International Journal of Pharmaceutical Research & Analysis

e-ISSN: 2249 – 7781
Print ISSN: 2249 – 779X

www.ijpra.com

DEVELOPMENT OF A STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF TRIMETHOPRIM, SULFAMETHOXAZOLE AND METYHL PARABEN IN ORAL SUSPENSION

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ABSTRACT

A gradient Simultaneous estimation by RP-HPLC Method were developed and validated for the quantification of Trimethoprim, Sulfamethoxazole and methyl paraben at single wavelength (275nm) in order to assess assay and in vitro drug release profile of drug from Oral Suspension formulation. A gradient elution of samples performed on Lichrospher 60-RP select B, 5µm, Lichrocart MERCK (250 × 4.0 mm, 5µm) with buffered mobile phase consisting solvent A of KH_2PO_4 0.05M , solvent B (Acetonitrile) and solvent C (Methanol) in ratio of (75:15:10) (v/v/v) delivered at flow rate 1.0 mL/min. The average retention time for Trimethoprim, Sulfamethoxazole and methyl paraben were found to be 6.4 min, 16.8 min and 26.6 min. The proposed method was validated for selectivity, precision, linearity and accuracy. The assay methods were found to be linear from 140-260 µg/ml for Sulfamethoxazole, 28-52µg/ml for Trimethoprim and 7.0-13.0µg/ml for methyl paraben. In addition filter suitability, standard and sample solution stability was demonstrated. The validated method is suitable for quality control applications and its advantages over the already existing methods are simplicity and reduced analysis time.

Keywords: RP-HPLC Method; Simultaneous Estimation; Stability; Sulfamethoxazole; Trimethoprim.

INTRODUCTION

Sulfamethoxazole Fig. (A1) is N1-(5-methylisoxasole-3-il) sulfanilamide. The molecular formula is $C_{10}H_{11}N_3O_3S$ and molecular weight 253.3 g/mol (SM) and Trimethoprim (TM) Fig. (A2) is 5-(3, 4, 5-trimethoxybenzyl) pyrimidin-2, 4-diyldiamine. The molecular formula is $C_{14}H_{18}N_4O_3$ molecular weight 290.3 g/mol. is a broad spectrum anti-microbial agent composed of a fixed combination of a diamino-pyrimidine and a sulphon-amide [1]. It was developed by the systematic investigation of a series of compounds known to be specific enzyme inhibitors of bacterial folate synthesis. It has a wide range of activity against both Gram-positive and Gram negative aerobic bacteria: chlamydia, actinomycetes and Protozoa [2-5]. Many anaerobic organisms including *Bacteroides fragilis*, can be shown to be susceptible *in vitro* as well [6].

A synergy or summation effect between the two drugs (TM and SM in a 1: 5 ratio) has been demonstrated both *in vitro* and in most studies [7]. A general approach for the determination of TM and SM is high performance liquid chromatography (HPLC) analysis because HPLC provides adequate sensitivity and precision for monitoring therapeutic steady state concentration [8-10]. We report here an HPLC method capable of quantifying TM, SM and methyl paraben simultaneously.

MATERIALS AND METHODS

Chemicals and Reagents

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An analytically pure sample of Oral Suspension was procured as gift sample from officinal pharmaceutical (Morocco). Methanol and Acetonitrile (HPLC grade) were procured from Merck Specialist. Ultra pure water (HPLC-grade) was obtained from Merck. Potassium dihydrogen phosphate (AR grade, purity 99.6%) was procured from Merck. Oral Suspension formulations (office in Morocco.) were procured from a local pharmacy with labeled amount TM 8.0 mg/ ml, SM 40.0 mg/ ml and methyl paraben 1.0 mg/ ml.

Instrumentation and Chromatographic Conditions

Chromatographic separation was achieved by using Lachrom system for quantification of Oral Suspension consisted of a LaChrom L-7100 Merck Hitachi Pump, LaChrom L-7200 Merck Hitachi Autosampler and LaChrom L-7400 Merck Hitachi UV Detector. The chromatogram peaks were quantified by means of PC Multi- System Manager Software (Merck- Hitachi Model D-7000). LC parameters are optimized by investigating the influence of the mobile phase, column temperature and detection wavelength. The initial separation is carried out on Lichrospher 60-RP select B reserved-phase column with mixtures of Methanol, Acetonitrile and Potassium dihydrogen phosphate 0.05M while the mobile phase with an isocratic elution method. Because the peak shapes are unsatisfactory sharpen peak shapes and improve analytical sensitivity and resolution. The optimum mobile phase was composed of KH_2PO_4 0.05M/Acetonitrile /Methanol.

In ratio of (75:15:10) (v/v/v) that was set at a flow rate of 1.0 ml/min. The mobile phase was degassed in an ultrasonic bath prior to use and filtered through 0.45µm membrane filter before pumping into HPLC system. The injection volume was 20 µl, and a chromatographic peak was detected at 275nm. Chromatography separation for analyte was achieved on Lichrospher 60-RP select B analytical column with 250 × 4.0 mm i.d. and 5 µm particle size.

Preparation of Mobile Phase

Preparation of Buffer KH_2PO_4 0.05M

3.4 g Potassium dihydrogen phosphate (AR grade, purity 99.6%) was dissolved in 500 ml distilled water and pH was adjusted to 5.6 with 0.5 M Potassium Hydroxide.

Mobile phase was a mixture of 100ml of Methanol, 150ml of Acetonitrile and 750ml of Potassium dihydrogen phosphate 0.05M adjusted to pH 5.6 with 0.5 M Potassium Hydroxide. Filtered through a 0.45µm nylon filter and degassed for 5min using an ultrasonicator.

Standard Solution Preparation

***Solution A*:**

Accurately weighed about 125mg of methyl paraben standard was taken in a 100ml volumetric flask and was dissolved in 20ml with methanol then it was sonicated for 10 minutes and it was diluted up to mark with methanol.

***Solution B*:**

A standard solution containing 50.0 mg of trimethoprim and 250.0 mg of sulfamethoxazole were weighed and transferred to 100 ml of volumetric flask and dissolved in the methanol then it was sonicated for 10 minutes and add 10 ml of solution A. The flask was shaken and volume was made up to mark with methanol to give a primary stock solution containing 2500µg/ml Sulfamethoxazole, 500µg/ml of trimethoprim and 125µg/ml of methyl paraben.

From the above solution 20 ml of solution B is pipetted out into a 250 ml volumetric flask and volume was made up to mark with mobile phase to give a solution containing 40µg/ml of Trimethoprim, 200µg/ml sulfamethoxazole and 10 µg/ml of methyl paraben.

Preparation of Sample Solution

About 3.1 g of oral suspension was weighed and transferred it in to a 200 ml volumetric flask, and was dissolved in 100 ml with mobile phase. Then it was sonicated for 15 minutes. The volume was made up with mobile phase and solution was centrifuged10 min at 4000 rpm/minute. 10 ml of this solution was further diluted to 25 ml with mobile phase. The solution was filtered through 0.45µm membrane. The amount of Trimethoprim, sulfamethoxazole and methyl paraben presents in oral suspension formulation was calculated by comparing the peak area of the standard.

METHOD VALIDATION [11-14]

Specificity

The specificity of the RP-HPLC method was determined by elution of TM, SM and methyl paraben. The tailing factor for peak obtained was satisfactory because it was less than 2%. The retention time for TM, SM and methyl paraben were found to be 6.4±0.1min, 16.6±0.1min and 26.6±0.1min for six replicates. The peak obtained for TM, SM and methyl paraben were sharp with clear baseline result of the method validation experiments are given in Table 1 and chromatograms of blank, placebo Oral Suspension and Atypical chromatograms of standard TM, SM and methyl paraben are shown in Fig 2.

Linearity and Range

Linearity of the method was evaluated by using 5 linearity solutions of different concentrations in the range of 140-260µg/ml for SM, 28-52 µg/ml for TM and 7.0-13 µg/ml for methyl paraben of the standard. Accurately measured aliquots of solution standard were taken in five different 200 ml volumetric flask and diluted up to the mark with the mobile phase such that the final concentrations of TM were 28 $\mu g\ ml^{-1}$, 34 $\mu g\ ml^{-1}$, 40 $\mu g\ ml^{-1}$, 46 $\mu g\ ml^{-1}$ and 52 $\mu g\ ml^{-1}$ of SM were 140 $\mu g\ ml^{-1}$, 170 $\mu g\ ml^{-1}$, 200 $\mu g\ ml^{-1}$, 230 $\mu g\ ml^{-1}$ and 260

µg ml^{-1} and of 7.0 µg ml^{-1}, 8.5 µg ml^{-1}, 10.0 µg ml^{-1}, 11.5 µg $ml^{-1}$and 13.0 µg ml^{-1}.

A 20 µl aliquot of each linearity solution was injected in triplicate. The peak area values were plotted against the corresponding analyses concentrations to obtain the linear calibration. The coefficients of these dependences were calculated to be 0.9993 of TM, 0.9996 SM and 0.9990 of methyl paraben are shown in Fig. 3. The standard solutions were prepared by diluting an appropriate volume of stock solution with mobile phase. Each solution was analyzed in triplicate.

Method Precision (Repeatability)

The precision of the instrument was checked by repeated injections and measurement of peak areas and retention times of solutions (n = 6) for 40µg/ml of TM, 200µg/ml of SM and 10 µg/ml of methyl paraben without changing the parameter of the proposed chromatographic method.

Intermediate Precision (Reproducibility)

The intraday and interday precision of the proposed method was determined by analyzing the Corresponding responses 3 different days over a period of 1 week for 40µg/ml of TM, 200µg/ml of SM and 10 µg/ml of methyl paraben. The result was reported in terms of relative standard deviation (% RSD). The intraday and interday precisions were determined and results are given in Table 2.

Accuracy

Accuracy of method was evaluated as a percentage of recovery obtained from analysis of sample spiked with known amount of TM, SM and methyl paraben (70 **%**, 100 **%** and 130 **%**). The accuracy was carried out three times at each level of recovery. The results of study along with its evaluation are given in Table 3.

Detection Limit and Quantification Limit

The Standard deviation of Y intercepts of regression lines were determined and kept in the following equation for the determination of LOD and LOQ. Detection limit=3.3σ/S; Quantitation limit = 10σ/S; where, σ is the Standard deviation of Y intercept of regression lines and S is the slope of calibration curve. The LOD was found to be 0.05µg/ml for TM, 0.08µg/ml for SM and 0.12µg/ml for methyl paraben. Limit of quantitation was found to be 0.17µg/ml for TM, 0.27µg/ml for SM and 0.40µg/ml for methyl paraben, respectively.

Robustness

It was observed that by making changes in chromatographic parameters, absolute difference between percent assay under altered condition and mean percent assay obtained during repeatability was not more than 2.0%. %RSD of area response and retention time was below 2%. The results of Robustness evaluation are shown in Table 4.

Table 1. Validation and System suitability parameters

Parameters	Trimethoprim	Sulfamethoxazole	Methyl paraben
Linearity	28- 52µg/ml	140-260 µg/ml	7.0- 13.0µg/ml
Slope	154010	72600	37235
Intercept	125032	29847	610.47
Coefficient of correlation	0.9996	0.9991	0.9990
Percentage curve fitting	99.84%	99.98%	99.72%
T_r	6.4±0.1 min	16.8±0.1 min	26.6±0.1 min
Tailing Factor	1.16	1.10	1.09
Resolution	-	18.83	7.93
LOD µg/ml	0.05 µg/ml	0.08 µg/ml	0.12 µg/ml
LOQ µg/ml	0.17 µg/ml	0.27 µg/ml	0.40 µg/ml
Theoretical plates Ph Eup	7285	6414	9719

Table 2. intraday and interday precisions

Intraday precision		Interday precision	%RSD	%RSD
*Mean %±SD		*Mean %±SD	Intraday	Interday
TM	98.3 ± 0.83	99.1 ± 0.54	0.83	0.54
SM	99.0 ± 0.77	99.2 ± 0.85	0.77	0.85
Methyl paraben	98.6 ± 0.97	97.0 ± 1.27	0.97	1.27

*Mean of six determination (n=6)

Table 3. Recovery studies of TM, SM and methyl paraben in Oral Suspension

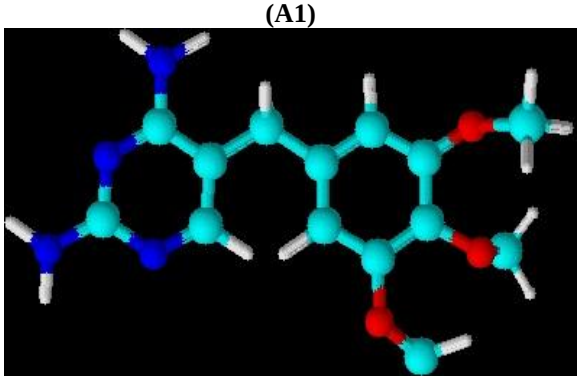
% Taken	%Recovery of	%RSD	Recovered of SM	%RSD	Recovery of MP	%RSD
70%	99.4	0.67	98.2	0.43	99.5	0.77
100%	99.0	0.46	98.5	0.82	98.9	0.51
130%	99.3	0.82	98.8	0.55	99.1	0.47

Table 4. Result of robustness studies

Method parameter	Altered condition	%Assay of TM	%RSD	%Assay of SM	%RSD	%Assay of Methyl paraben	%RSD
Flow rate	1.05 ml min^{-1}	99.33	1.26	99.32	0.64	99.75	0.44
	1.00 ml min^{-1}	99.43	0.54	98.89	0.47	99.44	0.58
	0.95 ml min^{-1}	100.54	0.45	99.64	0.75	100.32	0.33
Temperature	23 °C	99.44	0.59	100.12	0.47	99. 87	1.23
	25 °C	99.65	0.53	99.56	0.78	99.42	0.47
	27 °C	100.54	1.43	100.52	0.94	100.11	1.24
Wavelength (nm)	273 nm	99.52	0.68	99.12	0.67	99.80	1.42
	275 nm	99.97	0.64	98 .96	1.04	99.27	0.31
Column	277 nm	98.46	1.37	100.25	1.32	98.76	1.55
	Lot-1	99.56	0.34	99.11	0.81	99.16	0.84
	Lot-2	99.89	0.57	100.38	0.43	99.79	1.34

Fig 1. The chemical structure of sulfamethoxazole (A1) and trimethoprim (A2)

(A1) **(A1)**



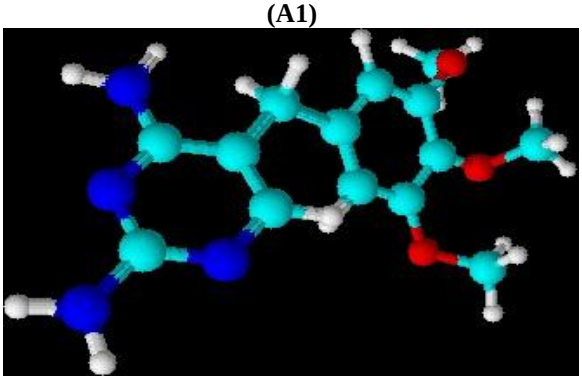


Fig 2. Chromatograms of blank (B1), Placebo tablet (B2) and Atypical chromatogram of standard Trimethoprim, Sulfamethoxazole and methyl paraben (B3).

(B1) **(B2)** **(B3)**

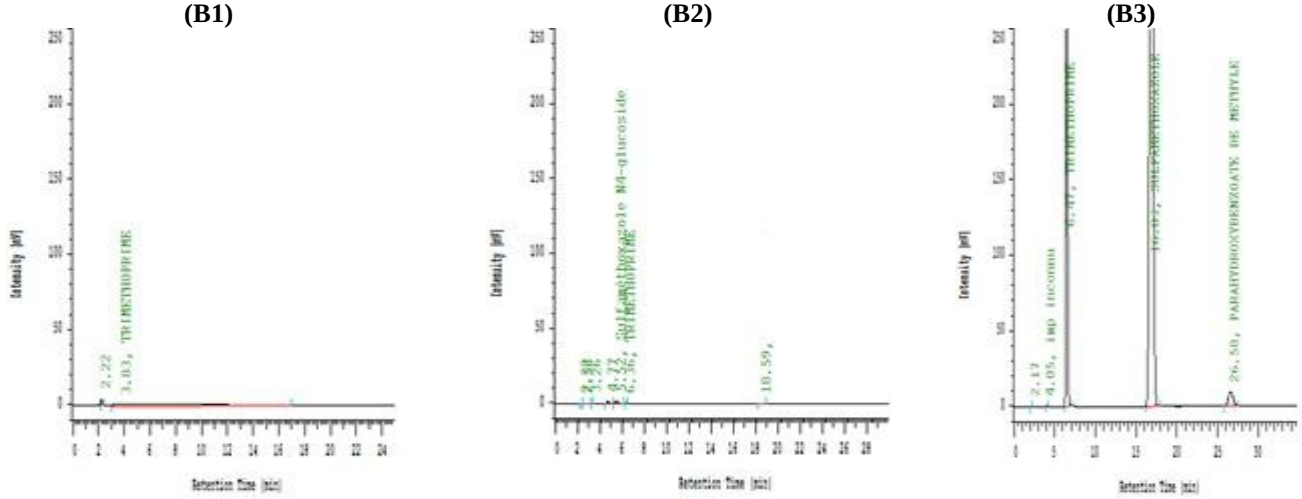
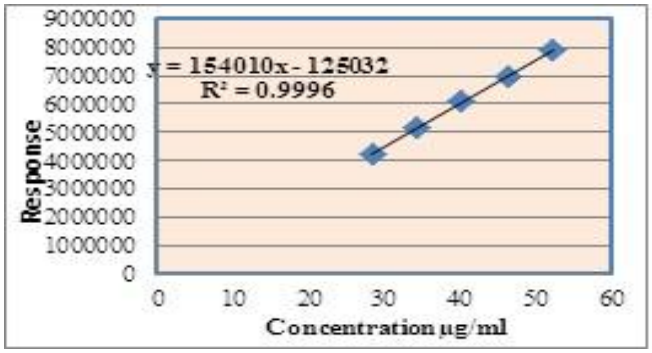
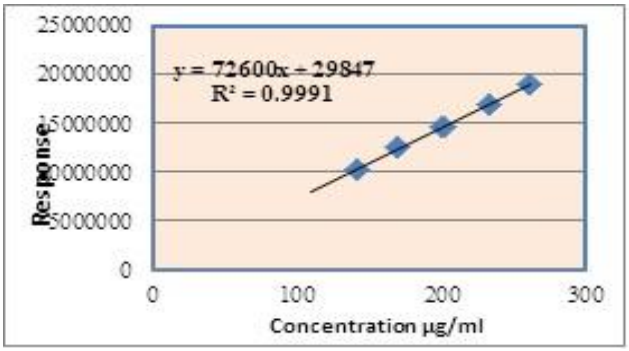


Fig 3. Linearity (calibration) curve of TM, SM and methyl paraben

TM



SM



Methyl paraben

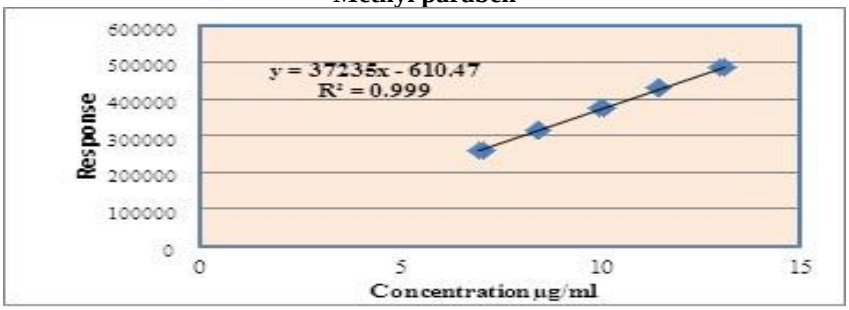
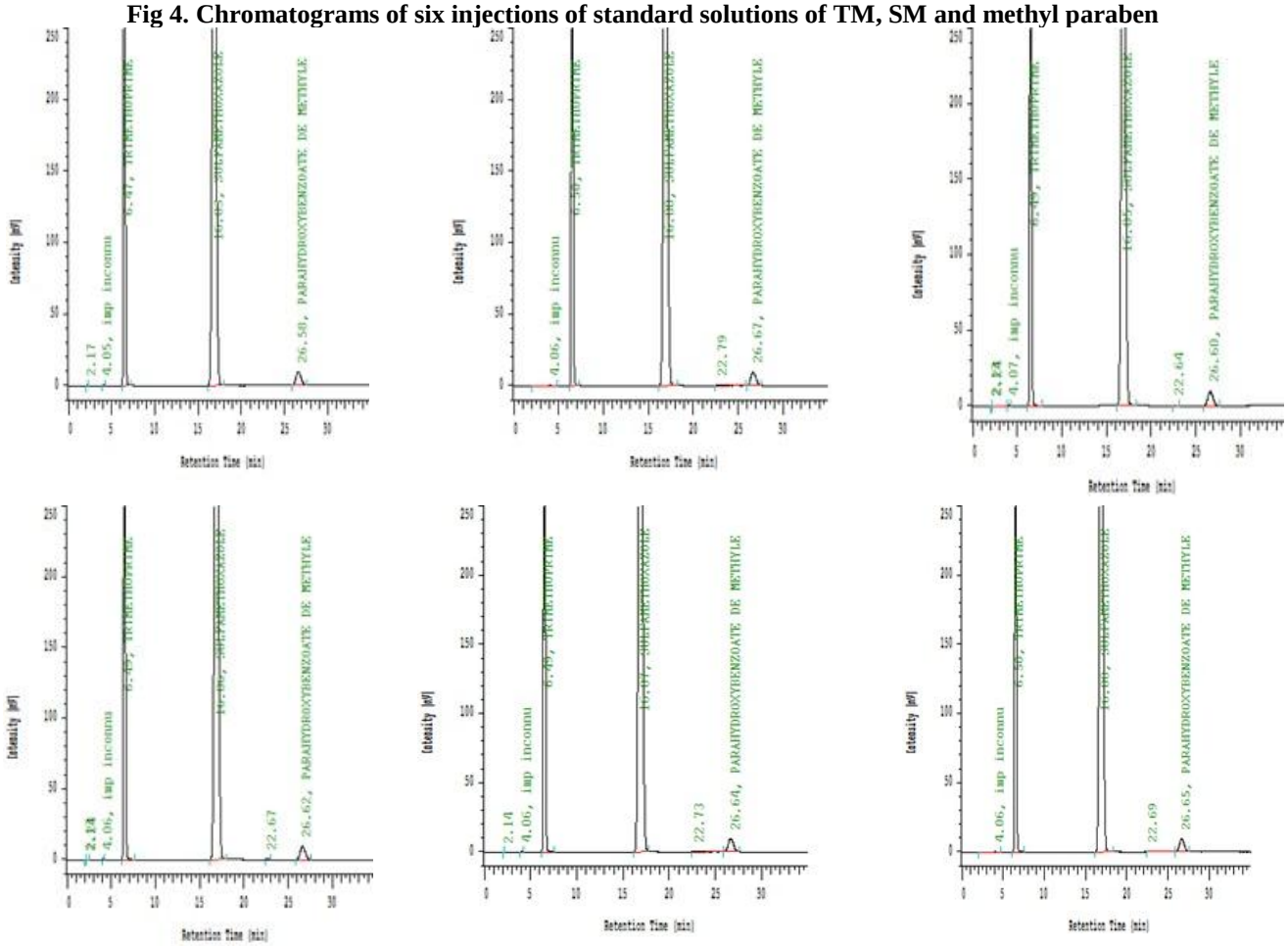


Fig 4. Chromatograms of six injections of standard solutions of TM, SM and methyl paraben



RESULTS AND DISCUSSION

In this method to optimize chromatographic parameters several mobile phase compositions were tried. A satisfactory separation, good peak symmetry and to achieve good retention time was obtained with economic mobile phase consisting of KH_2PO_4 0.05M/Acetonitrile /Methanol in ratio of (75:15:10) (v/v/v) adjusted to pH 5.6 with KOH. The flow rate was 1.0 ml/min with UV detection at 275 nm. The calibration curve was found to be linear in the range of 28 to 52µg/ml for Trimethoprim, 140-260µg/ml for Sulfamethoxazole and 7.0-13.0 µg/ml for methyl paraben. The linearity coefficient and percentage curve fitting slope were found to be 0.9996, 99.84% for Trimethoprim, 0.9991, 99.98% for Sulfamethoxazole and 0.9990, 99.72% for methyl paraben. The validation parameters are presented in Table 1. The LOD and LOQ for TM and SM were determined in the basis of peak response and slope of the regression equation. The LOD of the drug were found to be 0.05 µg/ml for Trimethoprim, 0.08 µg/ml for Sulfamethoxazole and 0.12 µg/ml for methyl paraben. The limit of quantification of TM, SM and methyl paraben was 0.17 µg/ml, 0.27 µg/ml and 0.40 µg/ml respectively. The low % RSD value for intraday and interday precisions revealed that the proposed method is reproductible and robust. No interfering peaks were found in the chromatogram indicating that the excipients used in Oral Suspension formulations did not interfere with the estimation of drug by the proposed HPLC method.

The % RSD value of assay determined under original conditions and robustness conditions was less than 2.0%, indicating that the developed method was robust.

System suitability was determined by performing the assay with the same sample repeatedly. The number of theoretical plates was found to be 7285 for TM, 6414 for SM and 9719 for methyl paraben. The tailing factor was found to be 1.16 for TM, 1.10 for SM and 1.09 for methyl paraben and it is indicating good and complete separation of the two components from each other with well defined base line.

CONCLUSION

Proposed study describes a new RP-HPLC method for the simultaneous estimation of these two drugs, Trimethoprim and Sulfamethoxazole in combination. The developed method is cheap, easy and it gives sharp peak with high resolution. The assay results are with the label claim of the formulation. The developed method is validated as per ICH guidelines using parameters like Accuracy, Precision Linearity and Range, Specificity, LOD, LOQ and Robustness. Hence the developed method is found to be satisfactory and it complies with all validation parameters. So this developed method can be used for the routine analysis in quality control laboratory of the drug in pharmaceutical formulation.

ACKNOWLEDGEMENT

I'm very thankful to Laboratory Assistance development Services[2] for providing the gift sample of pure drug of Trimethoprim and Sulfamethoxazole commercial Oral Suspension formulations. The authors are thankful to the University of Hassan II – Mohammedia, Faculty of Sciences and Technologies, Mohammedia, Morocco for providing necessary facilities to carry out this research work.

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