ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Developement and Validation of Spectrofluorimetric and Spectrophotometric Stability-Indicating Methods for Determination of Idrocilamide

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Manuscript Info

Manuscript History:

Received: 10 August 2013
Final Accepted: 25 August 2013
Published Online: September 2013

Key words:
Idrocilamide; NBD-Cl;
Iodine; KI|KIO_3;
Stability Indicating.

Abstract

Three simple, rapid and sensitive spectrofluorimetric and spectrophotometric methods were developed and validated for analysis of idrocilamide (***Idro***) in presence of its hydrolytic and oxidative degradants. The first method is based on reaction of ***Idro*** with 7-Chloro-4-Nitrobenz-2-Oxa-1, 3-Diazole (NBD-Cl) resulting in the formation of highly fluorescent product which was measured at 535 nm (λ_{ex} 460 nm). The high sensitivity of the method allowed its successful application to the analysis of ***Idro*** in spiked human plasma. The second method is based on charge transfer complex formation of the drug with iodine in dichloromethane. The reaction is followed spectrophotometrically by measuring the absorbance at 365 nm. The third method is based on the reaction of carboxylic acid group of hydrolytic degradant of ***Idro*** with a mixture of potassium iodate and iodide to form yellow colored product showing maximum absorbance at 350 nm. Under optimized experimental conditions, Beer's law is obeyed in the concentration ranges of 1–10, 50–500, and 25–250 μg mL^{-1}, with limits of detection; 0.05, 4.50 and 1.72 μg mL^{-1} for methods 1, 2 and 3 respectively. All the methods have been applied to the determination of ***Idro*** in pharmaceutical dosage form. The proposed methods could be applied for quality control analysis of idrocilamide.

Introduction

Idrocilamide (***Idro***), N-(2-Hydroxyethyl)-3-phenyl-2-propenamide, is a centrally acting muscle relaxant with anti-inflammatory effects (1–5). In the literature, few analytical methods were reported for its determination. These include spectrophotometry(6), TLC-densitometry and High Performance Liquid Chromatographic (HPLC) methods (7). Liquid chromatography with UV detection was used to investigate the pharmacokinetics parameters of ***Idro*** in both rabbits and human plasma (8,9). Depending on the fact, that spectrofluorimetric and spectrophotometric methods of analysis are usually rapid, sensitive and have the advantage of being inexpensive, the present work is directed to introduce simple fluorimetric or colorimetric methods for the determination of ***Idro*** in presence of its hydrolytic and oxidative degradants, pharmaceutical dosage form as well as in spiked human plasma.

2. Experimental Design

2.1. Instruments

- Spectrofluorimeter: Shimadzu RF – 1501 spectrofluorimeter with 1-cm quartz cells (Kyoto - Japan) was used for the spectrofluorimetric study.

- Spectrophotometer: Shimadzu UV-1601 PC, dual-beam UV-vis spectrophotometer (Kyoto - Japan), with matched 1 cm quartz cells, was used for spectrophotometric study.
- Thermostatically controlled water bath (Memmert - Germany).
- Sonicator (Memmert – Germany).
- Centrifuge (Thermo Electron Corporation-USA)

2.2. Materials and reagents

Idrocilamide was kindly supplied by Mina-Pharm Co. (Cairo, Egypt). Its purity was found to be 99.60% according to the manufacturer's method (potentiometric method using acetic anhydride in pyridine as a solvent and 1N sodium hydroxide as a titrant).

Srilane Cream, Batch No.5EE0720 manufactured by Minapharm Co. Cairo, Egypt under license from Merck, Lyon, France, and labeled to contain 5 g *Idro*/100 g cream.

7- Chloro- 4 -nitro-2-oxa- 1, 3- oxadiazole (NBD-Cl; Sigma Chemical Co., St. Louis, USA) was prepared as 0.2% (w/v) solution in methanol, freshly prepared daily. Iodine (Merck Limited, Mumbai, India) was prepared as 0.1% (w/v) solution in dichloromethane. 4.0 x 10^{-2} M Potassium iodide (Fluka Chemie AG, Switzerland) solution was freshly prepared in distilled water. 2.0 x 10^{-3} M Potassium iodate (Fluka Chemie AG, Switzerland) was also freshly prepared in distilled water. Human plasma was kindly supplied from VACSERA, Egypt. All solvents and other chemicals used throughout this study were of analytical grade.

2.3. Preparation of solutions

2.3.1. Standard drug solutions

2.3.1.1 ***NBD-Cl method***

Idrocilamide standard solution was prepared as 1 mg mL^{-1} solution in methanol.

2.3.1.2 ***Iodine method***

Idrocilamide standard solution was prepared as 1 mg mL^{-1} solution in dichloromethane.

2.3.1.3 ***KI | KIO_3 Method***

Idrocilamide hydrolytic degradant stock solution (Series A), (1mg mL^{-1})

Accurately weighed amount about 5 mg of ***Idro*** hydrolytic degradant namely 3-phenyl-acrylic acid (7) was transferred to 50-mL volumetric flask. The volume was completed with distilled water. Standard degradant working solutions (250, 500, 1000, 1500, 2000, 2500 µg mL^{-1}) were prepared by suitable dilution from the obtained stock solution of the degradant.

Idrocilamide stock standard solution (Series B), (1mg mL^{-1})

Accurately weighed amount about 50 mg of ***Idro*** was transferred into 50-mL volumetric flask, dissolved in 1 mL methanol and diluted to volume with distilled water. Standard working solutions (250, 500, 1000, 1500, 2000, 2500 µg mL^{-1}) were prepared by suitable dilution from the obtained standard stock solution of the drug.

2.3.2 Degradant solutions (1 mg mL^{-1})

Different degradants were laboratory prepared according to the reported method for ***Idro*** (7). Accurately weighed amount about 50 mg of ***Idro*** were refluxed with 50 mL 5 M hydrochloric acid at 100 ^{o}C for 2 hours or 2 M sodium hydroxide at 100 ^{o}C for 3 hours or 3% (w/v) hydrogen peroxide for 24 hours; for acid, alkaline and oxidative degradants respectively. The solutions were neutralized with 5 M sodium hydroxide or 2 M hydrochloric acid for acid and alkaline degradants respectively. The solutions were concentrated to about 20 mL and quantitatively transferred to 50-mL volumetric flasks. For oxidative degradant, the solution was evaporated to dryness on water bath. Then the residue was dissolved in 20 mL distilled water and quantitatively transferred to 50-mL volumetric flask. The volume were completed with distilled water for all the prepared degradants(7).

2.4. General analytical procedures

2.4.1. NBD-Cl method

2.4.1.1. Construction of calibration curve

Aliquots equivalent to 10 – 100 µg of ***Idro*** were separately transferred into a series of 20-mL test tubes. One milliliter of 0.1 M $NaHCO_3$ solution (pH 8 ± 0.2) was added followed by 1 mL of NBD-Cl solution (0.2%, w/v). The reaction solution was allowed to stand in thermostatically controlled water bath at 60 ± 5 ºC for 30 min. After cooling, the reaction mixture was acidified by adding 1 mL of 0.1 M HCl, transferred quantitatively to 10-mL volumetric flasks and completed to volume with methanol. The relative fluorescence intensity of the resulting solution was measured at 535 nm after excitation at 460 nm against reagent blank treated similarly.

2.4.1.2. Procedure for spiked human plasma

From aliquots equivalent to 25 – 475 µg mL^{-1} ***Idro*** standard solution, 1 mL was transferred into a series of centrifugation tubes. One milliliter of drug free plasma and 3 mL of acetonitrile were added to each tube successively and the solution was sonicated for 5 min. then the tubes were centrifuged at 4500 rpm for 20 min. One milliliter of the supernatants was transferred separately to a series of 10-mL volumetric flasks, evaporated to dryness and the residues were

dissolved each in 1 mL methanol. The procedure under 2.4.1.1 was followed.

2.4.2. Iodine method

2.4.2.1. Construction of calibration curve

Aliquots equivalent to 0.5 – 5.0 mg of ***Idro*** were separately transferred from their stock solutions into a series of 10-mL volumetric flasks. Two mL of 0.1% iodine were added in each flask at room temperature (25 ± 2 °C) and diluted up to the mark with dichloromethane. The absorbance was measured at 365 nm against the reagent blank. Each measurement was plotted against its corresponding concentration and the regression equation was calculated.

2.4.2.2. Determination of stoichiometry of the complex by Job' s method of continuous variation (10)

Into a series of 10-mL volumetric flasks 0.5 , 1, 1.5 , 2 , 2.5 ……4.5 mL of 5.2 $\times 10^{-3}$ M ***Idro*** solution were transferred followed by 4.5 , 4 ….0.5 mL of 5.2 $\times$ 10^{-3} M of iodine and the volume was completed with dichloromethane. The absorbance was measured at 365 nm each against its appropriate blank. The absorbance was plotted against mole fraction of the drug.

2.4.2.3. Determination of stability constant, molar absorbitivity and standard free energy change

Serial volumes of 0.7 - 1.2 mL of 5.2 x 10^{-3} M solutions of idrocilamide in dichloromethane were transferred into 10-mL volumetric flasks. To each flask, 2 mL of 5.2 x 10^{-4} M iodine in dichloromethane were added and continued as directed under *2.4.2.1.*

2.4.3. KI | KIO_3 Method

2.4.3.1. Construction of calibration curve

Aliquots equivalent to 0.25 - 2.50 mg of ***Idro*** hydrolytic degradants were separately transferred from its working solutions (series A), into 10-mL volumetric flasks, 2 mL of 2x10^{-3} M potassium iodate and 2.5 mL of 4.0x10^{-2} M potassium iodide were added and diluted to volume with distilled water. The difference in absorbance was measured at 350 nm. Each measurement was recorded against its corresponding concentration without hydrolysis and similarly treated. The values obtained were plotted against the corresponding concentrations. The regression equation was calculated.

2.4.4. Analysis of pharmaceutical formulation

An accurately weighed amount of cream equivalent to about 100 mg ***Idro*** was transferred to 200 mL beaker and dissolved in 50 mL methanol for NBD-Cl method and in 50 mL dichloromethane for iodine method. The solutions were stirred with magnetic stirrer for 15 min and transferred quantitatively to 100-mL volumetric flasks. For KI | KIO_3 method, an accurately weighed amount of cream equivalent to about 50 mg ***Idro*** was transferred to 100 mL beaker and dissolved in 10 mL methanol. The solution was evaporated and refluxed with 50 mL of 2 M NaOH at 100 ^{o}C for 3 hours. The solution was neutralized with 2 M hydrochloric acid and concentrated to about 10 mL then transferred quantitatively to 50-mL volumetric flask. The volumes were completed to the mark each with its corresponding solvents and then analyzed following the proposed procedures.

2.4.5. Analysis of laboratory prepared mixtures

Aliquots equivalent to 10 – 90 µg and to 0.25–2.25 mg were separately transferred from the standard solutions into 10-mL and 5-mL volumetric flasks for NBD-Cl and iodine methods respectively. To the previous solutions, complementary volumes of each degradant (hydrolytic and oxidative) were separately added to obtain solutions contained degradants in the range of 10 – 90 %. For KI | KIO_3 method; Aliquots equivalent to 0.125–1.125 mg of ***Idro*** hydrolytic degradants were separately transferred from its working solution (series A) into a series of 5-mL volumetric flasks. To the previous solutions aliquots equivalent to 1.125 - 0.125 mg of working standard solutions of ***Idro*** (series B) were added. Then analyzed following the proposed procedures.

Result and Discussion

3.1. Involved reaction, design and strategy for assays development

1- Reaction with NBD-Cl

NBD-Cl is an activated halide derivative that has been used as fluorogenic reagent for spectrofluorimetric determination of many pharmaceutical compounds (11 -15). Idrocilamide exhibits no native fluorescence, thus its derivatization with fluorogenic reagent was necessary for its spectrofluorimetric determination. The reaction of NBD-Cl with idrocilamide has not been investigated yet. Owing to the presence of labile chloride in the chemical structure of NBD-Cl, a daily fresh solution was prepared and tested in the present study. It was found that idrocilamide reacts with NBD-Cl and forms a yellow fluorescent derivative. This derivative exhibited maximum relative fluorescence intensity (λ_{em}) at 535 nm after its excitation at maximum wavelength (λ_{ex}) at 460 nm. The hydrolytic degradants of ***Idro*** contain 2-amino ethanol, which is small chain aliphatic amine and it does not react with NBD-Cl. This may be due to its lack of chromophore and conjugation compared with the parent drug. Moreover, it may react under different conditions. So, the proposed method extends to develop a stability indicating assay for the studied drug. The excitation and emission spectra for the reaction product of ***Idro*** with NBD-Cl are given in Figure (1).

The high sensitivity of the proposed method renders applicable to the in vitro determination of ***Idro*** in

biological fluids. Idrocilamide was successfully determined in human plasma samples. To avoid variation in background fluorescence, a simple deproteination of plasma samples with acetonitrile was performed followed by centrifugation; the clear supernatant containing ***Idro*** was analyzed.

The fluorescence concentration plot was found to be linear over a range of 0.5 – 9.5 µg mL^{-1} as shown in Figure (2). The reaction pathway between ***Idro*** and NBD-Cl was postulated to proceed as shown in Figure (3).

2- Reaction with σ acceptor (iodine)

Some *n*-donor drugs react with sigma electron acceptor iodine forming charge-transfer complex followed by tri- iodide ion pair formation. The immediate change of the violet color of iodine in dichloromethane (520 nm) to a lemon yellow upon reaction with ***Idro*** was taken as suggestive of charge transfer complex formation (16, 17) as illustrated in Figure (4). The appearance of absorption peak at 365 nm was attributed to the formation of a charge-transfer complex between ***Idro*** and iodine, having an ionized structure $DI^{+}....I^{-}_{3}$ in 1, 2-dichloroethane (Figure 5).

The stoichiometry of the reaction between ***Idro*** and iodine was studied by Job's method of continuous variations (10), indicates that the interaction occurs through the formation of 1:1 (donor: acceptor) complex.

The absorbance of ***Idro*** – iodine CT complex was used to calculate the association constant and molar absorbitivity using the Benesi - Hildebrand equation (18) which depends on the experimental condition that one of the two component species should be present in large excess, so that its concentration is virtually unaltered on formation of the complex.

$$\frac{[A\circ]}{A^{AD}} = \frac{1}{\varepsilon^{AD}} + \frac{1}{\varepsilon^{AD} K_C^{AD}} X \frac{1}{[D\circ]}$$

Where $[A_O]$ and $[D_O]$ are the total concentrations of the interacting species (acceptor and donor respectively), A^{AD} is the absorbance of the complex, ε^{AD} is the molar absorptivity of the complex and K_C^{AD} is the association constant of the complex.

From the above equation on plotting the values of $[A_O]/A^{AD}$ versus $1/[D_O]$ straight lines were obtained. The intercept and the slope were calculated and the equation is transformed into the following equations:

$[A_O]/A^{AD} = 1 \text{ X } 10^{-7}. 1/[D_O] - 0.0006.$

The standard free energy change of complexation (ΔG^{O}) is related to the association constant by the following equation (19):

$$\Delta G\circ = -2.303RT \log K_C^{AD}$$

Where ΔG^{O} is the Standard free energy of the complex (kcal/mol), R is the gas constant (1.987 cal $mol^{-1}deg^{-1}$), T is the temperature in Kelvin (273 + ^{o}C) and is the association constant for drug – acceptor complex (L/mol).

The high values of association constants are common in n-electron donors where the intermolecular overlap may be considerable.

3- Reaction with KIO_3|KI System

The spectrum of hydrolytic degradant of ***Idro*** in aqueous solution shows characteristic λ_{max} at 272 nm. The addition of aqueous solution of KIO_3 and KI to the hydrolytic degradant solution causes change in the absorption spectrum with a new characteristic band at 350 nm as shown in Figure (6). The reagent blank solution shows one peak at 275 nm and no peak at 350 nm. Also from this figure it is clear that, no reaction was observed between the intact drug and KIO_3|KI. So, this method was used for the stability studies of the drug.

A yellow color observed is due to the formation of I_2, which is immediately converted into tri-iodide ions in the presence of iodide ions ($I_2 + I^{-} \rightarrow I^{-}_3$) (20, 21) exhibiting absorption maxima at 292 nm and 350 nm. Idrocilamide hydrolytic degradant possesses –COOH group in its moiety and hence undergoes a similar reaction with iodate- iodide mixture. The reaction sequence is shown in Figure (7). The confirmatory test for the presence of iodine in the final solution of the drug is established by the appearance of blue color on addition of starch solution (22).

3.2. Optimization of the reaction conditions

The factors affecting the reaction conditions (pH, the concentrations of reagents, reaction time and temperature, and the diluting solvent) were performed by altering each variable in turn while keeping the others constant. The results are summarized in Table 1.

3.2.1. NBD-Cl method

For investigating the effect of NBD-Cl concentration and volume, the reaction was performed using different concentration (0.05-0.4 g %) and volumes 0.5 – 3 mL.

The highest readings were attained using 1 mL NBD-Cl at a concentration of 0.2% (w/v). At higher concentrations of NBD-Cl, the relative fluorescence intensity was not affected.

In order to determine the optimum temperature and time required for completion of the reaction, the derivatization reaction was carried out at room temperature (25 ± 5 ° C) and the induced RFI values were monitored at different time intervals. It was found that the reaction was very slow, and did not go

to completion in reasonable time; it required more than 1 hour, therefore, investigations were carried out at varying elevated temperatures (40 – 80 °C), and the intensities of the induced fluorescence were monitored for 60 min. The results indicated that the reaction was dependent on temperature and time, and the optimum condition was achieved by heating at 60 °C for 30 min. At higher temperatures, the maximum RFI was obtained in shorter times (~ 15 min), however rapid progressive decrease in the readings was observed as the reaction time increased. This was probably attributed to the degradation of the reagent at high temperature. This observation was coincident with the results that have been previously reported (23).

In order to generate the nucleophile from idrocilamide, the reaction should be carried out in alkaline medium. Different alkaline buffer systems (borate, phosphate, and carbonate) having pH range from (8 -11) were tested. The highest RFI was obtained when the reaction was carried out in carbonate buffer. With other buffers, either precipitation or white colloid occurred upon addition of NBD-Cl reagent solution, non reproducible results, and/or weak sensitivities were observed. Maximum readings were obtained at pH 8.2. At pH above 9, sharp decrease in the readings occurred. This was attributed probably to the increase in the amount of hydroxide ion that holds back the condensation reaction between idrocilamide and NBD-Cl.

In order to keep the high sensitivity for determination of idrocilamide, the subsequent experiments were carried out at pH 8.2.

Under the above mentioned conditions, significantly high fluorescence backgrounds were also observed. This was attributed to the hydrolysis of NBD-Cl to the corresponding hydroxyl derivative namely, 7-hydroxy-4-nitrobenzo-2-oxa-1, 3-diazole (NBD-OH) (24). The fluorescence of NBD-OH was found to be quenched by decreasing the pH of the reaction medium to less than one (25). Therefore acidification of the reaction mixture prior to measurement of the RFI was necessary to remarkable decrease the background fluorescence. Meanwhile, the reaction product was not affected, thus the sensitivity was ultimately increased. The concentration of HCl required for acidification was found to be 0.01 M in the final assay solutions (i.e. 1 mL of 0.1 M).

Upon diluting the reaction with water, colloids were obtained indicating the incomplete solubility of idrocilamide -NBD in water. Therefore, water could not be used for dilution. In order to select the most appropriate organic solvent for diluting the reaction solution, different solvents were tested: methanol, ethanol, isopropanol, acetone, and acetonitrile. The highest readings were obtained when methanol was used. Therefore, methanol was used for diluting the reaction mixture in the subsequent experiments.

The effect of time on the stability of the ***Idro***-NBD-Cl fluorescent derivative was studied by follow the RFI of the reaction solution at different time intervals. It was found that the RFI values remain constant for 1 hour.

Figure 1: Excitation and emission spectra of:
- Idrocilamide – NBD Cl reaction product, 1µg/mL (——).
-Hydrolytic degradant +NBD Cl, 1µg/mL (– – –).
-Oxidative degradant +NBD Cl, 1µg/mL, (– · – · –).
- Blank reagent (·····).
(λ_{em} 535 nm, λ_{ex} 460 nm).

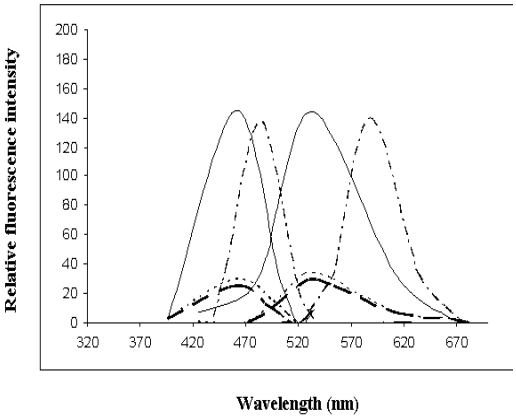
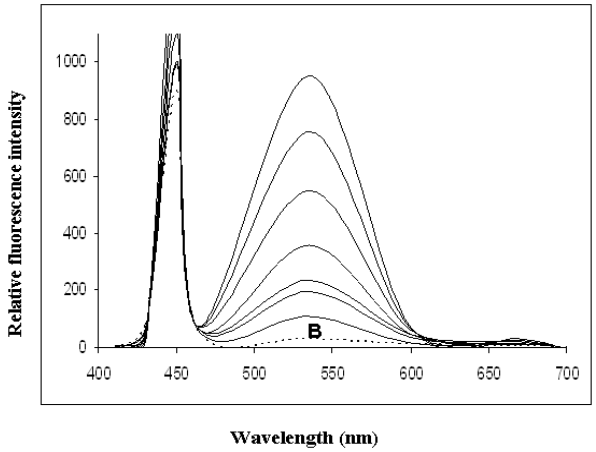


Figure 2: Fluorescence spectra of plasma spiked with idrocilamide - NBD-Cl (0.5 - 9.5 µg/ mL), B: control plasma. (λ_{em} 535 nm , λ_{ex} 460 nm).



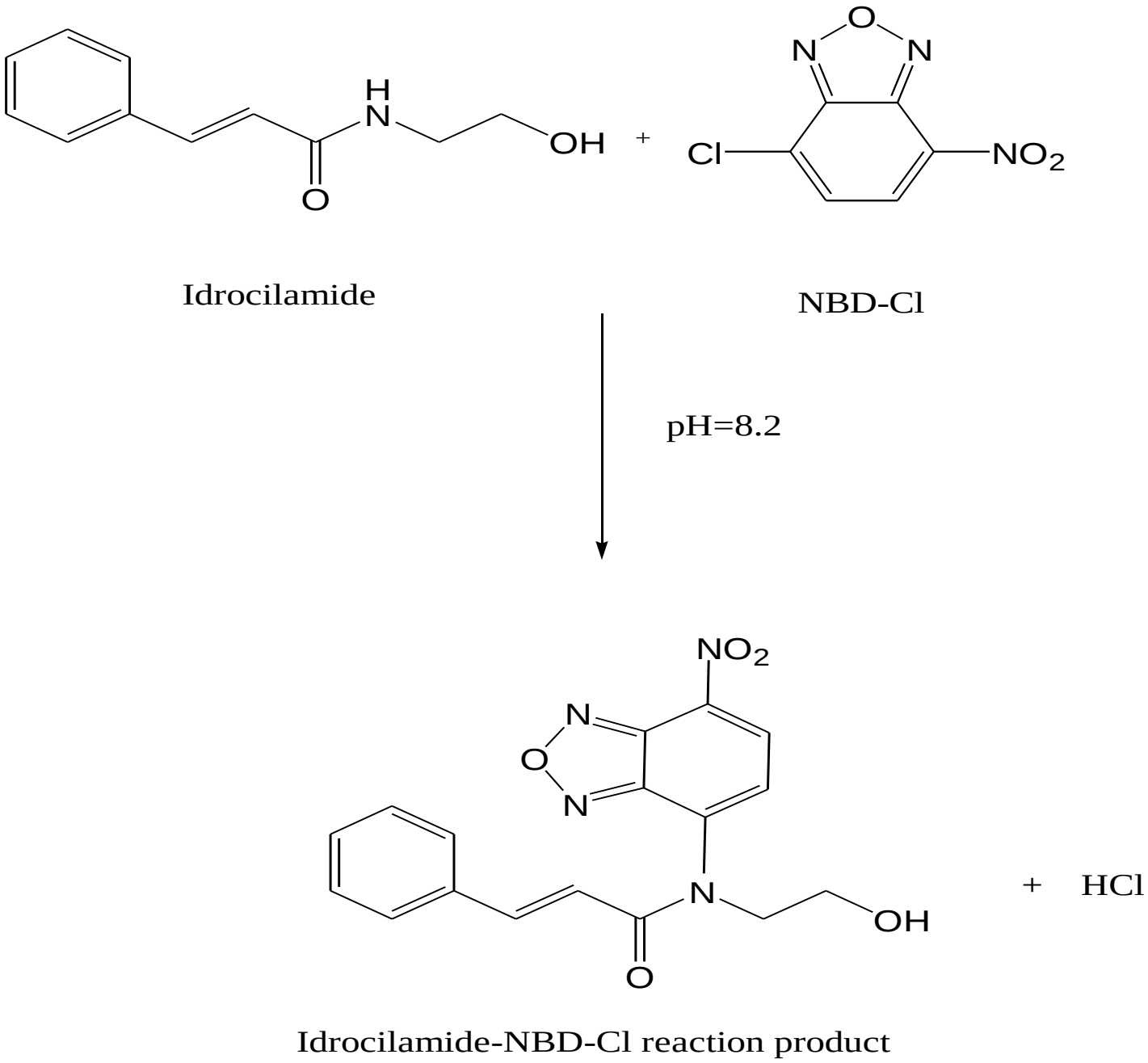


Figure 3: The suggested mechanism of reaction of idrocilamide and NBD-Cl.

Figure 4: Zero order absorption spectra of:
- Idrocilamide - iodine CT complex, 400 µg/ ml (_____).
- Hydrolytic degradant + iodine,450 µg /ml (_ _ _ _ _ _).
- Oxidative degradant + iodine,200 µg/ml (. . . . .).
- Idrocilamide in dichloromethane, 40 µg/ ml (━━━━).
- Blank reagent (·····).

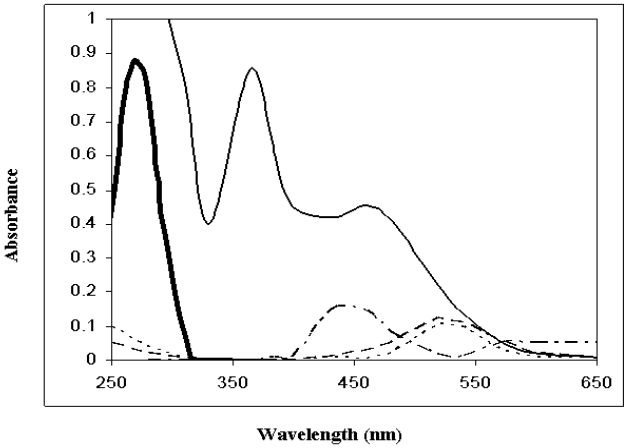


Figure 5: Suggested mechanism of the reaction between idrocilamide and iodine.

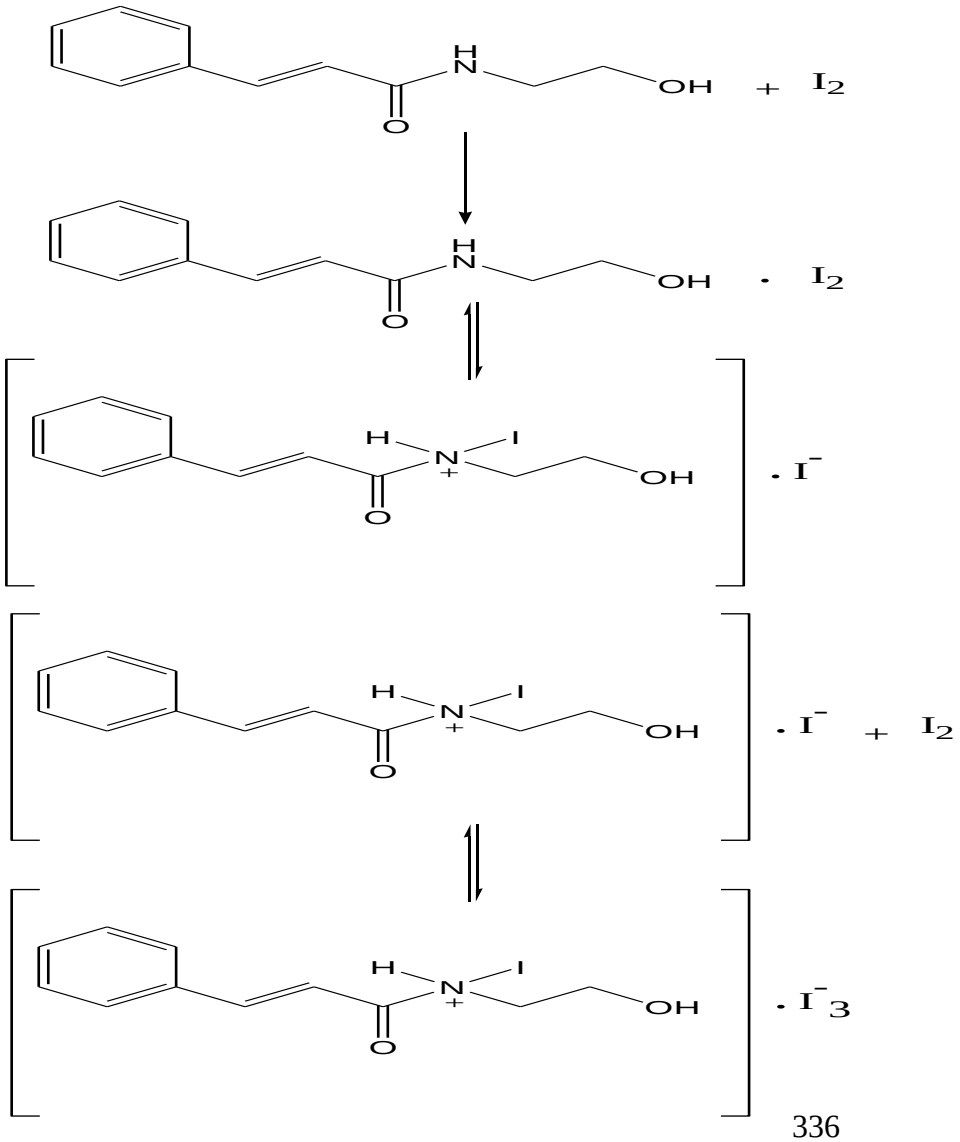


Figure 6: Zero order absorption spectra of:
- Idrocilamide degradant- KIO_3|KI reaction product, 150µg/ml (———).
- Idrocilamide KIO_3|KI reaction product,150µg/ml (_ . _ . _ .).
- Idrocilamide degradant in distilled water, 35 µg/ml (———).
- Blank reagent (…….).

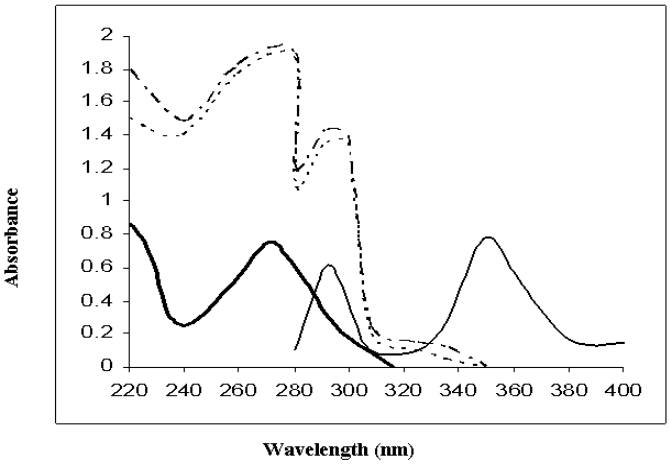
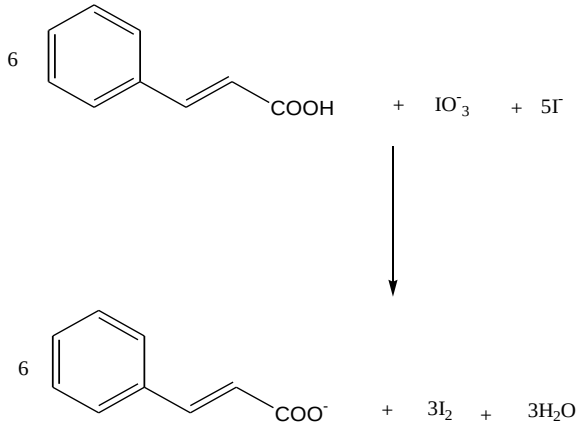


Figure 7: The suggested mechanism of reaction of idrocilamide hydrolytic degradant and KIO_3|KI.



3.2.2. Iodine method

In order to study the effect of the concentration of iodine on the absorbance of the charge transfer complex, varying concentrations (0.025% - 0.5%) and volumes (0.5 – 4 mL, 0.1% w/v) were investigated. The results indicated that 2 mL of 0.1% iodine gave the maximum absorbance and remained constant by further addition of iodine.

The colored product of charge transfer complex was formed and remained stable at room temperature for about 1 hr.

In order to select the most appropriate solvent, the reaction was carried out in different solvents as acetone, acetonitrile, methanol, ethanol,dichloromethane, 1, 4 dioxane and isopropanol. The best solvent was found to be 1, 2-dichloromethane.

3.2.3. KIO_3|KI method

For potassium iodate, varying concentrations (0.5 x 10^{-3} M - 4.5 x 10^{-3} M) and volumes (0.5 – 4 mL) were investigated. The results indicated that 2 mL of 2.0 X 10^{-3} M of potassium iodate gave the maximum absorbance.

For potassium iodide, varying concentrations ($1x10^{-2}$ M - $6x10^{-2}$ M) and volumes (0.5 – 4 mL) were investigated. The results indicated that 2.5 mL of $4.0x10^{-2}$ M potassium iodide gave the maximum absorbance.

The optimum reaction time was determined by monitoring the color development at room temperature (25 ± 2 ^{o}C). Complete color development was attained instantaneously and the developed color remained stable at room temperature for 120 minutes.

3.3. Development and validation of the analytical methods (26, 27)

3.3.1 Calibration curves, linearity, detection and quantification limits

Under the specified optimum reaction conditions, the calibration curves for ***Idro*** with the different analytical reagents employed in the present work were constructed.

The regression equations for the results were derived using the least-squares method. In all cases, Beer's law plots were linear with good correlation coefficients in the general concentration range(Table 2). The limits of detection(LOD) and quantification (LOQ) were determined, using the formula: LOD = 3.3 σ/ S and LOQ = 10 σ/ S, where σ = the standard deviation of the intercepts of regression lines, S = the slope of the calibration curve.

3.3.2. Precision

The precisions of the assays (repeatability and intermediate precision) were determined for ***Idro*** concentrations. Repeatability was evaluated by assaying freshly prepared solutions in triplicate using the proposed procedures (intra-assay precision). Intermediate precision was evaluated by assaying the concentrations mentioned above in triplicate on three successive days. The assays gave satisfactory results; the relative standard deviations (RSD) were less than 2%(Table 2). This level of precision of the proposed methods was adequate for the quality control analysis of ***Idro*****.**

3.3.4. Selectivity and specificity

The specificity of the methods was assessed by analyzing laboratory prepared mixtures of ***Idro*** and its hydrolytic and oxidative degradants (potential

organic impurities) in different proportions. The results show high selectivity of the methods, and no potential interference as shown in Table 3.

3.4.5. Accuracy

Statistical comparison between the proposed spectrofluorimetric, spectrophotometric and the manufacturer's methods using student's *t*-test and *F*-ratio at the 95% confidence level reveal insignificant difference(28) (Table 4). The accuracy was also assessed by applying the standard addition technique. Satisfactory results were obtained in good agreement with the labeled claim (Table 5).

Table 1: Optimum conditions for the interaction of idrocilamide with different reagents

Variables	NBD-Cl		Iodine		KI/KIO_3	
	Studied range	**Optimum conditions**	**Studied range**	**Optimum conditions**	**Studied range**	**Optimum conditions**
Reagent concentration	0.05 – 4 g %	0.2 g %	0.025 –0.5 g %	0.1 g %	$1X10^{-2}$ - $6X10^{-2}$ for KI $0.5X10^{-3}$- $4.5X10^{-3}$ for KIO_3	$4X10^{-2}$ $2X10^{-3}$
Reagent volume	0.5 – 3 mL	1 mL	0.5 – 4 mL	2 mL	0.5 – 4 mL 0.5 – 4 mL	2.5 mL 2.0 mL
Solvent [a]	Different solvent	Methanol	Different solvent	Dichloromethane	Different solvent	Distilled water
Time	120 minutes	100 minutes	120 minutes	60 minutes	120 minutes	120 minutes
Temperature	40 – 80 °C	60 °C	-	Room temperature	-	Room temperature
Buffers [b]	Different Buffers	NaHCO3	-	-	-	-
pH	8 – 11	8.2	-	-	-	-

[a] Acetone, acetonitrile, ethanol, methanol, 1, 4 dioxane, dichloromethane and isopropanol.

[b] Borate, phosphate and carbonate.

Table 2: Results of assay validation obtained by applying the proposed methods for the determination of idrocilamide in drug substance

Parameters	**NBD-Cl**	**Iodine**	**KI/KIO_3**
Linearity range (µg mL^{-1})	1.00 - 10.00	50 – 500	25.00 - 250.00
LOD(µg mL^{-1})	0.05	4.50	1.72
LOQ (µg mL^{-1})	0.18	14.78	5.70
Accuracy [a]			
Mean ± RSD% [b]	100.01 ±1.400	99.21 ± 0.841	99.69 ± 1.228
Mean ± RSD% [c]	99.31 ±1.544	99.56 ± 1.104	99.42 ± 0.935
Precision [d]			
Repeatability RSD%	0.34	0.624	0.964
Intermediate precision RSD%	0.39	1.075	1.790
Regression equation			
-Slope	95.573	0.0021	0.0041
SE of slope	1.35	$2.57X10^{-5}$	$5.58 X 10^{-5}$
Confidence limit of the slope [e]	92.08 - 99.06	$2.07X10^{-3}$ - $2.2X10^{-3}$	$3.9 X 10^{-3}$ - $4.2 X 10^{-3}$
-Intercept	38.108	0.0189	0.1514
SE of intercept	7.77	$6.9X10^{-3}$	$8.4 X 10^{-3}$
Confidence limit of the intercept[e]	18.11 - 58.09	$1.9X10^{-3}$ -$3.5X10^{2}$	0.127- 0.174
-Correlation coefficient (r)	0.9994	0.9995	0.9996
SE of estimation	10.925	0.011	0.010

[a] n = 6, [b] drug substance, [c] drug product.

[d] n = 9 for concentrations of 2.0, 4.0, 8.0 µg mL^{-1} and 50.0, 200.0, 400.0 µg mL^{-1} and 25.0, 100.0, 200.0 µg mL^{-1} for NBD-Cl, Iodine and KI/KIO_3 methods respectively.

[e] 95% confidence limit.

Table 3: Specificity of the proposed methods for the determination of Idrocilamide in laboratory prepared mixtures with its hydrolytic and oxidative degradants

Degradants %	NBD-Cl		Iodine		KI/KIO_3
	Hyd. Deg.	**Oxid. Deg.**	**Hyd. Deg.**	**Oxid. Deg.**	**Hyd. Deg.**
10	99.74	101.91	100.32	98.45	99.39
20	99.46	98.13	99.73	100.25	98.57
30	99.50	99.65	100.45	101.69	98.98
40	98.39	100.46	100.98	99.73	99.52
50	98.60	98.78	98.45	-	100.04
60	101.19	98.64	101.82	-	101.27
70	99.19	98.55	101.36	-	99.48
80	101.16	100.65	98.29	-	98.43
90	100.23	99.66	99.42	-	99.90
Mean [a] ± RSD%	99.71 ± 0.996	99.60 ± 1.230	100.09 ± 1.227	100.03 ± 1.339	99.50 ± 0.861

[a] Average of four different determinations.

Table 4. Statistical comparison between results obtained by applying the proposed methods and the reported methods for determination of idrocilamide in drug substance and drug product

Parameters	Drug product			Drug substance				
	KI/KIO_3	I_2	NBD-Cl	Reported[a] method	KI/KIO_3	I_2	NBD-Cl	Reported[b] method
Mean	99.42	99.56	99.31	99.13	99.69	99.21	100.01	99.46
SD	0.930	1.104	1.530	0.811	1.225	0.840	1.400	1.251
n	5	5	5	5	5	5	5	5
Variance	0.874	1.218	2.340	0.650	1.488	0.708	1.960	1.560
SE	0.418	0.493	0.684	0.362	0.545	0.376	0.626	0.560
t- value(2.306)[c]	0.054	0.142	0.169		0.869	0.157	1.224	
F-value(6.400)[c]	1.784	1.280	1.500		2.290	1.089	3.020	

[a]Manufacturer's potentiometric method.
[b]Manufacturer's HPLC method.
[c]The values between parenthesis are the theoretical values of t and F at (P = 0.05).

Table 5: Application of standard addition technique for determination of idrocilamide by the proposed methods

Drug product	Proposed methods	Recovery %[a] of claimed amount ± RSD%	Standard added (µg/mL)	Recovery [a] % of standard added
Srilane cream (5 g Idro/100 g cream)	NBD-Cl	99.31 ± 1.544	1	99.12
			2	100.62
			4	101.32
			8	98.36
			Mean ± RSD%	99.85 ± 1.356
	Iodine	99.56 ± 1.104	25	100.36
			50	98.56
			100	100.99
			150	98.02
			Mean ± RSD%	99.48 ±1.425
	KI/KIO_3	99.42 ± 0.935	25	98.73
			50	101.07
			100	99.36
			150	98.52
			Mean ± RSD%	99.42 ± 1.162

[a] Average of four different determinations.

3.4.6. Ruggedness and robustness

The ruggedness of the proposed methods was assessed by applying the procedures using two different instruments in two different laboratories at different elapsed time. Results obtained from lab-to-lab and day-to-day variations were found to be reproducible as RSD did not exceed 2%. Robustness of the procedures was assessed by evaluating the influence of small variation of experimental variables: concentrations of reagents, and reaction time, on the analytical performance of the method. In these experiments, one experimental parameter was changed while the other parameters were kept unchanged, and the recovery percentage was calculated each time. The small variations in any of the variables did not significantly affect the results; recovery percentages were 99.25 - 100.59% ± 0.76 - 1.05. This provided an indication for the reliability of the proposed methods during routine work.

3.5. Application of the methods to the analysis of dosage form

The obtained satisfactory validation results made the proposed procedures suitable for the routine quality control analysis of ***Idro***. The proposed and reported methods were applied to the determination of ***Idro*** in its cream. The results obtained by the proposed methods were statistically compared with those obtained by the reported method. In the t and F tests, no significant differences were found between the calculated and theoretical values of both the proposed and the reported methods at 95% confidence level (Table 4). This indicated similar precision and accuracy in the analysis of ***Idro*** in its cream. It is evident from

these results that all the proposed methods are applicable to the analysis of ***Idro*** in its cream with comparable analytical performance.

Conclusion

The proposed methods are simple, selective and accurate spectrofluorimetric and spectrophotometric methods for determination of ***Idro*** in drug substance, product, and in the presence of its degradants. To our knowledge the methods have not been reported before for analysis of ***Idro***. The methods are with comparable analytical performance devoid from any potential interference. This gives the advantage of flexibility in performing the analysis on any available instrument. Furthermore the spectrofluorimetric method has range sensitive enough for suggesting the ability of application of the method in biological samples, where low concentration levels are found. These encourage their successful use in routine analysis of this drug in quality control laboratories.

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