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Subtraction method for the high-performance liquid chromatographic measurement of plasma adenosine

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ABSTRACT

The measurement of plasma adenosine with traditional high-performance liquid chromatographic techniques is difficult because of its nanomolar concentration, its short half-life in blood, and because of the difficulty in isolating adenosine from interfering peaks in the chromatogram. To prevent loss of adenosine in the blood sample, a "stop solution" is used to prevent enzymatic degradation and cellular uptake. Peak-shifting techniques on fractionated samples to measure adenosine derivatives have been used in the past to avoid interfering peaks in the chromatogram. A new method has been developed by which nanomolar levels of plasma adenosine can be accurately measured despite co-eluting peaks in the chromatogram. In this method, plasma samples are collected with a stop solution, processed, and divided. Adenosine deaminase is added to part of the sample to form a blank. A computer program subtracts the blank chromatogram from the paired unknown, and the result is compared to adenosine standards prepared from the blank and subtracted in a similar fashion. With this subtraction method, the overall recovery of physiological concentrations of adenosine was 89% from dog blood, and the average coefficient of variation was 12%. In summary, the subtraction method of plasma adenosine measurement offers good recovery, reproducibility, and the ability to quantify low levels of adenosine despite interfering peaks in the chromatogram.

INTRODUCTION

The nucleoside adenosine has been suggested to be important in the control of coronary blood flow [1], the prevention of cardiac arrhythmias [2], and the inhibition of adrenergic effects [3]. These physiologic actions of adenosine are thought to be mediated through changes in interstitial adenosine concentration, interacting with a specific receptor for the nucleoside. However, current technology does not allow for direct measurement of interstitial adenosine concentration. Adenosine has been measured in several compartments, including plasma, to estimate interstitial adenosine levels.

Measurement of plasma adenosine concentration with traditional high-performance liquid chromatographic techniques is difficult for several reasons. Firstly, native adenosine concentration is in the nanomolar range, thus the signal-to-noise ratio for adenosine in plasma is quite low. Secondly, blood samples contain both enzymes and cellular uptake mechanisms which actively remove adenosine from the plasma sample. These biochemical sinks for adenosine result in a relatively short blood half-life of the nucleoside. Thirdly, plasma adenosine samples often require several purification procedures, including protein precipitation, before HPLC analysis can begin.

Another particular problem in the measurement of plasma adenosine concentration is the difficulty of isolating the nucleoside from interfering peaks on the chromatogram. Futhermore, plasma samples from different individuals will have different interfering peaks. A gradient that successfully isolates adenosine in plasma from one individual will often not be adequate for plasma from a second individual.

A new microbore HPLC method has been developed which allows for accurate quantification of plasma adenosine levels, despite co-eluting peaks in the chromatogram, and eliminates the

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problems of inconsistencies in the separation. In this method, plasma samples are collected with an enzymatic "stop solution" to prevent loss of the nucleoside to biochemical sinks. The sample is processed, purified, and divided. Part of the unknown sample is treated with adenosine deaminase to form a blank. A computer program is used to subtract the blank chromatogram from

PLASMA ADENOSINE ASSAY SAMPLE HANDLING PROTOCOL

Draw 4.3 ml blood + 5.7 ml stop solution

Centrifuge at 18,500 x g, 0°C, for 2 min

Remove supernatant, add to 200 μl cold 4M PCA in pre-weighed tube

Re-weigh tubes & centrifuge at 18,500 x g, 0°C for 10 min

Remove supernatant, add 200 μl of 1M Hepes

Titrate to pH 7.0-7.5 with cold KOH

Centrifuge at 18,500 x g, 0°C, for 10 min

Remove supernatant, bring to room temperature

SEP-PAK EXTRACTION

Wash column with 20 ml CH_3OH

Wash column with 10 ml H_2O

Draw sample through column with 7.5 cm Hg vacuum

Wash column with 500 μl H_2O

Elute with 3.5 ml CH_3OH, 7.5 cm Hg vacuum

BUCHLER EVAPORATION

Reconstitute in 600 μl H_2O, vortex

Remove 400 μl		Leave 200 μl of solution
Add 10 units adenosine deaminase, vortex		Add 2.5 μl 3M $(NH_4)_2SO_4$, vortex
Incubate room temp. 45 min		Incubate room temp. 45 min
Add 500 μl 100% CH_3OH, vortex		Add 250 μl 100% CH_3OH, vortex
Heat 1 h @ 75°C		Heat 1 h @ 75°C
Cool to room temp.		Cool to room temp.
Buchler evaporation		Buchler evaporation
Reconstitute 160μl Buffer A		Reconstitute 80μl Buffer A
Standard	Blank	Unknown
Remove 80 μl	Leave 80 μl	
Add 20 μl Std.	Add 20 μl Buffer A	Add 20 μl Buffer A
HPLC	HPLC	HPLC

Fig. 1. Flow diagram for the sample handling and processing of plasma adenosine samples for the subtraction method.

its paired unknown. Adenosine concentration is calculated from standards prepared from the blank and subtracted in a similar fashion. This subtraction method of plasma adenosine measurement offers good recovery, reproducibility, and the sensitivity required to measure nanomolar levels of adenosine in the plasma, even when interfering peaks are present on the chromatogram.

EXPERIMENTAL

Stop solution

An ice cold stock solution of *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) (Burroughs-Wellcome, Research Triangle Park, NC, USA), an adenosine deaminase inhibitor [4], is prepared by dissolving the compound in sterile saline to a concentration of 0.3 mg/ml. An ice cold stock solution of dipyridamole (Sigma, St. Louis, MO, USA), an inhibitor of cellular uptake [5], is prepared by dissolving the drug in methanol to a concentration of 1 mg/ml. This solution should be protected from light. Immediately before obtaining the plasma samples, the stop solution is prepared with 150 μl of stock EHNA, 1.5 ml of stock dipyridamole, and 148.35 ml of ice cold sterile saline, yielding a final stop solution concentration of 1 μM EHNA and 20 μM dipyridamole.

Sample procedure

A complete sample processing flow diagram is given in Fig. 1. A 10-ml syringe device [6] that mixes stop solution and blood in a fixed volume ratio is used. The final sample solution is comprised of 57% stop solution and 43% blood. This dilution results in a final concentration of 0.6 μM EHNA and 11 μM dipyridamole in the sample. After drawing the sample, a rapid centrifugation for 2 min at 18 500 *g* and 0°C (Tomy MTX-150 with TMA-3 rotor; Peninsula Labs., Belmont, CA, USA) is necessary to separate the plasma and stop solution mixture from the cellular elements. After centrifugation, the supernatant is removed and transferred to a tared tube containing 200 μl of of 4 *M* perchloric acid (PCA). The addition of acid decreases the sample pH to approximately 1.8 and precipitates plasma proteins. It is critical that no blood cells are transferred to the acid solution because of high cellular concentrations of phosphorylated adenine compounds that can form adenosine by hydrolysis and the release of intracellular adenosine from S-adenosylhomocysteine hydrolase that occurs under acidic conditions. Upon the addition of the supernatant to the acid solution, all enzymatic processes for adenosine metabolism are considered stopped, and the sample may be placed on ice and processed further at the investigator's convenience. From the hematocrit (hct), the tube weights, and the syringe blood/stop solution ratio (0.43), the plasma volume transferred to the PCA tube can be calculated from the following formula, assuming a solution density of 1 g/ml:

$$\text{plasma volume} = (\text{final weight} - \text{initial weight})\,(1 - \text{hct})\,(0.43)/(1 - 0.43\ \text{hct}). \quad (1)$$

After the plasma volume of the sample is determined, 200 μl of 1 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (p*K* 7.4) are added and the sample titrated to pH 7.0–7.5 with approximately 180 μl of 4 *M* potassium hydroxide. This recipe neutralizes the sample with little salt formation, thus avoiding loss of adenosine in the salt matrix. A neutral pH is necessary for further sample purification.

Sample purification

In order to remove unwanted chromatographic peaks and de-salt the sample, a C_{18} Sep-Pak (Waters Chromatography, Milford, MA, USA) purification is used (Fig. 1). Care must be taken to clean the Sep-Pak with methanol and water before use. To maximize adenosine recovery, the sample is drawn slowly through the Sep-Pak with a manifold (Baker SPE-21 Chamber, Phillipsburg, NJ, USA) and a vacuum of less than 12 cmHg. Adenosine is eluted with 100% methanol from the Sep-Pak column. Elution with pure methanol allows for rapid evaporation in the subsequent evaporation procedure.

Evaporation

Samples are dried in a Buchler vortex evaporator (Buchler Instruments, Fort Lee, NJ, USA) in 15 × 75 mm glass tubes. The vacuum pressure is manually increased from 25 cmHg to full vacu-

um in 25-cm increments every 10 min to prevent "bumping".

Enzyme treatment

After evaporation, the sample is ready to be divided for enzymatic treatment. The addition of adenosine deaminase to part of the sample removes adenosine, thus forming the paired blank which can be subtracted from the unknown. Each sample is reconstituted in water (Fig. 1) and divided 2:1. Water is used instead of HPLC buffer to retain the activity of adenosine deaminase (EC 3.5.4.4). The blank fraction is treated with 10 U of adenosine deaminase (Boehringer Mannheim, Indianapolis, IN, USA) in 3 *M* $(NH_4)_2SO_4$. The unknown fraction of the sample receives an equal volume (2.5 μl) of the adenosine deaminase vehicle. Both fractions are incubated at room temperature for 45 min. After incubation, adenosine deaminase is inactivated with 100% methanol and heating at 75°C for 45 min. After heating, both the unknown and the blank are allowed to cool to room temperature and then evaporated as above. Adenosine deaminase efficacy may be confirmed by adding approximately 200 pmol of adenosine to an identically collected and processed plasma sample just before splitting and enzyme treatment. The action of adenosine deaminase is confirmed by the loss of adenosine in this sample.

HPLC analysis

Both the unknown and the paired blank are reconstituted in an ion-pairing buffer (pH 5.8) consisting of 11 m*M* tetrabutylammonium hydrogensulfate and 61 m*M* potassium phosphate solution (buffer A) (Fig. 1). The blank is then further divided, one part remaining as a blank and the other receiving one of several standards of adenosine. Unknown, blank, and standard 75-μl aliquots are chromatographed at a flow-rate of 150 μl/min with a Hewlett-Packard 1090M diode-array system (Hewlett-Packard, Avondale, PA, USA) on a Brownlee 220 mm × 2.1 mm I.D. microbore C_{18} column (Applied Biosystems, Santa Clara, CA, USA). A linear gradient of 70% acetonitrile–water (v/v) solution (buffer B) reaching a final buffer B fraction of 12% at 15 min is used to elute the nucleoside against the aqueous ion-pairing solution. Acetonitrile is used instead of methanol for the organic solvent because it has a lower UV absorption at the absorption maximum of adenosine (258 nm). The detector records absorbance at 258 ± 6 nm with a reference of 300 ± 15 nm throughout the separation. Because the subtraction method requires very consistent chromatography between paired unknowns, blanks, and standards, the blank is injected in between its paired unknown and standard. Furthermore, temperature is controlled at 40°C to stabilize retention time. After each injection, the column is flushed for 5 min with 100% of the acetonitrile solution (buffer B) and re-equilibrated for 35 min with the ion pairing buffer A.

Data analysis

After obtaining the chromatograms of both the unknown and its paired blank, the data are superimposed using Hewlett-Packard chemstation software. Fig. 2 demonstrates the subtrac-

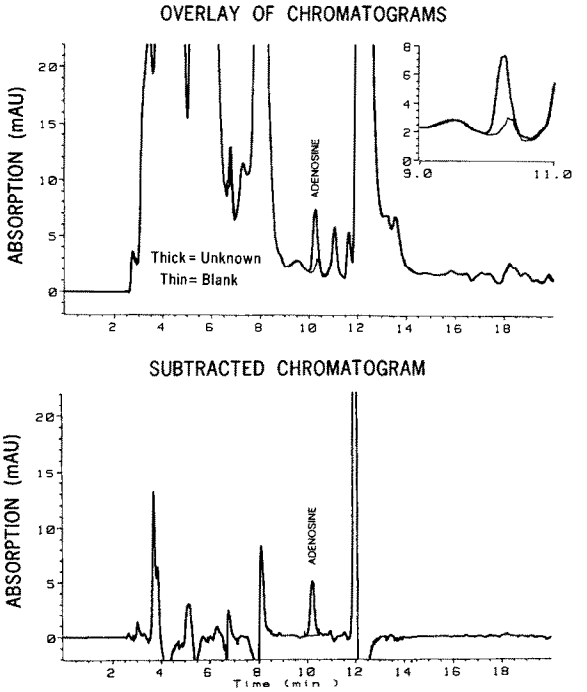

Fig. 2. Chromatograms obtained from dog plasma. The upper panel is the overlay of the unknown chromatogram (thick line) and the adenosine deaminase-treated blank (thin line). The inset is a magnification of the chromatogram in the adenosine region. The lower panel shows the resultant subtracted chromatogram (thick line) and integration baseline (thin line) where adenosine is quantified.

tion method for the plasma adenosine measurement. The upper panel shows the overlay of an adenosine sample (thick line) and its paired blank chromatogram (thin line). Usually in the overlay procedure, a well resolved peak that elutes just after adenosine is used as a reference point for superposing the blank chromatogram. After obtaining the best overlay, the blank chromatogram is subtracted from the unknown chromatogram point by point by the computer program. The lower panel of Fig. 2 shows the resultant subtracted chromatogram from which adenosine is quantified. In this example, the plasma adenosine concentration was 48 n*M*. Plasma adenosine is calculated by integrating the area of the subtracted chromatogram and comparing it to a calibration curve of standards of adenosine also determined by subtracting a plasma blank from a paired standard.

The calibration curve for adenosine is linear between 4 and 150 pmol injected (plasma concentrations approximately between 6 and 225 n*M*) with an r^2 of 0.9996. The plasma adenosine concentration is calculated by multiplying the measured number of picomoles injected by 4 (overall assay dilution factor), dividing by the plasma volume calculated from eqn. 1 and dividing the result by the recovery fraction (0.89). The detection limit of the subtraction method is 4 pmol of adenosine.

Assay recovery

To determine the recovery of adenosine throughout the sample handling and HPLC analysis, adenosine recoveries were done by adding physiological concentrations of adenosine to stop solution and collecting blood samples. Because the blood also contained some native adenosine, a control sample was collected simultaneously with the recovery sample. The control adenosine concentration ($\sim$10 n*M* in all experiments) was then subtracted from the recovered concentration to yield the final adenosine recovery.

RESULTS

Fig. 3 shows the recovery of adenosine throughout the entire assay procedure at physiologic concentrations of the nucleoside. Each recovery was done on a separate day and reflects different HPLC columns and Sep-Pak cartridges. The average (± S.D.) recovery for adenosine in canine blood was 89 ± 2.82%. The average (± S.D.) coefficient of variation was 12 ± 13%.

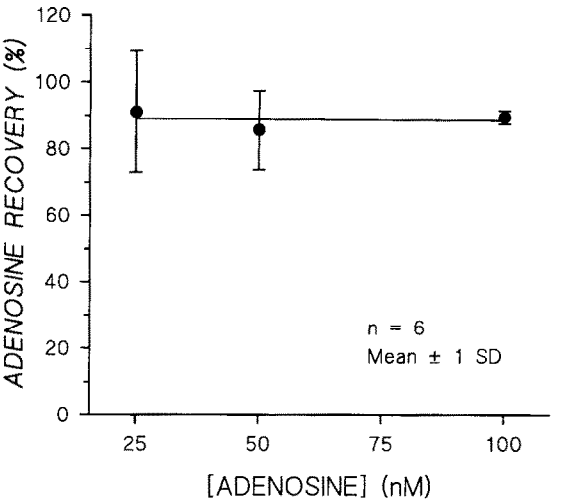

Fig. 3. Recovery of adenosine in dog blood. All recovery values calculated by subtraction method (see text for details). Values are mean ± S.D. The regression line through the points indicates an 89% recovery.

TABLE I

ADENOSINE VALUES IN ANESTHETIZED NORMOXIC DOGS

Sample	Adenosine concentration (mean ± S.D., n = 8) (n*M*)
Arterial adenosine	14.8 ± 8.48
Coronary venous adenosine	29.9 ± 7.49
Paired (v − a) difference	15.7 ± 7.38

Table I shows the average arterial plasma adenosine concentrations measured with the subtraction method from eight open-chest, chloralose-anesthetized dogs. All samples were taken under normoxic conditions and all dogs were treated with atropine (0.5 mg/kg intravenously).

DISCUSSION

Adenosine measurements in biologic compartments which are thought to be in steady state with the interstitial fluid have led to the hypothesis that adenosine is an important regulatory me-

tabolite. In particular, plasma measurements of adenosine have been used as an estimation of interstitial adenosine concentration. A new subtraction method for plasma adenosine measurement has been developed which alleviates many of the problems associated with previous HPLC assays. The subtraction method offers good recovery, high reproducibility, and a linear calibration of adenosine in plasma despite co-eluting peaks in the chromatogram.

Blood contains both cellular uptake mechanisms and enzymes which remove adenosine from the plasma. These biochemical sinks for adenosine contribute to the short half-life of adenosine in blood, which ranges from a few seconds in human [6–9] to 2–3 min in dog blood [5,8,10]. To prevent loss of adenosine to biochemical sinks in blood samples, adenosine must be collected with a stop solution. Some investigators have used stop solutions containing only dipyridamole at concentrations between 1 and 100 nmol/ml of blood. Adenosine measurements collected with only dipyridamole are sufficient for human samples [6,11] because of the low adenosine deaminase activity in human blood [12], but are inadequate for dog blood [13,14] because of substantial adenosine deaminase activity [12]. Other investigators have employed both dipyridamole and micromolar levels of either EHNA [8–10] or deoxycoformycin [15] in both human and dog blood. The stop solution/blood sample ratio used here contains adequate dipyridamole (11 μM) to prevent more than 90% of the cellular uptake in human blood [7] and to increase blood half-life of adenosine three-fold in dog blood [5]. The 0.6 μM EHNA in the blood sample prevents the 5–20% loss of blood adenosine by adenosine deaminase [4,12].

Adenosine formation may occur from the breakdown of adenine nucleotides via *ecto*-5′-nucleotidase [16]. The extracellular source of adenosine formation could lead to elevated levels of adenosine in the plasma sample. Recently, *ecto*-5′-nucleotidase inhibitors or cation chelators have been included into the stop solution of some plasma adenosine assays. Möser *et al.* [8] showed that addition of the *ecto*-5′-nucleotidase inhibitor α-,β-methyleneadenosine 5′-diphosphate (AOPCP) to the stop solution reduced the plasma adenosine concentration. Gewirtz *et al.* [17] demonstrated that the divalent cation chelator ethylenediaminetetraacetate (EDTA) also reduced plasma adenosine concentration, probably by inhibiting *ecto*-5′-nucleotidase. However, neither Ontyd and Schrader [6] nor Shryock *et al.* [9] found a change in plasma adenosine concentration when AOPCP was added to the stop solution. An attempt was made to incorporate EDTA into the stop solution used in the present subtraction method; however, in the presence of EDTA, the efficacy of adenosine deaminase to form a blank was decreased. Since complete removal of adenosine in the blank is essential in the subtraction method, EDTA was not used in the stop solution.

With most reversed-phase HPCL techniques, other compounds co-elute with adenosine in the chromatogram. To avoid these difficulties, some methods [5,10,17–20] have used adenosine deaminase to verify the adenosine peak. Measurements are often lost with this approach because of contaminating peaks in the adenosine region of the chromatogram [10]. Other investigators [6,8,9,11,14] have developed a peak shifting technique to measure plasma adenosine. In the peak shift method, the sample is initially chromatographed and the fraction containing adenosine, but not inosine, is collected. This fraction is then treated with adenosine deaminase to convert adenosine to inosine and chromatographed again, quantitating inosine with standards in buffer. Plasma adenosine has also been converted to uric acid by a similiar technique [13].

In the above assays that rely on fraction collecting and enzymatic peak shift, the recovery of adenosine from canine blood ranges from 40–60% [13] to 95–101% [8,10]. The lack of EHNA in the stop solution used by McKenzie *et al.* [13] could be a factor in their low recovery of blood adenosine. Other adenosine recoveries using peak-shifting techniques with human blood range from 87 to 89% [6,9,11]. Thus, the recovery of 89% using the present subtraction method in canine blood for physiologic levels of adenosine is similar to other assays.

Adenosine deaminase treatment removes adenosine from a portion of the sample and forms a blank which is subsequently subtracted from

both a paired unknown and standard. Since the adenosine subtraction assay is based on the formation of an adenosine-free blank, it is critical that enzymatic conversion of adenosine in the blank sample be complete. The degradation of adenosine in the subtraction method assay has been verified in four ways. Firstly, during every analysis, approximately 200 pmol of adenosine are added to an identically processed sample. With this large adenosine signal, a clear reduction in the adenosine peak is observed, and the residual peak is not different from that in native samples. Secondly, the area and peak height of the residual peak in the blanks is the same throughout an analysis from a given individual despite large differences in the initial adenosine concentration in the sample. If adenosine were not completely removed from the blank, the area of the residual peak would be larger for samples with more adenosine. Thirdly, the UV spectrum of the residual peak in the blank sample is different from that of adenosine. Fourthly, the linear calibration curve for adenosine in the presence of an interfering peak indicates that there is little adenosine in the blank. Therefore, it is very unlikely that any adenosine remains in the blank sample after adenosine deaminase treatment.

The subtraction method for plasma adenosine measurement has several advantages over previous assays. Firstly, there is no need for a fraction collector that is necessary for the peak shift method. Secondly, the subtraction method provides a linear calibration of adenosine in a plasma extract despite co-eluting peaks. By constructing adenosine standard curves in a plasma extract, a truer calibration would be expected. Most importantly, the subtraction method of plasma adenosine measurement alleviates the need for baseline resolution of the nucleoside from co-eluting peaks. Furthermore, subject-to-subject variability in HPLC peak separation, which may result in uninterpretable chromatograms, is not a problem when the co-eluting peak is subtracted. Although the present method is described for adenosine measurements in dog blood, the logic of the assay may be extended to other species with minor adjustments in the collection procedure [9], stop solution, or HPLC gradient.

In summary, the measurement of plasma adenosine by subtracting paired chromatograms offers good recovery, reproducibility, and direct quantification of plasma adenosine by standards in plasma, despite co-eluting peaks, without the use of a fraction collector.

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