sample throughput was reported. In a related work, Rezaee*et al* (2000) reported the use of 25 mM sodium phosphate buffer and acetonitrile for the determination of trimethoprim and sulphamethoxazole drug combinations in dosage forms with a run time of 16 minutes.

Various methods including spectroscopy, fluorimetry, polarography and HPLC have been used to analyse anti-microbial drugs. These methods require not only derivatization but also elaborate sample preparation, expensive equipment, and complicated solvent switching technique (Okine *et al*., 2006; Rade*et al*., 2008). The official UV spectroscopic method of analysis involving acetone and chloroform extraction of the active components of co-trimoxazole (sulphamethoxazole and trimethoprim) has been found to be quite laborious for routine laboratory analysis (British Pharmacopeia, 2000). This research work is designed to develop a simple, sensitive, accurate, and reproducible HPLC

analytical method for the simultaneous determination of sulphamethoxazole and trimethoprimin pharmaceutical formulations without any pre-extraction or elaborate derivatisation.

EXPERIMENTAL

Chemicals and Reagents

Pure standards of trimethoprim (99.89% purity) and sulphamethoxazole (100.67%) were provided by House of CHI Pharmaceuticals, in care of Hydrochrom Resources, Lagos, Nigeria. HPLC grade acetonitrile solvent was obtained from Chromasolv, a registered trademark of Sigma-Aldrich (Germany). HPLC grade methanol solvent was obtained from Scharlab S.L. Sentimenat, Spain. Sodium dihydrogenorthophosphate ($NaH_2PO_4.2H_2O$), and orthophosphoric acid (H_3PO_4) were of analytical grade. Highly purified (distilled) water was used throughout the analysis. Samples of cotrimoxazole tablets normal strength (label claim: sulphamethoxazole 400 mg/Trimethoprim 80 mg) and double strength (label claim: sulphamethoxazole 800 mg/Trimethoprim 160 mg) were purchased from Pharmacy outlets in Lagos, Nigeria.

Instrumentation and Chromatographic Conditions

The HLPC system used was Agilent HPLC 1100LC System (Agilent Technology) consisting of degasser – LC019, quartenary pump, autosampler – LC019 all Agilent 1100 series; LC- 019 column compartment of HP 1100 series, LC019 VWD (a double beam variable wavelength detector) detector and Agilent Technology Software: Agilent Chem. Station for LC/LC-MS.The HPLC experimental conditions were optimized on the octadecylsilane C_{18} chemically bonded column (100 x 4.6 mm i.d., 5 µm particles) from YMC Company Limited,Japan. The mobile phase composition was a combination of 50 mM NaH_2PO_4 buffer (adjusted to pH of 3.0 with 15.2 M H_3PO_4) and acetonitrile.

Selection of mobile phase

The optimum mobile phase ratio was prepared by mixing 1700 ml of phosphate buffer with 300 ml acetonitrile (85:15 v/v) in a 2000 ml volumetric flask, and sonicated for few minutes to expel bubbles and was then allowed to equilibrate at room temperature. About 1000 ml of the mixture was transferred into the solvent bottle (isocratic mode). A wavelength of 260 nm was chosen (most appropriate for the determination of the two active ingredients). The flow rate used was 1ml/minute. The injection volume was 10 µl at a column temperatureof 28^{o}C.This was achieved on an octyldecylsilane C_{18} column (100 x 4.6 mm, i.d. 5µm) using 50mM sodium phosphate buffer (adjusted to pH 3.0 with H_3PO_4) and acetonitrile (ACN) as the mobile phase. Various mixtures/ratios of 50mMNaH_2PO_4(pH 3.0) and acetonitrile were prepared and evaluated in combination of NaH_2PO_4(pH 3.0): ACN - 50:50, 60:40, 70:30, 75:25, 85:15 and 90:10 (v/v).

Preparation of standard solutions

The stock solution of the mixed standard of sulphamethoxazole and trimethoprim was prepared by weighing and dissolving 50 mg of sulphamethoxazole and 10 mg trimethoprim (5:1) in HPLC grade methanol solvent in a 50 ml-standard flask and made up to the mark with methanol as diluents to produce mixed standard containing 1000 mg/L of sulphamethoxazole and 200 mg/L of trimethoprim. Working standard solutions of the sulphamethoxazole/trimethoprim combination (400 mg/L /80 mg/L, 300 mg/L /60 mg/L , 200 mg/L /40 mg/L, 100 mg/L /20 mg/L, 50 mg/L /10 mg/L and 25 mg/L /5 mg/L) were prepared from the mixed standard stock solution in a 25 ml standard flask by taking 10, 7.5, 5.0 and 2.5 ml respectively of the mixed stock standard and made up to the mark with the diluent. Aliquot (12.5 ml) was taken from 100 mg/L to prepare 50 mg/L /10 mg/L and 12.5 mL from 50 mg/L concentration to prepare 25 mg/L /5 mg/L.

Sample Preparation

Ten (10) tablets of two brands of commercial samples of cotrimoxazole tablets, Brand A and Brand B were separately weighed and powdered. A 502.07mg portion of the powdered Brand A, equivalent of an average weight of one tablet,was accurately weighed, dissolved in a 100 ml standard flask and made up to the mark with methanol (diluent). The working (appropriate) sample solution was prepared by diluting this sample solution in a ratio of 1:20 and was filtered using 0.45 µm Whatman filter paper. A 1000 mg of the Brand B sample, equivalent of an average weight of one tablet was accurately weighed, dissolved in a 100 ml standard flask and made up to the mark with methanol (diluent). The working sample solution required for the analysis was prepared by diluting the initial sample solution in a ratio of 1:40 using a standard flask and filtered via a 0.45 µm Whatman filter paper.

Procedure for Analysis

Ten (10) µL each of the prepared mixed pure standards was injected in triplicates into the HPLC. These were done starting from the lowest concentration through to the highest concentration and their respective peak areas were recorded and the chromatogram obtained was used to prepare standard calibration curves for the two compounds. The corresponding concentrations were extrapolated from the standard calibration curves prepared.

Method Validation

Linearity/Range

Various concentrations of the mixed pure standards were investigated and chosen with defined increments to determine the highest concentration and appropriate concentration most suitable conforming to Beer-Lambert law all done in an effort to assess the linearity of the proposed method.

Specificity
The mixed pure standards and the sample test solutions containing the active ingredients in the presence of other matrices were all injected repeatedly at the same wavelength of 260 nm to ascertain the specificity of the optimized method.

Sensitivity
This was achieved in terms of limit of detection (LOD) and limit of quantification (LOQ). Series of dilutions of the mixed standard (25mg/L) sulphamethoxazole and (5mg/L) trimethoprim) of known concentrations were injected to ascertain the peak height that gave signal to noise ratio of 10 (LOQ) and signal to noise ratio of 3 (LOD).

Precision
Precision was determined through repeatability studies. Precision was determined in six replicate injections of SMZ (300 mg/L and TMP (60 mg/L) standard solutions and test sample solutions of 200 mg/L / 40mg/L on the same day (intra-day precision). The peak areas of each injection were recorded and statistically evaluated (Subrata *et al., 2011*).The results were expressed as %RSD of the measurements.

Accuracy and Recovery
Accuracy was determined through recovery studies. A 10ml aliquot of the test sample was set aside and its actual concentration determined before spiking. This same sample was spiked with 1mlknown amount of the pure sulphamethoxazole/trimethoprim (1000/200 mg/L) mixed standards. This spiked sample was injected into the HPLC and the actual concentration compared with the expected concentration.

RESULTS AND DISCUSSION

Selection of mobile phase
An optimised HPLC method for the simultaneous analysis of sulphamethoxazole and trimethoprim in pharmaceutical formulation was developed and validated. The ACN strength was investigated in the mobile phase combination of 50mM NaH_2PO_4(pH 3.0): ACN - 50:50, 60:40, 70:30, 75:25, 85:15 and 90:10 (v/v). The first three ratios gave run times below 3.5minutes and separation factor (α) less than 1.0 while the last three gave separation factors and run times of >1.0 and 4.5 min, >2.0 and 6.5 min, and >6 and 12 min respectively. The mobile phase ratio (isocratic mode) of 85:15 was chosen due to a fair run time and separation with respect to cost of analysis. The UV-detector wavelength of 254 nm, 260 nm and 270 nm were considered. The 260 nm and 270 nm wavelength gave the best detection for both active ingredients in the mixed combination, but the 260 nm was chosen due to reduced run time difference of 0.3 min.

Chromatographic Conditions

The optimum chromatographic (working) conditions were 50mM NaH_2PO_4**:** ACN mobile phase composition ratio of 85**:**15 (isocratic mode), 10 µL injection volume, and 260 nm wavelength of detection, flow rate 1ml/min and at ambient temperature. This gave good separation and resolution for the active ingredients (analytes) with the relatively more polar trimethoprim eluting before the relatively less polar sulphamethoxazole. The qualitative determination of trimethoprim and sulphamethoxazole gave individual retention times of 2.958 min and 6.654 min respectively. In their combined or mixed form, after proper resolution and separation, the mean retention times were 2.998±0.226 min for trimethoprim and 6.205±0.495 min for sulphamethoxazole, as shown in figures 1 to 4.

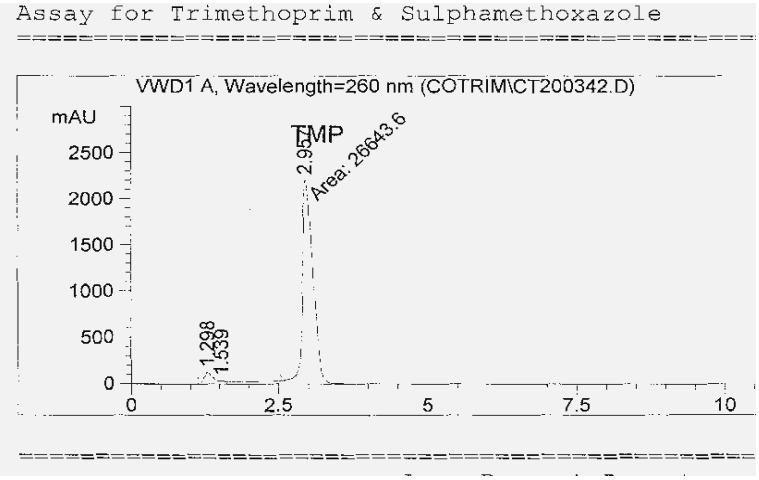


Figure 1: Chromatogram of trimethoprim pure standard

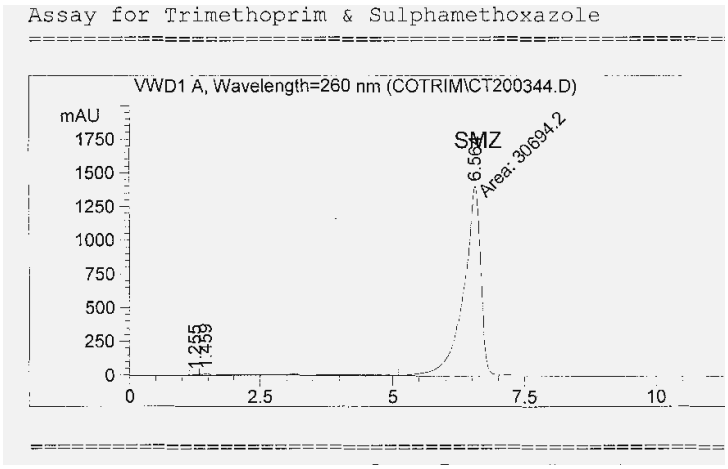


Figure 2: Chromatogram of sulphamethoxazole pure standard

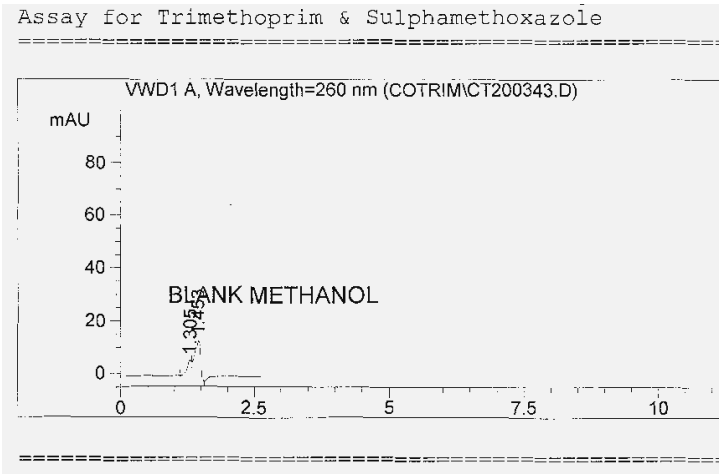
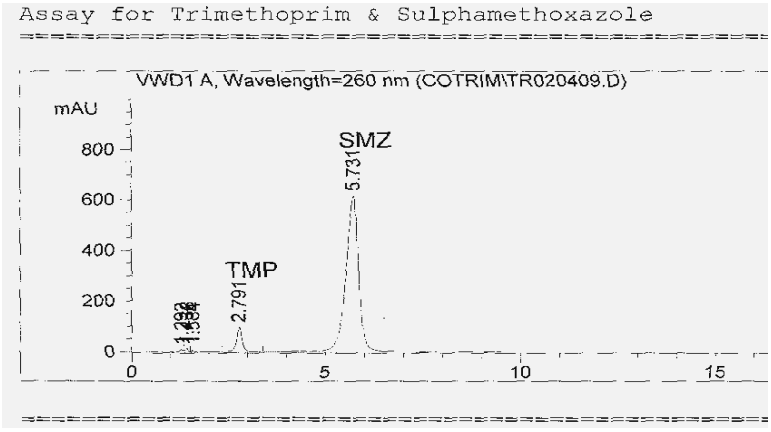
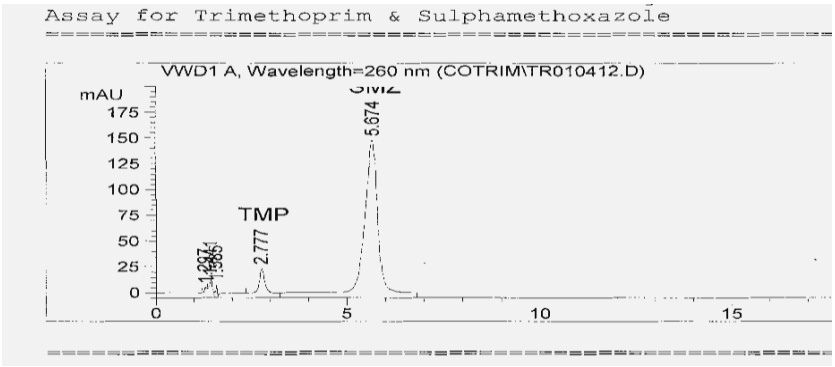


Figure 3: Chromatogram of Methanol solvent blank



(a)



(b)

Figures 4a and 4b: Chromatograms showing resolution and separation of pure mixed standard

Linearity and Range

The linearity of 5.0 -80.0 mg/L and 25.0-400.0 mg/L of trimethoprim and sulphamethoxazole standard solutions was investigated. The regression lines established in the tested rangeare shown in Figs 5 and 6. The regression analysis proved that the deviation of the intercepts on y-axis from origin is in compliance with USP and ICH recommendations of less than 2% (Ghanem and Abu-Lafi, 2013). The regression lines were R = 0.9980 for trimethoprim and 0.9975 for sulphamethoxazole as shown in Table 1.

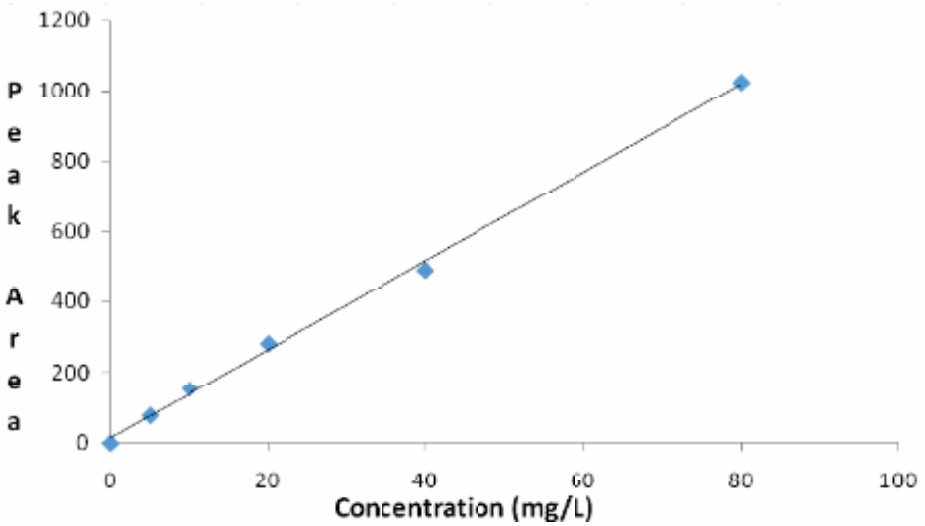


Figure 5: Linearity and range of Trimethoprim (TMP)

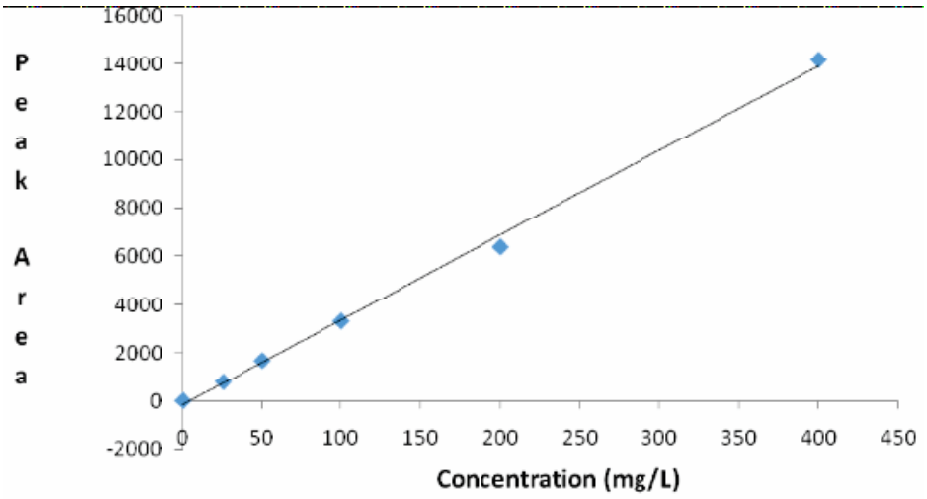


Figure 6: Linearity and range of Sulphamethoxazole (SMZ)

Table 1: Regression Statistics of Proposed Method

Active Ingredients	**Linearity range (mg/L)**	**(Correlation Coefficient)**	**Linear equation**	**y-intercept (%)**
TMP	5 – 80	0.9980	y=12.52x + 15.269	1.49%
SMZ	25 – 400	0.9975	y =35.16x - 154.73	1.09%

Specificity of the proposed method

The specificity of a method is the extent to which a particular analyte can unambiguously be detected and determined in a mixture without interference from other components in the mixture (Prichard and Barwick, 2008). The mixedpure standard and the sample test solutions were all injected at the same wavelength of 260 nm to ascertain the specificity of the proposed method. A comparison of the retention times of trimethoprim (TMP) and sulphamethoxazole (SMZ) in sample solutions and in the mixed standard solutions showed that they were identical.

Table 2: Specificity data showing retention characteristics

SN	**Mixed standard (TMP/SMZ) mins**	Brand A **sample (mins)**	Brand B **sample (mins)**
1.	3.233 / 6.607	3.202 / 6.593	3.244 / 6.699
2.	3.195 / 6.528	3.174 / 6.597	3.228 / 6.613

Table 2 showed that interference had no influence on the retention times of TMP and SMZ within the matrix. This proposed method would be appropriate for the determination of both ingredients in dosage forms without any elaborate pre-extraction step.

Precision of the proposed method

Precision was determined through repeatability studies. Six repeated injections of the test samples and mixed standard (300/60 mg/L) were carried out under the same analytical conditions using the same equipment. The test results and statistical evaluations of the replicate determinations of the active ingredients showed that the relative standard deviations (RSD) were between 0.69and2.61% as presented in Tables 3 and 4.

Table 3: Intra-Day Precision Studies on pure mixed standard

SN	TMP	SMZ
1	661.00	10620.00
2	652.00	10700.10
3	665.00	10668.00
4	662.00	10998.00
5	681.12	11314.30
6	648.21	10609.00
Mean	661.56	10818.23
SD	11.53	282.13
%RSD	1.74	2.61

Table 4: Intra-Day Precision Studies on Sample Solutions

	(Brand A SAMPLE)		(Brand **B** SAMPLE)	
SN	TMP	SMZ	TMP	SMZ
1	586.00	7289.00	591.00	8340.72
2	568.00	7400.10	601.00	8441.50
3	555.00	7370.40	599.00	8366.80
4	568.00	7391.40	582.00	8270.50
Mean	564.78	7362.73	593.25	8354.8
SD	6.52	50.71	7.50	61.17
%RSD	1.15	0.69	1.26	0.73

Accuracy of the proposed method

Accuracy was determined by the recovery study of a known amount of the pure mixed standards added to the samples of tablet dosage forms. A fixed volume of the working samples was injected before spiking and after spiking with the pure sulphamethoxazole/trimethoprim (1000/200 mg/L) mixed standard, and the recovery was calculated. %Recovery = (actual concentration/expected concentration) x 100. The accuracy as depicted by the recovery data of the assay for the two active ingredients is shown in Table 5.

Table 5: Recovery of sample of Trimethoprim and Sulphamethoxazole in spiked samples

Active ingredient	**%Recovery (Brand A)**	**%Recovery (Brand B)**
Trimethoprim	95.67%	91.93%
Sulphamethoxazole	103.98%	100.93%

Sensitivity of the proposed method

The sensitivity of this method was investigated through measurement of the limit of detection (LOD) and limit of quantification (LOQ) for trimethoprim and sulphamethoxazole at a signal to noise ratio of 3 and 10 respectively. It was accomplished by injecting a series of diluted solutions of known concentrations. The proposed method is capable of detecting 0.50 and 1.0 mµg/mL of TMR and SMZ respectively while 1.0 and 2.5 µg/mL could be quantified accurately as shown in Table 6.

Table 6: LOD and LOQ of Trimethoprim and Sulphamethoxazole

Analyte	**Limit of Detection (LOD) µg/ml**	**Limit of Quantification (LOQ) µg/ml**
Trimethoprim	0.50	1.00
Sulphamethoxazole	1.00	2.50

Applicability of the method to pharmaceutical formulations

This proposed and validated method was successfully applied to two commercial brands of sulphamethoxazole and trimethoprim tablet combinations. The brands were Brand A normal strength tablet (400mg /80mg) and Brand B double strength tablet (800mg /160mg). The levels of sulphamethoxazole and trimethoprim found in the dosage forms are shown in Table 7. Recovery studies of 91.93 and 103.98% were observed using the proposed method.

Table 7: Determination of sulphamethoxazole and trimethoprim in pharmaceutical formulation

Brand name	Label claim (mg)	SMZ (mg)/ TMP (mg)	% SMZ and TMP found	BP (2002)
Brand A	SMZ (400)	399.64	99.91%	92.5-105%
	TMP (80)	67.47	84.33%	
Brand B	SMZ (800)	830.43	103.80%	92.5-105%
	TMP (160)	158.86	99.29%	

DISCUSSION

The chromatographic evaluation and determination were conducted on an Agilent HPLC 1100LC System with UV detection at 260nm.The resolution was achieved at ambient temperature with 50mM sodium phosphate buffer and acetonitrile (85:15 volume/volume) maintained at 1ml/min flow rate on a reversed phase octyldecylsilane C18 column (100 x 4.6 mm, i.d., 5µm).The mean retention times for trimethoprim (TMP) and sulphamethoxazole (SMX) were 2.998 and 6.205 minutes respectively. The proposed HPLC method was evaluated for linearity/range, specificity, precision (repeatability), accuracy (recovery), and sensitivity. All validation results were within the allowed specifications of ICH/USP guidelines and BP (2002). There was a complete separation (qualitative identification) of both analytes from their interfering excipients.

This proposed method was found to be relatively cheap and fast with respect to cost-time benefit analysis as it deploys acetonitrile and phosphate buffer with runtime of 6 minutes compared with 16 mins reported by Rezaee *et al* (2000) and the Renn*et al* (1999) method that suffered from low sample throughput. This proposed method has some merits based on the validation procedure considered. The trimethoprim plot scatter had a correlation coefficient of 0.998 while that of sulphamethoxazole was 0.9975 (Figs 5 and 6, Table 1). The deviation of the y-intercept from zero was less than 2% in compliance with ICH and USP recommendations (Ghanem and Abu-Lafi, 2013).

There was improved sensitivity with respect to LOD (TMP, 0.5mg/L and SMZ, 1.0 mg/L) and LOQ (TMP, 1.0 mg/L and SMZ, 2.5mg/L), relative to the findings of 0.8mg/L LOD and 2.7mg/L LOQ for trimethoprim (Ghanem and Abu-Lafi, 2013). Harmita *et al*

(2012) in their HPLC analysis of trimethoprim and sulphamethoxazole observed that the relative standard deviations (%R.S.D) of both inter-day and intra-day precision analysis were less than 7.11% (TMP) and 6.15% (SMZ). This proposed method based on instrument precision, had intra-day precision of 1.74% (TMP) and 2.61% (SMZ).

The percentage recoveries obtained with respect to accuracy (91.93%, 95.67%, 100.93%, and 103.98%) were found to be consistent with the acceptable limits of between 98.0 and 102.0% (British Pharmacopeia, 2002). Assay of the contents of two commercial brands by HPLC yielded values that were consistent with BP (2002) monograph requirement for cotrimoxazole tablets (92.5% - 105%). The contents of sulphamethoxazole, (99.91-103.80%) and trimethoprim (84.33% - 99.29%) were observed to be in close agreements with the label claims, thus supporting the potential usefulness and application of this method. This shows that the proposed method would be well suited for quantitative detection and determination of the intended analytes.

CONCLUSION

An HPLC analytical method has been developed for the simultaneous determination of sulphamethoxazole and trimethoprim components of commercial brands of sulphamethoxazole/trimethoprim drug combination without any laborious extraction process. The method was found to be rapid, accurate, and stable for the simultaneous determination of the combined trimethoprim and sulphamethoxazole in pharmaceutical dosage forms in the presence of other excipients. This proposed HPLC method could, therefore, be applied for use in quality control and regular analysis of pharmaceutical dosage formulations.

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