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Simultaneous quantitation of nucleoside HIV-1 reverse transcriptase inhibitors by short-end injection capillary electrochromatography on a β-cyclodextrin-bonded silica stationary phase

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Abstract

As part of our on-going study of the analysis and quantitation of anti-HIV nucleosides, a capillary electrochromatography (CEC) method has been developed for the simultaneous quantitation of nucleoside HIV reverse transcriptase inhibitors (NRTIs), i.e. zidovudine (AZT), lamivudine (3TC), didanosine (ddA) and its administrated form (ddI), stavudine (d4T) and hivid (ddC). CEC on chiral stationary phase has mainly been dedicated to the separation of enantiomers. However, this paper explores an original application of a β-cyclodextrin-bonded silica packed column, taking advantage of the internal hydrophobicity of the polysaccharide to separate the NRTIs. The influence of several parameters (pH buffer, ionic strength, acetonitrile content, temperature and voltage) has been investigated using the short-end injection technique to achieve baseline separation in a short-time analysis before quantitation.

Keywords: Stationary phases, electrochromatography; Short-end injection; Nucleoside HIV-1 reverse transcriptase; Enzymes

1. Introduction

To date, the dideoxynucleosides AZT (3′-azido-3′-deoxythymidine), ddI (2′,3′-dideoxyinosine) or one of its metabolites ddA (2′,3′-dideoxyadenosine), ddC (2′,3′-dideoxycytidine), d4T (2′,3′-didehydro-3′-deoxythymidine) and 3TC (β-L-(−)-2′-deoxy-3′-thiacytidine) have been approved in HIV therapy [1]. The common metabolic pathway for the anabolism of these compounds brings them to the active form, e.g. 5′-triphosphorylated nucleosides, which act as HIV reverse transcriptase inhibitors. However, this process is nucleoside-dependent as each NRTI possesses its own pharmacokinetic properties, such as extracellular drug concentration and kinase activity. An accurate analysis of these anti-HIV nucleosides may therefore represent a useful therapeutic tool.

The reported methods for their quantitation in plasma or cells have mainly been performed by liquid chromatography with UV detection (HPLC–UV) [2,3], HPLC–RIA [4–6], or more recently by HPLC interfaced with tandem mass spectrometry (HPLC–MS/MS) [7–11] and capillary electrophoresis–tandem mass spectrometry (CE–MS/MS) [12,13].

Capillary electrochromatography (CEC), which

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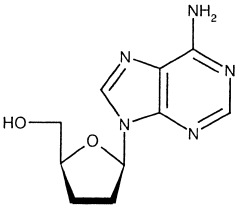
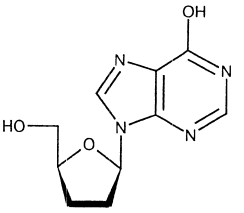
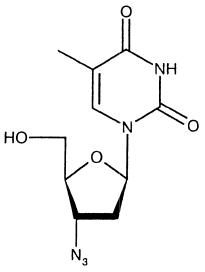


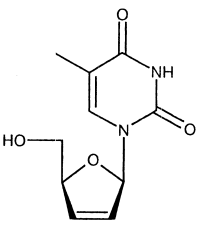
ddA, active form of ddI
pKa = 3.5



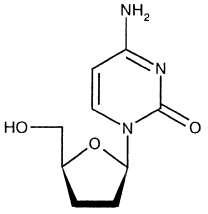
Didanosine, ddI
$pKa_1 = 1.5$; $pKa_2 = 8.9$



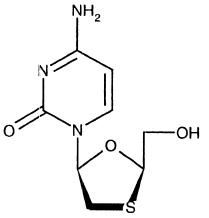
Zidovudine, AZT
pKa = 9.8



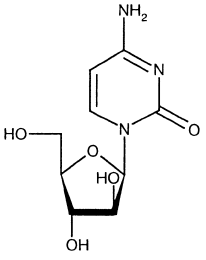
Stavudine, d4T
pKa = 9.8



Hivid, ddC
pKa = 4.4



Lamivudine, 3TC
pKa = 4.4



ArabinoCytidine, Ara-C (I.S.)
pKa = 4.4

Fig. 1. Structures of the anti-HIV nucleosides and the selected internal standard.

has been used to resolve nucleoside mixtures [14–17], has never been applied to the simultaneous analysis of NRTIs. CEC combines the advantages of both HPLC and CE techniques and is performed by applying a high voltage over a packed column, thus allowing the simultaneous separation of neutral and ionised species. According to the pKa of the heterocyclic moieties, the pool of the studied nucleosides constitutes a mixture of neutral and charged solutes, whatever the buffer pH.

Therefore, as part of our on-going antiviral research, this paper explores the possibility of using CEC, in combination with the short-end injection technique, to resolve a mixture of AZT, ddI, ddA, ddC, 3TC and d4T (Fig. 1), in a short-time analysis, using a β-cyclodextrin-bonded silica stationary phase. The influence of several experimental parameters (pH buffer, acetonitrile content, ionic strength, applied voltage, temperature) has been investigated, employing volatile reagents suitable for further CEC–MS/MS coupling.

2. Experimental

2.1. Capillary electrochromatography apparatus

CEC was performed on a Beckman-Coulter P/ACE MDQ (Fullerton, CA, USA) instrument. A 31.2 cm×75 μm I.D. fused-silica capillary was packed in our laboratory, using the slurry packing method [18,19] to obtain a 10 cm column length. β-Cyclose-2-OH® (5 μm) was a gift from Chiralsep (La Frenaye, France) and phenyl silica stationary phase (3 μm) was provided by Hypersil (Thermoquest, Les Ulis, France). The column was obtained by first pumping under 400 bars a slurry of phenyl silica particles which were then burned to produce the outlet frit, using a capillary burner (Electro-Kinetic Technologies, Broxburn, UK). The exceeding phase (on both sides of the frit) was then removed from the capillary. Afterwards, a slurry of β-Cyclose-2-OH® was pumped into the capillary until pressure dropped by itself; the filled capillary was then cut at the desired distance (10 cm) from the outlet frit; the inlet frit was obtained by tapping the capillary into silica particles which were sintered using the burner. After

packing, the chiral capillary column was flushed with water before conditioning with the mobile phase (acetic acid–ammonia buffer, pH 5/acetonitrile (80/20 v/v), 20 m*M* of total ionic strength). A small section of polyimide was removed next to the outlet frit to make a window for UV detection. Lastly, the packed capillary was installed in the P/ACE MDQ instrument and conditioned by applying successively increasing voltages (−5, −10 and −15 kV) during steps of 30 min. A 30 p.s.i. pressure was applied on both inlet and outlet vials during conditioning and analyses, to prevent the formation of air bubbles in the capillary.

All mobile phases were obtained by first preparing the background electrolyte (BGE) using Phœbus software (Sedere, Alfortville, France); the pH of the aqueous buffer was checked and adjusted on a Beckman pH meter (Model 10, Fullerton, CA, USA) before addition of the required amount of acetonitrile. Each solution was degassed by ultrasonication prior to use.

The analytes were electrokinetically injected (−10 kV, 1 s) using the short-end injection technique [17,20] and UV detection was performed at 254 nm.

2.2. Chemicals

Acetic acid and ammonia of analytical grade were purchased from Carlo Erba (Milan, Italy), triethylamine was obtained from Sigma (St Louis, MO, USA). Acetonitrile of HPLC quality was obtained from J.T. Baker (Deventer, Netherlands); HPLC quality water was prepared using Elgastat UHQ II system (Villeurbanne, France).

Zidovudine (AZT), stavudine (d4T) and didanosine (ddA) were synthesised as reported in the literature [21–23]. Lamivudine (3TC) was donated by Dr. C.K. Chu (UGA, Athens, GA, USA). Didanosine (ddI), 2′,3′-dideoxycytidine (ddC) and β-D-arabinocytidine (Cytosine-β-D-Arabinofuranoside, Ara-C) were supplied by Sigma. All nucleoside stock solutions (1 mg/ml) were prepared in water before aqueous dilution to the working concentration (10 μg/ml). The difference in acetonitrile content between the BGE and the sample induced a perturbation of the signal, which was considered as the EOF mobility marker [24].

3. Results and discussion

The fastest separations are obtained when the highest electric fields are applied on the shortest capillaries. To avoid long-time analysis due to ‘‘conventional injection’’, the effective length of the capillary can be minimised using the ‘‘short-end injection’’ technique. Thus, analyses were carried out on a 10 cm-packed CEC capillary, and the polarity of the applied voltage was switched (e.g. −10 kV for the injection step). A β-cyclodextrin-bonded silica stationary phase was used to seek new selectivities and additional interactions compared to conventional stationary phases (such as phenyl-bonded silica packing).

As previously reported, triethylammonium acetate (TEAA) buffer was preferred to ammonium acetate buffer as triethylamine gave sharper peaks, leading to better efficiencies [25].

3.1. Effect of the pH of the mobile phase

As in CE, solutes migrate through the CEC capillary with the electroosmotic flow (EOF), which strongly depends on the pH of the mobile phase. An increase in pH results in a faster EOF, due to a greater ionisation of the silanol groups present on the inner capillary wall and at the surface of the packing material. Fig. 2 illustrates the influence of the pH

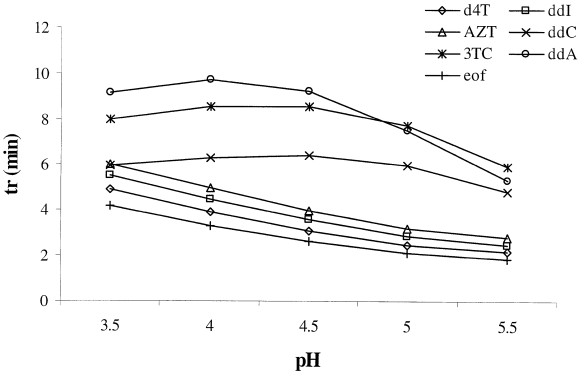


Fig. 2. Influence of the buffer pH on the retention times of the NRTIs in CEC. Column: 31.2 cm×75 μm I.D. (10 cm packed with β-Cyclose-2-OH®, 5 μm); mobile phase: TEAA/acetonitrile (90/10, v/v), 20 m*M* of total ionic strength; applied voltage: −15 kV; temperature: 20°C, UV detection: 254 nm; electrokinetic injection: −10 kV, 1 s; nucleoside concentration: 10 μg/ml.

(from 3.5 to 5.5) on the retention times of the different nucleosides.

It clearly appears that the nucleosides can be divided into two groups. On the one hand, d4T, ddI and AZT, which are neutral in this pH range, behave like EOF: their retention times decrease as pH increases. Since the electrophoretic mobility of these solutes is zero, the retention reflects a pure chromatographic process. On the other hand, the retention times of cationic species, ddC, ddA and 3TC, depend both on their own electrophoretic mobility and on their partition coefficient between mobile and stationary phases; when pH grows from 3.5 to 5.5, the apparent charge of each nucleoside decreases and the chromatographic aspect rapidly prevails over the electrophoretic phenomenon. 3TC and ddC are both cytidine derivatives; therefore, they exhibit the same trend of retention time evolution. The behavior of ddA is similar but the decrease in retention time occurs at a more acidic pH than for ddC or 3TC; this can be explained by their difference of charge since ddA (pK_a=3.5) switches from the positively charged form to the neutral one in a more acidic region than 3TC (pK_a=4.4) or ddC (pK_a=4.4). Finally, the sulphur atom in 3TC could be involved in an additional hydrogen-bond interaction with the β-cyclodextrin stationary phase, leading to a greater retention of 3TC than ddC.

As a conclusion, the retention of the studied nucleosides on β-Cyclose-2-OH® is mainly controlled by a chromatographic process (at the expense of the electrophoretic phenomenon) since cationic species are eluted after the EOF and neutral solutes, whatever the pH.

A 5.5 pH seems to be suitable to achieve baseline separation in a short-time analysis; moreover, as the apparent charge of cationic species is lowered, the silanophilic interactions of the solutes with the residual silanol groups are minimised and peak tailing is reduced.

3.2. Effect of acetonitrile percentage

In this study, the total ionic strength of the mobile phase (TEAA, pH 5/acetonitrile) was kept constant (10 m*M*) whatever the acetonitrile concentration.

As in reversed-phase HPLC, a general decrease in retention times of the solutes is observed when the acetonitrile content rises from 5 to 20% v/v (Fig. 3). This general trend can be explained by the growing apolarity of the eluent, enhancing the affinity of the analytes towards the mobile phase (at the expense of hydrophobic interactions with the β-CD). However, a change in the acetonitrile content of the mobile phase does not alter all nucleosides equally, highlighting once again the same two groups of NRTIs.

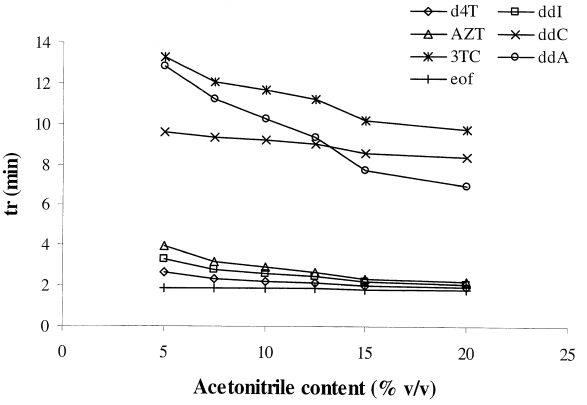


Fig. 3. Effect of acetonitrile content on the retention times of the NRTIs in CEC. Column: 31.2 cm×75 μm I.D. (10 cm packed with β-Cyclose-2-OH®, 5 μm); mobile phase: TEAA, pH 5/acetonitrile, 10 m*M* of total ionic strength; applied voltage: −15 kV; temperature: 20°C, UV detection: 254 nm; electrokinetic injection: −10 kV, 1 s; nucleoside concentration: 10 μg/ml.

Separation was achieved all over the studied range. However, it was expected that d4T, ddI and AZT would be coeluted beyond 20% acetonitrile v/v. As can be seen in Fig. 3, the elution order of these neutral solutes was not dependent on the acetonitrile percentage. Conversely, acetonitrile concentration was found to have a dramatic effect on ddC and ddA, since an inversion of elution order occurred around 13% v/v of acetonitrile. While increasing acetonitrile concentration, the relative hydronium concentration decreases, leading to an increase in the apparent pH of the mobile phase and a decrease in the pKa of bases [26]. Moreover, as the eluent becomes more hydrophobic, the growing affinity for the mobile phase seems to be larger for purine compounds (e.g. ddA) than for pyrimidine derivatives (e.g. ddC); finally, retention of the analytes is reduced when the apparent charge of the solutes is lowered since interactions with the silanol groups are reduced.

Therefore, a mobile phase containing 10% of acetonitrile provides a good separation of the NRTIs, while preserving an acceptable resolution between the cationic nucleosides 3TC, ddC and ddA.

3.3. Effect of the ionic strength of the buffer

The influence of the ionic strength of the mobile phase on nucleoside retention times was investigated (Fig. 4). As in CE, an increase in the ionic strength of the electrolyte results in a decrease in the EOF velocity and, consequently, in a rise in the retention times of the neutral solutes (d4T, ddI, AZT). Indeed, the thickness of the diffuse double layer decreases at high ionic strength, thus reducing zeta potential. In contrast, cationic solutes (ddC, 3TC, ddA) are eluted all the more rapidly since ionic strength increases from 5 to 25 m*M*. At low ionic strength (e.g. 5 m*M*), ionized solutes exhibit strong interactions with residual silanol groups of the stationary phase, leading to significant retention times and band broadening. When the ionic strength of the mobile phase is increased, silanol groups become less accessible for cationic species since TEA acts as a competing agent. Therefore, TEA reduces silanophilic interactions with charged analytes, decreasing retention of the nucleosides.

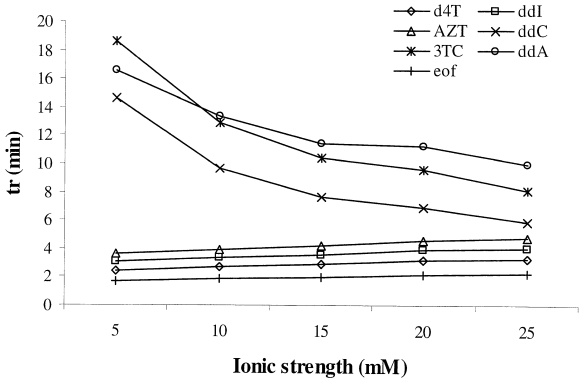


Fig. 4. Influence of the ionic strength of the mobile phase on the retention times of the NRTIs in CEC. Column: 31.2 cm×75 μm I.D. (10 cm packed with β-Cyclose-2-OH®, 5 μm); mobile phase: TEAA, pH 5/acetonitrile (95/5, v/v); applied voltage: −15 kV; temperature: 20°C, UV detection: 254 nm; electrokinetic injection: −10 kV, 1 s; nucleoside concentration: 10 μg/ml.

Subsequently, a 20 m*M* ionic strength buffer was found to be suitable for the analysis of those NRTIs.

3.4. Effect of the applied voltage

The velocity of the mobile phase directly depends on the applied voltage; thus, a fast EOF is obtained when a high voltage is applied. As solutes migrate faster in high electric fields, an increase in voltage results in a general decrease of the nucleoside retention times.

However, the range of applied voltage was not investigated beyond −25 kV since baseline separation was assumed to be lost at higher electric fields, owing to excessive Joule heating.

As a conclusion, baseline separation was achieved in a short-time analysis, without problems of Joule heating, by applying a −15 kV voltage.

3.5. Effect of the temperature

The increase of EOF velocity and electrophoretic mobilities with the temperature may be explained by a decrease in the mobile phase viscosity, leading to an increase in both ϵ_r/η ratio of the eluent and diffusion coefficients of the analytes. Besides, an increase in temperature affects the distribution constants of the solutes (between stationary and mobile phases), leading to a general decrease in the retention times. Nonetheless, temperature was not found to have a significant effect on retention times beyond 20°C.

Consequently, as resolution between all nucleosides was satisfactory, 20°C was selected as a proper operating condition to achieve good separation.

3.6. Final operating conditions

As shown in Fig. 5, baseline separation of the six nucleosides was achieved in a short–time analysis (<7 min) using TEAA, pH 5.5/MeCN (90/10, v/v), 20 m*M* of total ionic strength, as the final mobile phase. This optimised CEC separation was performed at 20°C, when applying a −15 kV voltage.

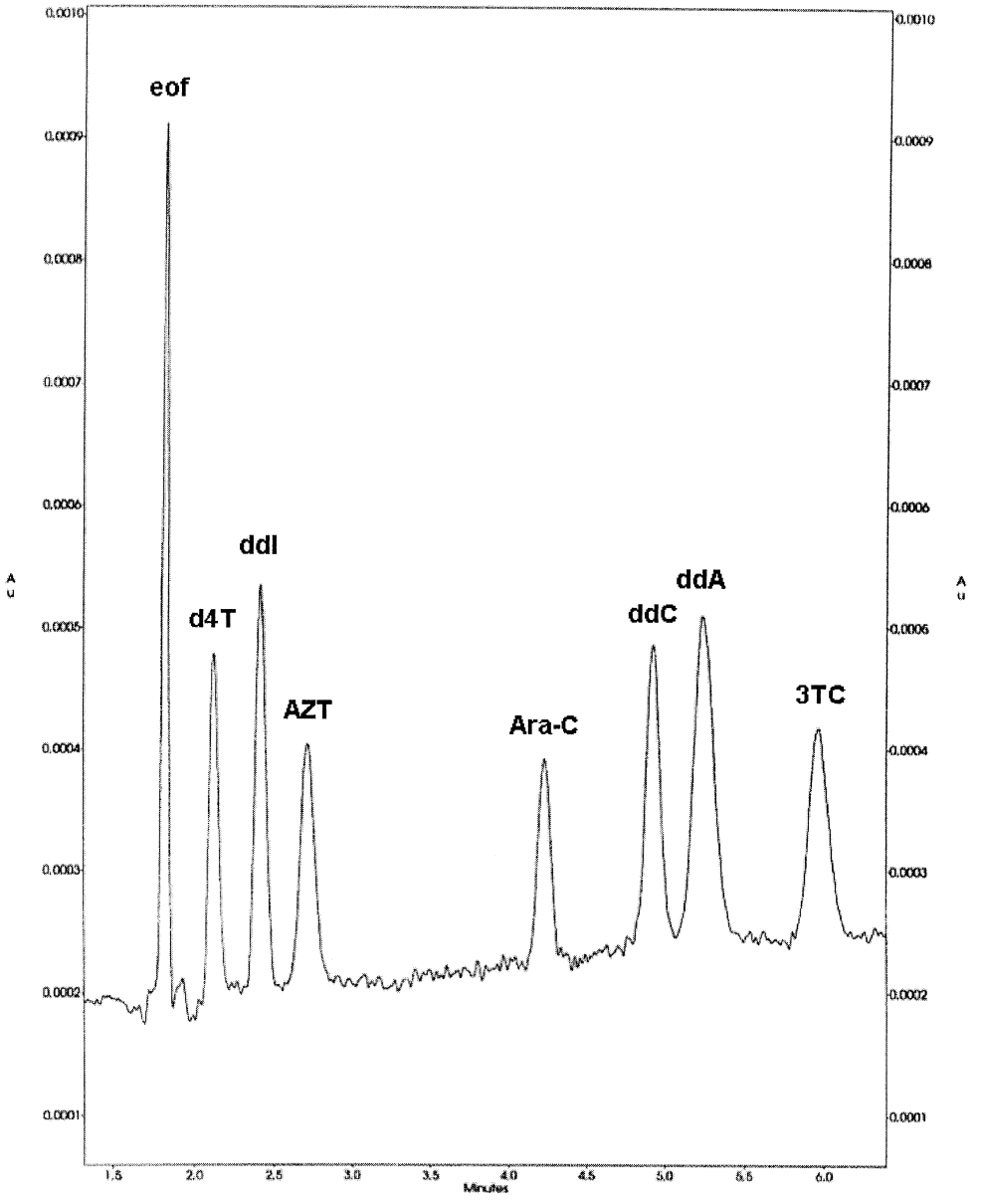


Fig. 5. Final CEC separation of the NRTIs. Column: 31.2 cm×75 μm I.D. (10 cm packed with β-Cyclose-2-OH®, 5 μm); mobile phase: TEAA, pH 5.5/acetonitrile (90/10, v/v), 20 m*M* of total ionic strength; applied voltage: −15 kV; temperature: 20°C, UV detection: 254 nm; electrokinetic injection: −10 kV, 1 s, nucleoside concentration: 10 μg/ml.

4. Validation study

4.1. Linearity

Since electrokinetic injection induces a bias for charged compounds, linearity was assessed using β-D-arabinocytidine (Ara-C) as an internal standard to improve the results. A good linear correlation for all nucleosides (r^2>0.996) was found in the investigated range of concentration (5–25 μg/ml).

Table 1
Repeatability ($n=10$) on retention times (t_r), relative retention times ($Rt_r=t_r/t_{eof}$), and corrected areas ($S_c=S/t_r$) of the six anti-HIV nucleosides (experimental conditions as in Fig. 5)

	t_r (min)	RSD (%)	Rt_r	RSD (%)	S_c	RSD (%)
d4T	2.218	0.73	1.174	0.13	105.10	1.36
ddI	2.545	0.76	1.347	0.20	123.23	2.22
AZT	2.870	0.71	1.519	0.22	82.15	2.64
ddC	5.046	0.55	2.670	0.15	65.80	4.99
ddA	5.782	1.24	3.060	0.68	81.75	3.63
3TC	6.285	0.49	3.326	0.24	47.08	1.61

4.2. Limit of detection (LOD) and limit of quantitation (LOQ)

Limits of detection (LOD) and quantitation (LOQ) were defined as a signal-to-noise ratio equal to 3 and 10, respectively. The range of LOQ reached by UV detection was of [2–3.5 μg/ml]. LOD between 0.6 and 1.1 μg/ml were achieved and checked experimentally.

4.3. Repeatability

A mixture of the six anti-HIV nucleosides (10 μg/ml of each compound) was used in these experiments. Repeatability studies on retention times (t_r), relative retention times (Rt_r) and corrected areas (S_c) are reported in Table 1.

An excellent accuracy on retention times was found for all nucleosides as the relative standard deviation (RSD) was generally less than 1%. The CEC method provided also a good repeatability on corrected areas since RSD never exceeded 5%.

These experimental results attest the reliability of the method and demonstrate that CEC is a powerful technique to perform simultaneous quantitative analysis of the six nucleosides.

5. Conclusion

CEC performed on a β-cyclodextrin-bonded silica stationary phase has proved to be an efficient tool to resolve an achiral mixture, taking advantage of the strong hydrophobic interactions between β-cyclodextrins and analytes. Indeed, baseline separation of six NRTIs (d4T, ddI, AZT, ddC, 3TC, ddA) was achieved in a short-time analysis (<7 min), using the short-end injection technique.

Based on the results obtained in a preliminary validation study, CEC appears to be suitable for both the qualitative determination and the assay of a mixture of nucleosides. Nevertheless, CEC analysis of cell extracts spiked with those NRTIs is currently under investigation in our laboratory to fully assess the matrice effects. CEC–MS/MS development is also necessary to achieve a selective and high-sensitive quantitation (expected NRTIs concentrations in plasma between 0.2 μg/ml and 0.6 μg/ml).

Full validation of this method in cell pool samples is still needed to make CEC a proper technique for the biological analysis of anti-HIV nucleosides.

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