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Kinetic study on the degradation of prazepam in acidic aqueous solutions by high-performance liquid chromatography and fourth-order derivative ultraviolet spectrophotometry[1]

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Abstract

A reversed-phase HPLC method was developed for the kinetic investigation of the acidic hydrolysis of prazepam which was carried out in hydrochloric acid solutions of 0.01, 0.1 and 1.0 M. In addition, a fourth-order derivative method for monitoring the parent compound itself was proposed and evaluated. One intermediate was observed by HPLC, which should be formed from breakage of the azomethine linkage. Further slow hydrolysis of the amide bond led to the benzophenone product that was isolated and identified. The mechanism of hydrolysis was biphasic, showing a consecutive reaction with a reversible step. Relative standard deviation was less than 2% for HPLC and less than 5% for the derivative method. Detection limits were 1.2×10^{-7} M for the former method and 6.7×10^{-7} M for the latter. Accelerated studies at higher temperatures were employed. Results of HPLC and fourth-order derivative methods were statistically the same.

Keywords: Prazepam; Kinetic study; Acidic hydrolysis products; High-performance liquid chromatography; Derivative spectrophotometry

1. Introduction

Chemical stability of pharmaceutical products is very important for analytical chemists because systematic kinetic studies on the decomposition of drugs using stability testing techniques is essential for the quality control of such products.

Prazepam [1], 7-chloro-1-(cyclopropylmethyl)-1,3-dihydro-5-phenyl-1,4-benzodiazepin-2-one, is a member of the 1,4-benzodiazepine series [2,3]. These compounds represent an important group of psychotherapeutic drugs used as anxiolytic, sedatives, sleep inducers, and skeletal muscle relaxants. They may undergo acid–base hydrolysis in aqueous solutions. Study of this hydrolysis [4–11] is of great importance since the absorption of these drugs in the gastrointestinal tract is affected by the chemical species involved.

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The aim of this work was to study kinetics of the acidic degradation of prazepam (Scheme 1: R_1 = cyclopropylmethyl, R_2 = chloro, R_3 = phenyl), which is one of the most commonly prescribed drugs for the treatment of anxiety, sleep and seizure disorders. However, this compound is very rarely mentioned in the literature [1,12–14]. A stability assay of this drug has been found [15] but nothing on the mechanism and kinetics of its hydrolysis in aqueous media. In this paper, a kinetic investigation of prazepam in acidic aqueous solutions by a reversed-phase HPLC method was reported. This method could determine **Ia** and its hydrolysis products simultaneously, as such it could be used as a stability-indicating technique. A mechanism for the acidic hydrolysis of prazepam under the experimental conditions used was also proposed. Finally, the possibility of using a derivative UV-spectrophotometry [16–25] in this chemical system was examined, because this technique has been proven to be fast, simple, inexpensive, solving difficult analytical problems as a resolution enhancing, background correcting and reducing matrix interference method.

2. Experimental

2.1. Equipment

A HPLC system consisted of a Waters model 600E gradient controller, a Rheodyne 7125 injector with a 20 μl loop and a PDA Waters model 996 photodiode array detector, set at 230 nm. The chromatographic apparatus is controlled by a Waters software package: Millennium version 2.0.

A Hitachi (Tokyo, Japan) double beam UV/VIS spectrophotometer (Model U-2000) was used for zero-, second- and fourth- order derivative spectrophotometric measurements. The operational conditions were as follows: scan mode, WL; data mode, ABS, with user baseline; start wavelength, 400 nm; stop wavelength, 220 nm; scan speed, 100 nm min^{-1} and medium response. In the derivative mode, a sensitivity factor S of 1–3 was applied depending on the smoothing required in each case.

A Heto water bath was used for accelerated kinetic studies.

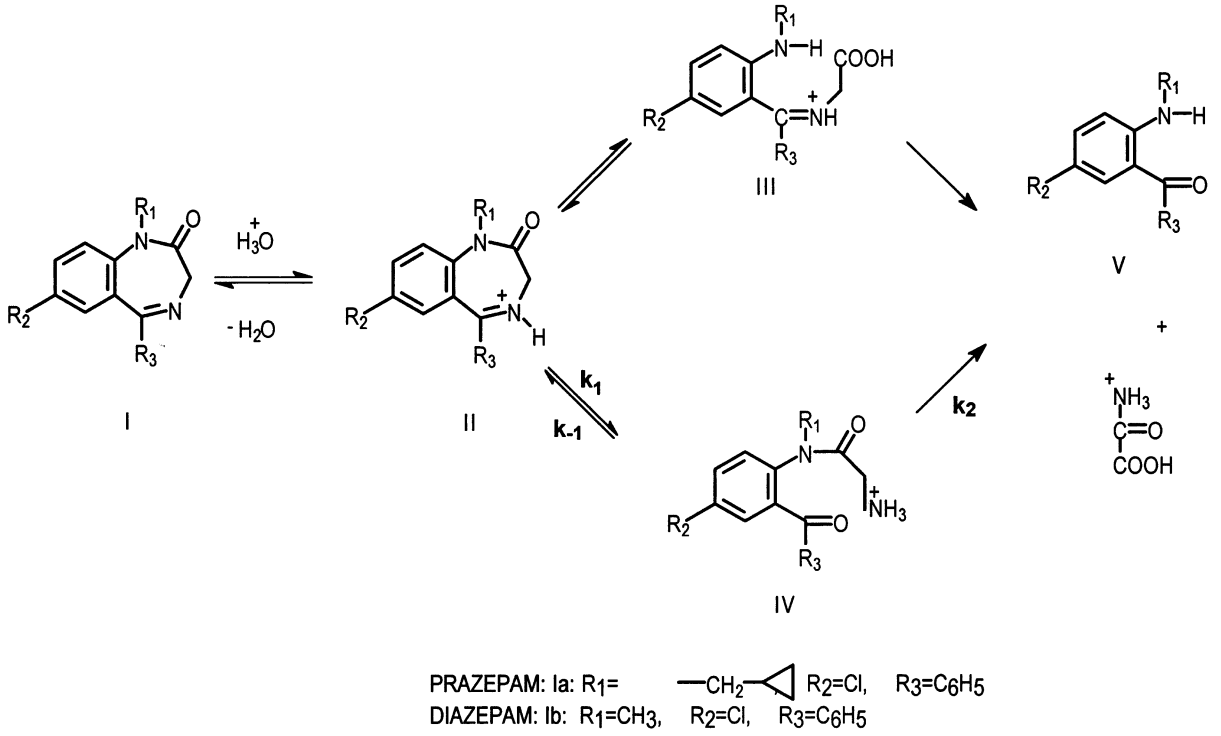
Structure elucidation of compound **Va** was based on the combination of results taken from the following methods: elemental analysis, done on a Perkin-Elmer CHN 2400 instrument; ^1H and ^{13}C NMR performed on a Varian Unity Plus-300 instrument; mass spectrometry using a GC-MS instrument, model VG-TRIO 1000, operated on EI mode at 70 eV and DIP (direct inlet probe) and IR spectroscopy done on a Perkin Elmer model 883 infrared spectrophotometer.

2.2. Reagents

Prazepam ($C_{19}H_{17}N_2ClO$, $M_r = 324.8$), **Ia**, of pharmaceutical purity grade, was kindly provided by Minerva Hellas, Athens, Greece.

N-(2-benzoyl-3-chlorophenyl)-*N*-cyclopropylmethyl-2-aminoacetamide ($C_{19}H_{19}N_2ClO_2$, $M_r = 342.8$), **IVa**, was isolated as follows: 0.750 g of **Ia** was dissolved in 30 ml of methanol and 150 ml HCl 0.1 N was added. The resulting mixture was heated under reflux for 2 h. Upon heating the mixture turned clear. The clear solution was cooled at room temperature and a cloudy yellow mixture formed (due to the low solubility of both prazepam and benzophenone in an aqueous environment). Finally, the precipitate was filtered and the solution was extracted five times by 50 ml aliquots of ethyl acetate and five times by 50 ml aliquots of diethyl ether. Attempts to isolate this product in pure crystalline form were unsuccessful due to its high reactivity at pH values above the pK_a of the starting compound. From HPLC data for **Ia**, **IVa** and **Va** before the extractions and for **IVa** after the extractions and the calibration curves for **Ia** and **Va**, the concentration of **IVa** in the final solution was approximately estimated at about 2.4×10^{-3} M. Once prepared it had to be used immediately to avoid further hydrolysis.

N-cyclopropylmethyl-2-amino-5-chloro-benzophenone ($C_{17}H_{16}NClO$, $M_r = 285.5$), **Va**, was isolated in crystals in the following way: 0.5 g of prazepam was dissolved in a minimum volume of methanol, 100 ml of 1.0 M hydrochloric acid were added and the solution was heated to 95°C for 10 h. When cooled to room temperature yellow crystals of **Va** precipitated (250 mg).



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

Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Lab-Scan sciences. Ammonium acetate (pro analysi) and hydrochloric acid (analytical reagent grade) were purchased from Merck. Water was distilled and deionized; it was purified by means of a Milli-Q Plus Water Purification System, Millipore, when used for HPLC experiments. All other reagents used were of analytical reagent grade.

Stock methanolic solution of **Ia** and **Va** (6.2×10^{-4} M) were prepared and stored in the dark under refrigeration. Working standard solutions of **Ia** were prepared daily in HCl for HPLC and derivative measurements in the ranges 3.08×10^{-6}–9.24×10^{-6} M and 6.2×10^{-6}–3.1×10^{-5} M, respectively and used for the construction of calibration curves. Moreover, for validation of the derivative method, working standard solutions of **IVa** and **Va** in the range 1.2×10^{-5}–3.7×10^{-5} M were prepared daily in 0.1 M HCl, as well as mixed working standard solutions of **Ia**, **IVa** and **Va** for recovery studies. In the latter case, standard solutions of **Ia** were prepared in the range of 0.6×10^{-5}–3.0×10^{-5} M containing, on one hand constant concentration of **IVa** or **Va** equal to 2.4×10^{-5} M or alternatively constant concentrations of **IVa** and **Va** equal to 1.2×10^{-5} M, each. Additionally, a series of standard solutions of **IVa** or **Va** were prepared in the range 1.2×10^{-5}–3.6×10^{-5} M containing a constant concentration of **Ia** equal to 1.2×10^{-5} M.

Standard solutions of **Ia** 1.5×10^{-4} M in 0.01, 0.1, 1.0 M HCl and 2.4×10^{-5} M in 0.1 M HCl were freshly prepared and used for prazepam accelerated kinetic studies by HPLC and derivative methods, respectively.

2.3. Measurement procedure

2.3.1. HPLC method

Chromatography was carried out on a BDS C-8 column (25.0 cm × 4.6 mm i.d. 5 μm particle size) (Shandon HPLC, UK). A mobile phase, consisting of acetonitrile:ammonium acetate 0.1 M,

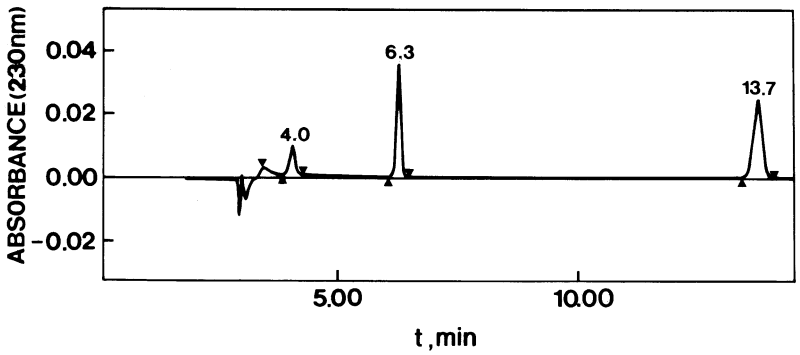


Fig. 1. Representative chromatogram of a mixture of **Ia**, **IVa** and **Va** at retention times of 6.3, 4.0 and 13.7 min, respectively. Chromatographic conditions: reversed-phase-HPLC on a C-8 BDS column; mobile phase: acetonitrile:ammonium acetate (0.1 M) in a ratio 68:32 and a photodiode-array detector set at 230 nm.

62:38, was filtered through a 0.45 μm Millipore filter and degassed under vacuum using a Millipore system. An isocratic elution was performed at a flow rate of 1.5 ml min^{-1} for the first 7.5 min and 1.9 ml min^{-1} until 15 min.

Retention times of **Ia**, **IVa** and **Va** were 6.3, 4.0 and 13.7 min, respectively.

All experiments were carried out at an ambient temperature of 25°C.

2.3.2. Derivative spectrophotometric method

Prepared working standard solutions of **Ia**, **IVa** and **Va** were measured against a blank solution of HCl 0.1 M. Measurement procedure was similar to that described elsewhere [25].

2.4. Kinetic investigation of the acidic hydrolysis

The instrumental setup for kinetic studies performed by HPLC was similar to that described in [26]. Experimental conditions used are shown in Tables 4 and 5 and in Fig. 3.

During the kinetic study at predetermined time intervals, 100 μl aliquots were removed from the flask and 2 ml of water were added followed by vigorous mixing. A volume of 20 μl was injected into the analytical column.

The procedure adopted for kinetic investigation using the derivative method was similar to that followed in other studies [25]. Experimental parameters are summarised in Table 4 and Fig. 4.

Treatment of kinetic data in both methods was carried out by a software package called MINSQ, (Version 4.03, Micro-Math Scientific Software, Salt Lake City, UT) following the procedure in Ref.[25].

3. Results and discussion

3.1. Structure elucidation of N-cyclopropylmethyl-2-amino-5-chloro-benzophenone

The purity of the precipitate was confirmed by TLC, HPLC and elemental analysis. This compound was the degradation product, *N*-cyclopropylmethyl-2-amino-5-chlorobenzone **Va**, m.p. = 81°C; [27,28] ^{1}H NMR ($CDCl_3$) 7.2–7.7 ppm (m, 7H, aromatics), 6.7 p.p.m. (d, 1H, aromatic), 3.05 (d, 2H, methylenics), 1.1–1.2 ppm (m, 1H), 0.8 ppm (q, 2H), 0.5 ppm (q, 2H), 8.5 (s, br, 1H, NH); ^{13}C NMR, 197.2 ppm carbonyl, 149.8 amine, 49.17 ppm methylenic; EI-MS m/z: 287 ($M^{\bullet+}$ + 2), 286($M^{\bullet+}$ + 1), 285($M^{\bullet+}$), 284 ($M^{\bullet+}$ − 1), 230 ($M^{\bullet+}$, − 55), 105 ($PhCO^{\bullet+}$), 77($Ph^{\bullet+}$), 55(cyclopropylmethyl$^{\bullet+}$); IR (nujol) 1650 cm^{-1} –C=O, 3310 cm^{-1} –NH; elemental analysis: (Calcd ($C_{17}H_{16}NOCl$): C, 71.45; H, 5.64; N, 4.90; Cl, 12.41; O, 5.60. Found: C, 71.32; H, 5.88; N, 4.98)

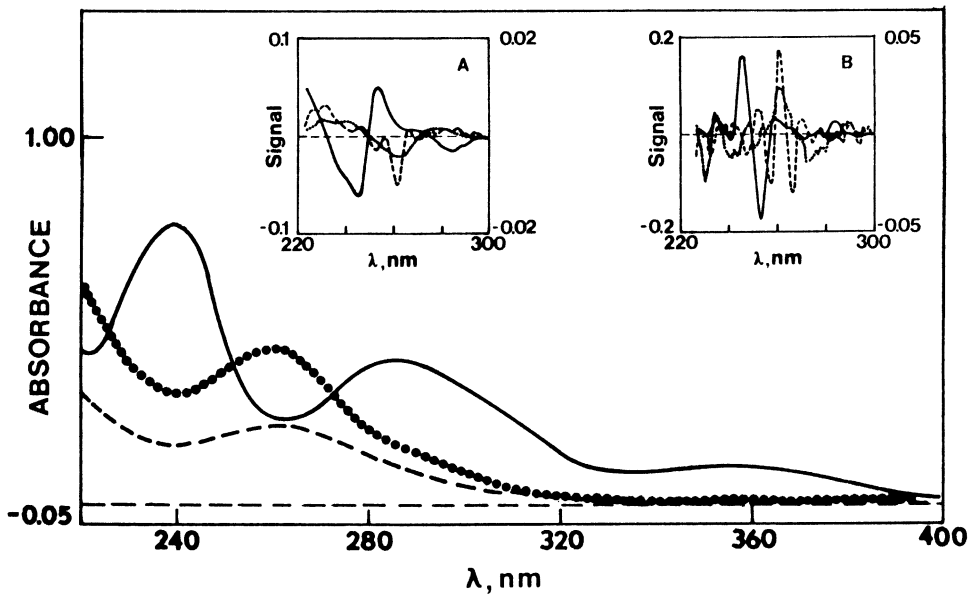


Fig. 2. Zero-order absorbance spectra (pathlength 1.0 cm) of **Ia** (solid line), **IVa** (dotted line) and **Va** (broken line) at concentrations equal to 2.4×10^{-5} M each in a 0.1 M hydrochloric acid solution. Their second-order derivative spectra (A), the left-hand y-axis -0.1–0.1 corresponds to **Ia** while the right-hand y-axis -0.02–0.02 corresponds to **Vα**. Their fourth-order derivative spectra (B), the left-hand y-axis -0.2–0.2 corresponds to **Ia** while the right-hand y-axis -0.05–0.05 corresponds to **IVa** and **Va**.

3.2. Chromatograms and spectral characteristics

A representative chromatogram of **Ia**, **IVa** and **Va** is shown in Fig. 1. It was obvious that a very good separation of these species was accomplished. This implied that using the HPLC method, any of these three species could be determined accurately without any interference from the other two.

Since spectrophotometric techniques have been faster, easier to use and less expensive than HPLC method, the possibility of applying such a technique in a complex chemical system was examined. It was clear that a zero-order UV-Vis technique could not be used because of the strong overlap of the three spectra shown in Fig. 2. As a result a derivative spectrophotometric technique was attempted.

In Fig. 2(A) the second-order derivative spectra of **Ia**, **IVa** and **Va** in HCl 0.1 M are presented. Distance, d2, between the maximum at 254 and the minimum at 244 nm in the spectrum of **Ia** was chosen as a trial signal to work with because it showed the least interference from the presence of **IVa** and **Va** compared with other graphical amplitudes. Direct determination of **IVa** or **Va** in the presence of **Ia** would be erroneous because the latter interfered considerably with the signal of the former.

In Fig. 2(B) the fourth-order derivative spectra of **Ia**, **IVa** and **Va** in a 0.1 M hydrochloric acid solution are shown. The major peak of **IVa** had almost disappeared. Again, considering the difference, in the y-axis, between these spectra, direct determination of **IVa** or **Va** in the presence of **Ia** would give false results. Yet, several distances between maxima and minima in the fourth-order derivative spectrum of **Ia** could be used as trial signals for the determination of prazepam with rather insignificant interference from the corresponding spectra of the degradation products, e.g. $d4_1$ (240–246 nm), $d4_2$ (246–253 nm) and $d4_3$ (253–260 nm).

3.3. Linearity and reproducibility

3.3.1. HPLC method

Under the experimental conditions described in

Table 1
Analytical parameters of the calibration curves of prazepam, **Ia**

Mode[a]		Concentration range of **Ia**, (M)	+concentration of **IVa** and **Va**, (M)	Selected distance (nm)	Regression equation[b]		
					Intercept (a ± s)	Slope (b ± s)	r (n)[c]
(a)	$d2_1(1)$			245–254 (2nd deriv., s = 1)	0.004 ± 0.003	4883 ± 166	0.998 (5)
	$d2_1(2)$			244–255 (2nd deriv., s = 2)	0.015 ± 0.005	16 600 ± 252	0.9997 (5)
	$d4_1(2)$			240–246 (4th deriv., s = 2)	0.014 ± 0.005	6783 ± 286	0.997 (5)
	$d4_2(2)$	$(0.6–3.0)\times10^{-5}$	—	246–253 (4th deriv., s = 2)	0.015 ± 0.003	13 433 ± 135	0.9998 (5)
	$d4_3(2)$			253–260 (4th deriv., s = 2)	0.010 ± 0.002	8100 ± 94	0.9998 (5)
	$d4_2(3)$			246–253 (4th deriv., s = 3)	0.044 ± 0.010	42 883 ± 541	0.9998 (5)
	$d4_3(3)$			253–262 (4th deriv., s = 3)	0.031 ± 0.007	26 800 ± 360	0.9997 (5)
(b)	$d2_1(1)$			245–254 (2nd deriv., s = 1)	−0.002 ± 0.004	4967 ± 198	0.998 (5)
	$d2_1(2)$			244–255 (2nd deriv., s = 2)	−0.007 ± 0.009	16 467 ± 488	0.9990 (5)
	$d4_1(2)$			240–246 (4th deriv., s = 2)	0.010 ± 0.005	6733 ± 246	0.998 (5)
	$d4_2(2)$	$(0.6–3.0)\times10^{-5}$	1.2×10^{-5}[d]	246–253 (4th deriv., s = 2)	0.019 ± 0.008	13 233 ± 448	0.998 (5)
	$d4_3(2)$			253–260 (4th deriv., s = 2)	0.010 ± 0.002	7917 ± 97	0.9998 (5)
	$d4_2(3)$			246–253 (4th deriv., s = 3)	0.051 ± 0.024	42 133 ± 1254	0.9990 (5)
	$d4_3(3)$			253–262 (4th deriv., s = 3)	0.059 ± 0.017	25 967 ± 881	0.998 (5)

[a] Measured distances as peak-trough amplitudes $d2_1(1)$, $d2_1(2)$ of the second-order derivative spectra and $d4_1(2)$, $d4_2(2)$, $d4_3(2)$, $d4_2(3)$, $d4_3(3)$ of the fourth-order spectra of **Ia**

[b] Distances $dm_l(j)$ fit in the regression equation $dm_l(j) = a + bc$, where $m = 2,4$, $l = 1,2,3$ and $j = 1,2,3$ as they appear under the 'mode' column in the above table, c is the concentration of **Ia** in M.

[c] n, number of points in each calibration curve, each point is the mean of two or three experimental measurements

[d] Presence of both **IVa** and **Va** in a concentration of 1.2×10^{-5} M, each.

(a) in the absence and (b) in the presence of degradation products **IVa** and **Va** in an acidic solution of HCl 0.1 M.

Table 2
Spectrophotometric determination of prazepam, **Ia**, in the presence of a constant concentration of its acidic-induced degradation products **IVa** and **Va**, in a solution of 0.1 M HCl

Experiment no.	Concentration of **Ia** added[a] (M)	Recovery (%)						
		$d2_1(1)$	$d2_1(2)$	$d4_1(2)$	$d4_2(2)$	$d4_3(2)$	$d4_2(3)$	$d4_3(3)$
1	0.6×10^{-5}	104.8	103.5	98.2	101.6	106.8	102.7	104.5
2	1.2×10^{-5}	102.1	101.7	101.0	100.0	99.1	98.9	102.6
3	1.8×10^{-5}	100.4	100.2	98.5	100.0	100.6	98.4	99.0
4	2.4×10^{-5}	100.9	99.6	99.8	100.1	100.5	99.1	98.8
Mean ± SD		102.1 ± 2.0	101.3 ± 1.7	99.4 ± 1.3	100.4 ± 0.8	101.8 ± 3.4	99.8 ± 2.0	101.2 ± 2.8

[a] Increasing amounts of **Ia** are added to a constant concentration of **IVa** and **Va** equal to 1.2×10^{-5} M, each and the recovery of **Ia** is measured.

a previous section, linear regression analysis of HPLC data gave the following equation for the calibration curve:

$$H = 170.13(\pm 1.06) \times C - 12.00(\pm 6.93),$$

$$r = 0.99994, \qquad \mathrm{SE} = 0.051 \qquad n = 5$$

where H was the chromatographic signal $\times 10^3$, C was the concentration of prazepam times 10^6 in M, r was the correlation coefficient, SE was the standard error of the estimate and n was the number of samples. A series of working standard solutions containing 3.08×10^{-6}, 6.16×10^{-6} and 9.24×10^{-6}M of prazepam in HCl were each measured four times and the relative standard deviation (RSD), was found to be 2.0, 1.5 and 1.9%, respectively. The statistical evaluation of the HPLC method revealed its good linearity and reproducibility and led to the conclusion that it could be reliably used for the kinetic investigation of prazepam.

The detection limit attained for prazepam, as defined by IUPAC [29] $\mathrm{DL}_{(k=3)} = K\cdot s_b/b$ (where b was the slope of the calibration graph and s_b was the standard deviation of the blank signal) was found to be 1.2×10^{-7} M.

3.3.2. Spectrophotometric measurements

Analytical parameters of the **Ia** calibration curves in both derivative modes are summarised in Table 1.

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

To ensure applicability of the derivative approach to the kinetic investigation of prazepam, a recovery study was conducted. Mixed standard solutions of **Ia**, **IVa** or **Va** were prepared, where concentration of **IVa** and/or **Va** remained constant and **Ia**, was varied. Representative results of this linear regression analysis were also included in Table 1. A t-test was applied to 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%). However, it was noticed that, in certain cases, intercept values were significant. For a better comparison, 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 **IVa** or **Va** and the recovery of **Ia** was measured and second, increasing amounts of **IV** and/or **Va** 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 it was concluded that as the concentration of **IV** and/or **Va** increased the

Table 3
Recovery of prazepam **Ia**, in the presence of its acidic-induced degradation product **Va**, in a solution of 0.1 M HCl

Experiment no.	Concentration of **Va** added[a] (M)	Recovery (%)						
		$d2_1(1)$	$d2_1(2)$	$d4_1(2)$	$d4_2(2)$	$d4_3(2)$	$d4_2(3)$	$d4_3(3)$
1	1.2×10^{-5}	99.8	99.4	98.9	100.2	99.6	99.3	101.5
2	1.8×10^{-5}	99.1	98.9	100.4	99.6	100.2	100.2	102.1
3	2.4×10^{-5}	100.6	100.9	100.9	101.2	100.5	98.6	102.6
4	3.0×10^{-5}	103.9	102.4	103.1	100.6	106.4	104.1	108.3
5	3.6×10^{-5}	105.2	106.1	101.8	100.8	108.1	106.2	110.2
Mean ± SD		101.7 ± 2.7	101.5 ± 2.9	101.0 ± 1.6	100.5 ± 0.6	103.0 ± 4.0	101.7 ± 3.0	104.9 ± 4.0

[a] Increasing amounts of **Va** are added to a constant concentration of **Ia** equal to 1.2×10^{-5} M and the recovery of **Ia** is measured against the same concentration of pure **Ia**.

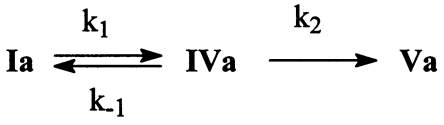
second-derivative method showed poor accuracy while the $d4_2$ (2) presented the best reproducibility and accuracy among the fourth-order derivative approaches. As a consequence this was the signal to be measured in the kinetic investigation of prazepam.

The same type of study was conducted for **IVa** and **Va** as well. However, as expected, there were no derivative signal (2nd- or 4th- order) free of significant interference from **Ia** that could be used for the determination of **IVa** or **Va**. As a result only the determination of **Ia** was feasible (in the presence of **IVa** and/or **Va**), using a fourth-order derivative approach.

Defining 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, DL for the fourth-order derivative method was 6×10^{-7}M and was derived from the fourth-order derivative spectrum of the blank and the calibration curve of **Ia**.

3.4. Kinetic investigation

Since a similar kinetic study on **Ia** has not been reported in the literature, the mechanism of degradation needed to be clarified. This was done using HPLC, where compounds were separated and determined at the same time. This kind of work was could not be carried out reliably by derivative spectrophotometry in such a complex reaction, because the number and the nature of the different species that were in solution were unknown and their UV-spectra looked very similar. Results of the HPLC method are illustrated in Fig. 3. Combining this data, with gathered knowledge of 1,4 benzodiazepines [4,8] and particularly diazepam **Ib** [5,7,10] (where $R_1 = CH_3$, $R_2 = Cl$ and $R_3 = C_6H_5$), which was chemically and structurally similar to **Ia** the fact that **Va** was identified, the following conclusions were drawn. In prazepam, hydrolysis of 1,2-amide bond leading to the intermediate **IIIa** was not favored because of the steric hindrance that the cyclopropylmethyl group showed to any attack from that site. In other words, **IVa** was the intermediate expected to be derived from the rupture of 4,5-azomethine linkage which seemed to occur in prazepam. We observed biphasic kinetics in **Ia** as it was clearly demonstrated in Fig. 4. The same behavior was reported for diazepam, [5]. Following these observations, it was evident that acidic hydrolysis of prazepam proceeded through an intermediate according to Scheme 2.



Scheme 2.

Comparison of kinetic results derived from HPLC and derivative methods, could only be performed using the signal of **Ia** because the latter method determined only prazepam in the presence of its degradation products. The estimated $(k_{obs})_1$ and $(k_{obs})_2$, the two corresponding slopes, were presented under several experimental conditions in Fig. 4. For the determination of $(k_{obs})_1$ and $(k_{obs})_2$, results were treated using MINSQ, and assumed as pseudo-first order reaction rate constants [25]. Values of $(k_{obs})_1$ and $(k_{obs})_2$, calculated by both methods are tabulated in Table 4 and their differences in most cases were statistically insignificant.

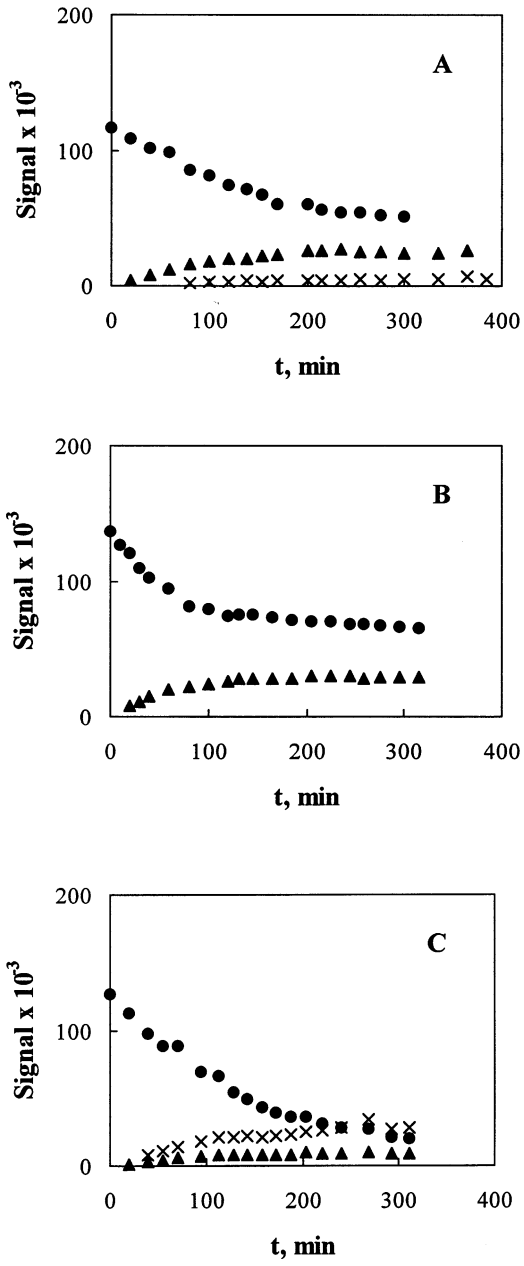


Fig. 3. Plot of the HPLC signal of: ●, **Ia**; ▲, **IVa** and ×, **Va** during an accelerated degradation study at 70°C in 0.1 M (A), 0.01 M (B) and 1.0 M (C) HCl solutions.

The reaction shown in Scheme 2 is a typical consecutive reaction with a reversible step. Since the general solutions were very complicated [30], in order to determine the rate constants k_1, k_{-1} and k_2 some assumptions were made to simplify them. In the case of 0.01 and 0.1 M hydrochloric acid solutions it was assumed that $k_2 \rightarrow 0$ thus $(k_{obs})_1 = k_1 + k_{-1}$ and k_1 and k_{-1} were calculated from the solutions for **Ia** and **IVa**. In the case of 1.0 M hydrochloric acid solutions, assuming that $k_{-1} \rightarrow 0$, thus $(k_{obs})_1 = k_1$, we calculated k_1 from the solution of **Ia** [26]. The results of this kinetic investigation were summarised in Table 5.

From the above study it is obvious that in the first two hydrochloric acid concentrations the equilibrium existing between **I** and **IVa** favored prazepam, a fact that is well shown in Fig. 3. In contrast, in 1.0 M hydrochloric acid solution the final product **Va** was favored as the complete hydrolysis proceeded much faster. This study also verified the complexity of the mechanism of acidic hydrolysis of 1,4-benzodiazepines. Another observation was the hydrolysis of prazepam even at very low pH values, was a very slow reaction, implying that it was not likely to take place in the stomach after oral administration. Finally, it was shown that although the derivative UV-spectrophotometric method performed well, it could only be used reliably for the determination of prazepam itself, while the HPLC method could be used successfully as a stability-indicating technique.

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Table 4
Comparison of the $(k_{obs})_1$ and $(k_{obs})_2$ values calculated by MINSQ using HPLC and fourth-order derivative methods

C_{HCl} (M)	θ (°C)	HPLC method				Fourth-order derivative method, $d4_2$ (2)			
		$[(k_{obs})_1 \pm SD] \times 10^3$ (min^{-1})	Correlation r_1 (n)[a]	$[(k_{obs})_2 \pm SD] \times 10^3$ (min^{-1})	Correlation r_2 (n)[a]	$[(k_{obs})_1 \pm SD] \times 10^3$ (min^{-1})	Correlation r_1 (n)[a]	$[(k_{obs})2 \pm SD] \times 10^3$ (min^{-1})	Correlation r_2 (n)[a]
0.01	70	6.5 ± 0.2	0.997 (7)	0.68 ± 0.04	0.995 (8)				
0.1	57	3.1 ± 0.2	0.98 (7)	0.90 ± 0.1	0.97 (7)	2.69 ± 0.07	0.997 (10)	0.9 ± 0.1	0.96 (9)
0.1	60	4.4 ± 0.2	0.997 (5)	1.00 ± 0.1	0.95 (9)	4.0 ± 0.3	0.98 (7)	0.96 ± 0.07	0.97 (14)
0.1	63	4.9 ± 0.2	0.998 (6)	1.80 ± 0.1	0.995 (8)	4.4 ± 0.4	0.98 (8)	1.4 ± 0.1	0.98 (9)
0.1	70	9.5 ± 0.4	0.997 (5)		—				
1.0	70	6.5 ± 0.3	0.995 (10)	4.80 ± 0.4	0.98 (8)				

[a] r is the 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.

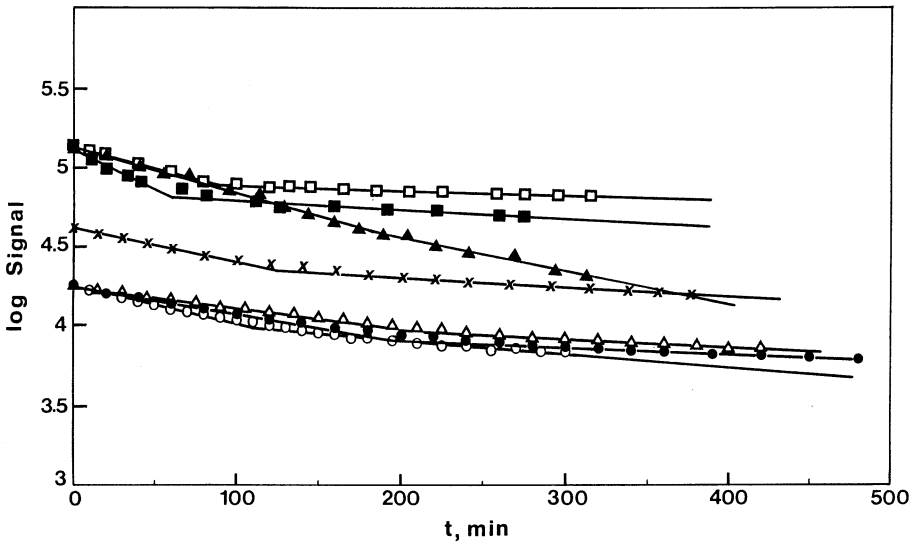

Fig. 4. Typical apparent first-order plots for the accelerated hydrolysis of prazepam in □, 0.01 M HCl; ■, 0.1 M HCl; ▲, 1.0 M HCl all three at 70°C by HPLC, ×, 0.1 M HCl at 63°C by HPLC and △, at 57°C; ●, at 60°C; ○, at 63°C; the last three in 0.1 M HCl by the fourth-order derivative approach. Signals were multiplied by proper factors to be fitted in the same graph.

Table 5
Results of kinetic investigation of prazepam 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	70	2.8	3.7	—
	57	1.0	2.1	—
0.1	60	1.4	3.0	—
	63	1.6	3.3	—
	70	3.0	6.5	—
1.0	70	6.5	—	16

prazepam. They also thank D.B. Kolovos for his contribution to the derivative method, Dr V. Roussis, Dr S. Koinis, D. Argyropoulos for their assistance in the MS and NMR facilities and G. Tsoutsoura-Kampyli for the graphic work. The main author expresses her gratitude to Dr M. Kondylis for his invaluable help.

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