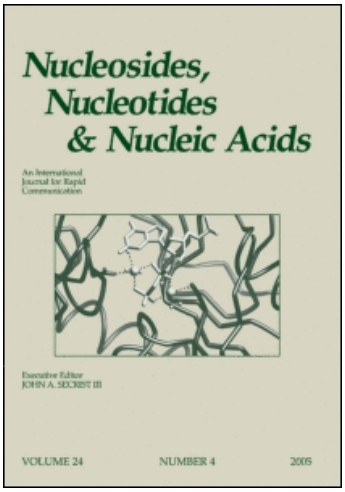

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 31 March 2001

To cite this Article Agrofoglio, L. A. , Cahours, X. , Tran, T. T. , Dessans, H. , Kieda, C. and Morin, P.(2001) 'ANALYSIS OF ANTI-HIV NUCLEOSIDE INHIBITORS BY CAPILLARY ELECTROPHORESIS-ELECTROSPRAY IONIZATION MASS SPECTROMETRY', Nucleosides, Nucleotides and Nucleic Acids, 20: 4, 375 — 381
To link to this Article: DOI: 10.1081/NCN-100002309
URL: http://dx.doi.org/10.1081/NCN-100002309

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(4–7), 375–381 (2001)

ANALYSIS OF ANTI-HIV NUCLEOSIDE INHIBITORS BY CAPILLARY ELECTROPHORESIS-ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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ABSTRACT

A method employing capillary electrophoresis (CE) with tandem mass spectrometry (MS) has been developed for the simultaneous determination, on one hand, of zidovudine (AZT) with stavudine (d4T), and on the other hand, of lamivudine (3TC) with a didanosine metabolite (ddA), four potent human immunodeficiency virus reverse transcriptase (RT-HIV) inhibitors. The influence of several parameters (pH and ionic strength of volatile formic acid-ammonia buffer) as well as the influence of magnesium cation upon electroosmotic flow, electrophoretic mobility and peak efficiency has been studied. The limit of detection (LOD) by this method is 2.5 ppb for AZT and 20 ppb for d4T, 2 ppb for ddA and 5 ppb for 3TC, respectively. This paper illustrates the current importance in CE-ESI/MS/MS technique as a complementary or substituted method to measure levels (at ng/mL) of anti-HIV drugs alone or in combination.

INTRODUCTION

Zidovudine (AZT, 3′-azido-3′-deoxythymidine), lamivudine (3TC, 2′-deoxy-3′- thiacytidine), stavudine (d4T, 2′,3′-didehydro-3′-deoxythymidine) and didanosine (ddI, 2′,3′-dideoxyinosine) (Fig. 1) are nucleoside reverse transcriptase

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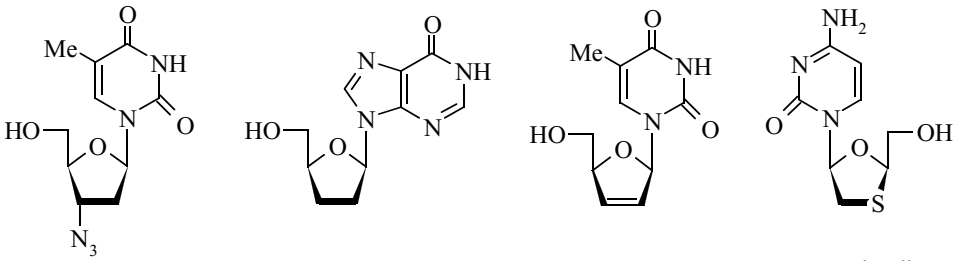


Figure 1. Structure of some FDA approved anti-HIV nucleosides.

inhibitors (NRTIs). Those synthetic nucleoside analogs have demonstrated potent and selective inhibition against the human immunodeficiency virus (HIV). In common with other nucleoside analogues, a NRTI undergoes intracellular phosphorylation, *via* monophosphate and diphosphate, to the active triphosphate metabolite which inhibits HIV reverse transcriptase and acts as chain terminator of the proviral DNA. Recently, the intracellular concentration of the active NRTI-triphosphate has been correlated directly with HIV viral load response (1). Therefore, the measurement of their concentration needs to be determined to establish a relationship between the dosage and therapeutic efficacy.

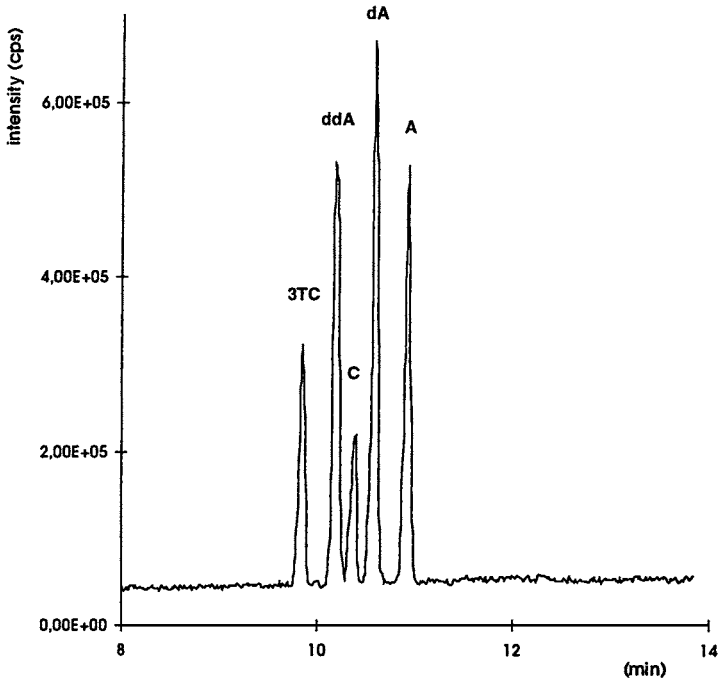
Several analytical methods have been developed for the determination of nucleosides or nucleotides in human biological fluids. In the field of HIV, analyses have been performed mainly by HPLC/RIA (2) or more recently by solid phase extraction (Sep-Pak cartridge) SPE/RIA (3,4) which was reported to improve the processing time for the measurement of AZT and 3TC. Nevertheless, the specificity of the antibodies developed against the nucleosides is essential for the sensitivity of these methods (HPLC/RIA or Cartridge/RIA). Other analytical procedures based on capillary electrophoresis or liquid chromatography interfaced with tandem mass spectrometry, CE/MS/MS (5) or LC/MS/MS (6,7) respectively, have been reported for the intracellular quantitation of the some NRTIs. In the last ten years, capillary electrophoresis (CE) has become a powerful analytical technique for the separation and quantification of pharmaceutical drugs (8), and we have thoroughly studied the improvement of CE resolution occurring with the addition of metal cations (magnesium, calcium, cadmium, zinc) to the buffer for the separation of nucleoside mixtures (9–11). Thus, as part of our on going anti-HIV research, we proposed two complementary CE systems (under acidic or alkaline conditions) to analyze by CE/MS/MS the active triphosphate derivatives of several NRTIs among a pool of natural nucleosides.

RESULTS AND DISCUSSION

Quantitative analysis in forensic and clinical toxicology or pharmacokinetic area has been revolutionized in the last five years by the development of

atmospheric pressure interfaces for mass spectrometry and the LC/MS coupling. At the moment, electrospray-type and APCI interfaces represent the sources of choices for a wide range of organic compounds. 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 (nL/min) 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 selected volatile formic acid-ammonia buffer allows simultaneous direct UV and ionspray MS detections either at acidic or alkaline conditions. In a first step, the composition and the flow rate of the sheath liquid have been optimized to promote better ionization of the analytes and reach better sensitivities. We found that the methanol-water (95/5 v/v) + 0.5% formic acid mixture was suitable as sheath liquid for both acidic and alkaline systems. In positive ionization mode, mass-spectra of these nucleosides exhibit abundant positive M-H^+ ions at m/z 230 for 3TC, 236 for ddA, 252 for dA, 268 for A and 244 for C. Otherwise, simultaneous UV detection was performed at 254 nm (close to the wavelength of maximum absorbance of each nucleoside). Firstly, the separation of natural or anti-HIV nucleosides by CE/ESI/MS has been investigated in acidic medium. Several volatile formic acid-ammonia buffers having the same ionic strength (10 mM) but different pH values varying in the 2–5 pH range were prepared and tested. In these conditions, 3TC, ddA, dA, A and C nucleosides behave as positively charged species and migrated faster than the electroosmotic flow (EOF), while U,T, AZT and d4T are uncharged and migrated slowly at the EOF velocity. In capillary electrophoresis, two main parameters (pH and ionic strength of buffer) determine the migration velocity of each solute according to its pKa value. Decreasing electrophoretic mobility of each nucleoside with pH may be explained by a decrease of its positive apparent charge, particularly near its pKa value. Optimum pH at 2.5 has been selected to avoid any comigration, to increase the solubility of these solutes and to provide better buffer capacity. As expected, the electrophoretic mobility of each nucleoside decreases with increasing ionic strength of buffer. A 10 mM ionic strength of buffer provided a sufficient buffer capacity and a reduced ionic strength suitable for MS detection. So, optimum CE/MS separation of 3TC, ddA, C, dA and A nucleosides mixture was achieved by using formic acid-ammonia (pH 2.5, ionic strength 10 mM), as shown in Figure 2. Resolutions between two consecutive solutes are always greater than 1.5. Neutral nucleosides (AZT, d4T, U and T) migrated at the EOF velocity and are detected at 85 minutes. Otherwise, CE-MS electrophoregram (total ionic current) of nucleosides mixture shows a better signal-to-noise ratio compared to the UV chromatogram.

Secondly, the separation of several anti-HIV nucleosides has been investigated in alkaline medium by CE/ESI/MS. The same formic acid-ammonia buffer has been used in the 9–11 pH range and with higher ionic strength values. After optimization of the resolution, pH 10 and 50 mM of ionic strength have been chosen. Then, AZT, d4T, G, U and T behave as negatively charged species and migrated *slower* than the EOF, while A, C, 3TC, ddA and dA are uncharged and migrated at the

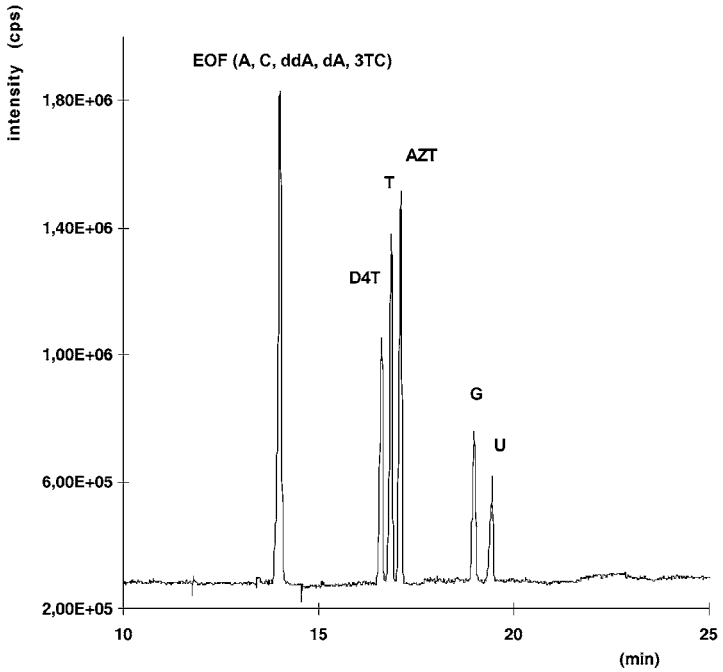


CE : capillary : 70 cm x 50 µm i.d. x 150 µm o.d. ; electrolyte formic acid ammonia (pH 2.5; 10 mM ionic strength) ; detection at 254 nm ; voltage : 25 kV ; temperature : 25 °C ; hydrodynamic injection : 20 s, 50 mbar; nucleoside concentration 10 mg L^{-1}.MS : sheath liquid : methanol-water (95/5 v/v) + 0.5 % formic acid at 5 $\mu L.min^{-1}$; ionspray voltage + 5 kV.

Figure 2. Separation of some antiviral nucleosides (3TC, ddA) by CE/MS with acidic system.

same velocity than EOF. Optimum CE/MS separation of d4T, AZT, T, G and U nucleosides mixture was achieved by using formic acid-ammonia (pH 10, ionic strength 50 mM), as illustrated in Figure 3.

Then, MS-MS detection has also been performed to get better limits of detection (LOD). In MS/MS mode, several nucleosides give the same major product ion due to the loss of the sugar moiety, such as ddA, dA and A (m/z 136), C and 3TC (m/z 112), and also, d4T, AZT and T (m/z 127). So, the analysis and quantitation of all anti-HIV nucleosides among the pool of natural nucleosides could not be performed by FIA/MS/MS, and a separation technique prior MS/MS detection is necessary. Calibration curves were determined in the 250–1000 ng/mL concentration range for UV detection and in the 5-1000 ng/mL concentration range for MS-MS detection, and good linear correlation coefficients were obtained (with MS/MS detection, 0.9981 and 0.9998 for ddA and 3TC, respectively) were obtained. Limits of detection (LOD) or quantitation (LOQ) with MS/MS are around 50-times lower than those obtained by CE/UV. So, CE/MS/MS allows to quantitate **2 ng/mL of ddA, 2.5 ng/mL of AZT, 5 ng/mL of 3TC and 20 ng/mL of d4T.**



CE : capillary : 95 cm x 50 µm i.d x 150 µm o.d ; electrolyte : formic acid-ammonia (pH 10 ; 50 mM ionic strength) ; detection at 254 nm ; applied voltage : + 25 kV ; 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.

Figure 3. Separation of some antiviral nucleosides (d4T, AZT) by CE/MS with alkaline system.

CONCLUSION

In summary, this work has demonstrated the possibility of measuring common anti-HIV nucleoside drugs from other natural nucleosides by using either one or two volatile formic acid/ammonia buffers (acidic system: pH 2.5, 10 mM ionic strength; alkaline system: pH 10, 50 mM ionic strength) in capillary electrophoresis coupled to mass spectrometry with an electrospray ionization. The limits of detection for ddA or AZT are 2 ng/mL and are similar to those attainable by HPLC-radioimmunoassay.

EXPERIMENTAL

All natural nucleosides were supplied by Sigma Chem. (St. Louis, MO, USA): adenosine (A), 2′-deoxyadenosine (dA), cytidine (C), 2′,3′-dideoxyadenosine (ddA), uridine (U), guanosine (G), thymidine (T). The antiviral nucleosides have been synthesized following reported papers or are gift from pharmaceutical companies. 3TC is a gift from Dr. C. K. Chu (UGA, Athens, USA). 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 were filtered before use through a polypropylene filter of 0.22 μm porosity (Prolabo, France).

Capillary electrophoresis 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, U.S.A.), using a fused-silica capillary of 37 cm $\times$ 75 μm i.d $\times$ 375 μm o.d, with a detector length (injection to detection) of 30 cm. The UV detection aperture was 100 μm $\times$ 800 μm. Solutes were injected at the anode by hydrodynamic injection for 10 s., under nitrogen overpressure (0.5 psi). 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. Detector time constant was 1 s and data acquisition rate was 20 Hz. All data were collected using an IBM PS/2 computer with an electrophoresis data calculation program.

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 (SEDERE, Orleans, 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).

The mass spectrometer utilized in all studies was a PE Sciex API 300 (Perkin Elmer Sciex, Toronto, Canada) triple quadrupole instrument equipped with an electrospray ionization (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) mixture and delivered by a Harvard Model 22 syringue pump (Harvard Apparatus, South Natick, MA, USA) at a flow rate of 5 μL/min. 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 amu. 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 $\times$ 50 μm i.d $\times$ 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.

ACKNOWLEDGMENTS

Support of this reasearch by a grant from the Agence Nationale de Recherches sur le SIDA (ANRS, France) and a grant from SIDACTION (France) is gratefully acknowledged.

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