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Kinetics and mechanism of acidic hydrolysis of nordazepam studied by high-performance liquid chromatography and fourth-order derivative ultraviolet spectrophotometry

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Abstract

A reversed-phase HPLC method was developed to study the acid-catalysed hydrolysis of nordazepam in hydrochloric acid solutions of 0.01, 0.1 and 1.0 M. One intermediate was observed, which was isolated and identified. The mechanism of hydrolysis appeared to be biphasic, showing a consecutive reaction with a reversible first step. Initial breakage of the azomethine bond, followed by a slow hydrolysis of the amide bond resulted to creation of the benzophenone product in strongly acidic solutions. A fourth-order derivative method for monitoring the parent compound itself was also proposed and evaluated, as well. Relative standard deviation was less than 2% for the HPLC and less than 5% for the derivative method. Detection limits for nordazepam, intermediate and final degradation product were 1.8×10^{-9} M, 2.1×10^{-9} M and 2.0×10^{-9} M, respectively, in the former method and 7.0×10^{-7} M for nordazepam in the latter. Estimation of k_1, k_{-1} and k_2 values was tried and results of HPLC and fourth-order derivative methods were compared.

Keywords: HPLC method; Derivative spectrophotometry; Nordazepam or nordiazepam or *N*-desmethyldiazepam; Acidic hydrolysis; Kinetics; Mechanism

1. Introduction

Chemical stability of pharmaceuticals (Connors et al., 1978) is a matter of growing concern in many analytical laboratories. This is because systematic kinetic studies on the decomposition of drugs using stability testing techniques is essential for their quality control.

Nordazepam (Mayer et al., 1974) 7-chloro-5-phenyl-2,3-dihydro-1*H*-benzo[*e*]diazepin-2-one,

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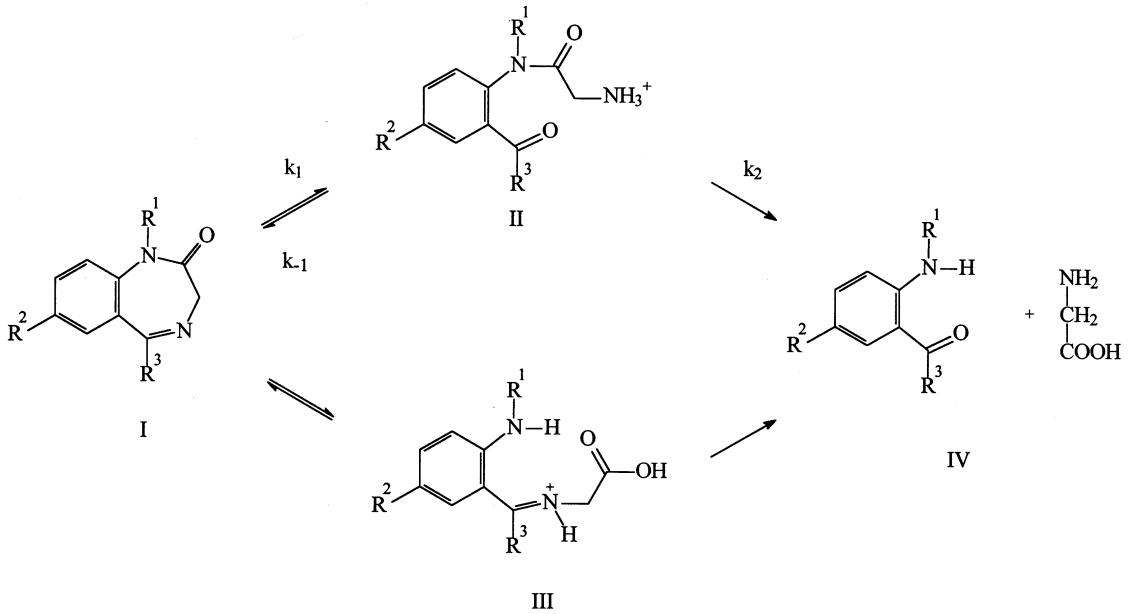

Nordazepam **Ia** R^1=H, R^2=Cl, R^3=C_6H_5

Scheme 1. General scheme of acidic hydrolysis of 1,4-benzodiazepinones in aqueous solutions.

belongs to the 1,4-benzodiazepinone class of tranquillizing agents. These compounds represent a large group of therapeutic drugs used as anxiolytic, sedatives, sleep inducers and skeletal muscle relaxants. They undergo hydrolysis in acidic aqueous solutions which may be important for their absorption in the gastrointestinal tract.

Owing to the importance of the mechanism of this reaction, degradation kinetics of such drugs has been studied by many authors (Maulding et al., 1975; Han et al., 1976, 1977a,b; Cho et al., 1983; Broxton et al., 1984; Broxton and Morrison, 1985; Pfendt et al., 1990; Moro et al., 1991; Yang et al., 1994). Even though there is a general scheme of their hydrolysis (Scheme 1), the reaction is complicated and in many cases the results are vague and not conclusive. Several 1,4-benzodiazepines, although similar in structure, are strongly different in their acidic hydrolysis kinetics. They form long- or short-lived intermediates, they show monophasic or biphasic kinetics.

In our work we intended to study kinetics of the acidic degradation of nordazepam or nordiazepam or *N*-desmethyldiazepam (Scheme 1, $R^1 = H$, $R^2 = Cl$, $R^3 =$ phenyl), which is a metabolite of many benzodiazepines, including chlordiazepoxide, chlorazepate, diazepam, medazepam and prazepam. It is a minor tranquillizer, orally given and although several methods have been reported for its determination (El-Bardicy et al., 1992a,b, 1993), nothing has been found on the mechanism and kinetics of its hydrolysis in aqueous media. Motivation of such study came from the fact that this compound behaves as a model in the series of 7-chloro-1,4-diazepin-2-ones because it is the least substituted one. Studying this drug, predictions can be made for other more substituted members of this group.

In this paper, we report a kinetic investigation of nordazepam in strongly acidic solutions. For this purpose, we have developed a reversed-phase HPLC method to determine **Ia** and its hydrolysis products **IIa** and **IVa** simultaneously, which can

be used as a stability-indicating method, as well. We have also evaluated a fourth-order derivative UV spectrophotometric technique (trying to cut in cost and time) for the determination of nordazepam in the presence of its hydrolysis products, showing its advantages and limitations. We propose a mechanism of this degradation reaction after the isolation and identification of the intermediate product. Finally, we give values of the calculated rate constants k_1, k_{-1} and k_2.

2. Experimental

2.1. Equipment

The high-performance liquid chromatographic system used, consisted of a Waters Model 501 pump and a Rheodyne Model 7125 injector with a 20-μl loop, which were coupled to a Waters Model 486 UV-Vis detector with a 8-μl flow cell operated at 230 nm and a Hewlett-Packard Model HP-3394A integrator.

A Hitachi (Tokyo, Japan) double beam UV/Vis spectrophotometer (Model U-2000) was employed for zero-, second-, and fourth-order derivative spectrophotometric measurements. The following operational conditions were utilised: scan mode, WL; data mode, ABS, with user baseline; start wavelength, 360 nm; stop wavelength, 220 nm; scan speed, 100 nm min^{-1} and medium response. In the derivative mode, sensitivity values (term related to the smoothing of the signal) equal to 1 and 2 were applied to the second- and fourth-order spectra.

A pH meter Metrohm, Model 654 Herisau performed all pH measurements.

A Heto water-bath facilitated for the accelerated kinetic studies.

IR-spectra were obtained using a Perkin Elmer model 883 infrared spectrophotometer.

All Nuclear Magnetic resonance spectra were recorded on a Varian Unity Plus-300. The isolated intermediate was dissolved in DMSO-d_6. ^{1}H- and ^{13}C NMR spectra were acquired using a frequency of 200 MHz and 50 MHz, respectively.

Mass spectral analysis was performed on a GC-MS instrument, Model VG-TRIO 1000 operated on EI mode at 70 eV and DIP (direct inlet probe); the ion source maintained at 230°C.

2.2. Reagents

Nordazepam ($C_{15}H_{11}N_2OCl$, $M_r = 270.72$) of pharmaceutical purity grade was kindly provided by Uni-Pharma Hellas A.E. (Athens, Greece), and was used without any further purification.

The intermediate degradation product ($C_{15}H_{13}N_2O_2Cl$, $M_r = 288.73$) was isolated in crystals in the following way: 0.750 g of nordazepam was dissolved in 30 ml of methanol and 150 ml HCl 0.1 M were added. The resulting mixture was heated under reflux for 2 h. Upon heating the mixture turned clear. The clear solution was then cooled to room temperature, a cloudy yellowish mixture formed (due to low solubility of both the intact molecule and the benzophenone, the aniline moiety of which does not form salt with dilute mineral acids, in aqueous conditions) was filtered and the solution extracted five times with 50-ml aliquots of ethyl acetate and five times with 50-ml aliquots of diethyl ether. The aqueous phase was neutralized with solid potassium hydrogen carbonate and the resulting mixture was extracted exhaustively with ethyl acetate. The organic phase was dried with anhydrous potassium sulphate, filtered and the solvent was removed under reduced pressure. The resulting solid is the intermediate hydrolysis product in the free base form along with a small amount of nordazepam. For a greater degree of purity, the solid was dissolved in a small volume of ether and the hydrochloric salt of the intermediate was precipitated as a yellow solid by adding an etheric solution of HCl. This was filtered quickly and desiccated over P_2O_5 under reduced pressure (400 mg). The whole process was monitored at every step by means of HPLC, in both phases (organic and aqueous) during the extraction procedure.

The final product ($C_{13}H_{10}NOCl$, $M_r = 231.68$) was bought from Aldrich. Moreover, it was isolated in crystalline form as follows: 0.5 g of nordazepam was dissolved in methanol and heated under reflux for 2 h along with 100 ml of HCl 1 M. The clear solution was left to cool at room temperature and was further refrigerated

overnight. The yellow crystals formed were collected by vacuum filtration, washed several times with water and dried under reduced pressure over P_2O_5. This process was also monitored by HPLC. The formed product was identical in every aspect with the commercial one (verified by mp, IR, MS and HPLC).

2.2.1. Derivative spectrophotometric method

All reagents used were of analytical reagent grade and distilled, de-ionized water was consumed.

Stock methanolic solution of nordazepam, **Ia**, 7.4×10^{-4} M was prepared by dissolving 0.0200 g of pharmaceutical grade **Ia** in 100 ml of methanol.

Stock methanolic solution of the intermediate, **IIa**, 7.4×10^{-4} M was prepared by dissolving 0.0213 g of the isolated product in 100 ml of methanol.

Stock methanolic solution of 2-amino-5-chlorobenzophenone, **IVa**, 7.4×10^{-4} M was prepared by dissolving 0.0171 g of the commercially available chemical in 100 ml of methanol.

Working standard solutions of **Ia** and **IIa** in the range of 3.7×10^{-6}–2.2×10^{-5} M and **IVa** in the range of 7.4×10^{-6}–2.2×10^{-5} M were prepared daily from the corresponding stock solutions by dilution in 0.01, 0.1 and 1.0 M hydrochloric acid solutions and used for construction of calibration curves of **Ia**, **IIa** and **IVa**.

Mixed working standard solutions of **Ia**, **IIa** and **IVa** were prepared for recovery studies as follows: standard solutions of **Ia** in the range of 3.7×10^{-6}–1.5×10^{-5} M containing constant concentration of **IIa** or/and **IVa** equal to 7.4×10^{-6} M each and series of standard solutions of **IIa** or **IVa** in the range of 3.7×10^{-6}–1.5×10^{-5} M containing a constant concentration of **Ia** equal to 7.4×10^{-6} M.

Standard solutions of **Ia** equal to 2.2×10^{-5} M in 0.01, 0.1 and 1.0 M HCl were freshly prepared for nordazepam accelerated kinetic studies.

2.2.2. HPLC method

All solvents were of HPLC grade and were purchased from Lab-Scan Science (Ireland). Ammonium acetate (pro analysi), potassium dihydrogen phosphate and hydrochloric acid (analytical reagent grade) were purchased from E. Merck (Germany). Water was deionised and further purified by means of a Milli-Q Plus water purification system (Millipore).

During the HPLC study we used the stock solutions prepared in the derivative procedure, diluted to the appropriate concentration.

Series of working standard solutions and mixed working standard solutions of **Ia**, **IIa** and **IVa** (1 + 1 + 1) were prepared in the range of 0.4×10^{-7}–1.0×10^{-7} M by appropriate dilution of each stock solution. These solutions were used for construction of calibration curves and statistical evaluation of the HPLC method.

2.3. Measurement procedure

2.3.1. HPLC method

Chromatographic separation was performed on a reversed phase hypersil MOS C-8 column (250 × 4.6 mm I.D., 5 μm particle size) Shandon HPLC (UK). The mobile phase, methanol/acetonitrile/0.005 M potassium dihydrogen phosphate and 0.1 M ammonium acetate buffer adjusted to pH 6.0 with glacial acetic acid (35 + 20 + 45), was filtered through a 0.45-μm Millipore filter and degassed under vacuum, prior to use. A flow rate of 1.5 ml min^{-1} with a column inlet pressure of 2500 psi was used in order to separate nordazepam from its degradation products. Peak heights were measured using a Hewlett-Packard Model HP3394A integrator. HPLC analysis was conducted at ambient temperature (24 ± 1°C).

Calibration curves of **Ia**, **IIa** and **IVa** were constructed using series of working and mixed working standard solutions of these compounds as described previously. The concentration range tested was 0.4–1.0×10^{-7} M for each one of the compounds. All of these compounds were analysed immediately after their preparation. Peak heights of the compounds were measured for their quantitative determination.

The over-all precision and accuracy of the assay was evaluated by analysing two series of mixed working solutions of **Ia**, **IIa** and **IVa** at concentrations of 0.5×10^{-7} and 1.0×10^{-7} M each, (1 + 1 + 1), in five replicates. The relative standard deviation %R.S.D. was determined in order to assess the precision of the method.

2.3.2. Derivative spectrophotometric method

Prepared working standard solutions of **Ia**, **IIa** and **IVa** were measured against a blank solution of HCl 0.01, 0.1 or 1.0 M. Measurement procedure was similar to that described elsewhere (Archontaki, 1995).

2.3.3. Kinetic investigation of the acidic hydrolysis

2.3.3.1. HPLC method. A 0.5-ml aliquot of the stock standard solution of nordazepam was transferred to a 200-ml volumetric flask and diluted to volume with the appropriate concentration of hydrochloric acid. This solution was transferred to a two-necked round-bottomed flask. One neck of the flask was fitted with a reflux condenser and samples were collected from the other neck. The entire flask assembly was submerged in a thermostated water-bath and the temperature was allowed to equilibrate prior to the addition of nordazepam solution. This procedure was repeated at different pH values and temperatures shown in the corresponding table.

During the kinetic study at predetermined time intervals, a 1.0-ml aliquot was removed from the flask and a 1.0-ml aliquot of the mobile phase was added, followed by vigorous mixing. Immediately after its preparation the sample solution was injected into the analytical column, in order to prevent further possible hydrolysis of nordazepam in the aqueous medium.

2.3.3.2. Derivative spectrophotometric method. Procedure is described elsewhere (Archontaki, 1995). Kinetic investigation took place under the following conditions: First in 0.01 M HCl at temperatures of 66, 70 and 74°C, where the total duration of the study was 360, 270 and 220 min, respectively. Second, in 0.1 M HCl at temperatures of 60, 63 and 68°C, where the total duration of the study was 480, 360 and 270 min, respectively. Third, in 1.0 M HCl at temperatures of 58, 62 and 66°C, where the total duration of the study was 430, 320 and 290 min, respectively.

Treatment of the data for both methods was carried out in the way mentioned elsewhere (Archontaki, 1995).

3. Results and discussion

3.1. Structure elucidation of N-(2-benzoyl-4-chlorophenyl)-2-aminoacetamide

The intermediate isolated during the degradation process of nordazepam was analysed by GC-MS, ^{1}H- and ^{13}C-NMR, and IR spectroscopy. The most characteristic data received are the following: EI-MS m/z, 289 ($\dot{M}^+$), 290 ($\dot{M}^+ + 1$), 291 ($\dot{M}^+ + 2$), 105 ($PhC\dot{O}^+$), 77 ($P\dot{h}^+$); ^{1}H-NMR (DMSO-d_6), 3.1 ppm (2H, amine), 4.2 ppm (2H, methylenic), 7.1–8.3 ppm (8H, aromatic) and 10.6 ppm (1H-amidic); ^{13}C-NMR (DMSO-d_6), 15 carbon atoms, 193 ppm (1C, ketone), 165 ppm (1C, amide), 123–136 ppm (12C, aromatic), 65 ppm (1C, methylenic); IR (KBr) 1707 cm^{-1} (strong, ketonic –C=O), 1660 cm^{-1} (strong, amidic –C=O).

Mass spectrometry showed us the molecular ion of this compound, which agrees with that expected from Scheme 1 ($M_r = 289.55$). In other words, the intermediate should be **IIa** or **IIIa**. Moreover, from the ^{13}C-NMR data shown above, there is a carbon atom at 193 ppm which should definitely belong to a ketone-carbonyl moiety, since only this group appears in the region beyond 180 ppm. As a result, intermediate **IIIa**, 2-[(*E*)-1-(2-amino-5-chlorophenyl)-1-phenylmethylidene-amido)acetic acid, is precluded because it contains an acid-carbonyl moiety that should be in the region of 170 ppm or less. The other carbon atom of the intermediate at 165 ppm should be the amidic one. ^{13}C-NMR spectrum of nordazepam showed peaks at 170 and 168 ppm corresponding to the amidic and azomethine carbon atoms. Also, in the IR spectrum of the intermediate the two strong peaks of similar intensity at 1707 and 1660 cm^{-1} are characteristic for the group –C=O coming from the ketone and amide part of this molecule. Nordazepam showed peaks at 1685 and 1650 cm^{-1} corresponding to amidic C=O and C=N, respectively. However, their intensity is very different. The former is strong while the latter one is rather medium. This is one more evidence that the observed intermediate is **IIa**. Furthermore, ^{1}H-NMR spectrum is consistent with the proposed structure.

In addition to the above spectral structure elucidation of the intermediate, we performed comparative color-reactions of **Ia**, **IIa** and **IVa** on TLC plates. More specifically, positive reactions with ninhydrin revealed the existence of an α-amino-acylo group in **IIa** and with 4,4′-methylenebis-*N*,*N*′-dimethylaniline in chlorine environment the existence of primary or secondary aliphatic amines in **Ia** and **IIa**. Finally, the last piece of evidence for the proposed structure **IIa** of the intermediate is the procedure of its isolation, e.g. the formation of a hydrochloric salt with etheric hydrochloric acid solution showing the existence of a non-aromatic amino group since the aniline moiety of 2-amino-chloro-benzophenone does not form salts with dilute mineral acids.

3.2. Chromatograms and spectral characteristics

A typical chromatogram obtained during the kinetic study on hydrolysis of **Ia**, is displayed in Fig. 1. **Ia** is eluted at 8.3 min, while the retention times of the two degradation products **IIa** and **IVa** are 7.1 and 15.4 min, respectively. Good separation of the three species of interest was accomplished, allowing determination of each one

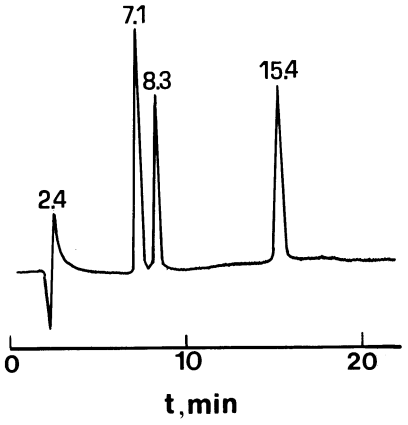

Fig. 1. Representative chromatogram of a mixture of **Ia**, **IIa** and **IVa** at retention times of 8.3, 7.1 and 15.4 min, respectively. Chromatographic conditions: reversed-phase HPLC on a C-8 column; mobile phase: methanol/acetonitrile/0.005 M potassium dihydrogen phosphate and 0.1 M ammonium acetate buffer adjusted to pH 6.0 with glacial acetic acid (35:20:45, v/l), and UV detector set at 230 nm.

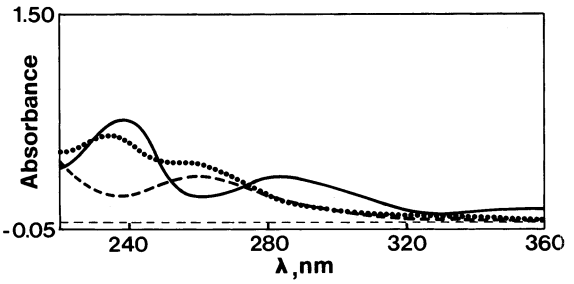

Fig. 2. Zero-order absorbance spectra (pathlength 1.0 cm) of **Ia** (solid line), **IIa** (dotted line) and **IVa** (broken line) at concentrations equal to 2.2×10^{-5} M each in a 0.1 M hydrochloric acid solution.

accurately without any interference from the other two.

Since spectrophotometric techniques are easier to use, faster and less expensive than HPLC method, we wanted to check their applicability to this chemical system. However, looking at the spectra of Fig. 2, it is obvious that zero-order UV-Vis technique is insufficient for determination of any of the above three species because of the strong overlap of the three spectra. In this situation, we decided to use the advantages of derivative spectrophotometry.

In Fig. 3, we see the second-order derivative spectra of **Ia, IIa** and **IVa** in 0.1 M HCl. We chose the distance, *d*2, between the maximum at 252 nm and the minimum at 244 nm in the

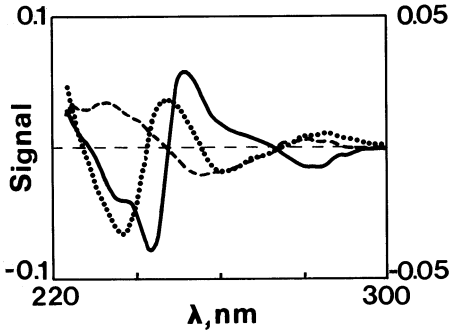

Fig. 3. Second-order derivative spectra (pathlength, 1.0 cm) of **Ia** (solid line), **IIa** (dotted line) and **IVa** (broken line) at concentrations of 2.2×10^{-5} M each in a 0.1 M hydrochloric acid solution. The left-hand *y*-axis (-0.1 -0.1) corresponds to **Ia**, while the right-hand *y*-axis (-0.05 -0.05) corresponds to **IIa** and **IVa**.

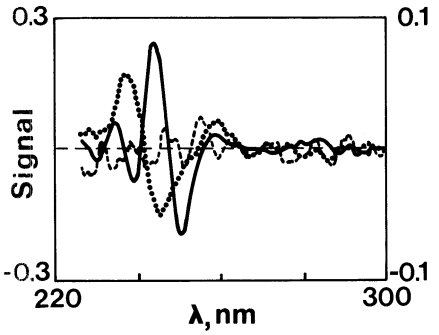

Fig. 4. Fourth-order derivative spectra (pathlength 1.0 cm) of **Ia** (solid line), **IIa** (dotted line) and **IVa** (broken line) at concentrations of 2.2×10^{-5} M each in a 0.1 M hydrochloric acid solution. The left-hand y-axis (-0.3 -0.3) corresponds to **Ia** while the right-hand y-axis (-0.1 -0.1) corresponds to **IIa** and **IVa**.

spectrum of **Ia** as a trial signal to work with because it shows a moderate interference from the presence of **IIa** and **IVa**. This is actually the smallest interference from **IIa** and **IVa** on **Ia** compared with other graphical amplitudes in the second-order derivative spectrum of **Ia**. Direct determination of **IIa** or **IVa** in the presence of **Ia** would be erroneous because the latter interferes with the signal of the former considerably, looking at the difference in the y-axis between these spectra.

In Fig. 4, we present the fourth-order derivative spectra of **Ia**, **IIa** and **IVa** in a 0.1 M hydrochloric acid solution. It is important to notice that the major peak of compound **IVa** has almost disappeared (a clear advantage of derivative spectrophotometry, as broad peaks tend to be eliminated in the derivatization process). Again, considering the difference in the y-axis of these spectra, direct determination of **IIa** and **IVa** in the presence of **Ia** would be erroneous. Meanwhile, distances, $d4_1$ and $d4_2$, between maxima and minima (244–251) and (244–239) nm, respectively, in the fourth-order derivative spectrum of **Ia** were used as trial signals for the determination of nordazepam with insignificant interference from the corresponding spectra of the degradation products.

3.3. Linearity and reproducibility

3.3.1. HPLC method

Under the experimental conditions described in a previous section linear regression analysis of HPLC data gave the following equations for the calibration curves of **Ia**, **IIa** and **IVa**:

$$H_{\mathbf{Ia}} = 101.8\ (\pm 0.8) \times C_{\mathbf{Ia}} - 0.80\ (\pm 0.6) \quad r = 0.9999,\ \text{S.E.} = 0.024,\ n = 5 \tag{1}$$

$$H_{\mathbf{IIa}} = 86.8\ (\pm 0.8) \times C_{\mathbf{IIa}} - 0.9\ (\pm 0.6) \quad r = 0.9998,\ \text{S.E.} = 0.022,\ n = 5 \tag{2}$$

$$H_{\mathbf{IVa}} = 30.2\ (\pm 0.3) \times C_{\mathbf{IVa}} - 0.2\ (\pm 0.2) \quad r = 0.9998,\ \text{S.E.} = 0.008,\ n = 5 \tag{3}$$

where $H_{\mathbf{Ia}}$, $H_{\mathbf{IIa}}$ and $H_{\mathbf{IVa}}$ are the peak heights of **Ia**, **IIa** and **IVa**, respectively; $C_{\mathbf{Ia}}$, $C_{\mathbf{IIa}}$ and $C_{\mathbf{IVa}}$ are their corresponding concentrations $\times 10^7$ times in M, r is the correlation coefficient, S.E. is the standard error of the estimate and n is the number of points in each calibration curve.

Moreover, data for precision and accuracy of this method were acquired using two series of mixed working standard solutions of **Ia**, **IIa** and **IVa**. %R.S.D. ($n = 5$) was less than 1.4, 1.5 and 2.0 for **Ia**, **IIa** and **IVa**, respectively. $\%E_r$ for both concentrations involved, mentioned in the experimental section was less than 2.0% in all cases. The above statistical evaluation of the HPLC method revealed its good linearity and reproducibility and led us to the conclusion that it could be used for the kinetic investigation of nordazepam reliably.

The limit of detection attained for **Ia**, **IIa** and **IVa** as defined by IUPAC (Winefordner and Long, 1983) $DL_{(k=3)} = k \times S_b/b$ (where b is the slope of the calibration graphs and S_b is the standard deviation of the blank signal) was found to be 1.8×10^{-9} M, 2.1×10^{-9} M and 2.0×10^{-9} M, respectively.

3.3.2. Derivative spectrophotometric method

Since derivative spectrophotometry is not an established technique as HPLC, we performed an extensive study on its capabilities in a complex system as the one presented here. We derived calibration curves of nordazepam, under the experimental conditions described previously, for

Table 1
Analytical parameters of the calibration curves of nordazepam, **Ia**, in the absence of degradation product **IIa** in acidic solutions of (A) 0.01 M HCl, (B) 0.1 M HCl, (C) 1.0 M HCl and in the presence of **IIa** in solutions of (D) 0.1 M HCl

Mode[a]	Concentration range of **Ia** (M)	Concentration of **IIa** (M)	Selected distance (nm)	Regression equation[b]		
				Intercept ($a \pm s$)	Slope ($b \pm s$)	r (n)[c]
(A) $d2_1(1)$	3.7×10^{-6}–2.2×10^{-5}	—	252–244 (2nd deriv., $s=1$)	0.000 ± 0.005	6217 ± 333	0.994 (6)
$d4_1(2)$			244–251 (4th deriv., $s=2$)	-0.012 ± 0.008	$21\,470 \pm 569$	0.999 (6)
$d4_2(2)$			244–239 (4th deriv., $s=2$)	-0.006 ± 0.009	$13\,433 \pm 135$	0.9998 (5)
(B) $d2_1(1)$	3.7×10^{-6}–2.2×10^{-5}	—	252–244 (2nd deriv., $s=1$)	-0.003 ± 0.002	7398 ± 128	0.9997 (4)
$d4_1(2)$			244–251 (4th deriv., $s=2$)	-0.0009 ± 0.0008	$22\,657 \pm 54$	0.99999 (4)
$d4_2(2)$			244–239 (4th deriv., $s=2$)	0.006 ± 0.008	$16\,526 \pm 557$	0.9990 (4)
(C) $d2_1(1)$	3.7×10^{-6}–2.2×10^{-5}	—	252–244 (2nd deriv., $s=1$)	-0.005 ± 0.003	6516 ± 175	0.9997 (6)
$d4_1(2)$			244–251 (4th deriv., $s=2$)	-0.018 ± 0.009	$20\,396 \pm 618$	0.99999 (6)
$d4_2(2)$			244–239 (4th deriv., $s=2$)	-0.007 ± 0.005	$14\,828 \pm 380$	0.9990 (6)
(D) $d2_1(1)$	3.7×10^{-5}–1.5×10^{-6}	7.4×10^{-6}	252–244 (2nd deriv., $s=1$)	0.002 ± 0.002	7310 ± 192	0.9995 (4)
$d4_1(2)$			244–251 (4th deriv., $s=2$)	-0.020 ± 0.003	$22\,261 \pm 251$	0.99990 (4)
$d4_2(2)$			244–239 (4th deriv., $s=2$)	-0.041 ± 0.003	$16\,675 \pm 282$	0.9997 (4)

[a] Measured distances as peak-trough amplitudes $d2(1)$, of the second-order derivative spectra and $d4_1(2)$, $d4_2(2)$ of the fourth-order spectra of **Ia**.
[b] Distances $dm_l(j)$ fit in the regression equation $dm_l(j) = a + bc$, where $m = 2, 4$, $l = 1, 2$, and $j = 1, 2$ as they appear under the 'mode' column in the above table
[c] n, number of points in each calibration curve; each point is the mean of three experimental measurements.

Table 2
Recovery of nordazepam, **Ia**, in the presence of its acidic-induced degradation product **IIa**

	Concentration of **Ia** (M)	Concentration of **IIa** added (M)	Recovery (%)		
			$d2_1(1)$	$d4_1(2)$	$d4_2(2)$
(A)[a]	3.7×10^{-6}	7.4×10^{-6}	108.7	97.7	60.8
	7.4×10^{-6}		104.1	99.4	84.2
	1.1×10^{-5}		101.1	100.1	95.8
	1.5×10^{-5}		99.5	100.2	102.4
	Mean $\pm$ s		103.4 ± 4.0	99.4 ± 1.2	85.8 ± 18.3
(B)[b]	7.4×10^{-6}	3.7×10^{-6}	—	101.6	87.0
		7.4×10^{-6}		98.6	76.1
		1.1×10^{-5}		99.8	71.4
		1.5×10^{-5}		100.0	70.7
	Mean $\pm$ s			100 ± 1.2	76.3 ± 7.0

[a] Increasing amounts of **Ia** are added to a constant concentration of **IIa** equal to 7.4×10^{-6} M and the recovery of **Ia** is measured against the same concentration of pure **Ia**.
[b] Increasing amounts of **IIa** are added to a constant concentration of **Ia** equal to 7.4×10^{-6} M and the recovery of **Ia** is measured against the same concentration of pure **Ia**.

the second-order derivative signal $d2(1)$ and for the fourth-order ones $d4_1(2)$ and $d4_2(2)$. Numbers 1 and 2 in parenthesis are instrumental parameters, related to the smoothing of spectra, increasing as the number goes from 1 to 10. The results derived by least-squares regression analysis, applied to the above standard curves, are presented in Table 1.

Measuring the same sample three times (in all concentrations) we calculated a relative standard deviation (R.S.D.) of less than 3% for the second-order and less than 5% for the fourth-order derivative spectra.

To ensure applicability of the derivative approach to the kinetic investigation of nordazepam, an interference study was conducted in mixed standard solutions of **Ia**, **IIa** or/and **IVa**, where concentration of **IIa** or/and **IVa** was kept constant and that of **Ia** was varied. Representative results in 0.1 M hydrochloric acid solutions are also included in Table 1. A t-test was applied in all cases and it was verified that differences between slopes from working and mixed standard solutions were statistically insignificant (at a confidence level of 95%). Even more, two sets of experiments were carried out and recoveries were calculated in two ways. First, increasing amounts of **Ia** were added to a constant concentration of **IIa** or/and **IVa** and the recovery of **Ia** was measured and second increasing amounts of **IIa** or

Table 3
Recovery of nordazepam, **Ia**, in the presence of its acidic-induced degradation product **IVa**

Concentration of **Ia** (M)	Concentration of **IVa** added[a] (M)	Recovery (%)		
		$d2_1(1)$	$d4_1(2)$	$d4_2(2)$
7.4×10^{-6}	3.7×10^{-6}	90.2	101.1	98.3
	7.4×10^{-6}	83.3	99.5	92.5
	1.1×10^{-5}	81.0	98.5	93.5
	1.5×10^{-5}	76.4	100.8	93.5
Mean $\pm$ s		82.7 ± 5.8	99.7 ± 1.0	94.4 ± 2.6

[a] Increasing amounts of **IVa** are added to a constant concentration of **Ia** equal to 7.4×10^{-6} M and the recovery of **Ia** is measured against the same concentration of pure **Ia**.

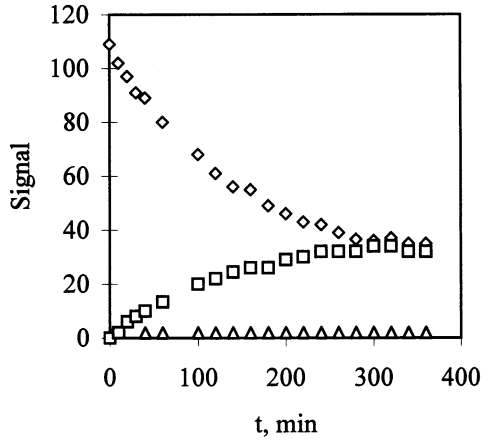

Fig. 5. Plot of the HPLC signal of nordazepam (◇), intermediate (□), and benzophenone (△) during an accelerated degradation study of **Ia** in a 0.01 M HCl solution at 66°C.

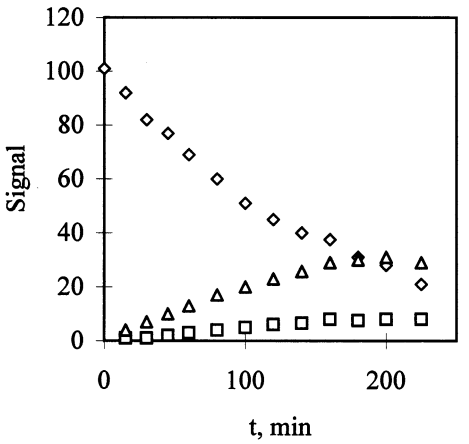

Fig. 7. Plot of the HPLC signal of nordazepam (◇), intermediate (□), and benzophenone (△) during an accelerated degradation study of **Ia** in a 1.0 M HCl solution at 66°C.

IVa were added to a constant concentration of **Ia** and the recovery of **Ia** was measured against a known concentration of pure **Ia**. Representative results were tabulated in Tables 2 and 3, respectively.

From both tables, we can see that on one hand the second-derivative method does not show good accuracy or sometimes it is not even detectable and on the other hand the most accurate and reproducible signal for the determination of nordazepam is the $d4_1(2)$.

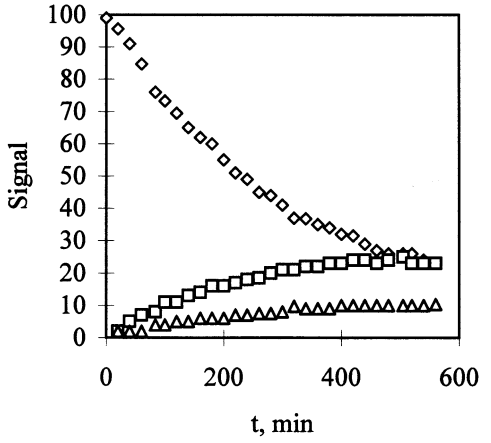

Fig. 6. Plot of the HPLC signal of nordazepam (◇), intermediate (□), and benzophenone (△) during an accelerated degradation study of **Ia** in a 0.1 M HCl solution at 60°C.

The same type of studies were conducted for **IIa** and **IVa**, as well. However, as expected, there was no derivative signal free of interference from **Ia**. As a result we did not present these data.

Defining the detection limit (DL) as the concentration that gives a signal equal to $b + 3S_b$, where b is the signal of the blank and S_b is its standard deviation, we estimated that DL for the developed fourth-order derivative method was 7×10^{-7} M.

3.4. Kinetic investigation

Accelerated stability measurements were performed under the experimental conditions described earlier. We tried to explore the mechanism of degradation of **Ia** in 0.01, 0.1 and 1.0 M hydrochloric acid solutions, relying on the data of HPLC. Derivative spectrophotometry cannot do this work in such a complex reaction. We have illustrated some results of the HPLC method in Figs. 5–7. There we can see the trend and behavior of this chemical system changing the concentration of hydrochloric acid solution. Taking into consideration the way of the isolation of the intermediate, MS, NMR and IR data and the positive specific reactions that took place, we believe that nordazepam proceeds through initial breakage of the azomethine linkage. In 0.01 and 0.1 HCl, we observed biphasic kinetics in **Ia** as it is demonstrated very clearly in Fig. 8.

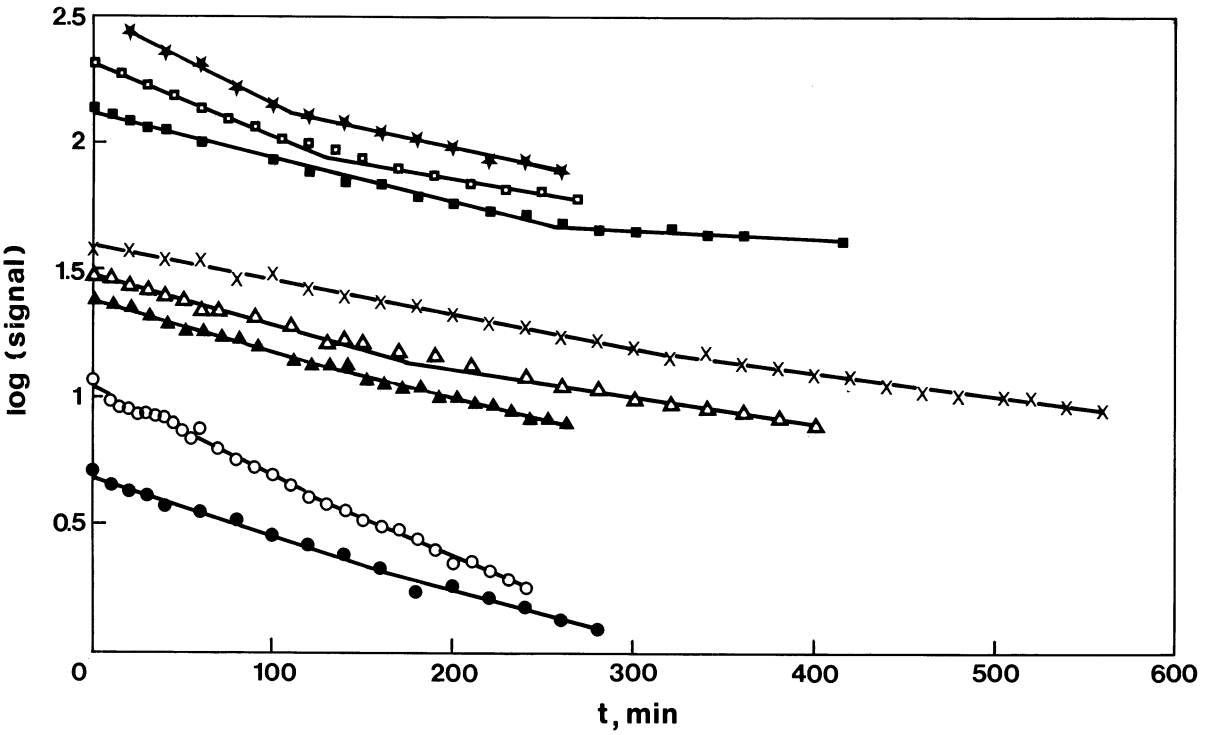

Fig. 8. Typical apparent first-order plots for the accelerated hydrolysis of nordazepam at (✱) 74°C, (□) 70°C and (■) 66°C, all three in 0.1 M HCl solution, by HPLC; (×) 60°C, (△) 63°C, both in 0.1 M HCl solution by HPLC, and (▲) 68°C in 0.1 M HCl solution by the fourth-order derivative approach; (○) 66°C in 1.0 M HCl solution by the derivative technique and (●) 62°C in 1.0 M HCl solution by HPLC. Signals were multiplied by proper factors to be fitted in the same graph.

In order to compare kinetic results derived by HPLC and derivative methods we used the signal of **Ia** because the latter method determines only nordazepam in the presence of its degradation products. The estimated $(k_{obs})_1$, slope of the first line, is shown under several experimental conditions in Fig. 8. Results were treated with a non-linear parametric method (MINSQ) for the determination of $(k_{obs})_1$, assumed as pseudo-first order rate constant. Values of $(k_{obs})_1$ calculated by this program for both methods, are tabulated in Table 4.

The reaction under study is a typical consecutive reaction with a reversible first step. Since the general solutions are very complicated (Espenson, 1981), we made some assumptions in order to simplify them. In hydrochloric acid solutions of 0.01 and 0.1 M, we assumed that $k_2 \rightarrow 0$, thus $(k_{obs}) = k_1 + k_{-1}$, and we calculated k_1 and k_{-1} from the solutions for **Ia** and **IIa**. In 1.0 M hydrochloric acid solutions, we assumed that $k_{-1} \rightarrow 0$, thus $(k_{obs}) = k_1$, and we calculated k_1 from the solution for **Ia**. The results of the kinetic investigation are summarised in Table 5. In the case of 0.01 M enough data were obtained in order to draw the Arrhenius plot, according to the equation: $k = A e^{-E_a/RT}$, where k is the reaction rate constant, A is a constant termed the frequency factor, E_a is the activation energy of the chemical reaction and T is the absolute temperature. In order to draw this plot, k_1 values were used as referred in Table 5. k_1 and $t_{1/2}$ values at 37°C were found to be 4.12×10^{-4} min^{-1} and 1683 min, respectively.

This study reveals the complexity of the mechanism of acidic hydrolysis of 1,4-benzodiazepines. Moreover, it shows us that in the general case, which representative is nordazepam, in very acidic

Table 4
Comparison of the $(k_{obs})_1$ values calculated by MINSQ using fourth-order derivative methods and HPLC

C_{HCl} (M)	θ (°C)	Fourth-order derivative method ($d4_1(2)$)		HPLC method	
		$[(k_{obs})_1 \pm s] \times 10^3$ (min^{-1})	Correlation r_1 (n)[a]	$[(k_{obs})_1 \pm s] \times 10^3$ (min^{-1})	Correlation r_1 (n)[a]
0.01	66	6.0 ± 0.3	0.98 (11)	4.7 ± 0.1	0.996 (9)
0.01	70	7.5 ± 0.3	0.990 (12)	6.6 ± 0.1	0.998 (8)
0.01	74	10.8 ± 0.6	0.98 (12)	7.7 ± 0.4	0.991 (6)
0.1	60	4.2 ± 0.1	0.990 (14)	2.8 ± 0.1	0.96 (17)
0.1	63	5.0 ± 0.1	0.990 (18)	3.54 ± 0.07	0.996 (16)
0.1	68	7.3 ± 0.4	0.98 (10)	—	
1.0	58	3.72 ± 0.04	0.995 (42)	—	
1.0	62	5.13 ± 0.07	0.995 (33)	4.8 ± 0.6	0.998 (16)
1.0	66	7.8 ± 0.2	0.990 (30)	6.6 ± 0.2	0.994 (13)

[a] r is a statistical parameter which is the square root of coefficient of determination calculated by MINSQ, and n is the number of experimental points included in the nonlinear fit (signal versus time) made by MINSQ.

Table 5
Results of kinetic investigation of nordazepam derived by the HPLC method

C_{HCl} (M)	θ (°C)	$k_1 \times 10^3$ (min^{-1})	$k_{-1} \times 10^3$ (min^{-1})	$k_2 \times 10^3$ (min^{-1})
0.01	66	2.4	2.2	—
0.01	70	3.2	3.4	—
0.01	74	3.7	4.0	—
0.1	60	1.5	1.3	—
	63	1.8	1.8	—
1.0	62	4.8	—	19
1.0	66	6.6	—	23

solutions their azomethine bond breaks first and the amidic one follows slowly. In addition to that, even though the fourth-order derivative approach can be used for the determination of nordazepam, it is not capable of calculating the complete set of kinetic parameters. Finally, the HPLC method developed can be used reliably for the determination of all three species of **Ia**, **IIa** and **IVa** as well as for the complete kinetic investigation of the acidic hydrolysis of nordazepam.

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