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Separation of nucleosides using capillary electrochromatography

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Abstract

The analysis of nucleosides and nucleotides have in most cases been performed by HPLC using either reversed-phase HPLC with gradient elution or using reversed-phase ion-pair chromatography. In this paper we have explored the possibility of using capillary electrochromatography (CEC) in order to avoid the use of gradients or ion-pairing reagents. CEC is in many ways comparable to HPLC, but CEC is theoretically able to provide better separations due to the higher efficiency caused by the flowfront being more plug-like as also is the case in CE, which is to be compared to the more parabolic flow observed in HPLC. The separation of six nucleosides (adenosine, cytidine, guanosine, inosine, thymidine and uridine) was investigated with respect to concentration of buffers, pH, amount of acetonitrile, temperature and voltage in order to optimise the separation. Baseline separation was achieved for the six nucleosides in less than 13 min using a background electrolyte consisting of (5 m*M* acetic acid, 3 m*M* triethylamine, pH 5.0)–acetonitrile (92:8, v/v). © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Electrochromatography; Mobile phase composition; Nucleosides

1. Introduction

Analysis of nucleosides, nucleotides and modified nucleosides can be very useful in the diagnosis of several serious diseases and metabolic disorders. Profiling of nucleosides in urine, serum or plasma shows noticeable differences between healthy subjects and individuals with various types of cancer [1]. The level of nucleosides and modified nucleosides have thus been proposed as cancer markers [2] and have also been proposed as diagnostic markers of human immunodeficiency virus (HIV) [3]. Also the level of nucleosides and nucleotides can be used to establish myocardial cellular energy status, and they are useful in the study of energy metabolism in cardiac tissue [4,5].

Several methods have been developed for the separation of nucleosides and nucleotides. The vast majority of these separations have been performed by using either reversed-phase high-performance liquid chromatography (HPLC) with gradient elution [2–4,6] or using reversed-phase ion-pair chromatography [1,5]. An HPLC method using immobilised enzyme reactors has also been developed [7] and for some modified nucleosides it has been attempted to develop an immunoassay for quantification [8].

Capillary electrochromatography (CEC) combines the two well known analytical techniques HPLC and capillary electrophoresis (CE) and is performed by applying high-voltage to a packed column (typically

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25–50 cm long and 50–150 μm I.D.). The combination of the two techniques provide an analytical technique with advantages from both HPLC and CE. CEC will thus have the ability to provide high selectivity (due to the variety of stationary and mobile phases), the ability to handle highly polar and neutral compounds, and the well characterised retention mechanisms of HPLC [9–12]. The technique has the potential of providing 5–10-times higher efficiencies [13] than in HPLC primarily due to the plug-like flow profile created by the electroosmotic flow (EOF) compared to the parabolic flow of HPLC [14–16]. Furthermore, CEC has the advantage that the flow is created in the column, which enables the use of packing materials with a diameter of 1.5 μm or even smaller without significant increase in backpressure [14]. The combination of HPLC and CE gives the analyst a larger number of parameters to adjust in order to obtain and optimise a separation than either of the two techniques provide on its own. However, one problem is that some of the variables overlap (e.g., if the buffer concentration or the amount of organic modifier is changed the flow will also change). In this paper we explore the possibility of using CEC for the separation of nucleosides in order to avoid the use of gradient elution or ion-pairing reagents.

2. Experimental

2.1. Apparatus

A Hewlett-Packard ^{3D}CE capillary electrophoresis system (Waldbronn, Germany) fitted with a 100 μm I.D. column with a packed bed length of 25 cm (CEC-Hypersil C_{18}, 3 μm obtained from Hewlett-Packard), was used for all the experimental work. The total column length was the length of the packed bed plus 8.5 cm of polyimide coated fused silica tubing. The column was conditioned with every new background electrolyte (BGE) for at least 2 h before any samples were injected. The conditioning was done by applying 20 kV over the capillary. Both inlet and outlet were pressurised at 10 bar during conditioning and analysis. No pair of BGE vials (inlet and outlet) were used for more than a total of 2 h run-time. Electrokinetic injection was used (10 kV for 3 seconds) and the detection was performed at 254 nm.

2.2. Reagents

Potassium hydroxide was purchased from Merck (Darmstadt, Germany), phosphoric acid was obtained from Riedel-de Haën (Seelze, Germany), triethylamine (TEA) was from Aldrich (Steinheim, Germany), and acetic acid was purchased from Baker (Deventer, Netherlands). All nucleosides were purchased from Sigma (St. Louis, MO, USA). Deionised water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA).

2.3. Procedures

The buffers containing acetic acid and TEA were adjusted to the desired pH with either 0.1 *M* potassium hydroxide or 0.05 *M* phosphoric acid. After the pH was adjusted, the buffer and the acetonitrile were mixed. The BGE was degassed by ultrasonication under vacuum for approximately 5 min before it was transferred to the inlet/outlet vials. The nucleosides were first dissolved in a stock solution containing 5–10 mg/ml in water (guanosine needed to be heated to 70°C to be dissolved) and then diluted 1:10 in the BGE (for the mixture of more than one nucleoside the dilution was still 1:10). Thiourea (Fluka, Neu-Ulm, Germany) was used in some electrochromatograms as a marker of the EOF.

The formation of air bubbles has previously been reported as a major problem in CEC work, particularly in the frits, but this problem can be almost completely solved by pressurising both inlet and outlet vials. Thus we never experienced air bubbles during the experimental work described.

3. Results and discussion

3.1. CEC of nucleosides

All CEC experiments were performed using a mixture of the six nucleosides; adenosine, cytidine, guanosine, inosine, thymidine and uridine (Fig. 1), and in most experiments with thiourea added as the EOF marker. A BGE (5 m*M* acetic acid, 2 m*M* TEA

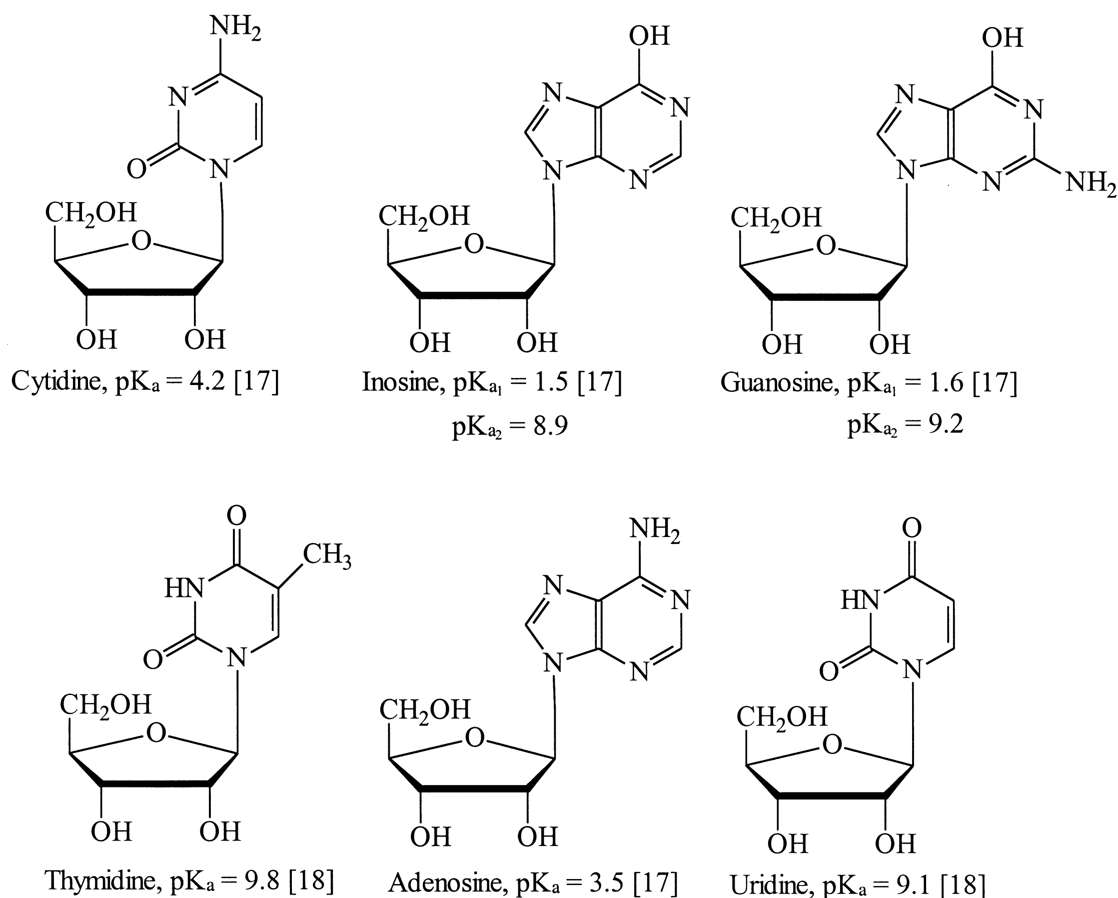

Fig. 1. Structure and pK_a values of the six nucleosides.

pH 5.0)–acetonitrile (90:10, v/v) that gave a reasonable separation was found from preliminary experiments. The optimisation of the method was performed by investigating one parameter at a time and then choosing the best possible combination. One of the reasons for this procedure was that in CEC no matter which parameter was being changed the flow would also change. Thus, concomitant change of several parameters occur which makes it harder to compare the results obtained.

3.2. Effect of buffer concentrations

To optimise the method the concentrations of acetic acid as well as TEA were changed (Fig. 2). A reasonably good separation of all six nucleosides were obtained when using 5 m*M* or 10 m*M* acetic acid (Fig. 2A). However, the use of 5 m*M* was superior because of the shorter run-time due to the lower ionic strength and thereby higher zeta potential. Furthermore, at 10 m*M* some peaks showed low efficiency.

TEA is known from HPLC to decrease peak tailing, and since the initial experiments proved that the nucleosides exhibit peak tailing TEA was added in order to reduce these problems. Using 2 m*M* TEA it was not possible to separate cytidine and thiourea (Fig. 2B). When using 3 m*M* or 4 m*M* TEA fair resolution was obtained, but the run-time was increased when using 4 m*M* TEA. For the final optimised method 5 m*M* acetic acid and 3 m*M* TEA were chosen.

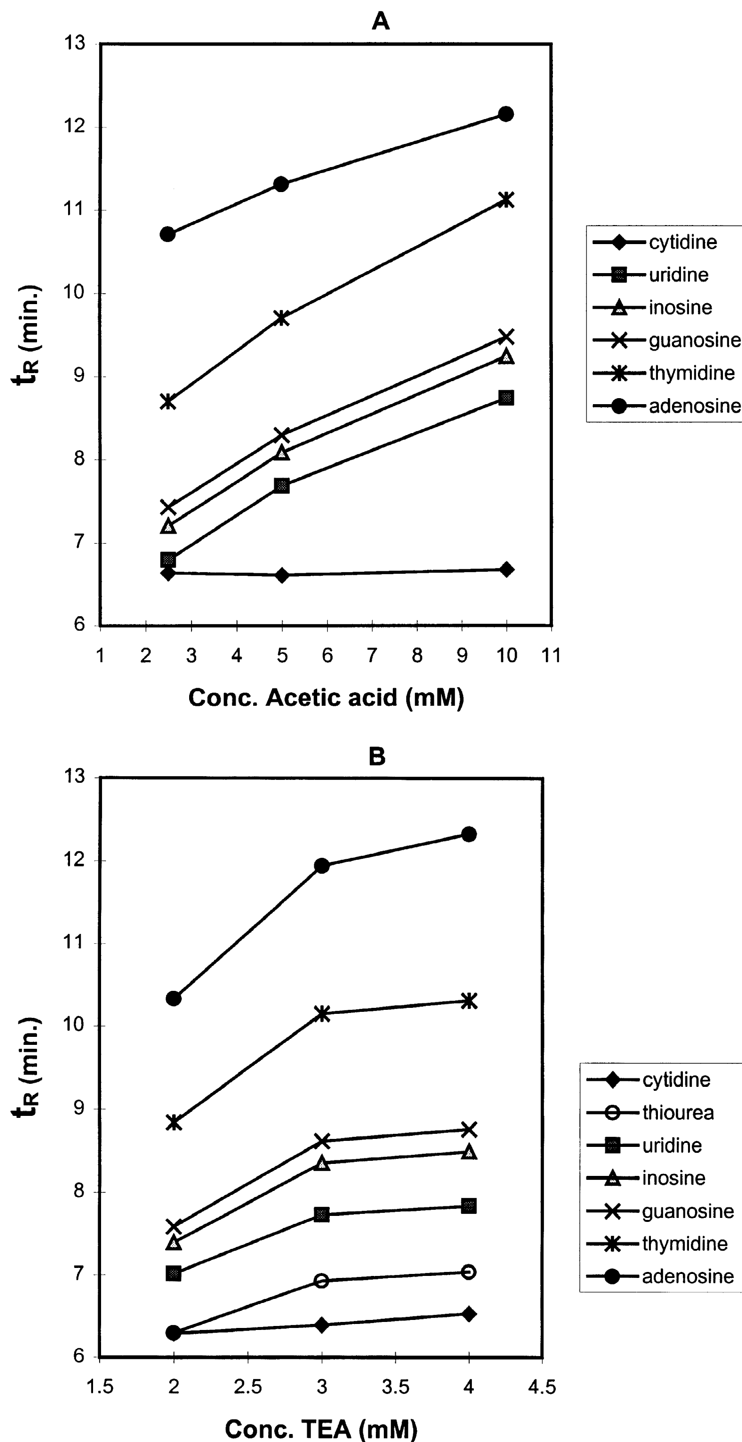

Fig. 2. (A) Effect of the acetic acid concentration in the BGE. Conditions: injection, 10 kV for 3 seconds; 25 cm×100 μm column (CEC-Hypersil C_{18}, 3 μm); mobile phase (acetic acid, 2 m*M* TEA, pH 5)–acetonitrile (90:10, v/v), 20 kV and 20°C. (B) Effect of TEA concentration in the BGE. Conditions: injection, 10 kV for 3 seconds; 25 cm×100 μm column (CEC-Hypersil C_{18}, 3 μm); mobile phase (TEA, 5 m*M* acetic acid, pH 5)–acetonitrile (90:10, v/v), 20 kV and 20°C.

3.3. Effect of pH

As expected the flow increased by increasing pH, an increase caused by the greater deprotonation of the silanol groups on the capillary wall and on the packing material. Four of the nucleosides (guanosine, inosine, thymidine and uridine) were not ionised at pH 5.0 and they generally remained in the same order of elution (Fig. 3). Their pK_a values [17,18] (Fig. 1) were not in the pH range being examined, and therefore they did not experience a change of charge in this pH range. Cytidine and adenosine have pK_a values of 4.2 and 3.5, respectively, and this caused cytidine and adenosine to change from being positively charge to being generally neutral when pH was changed from 4 to 6. Calculated under simplified conditions in dilute aqueous solution the mean charge of cytidine would be approximately; +0.61 at pH 4.0, +0.14 at pH 5.0, +0.015 at pH 6.0. For adenosine the mean charge would be approximately; +0.24 at pH 4.0, +0.03 at pH 5.0, +0.003 at pH 6.0. This explains the increased retention time (t_R) for cytidine when changing the pH from 4.0 to 5.0 in spite of the simultaneous increase in the EOF, as well as the limited change in retention time when changing from pH 5.0 to 6.0 (Fig. 3). It also explains the limited increase in t_R for adenosine from pH 4.0 to 6.0. The decrease in ionisation when increasing the pH results in a decrease in the electrophoretic migration and thus the reversed-phase mechanism would dominate the separation at higher pH. The best separation of the nucleosides was achieved at pH 6.0, but the run time was also increased by 15% compared to pH 5.0 due to the increased retention of adenosine. pH 5 was chosen as the optimal pH value since this provided the shortest analysis time, and still gave separation of all six nucleosides and separation from EOF, provided that 3 m*M* TEA was used in the final optimisation.

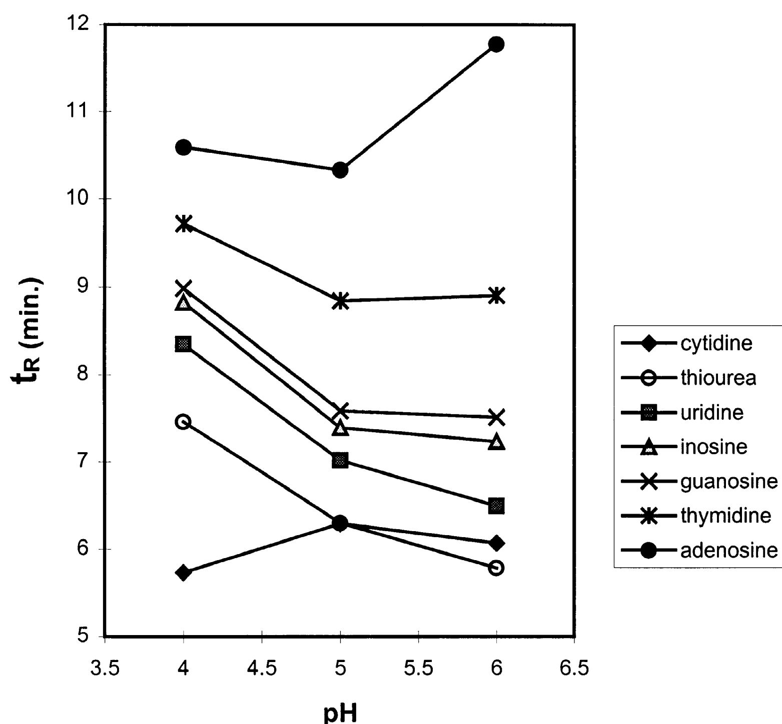

Fig. 3. Effect of the pH of the BGE. Conditions: injection, 10 kV for 3 seconds; 25 cm×100 μm column (CEC-Hypersil C_{18}, 3 μm); mobile phase (5 m*M* acetic acid, 2 m*M* TEA)–acetonitrile (90:10, v/v), 20 kV and 20°C.

3.4. Effect of temperature and voltage

When the temperature was increased the flow-rate was also increased as the viscosity was reduced at higher temperatures (Fig 4A). Increasing the temperature will also effect the distribution constants of the solutes, and this will normally result in decreasing retention and in some cases in a change in selectivity. An increase in the voltage also increased the flow-rate while the resolution was practically unchanged (Fig. 4B). The EOF was proportional to the voltage since the ions move faster in an increased electric field. With temperature and voltage raised beyond a certain level, the flow reached a point were the on-column time was insufficient for the separation to take place.

3.5. Effect of acetonitrile concentration

An increase in the acetonitrile concentration increased the flow-rate, partially because the ionic strength was decreased and partially because a greater amount of acetonitrile increases the EOF [19]. The increase in EOF observed at higher concentrations of acetonitrile is believed to be caused by changes in the viscosity and the zeta potential [20]. Increasing the amount of acetonitrile in the BGE also made the BGE electrolyte more apolar, and this changed the partitioning equilibrium, and thereby affected the selectivity and resolution and decreased the retention of the analytes. By increasing the acetonitrile concentration to 12% (v/v) no separation between inosine and guanosine was obtained (Fig. 5), whereas when the acetonitrile concentration was reduced to 8%, this separation was improved and baseline separation was achieved (Fig. 6). The peak shape particularly for uridine was also improved with the lower acetonitrile concentration. For the final method 8% of acetonitrile in the eluent was chosen.

3.6. Final optimisation

The final BGE consisted of (5 m*M* acetic acid, 3 m*M* TEA pH 5.0)–acetonitrile (92:8, v/v). For the final optimisation three different combinations of temperature and voltage were tested (Fig. 7), and the best separation was obtained at 25 kV and 20°C.

4. Preliminary validation

4.1. Linearity and limit of detection/quantitation

As shown in Table 1 all the nucleosides proved to exhibit good linearity in the range from their limit of quantitation (LOQ) (the lowest measured concentration within the linear range) and up to the highest used concentration (1 mg/ml). The limit of detection was defined as the signal-to-noise ratio of 3.

4.2. Repeatability

To achieve high repeatability in CEC it is very important to change the BGE more often than the run-buffer in CE since the ionic strength of the BGE usually is much lower in CEC than the ionic strength of the run-buffer in CE. This means that a pH gradient may occur much faster in CEC than in CE, due to the lower buffer-capacity, and the BGE will therefore have a relatively short lifetime. In these experiments 0.85 ml of BGE was used in each vial, and as mentioned above no pair of vials were used for more than 2 h of run-time. The short term repeatability (RSD$<$0.92%) as well as the long term repeatability (RSD$<$1.09%) of the retention time was good (Tables 2 and 3). However, the area repeatability was unsatisfactory, both the short term repeatability (RSD$<$13%) and the long term repeatability (RSD$<$23%). The area repeatability was considerably improved by calculating the relative area (using thymidine as an internal standard). The short term repeatability of the relative area was reduced to RSD$<$3.05%, and the long term repeatability corresponding to RSD$<$6.5%. This indicated that the injected volume was not repeatable and that the use of an internal standard was necessary, especially when quantitative analysis was performed.

The method was not fully validated since the analysis of nucleosides will be done in biological fluids. A full validation will not be performed until the method is applied to biological samples.

5. Conclusion

The work presented demonstrates that the six nucleosides can be baseline separated in a relatively

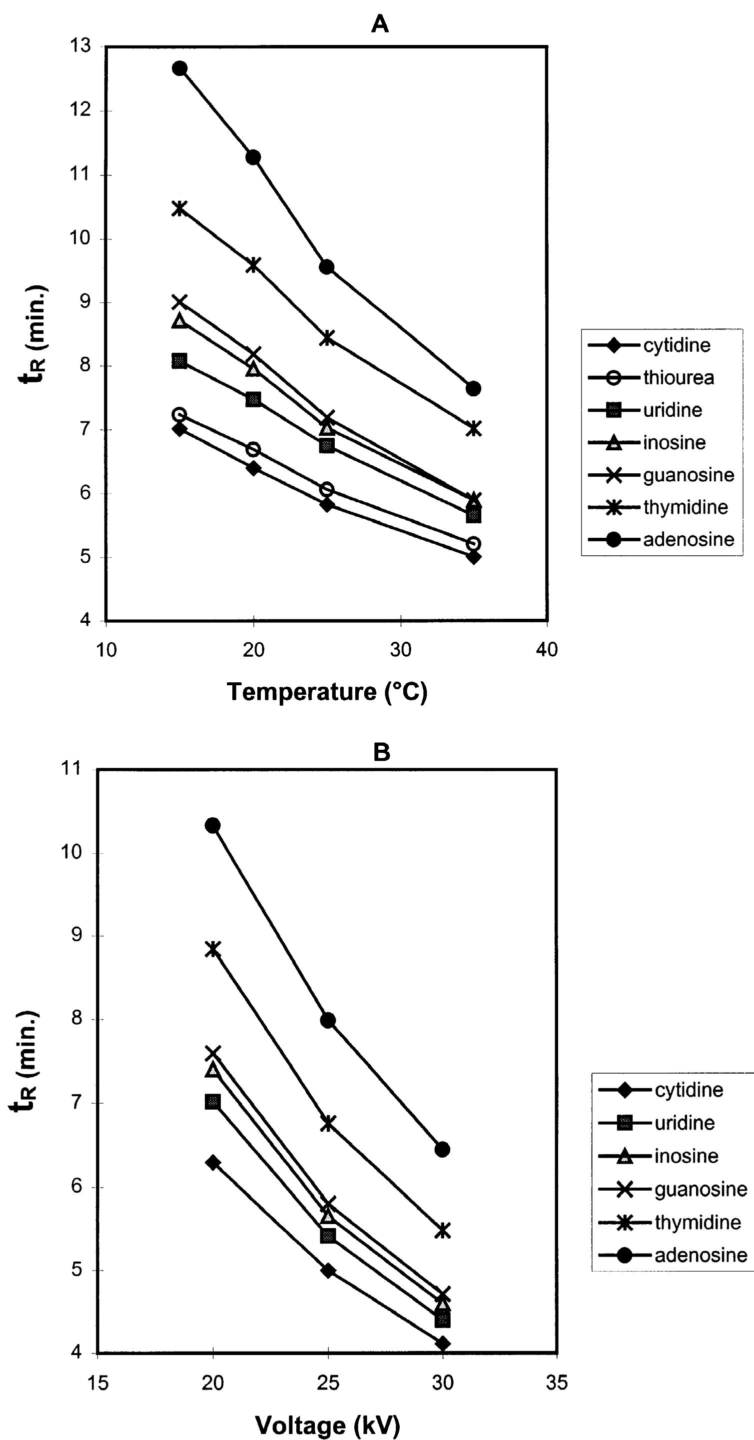

Fig. 4. Effect of (A) temperature and (B) voltage. Conditions: injection, 10 kV for 3 seconds; 25 cm×100 μm column (CEC-Hypersil C_{18}, 3 μm); mobile phase (5 m*M* acetic acid, 2 m*M* TEA, pH 5)–acetonitrile (90:10, v/v), 20 kV (A) or 20°C (B).

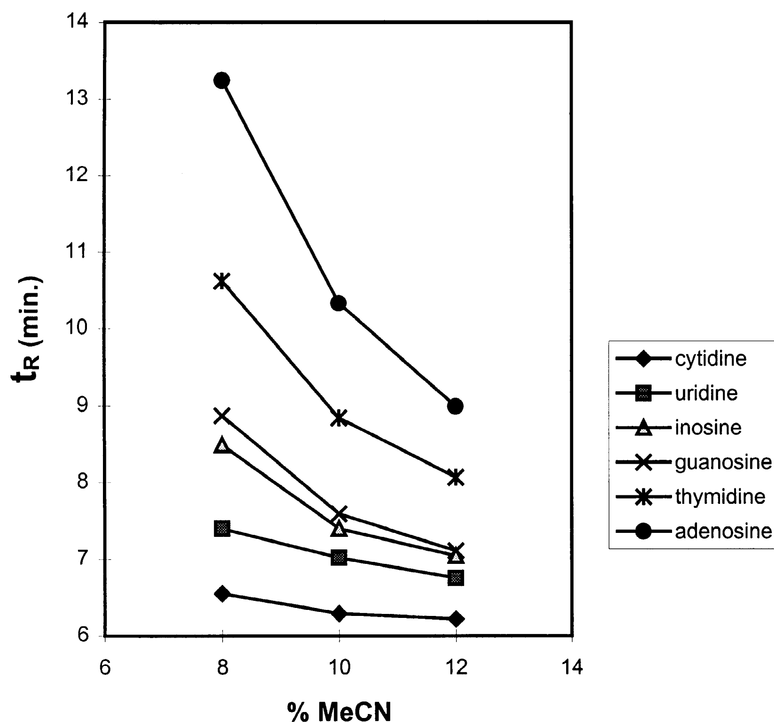

Fig. 5. Effect of the acetonitrile % in the BGE. Conditions: injection, 10 kV for 3 seconds; 25 cm×100 μm column (CEC-Hypersil C_{18}, 3 μm); mobile phase (5 m*M* acetic acid, 2 m*M* TEA, pH 5)–acetonitrile, 20 kV and 20°C.

short time (<13 min) by using CEC. The conventional HPLC methods require about twice that time to separate the nucleosides. The separation was performed without the use of a gradient or ion-pairing reagents. The method developed shows good retention time repeatability. However, the area repeatability is insufficient without the use of an internal standard. By using thymidine as internal standard the RSD of the short and long term area repeatability was improved to an acceptable level.

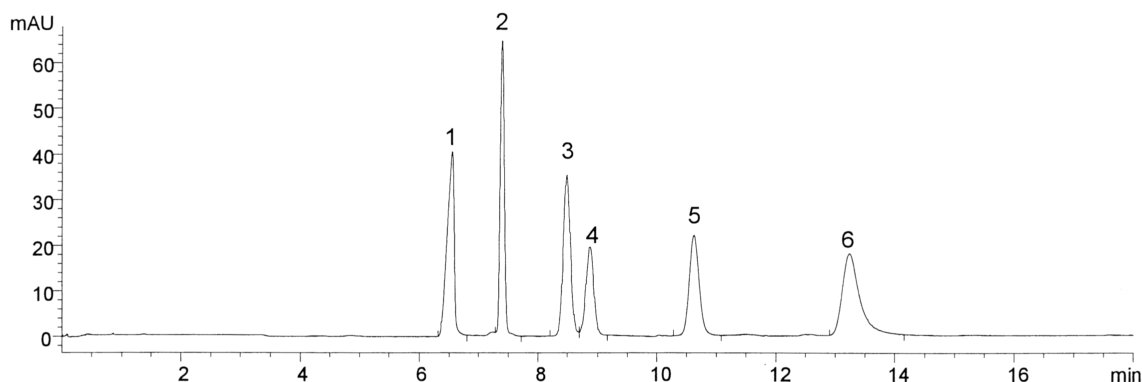

Fig. 6. Baseline separation of six nucleosides. Peaks: 1=cytidine, 2=uridine, 3=inosine, 4=guanosine, 5=thymidine, 6=adenosine. Conditions: injection, 10 kV for 3 seconds; 25 cm×100 μm column (CEC-Hypersil C_{18}, 3 μm); mobile phase (5 m*M* acetic acid, 2 m*M* TEA, pH 5)–acetonitrile (92:8, v/v), 20 kV and 20°C.

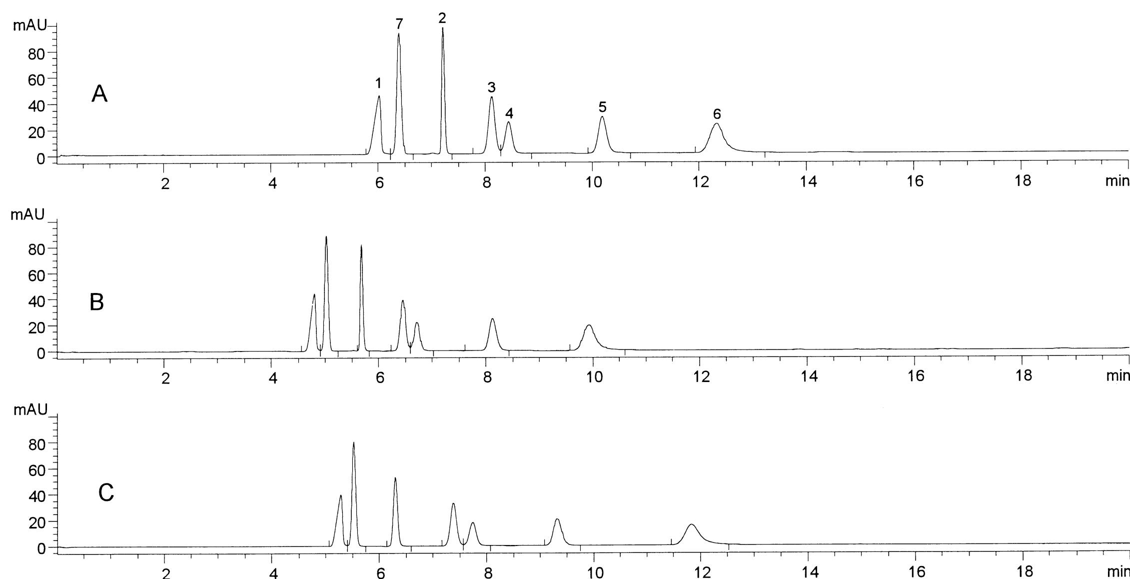

Fig. 7. Final optimisation of temperature and voltage. Order of peaks: 1=cytidine, 7=thiourea, 2=uridine, 3=inosine, 4=guanosine, 5=thymidine, 6=adenosine. Conditions: injection, 10 kV for 3 seconds; 25 cm×100 μm column (CEC-Hypersil C_{18}, 3 μm); mobile phase (5 m*M* acetic acid, 3 m*M* TEA, pH 5)–acetonitrile (92:8, v/v). (A) 25°C and 20 kV; (B) 25°C and 25 kV; (C) 20°C and 25 kV.

Table 1
Linearity, LOD and LOQ for the six nucleosides (experimental conditions as in Section 3.6)

	LOD (μg/ml)	LOQ (μg/ml)	r
Cytidine	1	5	0.9998
Uridine	1	5	0.9999
Inosine	1	5	0.9998
Guanosine	1	5	0.9998
Thymidine	1	5	0.9996
Adenosine	1	5	0.9999

Table 2
Short term repeatability ($n=10$) of the retention time (t_R), relative retention time (Rt_R), area and relative area (RArea) of the nucleosides

	RSD (%)			
	t_R	Rt_R	Area	RArea
Cytidine	0.09	0.86	9.70	3.05
Uridine	0.59	0.34	11.46	2.39
Inosine	0.82	0.11	11.53	1.22
Guanosine	0.84	0.08	11.41	1.69
Thymidine	0.92	I.S.	12.72	I.S.
Adenosine	0.29	0.66	10.53	2.42

Table 3
Long term repeatability (day 1, $n=10$; day 4, $n=10$;day 7, $n=10$) of the retention time (t_R), relative retention time (Rt_R), area and relative area (RArea) of the nucleosides

	RSD (%)			
	t_R	Rt_R	Area	RArea
Cytidine	0.28	1.08	20.63	2.03
Uridine	0.69	0.42	21.46	1.50
Inosine	0.94	0.16	21.25	4.99
Guanosine	0.96	0.15	22.42	2.24
Thymidine	1.09	I.S.	21.88	I.S.
Adenosine	0.38	0.77	20.69	6.50

This has also been the case in other CEC separations [9]. The area repeatability is improved to a level that makes CEC a suitable technique for biological analysis, however, if CEC is to be used for analysing pharmaceutical products and substances the repeatability still needs to be improved further.

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