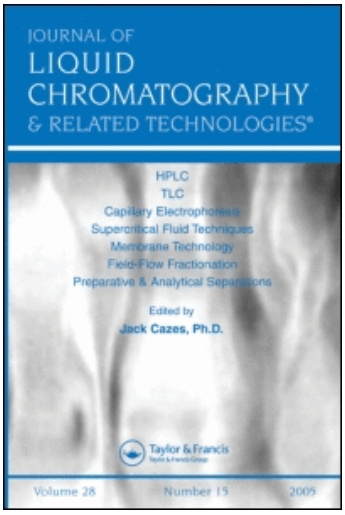


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# KINETIC INVESTIGATION ON THE DEGRADATION OF LORAZEPAM IN ACIDIC AQUEOUS SOLUTIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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## ABSTRACT

A high performance liquid chromatographic method was developed for the kinetic investigation on the acidic hydrolysis of lorazepam in 0.01, 0.1, and 1.0 M hydrochloric acid solutions. In this study, the simultaneous determination of lorazepam and its main degradation product was performed on a reversed phase BDS C-8 column. The mobile phase consisted of a mixture of methanol : acetonitrile : buffer solution containing 0.005 M $KH_2PO_4$ and 0.1 M $CH_3COONH_4$ adjusted to pH 6.0 with glacial acetic acid (35 : 20 : 45, v/v/v) pumped at a flow rate of 1.5

mL/min. The UV detector was operated at 230 nm. The main degradation product attained under the above mentioned experimental conditions was 6-chloro-4-(2-chlorophenyl)-2-quinazolinecarboxaldehyde which was isolated and identified by $^{1}H$-, $^{13}C$-NMR and mass spectrometry. The proposed method showed a relative standard deviation of less than 2.04 % and detection limits of 0.16 and 0.17 μg/mL for lorazepam and its main degradation product, respectively.

## INTRODUCTION

Lorazepam,[1,2] 7 - chloro - 5 - ( 2-chlorophenyl)-3-hydroxy-2,3- dihydro-1H-1,4-benzo diazepin-2-one ($C_{15}H_{10}Cl_2N_2O_2$, M.W.= 321.16), is a member of the 1,4-benzodiazepine series. These compounds are psychotherapeutic drugs widely used in the treatment of nervous diseases such as anxiety, insomnia, and epileptic convulsions.[3,4] Due to the therapeutic interest of these compounds, a number of analytical methods has been reported for their determination.[5-8]



Lorazepam, like most of the benzodiazepines, is hydrolysed in acidic aqueous solutions to a series of degradation products. The extensive use of this compound makes the kinetic study on this reaction a matter of great concern.

Previous work on the chemical stability of lorazepam has been reported. The polarographic behaviour of lorazepam has been investigated over a wide pH range, where 6-chloro-4-(2-chlorophenyl)-2-quinazolinecarboxaldehyde was the main degradation product formed on shelf storage of formulations.[9] Thin layer chromatography has also been used for the separation and determination of benzophenones obtained by acid hydrolysis of lorazepam and other benzodiazepines.[10,11] The stability of lorazepam at phosphate buffer (pH =7) was studied by a high performance liquid chromatographic method, where experimental data were evaluated by an analog-hybrid computer simulation.[12] An accelerated stability study of solid dispersions of lorazepam was also conducted at high temperature, humidity, and sunlight exposure.[13] A first-order derivative spectrophotometric method was also used to study the microwave-assisted hydrolysis of lorazepam.[14] However, nothing clear and conclusive has been reported in acidic environment.

The purpose of the present study was to develop a high performance liquid chromatographic method, allowing the simultaneous determination of the drug and its degradation products, in order to use it in the kinetic investigation of the acid-catalysed hydrolysis of lorazepam. The main degradation product of lorazepam in hydrochloric acid solutions of 0.01, 0.1, and 1.0 M, was isolated,

identified, and appeared to be 6-chloro-4-(2-chlorophenyl)-2-quinazolinecarboxaldehyde. Under more rigorous acidic conditions (HCl 4.0 M), lorazepam degraded to (2-amino-5-chlorophenyl)(2-chlorophenyl)methanone which was also isolated and identified.

## MATERIALS AND METHODS

### Apparatus

Chromatographic analysis was performed using a Waters pump Model 501, a Rheodyne injector Model 7125 with a 20-μL sample loop, a Waters UV-Vis detector Model 486 with a 8-μL flow cell, set at 230nm, and a Hewlett-Packard Model HP3394A integrator. The separation was performed on a reversed phase BDS C-8 column (250 x 4.6 mm i.d., 5μm particle size) (Shandon HPLC, U.K.). A pH meter (Metrohm, model 654 Herisau) was used for all pH measurements. A thermostated Heto water bath was used at $\theta \pm 0.2°C$ for the accelerated kinetic studies. IR-spectra were recorded on a Perkin-Elmer, Model 883 infrared spectrophotometer. Nuclear Magnetic resonance spectra were recorded on a Bruker, Model AC-200 spectrometer. Mass spectrometry was performed using a GC-MS instrument, model VG-TRIO 1000, operated at 70 eV and DIP (direct inlet probe).



### Methods

All chromatographic experiments were carried out at a flow rate of 1.5 mL/min. The mobile phase, methanol : acetonitrile : buffer solution adjusted to pH 6.0 with glacial acetic acid (35 : 20 : 45, v/v/v). The buffer solution was prepared as follows: 7.7 g $CH_3COONH_4$ and 0.68 g $KH_2PO_4$ were dissolved in 1000 mL of water (HPLC grade) giving final concentrations of 0.1 M $CH_3COONH_4$ and 0.005 M $KH_2PO_4$. The mobile phase was filtered through a 0.45 μm Millipore filter and degassed under vacuum prior to use. $^1$H-NMR and $^{13}$C-NMR spectra were acquired in $CDCl_3$, using a frequency of 200 MHz and 50 MHz, respectively.

### Materials

Lorazepam of pharmaceutical purity grade was kindly provided by Minerva Hellas A. E., Athens, Greece and was used without any further

purification. Solvents were of HPLC grade and were purchased from Lab-Scan Science Ltd., Ireland. Ammonium acetate (pro analysi), pottasium 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.

6-Chloro-4-(2-chlorophenyl)-2-quinazolinecarboxaldehyde, **CQA**, was isolated in crystalls as follows : 0.4 g of lorazepam was dissolved in the minimum volume of methanol, 100mL of 0.1 M hydrochloric acid were added and the solution was heated to 95°C for 6 hours. When cooled to room temperature, yellow crystals were precipitated. The crystals were washed several times with water and dried under reduced pressure over $P_2O_5$. The whole process was monitored at every step by means of HPLC.

The same procedure was repeated in order to prepare 2-amino-5-chlorophenyl)(2-chlorophenyl)methanone, with the exception that the solution was heated to 95°C with 4.0 N hydrochloric acid for 5 hours. The solution was left to cool at room temperature and was refrigerated overnight. The orange 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.



## Standard Solutions

Stock standard solution of lorazepam, **LRZ**, 1.0 mg/mL was prepared by dissolving the compound in methanol. This solution was stored in the dark under refrigeration and was found to be stable for several weeks. Stock standard solution of 6-chloro-4-(2-chlorophenyl)-2-quinazolinecarboxaldehyde, **CQA**, 0.25 mg/mL was also prepared in methanol, kept in deep freeze and used within two days of its preparation.

## Calibration Procedure

A series of mixed working standard solutions of **LRZ** and **CQA** in a ratio 1:1 was prepared by the appropriate dilution of the above mentioned stock standard solutions to 2 mL of HCl 0.005M. The concentration range tested was 0.5 to 1.8 μg/mL for each one of the compounds. All of these solutions were analysed immediately after their preparation. Peak heights of each compound were calculated for their determination.

The over-all precision of the assay was evaluated by analysing two series of working standard solutions containing 0.5 and 1.8 μg/mL respectively of a binary mixture of **LRZ** and **CQA** in a ratio 1:1 with eight replicates. The relative standard deviation (RSD%) was determined in order to assess the precision of the method, while the accuracy was assessed using the relative percentage error ($E_r$%).

### Kinetic Investigation of the Acidic Hydrolysis

A 4mL-aliquot of the stock methanolic solution of **LRZ**, 2.0 μg/mL, was transferred to a 200 mL round-bottomed flask containing 96 mL of HCL 0.1 M. The flask was placed in a thermostated water bath. This procedure was carried out at 55, 58, and 60°C.



The above mentioned treatment was repeated with hydrochloric acid solutions of 0.01 and 1.0 M at 60 and 53°C, respectively, to further investigate the acidic hydrolysis of **LRZ**.

During the kinetic study at predetermined time intervals, a 100 μL aliquot was removed from the flask and 1.0-mL of water 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 **LRZ** in the aqueous media, which might have led to erroneous results. The total time spent to follow the accelerated degradation reaction was 4.5 - 5 hours.

## RESULTS AND DISCUSSION

### Evaluation of the HPLC Method

### Chromatographic characteristics

A typical chromatogram obtained under the experimental conditions described in a previous section is displayed in Figure 1. **LRZ** was eluted at 4.6 min, while the main degradation product, **CQA**, appeared at 10.8 min. During this kinetic study, another product was also eluted at 16.3 min in very small quantities in hydrochloric acid solutions of 0.1 and 1.0 M.

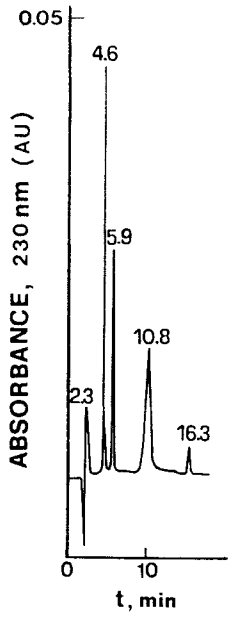



**Figure 1**. Separation of lorazepam (4.6 min), 2-amino-2,5-di-chlorobenzophenone (5.9 min), 6-chloro-4-(2-chlorophenyl)-2-quinazolinecarboxaldehyde (10.8 min), and an unidentified degradation product of lorazepam hydrolysis (16.3 min), using a BDS C-8 column; mobile phase of methanol : acetonitrile : 0.005 M $KH_2PO_4$ and 0.1 M $CH_3COONH_4$ adjusted to pH 6.0 with glacial acetic acid (35 : 20 : 45, v/v/v).



## Table 1

### Analytical Data of the Calibration Graphs for the Determination of Lorazepam LRZ, and 6-Chlorophenyl)-2-quinazolinecarboxaldehyde, CQA, by High Performance Liquid Chromatography

| Compound | Linearity Range (μg/mL) | Calibration Equation[a] | r[b] |
|---|---|---|---|
| LRZ | 0.5 - 1.8 | $H_{LRZ} = 0.354(\pm0.01)\times C_{LRZ} + 0.020(\pm0.018)$ | 0.9994 |
| CQA | 0.5 - 1.8 | $H_{CQA} = 0.179(\pm0.02)\times C_{CQA} + 0.010(\pm0.008)$ | 0.9990 |

[a] Peak height amplitude, H, versus concentraiton of each compound, C, in μg/mL; six standards. [b] Correlation coefficient.

**Table 2**

**Precision and Accuracy for the Determination of Lorazepam, LRZ, and 6-Chloro-4-(2-chlorophenyl)-2-quinazolinecarboxaldehyde, CQA, by High Performance Liquid Chromatography**

| Compound | Added (μg/mL) | Found (μg/mL) mean ± SD(n=8) | RSD %[a] | $E_r$(%)[b] |
|---|---|---|---|---|
| LRZ | 0.5 | 0.49 ± 0.01 | 2.04 | - 2.00 |
| LRZ | 1.8 | 1.77 ± 0.03 | 1.69 | - 1.67 |
| CQA | 0.5 | 0.50 ±0.01 | 2.00 | 0.01 |
| CQA | 1.8 | 1.81 ± 0.01 | 0.55 | 0.55 |

[a] Relative standard deviation.
[b] Relative percentage error.



Unfortunately, it was not easy to isolate it in considerable amounts so as to be able to identify it. (2-amino-5-chlorophenyl)(2-chlorophenyl)methanone, which was produced under more rigorous acidic conditions (4.0 M HCl), was eluted at 5.9 min.

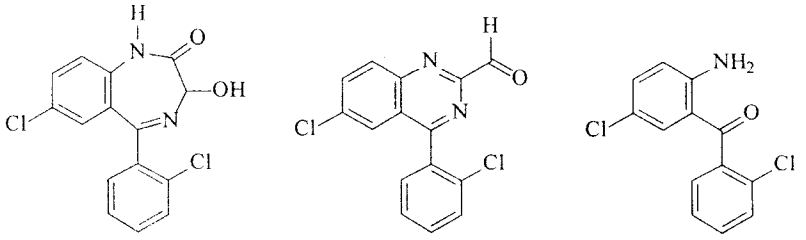
**Linearity and reproducibility**

Under the experimental conditions described in a previous section, linear relationship between the HPLC signal of **LRZ** and **CQA** and their concentrations was observed as shown in Table 1.

Moreover, data for the precision and accuracy of the method given in Table 2, indicated a relative standard deviation, % RSD = 0.55 to 2.04 and a relative percentage error $E_r$ = -2.0 to 0.55 % for the studied compounds.

The 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 **LRZ** and its main degradation product, **CQA**, reliably.

The limits of detection attained as defined by IUPAC[15] $DL_{(k=3)} = k \times S_b / b$, where b is the slope of the calibration graph and $S_b$ is the standard deviation of the blank signal, were found to be 0.16 and 0.17 μg/mL for **LRZ** and **CQA**, respectively.



I II III

**Scheme 1.** Structure of lorazepam **I**, (2-amino-5-chlorophenyl)(2-chloro- phenyl) methanone **II** and 6-chloro-4-(2-chlorophenyl)-2-quinazoline carboxaldehyde **III**.



## Structure Elucidation of the Degradation Products

### 6-Chloro-4-(2-chlorophenyl)-2-quinazolinecarboxaldehyde

($C_{15}H_8Cl_2N_2O$, M.W.= 303.15) IR (Nujol): 1720 $cm^{-1}$ (aldehyde), 1665 $cm^{-1}$ (azomethine bond) $^1$H-NMR (200 MHz, $CDCl_3$): 7.2 - 8.4 ppm (7H, aromatics), 10.4 ppm (s, 1 H, CHO). $^{13}$C-NMR(50 MHz, $CDCl_3$): 136-124 ppm (12 C, aromatics), 149 ppm (1C, C=N), 167 ppm (1C, C=N), 191 ppm (1C, CHO). MS: (m/z) $M^+$ 302 and ion fragments followed a pattern typical of compound containing two chlorine atoms.

### (2-Amino-5-chlorophenyl)(2-chlorophenyl)methanone

(M.W.= 266.13 $C_{13}H_9Cl_2NO$). This product was identified by mass spectrometry, the results obtained were similar to those obtained by Schillings et al[16]. MS: (m/z) $M^+$ 265. Structures of **LRZ** and its main degradation products **CQA** and (2-amino-5-chlorophenyl)(2-chlorophenyl)methanone are presented in scheme 1.

## Kinetic Investigation

In order to gain a comprehensive understanding on the mechanism of degradation of LRZ, accelerated stability measurements were performed at different pH values and temperatures. The rate of disappearance of LRZ as well as the rate of formation of degradation products was measured by HPLC under the experimental conditions described earlier.

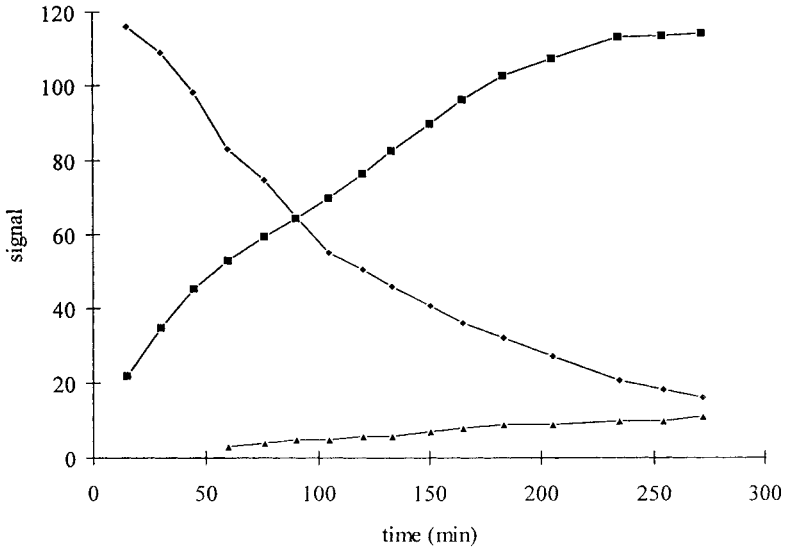



**Figure 2.** Plot of HPLC signal of : **LRZ** ♦, **CQA** ■ and the unknown product ▼, during an accelerated degradation study of **LRZ** in a 0.1 M HCl solution at 60°C.



Aqueous LRZ in 0.1 M HCl at 55, 58 and 60°C was subjected to HPLC analysis at t=0 min and different time intervals until 270 min. In the case of 0.1 M HCl and at 60°C, plots of experimental points versus time are presented in Figure 2, where it can be observed that **LRZ** was degraded to give **CQA**.

After 50 min, **CQA** was further degraded to give another product ($t_R$=16.3 min) in a very small quantity.

The rate of disappearance of **LRZ** followed first order kinetics as other authors have declared,[12] while the rate of appearance of **CQA** did not follow first order kinetics. Kinetic measurements were also performed in 0.01 M HCl at 60°C. Figure 3 shows the plots of the signal of **LRZ** and **CQA** against time.

During this study the rate of disappearance of **LRZ** was similar to the rate of appearance of **CQA** and both followed first-order kinetics, which confirmed that **CQA** did not degrade to other products.

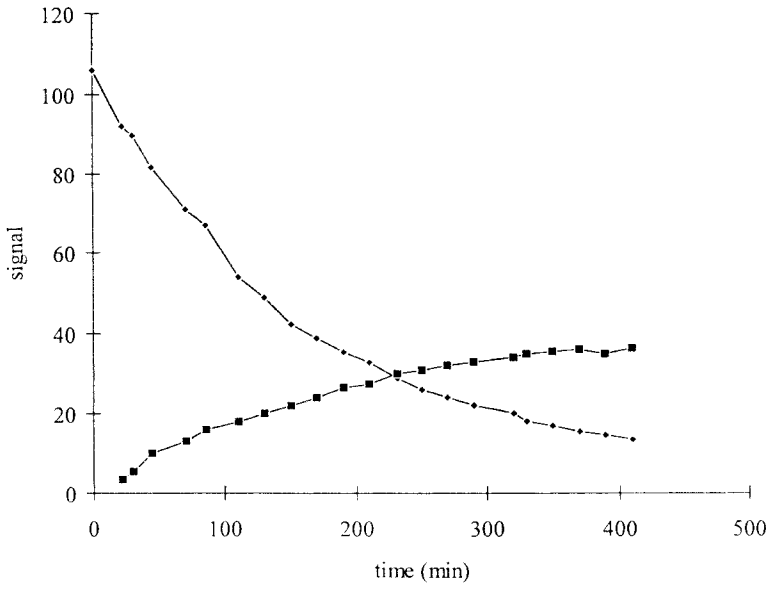



**Figure 3**. Plot of HPLC signal of : **LRZ** ♦ and **CQA** ■ during an accelerated degradation study of LRZ in a 0.01 M HCl solution at 60°C.



An additional kinetic study was also performed in 1.0 M HCl at 53°C and the results are presented in Figure 4. The fact, that in this study the rate of disappearance of **LRZ** was different than the rate of appearance of **CQA**, proves that this product dissociates further to other degradation products, as well. Apart from **CQA**, the degradation product eluted at 16.3 min also appeared, as previously mentioned in the case of 0.1 M HCl.

In the literature, most of the benzodiazepines in acidic aqueous solutions degrade to give benzophenone derivatives.[17] In order to investigate whether the degradation product eluted at 16.3 min was to (2-amino-5-chlorophenyl)(2-chlorophenyl)methanone, accelerated degradation of **LRZ** was conducted in 4.0 M HCl at 95°C. After 5 h, **LRZ** was degraded to form a product which was eluted at 5.9 min. This product was isolated, identified by mass spectrometry,[16] and found to be to (2-amino-5-chlorophenyl)(2-chlorophenyl)methanone. Therefore, the product at 16.3 min was a different degradation product presumably the 6-chloro-4-(2-chlorophenyl)-2-quinazolinecarboxylic acid or the [6-chloro-4-(2-chlorophenyl)-2-quinazolinyl] methanol as mentioned in the literature.[2]

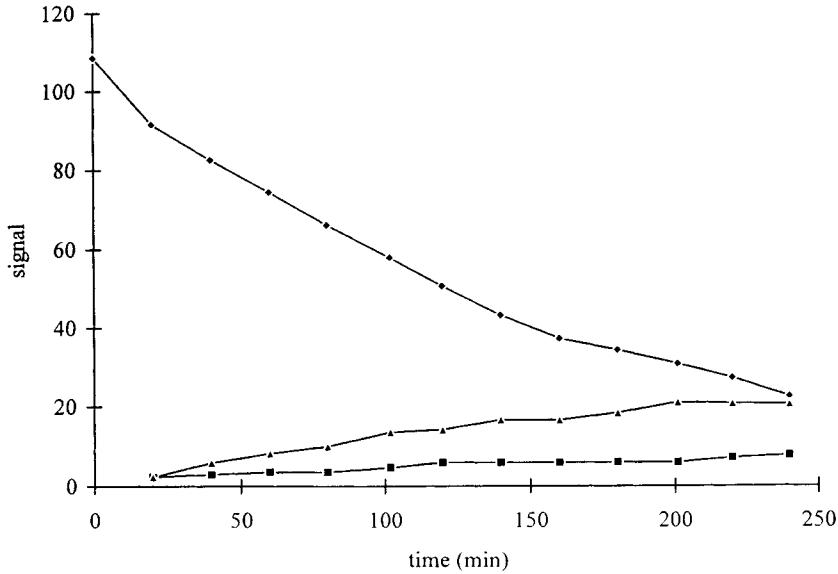



**Figure 4**. Plot of HPLC signal of : **LRZ** ♦, **CQA** ■ and the unknown product ▼, during an accelerated degradation study of **LRZ** in a 1.0 M HCl solution at 53°C.



Calculations of the observed rate constants $k_{obs}$ were accomplished by the MINSQ program, using the Simplex and Least squares subroutines. The models used were :

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

$$Y = p\,(1-e^{-(k_{obs})t}) \quad (2)$$

Equation 1 applies to reactants of a first order reaction, while equation 2 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 **LRZ** are presented in Table 3 and indicate that a good fit between calculated plots and experimental data was achieved. In the case of 0.1 M HCl enough data were produced in order to draw Arrhenious plots, according to the equation: $k = A\, e^{-Ea/RT}$, where k is the reaction rate constant, A is a constant

**Table 3**

**Results of the Kinetic Investigation of Lorazepam in Acidic Aqueous Solutions**

| $C_{HCl}$, M | θ°C | $K_{obs} \times 10^3$, $min^{-1}$ | r[a] |
|---|---|---|---|
| 0.01 | 60 | 5.4 | 0.996 |
| 0.01 | 55 | 4.5 | 0.998 |
| | 58 | 6.1 | 0.995 |
| | 60 | 7.8 | 0.994 |
| 1.0 | 53 | 6.2 | 0.997 |

[a] Correlation coefficient.



termed the frequency factor, $E_a$ is the activation energy of the chemical reaction and T is the absolute temperature. A linear relationship was achieved between log k and $T^{-1}$ with a slope of $-E_a/(R \times 2.303)$ and an intercept of log A. Based on this plot, $E_a$ was 23.78 kcal/mol, while $k_{obs}$ value at room temperature was $1.15 \times 10^{-4}$. The $t_{1/2}$ value (at 25°C) was derived from the relationship $t_{1/2} = 0.693/ k_{obs}$ and found to be 6011 min.

## CONCLUSIONS

In this investigation main degradation products of acidic hydrolysis of **LRZ** were isolated and identified. The optimum chromatographic conditions were chosen in order to separate **LRZ** and its degradation products; the HPLC method was found to be accurate and reproducible and it was successfully applied to the kinetic investigation on the hydrolysis of **LRZ** in acidic aqueous solutions.

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