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Spectrophotometric Assay of Ethionamide in Pharmaceuticals Using Picric Acid and Iodine as Charge-Transfer Complexing Agents

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Abstract
Two simple, rapid and sensitive spectrophotometric methods are described for the determination of ethionamide (ETN) in bulk drug and tablets. The methods are based on the reaction of ETN as n-donor with σ–acceptor iodine (method A) and π-acceptor, picric acid (method B) in chloroform. The resultant charge-transfer complexes were measured at 400 nm for iodine (method A) and at 420 nm for picric acid (method B). Different variables affecting the reaction were studied and optimized. Under the optimum reaction conditions linear relationships with good correlation coefficients were found between absorbance and concentration of ETN in the ranges, 1-20 and 2-45 µg mL^{-1}, for method A and method B, respectively. The limits of detection (LOD) and quantification (LOQ) were: 0.24 and 0.73 µg mL^{-1} (method A), and 0.96 and 2.92 µg mL^{-1} (method B). A Job's plot of absorbance *versus* the molar ratio of ETN to each of the acceptors under consideration indicated 1:1 (donor: acceptor) ratio. The methods were validated for accuracy, precision, selectivity, robustness and ruggedness. The proposed methods were applied successfully to the determination of ETN in tablets with good accuracy and precision and without interference from common additives. The results compared favorably with those of the official method.

Keywords
Ethionamide, Assay, Spectrophotometry, Charge-transfer complexes, Iodine, Picric acid.

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INTRODUCTION
Ethionamide is an isonicotinic acid derivative of thioamide class. It is the second line orally administered drug used in the treatment of clinical tuberculosis that has failed to respond to adequate first line therapy. Ethionamide is often combined with other anti-tuberculous agents for the treatment of multidrug resistant organisms. Ethionamide is active against *tubercle bacilli* that are growing with in human macrophages [1, 2]. It is used to cure tuberculosis, a disease that infects more than a third of the world's population [3]. Infections caused by Mycobacterium avium intracellular complex (MAI) and drug resistant mycobacterium are increasingly

common in different parts of the world and are fueled with spread of Acquired Immunodeficiency Syndrome (AIDS), as a result of this second line antimicrobial agents such as ethionamide are being used much more frequently.

The pharmacological effects of ETN has necessiated the development of analytical methods for its determination in body fluids and pharmaceuticals. The drug is official in the United States Pharmacopoeia [4] which recommends a UV-spectrophotometric method for its assay. The drug in body fluids has been assayed by a few liquid chromatographic [5-11] and fluorimetric [12] methods. In addition to one fluorimetric method [12], a few titrimetric [13-15] and spectrophotometric methods have been adapted for the assay ETN in pharmaceuticals.

Spectrophotometric assays have been through extractive ion-pair formation with Alizarin Violet 3B and Alizarin Brilliant Violet R [16], redox reaction with $KMnO_4$ [17] and N-bromosuccinimide-celestein blue [18], orange red complexation with sodium nitroprusside [19,20], coloured product formation with 2,3-dichloro-1,4-naphathaquinone [21,22], ternary complexation with pyridylazo resorcinol-vanadium(V) [23], thionine compound formation with *p*-phenylenediamine-zinc-iron(III) [24], complex formation with osmic acid [25], hydroxylamine-iron(III) [26], Folin-Ciocalteu and iron(III)-ferricyanide [27], ion association with bromocresol green and bromocresol purple [28]. In addition, a kinetic spectrophotometric method [29], based on the catalytic effect of ETN on the reaction between sodium azide and iodine and uv- spectrophotometry of ETN in phosphate buffer medium [30] have also been reported. However, the above spectrophotometeric methods suffer from such disadvantages as narrow linear range of response, low sensitivity, longer contact time for full colour development, and involve multiple reaction steps and prior extraction of the coloured product. Thus, simple and sensitive spectrophotometric methods are required for the determination of ETN in pharmaceuticals. This is fulfilled in the investigation by the application of charge-transfer complexation reaction with iodine (a σ- acceptor) and picric acid (a π- acceptor).

The molecular interactions between electron donors and acceptors are generally associated with the formation of intensely coloured charge-transfer complexes, which absorb radiation in the visible region [31]. The spectrophotometric methods based on these interactions are usually simple and convenient because of the rapid formation of the complexes. ETN, being an amide is a good n-donor and will form charge-transfer complexes with σ- or π- acceptors.

Iodine, a σ- acceptor is known to yield charge-transfer complex and tri-iodide ion-pair with a variety of electron-donors [32-34], and picric acid, a π- acceptor is also reported to form charge-transfer complex, and finally coloured radical anion with a number of donor molecules [35,36]. Based on these interactions, simple, facile, sensitive and selective spectrophotometric methods have been developed for the assay of diverse compounds of pharmaceutical importance [32-45]. This study applies these interactions successfully for developing direct, rapid and inexpensive spectrophotometric methods for ETN in bulk drug and tablets.

EXPERIMENTAL

Apparatus: Absorbance measurements were made with a Systronics model 166 digital spectrophotometer (Ahmedabad, India) equipped with 1 cm matched quartz cells.

Materials and reagents*:* All reagents used were of analytical reagent grade and spectroscopic grade organic solvents were used throughout the investigation.

Solutions of 0.2% iodine (Merck Ltd., Mumbai, India) and 0.1% picric acid [PA] (S.D. Fine Chem., Mumbai, India) were prepared separately in chloroform.

Standard ETN solution (50 & 100 μg mL^{-1}): Pharmaceutical grade ETN (99.7% pure) was procured from Lupin Laboratories Pvt. Ltd., Mumbai, India, and was used as received. Ethide-250 (Lupin Ltd., Jammu, India) and Mycotuf-250 (Cadila (Le Sante) Pharma. Ltd., Bangalore, India) tablets were purchased from local markets.

A stock standard solution containing 100 μg mL^{-1} ETN was prepared by dissolving 10 mg of pure drug in chloroform, and diluted to volume with the same solvent in a 100 mL volumetric flask and used in method B. This was diluted to get a working concentration of 50 μg mL^{-1} was prepared by diluting with chloroform for method A.

GENERAL PROCEDURES

Procedure for bulk drug

Method A: Varying aliquots: 0.1, 0.25, 0.5, 1.0, 1.5 and 2.0 mL of 50 μg mL^{-1} standard ETN solution were accurately transferred into 5 mL calibrated flasks using a micro burette, and the total volume was adjusted to 2 mL with chloroform. One mL of 0.2% iodine was added to each flask and the content was diluted to the mark with chloroform and mixed well. The absorbance of each solution was measured at 400 nm against the reagent blank after 5 min.

Method B: Different aliquots: 0.1, 0.25, 0.5, 1.0, 1.5, 2.0 and 2.25 mL of 100 μg mL^{-1} standard ETN solution were accurately added to 5 mL standard flasks using a micro burette, and the total volume was adjusted to 2.25 mL with chloroform. One mL of 0.1% picric acid was added to each flask and the content was diluted to the mark with chloroform and mixed well. After five minutes, the absorbance of each solution was measured at 420 nm against the reagent blank.

A standard graph was constructed by plotting the absorbance *versus* concentration of ETN and the unknown concentration was computed from the regression equation.

Procedure for tablets*:* Tablet powder equivalent to 5 mg of ETN was accurately weighed and transferred into a 50 mL standard flask and shaken with 20 mL of chloroform for 15 min. The volume was brought to the mark with chloroform and mixed. It was filtered using a Whatman No. 42 filter paper and used in method B. The filtrate was diluted to get 50 μg mL^{-1} ETN for method A. One mL each of tablet extract was analyzed in five replicates following the recommended procedure.

Procedure for selectivity study: A placebo blank containing starch (35 mg), acacia (45 mg), hydroxyl cellulose (55 mg), magnesium stearate (45 mg), sodium citrate (30 mg), talc (40 mg) and sodium alginate (35 mg) was prepared by mixing all the components into a homogeneous mixture. A 100 mg of the placebo blank was accurately weighed and its solution was prepared as described under '*tablets*', and then subjected to analysis following the recommended procedures.

A synthetic mixture was prepared by adding 5 mg ETN to 5 mg placebo blank, and the mixture was homogenized. Following the procedure used for tablets, the placebo blank and synthetic mixture solutions were prepared, and a suitable aliquot was subjected to analysis by method B (n=5) after appropriate dilution by method A.

RESULTS AND DISCUSSION

Spectral characteristics: In method A, ETN reacts with iodine and gives a wine red chromogen that exhibits a maximum absorption at 400 nm in chloroform. This can be attributed to the formation of charge-transfer complex between ETN (D) (Scheme 1) and iodine (A) followed by the formation of tri-iodide complex ion, which probably due to the dissociation of ETN-iodine complex in chloroform was as suggested in Scheme 1 and is in conformity with previous reports [32-45].

In method B, the interaction between ETN with picric acid in chloroform gave an intense yellow colored chromogen with strong absorption at 420 nm due to the formation of the free radical anion. The interaction between ETN and n-donor and picric acid, a π-acceptor, is a charge-transfer complexation reaction occurring according to the Scheme 2, which is supported by the findings of previous workers [35-45].

Absorption spectra: The absorption spectrum of colored product formed in either method was recorded at 370-470 nm against the corresponding reagent blank. The resulting colored products showed maximum absorbance at 400 and 420 nm for ETN-I_2 and ETN-PA complexes, respectively (Fig. 1).

OPTIMIZATION OF REACTION CONDITIONS

Choice of solvent: Different solvents, such as dichloromethane, chloroform, acetonitrile, benzene, 1,2-dichloroethene, 1,4-dioxan and methanol were tried as the reaction medium, and the reaction of ETN with iodine or picric acid was followed (Fig. 2). In both methods, chloroform was best suited as the reaction medium as well as the diluent.

Effect of reagent concentration: The effect of acceptor concentration on complex formation was studied. The study showed that one mL of acceptor solution was adequate in both methods. Though larger volumes did not affect complex formation, higher blank absorbance was observed in both methods (Fig. 3).

Effect of reaction time, and stability of colour: Though complex formation in both methods was rapid, constant absorbance was obtained after 5 min and the coloured products were stable for 60 min in method A and 4 hours in method B, beyond which, slight increase in absorbance was noticed (Fig. 4).

Stoichiometric relationship*:* Job's method of continuous variations was used to establish the composition of the CT complex [46]. Solutions equivalent to 1.97×10^{-4}M ETN and iodine for method A; and 6.02×10^{-4}M ETN and picric acid in method B were prepared in chloroform by dissolving the calculated quantities. A series of solutions was prepared in which the total volume of ETN and acceptor was kept at 3 mL in 5 mL calibrated flask. The contents were mixed well; the volume was completed to the mark with chloroform. The absorbance of the resulting solution was measured after 5 min at the respective λ_{max} *vs* chloroform blank. The resulting plot (Fig. 5) shows that the CT interaction occurs on an equimolar basis (1:1 reaction stoichiometry), owing to the presence of one basic nitrogen in ETN.

The conditional stability constant (K_f) of the CT complex was calculated from the continuous variations data using the following equation [47].

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$$K_f = \frac{A/A_m}{[1-A/A_m]^{n+2} C_M (n)^n}$$

where, A and Am are the observed maximum absorbance, and the absorbance value when all ETN present is associated, respectively. C_M is the molar concentration of ETN at the maximum absorbance and n is the stoichiometry with which iodine/PA complexes with ETN. The log K_f values were found to be 8.12 and 7.02 for method A and method B, respectively.

METHOD VALIDATION

Analytical parameters: A linear correlation was found between absorbance at λ_{max} and concentration of ETN in the ranges given in Table 1. The slopes, intercepts and correlation coefficients of the linear plots (Fig. 6) are presented in Table 1. The optical characteristics such as Beer's law limits, molar absorptivity, Sandell sensitivity values [48], limits of detection (LOD) and quantification (LOQ) of both methods [49] are also given in Table 1.

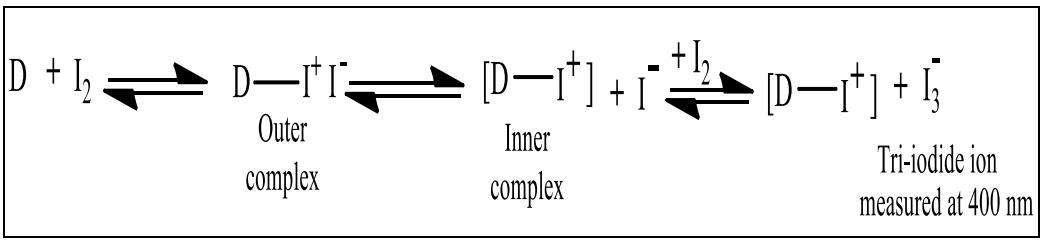
Accuracy and precision: To assess the accuracy and precision of the proposed methods, pure ETN solution at three different concentration levels were prepared and analyzed in seven replicates during the same day and on five consecutive days and the results are presented in Table 2. The percentage relative error (%RE) was ≤ 2.50 indicated that the accuracy of the methods was satisfactory. Percentage relative standard deviation (%RSD) for intra-day was ≤ 1.63 and for inter-day it was ≤ 1.55 indicating good precision and usefulness of the proposed methods.

Robustness and ruggedness: To evaluate the robustness of the proposed methods, two experimental variables: reagent volume and contact time were slightly varied, and the effect of change on complex formation was studied. The results of this study are presented in Table 3 and indicated that the proposed methods are robust. To determine ruggedness analysis was performed by three different analysts and using three different cuvettes by the same analyst. From the %RSD values presented in Table 3, it is concluded that the proposed methods are rugged.

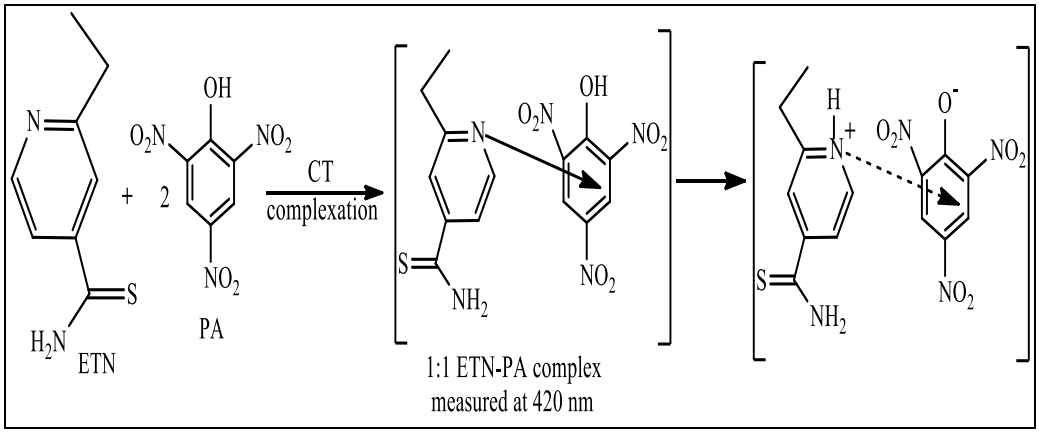
Selectivity study: The absorbance of the CT complex with the placebo blank was almost equal to the absorbance of the reagent blank suggesting no interference. The percent recoveries of ETN from the synthetic mixture solution were 97.95±0.92 and 101.5±1.54 for method A and method B, respectively. This reflects the selectivity of the methods in the presence of the tablet excipients.

Application to tablets: The proposed methods were applied to the determination of ETN in tablets. The results obtained were statistically compared with those of the official method [4], by applying the Students *t*-test for accuracy and *F*-test for precision. As can be seen from the Table 4, the calculated *t* and *F*-values at 95% confidence level did not exceed the tabulated values of 2.77 and 6.39 respectively (n=5). Thus, there is no difference between the proposed methods and the official method with respect to accuracy and precision.

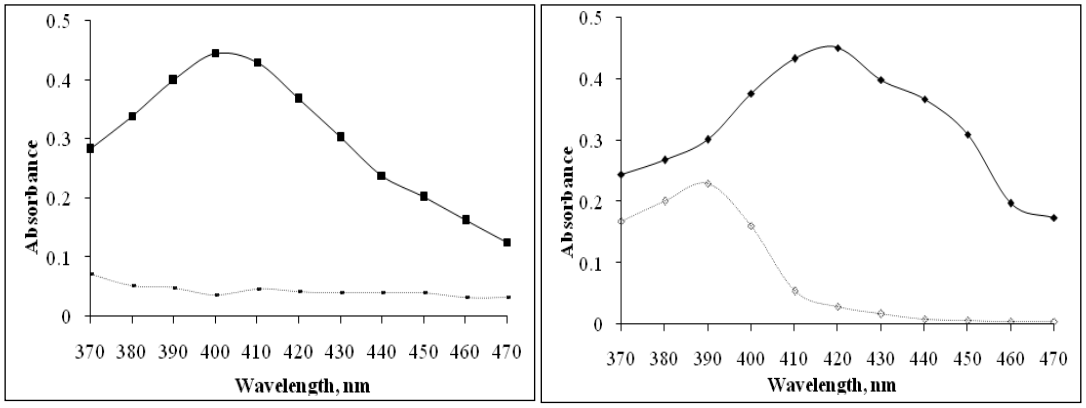
Accuracy by recovery study: The study was done by spiking the pre-analyzed tablet powder with pure ETN at three different levels and the total was determined by the proposed methods. Each test was repeated three times. The % recovery values ranged between 97.96 and 102.7% with standard of ≤ 1.52%. Closeness of the results to 100% showed the fairly good accuracy of the methods and non-interference from the co formulated substances in the assays. The results are shown in Table 5.



Scheme 1. Pathway of CT complex formation between ETN and iodine in chloroform

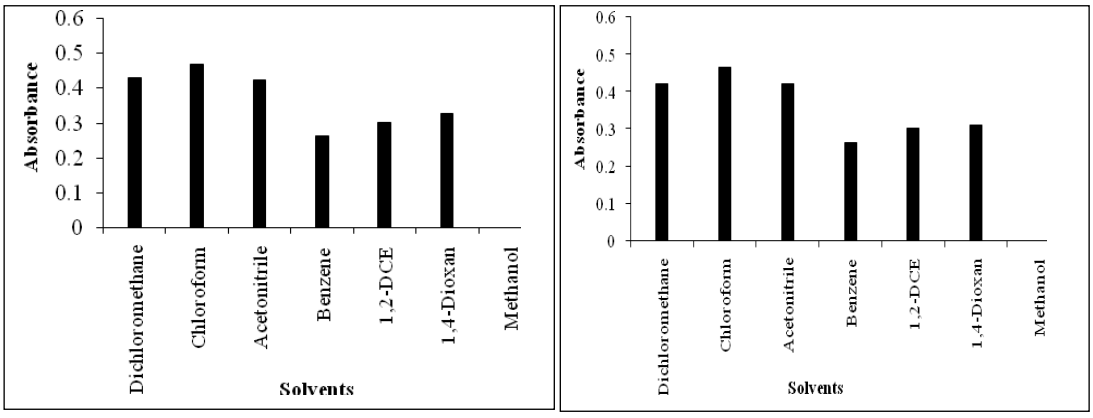


Scheme 2. Reaction pathway for the formation of electron donor-acceptor complex due to ETN and picric acid (PA) interaction



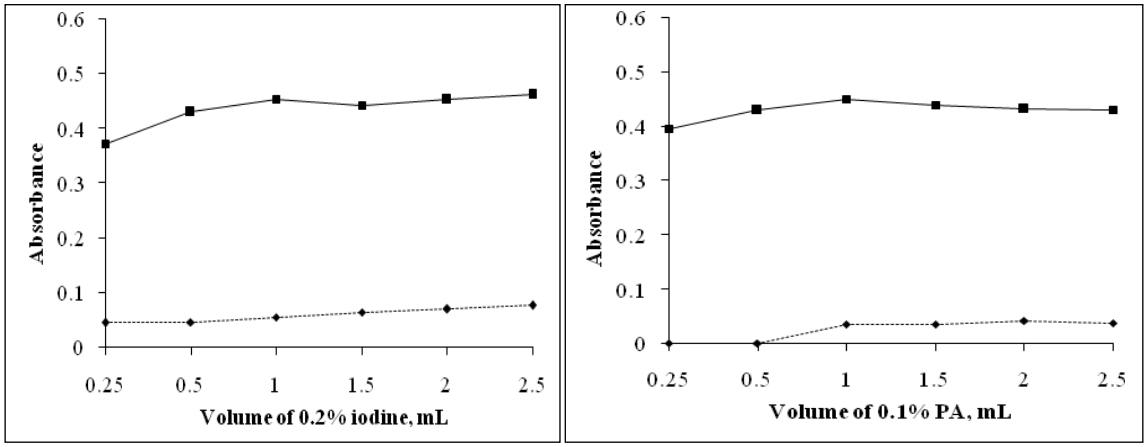
(a) **(b)**

Fig. 1. Absorption spectra of: (a) ETN-iodine complex (–■–) [10 µg mL^{-1} ETN] against blank (-♦-) and (b) ETN-PA complex (–■–) [20 µg mL^{-1} ETN] against blank (-Δ-)



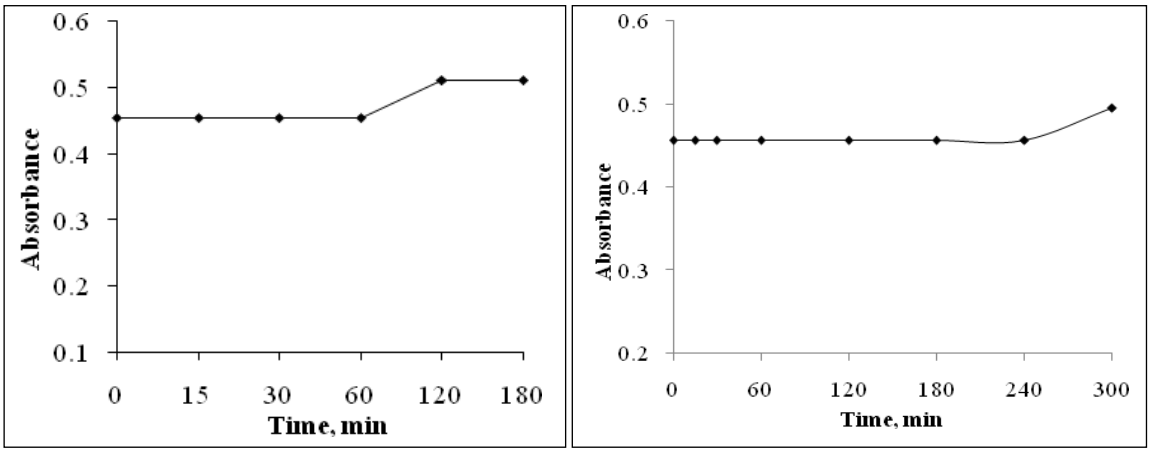
(a) **(b)**

Fig. 2. Effect of different solvents: (a) ETN-iodine complex (–■–) [10 µg mL^{-1} ETN] and blank (-♦-); (b) ETN-PA complex (–■–) [20 µg mL^{-1} ETN] and blank (-♦-)



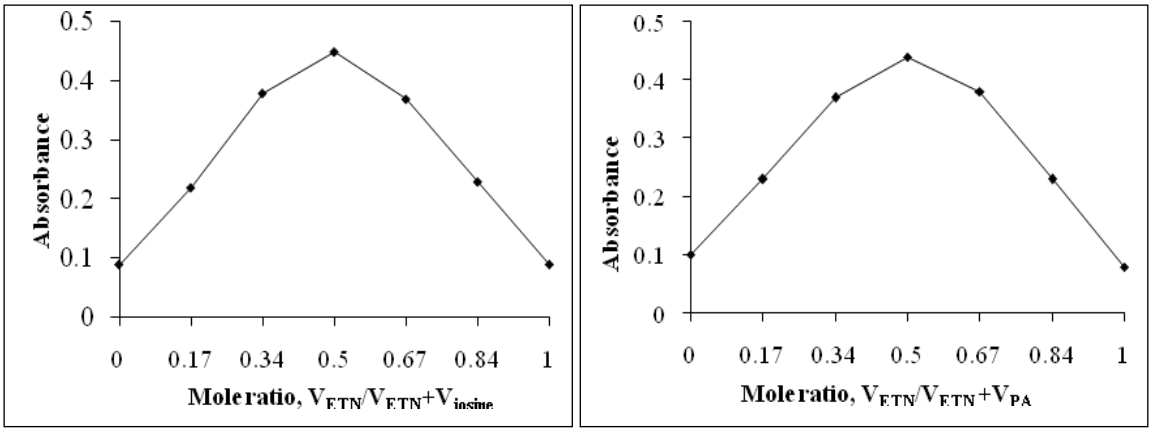
(a) (b)

Fig. 3. Effect of volume of: (a) 0.2% iodine in method A (10 µg mL^{-1} ETN) (b) 0.1% picric acid in method B (20 µg mL^{-1} ETN)



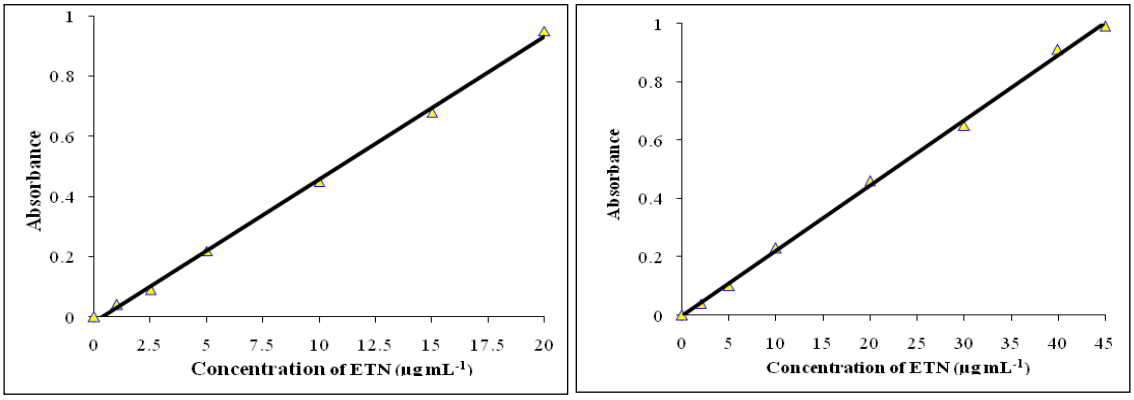
(a) (b)

Fig. 4. Effect of reaction time: (a) method A (10 µg mL^{-1} ETN) (b) method B (20 µg mL^{-1} ETN)



(a) (b)

Fig. 5. Job's plots for: (a) ETN-iodine complex (1.97×10^{-4}M) and (b) ETN-picric acid complex (6.02×10^{-4}M)



(a) **(b)**

Fig. 6. Calibration curves: (a) method A and (b) method B

Table 1: Sensitivity and regression parameters

Parameter	Method A	Method B
λ_{max}, nm	400	420
Colour stability, h	1 h	4 h
Linear range, µg mL^{-1}	1–20	2–45
Molar absorptivity (ε), L mol^{-1} cm^{-1}	7.15×10^{3}	3.62×10^{3}
Sandell sensitivity*, µg cm^{-2}	0.0495	0.0459
Limit of detection (LOD), µg mL^{-1}	0.24	0.96
Limit of quantification (LOQ), µg mL^{-1}	0.73	2.92
Regression equation, Y**		
Intercept (a)	-0.0213	-0.0018
Slope (b)	0.0478	0.0223
Standard deviation of a (S_a)	2.28×10^{-4}	1.2×10^{-5}
Standard deviation of b (S_b)	1.72×10^{-4}	1.41×10^{-4}
Regression coefficient (r)	0.9994	0.9993

***Limit of determination as the weight in µg mL^{-1} of solution, which corresponds to an absorbance of A = 0.001 measured in a cuvette of cross-sectional area 1 cm^2 and l = 1 cm.**
****Y=a+bX, where Y is the absorbance, X concentration in µg mL^{-1}, a intercept and b slope.**

Table 2: Results of intra-day and inter-day accuracy and precision study

Method	ETN taken (µg mL^{-1})	Intra-day accuracy and precision (n=7)			Inter-day accuracy and precision (n=5)		
		ETN found[a] (µg mL^{-1})	RSD[b] %	RE[c] %	ETN found[a] (µg mL^{-1})	RSD[b] %	RE[c] %
A	5.0	4.92	1.21	1.60	5.07	1.55	1.40
	10.0	9.81	1.25	1.90	10.25	0.93	2.50
	15.0	15.14	0.98	0.94	14.81	1.38	1.27
B	10.0	9.89	1.63	1.10	10.21	1.32	2.10
	20.0	20.25	1.10	1.25	20.36	0.89	1.80
	30.0	29.42	0.91	1.94	29.32	1.18	2.27

[a] Mean value of n determinations; [b] Relative standard deviation (%); [c] Relative error (%).

Table 3: Results of method robustness and ruggedness study expressed as intermediate precision (%RSD)

Method	ETN taken (µg mL^{-1})	Method robustness			Method ruggedness	
		Parameters altered				
		Volume of iodine, (n=3)	Volume of PA, (n=3)	Reaction time (n=3)	Inter-analysts, (n=3)	Inter-cuvettes, (n=3)
	5.0	2.05	-	1.71	1.54	1.61
A	10.0	1.13	-	1.54	1.23	1.82
	15.0	2.16	-	2.03	2.85	2.08
	10.0	-	0.95	1.64	1.02	1.32
B	20.0	-	1.23	1.88	0.99	1.29
	30.0	-	1.35	1.07	1.49	2.15

In both methods, volumes of reagent were 1 and 1±0.1 mL and reaction times were 5 and 5±1 min.

Table 4: Results of analysis of tablets by the proposed methods

Tablets analyzed	Label claim mg/tablet	Found* (Percent of label claim±SD)		
		Official method	Proposed methods	
			Method A	Method B
Ethide-250	250	98.9±1.08	99.45±1.02 *t* = 0.83 *F* = 1.12	99.12±1.32 *t* = 0.29 *F* = 1.49
Mycotuf-250	250	101.3±0.75	102.1±1.31 *t* = 1.18 *F* = 3.05	101.9±0.96 *t* = 1.10 *F* = 1.64

*Mean value of five determinations.
Tabulated *t*-value at the 95% confidence level is 2.77.
Tabulated *F*-value at the 95% confidence level is 6.39.

Table 5: Results of recovery study *via* standard addition method with tablet

Method	Tablet studied	ETN in tablet µg mL^{-1}	Pure ETN added µg mL^{-1}	Total found µg mL^{-1}	Pure ETN recovered* Percent±SD
		4.97	2.5	7.33	98.13±0.96
A	Ethide-250	4.97	5	10.16	101.9±0.48
		4.97	7.5	12.81	102.7±0.69
		4.96	2.5	7.31	97.91±0.59
B	Ethide-250	4.96	5	10.15	101.9±0.79
		4.96	7.5	12.23	98.13±1.14

*Mean value of three determinations.

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