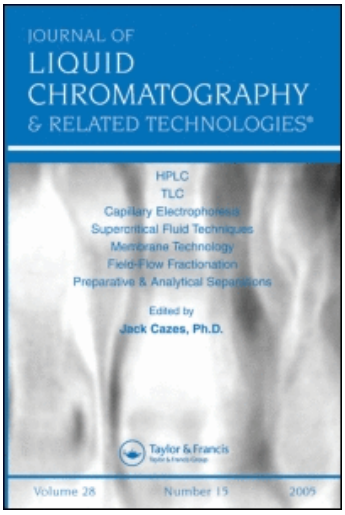

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AN IMPROVED ASSAY FOR ADENOSINE IN RAT BRAIN MICRODIALYSATES USING MICROBORE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-performance liquid chromatographic method using a microbore system consisting of a microbore column (100 mm - 1mm I.D.- 3 μm), microbore pump (60 μl/min) and UV absorbance detector has been developed for the determination of adenosine in rat brain microdialysis samples. UV absorbance of adenosine at 259 nm was used to measure adenosine. The limit of detection (LOD) was 50 pg and the limit of quantitation (LOQ) was 10 ng/ml. The methodology is applicable for the quantitation of adenosine in small volumes (3 - 10 μl) of microdialysate and does not require sample preparation.

INTRODUCTION

Adenosine, a purine nucleoside, is an important regulator of physiologcal functions throughout the body (1, 2). Because of the role adenosine plays during ischemia, there has been an increasing interest in measuring levels of adenosine in interstitial cellular space using microdialysis techniques (3). Hence, an accurate and sensitive method for the determination of adenosine concentrations in very small amount of dialysate (5 - 10 μl) is necessary.

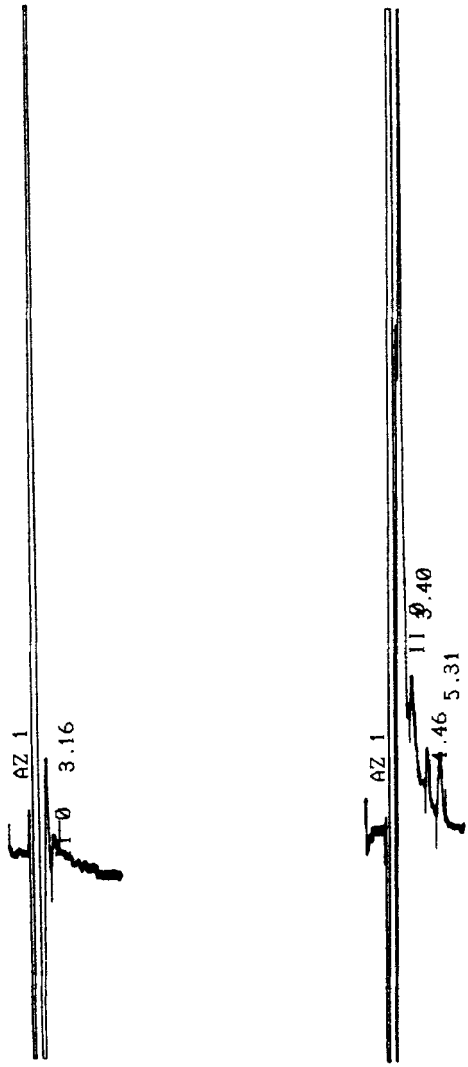
A number of procedures have already been described for the measurement of adenosine in biological fluids, including high-performance liquid chromatography with fluorometric (2), electrochemical (1), spectrometric (4, 5) and microbore-column isocratic (Brownlee RP-18, 220 mm - 2.1 mm I.D.- 7 μm) (6) methods. The problems of using fluorometric and electrochemical detectors are that sophisticated procedures of sample derivatization and/or enzymatic conversion are required prior to chromatography. This can result in errors, is time-consuming , and may require large sample volumes. Also, other metabolites of interest cannot be assayed. In our hands, the previously reported spectrophotometric method using the Brownlee microbore column (6) did not have sufficient sensitivity to detect adenosine in rat brain microdialysates. In the present study, we found an alternative and more reliable method of increasing the sensitivity using a microbore solvent delivery system and a SepStik-microbore column.

MATERIALS AND METHOD

The HPLC-system consisted of microflow-pump (Applied Biosystems, ABI-140 B Solvent Delivery Systems, Foster City, CA, U.S.A.), microcell-UV detector (ABI-785) and microbore-autosampler (HP-1050, Kennet Square, PA, U.S.A.) equipped with a SepStik-RP-18 microbore column (100 x 1 mm I.D.- 3 μm, Bioanalytical Systems, BAS, West Lafayette, Indiana, U.S.A.) attached to a 1 mm in-line filter kit (0.2 μm-membrane, MF-8952, BAS) as guard filter to the protection against particulates. To obtain high sensitivity and to minimize peak broadening, the connection tubings were kept as short as possible, and the column was directly connected to the injector. The integrator was a Waters-746 (Milford, MA, U.S.A.).

The chemicals used were HPLC/analytical grade. Acetonitrile and potassium phosphate were obtained from Fisher (Fairlawn, NJ, U.S.A.). Adenosine was purchased from Sigma (St. Louis, MO, U.S.A.). Milli-Q Water (Millipore, Bedford, MA, U.S.A.) was used for preparation of the mobile phase.

The mobile phase contained 10 mM potassium dihydrogenphosphate with 3 % (v/v) acetonitrile and adjusted to pH 3.5 with 10 % phosphoric acid. This solution was filtered using a 0.45-μm HV-filter (Millipore, Bedford, MA, U.S.A.) and degassed with helium for 10 min before use. Chromatography was carried out at room temperature using a mobile phase of 50/50 (v/v) in both syringe-pumps at the total flow-rate of 60 μl/min. Ten μl of the dialysate were directly injected into the HPLC-system. The chromatographic condition was set at the range of 0.1 a.u.f.s.. The standard solutions were prepared by *successive 1: 2 dilution*, and encompassed a range from 10 to 3125 ng/ml (37 - 1174 nM). Variance stablized regression analysis of calibration standards were used for statistical analysis of the data (7).



(A) (B)

FIGURE 1. Chromatogram showing the retention time for adenosine.
(A) Blank saline. (B) Dialysate at the time of 1.00 hr (Rt. : 5.31).

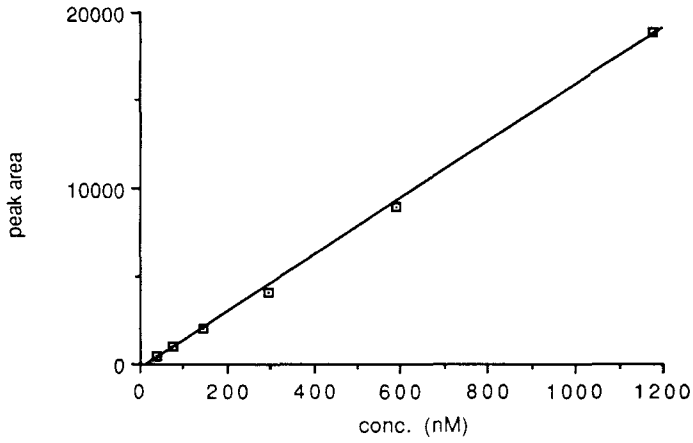


FIGURE 2. Standard curve for adenosine

TABLE 1

Variance stablized regression analysis of calibration standards.

std. conc.(nM)	peak area	back calc.	% residual
36.7	446	37.18	1.3157
73.4	1001	74.06	0.9029
146.8	1979	139.1	-5.2786
293.6	4021	274.7	-6.4229
587.2	8890	598.3	1.8884
1174.4	18902	1263.6	7.5944
mean % residual:			± 3.9005
slope:			15.0489

TABLE 2

Intra- and inter-day precision of adenosine in water (n = 3).

std. conc. (nM)	36.7	73.4	146.8	293.6	587.2	1174.4
Intra-day precision (peak area)						
mean	37.9	71.05	137.2	282.9	608.5	1251
stdev	0.95	3.16	7.82	7.2	19.42	28.3
C.V. %	2.51	4.44	5.70	2.55	3.19	2.26
Inter-day precision						
mean	37.23	74.39	137.2	272.7	602.6	1272
stdev	1.62	6.07	3.66	14.59	24.99	52.7
C.V. %	4.35	8.16	2.67	5.35	4.15	4.14

RESULTS

Adenosine was eluted at 5.31 min (Fig. 1).The standard curve was linear throughout the range tested (r = 0.9993) (Fig. 2) and the mean % residual was 3.9 (Tab. 1). The minimum detectable amount (at a signal-to-noise ratio 3:1) was 50 pg for adenosine. The intra- and inter-day precision was < 10 % (C.V.) (Tab. 2). The method was used to measure adenosine in dialysates from rat brain and the conc. ranges from 37 to 1433 nM (9.9 - 3807 ng/ml) (Fig. 3).

CONCLUSIONS

We have developed a highly sensitive HPLC-microbore method for the quantitation of adenosine in rat brain microdialysates. The level of sensitivity (LOD = 50 pg) was thirteen times higher than the previously reported level of sensitivity attained using a Brownlee microbore column (6). The reason for the higher sensitivity is that the SepStik-column used has a shorter (100 mm) length, narrower bore (1 mm I.D.) and smaller particle size (3 μm) of packing material. This offers the smallest void volume and reduces the drug dispersion in the column. The direct connection between the injector and the SepStik-column also reduces the dead volume to increase the sensitivity. The Microbore Solvent Delivery System offers reduced solvent consumption (1 : 50, compared with the

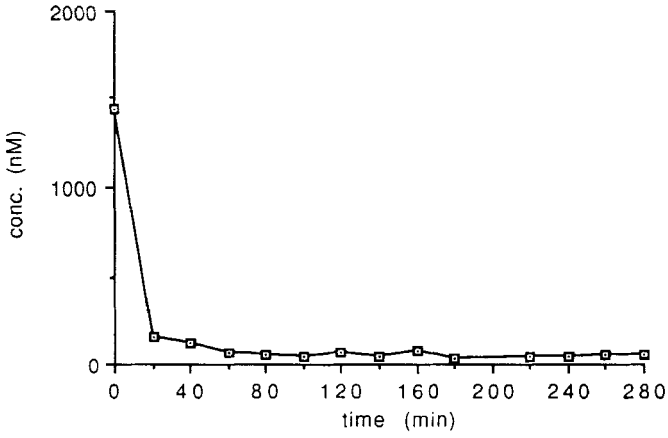


FIGURE 3. The change with time of the adenosine concentration in intracerebral microdialysates of rat brain

conventional solvent delivery systems), reduced disposal cost and therefore helps the environment. In summary, the assay described has the advantage of being simple and possesses good linearity, precision and superior sensitivity.

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