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Determination at ppb level of an anti-human immunodeficiency virus nucleoside drug by capillary electrophoresis–electrospray ionization tandem mass spectrometry

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Abstract

Capillary electrophoresis coupled with tandem mass spectrometry was used to indirectly separate and quantify the active metabolite of the anti-human immunodeficiency virus (anti-HIV) didanosine drug. The influence of several parameters (pH and ionic strength of volatile formic acid–ammonia buffer) upon electroosmotic flow, electrophoretic mobility and peak efficiency of several nucleosides (A, dA, ddA, C) has been studied. This paper illustrates the current importance in CE–MS technique as a complementary or substituted method to the known HPLC–radioimmunoassay or HPLC–UV method to measure levels of anti-HIV drugs. The limit of detection for 2′,3′-dideoxyadenosine by this method is 2 $\mu g\ l^{-1}$ in a formic acid–ammonia buffer (pH 2.5, 10 m*M* ionic strength).This methodology could be used to perform simultaneous detection of two or more anti-HIV nucleosides, such as stavudine or didanosine in combination therapy.

Keywords: Pharmaceutical analysis; Capillary electrophoresis; Mass spectrometry; Nucleosides; Didanosine; Didexoyadenosine-5′-triphosphate

1. Introduction

The didanosine (2′,3′-dideoxyinosine; ddI) is a dideoxy analogue of the purine nucleoside inosine that potently inhibits the replication of the human immunodeficiency virus (HIV) [1]. In common with other nucleoside analogues, the parent compound ddI requires intracellular metabolism to the active triphosphate, 2′,3′-dideoxyadenosine-5′-triphosphate (ddATP), which acts as a competitive inhibitor of HIV reverse transcriptase or as a DNA chain terminator [2] (Fig. 1).

Its antiviral activity is dependent on competition with endogenous 2′-deoxyadenosine 5′-triphosphate (dATP) for binding to the viral reverse transcriptase (RT-HIV) [3]. Recently, the intracellular concentration of the triphosphate form of nucleoside reverse transcriptase inhibitors (NRTIs) has been correlated directly with HIV viral load response [4]. Therefore, the concentration of the active triphosphate species needs to be determined to establish a relationship between the dosage and therapeutic efficacy. To date, there is no direct method for the intracellular quantification of the triphosphates of NRTIs. Typically, after separation by high-performance liquid chromatography (HPLC) or solid-phase extraction (SPE) from other metabolites, phosphate groups of an antiviral NuTP were removed via an enzymatic pathway. The corresponding nucleoside was quantified by several analytical methods, leading in return the concentration of the triphosphate (Fig. 1). Sever-

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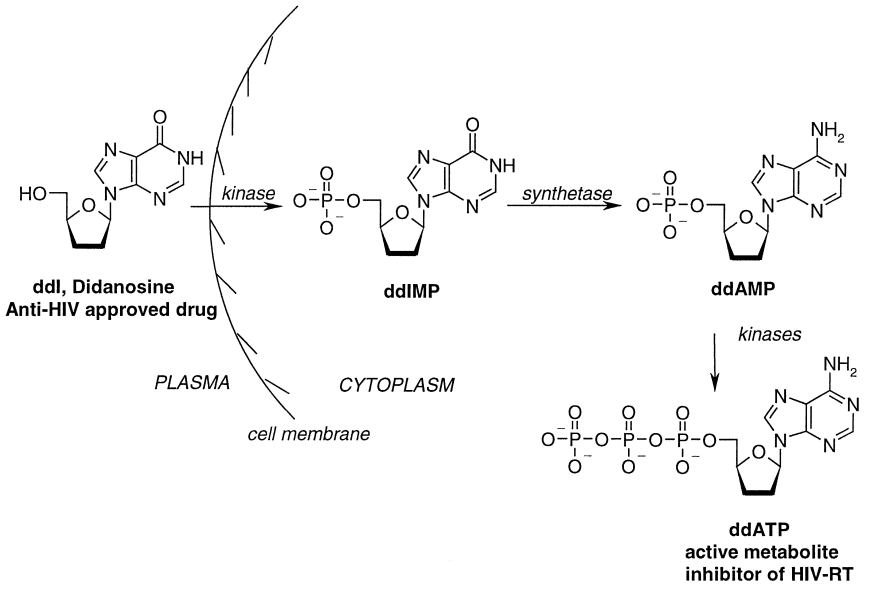

Fig. 1. Simplified activation pathway of ddI into ddATP.

al approaches have been reported for the indirect quantification of some anti-HIV nucleoside drugs, including a combination of HPLC with UV detection or coupled with radioimmunoassay (RIA) [5–8]. However, the lack of sensitivity and the selectivity of these methods make these techniques not enough efficient for clinical monitoring therapeutics in physiologic fluids at low concentrations levels. In addition, these techniques were also time consuming since it took more than 50 min for each sample to be analyzed by HPLC, 6 h by RIA and more than 3 months to obtain good quality polyclonal antiserum (RIA). Recently, an SPE approach (Sep-Pak cartridge/RIA) was reported to improve the processing time for measurement of others NTRIs [9]. However, this method based on specificity of the antibodies could not determine two or more anti-HIV nucleosides simultaneously (Fig. 2).

In the last decade, capillary electrophoresis (CE) has assumed an increasing role in the pharmaceutical industry. CE has many attractive features for the separation and quantification of drugs such as rapid analysis time, high peak efficiencies, great resolutions, small sample volume requirements and low solvent consumption. It has been used for the analysis of ribonucleoside mixtures by capillary electrochromatography on non-encapped phenyl-bonded silica phase [10] or by capillary zone electrophoresis [11,12]. Quantitative bioanalytical chemistry has been revolutionized in the past 10 years by

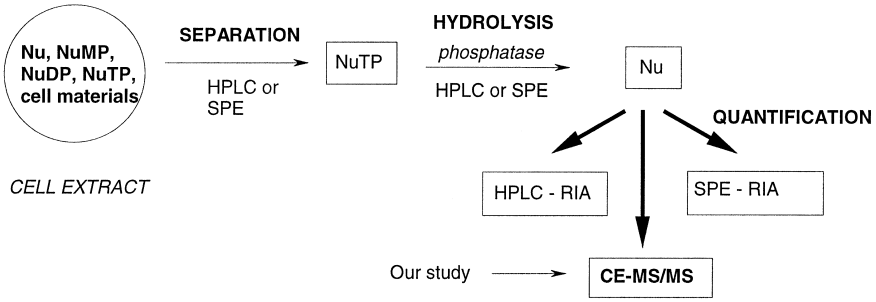

Fig. 2. Typical procedure for the quantification of anti-HIV NuTP.

the development of liquid interfaces for tandem mass spectrometry (MS–MS). The immense selectivity of this technique stems for MS–MS detection. Several liquid interfaces for mass spectrometry have been used to study nucleosides [13–15] and electrospray ionization (ESI) is the most commonly used interface; MS–MS provides a specific and selective method in which a selected fragment ion is used for the quantification analysis.

As part of our ongoing anti-HIV research, we describe a method combining a CE–MS–MS procedure to develop a sensitive and selective method, first for the indirect measurement of ddATP with sensitivities frequently comparable to those attainable by RIA then later for the direct quantification of the triphosphate. The CE–MS–MS method we developed was suitable to separate and quantify, at the ppb level, concentration of ddA among a mixture of nucleosides including A and dA, two major structural nucleoside analogues. In this work, the basic electrophoretic properties of ddA were studied as well as the measurement of this drug by CE–MS–MS in a model mixture with adenosine and 2′-deoxyadenosine.

2. Experimental

2.1. Chemicals

All nucleosides were supplied by Sigma (St. Louis, MO, USA): adenosine (A), 2′-deoxyadenosine (dA), cytidine (C), 2′,3′-dideoxyadenosine (ddA), uridine (U), guanosine (G), thymidine (T). The chemical structures of these solutes are given in Fig. 3 and their names and abbreviations are also mentioned. Formic acid and ammonia were of analytical grade and obtained from Sigma. The water used for the preparation of electrolytes was of HPLC quality obtained from Elgestat UHQ II system (Villeurbanne, France). All electrolytes and washing solutions

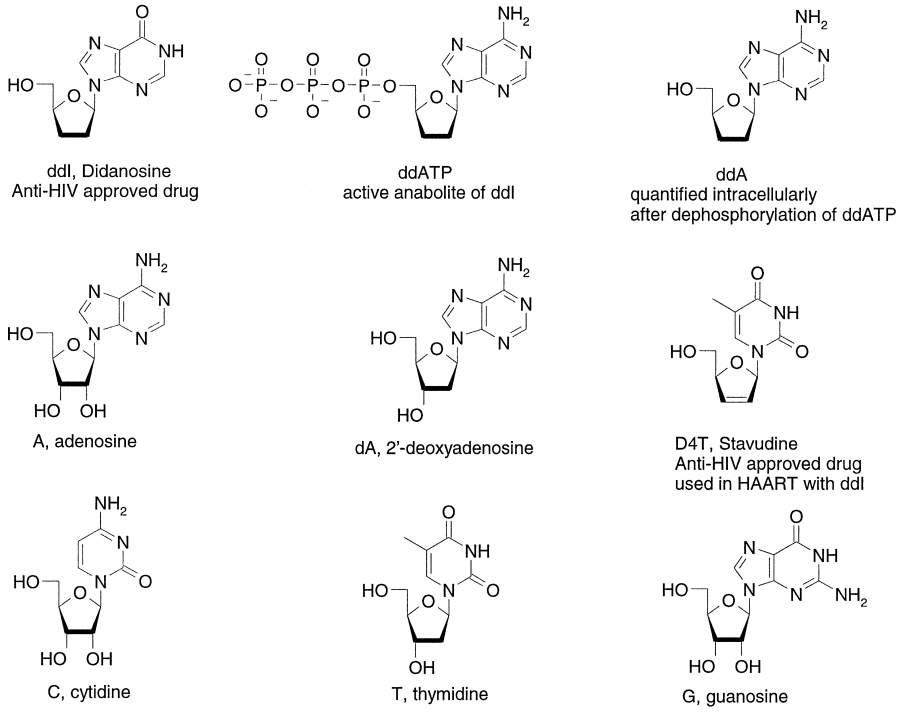

Fig. 3. Chemical structures, names and abbreviation of several anti-HIV nucleosides. ddI: 2′,3′-dideoxyinosine; ddATP: 2′,3′-dideoxyadenosine 5′-triphosphate; ddA: 2′,3′-dideoxyadenosine; A: adenosine; dA: 2′-deoxyadenosine; D4T: 2′,3′-didehydro-3′-deoxythymidine; C: cytidine; T: thymidine; G: guanosine.

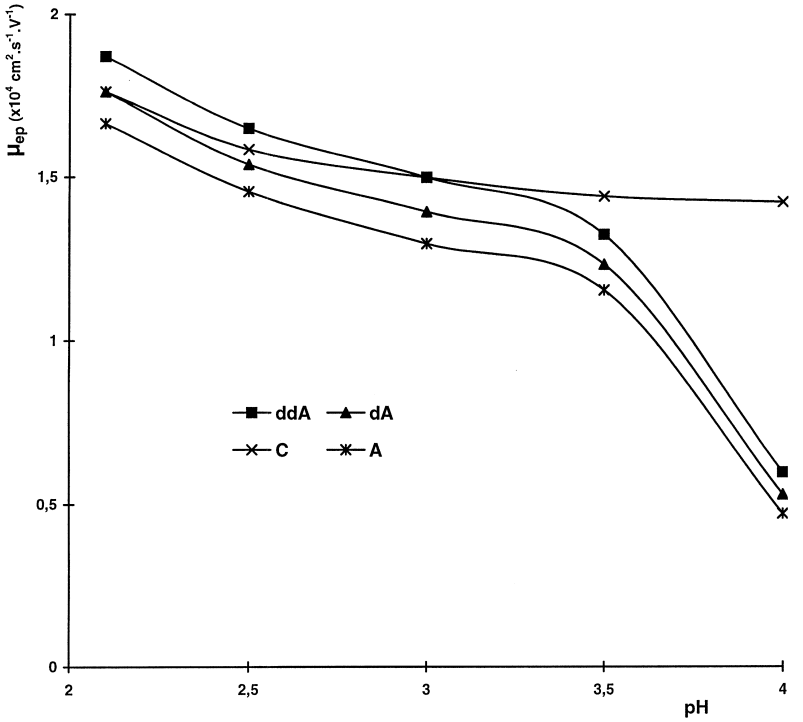

Fig. 4. Influence of the pH of formic acid–ammonia buffer (10 m*M* ionic strength) on electrophoretic mobility of nucleosides. Fused-silica capillary dimensions: 37 cm×75 μm I.D.; electrolyte: formic acid–ammonia (10 m*M* ionic strength); UV detection at 254 nm; applied voltage: +20 kV; temperature: 25°C; hydrodynamic injection: 10 s; nucleoside concentration: 10 mg l^{-1}.

were filtered before use through a polypropylene filter of 0.22 μm porosity (Prolabo, France).

2.2. CE instrument

CE separation was carried out on a P/ACE 5000 apparatus during CE–UV experiments and on a P/ACE MDQ during CE–ESI-MS studies (Beckman–Coulter, Fullerton, CA, USA). Solutes were injected at the anode by hydrodynamic injection. Separations were achieved at constant temperature (25°C) by immersion in a cooling liquid circulating in the cartridge under constant applied voltage (+20 kV). Direct UV detection was performed at 254 nm.

The optimization of nucleoside separation depends upon the ionic strength and the pH value of the electrophoretic buffer. Several formic acid–ammonia buffers were prepared at fixed pH and ionic strength by using Phoebus software (Beckman–Coulter, Villepinte, France); this application program is designed to help in the preparation of a buffer for which the user has selected the pH and ionic strength. Then, the pH of each predicted buffer was checked on a Beckman pH meter (Model ϕ 10, Fullerton, CA, USA).

2.3. Mass spectrometer and interface

The mass spectrometer utilized in all studies was a PE Sciex API 300 (Perkin-Elmer Sciex, Toronto, Canada) triple quadrupole instrument equipped with an ESI source operating at room temperature. The PE Sciex ESI source with a coaxial sheath liquid interface was used. The sheath liquid was composed of 0.5% formic acid added to methanol–water (95:5, v/v) and delivered by a Harvard Model 22 syringe pump (Harvard Apparatus, South Natick, MA, USA) at a flow-rate of 5 μl min^{-1}. The electrospray needle was maintained at +5 kV in the positive mode. Mass spectra were acquired using a dwell time of 1 ms per step of 0.2 u. A Macintosh computer was used for

instrument control, data acquisition and data processing using LC_2 Tune software.

For CE–ESI-MS studies, the fused-silica separation capillary has 70 cm×50 μm I.D.×150 μm O.D. geometrical dimensions. The outside coating of polyimide of the fused-silica capillary was removed 0.5–1 cm from the end.

3. Results and discussion

In this study, the separation of ddA from dA, A and C by CE was investigated. Direct UV detection was performed at 254 nm (close to the wavelength of maximum absorbance of each nucleoside). Several volatile formic acid–ammonia buffers having the same ionic strength (10 m*M*) but different pH values varying in the 2–5 pH range were prepared with the help of Phoebus software and used for CE experiments. The influence of pH upon the electrophoretic mobilities of four nucleosides is reported in Fig. 4. In this acidic pH range, ddA, dA, A and C nucleosides behave as positively charged species. Indeed, pK_a values of cytidine and adenosine at 25°C are equal to 4.1 and 3.5, respectively. Additionally, decreasing electrophoretic mobility of each nucleoside with pH may be explained by a decrease of its positive apparent charge, particularly near its pK_a value. Furthermore, it should be noted that acidic pH values result in co-migration of C and dA and that an increase of buffer pH undergoes a modification of migration order between C and dA. However, Fig. 5 indicates that pH values greater than 3 undergo smaller peak efficiencies due probably to a reduced solubility of nucleosides under neutral form compared to cationic form. In addition, pH 2.5 has been selected rather than pH 3 due to a better buffer capacity.

Secondly, the effect of buffer ionic strength upon nucleoside retention has been determined (Fig. 6). As expected, the electrophoretic mobility of each nucleoside decreases with an increase in ionic strength, but no improvement of the resolution occurs at high ionic strength values. So, an ionic strength of 10 m*M* has been selected due to a

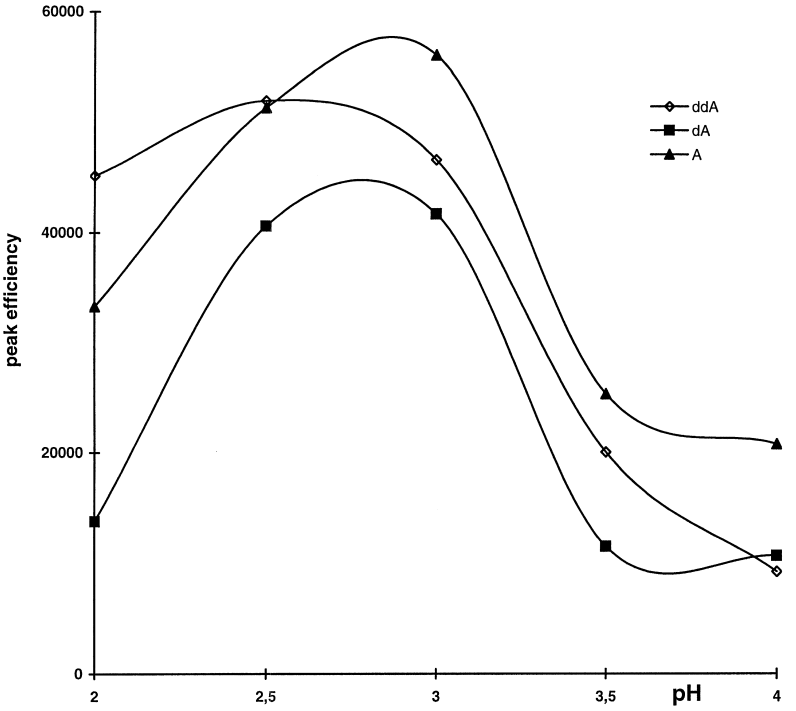

Fig. 5. Variation of peak efficiency expressed in theoretical plates versus buffer pH. Experimental conditions as in Fig. 4.

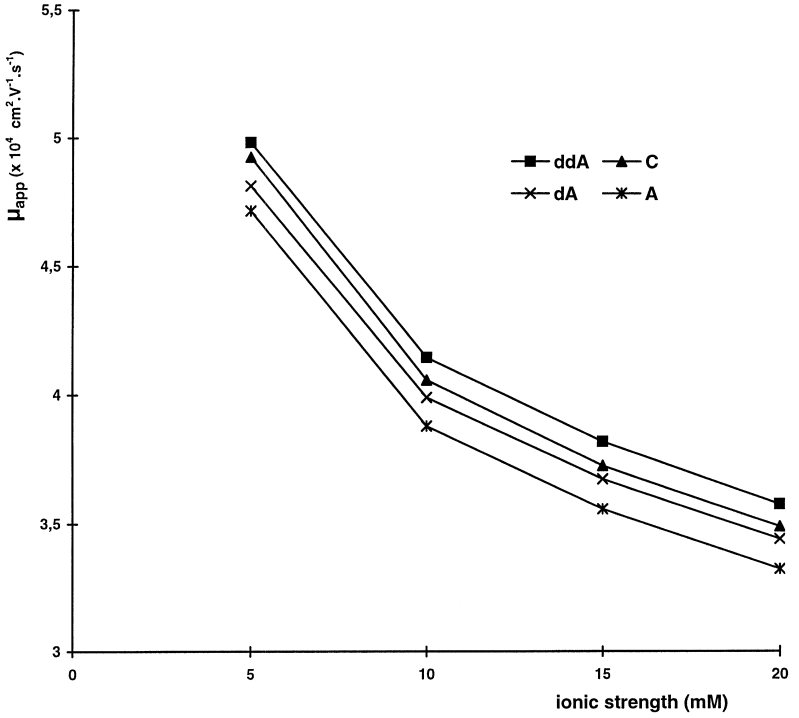

Fig. 6. Influence of the ionic strength of formic acid–ammonia buffer (pH 2.5) on electrophoretic mobility of nucleosides. Experimental conditions as in Fig. 4 except pH 2.5 and variable ionic strength of formic acid–ammonia buffer.

sufficient buffer capacity and a moderate ionic strength appropriate with MS detection.

Finally, the overall separation of the four nucleosides (ddA, C, dA and A) was achieved in less than 5 min by using formic acid–ammonia (pH 2.5, ionic strength 10 m*M*), as shown in Fig. 7. Resolution between two consecutive solutes are always greater than 1.5. Besides, ddA migrates faster than dA and A in formic acid–ammonia buffer (pH 2.5, 10 m*M* ionic strength). As adenosine and two derivatives (dA and ddA) only differ by the number of hydroxy groups on ribose, the difference of migration velocity mainly depends upon the molecular size of the nucleoside. Neutral nucleosides (U, T) migrate slowly at the electroosmotic flow (EOF) velocity ($1.08 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}) and are detected at 85 min.

CE–MS coupling requires the use of volatile electrolyte systems to ensure compatibility with the mass spectrometer, the addition of a sheath liquid to compensate the low flow-rate of the CE system (nl min^{-1}) in order to increase the stability and the production of the spray, the use of a nebulization gas to stabilize the spray formation and the adjustment of the position between the CE capillary, into the stainless steel. The volatile formic acid–ammonia buffer allows simultaneous direct UV and ionspray MS detection. Several parameters (composition and flow-rate of sheath liquid) have been optimized. The mass spectra of these compounds exhibit very abundant positive $M-H^+$ ions (at m/z 236 for ddA, 252 for dA, 268 for A and 244 for C). The CE–MS electropherogram (total ionic current) of nucleoside mixture shows as expected an increase in the signal-to-noise ratio (Fig. 8).

MS–MS detection has also been performed to reach lower detection limits for these nucleosides. Studied nucleosides give common product positive ions due to the loss of the sugar moiety at m/z 136 for ddA, dA and A and at m/z 112 for C. Quantitative analysis were both achieved in CE–UV and CE–MS–MS. For example, calibration curves were

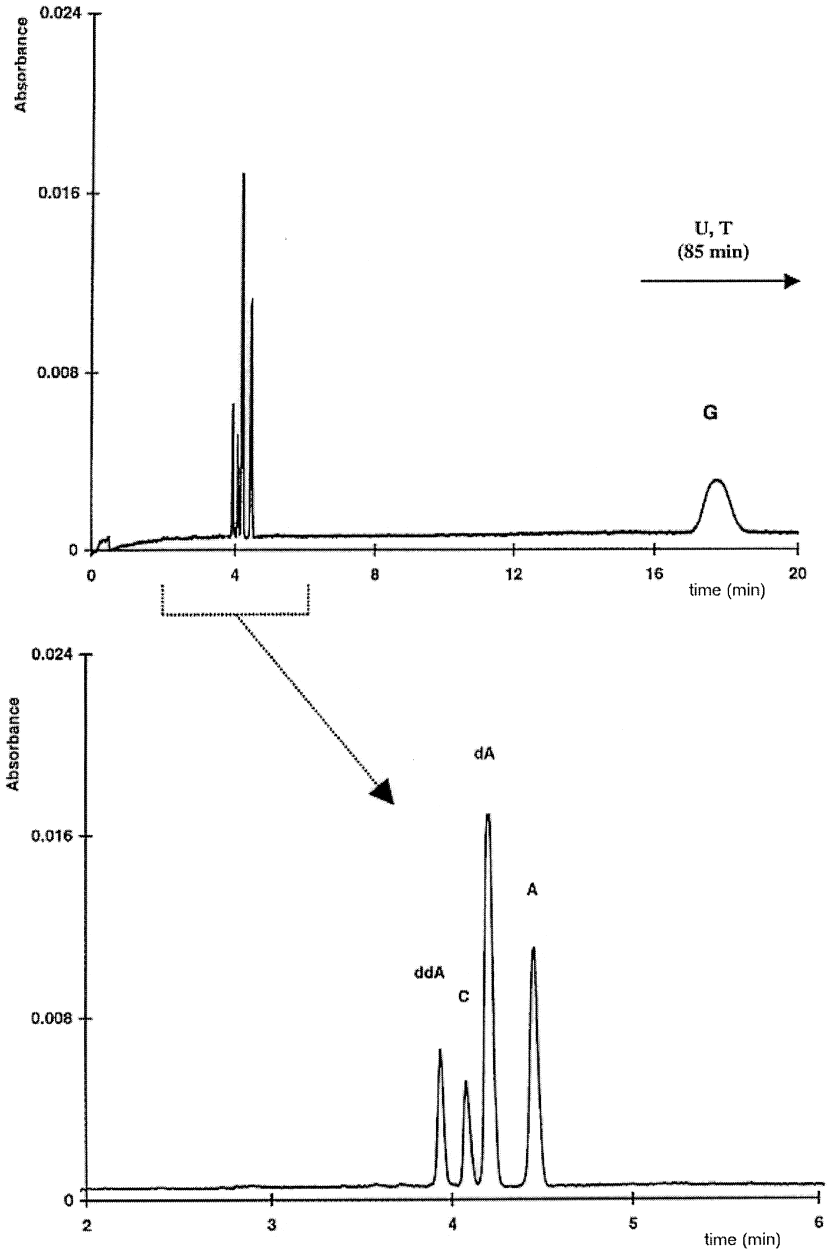

Fig. 7. Separation of antiviral nucleosides by capillary electrophoresis with UV detection. Fused-silica capillary dimensions: 37 cm×75 μm I.D.; electrolyte: formic acid–ammonia (10 m*M* ionic strength); UV detection at 254 nm; applied voltage: +20 kV; temperature: 25°C; hydrodynamic injection: 10 s; nucleoside concentration: 10 mg l^{-1}.

determined in the 0.25–10 mg l^{-1} concentration range for UV detection and in the 0.005–1 mg l^{-1} concentration range for MS–MS detection; good linear correlation coefficients (for ddA, 0.9997 and 0.9981 respectively) were obtained. Limits of detection (LODs) and quantitation (LOQs) of ddA are

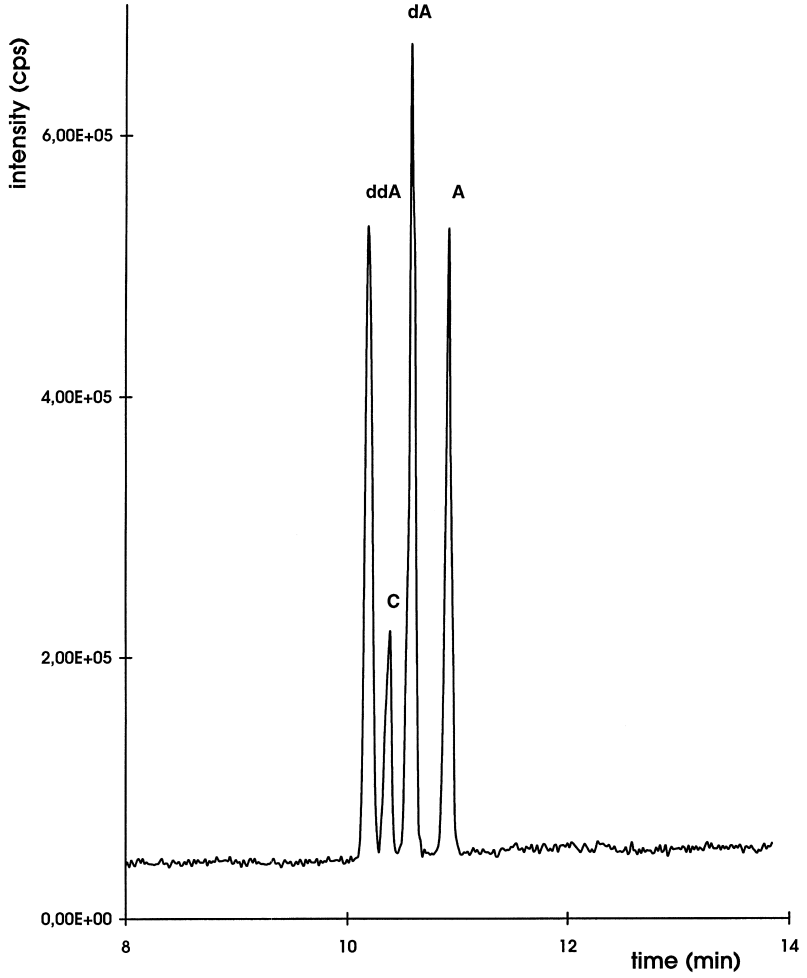

Fig. 8. Separation of antiviral nucleosides by capillary electrophoresis with MS detection. Fused-silica capillary dimensions: 70 cm×50 μm I.D.×150 μm O.D.; electrolyte: formic acid–ammonia (10 m*M* ionic strength); UV detection at 254 nm; applied voltage: +25 kV; temperature: 25°C; hydrodynamic injection: 20 s (50 mbar); nucleoside concentration: 10 mg l^{-1}; MS sheath liquid: methanol–water (95:5, v/v)+0.5% formic acid at 5 μl min^{-1} flow-rate; ionspray voltage: +5 kV.

reported in Table 1. A lower concentration LOD can be achieved by CE–MS–MS (2 μg l^{-1}) compared to CE–UV (100 μg l^{-1}).

4. Conclusion

This work has demonstrated the possibility of measuring ddA from other nucleosides by CE–UV or CE–MS–MS. Baseline separation was achieved in less than 12 min. CE–MS–MS allows one to measure ddA with an LOD of 2 ppb [e.g., 50-times lower than the LOD obtained by CE–UV (100 μg l^{-1})] using the formic acid–ammonia buffer (pH 2.5, 10 m*M* ionic strength). The analyses of other NRTIs (D4T: 2′,3′-didehydro-3′-deoxythymidine; 3TC: 2′,3′-dideoxy-3′-thia-*b*-L-cytidine; AZT: 3′-azido-3′-deoxythymidine) separately or in combination have been also realized by this technique and results will be published elsewhere [16]. We are currently inves-

Table 1
Comparison of ddA quantitation by CE–UV and by CE–MS–MS[a]

	Concentration range (mg l^{-1})	LOQ concentration (μg l^{-1})	LOD concentration (μg l^{-1})	Injected amount (fmol)
CE–UV	0.25–10	250	100	2.7
CE–MS–MS	0.005–1	5	2	0.2

[a] Same experimental conditions as in Fig. 7 for CE–UV, and as in Fig. 8 for CE–MS–MS.

tigating the direct and simultaneous quantification of the triphosphate ddATP and D4T-TP, the active form of anti-HIV nucleosides ddI and D4T used in combination by CE–MS–MS in cell pool samples. Moreover, this method allows us to determine the ratio of ddA to dA (e.g., in return of ddATP to dATP); of great importance for the antiviral activity of this drug.

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References

[1] P.A. Furman, J.A. Fyfe, M.H. St. Clair, K. Weinhold, J.L. Rideout, G.A. Freeman, S. Nusinoff Lehrman, D.P. Bolegnesi, S. Broder, H. Mitsuya, D.W. Barry, Proc. Natl. Acad. Sci. USA 83 (1986) 8333.
[2] D. Faulds, R.N. Brogden, Drugs 44 (1992) 94, and references cited therein.
[3] H. Mitsuya, R. Yarchoan, S. Broder, Science 249 (1990) 1533.
[4] J.-P. Sommadossi, M.A. Valentin, X.J. Zhou, M.Y. Xie, J. Moore, V. Clavez, M. Desa, C. Katlama, in: The 5th Conference on Retroviruses and Opportunistic Infections, Chicago, IL, 1998, p. 146, Abstract 262.
[5] H. Kuster, M. Vogt, B. Joos, V. Nadai, R. Luthy, J. Infect. Dis. 164 (1991) 773.
[6] J.T. Slusher, S.K. Kuwahara, F.M. Hamzeh, L.D. Lewis, D.M. Kornhauser, P.S. Lietman, Antimicrob. Agents Chemother. 36 (1992) 2473.
[7] M. DeRemer, R. D'Ambrosio, D. Gene, Antimicrob. Agents Chemother. 40 (1996) 1331.
[8] D. Lowe Walters, D.L. Jacobs, J.E. Tomaszewski, S. Graves, J. Pharm. Biomed. Anal. 19 (1999) 955.
[9] B.L. Robbins, T.T. Tran, F.H. Pinkerton, F. Akeb, R. Guedj, J. Grassi, D. Lancaster, A. Fridland, Antimicrob. Agents Chemother. 42 (1998) 2656.
[10] X. Cahours, Ph. Morin, L. Agrofoglio, M. Dreux, J. High Resolut. Chromatogr. 23 (2000) 138.
[11] A.S. Cohen, S. Terabe, J.A. Smith, B.L. Karger, Anal. Chem. 59 (1987) 1021.
[12] R. Singhal, J. Xian, O. Otim, J. Chromatogr. A 756 (1996) 263.
[13] E. Font, S. Lasanta, O. Rosario, J.F. Rodriguez, Nucleosides Nucleotides 17 (1998) 845.
[14] H.K. Jajoo, S.M. Bennett, D.M. Kornhauser, J. Chromatogr. 577 (1992) 299.
[15] E.L. Esmans, D. Broes, I. Hoes, F. Lemière, K. Vanhoutte, J. Chromatogr. A 794 (1998) 109.
[16] X. Cahours, H. Dessans, Ph. Morin, M. Dreux, L. Agrofoglio, Electrophoresis, submitted for publication.