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Kinetic Spectrophotometric Determination of Isoxsuprine in Dosage Forms Through Derivatisation with 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)

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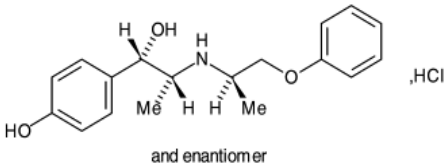
Abstract

A simple and sensitive kinetic spectrophotometric method was developed for the determination of isoxsuprine in pharmaceutical preparations. The method is based upon a kinetic investigation of the coupling reaction between isoxsuprine HCl and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) in borate buffer of pH 7.8 for a fixed time of 30 min. The absorbance of the yellow coloured product was measured at 395 nm. The absorbance- concentration plot was rectilinear over the range of 2-20 μg mL^{-1} (r= 0.9994) with minimum detectability of 0.6 μg mL^{-1} (1.75 $x10^{-6}$M). The different experimental parameters affecting the development and stability of the colour were carefully studied and optimized. The determination of isoxsuprine by the fixed-concentration and rate-constant methods is also feasible with the calibration equations obtained, but the fixed time method has been found to be more applicable. The proposed method was further applied to the determination of the drug in formulations. The results obtained were in good agreement with those obtained using a reference method. The reaction pathway was proposed.

Keywords

Kinetic determination, isoxsuprine HCl, (NBD-Cl), pharmaceutical preparations.

Introduction



Scheme 1: Structural Formula of Isoxsuprine HCl

Isoxsuprine (ISX), 4-Hydroxy-α-[1-[(1-methyl-2-phenoxy-ethyl)amino]ethyl] benzenemethanol, is a vasodilator that produces the effects of β-adrenoceptor stimulation and α-adrenoceptor antagonism; the former effect is the more predominant. It is used in the treatment of cerebral and peripheral vascular diseases. It is also used to arrest premature labor [1].

Several analytical methods have been reported for the determination of ISX in raw material, dosage forms and biological fluids. A good guide to the reported methods of this drug published up to 1997 is found in the comprehensive review written by Belal et al [2]. The more recent publications include spectrophotometry [3-6], polarography [7], HPLC [8,9]and LC-MS [10]. All these methods are either insufficiently sensitive [4,5] or tedious and require highly sophisticated and dedicated instrumentation [7-10]. This led us to study the reaction of ISX with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) to develop simple and sensitive spectrophotometric method for the determination of ISX in pharmaceutical preparations. ISX contains a secondary aliphatic amino group which is suitable for derivatization with chromogenic reagents such as NBD-Cl. The latter is a useful derivatizing agent for primary and secondary amines, thiol, etc. Several pharmaceutical compounds have been determined through this approach [11-16].

The literature is still poor in analytical procedures based on kinetics, especially for pharmaceuticals and biological fluids. However, some specific advantages in the application of kinetic methods can be expected such as: selectivity due to the measurement of the evolution of the absorbance with the time of the reaction instead of the measure of a concrete absorbance value [17]. Some pharmaceutical

compounds were determined through kinetic approach [18-20]. ISX was determined kinetically before through oxidation with alkaline potassium permanganate [20], however this method is not specific since $KMnO_4$ can oxidize both of ISX and its degradation products such as 4-hydroxybenzaldehyde, p-hydroxyacetophenone, 4-hydroxybenzylalcohol, and p-hydroxy benzoic acid. On the other hand the proposed method proved be specific for determination of ISX in the presence of its degradation products, and so could be considered as stability indicating method.

Experimental

Reagents and Materials

All chemicals used were of Analytical Reagent Grade. ISX HCl pure sample (Batch # 191401) was purchased from Sigma, (Saint Louis, MO, USA). Tablets containing the drug: Duvadilan tablets (product of Pharco Pharmaceuticals. Alexandria, Egypt, Batch # 198) labled to contain 20 mg each and Vascular tablets (product of South Egypt Drug Industries Co. 6 October City, A.R.E., Batch # 798140) labeled to contain 20 mg each were obtained from commercial sources in the local market.

•4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was purchased from Sigma (St. louis USA). A stock solution containing 1 $mg.mL^{-1}$ was freshly prepared in methanol.

• Borate buffer (0.2 M, pH 7.8).

• Methanol, AR grade (Aldrich, USA)

• HCl (Aldrich-Chemie, Germany).

Standard Solutions: A stock solution of ISX HCl was prepared by dissolving 20.0 mg of ISX HCl in 100 mL of distilled water and was further diluted with the same solvent as appropriate. The standard solutions were stable for seven days when kept in the refrigerator.

Apparatus

UV- VIS 1601, Shimadzu recording Spectrophotometer (P/ N 206-67001) equipped with kinetic accessory and provided with temperature controlled cell (TCC-240A). Recording range from 0 to 1.0 absorbance unit, wavelength (nm) 395, factor=1, number of cells 1, reaction time 30 min.

Procedures

Recommended Procedure

Transfer aliquots of ISX working standard solution into a series of 10 ml volumetric flasks so that the final concentration is in the range of 2- 20 μg mL^{-1}. Add 5 mL of borate buffer (pH 7.8), then add 1.1± 0.1 mL of 0.1% NBD-Cl and mix well. Heat at 60 ^{o}C ± 2 ^{o}C for 30 ± 2 min in a thermostatically-controlled water bath, then cool. Add 0.2 mL of HCl. Complete to the mark with distilled water. Measure the absorbance of the solution at 395 nm against a reagent blank. Plot the measured absorbance vs the final concentration to get the calibration curve. Alternatively, derive the corresponding regression equation.

Analysis of Commercial Tablets

Weigh and pulverize twenty tablets. Transfer a weighed quantity of the powder equivalent to 20 mg of ISX HCl into a small conical flask, extract with 3 x 30 mL of distilled water. Filter the extract into 100 mL volumetric flask. Wash the conical flask with few mLs of distilled water. Pass the washings into the same volumetric flask and complete to the mark with the same solvent. Transfer aliquots covering the working concentration range into 10 mL volumetric flasks. Proceed as described under Recommended Procedure. Determine the nominal content of the tablets either from the calibration curve or using the corresponding regression equation.

Results and Discussion

4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), as an electroactive halide reagent, was first introduced as an analytical reagent for the determination of some amines and amino acids [11]. In recent reports. NBD-Cl was further used as a chromogenic reagent for the determination of some primary and secondary amines [12-16]. In the present study, ISX was found to react with NBD-Cl in borate buffer of pH 7.8 producing a yellow color with maximum absorbance at 395 nm. (Fig. 1).

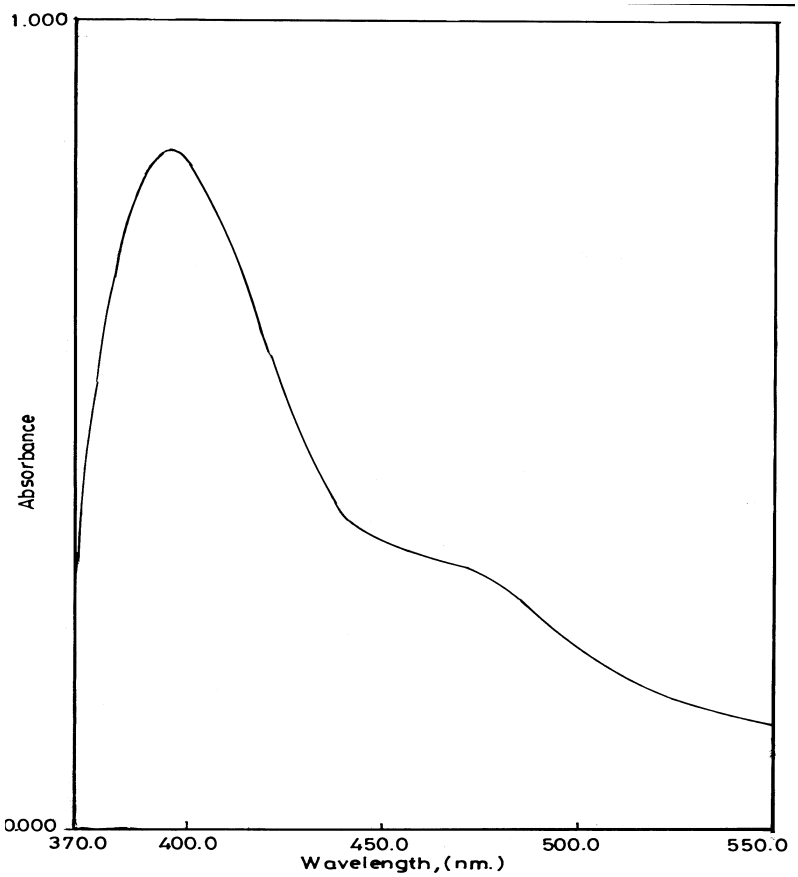


Fig. 1. Absorption spectrum of the reaction product of isoxsuprine HCl (20 µg mL^{-1}) with NBD-Cl at pH 7.8.

Study of experimental conditions

The spectrophotometric properties of the colored product as well as the different experimental parameters affecting the color development and its stability were carefully studied and optimized. Such factors were changed individually while the others were kept constant. The factors include pH, type of buffer, temperature, time of heating, effect of sensitizers and surfactants.

The influence of pH on the absorbance value of the reaction product was evaluated. Maximum absorbance value was obtained at pH 7.8 after which the absorbance of the reaction product began to decrease gradually until pH 9.5. Therefore, pH of 7.8 was chosen as the optimum pH (Fig. 2). Other buffers having the same pH value such as phosphate buffer and hexamine buffer were tried and compared with 0.2 M borate buffer. The borate buffer was found to be superior to the phosphate and hexamine buffers having the same pH value since it gave the highest absorbance value. This is probably, because the rate of hydrolysis of NBD-Cl to NBD-OH was much slower. This result is in agreement with that of Miyano et al. [21].

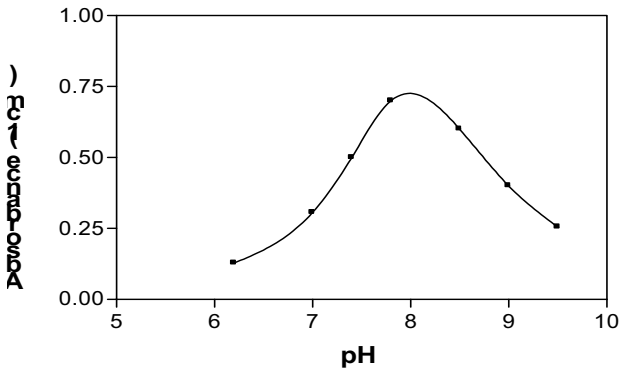


Fig. 2. Effect of pH on the absorbance value of the reaction product of isoxuprine HCl (16 μg mL^{-1}) with NBD-Cl

The influence of the concentration of NBD-Cl was studied using different volumes of 0.1% solution of the reagent. It was found that, the reaction of NBD-Cl

with ISX started upon using 0.2 mL of the reagent in the presence of borate buffer of pH 7.8. Increasing the volume of the reagent, produces a proportional increase in the absorbance of the reaction product up to 1 mL and remains constant till 1.2 mL, after which further increase produces a gradual decrease in the absorbance value. Therefore, 1.1 ± 0.1 mL of 0.1% of NBD-Cl solution was chosen as the optimal volume of the reagent (Fig. 3).

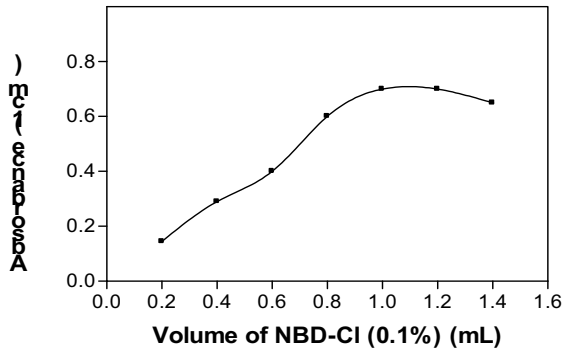


Fig. 3. Effect of volume of NBD-Cl (0.1%) on the absorbance value of the reaction product of isoxuprine HCl (16 mg mL^{-1}) at pH 7.8.

Different temperature settings were used with constant heating time. Increasing the temperature was found to produce a proportional increase in the absorbance of the reaction product up to boiling. It was found that heating at a relatively lower temperature (60^{o}C) for longer period (30 min) was better than heating at a higher temperature for a shorter period of time, regarding the reproducibility of the absorbance.

The time of heating is an essential part of the experiment.

Different time intervals were tested to ascertain the time after which the solution attained its highest absorbance value. It was found that after 30 min, the reaction product reached the highest absorbance value (Fig. 4). It was observed that heating time for 30 min is adequate and the absorbance value of the reaction product is stable for about 40 min. at room temperature.

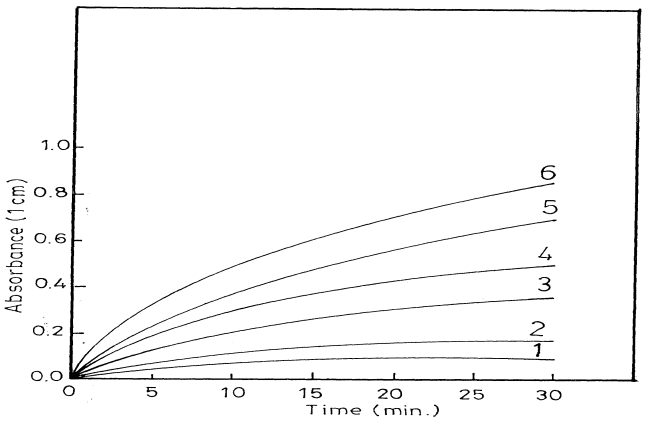


Fig. 4 . Effect of time on the absorbance of isoxsuprine HCl

(1) 5.920 x 10^{-6} M

(2) 1.184 x 10^{-5} M

(3) 2.368 x10^{-5}M

(4) 3.552 x 10^{-5} M

(5) 4.736 x 10^{-5} M

(6) 5.920x10^{-5}M.

Acidification of the reaction mixture prior to measurement of the absorbance Value remarkadly decreased the background absorbance due to the formation of NBD-OH without affecting the drug-reagent adduct, hence the sensitivity was increased. Therefore, the absorbance of the hydrolysis product of NBD-Cl, namely, 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH) is quenched by decreasing the pH of the reaction medium to less than 1.

Preliminary attempts had been carried out to enhance the quantum yeild by the addition of some sensitizers and surfactants, Different sensitizers such as quinine, fluorescein and rhodamine-B, at concentrations of 5 µg mL^{-1} were tested by adding tothe reactants mixture before heating. Outstanding inhibitory effects were observed (Tab. 1) as these sensitizers reacted with NBD-Cl.

In the same manner, the effect of surfactants on the color development was studied. Different sufactants (cetrimide, gelatine and sodium lauryl sulphate) at three different concentrations 2.5, 7.5 and 15 μg mL^{-1}, were tested by adding to the reaction mixture prior to heating. All tested surfactants reacted with NBD-Cl producing an inhibitory effect, as evident from low absorbance reading. The results obtained are given in Table 1.

Surfactant / Sensitizer	Concentration (μg mL^{-1})	Absorbance
No surfactant	0	0.420
Cetrimide	2.5	0.430
Sodium lauryl sulfate	2.5	0.247
Gelatin	2.5	0.333
Cetrimide	7.5	0.214
Sodium lauryl sulfate	7.5	0.314
Gelatin	7.5	0.297
Cetrimide	15	0.230
Sodium lauryl sulfate	15	0.300
Gelatine	15	0.316
No sensitizer	0	0.420
Quinine	5	0.292
Fluorescein	5	0.421
Rhodamine-B	5	0.390

Tab 1: Effect of surfactants and sensitizers on the performance of the proposed method after heating at 60 ^{o}C for 30 min

Analytical Performance

The rate of the reaction was found to be concentration-dependent. The rate of the reaction was followed at 60 ^{o}C with various concentrations of the drug in the

range of 2-20 μg mL^{-1}, keeping NBD-Cl and borate buffer concentrations constant (Fig. 5).

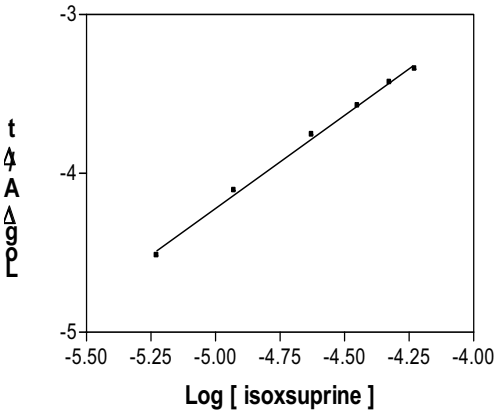


Fig. 5. plot of Log ΔA/ Δt (reaction rate) versus log [isoxsuprine]

The reaction rate was found to obey the following equation:

$$\text{Rate} = K` [\text{ ISX. HCl}]^n \quad (1)$$

where k`is the pseudo-order rate constant and n is the order of the reaction.

The rate of the reaction may be estimated by the variable-time method [22] as ΔA/Δt, where A is the absorbance and t is the time in seconds. Taking logarithms of rates and concentrations (Table 2) equation (1) is transformed into:

$$\text{Log (rate)} = \text{Log } \Delta A/\Delta t = \text{Log } K` + n \log [\text{ISX. HCl}] \quad (2)$$

Regression of log (rate) versus log [ISX. HCl] gave the regression equation;

$$\text{Log rate} = 1.638 + 1.172 \log C \quad (r = 0.9984)$$

Hence K` =43.45 Sec^{-1} and the reaction is first order (n =1.17) with respect to isoxsuprine concentration.

Log ΔA/Δ t	Log [ISX. HCl]
-4.519	-5.227
-4.109	-4.927
-3.757	-4.626
-3.575	-4.449
-3.429	-4.325
-3.344	-4.227

Tab. 2. Logarithms of rates for different concentrations at room temperature at 395 nm.

Evaluation of the kinetic methods:

The quantitation of ISX. HCl under the optimized experimental conditions outlined above, would result in a pseudo-first order with respect to its concentrations. However, the rate will be directly proportional to isoxsuprine concentration resulting in a pseudo-first order rate equation as follows:

$$\text{Rate} = K` [\text{ ISX. HCl }] \qquad (3)$$

where K` is the pseudo-first order rate constant. Several experiments were then carried out to obtain isoxsuprine concentration from the rate data according to equation (3).

Initial rate, rate constant, fixed concentration and fixed time methods [23-24] were attempted and the most suitable analytical method was selected taking into account the applicability, the sensitivity, the intercept and the correlation coefficient (r).

The Rate constant method:

Graphs of log absorbance versus time for ISX. HCl concentration in the range of 1.184×10^{-5} to 5.920×10^{-5} M were plotted and all appeared to be

rectilinear. Pseudo-first order rate constant (K`) corresponding to different ISX. HCl concentrations (C) were calculated from the slopes multiplied by –2.303 and are presented in Table 3. Regression of (C) versus K` gave the following equation:

K` = -3.776 x 10-4 + 3.683 C (r = 0.9841)

K` (S^{-1})	[ISX. HCl]
3.455×10^{-4}	1.184×10^{-5} M
2.879×10^{-4}	2.368×10^{-5} M
2.303×10^{-4}	3.552×10^{-5} M
1.974×10^{-4}	4.736×10^{-5} M
1.727×10^{-4}	5.920×10^{-5} M

Tab. 3. Values of K` calculated from slopes of log A versus t graphs at 395 nm.

The Fixed concentration method:

Reaction rates were recorded for different ISX. HCl concentrations in the range of $2.368 \times 10^{-5} – 5.920 \times 10^{-5}$ M. A preselected value of the absorbance (0.3) was fixed and the time was measured in seconds. The reciprocal of time (1/t) versus the initial concentration of ISX. HCl (Table 4) was plotted and the following equation of the calibration graph was obtained:

$1/t = -7.456 \times 10^{-4} + 67.465$ C (r = 0.9843)

1/t (S^{-1})	[ISX. HCl]
7.937×10^{-4}	2.368×10^{-5} M
1.852×10^{-3}	3.552×10^{-5} M
2.222×10^{-3}	4.736×10^{-5} M
3.333×10^{-3}	5.920×10^{-5} M

Tab. 4. Values of reciprocal of time taken at fixed absorbance for different rates of variable concentrations of ISX. HCl at constant concentrations of NBD-Cl and borate buffer.

The Fixed-time method:

Reaction rates were determined for different concentrations of ISX. HCl. At a pre -selected fixed-time, which was accurately determined, the absorbance was measured. Calibration graphs of absorbance versus initial concentration of ISX. HCl were established at fixed times of 5, 10, 15, 20 and 30 min. with regression equations assembled in Tab. 5.

Time (min.)	Regression equation	Correlation Coefficient (r)
5	$A = 5.096 \times 10^{-3} + 0.015\ C$	r = 0.9948
10	$A = 1.848 \times 10^{-3} + 0.027\ C$	r = 0.9985
15	$A = 2.792 \times 10^{-3} + 0.032\ C$	r = 0.9972
20	$A = -7.826 \times 10^{-3} + 0.036\ C$	r = 0.9993
30	$A = 1.562 \times 10^{-4} + 0.043\ C$	r = 0.9994

Tab. 5. Regression equation for ISX. HCl at different fixed time over the range 5.920×10^{-6} to 5.920×10^{-5} M at 395 nm.

It is clear that, the slope increases with time and the most acceptable values of the correlation coefficient (r) and the intercept were obtained for a fixed time of 30 min, which was therefore chosen as the most suitable time interval for measurement.

After optimizing the reaction conditions, the fixed time method was applied to the determination of isoxsuprine in pure form over the range of 2-20 μg mL^{-1}. Analysis of the data gave the following equations:

$A = 1.562 \times 10^{-4} + 0.043\ C$ (r = 0.9994)

where A is the absorbance at 395 nm.

and C is the concentration in μg mL^{-1}.

The limit of quantification (LOQ) was determined by establishing the lowest concentration that can be measured with acceptable accuracy and precision and was found to be 2 μg mL^{-1}.

The limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected and was found to be 0.6 μg mL^{-1} (1.75×10^{-6} M).

Validation of the method

The method was tested for linearity, specificity, precision and reproducibility.

Linearity:

By using the above spectrophotometric procedures, linear regression equations were obtained. The regression plots showed that there was a linear dependence of the absorbance value on the concentration of the drug over the range of 2-20μg mL^{-1}. The validity of the method was evaluated by Statistical evaluation of the regression lines. It was found that the standard deviation of the residuals ($S_{y/x}$) is 8.471×10^{-3}, the standard deviation of the intercept (S_a) is 5.024×10^{-3} and standard deviation of the slope (S_b) is 5.430×10^{-4}. The small values of the

figures point out to the high precision of the proposed method and low scattering of the points of the calibration curve and high accuracy.

The proposed method was applied to the assay of a pure sample of ISX. HCl. The results obtained by the proposed method were compared with those given by a reference method [25]. Statistical analysis [26] of the results obtained by both methods using Student's t- test and variance ratio, F- test, reveals no significant difference in the performance of the two methods, regarding the accuracy and precission, respectivily (Tab.6).

Precision and Repeatability:

The within days precision was evaluated through replicate analysis of sample spiked with 16 µg mL^{-1}. The percentage recoveries based on four separate determinations ranged from 98.90 to 99.88 with mean percentage recovery of 98.96 $\pm$ 0.88, thus indicating the high precision of the method.

The inter-day precision on four successive days was evaluated through replicate analysis of sample spiked with 16 µg mL^{-1}. The percentage recoveries based on the average of four separate determinations were 98.92 $\pm$ 0.93.

Specificity of the proposed method

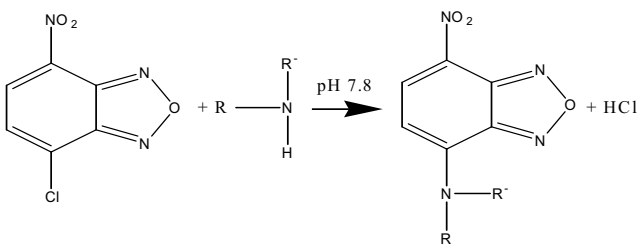
The major degradation products of ISX. HCl are: 4-hydroxybenzaldehyde, p-hydroxyacetophenone, 4-hydroxybenzyl alcohol, and p-hydroxybenzoic acid [27] did not interfere with the determination of ISX by the proposed method, since the main reaction depends on the presence of secondary aliphatic amine. So, the proposed method proved to be specific for intact molecule in the presence of its degradation products . Hence the proposed method can be considered as stability indicating assay for the determination of ISX in the presence of its degradation products. Additive excipients commonly incorporated in tablet formulations did not interfere with the proposed method as experimentally checked (Tab. 6).

Pharmaceutical applications

The proposed method was further applied to the determination of ISX in its tablets dosage form. Common tablets excipients such as talc, lactose, starch, avisil, gelatine and magnesium stearate did not interfere with the assay. The results obtained were compared with those given using a reference method [25]. Statistical analysis [26] of the results using Student's t-test and variance ratio F-test, revealed no significant difference between the two methods at the 95 % confidence level regarding accuracy and precision, respectively. The results obtained are abridged in Tab. 6.

Mechanism of the reaction

The stoichiometry of the reaction was studied adopting the limiting logarithmic method [28]. The absorbance of the reaction product was alternatively measured in the presence of excess of NBD-Cl and ISX. A plot of log absorbance versus log [NBD-Cl] and log [ISX] gave straight lines, the values of the slopes are 0.980 and 1.006 respectively (Fig. 6). Hence, it is concluded that, the molar reactivity of the reaction is 0.980 / 1.006,i.e. the reaction proceeds in the ratio of 1: 1, confirming that one molecule of the drug denses with one molecule of NBD-Cl . The drug reacts via the secondary amino group with the chlorine atom of the reagent with the liberation of hydrochloric acid. Based on the obtained molar reactivity, the reaction pathway is proposed to proceed as follows:



Scheme 2: Proposal reaction pathway between NBD-Cl and ISX

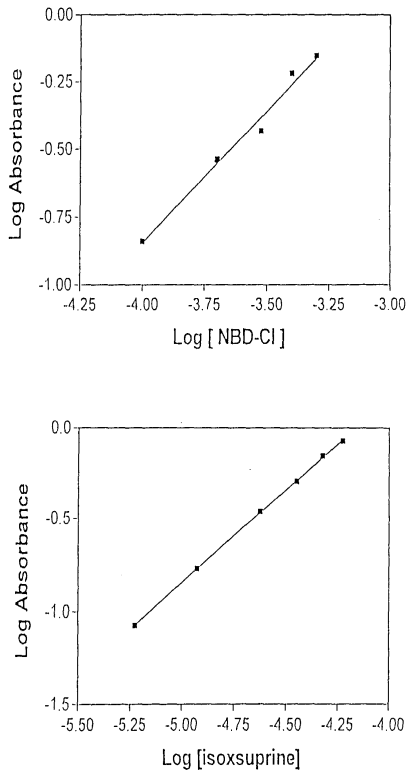


Fig. 6 . Limiting logarithmic plots for the molar ratio.

(A) Log A vs. Log [NBD-Cl] (B) Log A vs. log [isoxsuprine]

Conclusion

Different methods were established to determine isoxsuprine concentration kinetically, the reaction rate method, rate constant and fixed time methods were applied. Applying the fixed time method, it is clear that the slope increased with time and the most acceptable values of correlation coefficients (r) and intercepts were obtained for a fixed time of 30 min. which was therefore chosen as the most suitable time interval for measurements. The proposed method is selective for the determination of the drug in the presence of its degradation products.

preparations	µg taken	µg found	% recovery	Reference method [25]
*ISX HCl (pure form)	2.00	1.961	98.05	
	4.00	3.950	98.75	
	8.00	8.019	100.24	
	12.00	11.834	98.62	
	16.00	16.275	101.72	
	20.00	19.648	98.24	
-Mean found (%) ±SD.			99.27 ±1.43	99.89 ±0.78
-Variance.			2.04	0.61
-Student's t-Value.			0.69 (2.37)	
-Variance ratio F-test.			3.36 (5.79)	
** ISX HCl (Dosage Forms)				
1-Vascular tablets[a]	4.00	3.978	99.45	100.25
(ISX.HCl, 20 mg/tablet)	8.00	8.068	100.85	99.75
	12.00	11.971	99.76	100.08
	16.00	16.066	100.41	
$\bar{X}$ ± SD.			100.12 ± 0.63	100.03± 0.25
t-value			0.10 (2.57)	
F-value			6.29 (9.55)	
2-Duvadilan tablets[b]	4.00	3.971	99.28	100.05
(ISX.HCl, 20 mg/tablet)	8.00	7.961	99.51	99.79
	12.00	11.971	99.76	99.96
	16.00	16.014	100.09	
$\bar{X}$ ± SD.			99.66 ± 0.35	99.93 ± 0.13
t-value			1.79 (2.57)	
F-value			7.25 (9.55)	

Tab. 6. Application of the proposed method to the determination of ISX HCl in in pure form and dosage forms using fixed time method.

N.B.:

Figure in parentheses are the tabulated values of t and F respectively at p = 0.05 [26]

[a] is product of South Egypt Drug Industries Co. 6 October City, A.R.E.

[b] is product of Pharco Pharmaceuticals. Alexandria, Egypt.

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