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Spectrophotometric determination of isoxsuprine hydrochloride using 3-methyl-2-benzothiazolinone hydrazone hydrochloride in spiked human urine and pharmaceuticals

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ABSTRACT

Two selective and sensitive spectrophotometric methods are proposed for the determination of isoxsuprine hydrochloride (ISX) in spiked human urine and in pharmaceuticals. The methods are based on the oxidative-coupling reaction between 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) and ISX in the presence of $Ce(SO_4)_2$. The novelty of the proposed reaction is the formation of two different colored chromogens at two different pHs. The resulting product at pH < 1.5 is a red colored chromogen peaking at 500 nm (method A) and that formed between the pH 3.85 and 4.15, is violet colored with an absorption maximum at 580 nm (method B). In both the methods, absorbance of the chromogen is found to increase linearly with the concentration of ISX as is corroborated by the correlation coefficients of 0.9989 and 0.9970, and the systems obey Beer's law over the ranges of 1.4–21.0 and 1.0–15.0 μg ml^{-1}, for method A and method B, respectively. The calculated molar absorptivities are 1.08×10^4 and 1.78×10^4 l mol^{-1} cm^{-1} for method A and method B, respectively with corresponding Sandell sensitivity values of 0.0311 and 0.0190 μg cm^{-2}. The reaction stoichiometry, in both the methods, was evaluated by the limiting logarithmic method and was found to be 1:1 (ISX:MBTH). The methods were successfully applied to the determination of ISX in spiked human urine and pharmaceutical formulation.

1. Introduction

Isoxsuprine hydrochloride (ISX) is chemically known as 4-hydroxy-α-[1-[(1-methyl-2-phenoxyethyl) amino] ethyl] benzenemethanol hydrochloride (Scheme 1). It is used in the treatment of cerebral and peripheral vascular disease, and to arrest premature labor [1]. The official method [2] recommends UV-spectrophotometric measurement of aqueous solution of ISX at about 300 nm, while the British Pharmacopoeia [3] recommends a visual non-aqueous titration with $HClO_4$ as titrant and 1-naptholbenzein as the indicator.

Ultra-violet spectrophotometry [4,5], fluorimetry [6], chemiluminescence spectrometry [7], ion-selective electrode-based potentiometry [8], polarography [9], high-performance liquid chromatography (HPLC) [10–12], gas chromatography (GC) [13], liquid chromatography–mass spectrophotometry [14], gas chromatography–mass spectrophotometry [15] and affinity chromatography [16] have been employed for determining ISX in pharmaceutical dosage forms. Many of these techniques are deficient in simplicity, cost-effectiveness and easy access.

The most widely used technique for the assay of ISX in pharmaceuticals has been visible spectrophotometry and methods based on such diverse color reactions as redox [17–19], redox followed by chelation [20–23], condensation [24–26], oxidative-condensation [27,28], oxidative-coupling [29,30], diazotization and coupling [31–35], ion-pair complexation [36–39], nitration followed by Meisenheimer complexation [40], nitrosation [41], nitrosation followed by chelation [41,42] and derivatization [43,44]. Determination of ISX by kinetic spectrophotometry based on redox [45], derivatization [46] and oxidative-condensation [47] reactions has also been reported by several workers. However, many of the above methods suffer from one or the other disadvantage like poor sensitivity, poor selectivity, narrow linear dynamic range, longer contact time, rigid experimental conditions, multi-step reaction, heating or cooling step, liquid–liquid extraction step, use of organic solvent or expensive chemical and/or complicated experimental setup as can be seen from the performance characteristics of the published methods complied in Table 1.

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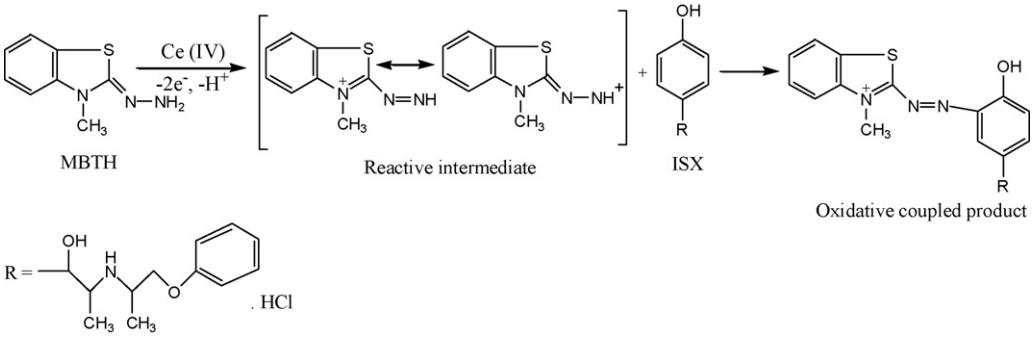

Scheme 1. Probable reaction scheme.

With the growing number of analysts proposing visible spectrophotometric methods for the determination of compounds of pharmaceutical importance and lack of access to published works could lead to unnecessary duplication of the reactions. Since 1980, out of 31 published articles dealing with the spectrophotometric assay of ISX, 23 are in Internet inaccessible journals. Hence, we have compiled a comprehensive methodology which is followed in all proposed methods in the form of a table (Table 1).

In this work, the well-known oxidative-coupling reaction using cerium (IV) and 3-methyl-2-benzothiazolinone hydrazone hydrochloride has been extended to the assay of ISX. Though the reaction was earlier used by Anon [30] for the determination of ISX in pharmaceuticals, the novelty of the present methods is the formation of two different colored chromogens of the same product at different pHs, which exhibit distinct λ_{max} at 500 and 580 nm. Change in the pH also resulted in the different quantification ranges of ISX with different molar absorptivity values. Further, the proposed chemo selective methods have many advantages over the above mentioned physico-methods [10–16] in terms of simplicity, cost-effectiveness and freedom from cumbersome instrumentations.

2. Experimental

2.1. Apparatus

A Systronics model 106 digital spectrophotometer provided with 1-cm matched quartz cells were used for absorbance measurements. All chemicals used in the experiment were of analytical reagent grade and distilled water was used to prepare the solutions.

2.2. Reagents

A stock solution of 0.1% MBTH (Alfa aeasar, USA) was prepared in water for method B and further diluted with water to get 0.05% for method A. A 0.2% $Ce(SO_4)_2$ (Loba chemie, Mumbai, India) solution was prepared in 0.5 M sulfuric acid. Ethylenediamine tetraaceticacid disodium salt (5×10^{-3} M EDTA, Merck, Mumbai, India) was prepared by dissolving 0.093 g of the salt in 50 ml of water. Sodium acetate (2 M, Merck, Mumbai, India) and potassium hydrogen phthalate (0.05 M, Loba Chemie, India) were prepared separately in water. Sulfuric acid (Merck, India, Sp. gr. 1.84) and hydrochloric acid (Merck, Mumbai, India; Sp. gr. 1.18) were diluted appropriately with water. Carbonate–bicarbonate buffer of pH 9.4 was prepared by dissolving 1.325 g of Na_2CO_3 (Merck, Mumbai, India) and 1.050 g of $NaHCO_3$ (Merck, Mumbai, India) in a 25 ml standard flask with water. A stock standard solution of 500 $\mu g\,ml^{-1}$ ISX was prepared by dissolving 50 mg of pure ISX (Juggat Pharma, India) in water, and made up to volume in a 100 ml calibrated flask with the same solvent. It was further diluted to get working concentrations of 70 and 50 $\mu g\,ml^{-1}$ ISX with the same solvent for method A and method B, respectively. The aqueous solution of ISX is reported to remain stable for 323 days [48].

2.3. Procedures

2.3.1. Method A

Different aliquots of a standard ISX solution (0.20–3.00 ml, 70 $\mu g\,ml^{-1}$) were transferred into a series of 10 ml calibrated flasks using micro burette and the total volume was adjusted to 3.0 ml with water. Two millilitres of 0.05% solution of MBTH was added to each flask followed by 2 ml of 0.2% solution of $Ce(SO_4)_2$ and diluted to volume with water. The absorbance of the resulting color was measured against the reagent blank at 500 nm.

2.3.2. Method B

Into a series of 10 ml calibrated flasks, 0.20–3.00 ml of 50 $\mu g\,ml^{-1}$ of a standard ISX solution were added using micro burette and the total volume was made up to 3.0 ml with water. To each flask was added 1.5 ml of 0.1% solution of MBTH and 2 ml of 0.2% $Ce(SO_4)_2$ followed by 1 ml of 5×10^{-3} M EDTA and 1.5 ml of 2 M sodium acetate. Finally, the mixture was diluted to the volume with 0.05 M potassium hydrogen phthalate and the absorbance of the resulting color was measured against the reagent blank at 580 nm.

2.3.3. Procedure for spiked human urine

Five millilitres of ISX free urine taken in a 125 ml separating funnel was spiked with 10 ml of aqueous solution containing 2.5 mg of pure ISX and to the same solution, 5 ml of carbonate–bicarbonate buffer of pH 9.48 was added followed by 20 ml of ethylacetate. The content was shaken for 15 min. The lower aqueous layer was discarded and the upper organic layer was collected in a beaker containing anhydrous sodium sulphate. The water-free organic layer was transferred into a dried beaker and evaporated on a hot water bath. The dry residue was reconstituted with 2 ml of 1 M HCl and transferred into a 25 ml calibrated flask, and diluted to the mark with water. An aliquot of resulting solution was analyzed following the procedures described above.

2.3.4. Procedure for tablets

Twenty tablets containing ISX were weighed and ground into a fine powder. An amount of powder equivalent to 10 mg of ISX was weighed into a 100 ml calibrated flask, 40 ml of water added and the mixture was shaken for 20 min; then the volume was made up to the mark with the same solvent, mixed well and filtered using Whatman No. 42 filter paper. The first 10 ml portion of the filtrate was discarded in order to avoid small dilution in the concentration of ISX because of the wetted filter paper. The resulting (100 $\mu g\,ml^{-1}$) ISX solution was diluted appropriately with water to get the working concentrations (50 and 70 $\mu g\,ml^{-1}$ ISX) and subjected to analysis following the procedures described earlier.

Table 1
Comparison of the performance characteristics of the proposed methods with the existing methods for isoxsuprine hydrochloride.

S. no.	Reagent(s)	λ_{max} (nm)	Range ($\mu g\ ml^{-1}$)	ε ($l\ mol^{-1}\ cm^{-1}$)	LOD ($\mu g\ ml^{-1}$)	Remarks	Reference
1	Folin–Ciocalteu reagent	650	–	–	–	Not selective	[17]
2	Molybdophosphoric acid	680	20.0–120.0	–	–	Less selective, less sensitive	[18]
3	Sodium cobaltinitrite	–	–	–	–	Requires boiling for 20 min	[19]
4	$FeCl_3$ and 1,10 phenanthroline	510	1–10	2.47×10^4	–	Involves boiling for 15 min	[20]
5	$FeCl_3$ and 1,10 phenanthroline	490	1–10	5.07×10^4	–	Involves boiling for 15 min	[21]
6	$FeCl_3$ and 2,2′ bipyridine	520	20.0–100.0	1.0×10^3	–	Involves boiling for 15 min, less sensitive, narrow linear range	[22]
7	$FeCl_3$ and 2,4,6-tri-2-pyridyl-1,3,5-triazine	595	0.4–2.0	–	–	Involves boiling for 15 min, narrow linear range	[23]
8	p-Aminophenol	635	10–110	–	–	35 min reaction time, less sensitive	[24]
9	4-Aminophenol	–	10.0–120.0	–	–	60 min contact time, use of organic solvent, less sensitive	[25]
10	4-Aminophenazone	502	3.0–24.0	–	–	Narrow linear range	[26]
11	4-Amino-antipyrine and potassium hexacyanoferrate	510	1–18	1.20×10^4	0.071	Critical dependence on 4-amino-antipyrine concentration	[27]
12	4-Amino-antipyrine and potassium hexacyanoferrate	507	1–60	–	0.30	Requires automated flow injection analysis assembly	[28]
13	N,N-Dimethyl-p-phenylenediamine dihydrochloride	620	10.0–40.0	–	–	Involves extraction step, less sensitive, narrow linear dynamic range, use of organic solvent	[29]
14	3-Methyl-2-benzothiazolinone hydrazone hydrochloride and $(NH_4)_2SO_4.Ce(SO_4)_2$	510	1.0–15.0	–	–	Strict control of acid concentration	[30]
15	p-Aminoethyl benzoate	460	1–12	2.17×10^4	–	Use of organic solvents, mixture of concentrated acids used for diazotization	[31]
16	Sulfanilic acid and $NaNO_2$	440	0.8–8.0	–	–	Measurement at shorter wavelength	[32]
17	Sulfanilic acid and $NaNO_2$	440	1–20	9.6×10^3	–	Measurement at shorter wavelength	[33]
18	4-Nitroaniline	480	1.0–10.0	–	–	Diazotization carried out at ~0 °C	[34]
19	Sulfanilic acid and $NaNO_2$	440	1.0–20.0	–	–	Measurement at shorter wavelength	[35]
20	Bromophenol blue, bromocresol purple, bromocresol green, bromothymol blue and methyl orange	420	–	–	–	Judicious pH control required, involve extraction step with organic solvent	[36]
21	Neutral red	517	10.0–50.0	–	–	Less sensitive, narrow linear dynamic range, involve extraction step with organic solvent	[37]
22	Fast green	630	0.4–5.0	–	–	Involves strict pH control, involve extraction, use of organic solvent	[38]
	Orange II	495	1.0–20.0				
23	Metanil yellow	407	2.0–12.0	–	–	Strict pH control required, involves extraction step, use of organic solvent	[39]
24	$NaNO_2$ and acetone	385	4.8–16.0	–	–	Measurement at shorter wavelength, uses HNO_3–H_2SO_4 mixture, requires boiling for 20 min, less sensitive, narrow linear dynamic range	[40]
25	$NaNO_2$	405	10.0–50.0	–	–	Involve heating and extraction into organic solvent, less sensitive, narrow linear range, measurement at shorter wavelength	[41]
	Copper acetate	525	20.0–70.0				
	$CoCl_2$	356	16.0–80.0				
26	$NaNO_2$ and copper acetate	525	8.0–96.0	–	–	Requires boiling for 25 min, less sensitive	[42]
27	3,5-Dichloro-p-benzoquinonechlorimine	610	2.5–20.0	–	–	20 min reaction time, use of organic solvent, strict pH control and expensive reagent	[43]
28	2,6-Dichloroquinone chlorimide	610	1.2–16.8	–	–	Uses of expensive reagent	[44]
29	$KMnO_4$	610 and 525	0.5–4.0	–	0.05	Kinetic studies requiring judicious control of experimental variables	[45]
30	4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole		2–20	–	0.6	Kinetic studies requiring judicious control of experimental variables	[46]
31	1-Nitro-2-napthol and Ce (IV) or Pb (IV).	540 and 510	33.78–270.27	–	–	Kinetic studies, involves flow injection set up, rigorous control of experimental variables, less sensitive	[47]
32	Oxidative-coupling reaction with MBTH in the presence of $Ce(SO_4)_2$ at					Non-rigid experimental conditions, selective and sensitive, wide linear dynamic range, free from heating and extraction step, no use of organic solvent	Proposed methods
	(a) pH below 1.5, λ_{max} = 500 nm		1.4–21.0	1.08×10^4	0.17		
	(b) pH between 3.85 and 4.15, λ_{max} = 580 nm		1.0–15.0	1.78×10^4	0.05		

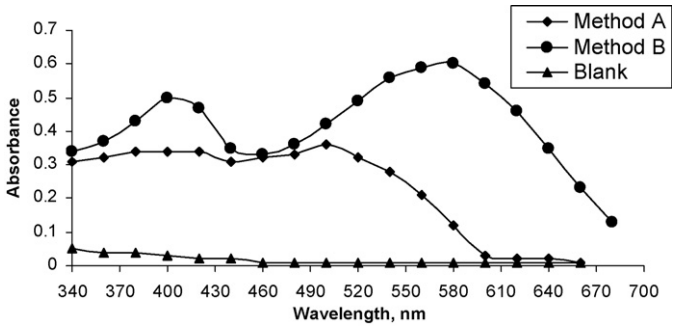

Fig. 1. Absorption spectra for method A (10.5 μg ml^{-1} ISX), method B (12.5 μg ml^{-1} ISX) and their common blank.

3. Results and discussion

3.1. Chemistry

Since the discovery of azo dye obtainable by the oxidative-coupling of MBTH with phenols by Hunig et al. [49], its application in the quantitative determination of phenol and substituted phenols has been used to the greatest advantage. The chemistry behind this reaction is the oxidation of MBTH (loss of two electrons) to a desirable reactive coupling species that attaches itself to either at ortho or para position relative to the phenolic OH group resulting in the formation of intensely colored oxidative-coupling product (Fig. 1).

As ISX possesses para substituted phenolic group, the suitability of MBTH in combination with different oxidants like ferric chloride, potassium ferricyanide and Ce $(SO_4)_2$ was examined and it was found that only $Ce(SO_4)_2$ gave a positive reaction. The oxidative-coupling reaction was carried out at pH < 1.5 resulting in the formation of red color chromogen peaking at 500 nm (Fig. 1, method A). When the pH of the same red colored coupled product was raised to 3.85–4.15, a bathochromic shift to 580 nm was observed due to the formation of violet colored product (Fig. 1, method B). We can also see the formation of orange colored oxidative coupled product at the same time (peaking at 400 nm; Fig. 1, method A and method B), which is masked by red and violet colored chromogens. The possibility of absorption due to the yellow colored $Ce(SO_4)_2$ at 400 nm was ruled out as inferred from the absorption spectra of subsequent blank (Fig. 1, blank). The orange colored oxidative coupled product at 400 nm is of less analytical importance as far as sensitivity as well as stability of the product is concerned. The formation of all the three colored coupled products is contrary to that of the more general observation by Gasparic et al. [50], that the color obtained with p-unsubstituted phenols with MBTH in the presence of oxidant was usually orange to red and p-alkyl substituted phenol derivatives were violet. Since ISX possesses para substituted phenolic group, the oxidative-coupling reaction with MBTH occurs only at ortho position.

3.1.1. Reaction stoichiometry

Though both the ortho positions are vacant, the substitution occurs only at one position as confirmed by 1:1 (ISX:MBTH) reaction stoichiometry in both the methods. The stoichiometry of the reaction was studied adopting the limiting logarithmic method [51]. Two straight lines were obtained upon using increasing concentrations of MBTH while keeping the concentration of ISX constant (Fig. 2a) and also upon using increasing concentrations of ISX while keeping the concentration of the MBTH constant (Fig. 2b). The slopes of the two lines are 1.09 and 1.02 for method A; 1.10 and 0.98 for method B. This means that the reaction proceeds in a molar ratio of 1.09:1.02 for method A and 1.10:0.98 for method B, i.e. in a ratio of ≈1:1 in both the methods. Hence, based on 1:1 (ISX:MBTH) reaction stoichiometry, a probable reaction scheme has been proposed (Reaction Scheme 1).

3.1.2. Ce (III)–EDTA complex

However, the difficulty in method B was precipitation of cerium when the pH was raised above 1.0. The cause of the precipitation was perceived as formation of cerium(IV) hydroxide by Friestad et al. [52]. The present authors would rather suggest the formation

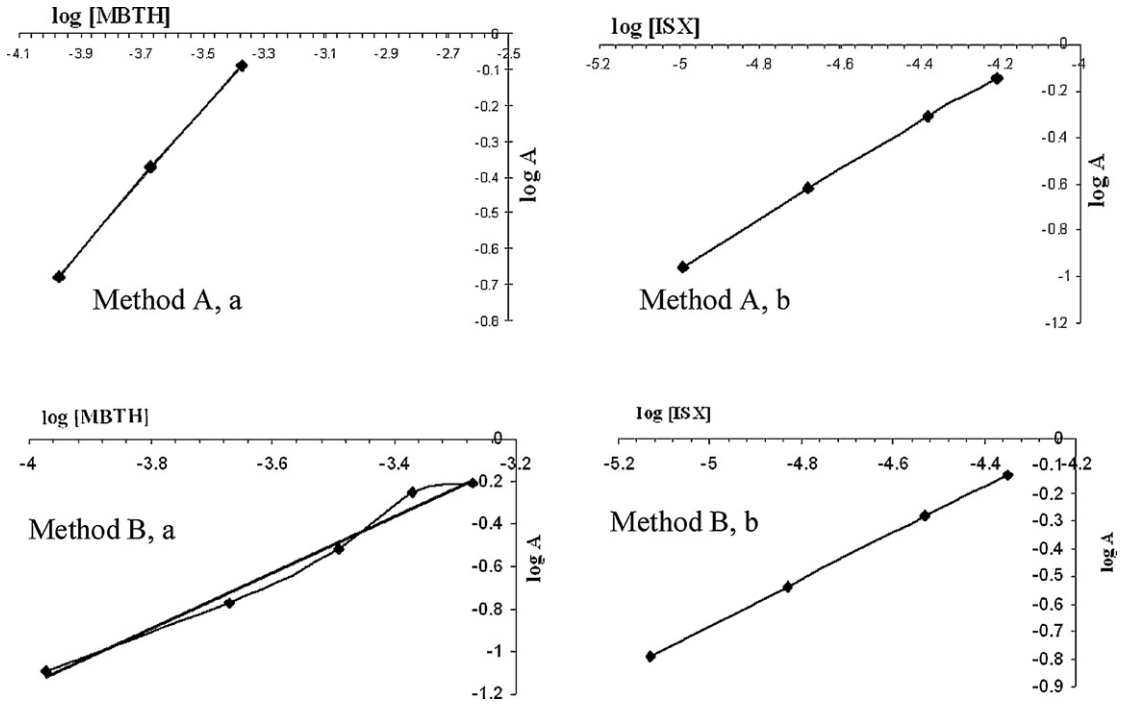

Fig. 2. (a) Limiting logarithmic plots for the molar reactivity of MBTH with ISX: log absorbance vs. log [MBTH] at which [ISX] kept constant. (b) Log absorbance vs. log [ISX] at which [MBTH] kept constant.

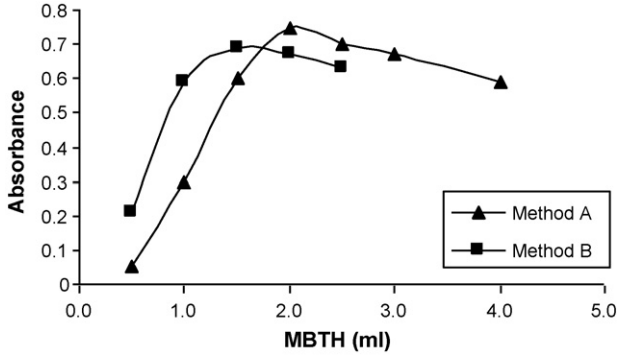

Fig. 3. Effect of 0.05% MBTH in method A (21.0 μg ml^{-1} ISX) and effect of 0.1% MBTH in method B (12.5 μg ml^{-1} ISX).

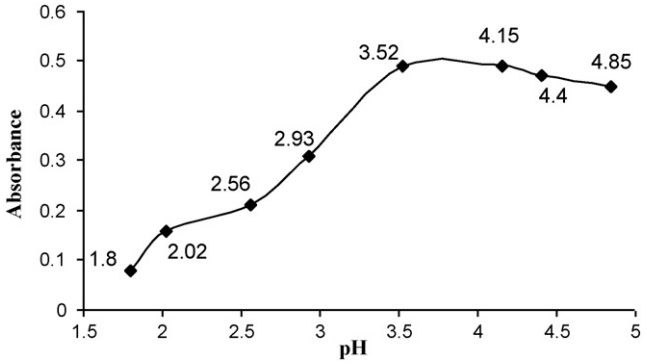

Fig. 5. Effect of pH on maximum color development in method B (7.5 μg ml^{-1} ISX).

cerium(III) hydroxide, a reduced form of cerium(IV) sulfate which is predominant in the reaction mixture after the oxidation of MBTH. This was confirmed by the absence of any precipitate formation when EDTA was added before raising the pH. Addition of EDTA resulted in the formation of highly stable Ce(III)–EDTA complex; log K = 15.9 [53].

3.2. Optimization of experimental variables

Various experimental variables were optimized to achieve maximum sensitivity.

3.2.1. Effect of reagent concentration

The amount of MBTH required to develop maximum color intensity was found to be 2.0 ml of 0.05% and 1.5 ml of 0.1% for method A and method B, respectively (Fig. 3). Beyond this optimum volume of MBTH, the absorbance of the colored product decreased and the blank color intensity increased due to the self-coupling of MBTH [54]. Beyond the optimum concentration of Ce $(SO_4)_2$ (Fig. 4) in method A (4 ml of 0.1% or 2 ml of 0.2%) and in method B (2 ml of 0.2%), there occurs further oxidation of the coupled product resulting in the decrease in absorbance.

3.2.2. Effect of pH

When the pH of the red colored coupled product was raised above 1.8, a bathochromic shift towards 580 nm occurred with the gradual appearance of violet colored chromogen. The effective pH yielding maximum absorbance was between 3.85 and 4.15 (Fig. 5). Therefore, pH 4.0 was chosen. The formation of precipitate due to the increase in pH was overcome by addition of 1 ml of 5×10^{-3} M EDTA before raising the pH. Use of 0.05 M potassium hydrogen phthalate as a buffer of pH 4.0 [55], was essential since the violet colored chromogen does not remain stable in unbuffered medium.

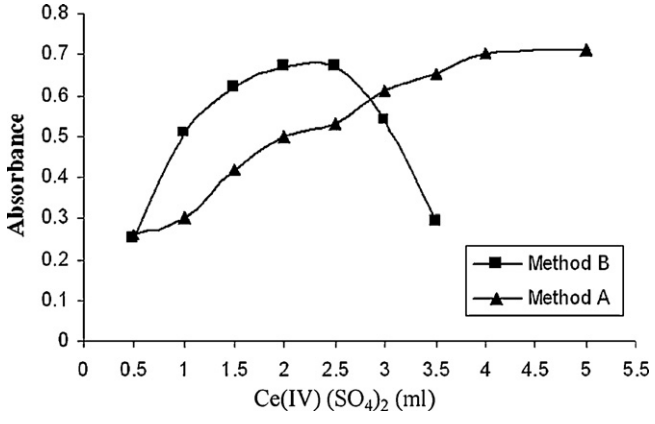

Fig. 4. Effect of 0.1% $Ce(SO_4)_2$ in method A (21.0 μg ml^{-1} ISX) and effect of 0.2% $Ce(SO_4)_2$ in method B (12.5 μg ml^{-1} ISX).

3.2.3. Order of addition

After optimizing all other experimental variables, further experiments were performed to ascertain the influence of sequencing the addition of reactants on the color development by measuring the absorbance (for 14 μg ml^{-1} ISX) based on following three orders of addition:

Order 1: ISX + MBTH + $Ce(SO_4)_2$: A = 0.45.
Order 2: ISX + $Ce(SO_4)_2$ + MBTH: A = 0.02.
Order 3: MBTH + $Ce(SO_4)_2$ + ISX: A = 0.23. The order of addition number 1 is recommended.

3.3. Method validation

3.3.1. Analytical data

A linear relationship was obtained between absorbance and concentration of ISX in both the methods. The linear regression equations, $Y = a + bX$ (where Y = absorbance of 1-cm layer of solution; a = intercept; b = slope and X = concentration in μg ml^{-1}), for the methods were obtained by the method of least squares. The Beer's law range, molar absorptivity, correlation coefficient, variance, confidence limits for slope and intercept for both the methods are summarized in Table 2. The limits of detection (LOD) and quan-

Table 2
Regression and analytical parameters.

Parameter	Method A	Method B
λ_{max} (nm)	500	580
Beer's law limits (μg ml^{-1})	1.4–21.0	1.0–15.0
Color stability (min)	120	90
Molar absorptivity (l mol^{-1} cm^{-1})	1.08×10^4	1.78×10^4
Sandell sensitivity[a] (μg cm^{-2})	0.0311	0.0190
Limit of detection (μg ml^{-1})	0.17	0.05
Limit of quantification (μg ml^{-1})	0.52	0.17
Regression equation, Y[b]		
Intercept (a)	−0.0056	0.0239
Slope (b)	0.0334	0.0492
Correlation coefficient (r)	0.9989	0.9970
Standard deviation of intercept (S_a)	0.0086	0.0154
Variance (S_a^2)	7.4×10^{-5}	2.4×10^{-4}
$\pm tS_a/\sqrt{n}$	0.0079	0.0142
Standard deviation of slope (S_b)	0.0007	0.0017
$\pm tS_b/\sqrt{n}$	0.0006	0.0016

[a] The sensitivity parameter in μg cm^{-2} ISX corresponding to an absorbance of 0.001 measured in a cuvette of cross-sectional area 1 cm^2 and L = 1 cm.
[b] $Y = a + bX$, where Y is the absorbance and X concentration in μg ml^{-1}.
$\pm tS_a/\sqrt{n}$ = confidence limit for intercept, $\pm tS_b/\sqrt{n}$ = confidence limit for slope.

Table 3
Intra-day and inter-day precision and accuracy evaluation.

ISX taken μg ml^{-1}	Intra-day (n = 7)			Inter-day (n = 5)		
	ISX found[a] (μg ml^{-1})	Precision[b]	Accuracy[c]	ISX found[a] (μg ml^{-1})	Precision[b]	Accuracy[c]
Method A						
3.50	3.57	2.65	1.94	3.34	2.85	4.57
10.50	10.68	4.19	1.74	10.23	3.36	2.57
17.50	17.78	1.16	1.60	17.33	2.13	0.97
Method B						
2.50	2.56	2.40	2.40	2.58	2.50	3.20
7.50	7.63	2.25	1.68	7.34	2.12	2.13
12.50	12.62	3.11	0.94	12.44	3.24	0.48

[a] Mean of n determinations.
[b] Relative standard deviation (%).
[c] Bias %: {(found − taken)/taken} × 100.

titation (LOQ) was calculated according to International Conference on Harmonization, 2005 guidelines, and presented in Table 2.

The significance of correlation coefficients was evaluated by calculating the t-values using the following formula [56]:

$$t = \frac{|r|\sqrt{n-2}}{\sqrt{1-r^2}}$$

The calculated t-value was then compared with the tabulated value at 95% significance level, using a two-sided t-test and $(n-2)$ degrees of freedom. The null hypothesis, in this case, showed that there was no correlation between the measured absorbance (Y) and the concentration (X). Since the calculated t-values were 73.77 and 44.59 for method A and method B respectively, which are greater than the tabulated value (2.57), the null hypothesis was rejected and was concluded that a significant correlation did exist between Y and X. As expected, the closer $|r|$ is to 1, i.e. as the straight-line relationship becomes stronger, the higher the values of t that are obtained.

3.3.2. Precision and accuracy

The precision of the methods was calculated in terms of the intermediate precision (intra-day and inter-day) [57]. Three different concentrations of ISX were analyzed in seven replicates during the same day (intra-day precision) and five consecutive days (inter-day precision). The RSD (%) values of intra-day and inter-day studies showed that the precision was good (Table 3). The accuracy of the analytical method expresses the closeness between the reference value and the found value. Accuracy was evaluated as percentage relative error between the measured concentrations and taken concentrations for ISX (Bias %). The results obtained are compiled in Table 3 and shows that the accuracy was good.

3.3.3. Selectivity

A study of some potential interference in the present proposed methods was performed by selecting the excipients often used in pharmaceutical formulations or as possible co-active substance. Selectivity was evaluated by both placebo blank analysis and recovery studies. The placebo blank, a commonly employed tablet excipients, consisting of 20 mg sodium alginate, 30 mg mag-

Table 4
Method robustness and ruggedness.

ISX taken (μg mL^{-1})	Robustness (% RSD)			Ruggedness (%RSD)	
	pH[a]	mL of X % MBTH[b]	Y mL of 0.2% $Ce(SO_4)_2$	Inter instruments (n = 3)	Inter analysts (n = 4)
Method A					
5.00	–	2.18	0.72	3.51	2.22
10.00	–	1.07	0.28	2.16	1.97
15.00	–	1.18	0.41	3.15	1.28
Method B					
4.00	2.11	1.02	0.12	2.35	1.25
6.00	1.02	0.07	0.33	2.23	2.30
8.00	1.15	0.11	0.75	2.11	1.54

[a] pH in method B: 3.85, 4.00 and 4.10.
[b] Method A: 1.80, 2.00 and 2.10 ml of 0.05% MBTH. Method B: 1.30, 1.50 and 1.65 ml of 0.1% MBTH, Y = 1.80, 2.00 and 2.10 ml of 0.2% $Ce(SO_4)_2$ for method A and method B.

Table 5
Results of assay of tablets and statistical evaluation.

Tablets/combination tablet analyzed	Label claim	Found[a] (Percent of label claim ± SD)		
		Reference method	Method A	Method B
Tidilan[b]	100 mg/Tab	100.3 ± 0.78	100.8 ± 1.34 t = 0.74 F = 2.95	99.18 ± 1.68 t = 1.44 F = 4.64
Tidilan[b]	40 mg/Tab	101.3 ± 1.04	100.7 ± 1.85 t = 0.66 F = 3.16	101.6 ± 1.54 t = 0.37 F = 3.28

[a] Mean value of five determinations.
[b] Marketed by: Juggat Pharma, Bangalore 560074, India.
Tabulated t-value at the 95% confidence level is 2.78; tabulated F-value at the 95% confidence level is 6.39.

nesium stearate, 20 mg lactose, 20 mg acacia, 50 mg talc and 30 mg starch, but without ISX, was prepared and analyzed as described under the procedures. The resulting absorbance readings for both the methods were same as the reagent blank, inferring no interference from the placebo. It was further confirmed by carrying out recovery study from synthetic mixture prepared by adding 10 mg of ISX to 50 mg of the placebo blank. The active component was then extracted into water as described under "procedure for tablets". The percent recoveries of ISX were 100.93 ± 0.63 and 102.56 ± 0.26 for method A and method B, respectively. This confirms the selectivity of methods in the presence of the commonly employed tablet excipients.

3.3.4. Robustness and ruggedness

For the evaluation of the method robustness, two important experimental variables, such as pH and reagent concentration, were slightly varied deliberately. The analysis was performed at the intentionally varied experimental conditions by taking four different concentrations of ISX and found to remain unaffected as shown by the RSD values between the range of 0.07 and 2.18%. Method ruggedness was expressed as the RSD of the same procedure applied by four different analysts as well as using two different spectrophotometers. The results are shown in Table 4.

3.3.5. Application to analysis of spiked urine sample and pharmaceutical formulations

The proposed methods were successfully applied to the determination of ISX in spiked urine sample with mean percent recovery of 97.50 ± 0.82 ($n = 5$) and 104.4 ± 0.73 ($n = 5$), for method A and method B, respectively, and two representative tablets (Table 5). The results obtained were statistically compared with those of the official method [2] by applying the Student's *t*-test for accuracy and *F*-test for precision. The official method consisted of extraction of ISX from the matrices into aqueous solution and absorbance measurement at about 300 nm. As can be seen from Table 5, the calculated *t*-value and *F*-value at 95% confidence level did not exceed the tabulated values of 2.78 and 6.39 respectively, for four degrees of freedom. The results indicated that there is no difference between the proposed methods and the official method with respect to accuracy and precision. Accuracy of the proposed methods was further confirmed by standard-addition procedure. Pre-analyzed tablet powder (Tidilan 40 mg) was spiked with pure ISX at three different concentration levels (50, 100 and 150% of the quantity present in the tablet powder) and the total was found by the proposed methods. The percent pure ISX recovered ranged from 96.34 to 105.1% with a standard deviation of 2.6–3.8% for two degrees of freedom at each level.

4. Conclusions

Two new spectrophotometric methods for the determination of isoxsuprine hydrochloride in bulk drug and in tablets were developed and validated as per the current ICH guidelines. The methods are based on well-characterized oxidative-coupling reaction involving the use of cerium (IV) and MBTH as reagents. The methods are simple, rapid and cost-effective compared to most published methods for isoxsuprine hydrochloride. Of the non-chromatographic methods, the UV-spectrophotometric methods require either an automated analyzer [4] or applicable to multi-component mixture [5]. The methods based on luminescence spectrometry [6,7], though sensitive, require expensive experimental setup. The ISE-potentiometric method [8] is indirect and is less sensitive (linear range 33.8–1690 $\mu g\,ml^{-1}$) whereas the reliability and precision of the results by polarography [9] depend on the capillary characteristics which are often not reproducible. The chromatographic methods [10–16], no doubt, are sensitive and selective, but require expensive instruments and solvents besides involving several clean-up procedures.

The reported visible spectrophotometric methods based on redox reactions are less selective and less sensitive [17–19] in addition to involving a boiling step [18,19] while all the procedures based on redox-chelation reactions [20–23] require boiling for 15–20 min. Methods based on condensation reactions suffer from poor sensitivity [24–26], narrow linear range [26] and require organic solvent medium [25]. Oxidative-condensation reaction-based procedures [27,28], though sensitive, are devoid of simplicity since a critical reagent condition is involved [27] or an automated flow injection assembly is required [28]. Liquid–liquid extraction step, poor sensitivity, narrow linear range and use of organic solvent medium are the drawbacks of the methods based on oxidative-coupling reaction [29,30]. Sensitive procedures using diazo-coupling reactions [31–35] require a near zero temperature for diazotization step and the measurement is made at shorter wavelengths where the interference from the co-formulated substances is far more than at longer wavelength. Extractive-spectrophotometric methods [36–39] based on ion-pair complexation reactions are both sensitive and selective, but they are tedious, labor-intensive, time-consuming, and very prone to loss of analyte. They require strict pH control and large amounts of high purity solvents, which are often hazardous and results in the production of toxic lab waste. Besides having a narrow linear dynamic range, the method based on nitration-Meisenheimer complexation reaction [40] requires drastic experimental conditions like use of conc. H_2SO_4–HNO_3 mixture and boiling for 20 min. All the procedures utilizing nitrosation and nitrosation–chelation reactions [41,42] lack sensitivity and require extraction step with an organic solvent [41]. Derivatization reaction-based methods [43,44] use expensive reagents and need longer contact times. The kinetic spectrophotometric methods [45–47] rely on judicious control of many experimental variables such as temperature, pH and ionic strength and often very prone to inaccuracy and imprecision.

In contrast to the above published visible spectrophotometric methods [17–47], the proposed methods using cerium (IV) and MBTH reagents can be applied at ambient temperature, color development is instantaneous and neither involves complicated extraction procedure nor use organic solvents. They are free from rigid experimental variables and employ inexpensive and easily available chemicals and instrument. The present methods have wide linear dynamic ranges and are more sensitive than most of other published methods. The methods can measure concentrations down to 1.4 and 1.0 $\mu g\,ml^{-1}$ with good precision and accuracy. The minimum detectable limits are 0.17 and 0.05 $\mu g\,ml^{-1}$ which are better than those reported for other methods including some non-spectrophotometric techniques. This was exploited for the determination of ISX in spiked human urine with excellent recoveries which can be regarded as a striking feature of the proposed methods. Between the two methods, method B with ε value of $1.78 \times 10^4\,l\,mol^{-1}\,cm^{-1}$ is more sensitive than method A ($\varepsilon = 1.08 \times 10^4\,l\,mol^{-1}\,cm^{-1}$). Although the proposed methods seem less sensitive than some of the published methods in terms of molar absorptivity, in both the methods, measurement is made at longer wavelengths. This is a decisive advantage since the interference from the co-formulated substances will generally be far less at longer wavelengths. With these two methods one gains the advantages of speed, low-cost, environmental protection without sacrificing either accuracy and precision or selectivity and sensitivity. The analysts are encouraged to develop visible reflectance spectrophotometric methods based on the proposed reactions which will be a step towards green analytical chemistry.

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