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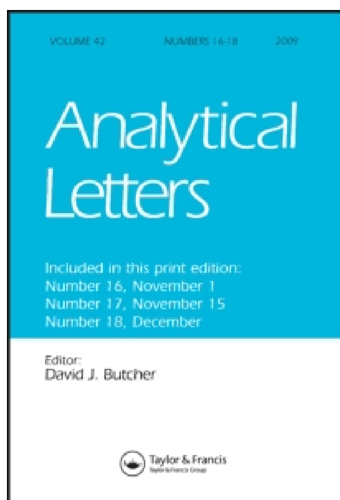

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Comparison of Ion-Pairing and Reversed Phase Liquid Chromatography in Determination of Sulfamethoxazole and Trimethoprim

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SEPARATIONS

Comparison of Ion-Pairing and Reversed Phase Liquid Chromatography in Determination of Sulfamethoxazole and Trimethoprim

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Abstract: Two simple, rapid, and sensitive HPLC methods have been developed for the simultaneous determination of sulfamethoxazole and trimethoprim in their pure and dosage forms, one utilizing reversed phase HPLC and the other ion-pair HPLC. In the reversed phase HPLC method (A) the mobile phase consists of 0.05% aqueous solution of formic acid with pH adjusted to 4.5 ± 0.2 with triethylamine : acetonitrile:tetrahydrofuran 50 : 49 : 1 (v/v), and the mobile phase pumped at flow rate of $1.0\,ml\,min^{-1}$. An Appolo LC18 column (5.0 μm), 250 mm length × 4.6 mm diameter, was utilized as the stationary phase. Detection was affected spectrophotometrically at 254 nm. In the ion-pair HPLC method (B) the mobile phase consisted of methanol : buffer 35 : 65 (v/v) with the buffer composed of potassium dihydrogen phosphate 0.3 M and sodium heptan sulfonic acid 5.0 mM. To 500 ml of buffer was added 2.0 ml triethylamine, and then the pH was adjusted to 5.0 with phosphoric acid, and the mobile phase was pumped at a flow rate of $1.2\,ml\,min^{-1}$. A Hypersil C_{18} column (5.0 μm), 150 mm length × 4.6 mm diameter, was utilized as the stationary phase. Detection was affected spectrophotometrically at 254 nm. Linearity ranges for sulfamethoxazole and trimethoprim were 1.0–110 and 1.5–98 $\mu g\,ml^{-1}$, respectively, with method A and 0.5–100 and

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1.0–125 μg ml^{-1}, respectively, with method (B). Minimum detection limits obtained were 0.1969 and 0.3451 μg ml^{-1} for sulfamethoxazole and trimethoprim, respectively, with method A, and 0.1377 and 0.2454 μg ml^{-1} with method (B). The proposed methods were further applied to the analysis of tablets containing the two drugs, and the results were satisfied.

Keywords: Dosage forms, ion-pair, reversed phase liquid chromatography, sulfamethoxazole, trimethoprim

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INTRODUCTION

Sulfamethoxazole is 4-amino-N-(5-methyl-3-isoxazolyl) benzenesulfonamide, N 1-(5-methyl-3-isoxazolyl) sulfanil amide (see Fig. 1); its action is primarily bacteriostatic, although it may be bactericidal where concentrations of thymine are low in the surrounding medium. The sulfonamides have a broad spectrum of action, but the development of widespread resistance (see below) has greatly reduced their usefulness, and susceptibility often varies widely even among nominally sensitive pathogens. It is rare to find a method to determine sulfamthoxazole fluids, viz. spectrophtometry (Numan et al. 2002; Mahedero, Galeano Diaz et al. 2002; Sun et al. 2001; Liu et al. 2000; Husain et al. 1995), HPLC (Berzas-Nevado et al. 2001; Dost et al. 2000; Vinas et al. 1996), capillary electrophresis (You et al. 1998), or polarographic (Liang et al. 2000).

Trimethoprim (5-(3,4,5-trimethoxybenzyl) pyrimidine-2,4-diamine) (Fig. 1) has low antimicrobial activity and is used as an antibiotic for protozoal infections. Although the spectrum of activity of trimethoprim includes numerous gram-negative and gram-positive bacteria, in long-term trimethoprim monotherapy development of bacterial resistance has been observed. Trimethoprim is, therefore, used not only for monotherapy but also combined with sulfonamides, which have approximately the same pharmacokinetic properties. The most widely applied multi-component drug is co-trimoxazole (Biseptol), which combines trimethoprim with sulfamethoxazole. The maximum synergistic effect is observed when the sulfamethoxazole-to-trimethoprim ratio is 5 : 1.

Literature surveys show that several techniques have been developed for determination of such a mixture, for example, spectrophotometry (Goebel et al. 2005; Tomsu et al. 2004; Fernandez de Cordova et al. 2003; Cruces Blanco et al. 1999; Ribone et al. 1999; Hassouna 1997; Altesor et al. 1993), HPLC (Pereira and Cass 2005; Kulikov et al. 2005; Berzas-Nevado et al. 2005; Akay and Ozkan 2002; Kebriaeezadeh et al. 2000), thin layer (Feng et al. 1994), capillary electrophoresis (Fan et al.

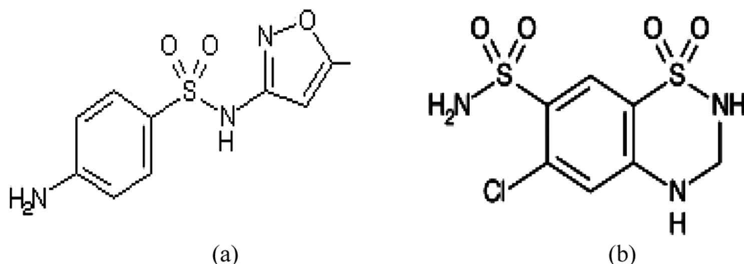

Figure 1. Chemical structures of sulfamethoxazole (a) and trimethoprim (b).

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2005; Chen et al. 2004; Teshima et al. 2004), potentiometry (Abdul-Kamal-Nazer et al. 2001), enthalpic determination (Issa et al. 1998), and voltametry (Kotoucek et al. 1997).

EXPERIMENTAL

Equipment

Liquid chromatography was performed with Hewlett–Packard equipment (Agilent Technologies, Waldbronn, Germany) comprising a Series 1050 pump, a Series 1050 variable-wavelength spectrophotometric detector, and a Series 3395 integrator. A Beckman Instruments (Fullerton, CA, USA) U50 pH meter was used for pH control; the instrument was previously calibrated against standard buffer solutions of pH 2.0, 4.0, and 7.0.

Drugs

Sulfamethoxazole batch no. 337/1105 and trimethoprim batch no. 2005001 were kindly supplied by Memphis Pharmaceutical Company, Pharmaceutical dosage forms were bought from local market.

Reagents

All the following chemicals and reagents were HPLC grade: methanol HPLC grade, Rediel de Haen, Germany; tetrahydrofuran HPLC grade, Rediel de Haen, Germany; Phosphoric acid 85% anal. R Merck, Darmastadt, Germany; sodium heptan sulfonic acid HPLC grade; formic acid 70% anal, R Merck, Darmastadt, Germany; potassium dihydrogen phosphate acid; and 6-triethyamine, Rediel De Haen, Germany.

Solution Preparation

Stock and Working Standard Solution

Sulfamethoxazole stock solution containing 0.5 mg ml^{-1} in methanol was prepared by weighing 50 mg of sulfamethoxazole in a 100 ml volumetric flask and diluted to the mark with methanol (standard stock 1). Working standard solution of sulfamethoxazole was prepared by diluting 2.0 ml from standard stock solution 1 to 100 ml with mobile phase A or B for method A or method B, respectively.

Trimethoprime stock solution containing 0.5 mg ml^{-1} in methanol was prepared by weighing 50 mg of trimethoprime in a 100 ml volumetric flask and diluted to the mark with methanol (standard stock 2). Working standard solution of trimethoprim was prepared by diluting 2.0 ml from standard stock solution 2, to 100 ml with mobile phase A or B for method A or method B, respectively.

Preparation of Buffer Solutions

A – Formate buffer: to one liter of double distilled water was added 0.5 ml formic acid and then the pH was adjusted to 4.5 ± 0.2 with triethylamine.

B – Dissolve exactly 4.0 gm KH_2PO_4 previously dried at 120°C for two hours and 500 mg sodium heptan sulfonic acid in 500 ml DI H_2O, add 2.0 ml triethylamine and adjust pH to 5.0 ± 0.2 with H_3PO_4.

Application of Pharmaceutical Dosage Forms

About twenty tablets were weighed and powdered. For method (A) reversed phase HPLC, an amount of powder equivalent to 50 mg of sulfamethoxazole was transferred into 100 ml volumetric flask, and dissolved in methanol, sonicated for 5.0 min., cooled to room temperature then completed to the mark with methanol. (Test stock solution). Stock solution of test was filtered to remove any insoluble substance; then 2.0 ml of the filtrate was diluted to 100 ml with mobile phase A. For method (B) ion-pairing mobile phase, the preparation was the same as method (A) but the second dilution was performed with mobile phase (B).

Suspensions

For suspensions, 2.0 ml of suspension was transferred to a 100 ml volumetric flask, 20 ml methanol was added, and the mixture was sonicated

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for 5.0 min, cooled to room temperature, and then the volume was completed to the mark with methanol. Stock solution of test was filtered to remove any insoluble substance, and then 1.0 ml of the filtrate was diluted to 100 ml with mobile phase A or B.

Chromatographic Conditions

Method (A) Reversed Phase HPLC

Chromatography was performed on a 250 mm length $\times$ 4.6 mm diameter, 5.0 μm particle size, Apollo C_{18} reversed-phase column packed with dimethyloctadecylsilyl bonded amorphous silica. The mobile phase was 0.05% aqueous solution of formic acid with adjusted pH to 4.5 ± 0.2 with triethylamine : acetonitrile : tetrahydrofuran 50 : 49 : 1 (v/v), and the mobile phase was filtered through 0.45 μm nylon filter, degassed for 15 min, and then pumped at a flow-rate of 1.0 ml min^{-1}. The column was kept at 25.0 ± 2.0°C during the analysis; the detection wavelength was 254 nm, and the injection volume was 20 μl.

Method (B) Ion-Pairing HPLC

Chromatography was performed on a 150 mm length $\times$ 4.6 mm diameter, 5.0 μm particle size, hypersil ODS reversed phase column (Merck, Darmstadt, Germany). The mobile phase was methanol : buffer 65 : 35 (v/v), where the buffer consisted of 4.0 gm KH_2PO_4 and 500 mg sodium heptan sulfonic acid in 500 ml DI H_2O. After adding 2.0 ml triehylamine and adjusting the pH to 5.5 with H_3PO_4, the mobile phase was filtered through 0.45 μm nylon membrane filter, degassed for 15 min, and then pumped at a flow-rate of 1.2 ml min^{-1}. The column was kept at 25.0 ± 2.0°C during the analysis; the detection wavelength was 254 nm, and the injection volume was 20 μl.

RESULTS AND DISCUSSIONS

The cationic nature of sulfamethaxazole and trimethoprim leads to broad asymmetric peaks in reversed phase HPLC with aqueous-organic mobile phases and conventional C_{18} columns because of the ionic interaction with the alkyl-bonded reversed-phase packing. Also, use of methanol or acetonitrile leads to unresolved and high tailing peaks.

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Method (A) Reversed Phase HPLC

In order to affect the simultaneous elution of two peaks under isocratic conditions, the mobile phase composition (organic modifier, flow rate, ionic strength and pH) was investigated. The reversed phase HPLC method (A) used a low pH formic acid solution with acetonitrile and tetrahydrofuran as an organic modifier with addition of triethylamine as blocking agent and tetrahydrofuran as organic modifier to block the residual silanol interaction and reduce tailing; triethylamine is also used here in pH adjustment. The mobile phase (A) was a 0.05% aqueous solution of formic acid with adjusted pH to 4.5 ± 0.2 with triethylamine: acetonitrile : tetrahydrofuran 50 : 49 : 1 (v/v). The mobile phase composition was optimized, and under the described conditions the two components were defined, resolved, and free from tailing; the tailing factors were < 1.20 for all peaks. The elution order was trimethoprim ($t_R = 4.464$) and sulfamethoxazole ($t_R = 7.0067$) (Fig. 2a).

Method (B) Ion-Pairing Mobile Phase

In the ion-pairing HPLC method we used an ion-pairing substance, sodium heptan sulfonic acid, and phosphate buffer solution with methanol to decrease the residual silanol interaction and decrease tailing. The mobile phase was methanol : buffer 35 : 65 (v/v), where the buffer consisted of 4.0 gm KH_2PO_4 and 500 mg sodium heptan sulfonic acid in 500 ml DI H_2O; 2.0 ml triehylamine was added and then the pH adjusted to 5.0 with H_3PO_4 to optimize the mobile phase composition. Under the described condition the two components were well defined, resolved, and free from tailing. The elution order was sulfamethoxazole ($t_R = 3.541$) and trimethoprim ($t_R = 4.724$) (Fig. 2b).

Validity of the Methods

Specificity

The selectivity of both methods was checked two ways, the first by comparison of the chromatograms obtained from Sutrim samples and the corresponding placebo. The additives of the tablets are practically insoluble in methanol or in both mobile phases, whereas the active constituents are freely soluble in methanol and in both mobile phases. The chromatograms obtained from Sutrim samples and corresponding placebo are shown in Fig. 3. It is clear that there is no peak in the placebo

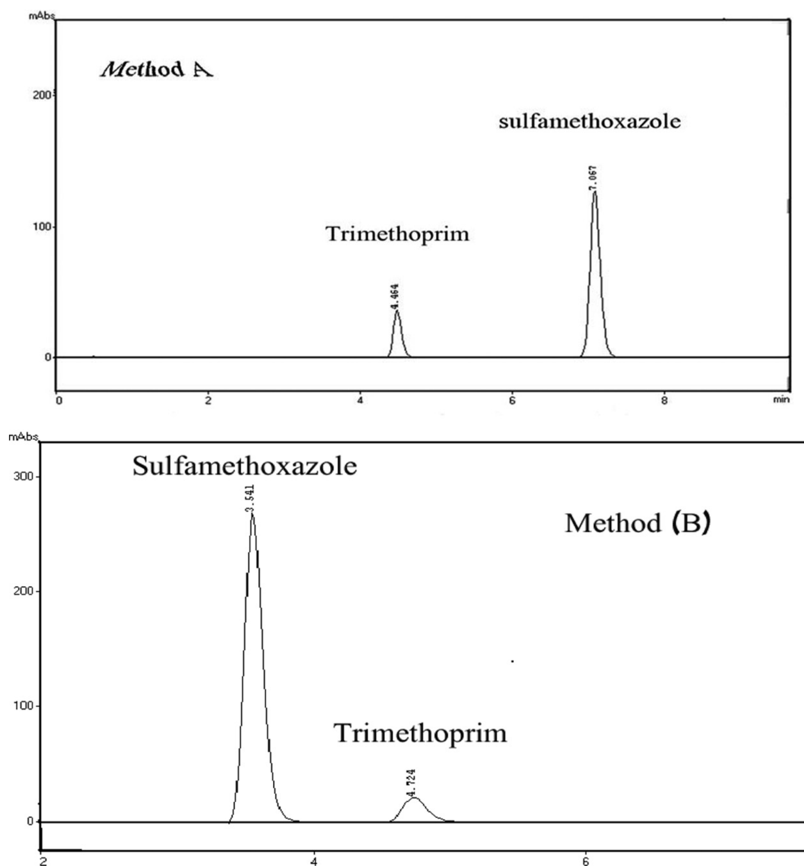

Figure 2. Separation of sulfamethoxazole and trimethoprim on a Hypersil C18 column using methods A and B.

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chromatogram, whereas two separated peaks appear in the Sutrim chromatogram The second way of checking the selectivity of both methods was by standard addition method in which a known concentration of the analyte was added to previously analyzed pharmaceutical preparation.

Linearity

The linear correlation between area under peaks and compound concentrations was checked for each component using both methods. Data for

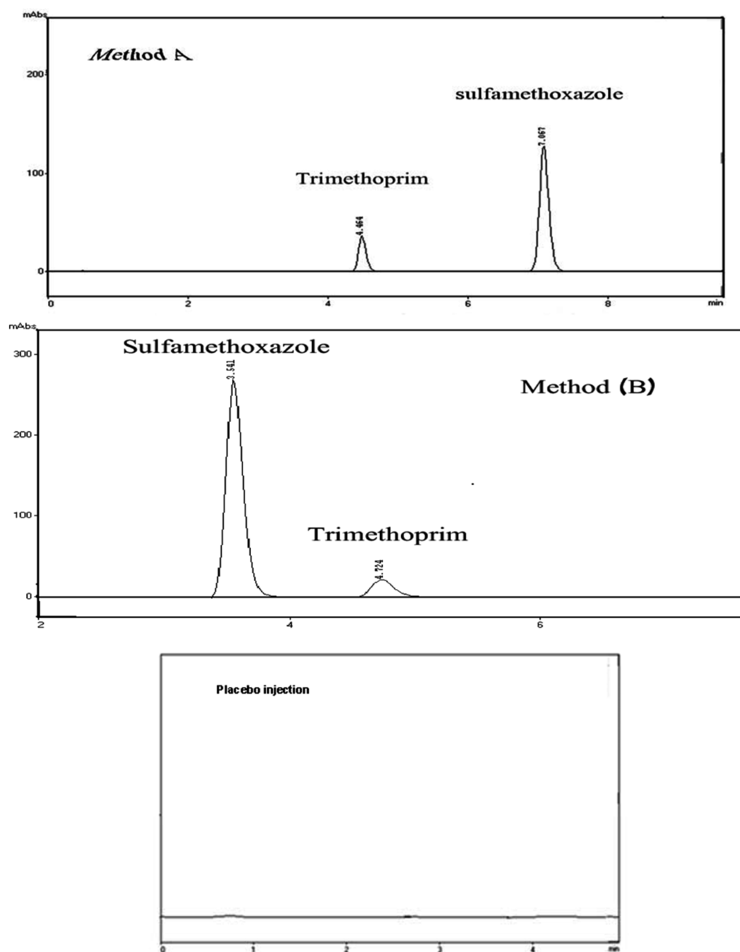

Figure 3. Chromatogrames obtained during specificity for separation of sulfamethoxazole and trimethoprim with mobile phase (A), and mobile phase (B), then placebo injection on a Hypersil C_{18} column.

six different concentrations ranging from 1.0–110 μg ml^{-1} for sulfamethoxazole and 1.5–98 μg ml^{-1} for trimethoprim with method (A), and from 0.5–100 μg ml^{-1} for sulfamethoxazole and 1.0–125 μg ml^{-1} for trimethoprim with method (B) were collected and analyzed. Each solution was injected five times and then the least squares method was used for calculation of the slope, intercept, and correlation coefficient (r) for both compounds with both mobile phases. The correlation between the

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Table 1. Characteristics of the methods used in assay of sulfamethoxazole and trimethoprim

	Reversed phase HPLC (method A)		Ion-pairing (method B)	
Parameter	SMX	TMP	SMX	TMP
Linearity range $\mu g\ ml^{-1}$	1.0–110	1.5–98	0.5–100	1.0–125
Slope	22.8542	10.4317	25.063	11.523
Intercept (a)	38×10^3	15×10^3	47×10^3	18×10^3
Correlation coefficient	0.9899	0.9986	0.9978	0.9998
Detection limit $\mu g\ ml^{-1}$	0.1969	0.3451	0.1377	0.2454
Quantification limit $\mu g\ ml^{-1}$	0.6533	1.1572	0.4591	0.8180
Capacity factor	6.41	3.83	2.68	4.23
Tailing factor	1.16	1.10	1.13	1.18
Theoretical plate no.	10,967	8,465	6,421	8,651

Regression equation: $A = a + bC$, where A is the area under peak and a, is the intercept.

analyte concentration and peak area is described by linear regression equations with high value of correlation coefficient (r). All results are listed in Table 1.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated in accordance with 3.3 s/m and 10 s/m criteria, respectively, where s is standard deviation of peak area (for five replicates) for analyte and m is the slope of the calibration plot, determined from the linearity investigation. The LOD and LOQ obtained are listed in Table 1.

Accuracy

In order to determine the accuracy of the proposed methods, solutions containing six different concentrations of sulfamethoxazole and trimethoprim were prepared and analyzed; the results obtained from these investigations are summarized in Table 2.

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Table 2. Evaluation of accuracy of the proposed methods

		Reversed phase HPLC method A			Ion-pairing method-B		
Drugs	Theoretical conc.	Found	Recovery*	RSD %	Found	Recovery*	RSD %
SMX	5.00	5.01	100.20	1.021	5.06	101.20	1.052
	55.80	55.73	99.87	0.987	55.85	100.09	1.251
	80.0	81.01	101.26	0.991	80.52	100.65	1.067
	96.5	97.21	100.74	1.001	96.370	99.87	1.039
TMP	3.00	2.90	96.67	1.051	2.91	97.00	0.981
	69.50	69.3	99.71	1.102	69.43	99.90	1.020
	98.0	98.01	100.01	0.986	97.99	99.99	1.046
	110	110.31	100.28	1.032	110.5	100.45	1.025

*Average of five determinations.

Precision

Repeatability and reproducibility expressed as RSD% were characterized by spread data from replicate determination. The intra-day precision (repeatability or accuracy) of both methods was evaluated by analysis of the mean of five replicates of three different reference standard solutions containing 90%, 100%, and 110% of the labeled amount of sulfamethoxazole (400 mg) and trimethoprim (80 mg) present in Sutrim tablets. The inter-day precision (accuracy or repeatability) of both methods was evaluated by analysis of freshly prepared reference solution (second dilution, i.e., 100%) of the labeled amount on three different days and results obtained from this analysis are listed in Table 3 as mean recovery (%). The results showed that there is no difference either within day or between days, implying that the reproducibility of both methods was good.

Robustness

The methods were found to be robust although small deliberate changes in method conditions did have some effect on the chromatographic behavior of the solutes. The chromatographic condition investigated was mobile phase composition, pH, flow rate, column temperature, and detected wavelength. The results of robustness analysis showed that although small changes in the mobile phase pH has no significant effect on the retention time for sulfamethoxazole and

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Table 3. Summary of repeatability (intra-day) and reproducibility (inter-day) precision data for sulfamethoxazole and trimethoprim with mobile phase (A) and mobile phase (B)

	Intra-day		Inter day amount recovered ± RSD					
	Mean ± RSD		After two days		After five days		After ten days	
Theoretical conc. μg ml^{-1}	A	B	A	B	A	B	A	B
SMX								
20.0	100.51 ± 1.0	100.36 ± 0.96	100.50 ± 0.89	100.32 ± 0.99	100.10 ± 0.91	99.99 ± 0.68	98.95 ± 0.66	100.10 ± 0.58
55.0	100.32 ± 0.37	99.95 ± 0.49	100.31 ± 0.93	100.01 ± 0.76	100.01 ± 0.19	99.10 ± 0.26	99.97 ± 0.82	98.91 ± 0.73
93.0	100.10 ± 0.39	100.68 ± 0.55	100.11 ± 0.69	100.52 ± 0.61	100.06 ± 0.88	100.39 ± 0.17	99.23 ± 0.24	100.19 ± 0.44
TMP								
5.0	100.15 ± 0.62	100.06 ± 0.79	100.16 ± 0.28	100.10 ± 0.93	99.89 ± 0.23	100.0 ± 0.14	99.1 ± 1.0	99.6 ± 0.73
40.0	99.50 ± 0.85	101.00 ± 0.64	99.71 ± 0.33	100.62 ± 0.27	99.13 ± 0.71	100.09 ± 0.83	98.26 ± 0.99	99.82 ± 0.96
60.0	100.25 ± 0.13	99.97 ± 0.62	100.2 ± 1.0	100.01 ± 0.29	100.26 ± 0.19	99.22 ± 0.69	98.49 ± .37	98.82 ± 0.79

A: refer to method A, reversed phase HPLC.
B: refer to method B, ion-pair HPLC.

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Table 4. Effect of column temperature and mobile phase pH on the chromatographic behavior of sulfamethoxazole and trimethoprim with method A and B

	Mobile phase pH					Column temperature		
Parameters	3.0	4.0	4.5	5.0	5.5	20°C	25°C	30°C
	Method (A) reversed phase HPLC							
SMX								
K	5.83	6.19	6.41	6.42	6.48	8.13	6.56	5.46
As	2.01	1.19	1.15	1.15	1.25	1.18	1.16	1.12
N	8711	9485	10178	10069	9759	10207	10178	9699
TMP								
K	3.01	3.60	3.80	3.85	4.10	4.78	3.83	2.55
As	1.32	1.15	1.12	1.27	1.35	1.16	1.12	1.11
N	6120	6390	6585	6599	6610	6730	6585	5987
	Method (B) Ion-pair-HPLC							
SMX								
K	2.26	2.52	2.68	2.70	2.71	3.35	2.68	2.26
As	1.06	1.08	1.13	1.13	1.23	1.16	1.13	1.13
N	5612	6150	6523	6547	6564	6580	6523	6469
TMP								
K	3.16	3.98	4.23	4.25	4.30	4.39	4.23	4.19
As	1.09	1.13	1.16	1.17	1.29	1.21	1.16	1.09
N	6853	8303	8825	8879	8896	9050	8825	8720

Table 5. Summary of system suitability tests

	Method (A)		Method (B)	
Parameter	SMX	TMP	SMX	TMP
K	6.41	3.83	2.68	4.23
R	7.01	4.43	2.43	4.06
N	10967	8465	6421	8651
As	1.15	1.12	1.13	1.18
RSD* (peak areas)	0.99	1.06	1.16	1.21
RSD* (retention time)	0.16	0.23	0.35	0.29

*RSD for five determinations.

Where K: is capacity factor; N: No. of theoretical plates; R: is resolution time; and As: is asymmetry factor.

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trimethoprim with both methods, changes in the flow rate is undesired as it leads to increasing peak tailing of sulfamethoxazole and increasing of the broadening of trimethoprim peak. Changing the column temperature has a larger effect on the chromatographic behavior of the two peaks in both methods than mobile phase pH and flow rate as recorded in Table 4. The reduction in the concentration of sodium heptan sulfonate in method (B) lead to partial deterioration in the behavior of both solutes, but the decrease in the concentration of tetrahydrofuran in mobile phase (A) also lead to deterioration of the chromatographic behavior of both solutes. Finally, altering the wavelength detection had no effect on the chromatographic behavior of the solutes with either method.

System Suitability

According to United States Pharmacopoeia (2004), system suitability tests are an integral part of the liquid chromatographic method. System suitability tests were used to verify that resolution and reproducibility were adequate for the analysis performed. The parameters of this test were column efficiency, asymmetry of chromatographic peak, peak resolution repeatability as RSD of peak area for six injections, and reproducibility of retention as RSD of retention time. The results of these tests and their acceptance criteria according to USP regulations are listed in Table 5. From the results obtained by comparison with the specification set for the methods, we can draw conclusions about the suitability of the system for analysis.

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Table 6. Determination of sulfamethoxazole and trimethoprim in pharmaceutical dosage forms by the proposed methods

				Method (A) Reversed phase HPLC				Method (B) Ion-pair-HPLC			
		Labeled mg per tablet		SMX		TMP		SMX		TMP	
Drugs	Company	SMX	TMP	Found	Recovery*	Found	Recovery*	Found	Recovery*	Found	Recovery
Entrim Tab.	Nile	400	80	402.41	100.60	81.01	101.26	402.59	100.65	80.75	100.94
Sutrim Tab.	Memphis	400	80	395.63	98.91	76.92	96.15	398.51	99.63	79.51	99.39
Sutrim Susp.	Memphis	200	40	201.22	100.61	40.02	100.05	199.50	99.75	40.67	101.68
Septazole Tab.	Alexandaria	400	80	405.16	101.29	80.76	100.95	400.71	100.18	79.37	99.21
Septazole Susp.	Alexandaria	200	40	196.33	98.17	39.00	97.50	201.24	100.62	41.56	103.90
Chemotrim Tab.	El-Kahira	400	80	403.78	100.95	77.52	96.90	402.36	100.59	80.66	100.83
Chemotrim Fort tab.	El-Kahira	800	160	810.57	101.32	165.22	103.26	803.93	100.49	164.29	102.68
Chemotrim Susp.	El-Kahira	200	40	203.14	101.57	38.05	95.13	198.33	99.17	40.05	100.13

*Average of five determinations.

Analytical applications

Pharmaceutical formulations containing sulfamethoxazole and trimethoprim were analysed successfully by the proposed method with a good recovery. Results are recorded in Table 6 confirming that the proposed method is not liable to interference by tablet and suspension fillers, excipients and additives usually formulated with tablets and suspensions. The proposed method is highly sensitive, therefore, it could be used easily for the routine analysis of pure form and in its pharmaceutical formulation.

CONCLUSION

Two simple, sensitive, accurate, reproducible, and precise liquid chromatographic methods for assay of sulfamethoxazole and trimethoprim in pure and bulk forms have been developed and validated. The advantages of the proposed methods are the lower detection limits and higher quantification limits, which permit a wide range of analysis.

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