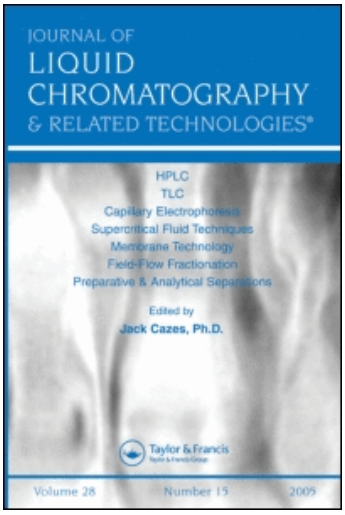

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Analysis of Dideoxyadenosine Triphosphate by Capillary Electrophoresis with Fluorescence Detection. Derivatization Through the Adenine Group

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Abstract: The adenine base of 2′,3′-dideoxyadenosine-5′-triphosphate (ddATP) was chosen as the target for precapillary derivatization for its analysis by CE with fluorescence detection. Several fluorophores, such as chloroacetaldehyde, 9-fluorenylmethyloxycarbonylchloride (FMOC), fluorescamine (Fluram), *o*-phthaldialdehyde (OPA) and complexation with terbium (Tb), gadolinium (Gd), and phenanthroline (phen) were tried. Precapillary derivatization of ddATP with chloroacetaldehyde showed poor peak shape for the ε-ddATP adduct and degradation peaks of ε-adenine, possibly due to the low sample pH of 4.0. Precapillary derivatization of ddATP with OPA/β-mercaptoethanol, Fluram, and FMOC showed no fluorescent derivatization products. Complexation of ddATP with Tb-Gd-phen produced a sharp fluorescent peak. The sensitivity of the Tb-Gd-ddATP-phen complex was found to be 20 times higher than UV detection of ddATP.

Keywords: 2′,3′-Dideoxyadenosine-5′-triphosphate, CE, Didanosine, Fluorescence detection, Human immunodeficiency virus, Precapillary derivatization

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INTRODUCTION

Didanosine (2′,3′-dideoxyinosine; ddI) requires intracellular metabolism, via monophosphate and diphosphate, to the active triphosphate, 2′,3′-dideoxyadenosine-5′-triphosphate (ddATP), which acts as a competitive inhibitor of human immunodeficiency virus reverse transcriptase (HIV-RT).[1] ddATP determination appears necessary for therapeutic drug monitoring of didanosine because the intracellular ddATP level did not correspond with levels of ddI measured in plasma.[2] Quantitation of the very low levels ($10 - 50$ fmol per 10^6 cells) of the active anabolite 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. Some of the analytical methods such as radioimmunoassay,[3] LC-MS[2,4–6] and CE-MS[1,7] have been applied before. They have advantages and/or disadvantages. A micellar electrokinetic capillary chromatography (MEKC) method with fluorescence detection has recently been developed by our group to measure ddATP after precapillary derivatization through the phosphate group with dansylethylenediamine (dansyl EDA) for 90 min.[8] Upon dansylation the sensitivity of fluorescence detection of ddATP was 160 times higher than UV detection. The aim of the present work was to try other possibilities of precapillary derivatization for ddATP, which might improve the rate of sample preparation and achieve better sensitivity. Hence, the adenine base of ddATP was chosen as the target for precapillary derivatization. Several fluorophores such as chloroacetaldehyde, 9-fluorenylmethyloxycarbonylchloride (FMOC), fluorescamine (Fluram), *o*-phthaldialdehyde (OPA) and complexation with terbium (Tb), gadolinium (Gd) and phenanthroline (phen) were tried.

EXPERIMENTAL

Chemicals

Ammonium acetate anhydrous, lithium dodecyl sulfate (LiDS), sodium tetraborate decahydrate, phosphoric acid, OPA, β-mercaptoethanol, FMOC, methanol and 1,10-phenanthroline monohydrate were purchased from Acros Organics (Geel, Belgium). Ammonium hydroxide solution, sodium hydroxide pellets and hexamethylenetetramine (HMTA) were purchased from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). Terbium(III) oxide (Tb_2O_3), terbium(III) chloride ($TbCl_3$), gadolinium(III) oxide (Gd_2O_3) and gadolinium(III) chloride ($GdCl_3$) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Hydrochloric acid was from Chem-Lab MV (Zedelgem,

Belgium). Chloroacetaldehyde solution and sodium acetate trihydrate were from Fluka Chemika (Buchs, Switzerland). Acetonitrile (ACN) was purchased from Fisher Scientific (Leicestershire, UK) and acetone was from Merck Eurolab nv/sa (Leuven, Belgium). Fluram was obtained from Serva Electrophoresis GmbH (Heidelberg, Germany). ddATP stock solution (5 μmol/~2.4 mg per 500 μL) was purchased from Biolog Life Science Institute (Bremen, Germany). All solutions were prepared with ultrapure Milli-Q water (Millipore, Milford, MA, USA). The background electrolyte (BGE) was filtered with a 0.2 μm Uniflo 25 RC filter (Schleicher & Schuell MicroScience GmbH, Dassel, Germany).

Instrumentation

An Agilent CE G1600A system (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) with Argos 250 B fluorescence detector from Flux Instruments AG (Basel, Switzerland) was used. Uncoated fused-silica capillary was obtained from Polymicro Technologies (Phoenix, AZ, USA). The Eppendorf 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 as prescribed in Ref. [9].

Electrophoresis

An uncoated fused-silica capillary with a total length (L_T) of 40 cm, effective length (L_E) of 24 cm, and an i.d. of 75 μm was used. 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. The capillary was rinsed with background electrolyte (BGE) for 3 min in between runs. All the washings were performed by applying a pressure of 138 kPa. The inlet/outlet outlet vials were renewed for every run.

Capillary Zone Electrophoresis (CZE)

The electrophoretic conditions include the use of a BGE containing 100 mM ammonium acetate pH 9.0 (adjusted with 1 M ammonia solution), an applied voltage of 8.4 kV (normal polarity) and the capillary temperature maintained at 15°C. The current corresponding to these conditions was 99 μA.

MEKC

The electrophoretic conditions include the use of a BGE containing 70 mM LiDS and 140 mM ammonium acetate pH 9.0 (adjusted with 1 M ammonia 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 100 μA.

Complexation Procedure

To 1.0 μL of 10 mM $TbCl_3$ pH 7 (adjusted with dilute NaOH), 1.0 μl of ddATP stock solution (5 μmol/~2.4 mg per 500 μL), 50 μL of 1 mM phen solution, 2.5 μL of 10 mM $GdCl_3$ pH 7 (adjusted with dilute NaOH) and 25 μL of 10% m/v HMTA buffer pH 7.0 (adjusted with 6 M HCl) were added and made up to 250 μL with water. It was then mixed at 450 rpm for 90 min at 25°C using an Eppendorf Thermomixer comfort. A diluted solution of ddATP was used for complexation at lower concentrations (1.9–3.8 μg/mL).

RESULTS AND DISCUSSION

The MEKC method and CZE method developed recently for the analysis of ddATP as dansyl EDA derivative[8] were applied for the present work. The adenine base of ddATP was targeted for precapillary derivatization.

Precapillary Derivatization with Chloroacetaldehyde

Haloaldehydes such as chloroacetaldehyde[10,11] and bromoacetaldehyde[10] have been used as derivatizing agents for the analysis of adenine containing nucleotides. The derivatization of the adenine base with chloroacetaldehyde to form a fluorescent 1,N^6-etheno adduct has been employed for the present work. Figure 1 shows the probable derivatization reaction for ddATP with chloroacetaldehyde.

The derivatization procedure proposed by Dai et al.[11] was applied for the present work. The procedure was as follows: to 32 μL of chloroacetaldehyde (3.6 M, pH 4.0) in acetate buffer, 68 μL of ddATP solution (15.2 μg/mL) was added and the mixture was incubated for 60 min at 60°C using an Eppendorf Thermomixer comfort (450 rpm). The sample was cooled at 0°C for 10 min before injection. Chloroacetaldehyde (3.6 M, pH 4.0) in acetate buffer was prepared by diluting 10.2 mL of 45% (m/m) aqueous chloroacetaldehyde with 6.4 mL of 1.0 M sodium

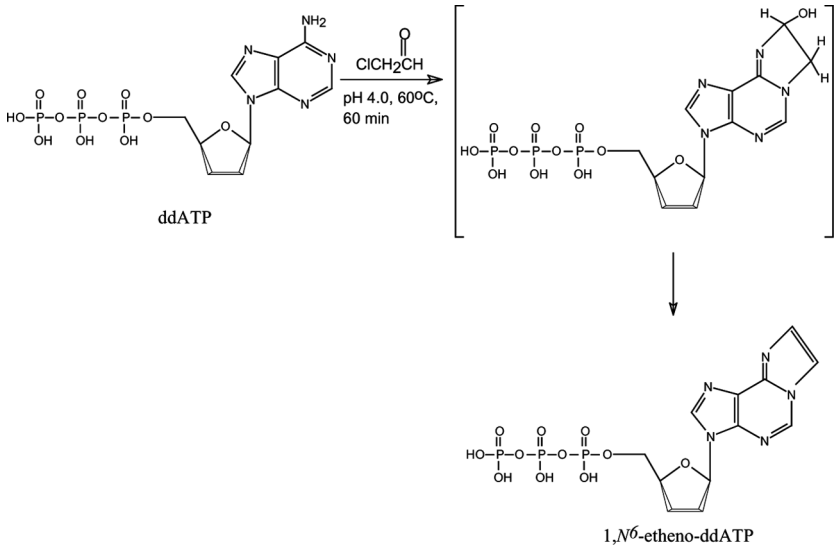

Figure 1. Chemical derivatization of ddATP with chloroacetaldehyde to form 1,N^6-etheno-ddATP.

acetate (buffer pH 4.0). The pH of this solution was then adjusted to pH 4.0 by adding 3.35 mL of 1.0 M sodium hydroxide.

The electrophoretic conditions used were as described in section 2.3.1. Detection was performed at an excitation wavelength of 300 nm using a monochromator and an emission wavelength above 418 nm using a 418 nm Cut-off Long-pass emission filter. The photomultiplier tube (PMT) voltage was 750 V. The analysis of the reaction mixture showed poor peak shape for N^6-etheno-ddATP adduct (ε-ddATP) and degradation peaks of ε-adenine, possibly due to the low sample pH of 4.0. The amount of degradation decreased when the sample pH was adjusted to 7.0 after the derivatization. However, 20% of the sample degraded to ε-adenine, see Fig. 2.

Precapillary Derivatization with OPA, Fluram, and FMOC

OPA and Fluram have been used extensively to derivatize primary aliphatic amine containing compounds, while FMOC has been used to derivatize both primary and secondary aliphatic amine containing compounds.[12–14] There have been also some reports for the derivatization of aromatic amine containing compounds using these tags.[15–17] The reactions were completed in $\leq$5 min.

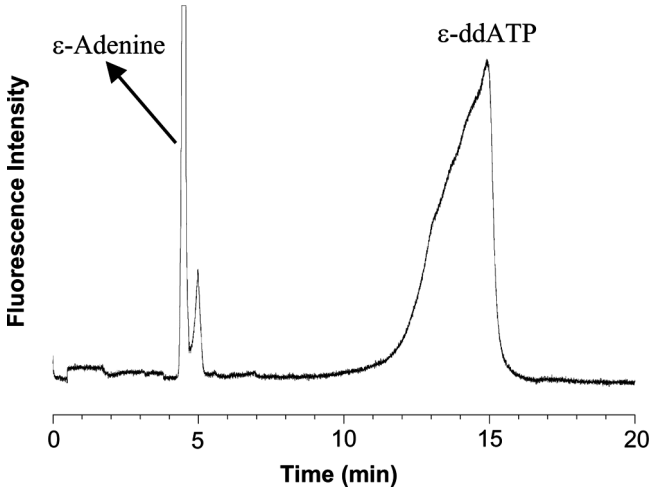

Figure 2. A typical electropherogram showing the derivatized ε-ddATP and its degradation product ε-adenine. Electrophoretic conditions – as mentioned in the text; fluorescence detection with λ_{exc} of 300 nm, λ_{em} of above 418 nm (Cut-off Long-pass filter) and PMT voltage of 750 V; sample: 10.3 μg/mL ddATP; injection: 5.0 s × 3.5 kPa.

Two derivatization strategies with OPA/β-mercaptoethanol at high and low reaction pH were tried. Firstly, 10 μL of ddATP (47.5 μg/mL) was reacted with 50 μL of 3.7 mM OPA in 20 mM borate and methanol (98:2, *v*/*v*) and 40 μL of 0.5% β-mercaptoethanol. The 2% methanol was required for the solubility of the OPA reagent. The reaction mixture was mixed for 1 min at room temperature before injection. The electrophoretic conditions used were as described in another section. Detection was performed at an excitation wavelength of 350 nm using a monochromator or a UG 11 broad band excitation filter (240–400 nm) and an emission wavelength above 418 nm using a 418 nm Cut-off Long-pass emission filter. The PMT voltage was 900 V. The analysis of the reaction mixture showed no fluorescent derivatization product peak and the unreacted ddATP peak was found consistently by UV detection. Other fluorescent peaks were found in the electropherogram which originated from the blank reaction mixture but were not found when the two reagents OPA and β-mercaptoethanol were injected individually. Increasing the reaction time up to 2 h did not help either. Secondly, to investigate the influence of pH, the post column fluorescence derivatization reported for the determination of several sulphonamides at low pH by dissolving the two reagents in methanol and 0.7 M H_3PO_4 (98:2, *v*/*v*)[17] was adapted for the

present precapillary reaction. 10 μL of ddATP (47.5 μg/mL) was reacted with 20 μL of 20 mM β-mercaptoethanol and 70 μL of 10 mM OPA in 0.7 M H_3PO_4 and 2% methanol. The reaction was mixed at 40°C for 10 min before injection. The analysis of the reaction mixture showed no fluorescent derivatization product peak and no unreacted ddATP peak in the UV window, probably due to its degradation at low sample pH.

Consequently, precapillary derivatization of ddATP was tried with Fluram. 10 μL of ddATP (47.5 μg/mL) in 200 μl of sodium acetate buffer pH 7 (adjusted with 0.01 M glacial acetic acid) was reacted with 50 μL of 10 mM Fluram in acetone. The reaction mixture was mixed for 5 min at 25°C before injection. The electrophoretic conditions used were as described in another section. Detection was performed using a UG 11 broad band excitation filter (240–400 nm) and an emission wavelength above 495 nm using a 495 nm cut-off long-pass emission filter. The PMT voltage was 800 V. The analysis of the reaction mixture showed no fluorescent derivatization product peak and the unreacted ddATP peak was found consistently by UV detection. No significant improvement in the reaction was observed by increasing the reaction temperature to 50°C. The excitation and emission wavelengths were changed to 390 nm (monochromator) and above 450 nm (450 nm cut-off long-pass emission filter), respectively, but no new fluorescent peaks were observed.

Precapillary derivatization of ddATP with FMOC was tried by reacting 35 μl of ddATP (13.6 μg/mL) in 90 μL of ACN with 125 μL of 10 mM FMOC. The presence of ACN in the reaction mixture was required for the solubility of the FMOC reagent. The reaction mixture was mixed for 45 s at room temperature before injection. The electrophoretic conditions used were as described in another section. Detection was performed at an excitation wavelength of 260 nm using a monochromator and an emission wavelength above 295 nm using a 295 nm Cut-off Long-pass emission filter. The PMT voltage was 850 V. An injection of the reaction mixture for 20 s at 3.5 kPa produced an overloaded fluorescent peak, which was eluting throughout the run; therefore the injection time was reduced from 20 to 5 s. The overloaded peak was from the excess reagent and no fluorescent product peak was found. In order to check if the product peak was comigrating with the huge reagent peak, pentane was used to extract the excess reagent. After pentane extraction, the excess reagent peak disappeared but no additional fluorescent product peak was found. The current was also not stable throughout the run, probably due to traces of pentane left in the reaction mixture. The use of 5 mM borate with ACN (1:1, *v*/*v*) as reaction solvent and an increase in reagent concentration from 10 to 50 mM FMOC produced no improvement in the reaction.

Complexation with Tb-Gd-phen

A new co-luminescence system has been reported for the determination of ATP as a Tb-Gd-ATP-phen complex using a spectrofluorimeter and it was proposed to be one of the most sensitive fluorimetries of ATP.[18] The fluorescence intensity was reported to be enhanced when Gd^{3+} was added to the Tb-ATP-phen system. For the present work, similar complexation was tried for ddATP but in small volumes to minimize sample dilution. The complex is formed by the combination of a ddATP molecule with two terbium metal ions and six phenanthroline ligand molecules. Each terbium metal ion binds with three phenanthroline molecules. Similarly, a Gd-ddATP-phen complex can be formed and it aggregates together with the Tb-ddATP-phen complex to form a large congeries. Each Tb-ddATP-phen complex is surrounded by many Gd-ddATP-phen complex molecules.[19]

The initial complexation procedure used was as follows: to 1.0 μL of 10 mM Tb_2O_3, 1.0 μL of ddATP stock solution (5 μmol/~2.4 mg per 500 μL), 50 μl of 1 mM phen solution, 2.5 μL of 10 mM Gd_2O_3 and 25 μL of 10% m/v HMTA buffer pH 7.0 (adjusted with 6 M HCl) were added and made up to 250 μL with water. The mixture was then mixed for few seconds at room temperature before injection. The 10 mM Tb_2O_3 and Gd_2O_3 solutions were prepared by dissolving the corresponding oxides (99.9%) in concentrated HCl and diluting with deionized water. The electrophoretic conditions used were as described in another section. Detection was performed at an excitation wavelength of 300 nm using a monochromator and an emission wavelength above 495 nm using a 495 nm cut-off long-pass emission filter. The PMT voltage was 1139 V. The analysis of the complexation mixtures showed no fluorescent peaks, probably due to the low sample pH of 3.4. Although 10% m/v HMTA buffer pH 7.0 was used for the complexation reaction, the presence of HCl as solvent for Tb_2O_3 and Gd_2O_3 brought the final sample pH to 3.4. Since ddATP is highly acid labile, the complexation reaction was tried by replacing Tb_2O_3 and Gd_2O_3 with $TbCl_3$ and $GdCl_3$ that are very soluble in water. However, the pH of 10 mM $TbCl_3$ and 10 mM $GdCl_3$ in water was ~4.9 and 4.2, respectively. Therefore, the pH of these solutions was adjusted to 7 with dilute NaOH before making up the volume. The analysis of the reaction mixture showed a very sharp fluorescent peak (see Fig. 3). However, in subsequent injections more than one fluorescent peak was found, probably due to degradation of the complex in the presence of micelles in the BGE. It has been reported that the fluorescence intensity of the ligand 1,10-phenanthroline accommodated in the micellar core is significantly reduced relative to that in water.[19]

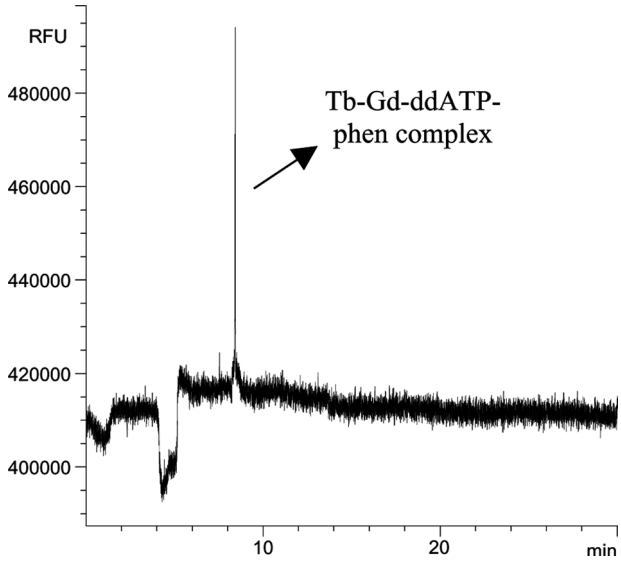

Figure 3. A typical electropherogram showing the initial MEKC analysis of Tb-Gd-ddATP-phen complex. Refer to the text for electrophoretic conditions; fluorescence detection with λ_{exc} of 300 nm, λ_{em} of above 495 nm (Cut-off Long-pass filter) and PMT voltage of 1139 V; sample: 19 μg/mL ddATP; injection: 20.0 s × 3.5 kPa.

Therefore, the CZE method described in another section was used for further investigations. The CZE analysis showed no degradation of the sharp complex peak, however, it was observed to be migrating close to a baseline deformation also present in the blank. The reaction time was optimized further and at 25°C, it took 90 min for the complexation to be finished. In order to improve the reaction rate, 35°C was tried but the complex was found to be unstable at this temperature. The finally optimized procedure for complexation with Tb-Gd-phen was as described in section 2.4. Figure 4 shows an overlay of CZE electropherograms showing the Tb-Gd-ddATP-phen complex peak at 19 μg/mL and 3.8 μg/mL concentrations together with a blank run. The blank injection was done with the same components as the sample complexation mixture but without ddATP.

Unlike the high sensitivity achieved for ATP as a Tb-Gd-ATP-phen complex,[18] the sensitivity of the Tb-Gd-ddATP-phen complex was found to be 20 times higher than UV detection of ddATP. This can be due to the difference in sample pH for ATP and ddATP complexation, respectively.

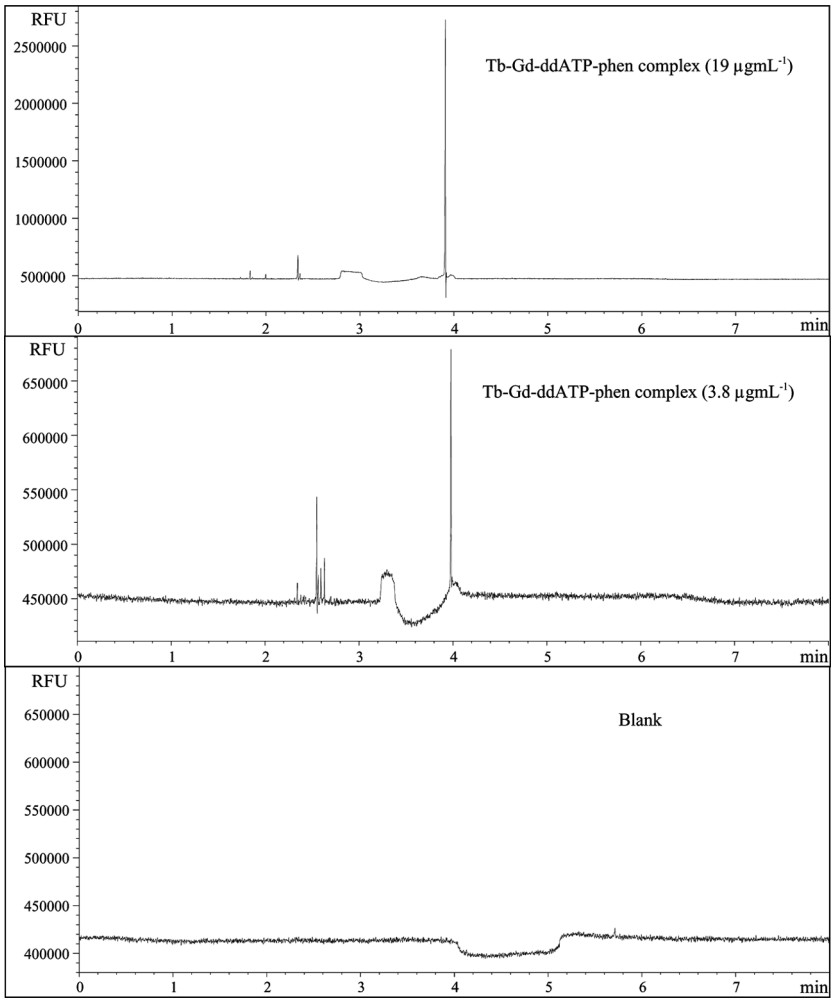

Figure 4. A typical electropherogram showing the Tb-Gd-ddATP-phen complex peak at 19 μg/mL and 3.8 μg/mL concentrations in the upper two panels, respectively and a blank run in the lower panel. Electrophoretic conditions and complexation procedure are as reported in the text; fluorescence detection with λ_{exc} of 300 nm, λ_{em} of above 495 nm (Cut-off Long-pass filter) and PMT voltage of 1139 V; injection: 20.0 s×3.5 kPa.

CONCLUSIONS

Precapillary derivatization of ddATP with chloroacetaldehyde showed poor peak shape for the ε-ddATP adduct and degradation peaks of ε-adenine, possibly due to the low sample pH of 4.0. Precapillary

derivatization of ddATP with OPA/β-mercaptoethanol, Fluram and FMOC showed no fluorescent derivatization products. Complexation of ddATP with Tb-Gd-phen produced a sharp fluorescent peak. The sensitivity of the Tb-Gd-ddATP-phen complex was found to be 20 times higher than UV detection of ddATP. It can be concluded that the derivatization of adenine base might require an acidic pH, whereas, ddATP was found to be very acid labile and hence no pH lower than 7.0 can be used.

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