Ethambutol-Cobalt (II) ions complexation spectral characteristics and applications for quantitative analysis

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Abstract: Ethambutol (EMB) has no significant absorption characteristics beyond 200 nm due to its aliphatic saturated nature. Thus, direct EMB determination in the UV range is not feasible due to its very low molar absorptivity and must requiring a derivatization reaction. Since EMB can act as a chelating agent that disrupts one of several metal-containing enzyme systems in the nucleic acid structure of mycobacteria. This chelating property can be used for quantitative analysis of EMB by formation of UV-Vis absorbing metal complexes. The study in this work describes simple, accurate and sensitive spectrophotometric procedure for the determination of EMB in its pure form and in pharmaceutical formulations. The method depends on the interaction of EMB with cobalt (II) ions in presence of ammonia solution (4 N). The absorbance was measured either at 250, 348 or 521 nm respectively. All parameters affecting the reaction were carefully studied and optimized. Beer's-Lambert's law was obeyed within the concentration ranges 5-25, 40-240 and 100-700 μg ml^{-1} for the three studied wavelengths respectively. The developed method was validated according to the ICH guidelines and applied to pharmaceutical formulation analysis with good recovery ranges. The results were compared to those obtained by an official method and found satisfactorily matched and no significant differences were found within the 95% confidence level. The proposed procedures were suitable for simple routine work and quality control analysis of EMB.

Keywords: Ethambutol, Cobalt (II), spectrophotometry, metal complexation.

INTRODUCTION

Since its first description at 1961, EMB, [(+) 1-butanol-2, 2-(1, 2-ethane diyldiimino)-bis-dihydrochloride] (fig. 1) has been widely used as bacteriostatic antimicrobial agent in the treatment of infections caused by Mycobacteria (C. D. Wells, 2007). The first line TB treatment is based on four drugs: isoniazid, rifampicin, pyrazinamide and EMB, which are available in cheap generic forms and are effective if taken as prescribed (F. Klaus). EMB acts through its chelating abilities that can disrupt one of the metal-containing enzyme systems in the nucleic acid structure of mycobacteria.

Many procedures have been presented for the quantification of EMB in various matrices, from simple analytical procedures such as titrimetry (Feng *et al*., 1987) and electrochemical methods (Chen Chao *et al*., 2008) to the more advanced ones. Most of these advanced techniques focus on separations under chromatographic (Ali *et al*., 2007) or electrophoretic conditions (Hsieh *et al*., 2006).

Spectrophotometric procedures using different reagents have also been cited. The reported spectrophotometric methods mostly involved the use of reagents such as copper (II) ions (Burger *et al*., 1969), hydroquinone (Mahrous, 1992), 2, 4-dinitro-1-fluorobenzene (Shingbal *et al*., 1982) and iodine (Tan *et al*., 1977) or others (Hassan *et al*., 1992). Most of these methods were suffering from the low sensitivity, fastidious reaction conditions and/or high time consumption.

Reported methods that utilizing copper (II) ions for the spectrophotometric determination of EMB specifically that did by Hassan and Shalaby via complexation reaction between the copper phosphate and EMB in medium of borate buffer (pH 9.2) and read the absorbance of the final solution at 640 nm. The methods involved steps such as heating, cooling and filtration before the absorbance reading, making sample preparation procedure long and arduous, in addition to the markedly low sensitivity of the method and it only determines EMB at one wavelength.

Within this context, the present work studies the development, optimization and validation of a new, fast and simple alternative spectrophotometric method for the determination of EMB in pharmaceutical formulations. The proposed method depends on the interaction between EMB and cobalt (II) ions in presence of ammonia solution. EMB can be determined at three different maxima (250, 348 and 521 nm) respectively. The three wavelengths showed variable sensitivity limits but considerable good accuracy and precision limits as well as increased selectivity for the determination of EMB in its pure form and in pharmaceutical formulations. The results showed good recoveries and were favorably comparable with the results obtained from the official method. The proposed method is simple, time saving and of low cost.

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MATERIALS AND METHODS

Apparatus

The measurements of absorption spectra were made in a double-beam in time UV-Vis spectrophotometer system (model UV-1601 PC, Shimadzu, Tokyo, Japan) using quartz cells of optical path length equal to 1.0 cm.

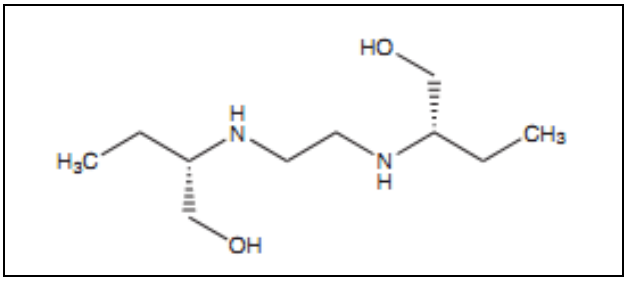

Fig. 1: Chemical structure of Ethambutol

Chemicals and reagents

Ethambutol was kindly supplied by Memphis/Zoga, Cairo, Egypt. All solvents and other chemicals used throughout this study were of analytical grade.

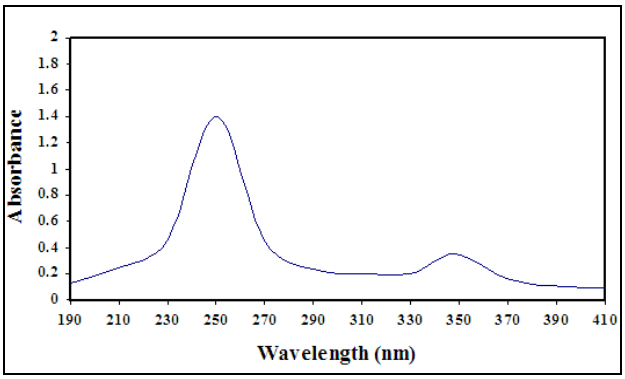

Fig. 2: Absorption spectrum of EMB-cobalt (II) complex, (20 µg ml^{-1}) formed immediately with 0.5 % w/v cobalt sulfate solution and 0.2 ml of 4 N ammonia solution.

Cobalt sulfate (The General Chemical & Pharmaceutical Co. LTD, Sudbury, Middlesex, England).

Pharmaceutical formulations

Etibi® 500 tablets (Memphis/Zoga, Cairo, Egypt) were purchased from the local market, each tablet is labeled to contain 500 mg of ethambutol dihydrochloride.

Procedures

Cobalt sulfate solutions

Methanolic solutions of cobalt sulfate containing 0.5 and 3.0 g% w/v were prepared by dissolving an accurately weighed amounts (0.5 and 3grams) of cobalt sulphate in 100 ml of methanol.

Preparation of stock standard solutions

An accurately weighed amount of EMB (150 mg) was transferred to 50 ml calibrated flask and dissolved in methanol and shaked well with about 40 ml until dissolved then completed to the mark with methanol to provide stock solution with a concentration of 3 mg ml^{-1}. The standard solution either used as it is or further diluted with methanol to give the appropriate working standard solutions.

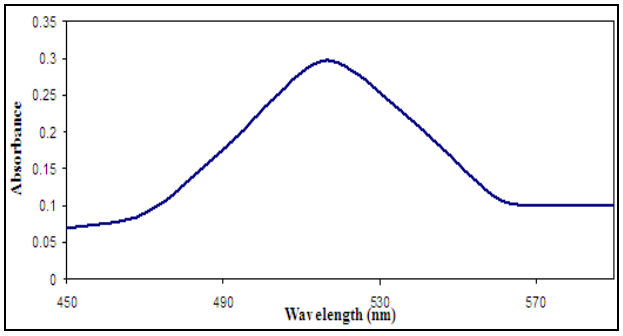

Fig. 3: Absorption spectrum of EMB-cobalt (II) complex (200 µg ml^{-1}) formed after 30 minutes with 3.0 % cobalt sulfate and 0.2 ml of 4 N ammonia solutions.

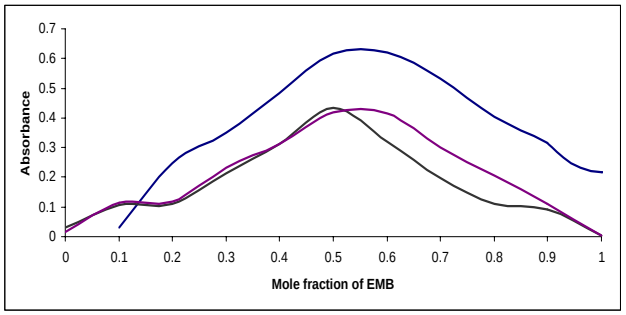

Fig. 4: Job's plot of continuous variation of ethambutol-cobalt complexes.

Preparation of sample solution

Ten tablets of Etibi® 500 were accurately weighed, finely powdered and mixed thoroughly. An accurately weighed quantity of the powdered tablets equivalent to 150 mg of EMB was quantitatively transferred into 50-ml calibrated flask containing about 40 ml methanol, shaked well for 5 minutes and sonicated for 10 minutes then completed to the mark with methanol. The solution was then filtered using double filter paper and the first proportion of the filtrate was discarded. The rest of filtrate was collected and used as stock sample solution.

Determination procedure

An aliquot of 1 ml of either working standard or sample solution was transferred into 10-ml calibrated flask. A volume of 1 ml of either 0.5 g% w/v (in case of 250 and 348 nm measurements), or 3 g% w/v (in case of 521 nm measurements) of cobalt sulfate solution was added followed by 0.2 ml of 4N ammonia solution. The solution was diluted to the mark with methanol or water. The absorbance was measured immediately after dilution with solvent either at 250 and 348 nm or after 35 minutes at 521 nm against a blank solution treated concurrently.

RESULTS

Spectral characteristics

After the preliminary studies to confirm the formation of EMB-Co (II) complexes, the spectral characteristics for the so formed complexes were investigated. Figs. 2 and 3 show the absorption spectra of the produced complexes by the reaction of EMB with cobalt sulfate in presence of ammonia solution.

Optimization of variables affecting the complexation reaction

Effect of concentration of cobalt sulfate

Concentrations of cobalt sulfate solution between 0.1 and 5 g% w/v were tested in order to select the concentration that give the most intense constant and reproducible readings. No significance change in the absorbance readings were observed on changing the volume of the selected concentrations of cobalt sulfate from 0.5 to 1.5 ml thus one milliliter was selected for all measurements.

Table 1: Effect of different solvents on the obtained reaction product under the proposed reaction conditions at the three studied maxima

Solvent	25 µg ml^{-1}		120 µg ml^{-1}		400 µg ml^{-1}	
	λ_{max}	A*	λ_{max}	A*	λ_{max}	A*
Water	250	0.110	348	0.332	521	0.413
Methanol	250	0.830	337	0.381	525	0.428
Ethanol	250	0.740	330	0.381	525	0.400
Isopropanol	251	0.601	327	0.391	531	0.334
Acetonitrile	253	0.540	326	0.310	534	0.376
DMF	257	0.463	339	0.307	535	0.356
DMSO	261	0.450	332	0.371	526	0.359
Acetone	255	0.452	340	0.198	525	0.362
Chloroform	252	0.511	339	0.225	532	0.352
1,4-dioxan	250	0.491	332	0.343	528	0.380

*Average of five determinations

Table 2: Data of continuous variation method at the three studied maxima

No.	Mole fraction of Co (II)*	Mole fraction of EMB (II)*	Molar ratio Co:EMB	Absorbance (corrected mean values)		
				250 nm	348 nm	521 nm
1	1.0	0.0	10.0	0.030	0.029	0.015
2	0.9	0.1	9.0	0.246	0.105	0.115
3	0.8	0.2	4.0	0.350	0.110	0.117
4	0.7	0.3	2.33	0.485	0.213	0.233
5	0.6	0.4	1.5	0.618	0.313	0.311
6	0.5	0.5	1.0	0.620	0.434	0.419
7	0.4	0.6	0.67	0.531	0.318	0.416
8	0.3	0.7	0.43	0.403	0.199	0.301
9	0.2	0.8	0.25	0.315	0.109	0.206
10	0.1	0.9	0.11	0.215	0.092	0.109
11	0.0	1.0	0.0	0.005	0.004	0.004

*Concentrations of 1 x 10^{-4} M for measurements at 250 nm, 1 x 10^{-3} M for measurements at 348 nm and 1 x 10^{-2} M for measurements at 521 nm were used.

Table 3: Experimental and calculated results for the Scatchard diagram

No.	EMB µmoll^{-1}	Co (II) µmoll^{-1}	A_{mean}	Δ A	[Co. EMB] µmoll^{-1}	[Co. EMB] /[Co]
1	0.00	120	0.015	-----	-----	-----
2	100	20	0.125	0.110	11.34	0.567
3	100	40	0.190	0.175	18.04	0.451
4	100	60	0.265	0.250	25.77	0.429
5	100	80	0.335	0.320	32.99	0.412
6	100	100	0.445	0.430	44.33	0.443
7	100	120	0.390	0.375	38.66	0.322

Effect of ammonia concentration

Results showed that absorbance increased significantly by increasing the concentration of ammonia solution up to 3 N then become approximately constant. Concentrations more than 5 N of ammonia solutions cause a slight decrease in the absorbance intensities. No significant changes in the absorbance readings were observed on changing the volume of the selected concentrations of ammonia solution from 0.1 to 0.5 ml. Thus 0.2 ml was selected for all measurements.

Reaction time and product stability

The reaction products were formed immediately at room temperature (25±2ºC) and absorbance measured at 250 and 348 nm reached maximum within one minute and kept constant for at least 60 min. While for the colored product measured at 521 nm, the absorbance increased gradually with time and maximum intensity was reached after about 30 min. The increase of temperature (up to 80ºC) didn't reduce the reaction time significantly, so the measurements done at room temperature after 30 minutes.

Table 4: Characteristics of the calibration graph and quantitative parameters for determination of EMB using the proposed procedures

Parameter	I	II	III
Wavelength, nm	250	348	521
Time (min.)	0.00	0.00	30
Co (II) g% w/v	0.5	0.5	3.0
Media	4 N ammonia		
Diluting solvent	Methanol	Water	Water
Experimental linearity range (μg ml^{-1})	5.0-25.0	40.0-240.0	100.0-700.0
Molar absorptivity ($L.mol^{-1}.cm^{-1}$)	9700	800	300
Intercept ±SD	$0.032 \pm 5 \times 10^{-3}$	$0.107 \pm 2.5 \times 10^{-4}$	$0.079 \pm 1.0 \times 10^{-4}$
Slope ± SD	$0.034 \pm 4.0 \times 10^{-4}$	$0.002 \pm 1.6 \times 10^{-5}$	$0.001 \pm 1.1 \times 10^{-5}$
Correlation coefficient (r)	0.9996	0.9999	0.9996
Determination coefficient (r^2)	0.9992	0.9998	0.9992
Limit of detection (LOD, μg ml^{-1})	0.82	2.64	15.5
Limit of quantification (LOQ, μg ml^{-1})	4.35	8.81	52.6

Table 5: Intra-day and inter-day precision of the proposed method for analysis of EMB at three concentration levels respectively.

Wavelength (nm)	Concentration μg ml^{-1}	Intra-day precision		Inter-day precision	
		% Found*	RSD*	% Found*	RSD*
250	10	101.4	0.7	100.5	0.7
	15	99.31	0.3	101.6	0.3
	25	100.7	1.1	99.00	0.5
348	80	100.2	0.9	102.9	0.5
	120	97.35	0.6	98.06	0.8
	160	96.96	1.0	99.84	0.5
521	200	101.6	0.7	99.60	1.5
	400	98.60	0.6	100.5	0.6
	600	96.10	1.0	96.60	1.0

*Average percent of six replicates, RSD: Relative standard deviation.

Table 6: Accuracy of the proposed method for the analysis of EMB in Etibi® 500 tablets at three concentration levels

Wavelength (nm)	Concentration levels analyzed μg ml^{-1}	% Recovery*	RSD*
250	10	100.5	1.0
	15	99.51	0.3
	25	100.6	0.7
348	80	100.2	1.6
	120	99.33	0.9
	160	98.78	1.2
521	200	98.61	1.4
	400	100.3	1.0
	600	96.49	1.3

*Average percent of six observations.

Effect of diluting solvents

Several solvents were tested to be used as final dilution solvent and their effects on the maxima position and absorption intensities were studied and recorded. From the results listed in table 1, it was observed that maximum absorption intensities were obtained with either methanol or ethanol for measurements done at 250 nm, while for 348 and 521 nm measurements, many solvents could be used.

Stoichiometry of the formed complexes

The continuous variation method or Job's method (D. A. Skooge *et al*., 2007) was used in order to establish the stoichiometry of the predominant complex.

$$aCo^{2+}{}_{(Aq)} + b\ EMB_{(aq)} \longrightarrow Co_b.EMB_{a(aq)}$$

A series of solutions were prepared by mixing equimolar (1×10^{-4} M in case of measurements done at 250 nm, 1×10^{-3} M for measurements at 348 nm and 1×10^{-2} M for measurements at 521 nm) quantities of Co (II) and EMB as shown in table 2. The ammonia concentration and volume was kept constant according to the optimization reaction conditions. Absorbances were monitored at 250, 348 and 521 nm respectively. The results showed that the maximum absorbance reached in Co (II) molar fraction of 0.5 corresponding to the stoichiometry of 1:1 of the predominant complex (fig. 4).

The Co.EMB formation constant

Rigorously to obtain the formation constant of Co.EMB complex (K_f)**,** it must be considered the activities of the species involved in the chemical equilibrium. However,

Table 7: Analysis of Etibi® 500 tablets by proposed spectrophotometric and official methods using standard addition method

Wavelength (nm)	Proposed method			Official method**			t-value	F-value
	Added (mg)	Found (mg)	% Recovery ± RSD*	Added (mg)	Found (mg)	% Recovery ± RSD*		
250	0.0	0.0	99.51±0.3	0.0	0.0	97.92±1.75	0.26	1.03
	10.0	9.76	97.60±0.5	10.0	9.95	99.50±0.38	0.88	2.82
	25.0	24.6	98.40±0.5	25.0	24.31	97.24±0.62	0.46	1.36
	50.0	50.3	100.6±0.7	50.0	49.77	99.54±0.83	0.49	3.93
348	0.0	0.0	99.33±1.2	0.0	0.0	97.92±1.75	1.41	1.10
	25.0	25.2	100.8±1.5	25.0	24.31	97.24±0.62	2.11	4.51
	50.0	49.1	98.20±0.5	50.0	49.77	99.54±0.83	1.82	3.42
	100.0	97.7	97.70±0.8	100.0	96.11	96.11±0.71	1.32	1.62
521	0.0	0.0	100.3±1.3	0.0	0.0	97.92±1.75	1.13	1.32
	25.0	24.9	99.60±1.5	25.0	24.31	97.24±0.62	0.20	2.14
	50.0	49.5	99.00±1.3	50.0	49.77	99.54±0.83	1.17	1.75
	100.0	97.0	97.00±0.7	100.0	96.11	96.11±0.71	2.13	1.14

*Average of five replicates. **Non aqueous titration according to USP.

Table 8: Robustness studies for the proposed method.

Variable studied	% Recovery ± RSD*		
	At 250 nm[1]	At 348 nm[2]	At 521 nm[3]
Cobalt (II) ions conc.			
Recommended conc. + 10% of the labeled amount	98.82 ± 0.8	101.2 ± 1.2	99.75 ± 0.7
Recommended conc. + 10% of the labeled amount	99.71 ± 0.8	98.75 ± 1.7	99.58 ± 1.1
Cobalt (II) ions volume			
Recommended volume + 0.1ml	101.3 ± 1.3	98.39 ± 1.1	98.43 ± 0.8
Recommended volume - 0.1ml	102.3 ± 0.8	103.4 ± 1.1	98.94 ± 0.9
Ammonia solution conc.			
4.2 N	97.33 ± 1.2	99.34 ± 1.3	98.84 ± 1.0
3.8 N	98.11 ± 1.1	103.9 ± 0.4	100.6 ± 0.8
Ammonia solution volume			
Recommended volume + 0.02 ml	98.32 ± 1.1	98.23 ± 0.9	98.32 ± 1.2
Recommended volume - 0.02 ml	98.56 ± 0.9	100.5 ± 0.9	100.3 ± 0.5
Reaction time			
37 min	Immediate	Immediate	101.6 ± 0.5
33 min	Immediate	Immediate	97.68 ±0.9

*Average of five replicates. 1, 2 and 3: Drug concentrations used 25, 120 and 400 µg ml^{-1} respectively.

for the diluted solutions, the coefficient of activity is closed to the unity and to the species concentration in the chemical system. Thus K_f can be determined according to the following equation:

$$K_f = [Co.EMB] / [Co^{2+}][EMB] \quad (1)$$

A set of solutions containing constant EMB concentration and constantly increased amounts of Co (II) in presence of 4 N ammonia solution (table 3) was performed. The total Co (II) concentration is equal to:

$$[EMB] = [EMB]_0 - [Co.EMB] \quad (2)$$

Now the equilibrium expression, Equation (1), can be rearranged as follows:

$$[Co.EMB]/[Co]=K_f[EMB]=K_f([EMB]_0-[Co.EMB]) \quad (3)$$

The complex concentration formed was calculated by Scatchard approach. The plot of [Co.EMB]/[Co] versus [Co.EMB] was fitted by linear regression model, obtaining a slope which corresponding the $-K_f$ value (fig. 5).

Validation of the proposed method

Linearity, detection and quantification limits

Under the established optimal reaction conditions, concentrations of EMB were found to be proportional to the absorbance obtained for the reaction product. Typical Beer-Lambert's law plots were obtained. The linearity of the produced products was found in the range of 5-25, 40-240 and 100-700 μgml^{-1} for the three measured maxima (250, 348 and 521 nm) respectively. Table 4 showed the regression parameters for the obtained data at three wavelengths, in addition to the detection, quantification limits, molar absorptivities, correlation and determination coefficients calculated.

Precision

The precision of the proposed method was checked by analysis of six separate working standard EMB solutions. Intra-day and inter-day precision parameters were determined. The results (table 5) showed that relative standard deviations were less than 2 % in all cases, indicating good repeatability of the proposed method.

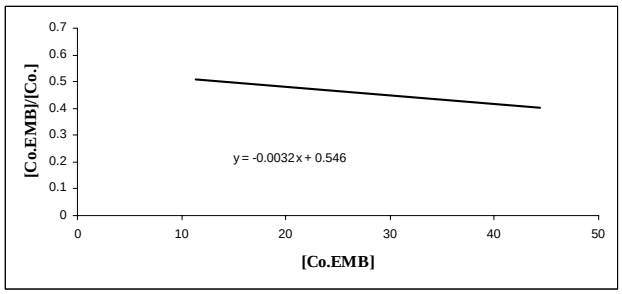

Fig. 5: Scatchard diagram for the binding of Co (II) to EMB

Accuracy

The accuracy of the developed method for the analysis of EMB commercial dosage forms was checked by measuring the absorbance at three concentration levels which covers low, medium and high levels to the calibration curves. Table 6 shows good percentage recoveries ranging from 98.60 to 101.5%. Recovery studies were also performed by using standard addition method (D. Harvey, 2000). Results obtained in table 7; indicated the good percent recoveries obtained by the standard addition method and confirm the absence of the interference due to the common excipients and additives. Results obtained agree well with those of the official method as shown by t- and F- tests at 95% confidence level.

Robustness

Slight variation (about ± 10% of the labeled values) in several parameters such as cobalt concentration, ammonia concentration, heating and reaction time were found not to significantly affect the performance of the developed method. Table 8 shows the obtained results that confirm that the developed method is robust with respect to the studied parameters.

DISCUSSION

The absorption maxima of the formed products at room temperature were found to be at 250, 348 and 521 nm. It was found that the optimal concentration of cobalt sulfate was 0.5 g% w/v for measurements done at 250 and 348 nm, while at 521 nm required a more concentrated solution (3 g% w/v) to develop the most intense colored product. 0.2 ml of 4 N ammonia solution was used as an alkaline medium. For the final dilution of the reaction product, methanol and water were selected. The complexes formed and measured immediately at 250 and 348 nm, while at 521 nm, it was measured after 30 minutes. EMB reacted with cobalt (II) ions in a 1:1 ration according to Job's method of continuous variation under the mentioned reaction conditions. According to the results obtained by performing precision, accuracy and robustness study, the developed procedures are of good repeatability, selective and robust.

In conclusion, EMB is an important anti-tuberculostic drug under use until now in the combinations for treatment of TB. Due to its aliphatic nature; it is difficult to be determined spectrophotometrically. The present procedures that depending on chelation with cobalt (II) ions is so simple and highly selective for EMB determination spectrophotometrically. At least three maxima can be used for determination of the same sample with different sensitivity and accuracy levels. There is no need for prior extraction and reagents used are stable for long time. Therefore; the proposed method procedures are suitable for routine determination of EMB in various pharmaceutical preparations.

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