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A novel spectrofluorimetric determination of four anti-TB drugs in their pure and pharmaceutical dosage forms by quenching effect on the fluorescence of NBS-phenothiazine product

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Abstract

Simple and sensitive method has been developed and validated for determination of ethambutol (EMB), isoniazid (INH), pyrazinamide (PZA) and rifampicin (RIF) in pure and pharmaceutical dosage forms. The method is based on measuring the quenching effect of studied drugs on the fluorescence intensity of NBS-phenothiazine oxidation product (NBS-Phz) in a buffered medium (pH 7, λ_{ex} 271 and λ_{em} 375 nm). Different factors affecting the reaction were studied and optimized. Under the optimized conditions, linear relationships with good correlation coefficients (0.9995-0.9999) were obtained. The limits of detection were 0.139, 6.39 x 10^{-3}, 0.029 and 0.180 µg ml^{-1} for EMB, INH, PZA and RIF respectively. The precision of the method was satisfactory with relative standard deviation less than 2 %. Good accuracy was assessed with recovery percentages ranged from 97.24 to 101.2 %. The results were favorably compared with those of the official method. Therefore, the developed method provides applicable tool for determination of studied drugs in pure, pharmaceutical formulation and in quality control laboratories.

Keywords: Ethambutol, Isoniazid, Pyrazinamide, Rifampicin, Fluorescence quenching, NBS, Phenothiazine.

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1. INTRODUCTION

In the last decade, tuberculosis (TB) has remerged as one of the leading causes of death (nearly 3 million deaths annually). A major public health problem worldwide, TB is now global emergency. EMB, INH, PZA and RIF are the first line anti-TB drugs (Fig. 1). The standard treatment for TB is to treat the patient with a combination of these four compounds for two months, followed by INH and RIF alone for an additional four months. For more than 50 years, TB has been treated with combination drug therapy and there are a number of available combination drug products with different drug contents and composition. These compounds are used in combination because they have different modes of action [1-3].

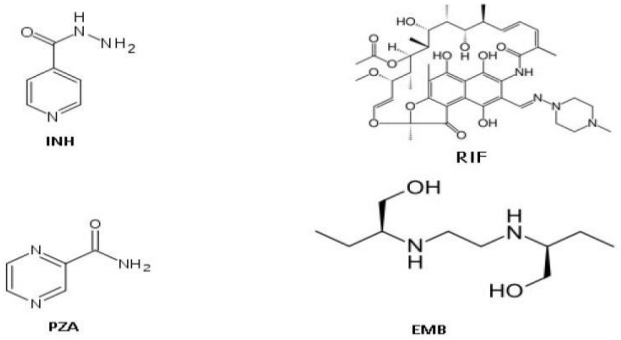


Figure 1: Chemical structures of studied anti-TB drugs

Owing to the therapeutic importance of these drugs, numerous different analytical methods have been developed for their quantitative determination in pure, pharmaceutical dosage forms and/or biological fluids. These methods are; liquid chromatography [4-7], thin layer chromatography [8, 9], gas chromatography [10], capillary electrophoresis [11-13], electrochemically [14-16], spectrophotometry [17-20], spectrofluorimetry [21, 22] and chemiluminescence methods [23-25]. These methods, suffer from their sophisticated instrumentation and high-analysis cost. Moreover, these instruments are not available in most quality control laboratories specially, third world countries.

In general, spectrofluorometry is considered one of the most convenient analytical techniques, because of its inherent simplicity, low cost, and wide availability in most quality control laboratories. To the best of our knowledge, there were no reported spectrofluorimetric methods on PZA or one method for the four studied drugs. For these reasons, the present study describes simple, sensitive and economical spectrofluorometric method for the analysis of four anti-TB drugs in their pure and pharmaceutical dosage forms. The proposed method is based on measuring the quenching effect of these drugs in buffered medium (pH 7) on the fluorescent reaction product between NBS and phenothiazine.

2. EXPERIMENTAL

2.1. Apparatus:

RF-5301 PC spectrofluorimeter (Shimadzu, Japan), with 1-cm matched quartz cells, were used for all measurements. The spectrofluorimeter was set at excitation and emission slit width of 3 nm.

2.2. Materials and reagents:

RIF and INH working reference standards were gifted from Pharco, Cairo, Egypt. PZA working reference standard was kindly supplied from Amoun, Cairo, Egypt and EMB by Memphis/ Zoga, Cairo, Egypt. Phenothiazine (Sigma chemical Co., USA) freshly prepared ethanolic solution (5 x 10^{-4} M). N-bromosuccinimide (Merck, Germany) freshly prepared aqueous solution (10^{-3} M). Teorell and Stenhagen

Pharmaceutical preparations	Ingradients	Nominal content, mg	Manufacturer
Etibi® 500 tablets	EMB	500 mg/tablet	Memphis/ Zoga, Cairo, Egypt
Isocid fort® tablets	INH	200 mg/tablet	Cid, Cairo, Egypt
Rifampicin® capsule	RIF	300 mg	El Nasr Pharm. Chem. Co., ADWIC
Rimactane® capsule Rimactane® suspension	RIF	300 mg 20 mg/ml	Novartis Pharm. Co.
Rifactine® capsule Rifactine® suspension	RIF	300 mg 2 g/100 ml	Medical Union Pharm. Co., (MUP)
Rifadin® capsule	RIF	300 mg	Nile Co. for Pharm. & Chem. Ind.
P.T.B tablets®	PZA	500 mg	Amoun Pharm. Co.

Table 1: Commercial pharmaceutical preparations analyzed by the proposed spectrofluorimetric method

buffer solution [26] of the pH range 2-10 was prepared in freshly boiled and cooled distilled water. The buffer composed of phosphoric acid, citric acid, 1 M sodium hydroxide, adjusted to the required pH with 0.1 M hydrochloric acid.

All solvents and other chemicals used throughout this study were of analytical grade. Double distilled water has been used.

2.2.1. Pharmaceutical dosage forms:

Different pharmaceutical formulations analyzed (Table 1), were purchased from the local market.

2.3. Preparation of solutions:

2.3.1. Stock standard solutions:

An accurately weighed amount (25 mg) of each studied drug was transferred into a 25-ml volumetric flask. The

powder was dissolved in 10-ml methanol. The solution was then diluted to the mark with the same solvent to obtain a working standard solution of 1 mg ml^{-1} of each of the studied drugs. Further dilutions were made to obtain the suitable drug concentrations; 1-4, 0.1-0.35, 0.1-1 and 1-5 µg ml^{-1}. These solutions were found to be stable for at least 1 weak when kept in the refrigerator.

2.3.2. Sample preparation:

Tablets

Ten tablets of each formulation were weighed and finely powdered. A quantity of the mixed powder equivalent to 25 mg of each drug was transferred into a 25-ml calibrated flask, dissolved in 10-ml methanol, swirled and sonicated for 10 min, completed to volume with the same solvent, shaken well for 10 min, and filtered. The procedure was then completed as described for preparation of stock standard solutions.

Capsules

The contents of ten capsules were evacuated and accurately weighed and finely powdered. A portion of the powder equivalent to 25 mg of RIF was weighed and quantitatively transferred into a 25 ml volumetric. The powder was then dissolved in a suitable volume of methanol, shacked and sonicated for 10 min. The solution was then filtered to be used in further experiments.

Suspension

A suitable volume was quantitatively transferred (equivalent to 25 mg of RIF) into a 25 ml volumetric flask containing 10 ml methanol. The solution was shacked and sonicated for 10 min., and then the volume was made up to the mark with the same solvent. The solution was then filtered to give a concentration of 1 mg ml^{-1}.

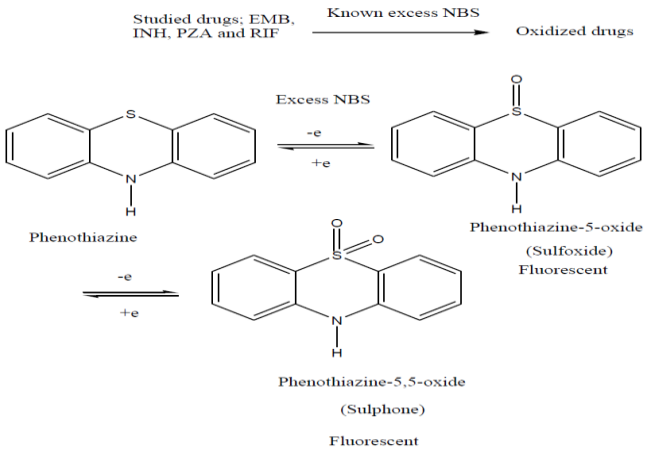
2.4. Determination procedure:

Accurately transfer one milliliter of standard or sample solution into 10 ml volumetric flask; add one milliliter of the buffer pH 7 and mix well. Then add one milliliter of 10^{-3} M NBS. Allow the mixture to stand for 15 min. and finally add 1 ml of 5 x 10^{-4} M phenothiazine stand at ambient temperature for 5 min. complete the solution to the mark with distilled water. Measure Δ RIF at λ_{em} 375 nm (λ_{ex} 271 nm) against blank treated similarly.

3. RESULTS AND DISCUSSIONS

3.1. Method development:

N-bromosuccinimide has been extensively used as brominating and oxidizing agent for many organic compounds [27, 28]. In the present work, each studied drug was treated with excess amount of NBS. The excess of NBS was then determined using phenothiazine reagent which undergoes oxidation to the fluorescent products (sulfoxide or sulphone) [29]. These products could be measured fluorimetrically at λ_{ex} 271 nm and λ_{em} 375 nm according to scheme 1. Fig. 2 illustrates the excitation and emission spectra of the studied drugs (3 µg ml^{-1}) and their quenching effects.



Scheme 1: The suggested reaction mechanism of the proposed spectrofluorimetric method

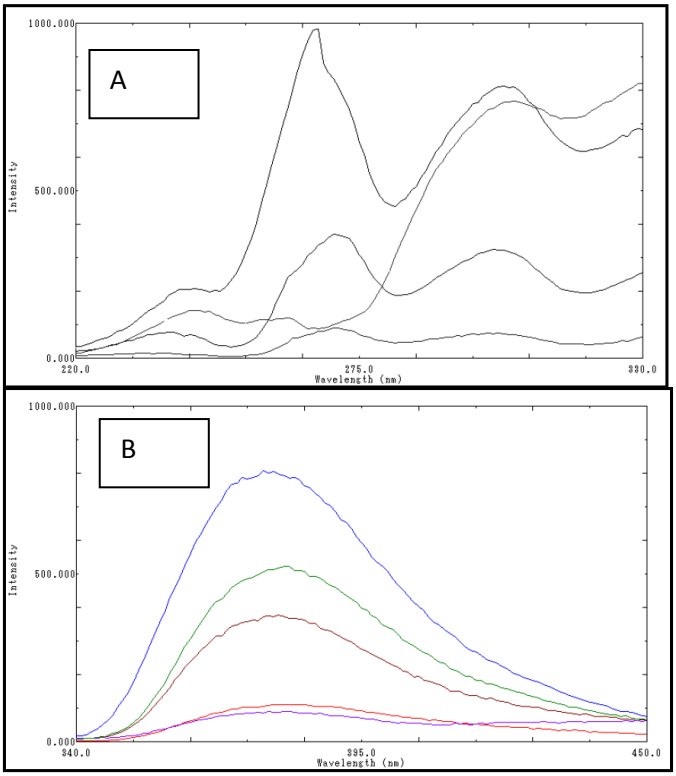


Figure 2: a) Excitation and b) Emission spectra of NBS-Phenothiazine product in absence (blank) and presence of studied drugs

3.2. Optimization of reaction conditions:

All factors affecting the reaction were optimized by altering one variable and keeping the others constant by using 10 µg ml^{-1} of each studied drug.

These factors include:

3.2.1. NBS concentration:

The effect of different concentrations of N-bromosuccinimide (10^{-5} to 10^{-2} M) were tested for the effect on Δ RFI of NBS-Phz product (Fig. 3). It was found that maximum relative fluorescence intensity difference obtained upon using 10^{-3} M NBS.

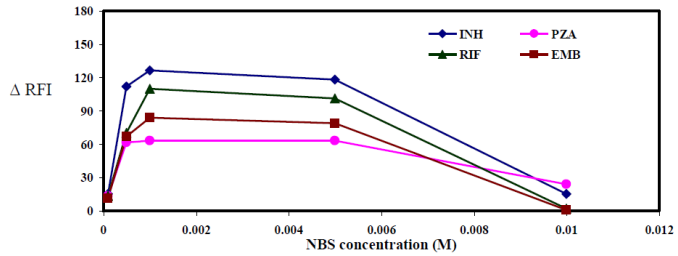


Figure 3: Effect of NBS concentration (M) on Δ RFI of the produced oxidized phenothiazine in presence of drugs under investigation

3.2.2. Phenothiazine concentration:

Various concentrations of phenothiazine ranged from 10^{-5} to 10^{-2} M were examined. Maximum response was obtained upon using 5 x 10^{-4} M phenothiazine in ethanol (Fig.4).

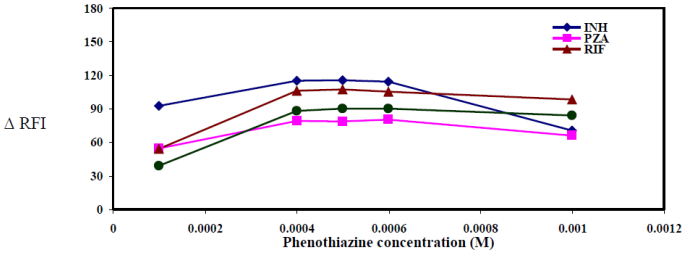


Figure 4: Effect of phenothiazine concentration (M) on Δ RFI of NBS-Phenothiazine product in presence of anti-TB drugs

3.2.3. pH:

For investigating the effect of pH, the reaction was performed at different pH values (2-10). The results indicated that the fluorescence intensity difference was pH dependent (Fig.5.). The optimum pH was found to be 7.0.

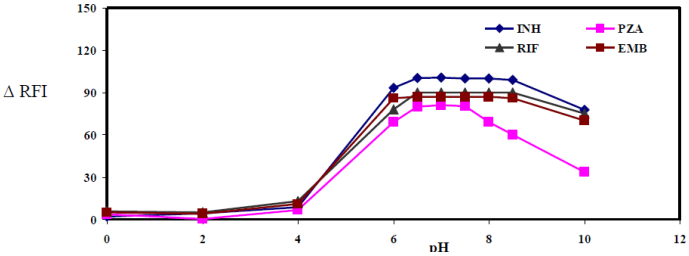


Figure 5: Effect of pH on Δ RFI of NBS-Phenothiazine product in presence of studied drugs

3.2.4. Reaction time and temperature:

It was found that high temperature leading to unstable oxidized form of phenothiazine according to the practical study (Fig. 6).

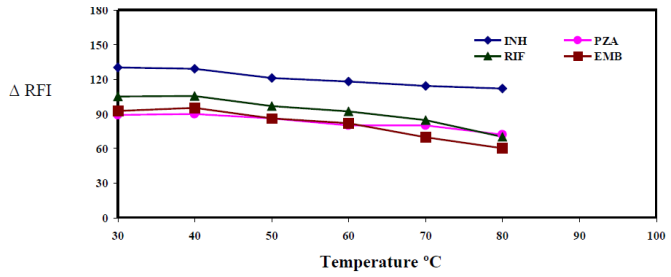


Figure 6: Effect of temperature on Δ RFI measured at λ_{ex} 271 and λ_{em} 375 nm

Therefore reaction at ambient temperature was selected. Fifteen minutes was selected as the reaction time of choice between each drug and NBS.

3.2.5. Reaction time between phenothiazine and NBS:

Reaction time between NBS and phenothiazine was studied for anti-TB drug through 25 min. Five minutes standing was chosen before final dilution with distilled water. The stability time of the oxidized product was studied by carrying out the general assay procedure varying standing time from 5 to 60 min. It was observed that the produced relative fluorescence intensity difference of the reaction product remained stable at least 1 hr.

3.2.6. Diluting solvent:

In order to select the most appropriate solvent for dilution, different solvents were tested: water, methanol, ethanol, dimethylformamide and dimethylsulphoxide. Water was found to be an ideal diluting solvent (Table 2) as it afforded maximum sensitivity, and therefore it was selected for further investigations.

Solvent	λ_{ex}/ λ_{em} (nm)	Δ RFI[a]			
		EMB	INH	PZA	RIF
Water	271/ 375	101	127	74	107
Methanol	272/ 370	94	103	72	99
Ethanol	274/ 371	92	64	30	97
DMSO	275/ 376	82	64	41	85
DMF	274/ 369	77	59	41	82

Table 2: Effect of different solvents on λ_{ex}/ λ_{em} and Δ RFI caused by the reaction of studied drugs with NBS and phenothiazine

[a] Values for all solvents are mean of three determinations; the RSD for the readings were < 2%

3.3. Validation of the proposed method:

The method was validated according to International Conference on Harmonization guidelines (ICH) on the validation of analytical methods, in terms of linearity, precision, limit of detection, limit of quantitation, robustness, accuracy and analysis of marketed formulations [30].

3.3.1. Linearity and sensitivity:

Under the specified optimum reaction conditions, the calibration curves for anti-TB drugs were constructed by measuring a series of six concentrations of the standard solutions. All measurements were carried out using six replicate measurements (n=6). The assays were carried out according to the general procedure previously established for each drug. In all cases, standard curves were linear with acceptable intercepts and very good correlation coefficients in the general concentration range of 1-4, 0.1-0.35, 0.1-1 and 1-5 µg ml^{-1} for EMB, INH, PZA and RIF respectively (Table 3). The LOD was 0.139, 6.39 x 10^{-3}, 0.029 and 0.180 µg ml^{-1}, while the LOQ was 0.420, 0.019, 0.088 and 0.546 µg

ml^{-1} for EMB, INH, PZA and RIF respectively. Limits of detection (LOD) and limits of quantitation (LOQ) were determined using the formula: LOD or LOQ = kSD_a/b, where, k = 3.3 for LOD and 10 for LOQ, SD_a is the standard deviation of the intercept, and b is the slope.

Parameter	EMB	INH	PZA	RIF
Linearity range (µg ml^{-1})	1-4	0.1-0.35	0.1-1	1-5
Correlation coefficient (r)	0.9995	0.9999	0.9996	0.9996
Intercept ± SD*	-2.23 ± 0.336	-6.9 ± 0.275	-0.644 ± 0.657	6.9 ± 0.546
Slope ± SD*	0.008 ± 1.3 x 10^{-5}	0.142 ± 1 x 10^{-3}	0.075 ± 1 x 10^{-3}	0.010 ± 1.6 x 10^{-5}
LOD (µg ml^{-1})	0.139	6.39 x 10^{-3}	0.029	0.180
LOQ (µg ml^{-1})	0.420	0.019	0.088	0.546

Table 3: Quantitative parameters for the proposed spectrofluorometric method for the analysis of studied anti-TB drugs

* Mean of six replicates

3.3.2. Precision:

The precision of the method was estimated by measuring six replicate samples of each drug within the same day and on different days. Three concentrations were selected which covers low, medium and high levels to the calibration curves (1, 2, 4), (0.1, 0.2, 0.3), (0.2, 0.4, 0.6) and (2, 3, 4) µg ml^{-1} for EMB, INH, PZA and RIF respectively. The assays gave satisfactory results ranged from 98.18 to 101.5 with relative standard deviation less than 2 % (Table 4). This level of precision of the proposed method was adequate for the quality control analysis of the first line anti- TB drugs in their pharmaceutical dosage forms.

Authentic drug	Concentration (µg ml^{-1})	Intra-day precision % Found ± RSD*	Inter-day precision % Found ± RSD*
EMB	1	98.51 ± 1.20	98.91 ± 1.40
	2	99.73 ± 1.75	99.53 ± 1.20
	4	100.4 ± 0.71	98.75 ± 0.88
INH	0.1	101.3 ± 0.92	98.42 ± 0.97
	0.2	98.18 ± 0.79	101.5 ± 1.03
	0.3	99.90 ± 0.66	99.66 ± 0.71
PZA	0.2	98.67 ± 1.79	100.3 ± 1.96
	0.4	98.44 ± 1.29	98.96 ± 0.93
	0.6	98.58 ± 0.87	98.76 ± 0.82
RIF	2	99.75 ± 1.42	98.85 ± 0.90
	3	98.33 ± 0.64	98.88 ± 0.98
	4	99.00 ± 0.90	100.6 ± 0.77

Table 4: Intra-day and inter-day precision of the proposed spectrofluorimetric method at three concentration levels

* Standard deviation of six replicates

3.3.3. Robustness:

Robustness of the procedure was assessed by evaluating the influence of small variation in experimental variables: NBS and phenothiazine concentrations, pH, drug-NBS and NBS-phenothiazine reaction times on the analytical performance of the method. In these experiments, one experimental parameter was changed while the other parameters were kept unchanged, and the recovery percentage was calculated each time (Table 5). The small variations in any of the variables didn't significantly affect the results where the percentage recovery ranged from 96.15 to 104.5 with SD less than 2 %. This gave an indication for the reliability of the proposed method during routine work.

Variable studied	% Recovery ± SD*			
	EMB[a]	INH[b]	PZA[c]	RIF[d]
NBS concentration				
0.0011 M	100.6 ± 1.23	97.65 ± 1.13	97.61 ± 1.38	104.5 ± 0.58
0.0009 M	96.54 ± 0.47	96.95 ± 1.02	97.95 ± 1.29	98.92 ± 0.57
Phenothiazine concentration				
0.00052 M	100.8 ± 1.67	100.5 ± 0.40	97.15 ± 0.58	100.9 ± 1.00
0.00048 M	97.88 ± 1.39	99.89 ± 1.76	97.92 ± 1.01	96.33 ± 1.49
pH				
7.5	99.62 ± 1.42	98.12 ± 0.89	97.59 ± 0.77	99.91 ± 0.99
6.5	100.8 ± 0.93	99.06 ± 0.89	98.06 ± 0.87	99.98 ± 1.09
Reaction time between drug and NBS				
17 min.	102.2 ± 1.22	97.62 ± 0.39	96.37 ± 0.70	104.4 ± 0.52
13 min.	100.6 ± 0.94	99.06 ± 0.73	96.15 ± 0.34	100.6 ± 1.15
Reaction time between NBS and phenothiazine				
6 min.	101.4 ± 0.46	99.88 ± 1.13	98.26 ± 0.69	102.5 ± 1.10
4 min.	98.41 ± 0.46	96.59 ± 0.73	97.48 ± 1.20	96.98 ± 1.69

Table 5: Influence of small variation in the assay conditions on the analytical performance of the proposed method for analysis of the four anti-TB drugs

* Average of three determinations.

[a, b, c, d] : the concentration of drugs used were 2, 0.2, 0.4 and 3 µg ml^{-1} respectively

3.3.4. Analysis of pharmaceutical dosage forms and accuracy testing:

The available pharmaceutical dosage forms were subjected to the analysis by the proposed, as well as the official methods [31-34] and the obtained results were statistically compared with each other. The mean percentage recoveries relative to the labeled amounts, obtained by the proposed method ranged from 97.24 to 101.2 % (Table 6). With respect to *t*- and *F*-tests, no significant differences were found between the calculated and theoretical values of both the proposed and official methods at 95% confidence level. This indicated similar accuracy and precision in the analysis of studied drugs in tablets (Table 6). Also, the accuracy was checked by using standard addition method [35]. A fixed weight of tablet or capsule equivalent to 25 mg of each drug was used, then (5, 10 and 15 mg) of pure drug were added separately.

Dosage form	Proposed method	Official method
	% Recovery ± SD[a]	% Recovery ± SD[a]
Etibi® 500 tablets	99.53 ± 1.82[b] *t = 2.26* *F = 2.94*	96.79 ± 1.06
Isocid fort® tablets	97.24 ± 0.97 *t = 0.86* *F = 2.55*	98.15 ± 1.54
P.T.B® tablets	98.96 ± 1.25 *t = 0.30* *F = 4.44*	99.64 ± 2.63
Rifadin® capsule	101.2 ± 2.02 *t = 0.47* *F = 1.91*	100.5 ± 1.46
Rifampicin® capsule	99.91 ± 0.99 *t = 0.48* *F = 3.86*	99.31 ± 1.94
Rimactane® capsule	100.2 ± 2.23 *t = 0.91* *F = 2.70*	98.85 ± 1.36
Rifactine® capsule	97.64 ± 1.12 *t = 0.82* *F = 4.54*	98.89 ± 2.39
Rifactine® suspenion	100.2 ± 1.51 *t = 0.30* *F = 2.36*	99.76 ± 2.32
Rimactane® suspension	98.60 ± 1.49 *t = 0.39* *F = 1.37*	98.17 ± 1.27

Table 6: Determination of studied anti-TB drugs in their dosage forms by the proposed spectrofluorimetric and official methods

* Average of three determinations

** Theoretical values for t and F at 95 % confidence limits (t = 2.78) and (F = 19) respectively

Drug/ dosage form	Added (mg)	Found(mg)	% Recovery	SD*
EMB/ Etibi tablets	5	4.97	99.40	2.00
	10	9.78	97.80	1.44
	15	15.3	102.0	1.83
INH/ Isocid fort tablets	5	5.02	100.4	1.22
	10	9.98	99.80	1.76
	15	15.2	101.1	1.00
PZA/ P.T.B tablets	5	5.02	100.4	1.00
	10	10.02	100.2	1.85
	15	14.92	99.47	0.56
RIF/ Rimactane capsule	5	5.10	102.0	1.39
	10	10.2	102.0	1.73
	15	14.9	99.33	1.83

Table 7: Recovery studies for the determination of anti-TB drugs using standard addition method by the proposed spectrofluorimetric method

* Average of five determinations

4. CONCLUSION

The present study described a validated spectrofluorimetric method for the analysis of four antituberculous drugs in their available dosage forms. The method was simple, rapid, accurate, and reliable for the determination of studied drugs without interference from the common excipients, the proposed method is of great value in quality control analysis of these drugs owing to its improved simplicity, sensitivity, low-cost, and its independence on expensive instruments, or critical analytical reagents.

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