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Use of Validated Stability-Indicating Chromatographic Methods for Quantitative Analysis of Idrocilamide in a Pharmaceutical Formulation

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Summary. Two sensitive and selective chromatographic methods have been developed and validated for analysis of idrocilamide in the presence of its degradation products. Forced degradation studies were performed using HCl, NaOH, and 3% H_2O_2. The first method is based on thin-layer chromatographic separation of the intact drug from its degradation products, followed by densitometric measurement. The second method is based on isocratic reversed phase high-performance liquid chromatographic separation of the drug from its degradation products on a C_{18} column. The HPLC method was used to investigate the kinetics of alkaline degradation of the drug at different temperatures.

Key Words: idrocilamide, TLC–densitometry, HPLC, stability studies, kinetics

Introduction

Idrocilamide (Idro; *N*-(2-hydroxyethyl)-3-phenyl-2-propenamide [1]) is a centrally acting muscle relaxant with anti-inflammatory effect [2–5]. Liquid chromatography with UV detection was used to investigate the pharmacokinetics of Idro in both rabbits and human plasma [6, 7].

The International Conference on Harmonization (ICH) guidelines recommend stress testing to elucidate the inherent stability of an active substance. Acid, alkaline, and oxidative stability should be assessed [8, 9]. Because no methods have been published for analysis of Idro in the presence of its degradation products, the objective of this work was to establish densitometric TLC and HPLC methods for this purpose. The HPLC method was also used to investigate the kinetics of the alkaline degradation process.

Experimental

Materials and Reagents

Idro, certified to contain 99.60%, was kindly supplied by Mina-Pharm (Cairo, Egypt). Srilane cream was manufactured by Mina-Pharm under license from Merck, Lyon, France. The drug product contains 5 g Idro per 100 g cream. 2-Aminoethanol (Deg II; labeled to contain 98.00%) was purchased from Merck (Munich, Germany). Other chemicals used were HPLC or analytical grade. Methanol, ethanol, and acetonitrile were from Lab-Scan (Eire). Ninhydrin was from Sigma (USA), chloroform and hydrochloric acid from Fischer Scientific (UK), and toluene, ammonia, orthophosphoric acid, hydrogen peroxide, and sodium hydroxide from Adwic (Egypt**)**.

Deg I and Deg III were isolated by preparative TLC (which enables isolation of quantities from 1–50 μg).

Water for HPLC was prepared by double glass distillation and filtration through a 0.47 μm membrane filter (Alltech Associates, USA).

Densitometric TLC Method

TLC was performed on 20 × 20 cm^2 aluminum-foil plates precoated with 0.2 mm layers of silica gel F_{254} (Macherey–Nagel, Germany). Samples were applied to the plates with a 25-μL Hamilton micro-syringe. For detection and quantification 10 μL each of the sample solution and standard solutions were applied. Ascending development of the plates, with toluene–methanol–chloroform–10% ammonia 5:3:6:0.1 (v/v) as mobile phase, was performed in the usual way. After development the plates were air-dried then scanned at 274 nm by means of a Shimadzu (Tokyo, Japan) model CS-9301 PC dual-wavelength flying-spot densitometer in reflection photo mode, zigzag scan mode, and swing width = 10.

Isocratic HPLC Method

HPLC was performed with Waters (USA) equipment comprising model 600 LC series pump, model 600 controller unit, model 486 tunable absorbance detector, injection valve with 20-μL constant-volume loop, and model 746 data module. Compounds were separated on a 25 cm × 4.6 mm, 5-μm particle, Luna C_{18} column from Phenomenex (CA, USA). The mobile phase was methanol–acetonitrile–water 60:10:30 (v/v) adjusted to pH 4 with orthophosphoric acid. The flow rate was 1 mL min^{-1}. All analyses were performed at ambient temperature. Detection was at 274 nm.

Acid, Alkali, and Hydrogen Peroxide-Induced Degradation

Idro (50 mg, accurately weighed) was placed in three round-bottomed flasks containing 50 mL 5 M HCl, 2 M NaOH, or 3% H_2O_2 and heated under reflux for 2, 3, or 24 h, respectively, at 100°C. Each solution was then neutralized, evaporated to approximately 10 mL, quantitatively transferred to a 50 mL volumetric flask, and diluted to volume with distilled water.

Degradation products were identified by comparison with standards and by use of FT-IR and mass spectrometry, performed in the Micro Analytical Center, Cairo University. FT-IR spectra in the range 4000–400 cm^{-1} were recorded on a Bruker Instruments (Rheinstetten/Karlsruhe, Germany) Vector 22 spectrometer, using KBr pellets. Mass spectrometry was performed with a Shimadzu GCMS-QP-1000 EX quadrupole spectrometer. EI ionization was performed with an electron energy of 70 eV. The ion-source temperature was 200.00°C, ACQ mode was scan, and scan speed was 769 U s^{-1}.

Standard Solutions and Calibration Graphs

Standard solutions were prepared by diluting a stock solution (1 mg mL^{-1}) of the drug with methanol for TLC and with mobile phase HPLC. The concentration ranges were 25–1000 μg mL^{-1} and 0.02–3 μg mL^{-1} for TLC and HPLC, respectively. After chromatography, average peak areas were calculated and plotted against concentration.

Laboratory-Prepared Mixtures

Laboratory-prepared mixtures of Idro and its degradation products in the concentration ranges 10–90% for TLC and 1–99% for HPLC were prepared and analyzed, and percentage recovery was calculated.

Application to Pharmaceutical Formulation

An accurately weighed amount of cream equivalent to 100 mg Idro was dissolved in 80 mL methanol and stirred with a magnetic stirrer for 15 min. The solution was transferred quantitatively to a 100-mL volumetric flask and diluted to volume with methanol. The procedure was then completed as described under construction of calibration graphs.

Kinetic Investigation of Alkali-Induced Degradation

Idro (50 mg, accurately weighed) was dissolved in 50 mL 2 M NaOH, and 5-mL volumes were separately transferred to a series of stoppered test tubes which were placed in a thermostatic water bath at different temperatures (90, 80, 70, 60, and 50°C) for different times. The contents of each tube were then neutralized and transferred to 50 mL volumetric flasks and diluted to volume with mobile phase. The same Idro solution (0.1 mL) was separately transferred to a 10-mL volumetric flask and diluted to volume with mobile phase. All the solutions were then analyzed by the HPLC method.

Results and Discussion

Densitometric TLC Method

The experimental conditions mobile phase, scan mode, and wavelength were optimized. Complete resolution was achieved by use of toluene–methanol–chloroform–10% ammonia 5:3:6:0.1 (*v*/*v*) *(Fig. 1)*.

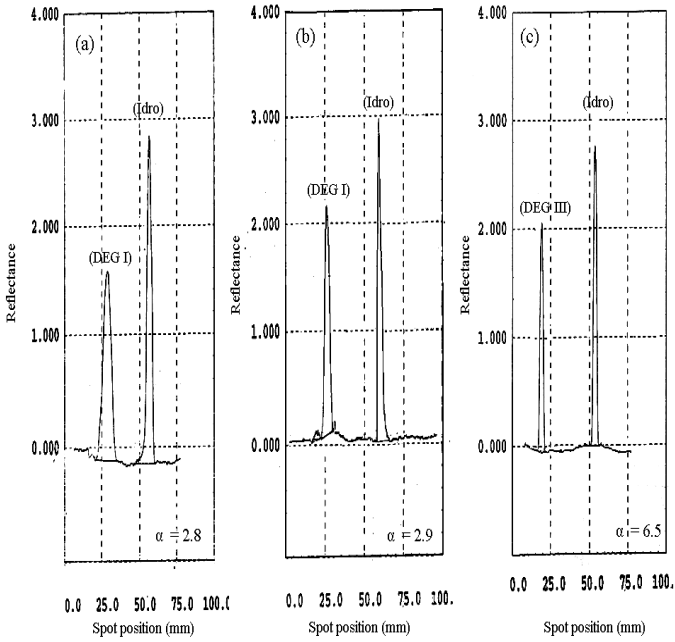

Fig. 1. Chromatograms obtained from mixtures of 10 μg per spot idrocilamide and 5 μg per spot of its degradation products after (a) acidic, (b) alkaline, and (c) oxidative degradation. α = selectivity

Table I. Assignment of IR and MS spectral data of idrocilamide and its degradation products[a]

Intact Idro	Deg I	Deg III
IR data		
Band at 3375 cm^{-1} (νN–H) of amide group	Band at 3443 cm^{-1} (νO–H) of COOH	Band at 3391 cm^{-1} (νO–H) of COOH
Band at 3301 cm^{-1} (νO–H) alcohol	Band at 1640 cm^{-1} (νC=O) of COOH	Bands at 1654 and 1703 cm^{-1} (νC=O) and (N–H) of amide group
Bands at 1605 and 1653 cm^{-1} (νC=O) and (νN–H) of amide group		Band at 1871 cm^{-1} (νC=O) of COOH
		Band at 3567 cm^{-1} (νN–H) of amide group
MS data		
m/z = 191, M^+ corresponding to $C_{11}H_{13}NO_2$	m/z = 149, $[M + H]^+$ corresponding to $C_9H_8O_2$	m/z = 206, $[M + H]^+$ corresponding to $C_{11}H_{11}NO_3$

[a]Deg II, 2-aminoethanol, is a volatile liquid and could not be isolated for IR and MS analysis

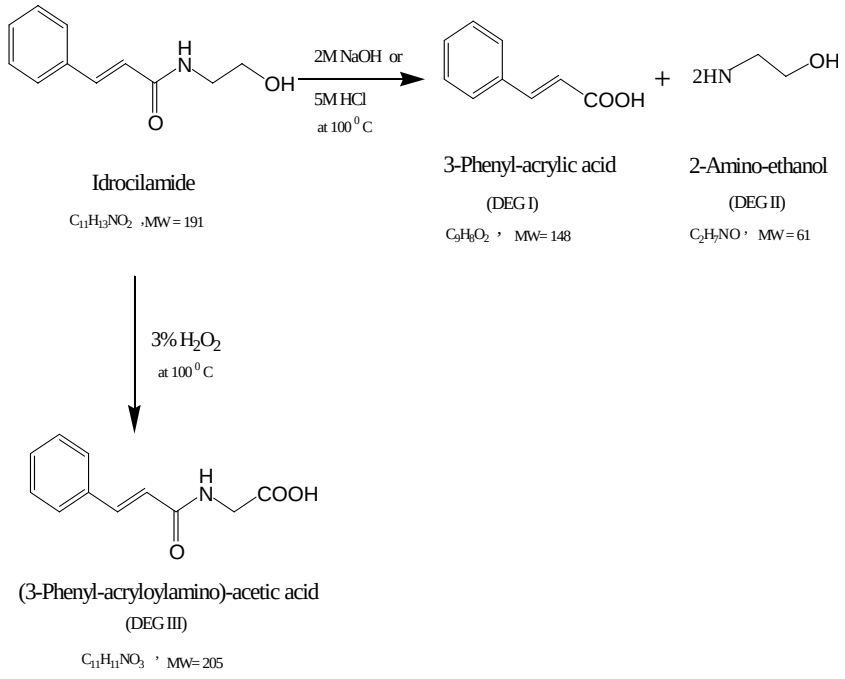

Fig. 2. Suggested pathways to the degradation products of idrocilamide in 5 M HCl, 2 M NaOH, and 3% H_2O_2

Two spots of degradation products were obtained after acidic or alkaline degradation – 3-phenylacrylic acid (DEG I), R_F 0.21, and 2-aminoethanol (DEG II), R_F zero. One degradation product was obtained after oxidative degradation – 3-(3-phenylacryloylamino)propionic acid (DEG III), R_F 0.09. The R_F of Idro was 0.59. 2-Aminoethanol was detected by use of ninhydrin [10] and identified by use of a reference standard. FT-IR and MS data for the degradation products are listed in *Table I*. Suggested pathways to the degradation products are presented in *Fig. 2*.

Isocratic HPLC Method

To optimize the conditions used for the HPLC assay, several mobile phase compositions were tried. Satisfactory separation was achieved by using methanol–acetonitrile–water 60:10:30 (*v*/*v*), pH 4 adjusted with orthophosphoric acid as mobile phase. Increasing the acetonitrile concentration to more than 20% led to inadequate separation of Idro from its degradation products. Reducing the acetonitrile concentration to <5% resulted in separation with excessive tailing and increased retention time. The specificity of the HPLC method is illustrated in *Fig. 3*.

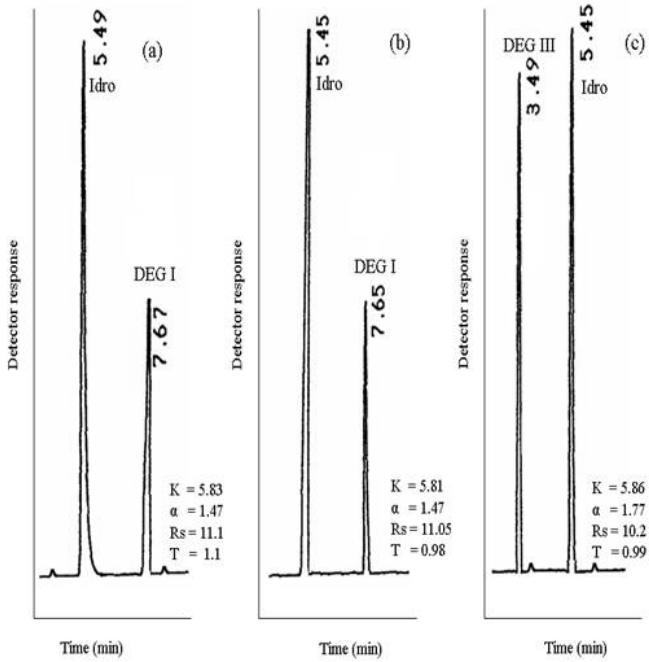

Fig. 3. Chromatograms obtained from mixed solutions (1 μg mL^{-1}) of idrocilamide and the degradation products obtained after treatment with acid (a), alkali (b), and hydrogen peroxide (c). *K* is the capacity factor, α the selectivity, R_S the resolution, and *T* the tailing factor

Method Validation [11, 12]

The methods were validated in accordance with ICH guidelines by documenting their linearity, accuracy, precision, and limits of detection and quantification *(Table II)*. Analysis of different concentrations of the drug showed the linearity of the methods was good. Accuracy was based on mean measured concentrations (n = 6) as a percentage of the actual concentration *(Table III)*. The results obtained were compared statistically with those obtained from other reported methods and no significant difference

Table II. Results from validation of the TLC and HPLC methods for analysis of idrocilamide in drug substance

	HPLC	TLC
Linear range	0.02–3.00 μg mL^{-1}	0.25–10.00 μg per spot
Accuracy[a]		
Mean ± RSD%[b]	99.74± 1.73	99.88 ± 1.75
Mean ± RSD%[c]	99.56 ±1.08	99.36 ± 0.71
Precision		
Intra-day RSD (%)[d]	0.45	0.65
Inter-day RSD (%)[d]	0.20	1.15
LOD	8.7 × 10^{-4} μg mL^{-1}	0.006 μg per spot
LOQ	2.6 × 10^{-4} μg mL^{-1}	0.042 μg per spot
Specificity (mean ± RSD, %)	100.30 ± 1.11	99.53 ± 0.97
Regression equation[e]		
Slope	28.1 × 10^4	9.64 × 10^2
SE of the slope	37.03 × 10^2	12.80
Confidence limit of slope[f]	27.21 × 10^4–29.02 × 10^4	9.31 × 10^2–9.97 × 10^2
Intercept	12.6 × 10^3	1.95 × 10^3
SE of intercept	29.99 × 10^2	72.01
Confidence limit of intercept[f]	5.39 × 10^3–20.07 × 10^3	17.69 × 10^2–21.4 × 10^2
Correlation coefficient (r)	0.9991	0.999

[a] n = 6
[b] Drug substance
[c] Drug product
[d] n = 9
[e] $Y = a + bC$, where C is the concentration in μg per spot for TLC and μg mL^{-1} for HPLC, and Y is the peak area
[f] 95% confidence limit

was found *(Table IV)*. Precision was assessed by determining RSD (%) values for intra-day and inter-day analysis (n = 9) over three days. RSD values were <2%. The limits of detection and quantification were estimated as the amounts for which signal-to-noise ratios were 3 and 10, respectively.

Table III. Accuracy of the TLC and HPLC methods for analysis of idrocilamide in drug substance and drug product

HPLC			TLC		
Conc. taken (μg mL^{-1})	Drug substance	Drug product	Conc. taken (μg per spot)	Drug substance	Drug product
	Recovery (%)[a]			Recovery (%)[a]	
0.06	98.38	101.5	1.5	99.93	99.201
0.12	98.05	98.7	3	98.28	99.501
0.14	99.54	99.3	5	102	98.702
0.2	101.35	99.1	7	101.26	100.50
0.7	101.96	99.2	9	98.11	98.90
Mean± RSD%	100.05± 1.58	99.65±0.55	Mean± RSD%	99.11±1.74	99.36± 0.705

[a]Average from four different experiments. For drug product, recovery is given as a percentage of the amount claimed

Table IV. Statistical comparison of results obtained by use of the proposed TLC and HPLC methods and the reported method for analysis of idrocilamide in drug substance

	Reported method[a]	HPLC	TLC
Mean	99.13	100.05	99.11
SD	0.805	1.58	1.747
n	5	5	5
Variance	0.65	2.49	3.05
SE	0.36	0.706	0.781
t (2.306)[b]		0.834	0.872
F (6.400)[b]		3.83	4.709

[a]Potentiometric assay (using acetic anhydride in pyridine, 15% by volume as solvent and 1 M sodium hydroxide as titrant) obtained from Mina Pharm (Egypt) registration file by personal communication

[b]The values in parentheses are the theoretical values of t and F for P = 0.05

The specificity of the methods was assessed by analyzing a synthetic mixture of Idro with its degradation products in different proportions ranging from 10–90% and from 1–99% for the TLC and HPLC methods, respectively. Percentage recovery was calculated. RSD was less than 2% *(Table V)*.

Table V. Specificity of the TLC and HPLC methods for analysis of laboratory-prepared mixtures of idrocilamide and its degradation products

HPLC		TLC	
Degradation (%)	Idrocilamide recovery (%)[a]	Degradation (%)	Idrocilamide recovery (%)[a]
1	100.15	10	98.53
10	98.76	20	99.15
30	99.15	40	100.98
50	100.56	60	100.34
70	101.77	80	99.59
99	100.31	90	98.66
Mean ± RSD (%)	100.30 ± 1.11	Mean ± RSD (%)	99.53 ± 0.97

[a]Average, $n = 4$

The robustness of the methods was assessed by evaluating the effect of small changes of the experimental conditions, for example organic solvent strength, pH, and flow rate, by measurement of chromatographic resolution. The results indicated the method was not affected by small changes in the conditions used, indicating the reliability of the methods during routine work.

The solutions were stable for 14 days when stored in tightly capped volumetric flasks, protected from light on a laboratory bench and under refrigeration.

System-suitability test data (capacity factor, K, selectivity, α, resolution, R_S, and tailing factor, T) were calculated to ensure the system was working correctly during analysis. The results are shown in *Fig. 3*.

Analysis of Pharmaceutical Formulation

The methods were used for analysis of the drug in its pharmaceutical formulation. Satisfactory results were obtained *(Table III)*. Statistical comparison of results from the proposed and from a reported method indicated there was no significant difference *(Table IV)*.

Kinetic Investigation

The effect of temperature on alkaline degradation was studied. Between 50 and 90°C the degradation process followed pseudo first-order kinetics. By plotting log K_{obs} values against $1/T$, Arrhenius plots were obtained which were linear in the selected temperature range (*Fig. 4*). From the slope of the straight line, it was possible to calculate the apparent first-order degradation rate constant and the half-life at each temperature for alkaline degradation of Idro *(Table VI)*. The activation energy was calculated and found to be 13.1 kcal mol $^{-1}$. This value was suggestive of intermediate instability.

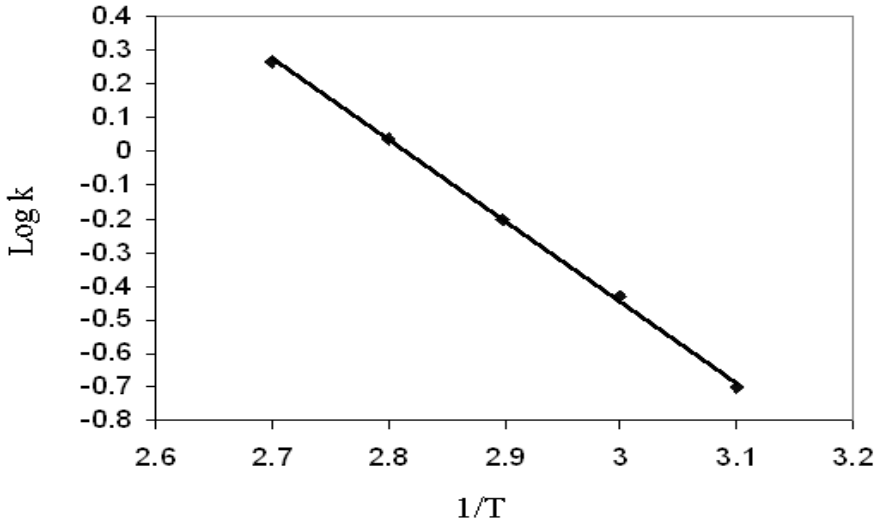

Fig. 4. Arrhenius plot for alkaline (2 M NaOH) degradation of idrocilamide

Table VI. Degradation rate constant (K_{obs}) and half-life ($t_{1/2}$) for idrocilamide in 2 M NaOH

$t_{½}$ (h)	K_{obs} (h^{-1})	Temperature (°C)
0.38	1.84	90
0.63	1.11	80
1.20	0.58	70
1.88	0.37	60
3.77	0.18	50

Conclusion

The TLC and HPLC methods proposed enable simple, accurate, and reproducible quantitative analysis of Idro in this pharmaceutical preparation without interference from excipients, and in the presence of the products from acidic, alkaline, and oxidative degradation products. These methods can be used for simple accelerated stability studies to predict the expiry dates of pharmaceuticals. Both methods complied with the validation guidelines of the International Conference on Harmonization and could be used for purity testing, stability studies, quality control, and routine analysis of idrocilamide.

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