Investigation of a New Detection Method for Adenosine: Binding Studies and Reaction Kinetics

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Motivation

Adenosine, a purine ribonucleoside, is an important neuromodulator involved with sleep, locomotion, hibernation and is linked to neurological disorders.[1-3]

Microdialysis techniques are typically used in *in vivo* adenosine studies and result in small sample sizes (µL) and low analyte concentrations (nM).[3]

Current adenosine detection methods use reverse phase liquid chromatography coupled with UV detection (HPLC) that require injection volumes of 3-10 µL and can provide low temporal resolution[4].

The goal of this research is to develop a new adenosine detection method using laser-induced fluorescence capillary electrophoresis (LIF-CE). LIF-CE is a technique that can analyze lower sample volumes (nL) in high resolution providing a more sensitive detection method for adenosine studies.

LIF-CE Method

LIF-CE is a powerful analytical separation technique based on the migration of charged molecules under the influence of an applied voltage and electroosmotic flow (EOF).[5] Analytes can be identified by migration time and quantified by signal integration.

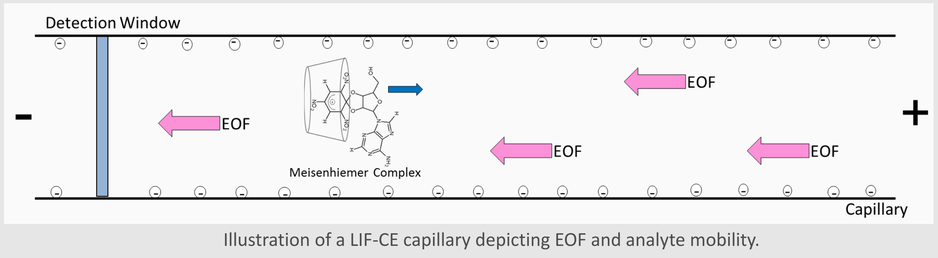
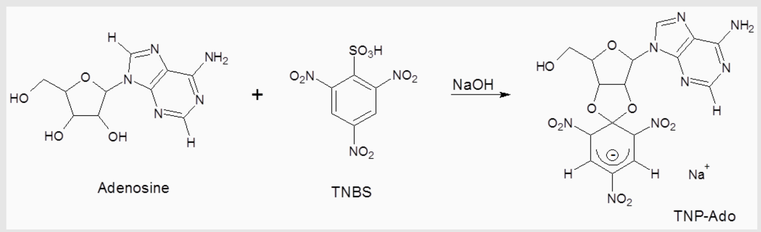


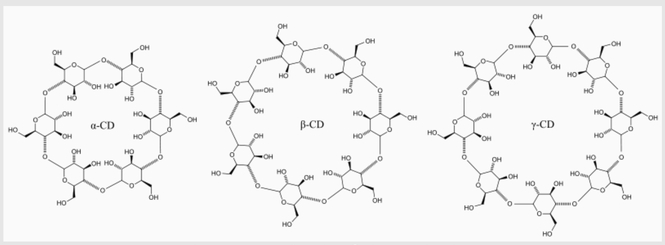
Illustration of a LIF-CE capillary depicting EOF and analyte mobility.

Adenosine is reacted with trinitrobenzenesulfonic acid (TNBS) to form trinitropheylated-adenosine (TNP-Ado), a stable Meisenheimer complex. TNP-Ado exhibits two absorbance maxima at 408 nm and 476 nm and a single fluorescence emission maximum in the 530-560 nm range.[6]

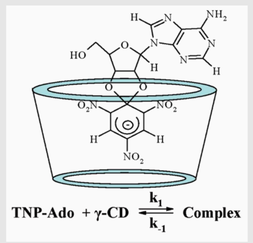


Reaction scheme of the TNP-Ado derivative.

The TNP-Ado derivative experiences enhanced fluorescence in the presence of cyclodextrins. Cyclodextrins are 6, 7 and 8 membered glucose rings with nonpolar cavities which form inclusion complexes with smaller molecules. γ-Cyclodextrin enhances fluorescence by a factor of 110 compared to the absence of cyclodextrin in water.[7]



Structures of α-CD, β-CD, and γ-CD.



TNP-Ado complexed with γ-CD

In order to develop a reliable LIF-CE detection method for adenosine, the binding interactions between TNP-Ado and various cyclodextrins were investigated along with the reaction kinetics of adenosine in artificial cerebral spinal fluid (ACSF) and TNBS.

The LIF-CE instrumental parameters were 48 cm total length (28 cm detector) x 50 µm inner diameter bare-fused silica capillary, 20 mM borate buffer pH 9, 10 kV, 50 mbar 3 second injection for the binding studies, 50 mbar 1 second injection for the reaction kinetics studies and 25 mW 405 nm laser.

References

(1) Porkka-Heiskanen, T.; Kalinchuk, A. *Sleep Biol Rhythms* 2011, 9(1), 18-23. (2) Quiroz, C. et al. *Exp Neuro* 2010, 224, 292-297. (3) Jinka, T. R. et al. *J Neurosci* 2011, 31(30), 10752-10758. (4) Chen, L.S.; Fujitaki, J.; Dixon R. *J Liq Chromatogr* 1993, 16, 2791-2796. (5) Weinberger, R. Ed. Practical Capillary Electrophoresis; Academic Press: San Diego, USA, 2000. (6) Hiratsuka, T. *Biochim. Biophys. Acta* 1982, 719, 509–517 (7) Green, T. K. et al. *J Org Chem* 2010, 75, 4048-4055. (8) Azzam, K. M.; Saad, B.; Aboul-Enein, H. Y. *Electrophoresis* 2010, 31, 2957-2963.

Binding Studies

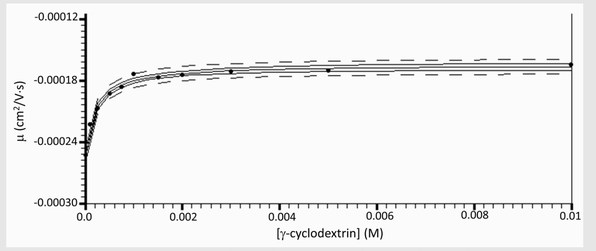
Assuming a 1:1 association of TNP-Ado and cyclodextrin, binding constants can be determined by the electrophoretic mobility and integrated fluorescence of TNP-Ado with respect to the concentration of cyclocextrin.[8]

$$\mu_i = \frac{\mu_f + \mu_c K[C]}{1 + K[C]}$$

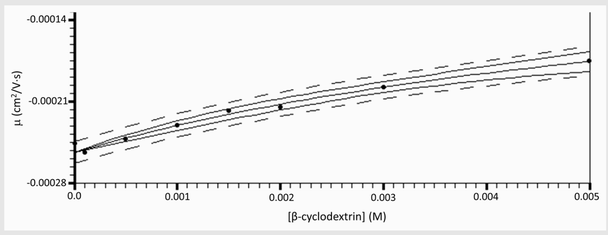
$$f_i = \frac{f_f + f_c K[C]}{1 + K[C]}$$

μ_i / f_i = complexed TNP-Ado mobility or fluorescence
μ_f / f_f = uncomplexed TNP-Ado mobility or fluorescence
μ_c / f_c = complexed TNP-Ado mobility or fluorescence at 10mM cyclodextrin
C = cyclodextrin concentration
K = binding constant

Binding studies for TNP-Ado (10 µM) with γ-cyclodextrin were determined at 15, 20, and 25 °C and with β-cyclodextrin at 25 °C by 2 parameter nonlinear curve fitting. Cyclodextrin concentrations ranged from 0 to 10 mM in a 20 mM borate buffer system at pH 9.



Electrophoretic mobility of TNP-Ado (10 µM) as a function of γ-CD at 25°C.



Electrophoretic mobility of TNP-Ado (10 µM) as a function of β-CD at 25°C.

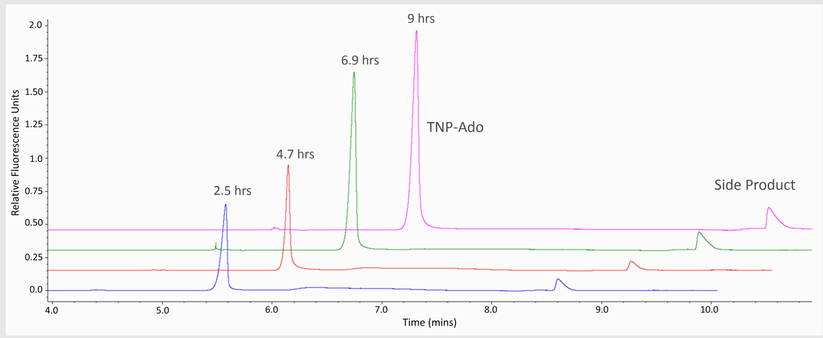
Temperature(°C)	Electrophoretic Mobility K (M^{-1})		Integrated Fluorescence K (M^{-1})	
	γ-Cyclodextrin	β-Cyclodextrin	γ-Cyclodextrin	β-Cyclodextrin
25	5395 +/- 631	136 +/- 45	1639 +/- 359	297 +/- 142
20	4590 +/- 463	-	1522 +/- 654	-
15	5131 +/- 1201	-	2270 +/- 705	-

The electrophoretic mobility binding constant for TNP-Ado and γ-CD is approximately 39 orders of magnitude larger than that of TNP-Ado and β-CD.

From the binding studies, it can be concluded that TNP-Ado has an higher affinity for γ-CD than β-CD and temperature had no significant effect on the binding constants determined by either electrophoretic mobility or integrated fluorescence.

Reaction Kinetics

The derivatization of adenosine in ACSF must be optimized to ensure TNP-Ado formation in future biological studies. A 100 µM sample of adenosine in 0.5 mL ACSF was reacted with 20 µL of 1 M NaOH and 10 µL of TNBS in H_2O and monitored with LIF-CE over a 13 hour period.



Adenosine (100 µM) derivatization in ACSF at 2.5 hrs, 4.7 hrs, 6.9 hrs and 9 hrs at 25 °C.

Preliminary results show the formation of TNP-Ado in ACSF and a side product, most likely picric acid. It can be concluded that the derivatization of adenosine is possible in ACSF and the LIF-CE method is able to baseline resolve the TNP-Ado signal from side products in ACSF.

Future Directions and Acknowledgements

Future research will focus on further optimizing the adenosine reaction in ACSF using realistic quantities of adenosine (< 10 µM).

An internal standard possibly, N^6-cyclohexyladenosine, will be derivatized with TNBS and and used to quantify the TNP-Ado conversion by minimizing error due to injection variability. Other reaction conditions such as temperature, solvent and various bases will also be investigated.

Once the reaction method is optimized in ACSF it will be applied to *in vitro* and *in vivo* hibernation studies.

Thank you to Dr. Tom Green, Dr. Bill Simpson and the Department of Chemistry and Biochemistry. Funding source NIH NINDS R15NS074422.