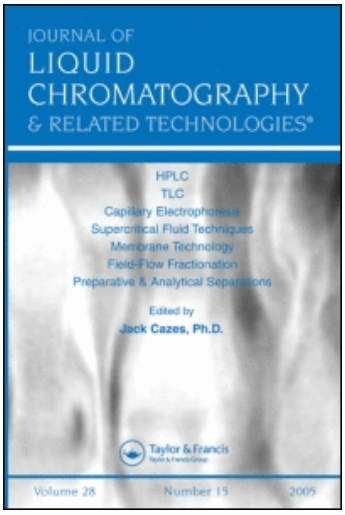

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

DEVELOPMENT OF A CAPILLARY ELECTROPHORESIS PRECONCENTRATION METHOD FOR THE ANALYSIS OF DIDEOXYADENOSINE TRIPHOSPHATE

Swapna Mallampati[a]; Kris Wolfs[a]; Murali Mohan Pendela[a]; Jos Hoogmartens[a]; Ann Van Schepdael[a]
[a] Laboratory for Pharmaceutical Analysis, K.U. Leuven, Leuven, Belgium

Online publication date: 24 March 2010

To cite this Article Mallampati, Swapna , Wolfs, Kris , Pendela, Murali Mohan , Hoogmartens, Jos and Schepdael, Ann Van(2010) 'DEVELOPMENT OF A CAPILLARY ELECTROPHORESIS PRECONCENTRATION METHOD FOR THE ANALYSIS OF DIDEOXYADENOSINE TRIPHOSPHATE', Journal of Liquid Chromatography & Related Technologies, 33: 6, 802 — 817
To link to this Article: DOI: 10.1080/10826071003684281
URL: http://dx.doi.org/10.1080/10826071003684281

Journal of Liquid Chromatography & Related Technologies, 33:802–817, 2010

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826071003684281

Taylor & Francis
Taylor & Francis Group

DEVELOPMENT OF A CAPILLARY ELECTROPHORESIS PRECONCENTRATION METHOD FOR THE ANALYSIS OF DIDEOXYADENOSINE TRIPHOSPHATE

Swapna Mallampati, Kris Wolfs, Murali Mohan Pendela, Jos Hoogmartens, and Ann Van Schepdael

Laboratory for Pharmaceutical Analysis, K.U. Leuven, Leuven, Belgium

□ *The compound 2′,3′-dideoxyadenosine-5′-triphosphate (ddATP) is the intracellular active metabolite of the antiretroviral drug didanosine. Since micellar electrokinetic capillary chromatography with fluorescence detection (MEKC-fluo) by means of precapillary derivatization allowed quantitation of ddATP only in pmol range, development of a more sensitive CE method was intended. CE preconcentration based on electrophoretic effects was tested for additional sensitivity enhancement. Large volume sample stacking (LVSS) using diethylenetriamine (DETA) as an EOF suppressant (ES) in combination with MEKC-fluo was found to be better, but the presence of excess unreacted reagent in the sample hindered the separation of the derivatized nucleotides. Some miscellaneous effects and precapillary derivatization of ddATP at low scale improved the sensitivity of the MEKC-fluo method by minimizing dilution of the sample, which allowed reaching the fmol level that can be found in cells (LOQ= 10 fmol/sample). However, cell studies showed a drastic decrease in fluorescence intensity of the derivatized ddATP peak in the presence of peripheral blood mononuclear cells. Therefore, LVSS-ES using DETA was combined with CZE-UV for the preconcentration of underivatized compounds, which showed promising results with the sensitivity of ddATP being 160 times higher compared to CZE-UV detection without stacking.*

Keywords CE, 2′,3′-dideoxyadenosine-5′-triphosphate, diethylenetriamine, fluorescence detection, large volume sample stacking, preconcentration

INTRODUCTION

The compound 2′,3′-dideoxyadenosine-5′-triphosphate (ddATP) is the intracellular active metabolite of the antiretroviral drug didanosine (ddI). ddATP determination appears crucial for therapeutic drug monitoring of ddI because the intracellular ddATP level did not correspond with levels of ddI measured in plasma.[1] Quantitation of the very low levels

Address correspondence to Dr. Ann Van Schepdael, Laboratory of Pharmaceutical Analysis, K.U. Leuven, O&N II, PB 923, Herestraat 49, 3000 Leuven, Belgium. E-mail: ann.vanschepdael@pharm.kuleuven.be

(10–50 fmol per 10^6 cells) of the active anabolite that can be found in the target peripheral blood mononuclear cells (PBMCs) requires an assay that is sensitive and specific enough to distinguish the compound from endogenous nucleotides and nucleosides.[2] Analytical methods such as radioimmunoassay,[3] LC-MS,[1,2,4] and CE-MS[5,6] have been applied before. They have advantages and disadvantages. In order to establish a sensitive CE method, we have lately examined fluorescence detection which requires prior derivatization.[7] A selective micellar electrokinetic capillary chromatography method with fluorescence detection (MEKC-fluo) had been developed, which was successfully separating the dansyl ethylenediamine (dansyl EDA) derivative of ddATP from the derivatives of other metabolites of ddI such as 2′,3′-dideoxyinosine-5′-monophosphate (ddIMP), 2′,3′-dideoxyadenosine-5′-monophosphate (ddAMP), and 2′,3′-dideoxyadenosine-5′-diphosphate (ddADP), and the endogenous nucleotides 2′-deoxyadenosine-5′-triphosphate (dATP) and adenosine-5′-triphosphate (ATP).[7] Upon dansylation, the sensitivity of ddATP with MEKC-fluo (LOQ = 12 ng/mL) was reported to be 160 times higher than capillary zone electrophoresis with UV detection (CZE-UV) and was better than the CE-MS method (LOQ = 50 ng/mL),[6] however, it would allow intracellular quantitation of ddATP only in the pmol range.[7] Therefore, for the present work, further improvement in sensitivity was aimed to combine MEKC-fluo via precapillary derivatization or CZE-UV for underivatized nucleotides with on-line capillary focusing methods based on electrophoretic effects for preconcentration.

Sample stacking in CE has been a sophisticated tool based on simple principles, which stem from inherent effects in electromigration systems. It enables the on-line accumulation of diluted analytes from a long injected sample zone into a much shorter sharp zone with much higher concentrations of these analytes to be separated and detected.[8] Extensive reviews are available, which give a clear overview of basic principles, diverse types, and advances of in-line preconcentration techniques for enhancing the sensitivity in CE.[8–10] To date, a number of stacking or in-line preconcentration techniques based on field strength induced changes in velocity [field amplified sample stacking (FASS), field amplified sample injection (FASI), isotachophoretic stacking (ITP), and countercurrent electroconcentration, or chemically induced changes in velocity (dynamic pH junction, sweeping with pseudophases) or physically induced changes in velocity (membrane filtration) have been developed.[9]

FASS is the simplest on-column concentration technique to increase CE sensitivity. It is achieved simply by having the conductivity of the electrolyte at least ten times higher than that of the sample. When high voltage is applied across the capillary, the sample ions experience higher field strength than the electrolyte ions due to the difference in conductivity

between the zones. This causes the sample ions to migrate quickly to the sample electrolyte interface. Here, the field strength of the ions is lower, and the sample ions slow down when they pass the boundary between the sample and the electrolyte compartments and stack into a zone at the sample electrolyte interface, which is much narrower than the original sample plug. FASS's restriction of the sample injection volume to around 5% of the total capillary volume led to the discovery of two primary modes of large volume sample stacking (LVSS), with and without polarity switching, which allow injection up to 100% of the capillary volume without causing loss in efficiency. LVSS with polarity switching (LVSS-PS) removes the sample matrix via EOF, while the stacked zone gradually moves back towards the inlet.[11–13] It is critical during this approach that the polarity of the system is reversed for the separation before the stacked analyte zone exits the inlet of the capillary. LVSS without the need of polarity switching can be performed by simply suppressing or changing the direction of the EOF during the stacking/matrix removal process by including a dynamic EOF suppressant (ES) or reversal agent in the separation electrolyte (LVSS-ES).[14–17] This ensures that no analytes are lost from the inlet of the capillary as the transition from stacking to separation is controlled chemically, and not via manually changing the voltage.[9]

EXPERIMENTAL

Materials

Ammonium acetate anhydrous, lithium dodecyl sulfate (LiDS), hydroxypropyl methyl cellulose (HPMC), and methanol were purchased from Acros Organics (Geel, Belgium). Ammonium hydroxide solution and sodium hydroxide pellets were purchased from Sigma–Aldrich Laborchemikalien (Seelze, Germany). Hydrochloric acid was from Chem-Lab MV (Zedelgem, Belgium). Dansyl EDA was purchased from Toronto Research Chemicals (Ontario, Canada). 3-(N,N-Dimethylpalmitylammonio)-propanesulfonate (SB3-16), anhydrous magnesium chloride, and hexamethonium hydroxide (HM) solution were obtained from Fluka Chemika (Buchs, Switzerland). 1-Methylimidazole, *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDAC), diethylenetriamine (DETA), hydroxyethylcellulose (HEC), poly(ethyleneimine) solution 50% w/v solution (PEI), and the reference standards of dATP and ATP were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). ddATP was purchased from Biolog Life Science Institute (Bremen, Germany). All solutions were prepared with ultrapure Milli-Q water (Millipore, Milford, MA, USA). The BGE was filtered with a 0.2 µm Uniflo 25 RC filter (Schleicher & Schuell MicroScience, Dassel, Germany).

Instrumentation

CE experiments involving UV detection were performed on a Beckman P/ACE MDQ instrument equipped with a photo diode array detector from Beckman Coulter (Fullerton, CA, USA). Data acquisition was done by means of 32 Karat^{TM} version 5.0 software (Fullerton, CA, USA). CE experiments involving fluorescence detection were performed on an Agilent CE G1600A system (Agilent Technologies, Waldbronn, Germany) with Argos 250 B fluorescence detector from Flux Instruments AG (Basel, Switzerland). The uncoated fused silica capillary was obtained from Polymicro Technologies (Phoenix, AZ, USA). The Thermomixer comfort used for derivatization reactions was from Eppendorf AG (Hamburg, Germany). The pH measurements were performed on a Metrohm 691 pH meter (Herisau, Switzerland). To ensure consistent results, it was calibrated before each measurement with reference buffer solutions.[18]

Electrophoresis

A new capillary was conditioned at 50°C by rinsing with 1 M NaOH, 0.1 M NaOH, and water for 5 min each. At the beginning of each day prior to the analyses, the capillary was activated by flushing with 1 M NaOH, 0.1 M NaOH, and water for 5 min each at 25°C. All the washings were performed by applying a pressure of 138 kPa. The inlet/outlet vials were renewed for every run.

MEKC-Fluo

The primary electrophoretic conditions with fluorescence detection and the precapillary derivatization with dansyl EDA through the phosphate group of ddATP were as described in Ref.[7]. They include the use of an uncoated fused silica capillary with an L_T of 40 cm, an L_E of 24 cm and an id of 75 μm, a BGE containing 70 mM LiDS and 140 mM ammonium acetate at pH 9.0 (adjusted with 2 M ammonium hydroxide solution), an applied voltage of 7.0 kV (normal polarity) and the capillary temperature maintained at 15°C. The current corresponding to these conditions was about 125 μA. In between runs, the capillary was rinsed with 0.1 M NaOH for 2 min followed by a rinse with the BGE for 3 min. The samples were hydrodynamically injected for 20.0 s × 3.5 kPa. Detection was performed at an excitation wavelength of 310 nm using a monochromator and at an emission wavelength above 495 nm using a 495 nm Cut off Long pass emission filter. The 495 nm Cut-off Long-pass emission filter used in the present study has a maximum transmission of 99.9% at 555 nm. The photomultiplier tube (PMT) voltage was 1139 V. The composition of the BGE and the injection time were varied for preconcentration experiments as described in the results and discussion section, where necessary.

LVSS-ES with CZE-UV

The optimized electrophoretic conditions include the use of an uncoated fused silica capillary with a total length (L_T) of 40 cm, effective length (L_E) of 30 cm, and an id of 75 μm, a BGE containing 7 mM DETA, and 40 mM ammonium acetate at pH 9.0 (adjusted with 2 M ammonium hydroxide solution), an applied voltage of 20 kV (reverse polarity), and the capillary temperature maintained at 15°C using liquid coolant. The current corresponding to these conditions was about −85 μA. To ensure repeatable separations, the BGE containing DETA should be prepared fresh daily. The capillary was rinsed with BGE for 2 min in between runs. The samples were hydrodynamically injected for 93.0 s × 8.3 kPa. Online detection was performed by UV light at 205 nm. The spiked sample was prepared by diluting stock solutions of all the compounds to 100 μL to obtain 2 μg/mL of ATP; 1 μg/mL of ddAMP; 0.7 μg/mL of ddADP; 0.6 μg/mL of ddIMP, and 0.1 μg/mL of dATP and ddATP.

PBMC Sample Treatment

Human PBMC sample pellets were purchased from Biopredic international (Rennes, France) and stored at −80°C. Before use, each PBMC sample vial containing 10×10^6 cells was transferred to 4°C and spiked with different concentrations of ddATP (10 fmol/sample – 500 pmol/sample). The spiked PBMCs were lysed in 400 μL of cooled buffer (0.1 M 1-methylimidazole buffer pH 8 – methanol; 30:70% *v*/*v*) stored at −20°C. After cell lysis, the cell lysate was centrifuged for 30 min at 14500 rpm with intermittent cooling every 10 min at 4°C for 5 min duration. The supernatant was taken and the methanol content was evaporated using a TurboVap evaporator (Zymark, France) at 25°C for 10 min at 2 psi and 20 min at 5 psi. The remaining fraction was made up to 200 μL with 0.1 M 1-methylimidazole buffer pH 8.0 (adjusted with 0.1 M HCl) and was reacted with 10 μL of 140 mM EDAC in 1-methylimidazole buffer and 40 μL of 30 mM dansyl EDA in DMF. The reaction was mixed in the dark at 450 rpm for 90 min at 60°C using an Eppendorf Thermomixer comfort, cooled at 0°C for 5 min and centrifuged for 60 s before injection.

RESULTS AND DISCUSSION

Additional Sensitivity Enhancement

In order to gain additional sensitivity, on-line capillary focusing methods based on electrophoretic effects such as field strength induced changes in velocity (tITP or FASS)[9] were tested. In order to combine them with

MEKC-fluo, several limitations need to be surpassed such as the presence of excess unreacted reagent in the sample, the partial charge neutralization of the phosphate group of ddATP whilst derivatization, and lack of the possibility to modify the sample conductivity. LVSS-ES was found to be a convenient preconcentration strategy for derivatized nucleotides in combination with MEKC-fluo compared to tITP and LVSS-PS, which showed only huge overloaded peaks of reagent and no sample peaks (data not shown).

LVSS-ES of Derivatized Nucleotides in Combination with MEKC-Fluo

Precapillary derivatization was combined with LVSS using EOF suppressants (ES) to pump the sample matrix out of the capillary without the need for PS. For the present work, LVSS using low pH BGE to suppress the EOF was not considered due to acid instability of ddATP. Initially, EOF suppressants such as HPMC and HEC were tried, which dynamically surface coat the capillary by adsorption to the silica wall via hydrogen bonding (hydroxyl or ether group) of the polymers[19] and increase the viscosity of the BGE. HPMC showed poor solubility in the BGE, therefore HEC was preferred. An increase in HEC concentration to 0.1% decreased the EOF to a constant value but it was not significant enough, probably due to the presence of anionic micelles in the BGE. Although HEC is a nonionic hydrophilic polymer it is being charged by the anionic micelles present in the BGE due to their interaction or adsorption on to the polymer.[20] To verify this, EOF suppression using HEC was checked both in non micellar BGE (NMBGE) and micellar BGE (MBGE). The EOF mobility was calculated using 70% methanol. In the NMBGE, a concentration of 0.05% HEC decreased the EOF mobility from 16 to $\sim 9\,cm^2V^{-1}s^{-1}$, however, removing the micelles from the BGE would bring the sensitivity down.[7] In the MBGE, the EOF mobility decreased from 19 to $\sim 15\,cm^2V^{-1}s^{-1}$. For additional EOF suppression in the MBGE containing HEC, 0.5–2.5 μg/mL of PEI was added and the EOF was significantly suppressed in the presence of micelles but PEI was found to precipitate. The use of zwitterionic surfactants to suppress the EOF by forming a hemimicelle layer on the capillary surface has been reported for stacking of either positively or negatively charged analytes.[16,21–23] Hence, SB3-16 was tried to suppress the EOF. In contrast the EOF was found to increase with an increase in concentration of SB3-16. This could be due to the presence of lithium dodecyl sulfate (LiDS) in the BGE, which incorporates into the zwitterionic hemimicelle layer, making it charged. The high buffer pH or the high LiDS concentration of the BGE can also disrupt the negatively charged hemimicelle by electrostatic repulsion with the capillary wall.[22]

Divalent metal cations (M^{2+}), alkylammonium ions, and alkylamines have been reported to suppress the EOF by undergoing dynamic ion exchange at the capillary wall with the buffer cations that are intimately associated with the silanols.[24] Precipitation is a concern with most of the M^{2+},[24] hence, to start with, HM in its hydroxide form and DETA were tried. An increase in HM concentration from 0 to 1 mM did not show any significant EOF suppression, whereas, an increase in concentration of DETA from 0 to 10.8 mM showed a significant decrease in EOF mobility from ~22 to ~5 $cm^2V^{-1}s^{-1}$, however, an increase in the BGE pH also. Recently, the use of cation additives to control the EOF in MEKC has been explored and Mg^{2+} was reported to suppress the EOF to the highest degree compared to monovalent cations.[25] A concentration of 2 mM $MgCl_2$ significantly decreased the EOF mobility and the methanol peak was not found even after 90 min. For combining LVSS-ES with precapillary derivatization, either 2 mM Mg^{2+} or 7.5 mM DETA were employed as EOF modifiers, which showed significant suppression in the presence of micelles. Other electrophoretic conditions were as mentioned in the "MEKC-fluo" section in Materials and methods except for the following changes: reverse polarity instead of normal polarity (−7 kV instead of 7 kV) and injection time increased from 20 to 120 s (up to the fluorescence flow cell window). However, at these conditions high currents were produced especially with Mg^{2+} containing BGE (I = −150 μA). The high currents were not suitable for the LVSS-ES stacking causing the current to drop during the run. Decreasing the voltage did not show much improvement with the Mg^{2+} system but it did with the DETA system. A voltage of −4 kV (I = −67 μA) was used, however, with an increase in run time. The run times were also longer due to the counter-EOF conditions.[24] The analysis of the reaction mixture showed two huge reagent peaks and the dansyl EDA derivatives of ATP, dATP, and ddATP being partially separated from the reagent peaks, see Fig. 1. The LOQ values couldn't be determined for the dansyl EDA derivatives of ddATP and dATP as they were migrating as shoulder peaks to the reagent or blank peaks which hindered their separation. A concentration of 30 mM excess dansyl EDA was required for the reaction and the use of lower concentrations had an impact on the reaction yields. Separation at lower injection times could be investigated for better separation and reduced reagent peaks, however, with decrease in sensitivity.

Miscellaneous Effects

Some miscellaneous effects gained additional sensitivity enhancement, which was fifty times higher (LOQ = 0.24 ng/mL) compared to the reported MEKC-fluo method[7] for the dansyl EDA derivative of ddATP.

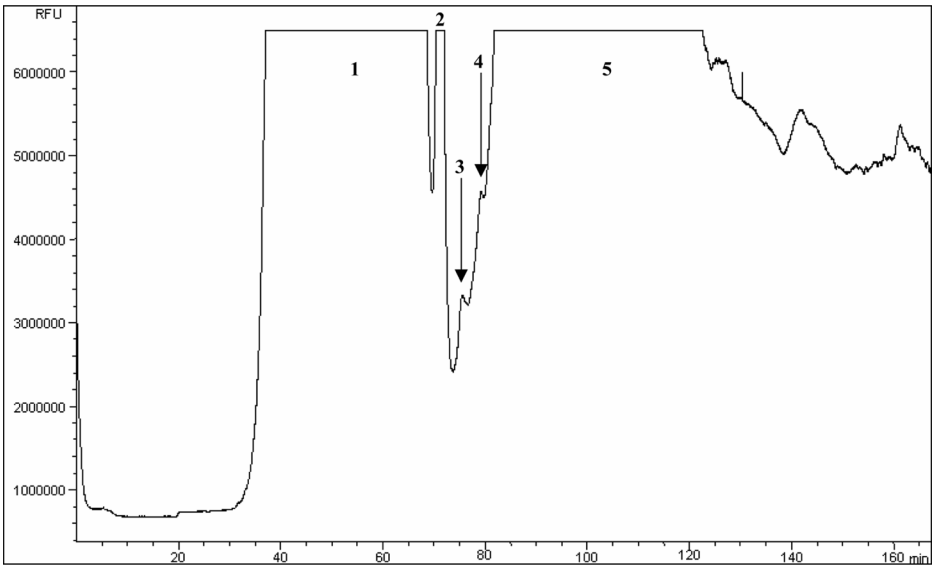

FIGURE 1 A typical electropherogram obtained by LVSS-ES after precapillary derivatization using DETA as an EOF suppressant. Capillary: uncoated fused-silica capillary ($L_T = 40$ cm, $L_E = 24$ cm, id = 75 μm); BGE: 7.5 mM DETA, 70 mM LiDS and 140 mM ammonium acetate at pH 9.0; applied voltage: 4.0 kV (reverse polarity); capillary temperature: 15°C (I = −67 μA); injection: 120.0 s × 3.5 kPa; fluorescence detection with λ_{exc} of 310 nm, λ_{em} of above 495 nm (Cut-off Long-pass filter) and PMT voltage of 1139 V; sample: 2 μg/mL ATP, 24 ng/mL dATP and 12 ng/mL ddATP; Peaks 1&5: excess reagent, 2: dansyl EDA derivative of ATP, 3: dansyl EDA derivative of dATP, 4: dansyl EDA derivative of ddATP.

They include capillary effects, the use of centrifugation instead of filtering the sample before injection, the use of pure reagent grade DMF, preferably N_2 flushed for dissolving the reagent dansyl EDA, and storage of 1-methylimidazole in a dry and inert atmosphere (under N_2). The use of a new batch of capillary showed better sensitivity, which was a few orders of magnitude higher than the old batch. This could be due to a difference in capillary walls producing different background signals, which can have an impact on the *S/N*.[26] Centrifugation instead of filtering the sample through 0.2 μm membrane filter yielded better sensitivity, probably because of loss of sample due to some adsorption of the dansyl EDA derivatives to the filter membrane. These miscellaneous effects would allow quantitation of ddATP in the higher fmol range (125 fmol/sample). For the quantitation of ddATP in cells at least two times better sensitivity will be required.

Derivatization at Low Scale

As a last step to enhance the sensitivity the reaction volume was decreased from 250 to 125 μL. A 1.0 μL aliquot of ddATP standard solution (23.8 ng/mL) taken in 100 μL of 1-methylimidazole buffer (0.1 M, pH 8.0)

was reacted with 5 μL of 140 mM EDAC in 1-methylimidazole buffer and 20 μL of 30 mM dansyl EDA in DMF. The mixture was stirred in the dark at 450 rpm for 90 min at 60°C using a Thermomixer and cooled at 0°C for 5 min before injection. The analysis of the reaction mixture showed an increase in sensitivity of the dansyl EDA derivative of ddATP, see Fig. 2. An improved sensitivity (LOQ = 10 fmol/sample) was achieved which was 12.5 times higher compared to the miscellaneous effects-MEKC-fluo

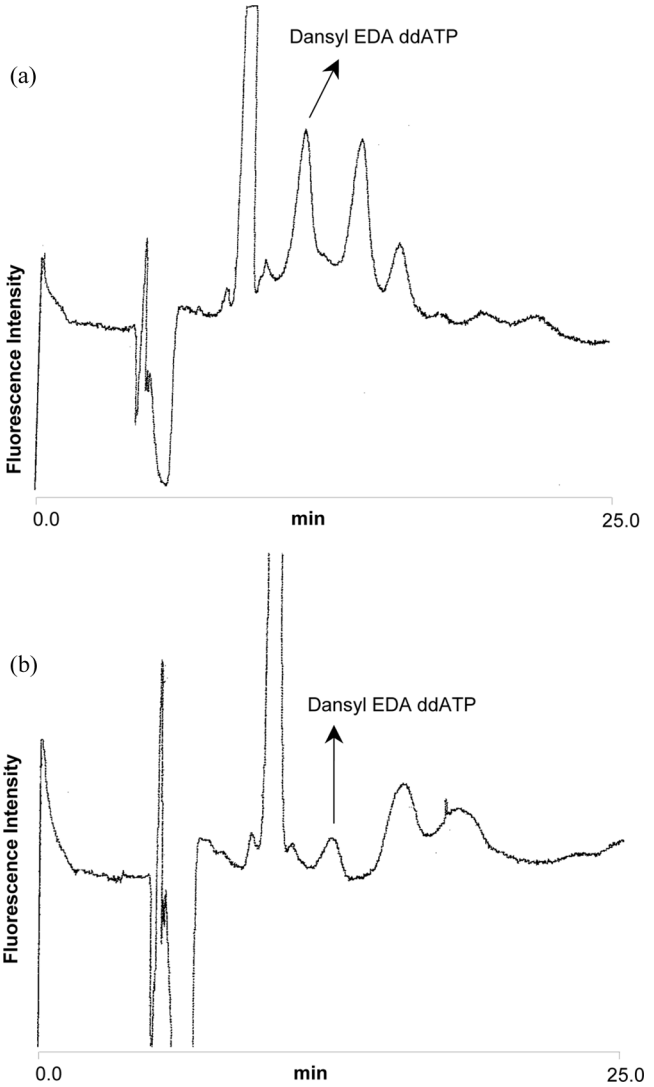

FIGURE 2 A typical electropherogram obtained by precapillary derivatization at low scale. (a) 50 fmol of ddATP/sample (b) 10 fmol of ddATP/sample. Electrophoretic conditions were as described in the "MEKC-fluo" section in Materials and Methods.

method (LOQ = 125 fmol/sample). This sensitivity enhancement would allow quantitation of ddATP at concentrations found in the cells and therefore spiking experiments were performed in the following section.

Cell Studies

Peripheral blood mononuclear cells (PBMCs) were chosen as the target cells. Initially, PBMCs available in suspension were purchased; however, the selectivity was very poor with overloaded derivatized extracellular protein peaks co-migrating with the dansyl EDA derivatives. Hence, PBMC samples in the form of pellets, which were more thoroughly washed to remove the extracellular matter, were preferred. The PBMC sample treatment was optimized and was as described in the "PBMC sample treatment" section in Materials and Methods. The 1-methylimidazole buffer used for derivatization could also be used as cell lysis buffer. Unfortunately, the analysis of the reaction mixture containing 10 fmol/sample of ddATP and blank cell lysate showed similar peaks and the dansyl EDA derivative of ddATP is probably co-migrating with the blank endogenous peaks. This did not improve even when the sample concentration was increased from 10 to 500 fmol/sample. Therefore, the stability or recovery of the dansyl EDA derivative of ddATP in the presence of PBMCs was studied at a high concentration of 500 pmol/sample ddATP. The analysis of the reaction mixture showed a peak visible for the dansyl EDA ddATP, but its fluorescence intensity was only 30% of the originally expected value obtained without the cells, see Fig. 3. This decrease could be due to fluorescence quenching of the dansyl EDA ddATP in the presence of cells or due to poor recovery.

Although high sensitivities were achieved by combining miscellaneous effects and derivatization at low scale with the MEKC-fluo method, the cell studies show that it is not a reliable method for the intracellular analysis of ddATP.

LVSS-ES of Underivatized Nucleotides in Combination with CZE-UV

The CZE-UV method developed in our previous work[7] was combined with LVSS-ES to check the sensitivity enhancement for the underivatized compounds. Similar electrophoretic conditions were used initially, except for the following changes: 75 μm id capillary instead of 50 μm and the applied voltage was adapted accordingly from 15.0 to 8.4 kV; reverse polarity instead of normal polarity and sample injections were performed up to the UV window by applying a pressure of 8.3 kPa for 93 s. LVSS-ES was performed using Mg^{2+} or DETA as EOF suppressants. With 2 mM Mg^{2+} as EOF suppressant sharp intense peaks were found at 205 nm but

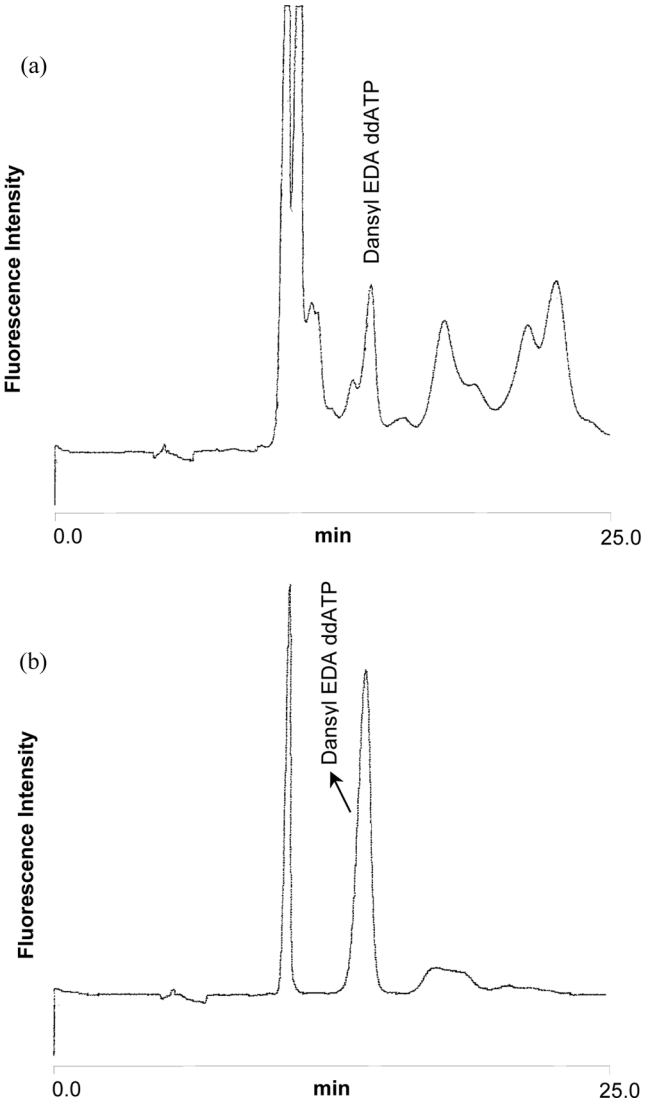

FIGURE 3 Comparison of fluorescence intensity of dansyl EDA derivative of ddATP peak (a) in the presence of 10×10^6 cells (b) without cells. Electrophoretic conditions were as described in the "MEKC-fluo" section and sample treatment as described in the "PBMC sample treatment" section in Materials and Methods. Sample: 500 pmol of ddATP/sample.

less intense peaks at 260 nm. This might be due to some complexation between the nucleotides and Mg^{2+}. The samples had to be dissolved in 2 mM of $MgCl_2$ instead of water to decrease EOF mismatch between the sample and the BGE. This LVSS-ES system could also be combined with sweeping. Maximum sensitivity was targeted by varying the concentration of LiDS from 0 to 70 mM in the BGE and a concentration of 50 mM was

found to be optimal. However, the analysis of a spiked mixture containing all the compounds showed only one peak, whereas individual analysis except for ATP showed peaks at different migration times, see Fig. 4. This phenomenon couldn't be explained because increasing the concentration of $MgCl_2$ in the sample and BGE to 8 mM for more complexation to occur, showed noisy baselines.

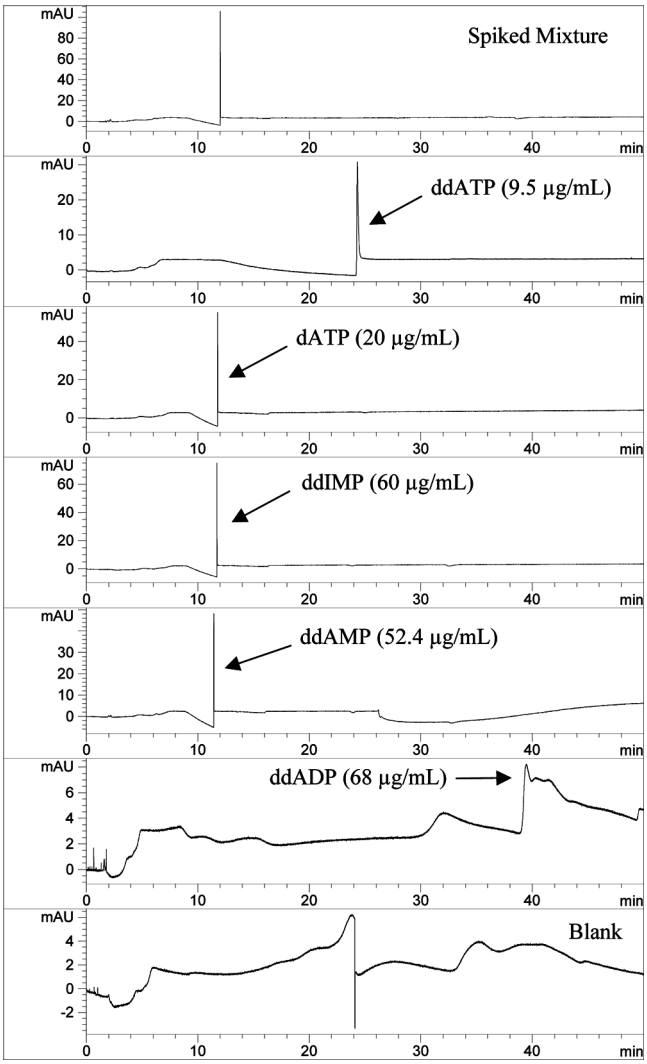

FIGURE 4 An overlay of electropherograms obtained by LVSS-ES using Mg^{2+} as the EOF suppressant combined with CZE-UV detection. Capillary: uncoated fused-silica capillary ($L_T = 40$ cm, $L_E = 31.5$ cm, id = 75 µm); BGE: 2 mM $MgCl_2$ and 100 mM ammonium acetate at pH 9.0; applied voltage: −8.4 kV (reverse polarity); capillary temperature: 15°C (I = ∼ −92 µA); injection: 160.0 s × 3.5 kPa; UV detection at 205 nm.

Subsequently, LVSS-ES was performed using DETA as the EOF suppressant. A concentration of 7 mM DETA was found to be optimal (reduction of EOF from ~20 to ~11 $cm^2V^{-1}s^{-1}$) and all the compounds eluted within 75 min with increased sensitivity. However, the compound ddADP was found to be co-migrating with ATP and ddAMP with ddATP. Optimum and rapid separations were targeted by decreasing the ammonium acetate concentration from 100 to 40 mM and increasing the voltage from 8.4 to 20 kV. A concentration of 40 mM ammonium acetate and an applied voltage of 20 kV allowed baseline separation of all the compounds within 50 min, see Fig. 5. A voltage higher than 20 kV led to high currents and was not favorable for the LVSS-ES stacking causing the current to drop during the run. The sensitivity was a few orders of magnitude higher at 205 nm compared to 260 nm. To further enhance the sensitivity, the capillary length was increased from 40 to 50 cm so that the sample injection plugs length can be increased from 30 to 40 cm (effective length). However, there was an increase in migration time of ddATP with a decrease in sensitivity although similar field strengths were applied. Subsequently, the method was also combined with sweeping by adding LiDS to the BGE. However, at a concentration of 10 mM LiDS, the migration times drastically increased. Only 2 out of 6 compounds were eluted at 100 and 150 min, respectively, and the remaining compounds were not eluted even after 200 min. The finally optimized electrophoretic conditions were as

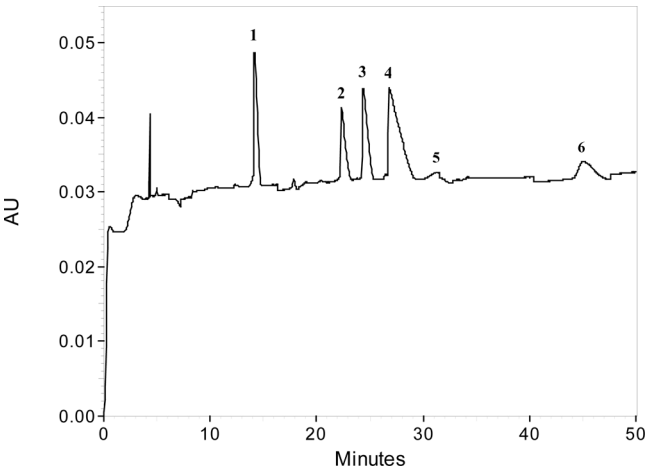

FIGURE 5 A typical electropherogram obtained by LVSS-ES combined with CZE-UV detection. Capillary: uncoated fused-silica capillary ($L_T = 40$ cm, $L_E = 30$ cm, id = 75 μm); BGE: 7 mM DETA and 40 mM ammonium acetate at pH 9.0 (adjusted with 2 M ammonium hydroxide solution); applied voltage: 20.0 kV (reverse polarity); capillary temperature: 15°C (I = −84 μA); injection: 93.0 s × 8.3 kPa; UV detection at 205 nm; sample: 2 μg/mL ATP, 1 μg/ml of ddAMP, 0.7 μg/ml of ddADP, 0.6 μg/ml of ddIMP and 0.1 μg/mL of dATP and ddATP; Peaks 1: ddIMP, 2: ddADP, 3: ddAMP, 4: ATP, 5: dATP, 6: ddATP.

mentioned in "LVSS-ES with CZE-UV" section in Materials and Methods. With LVSS-ES combined to CZE-UV detection, the LOQ for underivatized ddATP was found to be 12 ng/mL (2.5 pmol/sample), which was 160 times lower than CZE-UV detection without stacking (LOQ = 1.9 μg/mL). A similar sensitivity enhancement as achieved for the dansyl EDA derivatized ddATP with MEKC-fluo[7] was thus possible with the LVSS-ES combined to CZE-UV for the underivatized ddATP. However, this method would allow intracellular quantitation of ddATP only in the pmol range. Other concerns include unstable baseline causing deformation of peaks at low concentrations due to the adsorption-desorption kinetics of DETA[15] and repeatability of the migration times. Due to the very low concentrations of the volatile DETA in the BGE, it needs to be prepared fresh daily for repeatable separations. This volatile system can be coupled to MS for further sensitivity enhancement.

CONCLUSIONS

LVSS-ES using DETA as an EOF suppressant was found to be a good preconcentration strategy in combination with MEKC-fluo, but the presence of excess unreacted reagent in the sample hindered the separation of the dansyl EDA derivatives. Some miscellaneous effects gained additional sensitivity enhancement (LOQ = 0.24 ng/mL), which was 50 times higher compared to the earlier MEKC-fluo method (LOQ = 12 ng/mL). Precapillary derivatization of ddATP at low scale further improved the sensitivity by minimizing sample dilution, which allowed reaching the fmol level that can be found in cells (LOQ = 10 fmol/sample). Unfortunately, studies in which cells were spiked with ddATP showed a significant decrease in fluorescence intensity of the dansyl EDA ddATP peak in the presence of PBMCs and the recovery was found to be only 30%. This concluded that MEKC-fluo is not a reliable method and other approaches will thus need to be followed in order to analyze the intracellular ddATP levels.

LVSS-ES using DETA was successfully combined with CZE-UV detection for the underivatized compounds and the sensitivity of ddATP was 160 times higher than the CZE-UV detection without stacking. This volatile on-line preconcentration method can be combined with MS for further sensitivity enhancement.

ACKNOWLEDGMENTS

Funding of this work by the Flemish Fund for Scientific Research (Research Project G.0355.06) is gratefully acknowledged. S.M thanks the Onderzoeksfonds K.U.Leuven/Research Fund K.U.Leuven for financial

support. The authors also acknowledge the help of Prof. P. Annaert (Laboratory for Pharmaceutical Technology and Biopharmacy, K.U.Leuven) with PBMC cell counting.

REFERENCES

1. Becher, F.; Landman, R.; Mboup, S.; Kane, C.N.T.; Canestri, A.; Liegeois, F.; Vray, M.; Prevot, M.H.; Leleu, G.; Benech, H. Monitoring of didanosine and stavudine intracellular trisphosphorylated anabolite concentrations in HIV-infected patients. Aids **2004**, *18*(2), 181–187.
2. Becher, F.; Pruvost, A.; Gale, J.; Couerbe, P.; Goujard, C.; Boutet, V.; Ezan, E.; Grassi, J.; Benech, H. A strategy for liquid chromatography/tandem mass spectrometric assays of intracellular drugs: Application to the validation of the triphosphorylated anabolite of antiretrovirals in peripheral blood mononuclear cells. J. Mass Spectrom. **2003**, *38*(8), 879–890.
3. Le Saint, C.; Terreux, R.; Duval, D.; Durant, J.; Ettesse, H.; Dellamonica, P.; Guedj, R.; Vincent, J.P.; Cupo, A. Determination of ddATP levels in human immunodeficiency virus-infected patients treated with dideoxyinosine. Antimicrob. Agents Chemother. **2004**, *48*(2), 589–595.
4. Becher, F.; Pruvost, A.; Goujard, C.; Guerreiro, C.; Delfraissy, J.F.; Grassi, J.; Benech, H. Improved method for the simultaneous determination of d4T, 3TC and ddI intracellular phosphorylated anabolites in human peripheral-blood mononuclear cells using high-performance liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. **2002**, *16*(6), 555–565.
5. Cahours, X.; Dessans, H.; Morin, P.; Dreux, M.; Agrofoglio, L. Determination at ppb level of an anti-human immunodeficiency virus nucleoside drug by capillary electrophoresis-electrospray ionization tandem mass spectrometry. J. Chromatogr. A **2000**, *895*(1–2), 101–109.
6. Bezy, V.; Chaimbault, P.; Morin, P.; Unger, S.E.; Bernard, M.C.; Agrofoglio, L.A. Analysis and validation of the phosphorylated metabolites of two anti-human immunodeficiency virus nucleotides (stavudine and didanosine) by pressure-assisted CE-ESI-MS/MS in cell extracts: sensitivity enhancement by the use of perfluorinated acids and alcohols as coaxial sheath-liquid make-up constituents. Electrophoresis **2006**, *27*(12), 2464–2476.
7. Mallampati, S.; Van Aerschot, A.; Hoogmartens, J.; Van Schepdael, A. Analysis of dideoxyadenosine triphosphate by capillary electrophoresis with fluorescence detection. I. Derivatization through the phosphate group. Electrophoresis **2007**, *28*(21), 3948–3956.
8. Mala, Z.; Krivankova, L.; Gebauer, P.; Bocek, P. Contemporary sample stacking in CE: A sophisticated tool based on simple principles. Electrophoresis **2007**, *28*(1–2), 243–253.
9. Breadmore, M.C. Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips. Electrophoresis **2007**, *28*(1–2), 254–281.
10. Gebauer, P.; Mala, Z.; Bocek, P. Recent progress in capillary ITP. Electrophoresis **2007**, *28*(1–2), 26–32.
11. Chien, R.L.; Burgi, D.S. Sample stacking of an extremely large injection volume in high-performance capillary electrophoresis. Anal. Chem. **1992**, *64*(9), 1046–1050.
12. Albert, M.; Debusschere, L.; Demesmay, C.; Rocca, J.L. Large-volume stacking for quantitative analysis of anions in capillary electrophoresis. 1. Large-volume stacking with polarity switching. J. Chromatogr. A **1997**, *757*(1–2), 281–289.
13. Chien, R.L.; Burgi, D.S. On-column sample concentration using field amplification in CZE. Anal. Chem. **1992**, *64*(8), A489–A496.
14. Burgi, D.S. Large-volume stacking of anions in capillary electrophoresis using an electroosmotic flow modifier as a pump. Anal. Chem. **1993**, *65*(24), 3726–3729.
15. Albert, M.; Debusschere, L.; Demesmay, C.; Rocca, J.L. Large-volume stacking for quantitative analysis of anions in capillary electrophoresis. 2. Large-volume stacking without polarity switching. J. Chromatogr. A **1997**, *757*(1–2), 291–296.
16. Baryla, N.E.; Lucy, C.A. pH-Independent large volume sample stacking of positive or negative analytes in capillary electrophoresis. Electrophoresis **2001**, *22*(1), 52–58.
17. Kim, B.; Chung, D.S. Large-volume stacking in capillary electrophoresis using a methanol run buffer. Electrophoresis **2002**, *23*(1), 49–55.

18. *European Pharmacopoeia*. European Directorate for the Quality of Medicines of the Council of Europe: Strasbourg, France, 2008.
19. Tian, H.J.; Landers, J.P. Hydroxyethylcellulose as an effective polymer network for DNA analysis in uncoated glass microchips: Optimization and application to mutation detection via heteroduplex Analysis. Anal. Biochem. **2002**, *309*(2), 212–223.
20. Ridell, A.; Evertsson, H.; Nilsson, S. Influence of counterion on the interaction of dodecyl sulfates and cellulose ethers. J. Colloid Interface Sci. **2002**, *247*(2), 381–388.
21. Yeung, K.K.C.; Lucy, C.A. Suppression of electroosmotic flow and prevention of wall adsorption in capillary zone electrophoresis using zwitterionic surfactants. Anal. Chem. **1997**, *69*(17), 3435–3441.
22. Yeung, K.K.C.; Lucy, C.A. Improved resolution of inorganic anions in capillary electrophoresis by modification of the reversed electroosmotic flow and the anion mobility with mixed surfactants. J. Chromatogr. A **1998**, *804*(1–2), 319–325.
23. Baryla, N.E.; Lucy, C.A. Simultaneous separation of cationic and anionic proteins using zwitterionic surfactants in capillary electrophoresis. Anal. Chem. **2000**, *72*(10), 2280–2284.
24. Melanson, J.E.; Baryla, N.E.; Lucy, C.A. Dynamic capillary coatings for electroosmotic flow control in capillary electrophoresis. TRAC – Trends Anal. Chem. **2001**, *20*(6–7), 365–374.
25. Qu, Q.; Tang, X.Q.; Mangelings, D.; Wang, C.Y.; Yang, G.J.; Hu, X.Y.; Yan, C. Control of Electroosmotic flow by a cation additive to enhance the separation of amino acids by micellar electrokinetic chromatography. J. Chromatogr. B **2007**, *853*(1–2), 31–37.
26. Khaledi, M.G. *High-Performance Capillary Electrophoresis: Theory, Techniques and Applications*, John Wiley & Sons: New York, 1998.