DEVELOPMENT AND VALIDATION OF LC-MS METHOD FOR SIMULTANEOUS DETERMINATION OF PYRIMETHAMINE AND SULFADOXINE IN THE PHARMACEUTICAL PREPARATION

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ABSTRACT

A simple, sensitive, accurate and precise liquid chromatography mass spectrometry (LC-MS) method was developed for determination of pyrimethamine and sulfadoxine in pharmaceutical formulation. The chromatographic separation was performed on Phenomenex C18 column (I.D. 250 mm x 4.6mm, 5µm) and the column oven temperature was set at 30 ºC, with an injection volume of 5 µl, acetonitrile and Phosphate buffer in the ratio 75: 25 (v/v) at pH 2.5 as mobile phase. The Electrospray Ionization (ESI) was performed using nitrogen gas to assist nebulization (flow rate was set at 1.0 mL/min), the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. As part of the method validation, specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, precision and robustness were determined. The limit of detection and limit of quantification for pyrimethamine and sulfadoxine were 0.69 and 0.82 ng/mL, and limit of quantification for pyrimethamine and sulfadoxine were 2.29 and 2.73 ng/mL respectively. The intra- and inter-day precisions were lower than 0.53% while the accuracy ranged from 99.99% to 100.11%. The admissible robustness indicates that the method remains unaffected by small but deliberate variations.

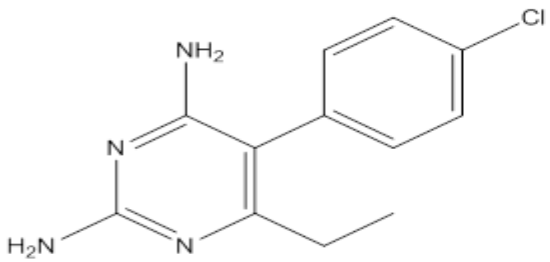
Keywords: Pyrimethamine, Sulfadoxine, Pharmaceutical formulation, ICH, LC-MS method, validation.

1. Introduction

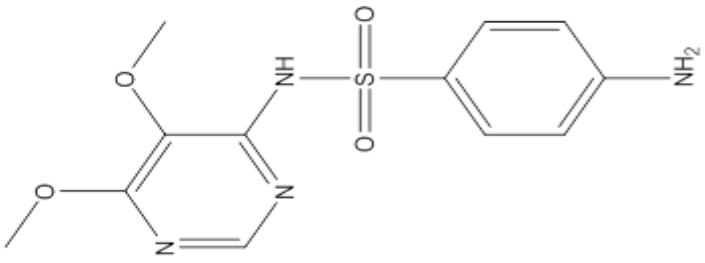
Pyrimethamine, 5-(4-chlorophenyl)-6-ethyl- 2,4-pyrimidinediamine, is an antiparasitic prescription medicine approved by the U.S. Food and Drug Administration (FDA) for the treatment of Toxoplasma gondii infection (toxoplasmosis) (The United States Pharmacopoeia 2011, Martindale 2009). Pyrimethamine is Soluble in DMSO (10 mg/ml at 25° C), hot ethanol, methanol, water (partly), acetonitrile, and dilute mineral acids. It has a molecular formula of $C_{12}H_{13}ClN_4$, and molecular weight of 248.71 g/mol as shown in **figure (1)** (Akwasi et al. 2018, Pai et al. 2016). Sulfadoxine, 4-Amino-N-(5,6-dimethoxy-4-pyrimidinyl)benzenesulfonamide, is an ultra-long-lasting sulfonamide used in combination with pyrimethamine to treat malaria (The United States Pharmacopoeia 2011, Martindale 2009). Sulfonamide is very slightly soluble in water; slightly soluble in alcohol and in methanol; practically insoluble in ether; soluble in dilute mineral acids and in solutions of alkali hydroxides and carbonates. It has a molecular formula of $C_{12}H_{14}N_4O_4S$, and molecular weight of 310.33 g/mol as shown in **figure (1)** (Anil et al. 2016, Sushil et al. 2021). Pyrimethamine and sulfadoxine is official in USP Pharmacopoeia, and world malaria report (Bergqvist et al. 1991). There are different analytical techniques applied for the determination of pyrimethamine and sulfadoxine as a combination in the pharmaceutical formulation such as spectrophotometric determination (Onah et al. 2002), HPTLC (Meena et al. 2013, Khuluza et al. 2016), HPLC chromatographic method (Márquez et al. 2012, Mwalwisi et al. 2016, Venkata et al. 2019). The main purpose of this work is to establish a sensitive, accurate and precise LC–MS method for the simultaneous determination of pyrimethamine and sulfadoxine in bulk powder and pharmaceutical preparation. The developed method was validated in accordance with ICH guidelines Q2 (R1) ((ICH Q2 (R1) 2005).

Figure (1): structural formula of pyrimethamine and sulfadoxine.

2. Experimental:



Pyremethamine



Sulphadoxine

2.1. Apparatus:

A Shimadzu LC-MS 2020 (Shimadzu, Japan) equipped with binary solvent delivery system, column compartment and a VWD-3400RS UV/Vis detector (Thermo Scientific, USA) was used for all analysis. All instrumentation data were collected and synchronized by Lab solutions software (Version 7.1) from Shimadzu, Japan.

2.2. Materials:

2.2.1. Pure sampels:

Reference standards of sulfadoxine (Chegshu Jinshen Med, China) and pyrimethamine (Xinda Pharma & Chem. Co. Ltd., China) were given by Trade Winds Pharmaceutical Company Ltd. in Ghana.

2.2.2.Pharmaceutical preparation:

Fansidar® tablet, each tablet contains sulfadoxine-500 mg and pyrimethamine-25 mg was obtained as gift samples (F. Hoffmann-La Roche Ltd. Basel, Switzerland, Distributed by: Roche Laboratories, Batch number. NDC-0004-0161-03, and expire date November- 2025).

2.3. Chemicals and reagents:

- Acetonitrile was purchased from Sigma-Aldrich (Steinheim, Germany).
- Monosodium phosphate (Sigma-Aldrich, Steinheim, Germany).
- Disodium phosphate (Sigma-Aldrich, Steinheim, Germany).
- Deionized water (WP 4100 reagent grade water purifier-SMEG) was used for standard and sample preparations.
- Phosphate buffer prepared using sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate dihydrate with pH (2.5) and dilute phosphoric acid (all these chemicals were purchased from Sigma-Aldrich, Germany) as prescribed in British pharmacopeia (The British Pharmacopoeia 2011).

2.4. Standard solutions:

2.4.1. Standard solutions of pyrimethamine:

A stock standard solution of pyrimethamine (1.0 mg/mL) was prepared by dissolving 100 mg of the drug powder in 100 mL of the mobile phase (acetonitrile and Phosphate buffer (pH 2.5) in the ratio 75: 25 (v/v)). Working standard solution (100µg/mL) was prepared by dilution of the stock solution with mobile phase.

2.4.2. Standard solutions of sulfadoxine:

A stock standard solution of sulfadoxine (1.0 mg/mL) was prepared by dissolving 100 mg of the drug powder in 100 mL of the mobile phase. Working standard solution (100µg/mL) was prepared by dilution of the stock solution with mobile phase.

2.5. Procedures:

2.5.1. Chromatographic conditions:

The chromatographic separation was performed on Phenomenex C18 column (I.D. 250 mm x 4.6mm, 5µm) and the column oven temperature was set at 30 ºC, with an injection volume of 5 µl, was used for the chromatographic separation. The instrument was operated by switching electrospray ionization (ESI) source in positive and negative ionization modes in a single run. The mobile phase was a mixture of acetonitrile and Phosphate buffer (pH 2.5) in the ratio 75: 25 (v/v). The ESI was performed using nitrogen gas to assist nebulization (flow rate was set at 1.0 mL/min).

2.5.2. Construction of the calibration graph:

Aliquots of 5 µL of analytes standard solutions at six different concentrations (5–30 ng/mL) of Pyrimethamine and (50–300 ng/mL) of sulfadoxine were injected into LC-MS system. The procedure was carried out in triplicate for each concentration. The analyte peak area obtained was plotted against the corresponding concentration of the analyte (expressed as ng/mL).

2.5.3. Validation of the procedure:

- **Linearity and range:**

The general procedure of the method under **"**2.5.2**"** was repeated. Calibration graphs were constructed by plotting the peak area values of Pyrimethamine and sulfadoxine versus their concentrations in ng/mL; alternatively, the regression equations were derived.

- **Limits of detection and quantitation:**

The limit of detection (LOD) and the limit of quantitation (LOQ) values were calculated according to ICH guidelines using signal to noise ratio.

A signal to noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit and 10:1 is atypical signal to noise ratio for estimating the quantification limit.

- **Accuracy and precision:**

Accuracy of the method, calculated as the mean percent recovery (%R), was assessed by applying the described procedure for triplicate determination of three concentration levels covering the linearity range (5, 10, 25 ng/mL) for Pyrimethamine and (50, 150, 300 ng/mL) for sulfadoxine.

Precision of the method, calculated as the percent of relative standard deviation (% RSD), was assessed by applying the described procedure for triplicate determination of three concentration levels covering the linearity range (5, 10, 25 ng/mL) for

Pyrimethamine and (50, 150, 300 ng/mL) for sulfadoxine, within one day for repeatability and on three successive days for intermediate precision.

- **Specificity:**

The specificity of the method was assessed by applying the proposed procedure for determination of pyrimethamine and sulfadoxine in their synthetic mixtures. The specificity of the method was also determined by applying the standard addition technique through adding known quantities of pyrimethamine and sulfadoxine in their pure forms to already analyzed pharmaceutical preparation, then the percent recovery (**%R**) of the pure added concentrations was calculated.

- **System suitability:**

System suitability test was applied to a representative chromatogram to check various parameters such as the number of theoretical plates (N), resolution factor (Rs) and tailing factor (T). These parameters were calculated according to FDA regulations.

- **Robustness:**

The robustness of the method was evaluated by slight changes in the chromatographic conditions such as flow rate (±0.1 mL/min), and LC temperature (±1%). In each case only one parameter was changed while other conditions were kept constant.

2.5.5. Procedure for pharmaceutical preparation:

i. Preparation of pharmaceutical sample:

Ten Fansidar® tablets (each tablet contains 25mg of pyrimethamine and 500 mg of sulfadoxine) were weighed and then finely powdered. Appropriate weight of the powder equivalent to one tablet was accurately weighed, transferred to 100- mL volumetric flask and About 50 ml of mobile phase was added to the flask and sonicated for 15 min to disperse the contents completely and filtered using Whatman filter paper (no. 45). The residue was washed three times with 10 mL mobile phase and The volume was made up to the mark with the mobile phase to obtain sample stock solution labeled to contain 0.25 mg mL^{-1} pyrimethamine and 5 mg/mL of sulfadoxine. This stock solution was diluted with mobile phase to obtain a series of test sample solutions containing pyrimethamine and sulfadoxine.

ii.Analysis of pharmaceutical sample:

The general procedure under "2.5.2" was repeated using aliquots covering the working concentration range. The content of the tablet was determined from the corresponding regression equation.

3. Results & discussions:

3.1. Optimization of experimental conditions:

The chromatographic separation was optimized after taking into account the resolution between the drugs. The column was performed by a flow rate of 1.0 mL/min and an injection volume of 5 µL. After optimization of the chromatographic conditions, well-defined symmetrical peaks were obtained, as shown in **figure (2)**. From this chromatogram it was found that pyrimethamine and sulfadoxine were clearly separated and their corresponding peaks were sharply developed at retention times of 4.59± 0.014 and 3.18± 0.012 min respectively. The ESI was performed using nitrogen gas to assist nebulization (flow rate was set at 1.0 L/min), the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. Con voltage at 30 and 40 V for pyrimethamine and sulfadoxine respectively, [M + H]+ of pyrimethamine and sulfadoxine were 249.10 and 310.33 respectively and temperatures of Curved Desolvation Line (CDL) and heat block at 250 ºC and 300 ºC were used as shown in **figure (3,5)**. The product ions of pyrimethamine were detected at 124 and 213 m/z and the product ions of sulfadoxine were detected at 108 and 247 m/z as shown in **figure (4, 6).** All instrumentation data were collected and synchronized by Lab solutions software.

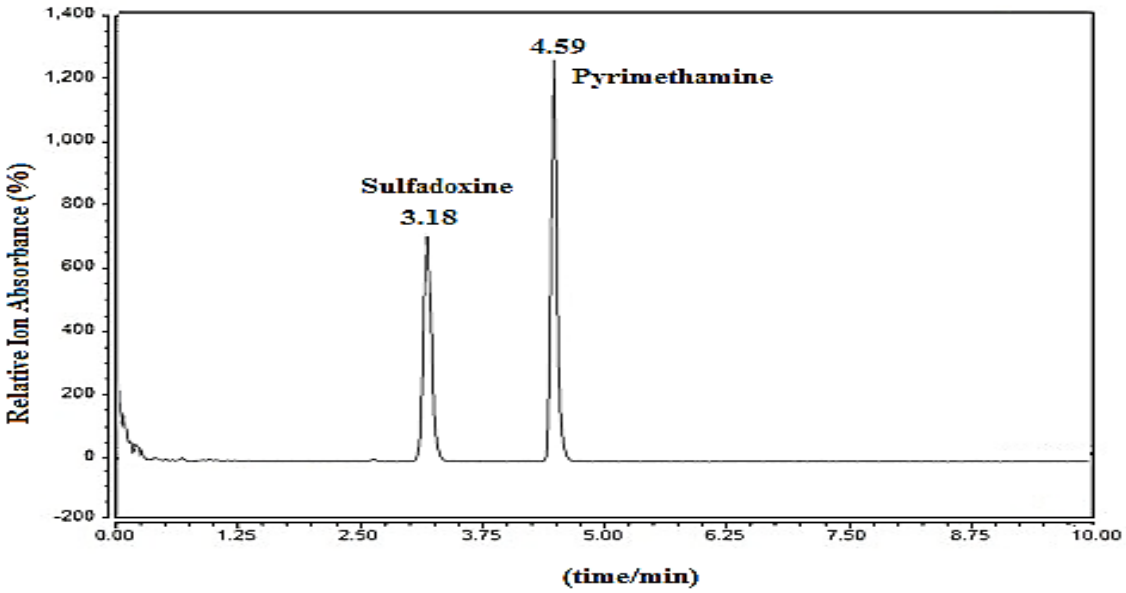


Figure (2): LC chromatogram of sulfadoxine (100 ng/ml) and pyrimethamine (20 ng/mL).

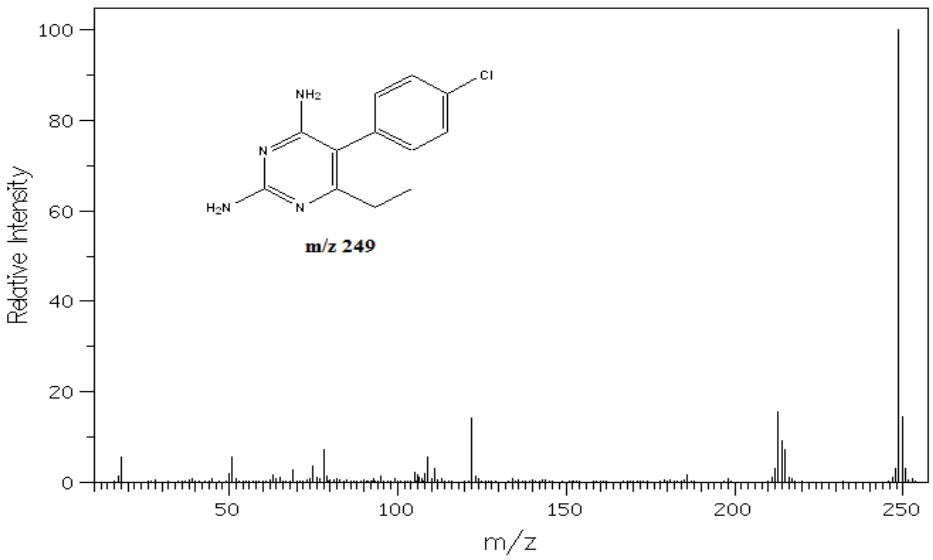


Figure (3): Mass spectrum of pyrimethamine.

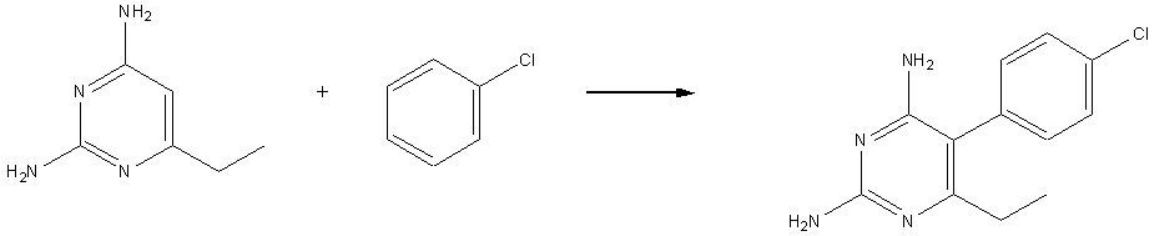


Figure (4): Fragmentation pattern of pyrimethamine.

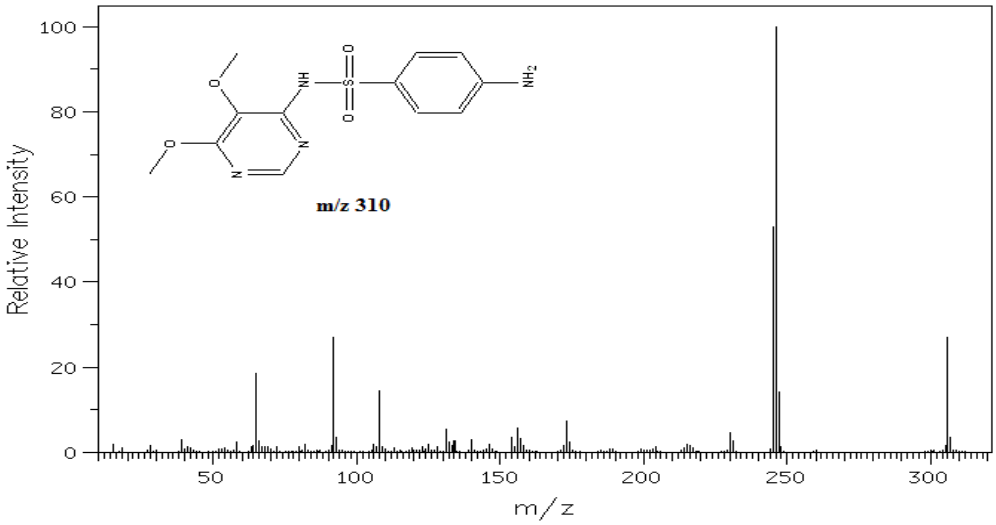


Figure (5): Mass spectrum of sulfadoxine.

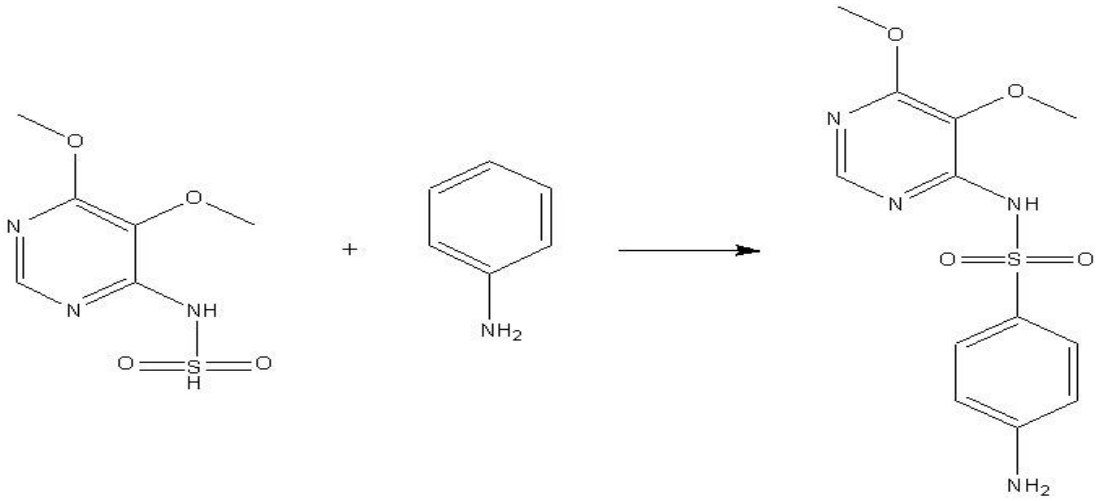


Figure (6): Fragmentation pattern of sulfadoxine.

3.2. Method validation:

- **Linearity and range:**

Under the described experimental conditions, the calibration graphs for the method were constructed by plotting the peak area values of each drug versus concentrations in ng/mL. The regression plots were found to be linear over the range of 5-30 ng/mL for pyrimethamine and 50-300 ng/mL sulfadoxine, as shown in **figures (7, 8)**. The regression data were presented in **table (1)**.

- **Limits of detection and quantitation:**

LOD and LOQ values were calculated and the obtained results indicated the sensitivity of the proposed method for analysis of the studied drugs as shown in **table (1).**

- **Accuracy and precision:**

Accuracy of the proposed method was indicated by the obtained good %R as shown in **table (1)**. The small values of %RSD indicated high precision of the method as shown in **table (1)**.

- **Specificity:**

LC chromatogram revealed that pyrimethamine and sulfadoxine were well clearly separated from each other confirming the selectivity and specificity of the method. Moreover the standard addition technique was also applied to check the effect of the matrix on the separation of both drugs. The obtained results as shown in **table (2)** proved that the proposed method could selectively analyze the drugs without any interference.

- **System suitability:**

System suitability parameters must be checked to ensure that the system is working correctly during the analytical process. The results, as shown in **table (3)**, revealed that the described chromatographic conditions allowed complete base line separation between pyrimethamine and sulfadoxine peaks with minimum tailing. The obtained values were within the acceptance reference values according to FDA regulations.

- **Robustness:**

The described minor changes did not affect the separation and resolution of pyrimethamine and sulfadoxine, confirming robustness of the procedure, as shown in **table (1)**.

3.3. Pharmaceutical applications:

The proposed LC-MS method was applied for the simultaneous determination of pyrimethamine and sulfadoxine in **Fansidar**® tablet. Satisfactory results were obtained in good agreement with the label claim, indicating no interference from excipients and additives which was confirmed by the results of the standard addition technique. The obtained results were statistically compared to those obtained by the reported method (Akwasi et al. 2018). No significant differences were found by applying *t*-test and *F*-test at 95% confidence level, indicating good accuracy and precision of the proposed method for the analysis of the studied drugs in their pharmaceutical dosage form, as shown in **table (4)**.

This method was more sensitive and accurate than the reported method for simultaneous determination of pyrimethamine and sulfadoxine. The linearity of the proposed method was more accurate and precise than the reported method. The determination coefficient of the proposed method was 0.9998 and 0.9999 for pyrimethamine and sulfadoxine respectively.

Table (1): Regression and validation data for determination of pyrimethamine and sulfadoxine by the proposed LC-MS method:

Parameter	Pyrimethamine	Sulfadoxine
Linearity range (ng/mL)	5 – 30	50 – 300
LOD (ng/mL)	0.69	0.82
LOQ (ng/mL)	2.29	2.73
Regression parameter*	Y= a+ b C	Y= a+ b C
Determination coefficient	0.9998	0.9999
Slope (b)	15878	18222
Intercept (a)	660.87	- 4421.5
Accuracy (mean)	99.99	100.11
Precision (% RSD)		
Repeatability	0.394	0.447
Intermediate precision	0.533	0.492
Robustness (%RSD)		
Flow Rate (±0.1mL)	0.298	0.455
LC temperature (±1 ^{O}C)	0.349	0.543

*Y= a + bC, where Y is the peak area and C is the concentration in ng/mL.

Table (2): Recovery study of pyrimethamine and sulfadoxine by applying standard addition technique:

Drug	Pharmaceutical taken (µg/mL)	Pharmaceutical Found (µg/mL)	Pure added (µg/mL)	Pure found (µg/mL)	%Recovery
Pyrimethamine	10	9.97	8	7.92	99.00
			10	9.86	98.60
			12	12.05	100.42
	Mean ± % RSD				99.34 ± 0.492
Sulfadoxine	100	99.89	80	79.91	99.90
			100	100.08	100.08
			120	119.84	99.86
	Mean ± % RSD				99.94 ± 0.472

Table (3): System suitability test for pyrimethamine and sulfadoxine

Acceptance criteria	Results	
	Pyrimethamine	Sulfadoxine
The %RSD for five replication injections	0.542	0.434
Resolution	1.25	
The Tailing factor	1.55	1.48
Theoretical Plates	2972	2887

Table (4): Results obtained for determination of pyrimethamine and sulfadoxine in Fansidar tablet and comparison with the reported method.

Parameter	Proposed method		Reported method *	
	Pyrimethamine	Sulfadoxine	Pyrimethamine	Sulfadoxine
n[a]	5	5	5	5
%R	99.34	99.94	100.24	100.32
%RSD	0.492	0.472	0.792	0.884
SD	0.488	0.472	0.793	0.886
Variance	0.238	0.223	0.630	0.786
Student's t-test (2.306)b	1.409	1.347	——	------
F-value (6.388)b	2.647	3.524	——	-----

[a] Experiments number.

[b] Tabulated values of "t "and "F" at (P = 0.05).

*HPLC method using C-18 column combined with a mixture of acetonitrile: phosphate buffer (75:25 v/v) mobile phase, pH adjusted with H_3PO_4 to be 2.5. The flow rate was 1.0 mL/min (Akwasi et al. 2018).

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التطوير والتحقق من صحة طريقة LC-MS للتقدير المتزامن للبيريميثامين والسلفادوكسين في المستحضرات الصيدلانية

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تم تطوير طريقة بسيطة وحساسة ودقيقة لقياس الطيف الكتلي السائل (LC-MS) لتقدير البيريميثامين والسلفادوكسين في المستحضرات الصيدلانية. تم إجراء الفصل الكروماتوجرافي على عمود Phenomenex C18 (٢٥٠ مم × ٤.٦ مم، ٥ ميكرومتر) وتم ضبط درجة حرارة فرن العمود على ٣٠ درجة مئوية، مع حجم حقن قدره ٥ ميكرولتر، ومحلول أسيتونيتريل وفوسفات بنسبة ٧٥: ٢٥ (v / v) عند درجة الحموضة ٢.٥ كمرحلة متنقلة. تم إجراء ESI باستخدام غاز النيتروجين للمساعدة في الإرذاذ (تم ضبط معدل التدفق عند ١.٠ لتر/دقيقة)، وتم تشغيل مطياف الكتلة في وضع مراقبة التفاعلات المتعددة (MRM). كجزء من التحقق من صحة الطريقة، تم تحديد (LOD) و(LOQ). وكان (LOD) للبيريميثامين والسلفادوكسين ٠.٦٩ و ٠.٨٢ نانوغرام / مل على التوالي، وكان (LOQ) للبيريميثامين والسلفادوكسين ٢.٢٩ و ٢.٧٣ نانوغرام / مل على التوالي. وكانت %RSD أقل من ٠.٥٣% بينما تراوحت accuracy من ٩٩.٩٩% إلى ١٠٠.١١%. الطريقة المقترحة حصلت على فصل جيد ودقة تامة .تم التحقق من صحة الطريقة المقترحة وفقًا لإرشادات ICH وتم تنفيذها لتحديد الأدوية المذكورة في صيغتها الصيدلانية.

الكلمات المفتاحية: بيريميثامين، سلفادوكسين، المستحضرات الصيدلانية، ICH، طريقة LC-MS، التحقق من الصحة.