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Validated stability-indicating HPLC method for the determination of pridinol mesylate. Kinetics study of its degradation in acid medium

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ABSTRACT

The stability of pridinol mesylate (PRI) was investigated under different stress conditions, including hydrolytic, oxidative, photolytic and thermal, as recommended by the ICH guidelines. Relevant degradation was found to take place under acidic (0.1N HCl) and photolytic (visible and long-wavelength UV-light) conditions, both yielding the product resulting from water elimination (ELI), while submission to an oxidizing environment gave the *N*-oxidation derivative (NOX). The standards of these degradation products were synthesized and characterized by IR, ^{1}H and ^{13}C NMR spectroscopy. A simple, sensitive and specific HPLC method was developed for the quantification of PRI, ELI and NOX in bulk drug, and the conditions were optimized by means of a statistical design strategy. The separation employs a C_{18} column and a 51:9:40 (v/v/v) mixture of MeOH, 2-propanol and potassium phosphate solution (50 mM, pH 6.0), as mobile phase, delivered at 1.0 ml min^{-1}; the analytes were detected and quantified at 220 nm. The method was validated, demonstrating to be accurate and precise (repeatability and intermediate precision levels) within the corresponding linear ranges of PRI (0.1–1.5 mg ml^{-1}; r = 0.9983, n = 18) and both impurities (0.1–1.3% relative to PRI, r = 0.9996 and 0.9995 for ELI and NOX, respectively, n = 18). Robustness against small modifications of pH and percentage of the aqueous mobile phase was ascertained and the limits of quantification of the analytes were also determined (0.4 and 0.5 μg ml^{-1}; 0.04% and 0.05% relative to PRI for ELI and NOX, respectively). Peak purity indices (>0.9997), obtained with the aid of diode-array detection, and satisfactory resolution (R_s > 2.0) between PRI and its impurities established the specificity of the determination, all these results proving the stability-indicating capability of the method. The kinetics of the degradation of PRI in acid medium was also studied, determining that this is a first-order process with regards to drug concentration, with an activation energy of 25.5 Kcal mol^{-1} and a $t_{1/2}$ = 10,830 h, in 0.1N HCl at 38 °C.

1. Introduction

Pridinol mesylate (PRI) is a central anticholinergic drug, with useful muscle relaxant properties [1]. The drug (Fig. 1), is used alone in patches [2] as a myotonolytic and spasmolytic agent in anti-stress therapy [3]; however, PRI is most frequently formulated in association with non-steroidal anti-inflammatory drugs, including diclofenac, piroxicam and meloxicam, for the treatment of muscular contractures and low back pain [1,4].

GC–MS [5,6] and CE [7] have been reported for the determination of PRI in biological fluids and we have recently disclosed an HPLC method for the simultaneous determination of PRI and meloxicam in their pharmaceutical association [8]. However, a literature search revealed that none of the most recognized Pharmacopoeias includes this drug, and that there is no information regarding the stability of PRI and its quantification in bulk drug.

Regulatory agencies recommend the use of stability-indicating methods [9] (SIMs) for the analysis of stability samples [10]; this requires stress studies in order to generate the stressed samples, method development and method validation [11]. With the advent of the International Conference on Harmonization (ICH) guidelines [12], requirements for the establishment of SIMs have become more clearly mandated.

Environmental conditions, including light, heat, humidity, and the susceptibility of the substance towards hydrolysis or oxidation can play an important role in the production of impurities. Stress testing can help identifying degradation products and provide important information about the intrinsic stability drug substances [13].

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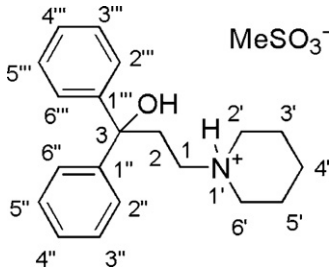


Fig. 1. Chemical structure of pridinol mesylate.

Therefore, herein we report the results of a stability study of PRI with the aim of determining the extent of the influence of different stress conditions on the stability of this active pharmaceutical ingredient. We also disclose the chemical synthesis and characterization of the observed degradation products, a kinetics study of the degradation of PRI under acidic conditions and, fulfilling the integral aim of this study, the development and validation of a stability-indicating HPLC method for the quantification of PRI and its relevant degradation impurities in bulk material.

2. Experimental

2.1. Instrumentation

IR spectra were obtained with the sample as a thin film held between two NaCl plates or as a KBr pellet (solid sample), with the aid of a Shimadzu Prestige 21 FT-IR spectrophotometer; ^{1}H and ^{13}C NMR spectra were acquired in $CDCl_3$, employing a Bruker Avance 300 spectrometer; chemical shifts are expressed in ppm, downfield from tetramethylsilane, used as internal standard and coupling constants (J) are expressed in Hertz. Signals are abbreviated as follows: s = singlet; d = doublet; t = triplet; m = multiplet; b = broad signal. The melting point of NOX (uncorrected) was recorded on an Ionomex hot stage apparatus. Photolytic stress studies were performed in a photostability chamber.

The HPLC system used was described before [8]. The specificity studies were performed in a HP 1100 HPLC system, with the same chromatographic column (C_{18} Luna, Phenomenex, 250 mm × 4.6 mm, 5 μm particle size), employing a photodiode array (PDA) detector. Statistical treatment of the data was performed with the SPSS v. 9 application software. Experimental designs were processed employing Design Expert v. 7.

2.2. Chemicals and materials

The experiments were performed with pharmaceutical-grade PRI and analytical-grade reagents. HPLC-grade solvents were employed for the chromatographic analyses. Phosphate solutions were prepared according to the USP 30 [14], employing double distilled water. Liquids were filtered through 0.45 μm nylon filters before use.

Stock standard solutions of PRI (10 mg ml^{-1}), ELI (2.96 mg ml^{-1}) and NOX (2.96 mg ml^{-1}) were prepared in MeOH and demonstrated to be stable at least 90 days at 4 °C. Solutions for analyses containing mixtures of the analytes were prepared immediately before use, by appropriate dilutions in volumetric flasks of the stock solutions or degraded samples with mobile phase. All the solutions were protected from light throughout the experiments.

2.3. Chromatographic conditions

TLC analyses to monitor drug degradation were developed with CH_2Cl_2:MeOH:Et_3N (92:7:1, v/v/v) for photolytic and acid degradation and with MeOH:NH_4OH (98.5:1.5) for degradation under oxidizing conditions and examined under short-wavelength UV-light (254 nm) or by exposure to vapors of iodine. In the optimized procedure, the mobile phase used for the HPLC separation was a 51:9:40 (v/v/v) mixture of MeOH, 2-propanol and potassium phosphate (50 mM, pH 6.0), pumped at room temperature, at a flow rate of 1.0 ml min^{-1}. The detection was accomplished at 220 nm [8].

Table 1
Hydrolytic, oxidizing and photolytic stress testing conditions for PRI

Stress condition	Solvent	Time (days)	Temperature (°C)	Result
Hydrolytic				
Neutral	H_2O	60	Room temperature	Stable
	H_2O	60	40	Stable
	H_2O	60	60	Stable
Acidic	0.1N HCl	60	Room temperature	ELI
	0.1N HCl	7	70	ELI
Basic	0.1N NaOH	60	Room temperature	Stable
	0.1N NaOH	60	70	Stable
Oxidizing	0.3% H_2O_2	3	Room temperature	NOX
	0.3% H_2O_2	8	Room temperature	NOX
	0.3% H_2O_2	60	Room temperature	NOX
	0.3% H_2O_2	2	40	NOX
	0.3% H_2O_2	7	40	NOX
	0.3% H_2O_2	14	70	Irrelevant degradation
Photolytic				
Short-wavelength UV-light	H_2O	0.33	Room temperature	Irrelevant degradation
Long-wavelength UV-light	H_2O	5	Room temperature	Stable
Long-wavelength UV-light	H_2O	14	Room temperature	ELI
Photolytic visible-light	H_2O	14	Room temperature	ELI
Solid state				
Photolitic visible light	–	60	Room temperature	ELI
Humidity (65%)	–	60	30	Stable
Dry heat	–	60	70	Stable

2.4. Forced degradation of pridinol mesylate. Stressed samples

Multiple stressed samples were obtained as indicated below. They were chromatographed along with a non-stressed standard sample.

2.4.1. Hydrolytic conditions. Acid, base and water-induced degradation

Solutions containing 10 mg ml^{-1} of the drug were prepared in 0.1N HCl, 0.1N NaOH and water, respectively. These were subjected to the conditions mentioned in Table 1 and periodically analyzed by TLC for the appearance of additional spots. For HPLC analyses, 1.0 ml aliquots of the above solutions were transferred to 10 ml volumetric flasks, neutralized as needed (0.1N HCl or 0.1N NaOH), and each sample diluted to the mark with MeOH.

2.4.2. Oxidizing conditions. Hydrogen peroxide-induced degradation

Solutions of PRI (10 mg ml^{-1}) were prepared in water containing 0.3% v/v of H_2O_2, treated in the dark under the conditions shown in Table 1 and periodically analyzed for the appearance of additional spots by TLC. For HPLC analyses, 1.0 ml aliquots of the stressed samples were transferred to 10 ml volumetric flasks and diluted to the mark with MeOH.

2.4.3. Dry heat degradation studies

The powdered drug was spread in a flat-bottomed tube to give a homogeneous layer (<5 mm thick) and subjected to the conditions indicated in Table 1. For periodic TLC and HPLC analyses, 10 mg of the solid were dissolved with 10 ml MeOH (volumetric flask).

2.4.4. Photolytic studies. Exposure to artificial light

Solutions of PRI (10 mg ml^{-1}) in water were placed in Pyrex (visible and long-wavelength UV-light) or quartz vessels (short-wavelength UV-light) and exposed to forced irradiation (at 15 cm from the sources) in a 40 cm × 30 cm × 30 cm chamber fitted with either four Philips F4T5/D daylight fluorescent lamps (6500 K) or four Philips G4T5 short-wavelength UV-lamps (4 W each). Irradiation with long-wavelength UV-light was carried out with a Philips MLW black-light lamp (160 W). For chromatographic analyses, 1 ml samples were periodically withdrawn and diluted to 10 ml with MeOH.

2.5. Synthesis and spectrometric identification of the degradation products

2.5.1. Preparation of 1-(3,3-diphenylprop-2-en-1-yl)piperidine (ELI)

PRI (150 mg) was dissolved in 0.1N HCl (15 ml) and placed at 70 °C for 7 days in the dark, when TLC [MeOH:Et_3N (99.5:0.5, v/v)] indicated complete absence of starting material. Then, the solution was treated with saturated Na_2CO_3 (pH 9) and extracted with EtOAc (3 × 20 ml). The combined organic extracts were washed with brine, dried (Na_2SO_4), concentrated under reduced pressure, and the residue was chromatographed, yielding ELI (96 mg, 97%), as a clear, yellowish oil. IR (film, ν): 3055, 2934, 2851, 1610, 1442, 1368, 1298, 119, 1039 and 701 cm^{-1}; ^{1}H NMR (δ): 1.40-1.47 (bs, 2H, H-4′), 1.61 (ddd, 4H, J = 5.6, 5.7 and 11.3, H-2′ and H-6′), 2.40 (bs, 4H, H-3′ and H-5′), 3.05 (d, 2H, J = 6.8, H-1), 6.28 (t, 1H, J = 6.8, H-2) and 7.15-7.45 (m, 10H aromatics); ^{13}C NMR (δ): 24.30 (C-4′), 26.03 (2C, C-3′ and C-5′), 54.67 (2C, C-2′ and C-6′), 58.15 (C-1), 126.83 (C-3), 127.07 and 127.16 (2C, C-4″ and C-4‴), 127.23 (2C, C-3″ and C-5″), 128.13 and 128.15 (2C each, C-2″, C-6″, C-3‴ and C-5‴), 129.85 (2C, C-2‴ and C-6‴), 139.84 (C-3), 142.15 (C-1‴) and 143.53 (C-1″).

2.5.2. Preparation of 1,1-diphenyl-3-piperidin-1-ylpropan-1-ol (NOX)

A stirred solution of PRI (67 mg, 0.17 mmol) in MeOH (8 ml) was treated with 80% magnesium bis(monoperoxyphthalate) hexahydrate (MMPP, 0.126 g, 0.2037 mmol) at room temperature for 90 min [15]. After complete transformation of the starting material [TLC, MeOH:CH_2Cl_2:Et_3N (92:7:1, v/v/v)], the organic solvent was removed under reduced pressure; the residue was basified with saturated Na_2CO_3 (pH 9) and extracted with EtOAc (3 × 20 ml). The combined organic layers were washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure. The residue was chromatographed, furnishing NOX (38 mg, 77%) as a solid of melting point 208–209 °C. IR (KBr, ν): 3286, 2976, 1648, 1446, 1207, 1063, 957, 878, 748 and 694 cm^{-1}; ^{1}H NMR (δ): 1.10–1.40 (bs, 1H, OH), 1.33 (ddt, 1H, J = 3.8 11.5 and 13.2, H-4′), 1.56 (dt, 2H, J = 3.8, and 14.3, H-3′ and H-5′), 1.72 (dt, 1H, J = 3.8 and 13.2, H-4), 2.27 (ddt, 2H, J = 3.8, 11.5 and 14.3, H-3′ and H-5′), 2.81 (dd, 2H, J = 2.8 and 11.3, H-2), 2.98 (dt, 2H, J = 2.8 and 11.3, H-1), 3.33 (bdd, 4H, J = 3.8 and 14.3, H-2′ and H-6′), 7.16 (dt, 1H, J = 1.1 and 7.3, H-4‴), 7.18 (dt, 1H, J = 1.1 and 7.3, H-4″), 7.28 (dd, 2H, J = 7.3 and 8.4, H-3″ and H-5″), 7.29 (dt, 2H, J = 7.3 and 8.4, H-3‴ and H-5‴), 7.52 (dd, 2H, J = 1.1 and 8.4, H-2″ and H-6″) and 7.53 (dd, 2H, J = 1.1 and 8.4, H-2‴ and H-6‴); ^{13}C NMR (δ): 20.96 (2C, C-3′ and C-5′), 22.26 (C-4′), 35.70 (C-2), 65.64 (C-2′ and C-6′), 66.74 (C-1), 75.04 (C-3), 126.31 (6C, C-2″, C-4″, C-6″, C-2‴, C-4‴ and C-6‴), 128.03 (4C, C-3″, C-5″, C-3‴ and C-5‴) and 148.12 (2C, C-1″ and C-1‴).

2.6. Validation of the developed HPLC method

System suitability parameters were evaluated using a solution of PRI (1.00 mg ml^{-1}) containing 1% of each impurity, dissolved in the mobile phase. Five replicates were injected.

To establish linearity and range, the stock solutions containing 10 mg ml^{-1} PRI and 2.96 mg ml^{-1} NOX and ELI in MeOH were diluted to yield combined solutions (six levels) containing PRI (0.10–1.50 mg ml^{-1}), ELI and NOX (0.05–1.30% each, with regards to PRI). The samples were injected in triplicate. Repeatability was ascertained by analyzing solutions of PRI (0.26, 0.78 and 1.30 mg ml^{-1}) and combined solutions containing ELI (1.00, 6.99 and 12.00 μg ml^{-1}), NOX (1.90, 7.00 and 12.36 μg ml^{-1}) and 1 mg ml^{-1} PRI. The intermediate precision experiment was carried out with the same solutions, in two different days, by three independent analysts.

Accuracy was determined by spiking a solution of PRI (0.71 mg ml^{-1}) with known amounts of drug to obtain 0.86, 1.00 and 1.15 mg ml^{-1} PRI. The same method was utilized for impurities; the original solutions containing 0.20% of both degradation products (with regards to 1.00 mg ml^{-1} PRI) were spiked with known amounts of the impurities to obtain impurity levels of 0.40%, 0.60% and 0.80%. These solutions were analyzed in triplicate.

Specificity of the method was established in stressed solutions employing a PDA detector and also by determining the peak purity index of all peaks. The robustness regarding pH of the phosphate solution (5.90–6.09), proportion of the organic phase (65–71%) and flow rate (0.95–1.05 ml min^{-1}) was evaluated by means of a central composite design coupled to a response surface study. The sample was a solution containing 7 μg ml^{-1} of each impurity and 1.00 mg ml^{-1} PRI. The limits of quantification (LOQ) were determined as the lowest analyte concentration that can be determined with R.S.D. ≤10%.

2.7. Kinetics of the degradation of PRI under acid conditions

The study was carried out at 38 °C or 70 °C with solutions containing 100 mg PRI, in 10 ml of 0.1, 0.2 and 0.4N HCl. At pre-

established times, 0.50 ml aliquots of the solutions were transferred into 5 ml volumetric flasks, neutralized with 0.2 N NaOH and diluted to the mark with mobile phase. Samples were analyzed by HPLC, employing the developed SIM.

3. Results and discussion

3.1. Stress testing of PRI

Stress testing provides evidence on how the quality of a drug may be affected under the influence of different stress conditions. Drug decomposition may result in loss of potency and advent of possible adverse effects due to the formation of degradation products [16]. The tests were performed on 10 mg ml^{-1} solutions of PRI using various conditions, including hydrolytic, photolytic and oxidizing setups; in addition, the solid was exposed to dry heat, light and humidity. Analyses revealed the drug behavior, as summarized in Table 1.

Under neutral and basic conditions, the samples demonstrated to be stable, even at the end of a 60 days period of heating at 70 °C; however, exposure to visible (fluorescent lamps) or long-wavelength UV-light (black-light) during 14 days, furnished a product, which resulted more retained than PRI in the chromatogram and was spectroscopically (UV and NMR) and chromatographically (HPLC and TLC) identified as ELI. On the other side, exposure of the drug to short-wavelength UV-light resulted in its rapid decomposition into numerous unidentifiable products. In acidic media, PRI also furnished ELI as the sole degradation product, the concentration of which increased with time, acid concentration and temperature more markedly than under the above photolytic conditions.

On the other side, reaction of the drug with hydrogen peroxide furnished a compound which eluted before PRI in the HPLC run, and was spectroscopically and chromatographically identified as NOX. However, prolonged exposure to the oxidizing agent resulted in progressive loss of PRI and generation of irrelevant degradation products. In the solid state, PRI was stable during 60 days, when subjected to dry heat (70 °C) and also when stored during two months at room temperature and 65% relative humidity. However, exposure to visible light during 60 days resulted in partial decomposition, leading to ELI and minor amounts of NOX.

3.2. Synthesis and characterization of the degradation products

Scheme 1 illustrates the syntheses of ELI and NOX from PRI. The preparation of the former was conveniently carried out by treatment with 1N HCl, while synthesis of NOX was more efficiently performed when PRI was subjected to oxidation with MMPP in MeOH. This oxidizing reagent effected a cleaner transformation, avoiding generation of by-products, formed when hydrogen peroxide was employed, which difficulted isolation of NOX. Both impurities were unequivocally characterized by IR, ^{1}H NMR and ^{13}C NMR spectroscopy.

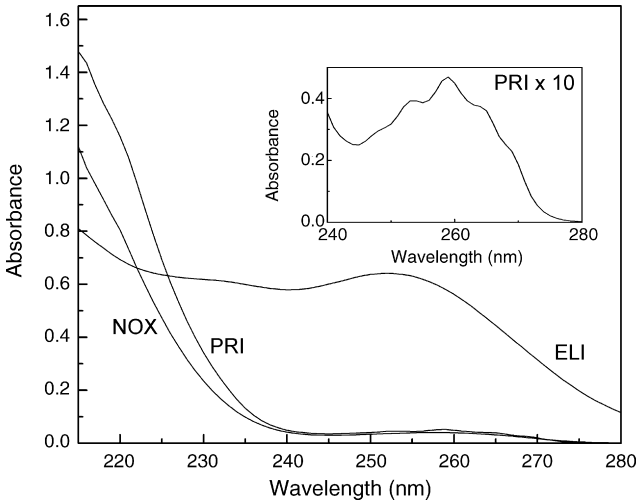


Fig. 2. The UV spectra, in mobile phase, of pridinol (0.50 mg l^{-1}), ELI (0.25 mg l^{-1}) and NOX (0.35 mg l^{-1}).

In its IR spectrum, ELI lacked the broad signal corresponding to the OH group found in PRI at 3150 cm^{-1}, while in its ^{1}H NMR spectrum it evidenced the presence of a single vinylic proton resonating at 6.28 ppm, coupled to a methylene group, observed as a doublet at δ 3.05. The ^{13}C NMR spectrum of ELI showed two sp^{2} carbons at 126.83 and 126.83 ppm replacing the methylene group (δ 35.20) and the quaternary carbon bearing a tertiary alcohol signal (78.98 ppm), found in the stating material.

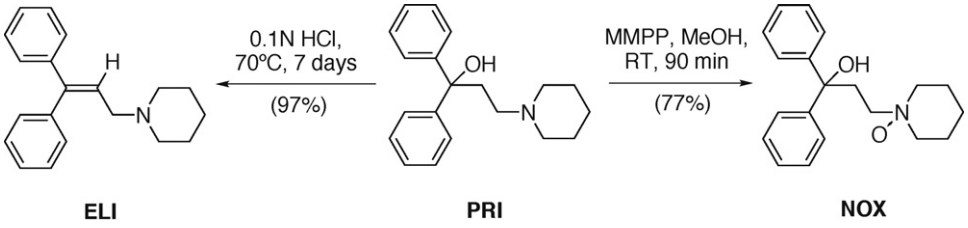
On the other side, the IR spectrum of NOX exhibited the band corresponding to the tertiary alcohol (3286 cm^{-1}) and its ^{1}H NMR spectrum showed a general deprotection of the hydrogen atoms, which was more evident among the three methylene groups attached to the nitrogen, also allowing the differentiation between pseudoaxial (1.33 and 2.27 ppm) and pseudoequatorial (1.56 and 1.72 ppm) hydrogens in the piperidine ring. The ^{13}C NMR spectrum revealed a marked deprotection (>10 ppm) of the carbons attached to nitrogen, with a slight protection (≅5 ppm) of the carbons two atoms away from this heteroatom.

3.3. Development of a RP-HPLC method for the determination of PRI, ELI and NOX

With standards of the synthetic impurities in hand, a chromatographic method for their separation and quantification on a C_{18} column was next developed, rationally selecting the detection wavelength and the composition of the mobile phase.

3.3.1. Selection of the detection wavelength

In common with other diphenylmethane derivatives, PRI lacks good chromophors [17]. Its UV spectrum in mobile phase exhibits a relative absorption maximum at 259 nm (Fig. 2); however, at this wavelength the method was not sensitive enough and detection of the degradation impurities proved difficult.



Scheme 1. Synthesis of relevant impurities ELI and NOX from PRI.

Therefore, and in order to favor instrumental sensitivity and precision, detection at lower wavelengths was explored, finding that 220 nm offered good linearity and high response, constituting the best alternative. At longer wavelengths, sensitivity of the method markedly decreased for NOX making difficult the quantification of NOX; on the other hand, at shorter wavelengths, increasing absorption due to the mobile phase made the determinations less precise.

3.3.2. Selection of the mobile phase composition

Different mobile phases consisting of 50 mM potassium phosphate (at various pH values) and 85:15 (v/v) methanol:2-propanol mixtures [8], conforming a central composite design, were coupled to a response surface methodology study in order to rationally examine the effects of pH and proportion of the aqueous phase on the retention time of PRI, the resolution between each degradation product and PRI and the run time of the separation, at a flow rate of 1.0 ml min^{-1}, employing a C_{18} column.

The central composite design contains embedded factorial or fractional factorial designs with center points that are augmented with a group of axial (star) points that allow estimation of curvature [18]. The star points represent extreme values (low and high) for each factor in the design. On the other hand, response surface methodologies mathematically fit the experimental domain studied in the theoretical design through a response function [19]. These designs are capable of revealing effects and interactions of the variables. When the responses of interest are expressed in a model as a continuous function of the studied variables, they bring to light, graphically or mathematically, regions of the experimental domain where combination of the variables satisfy the desired criteria.

Derringer's desirability (D) function [20] was applied in order to simultaneously optimize all the objective responses and find the optimum chromatographic conditions. The combination of factors producing the maximum desirability (D = 75%) was 68% of the organic phase and 32% of 50 mM phosphates at pH 6.0 (Fig. 3).

Under these conditions, the retention times of NOX, PRI and ELI were 4.64, 7.15 and 12.97 min, respectively, and a clear resolution of the analytes was achieved. Typical chromatograms obtained for the bulk drug and stressed samples (Fig. 4).

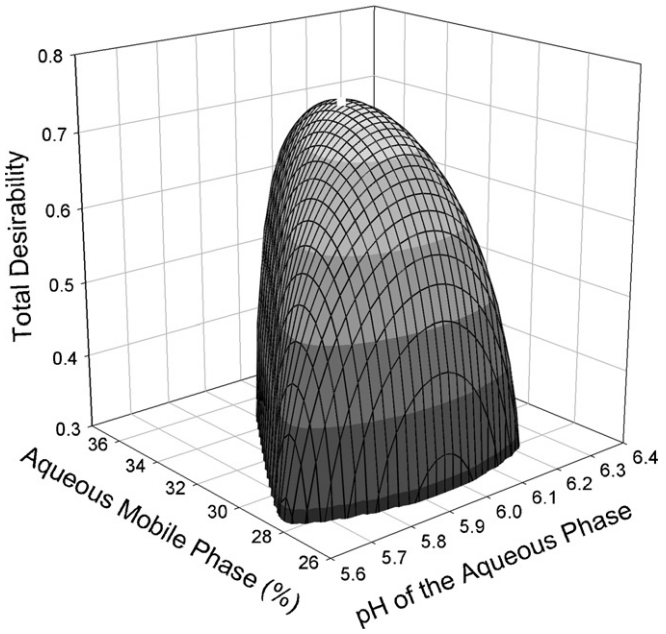


Fig. 3. Response surface plot of the optimization of the mobile phase composition. The white dot atop of the surface represents the most desirable conditions.

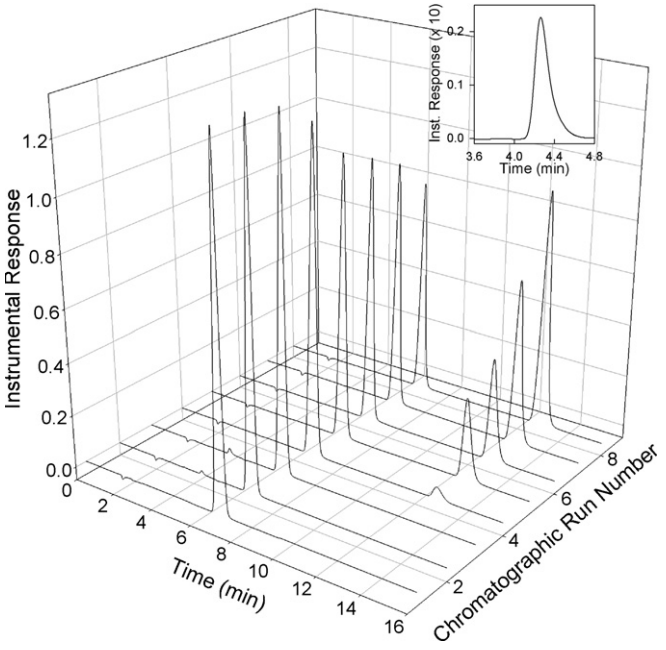


Fig. 4. Typical HPLC chromatograms of PRI and its degradation products under the optimized chromatographic conditions: (1) unstressed sample; (2) and (3) degradation under oxidizing conditions; (4–8) degradation under acid conditions, at different times.

3.4. Validation of the method for the determination of PRI, ELI and NOX

The optimized RP-HPLC method was validated according to ICH guidelines [21], with regards to specificity, accuracy, precision (repeatability and intermediate precision levels), range, linearity and robustness. System suitability features were also assessed.

3.4.1. System suitability test

The test was performed according to USP 30 [14] and BP 2007 [22] indications. The observed R.S.D. values (0.50%, 0.46% and 0.28% for PRI, ELI and NOX, respectively), were within the usually accepted values (≤2%). Capacity (k) and selectivity (α) factors, theoretical plates per meter, USP resolutions (R_s) and tailing factors (T_f) were also determined. Capacity factors were calculated employing the

Table 2
HPLC system suitability parameters

Analyte	k	α[a]	Resolution (R_s)[a,b]	Tailing factor (T_f)[c]	Efficiency (plates m^{-1})[b]	R.S.D. (%) for 5 separate injections
PRI	1.9	1.0	1.0	1.90	13,900	0.50
NOX	0.68	2.56	6.6	1.60	14,200	0.28
ELI	3.84	2.19	8.8	1.32	21,700	0.46

[a] With respect to the peak of PRI.
[b] Calculated according to the BP.
[c] Calculated according to the USP.

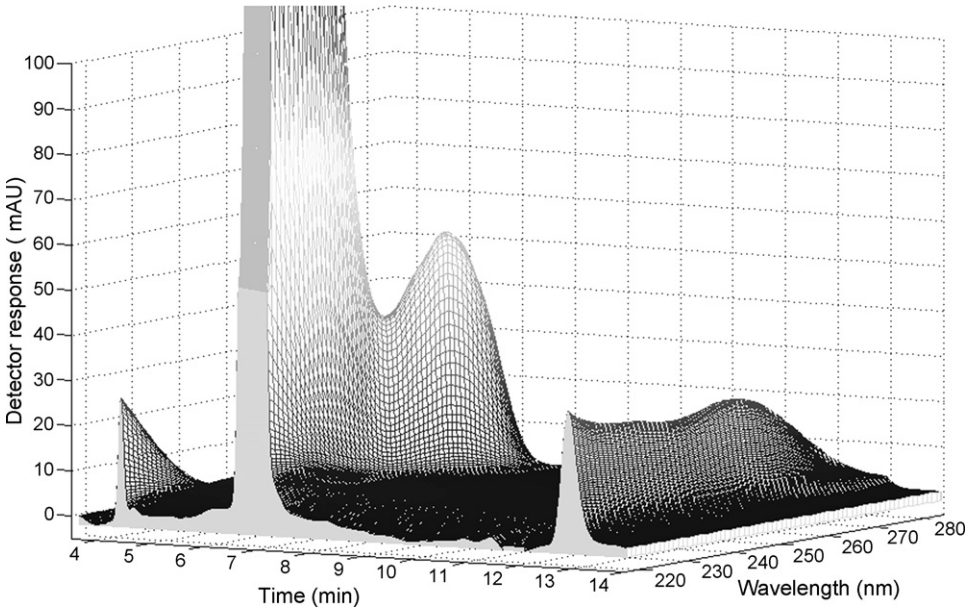


Fig. 5. Method specificity. HPLC-DAD analysis impurities NOX (13 μg ml^{-1}) and ELI (13 μg ml^{-1}) in the presence of PRI (1.00 mg ml^{-1}).

t_0 value experimentally determined by injecting the mobile phase. The results obtained (Table 2), were all within acceptable limits.

3.4.2. Specificity

The peak purity indices of the analytes in stressed solutions, determined with a PDA detector [23–25] under the optimized chromatographic conditions, were found to be better than 0.9997, indicating that no additional peaks were co-eluting with each of the analytes and evidencing the ability of the method to assess unequivocally the analytes of interest in the presence of potential interferences. Baseline resolution was achieved for all investigated compounds. The FDA guidance indicates that well-separated peaks, with resolution, $R_s > 2$ between the peak of interest and the closest eluted peak, are essential for reliable quantification [26]. All the peaks met this specification; visibly confirmed in Fig. 5.

3.4.3. Range and linearity

Solutions of PRI, covering the interval 10–150% of expected concentrations of the analyte, were employed. This is wider than the recommended range (80–120%), having been designed to also allow the valid determination of PRI in heavily degraded samples.

Taking into account that typical impurity tolerance levels currently range between 0.1 and 1.0%, and that identification of impurities below the 0.1% level is not considered to be necessary unless the potential impurities are expected to be unusually potent or toxic, linearity of the impurities was assessed employing solutions of ELI and NOX at concentration levels ranging from 0.05 to 1.3%, with regards to PRI (1.00 mg ml^{-1}).

The plots of area under the curves (AUC) of the peak responses of the analytes against their corresponding concentrations, they fitted straight lines responding to Eqs. [(1)–(3)].

$$\text{AUC} = 0.01(\pm0.03) \times 10^8 + 2.49(\pm0.03) \times 10^8 \times [\text{PRI, mg ml}^{-1}],$$
$$r = 0.9983(n = 18) \quad (1)$$

$$\text{AUC} = 0.20(\pm0.42) \times 10^6 + 7.25(\pm0.05) \times 10^6 \times [\text{ELI, \%}],$$
$$r = 0.9996(n = 18) \quad (2)$$

$$\text{AUC} = -0.02(\pm0.01) \times 10^6 + 2.00(\pm0.01) \times 10^6 \times [\text{NOX, \%}],$$
$$r = 0.9995(n = 18) \quad (3)$$

The y-intercepts were close to zero, with their confidence intervals containing the origin. The correlation coefficients (r) were 0.9983, 0.9996 and 0.9995 for PRI, ELI and NOX, respectively; these exceeded 0.98, the acceptance threshold suggested for linearity of procedures for the determination of impurities content in bulk drug [27]. Furthermore, the plot of the residuals exhibited random patterns, with the residuals passing the normal distribution test ($p < 0.05$), all of which evidenced that the method was linear in the tested range.

3.4.4. Precision

Precision was considered at the repeatability and intermediate precision levels. In order to verify repeatability, independent samples containing three concentration levels of the analytes were injected in triplicate, at random, by the same operator.

Table 3
Results of the determination of method repeatability

PRI			ELI			NOX		
Added (mg ml^{-1})	Recovered ± S.D. (mg ml^{-1})[a]	R.S.D. (%)	Added (μg ml^{-1})[b]	Recovered ± S.D. (μg ml^{-1})[a]	R.S.D. (%)	Added (μg ml^{-1})[b]	Recovered ± S.D. (μg ml^{-1})[a]	R.S.D. (%)
0.26	0.267 ± 0.002	0.63	1.00	1.05 ± 0.04	3.40	1.90	1.94 ± 0.03	1.73
0.78	0.789 ± 0.002	0.20	6.99	6.66 ± 0.14	2.10	7.00	7.27 ± 0.04	0.58
1.30	1.296 ± 0.001	0.11	12.00	11.60 ± 0.12	1.80	12.36	12.36 ± 0.01	0.11

[a] Triplicate injection of two independent samples at each concentration level.
[b] Final concentrations of the impurities in a mixture containing 0.78 mg ml^{-1} PRI.

Table 4
Two-way ANOVA results of the intermediate precision of the HPLC method

Analyte[a]	Source of variation	Sum of squares	Degrees of freedom	Mean square	*F*-ratio[b]
PRI	Between analysts	1.52	2	0.76	0.381
	Between days	2.68	1	2.68	1.343
	Days * Analysts	0.76	2	0.38	0.191
	Residual	59.90	30	2.48	
	Total	64.86	35		
ELI	Between analysts	26.26	2	13.13	0.397
	Between days	43.76	1	43.73	1.322
	Days * Analysts	14.67	2	7.34	0.803
	Residual	933.19	30	33.11	
	Total	1077.88	35		
NOX	Between analysts	1.11	2	0.56	0.439
	Between days	0.05	1	0.05	0.036
	Days * Analysts	0.29	2	0.14	0.113
	Residual	38.01	30	1.27	
	Total	39.46	35		

[a] Six independent samples containing three analyte levels were injected at random, by two different analysts in two different days.
[b] $F_{(0.95,\,1,\,30)} = 4.171$; $F_{(0.95,\,2,\,30)} = 3.316$.

The observed recoveries of PRI and its impurities were almost quantitative (Table 3), with all R.S.D. values complying with the acceptance criteria (<2.0% for PRI and <10% for ELI and NOX) [27].

The intermediate precision was determined simultaneously for PRI and its impurities. It was calculated as the inter-day variation produced on successive days by injecting six independent samples containing PRI and the impurities at three concentration levels, by three analysts. The drug recoveries and R.S.D. values in the inter-day assay, on the basis of the external calibration were $101.8 \pm 1.0\%$, $101.2 \pm 0.6\%$ and $99.7 \pm 0.3\%$ for PRI; $108.4 \pm 3.7\%$, $98.5 \pm 2.4\%$ and $97.9 \pm 1.2\%$ for ELI, and $102.0 \pm 0.9\%$, $104.1 \pm 0.5\%$ and $103.1 \pm 0.2\%$ for NOX, at the low, medium and high concentration levels, respectively. A two-way ANOVA of the data (Table 4) indicated that there is not significant difference between days and between analysts, confirming that the method is precise under the tested conditions.

3.4.5. Accuracy

Accuracy was evaluated by the simultaneous determination of the analytes in solutions prepared by the standard addition method. Recoveries of the added analytes were determined from their calibration curves. The results (Table 5) revealed low bias and essentially quantitative recoveries, indicating that the method enables the accurate determination of the analytes.

3.4.6. Robustness

The effects of deliberate variations in the pH and composition of the aqueous phase and the flow rate on drug recoveries were evaluated employing an experimental design. It was observed (Fig. 6) that the recovery of NOX increased with decrease in either pH, proportion of organic phase or flow rate, being less affected by the third factor. On the other hand, the recovery for ELI increased when pH, proportion of aqueous phase and flow rate were decreased, being the latter the most determining factor, followed by the pH of the aqueous phase. Low, acceptable variations in the percentage of recovery of PRI were found under the different conditions (PRI recovery rate of $101.4 \pm 1.9\%$). They evidence that there are no significant effects, except some changes noticed with pH of the aqueous mobile phase, a reduction of which resulted in lower separation efficiency and reduced retention times. However, all these variations were within acceptable limits. In addition, resolution between PRI and each of both impurities (>2) and the tailing factors (≤ 2) within the experimental domain were considered satisfactory, indicating method robustness.

3.4.7. Limits of detection and quantification

The limits of quantification (LOQ) [9,21] of ELI and NOX values were determined by the procedure recommended by Épshtein for impurities [27]. This consists in plotting the R.S.D. values of repeated determinations of the analyte in the neighborhood of the LOQ against their concentrations; LOQ is the lowest analyte concentration that can be determined with a given R.S.D. (10–20% according to different sources) [28]. For an R.S.D. of 10%, LOQ values for ELI and NOX were found to be 0.04 and 0.5 $\mu g\,ml^{-1}$ (0.04% and 0.05% relative to PRI, respectively) [27]. Accordingly, detection limits (3.3 times lower than the corresponding LOQs) of ELI and NOX were estimated as 0.013% and 0.017% relative to PRI.

3.5. Application of the HPLC method to samples of PRI-bulk substance

The validated HPLC method was applied to three different lots of unstressed samples of PRI, and also to stressed samples of these lots. As observed (Table 6), degradation products were observed only in stressed samples. Interestingly, exposure to visible light produced

Table 5
Results of the determination of the accuracy of the method

	PRI				ELI				NOX			
Conc. (%)[a]	71	86	100	115	0.2	0.4	0.6	0.8	0.2	0.4	0.6	0.8
Found (%)	72.1	86.7	101.5	116.3	0.205	0.401	0.606	0.796	0.210	0.401	0.610	0.790
Recovery (%)[b]	101.5	100.8	101.5	101.1	102.3	100.3	101.0	99.5	104.9	100.2	101.6	98.7
R.S.D. (%)	0.2	0.2	0.2	0.1	2.6	0.3	0.03	0.1	1.0	0.6	1.2	0.1
Bias (%)	+1.5	+0.8	+1.5	−1.1	+2.3	+0.3	+1.0	−0.5	+4.9	+0.1	+1.6	−1.3

[a] Final sample concentration, expressed as percentage relative to PRI (1.00 mg ml^{-1} solution), after fortification of a solution containing 71% PRI and 0.2% of each impurity.
[b] Samples were injected in triplicate.

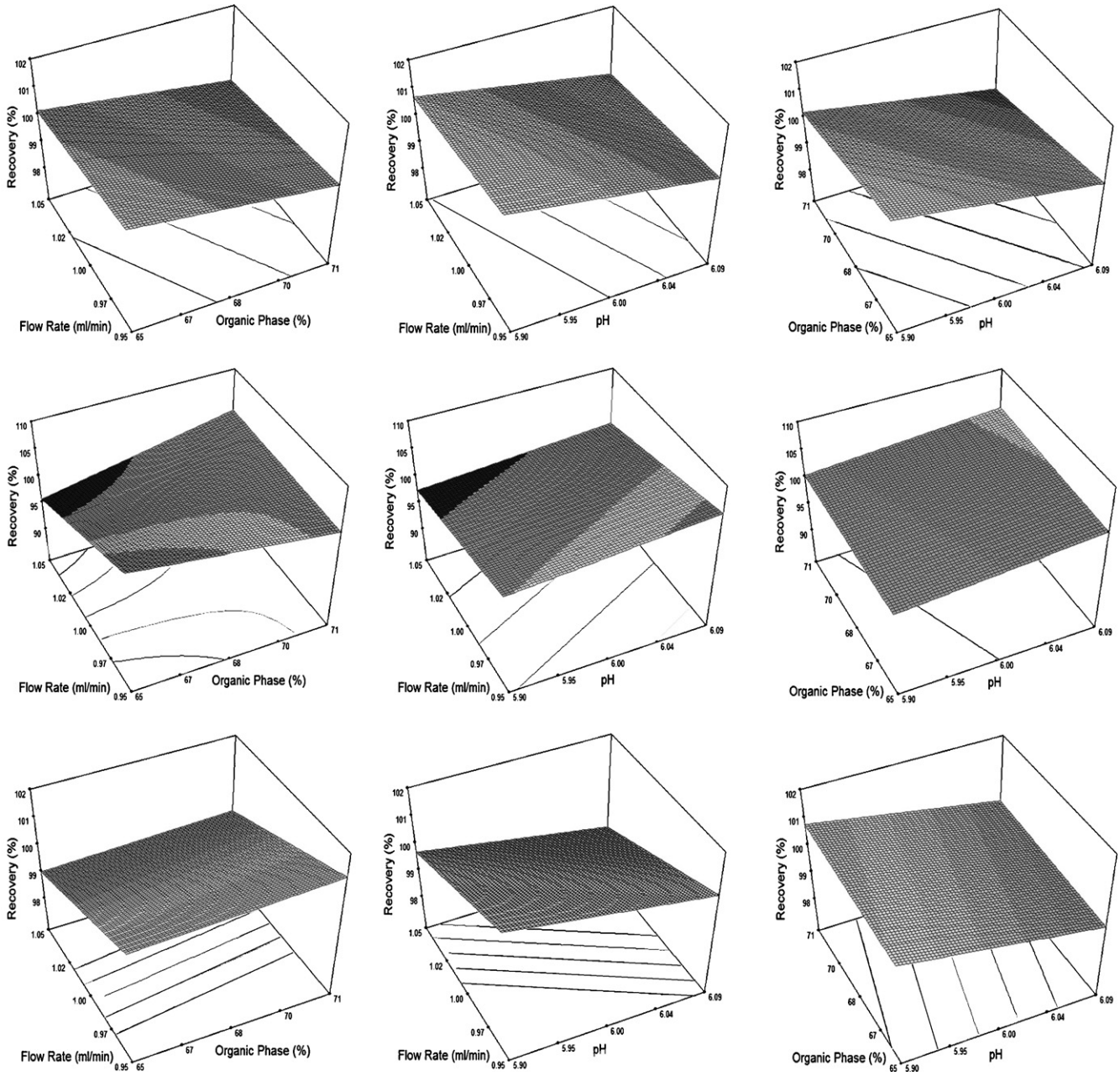


Fig. 6. Method robustness for the determination of NOX (above), PRI (middle) and ELI (below). Plots represent the system's response corresponding to the following values of the third variable: flow rate = 1.0 ml min^{-1}; organic phase = 68% and pH 6.0.

Table 6
Determination of ELI and NOX in stressed and unstressed samples of PRI

Sample	Stress condition	ELI (%)	NOX (%)
PRI-1	Unstressed	–	–
PRI-2	Unstressed	–	–
PRI-3	Unstressed	–	–
PRI-1	0.1N HCl, room temperature, 1 day	<LOQ	–
PRI-3	0.1N HCl, room temperature, 6 days	0.28	–
PRI-1	Visible light, H_2O, 14 days	0.70	–
PRI-1	Black-light, H_2O, 5 days	<LOQ	–
PRI-1	Black-light, H_2O, 14 days	0.13	–
PRI-1	0.3% H_2O_2, room temperature, 1 day	–	0.12
PRI-2	0.3% H_2O_2, room temperature, 2 days	–	0.22

0.7% ELI after 2 weeks, while reaction with H_2O_2 gave 0.22% NOX after 2 days.

3.6. Degradation kinetics of PRI under acid conditions

Solutions of PRI were degraded at 38 and 70 °C in 0.1N HCl; additional degradations were carried out at 70 °C in 0.2 and 0.4N HCl. The chromatograms obtained revealed that the peak area of PRI was reduced with time. The semi-logarithmic plots of concentration of PRI (mg ml^{-1}) against time in 0.1N HCl (Fig. 7) indicated an apparent first-order degradation behavior. Use of Eq. (4) [29], where $[A_0]$ is the concentration of PRI at the time $t = 0$ and $[A]_t$ is its concentration at time t, allowed calculation of the degradation rate constants (k), as the slopes of the lines, obtained by linear regression analysis. These were found to be 6.4 ($\pm$0.8) $\times$ 10^{-5} h^{-1} and 2.99

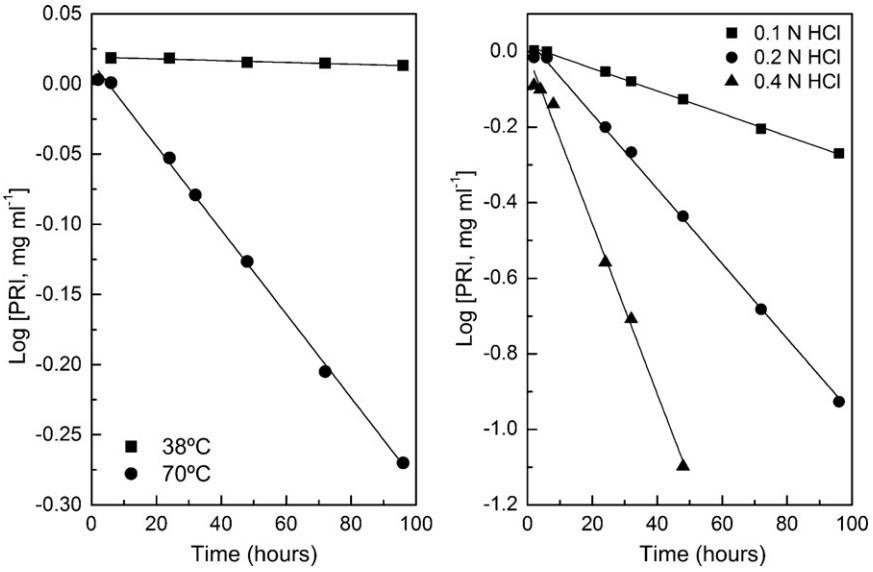


Fig. 7. (Left) Semi-logarithmic plot of the degradation of PRI against time, when exposed to 0.1N HCl at 38 °C (■) and 70 °C (●). (Right) Semi-logarithmic plot of the degradation of PRI at 70 °C *versus* time of exposure to HCl at concentrations 0.1N (■), 0.2N (●) and 0.4N (▲).

$(\pm 0.05) \times 10^{-3}\ h^{-1}$ ($r = 0.9988$) at 38 and 70 °C, respectively. The residual plots showed the absence of trends or correlations, representing only the experimental error. The half-life periods ($t_{1/2}$) were calculated according to Eq. (5) [29], and found to be about 10,830 and 232 h, respectively.

$$\log [A]_t = \frac{\log[A_0] - kT}{2.303} \quad (4)$$

$$t_{1/2} = \frac{0.693}{k} \quad (5)$$

$$\log \left(\frac{k_1}{k_2}\right) = \frac{E_a(T_2 - T_1)}{2.303RT_1T_2} \quad (6)$$

Application of Eq. (6), derived from the Arrhenius equation, where T_1 and T_2 are the absolute reaction temperatures (311 and 343 K) and R is the gas constant (1.987 cal mol^{-1}), resulted in an activation energy (E_a) for the acid-mediated degradation process of 25.5 Kcal mol^{-1}.

The activation energy (E_a) of the acid-mediated degradation process (25.5 Kcal mol^{-1}) was obtained with Eq. (4), derived from the Arrhenius equation, where T_1 and T_2 are the absolute reaction temperatures (311 and 343 K) and R is the gas constant (1.987 cal mol^{-1}).

On the other side, the reaction rates in acid medium at 70 °C were markedly dependent on the concentration of HCl (Fig. 7) with observed rate constants [2.99 $(\pm 0.05) \times 10^{-3}\ h^{-1}$ (0.1N), $(9.9 \pm 0.2) \times 10^{-3}\ h^{-1}$ (0.2N) and $(22.5 \pm 0.8) \times 10^{-3}\ h^{-1}$ (0.4N)] increasing linearly ($r = 0.9997$, $p < 0.05$) with an increase in acid concentration.

4. Conclusions

A stability study on pridinol mesylate was carried out and an efficient HPLC method for the quantification of PRI and its degradation products in bulk drug was developed and validated. The results of stress testing of the drug, undertaken according to the ICH guidelines, revealed that degradation products were formed under acidic, photolytic (visible light) and oxidizing conditions. The degradation products, identified as the dehydration and *N*-oxidation derivatives, were synthesized in good yields and spectroscopically characterized.

The kinetics of the acid-catalyzed degradation of pridinol was also studied in 0.1N HCl and found to be of first-order in analyte's concentration, with $t_{1/2}$ = 10,830 and 232 h at 38 and 70 °C, respectively, and an activation energy of 25.5 Kcal mol^{-1}. Degradation rates increased linearly with increased HCl concentrations in the 0.1–0.4N range.

Validation experiments provided proof that the HPLC analytical method is linear in the proposed working ranges for the three analytes, as well as accurate, precise (repeatability and intermediate precision levels), and specific, being able to separate the main drug from its degradation products. The proposed method was also found to be robust with respect to small variations in pH of the phosphate solution and composition of the mobile phase. Due to these characteristics, the method has stability-indicating properties for pridinol and, being fit for its intended purpose, it may find application for the routine analysis of this active pharmaceutical ingredient and its degradation impurities, in bulk substance.

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