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Il Farmaco 58 (2003) 513–519
IL FARMACO
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A validated spectrophotometric and liquid chromatography method for determination and purity evaluation of 4-methoxy-2-[2-hydroxy-3(4-phenyl-1-piperazinyl)]propyl-2,3-dihydro-6-methyl-1,3-dioxo-1*H*-pyrrolo[3,4-c]pyridine

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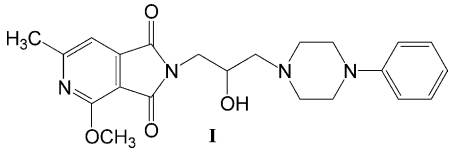
Received 4 December 2002; accepted 15 March 2003

Abstract

A spectrophotometric analysis of the UV-spectrum of 4-methoxy-2-[2-hydroxy-3(4-phenyl-1-piperazinyl)]propyl-2,3-dihydro-6-methyl-1,3-dioxo-1*H*-pyrrolo[3,4-c]pyridine (**I**) in 0.01 M HCl was performed by determining the values of specific absorption coefficients at the following analytical wavelengths: 224, 285 and 348 nm. The separation by means of TLC of compound **I** and of its five decomposition products was also studied. Silica gel coated plates (60 F_{254}) were used and the mobile phase was consisted of butanol–acetic acid (1.05 kg/l)–water (80:12:30, v/v/v). The HPLC method (LiChrosorb® 100 RP-18 column 250 × 4.0 mm I.D., dp = 5 μm; mobile phase: acetonitrile–0.01 M phosphate buffer ($H_3PO_4 + KH_2PO_4$; pH 3) (50:50, v/v-phase A) or (30:70, v/v-phase B) was validated by determination of the following parameters: selectivity, precision, linearity, stability of the analite and LOD as well as LOQ. Kinetic studies of the decomposition process of **I** in both acidic and alkaline environments indicate instability of the imide group.

Keywords: 4-Alkoxy-2,3-dihydro-6-methyl-1,3-dioxo-1*H*-pyrrolo[3,4-c]pyridine derivatives; UV; HPLC; TLC; Stability



I

1. Introduction

The starting issue for the study was the observation that *N*-[2-hydroxy-3(4-phenyl-1-piperazinyl)]propyl derivatives of 2-methoxy(ethoxy)-6-methyl-3,4-pyridinedicarboxyimides [1,2] and 2,3-dihydro-1,3-dioxo-1*H*-pyrrolo[3,4-c]pyridine (3,4-pyridinedicarboximide) [3,4] cause in animal tests significant analgetic activity and do not display any noticeable toxic effects (LD_{50} > 2000 mg/kg). Within the scope of a study aiming at establishing the structure/activity relationship in this group of imides, the following structural modifications were made: an introduction of $-OCH_3$ or $-CF_3$ groups into the phenyl substituent at *N*-4 of the piperazine fragment; removal of the –OH group from the propyl chain with a concommitant introduction of $-OCH_3$ into the phenyl residue at position N-4 of piperazine; replacement of the propyl chain by a butyl chain; insertion of $-OC_2H_5$ or a heteroaryl substituent in place of $-OCH_3$ at position C-2 of pyridine ring. The so far conducted pharmacological studies indicate that after insertion of $-CF_3$ into the phenyl residue, the so modified compounds revealed weaker analgetic activity than the reference substance: 4-methoxy-2-[2-hydroxy-3(4-phenyl-1-piperazinyl)]-propyl-2,3-dihydro-6-methyl-1,3-dioxo-1*H*-pyrrolo[3,4-c]pyridine (**I**) which displayed the strongest analgesic potency. In the 'hot-plate' test

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0014-827X/03/$ - see front matter
doi:10.1016/S0014-827X(03)00062-4

the imide **I** produced a significant effect up to the dose of 12.5 mg/kg and possessed very strong activity in the 'writhing syndrome' test. The most potent effect was produced by the imide **I** which was effective up to a dose of 0.39 mg/kg. The replacement of the methoxy group by the ethoxy one caused almost fourfold decrease of the analgesic activity in this test. Similarly, for the derivatives with $-CF_3$ group in the phenyl ring, the imide with the methoxy group in the pyridine ring was more active than its ethoxy analog. The introduction of trifluoromethyl or methoxy groups into the phenyl substituent at N-4 of piperazine in the compound **I** also weakens the analgetic properties [5,6]. Pharmacological studies pertaining to this group of compounds are under way. It would be of considerable importance to undertake studies aiming at evaluation of the stability of these compounds as well as at describing the mechanisms of their decomposition both when in aqueous solution and in solid state. For that purpose, the authors developed an analytical method to study the process of decomposition of substance **I** and the formation of degradation products that eventually may be present as contaminants of the main substance.

2. Experimental

2.1. Chemicals and reagents

The compound, 4-methoxy-2-[2-hydroxy-3(4-phenyl-1-piperazinyl)]propyl-2,3-dihydro-6-methyl-1,3-dioxo-1*H*-pyrrolo[3,4-c]pyridine:

- $C_{22}H_{26}N_4O_4$
- molecular mass: 410.47
- elementary composition [Calc. (Found)]: C, 64.23 (64.49); H, 6.57 (6.41); N, 13.62 (13.72)%
- melting point: 189–191 °C

in this paper designated as **I**. It was obtained by synthesis in the Department of Chemistry of Drugs at the Wroclaw University of Medicine. 4-Hydroxybenzoic acid methyl ester; $C_8H_8O_3$ (serving as the internal standard—i.s.) was a product of Sigma Chemical CO (St. Louis, MO). Other chemicals used were all of an analytical reagent grade.

2.2. Apparatus

The compounds studied were determined and their decomposition-traced by means of HPLC. The analytical system consisted of a Rheodyne 7120 20 μl fixed–loop injector, an LC 3-UV detector (Pye Unicam, UK), an L-6000 A pump (Merck–Hitachi, Germany) and an A/C transmitter with the software Chromed (Medson, Poland). LiChrosorb® 100 RP-18 column 250 × 4.0 mm I.D., dp = 5 μm (Merck) utilized was used. The flow rate was 1.0 ml/min. UV detection was carried out at 239 nm.

The spectrophotometric analysis was performed using the UV/VIS Lambda 20 (Perkin Elmer) spectrophotometer. The HPLC-MS analysis was made in the HPLC-MS Laboratory of the Faculty of Chemistry at Adam Mickiewicz University, Poznań, Poland. The analytical system consisted of a Waters 2690 chromatograph, a Photodiode Waters 936 detector, a Waters-micromass ZQ with ES ionization mass-spectrometer.

3. Results and discussion

3.1. Spectrophotometric analysis

The specific absorption coefficient ($a^{1\%}_{1\,cm}$) of **I** in 0.01 M HCl was determined by measuring the absorbances of six individual solutions at maximum absorption wavelengths $\lambda = 224$, 285 and 348 nm (Fig. 1) over a concentration ranges from 0.001160 to 0.001464% ($\lambda =$ 224 nm, $a^{1\%}_{1\,cm} = 657 \pm 6$, SD = 5.95, RSD = 0.91%, $n =$ 6) and from 0.0097 to 0.0103% ($\lambda = 285$ nm, $a^{1\%}_{1\,cm} = 68 \pm 1$, SD = 0.897, RSD = 1.31%, $n = 6$; $\lambda = 348$ nm, $a^{1\%}_{1\,cm} = 129 \pm 1$, SD = 1.17, RSD = 0.91%, $n = 6$).

3.2. Thin-layer chromatgraphy

Plates coated with an 0.2 mm thick layer of silica gel 60 F_{254} (Merck) served as the carrier for the stationary phase. The chromatographic solvent consisted of a mixture of butanol–acetic acid (1.05 kg/l)–water (80:12:30, v/v/v). The solvent system advanced approximately 15 cm in about 6 h. The above mentioned TLC-system ensured a chromatographic separation of **I** and its degradation products (**Ia**, **Ib**, **Ic**, **Id**, **Ie**) following hydrolysis in 0.1 M HCl and 60 °C (Table 1). The limit of detection (LOD) for **I** was found to be 1 μg (or 0.1 μg for detection in UV-light at 365 nm).

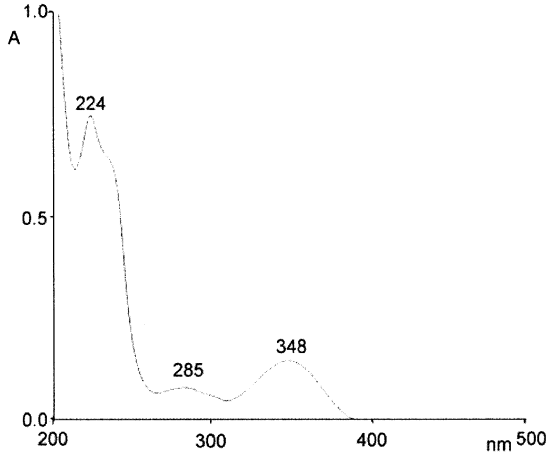


Fig. 1. UV-spectrum of **I** in 0.01 M HCl.

Table 1
Thin-layer chromatography (TLC)

Species	$R_f \pm \Delta R_f$	SD	RSD (%)	Observation
I	0.70±0.01	0.0191	2.75	blue fluorescence in UV-light at 365 nm
Ia	0.60±0.03	0.0494	8.31	
Ib	0.51±0.02	0.0207	4.08	
Ic	0.41±0.01	0.0141	3.45	yellow spot; green fluorescence in UV-light at 365 nm
Id	0.31±0.02	0.0232	7.61	violet-pink colour with ninhydrine reagent
Ie	0.30±0.02	0.0223	7.39	blue fluorescence in UV-light at 365 nm

3.3. High-performance liquid chromatography

The mobile phase consisted of acetonitrile and 0.01 M phosphate buffer ($H_3PO_4 + KH_2PO_4$; pH 3) (50:50, v/v-phase A) or (30:70, v/v-phase B). 4-Hydroxybenzoic acid methyl ester (0.5 mg/ml in methanol) was used as the internal standard—i.s. The retention time was ca. 3.6 min for i.s. and 5.3 min for **I** in mobile phase A or ca. 7.0 and 13.2 min in mobile phase B, respectively.

3.3.1. Optimization of the method, selectivity

A mixture of acetonitrile in phosphate buffer ($H_3PO_4 + KH_2PO_4$; pH 3) was used to optimize the chromatographic separation of **I** from its degradation products to ensure adequate characteristics, such as resolution and short time of analysis. Among the various contents of acetonitrile (28–60%), a 50% content was finally chosen for a determination of **I** and a 30% content of acetonitrile for purity assay and the kinetic analysis of degradation products. The plot of capacity factor (k') as a function of acetonitrile concentration is shown in Fig. 2. The addition of sodium 1-heptanosulfonate to the mobile phase did not affect the capacity factor of **I**. The exchange of acetonitrile for methanol caused the peak of **I** to disappear. The presence of acetonitrile in the mobile phase showed sharp symmetrical peaks of **I** and of its degradation products. The presence of phosphate buffer in the chromatographic solvent allowed a clear cut separation of **I** as well as of its degradation products (**IA**, **IB**, **IC**, **ID**) (Figs. 3 and 4). After complete degradation of **I** (0.1 M HCl, 60 °C) no peak corresponding to **I** was observed in the chromatogram (Fig. 4).

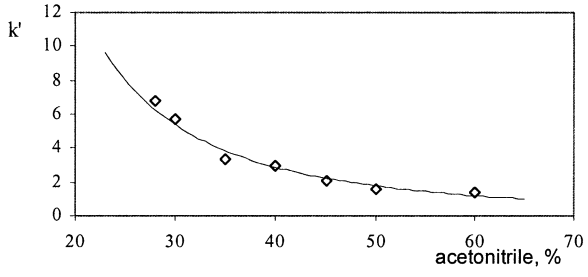


Fig. 2. Plot of k' as a function of acetonitrile in mobile phase.

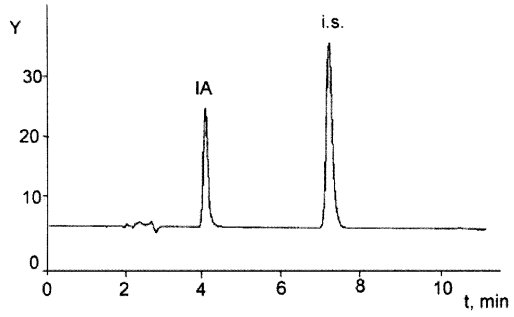


Fig. 3. Typical chromatogram showing the separation of **I** and its degradation product **IA** in basic medium after the hydrolysis (0.01 M NaOH, pH 10.95, 60 °C, $\mu = 0.5$ M) using the HPLC method (mobile phase B)—conditions listed in the text.

3.3.2. Stability of analite

The apparent pseudo-first-order rate constant for the hydrolysis of **I** (110 μg/ml) in 0.01 M HCl at temperature 25 °C was determined using the HPLC method (mobile phase A), according to the equation:

$$\ln(p_t/p_i) = \ln(p_o/p_i) - kt \quad (1)$$

where p_t and p_o are the peak areas of **I** at time t and at zero time, respectively; p_I is the peak area of the internal standard; k is the observed rate constant [$k = (5.14 \pm 0.71) \times 10^{-7}$ s^{-1}, $r = -0.9950$, $n = 6$]. For calculation of the values $t_{0.1}$ and $t_{0.5}$ the following equation was used:

$$t_{0.1} = 0.1054/k = 2.5 \text{ days} \quad (2)$$

$$t_{0.5} = 0.693/k = 15.5 \text{ days} \quad (3)$$

3.3.3. Precision and accuracy

A summary of the results regarding the precision and accuracy of the method obtained from the measured concentrations for the validation samples is given in Table 2. The precision of the method was determined through the analysis of six replicate injections of standard solution containing 25, 50 and 75 μg/ml of substance **I** dissolved in 0.01 M HCl. The RSDs ≤ 1% are satisfactory.

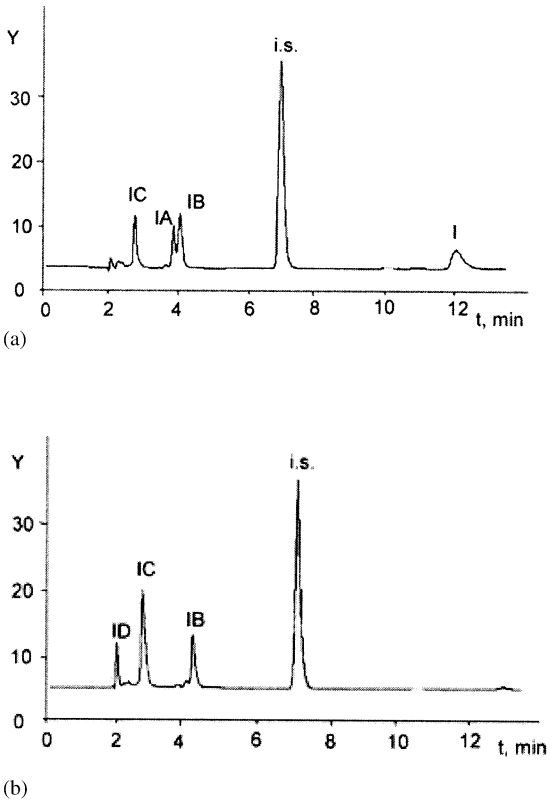


Fig. 4. Typical chromatogram showing the separation of **I** and its degradation products **IA**, **IB**, **IC**, **ID** in acidic medium after the hydrolysis (0. 1 M HCl, pH 1.11, 60 °C, $\mu = 0.5$ M, (a) after 57 h, (b) after 362 h) using the HPLC method (mobile phase B)—conditions listed in the text.

3.3.4. Linearity

The linearity between p/p_i (p, p_I—areas of substance **I** and i.s.) and concentrations of **I** in 0.01 M HCl ranging from 5.5 to 220.0 μg/ml was evaluated. The internal standard was added to the solutions under investigation in at a constant concentration of 50 μg/ml. Linearity was also examined for 3 consecutive days in solutions of the same concentration prepared from the stock solution. The equation for the calibration curve is: $y = (0.0242 \pm 0.0002)x$; (for the equation $y = ax + b$, the value b is insignificant). The calculated correlation coefficient was > 0.999, thus indicating good linearity.

3.3.5. Sensitivity

The detection limit depends on two parameters, such as the composition of the mobile phase and the detection mode as for instance the UV detection. In this study the detection limit (LOD) was approximately 1.65 μg/ml and the limit of quantification (LOQ) was 5.5 μg/ml.

3.4. Kinetical interpretation of *I* degradation in aqueous solutions

The solutions of **I** (0.338 mg/ml) in either hydrochloric acid (0.1 M, pH 1.11, ionic strength $\mu = 0.50$ M) or in sodium hydroxide solution (0.01 M, pH 10.95, ionic strength $\mu = 0.50$ M) were stored at 60 or 37 °C and concentration changes (represented by the ratios: p_t/p_i) of substance **I** in the course of time were recorded (Fig. 5). To each 1.0 ml sample to be analyzed, 1.0 ml of i.s. (0.15 mg/ml) and 1.0 ml of water of the sample were added. The pseudo-first-order rate constants were determined using the HPLC method (mobile phase B), according to the equations:

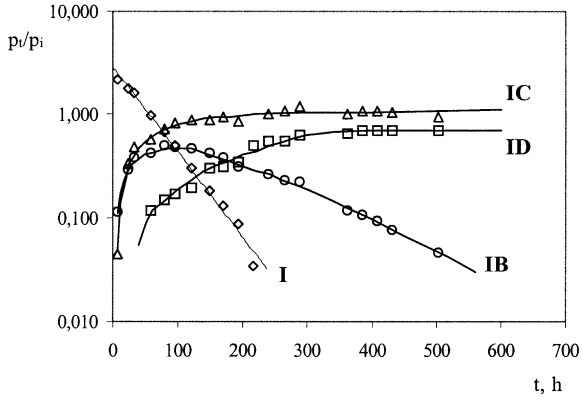


Fig. 5. Plots of p_t/p_i as a function of time for decomposition of **I** (0.338 mg/ml), formation and decomposition of **IB**, formation of **IC** and **ID** in acidic medium (0. 1 M HCl, pH 1.11, 60 °C, $\mu = 0.5$ M).

Table 2
Accuracy and precision of the measurement of **I** in 0.01 M HCl ($n = 6$)

Day of analysis	Nominal concentration (μg/ml)	Measured concentration (μg/ml)	SD	RSD (%)
	25.0	25.1 ± 0.1	0.132	0.53
0 day	50.0	50.7 ± 0.3	0.264	0.52
	75.0	75.4 ± 0.2	0.219	0.29
	25.0	24.6 ± 0.1	0.136	0.55
1 day	50.0	50.4 ± 0.3	0.299	0.59
	75.0	75.4 ± 0.2	0.149	0.20
	25.0	24.9 ± 0.3	0.254	1.00
Inter day	50.0	50.5 ± 0.4	0.420	0.83
	75.0	75.3 ± 0.7	0.733	0.97

Table 3
The observed rate constants for the degradation of **I** and formation or degradation of its products

Decomposition terms	Compounds	t_R (min)	$k \pm \Delta k$ (s^{-1}); $t_{0.1}$, $t_{0.5}$	$-r$	n
0.1 M NaOH, pH 10.95, $\mu = 0.50$ M	**IA**	3.8			
0.1 M HCl, pH 1.11, $\mu = 0.50$ M, 60 °C	**I**	13.2	$k_1 = (6.20 \pm 0.31)\ 10^{-6}$ $t_{0.1} = 5$ h $t_{0.5} = 31$ h	0.9974	10
	IB	4.2	$k_1 = (6.58 \pm 0.78)\ 10^{-6}$ $k_2 = (1.67 \pm 0.12)\ 10^{-6}$	0.9963 0.9945	6 12
	IA	3.8			
0.1 M HCl, pH 1.11, $\mu = 0.50$ M, 37 °C	**I**	13.2	$k = (5.43 \pm 0.72)\ 10^{-7}$ $t_{0.1} = 2.2$ days $t_{0.5} = 14.8$ days	0.9912	8

Table 4
Tabele 4 The observed ions in the HPLC-MS analysis

Decomposition terms	t_R (min)	ES^- (m/z)	ES^+ (m/z)	Molecular mass	Symbol of product
0.1 M NaOH, pH 10.95, $\mu = 0.50$ M, 60 °C	8.7	427	429	428	**IA**
0.1 M HCl, pH 1.11, $\mu = 0.50$ M, 60 °C	14.2		411	410	**I**
	9.0	427	429	428	**IB**
	8.4		236	235	**IC**
	5.9	210	212	211	**ID**
	5.9	166		167	$M_{ID}-CO_2$
	5.9	122		123	$M_{ID}-2CO_2$

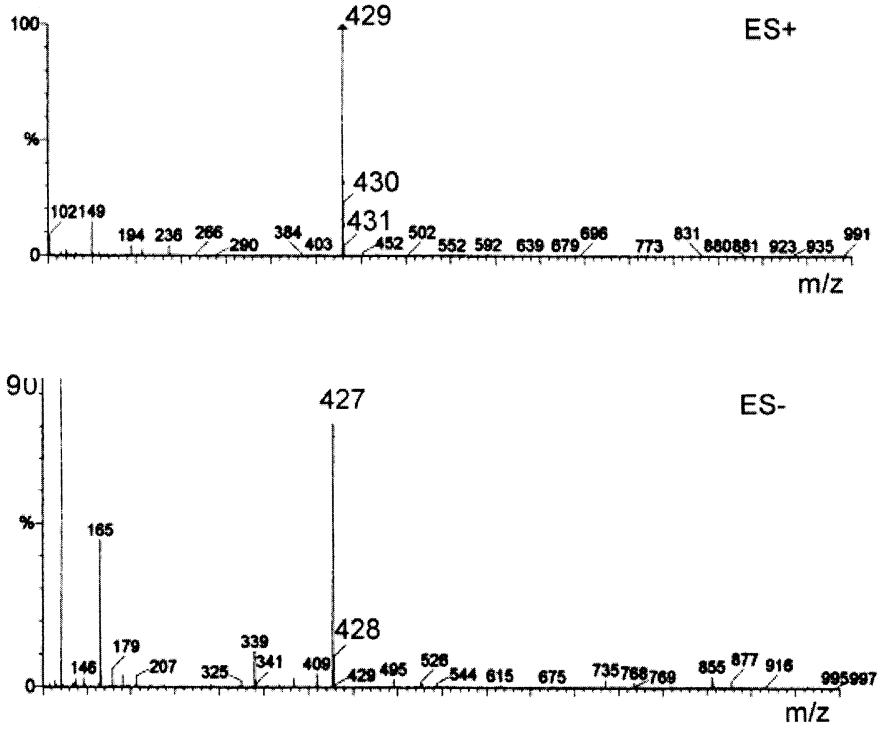


Fig. 6. Mass spectra of product degradation **IA**.

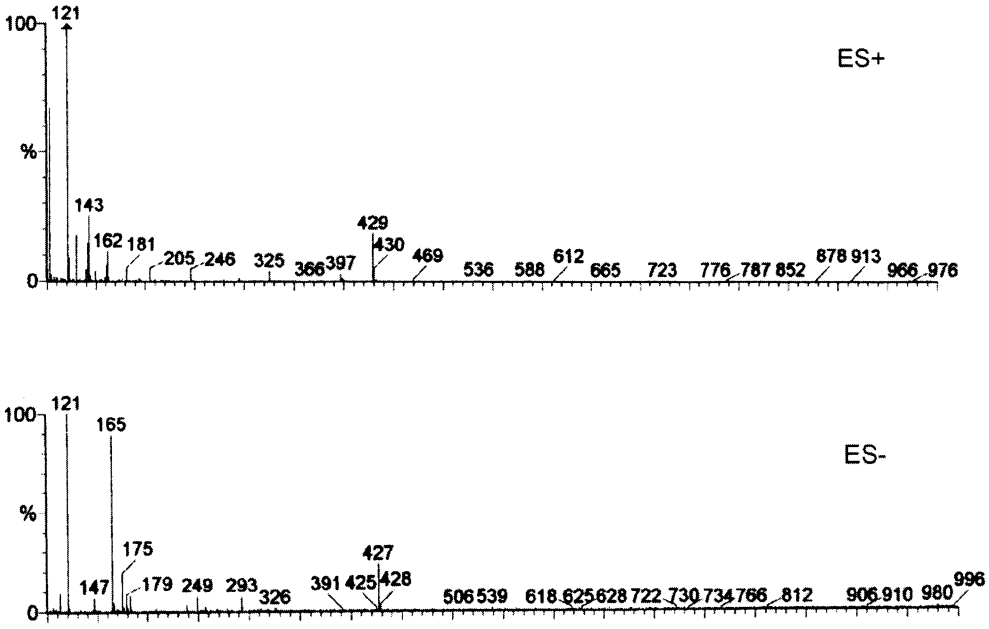
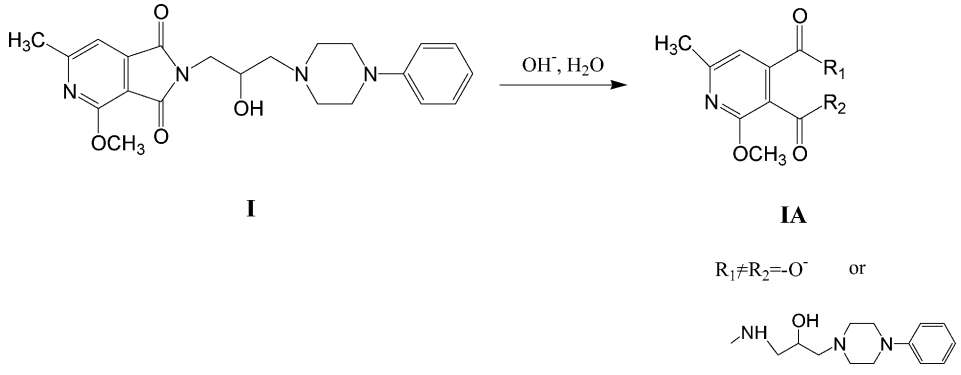
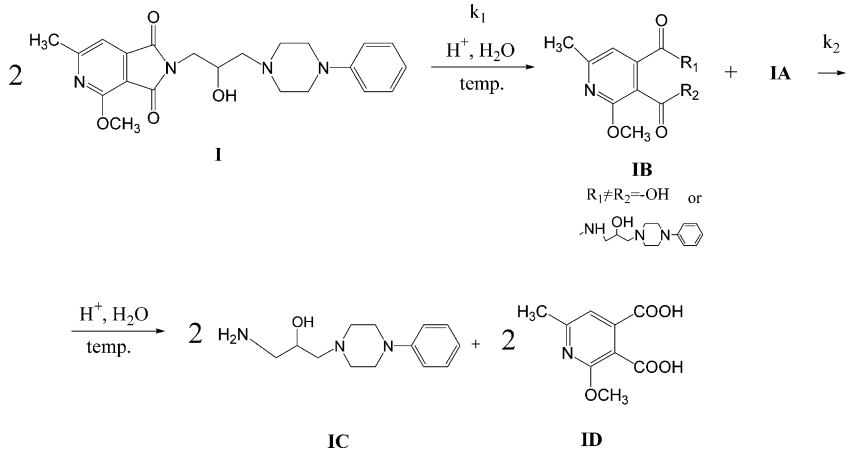


Fig. 7. Mass spectra of product degradation **IB**.



Scheme 1. Possible mechanism of degradation of **I** in basic medium.



Scheme 2. Possible mechanism of degradation of **I** in acidic medium.

$$\ln P_{t,\mathbf{I}} = \ln P_{o,\mathbf{I}} - k_1 t \quad (4)$$

$$\ln P_{t,\mathbf{IB}} = \ln P_{o,\mathbf{IB}} - k_2 t, \text{ for } t > t_{max} \quad (5)$$

$$\ln(P'_t - P_t)_{\mathbf{IB}} = \ln(P'_o - P_o)_{\mathbf{IB}} - k_1 t, \text{ for } t < t_{max} \quad (6)$$

$P = p/p_i$, the P' values was calculated by Eq. (5).

The observed rate constants k_1 (degradation of **I** and formation of **IB**) did not show any significant statistical differences (Table 3). In the basic solution, the decomposition of **I** occurs in statu nascendi resulting in the formation of **IA**.

The HPLC-MS analysis [column: LiChrosorb® 100 RP-18, 250 × 4.0 mm I.D., dp = 5 μm (Merck); mobile phase: acetonitrile–water–10% formic acid (40:55:5, v/v/v); flow-rate: 0.5 ml/min] suggests a degradation (hydrolysis) of the two imide bonds at N-2. The degradation products **IA** and **IB** were characterized by $M = 428$ (Table 4, Figs. 6 and 7). These compounds were found to be products of acid-catalysed hydrolysis of **I**. In the basic solution only product **IA** was observed.

4. Conclusion

The aim of this study was to determine specific absorption coefficients for **I** in 0.01 M HCl, at analytical wavelengths of 224, 285 and 348 nm and to specify of experimental conditions for TLC and HPLC separations of compound **I** and its degradation products. Both separation methods have proved adequate for the evaluation of purity of **I**. When separated by means of TLC, five degradation products of **I** were found (**Ia**, $R_f = 0.60$; **Ib**, $R_f = 0.51$; **Ic**, $R_f = 0.41$; **Id**, $R_f = 0.31$; **Ie**, $R_f = 0.30$), whereas HPLC revealed only four such products (**IA**, $t_R = 3.8$ min; **IB**, $t_R = 4.2$ min; **IC**, $t_R = 3.0$ min; **ID**, $t_R = 2.0$ min). Kinetic studies of the degradation of **I** as well as of the formation of degradation products and of their further decomposition have shown that during the early stage of these processes, in either an acidic or basic at an increasing temperature an instant hydrolysis of the imide bonds is obserwed resulting in the formation of a –COOH in position 3 or 4 of the pyridine ring (product **IA** or **IB**, $M = 428$; substrate **I**, $M = 410$). In an alkaline medium, the product **IA** does not undergo further degradation and is remained the only additional product formed in this study (Scheme 1). In these conditions **IA** is formed instantly—in statu nascendi—at room temperature. In an acidic medium (0.1 M HCl, pH 1.11, $\mu = 0.5$ M, 60 °C) the product **IA** is formed with a ca. 7% yield and in this medium the product **IB** is the major product of the degradation of **I**. A positive staining with the ninhydrine reagent of a thin-layer chromatogramme indicated that one of the subsequent degradation product is an amino derivative resulting from hydrolysis of an amide group–probably in substance **IA** or/and **IB** (Scheme 2). Studies aiming at identification of the chemical structure of the individual degradation products of **I** are in progress.

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