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Acidic hydrolysis of bromazepam studied by high performance liquid chromatography. Isolation and identification of its degradation products

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Abstract

A kinetic study on the acidic hydrolysis of bromazepam was carried out in 0.01 M hydrochloric acid solution at 25 and 95°C. A reversed-phase HPLC method was developed and validated for the determination of bromazepam and its degradation products. Bromazepam degraded by a consecutive reaction with a reversible first step. Two degradation products were isolated and identified by infrared, ^{1}H and ^{13}C nuclear magnetic resonance and mass spectroscopy. Spectroscopic data indicated that *N*-(4-bromo-2-(2-pyridylcarbonyl)phenyl)-2-aminoacetamide was the intermediate degradation product of this acid hydrolysis, whereas 2-amino-5-bromophenyl-2-pyridylmethanone was the final one. Therefore, the mechanism of this acid-catalysed hydrolysis involved initial cleavage of the 4,5-azomethine bond, followed by slow breakage of the 1,2-amide bond. Statistical evaluation of the HPLC method revealed its good linearity and reproducibility. Detection limits were 3.8×10^{-7} M for bromazepam, 6.25×10^{-7} M for the intermediate and 8.16×10^{-7} M for the benzophenone derivative.

Keywords: Bromazepam; Acidic-hydrolysis; Isolation and identification of degradation products; Reversed-phase high performance liquid chromatography; Mechanism and kinetic measurements

1. Introduction

Bromazepam (7-bromo-5-(2-pyridyl)-2,3-dihydro-1*H*-benzo[*e*]1,4-diazepin-2-one, **BMZ**) is a member of the 1,4-benzodiazepine series. These drugs are widely used as minor tranquillisers, sleep inducers, sedatives and muscle relaxants [1–3] and several methods have been reported for their analytical determination [4–8].

Bromazepam was first synthesised by Fryer in 1964 [9], and it is increasingly used up to today. The presence of the pyridine moiety in its molecule is responsible for its unique physicochemical properties. Its main metabolic route involves hydroxylation at C_3 and hydrolysis of the heterocyclic ring [10,11]. After therapeutical administration only the parent compound is present in blood, whereas 2-amino-5-bromophenyl-2-pyridylmethanone and hydroxylated metabolites are excreted in the urine [12,13].

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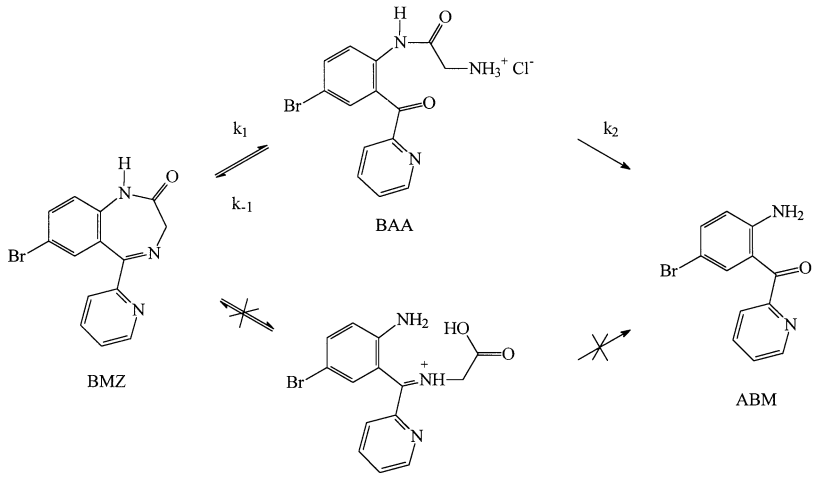

Scheme 1. Possible degradation pathway of the acidic hydrolysis of bromazepam in aqueous solutions.

Bromazepam, like most of the benzodiazepine derivatives, is hydrolysed in acidic aqueous solutions leading to a series of degradation products. Because of the extensive use of this compound, a study on the mechanism and kinetics of this reaction is a matter of great concern.

In order to analyse bromazepam contained in clinical samples, such as blood and urine, it was often hydrolysed under strongly acidic conditions to form fluorescent 2-amino-5-bromophenyl-2-pyridylmethanone [14–16].

Previous work on chemical stability of **BMZ** in acidic aqueous media has been reported [17–20]. As it is stated in these articles, the mechanism of this acid-catalysed hydrolysis involves initial cleavage of the 1,2-amide bond and then breakage of the 4,5-azomethine bond to give 2-amino-5-bromophenyl-2-pyridyl and glycine. However, the pathway involved in this reaction has not been clearly demonstrated by isolation and identification of its degradation products.

The purpose of the present study was to develop a high performance liquid chromatographic method (HPLC), allowing determination of **BMZ** and its degradation products simultaneously and then to use this method in the kinetic investigation of this acid-catalysed hydrolysis. It was also desirable to gain a more comprehensive understanding of the mechanism of this reaction, isolating and identifying the degradation products. The latter was imposed by the fact that similar 1,4-benzodiazepin-2-ones were hydrolysed by initial breakage of the 4,5-azomethine bond while in the case of **BMZ** initial rupture of the 1,2-amide bond was claimed without sufficient justification.

In our work we proved that hydrolysis of **BMZ** proceeded through the intermediate *N*-(4-bromo-2-(2-pyridylcarbonyl)phenyl)-2-aminoacetamide leading to the final products 2-amino-5-bromophenyl-2-pyridylmethanone and glycine (Scheme 1). We also showed that under the experimental conditions used, the creation of the intermediate was very fast while the creation of the final product was rather slow.

2. Experimental section

2.1. Equipment

An HPLC system consisted of a Waters pump Model 501 and a Rheodyne injector Model 7125 with a 20-μl loop, which were coupled to a Waters UV–vis detector Model 486 with a 8-μl flow cell operated at 230 nm and a Hewlett-Packard Model HP 3394A integrator.

A pH meter (Metrohm, Model 654 Herisau) was used for all pH measurements.

A thermostated Heto water bath was used at 95 ± 0.2°C for the accelerated kinetic studies.

Structure elucidation of *N*-(4-bromo-2-(2-pyridylcarbonyl)phenyl)-2-aminoacetamide was based on the combination of the following results: IR-spectra obtained on a Perkin-Elmer, Model 883, infrared spectrophotometer as KBr pellets; ^{1}H and ^{13}C-NMR spectra on a Bruker, Model AC-200 spectrometer (200 MHz), the samples were dissolved in DMSO-d_6; mass spectra performed on a Shimadzu GC–MS combined set-up, Model VG-TRIO 1000, operated on EI mode at 70 eV and DIP (direct inlet probe).

2.2. Materials and reagents

Bromazepam and lorazepam of pharmaceutical purity grade were kindly provided by Roche Hellas A.E. and Minerva Hellas A.E. pharmaceutical companies and were used without any further purification. All solvents were of HPLC grade and were purchased from Lab-Scan Science Ltd., Ireland. Ammonium acetate (pro analysi), potassium dihydrogen phosphate and hydrochloric acid (analytical reagent grade) were purchased from E. Merck Ltd., Germany. Water was deionised and further purified by means of a Milli-Q Plus Water Purification System, Millipore Ltd.

N-(4-bromo-2-(2-pyridylcarbonyl)phenyl)-2-aminoacetamide, **BAA**, was isolated in crystals as follows: an amount of 0.750 g of **BMZ** was dissolved in 30 ml of methanol and 150 ml 0.1 M HCl were added. The resulting mixture was heated under reflux for 4 h. Upon heating the mixture turned clear. The clear solution was then cooled to room temperature, the cloudy yellowish mixture formed (due to low solubility of **BMZ** and the 2-amino-5-bromophenyl-2-pyridylmethanone, **ABM**, in aqueous conditions) was filtered and the solution extracted five times with 50-ml aliquots of ethyl acetate and 5 times with 50-ml aliquots of diethyl ether. The organic phase was dried with anhydrous potassium sulfate, filtered and the solvent was removed under reduced pressure. The resulting 0.400 g solid was collected and desiccated over P_2O_5 under reduced pressure. The whole process of isolation was monitored at every step by means of HPLC, in both phases (organic and aqueous) during the extraction procedure.

2-amino-5-bromophenyl-2-pyridylmethanone was isolated in crystalline form as follows: an amount of 0.500 g of **BMZ** was dissolved in methanol and heated under reflux for 2 h along with 100 ml 1.0 M HCl. The clear solution was left to cool to 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 isolation process was also monitored by HPLC, as previously mentioned.

Stock methanolic solutions of bromazepam, **BMZ**, 2.0×10^{-4} M and lorazepam, **LRZ**, 2.0×10^{-4} M were prepared by dissolving the compounds in methanol. These solutions were stored in the dark under refrigeration and were found to be stable for several weeks. Stock methanolic solutions of *N*-(4-bromo-2-(2-pyridylcarbonyl)phenyl)-2-aminoacetamide ($C_{14}H_{12}N_3O_2Br$, MW = 334.17), **BAA**, 1.5×10^{-4} M and 2-amino-5-bromophenyl-2-pyridylmethanone ($C_{12}H_9N_2OBr$, MW = 277.12), **ABM**, 1.5×10^{-4} M were also prepared, kept in the deep freeze and used within 2 days of their preparation.

Working standard solution of **LRZ** (2.5×10^{-6} M) was prepared by the appropriate dilution of the corresponding stock standard solution in water.

A series of mixed working standard solutions of **BMZ**, **BAA** and **ABM** in a ratio 1:1:1 were prepared by the appropriate dilution of the above mentioned stock standard solutions to 2 ml in 0.005 M HCl. In each sample 1.5×10^{-6} M of the internal standard, **LRZ** was added.

2.3. Measurement procedure

The separation was performed on a reversed phase ODS C-8 column (250x4.6 mm i.d., 5 μm particle size) Shandon HPLC, UK. The mobile phase, methanol:acetonitrile:mixture of potassium dihydrogen phosphate, 5×10^{-3} M, and ammonium acetate 0.1 M adjusted to pH 6.2 with

glacial acetic acid (26.5:21.5:52, v/v/v), was filtered through a 0.45 μm Millipore filter and degassed under vacuum prior to use. A flow rate of 0.8 ml/min with a column inlet pressure of 2000 psi was used in order to separate **BMZ** and its degradation products. Peak heights were measured and HPLC analysis was conducted at ambient temperature (24 ± 1°C).

Calibration curves of **BMZ**, **BAA**, and **ABM** were constructed using the series of mixed working standard solutions of the above components, as described previously. The concentration range tested was from 0.5 to 2.0×10^{-6} M for each one of the compounds. All of these solutions were analysed immediately after their preparation. Ratios of the peak height of each compound to that of the internal standard were calculated for the determination of these compounds.

The overall precision of the assay was evaluated by analysing two series of mixed working standard solutions of **BMZ**, **BAA**, and **ABM** at concentrations of 0.5×10^{-6} M and 2.5×10^{-6} M each in a ratio 1:1:1 in eight replicates. The relative standard deviation (% R.S.D.) was determined in order to assess the precision of the method and the accuracy was expressed by the relative percentage error (E_r %) as defined in Table 2.

2.4. Kinetic investigation of the acidic hydrolysis

A 0.5-ml aliquot of stock methanolic solution of **BMZ** was transferred to a 200-ml volumetric flask and diluted to volume with 0.01 M 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 **BMZ** solution. This procedure was performed in 0.01 M HCl at 25 and 95°C; an indicative degradation study was also carried out in 1.0 M HCl at 25°C.

During the kinetic study, at predetermined time intervals, 1.0-ml aliquots were removed from the flask and 1.0-ml aliquots of the working standard solution of **LRZ**, used as internal standard, were 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 **BMZ** and **LRZ** in the aqueous media, which may lead to erroneous results.

A typical chromatogram obtained during the kinetic study of **BMZ** hydrolysis is illustrated in Fig. 1. **BMZ** was eluted at 10.10 min, while the internal standard, **LRZ** at 17.73 min. The retention times for the two degradation products, **BAA** and **ABM**, were 8.98 and 19.04 min, respectively. Good separation of the three species of interest was accomplished, allowing the determination of each one accurately without any interference from the others.

Decrease of **BMZ** as well as formation and decrease of the degradation products were fol-

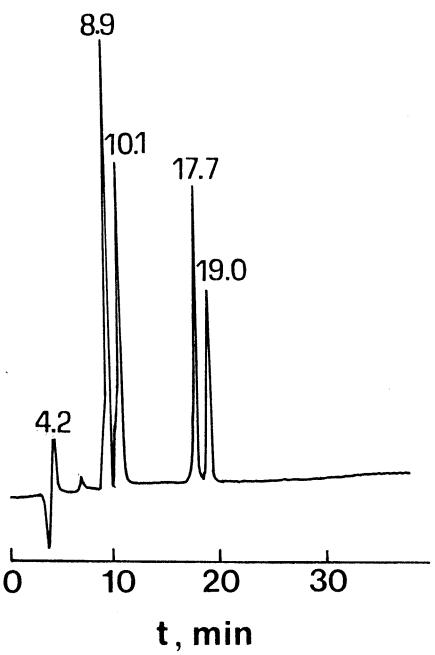

Fig. 1. A representative chromatogram of a mixture of **BMZ**, **LRZ**, **BAA** and **ABM** at retention times of 10.1, 17.7, 8.9 and 19.0 min, respectively. Chromatographic conditions: Reversed-phase HPLC on a C-8 ODS column; mobile phase: methanol:acetonitrile—5×10^{-3} M potassium dihydrogen phosphate and 0.1 M ammonium acetate adjusted to pH = 6.2 (26.5:21.5:52, v/v/v) and a UV detector set at 230 nm.

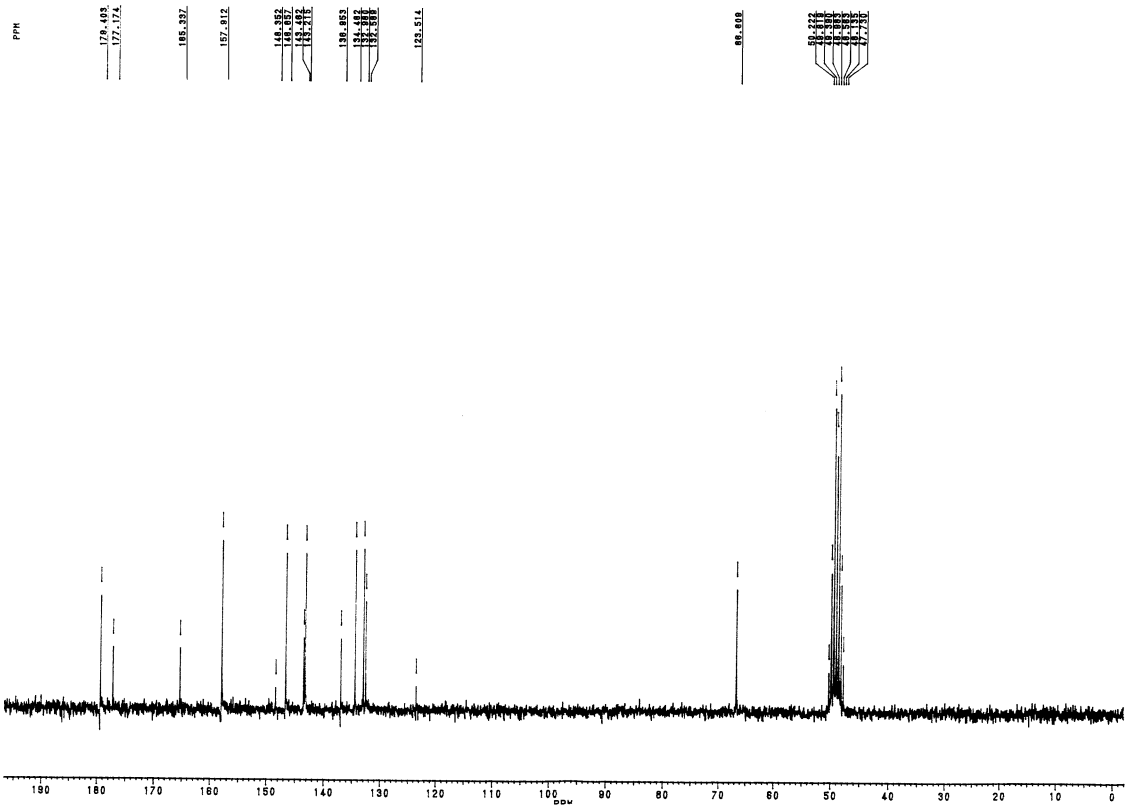

Fig. 2. ^{13}C-NMR spectrum of *N*-(4-bromo-2-(2-pyridylcarbonyl)phenyl)-2-aminoacetamide.

lowed in order to investigate the kinetics of the degradation reaction.

Treatment of kinetic data was carried out by non-linear regression analysis using the software package MINSQ (version. 4.03, Micro-Math Scientific Software, Salt Lake City, UT, USA) in the way mentioned elsewhere [21].

3. Results and discussion

3.1. Structure elucidation of the degradation products

As shown in Scheme 1, the intermediate degradation product expected from the general scheme of hydrolysis of 1,4-benzodiazepines, should be either the 2-[(*E*)-1-(2-amino-5-bromophenyl)-1-(2-pyridyl)methylideneamino]acetic acid or *N*-(4-bromo-2-(2-pyridylcarbonyl)phenyl)-2-aminoacetamide and the final one should be 2-amino-5-bromophenyl-2-pyridylmethanone.

Previous investigators, working under similar experimental conditions and based on polarographic data have proposed that the intermediate degradation product should have been the 2-[(*E*)-1-(2-amino-5-bromophenyl)-1-(2-pyridyl)methylideneamino]acetic acid and the final one 2-amino-5-bromophenyl-2-pyridylmethanone. In addition to that, **BMZ** hydrolysis by intestinal microflora was also studied [22], where 2-amino-5-bromophenyl-2-pyridylmethanone was isolated, and identified by GC–MS as the final degradation product.

^{13}C-NMR spectral data for **BMZ** revealed an amidic carbon atom at 172 ppm and an azomethine one at 163 ppm [10].

Table 1
Analytical data of the calibration graphs for the determination of bromazepam, intermediate degradation product and benzophenone by HPLC

Compounds	Retention time (min)	Linearity range ($\times 10^{-6}$ M)	Calibration equation[a]	r[b]
Bromazepam	10.10	0.5–2.0	$R = 0.79(\pm 9.6\times10^{-3})\times C - 0.07(\pm 0.02)$	0.9998
Intermediate	8.98	0.5–2.0	$R = 0.47(\pm 7.05\times10^{-3})\times C - 0.05(\pm 9.79\times10^{-3})$	0.9997
Benzophenone	17.73	0.5–2.0	$R = 0.35(\pm 6.86\times10^{-3})\times C - 0.04(\pm 9.52\times10^{-3})$	0.9994

[a]Ratio of the peak height of each compound to that of the internal standard vs. concentration of each compound 10^6 times in M; five standards.
[b]Correlation coefficient.

The ^{13}C-NMR spectrum of the isolated intermediate is shown in Fig. 2. In our study, ^{13}C-NMR data, [66 ppm (1C, methylenic), 123–165 ppm (11C aromatic), 177 ppm (amide), 179 ppm (ketone)], indicated the existence of 14 carbon atoms in this molecule that verified the proposed molecular formula and two carbonyl groups at 177 and 179 ppm. These two peaks was believed to belong to the amidic and the carbonyl groups of this compound, respectively. If the intermediate product of hydrolysis were 2-[(*E*)-1-(2-amino-5-bromophenyl)-1-(2-pyridyl)methylideneamino]-acetic acid (Scheme 1), as other investigators claimed [17–20], then there would have been only one carbonyl peak in the far region of the spectrum and the azomethine carbon would have been in the 160 ppm region. Thus, it was conclusive that the two peaks appearing at 179 and 177 ppm belonged definitely to the amide and the carbonyl groups of this compound. Also, ^{1}H-NMR spectrum of the intermediate degradation product was consistent with the proposed structure [2.0 ppm (2H, amine), 4.3 ppm (2H, methylenic), 7.1–8.5 ppm (7H, aromatic), 10.6 ppm (1H, amidic)].

Furthermore, IR spectrum of intermediate, revealed the existence of two carbonyl groups, one ketone (1690 cm^{-1}) and one amide (1650 cm^{-1}), whereas the acid form of the intermediate would have had only one, the carbonyl peak of the acid.

Regarding the final product, its EI–MS spectrum showed a strong molecular ion at m/z 277. Combining this with the results taken by elemental analysis and the structure elucidation presented by other researchers [22], it was clear that this product was the 2-amino-5-bromophenyl-2-pyridylmethanone.

Elemental analysis performed for both of the degradation products [intermediate: C (50.12%), H (3.6%), N (12.07%); final: C (52.07%), H (3.20%), N (10.01%)] supported the proposed molecular formulas of the aforementioned compounds.

3.2. Evaluation of the HPLC method

Under the experimental conditions described in a previous section, linear relationship between the HPLC signal of **BMZ**, **BAA** and **ABM** and their concentrations was observed as shown in Table 1. Moreover, data for the precision and accuracy, given in Table 2, indicated % R.S.D. = 1.37–3.92 and $E_r = -2.7$–2.0% for the studied compounds.

The above statistical evaluation of the HPLC method revealed its good linearity and reproducibility and led us to the conclusion that it could have been used for the kinetic investigation of **BMZ** and its degradation products reliably.

The limit of detection attained, as defined by IUPAC [23] $DL_{(k=3)} = k \times S_b/b$, where b is the slope of the calibration graph and S_b is the standard deviation of the blank signal was found to be 3.8×10^{-7} M for **BMZ**, 6.25×10^{-7} M for **BAA** and 8.16×10^{-7} M for **ABM**.

Table 2
Accuracy and precision for the determination of bromazepam, intermediate degradation product and benzophenone by HPLC

Compound	Nominal concentration ($\times 10^{-6}$ M)	Assayed concentration ($\times 10^{-6}$ M)[a]	R.S.D. %[b]	E_r %[c]
Bromazepam	0.5	0.49 ± 0.01	2.05	−2.0
	1.5	1.46 ± 0.02	1.37	−2.7
Intermediate	0.5	0.49 ± 0.01	2.08	−2.0
	1.5	1.47 ± 0.03	2.04	−2.0
Benzophenone	0.5	0.51 ± 0.02	3.92	2.0
	1.5	1.52 ± 0.03	1.97	−2.7

[a]Mean ± S.D. ($n = 8$).
[b]Percentage relative standard deviation (R.S.D).
[c]Relative percentage error = [(assayed concentration − nominal concentration)/nominal concentration] × 100.

3.3. Kinetic investigation and proposed degradation pathway

To examine kinetics of acidic hydrolysis of **BMZ** hydrochloric acid solutions of 1.0, 0.1 and 0.01 M were used. However, degradation was very fast in the first two cases, thus the kinetics were very difficult to follow accurately. In particular, working with 1.0 M HCl at 25°C, it was observed that 20 min after the initiation of the reaction only 20% of **BMZ** was present in the reaction medium. Thus, the majority of **BMZ** was actually degraded to give mostly intermediate **BAA** and a very small quantity of **ABM**. Forty min after the initiation of hydrolysis only 10% of **BMZ** existed in the reaction mixture, while intermediate **BAA** started to degrade to **ABM**. The acid-hydrolysis of **BMZ** was completed within 50 min, where only **BAA** and small amounts of **ABM** were present in the reaction mixture.

More detailed study was conducted in 0.01 M HCl at 25°C, where degradation was slower. Plot of the signals of **BMZ** and **BAA** vs. time measured by HPLC is shown in Fig. 3. Formation of **ABM** was not observed. In order to be able to follow degradation of **BAA** to **ABM** as well, an accelerated hydrolysis study was carried out at higher temperature.

Bromazepam was left to hydrolyse in 0.01 M HCl at 95°C and the reaction was followed for 1300 min. Plots of the HPLC signals of the three species vs. time are presented in Fig. 4. During the first 20 min, **BMZ** decreased rapidly to **BAA** while the **BAA** increased very fast. Then, both **BMZ** and **BAA** decreased slowly up to 300 min and for the rest of the time they become equal and decrease at the same rate. The final product **ABM** appeared after the first 20 min and increased slowly.

Based on the results obtained from the structure elucidation of degradation products and the above kinetic study, a two-step sequential hydrolysis reaction could be proposed with a reversible first step (Scheme 2).

The first step should have been reversible because in the beginning of hydrolysis there is a rapid change in concentration of both **BMZ** and **BAA** while the final product **ABM** did not participate.

The rate constants of the above reactions k_1, k_{-1} and k_2 could be calculated from the following differential equations:

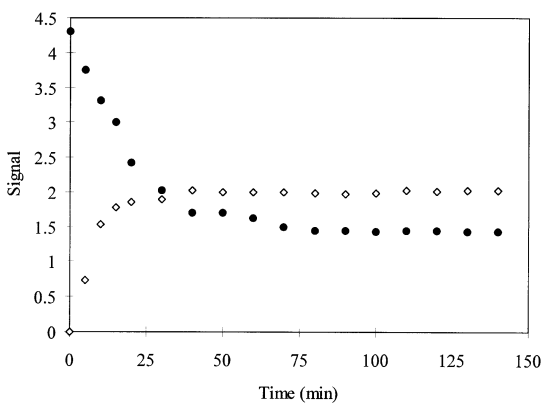

Fig. 3. Plot of the HPLC signal of: **BMZ** •, and **BAA** ◇ during an accelerated degradation study of **BMZ** in a 0.01 M HCl solution at 25°C.

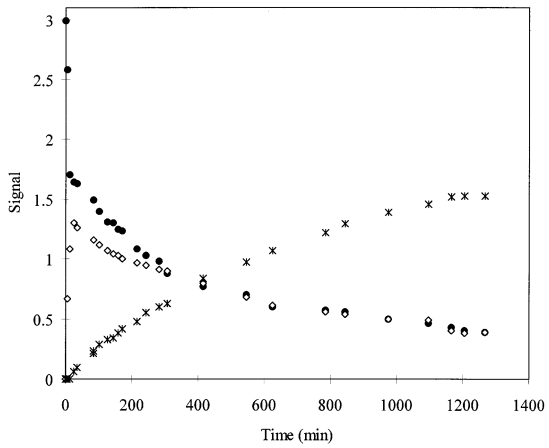

Fig. 4. Plot of the HPLC signal of: **BMZ** •, **BAA** ◇, and **BPH** * during an accelerated degradation study of **BMZ** in a 0.01 M HCl solution at 95°C.

$$-\frac{d[A]}{dt} = k_1[A] - k_{-1}[B] \quad (1)$$

$$\frac{d[B]}{dt} = k_1[A] - k_{-1}[B] - k_2[B] \quad (2)$$

$$\frac{d[C]}{dt} = k_2[B] \quad (3)$$

where A, B and C represent bromazepam, intermediate and final degradation product, respectively.

General solutions of the above equations [24] (for $[B]_0 = 0$ and $[C]_0 = 0$) are:

$$[A] = \frac{k_1[A]_0}{\lambda_2 - \lambda_3}\left[\frac{\lambda_2 - k_2}{\lambda_2}\exp(-\lambda_2 t) - \frac{\lambda_3 - k_2}{\lambda_3}\exp(-\lambda_3 t)\right], \quad (1a)$$

$$[B] = \frac{k_1[A]_0}{\lambda_2 - \lambda_3}[\exp(-\lambda_3 t) - \exp(-\lambda_2 t)], \quad (2a)$$

$$[C] = [A]_0 - [A] - [B] \quad (3a)$$

Scheme 2. Proposed degradation pathway of the acidic hydrolysis of bromazepam in aqueous solutions.

where $\lambda_2 = (p+q)/2$ and $\lambda_3 = (p-q)/2$ with $p = k_1 + k_{-1} + k_2$ and $q = (p^2 - 4k_1k_2)^{1/2}$.

Since the above solutions were very complicated, some assumptions were made to simplify them.

In the case of 0.01 M HCl and 25°C it was assumed that $k_2 = 0$, thus $\lambda_2 = k_1 + k_{-1}$ and $\lambda_3 = 0$, then:

$$[A] = \frac{k_1[A]_0}{k_1 + k_{-1}} e^{-(k_1 + k_{-1})t} \quad (1b)$$

$$[B] = \frac{k_1[A]_0}{k_1 + k_{-1}}(1 - e^{-(k_1 + k_{-1})t}) \quad (2b)$$

Transforming Eq. (1b) and plotting $\ln[A]$ (or ln of any measurable quantity proportional to $[A]$) vs. time, a linear plot was obtained with slope equal to the sum $(k_1 + k_{-1})$, which was the apparent or observed rate constant [24,25]. Similar transformation could be made in Eq. (2b). Then, k_1 and k_{-1} were calculated using Eq. (1b) and Eq. (2b) working with HPLC signals of **BMZ** and **BAA**.

In the case of 95°C the hydrolysis of **BMZ** to **BAA** was extremely fast during the first 20 min. Therefore, it was assumed that the rate determining step was defined by the second reaction $(k_{obs})_2 = k_2$. This should have been true because using Eq. (1a) or Eq. (2a) or Eq. (3a) for time longer than 20 min, the estimated $(k_{obs})_2$ received the same value.

Calculations of the observed rate constants k_{obs} were carried out by the MINSQ program, using the least squares and simplex subroutines. The models used were:

$$Y = pe^{-(k_{obs})t} \quad (4)$$

$$Y = p(1 - e^{-(k_{obs})t}) \quad (5)$$

Eq. (4) applies to reactants of a first order reaction, while Eq. (5) applies to products of the same type of reaction; where Y represents the signal measured at time t and p is a constant factor under certain experimental conditions. Average results of the rate constants obtained from the kinetic studies of **BMZ** are presented in Table 3 and indicated that a good fit between calculated plots and experimental data was achieved.

Table 3
Results of kinetic investigation of bromazepam derived by the HPLC method

C_{HCl} (M)	θ (°C)	$k_1 \times 10^2$ min^{-1}	$k_{-1} \times 10^2$ min^{-1}	$k_2 \times 10^3$ min^{-1}	r_1	r_1	r_2
0.01	25	3.1	2.2	—	0.991	0.991	—
0.01	95	—	—	1.4	—	—	0.9992

4. Conclusions

The degradation pathway of **BMZ** in strongly acidic solutions was investigated in our laboratory. *N*-(4-Bromo-2-(2-pyridylcarbonyl)phenyl)-2-aminoacetamide was the intermediate product of the reaction as proved after its isolation and identification by IR, ^{1}H-, ^{13}C-NMR and mass spectrometry and not the 2-[(*E*)-1-(2-amino-5-bromophenyl)-1-(2-pyridyl)methylideneamino)acetic acid as it was believed up to this time. This showed clearly that the mechanism of this hydrolysis involved initial cleavage of the 4,5-azomethine bond and not of the 1,2-amide bond, as was reported in the literature. Moreover, degradation of bromazepam, under the experimental conditions used, involved a two-step sequential reaction with a fast reversible first step.

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