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Stability study of selected adenosine nucleosides using LC and LC/MS analyses

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Abstract

The stability of the naturally occurring nucleoside, adenosine, and two synthetic chlorine-containing analogues, 2-chloroadenosine and 5′-chloro-5′-deoxyadenosine was studied using high performance liquid chromatography (LC) and liquid chromatography in combination with mass spectrometry (LC/MS). The stability of the examined nucleosides over pH range of 2–10 and at temperatures 40, 60 and 80°C was measured using an LC method, whereas the products of hydrolysis were identified using LC/MS. The LC data indicated that the hydrolysis of the nucleosides followed pseudo-first order kinetics. The MS data proved that the fragment ions at m/z 136.3 and 170.3 referred to the hydrolytic products, adenine and 2-chloroadenine, respectively. The calculated values of the hydrolysis rate constant and half-life indicated that the presence of chlorine atom in the nucleoside base moiety increases apparently the stability of 2-chloroadenosine against acid hydrolysis compared to 5′-chloro-5′-deoxyadenosine and adenosine.

Keywords: Adenosine; 2-Chloroadenosine; 5′-Chloro-5′-deoxyadenosine; LC; LC/MS; Stability study

1. Introduction

Currently, intensive research is focussed on naturally occurring nucleosides and their synthetic analogues. Adenosine constitutes the basic moiety of RNA and is considered the main substrate of adenosine receptors [1,2]. Synthetic adenosine analogues are being widely used in biomedical sciences and in medicine. Cladribine, adenosine deaminase resistant analogue, is clinically used in the treatment of hairy cell leukemia [3] and lymphoproliferative diseases [4,5]. Cladribine is enzymatically metabolized by deglycosylation in biological systems to 2-chloroadenine [6]. 2-Chloroadenosine is another synthetic adenosine analogue that serves as adenosine receptor ligand [7] and as a nucleoside transport inhibitor [8]. It markedly potentiates the activation of phospholipase C enzyme induced by methoxamine [9]. 5′-Chloro-5′-deoxyadenosine is a synthetic purine analogue with a chloro-substitution of ribose sugar moiety [10].

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The stability and degradation of nucleosides are of particular interest. These compounds are susceptible to hydrolysis, which may be followed by other degradation processes, such as deamination. The degradation rates of cytosine nucleosides [11], adenosine in 0.9% sodium chloride solution [12] and in cardioplegic potassium solutions [13] were reported. It is well known that nucleosides may undergo partial or complete nucleoside bond hydrolysis to yield their corresponding sugars and nucleoside bases. Substitution of the parent adenosine molecule with an electronegative atom such as chlorine atom, either in the base or in sugar moiety, alters the stability and subsequently the duration of action and resistance of the nucleoside to hydrolysis. In literature, the attention was paid mainly to naturally occurring nucleosides as adenosine and their clinically applicable synthetic 2′-deoxy or 2′-fluoro derivatives [14–16]. Still, the degradation of adenosine and its synthetic analogues is interesting because of their biological activities and because of biological activities of degradation products.

The availability of modern, highly sensitive and selective chromatographic techniques such as LC and LC/MS permits identification of the hydrolytic products, quantitation and evaluation of the stability of the examined nucleosides. As reported in the literature, only one article dealing with the application of LC/MS in investigating DNA adducts formed by 2-chloroxirane [17] was traced. This work aimed to show the high potential of applying modern instrumental method such as LC/MS and LC/MS/MS to the analysis of natural and synthetic nucleic acid components. This paper reports on the assessment of the stability of adenosine and two selected synthetic chloro substituted nucleoside analogues, 2-chloroadenosine and 5′-chloro-5′-deoxyadenosine (as models), in aqueous buffer solutions over pH range 2–10 and at temperatures 40, 60 and 80°C. A stability-indicating LC procedure was applied for measurement of the kinetic parameters of hydrolysis of the nucleosides. The hydrolytic products were identified using LC/MS analysis.

2. Experimental

2.1. Materials

Adenosine, adenine (Sigma, St. Louis, MO) and 2-chloroadenosine (Research Biochemicals, USA) were used as received. 5′-Chloro-5′-deoxyadenosine was prepared according to a reported method [10]. The chemical formulas of these nucleosides are shown in Fig. 8. Other chemicals were of analytical grade and the solvents were of LC grade. Stock solutions of the examined nucleosides and adenine base were prepared in methanol at concentrations (1 mg ml^{-1}) and were stored at 4°C.

2.2. LC analyses

LC measurements were performed using an isocratic LC system (Waters 2690 Separation Module, USA) equipped with a variable wavelength detector (Waters™ 486, Tunable Absorbency Detector) and auto-sampler (Waters, USA). Chromatographic separations were achieved at ambient temperature using Hypersil® BDS, C18, 5 μ, 150 × 4.6 mm column. The mobile phase consisted of methanol and 1% acetic acid solution in a ratio 4:1. The flow rate was 1.5 ml min^{-1} and the effluent was monitored at 265 nm. Quantitative measurements were based on peak area measurements processed by the instrument build-in Millennium software.

2.3. Stability studies

2.3.1. Effect of pH

Separate aqueous solutions of adenosine, 2-chloroadenosine and 5′-chloro-5′-deoxyadenosine (1 mg ml^{-1}) were prepared in 0.1 M HCl solution and kept in a thermostatically controlled water bath at 80°C. Samples (50 μl) were withdrawn at zero time and then at 1-h intervals for 6 h. The samples were diluted to 1 ml with methanol, vortexed for 30 s and 10 μl aliquots were injected. The concentration of the remaining nucleoside was determined from a linear regression equation relating the peak area of the nucleoside to concentration. The same procedure was repeated using buffer solutions at pH 3, 7 and 10, respectively.

2.3.2. Effect of temperature

Separate aqueous solutions of the nucleosides were prepared in 0.1 M HCl solution (1 mg ml^{-1}) and were kept at 40, 60 and 80°C, respectively. Samples were withdrawn at different time intervals and were treated as previously described.

2.4. LC/MS analyses

The LC/MS data of the investigated compounds were determined using LC/MS system. LC separations were made using LC pump (Spectra System P 2000, USA) connected to Hypersil® BDS column. The mobile phase consisted of methanol and 1% acetic acid solution (4:1) and was pumped at a flow rate 1 ml min^{-1}. Injection of the samples was manually done using 10 μl loop size. Mass spectrometric analyses were performed using mass spectrometer (Finnigan MAT, USA) operating in APCI mode. The APCI conditions were: vaporization temperature 450°C, sheath gas flow 80 ml min^{-1}, discharge current 5 μA and discharge potential 4.38 kV. The mass spectrometer was programmed to detect positive ions of mass/charge ratio (m/z) in the range 50–500. Analytical data were processed by LCQ software.

LC/MS analysis was performed by injecting 10 μl aliquot of 50 μg ml^{-1} solution of either methanolic or acid-treated solution of adenosine, 5′-chloro-5′-deoxyadenosine and 2-chloroadenosine, respectively. The recorded LC/MS spectra were compared with that of adenine prepared at concentration 50 μg ml^{-1}.

3. Results

3.1. The LC analyses of adenosine and 5′-chloro-5′-deoxyadenosine

Several trials have been made to select the proper conditions for the separation of the examined nucleosides and their acid-induced degradation products. The type of column as well as the mobile phase composition was varied to achieve rapid, sensitive and selective resolution of the nucleosides and the hydrolytic products. Initially, two types of ODS column with or without treatment were employed. The base deactivated silica (BDS) column provided better resolution of purine nucleosides and their degradation compounds than the untreated ODS column using either LC or LC/MS analysis. The ODS column exhibited unresolved broad peaks of the nucleoside with the hydrolytic product. The composition as well as the pH of mobile phase were optimized. A mobile phase composed of methanol and 1% acetic acid solution at 4:1 ratio and pH 3.8 gave rapid, sharp and highly resolved peaks of the nucleosides and degradation products. The relatively high content of methanol in the mobile phase resulted in sharp peaks of analytes at shorter time of analysis.

Under the selected chromatographic conditions, adenosine and 5′-chloro-5′-deoxyadenosine exhibited sharp peaks at 1.5 and 1.8 min, respectively. The acid-induced degradation product of adenosine and 5′-chloro-5′-deoxyadenosine appeared at 2.4 min (Fig. 1a,b). The latter corresponds to adenine base, whose LC chromatogram showed a well-defined peak at 2.4 min (Fig. 1c).

3.2. The LC/MS/MS analysis of 2-chloroadenosine

Under the conditions showed above, the developed LC method fails to separate 2-chloroadenosine and its acid-induced degradation product (Fig. 2) due to their similar retention times and identical UV absorption patterns. The high selectivity and sensitivity of LC/MS were utilized for detection and quantitation of each compound in the presence of the other one using LC/MS/MS mode. Programming of LC/MS scanning at the parent ion of 2-chloradenosine (m/z 302.7) makes it possible to detect any nucleoside in the presence of its acid-induced degradation product. Adjustment of the LC/MS scanning mode at the parent ion of 2-chloroadenine (m/z 170.3) allows detection of the base without interference of nucleoside (Fig. 3). Additionally, programming of the relative collision energy at 2% for the measured parent ion increases significantly the sensitivity of LC/MS for detection of the nucleoside and its degradation product.

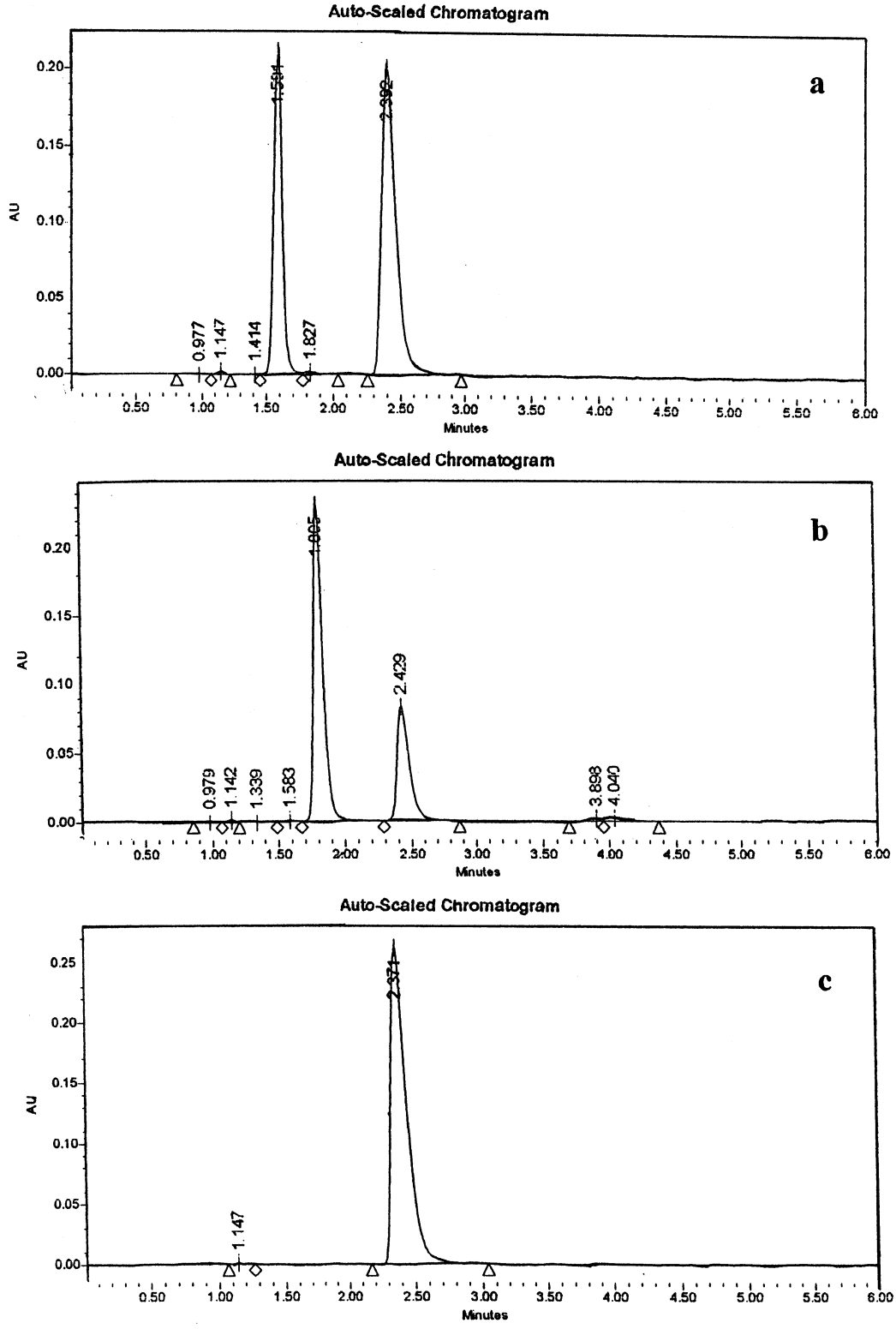

Fig. 1. Liquid chromatography (LC) chromatograms (50 μg ml^{-1}) of degraded solutions of adenosine (a), 5′-chloro-5′deoxyadenosine (b) and of free adenine base (c).

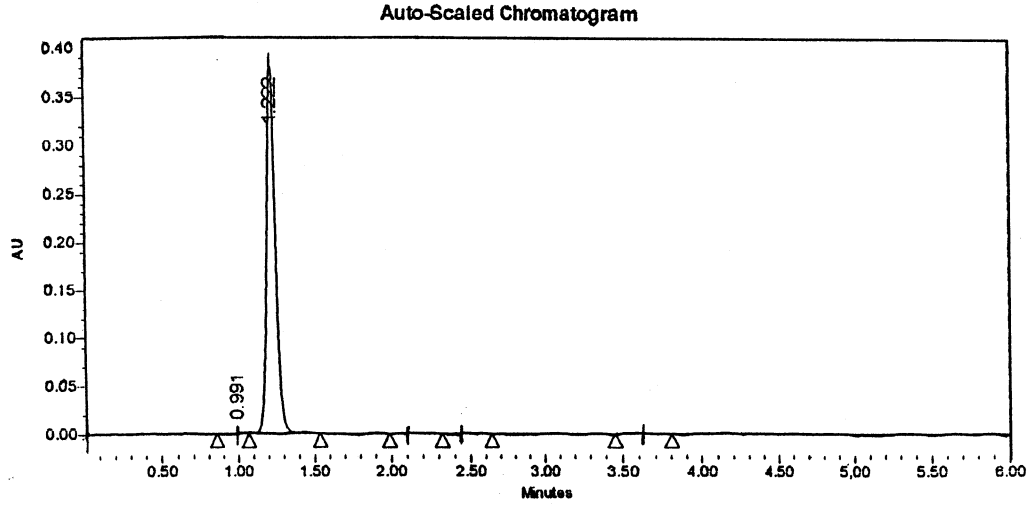

Fig. 2. Liquid chromatography (LC) chromatogram (50 μg ml^{-1}) of an acid-treated solution of 2-chloroadenosine.

3.3. Stability study

The stability of adenosine and its synthetic analogues, 2-chloroadenosine and 5′-chloro-5′-deoxyadenosine was evaluated under acidic and basic conditions at different temperatures. Initially, an accelerated stability study was conducted in 0.1 M HCl solution at 80°C for a period of 6 h. LC chromatograms showed two consecutive peaks at 1.5 and 2.4 min for adenosine and 1.8 and 2.4 min for 5′-chloro-5′-deoxyadenosine, respectively (Fig. 1a,b). In LC/MS/MS, 2-chloroadenosine peak was monitored at m/z 302.7, whereas the acid degradation product was monitored at m/z 170.2 (Fig. 3). Based on peak area measurements using either LC or LC/MS/MS, a prominent decrease in the peak area of the nucleoside as a function of time at 80°C was observed. At the same time, a significant increase of the peak area of the hydrolytic product was recorded. Under the selected experimental conditions (0.1 M HCl/80°C), the hydrolysis of the examined nucleosides followed the kinetics of the pseudo-first order reaction (Fig. 4). The kinetic parameters for the hydrolysis of adenosine, 5′-chloro-5′-deoxyadenosine and 2-chloroadenosine at different pH and temperatures were summarized in Tables 1 and 2. As shown in these tables, the examined nucleosides were relatively stable at pH 3, 7 and 10 at 80°C, however, they were readily hydrolyzed in 0.1 M HCl solution. Moreover, the stability of the nucleosides was influenced by temperature changes. Elevation of temperature and decrease of pH seriously influenced the stability of adenosine. The increased stability of 2-chloroadenosine and 5′-chloro-5′-deoxyadenine may be attributed to the presence of chlorine atom in the adenine base moiety or in the sugar moiety, respectively. This chlorine atom substitution stabilizes the nucleoside molecule against hydrolysis through the intramolecular hydrogen bonding.

3.4. LC/MS analysis

Although the LC data were clear regarding the stability of adenosine and 5′-chloro-5′-deoxyadenine, the LC analyses did not provide definite proof of the nucleoside degradation including the molecular masses of the hydrolytic products. The application of LC/MS or LC/MS/MS permits identification of the nucleosides and their hydrolytic products even in the case when the adequate LC separation is not achieved. Adenosine (MW 267) displayed a peak at 2.82 min under the selected experimental conditions. This corresponds to a parent ion (m/z 268), whereas the hydrolytic product eluted at 3.99 min referred to the parent ion of m/z 136.3 and frag-

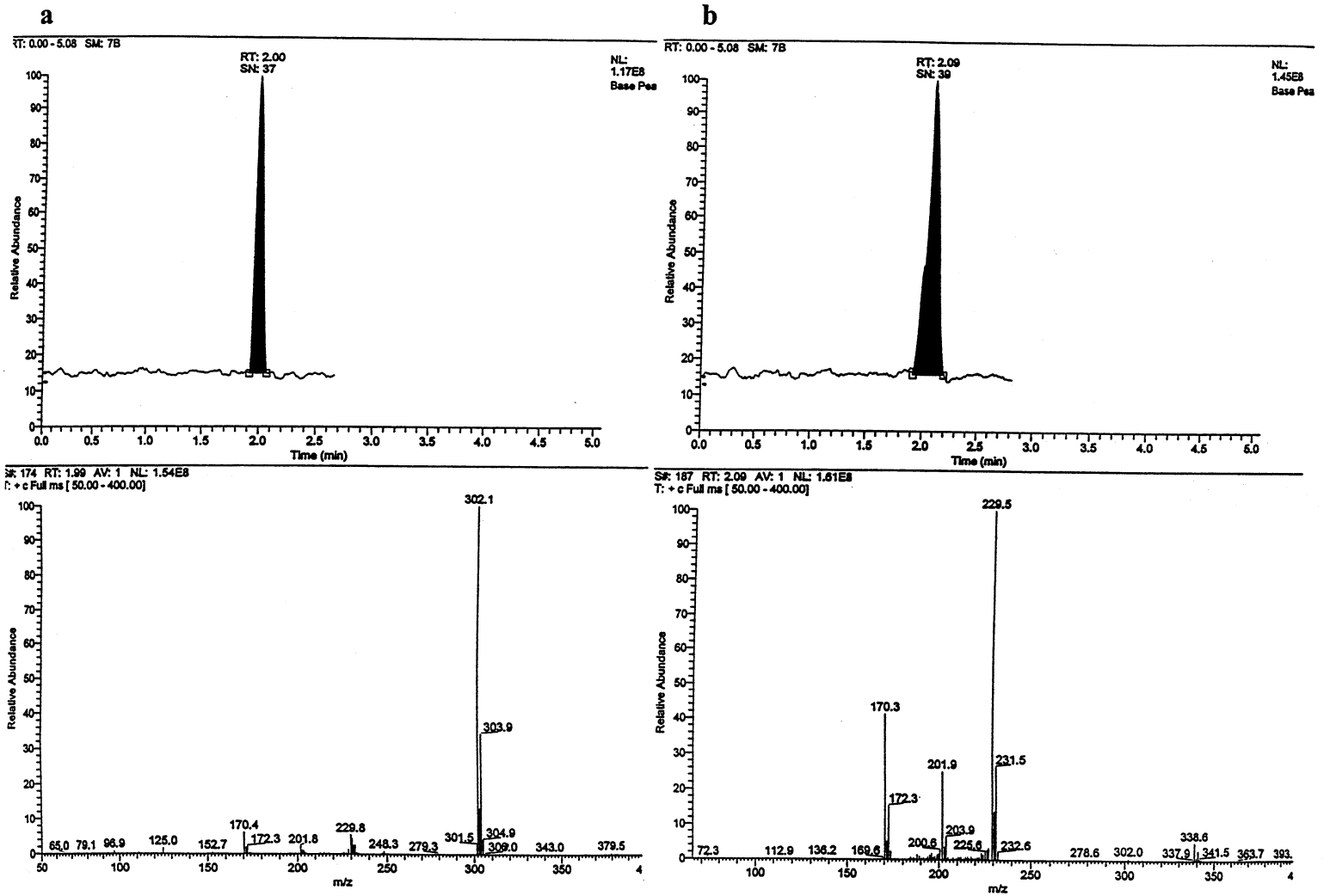

Fig. 3. The liquid chromatography (LC)/mass spectrometry (MS)/MS chromatogram (50 μg ml^{-1}) of 2-chloroadenosine.

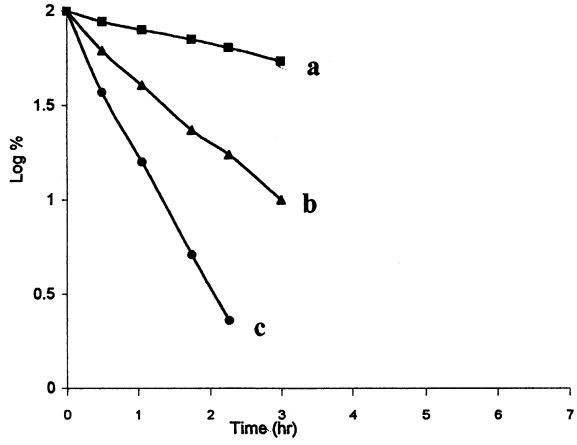

Fig. 4. Pseudo first-order plots of adenosine (a), 5′-chloro-5′-deoxyadenosine (b) and 2-chloroadenosine (c) in 0.1 M HCl/80°C.

Table 1
Kinetic parameters for the degradation of adenosine nucleosides at pH 3, 7 and 10 and the temperature of 80°C

	pH 3	pH 7	pH 10
Adenosine			
K_{hyd} (h^{-1})	0.05 ± 0.020	0.02 ± 0.018	0.04 ± 0.023
$t_{1/2}$ (h)	1.4 ± 0.055	3.5 ± 0.300	1.7 ± 0.095
2-Chloroadenosine			
K_{hyd} (h^{-1})	0.3 ± 0.055	0.2 ± 0.046	0.2 ± 0.031
$t_{1/2}$ (h)	2.3 ± 0.421	3.4 ± 0.707	3.4 ± 0.484
5′-Chloro-5′-deoxyadenosine			
K_{hyd} (h^{-1})	0.1 ± 0.049	0.1 ± 0.015	0.2 ± 0.024
$t_{1/2}$ (h)	6.8 ± 2.883	7.1 ± 1.199	3.4 ± 0.372

ment ions m/z of 195.5 and 167.8 (Fig. 5). The LC peak with parent ion at m/z 136.3 served as a definite proof of the hydrolytic product, adenine (MW 135) (Fig. 6). The ions at 195.5 and 167.8 are probably referred to the higher fragments of adenine base with methanol [18].

The LC/MS chromatogram of the acid-treated solution of 5′-chloro-5′-deoxyadenosine (MW 285) showed LC signals at 2.99 and 3.99 min, respectively. These correspond to the molecular ions of the nucleoside (m/z 286.1) and of adenine base (m/z 136.3), respectively (Fig. 7). The ions of the higher m/z ratios (195.5 and 167.8) also coincide with the products of adenine with methanol [18]. The LC/MS/MS of the acid-hydrolyzed solution of 2-chloroadenosine (MW 301.7) displayed the parent ions at m/z of 302.1 and 170.3, which reflected the presence of the parent nucleoside and 2-chloroadenine, respectively (Fig. 3).

4. Discussion

The stability of naturally occurring nucleoside, adenosine, and the synthetic analogues, 2-chloroadenosine and 5′-chloro-5′-deoxyadenosine was studied under various acidic and basic conditions and at different temperatures. The developed LC procedure using BDS C18 column and mobile phase composed of methanol and 1% acetic acid solution at 4:1 ratio, was proved to be a stability-indicating method as it permit detection and quantitation of the nucleosides in the presence of their acid-induced degradation products. In the case of 2-chloroadenosine, LC was not suitable for quantification of the nucleoside in the presence of its degradation product. However, LC/MS/MS was successfully used in the kinetic studies. Accelerated stability studies of the investigated nucleosides in 0.1 M HCl solution at 80°C showed that the hydrolysis of the nucleosides followed pseudo-first order kinetics. The calculated values of the hydrolysis rate constants and

Table 2
Kinetic parameters for the degradation of adenosine nucleosides in M HCl solution at 40, 60 and 80°C, respectively

Temperature (°C)	A* (k_{hyd} (h^{-1})/$t_{1/2}$ (h))	5′-CIA* (k_{hyd} (h^{-1})/$t_{1/2}$ (h))	2-CIA (k_{hyd} (h^{-1})/$t_{1/2}$ (h))
40	0.02/34.6	0.01/69.3	0.01/69.3
60	0.05/13.9	0.02/34.6	0.03/23.1
80	1.9/0.36	0.20/3.5	0.04/17.3

* A = adenosine; 5′-CIA = 5′-chloro-5′-deoxyadenosine; 2-CIA = 2-chloroadenosine.

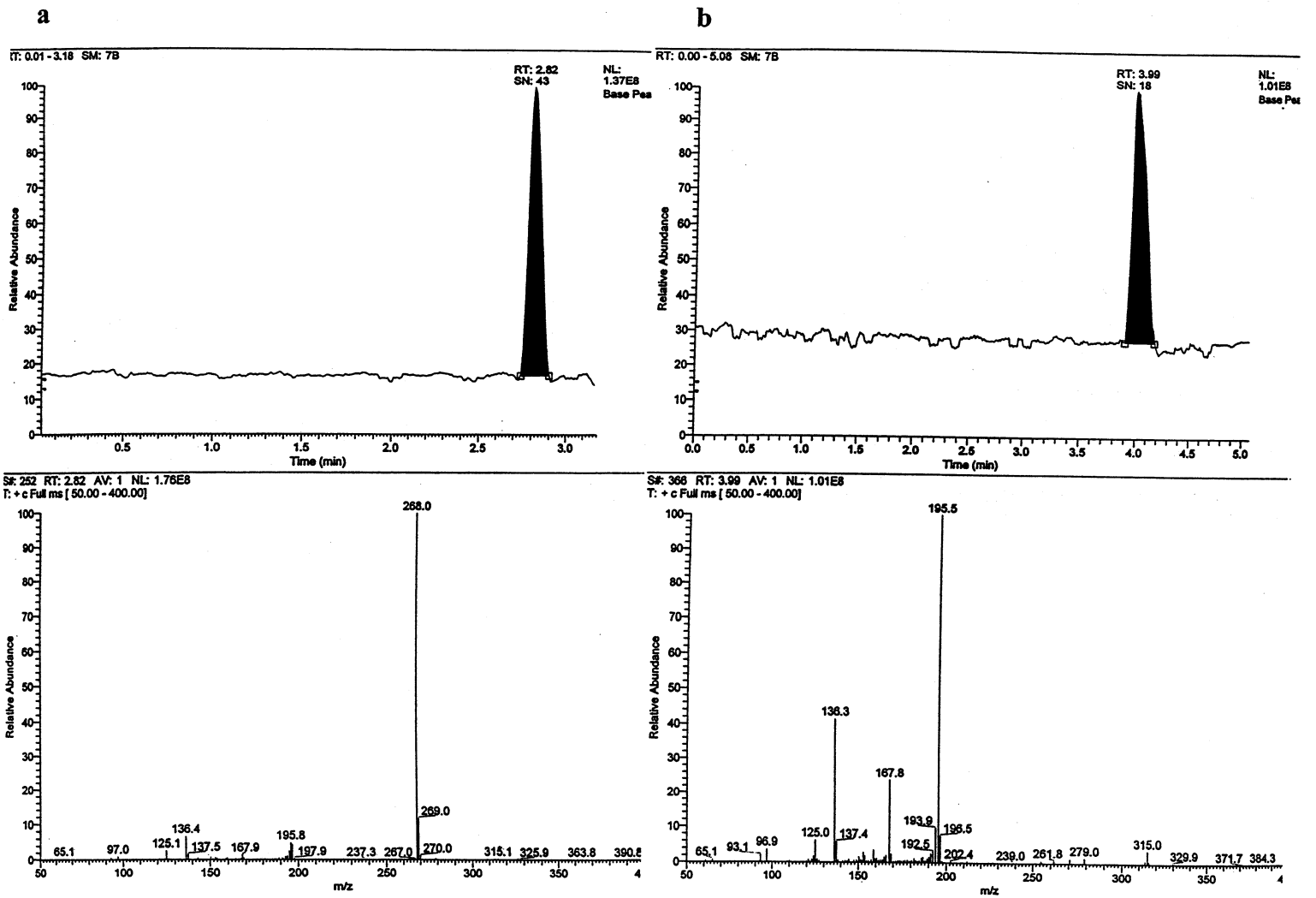

Fig. 5. Liquid chromatography (LC)/mass spectrometry (MS) chromatogram of adenosine (a) and its acid-induced degradation product adenine (b) (50 μg ml^{-1}).

of half-life showed that adenosine was hydrolyzed at relatively faster rate compared to 5′-chloro-5′-deoxyadenosine and 2-chloroadenosine. The presence of chlorine atom, in ribose or in the adenine base moiety, increases apparently the stability of the nucleoside against acid hydrolysis. LC/MS and LC/MS/MS analyses permit detection and identification of the hydrolytic products, adenine and 2-chloroadenine, in the presence of their parent nucleosides even when present at low concentrations or when they were not properly separated by LC, as seen in 2-chloroadenosine. The positive ions at m/z 268, 302.1 and 286.1 referred to adenosine, 2-chloroadenosine and 5′-chloro-5′-deoxyadenosine, respectively. The protonated ions at m/z 136.3 and 170.3 correspond to adenine and 2-chloroadenine, respectively. A schematic diagram showing the major ions in the mass spectra of adenosine nucleosides and their acid-induced hydrolytic products is shown at Fig. 8.

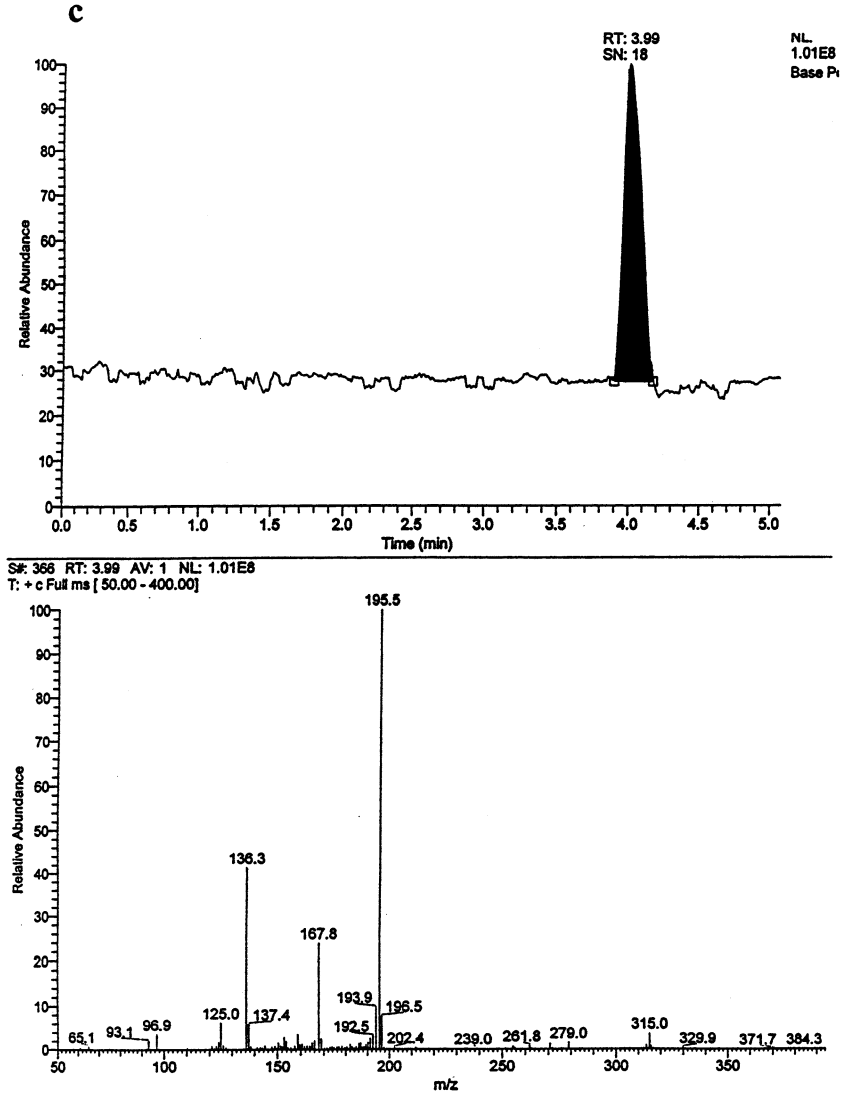

Fig. 6. Liquid chromatography (LC)/mass spectrometry (MS) chromatogram of adenine (50 μg ml^{-1}).

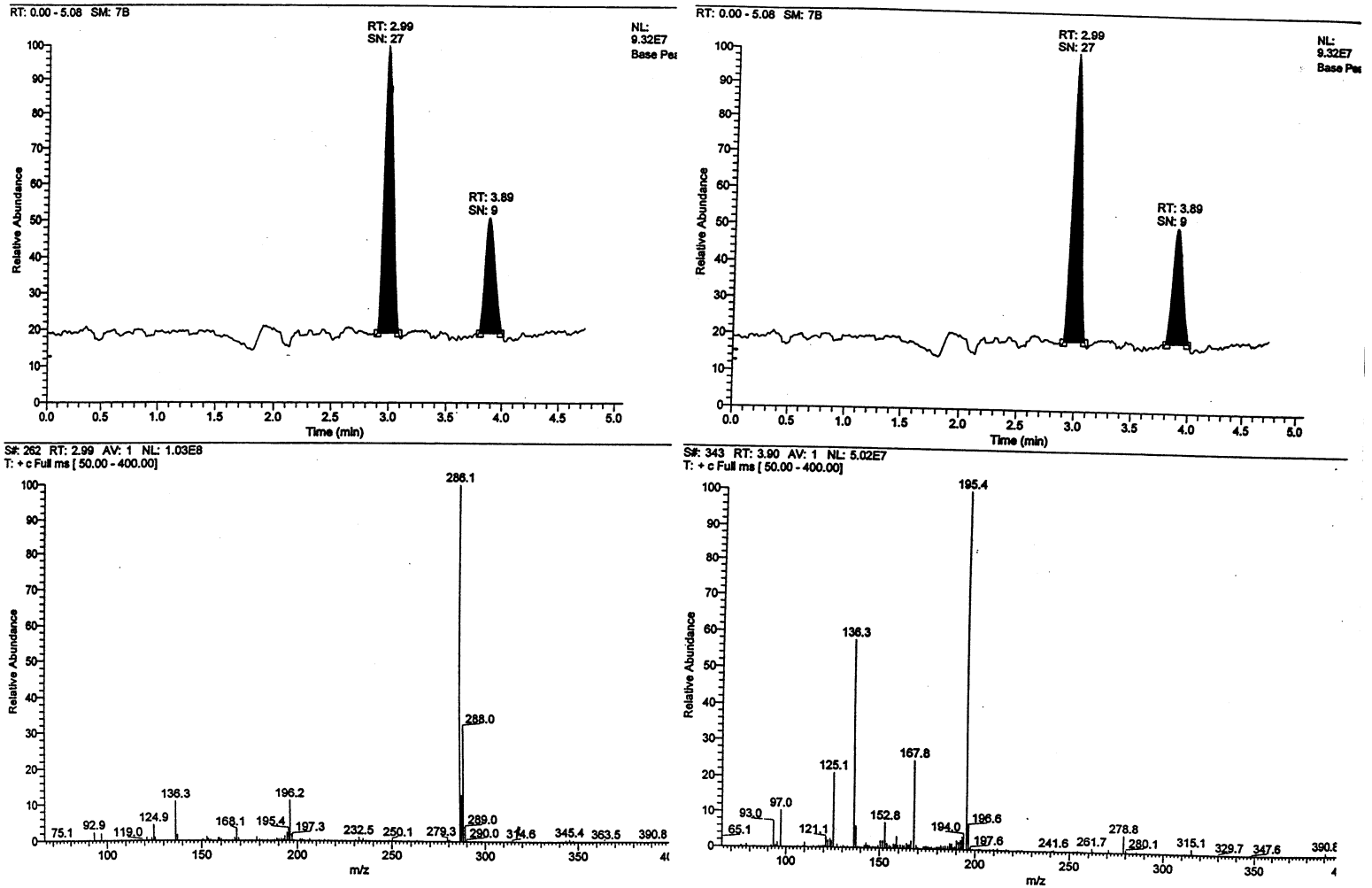

Fig. 7. Liquid chromatography (LC)/mass spectrometry (MS) chromatogram of 5′-chloro-5′-deoxyadenosine (a) and its acid-induced degradation product (adenine) (b) (50 μg ml^{-1}).

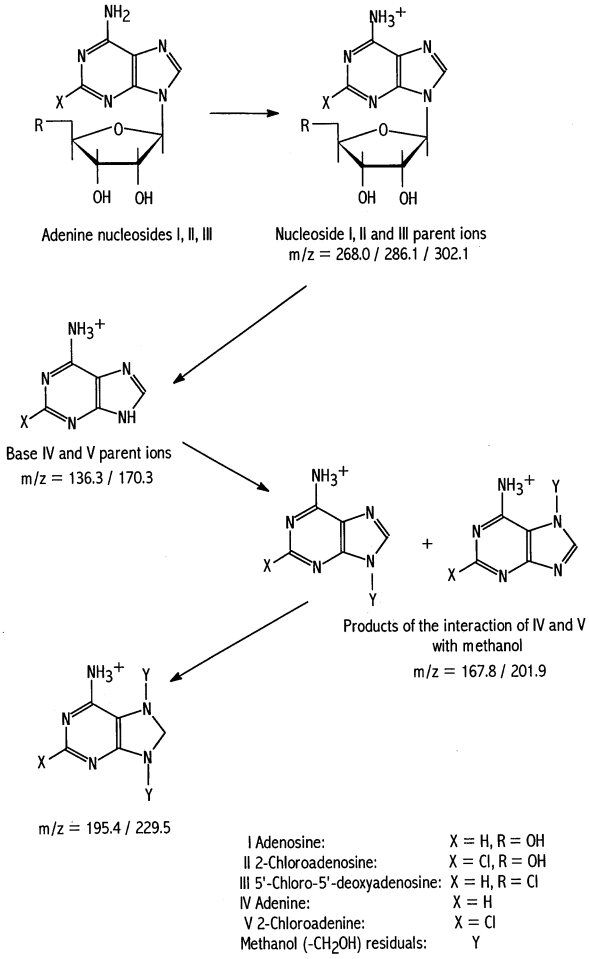

Fig. 8. Structures and major ions in mass spectra of adenosine nucleosides and their hydrolytic products.

5. Conclusions

The stability of adenosine and its synthetic analogues was evaluated at 40, 60 and 80°C in 0.1 M HCl and buffer solutions at pH 3, 7 and 10. Adenosine nucleosides exhibited prominent degradation only in 0.1 M HCl solution at 80°C. Kinetic analysis of the LC or LC/MS/MS records revealed relatively high stability of 5′-chloro-5′-deoxyadenosine and 2-chloroadenosine compared to adenosine. The process of hydrolysis was elucidated by the mass spectrometry method in the LC/MS or LC/MS/MS configuration. The parent ions of the hydrolytic products, adenine and 2-chloroadenine, were identified at m/z of 136.3 and 170.3, respectively.

Acknowledgements

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