Validation A New RP-HPLC Method For Simultaneous Determination Of Metronidazole And Spiramycin In Bulk Drug And Pharmaceutical Formulations

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Abstract

A new RP-HPLC method was developed and validated for Quantitative determination of Spiramycin and Metronidazole in bulk drugs and in pharmaceutical formulations. The separation was done on a CAPCELL PAK C18 column (250 mm x 4.6 mm ID x 5µm), and the detection was performed at 232 nm using UV detector. The mobile phase was a mixture of Methanol–Acetonitrile–Aqueous phosphate buffer (pH 2.64; 0.05 M) (45: 11: 44; v/v) pumped at ambient temperature with a flow rate of 0.9 ml/min, the calibration curve was linear from 5 to 40 µg/ml for both drugs with $R^2 > 0.999$. The detection limit (DL) and quantitation limit (QL) were found to be 0.381 and 1.27 µg/ml for Metronidazole and 0.488 and 1.629 µg/ml for Spiramycin, respectively. Accuracy (mean recovery: 100.11, 100.8% for Metronidazole and Spiramycin, respectively) and precision were found to be satisfactory. The optimized method was validated according to The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines [1]. The use of the gradient elution enhanced the separation between the two mentioned drugs in a simple, sensitive, fast and accurate manner within a short

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analysis time. The proposed method can be used for quality control assay of Spiramycin and Metronidazole in bulk drugs and in pharmaceutical formulations as a result of the ability of the method to separate both drugs from its excipients.

Keywords: Metronidazole, Spiramycin, RP-HPLC Method, Method Validation

صلاحية طريقة جديدة باستعمال الكروماتوغرافيا السائلة ذات الطور العكوس للتحديد المتزامن لميترونيدازول وسبيرامايسين في المادة الأولية الدوائية والمستحضرات الصيدلانية

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الملخص

طورت طريقة RP–HPLC جديدة وتُحُقِّق من صحتها من أجل التحديد الكمي للسبيرامايسين والميترونيدازول في الأدوية السائبة وفي المستحضرات الصيدلانية. أُجري الفصل على عمود CAPCELL PAK C18 (250 mm x 4.6 mm ID x 5µm)، وأُجري الكشف عند 232nm باستعمال كاشف UV. كان الطور المتحرك عبارة عن خليط من محلول ميثانول –أسيتونيتريل – محلول موقي فوسفاتي (pH 2.64؛ 0.05 M) (45: 11: 44 ; v/v) ضُخَّ عند درجة حرارة محيطة بمعدل تدفق 0.9 ml/min ، كان منحنى المعايرة خطيًا من 5 إلى 40 µg/ml لكلا الدواءين مع R^2> 0.999. عُثِرَ على حد الكشف الكيفي (DL) وحد الكشف الكمي (QL) ليكون 0.381 و 1.27µg/ml للميترونيدازول و0.488 و 1.629µg/ml لسبيرامايسين، على التوالي. نتائج صحة التحليل (متوسط الاسترداد: 100.11، 100.8% للميترونيدازول وسبيرامايسين، على التوالي) والدقة كانت مرضية. تُحُقِّقَ من صحة الطريقة المثلى وفقًا لإرشادات المجلس الدولي لمواءمة المتطلبات الفنية للمستحضرات الصيدلانية للاستخدام البشري ICH. عزز استخدام الشطف المتدرج الفصل بين العقارين المذكورين بطريقة بسيطة وحساسة وسريعة ودقيقة في وقت تحليل قصير. يمكن

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استخدام الطريقة المقترحة لضبط جودة تحديد سبيرامايسين وميترونيدازول في الأدوية السائبة وفي المستحضرات الصيدلانية نتيجة لقدرة الطريقة على فصل كلا العقارين عن سواغاتهما.

الكلمات المفتاحية: ميترونيدازول–سبيرامايسين–طريقة كروماتوغرافية سائلة–صلاحية طريقة (Validation Method) .

Introduction

Metronidazole (MEZ) (Figure 1) is the reference agent of the nitroimidazole anti-infective family (antibiotic drug) [2, 3]. It is chemically designated as 2-(2-methyl-5-nitro-1H- imidazol- 1-yl) ethanol [2, 4], OR 1-(2-hydroxyethyl)-2-methyl-5- nitroimidazole [3]. In humans it is used mainly in the treatment of infections caused by anaerobic bacteria and protozoa [2, 3, 4], and it has a radio-sensitizing effect on hypoxic tumour cells [2, 5]. It has also been used as an antiparasitic in veterinary work [2]. It is an amebicide, and it is the drug of choice for first episodes of mild-to-moderate Clostridium difficile infection. In addition or alternatively, the metronidazole metabolites are taken up into bacterial DNA, and form unstable molecules. This function only occurs when metronidazole is partially reduced, and because this reduction usually happens only in anaerobic cells, it has relatively little effect upon human cells or aerobic bacteria [3].

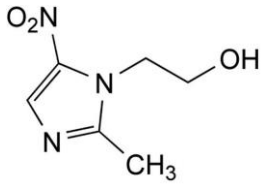


Figure 1 Chemical formula structures of Metronidazole

Spiramycin (SPI) (Figure 2) is chemically designated as (6R,7R,9R,10R,11E,13E,16R)-10-{[(2R,5S,6R)-5-(dimethylamino)-6-methyltetra-hydro-2H-pyran-2-yl]oxy}-5,9,16-trimethyl-2-oxo-7-(2-oxoethyl)oxacyclohexadeca-11,13-dien-6-yl 3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methyl-α-L-ribo-hexopyranosyl)-3-(dimethylamino)-α-D-glu-copyranoside [6], It belongs to the class of 16-membered macrolide antibiotics and it is considered to be a medium-spectrum antibiotic with high effectiveness against Gram-positive bacteria[7], and it is effective against many of the organisms found in obstetric and gynaecological infections including chlamydiae, mycoplasmas, Bacteroides melaninogenicus and almost all streptococci not belonging to group D. It is active against most gonococci, Listeria monocytogenes, clostridia and most staphylococci. Aerobic Gram-negative bacilli are resistant to Spiramycin [8].

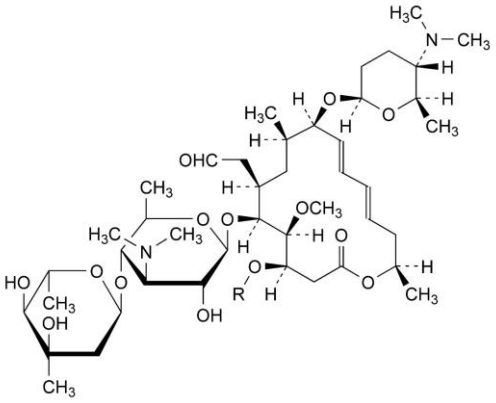


Figure 2 Chemical formula structures of Spiramycin

The combination of MEZ and SPI has been developed on the basis of the complementarity of the antibacterial activity of both compounds in vitro on the range of bacteria involved in dental infections [9], and upper respiratory tract infections [10]. Several methods have been reported for the determination of either MEZ or SPI alone, each in its single dosage form (not in combination), or in combination with other drugs. MEZ determined by short-wavelength Near-infrared spectroscopy [11], voltammetry [12, 13], thin-layer chromatography (TLC)[14], using spectrophotometr [15], flow injection chemiluminescence analysis [16], nuclear magnetic resonance spectrometry (NMR) [17], capillary electrophoresis [18], gas chromatography and HPLC methods either alone [19, 20], or in the presence of its metabolites [21], or its degradation product [22], in addition to its mixture with other drugs [5]. SPI Was also determined using several techniques such as HPLC [23, 24, 25], capillary electrophoresis [26, 27], voltammetry [28], TLC [29, 30, 31, 32], and immunological assay [33]. Several methods have been reported for the determination of SPI and MEZ mixture [2, 10, 34, 35, 36, 37]. A comprehensive literature search revealed the lack of any *gradient RP-HPLC* Methods for the simultaneous determination of SPI and MEZ as bulk drug and as pharmaceutical dosage forms. Therefore, the principal aim of this study was to develop a new, simple, economical, precise and stable *RP-HPLC method* with a wide linear range and good sensitivity for assay of both MEZ and SPI in the bulk drug and in the pharmaceutical dosage forms (tablets).

1. Materials and methods

1.1 Chemicals and solutions

Spiramycin reference material was purchased from Wuxi Fortune Pharmaceutical Co., Ltd, China; was kindly supplied from (Drug Control Directorate), (purity ≥ 99%). Metronedazol reference material was purchased from (Hubei Hongyuan), batch No (0121610063) (purity ≥ 99.7%) was obtained from (China). Methanol and Acetonitrile, HPLC grade (Merck, Germany). Di-sodium Hydrogen Orthophosphate Dihydrate (HiMedia, India). All other reagents used in this study were of analytical grade. Purified water was used for making the solutions.

The studied dosage form were Spirazole tablets® of Balsam Pharma (batch no. 14; 52; 017) contains (750,000IU), equivalent to 182.926mg SPI (Potency: 4100IU/mg) and 125mg MEZ, HaSy-Dent tablets® of Nawras Pharmaceutical industries (batch no. 06) contains (750,000IU), equivalent to 182,926mg SPI (Potency: 4100IU/mg) and 125 mg MEZ. Were purchased from the market.

1.2 Chromatographic conditions

Analyses were performed with a HPLC system (Shimadzu, Kyoto, Japan) equipped with two pumps LC-20AD, DGU- 20A3 degasser, an SPD-20AV (UV–VIS) detector. The chromatographic separation was achieved using a CAPCELL PAK C18 column (250mm × 4.6mm ID × 5µm, Shiseido, Japan). The out-put signal was monitored and processed using (Shimadzu LCsolution) software. The mobile phase comprised of a mixture of Methanol – Acetonitrile – Aqueous phosphate buffer (pH 2.64; 0.05M) (45: 11: 44, v/v), Acetonitrile was initially started at 11% then it was gradient up to 14% over 0.9min (On whose account the buffer solution), then it was isocratically held for 5min (5:9min). The pH of the phosphate buffer solution was adjusted to 2.64 using orthophosphoric acid as diluent. The mobile phase was filtered through 0.45 micron membrane filter, the samples were also filtered and degassed in ultrasonic bath. The flow rate was 0.9mL/min. All determinations were performed at ambient temperature and the detection wavelength was 232nm. The injection volume was 20µL. The column was equilibrated for 60min prior to the injection of the drug solution.

1.3 Preparation of standard solution

62.5mg of Spiramycin Reference Standard and Metronidazole Reference Standard were accurately weighed separately, transferred in a 50mL volumetric flask, dissolved with the initial mobile phase with the help of

ultrasonic bath for about (10min) and diluted to 50mL with the diluent. From this stock solution, 5mL was transferred in a 50mL volumetric flask and diluted with the diluent. This final solution contained 125µg/mL of Spiramycin and Metronidazole. Working solutions of SPI and MEZ were prepared by an additional dilution of their stock standard solutions to reach the concentration range (5-40µg/ml) for both MNZ and SPY.

1.4 Preparation of sample solution

The contents of 20 tablets of Spirazole tablets® and HaSy-Dent tablets® drugs were separately ground in a mortar to form a homogeneous powder. A portion of each finely powdered drug, equivalent to 91.46mg SPI and 62.5mg MEZ was accurately weighed and transferred into a 50mL volumetric flask and dissolved with the initial mobile phase composition in an ultrasonic bath for 20min. The solutions was cooled then diluted to volume with the same solvent and filtered. Further dilutions of the prepared solutions were carried out using the initial mobile phase composition to reach tablet solutions containing 20µg/mL MEZ and 29.27µg/ml SPI.

1.5 Calculation

Sample solutions were analyzed under the specified chromatographic conditions and the concentrations of each drug were calculated from the corresponding regression equation.

1.6 Method validation

The performance of the proposed method was validated according to ICH guidelines [ICH, Q2A, 1995; ICH, Q2 (R1), 1995; ICH, Q2B, 1996].

2. Results and discussion

2.1 System suitability

System suitability tests are an integral part of gas and liquid chromatographic methods. These tests are used to verify that the chromatographic system is adequate for the intended analysis. Capacity factor (k´), plate number (N), tailing factor (T), and RSD were evaluated (Equation 1, 2, 3, 4, 5) for five replicate injections of the drugs at a concentration of 20 µg/ml. The results given in Table 1, 2 and Figure 3 were within acceptable limits (FDA, 1994).

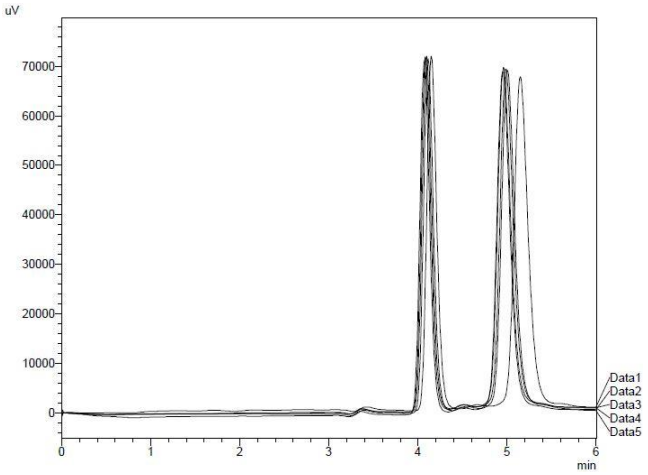


Figure 4 Chromatogram of reference standard mixture of MEZ and SPI for five replicate injections (20 µg/ml).

Table 1 chromatogram parameters of reference standard mixture of MEZ and SPI.

peak area		retention time	
MEZ	SPI	MEZ	SPI
563007	726534	4.147	5.094
562081	725248	4.11	5.001
561909	727632	4.094	4.984
558816	725570	4.082	4.968
555958	724641	4.069	4.969

$$N = 16\left(\frac{t_R}{W_b}\right)^2 \quad (1) \qquad k' = t_R - t_m / t_m \quad (2)$$

N: Theoretical plate k´: Capacity factor t_R: retention time

t_m: void time (dead time) W_b: peak width

$$T = (a+b)/2a \qquad (3)$$

T: Tailing factor

a: the distance from the leading edge of the peak to the peak midpoint measured at 5% of peak height.

b: the distance from the peak midpoint to the trailing edge of the peak measured at 5% of peak height.

$$\alpha = k'_b / k'_a \qquad (4)$$

α :selectivity factor

k'_b: Capacity factor of the studied substance SPI.

k'_a: Capacity factor of the studied substance MEZ.

$$RSD = \bar{X}/SD \qquad (5)$$

$\bar{X}$: The average of the measured values.

SD: Standard deviation of the measured values.

Table 2. Results from system suitability studies

Property	Values		Required limits
	MEZ	SPI	
RSD of peak area	0.005213	0.001618	RSD < 1 % for n ≥ 5
RSD of retention time	0.007346	0.010494	RSD < 1 % for n ≥ 5
Tailing factor (T)	1.23	1.27	T < 2
Theoretical plate (N)	3979.475	3464.638	plate N > 2000
Capacity factor (k´)	1.113608	1.578969	
selectivity factor (α) =	1.417886		

2.2 Method validation

2.2.1 Specificity

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components [USP38, 2015, General chapter < 1225 >].The specificity of the HPLC method is illustrated by the complete separation of the compounds peaks in the presence of tablets excipients. The peaks obtained were regular and symmetry and had clear baseline separation (Figure 7a).

2.2.2 Accuracy

To study the accuracy of the method, recovery experiments were carried out. Known quantities of the pure drugs of MEZ and SPI were used to make samples at the levels of 25%, 100%, and 200%, and were assayed by the proposed method. Accuracy was calculated as the percentage of recovery and the results are shown in Table 3.

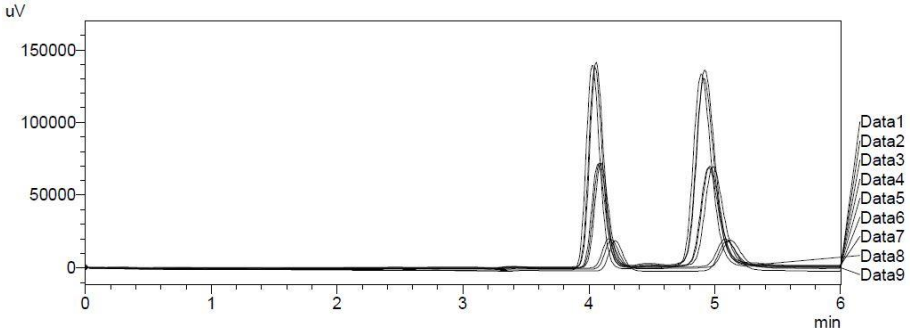
$$\text{Recovery \%} = \left(\frac{\text{amount of substance actually collected}}{\text{Labeled amount}}\right) \times 100$$

Table 3. Accuracy of the method in the range studied

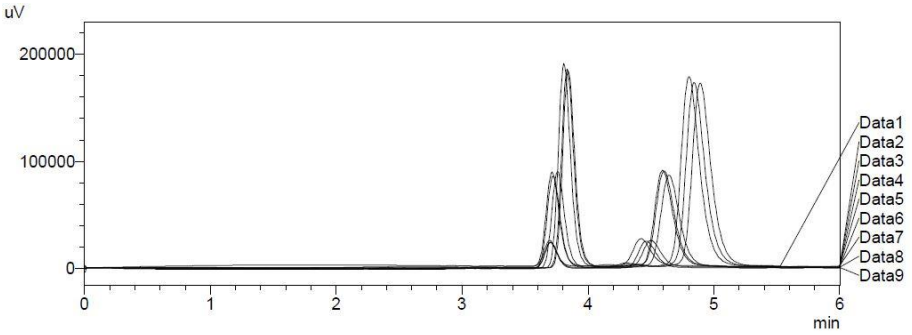
Level of addition (%)	Concentration (µg/ml)	Recovery (%)	
		SPI	MEZ
25	5	101.57	99.911
100	20	101.63	101.85
200	40	99.34	98.57
Mean recovery (%) =		100.85	100.11

2.2.3 Precision

The precision was demonstrated at two levels: repeatability (Intra-day precision) and intermediate precision (Inter-day precision). Each level of precision was investigated by 3 sequential replicate of injections of three concentrations of 5, 10 and 40 µg/ml of MEZ and SPI. The precision was expressed as relative standard deviation (RSD). The results of two levels of precision are shown in Table 4 which indicated satisfactory precision of the proposed methods.



(A) 1) MEZ, 2) SPI



B) 1) MEZ, 2) SPI

Figure 4 Chromatogram of reference standard mixture of MEZ and SPI for nine replicate injections (5, 20, 40 µg/ml).
A: Repeatability B: Intermediate precision

Table 4. The precision of the method in the range studied.

Precision	result				
	Concentration (µg/ml)	RSD of peak Area		RSD of Retention time	
		MEZ	SPI	MEZ	SPI
Repeatability	5	0.0074	0.0069	0.0062	0.0039
	20	0.0032	0.0017	0.0034	0.0043
	40	0.0093	0.0076	0.0036	0.0029
Intermediate precision	5	0.0056	0.0089	0.0010	0.0087
	20	0.0107	0.0077	0.0052	0.0110
	40	0.0073	0.0063	0.0046	0.0091

2.2.4 Detection limit (DL) and quantitation limit (QL)

The SD of the response and the slope was used for estimation of the DL and QL, respectively (Equation 6, 7) (ICH, Q2B, 1996). So, the limit of detection and the limit of quantitation were determined to be 0.381 and 1.27 µg/ml for Metronidazole and 0.488 and 1.629 µg/ml for Spiramycin, respectively.

$LOD = 3.3\ \sigma/S$ (6)

$LOQ = 10\ \sigma/S$ (7)

σ: the SD of response; S: the slope of the calibration line

2.2.5 Linearity and range

The linearity of measurement was evaluated by analyzing different concentrations of the standard solutions of MEZ and SPI. Calibration curve was constructed by plotting average peak area against concentration and then the regression equation was computed. The summary of linearity parameters is shown in Table 5 and Figures 5 and 6. As demonstrated in Figures 5 and 6 the calibration curve covers concentrations of 25% to 200% of the test concentration (5 to 40 µg/ml) of each MNZ and SPI.

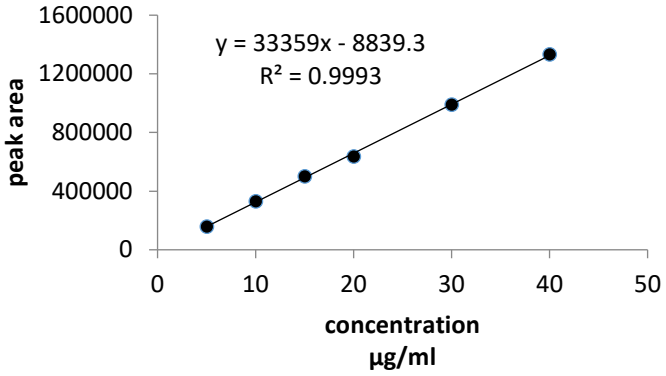


Figure 5. Calibration curve of MEZ

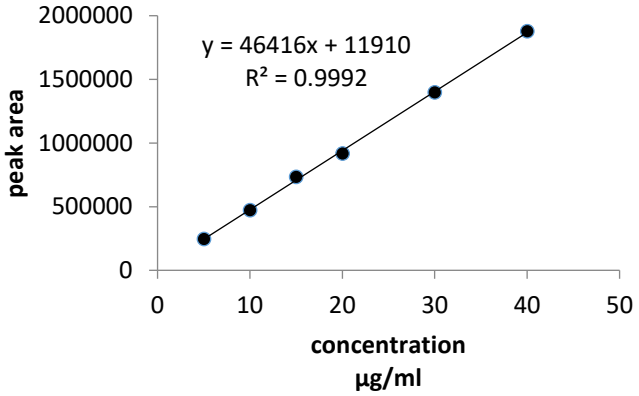


Figure 6. Calibration curve of SPI.

Table 5. Regression line parameters for analytical procedural

Linearity parameter	Results	
	MEZ	SPI
Linearity range	5 to 40 µg/mL	5 to 40 µg/mL
Correlation coefficient	0.9993	0.9992
Slope	33359	46416
Y Intercept	8839.3	11910

2.2.6 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [ICH, Q2 (R1), 1995]. Robustness (Table 6) was performed in 100% test concentration (20 µg/ml) of each MEZ and SPI and was explored using pH of mobile phase, flow rate, wave length.

Table 6 Results of robustness study.

parameter	Variation	Observed value									
		RSD of area		RSD of R.T		RSD of Tailing factor (T)		Capacity factor (k´)		Theoretical plate (N)	
		MEZ	SPI	MEZ	SPI	MEZ	SPI	MEZ	SPI	MEZ	SPI
Flow rate	1ml/min	0.0140	0.0220	0.0040	0.0054	0.0021	0.044	0.709	1.129	5433	3035
	0.8ml/min	0.0009	0.0288	0.0008	0.0009	0.0016	0.042	1.141	1.642	5221	5364
pH of M. Phase	2.75	0.0042	0.0076	0.0049	0.0096	0.0027	0.018	0.916	1.378	7653	3786
	2.54	0.0070	0.0138	0.0021	0.0277	0.0024	0.040	0.898	1.356	5425	4264
Wave length	237	0.0013	0.0036	0.0011	0.0070	0.0012	0.003	0.937	1.382	5123	3797
	227	0.0037	0.0015	0.0097	0.0454	0.0105	0.026	0.932	1.339	5622	3219

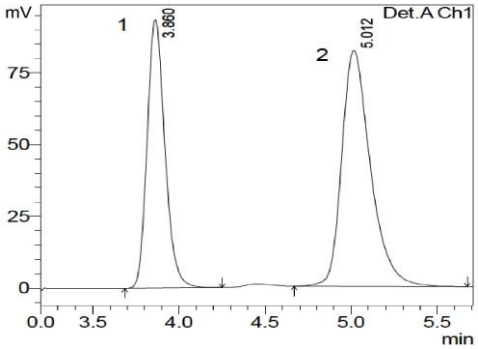
2.3 Mobile phase stability

The stability of the mobile phase was evaluated, so the mobile phase was stored at 5 C°. For 1 week. The aged mobile phase was compared using a freshly prepared one. The mobile phase was stable up to 1 week at 5 C°.

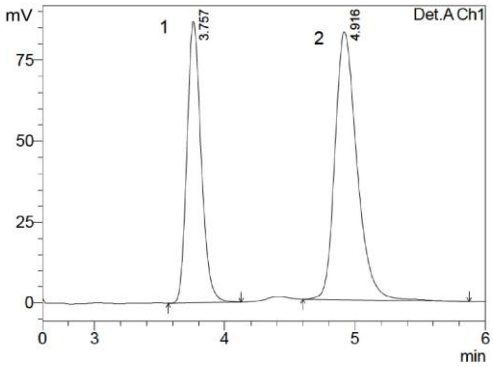
2.4 Standard solution stability

The stability of the standard solution was tested by leaving the standard solutions in tightly capped volumetric flasks, protected from light, on a

laboratory bench and in the refrigerator. There were no significant changes in the peak area, retention time, and symmetry of the peak of MEZ and SPI for 24 h when kept at room temperature and for 1 week when store refrigerated at 4 C°. Figure 7b shows the chromatogram of standard solution after 1 week.



(a) 1= MEZ, 2= SPI



(b) 1 = MEZ, 2= SPI.

Figure 7. (a) Chromatogram of reference standard mixture of MEZ and SPI.
(b) Chromatogram of reference standard mixture of MEZ and SPI after 1 week, at 4 C°.

2.5 Analysis of marketed products

The validated method was applied for the analysis of MEZ and SPI in their commercial dosage forms. Amounts obtained was more than 99% for MEZ and SPI. Results are summarized in Table 7.

Table 7 Result of MEZ and SPI in marketed product

Marketed formulation	Labeled amount (µg/mL)		Amount found %	
	MEZ	SPI	MEZ	SPI
Spirazole tablets®	20.01	29.28	101.18	100.06
HaSy-Dent tablets®	20.0	29.27	101.75	110.78

3. Conclusion

The present developed method is sensitive, rapid, robust, precise and accurate. Application of this method for the analysis of tablets shows that any of the sample components (Starch, croscarmellose sodium, Colloidal silica, Magnesium stearate, Microcrystalline cellulose), no interfere with the analytical determination. This indicates that the proposed method could be used for the determination of MEZ and SPI either in bulk powder or in pharmaceutical formulations (tablets).

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