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# Liquid chromatographic separation of diamino analogues of 2′- or 3′-deoxyadenosine from adenine on a poly(styrene–divinylbenzene) polymer column

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**Abstract**

A liquid chromatographic method that can separate each of a series of diamino analogues of 2′- or 3′-deoxyadenosine from their main degradation product, adenine, is described. A PLRP-S 1000 Å (8 μm) 250 mm × 4.6 mm I.D. column was used at 60°C. The method was developed by systematic evaluation of the influence of the mobile phase pH, the type and concentration of the organic modifier, the concentration of the ion-pairing agent and the buffer. The mobile phase consisted of tetrahydrofuran–0.2 *M* sodium octanesulphonate–0.2 *M* potassium phosphate buffer (pH 2.0)–water (15:25:30:30, v/v).

## 1. Introduction

Currently intensive research is focused on new drugs for the treatment of AIDS and a variety of nucleosides with activity against HIV have been discovered. Diamino analogues of 2′- or 3′-deoxyadenosine (**1–4**) (Fig. 1) were synthesized as part of a structure–activity relationship study of analogues of 2′,3′-dideoxyadenosine that are active against HIV, but none of them had substantial antiretroviral or cytostatic activity [1]. Although many studies have been published on the stability of normal deoxynucleosides, little is known about the stability of the amino-substituted congeners. We therefore considered it of interest to carry out stability studies on **1–4**. Knowledge of the stability might be useful when carrying out derivatization reactions with these nucleosides. In addition, compounds **1–4** are potential candidates for incorporation into oligodeoxynucleotides, serving as enzymatically stable substitutes for the natural deoxynucleosides. Stability studies on **1–4** necessitated an analytical method that could separate each of them from their main degradation product, adenine (A), and such a method had not previously been reported.

## 2. Experimental

### 2.1. Samples and reagents

The synthesis of compounds **1–4** has been described elsewhere [1]. Adenine, sodium octanesulphonate, methanol and acetonitrile were

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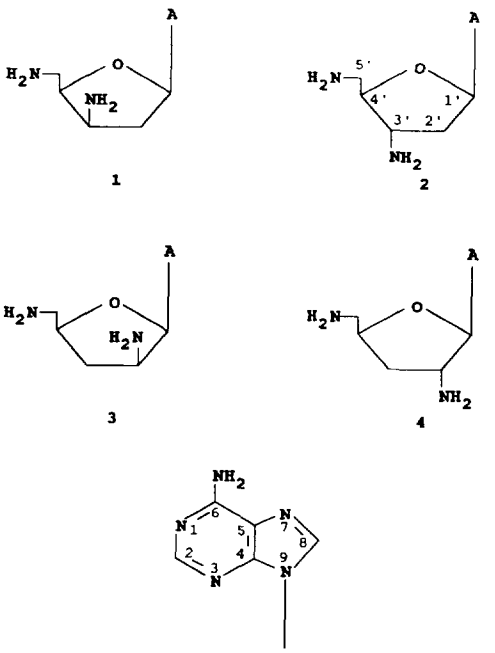



Fig. 1. Structures of diamino analogues of 2′- or 3′-deoxy adenosine.

purchased from Janssen Chimica (Beerse, Belgium). Methanol and acetonitrile were redistilled before use. HPLC-grade tetrahydrofuran was obtained from Rathburn (Walkerburn, UK). Water was distilled twice in glass apparatus. All other reagents were of analytical-reagent grade (Janssen Chimica).

### 2.2. Liquid chromatography

The liquid chromatographic apparatus consisted of an SP 8700 XR solvent-delivery system (Spectra-Physics, San Jose, CA, USA), used at a flow-rate of 1 ml/min, a Model CV-6UH-Pa-N-60 injector (Valco, Houston, TX, USA) equipped with a 20-$\mu$l loop, a Waters (Milford, MA, USA) Model 440 detector set at 254 nm and a Hewlett-Packard (Avondale, PA, USA) Model 3396 integrator. The stationary phases examined were RSil $C_{18}$ LL (5 $\mu$m) (Alltech, Deerfield, IL, USA), Spherisorb ODS-1 (10 $\mu$m) (Phase Separations, Queensferry, UK), Chromspher $C_8$ (5 $\mu$m) (Chrompack, Middelburg, Netherlands), PRP-1 (7–9 $\mu$m) (Hamilton, Reno, NV, USA), Rogel 80 Å (8 $\mu$m) (Bio-Rad Labs., Richmond, CA, USA), PLRP-S 100 Å (8 $\mu$m), PLRP-S 300 Å (8 $\mu$m) and PLRP-S 1000 Å (8 $\mu$m) (Polymer Laboratories, Church Stretton, Shropshire, UK), all in 250 mm × 4.6 mm I.D. columns, equilibrated by means of a water-bath. The mobile phase consisted of tetrahydrofuran–0.2 *M* sodium octanesulphonate–0.2 *M* potassium phosphate buffer (pH 2.0)–water (15:25:30:30, v/v). Samples of **1** used for kinetic studies were prepared by diluting 0.1 ml of a $5 \cdot 10^{-3}$ *M* stock standard solution with 4.9 ml of 0.1 *M* glycine hydrochloride buffer (pH 1.15) and ionic strength 0.4 (adjusted with KCl before measuring the final pH). Aliquots (0.5 ml) of this solution were put in vials, capped and degraded in a Memmert (Schwabach, Germany) oven. The vials were removed and quenched with 0.5 ml of 0.1 *M* KOH to give a final concentration of $5 \cdot 10^{-5}$ *M* (the amount injected was 0.25 $\mu$g).

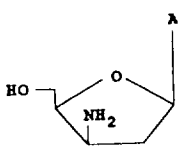
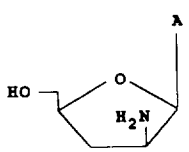
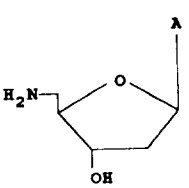
## 3. Results and discussion

Compound **1** was used to develop the method. Monoamino analogues of 2′- or 3′-deoxyadenosine have been well separated from adenine on a silica-based reversed-phase column with the use of a silanol-masking agent [2]. Similar studies were carried out for diamino analogues of 2′- or 3′-deoxyadenosine, whereby different types of silica-based reversed-phase columns, namely RSil $C_{18}$ LL (5 $\mu$m), Spherisorb ODS-1 (10 $\mu$m), and Chromspher $C_8$ (5 $\mu$m), were tested using either a silanol-masking agent (tetramethylammonium phosphate) or an ion-pairing agent (sodium octanesulphonate). There was good resolution, but considerable peak tailing with these columns owing to the high interaction between the basic amino groups and the acidic silanol groups and so a poly(styrene–divinylbenzene) polymer [PLRP-S 1000 Å (8 $\mu$m)] column was chosen for further method development. By using an ion-pairing agent, there was good resolution between **1** and

adenine. Therefore, the emphasis in optimizing the mobile phase was on improving the peak symmetry, particularly that of the nucleoside, because kinetic calculations are based on the peak areas of **1–4**. The mobile phase was developed by systematic investigation of the influence of the pH, the type and concentration of the organic modifier and the concentration of the ion-pairing agent and the buffer.

The $pK_a$ of adenine (N1) is 4.18 [3] but the $pK_a$ values of **1–4** have not previously been reported. It is expected that the $pK_a$ values of N1 of **1–4** would be in a similar range to those of monoamino analogues of 2′- or 3′-deoxyadenosine, some of which have been previously determined by NMR and are given in Table 1 (results from Ref. [4]). Fig. 2 shows the influence of the mobile phase pH on the chromatographic parameters of the separation of **1** and A. All

Table 1
$pK_a$ values of some monoamino analogues of 2′- or 3′-deoxyadenosine (from Ref. [4])

| Compound | $pK_a$ (N1) | $pK_a$ ($-NH_2$) |
|---|---|---|
|  | 3.1 | 9.0 |
|  | 3.3 | 6.2 |
|  | 4.1 | 9.0 |

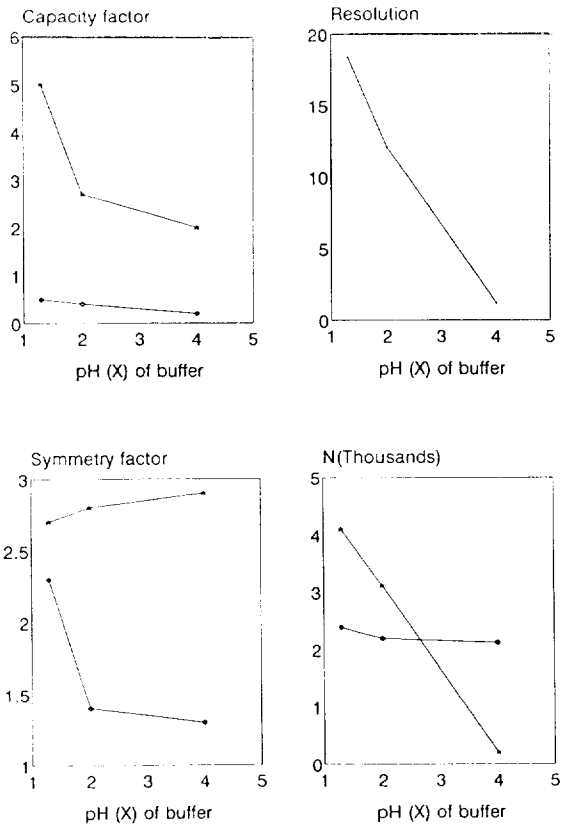



Fig. 2. Influence of mobile phase pH on the chromatographic parameters of separation of (●) adenine and (★) **1**. Mobile phase, acetonitrile–0.02 *M* sodium octanesulphonate–0.2 *M* potassium phosphate buffer–water (10:15:5:70, v/v). Column temperature, 70°C. $N$ = plate number.

chromatographic parameters were calculated using equations from the European Pharmacopoeia [5]. At the low pH values used in this study, the sugar amino groups are fully protonated. There is an increase in the retention of **1** and A with decrease in pH owing to increased protonation of the heterocyclic ring and hence more ion pairing. The peak symmetry ($A_s$) of **1** at pH 2.0 is almost the same as that at pH 1.3, yet $A_s$ of adenine is greatly improved at pH 2.0. Therefore, pH 2.0 was chosen for further work.

The column was heated to reduce the backpressure. When temperature was increased, there was an overall decrease in retention but little change in selectivity (results not shown). A temperature of 60°C was found to be the best

because it gave the best peak symmetry of **1** and it was used in all subsequent experiments.

The influence of organic modifiers and their concentrations on the chromatographic parameters of **1** and A was investigated. Methanol gave very poor peak shapes. Tetrahydrofuran produced a large improvement in peak symmetry compared with acetonitrile. Tetrahydrofuran (7%) was used for further work because it gave the best peak symmetry of adenine and **1**.

The effects of different concentrations of 0.02 *M* sodium octanesulphonate and 0.2 *M* potassium phosphate buffer (pH 2.0) on the chromatographic parameters of adenine and **1** are shown in Figs. 3 and 4, respectively. As expected, $k'$ increases with increase in concentration of the ion-pairing agent owing to the reduced polarity of A and **1** after the formation of an ion pair. Increasing the buffer concentration increases the dissociation of the ion pairs, thereby causing a decrease in retention [6]. The effect of changing the ion-pairing agent and the buffer concentration on $k'$ is much greater for **1** than for A. This is due to, amongst other factors, the fact that **1** has more protonation sites (and hence more potential pairing sites) than adenine [6]. The best results were obtained by use of 10% of 0.02 *M* sodium octanesulphonate and 5% of 0.2 *M* potassium phosphate buffer (pH 2.0).

Hence samples of **1–4** dissolved in water could

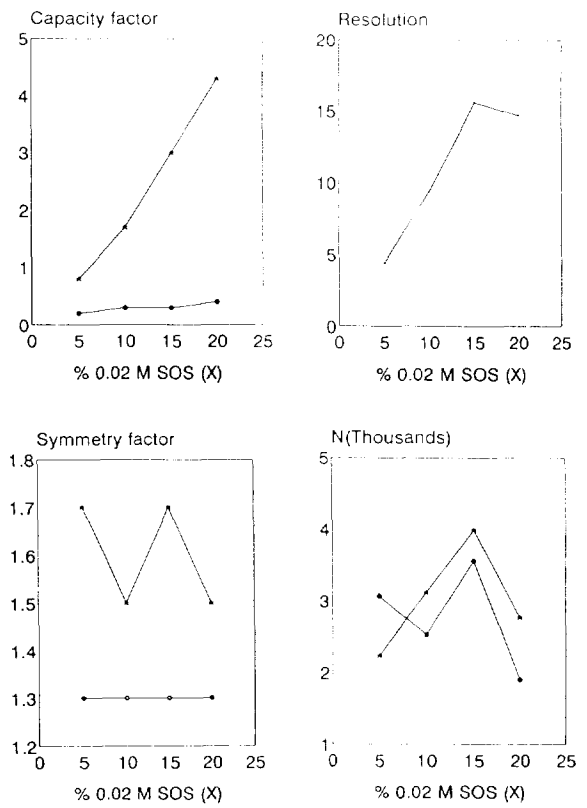



Fig. 3. Influence of proportion of 0.02 *M* sodium octanesulphonate on the chromatographic parameters of the separation of (●) adenine and (★) **1**. Mobile phase, tetrahydrofuran–0.02 *M* sodium octanesulphonate–0.2 *M* potassium phosphate buffer (pH 2.0)–water (7:*X*:5:88 − *X*, v/v); column temperature, 60°C. *N* = plate number; SOS = sodium octanesulphonate.

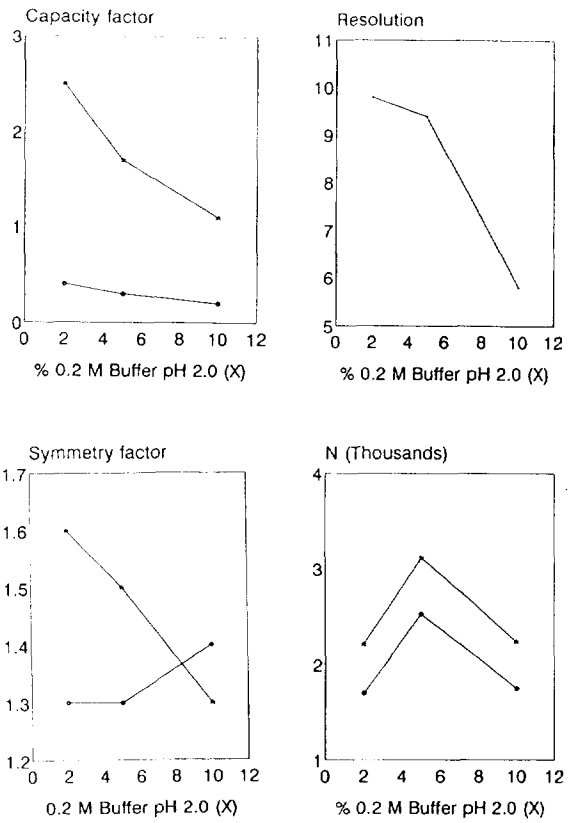



Fig. 4. Influence of proportion of 0.2 *M* potassium phosphate buffer on the chromatographic parameters of the separation of (●) adenine and (★) **1**. Mobile phase, tetrahydrofuran–0.02 *M* sodium octanesulphonate–0.2 *M* potassium phosphate buffer (pH 2.0)–water (7:10:*X*:83 − *X*, v/v); column temperature, 60°C. *N* = plate number.

be analysed on a PLRP-S 1000 Å (8 μm) column equilibrated at 60°C and a mobile phase consisting of tetrahydrofuran–0.02 *M* sodium octanesulphonate–0.2 *M* potassium phosphate buffer (pH 2.0)–water (7:10:5:78, v/v).

When a sample of **1** used for kinetic studies was analysed with the mobile phase developed above, there was a shoulder on the peaks (Fig. 5a). This is contrary to studies done on monoamino analogues of 2′- or 3′-deoxyadenosine, whereby a sample dissolved in water and one for kinetic studies could both be analysed on a Spherisorb ODS-1 column using a mobile phase containing acetonitrile–0.2 *M* tetramethylam-

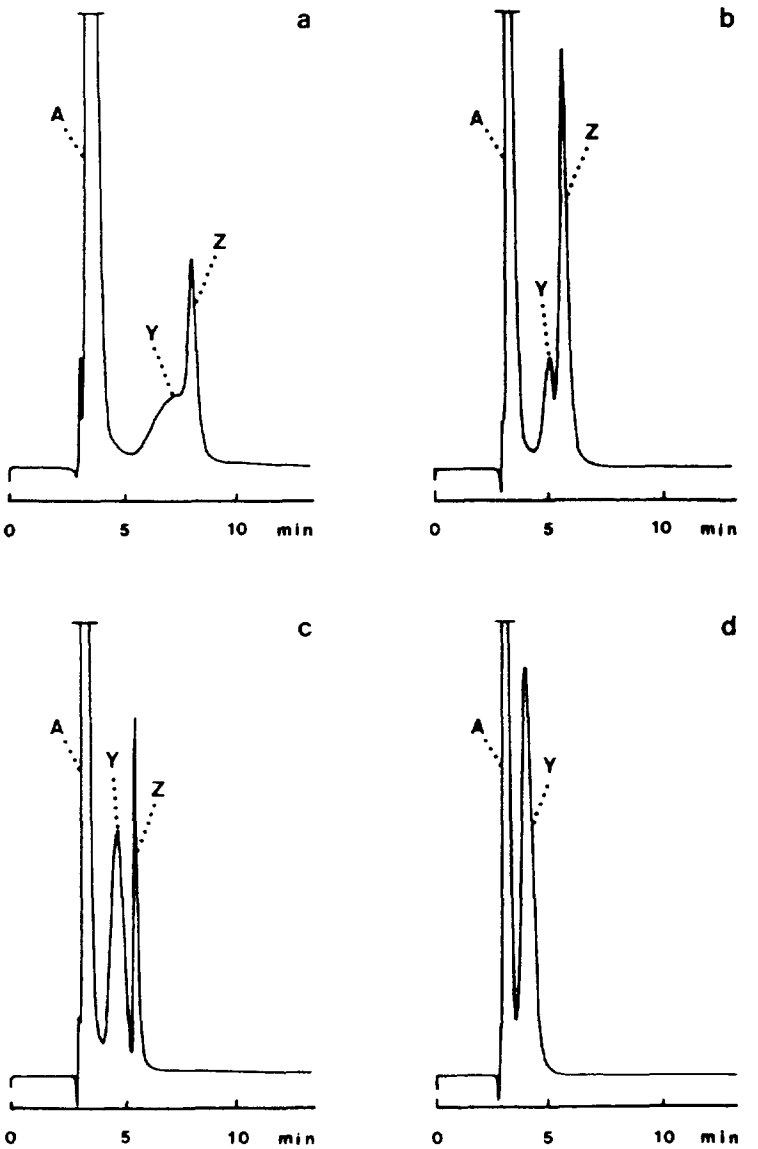



Fig. 5. Liquid chromatograms showing effect of buffer. Sample, **1** degraded at pH 1.15 and 100°C for 1.5 h; mobile phase, tetrahydrofuran–0.02 *M* sodium octanesulphonate–0.2 *M* potassium phosphate buffer (pH 2.0)–water (7:10:*X*:83 − *X*, v/v). *X* = (a) 5; (b) 15; (c) 20; and (d) 30. A = adenine.

monium phosphate (pH 6.0)–0.2 *M* potassium phosphate buffer (pH 6.0)–water (5:0.5:5:89.5, v/v) [2]. The appearance of shoulders on both the peaks corresponding to adenine and **1** suggested that the shoulders were due to chromatographic effects rather than the presence of other degradation products. Indeed, an undegraded sample of **1** also exhibited a similar shoulder on analysis with the mobile phase described above. Compounds **1**–**4** behaved similarly. It was suspected that the peak shoulders were due to insufficient buffering of the sample by the mobile phase. As the mobile phase buffer was increased to solve this problem, two distinct peaks (Y and Z) that corresponded to **1** were formed. As the buffer concentration increased, the size of peak Y increased while that of peak Z decreased until finally only one peak (Y) was left (Fig. 5b–d). In ion-pair systems, $k'$ is determined mainly by reversed-phase and ion-pair processes. Increasing buffer concentration increases the total cation concentration, resulting in reduced ion pairing [6], and this coincides with decrease in the retention and size of peak Z. In addition, this causes a second process (most likely a reversed-phase mechanism), responsible for peak Y, to become more predominant. This phenomenon disappeared when, in addition to increasing the buffer concentration, the amount of ion-pairing agent was increased until the stationary phase was saturated.

The influence of higher concentrations of the ion-pairing agent on the chromatographic parameters for the separation of **1** and adenine are shown in Fig. 6. There is a linear increase in $k'$ with increase in amount of the ion-pairing agent up to about 20% of 0.2 *M* sodium octanesulphonate, after which the graph flattens owing to saturation of the stationary phase. Below 25% of 0.2 *M* sodium octanesulphonate there are peak shoulders and split peaks. Different concentrations of the buffer were also investigated (data not shown). Use of 25% of 0.2 *M* sodium octanesulphonate and 30% of 0.2 *M* potassium phosphate buffer (pH 2.0) gave the best peak symmetry of adenine and **1**. The tetrahydrofuran concentration was then adjusted to give reasonable retention.

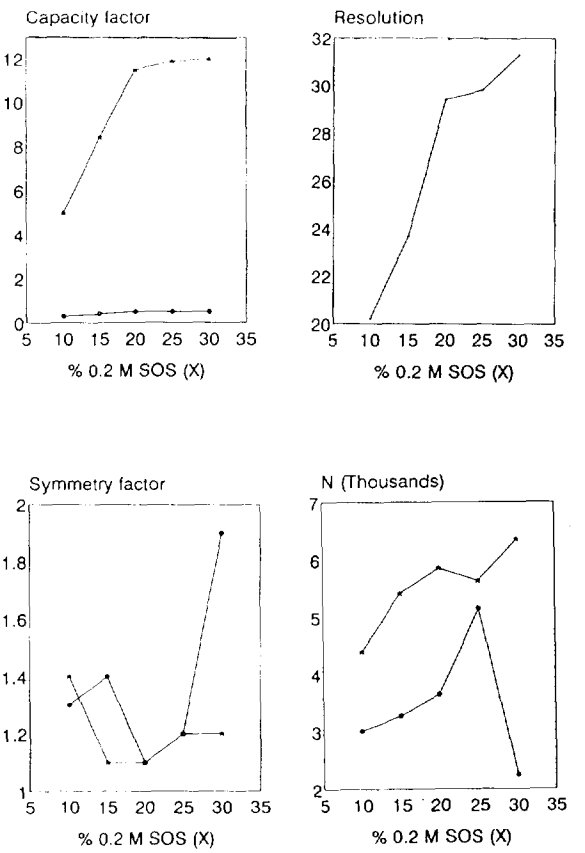



Fig. 6. Influence of proportion of 0.2 *M* sodium octanesulphonate on the chromatographic parameters of the separation of (●) adenine and (★) **1**. Mobile phase, tetrahydrofuran–0.2 *M* sodium octanesulphonate–0.2 *M* potassium phosphate buffer (pH 2.0)–water (10:*X*:30:60 – *X*, v/v); column temperature, 60°C. N = plate number; SOS = sodium octanesulphonate.

The best mobile phase for the analysis of samples used for kinetic studies consists of tetrahydrofuran–0.2 *M* sodium octanesulphonate–0.2 *M* potassium phosphate buffer (pH 2.0)–water (15:25:30:30, v/v).

The linearity of the detector response was tested in the range $5 \cdot 10^{-6}$ *M* (1.25 μg/ml) to $10^{-4}$ *M* (24.9 μg/ml) of **1** using four calibration points (twelve data points). The following relationship was obtained: $y = 14817x + 203$ ($y$ = peak area/1000 and $x$ = concentration injected in μg/ml); $r = 0.9988$; standard error of $y$

estimate = 243; standard deviation of slope = 252. The method was found to be repeatable (R.S.D. = 1.0%; $n = 4$). The limit of detection was 0.05 ng at a signal-to-noise ratio of 3. The limit of quantification was 5 ng ($n = 4$; R.S.D. = 14%).

Results obtained with this mobile phase and other columns are given in Table 2. Compound **1** tailed too much on the PLRP-S 300 Å (8 μm) column. On the PLRP-S 100 Å (8 μm) and Rogel 80 Å (8 μm) columns there was front tailing and this could cause inaccurate integra-

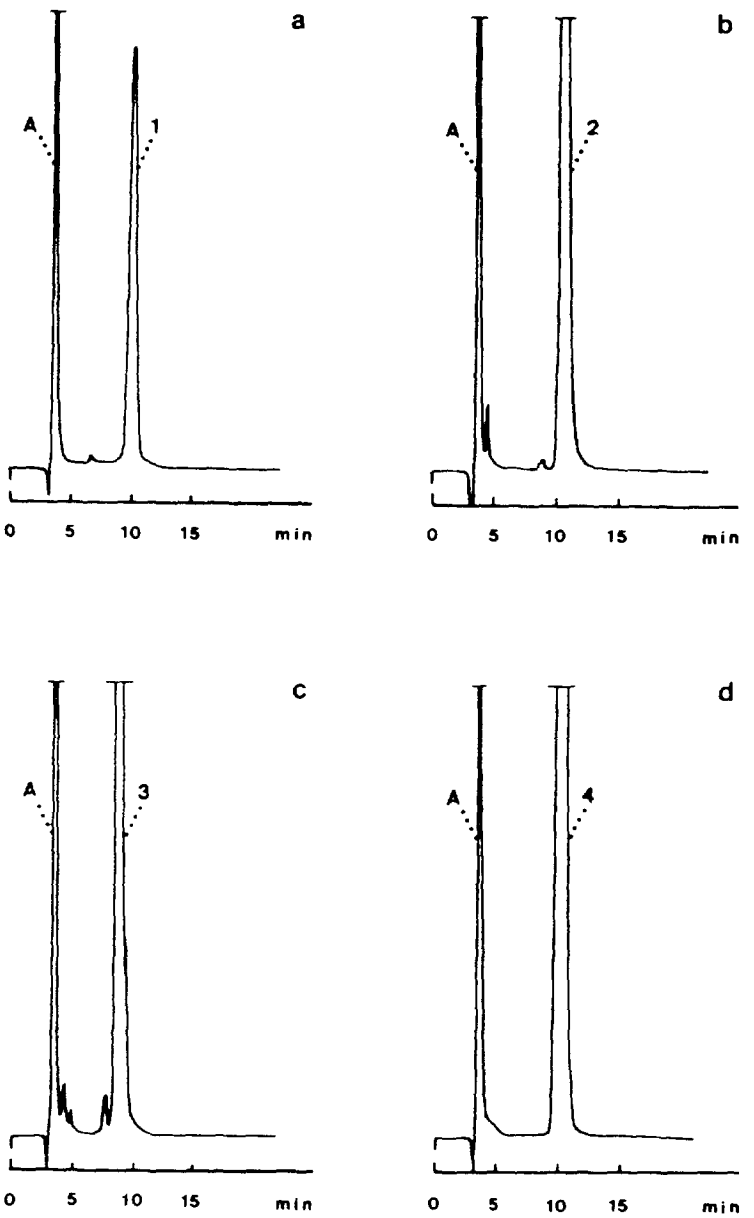



Fig. 7. Liquid chromatograms of **1–4** partially degraded at pH 1.15. Mobile phase: tetrahydrofuran–0.2 *M* sodium octanesulphonate–0.2 *M* potassium phosphate buffer (pH 2.0)–water (15:25:30:30, v/v).

Table 2
Chromatographic parameters of the separation of adenine and **1** on polymer columns from different manufacturers

| Column | Capacity factor | | Resolution, adenine/**1** | Peak Symmetry, **1** | Plate number | |
|---|---|---|---|---|---|---|
| | Adenine | **1** | | | Adenine | **1** |
| PLRP-S 1000 Å (8 μm) | 0.3 | 3.2 | 13.6 | 1.3 | 1490 | 4530 |
| PLRP-S 300 Å (8 μm) | 0.5 | 6.1 | 10.4 | 3.4 | 1940 | 900 |
| PLRP-S 100 Å (8 μm) | 0.7 | 8.4 | 16.8 | 0.8 | 1670 | 2550 |
| PRP-1 (7–9 μm) | 0.8 | 10.5 | 25.6 | 1.3 | 2240 | 5720 |
| RoGel 80 Å (8 μm) | 1.1 | 13.6 | 23.3 | 0.8 | 1990 | 4300 |

Mobile phase, tetrahydrofuran–0.2 *M* sodium octanesulphonate–0.2 *M* potassium phosphate buffer (pH 2.0)–water (15:25:30:30, v/v); flow-rate, 1 ml/min; column temperature, 60°C; detection at 254 nm.

Table 3
Chromatographic parameters for the separation of adenine and **1–4**

| Compound | Capacity factor | | Resolution, adenine/N | Peak Symmetry, N | Plate number | |
|---|---|---|---|---|---|---|
| | Adenine | N | | | Adenine | N |
| **1** | 0.3 | 3.2 | 13.6 | 1.3 | 1490 | 4530 |
| **2** | 0.3 | 3.4 | 15.0 | 1.5 | 1140 | 4290 |
| **3** | 0.3 | 2.6 | 12.3 | 1.5 | 960 | 4280 |
| **4** | 0.3 | 3.0 | 13.6 | 1.4 | 960 | 4550 |

Mobile phase, tetrahydrofuran–0.2 *M* sodium octanesulphonate–0.2 *M* potassium phosphate buffer (pH 2.0)–water (15:25:30:30, v/v); flow-rate, 1 ml/min; column, PLRP-S 1000 Å (8 μm); column temperature, 60°C, detection, 254 nm. N = nucleoside.

tion of the peaks. The PRP-1 (7–9 μm) column gave good symmetry of **1** and adenine, but high retention, and therefore the PLRP-S 1000 Å (8 μm) column was chosen for the analysis of **1–4**. Table 3 gives the results obtained and Fig. 7 shows typical chromatograms.

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