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Novel Reagents for the Spectrophotometric Determination of Isoniazid in Pure and Pharmaceutical Formulations

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ABSTRACT

New, simple and cost effective spectrophotometric methods have been developed for the quality assessment of isoniazid (INH), in bulk and in pharmaceutical formulations by using novel reagents. The proposed methods involve the utility of ethyl 2-[(E)-(4-hydroxyphenyl) diazenyl]-3-oxobutanoate and benzil as novel reagents for the determination of INH. Developed methods are based on the condensation reaction of INH with reagents to give orange colored chromogen with an absorption band at 454 nm (for method A) and 436 nm (for method B). The applicability of these methods is demonstrated by the determination of the studied drug in commercial tablets and the results are statistically evaluated. The new procedures described in this paper are fast, convenient and have the novelty of carrying out the quantitative determination of INH in pure and in pharmaceutical formulation.

KEYWORDS: Isoniazid; Spectrophotometry; Ethyl 2-[(E)-(4-hydroxyphenyl) diazenyl]-3-oxobutanoate; Benzil, Condensation reaction.

1. INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by a bacterium called Mycobacterium tuberculosis [1]. According to the World Health Organization (WHO), nearly two billion people have been exposed to the tuberculosis pathogen [2]. Annually, eight million people become ill with TB [3]. Once the infection is acquired, it usually remains dormant in the lungs for up to many years. Later, the infection may become active in the lungs and sometimes spreads throughout the body [4]. Currently rifampin, isoniazid, pyrazinamide and ethambutol are used for the treatment of TB. Among these INH is recommended as major medication for the effective treatment of TB because it is highly active, inexpensive and without significant side effects [5-6]. INH chemically, pyridin-4-carboxylic acid hydrazide (Fig. 1) is an antibacterial prescription medicine approved by the U.S. Food and Drug Administration (FDA) for the prevention and treatment of TB [7]. INH is a synthetic analog of pyridoxine which has been most commonly used anti-TB since 1952 [8].

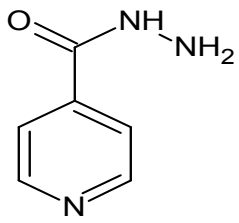


Fig. 1. Chemical structure of INH.

At therapeutic levels INH is bactericidal against actively growing intracellular and extracellular Mycobacterium tuberculosis organisms [9]. Because of its significant bactericidal activity, it has become a critical component of the first-line anti-TB regimens. It is widely used together with other anti-TB drugs like rifampin, pyrazinamide and ethambutol for the chemotherapy of tuberculosis [10]. INH is normally taken orally but it may be administered intramuscularly to critically ill patients. Within one to two hours after oral administration, INH produces peak blood levels which decline to 50 percent or less within six hours. It is rapidly absorbed and diffuses into all body fluids, tissues, organs and excreta (saliva, sputum and feces) [11]. However, powerful new anti-TB drugs with new mechanism of action have not been developed in the last 40 years. Therefore, INH is still considered to be a first line drug for chemotherapy of tuberculosis [12].

The recommended therapeutic doses of INH for adults are 300 mg daily for 6 months to 1 year and for children are 10 mg/kg (maximum 300 mg) daily for 6 months to 1 year [13]. Over dosage may lead to nausea, vomiting, dizziness, blurred vision and slurring of speech occur within 30 minutes to 3 hours of over dosage [14]. Repeated dosing of INH may cause severe toxicity, therefore it is an important issue to establish a standard method for monitoring residual drug in pure and in pharmaceutical formulations. All these aspects encouraged us to develop a new direct analytical procedure for the routine analysis of INH.

In the past few decades, very few elegant instrumental techniques such as spectrophotometry [15-21], polarography [22], coulometry [23], high-performance liquid chromatography [24] and fluorimetry [25] methods have been reported for the analysis of INH. Among these, spectrophotometry is the most important method, which is widely used for wide variety of materials. The greatest use of spectrophotometry lies in its application to quantitative measurements because of its high sensitivity, precision and relatively low cost of instrumental purchase and operation. In the present work, we came up with the two new visible spectrophotometric methods for the determination of INH in pure and pharmaceutical formulations. Through this work we introduced ethyl 2-[(E)-(4-hydroxyphenyl) diazenyl]-3-oxobutanoate and benzil as two novel chromogenic reagents for the spectrophotometric determination of INH in pure and dosage forms. In these developed methods drug is made to quantitatively react with molecules containing chromophore, resulting in the formation of the products with more extended conjugation. Absorbance of newly formed colored product can be successfully analyzed by visible spectrophotometry.

2. EXPERIMENTAL

2.1. Apparatus

A UV-Visible spectrophotometer (SHIMADZU, UV 2550 Japan) was used with 1 cm quartz cells for recording the spectra and making the analytical measurements.

2.2. Synthesis of chromophore

4-Aminophenol (0.01 mol) was dissolved in 8N hydrochloric acid solution (15 mL) and cooled to 0^0C. To this solution added cold aqueous solution of sodium nitrite (0.02 mol) drop wise. The resultant diazonium salt solution thus formed was further added to a cooled ethanolic solution of ethyl acetoacetate in presence of sodium acetate (0.05 mol). The reaction mixture was stirred for 2 hours and resulting solid was filtered, dried and purified by recrystallization from ethanol to afford compound (1). The structure of synthesized compound was confirmed by mass spectroscopy. The molecular ion peak at m/z=250.14 confirmed the formation of ethyl 2-[(E)-(4-hydroxyphenyl) diazenyl]-3-oxobutanoate (1). The mass spectrum of compound (1) was given in Fig. 2.

2.3. Reagents

The entire reagents were chemically pure grade and were used without further purification. INH bulk drug was obtained as gift sample from CAD Pharma Inc Bangalore, India. Pharmaceutical formulations of INH were obtained commercially. Stock solution of INH (1000 μg mL^{-1}) was prepared by dissolving 0.1 g of INH in distilled water and making the volume to 100 mL in a standard volumetric flask. The stock solution was diluted approximately to get working concentration. A stock solution of 2×10^{-3} M 2-[(E)-(4-hydroxyphenyl) diazenyl]-3-oxobutanoate was prepared by dissolving 0.5 g of reagent (synthesized) in 100 mL ethanol.

Stock solution of 5×10^{-3} M of benzil (Merck) was prepared by dissolving 1 g of reagent in 100 mL ethanol.

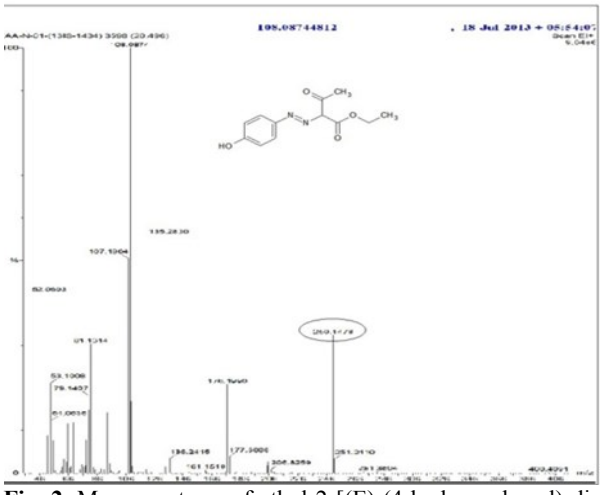


Fig. 2. Mass spectrum of ethyl 2-[(E)-(4-hydroxyphenyl) diazenyl]-3-oxobutanoate (1).

2.4. Recommended Procedures

2.4.1. Method A

Aliquots containing 5.00–30 μg mL^{-1} of INH were transferred into a series of 10 mL volumetric flasks. To each flask, 1 mL of reagent and 0.5 mL of 2 N HCl were added and heated to 60^0C for 5 minutes and cooled to room temperature. Afterwards reaction mixture was diluted to 10 mL by using ethanol. Obtained red colored adduct of INH was measured at 454 nm against the reagent blank.

2.4.2. Method B

Aliquots containing 10.00–50.00 μg mL^{-1} of INH were transferred into a series of 10 mL volumetric flasks. To each flask, 1 mL of reagent and 0.5 mL of 2 M NaOH were added and heated to 100^0C for 10 minutes and cooled to room temperature. Afterwards reaction mixture was diluted to 10 mL by using ethanol. Obtained orange colored adduct of INH was measured at 436 nm against the reagent blank.

2.5. Preparation of pharmaceutical formulation

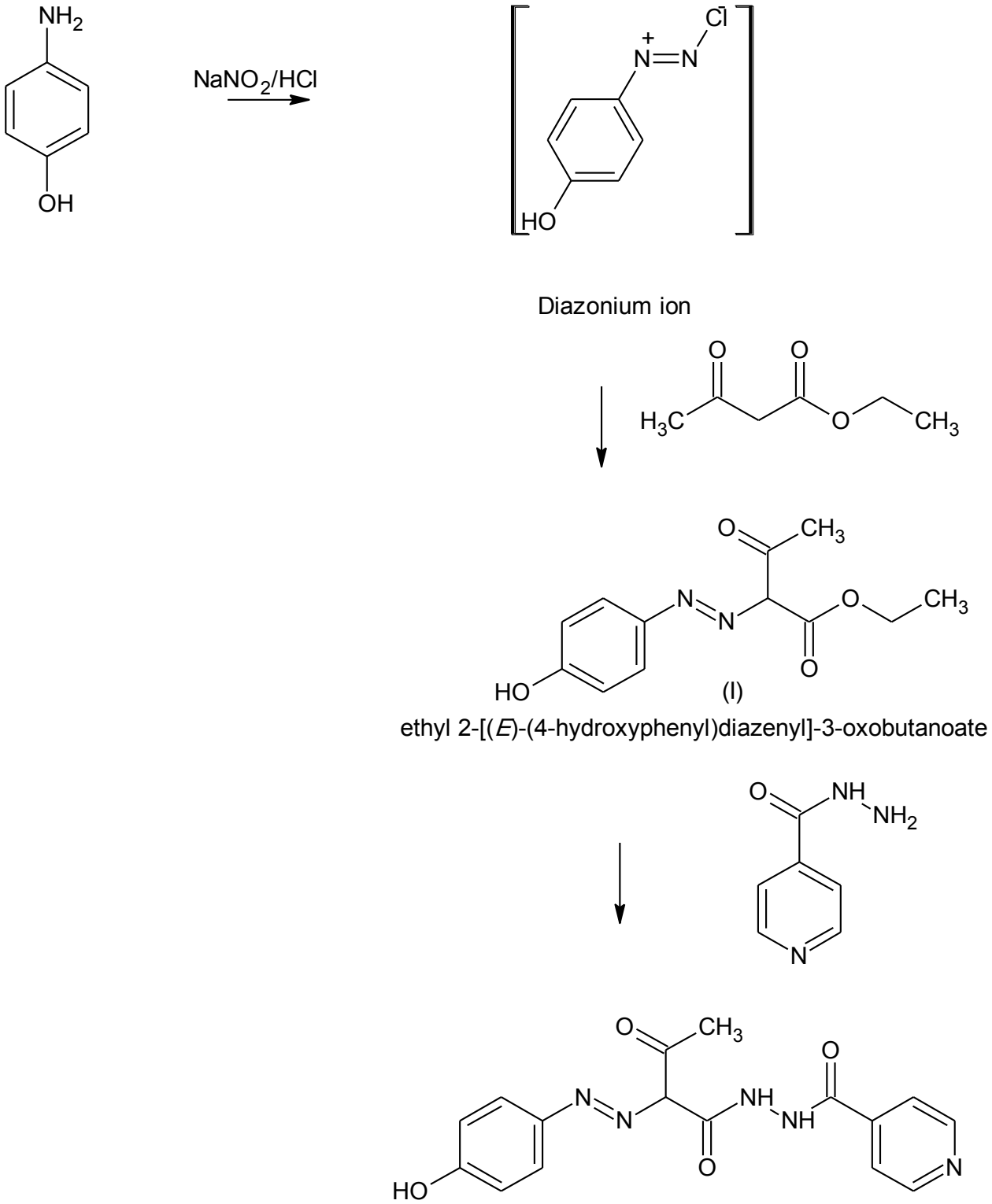
Commercial tablets of INH (Isokin 300 mg, Solonex 300 mg Ipcazide 100 mg) were analyzed by the proposed methods. All the tablets were crushed separately in a mortar and dissolved in ethanol, solution was filtered through whatman filter paper No. 41 and diluted quantitatively with ethanol to obtain a suitable concentration for the analysis. A convenient aliquot was then subjected to the analysis by using proposed methods.

3. RESULT AND DISCUSSION

3.1. Determination of INH by using ethyl 2-[(E)-(4-hydroxyphenyl) diazenyl]-3-oxobutanoate (method A)

In method A, novel diazenyl compound is synthesized and utilized as a chromogen for the analysis of INH. 2-

[(E)-(4-Hydroxyphenyl) diazenyl]-3-oxobutanoate is synthesized by the diazotization of 4-aminophenol and then diazotized product was coupled with a good coupling component namely ethyl acetoacetate in alkaline solution. In the obtained diazenyl compound two reactants are linked by a nitrogen bridge. According to the principles of spectroscopy when aromatic moieties are substituted with chromophoric groups, this may significantly extend the conjugation system which is responsible for the development of color [24]. Azo compounds contain a highly delocalized system of electrons (Scheme 1), which results in the formation of new orange colored adduct which measured at 454 nm (Fig. 3).



Scheme 1. Condensation reaction of INH with diazenyl compound.

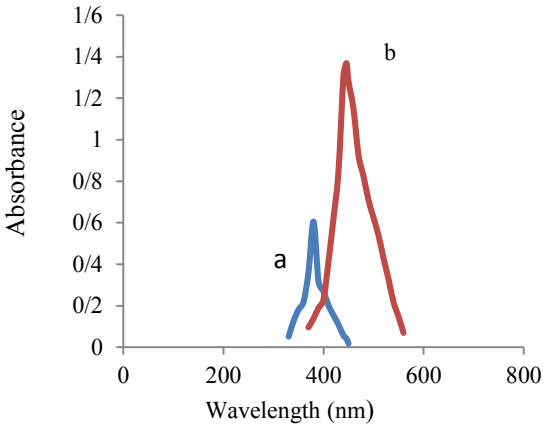


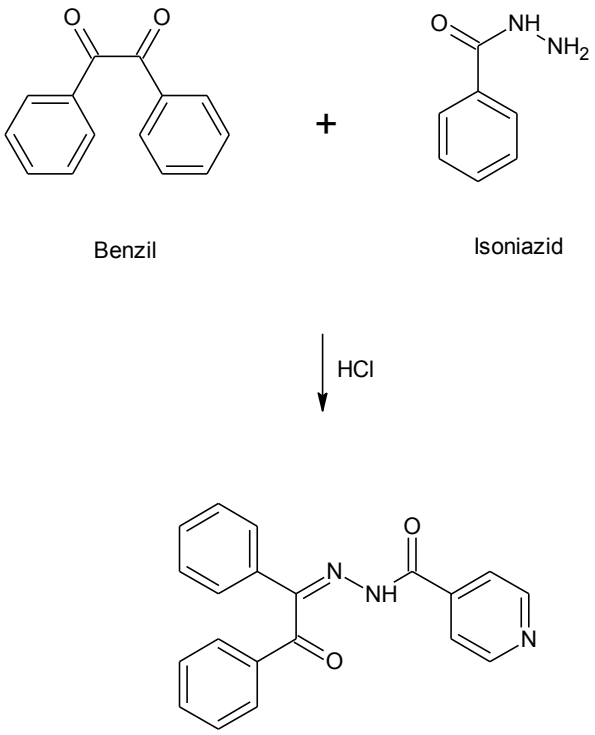
Fig. 3. Absorption maximum for (a) blank (b) method A.

3.1.1. Choice of coupling reagent

A critical study has been done with of several coupling reagents, most of which have not previously been used. The reagents tested are: acetyl acetone, malononitrile, ethyl cyano acetate, ethyl acetoacetate. Useful analytical results obtained by the diazenyl compound synthesized from ethyl acetoacetate. Therefore, this reagent was selected as a good coupling reagent to get diazenyl compound which further reacts with INH to give colored product.

3.2. Determination of INH by using benzil (method B)

In method B, INH reacts with benzil to form a colored Schiff base hydrazone (Scheme 2). Newly formed orange colored product showed maximum absorption at 434 nm (Fig. 4).



Scheme 2. Reaction of INH with benzil.

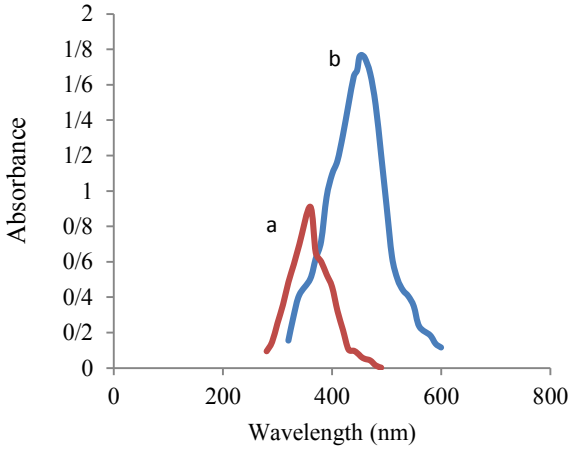


Fig. 4. Absorption maximum for (a) blank (b) method B.

3.3. Optimization of reaction conditions

To optimize the reaction conditions, we have investigated a number of parameters such as reagent concentration, effect of heating time and dilution solvents.

3.3.1. Effect of reagent concentration

To optimize concentration of the reagents, various volumes of 2-[(E)-(4-hydroxyphenyl) diazenyl]-3-oxobutanoate and benzil solutions are added to a fixed concentration of the drug. The influence of the concentration of 2-[(*E*)-(4-hydroxyphenyl) diazenyl]-3-oxobutanoate and benzil on the absorbance of the colored complex is examined in the range 1.0×10^{-3} M to 5.5×10^{-3} M. It is found that the absorbance of colored product increases as the concentration of reagent increases. It is evident from Fig. 5 and Fig. 6 that the maximum absorbance was obtained with 2×10^{-3} M 2-[(E)-(4-hydroxyphenyl) diazenyl]-3-oxobutanoate in method A and 5×10^{-3} M for benzil in method B. Above this concentration the absorbance remain unchanged. Thus 2×10^{-3} M of 2-[(E)-(4-hydroxyphenyl) diazenyl]-3-oxobutanoate and 5×10^{-3} M of benzil solutions are used in all measurements.

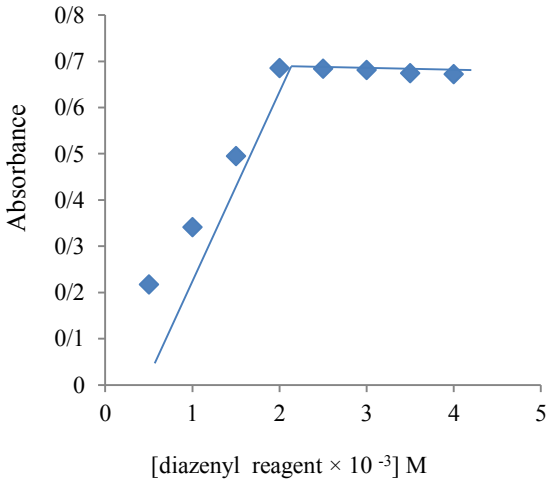


Fig. 5. Effect of reagent concentration on method A

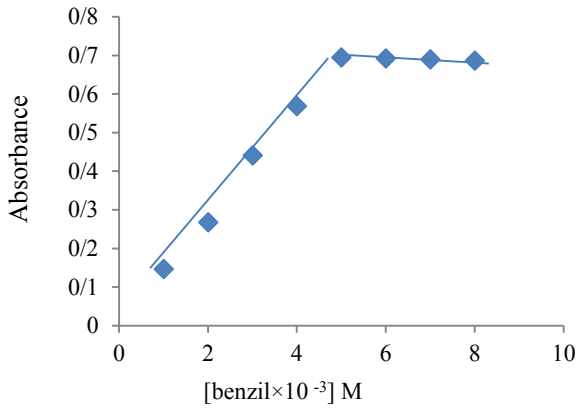


Fig. 6. Effect of reagent concentration on method B

3.3.2. Effect of heating time

The reaction between the drug and reagents are found to be slow at room temperature and required a longer time for completion. Hence, reaction is accelerated by carrying out the reactions at higher temperature. It is found that heating at 60^0C for 5 min and 100^0C for 10 min is sufficient to produce maximum color intensity for method A and method B, respectively. In both the methods produced color is stable up to 8 h.

3.3.3. Effect of solvents

Different solvents are used such as water, ethanol, methanol, acetonitrile and water but the best color is obtained using ethanol.

3.4. Validation of the methods

The method is validated with respect to linearity and range, accuracy and precision, limit of detection (LOD) and limit of quantification (LOQ), selectivity and robustness.

3.4.1. Linearity and range

A linear correlation is found between absorbance and concentration between the range of 5.00–30 µg mL^{-1} (Fig. 7) and 10.00–50.00 µg mL^{-1} (Fig. 8) with a correlation coefficient of 0.9987 and 0.9969 for the method A and method B, respectively. The analytical parameters such as molar absorptivity and Sandell's sensitivity values are calculated and given in Table 1. The table also has values for correlation coefficient, intercept, and slope. The LOD and LOQ are calculated according to ICH guidelines as LOD = 3.3 × σ/S and LOQ = 10 × σ/S, where σ is standard deviation and S is slope of calibration curve. The standard plot for adherence to Beer's law is given in Fig. 7 & Fig. 8.

3.4.2. Accuracy and Precision

The accuracy and precision of the methods are established by calculating the percentage relative error (% RE) and percentage relative standard deviation (% RSD) of the drug at different levels within working limits. Obtained results are illustrated in Table 2 and it shows a good recovery with a low RE and RSD value, which indicate the high accuracy and precision for the methods.

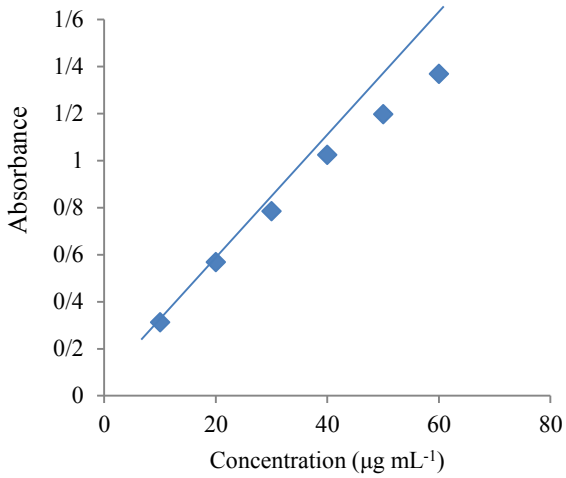


Fig. 7. Calibration curve for method A.

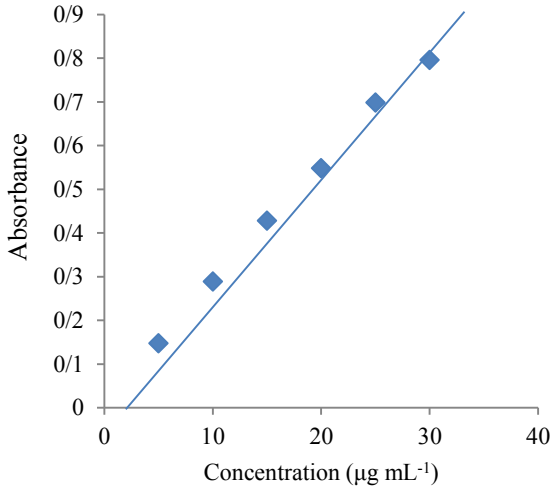


Fig. 8. Calibration curve for method B.

3.4.3. Selectivity

In the pharmaceutical analysis, it is important to test the selectivity towards the excipients and fillers added to the pharmaceutical preparations. Species that can occur in the real samples together with the drug were investigated. To investigate the effect of tablet fillers on the measurements involved in the methods, standard addition method was carried out. It is observed that excipients like starch, glucose and stearic acid did not interfere in the measurements.

3.4.4. Robustness

The robustness is the measure of reproducibility of the method which can be studied by varying the one or other reaction parameters. In the present work robustness is examined by evaluating the small variations in different experimental conditions such as heating temperatures (± 2° C), working wavelengths, volume and concentration of reagents. It is found that none of these variables had a significant effect on the determination of investigated drug.

Table 1. Spectral and Statistical data for the determination of INH

Parameters	Method A	Method B
λ_{max} nm	454	436
Beer's Law Limits (µg/ml)	5.00 – 30.00	10.00 -50.00
Molar Absorptivity (L mol^{-1} cm^{-1})	0.20 ×1 0^4	0.42 × 10^4
Sandell's Sensitivity (µg cm^{-2})	3.40 × 10^{-2}	3.20 × 10^{-2}
Limit of Detection* (µg mL^{-1})	0.6916	0.7045
Limit of Quantification * (µg mL^{-1})	2.1886	2.1343
Regression Equation**	Y= a + b X	Y= a + b X
Slope (b)	0.0262	0.0211
Intercept (a)	0.0251	0.1351
Correlation Coefficient (r)	0.9987	0.9969

* Calculated according to ICH guidelines

** Y is the absorbance and X concentration in µg mL-1Table 2 Evaluation of accuracy and precision

Table 2. Evaluation of accuracy and precision

Method A

Amount taken (µg mL^{-1})	Amount found* (µg mL^{-1})	RE (%)	SD (µg mL^{-1})	RSD (%)
5.00	4.91	1.80	0.04	0.93
10.00	9.82	1.80	0.12	1.22
15.00	14.81	1.26	0.17	1.14
20.00	19.84	0.80	0.30	1.51
25.00	24.70	1.20	0.32	1.29

Method B

Amount taken (µg mL^{-1})	Amount found* (µg mL^{-1})	RE (%)	SD (µg mL^{-1})	RSD (%)
10.00	10.04	-0.4	0.15	1.49
20.00	20.02	-0.01	0.03	0.14
30.00	29.38	2.06	0.50	1.70
40.00	39.47	1.32	0.68	1.72
50.00	49.37	1.26	0.37	0.74

* Mean value of five determinations RE - Relative Error; SD - Standard Deviation; RSD - Relative Standard Deviation.

3.5. Application to Pharmaceutical Formulations

The proposed methods have been successfully applied to the determination of INH in three different branded tablets. The content of the tablet formulations is calculated by applying suitable dilution factor. Results are given good recoveries of the drug in presence of formulation suggesting a noninterference from formulation excepients. The results for three pharmaceutical dosage forms are compared statistically with those of the tabulated value at 95% confidence level. The calculated Student's *t*-test does not exceed the tabulated value, indicating that there is no significant difference between the proposed methods and the tabulated value in respect to accuracy and precision. Results summarized in Table 3 suggest satisfactory recovery of the INH in pharmaceutical formulations. Hence, this method can be recommended for adoption in routine analysis of drug and pharmaceuticals.

Table 3. Result of assay of formulation by the proposed method

Brand name	Labeled amount (mg)	Found* ± SD using Method A	Found ± SD using Method B
Isokin®	300	300.58 ± 0.23 t = 0.15	299.85 ± 0.04 t = 0.09
Solonex®	300	299.90 ± 0.08 t = 0.09	300.06 ± 0.05 t = 0.39
Ipcazide®	100	100.04 ± 0.03 t = 0.13	100.06 ± 0.05 t = 0.15

*Mean value of five determinations

Tabulated t value at 95% confidence level is 2.

4. CONCLUSION

The proposed method is simple, rapid, inexpensive and sensitive for the determination of INH in bulk as well as in marketed form (capsules). There is no requirement of any sophisticated apparatus as in chromato-

graphic methods. Through this work we introduced novel chromogenic reagent for the spectrophotometric determination of INH. These methods have been validated in terms of its sensitivity, simplicity, reproducibility, precision, accuracy and stability of the colored species for ≥ 8 h suggesting its suitability for the routine analysis of INH in pure form and in bulk.

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