Measurement of adenosine by capillary zone electrophoresis with on-column isotachophoretic preconcentration

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Abstract

An on-column isotachophoretic (ITP)–capillary electrophoresis (CE) system capable of preconcentrating polyhydroxyl species is reported. The ITP–CE system utilizes borate complexation of the neutral diol species to form anionic compounds that can be directly separated by CE. Borate buffer functions as both the terminating electrolyte for the ITP preconcentration and the operating buffer for the subsequent CE separation. Isotachophoretic preconcentration allows injection volumes as large as 50% of the column volume, without compromising separation integrity, to yield detection limits about 70-fold lower than direct CE separation (with borate operating buffer). In this paper we also present an application of the ITP–CE system, with laser-induced fluorescence (LIF) detection, to the quantitative analysis of adenosine from urine. Nanomolar concentration levels of adenosine are successfully derivatized with chloroacetaldehyde (CAA) to form a fluorescent derivative whose spectral characteristics match the He–Cd laser. The technique is shown to be capable of quantitative measurement of adenosine as low as 10^{-9} M, the levels expected in plasma and urine.

Keywords: Capillary electrophoresis; Isotachophoresis; Adenosine

1. Introduction

Adenosine has widespread effects on coronary and cerebral circulations [1], control of blood flow [2,3], prevention of cardiac arrhythmias [4] and nerve-tissue functions such as the inhibition of neutrotransmitter release and the modulation of adenylate cyclase activity [5]. It has been suggested that these physiological actions are due to the interactions of adenosine with specific receptors, which are mediated by the changes of interstitial adenosine concentration [1]. To understand the involvement of adenosine in these systems, reliable analytical methods must be available to determine adenosine concentrations in plasma, tissue extracts, or urine.

Several methods, including HPLC with UV [5–7] or fluorescence detection [8–12] and radioimmunoassay [15,16], have been developed to quantitate plasma and urinary levels of adenosine with reported results varying between 10–100 nM [4–14]. Radioimmunoassay can provide very sensitive, quantitative results from nanomo-

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lar concentration levels, however, the inconvenience of using radioactive materials limits its applicability. The HPLC methodologies tend to be cumbersome with borderline sensitivity and subject to artifacts and interferences. HPLC with UV detection lacks the sensitivity required to accurately quantitate plasma levels of adenosine. Although fluorescence detection can provide suitable sensitivity for adenosine analysis by HPLC, the use of large blood samples required for HPLC may interrupt the in vivo equilibrium, particularly in small animals.

Capillary electrophoresis is a powerful tool for analyzing ionic species based on their electrophoretic mobility differences. The use of capillary-scale columns provides several advantages over conventional-scale separation methods that have been set-out elsewhere [17,18]. The minimal separation volumes result in mass detection limits at the femtomole level, with appropriate detection [19,20]. However, due to volumetric restrictions, the corresponding concentration detection limits are not as impressive.

Concentration detection limits of CE can be improved by increasing the sample capacity of the system. Sample stacking in which samples are dissolved in a low electrical conductivity solvent, has been successfully used to improve the sensitivity of CE [21–23]. The effectiveness of stacking is limited by the trade-off of resolution and injection plug length [24]. Chromatographic concentration is another method for on-line CE detection limit enhancement involving large sample loading. Although this technique can improve sensitivity up to 1000-fold [25], the cost and difficulties in reproducibility of manufacture of on-line systems restrict its applications [24].

Isotachophoresis allows for larger sample loading than free-solution CE and has been successfully utilized on-line with CE to improve concentration detection limits [26–31]. By selecting proper leading and terminating electrolytes, large volumes of dilute sample can be focused into narrow bands. The narrow concentration pulses then serve as injection plugs for the free-zone CE separation. Two instrumental arrangements for ITP–CE have been described [30]. A coupled-column arrangement allows large sample loading and provides a sensitivity improvement up to 1000-fold, but requires a sophisticated experimental design [26–30]. In the second arrangement ITP is directly coupled, on-column, to CE with automatic transition to a CE separation mechanism, providing 50–100 fold concentration enhancements without any modification of the CE instrumentation [30,31].

Since only charged species can be directly analyzed by CE, neutral species will require an alternative means for separation. Micellar electrokinetic chromatography (MEKC) has been demonstrated for separation of neutral ribonucleosides [32,33]. The MEKC technique provides similar advantages as CE, such as high separation efficiency [34] and minimal sample requirements [35], but moderate concentration detection limits restrict the applications for trace samples. Furthermore, surfactants in the buffer solution may affect the reproducibility. Another option, for certain polyhydroxyl compounds, is the use of borate additives to the operating buffer. Borate will complex with polyhydroxyl compounds containing the cis-diol moiety to form anionic complexes which have a higher electrophoretic mobility than free boric acid [36,37]. With these properties, borate buffer has been used as an operating buffer to separate ribonucleosides in gel electrophoresis [38] and carbohydrates by CE [39–41]. Also, mobility differences between boric acid and the complexes makes it an attractive terminating electrolyte for the ITP separation of polyols [36,37].

Here we will describe an on-column transient ITP–CE system for analysis of borate complexes and applications to adenosine analysis from biological samples. In this system, borate functions as both operating buffer in which charged complexes from the neutral adenosine are formed, and as a suitable terminating electrolyte for the ITP preconcentration step. Laser-induced fluorescence detection is used to spectroscopically differentiate adenosine from other ribonucleosides that represent possible interferences, as their 1,N^6-etheno derivatives, and improve concentration detection limits to the sub-nanomolar range without complicated, user-intensive operation or instrumental modification.

2. Experimental

2.1. Chemicals

Adenosine (ADO), cytidine, guanosine, uridine, and chloroacetaldehyde (CAA) were purchased from Aldrich (Milwaukee, WI, USA). The sugar and catechol samples were purchased from Sigma (St. Louis, MO, USA). The commercial preparation of CAA was found by titration [42] to have a concentration of 7.5 *M*. All dilutions are based on this concentration. Trimethylchlorosilane (TMS) was obtained from HULS (Bristol, PA, USA) and hydroxyethylcellulose (HEC) was from Fluka (Ronkonkoma, NY, USA). Electrophoretic grade Tris(hydroxymethyl)aminomethane (Tris) was purchased from Sigma and other analytical grade reagents were from Fisher Scientific (Pittsburgh, PA, USA). Calf intestinal adenosine deaminase, in 50% glycerol, was purchased from Sigma.

2.2. Apparatus

A home-built CE system was used in this work. Briefly, a Bertan (Hicksville, NY, USA) ±30 kV high-voltage power supply was used to provide the driving voltage across a 65-cm long, fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA), either 25 or 50 μm inner diameter. To eliminate the electroosmotic flow, the fused-silica capillaries were treated with TMS (procedure described below) and 0.1% HEC was incorporated in all operating buffers. The high-voltage end of the capillary was housed in a plexiglass box to prevent accidental electrical discharge to the operator. Prior to TMS treatment, a small portion of the fused-silica coating was removed with a flame to form a detection window 15 cm from the end of the capillary.

Detection was accomplished with an UV detector (Linear Instruments Model UVis200, Reno, NE, USA) or a home-built LIF system [43]. The LIF system consisted of a He–Cd laser (Liconix, Santa Clara, CA, USA) to provide about 6 mW of excitation power at 326 nm. The 415-nm emission fluorescence was isolated with spectral filters (Corion, Vermont, MA, USA) and

focused onto a photomultiplier tube. Optical mounts were from Newport (Irvine, CA, USA) and other optical components were from Melles Griot (Irvine, CA, USA). A low-noise current amplifier (Model SR750 Stanford Research Systems, Sunnyvale, CA, USA) was used to amplify and filter the signal. Data were collected and stored on an IBM PC clone microcomputer.

2.3. Capillary coating

The coating procedure was modified from the method described by Yao and Regnier [44]. Fused-silica capillaries were first treated with 1 *M* KOH for 15 min and washed with deionized water followed by methanol for 20 min each. The residual methanol was then evaporated by pulling air through the capillaries for 30 min. Trimethylchlorosilane (50%) in toluene was continuously drawn through the capillary by vacuum over an 8-h period. The capillary was then rinsed with methanol to remove residual TMS solution. The capillaries were washed with deionized water for 20 min and flushed with the operating buffer containing 0.1% HEC for 20 min to complete the coating and column equilibration procedure.

2.4. Sample derivatization

Aqueous ribonucleoside stock solutions were diluted to the appropriate concentration with 10 m*M* boric buffer. In 1.5 ml sample vials, 100 μl of 2 *M* aqueous CAA solution was added to 1 ml of nucleoside solutions. The sample vials were heated at 90°C for 50 min [9], and then stored in an ice bath.

2.5. Urine sample preparation

Urine samples were obtained from a healthy subject (Asian male, age 30). Freshly voided urine was collected into a chilled bottle containing sodium azide so the final azide concentration was 0.03% (w/v). After centrifugation to remove insoluble materials and particulates, 25 ml of the supernatant was mixed with 25 ml of 0.1 *M* phosphate buffer (pH 7.5), and put on ice.

To isolate adenosine, a boronic acid affinity column consisting of 0.8 ml immobilized boronic acid gel (Pierce, Affipak) was built in a 3-ml disposable syringe (Fisher Scientific). After saturating the column with 0.1 M carbonate buffer (pH 10), 10 ml of the buffered urine was loaded onto the column. The column was washed with 2 ml of 25 mM carbonate buffer (pH 10) and adenosine was then eluted with 9 ml of 0.1 M HCl. The eluent fraction containing adenosine was adjusted to approximately pH 7 with 0.5 M NaOH and applied onto a Sep-Pak C_{18} cartridge which had been prewashed with 5 ml of methanol followed by 5 ml of distilled water. The Sep-Pak was then washed with 1 ml of water, and the adenosine eluted with 1.5 ml of 25% methanol in 50 mM HCl (v/v). The aliquot was adjusted to a pH 6 with concentrated NaOH, and lyophilized. The dried sample was re-suspended in 1 ml of water and mixed with CAA solution for derivatization and further analysis.

A second 10-ml portion of buffered urine was incubated with adenosine deaminase (Sigma Chemical), 0.06 unit/ml for 50 min at 25°C to eliminate adenosine, and carried through the pretreatment procedure described above.

2.6. CE and ITP–CE

For CE analysis, samples were diluted in the operating buffer and about 7 nl was injected hydrodynamically (20 cm elevation for 25 s) at the cathodic end of capillary. For ITP–CE analysis, samples were diluted with 50% of 10 mM borate buffer (pH 10) in the leading electrolyte solution. After filling the capillary and both reservoirs with 80 mM borate buffer (pH 10), sample was injected from cathodic end of capillary by hydrodynamic pressure. The calculation of injection volume is based on the measured time for the sample to flow from the injection to the detection end of the capillary under constant pressure. All analyses were performed at 15 kV for 30 min. The current during CE operations was constant at 28 μA. In ITP–CE, the current started at 25 μA for 50% column volume injection, dropped to 10 μA, then returned to about 20 μA.

The leading electrolyte was prepared by titrating 180 mM HCl with Tris (1 M) to pH 7.5. The terminating electrolyte and operating buffer were 80 mM borate pH 10. Also, 10 mM borate pH 10 was used in sample preparation. All buffer and sample solutions contained 0.1% HEC and were filtered with 0.22-μm membrane filter (MSI, Westboro, MA, USA).

3. Results

To test the general performance of the ITP–CE system we examined the separation of various neutral polyol species. The direct CE separation (200 mM borate buffer) of amino-pyridine labeled sugars (peak identifications are given in the figure legend) with detection by UV absorbance at 240 nm is shown in Fig. 1a. Fig. 1b is the same mixture of sugars diluted 50-fold and preconcentrated on-line from a 50-fold larger injection volume (87 nl vs. 1.7 nl) by ITP. The detector sensitivity setting was identical in both runs. At this point, we do not have a clear understanding why xylose and ribose co-migrate in the ITP–CE arrangement. It may be due to inefficient de-stacking of the solute zones as detailed by Hjerten [23].
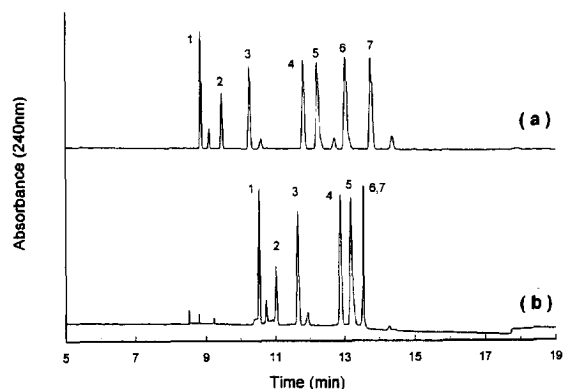

Fig. 1. (a) Direct CE (borate buffer) separation of amino-pyridine labeled reducing monosaccharides. Peaks: 1 = glucurone, 2 = *trans*-cinnamic acid, 3 = galactose, 4 = arabinose, 5 = glucose, 6 = ribose, 7 = xylose. All samples are $8.3 \cdot 10^{-4}$ M (injection volume 1.7 nl, electrokinetic). (b) The same sample diluted 50-fold and preconcentrated by on-line ITP–CE (injection volume 87 nl, hydrodynamic). Detection is UV at 240 nm.
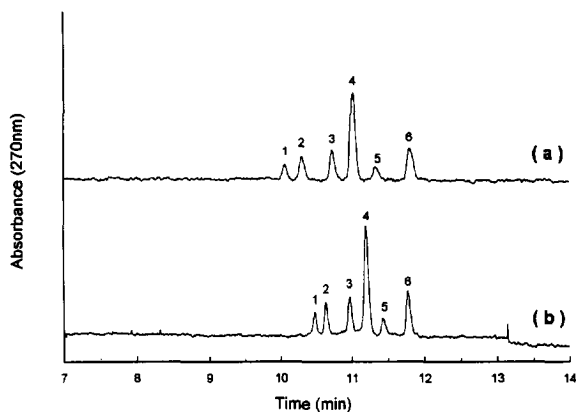
Fig. 2. (a) Separation of some catechols by direct CE in borate buffer. Peaks: 1 = pyrogallol, 2 = pyrocatechol, 3 = 3,4-dihydroxybenzaldehyde, 4 = 2,3-dihydroxybenzaldehyde, 5 = methyl catechol, 6 = 2,3-dihydroxynaphthalene at $6 \cdot 10^{-4}$ M (injection volume 1.7 nl, electrokinetic). (b) The same sample as Fig. 2a, diluted 50-fold and preconcentrated by on-line ITP-CE (injection volume 87 nl, hydrodynamic). Detection is UV at 270 nm.

Fig. 3. (a) CE separation of ribonucleosides. The ribonucleosides were dissolved in operating buffer (80 mM borate, pH 10, 0.1% HEC) with the following concentrations: A = adenosine $(3.1 \cdot 10^{-5}$ $M)$; U = uridine $(4.1 \cdot 10^{-5}$ $M)$; G = guanosine $(2.18 \cdot 10^{-5}$ $M)$; C = cytidine $(4.36 \cdot 10^{-5}$ $M)$. Injection: 7 nl. UV detection at 270 nm. (b) On-column transient ITP-CE separation of the ribonucleosides. The sample was diluted with 50% 10 mM borate (pH 10) in leading electrolyte (180 mM HCl, pH 7.5) to the following concentrations: A = adenosine $(1.24 \cdot 10^{-6}$ $M)$; U = uridine $(1.64 \cdot 10^{-6}$ $M)$; G = guanosine $(1.74 \cdot 10^{-6}$ $M)$; C = cytidine $(1.74 \cdot 10^{-6}$ $M)$. Injection: 490 nl. UV detection at 270 nm.

Similar results were obtained for 6 neutral catechols (peak identifications are given in the figure legend). Fig. 2a is the direct separation of the catechols by CE using 200 mM borate buffer. Fig. 2b depicts the ITP preconcentration of the same mixture, again diluted 50-fold from the sample in Fig. 2a. The injection volume was 50 times greater for the ITP-CE system. With both the sugars and catechols injections of up to 50% of the column volume can be made without sacrificing column performance. This leads to concentration detection limit enhancements of ca. 70-fold over free-solution CE.

Since our interests lie in the quantitation of adenosine, we investigated the ITP-CE system for the analysis of ribonucleosides. In Fig. 3a the free-solution CE separation profile of adenosine, uridine, guanosine and cytidine at the 10^{-5} M level in 80 mM borate buffer is shown. Thymidine does not have a favorable diol arrangement to form an anionic borate complex and therefore does not migrate in this system (data not shown). Fig. 3b demonstrates the on-line ITP preconcentration capability for the four ribonucleosides in Fig. 3a. Comparison of the peak areas and variances from the two electropherograms indi-
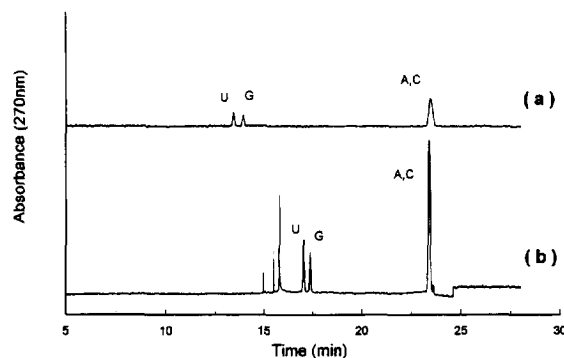
cates a 70-fold enhancement in concentration sensitivity for the ITP-CE, due to larger sample loading (injection volume about 490 nl) tolerated by the ITP-CE system. Calibration data for the ITP-CE system indicate concentration detection limits of $6 \cdot 10^{-7}$ M $(r^2 = 0.999)$ with UV detection. This level of detection is still inadequate for expected adenosine levels in plasma or urine. Furthermore, cytidine co-migration with adenosine interferes with adenosine quantitation.

Our approach to simultaneously improve concentration sensitivity and resolve the adenosine/cytidine co-migration problem is LIF detection. The mass sensitivity enhancements associated with LIF detection are well-known [19,45–47]. Furthermore, by appropriate selection of fluorogenic reagent, excitation and emission wavelengths it is possible to spectroscopically separate the adenosine signal from the cytidine response.

Since the primary amine moiety present on the base functionality of adenosine, cytidine and guanosine is deactivated by the ring system, conventional fluorogenic amine reagents are unsuitable for ribonucleoside analysis. Chloroacetaldehyde (CAA) is commonly used to form

1,N^6-etheno-derivatives of adenosine and cyti-dine for fluorometric analysis [10,48–50]. The structures of the etheno-derivatives of cytidine and adenosine are shown in Fig. 4.

The etheno-derivatives all feature an excita-tion maximum at 270 nm and emission maximum at 415 nm. However, the similarities in the excitation spectra of the various derivatives di-verge with increasing excitation wavelength. At 326 nm, the output wavelength of the He–Cd laser, etheno-adenosine (ϵ-ADO) still has suffi-cient fluorescence emission for sensitive analysis [51]. Cytidine, on the other hand, has no detect-able quantum yield when excited at 326 nm, as shown in Fig. 5. It is thus possible to selectively excite ϵ-ADO fluorescence in the presence of the co-migrating ϵ-cytidine.

To evaluate the selectivity of the LIF system for ϵ-ADO, two free-zonal electropherograms (no ITP preconcentration), in 80 mM borate buffer were generated. Peak areas of ϵ-ADO were measured for two reaction mixtures, the first containing only adenosine and the second containing adenosine and a large excess of the other ribonucleosides. The adenosine peak areas for each reaction mixture were identical, within experimental error, indicating that any contribu-tion to the fluorescence signal from other etheno-ribonucleoside derivatives is negligible. Further-
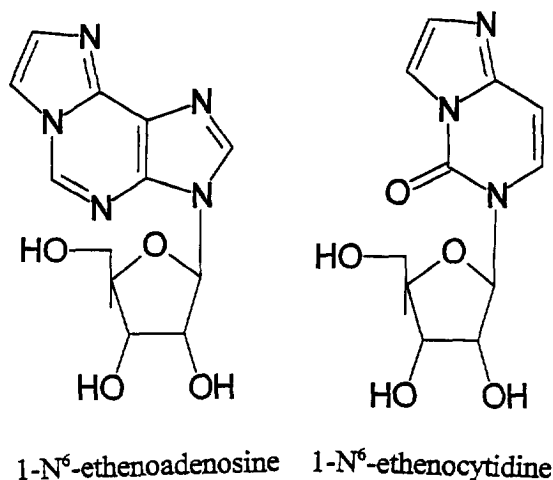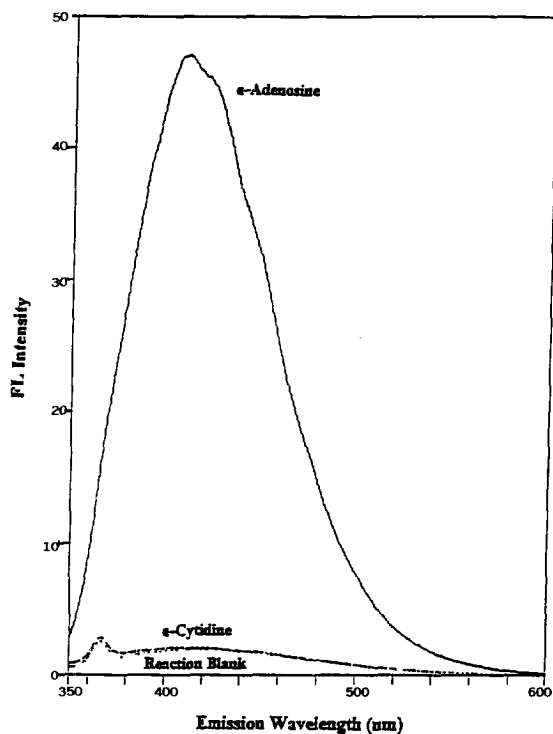
Fig. 5. Fluorescence emission spectra (excitation at 326 nm) of the etheno-derivatives of adenosine, cytidine, and a reac-tion blank. Samples were derivatized at 10^{-4} M, then diluted with 80 mM boric acid (pH 10) to concentrations: [ϵ-adeno-sine] = 4.6 · 10^{-6} M; [ϵ-cytidine] = 3.6 · 10^{-5} M.

more, there is no observable signal from any other ribonucleoside present in the reaction mixture. Therefore quantitation of adenosine with LIF detection can be carried out selectively in the presence other ribonucleosides.

Fig. 6 depicts the ITP–CE analysis of 1.6 · 10^{-9} M ϵ-ADO with LIF detection. The adenosine was derivatized at the 10^{-4} M level and diluted. The separation of ϵ-ADO derivatives by free-zonal CE analysis gave an efficiency of 8 000 plates; while the ITP preconcentration provides separation efficiency up to 400 000 plates. Adenosine laser-induced fluorescence calibration curves feature a dynamic range of 5 orders of magnitude ($r^2 = 0.999$) with concentration detec-tion limits of 5.4 · 10^{-10} M.

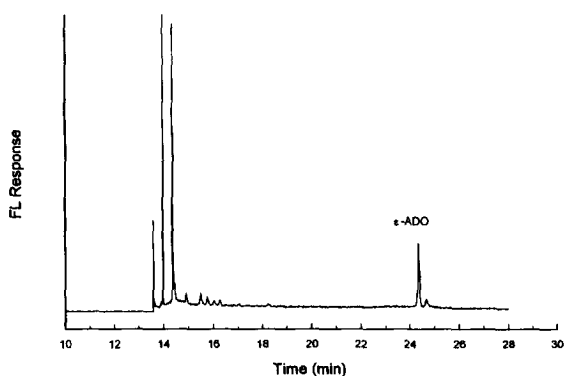To evaluate the CAA derivatization efficiency at the concentration levels expected from bio-

1-N^6-ethenoadenosine   1-N^6-ethenocytidine

Fig. 4. Structure of the etheno-adenosine and etheno-cytidine compounds.

Fig. 6. On-column transient ITP–CE analysis of $1.6 \cdot 10^{-9}$ M etheno-adenosine with LIF detection. The sample was diluted from 10^{-4} M reaction stock with 50% 10 mM boric acid (pH 10) in leading electrolyte (180 mM HCl, pH 7.5). Injection: 490 nl.

logical samples, the reaction was carried out on adenosine present at nanomolar concentrations. Fig. 7 depicts the electropherogram form the ITP–CE analysis of the ϵ-ADO produced by reaction of $5.4 \cdot 10^{-9}$ M ADO with 0.1 M CAA. The reaction mixture was diluted by 50% with leading electrolyte for the ITP–CE quantitation of ϵ-ADO. Even at a reaction concentration of $1.7 \cdot 10^{-9}$ M ADO, the ϵ-ADO signal is still observed with an average signal-to-noise (S/N) ratio of 7 ($n = 3$). Concentration detection limits

for ITP–CE analysis of ϵ-ADO derivatized in this manner (real analysis situation) are $9.8 \cdot 10^{-10}$ M.

To demonstrate the capabilities of the ITP–CE system in a realistic biological system, we measured adenosine levels in urine. The urine sample was collected and centrifuged immediately to remove any cells and insoluble material. The supernatant was separated into two fractions, one was eluted through a boronic acid gel column followed by a Sep-Pak C_{18} prior to derivatization, as detailed in Section 2. Recovery of ϵ-ADO through the Sep-Pak column was 40%. The second fraction was treated for 50 min with adenosine deaminase to remove adenosine, then passed through the boronic gel and Sep-Pak columns and treated exactly as the first fraction. The ITP–CE runs for both fractions are shown in Fig. 8, the adenosine deaminase treated fraction is overlaid on the fresh urine sample. The ϵ-ADO peak was identified by comparison of migration time with authentic standards and by spiking experiments. Conformation of the peak identity is indicated by the elimination of that peak upon treatment with adenosine deaminase (Figure 8, solid line). Quantitation based on adenosine recovery through the pretreatment steps and calibration curves indicate that the concentration of adenosine in this urine sample was 1.3 μM, which falls within the expected
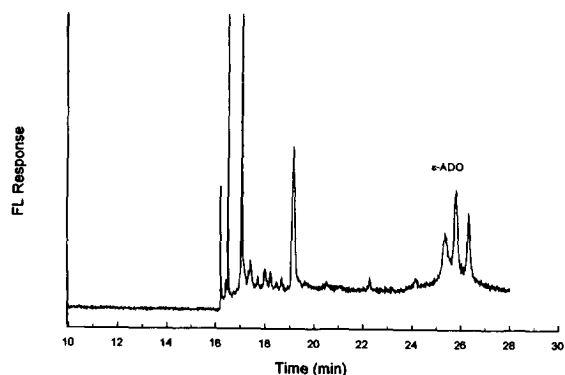

Fig. 7.On-column transient ITP–CE analysis of etheno-adenosine with LIF detection. The sample was $3 \cdot 10^{-9}$ M etheno-adenosine diluted from reaction of $6 \cdot 10^{-9}$ M ADO and 0.1 M CAA with leading electrolyte (180 mM HCl, pH 7.5). Injection: 490 nl.
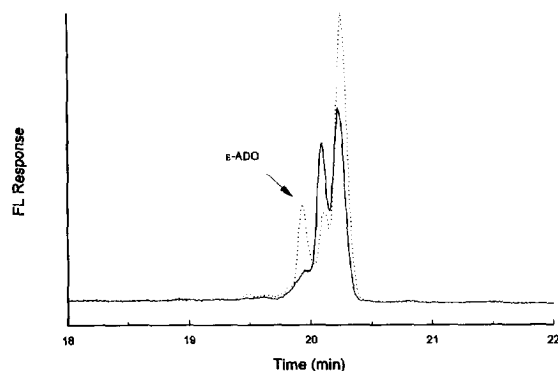

Fig. 8. Adenosine portion of the ITP–CE electropherogram of urine, pretreated as described in the Section 2. The dashed line is the urine sample. The solid line superimposed on the urine trace is the same urine sample treated with adenosine deaminase for 50 min to remove the adenosine.

range of 0.3–7 μM for urinary adenosine levels in humans.

4. Discussion

We are interested in improved means to quantitate plasma or uninary levels of adenosine. Capillary electrophoresis techniques offer attractive, often complementary, alternative separation modes to the well-established HPLC methodologies, while requiring less sample and providing faster analyses. However, as mentioned earlier, poor concentration detection limits pose a substantial obstacle to widespread acceptance of CE techniques for biological problems in which solutes are present in low concentration. In addition, we believe results from current methodologies may be skewed by platelet aggregation, cell lysing and adenosine uptake during the sampling process.

Several methods of on-line preconcentration for CE have been described [21–31]. Foret et al. [30,31] have presented two modes for on-column coupling ITP with CE. In the first, suitable leading and terminating electrolytes are used for the isotachophoretic preconcentration step. After the sample preconcentration is achieved, the terminating electrolyte is then replaced by the leading electrolyte, which also functions as operating buffer for CE separation. The second mode of ITP–CE is suitable when the CE operating buffer has a mobility lower than the sample ions, as in the case with the borate buffer system. The samples are supplemented with leading electrolyte while the column and buffer reservoirs contain the CE operating buffer (which functions as ITP terminator). In this case, isotachophoretic preconcentration of sample ions takes place at the beginning of the analysis with an automatic transition to CE mode separation as the ITP sample migrates through the column.

To our knowledge there have been no literature reports for coupling ITP and CE for polyol species. There are reports dealing with the analysis of polyol species by CE as anionic borate complexes using borate buffer or tetraborate buffer additives for direct free-zonal separation

[39–41]. In addition, ITP with borate acting as both the terminator and complexation agent for the analysis of catechols [36] has been demonstrated. The borate complexes feature higher electrophoretic mobilities than free boric acid [36,37], thus borate is a good terminator for the ITP as well as operating buffer for the CE. We have demonstrated here a novel ITP-CE system that is useful for uncharged cis-diol species.

In order to separate and quantitate adenosine by CE, relatively high concentrations of borate are required to drive the complexation equilibrium to favor the charged complex. However, ITP is most effective when the driving current is carried primarily by the sample and leading and terminating electrolytes (low background electrolyte concentration). We have found that the best results in coupling ITP and CE for separation of polyol species by borate complexation are achieved when a small amount (5 mM) of boric acid is incorporated in the sample solution.

For successful ITP preconcentration, electroosmotic flow must be eliminated. The popular polyacrylamide coating procedure described by Hjerten [52] is not suitable for the buffer system used in this work due to the base catalyzed hydrolysis of that coating which limits capillary lifetime to less than four runs. Our procedure for eliminating electroosmotic flow is a modified version of a report from Yao and Regnier [44] which involves adsorbing polymer buffer additives to alkyl-silane derivatized capillaries. We treat the capillary with TMS and incorporate 0.1% HEC into the operating buffer. In this manner, the electroosmotic flow was effectively eliminated as measured by the lack of migration of a neutral marker (acetone) after 45 min under a field of 300 V/cm. The TMS-coated capillary had a lifetime of over 100 working hours even with the basic buffer conditions described in this paper.

Here we have quantified adenosine in urine and demonstrated the capability of the ITP–CE technique to quantitate levels of adenosine from biological sources. The identification of the adenosine peak is based on migration time comparison with standard adenosine, spiking standard adenosine into the sample and the en-

zymatic metabolism of adenosine from the urine sample.

The electropherogram of the urine sample contains two unidentified components that migrate very closely with adenosine. As of yet we have not identified these two zones, but they seem to be related to adenosine. The relative peak areas of these solutes are sensitive to the presence of adenosine deaminase, they are retained by both the boronic acid gel and the C_{18} Sep-Pak and react with chloroacetaldehyde to form fluorescent derivatives with appreciable fluorescence when excited at 325 nm. An aliquot of urine treated as above, without CAA derivatization shows no peaks in this area of the electropherogram. The unidentified zones are not adenine, which does not migrate in our ITP–CE system or phosphate esters of adenosine (AMP, ADP or ATP), which are not retained by the Sep-Pak and migrate very rapidly in the ITP–CE system.

Removal of excreted cells and other particulate matter by centrifugation appears to be an important factor contributing to the relative peak-area ratio for the two unidentified zones. If the sample is not immediately centrifuged their relative peak areas are very sensitive to the presence of adenosine. In data not presented here, we found that if the naturally occurring adenosine is removed by addition of adenosine deaminase to a urine sample not subjected to centrifugation, the faster migration of these peaks increases dramatically in area. The addition of adenosine back to the urine sample, following inhibition of adenosine deaminase, results in the very rapid reduction in the size of that peak. Currently, we are looking further into the nature of these interactions, and the identity of these zones.

electrolyte for ITP preconcentration and is the running buffer for CE separation. Without any instrumentation modification, the borate on-column ITP–CE system improves both the sample loadability and sensitivity more than an order of magnitude over CE. The LIF detection system, with 326-nm excitation not only enhances the sensitivity by about 2 orders of magnitude over UV detection, it also allows the spectroscopic separation of the adenosine signal from other ribonucleosides, such as cytidine which co-migrates with adenosine. The concentration detection limits demonstrated here are the limits required for the analysis of plasma levels of adenosine. Furthermore, we have shown the ability to perform fluorogenic derivatization on analytes present at nanomolar concentration levels.

We have shown results from the analysis of adenosine in urine. The adenosine peak was identified by spiking with authentic standards. In addition, the identity was confirmed using adenosine deaminase to eliminate adenosine from the sample. The ITP–CE technique is shown to be a simple robust means of quantitating adenosine from urine.

The next step is to be the refinement of techniques for investigating more difficult samples, such as interstitial plasma adenosine concentrations. Production and metabolism of adenosine during the sampling process currently restricts adenosine analysis. We plan to address this issue through experimentation in the area of microdialysis and ultrafiltration sampling techniques, coupled with ITP–CE preconcentration and separation. Future experiments will hinge upon species differences in adenosine levels and the pharmacological role of adenosine.

5. Conclusions

With borate buffer, on-column transient ITP–CE of neutral sugars, catechols and ribonucleosides is demonstrated. In this system, the borate serves as the complexation reagent to charge neutral species, functions as terminating

Acknowledgments

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