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Aptamer affinity chromatography for rapid assay of adenosine in microdialysis samples collected in vivo

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Abstract

An anti-adenosine aptamer was evaluated as a stationary phase in packed capillary liquid chromatography. Using an aqueous mobile phase containing 20 m*M* Mg^{2+}, adenosine was strongly retained on the column. A gradient of increasing Ni^{2+} (to 18 m*M*), which is presumed to complex with nitrogen atoms in adenosine involved in binding to the aptamer, eluted adenosine in a narrow zone. Up to 6 μl of 1.2 μ*M* adenosine could be injected onto the 150-μm I.D.×7 cm long column without loss of adenosine. With UV absorbance detection, the detection limit was 30 n*M* or 120 fmol (4 μl injected). Samples could be repetitively injected with 4.6% relative standard deviation in peak area. Columns were stable to at least 200 injections. The adenosine assay, which required no sample preparation, was used on microdialysis samples collected from the somatosensory cortex of chloral hydrate anesthetized rats. Total analysis times were short enough that dialysate samples could be injected every 5 min. Basal dialysate concentrations of adenosine stabilized at 87±10 n*M* (n=5) with the probe operated at 0.6 μl/min.

Keywords: Microdialysis; Affinity adsorbents; Adenosine; Oligonucleotides

1. Introduction

Aptamers are oligonucleotides selected for tight binding to a target molecule from a combinatorial library of sequences. The selective binding of aptamers has suggested numerous analytical applications including affinity stationary phases for chromatography [1–3]. While antibodies have already been established as highly useful affinity stationary phases, aptamers have several advantages over antibodies making research into their use of interest. Advantages of aptamers relative to antibodies include: (1) in vitro selection and synthesis which minimizes use of animals, improves applicability to toxic compounds, and increases flexibility in selection conditions [4,5], (2) smaller size enabling higher density stationary phases [2], (3) higher stability, and (4) novel approaches for elution and immobilization based on oligonucleotide chemistry. Because of these potential advantages, several investigations have recently begun into aptamer affinity chromatography. In one example, the high affinity and selectivity of an aptamer was used for protein purification [1]. In an analytical scale application, an anti-adenosine

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aptamer was used as a stationary phase to separate several small molecules that contained the adenosine moiety [2]. This work illustrated the utilization of cross-reactivity to obtain selective retention and separation of a class of compounds. Aptamers have also been used as stationary phases in capillary electrochromatography to separate non-target species [3].

In addition to the applications discussed above, affinity phases may have application in chemical monitoring in which collected samples are rapidly assayed by affinity chromatography to provide temporally-resolved information on analyte concentration. The minimal sample preparation and separation time needed, even for a complex mixture, with an affinity phase is well-suited for this approach. In this work, we explore the use of an aptamer stationary phase for chemical monitoring of adenosine in the brain of live rats.

Adenosine is an important neuromodulator that has been implicated in regulation or mediation of a variety of neuronal phenomena including sleep, arousal, locomotion, neuroprotection, seizure susceptibility, and the effect of ethanol [6]. Pharmacological experiments have suggested several physiological events and experimental maneuvers that may alter adenosine concentration in the brain extracellular space including metabolic activity [7], brain temperature, electrical stimulation and prolonged wakefulness [8]. Direct monitoring of adenosine fluctuations under physiological conditions would be of utility in further characterizing the role of this purine in brain function and behavior. Microdialysis sampling combined with a simple, sensitive, and fast assay for adenosine is an attractive approach for in vivo monitoring.

The most popular assays for adenosine in microdialysis samples are based on reversed-phase HPLC coupled with UV-absorbance or fluorescence detection [7,9,10]. The selectivity of UV-absorbance detection is such that a fairly long separation time (23 min) is required to resolve adenosine from the dialysate mixture [9]. With the long time and sample preparation, dialysate may only be monitored at 60 min intervals. Fluorescence detection offers more selectivity; however, adenosine does not exhibit strong native fluorescence, therefore derivatization is required [7,10]. While this approach provides high sensitivity, the analysis time is over 60 min per sample. More recently, HPLC coupled with tandem mass spectrometry (MS–MS) has been successfully used to measure endogenous adenosine and its metabolites in dialysates [11]. Excellent signal-to-noise ratios and high analyte specificity were evident in this work. Fractions were collected every 40 min in this work; however, since the separation time was just a few min and the sensitivity was good, it may be possible to improve the temporal resolution using this approach [11]. While the HPLC–MS–MS method is highly effective, its requirement of a mass spectrometer makes it not readily available for experiments require routine monitoring. Therefore, a rapid and sensitive adenosine assay could significantly improve the tools available for studying adenosine function.

In this work, we demonstrate an aptamer-based chromatographic method that enables selective preconcentration and retention of adenosine from microdialysis samples with no sample preparation. The stationary phase aptamer is the same as that used previously for separation of multiple adenosine compounds [2], but the mobile phase is modified to allow selective retention of adenosine. The stationary phase is packed into a capillary chromatography column to improve the mass sensitivity and enable detection of adenosine in dialysates. As chemical monitoring requires multiple assays on a single column, we also investigate the stability of the phase to multiple analyses and gradient cycles.

2. Experimental

2.1. Chemicals and materials

Sodium phosphate monobasic, NaCl, $MgCl_2$, tris(hydroxymethyl)aminomethane (Tris), $ZnCl_2$, $NiCl_2$, and EDTA were obtained from Fisher Scientific (Pittsburgh, PA, USA). The 42-base aptamer (5′-GTG CTT GGG GGA GTA TTG CGG AGG AAA GCG GCC CTG CTG AAG-3′) was synthesized by Integrated DNA Technologies (Coralville, IA, USA). A biotin label was attached to the 3′ end of the aptamer through a 15-carbon spacer arm based on triethylene glycol. All other chemicals and standards were obtained from Sigma (St. Louis, MO,

USA). All solutions were prepared with deionized water purified by a Milli-Q plus system (Millipore, Marlborough, MA, USA).

2.2. Stationary phases and column packing

Column preparation was similar to our previous report [2]. Briefly, 300 μl of 10 μ*M* biotinylated aptamer in 20 m*M* Tris, 20 m*M* NaCl, 5 m*M* $MgCl_2$ at pH 6.6 was heated to 82 °C for 2.5 min and then removed from heat and allowed to reach ambient temperature (the heating and cooling cycle is performed to ''renature'' the aptamer which allows formation of the active confirmation). Then, 300 μl of a slurry of 5 μm diameter porous glass beads (500 Å pores) modified with streptavidin (MPG beads, CPG, Lincoln Park, NJ, USA) was centrifuged and supernatant removed (slurry was used as supplied by the manufacturer). The beads were mixed with the renatured aptamer solution and incubated at 4 °C overnight with moderate mixing.

The DNA-modified beads were packed into 13-cm lengths of 150-μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) as described previously [2,12]. The resulting columns were filled to a point 5 cm from the outlet end where a frit had been placed [7]. A detection window was prepared just past the frit by burning away a small section of polyimide prior to packing. Packed columns were rinsed with an aqueous buffer (20 m*M* Tris, 20 m*M* NaCl, 20 m*M* $MgCl_2$ at pH 6.6) for 1.5 h at a flow-rate of 5 μl/min before use.

2.3. Chromatography

All chromatography experiments were performed at room temperature (~25 °C) with an HPLC system consisting of a Eldex Micropro pump (Napa, CA, USA), a Valco C2 injection valve (Houston, TX, USA) and a TSP Spectra 100 UV detector (Spectra-Physics, USA) set at 254 nm. A flow-splitter consisting of a tee with one arm connected to a fused-silica capillary (30 cm×25 μm I.D.) was in-line between the pump and injection valve. The pump was set to generate 3500 p.s.i. at the column head resulting in a column flow-rate of 9 μl/min (1 p.s.i.=6900 Pa). Unless stated otherwise, mobile phase A was an aqueous solution of 20 m*M* Tris, 20 m*M* NaCl, 20 m*M* $MgCl_2$ at pH 6.6. Mobile phase B was an aqueous solution of 20 m*M* Tris, 20 m*M* NaCl, and 30 m*M* EDTA at pH 8.6 with either 20 m*M* $ZnCl_2$ for Zn^{2+} elution or 20 m*M* $NiCl_2$ for Ni^{2+} elution. Gradients are described in the text and figure legends. Mobile phase solutions were filtered with 0.22-μm PTFE membrane filter (MSI, Fisher Scientific, Atlanta, GA, USA) and degassed by sparging with He for at least 15 min prior to use. Injections were performed with the capillary column connected directly to the injection valve. The injection loop volume was 6–15 μl and the valve switched to inject position for 12–72 s depending on the injection volume desired. Microdialysis samples were loaded in a 6-μl loop directly from the microdialysis probe and injected for 24 s. All adenosine standard samples were dissolved in distilled water and prepared fresh daily. Signals from the UV detector were collected using a 486 DX2-66 MHz personal computer equipped with data acquisition board (National Instruments, Austin, TX, USA) controlled by locally written software.

2.4. Microdialysis

Microdialysis probes were prepared in-house as previously described [13]. The ''side-by-side'' probes had a 200-μm O.D. defined by the dialysis tubing (18 000 molecular-mass cut-off from Spectrum Laboratories (Deerfield, IL, USA) with 2 mm long sampling region and approximately 70 nl internal volume. During microdialysis sampling, artificial cerebral spinal fluid (aCSF) was perfused through the probe using a microsyringe pump (CMA/102, Acton, MA, USA) at 0.6 μl/min unless stated otherwise. The aCSF consisted of 145 m*M* NaCl, 2.68 m*M* KCl, 1.01 m*M* $MgSO_4$, and 1.22 m*M* $CaCl_2$. For in vitro tests of relative recovery, the probes were placed in solutions of known concentration of adenosine dissolved in aCSF and warmed to 37 °C. Relative recovery was 25±4% (n=5) at 0.6 μl/min. For in vivo and in vitro dialysis experiments, collected dialysate was analyzed for adenosine using the capillary LC method and quantified using an external calibration curve.

Male Sprague–Dawley rats (250–350 g) were anesthetized with chloral hydrate as described before [14]. The rat was mounted into a stereotaxic ap-

paratus (Kopf Instruments, Tujunga, CA, USA) and surgery was performed to expose the brain region of interest as described elsewhere [14]. The stereotaxic unit was used to implant the microdialysis probe in the somatosensory cortex using the following coordinates: 1.4 mm anterior of bregma, medial 5.0 mm of midline, and 2.7 mm down from dura. Probes were inserted at a rate of 500 μm/min to minimize tissue damage. Fractions were collected at 5-min intervals and injected directly onto the LC system.

3. Results and discussion

3.1. Development of elution conditions

The aptamer used in this work had been developed previously by in vitro selection [15] and had been immobilized for chromatography [2]. In developing a practical assay of adenosine based on this aptamer chromatography column, it was necessary to identify mobile phases that would allow control of retention. Because high sensitivity is needed for in vivo measurements, it was of interest to develop a method that would allow the adenosine to be concentrated on-column and then eluted in a narrow zone. On-column concentration requires retention of the target analyte even during large volume injections. Elution with a narrow zone requires a mobile phase that desorbs the target analyte with rapid kinetics. In addition, since it was desired to use the same column for multiple analyses, it was necessary to identify mobile phases that would allow the aptamer to remain stable.

Preliminary studies indicated that an aqueous buffer similar to the selection buffer would serve as a suitable weak mobile phase for adenosine on the aptamer column. Fortification with Mg^{2+} was also indicated based on the observation that during aptamer selection the adenosine affinity for some oligonucleotides was sensitive to [Mg^{2+}] [16]. In addition, in previous work, we had observed that affinity (measured as retention factor) of the 42-base DNA aptamer increased 3–4-fold as Mg^{2+} was increased from 5 to 20 m*M*. Increasing the concentration of Mg^{2+} up to 50 m*M* did not enhance binding further [2]. Chromatograms comparing retention of adenosine in an aqueous mobile phase containing Mg^{2+} to that with low Mg^{2+} and EDTA are shown in Fig. 1A. Although a mobile phase with high free Mg^{2+} concentration allows suitable retention of adenosine, isocratic elution results in a broad zone that would be difficult to detect at low levels. Therefore, it was necessary to identify a mobile phase gradient to effect elution of the adenosine.

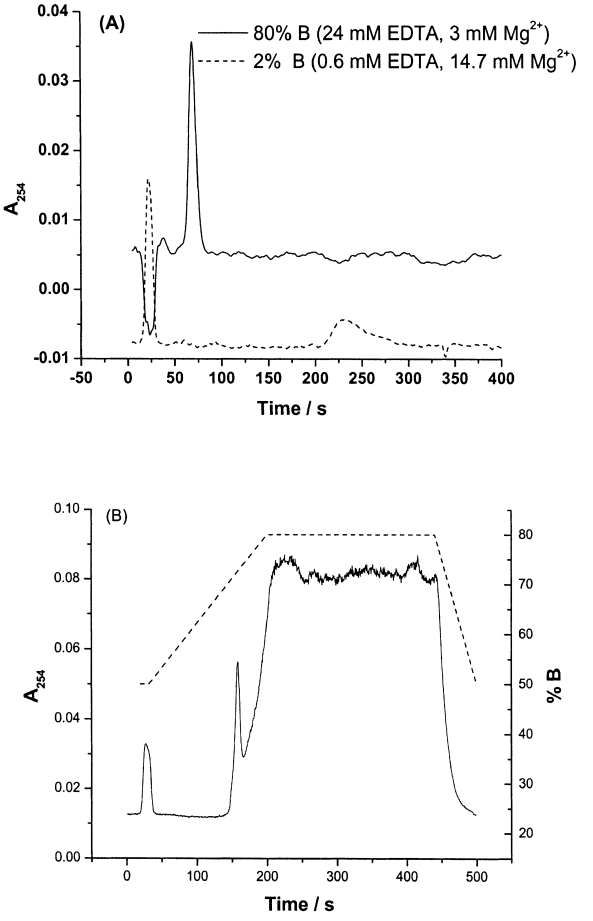

Fig. 1. (A) Chromatograms illustrating the effect of free Mg^{2+} on retention of adenosine on aptamer column. 1.3 μl of 5 μ*M* adenosine in water was injected and eluted isocratically with the mobile phase indicated. Mobile phase A consisted of 20 m*M* KH_2PO_4, 60 m*M* NaCl, 15 m*M* $MgCl_2$ at pH 6.6. Mobile phase B consisted of 20 m*M* KH_2PO_4, 60 NaCl, 30 m*M* EDTA, pH 8.6. Baseline disturbance at 25 s indicates the void time. (B) Chromatogram illustrating gradient elution of 2.5 μ*M* adenosine (1.3 μl injected) using the same mobile phases as in A. Dashed line indicates the gradient program. Adenosine elutes at 155 s. The large baseline disturbance is associated with EDTA.

3.2. Elution by chelation of Mg^{2+} with EDTA

The results in Fig. 1A suggest that manipulation of free Mg^{2+} concentration by removal of Mg^{2+} would be a suitable approach to gradient elution of adenosine; however, simple removal of Mg^{2+} from the mobile phase (after equilibration of a column with Mg^{2+} containing mobile phase) did not alter elution of adenosine from that with Mg^{2+} present indicating that the Mg^{2+} is tightly bound to the stationary phase and is only slowly is released. Therefore, a gradient of EDTA, which was expected to rapidly remove the Mg^{2+} from the aptamer, was explored (Fig. 1B). While an EDTA gradient does result in elution of a narrow adenosine zone, a large background disturbance is observed in the chromatograms due to the large change in EDTA concentration. The large background shift results in an unacceptably high detection limit of 250 n*M* (2 pmol injected) for adenosine.

3.3. Solution pH or ionic strength change

A common method of elution in affinity chromatography is to alter the mobile phase pH or ionic strength. In preliminary studies, we found that elution was unaffected by changing the pH from 2.5 to 7.5. A mobile phase with higher pH was not explored as it would decrease the stability of the glass support. Increasing the ionic strength resulted in modest alteration of the retention factor. When NaCl concentration was increased from 20 to 200 m*M*, the retention factor, *k*, decreased 50%; however, increases of NaCl concentration up to 750 m*M* did not further alter retention. In addition, the use of a high ionic strength mobile phase did not improve the peak shape. Given these unpromising results, and the observation that use of high salt concentrations tended to cause clogging in the capillary column, the use of ionic strength gradients was not further pursued.

3.4. Competitive elution of adenosine with divalent cations

Another approach to elution in affinity systems is to add a species that will compete for the binding site on either the stationary phase or the analyte. While an adenosine-like molecule is a potential candidate for such a competition, it is likely that any such molecule would make detection difficult by contributing to the background absorbance signal. Another approach is to interfere directly with the binding site. It has previously been demonstrated that the aptamer used in this work binds to adenosine in a structurally specific manner such that N7, N6 and N1 of the adenine moiety, as well as the 3′-hydroxyl and 5′-O of the ribose moiety of the nucleotide are involved in the interaction with the aptamer [15]. We hypothesized that blocking or adding a reagent to competitively bind these sites would dissociate the on-column aptamer–adenosine complex. Adenosine binds several divalent cations at these same sites suggesting that such species may be useful in a strong mobile phase [17,18]. In previous studies of metal ion binding to adenosine, it has been demonstrated that: (1) Ni^{2+}, Cu^{2+}, Co^{2+} and Cd^{2+} coordinate to adenosine through N1 and N7 sites with a preference for the N7 site; (2) Zn^{2+} binding is evenly distributed between N1 and N7 sites; and (3) Mn^{2+} prefers the N1 site [17,18]. Mg^{2+} also binds adenosine but the stability constant is much smaller than that of Zn^{2+} or Ni^{2+}. Therefore, we explored the use of these ions as possible mobile phase additives to elute adenosine.

Fig. 2 illustrates chromatograms from the isocratic elution of 5 μ*M* adenosine with an aqueous mobile phase containing: (a) 19 m*M* Mg^{2+}, (b) 1 m*M* Mg^{2+}, (c) 10 m*M* Zn^{2+}, 10 m*M* Mg^{2+}, and (d) 10 m*M* Ni^{2+}, 10 m*M* Mg^{2+}. The data show that addition of Zn^{2+} or Ni^{2+} results in much reduced retention and a narrow zone for adenosine. Ni^{2+} appears to be a slightly stronger mobile phase additive than Zn^{2+} as the retention factor is smaller with Ni^{2+} than with Zn^{2+} which is expected as the stability constant (log *K*) of Ni^{2+} with adenosine is 0.32 while Zn^{2+} with adenosine is only −0.17 [18].

The inset of Fig. 2 shows that divalent cation competition is much more effective than decomposing the complex by chelation of Mg^{2+} at eluting adenosine. This result may be because divalent cation competition directly targets the binding site of the analyte. In contrast, the Mg^{2+} may be involved in stabilizing the aptamer secondary structure. Therefore, chelation would involve removing it from the

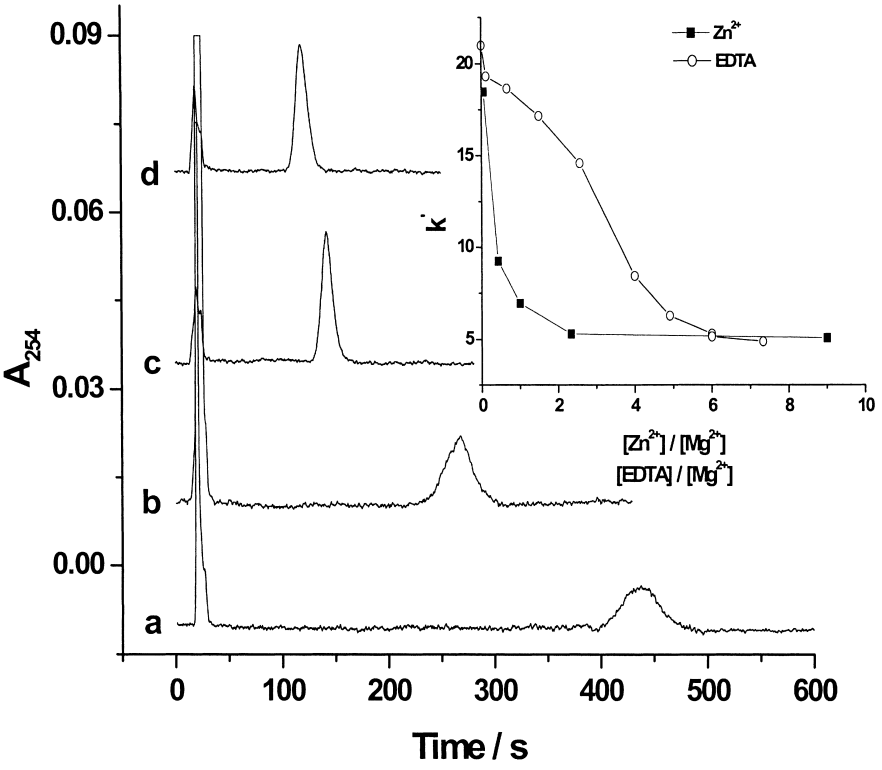

Fig. 2. Chromatograms illustrating effect of transition metals on retention of adenosine. First, 1 μl of 5 μ*M* adenosine was injected onto an aptamer column and eluted isocratically with mobile phases containing 20 m*M* Tris, 20 m*M* NaCl and (a) 19 m*M* $MgCl_2$, pH 6.6; (b) 1 m*M* $MgCl_2$, pH 6.6; (c) 10 m*M* $ZnCl_2$, 10 m*M* $MgCl_2$, pH 5.0; (d) 10 m*M* $NiCl_2$, 10 m*M* $MgCl_2$, pH 5.0. Inset: retention factor changes with the ratio of $[Zn^{2+}]$ to $[Mg^{2+}]$ and [EDTA] to $[Mg^{2+}]$ in the mobile phase under isocratic conditions. Data were obtained using a mobile phase of 20 m*M* Tris, 20 m*M* NaCl, 10 m*M* $MgCl_2$ at pH 5.0 with the EDTA or Zn^{2+} added to make the ratio indicated.

aptamer and subsequent loss of secondary structure. This process is likely to be slower than the Ni^{2+}–adenosine binding.

Based on these experiments, it was determined that gradient elution with $NiCl_2$ would be effective for adenosine determination. Fig. 3 illustrates a chromatogram resulting from gradient elution of adenosine. For this chromatogram, mobile phase A was 20 m*M* Tris, 20 m*M* NaCl and 20 m*M* $MgCl_2$, pH 6.6 and mobile phase B was an identical solution with 20 m*M* $NiCl_2$ substituted for $MgCl_2$ and adjusted to pH 3.45. (The adjustment in pH was found to be necessary to prevent precipitation during the gradient.) The gradient used was from 0 to 72 s, 2% B, a linear increase to 90% B from 72 to 150 s, and then a linear decrease to 2% B from 150 to 180 s. As shown in the figure, a symmetrical peak well-resolved from the baseline disturbance at the dead time was obtained with these conditions.

3.5. On-column preconcentration and elution

To obtain a suitable concentration detection limit, we explored on-column concentration of adenosine. On-column concentration is achieved by injecting large sample volume dissolved in weak mobile phase, allowing analytes to concentrate at the head of the column. Maximal concentration is obtained by maximizing the injection volume and simultaneously minimizing the volume of eluted peaks. To minimize the volume of the eluted peak, we used a relatively low flow-rate of 9 μl/min and a steep gradient slope of 74% increase in B per min. In affinity chromatography, the maximum injection volume is limited by

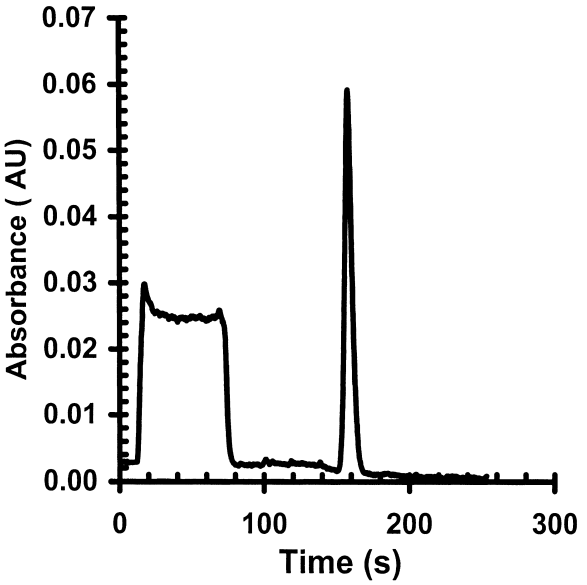

Fig. 3. Chromatogram illustrating gradient elution of 1.2 μ*M* adenosine (2 μl injected). Mobile phase A consisted of 20 m*M* Tris, 20 m*M* NaCl, 20 m*M* $MgCl_2$ at pH 6.6 and mobile phase B consisted of 20 m*M* Tris, 20 m*M* NaCl and 20 m*M* $NiCl_2$ at pH 3.45. The gradient was 2% B from 0 to 72 s with a linear increase to 90% B from 72 to 180 s.

the capacity of the binding sites on the column. We found that the peak area for adenosine increased proportionally with injection volume up to 6 μl of 1.2 μ*M* adenosine suggesting no loss of analyte or saturation of the stationary phase under these conditions. Above 6 μl leveling off of the peak area was observed indicating saturation of the column or loss of analyte. An injection volume of 4.0 μl was chosen for routine measurements. Using this injection volume, a linear increase in peak area was found in the concentration range of 50 n*M* to 3 μ*M* with a $r^2 > 0.99$ ($n = 7$). The detection limit, calculated as the concentration that would generate a signal-to-noise ratio of 3, was 30 n*M* or 120 fmol injected.

Under optimized elution conditions, adenosine was eluted within 3 min after injection (Fig. 3). The whole analysis time including column equilibration, injection and elution was 5 min per sample. This short analysis time enables injections to be made at 5-min intervals on the column as desired for microdialysis or other chemical monitoring applications. The precision and robustness of the method were evaluated using repetitive injections similar to that used in Fig. 3. The relative standard deviation of retention time, peak area, and peak height were 0.7, 4.6 and 5.7%, respectively, for 30 injections of 1 μ*M* adenosine. Columns could be used for over 200 injections without noticeable loss of retention suggesting good stability of the aptamer to the mobile phase gradient conditions used. Typically columns were broken or clogged before a loss of stationary phase performance was significant. While all the work reported here was obtained with manual operation of the injection valve, it is feasible to automate this process enabling on-line operation.

3.6. In vivo measurements

As a demonstration of the utility of this method adenosine levels were measured in the brain of anesthetized rats using this approach. Fig. 4 illustrates chromatograms obtained 30 and 200 min after probe implantation. The chromatograms are quite simple despite the complexity of the sample indicat-

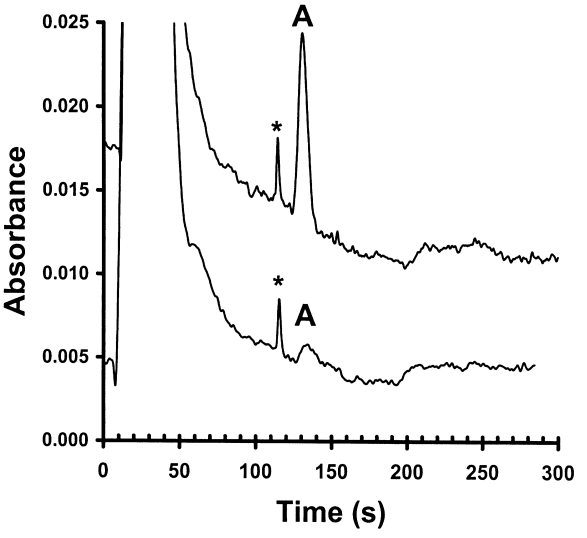

Fig. 4. Chromatograms from analysis of dialysate sampled from the anesthetized rat cortex. First, 4 μl of dialysate were injected and the sample separated using the gradient program of Fig. 1. Peaking matching the retention time of adenosine is marked with A. The peak marked with the asterisk is an unknown that occurred only in dialysate samples. Samples were collected 30 min (upper trace) and 200 min (lower trace) after the probe had been implanted.

ing the selectivity of the phase. As illustrated by the chromatograms, after initial implantation of the dialysis probe, the adenosine concentration tended to be high and eventually stabilized at a lower level. The mean concentration 10 min after the probe was implanted was 360±30 n*M* and at 90 min it was 87±10 n*M* (n=5). No further decreases were observed after 90 min. The higher initial levels are likely due to release of adenosine induced by damage caused during probe implantation. It can be difficult to compare dialysate concentrations from different experiments because of the effect of brain tissue and flow-rate on recovery and, in this case, the use of anesthesia. Nevertheless, the concentrations appear to be within the range expected as adenosine microdialysate concentrations have been reported to be between 10 and 460 n*M* depending upon the brain region, dialysis flow-rate, and consciousness of the animal [11,19,20].

4. Conclusions

The aptamer stationary phase provided good selectivity for retaining and separating adenosine from complex samples. The high selectivity combined with the robustness of the stationary phase suggests that such columns could be used for chemical monitoring or other forms of rapid, high-throughput analysis. As a demonstration of the application of aptamer phase, adenosine was successfully monitored in microdialysis samples collected from the brain of live rats. The method is considerably simpler and faster than other adenosine assays in common use. While not providing the multi-analyte capability of the recently developed HPLC–mass spectrometry assay for adenosine [18], the aptamer chromatography method is in principle less costly to use.

While antibodies remain a viable option for highly selective stationary phases, aptamers have a number of properties that make their development attractive including applicability to toxic or native compounds that are difficult to raise antibodies against, completely in vitro selection and synthesis thus obviating animal use, greater on-column stability, and flexibility in mobile phase selection. The present limitation of aptamers is that relatively few have been made available; however, this limitation is changing as improved methods of selection are developed.

Acknowledgements

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