Neeraj Kaul
Himani Agrawal
Pravin Maske
Janhavi Ramchandra Rao
Kakasaheb Ramoo Mahadik
Shivajirao S. Kadam

Department of Quality Assurance Techniques, Bharati Vidyapeeth Deemed University, Poona College of Pharmacy, Erandwane, Pune, Maharashtra State, India

Chromatographic determination of itopride hydrochloride in the presence of its degradation products

Two sensitive and reproducible methods are described for the quantitative determination of itopride hydrochloride (IH) in the presence of its degradation products. The first method is based on HPLC separation on a reversed phase Kromasil column [C_{18} (5-μm, 25 cm × 4.6 mm, ID)] at ambient temperature using a mobile phase consisting of methanol and water (70:30, *v/v*) adjusted to pH 4.0 with orthophosphoric acid with UV detection at 258 nm. The flow rate was 1.0 mL per min with an average operating pressure of 180 kg/cm^2. The second method is based on HPTLC separation on silica gel 60 F_{254} using toluene:methanol:chloroform:10% ammonia (5.0:3.0:6.0:0.1, *v/v/v/v*) as mobile phase at 270 nm. The analysis of variance (ANOVA) and Student's t-test were applied to correlate the results of IH determination in dosage form by means of HPLC and HPTLC methods. The drug was subjected to acid and alkali hydrolysis, oxidation, dry heat, wet heat treatment, UV, and photodegradation. The proposed HPLC method was utilized to investigate the kinetics of the acidic, alkaline, and oxidative degradation processes at different temperatures and the apparent pseudo-first-order rate constant, half-life, and activation energy were calculated. In addition the pH-rate profile of degradation of IH in constant ionic strength buffer solutions in the pH range 2–11 was studied.

Key Words: ANOVA; Student's t-test; Kinetics of degradation; pH-rate profile; Arrhenius plot; Activation energy

Received: January 27, 2005; revised: April 14, 2005; accepted: April 14, 2005

DOI 10.1002/jssc.200500047

1 Introduction

Itopride hydrochloride *N*-[*p*-2{2-(dimethylamino)ethoxy}-benzyl]varatramide hydrochloride (**Fig. 1**) occurs as white to pale yellowish white crystals or crystalline powder. It is very soluble in water, and freely soluble in methanol or in glacial acetic acid. It inhibits the enzyme acetylcholinesterase (AChE) and thereby prevents the hydrolysis of acetylcholine [1–3].

Very few analytical methods have been reported for its quantitative estimation. Mushiroda *et al.* [4] has reported an HPLC method with fluorescence detection. The limitation of this method is its use of a high ratio of buffer solution in the mobile phase that can cause damage to the reverse phase column and the elution time of itopride is more than 10 min. Takahara *et al.* [5] has reported an HPLC method for simultaneous determination of itopride (HSR-803) and its metabolites in human serum and urine using automated column switching.

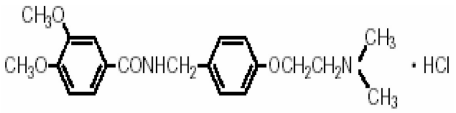

Figure 1. Structure of itopride hydrochloride.

The aim of the present work was to elucidate the inherent stability characteristics of the drug substance by subjecting it to the variety of suggested stress conditions as per International Conference on Harmonization (ICH) recommendations [6]. Therefore stability-indicating chromatographic methods are required for determination of IH in the presence of its degradation products and related impurities for assessment of purity of bulk drug and stability of its bulk dosage forms. The proposed HPLC and HPTLC methods were validated in compliance with ICH guidelines [7, 8] and its updated international convention [9]. Furthermore, the developed HPLC method was used to investigate the kinetics of the acidic, alkaline, and oxidative degradation processes by quantitation of drug at different temperatures, and to calculate the activation energy and half-life for IH degradation. The HPLC method was also utilized for pH-rate profile study of degradation of IH in constant ionic strength buffer solutions within the pH range 2–11 studied.

Correspondence: Prof. Janhavi Ramchandra Rao, Department of Quality Assurance Techniques, Bharati Vidyapeeth Deemed University, Poona College of Pharmacy, Erandwane, Pune-411038, Maharashtra State, India. Phone: +91 20 25437237. Fax: +91 20 25439383. E-mail:janhavirao@rediffmail.com.

2 Experimental

Pharmaceutical grade itopride hydrochloride (batch no: 5516680) was kindly supplied as a gift sample by Abbott India Ltd, Goa, India, and certified to contain 100.36% (*w/w*) on a dried basis. It was used without further purification. All other chemicals and reagents were of HPLC grade and were purchased from Merck Chemicals, India.

The HPLC system consisted of a pump (Jasco Model PU 1580 intelligent HPLC pump) with auto-injecting facility (AS-1555 sampler) programmed at 20 μL capacity per injection was used. The UV/VIS detector was a Jasco model UV 1575 operated at a wavelength of 258 nm. The software used was Jasco Borwin version 1.5, LC-Net II/ADC system. The columns used were Kromasil C-18 (250 mm × 4.6 mm, 5.0 μm) Flexit Jour Laborarories Pvt Ltd Pune, India and Finepak SIL-5, C-18 (250 mm × 4.6 mm, 5.0 μm) Jasco Corporation, Japan. The optimal composition of the mobile phase was determined to be methanol:water (70:30, *v/v*) adjusted to pH 4.0 with orthophosphoric acid. The flow rate was set to 1.0 mL/min and UV detection was carried out at 258 nm. The mobile phase and samples were filtered using a 0.45-μm membrane filter and degassed by ultrasonication prior to use. All determinations were performed at ambient column temperature.

In the HPTLC system the samples were applied as bands of 6-mm width using the spray-on technique with a Camag 100-μL sample syringe (Hamilton, Bonaduz, Switzerland) on a precoated silica gel aluminium plate 60 F-254 (20 × 10 cm) of 200-μm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a Camag Linomat IV applicator (Switzerland). A constant application rate of 0.1 μL/s was employed and the space between two bands was 6 mm. The slit dimension was kept at 5 × 0.45 mm and 10 mm/s scanning speed was employed. The mobile phase consisted of toluene:methanol:chloroform:10% ammonia (5.0:3.0:6.0:0.1 *v/v/v/v*) and 15 mL of mobile phase was used for chromatography. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25°C ± 2) and a relative humidity of 60% ± 5 in a humidity controlled chamber. The length of the chromatogram run was 9 cm. Subsequent to development, the TLC plates were dried in a current of air with the help of an air dryer in a wooden chamber with adequate ventilation. The flow of air in the laboratory was maintained unidirectional (laminar flow, towards exhaust). Densitometric scanning was performed on a Camag TLC scanner III in the reflectance-absorbance mode at 270 nm for all measurements and operated by CATS software (V 3.15, Camag).

2.1 Standard solutions and calibration graphs

Stock standard solution was prepared by dissolving 0.10 g of IH in 100 mL of methanol (1000 μg/mL). The standard solutions were prepared by dilution of the stock solution with methanol to reach a concentration range 10–100 μg/mL for HPLC and 50–1000 ng/μL for HPTLC.

2.2 Sample preparation

To determine the content of IH in conventional tablets (label claim: 50 mg IH per tablet), twenty tablets were weighed, their mean weight was determined, and they were finely powdered; powder equivalent to 50 mg IH was then weighed out. The equivalent weight of the drug was transferred into a 100 mL volumetric flask containing 50 mL methanol, sonicated for 30 min, and diluted to 100 mL with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min. Supernatant was taken and after suitable dilution the sample solution was then filtered using 0.45-μm filter (Millipore, Milford, MA). The above stock solution was further diluted to afford sample solutions at three different concentrations of 30, 60, 90 μg/mL and 200, 400, 800 ng/μL for HPLC and HPTLC, respectively.

2.3 Method validation

2.3.1 Precision

An amount of product powder equivalent to 100% of the label claim of IH was accurately weighed and assayed. System repeatability was determined by six replicate applications and six-fold measurement of a sample solution at the analytical concentration. The intra-day and inter-day variation for determination of IH was determined at three different concentration levels 300, 500, 700 ng/band and 30, 60, 90 μg/mL for HPTLC and HPLC, respectively.

2.3.2 Robustness

To evaluate the HPLC method robustness a few parameters were deliberately varied. Thus C_{18} columns from different manufacturers were used and the other parameters varied included the pH of the mobile phase, flow rate, percentage of methanol in the mobile phase, column temperature, and the amount of methanol of different lots. As described above, two analytical columns were used during the experiment. The robustness of the method was established at three different IH concentration levels, *viz.* 30, 60, 90 μg/mL. In HPTLC small changes were introduced in the mobile phase composition and the effects on the results were examined. Mobile phases having different compositions such as toluene:methanol:chloroform:10% ammonia (4.5:3.5:6.0:0.1, *v/v/v/v*), (5.5:2.5:6.0:0.1, *v/v/v/v*), (5.0:3.5:5.5:0.1, *v/v/v/v*), (5.0:2.5:6.5:0.1, *v/v/v/v*), (5.5:3.0:5.5:0.1, *v/v/v/v*), (4.5:3.0:6.5:0.1, *v/v/v/v*) and so on were tried and chromatograms were run. The amount of mobile phase, temperature, and relative humidity was varied in the range of ±5%.

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The plates were prewashed with methanol and activated at 110°C ± 5 for 2, 5, 7 min respectively prior to chromatography. Time from application to chromatography and from chromatography to scanning was varied from 0, 20, 40, and 60 minutes. The robustness of the method was determined at three different IH concentration levels, *viz.* 300, 500, 700 ng per band.

2.3.3 Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were respectively determined at a signal-to-noise ratio (*S/N*) of 3 and 10. LOD and LOQ were experimentally verified by diluting known concentrations of IH until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

2.3.4 Specificity

The specificity of the HPLC method was determined by complete separation of IH in the presence of its degradation products along with other parameters like retention time (t_r), capacity factor (k), tailing or asymmetry factor (T), etc.

The specificity of the HPTLC method was ascertained by analysing standard drug and sample. The band for IH in sample was confirmed by comparing the R_f and spectra of the band with that of a standard. The peak purity of IH was assessed by comparing the spectra at three different levels *i.e.*, peak start, peak apex, and peak end positions of the band.

2.3.5 Recovery studies

For both methods recovery studies was carried out by applying the method to a drug sample to which known amounts of IH corresponding to 80, 100, and 120% of the label claim had been added (standard addition method).

2.4 Forced degradation of IH

A stock solution containing 50 mg IH in 50 mL methanol was prepared. This solution was used for forced degradation to provide an indication of the stability indicating property and specificity of the proposed method. In all degradation studies the average peak area of IH after application (100 μg/mL for HPLC and 1000 ng/band for HPTLC) of seven replicates was obtained.

2.4.1 Preparation of acid- and base-induced degradation product

To 10 mL of methanolic stock solution, 10 mL each of 5N HCl and 5N NaOH were added separately. These mixtures were refluxed for 2 h at 70°C.

2.4.2 Preparation of hydrogen peroxide-induced degradation product

To 10 mL of methanolic stock solution, 10 mL of 6.0% *w/v* and 50% *w/v* of hydrogen peroxide was added separately. The solutions were heated in a boiling water bath for 10 min to completely remove the excess of hydrogen peroxide and then refluxed for 2.0 h at 70°C.

2.4.3 Dry heat and wet heat degradation product

The standard drug was placed in an oven at 80°C for 6 h to study dry heat degradation and the stock solution was refluxed for 2.0 h on a boiling water bath for wet heat degradation.

2.4.4 Photochemically and UV-degraded product

The photochemical stability of the drug was also studied by exposing the stock solution (1 mg/mL) to direct sunlight for 360 h on a wooden plank in the open air. The drug solution was also exposed to UV irradiation for 360 h in a UV stability chamber.

2.5 Detection of related impurities

Related impurities were determined by use of higher concentrations of the drug so as to detect and quantify them. IH (1000 mg) was dissolved in 100 mL of methanol, and this solution was referred to as sample solution (10 mg/mL). One mL of the sample solution was diluted to 100 mL with methanol and this solution served as standard solution (0.1 mg/mL). One microlitre of both the standard (100 ng/band) and the sample solution (10,000 ng/band) were applied to a TLC plate.

In order to detect and quantify the impurities present in IH using HPLC, low-concentration (5.0 μg/mL) and high-concentration (500.0 μg/mL) solutions of standard were prepared and 20 μL was injected in triplicate and the peak areas were correlated.

2.6 Kinetic investigation

An accurately weighed 100-mg portion of drug was dissolved in 100 mL of methanol. Separate 10-mL aliquots of this standard solution were transferred into separate 100-mL double-neck round-bottomed flasks and mixed respectively with 10.0 mL of 5.0 N NaOH, 5 N HCl, and 50% *w/v* hydrogen peroxide to obtain a final concentration of 500 μg/mL. The flasks were refluxed at different temperatures (40, 50, 60, 70, and 80°C) for acidic, basic, and oxidative degradation for different time intervals. At the specified time the contents of the flask were neutralized to pH 7.0 using predetermined volumes of 5.0 N HCl and 5.0 N NaOH and for oxidative degradation the excess of hydrogen peroxide was removed by heating on a water bath. The contents of the flask were quantitatively transferred to 50-mL volumetric flasks with the help of a micro-

syringe and appropriately diluted to volume with methanol and estimated by an HPLC method by one-point standardization using external standard. Each experiment was repeated three times at each temperature and time interval. Aliquots of 20 μL of each solution were chromatographed under the conditions described above and the concentration of the remaining IH was calculated at each temperature and time interval for the three replicates. Data were further processed and degradation kinetics constants were calculated.

2.7 pH-rate profile

Accurately weighed 100-mg portions of IH were transferred into 100 mL volumetric flasks and diluted to volume with constant ionic strength buffer solutions prepared as per Indian Pharmacopoeia. The pH values of buffer solutions used for the measurement of the pH-rate profile of the degradation of IH were as follows: pH 1.8, 2.8, 3.8, 4.6, 5.7, 6.8, 8.0, 9.2, 9.7, and 10.8. The pH values of these buffer solutions were checked before and after the reaction and were unchanged. The ionic strengths of the buffer solutions were adjusted with sodium chloride. Separate 10-mL aliquots of the buffer solution containing IH (200 μg/mL) were transferred into separate stoppered round-bottomed flasks. The flasks were then refluxed at 80°C for different time intervals. At the specified time interval the contents of the flasks were neutralized to pH 7.0 using 1 N sodium hydroxide or 1 N hydrochloric acid solution. The contents of the flasks were transferred into 100-mL volumetric flasks and diluted to volume with mobile phase. Aliquots of 20 μL of each solution were chromatographed under the conditions described above and the concentration of the remaining IH was calculated at each pH value and time interval.

3 Results and discussion

3.1 Optimization of procedures

HPLC method: After several trials it was found that methanol:water in a ratio of 70:30, *v/v* gave acceptable retention times but peak splitting was observed. The above mobile phase was tried along with its pH value changed from 3.0 to 5.0 by means of orthophosphoric acid. Finally pH 4.0 ± 0.2 was optimized at 258 nm.

HPTLC method: Different volumes of acetonitrile, acetone, and chloroform were tried. It was found that addition of 6 mL of chloroform to the ratio of toluene:methanol (5.0:5.0, *v/v*) gave good band characteristic with slight tailing and also the R_f value was too high; typical peak behavior was not observed. In order to overcome the tailing of the bands and to reduce the broadening at peak base, 0.1 mL of 10% *v/v* ammonia was added. The decrease in the R_f value was accomplished by adjusting the ratio of toluene and methanol to 5.0:3.0, *v/v*. Finally, mobile phase consisting of toluene:methanol:chloroform:10% ammonia in a ratio of 5.0:3.0:6.0:0.1, *v/v/v/v* was optimized and good resolution with an R_f value of 0.35 ± 0.02 for IH was obtained at 270 nm. The band appeared more compact and peak shape more symmetrical when the TLC plates were pretreated with methanol and activated at 110°C for 5 min. After the application of the bands, the TLC plates were pre-conditioned with ammonia vapor for 30 min, which is required to impart mildly polar nature to silica surface.

3.1.1 Linearity

Linearity was evaluated by analysing ten standard working solutions containing 50–1000 ng/band and 10–100 μg/mL twice in triplicate for HPTLC ($r^2 = 0.9995 \pm 1.50$, slope = 0.10 ± 0.06, intercept = 31.16 ± 1.14) and HPLC ($r^2 = 0.9999 \pm 1.64$, slope = 0.18 ± 0.08, intercept = 0.12 ± 0.05) respectively. For the HPTLC method the calibration graph was found to be linear, indicating adherence of the system to the Kubelka-Munk theory [10, 11]. For both proposed methods no significant difference was observed in the slopes of standard curves (ANOVA; $p < 0.05$).

3.1.2 Precision

HPLC method: The within-run precision and between-run precision were determined by assaying the tablets six times per day for six consecutive days and expressed as % RSD. The intra-day and inter-day precision were found to be 1.04 and 1.51, respectively.

HPTLC method: The repeatability of sample application and measurement of peak area were expressed in terms of % RSD and were found to be 1.68 and 1.13 for IH, respectively. The % RSD values of 1.54 and 1.63 respectively show that the proposed method provides acceptable intra-day and inter-day variation of IH.

3.1.3 Robustness of the method

HPLC method: Each factor selected (except columns from different manufacturers and solvents of different lots) to examine were charged at three levels (–1, 0, and 1). One factor at a time was changed to estimate the effect. Thus, replicate injections ($n = 6$) of mixed standard solution at three concentration levels were performed with small changes of six chromatographic parameters (factors). Results, presented in (**Table 1**), indicate that the selected factors remained unaffected by small variations of these parameters. No significant difference was found between the results from the two different columns. It was also found that methanol of different lots from the same manufacturer had no significant influence on the determi-

Table 1. Robustness evaluation[a)] of the HPLC method ($n = 6$).

Factor[b)]	Level	Chromatographic changes		
		t_r[c)]	k[d)]	T[e)]
A: pH of the mobile phase				
3.90	−1	3.22	2.23	1.26
4.00	0	3.30	2.25	1.28
4.10	1	3.35	2.30	1.29
Mean ± S.D ($n = 6$)		3.29 ± 0.06	2.26 ± 0.04	1.27 ± 0.02
B: Flow rate [mL/min]				
0.90	−1	3.35	2.28	1.31
1.00	0	3.30	2.25	1.28
1.10	1	3.25	2.22	1.16
Mean ± S.D ($n = 6$)		3.30 ± 0.05	2.25 ± 0.03	1.25 ± 0.08
C: %age of methanol in the mobile phase (*v/v*)				
68	−1	3.33	2.22	1.31
70	0	3.30	2.25	1.28
72	1	3.28	2.27	1.24
Mean ± S.D ($n = 6$)		3.30 ± 0.03	2.25 ± 0.03	1.27 ± 0.04
D: Temperature				
24	−1	3.32	2.29	1.33
25	0	3.30	2.25	1.28
26	1	3.28	2.21	1.26
Mean ± S.D ($n = 6$)		3.30 ± 0.02	2.25 ± 0.04	1.29 ± 0.04
E: Columns from different manufacturers				
Kromasil		3.30	2.25	1.28
Finepak		3.29	2.27	1.27
Mean ± S.D (n = 6)		3.29 ± 0.01	2.26 ± 0.01	1.28 ± 0.01
F: Solvents of different lots				
First lot		3.30	2.25	1.28
Second Lot		3.28	2.28	1.30
Mean ± S.D ($n = 6$)		3.29 ± 0.01	2.26 ± 0.02	1.29 ± 0.01

a) Average of three concentrations 30, 60, 90 μg/mL.
b) Four factors were slightly changed at three levels (1, 0, −1); each time a factor was changed from level (0) the other factors remained at level (0).
c) Retention time.
d) Capacity factor.
e) Tailing factor.

nation. Insignificant differences in peak areas and less variability in retention time were observed.

HPTLC method: The % RSD of peak areas was calculated for mobile phase composition, amount of mobile phase, temperature, relative humidity, plate pretreatment, time from application to chromatography, and time from chromatography to scanning, and was found to be 1.37, 1.29, 0.92, 1.21, 0.52, 0.37, and 0.42, respectively. The low values of % RSD (less than 2%) indicated the robustness of the method.

3.1.4 LOD and LOQ

HPLC method: The signal/noise ratios 3:1 and 10:1 were considered as LOD and LOQ, respectively. The LOD and LOQ were found to be 0.10 μg/mL and 0.50 μg/mL, respectively, for IH.

HPTLC method: The LOD and LOQ were found to be 30 ng/band and 50 ng/band, respectively, for IH.

3.1.5 Specificity

HPLC method: The specificity of the HPLC method was assessed by complete separation of IH in the presence of its degradation products. The average retention time ± standard deviation for IH was found to be 3.31 ± 0.05, for six replicates. The peaks obtained were sharp and have clear baseline separation.

HPTLC method: The peak purity of IH was assessed by comparing their respective spectra at peak start, peak apex, and peak end positions of the band, *i.e.*, *r* (start, middle) = 0.9997 and *r* (middle, end) = 0.9996. Good correlation ($r = 0.9997$) was also obtained between standard and sample spectra of IH.

3.1.6 Recovery studies

Both the proposed methods when used for extraction and subsequent estimation of IH from pharmaceutical dosage form after spiking with additional drug afforded recovery of 98–102% and mean recovery for IH from the marketed formulation was found to be 99.88% (% RSD 1.51) and 100.48% (% RSD 1.54), respectively.

3.2 Stability in sample solution

HPLC method: Three different concentrations of IH, 40, 60, and 80 μg/mL were prepared from sample solution and stored at room temperature for 3 days. They were then injected into the HPLC system and no additional peak was found in the chromatogram, indicating the stability of IH in the sample solution (% RSD of peak area 0.96).

HPTLC method: Solutions of three different concentrations, 400, 600, and 800 ng/band for IH were prepared from sample solution and stored at room temperature for 0.5, 1.0, 2.0, 4.0, and 24 h respectively. They were then applied on the same TLC plate; after development the densitogram was evaluated for additional bands if any. There was no indication of compound instability in the sample solution (% RSD of peak area 0.84).

3.2.1 Band stability

The time for which the sample is left to stand in the solvent prior to chromatographic development can influence the stability of the separated band and needs to be investigated for validation [12]. Two-dimensional chromatography using the same solvent system was used to detect any decomposition occurring during application and development. If decomposition occurs during development, peak(s) of decomposition product(s) will be obtained in both the first and the second direction of the run. No decomposition was observed during application and development.

3.3 Analysis of the marketed formulation

HPLC method: A peak at R_f 3.32 for IH was observed in the chromatogram of the drug samples extracted from tablets. The drug content was found to be 99.32% ± 1.45 (% RSD of 0.88) for IH. Statistical evaluation was performed using Student's *t*-test and the *F*-ratio at 95% confidence level.

HPTLC method: A band at R_f 0.36 for IH was observed in the densitogram of the drug samples extracted from tablets. The drug content was found to be 98.94% ± 1.26 (% RSD of 0.47) for IH.

3.4 HPTLC versus HPLC

Six different samples taken during in process control of tablet manufacturing were determined simultaneously by HPTLC and HPLC methods. Each sample was analyzed in duplicate. To test differences between the proposed HPTLC and HPLC methods, statistical tests were performed for the level of confidence 95% ($P = 0.05$). Two-way ANOVA was applied to test both method-sample interactions (interaction variation) and differences in the method precision (column variation). Since the within-cell variation (residual variation) is greater than interaction variation as well as column variations, the method-sample interaction and the differences between the methods are not significant. To test means (averages) a paired *t*-test was applied. The test removes any variations between samples [13]. The obtained value of t_{stat} is lower than two-tail t_{crit}, which leads to the conclusion that there is no significant difference between the means. The results of two way ANOVA and paired *t*-test are given in **Table 2** and **Table 3**, respectively.

3.5 Stability indicating property

3.5.1 Acid- and base-induced degradation product

In HPLC: The chromatograms of the acid-degraded sample showed two additional peaks at, respectively, t_r 2.34 and 4.41 min (amount of analyte recovered 3.59%) and base-degraded samples showed two additional peaks at, respectively, t_r 2.47and 4.30 min (amount of analyte recovered 33.20%).

In HPTLC: The densitogram of the acid-degraded sample showed three degraded peaks at, respectively, R_f 0.10, 0.23, and 0.65 (amount of analyte recovered 0.0%) and base-degraded samples showed two additional peaks at, respectively, R_f 0.28 and 0.43 (amount of analyte recovered 27.44%).

3.5.2 Hydrogen peroxide-induced degradation product

In HPLC: The sample degraded with 6% and 50% *w/v* hydrogen peroxide showed one additional peak at t_r

Table 2. Two-way ANOVA test of IH in six independent samples in duplicate by HPTLC and HPLC.

Sample	HPTLC[a)]		HPLC[a)]	
	1st Sampling	2nd Sampling	1st Sampling	2nd Sampling
1	98.94	99.16	99.32	100.08
2	99.51	100.39	99.44	99.48
3	98.79	98.77	99.81	100.22
4	100.64	100.48	100.62	98.97
5	99.32	98.94	98.99	100.35
6	99.89	100.61	100.18	99.84

ANOVA: Two-factor with replication

Summary	HPTLC	HPLC	Total
1 sample			
Count	6	6	12
Sum	597.09	598.36	1195.45
Average	99.515	99.72666667	99.62083333
Variance	0.46011	0.360146667	0.385062879
2 sample			
Count	6	6	12
Sum	598.35	598.94	1197.29
Average	99.725	99.82333333	99.77416667
Variance	0.72859	0.269386667	0.456262879
Total			
Count	12	12	
Sum	1195.44	1197.3	
Average	99.62	99.775	
Variance	0.552345455	0.2887	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P* value	F_{crit}[b)]
Sample	0.141066667	1	0.141066667	0.310337874	0.583654121	4.351250027
Columns	0.14415	1	0.14415	0.317121015	0.579602131	4.351250027
Interaction	0.019266667	1	0.019266667	0.042385466	0.838967948	4.351250027
Within	9.091166667	20	0.454558333			
Total	9.39565	23				

a) The results are presented as [%] of declared amount of IH per tablet.

b) $F_{stat} < F_{crit}$.

2.26 min with analyte recovery of 83.05 and 15.22%, respectively.

In HPTLC: The sample degraded with 6% and 50% *w/v* hydrogen peroxide showed two additional peaks at R_f values of 0.16 and 0.55 with analyte recovery of 74.81 and 22.70%, respectively.

3.5.3 Dry heat and wet heat degradation product

The samples degraded under dry heat conditions did not show additional peaks, either in HPLC or in HPTLC, and the peak area of the standard remained unchanged. Under wet heat conditions the peak area of the parent drug was considerably decreased in both the methods

Table 3. Average results of IH determination by HPTLC and HPLC and their correlation by paired t-test.

Sample	HPTLC[a)]	HPLC[a)]
1	98.94	100.32
2	99.86	100.12
3	100.09	99.79
4	99.72	100.38
5	98.97	98.82
6	100.26	99.18

t-Test: Paired two sample for means

	Variable 1	Variable 2
Mean	99.64	99.76833333
Variance	0.31612	0.409616667
Observations	6	6
Pearson correlation	0.011393812	
Hypothesized mean difference	0	
Df	5	
t_{stat}	**−0.371101791**	
P (*T t*) one-tail	0.362883207	
t Critical one-tail	2.015049176	
P (*T t*) two-tail	0.725766415	
t_{crit} **two-tail**	**2.570577635**	

***t*-stat** < ***t* critical**

[a)] The results are presented as [%] of declared amount of IH per tablet

with analyte recoveries of 26.17 and 30.60% in HPLC and HPTLC, respectively, thus indicating the conversion of standard IH to a non-chromophoric compound.

3.5.4 Photochemically and UV-degraded product

In both methods, the photo-degraded sample showed no additional peak and no change in peak area of standard when the drug solution was left in daylight for 360 h. The drug was degraded when exposed to UV irradiation for 360 h and showed one additional peak at t_R 2.15 min and R_f 0.53 respectively with analyte recovery of 70.54 and 65.12%, respectively.

Figure 2 shows the overlaid densitogram of IH along with all the degradation products resulting under different stress conditions.

3.6 Detection of the related impurities

The bands other than the principal band (IH) from the sample solution were not as intense as the principal band from the standard solution. The sample solution showed one additional band at R_f 0.53. However, the peak area of the additional band (10,000 ng/band, peak area = 641.16) was found to be much less than the peak area of the principal band from the standard solution (100 ng/band, peak area = 719.44).

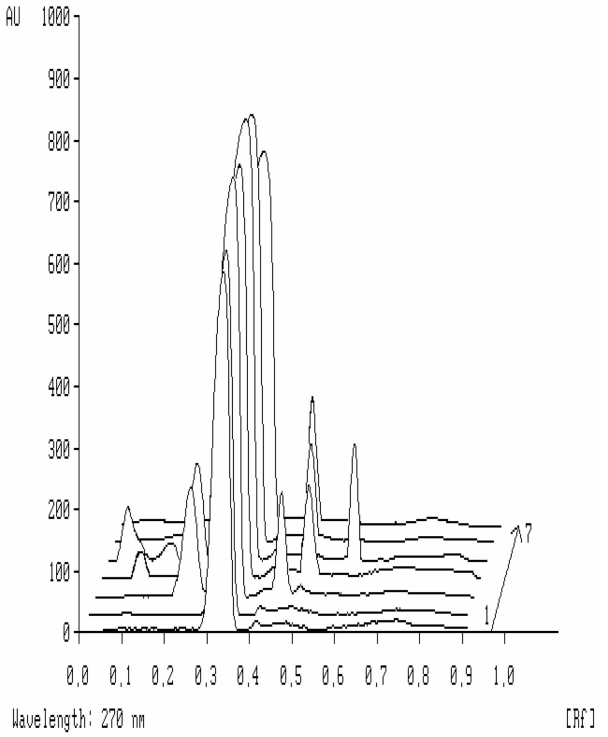

Figure 2. Overlaid densitogram of IH under various stress conditions. Track 1 = standard IH (1000 ng/band), Track 2 = IH in presence of excipients (1000 ng/band), Track 3 = standard IH treated with hydrogen peroxide (6.0% *w/v*, reflux for 1.0 h, temp. 70°C), Track 4 = standard IH treated with base (5N NaOH, reflux for 2 h, temp. 70°C), Track 5 = standard IH treated with acid (5N HCl, reflux for 2 h, temp. 70°C), Track 6 = IH and its impurity, Track 7 = standard IH exposed to UV irradiation for 360 h.

While injecting a higher concentration of standard IH drug solution (500 μg/mL) in triplicate, an additional peak was observed at t_r 2.15 min, which was considered as an unknown impurity associated with IH. The area of the additional peak (peak area = 12342.18) was found to be much less than for the standard solution (5 μg/mL, peak area = 182544.50).

It can be observed that the R_f value of hydrogen peroxide- and UV-degraded product closely matches the R_f value of an impurity present in the drug. This can be affirmed by similar observations in the case of HPLC where the t_R of an impurity closely matched that of a UV- and peroxide-degradation product. Therefore it is possible that during processing or storage the drug might have undergone oxidation or UV exposure to a small extent.

3.7 Degradation kinetics

The kinetics of degradation of IH was investigated in 5.0 N NaOH, 5 N HCl, and 50% *w/v* hydrogen peroxide, since

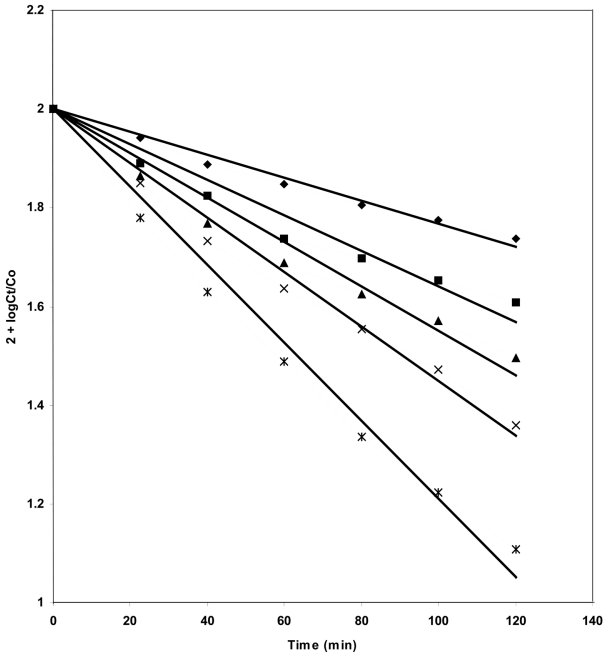

Figure 3. Pseudo first-order plots for the degradation of IH with 5 N HCl at various temperatures using HPLC method. Key: 80°C (∗), 70°C (×), 60°C (▲), 50°C (■), 40°C (●), C_t, concentration at time t; C_0, concentration at time zero.

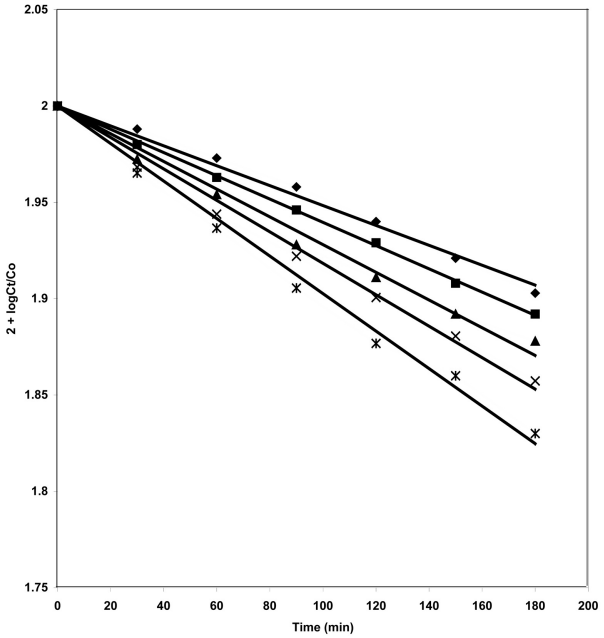

Figure 4. Pseudo first-order plots for the degradation of IH with 5 N NaOH at various temperatures using HPLC method. Key: 80°C (∗), 70°C (×), 60°C (▲), 50°C (■), 40°C (●), C_t, concentration at time t; C_0, concentration at time zero.

the decomposition rate of IH at lower strengths of HCl, NaOH, and hydrogen peroxide was too slow to afford reliable kinetic data. Each experiment was repeated three times at each temperature and time interval. The mean concentration of IH was calculated for each experiment. A regular decrease in the concentration of IH with increasing time intervals was observed. At the selected temperatures (40, 50, 60, 70, 80, and 90°C for acidic, alkaline, and oxidative degradation) the degradation process followed pseudo-first order kinetic as shown in **Fig. 3, Fig. 4, Fig. 5**. From the slopes of the straight lines it was possible to calculate the apparent first order degradation rate constant, half-life ($t_{1/2}$), and t_{90} (*i.e.*, time at which 90% of original concentration of the drug remains unchanged) at each temperature for acidic, alkaline, and oxidative degradation processes, as determined by the HPLC method (**Table 4**). Data obtained from first order kinetics treatment were further subjected to fitting in the Arrhenius equation;

$$\log K = \log A - E_a/2.303\,RT \quad (1)$$

Where K is the rate constant, A is the frequency factor, E_a is energy of activation (kcal mol^{-1}), R is the gas constant (1.987 kcal/deg mol), and T is the absolute temperature (K). A plot of (2 + log K_{obs}) values *versus* ($1/T \times 10^3$), the Arrhenius plot, was obtained (**Fig. 6**), which was found to be linear in the temperature range 40°C to 90°C. The activation energy and the Arrhenius frequency factor were calculated, respectively, for acidic, alkaline, and oxidative

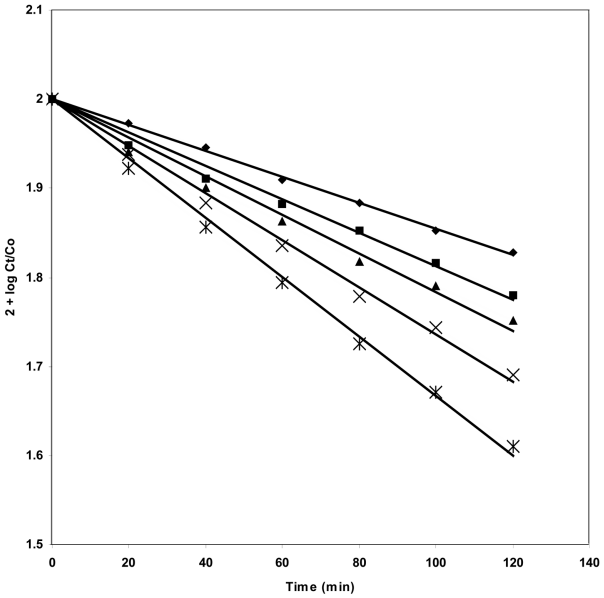

Figure 5. Pseudo first-order plots for the degradation of IH with 50% *w/v* hydrogen peroxide at various temperatures using HPLC method. Key: 80°C (∗), 70°C (×), 60°C (▲), 50°C (■), 40°C (●), C_t, concentration at time t; C_0, concentration at time zero.

degradation processes determined by the HPLC method. The method of accelerated testing of pharmaceutical products based on principles of chemical kinetics was used to obtain a measure of the stability of the drug under

Table 4. Degradation rate constant (K_{obs}), half-life ($t_{1/2}$), and t_{90} for IH in the presence of 5 N HCl, 5 N NaOH and 50 % *w/v* H_2O_2 determined by the HPLC method.

Temperature (°C)	K_{obs} [h^{-1}]	$t_{1/2}$ [h]	t_{90} [h]
In 5 N Hydrochloric acid			
40	0.0053	2.17	0.33
50	0.0083	1.39	0.21
60	0.0104	1.11	0.17
70	0.0129	0.89	0.14
80	0.0184	0.62	0.09
In 5 N Sodium hydroxide			
40	0.0012	9.62	1.46
50	0.0014	8.25	1.25
60	0.0016	7.22	1.09
70	0.0018	6.42	0.97
80	0.0023	5.02	0.76
In 50% *w/v* Hydrogen peroxide			
40	0.0035	3.30	0.50
50	0.0044	2.63	0.39
60	0.0051	2.26	0.34
70	0.0060	1.93	0.29
80	0.0076	1.52	0.23

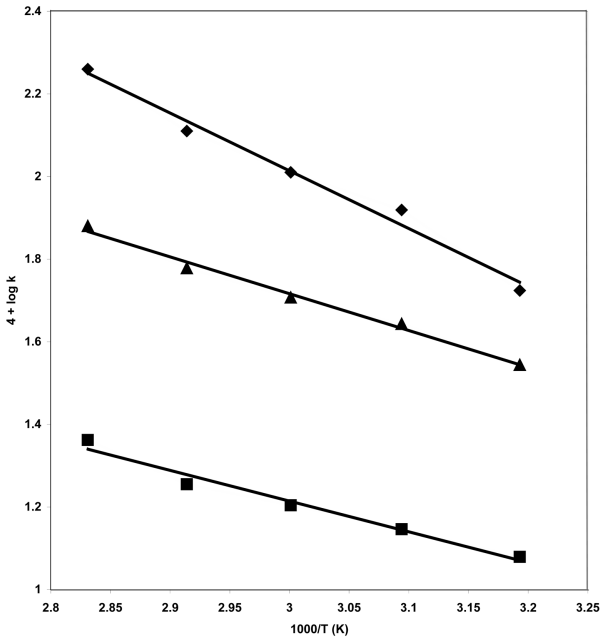

Figure 6. Arrhenius plot for the degradation of IH in presence of 5 N HCl. Key: (●), 50% *w/v* H_2O_2 (▲) and 5.0 N NaOH (■).

said conditions [14, 15]. The degradation rate constant at room temperature (K_{25}) is obtained by extrapolating to 25°C (where $1000/T = 3.356$) by inserting this into Eq. (1) and $t_{1/2}$ and t_{90} are calculated accordingly (**Table 5**).

Table 5. Summary of degradation kinetic data at 25°C using the HPLC method.

Parameters	In 5 N HCl	In 5 N NaOH	In 50% *w/v* H_2O_2
E_a (Kcal/deg mol)[a]	6.41×10^{-3}	3.41×10^{-3}	4.08×10^{-3}
K_{25} [h^{-1}][b]	3.23×10^{-2}	8.83×10^{-3}	2.50×10^{-2}
$t_{1/2}$ [h][c]	21	78.48	27.69
t_{90} [h][d]	3.25	11.89	4.20
A[e]	450	46.54	409

a) Activation energy.
b) Degradation rate constant.
c) Half-life.
d) Time left for 90% potency.
e) Arrhenius frequency factor.

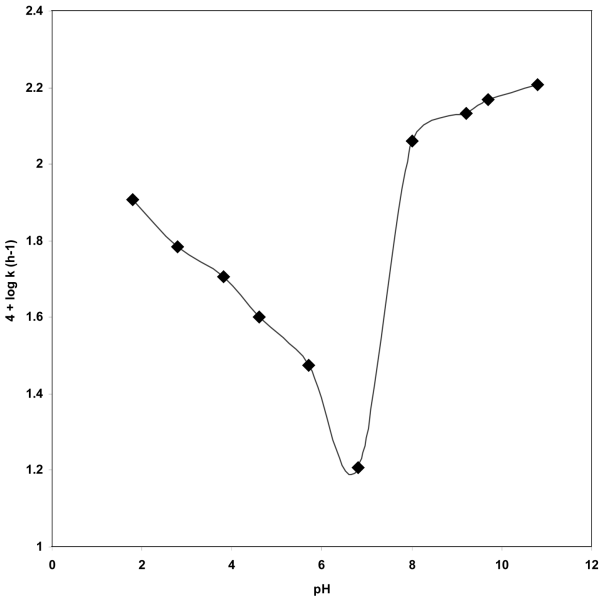

Figure 7. pH-rate profile for the decomposition of IH at constant ionic strength at 70°C.

The pH-rate profile of degradation of IH in constant ionic strength buffer solutions was studied at 80°C using the HPLC method (**Figure 7**). The apparent first order degradation rate constant and the half-life were calculated for each pH value (**Table 6**).

4 Concluding remarks

Both the chromatographic methods were validated in compliance with ICH guidelines. Six real samples of tablets were assayed simultaneously by HPTLC and HPLC methods and the results were correlated. The HPTLC method is simple and uses a minimal volume of solvents, compared to the HPLC method. Statistical tests indicate that the proposed HPTLC and HPLC methods reduce the duration of analysis and appear to be equally suitable for routine determination of IH in pharmaceutical

Table 6. Degradation rate constant (K_{obs}), half-life ($t_{1/2}$), and t_{90} for IH in constant ionic strength buffer at different pH values and a temperature of 70°C.

pH	K_{obs} [h^{-1}]	$t_{1/2}$ [h]	t_{90} [h]
1.8	0.008	1.44	0.22
2.8	0.006	1.93	0.29
3.8	0.005	2.31	0.35
4.6	0.004	2.88	0.44
5.7	0.003	3.85	0.58
6.8	0.002	5.77	0.88
8.0	0.012	0.96	0.15
9.2	0.014	0.83	0.13
9.7	0.015	0.77	0.12
10.8	0.016	0.72	0.11

formulations. It is one of the rare studies where forced decomposition was undertaken under all different suggested conditions. The method can be used to determine the purity of the drug available from various sources by detecting the related impurities and also in stability studies. The degradation rate constant, half-life, and t_{90} of IH can be predicted. As the method separates the drug from its degradation products, it can be employed as a stability indicating technique.

Acknowledgement

The authors thank Abbott India Ltd. for a gift sample of itopride hydrochloride. The authors are also grateful to Mr. Shailesh Bawaskar (General Manager-Service, Anatek Services Pvt. Ltd. Mumbai, India) and Mr. Dilip Charegaonkar (Managing Director, Anchrom HPTLC specialists, Mumbai, India), respectively, for providing facilities during research work.

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