

Sensitive Spectrophotometric Assay of Isoniazid in Pharmaceuticals using Cerium(IV) and Two Acid Dyes

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Summary

Two simple, rapid, selective and sensitive spectrophotometric methods are proposed for the assay of isoniazid (INH) in pure form and in tablets. The methods are based on the oxidation of INH by a measured excess of Ce(IV) in acid medium followed by determination of surplus oxidant by reacting with a fixed quantity of methyl orange (method A) or indigo carmine (method B) and measuring the absorbance at 520 or 610 nm. In both methods, the amount of Ce(IV) reacted corresponds to the amount of INH. The experimental parameters for the assay have been carefully optimized. In these methods, the absorbance is found to increase linearly with the concentration of INH at the respective wavelengths. Beer's law is obeyed over the ranges 0.3–3.0 and 0.5–7.0 $\mu\text{g mL}^{-1}$ for method A and method B respectively and the respective molar absorptivity values are 3.71×10^4 and $3.12 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$. The limits of detection and quantification are reported for both methods. The performance of the methods was validated according to the current ICH guidelines. The repeatability and intermediate precision, expressed as the RSD was less than 2%. The accuracy of the methods expressed as relative error was satisfactory. The proposed methods were applied to the analysis of tablet form of INH and the results agreed well with the label claim. No interference was observed from the common additives normally added to tablets. The results were statistically compared with those of an official method by applying the Student's t-test and F-test. The accuracy and validity of the methods were further ascertained by performing recovery studies using spike method.

Key Words: Isoniazid, assay, spectrophotometry, pharmaceuticals, spike.

Received: 18.01.2014

Revised: 16.04.2014

Accepted: 16.04.2014

Hassas Seryum (IV) Kullanma İlaç İzoniazid Spektrofotometrik Deneyi ve İki Asit Boyalar

Özet

Basit, hızlı, duyarlı ve seçici iki spektrofotometrik yöntem, saf formda ve tabletlerde izoniazid tayini (INH) için önerilmiştir. Yöntemler, INH'nin yükseltgenmesine ve asidik ortamda fazla Ce(IV) 'ün sabit miktardaki metil turuncu (yöntem A) ve indigo carmine (yöntem B) ile reaksiyonuna ve 520 veya 610 nm'deki absorpsiyonlarının ölçümüne dayanır. Her iki yöntemde de reaksiyona giren Ce(IV) miktarı, INH miktarına karşılık gelir. Analiz için deney parametreleri optimize edilmiştir. Bu yöntemlerde, absorpsiyonların ilgili dalga boylarında INH derişimi ile doğrusal şekilde arttığı bulunmuştur. Beer yasası, yöntem A ve B için sırasıyla 0.3-3.0 ve 0.5-7.0 $\mu\text{g mL}^{-1}$ aralıklarında geçerli olup molar absorpsiyon değerleri sırasıyla 3.71×10^4 ve $3.12 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ 'dir. Gözlenebilme ve tayin sınırları her iki yöntem için de rapor edilmiştir. Yöntemlerin performansı, mevcut ICH kurallarına göre doğrulanmıştır. Tekrarlanabilirlik ve kesinlik, bağıl standart sapma (BSS) cinsinden %2'den az bulunmuştur. Bağıl hata olarak ifade edilen yöntemlerin doğruluğu tatmin edicidir. Önerilen yöntem, tablet şeklindeki INH analizinde uygulanmış ve sonuçlar etiket değerleriyle uyumlu bulunmuştur. Tabletlere ilave edilen katkı maddeleri varlığında girişim gözlenmemiştir. Sonuçlar, istatistiksel olarak Student t-testi ve F- testi uygulanarak resmi yöntem ile karşılaştırılmıştır. Yöntemlerin doğruluğu ve geçerliliği, standart ilave etme yöntemi kullanılarak geri kazanım çalışmaları yapılarak bulunmuştur.

Anahtar Kelimeler: İzoniazid, analiz, spektrofotometre, ilaç, standart ilave etme.

INTRODUCTION

Isoniazid (INH; pyridine-4-carboxylic acid hydrazide) is a bacteriostatic drug which is now widely used together with other antituberculostatic agents for the chemotherapy of tuberculosis. It also seems to be active for extra-pulmonary illness such as meningitis and genito-urinary infections (1). The determination of isoniazid in pharmaceutical preparations has appeared especially attractive. Many methods such as titrimetry (2-5), voltammetry (6-17), amperometry (18,19), ISE-based potentiometry (20-25), spectrofluorimetry (26,27), chemiluminescence spectrometry (28,29), flow injection chemiluminescence spectrometry (30-37), liquid chromatography (38-42), gas chromatography (43-45), liquid chromatography-mass spectrometry (46), capillary electrophoresis (47-51), kinetic spectrophotometry (52-54), photometric titrimetry (55) and near infra red diffuse reflectance spectrometry (56) are found in the literature. Automated methods involving chemiluminescence spectrometry (57-59) and amperometry (60) have also been reported.

Several visible spectrophotometric methods have been reported for the determination of INH in pharmaceuticals and they are based on a variety of reactions and combination of reactions and include complex formation (61-63), charge-transfer and ion pair complex formation (64), condensation (65-68), nucleophilic substitution (69), diazo coupling (70,71), oxidative coupling (72,73), derivatization (74-77) and redox followed by complexation (78-81). However, these methods have their limitations in being practical tools for the determination of INH in pharmaceuticals.

The ability of cerium (IV) to oxidize organic pharmaceuticals and to destroy coloured dyes in acid medium has successfully been employed for the sensitive determination of several pharmaceuticals using spectrophotometry (82-91). But, no attention has been paid to the determination of INH by spectrophotometry based on this reaction scheme. The present investigation aims at developing sensitive and cost-effective methods for the determination of INH using cerium (IV) as an eco-friendly oxidizing agent, and two dyes, methyl orange and indigo

carmine, as auxiliary reagents. The methods are simple, rapid, accurate, precise, robust, rugged and finely sensitive and selective.

EXPERIMENTAL

Instrument

A Systronics model 166 digital spectrophotometer (Ahmedabad, India) with matched 1-cm quartz cells was used for all absorbance measurements.

Materials

All the reagents used were of analytical reagent grade and solutions were made in distilled water. Pharmaceutical grade INH certified to be 99.85% pure was kindly provided by Cipla India Ltd; Bangalore, India and was used as received, and Isokin-300 tablets (Pfizer Ltd. Hyderabad, India) were purchased from local market.

Reagents

a) **Cerium(IV) sulphate [Ce(IV)]**: An approximately 0.01 M solution was prepared by dissolving about 0.294 g of the chemical $\text{Ce}(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}$ (Loba Chemie Pvt. Ltd., Mumbai, India) in 0.5M sulphuric acid with the aid of heat and diluting to 100 mL with the same acid, and standardized using standard ferrous ammonium sulphate solution (92). It was diluted to obtain working concentration of 100 mg mL^{-1} Ce(IV) for method A and 180 mg mL^{-1} Ce(IV) for method B with the same solvent.

b) **Methyl orange (MO)**: A standard solution equivalent to $500 \text{ } \mu\text{g mL}^{-1}$ methyl orange was prepared by dissolving 58.8 mg of methyl orange (S. D. Fine Chem., Mumbai, India, 85% dye content) in water and diluting to 100 mL in a calibrated flask; and filtered using Whatman number 42 filter paper. It was diluted to get a $50 \text{ } \mu\text{g mL}^{-1}$ dye solution for use in method A.

c) **Indigo carmine (IC)**: A standard solution containing $1000 \text{ } \mu\text{g mL}^{-1}$ indigo carmine was prepared by dissolving 107.6 mg of indigo carmine (Loba Chemie Pvt. Ltd., Mumbai, India, 93% dye content) in water and diluting to the mark in a 100 mL volumetric flask. The solution was diluted with water to get a working concentration of $200 \text{ } \mu\text{g mL}^{-1}$ solution for use in method B.

d) **Sulphuric acid (5M):** Concentrated acid (S.D. Fine Chem, Mumbai, India, sp. gr. 1.84) was appropriately diluted with water to get the required concentration.

e) **Standard drug solution:** A stock standard solution of INH ($100 \mu\text{g mL}^{-1}$) was prepared by dissolving 10 mg of pure drug in 100 mL water in a calibrated flask, and the solution was diluted to get a working concentration of $10 \mu\text{g mL}^{-1}$ and $20 \mu\text{g mL}^{-1}$ INH and used in the assay.

General procedures

Method A (using methyl orange)

Varying volumes of 0.0, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL of $10 \mu\text{g mL}^{-1}$ INH solution (equivalent to 0.3–3.0 $\mu\text{g mL}^{-1}$ INH) were transferred into a series of 10 mL volumetric flasks and the total volume was brought up to 3 mL by adding water. Added 1.0 mL each of 5M sulphuric acid and $100 \mu\text{g mL}^{-1}$ Ce(IV) and mixed well. After a standing time of 10 min, 1.0 mL of $50 \mu\text{g mL}^{-1}$ MO was accurately added, content mixed and diluted up to mark with water and after 5 min, the absorbance was measured at 520 nm against reagent blank.

Method B (using indigocarmine)

Different aliquots (0.25–3.5 mL) of $20 \mu\text{g mL}^{-1}$ INH solution was accurately transferred in to 10 mL volumetric flasks using micro burette and diluted with distilled water to a volume of 3.5 mL. Then, 1.0 mL each of 5M sulphuric acid and $180 \mu\text{g mL}^{-1}$ Ce(IV) were added to each flask. The flasks were shaken and let stand for 5 minutes to ensure a complete oxidation of the drug. Subsequently, 1.0 mL of 200 ppm indigocarmine solution was added to each flask and diluted to the mark with water and mixed. The absorbance of each solution was then measured at the wavelength corresponding to the absorption maximum, i.e. $\lambda_{\text{max}} = 610 \text{ nm}$ Vs blank after five min.

Standard graph was prepared by plotting the absorbance *versus* INH concentration, and the concentration of the unknown was computed from the respective regression equation derived using the absorbance-concentration data.

Procedure for tablets

Twenty tablets were weighed and ground into a fine powder. An accurately weighed quantity containing 10 mg of INH was transferred to a 100 mL volumetric flask, 60 mL water added, shaken well for 20 minutes and made up to mark with water, then filtered. This solution was diluted to 10 and $20 \mu\text{g mL}^{-1}$ INH concentrations with water and analyzed by the recommended procedures.

Procedure for placebo blank and synthetic mixture analyses

A placebo blank containing starch (10 mg), acacia (15 mg), hydroxyl cellulose (10 mg), sodium citrate (10 mg), talc (20 mg), magnesium stearate (15 mg) and sodium alginate (10 mg) was prepared by mixing all components into a homogeneous mixture. A 5 mg of the placebo blank was accurately weighed and its solution was prepared as described under 'tablets', and then subjected to analysis by following the general procedures.

A synthetic mixture was prepared by adding about 10 mg of pure INH to 10 mg of the above mentioned placebo blank and the mixture was homogenized. Following the same procedure described under 'procedure for tablets' the synthetic mixture solution was prepared and a suitable aliquots were subjected for analysis by both methods following the general recommended procedures.

RESULTS AND DISCUSSION

The proposed spectrophotometric methods are indirect based on the ability of Ce(IV) to oxidize INH and to bleach the color of the dye methyl orange in method A or indigocarmine in method B (90, 91). INH is let to react with a known excess of Ce(IV) in acid medium. The unreacted Ce(IV) found in excess over INH is quantified by adding methyl orange or indigocarmine as in Fig. 1 and monitoring the absorbance of the solution at 520 nm or 610 nm as shown in Fig. 2.

In either method, the absorbance increased linearly with increasing concentration. INH, when added in increasing amounts to a fixed amount of Ce(IV), consumed the latter and there occurred a concomitant

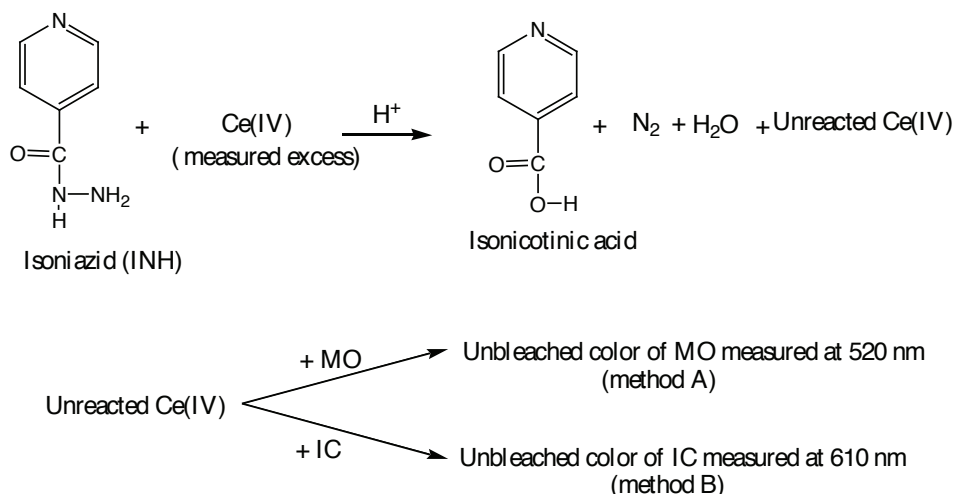


Figure 1. The tentative reaction scheme.

fall in its concentration. When fixed amount of either dye was added to decreasing amounts of oxidant, a concomitant increase in the concentration of dye resulted. This was observed as a proportional increase in absorbance at the respective λ_{max} with increasing concentration of INH (Fig. 3 and 4).

Method development

Spectral characteristics

The orange-red color of the unreacted MO in acid medium absorbed maximally at 520 nm (method A) and blue color of unreacted IC in acid medium peaked at 610 nm (method B). The absorption spectra of both the methods are presented in Fig. 2.

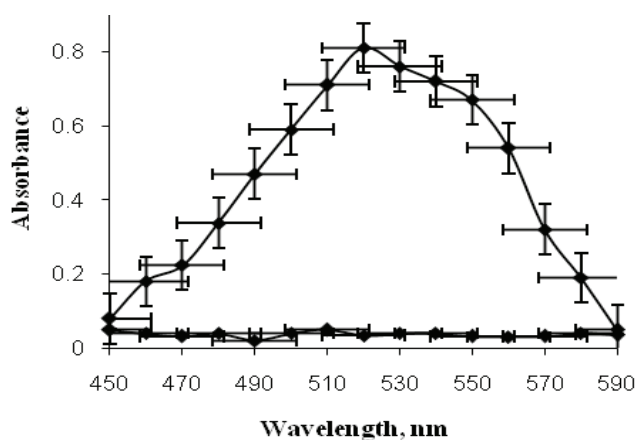
Optimization of experimental variables

a) Selection of dye concentration

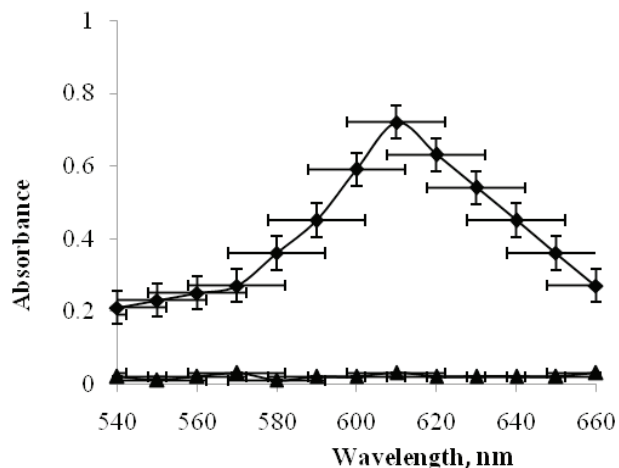
Preliminary experiments were performed to fix the upper limits of the MO and IC and they were found to be 5 and 20 $\mu\text{g mL}^{-1}$ for MO and IC, respectively, and hence 1 mL each of 50 and 200 $\mu\text{g mL}^{-1}$ MO and IC were used in the investigation.

b) Study of Ce(IV) concentration

A Ce(IV) concentration of 10.0 $\mu\text{g mL}^{-1}$ was found to destroy the red colour due to 5 $\mu\text{g mL}^{-1}$ methyl orange whereas in the case of 20 $\mu\text{g mL}^{-1}$ indigo carmine, 18.0 $\mu\text{g mL}^{-1}$ Ce(IV) was sufficient to bleach the blue colour in acid conditions. Hence, different amounts of INH were reacted with 1.0 mL of 100 μg



(a) MO-method A (3.0 $\mu\text{g mL}^{-1}$ INH)



(b) IC- method B (6.0 $\mu\text{g mL}^{-1}$ INH)

Figure 2. The Absorption spectra for method A and method B

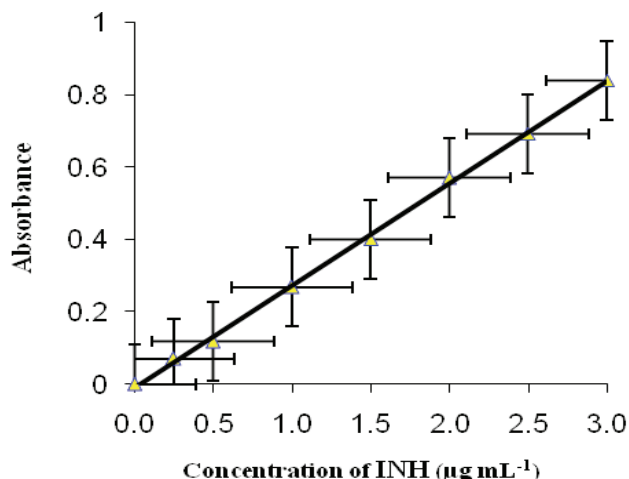


Figure 3. Calibration plot for method A

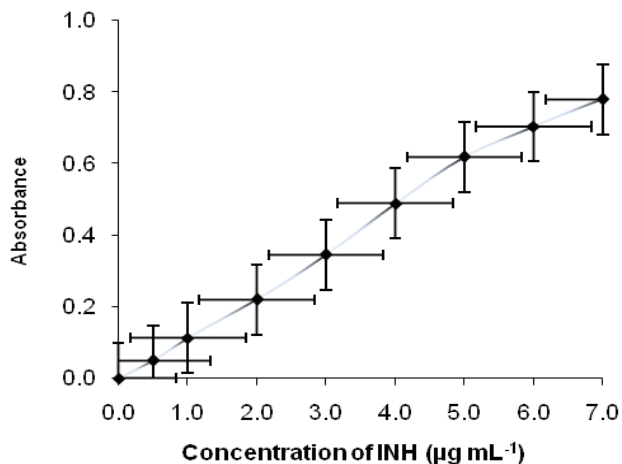


Figure 4. Calibration curve for method B

mL⁻¹ oxidant in method A and 1.0 ml of 180 µg mL⁻¹ oxidant in method B before determining the residual Ce(IV) as described under the respective procedures.

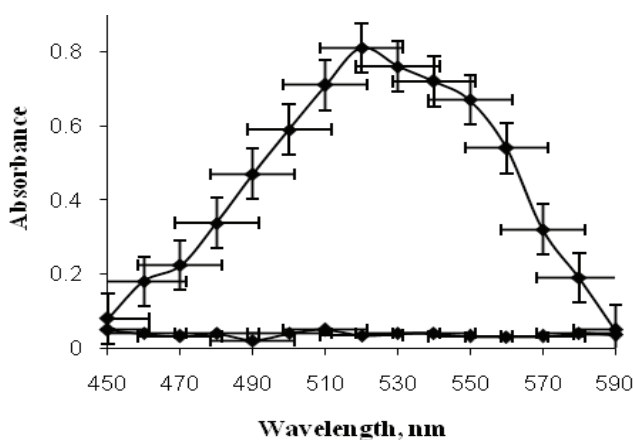
c) Selection of reaction medium

The reaction between INH and Ce(IV) was performed in different acid media *viz.*, sulphuric acid, hydrochloric acid, nitric acid and perchloric acid. Sulphuric acid was found to be the ideal medium for the oxidation of INH by Ce(IV) as well as the latter's determination employing either dye. The effect of acid concentration on the reaction between INH and Ce(IV) was studied by varying the concentration of H₂SO₄ keeping the concentrations of Ce(IV) and

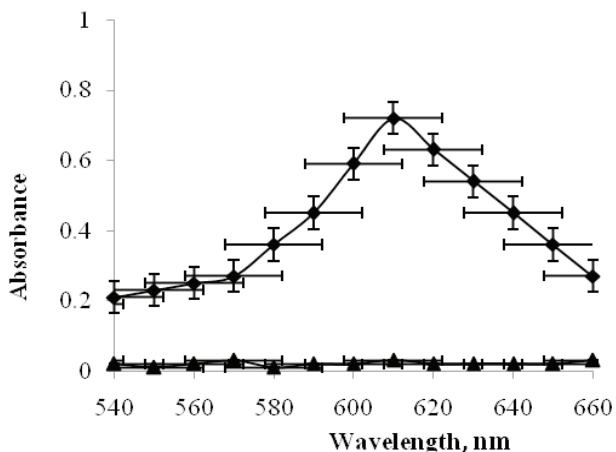
drug fixed. Higher the acid concentrations showed lower sensitivity, hence 1.0 mL of 5M H₂SO₄ in a total volume of 10 mL was found optimal.

d) Study of reaction time and stability

Under the optimized experimental conditions, for a quantitative reaction between INH and Ce(IV), contact time of 5 min was found necessary in both methods at room temperature. After addition of dye, the reaction between Ce(IV) and dye was instantaneous (bleaching) and absorbance of the unbleached dye was stable at least 12 hour in method A and 30 min in method B (Given in Fig. 5).



(a) Reaction stability for method A (3.0 µg mL⁻¹ INH)



(b) Reaction stability for method B (4.0 µg mL⁻¹ INH)

Figure 5. The reaction time and stability for method A and method B

VALIDATION STUDIES

The methods were validated according to the procedures described in ICH guidelines for the validation of analytical methods. The limits of detection (LOD) and quantitation (LOQ) were calculated according to the ICH guidelines [93] using the formulae:

$$\text{LOD} = 3.3 S/b \text{ and } \text{LOQ} = 10 S/b$$

where S is the standard deviation of blank absorbance values, and b is the slope of the calibration plot.

A linear correlation was found between absorbance at λ_{max} and concentration of INH in the ranges given in Table 1. Regression analysis of the Beer's law data using the method of least squares was made to evaluate the slope (b), intercept (a) and correlation coefficient (r) for each system and the analytical results obtained from this investigations

are presented in Table 1. The optical characteristics such as Beer's law limits, molar absorptivity and Sandell sensitivity values (93) of both the methods are also given in Table 1. The high values of ϵ and low values of Sandell sensitivity and LOD indicate the high sensitivity of the proposed methods.

a) Intra-day and inter-day precision and accuracy

The precision and accuracy of the proposed methods were studied by repeating the experiment seven times within the day to determine the repeatability (intra-day precision) and five times on different days to determine the intermediate precision (inter-day precision). The assay was performed for three levels of analyte in each method. The results of this study are summarized in Table 2. The percentage relative standard deviation (%RSD) values were in the range 0.89-1.98% indicating good precision of the methods. Accuracy was evaluated as percentage relative error (%RE) between the measured mean concentrations

Table 1. Sensitivity and regression parameters

Parameter	Method A	Method B
λ_{max} , nm	520	610
Color stability	12 hr	30 min
Linear range, $\mu\text{g mL}^{-1}$	0.3–3.0	0.5–7.0
Molar absorptivity (ϵ), $\text{L mol}^{-1} \text{cm}^{-1}$	3.71×10^4	1.56×10^4
Sandell sensitivity*, $\mu\text{g cm}^{-2}$	0.0037	0.0088
Limit of detection (LOD), mg mL^{-1}	0.08	0.03
Limit of quantification (LOQ), mg mL^{-1}	0.24	0.09
Regression equation, Y**		
Intercept (a)	0.0138	-0.0017
Slope (b)	0.284	0.117
Standard deviation of a (S_a)	9.98×10^{-3}	9.92×10^{-4}
Standard deviation of b (S_b)	3.98×10^{-2}	2.03×10^{-2}
Regression coefficient (r)	0.9993	0.9968

* Limit of determination as the weight in $\mu\text{g mL}^{-1}$ of solution, which corresponds to an absorbance of $A = 0.001$ measured in a cuvette of cross-sectional area 1 cm^2 and $l = 1 \text{ cm}$.

** $Y = a + bX$, Where Y is the absorbance, X is concentration in $\mu\text{g mL}^{-1}$, a is intercept and b is slope.

and taken concentrations of INH, and it was $\leq 3\%$ demonstrating the high accuracy of the proposed methods. Bias {bias% = [(Concentration found – known concentration) \times 100 / known concentration]} was calculated at each concentration and these results are also presented in Table 2 in terms of % RE.

b) Selectivity of the proposed methods

In the analysis of placebo blank solution the absorbance in each case was equal to the absorbance of blank which revealed no interference. To assess the role of the inactive ingredients on the assay of INH, the general procedure was applied on the synthetic mixture extract by taking three different concentrations of INH. The percentage recovery values were in the range 96.3–102.7% with RSD $< 3\%$ indicating clearly the non-interference from the inactive ingredients in the assay of INH.

c) Robustness and ruggedness

Robustness of the methods was evaluated by slightly varying two important experimental variables, viz., the amount of acid and reaction time, were, and the capacity of the methods was found to remain unaffected by small deliberate variations. The results of this study are presented in Table 3 and indicate that the proposed methods are robust. Method ruggedness is expressed as %RSD of the same procedure applied by three analysts and using three different spectrophotometers by the same analyst. The inter-analysts' and inter-instruments' RSD values were $\leq 3.12\%$ indicating ruggedness of the proposed methods. The results of this study are presented in Table 3.

d) Application to tablets

The proposed methods were applied to the quantification of INH in commercial tablets. The

Table 2. Evaluation of intra-day and inter-day accuracy and precision

Method	INH taken ($\mu\text{g mL}^{-1}$)	Intra-day accuracy and precision (n=7)			Inter-day accuracy and precision (n=5)		
		INH Found ^a ($\mu\text{g mL}^{-1}$)	RSD ^b %	RE ^c %	INH found ($\mu\text{g mL}^{-1}$)	RSD ^b %	RE ^c %
A	1.5	1.47	1.32	2.04	1.46	1.49	2.67
	2.0	2.04	1.65	1.96	2.06	1.13	3.00
	2.5	2.44	1.98	2.40	2.55	1.24	2.00
B	2.0	2.04	1.36	2.00	2.05	1.32	2.50
	4.0	4.09	0.92	2.25	4.11	0.89	2.75
	6.0	5.92	1.45	1.34	6.10	1.92	1.67

^a Mean value of 7 determinations; ^b Relative standard deviation (%); ^c Relative error (%).

Table 3. Method robustness and ruggedness expressed as intermediate precision

Method	Robustness Ruggedness				
	Nominal concentration	Reaction times* (n=3)	Volume of acid [#]	Inter- analysts (n=3)	Inter- instruments (n=3)
A	1.5	2.69	2.16	1.09	2.52
	2.0	3.12	2.54	1.18	2.24
	2.5	2.92	2.62	0.96	1.87
B	2.0	1.74	1.02	0.78	2.13
	4.0	2.12	1.24	1.11	2.72
	6.0	2.18	1.95	1.07	2.95

* Reaction time was 5 ± 1 min, [#] Volume of H_2SO_4 , 1 ± 0.1 mL

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

Tablets analyzed	Label claim (mg/tablet)	Found* (Percent of label claim \pm SD)		
		Official method	Proposed methods	
			Method A	Method B
Iskin-300	300	103.5 \pm 1.25	102.5 \pm 1.36 $t = 1.21$ $F = 1.18$	101.3 \pm 1.45 $t = 2.58$ $F = 1.35$

* 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.

tablets were assayed by the official BP method [94] which describes a titration of the drug with potassium bromate in presence of potassium bromide using methyl red indicator. The results obtained by the proposed methods agree well with the claim and also are in agreement with those by the official method. Statistical analysis of the results did not detect any significant difference between the performance of the proposed methods and reference method with respect to accuracy and precision as revealed by the Student's t -value and variance ratio F -value. The results of assay are given in Table 4.

e) Recovery studies

To further assess the accuracy of the methods, recovery experiments were performed by applying the standard-addition technique. The recovery was assessed by determining the agreement between the measured standard concentration and added known

concentration to the sample. The test was done by spiking the pre-analyzed tablet INH with pure INH at three different levels (50, 100 and 150% of the content present in the preparation and the total was found by the proposed methods. Each test was repeated three times. In both the cases, the recovery percentage values ranged between 98.7 and 102.6% with standard deviation in the range 1.05-1.97%. Closeness of the results to 100% showed the fairly good accuracy of the methods. The results are shown in Table 5.

CONCLUSIONS

In the present work two simple, sensitive, precise, robust and accurate spectrophotometric methods are described. The methods have been applied successfully to the determination of INH in tablets without interferences from the common additives. The procedures use inexpensive reagents with excellent shelf life, and are available in any analytical

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

Method	Tablets studied	INH in tablet $\mu\text{g mL}^{-1}$	Pure INH added $\mu\text{g mL}^{-1}$	Total found $\mu\text{g mL}^{-1}$	Pure INH recovered* Percent \pm SD
A	Isokin-300	1.02	0.5	1.50	98.7 \pm 1.05
		1.02	1.0	2.00	99.2 \pm 1.29
		1.02	1.5	2.55	101.3 \pm 1.07
B	Isokin-300	2.03	1.0	3.08	101.5 \pm 1.17
		2.03	2.0	4.13	102.6 \pm 1.97
		2.03	3.0	5.12	101.9 \pm 1.25

* Mean value of three determinations.

laboratory. The proposed methods are of great values in quality control determination of isoniazid because of its adequate accuracy, reliability, low cost, and also because the instruments used were inexpensive and non-sophisticated, critical reagents are not required. Even though both methods found better, method A is superior to method B due to the high stability and sensitivity, and low value of Sandell sensitivity.

Acknowledgement

The authors gratefully acknowledge the receipt of pure isoniazid as a gift from quality control manager, Cipla Ltd., Bangalore, India and the authorities of the University of Mysore, Mysore, for permission and research facilities. Prof. K. Basavaiah thanks UGC, New Delhi, for the award of UGC-BSR faculty fellowship (BFF). We do not have any conflict of interest with the commercial identities mentioned in this article.

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