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Analysis of submicromolar concentrations of adenosine in plasma using reversed phase high-performance liquid chromatography

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Abstract: A method is described for the determination of adenosine in small samples of plasma (< 1 ml) using reversed-phase high-performance liquid chromatography (HPLC) in either a simple isocratic or a gradient elution system which gives a clear separation of adenosine from other plasma constituents. Acetone is used to deproteinize plasma and chloroform to remove unwanted lipid soluble material prior to HPLC. 6-Methyladenosine is used as an internal standard for making corrections for changes in concentration during sample processing. Adenosine in plasma could be reliably detected at concentrations lower than its minimum effector concentration as a vasodilator (4×10^{-8} mol l^{-1} using the isocratic system and 1.9×10^{-8} mol l^{-1} with gradient elution). The recoveries of adenosine added to blood at concentrations ranging from 2×10^{-8} mol l^{-1} to 1.4×10^{-6} mol l^{-1} were from 101.4 ± 16.9% (n = 4) to 100.0 ± 3.6% (n = 5). The present method provides a simple, sensitive and selective assay for submicromolar concentrations of adenosine in plasma with good recovery.

Keywords: *Adenosine; vasodilators; reversed-phase high-performance liquid chromatography.*

Introduction

Adenosine has been reported to be released in many tissues and to play an important role in the control of blood flow in several vascular beds such as the myocardium, brain, skeletal muscle, gastrointestinal tract and kidney [1]. The implication of adenosine in vasodilator mechanisms requires an accurate measurement of adenosine in tissues and blood. Tissue adenosine contents have been measured either by direct enzymatic assay [2–6] or by thin-layer chromatography (TLC) followed by enzymatic analysis [7–10] and found to be about 2×10^{-6} mol kg^{-1} tissue. When these techniques were used to assay adenosine in venous blood returning from these tissues adenosine concentrations were reported to be an order of magnitude less ($1–2 \times 10^{-7}$ mol l^{-1}) and are very close to the

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detection limits of the above assays giving a poor reproducibility of the results. Thus, there is still controversy over whether adenosine is released into the venous effluent from contracting skeletal muscle, for example [6, 9, 11].

The technique of high-performance liquid chromatography (HPLC) provides a quantitative, sensitive and selective method for detecting adenosine in tissues such as cardiac myocytes [12], rat liver [13], rat heart [14], skeletal muscle [15] and erythrocytes [16] and also for many nucleosides including the breakdown products of adenosine (inosine and hypoxanthine) in biological fluids [17–18]. Several investigators have used HPLC methods for the detection of adenosine in plasma but have not been able to detect endogenous adenosine [19–22]. However, there have been only two reports of values in human plasma of 3×10^{-7} mol l^{-1} and 2×10^{-7} mol l^{-1} [23, 24], respectively, using HPLC. The former method of extraction used acid precipitation (perchloric acid) which can lead to co-precipitation of nucleosides and hence a poor recovery of adenosine [25]. It also leaves a multitude of unidentified peaks, necessitating the use of enzymatic peak shift determination on every sample to ensure specificity. The latter method [24] required complex pre-treatment of samples on a boronate affinity gel.

The purpose of the present investigation was to develop a rapid and simple procedure for the selective determination of adenosine in small samples of blood. For our method, deproteinization by organic solvent (acetone) was chosen [26]. Interference by lipid-soluble material during chromatography was reduced by extraction of the deproteinized plasma with chloroform [27]. The chromatography of samples so treated was based on that described [19] but with the addition of an internal standard (6-methyladenosine) to correct each individual plasma sample for losses, dilution or concentration during extraction.

Experimental

Plasma sample preparation

Blood samples (1.5 ml) were rapidly collected through cannulae placed in arteries and veins of dogs maintained under deep surgical anaesthesia with pentobarbitone sodium (Sagatal, May & Baker Ltd, 40 mg kg^{-1}). Blood was placed in pre-cooled tubes at 0–0.5°C. After centrifugation for 15 s at 13,000 ***g*** (Eppendorf 5412), aliquots of plasma (0.5 ml) were deproteinized by pipetting into ice-cold acetone (3 ml) containing 6-methyladenosine (10^{-9} mol). The total time from sampling to deproteinization was usually less than 2 min. Samples were spun for 5 min at 750 ***g*** to sediment denatured proteins. Chloroform (3 ml) was then added to the supernatant to extract lipid-soluble material and the aqueous layer was separated by centrifugation (5 min at 450 ***g***). The aqueous layer was taken for further analysis and bubbled with compressed air for 5 min at 70°C to remove residual chloroform. These steps in the extraction process served firstly to concentrate adenosine and secondly to avoid masking of the adenosine peak observed in samples treated only with acetone (Fig. 1).

Samples were kept at −20°C and rewarmed to room temperature prior to injection into the HPLC system. Total time for sample processing (removal of blood sample to storage at −20°C) was 20–60 min. To determine recovery the extraction procedure was carried out on blood or plasma samples to which known quantities of radioactive ^{14}C-adenosine and non-radioactive adenosine were added. Adenosine was measured by HPLC and ^{14}C-adenosine (added to give a final concentration of 1 mCi l^{-1} in 0.5 ml sample) was measured in a Triton–Toluene scintillant (Vickers Chemicals, Pool in

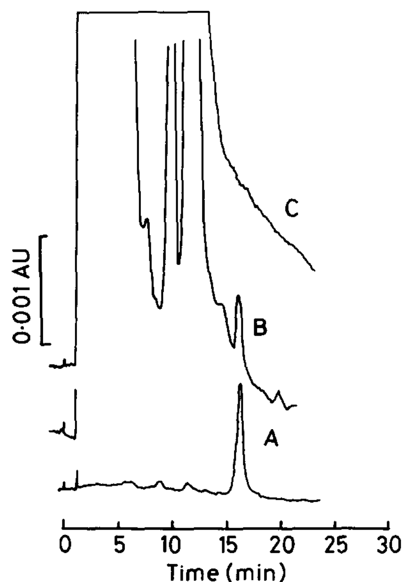

Figure 1
Representative chromatogram of adenosine standard in saline (1×10^{-6} mol l^{-1}) (A), a plasma sample treated with acetone and chloroform and then spiked with adenosine (8×10^{-7} mol l^{-1}) (B) and a spiked plasma sample treated with acetone but without chloroform extraction (C). Note masking of adenosine peak in C. Chromatographic conditions were as in IV, Table 1.

Wharfedale, Yorks) using a liquid scintillation counter (Kontron SL 3000). Quench corrections were made using external standard ratio.

High-performance liquid chromatography (HPLC)

Samples (100 μl) were injected into the HPLC system via a Rheodyne 7125 valve. Solvent was delivered as shown in Table 1 via a pump (model 300/02, Applied Chromatography Systems Ltd (ACS), Luton, Beds, UK) controlled by a decilinear gradient programmer (model 750.36, ACS Ltd). Adenosine and methyladenosine were detected by their absorbance at 254 nm in a selectable wavelength UV monitor (model 750.11, ACS Ltd) set at 0.005 A.U. full scale. Output was to a pen recorder (Phillips PM 8251) set at 10 mV full scale deflection. The individual peaks in chromatograms were identified by combination of four commonly used methods [25, 28]: (i) their retention times, (ii) absorbance ratios at two wavelengths, (iii) co-chromatography, and (iv) peak shift using adenosine deaminase. The areas under peaks were measured either by an integrator (model 308, Laboratory Data Control, Stone, Staffs, UK) or occasionally by cutting out peaks and weighing.

Concentrations of adenosine in plasma were calculated from chromatograms by integrating peak height (μV) with respect to time (s), using the equation $C_{\mathrm{Ado}} = C_{\mathrm{IS}}(A_{\mathrm{Ado}}/A_{IS})$ where C = concentration, A = integrated peak area, Ado = Adenosine and IS = internal standard.

Chromatographic conditions

The isocratic system requires simple chromatographic apparatus and separation was achieved under the conditions shown in Table 1 using a 5-μm ODS column (Spherisorb). Better resolution was obtained using a 3-μm ODS column (Spherisorb) under the isocratic conditions shown in system II (Table 1). In the gradient systems III and IV (Table 1) a higher column temperature was maintained and the proportion of methanol reduced in order to improve resolution. Adenosine was eluted isocratically for

Table 1
Choice of column and solvent conditions for isocratic or gradient elution of adenosine

System	Elution	Column	Guard column	Temp.	Solvent flow rate (ml min^{-1})	Isocratic step: Time (min)	Isocratic step: MeOH (%v/v)	Isocratic step: KH_2PO_4 (mmol l^{-1})	Time for gradient (min)	Solvent composition after gradient: MeOH (%v/v)	Solvent composition after gradient: KH_2PO_4 (mmol l^{-1})	Retention times of adenosine and 6-methyladenosine (min): Adenosine	Retention times of adenosine and 6-methyladenosine (min): 6-Methyladenosine
I	iso	5 μm ODS (25 cm)	50 μm ODS (2 cm)	20°C	2.0	22	12.0	8.50	—	—	—	7.7 ± 0.05 (n = 31)	18.1 ± 0.2 (n = 26)
II	iso	3 μm ODS (15 cm)	5 μm ODS (5 cm)	30°C	1.6	48	4.0	9.00	—	—		12.0 ± 0.1 (n = 14)	40.4 ± 0.5 (n = 13)
III	grad	3 μm ODS (15 cm)	5 μm ODS (5 cm)	35°C	1.0	21	3.0	9.70	7	17.0	8.3	20.9 ± 0.1 (n = 17)	32.5 ± 0.1 (n = 17)
IV	grad	3 μm ODS (15 cm)	5 μm ODS (5 cm)	45°C	1.0	18	1.3	9.87	6	13.3	8.7	17.8 ± 0.1 (n = 12)	30.3 ± 0.1 (n= 12)

All columns were filled with Spherisorb ODS of the particle diameter indicated and the length of the column is shown in brackets. Columns were water jacketed and maintained at the temperature indicated. Elution was isocratic (iso) or gradient (grad). The solvent composition of methanol plus KH_2PO_4, pH 5.5 during the isocratic phase and the gradient are shown.

the periods shown before a gradient of increasing methanol was applied. A further isocratic step of 6 or 7 min followed to elute methyladenosine on a stable baseline well separated from endogenous peaks in plasma. The column was regenerated by reversing the gradient prior to application of the next sample.

Columns were regenerated weekly by washing in methanol. Column performance did not deteriorate over a 3-month period estimated by the calculated plate height using adenosine standards. After this period, column life was limited by increasing resistance despite the presence of the guard column which was changed about every 6 weeks. Columns were discarded when back pressure exceeded 4000–5000 psi.

Solvents and reagents

HPLC solvents were prepared from Analar methanol (BDH and a stock solution of Analar potassium orthophosphate (0.5 mol l^{-1} BDH) in analytically pure water (>18 meg ohm) from a Milli-Q water purifying system (Millipore UK Ltd). The pH of the solution was adjusted with a 10 mol l^{-1} solution of laboratory grade potassium hydroxide to give 5.5 on dilution to 10 mmol l^{-1}. All solvents for HPLC were filtered, vacuum degassed and degassed with helium before use. Plasma samples were extacted with "special for chromatography" grade acetone and chloroform (BDH). Adenosine (MW 267.2) and 6-methyladenosine (6-methyl aminopurine ribofuranoside) for standard solutions were obtained from Sigma Chemical Co. Uniformly labelled ^{14}C-adenosine was obtained from Amersham International, Amersham, Bucks. All columns were obtained from Phase Separations Ltd, Queensferry, Clwyd, UK.

Results

Use of internal standard

In order to correct changes in adenosine concentration during sample processing a known amount (1×10^{-9} mol) of 6-methyladenosine was added to each sample prior to extraction, as described in Methods. The validity of this procedure was checked first by extracting standard solutions (10^{-6} to 10^{-5} mol l^{-1}). The ratio of adenosine concentration in the extract to that of methyladenosine was 0.937 ± 0.032 (n = 21). Secondly, samples containing known amounts of adenosine and methyladenosine were concentrated by varying degrees by means of evaporation. Changes in adenosine concentration were closely paralleled by those of 6-methyladenosine ($r^2 = 0.96$) which was therefore used routinely to correct each sample for changes in concentration. The use of 6-methyladenosine as an internal standard has an advantage in that it can be analysed simultaneously using the same technique. It is an analogue of adenosine but is more lipid-soluble, giving longer retention times than any other components in the sample (Figs 2–4) and is thus clear of endogenous plasma peaks.

Specificity of chromatography

Unknown peaks were identified by retention time, co-chromatography, absorption ratio and peak shift. The standard adenosine peak was moved from a minimum retention time of 7.71 ± 0.05 min (capacity ratio, $k^1 = 5.43$) to 20.9 ± 0.13 min ($k^1 = 18.0$) by varying the solvent composition (Table 1). The unknown peaks in plasma samples moved correspondingly. As a further check, samples were chromatographed once and then co-chromatographed after being 'spiked' with a known quantity of adenosine. Unknown and 'spiked' standard peaks were always coincident. The recovery (104.2 ± 14.6%) of

this added adenosine was in good agreement with the values obtained for the recoveries of adenosine added prior to sample processing (Table 2).

The ratios of the absorbance at 254 and 280 nm [30] for standard solutions of adenosine and 6-methyladenosine were 4.42 ± 0.13 (n = 9) and 1.88 ± 0.04 (n = 6) respectively and the corresponding values for adenosine and 6-methyladenosine added to plasma samples were 4.59 ± 0.16 (n = 9) and 1.91 ± 0.01 (n = 14). Endogenously produced adenosine in plasma had a ratio of 4.41 ± 0.19 (n = 13) and was not significantly different from that of standard solutions.

Plasma samples containing significant amounts of endogenously produced adenosine ($3.96 \pm 0.36 \times 10^{-8}$ mol l^{-1}, $n = 4$, $p < 0.002$) were treated with adenosine deaminase [31] and re-chromatographed. The adenosine concentration calculated from the residual peak area ($8.5 \pm 5.3 \times 10^{-9}$ mol l^{-1}) was not significantly different from zero, indicating that the unknown peaks were adenosine.

Thus, all the above methods (retention time, co-chromatography, absorbance ratio and peak shift) used to identify peaks indicated that the unknown peaks given by blood samples represented adenosine.

Effect of chromatographic conditions on resolution of adenosine peaks

In the isocratic system I (Table 1) adenosine in standard solution eluted at 7.71 min (capacity ratio k^1 = 5.43) and was clearly separated from its breakdown products, hypoxanthine and inosine, which eluted at 3.06 ± 0.02 (n = 6) min (k^1 = 1.55) and 3.32 ± 0.03 (n = 11) min (k^1 = 1.78) respectively. 6-Methyladenosine eluted at 18.1 ± 0.2 min (k^1 = 14.08) (Fig. 2). The adenosine peak in plasma samples (Fig. 2), however, was

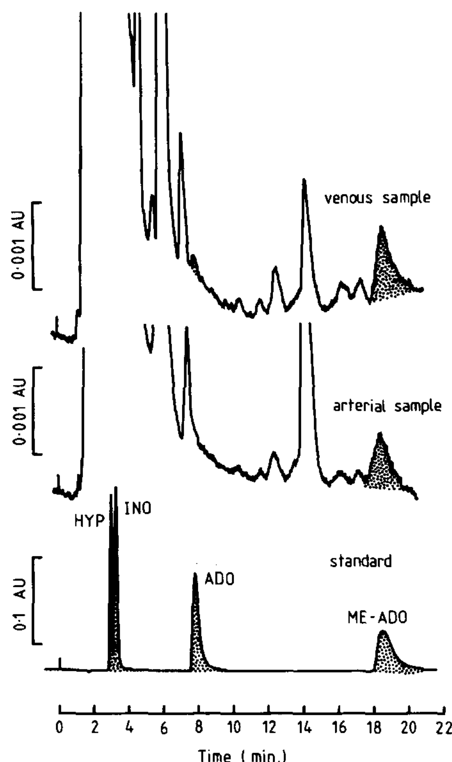

Figure 2
Representative chromatograms of extracted venous and arterial plasma samples using chromatographic system I as described in Table 1. The amount calculated from the area under the adenosine peak (shaded, 7.8 min) was corrected for concentration and losses during processing using the area under the internal standard peak, 6-methyladenosine (shaded, 18.2 min) as described in the text. Standard solutions (10^{-4} mol l^{-1}) of hypoxanthine (HYP), inosine (INO), adenosine (ADO) and 6-methyladenosine (ME-ADO) are shown. Arterial and venous plasma samples were taken after 20 min contraction of dog hind limb muscles [33]. Venous plasma adenosine concentration was 2.6×10^{-7} mol l^{-1} whilst there was no detectable adenosine peak in the arterial sample.

not fully separated from the falling phase of the previous peak which was noted in earlier work [19]. In venous plasma samples from contracting skeletal muscle, adenosine (2.6×10^{-7} mol l^{-1}) could be detected but no adenosine peak was produced by the arterial plasma flowing to the muscles (Fig. 2).

The resolution of the adenosine peak under isocratic conditions could be improved by using a 3 μm ODS column (system II, Table 1). Adenosine eluted at 12.0 ± 0.1 min ($k^1 = 11.0$). Because the methanol content was lower, methyladenosine did not elute until 40.4 ± 0.5 min ($k^1 = 39.4$). The venous plasma samples from contracting skeletal muscle contained 1.9×10^{-7} mol l^{-1} adenosine and the arterial plasma 8.2×10^{-8} mol l^{-1} (Fig. 3).

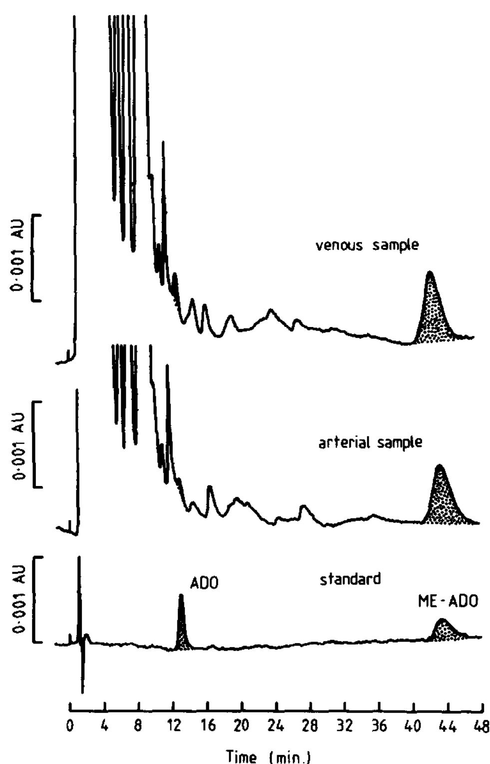

Figure 3
Representative chromatograms of extracted venous and arterial plasma samples using chromatographic system II as described in Table 1. The adenosine peak appeared at 12.1 min and methyladenosine at 43.0 min. Venous plasma adenosine concentration was 1.9×10^{-7} mol l^{-1} and arterial was 8.2×10^{-8} mol l^{-1}. Samples were taken after 10 min contraction of dog hind limb muscles. Abbreviations as in Fig. 2. The concentration of standards was 10^{-6} mol l^{-1}.

Adenosine peaks were fully resolved from previous peaks (Fig. 4) by lowering the methanol concentration in the isocratic step of elution (system III, Table 1) such that adenosine was retained on the column longer (20.9 min, $k^1 = 19.8$). Hypoxanthine (4.4 min, $k^1 = 3.00$) and inosine (7.2 min, $k^1 = 5.54$) were clearly separated. A gradient of increasing methanol was required, however, to elute 6-methyladenosine which combined with the better resolution by the 3 μm ODS column resulted in a much sharper peak (Fig. 4). Adenosine in arterial and venous plasma from contracting muscle was 2.1×10^{-7} mol l^{-1} and 8.2×10^{-7} mol l^{-1} respectively. Decreasing the gradient time (from 7 to 6 min) and increasing the temperature (from 35 to 45°C), resulted in similar chromatography (not shown) but reduced analysis time from 45 min to 37 min (see systems III and IV, Table 1). We therefore used system III and IV for subsequent analysis.

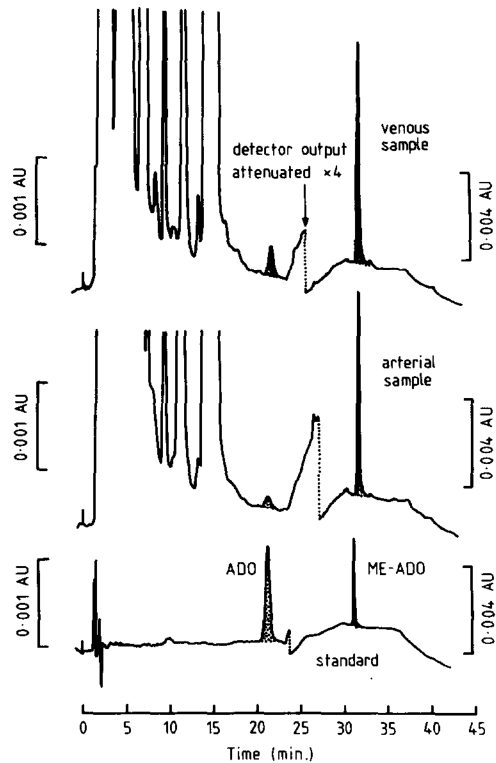

Figure 4
Representative chromatograms of extracted venous and arterial plasma samples using chromatographic system III as described in Table 1. The adenosine peak appeared at 21.5 min and methyladenosine at 31.5 min. Venous plasma adenosine concentration was 4.0×10^{-7} mol l^{-1}, arterial 1.3×10^{-7} mol l^{-1}. Samples were taken after 5 min contraction of an isolated gracilis muscle. The abbreviations are as in Fig. 2 and standard concentrations as in Fig. 3. Note that the resolution of adenosine peaks is better than that in Figs 2 and 3.

Standard curves

The amounts quantitated by measuring the area under the peak either by electronic integrator or by tracing the peak and weighing the paper were similar ($r^2 = 0.995$). Standard curves obtained using all chromatographic systems were superimposable. Calibration graphs of peak area versus concentration were linear from 10^{-4} mol l^{-1} to 10^{-7} mol l^{-1} adenosine and methyladenosine using the isocratic system and to 10^{-8} mol l^{-1} adenosine and methyladenosine using the gradient elution system.

Recovery of adenosine using HPLC

Samples of arterial blood cooled to 0°C were found to contain $3.1 \pm 0.8 \times 10^{-8}$ mol l^{-1} adenosine (Table 2). Known amounts of adenosine giving concentrations from 1.5×10^{-7} to 10^{-6} mol l^{-1} were added to the blood and plasma samples and were processed as described in Methods. After subtracting the small amount of endogenous adenosine in arterial samples the recovery of adenosine was calculated. The recoveries of adenosine at concentrations similar to that seen in venous blood from contracting muscle (1.5×10^{-7} mol l^{-1} and 3.2×10^{-7} mol l^{-1}) were 91.2% ± 14.6% and 93.7% ± 6.8% respectively (Table 2). In the micromolar range, however, adenosine recovery was 100.9% with a standard error of only 3.9% (Table 2). Since endogenous adenosine did not allow recoveries to be estimated in blood at lower concentrations, this was performed on plasma to which about 10^{-8} mol l^{-1} adenosine was added to plasma simultaneously with acetone. The isocratic system resolved adenosine at a concentration of 4.1×10^{-8} mol l^{-1} with a recovery of 106.8% ± 20.6% and the gradient method provided a resolution even at 1.86×10^{-8} mol l^{-1} with a recovery of 101.0 ± 16.9% ($n = 4$).

Table 2
Recovery of adenosine added to blood samples and analysed by HPLC

	Concentration of adenosine (mol l^{-1}) added to blood at 0°C				Concentration of adenosine (mol l^{-1}) added to blood at 37°C				
	0×10^{-7}	1.5×10^{-7}	3.2×10^{-7}	1.41×10^{-6}	0×10^{-7}	2.32×10^{-7}	4.91×10^{-7}	1.18×10^{-6}	2.21×10^{-6}
Expt I	0	1.34	3.21	1.42	11.1	11.2	9.41	2.21	3.03
	0	1.91	2.91	1.42	7.94	11.1	13.5	1.73	3.25
	1.0	1.61	4.39	1.32	6.49	8.39	15.4	1.33	3.18
	0	0.80	2.82	1.63	5.36	14.2	16.5	2.57	3.19
	0	1.09	3.19	1.49	7.91	7.71	18.0	1.47	2.78
Expt II	0.55		3.74		11.3		14.7	1.86	
	0.59		5.21		6.4		13.4		
	0.51		3.81		7.6		13.9		
	0		3.05		7.1		14.4		
	0		2.30		8.7		11.9		
Expt III	0.38				7.7		10.7		
	0.38				5.6				
	0.45								
	0.46								
Measured value SEM	0.31×10^{-7} ± 0.08	1.56×10^{-7} ± 0.19	3.45×10^{-7} ± 0.27	1.46×10^{-6} ± 0.51	7.76×10^{-7} ± 0.54	10.5×10^{-7} ± 1.15	13.5×10^{-7} ± 0.77	1.85×10^{-6} ± 0.18	3.08×10^{-6} ± 0.85
Recovery (%) SEM	—	91.2% ± 14.6	93.7% ± 6.8	100.9% ± 3.6	—	95.7% ± 47.8	115.8% ± 12.8	85.8% ± 15.6	106.9% ± 3.8
(n)	(14)	(5)	(10)	(5)	(12)	(5)	(11)	(6)	(5)

Adenosine was added to freshly drawn whole blood at the concentrations indicated at either 0°C or 37°C and adenosine and 6-methyladenosine analysed by HPLC using gradient elution as described in Table 1. The endogenous adenosine found in fresh blood was subtracted from the measured values and the recoveries of adenosine were calculated as a percentage of that added. Values are means and S.E. with the number of determinations shown in brackets. All mean values were significantly different from zero ($p < 0.01$ or less).

Recoveries of adenosine were also tested at higher concentrations (2.3×10^{-7} mol l^{-1} to 2.2×10^{-6} mol l^{-1}) added to blood at 37°C and then cooled to 0°C and deproteinized. This may more nearly represent the addition of adenosine to blood at tissue level. However, at 37°C endogenous adenosine rose some 25-fold (7.76×10^{-7} mol l^{-1}, Table 2), due probably to a release from platelets. The recoveries ranged from 85.8% to 115.8% (Table 2).

As an additional check on both retention times of unknown adenosine peaks and sample recovery, a known quantity of adenosine (5×10^{-11} mol) was added to experimental plasma samples after they had been chromatographed once. The recovery was calculated by comparing the difference in concentrations between the original and spiked samples with the amount of adenosine added. The recovery from chromatography was 104.2 ± 14.5% ($n = 14$).

Recovery of ^{14}C-adenosine

Recovery from the extraction process was checked independently of chromatography. ^{14}C-Adenosine was added to adenosine standards (10^{-7} mol l^{-1} to 10^{-5} mol l^{-1}) in either saline, plasma or plasma substitutes (dextran and haemacell) and extracted. The volume of aqueous phase recovered from the extraction process was 54.5 ± 1.6% of the original volume (0.5 ml) (Table 3). The concentration of adenosine in the extracted samples rose, however, to 144.2 ± 4.9% of its initial value. This concentration occurred largely in the final gassing phase to remove residual chloroform, and whilst it improved the sensitivity by leaving a solution about 50% more concentrated than plasma to be applied to the column, it introduced variability which had to be corrected by the use of an internal

Table 3
Recovery of ^{14}C-adenosine after sample preparation

	Extraction in saline			Extraction in plasma and plasma substitutes	
Concentration of adenosine added (mol l^{-1})	Volume recovery (% initial)	Adenosine recovery (% initial)	Concentration change (% initial)	Solvent	Concentration change (% initial)
10^{-5}	57.2 ± 2.9 (11)	73.7 ± 2.2 (11)	128.6 ± 3.8 (11)	Plasma	127.7 ± 2.2 (6)
10^{-6}	53.8 ± 1.6 (7)	73.7 ± 4.3 (8)	156.3 ± 10.5 (7)	Dextran	140.4 ± 10.4 (8)
10^{-7}	49.6 ± 2.0 (5)	75.1 ± 6.0 (6)	161.8 ± 5.9 (5)	Haemacell	124.2 ± 8.9 (8)
Mean	54.5 ± 1.6 (23)	74.0 ± 2.1 (25)	144.2 ± 4.9 (23)	P = NS	130.8 ± 5.1 (22)

^{14}C-Adenosine (5×10^{-4} μCi ml^{-1}) was added to non-radioactive adenosine solutions at concentrations from 10^{-4} mol l^{-1} to 10^{-7} mol l^{-1} in saline, water, plasma, dextran or haemacell. The volume of sample recovered was assessed by weight, adenosine recovery and final concentration by counting the ^{14}C in a liquid scintillation counter. Values are expressed relative to their initial starting value; the number of determinations is in brackets.

standard (see Table 2). The amount of radioactive adenosine recovered in the aqueous phase was 74.0 ± 2.1%. The recoveries from the plasma, plasma substitutes and saline vehicles were similar over the range 10^{-7} to 10^{-5} mol l^{-1} (Table 3).

Breakdown of adenosine by blood

A known concentration of adenosine (2×10^{-6} mol l^{-1}) was added to either saline or whole blood and incubated at 37°C and samples were processed as described in Methods. There was no significant change in adenosine concentration in saline (2.23 ± 0.2 and 2.01 ± 0.12×10^{-6} mol l^{-1} at 0 and 15 min respectively). However, in whole blood, plasma adenosine concentration was similar to that in saline at time 0 (2.18 ± 0.22×10^{-6}) but fell to 0.17 ± 0.03×10^{-6} mol l^{-1} over 15 min with a half-life of 3.5 min.

Discussion

The main features of adenosine analysis by the methods outlined in this paper are: (1) a high sensitivity of detection in plasma down to 2×10^{-8} mol l^{-1}, a value below its minimum effective vasodilator concentration [29]; (2) only 0.5 ml of plasma is required; (3) the changes due to concentration or dilution during extraction are corrected by the incorporation of an internal standard (6-methyladenosine).

There are few reports of the use of HPLC for plasma adenosine analysis. Plasma adenosine could be detected in patients deficient in adenosine deaminase [19] although no endogenous adenosine could be detected in normal subjects. Plasma adenosine levels of up to 1×10^{-5} mol l^{-1} have been reported in patients treated with adenosine deaminase inhibitors [21] but again no values were obtained for normal blood. Our chromatographic systems III and IV in particular give a better separation of peaks (see Fig. 4), and adenosine (3.1 ± 0.8×10^{-8} mol l^{-1}) could be detected reliably in normal plasma of dogs (Table 2). Adenosine (2×10^{-7} mol l^{-1}) has been reported in normal human plasma [23, 24] but these methods required larger volumes of blood (5 ml) and complex pretreatment on a boronate affinity gel column not suitable for handling large numbers of samples.

Recovery of adenosine from plasma

The recovery of adenosine was first checked independently of chromatography using ^{14}C-adenosine. The steps involved in sample processing resulted in an apparent recovery over a wide range of concentrations (10^{-5} mol l^{-1} to 10^{-7} mol l^{-1}) of 144.2 ± 4.9% (Table 3). Thus the solution which is applied to the column is more concentrated than the original plasma which improved the sensitivity. Because a proportion of the aqueous phase was lost during processing (see Table 3) in absolute terms only 74% of the original adenosine was recovered in any solvent (plasma, haemacell, dextran or saline). This dictated to us the use of an internal standard which undergoes similar changes during extraction as does adenosine in order to make appropriate correction for losses during processing.

6-Methyladenosine was chosen as the internal standard for two reasons. Firstly, adenosine is one of the most lipid-soluble nucleosides [28] and as sample processing involves lipid extraction it was important to choose a standard with similar solubilities in organic solvents to adenosine. Secondly, there are no endogenous plasma peaks at the time 6-methyladenosine elutes. Thus there was no co-elution of unknown compounds with the internal standard.

Previous reports on recoveries of nucleosides added to plasma showed values of some 54–58% [32] using perchloric acid or trichloracetic acid deproteinization. Ammonium sulphate precipitation produced better recoveries (98%) but produced sample dilution and did not remove all protein. The major advantages of our technique is that it produced a good separation of peaks during chromatography and good recoveries (Figs 2–4). Samples were corrected for losses, concentration or dilution during processing using the internal standard 6-methyladenosine as described. In all, 91.2–100.9% of adenosine was recovered from blood cooled to 0°C over a range of concentrations from 1.5×10^{-7} mol l^{-1} to 1×10^{-6} mol l^{-1} (Table 2). At concentrations nearer the detection limit of the assay, around 10^{-8} mol l^{-1}, recoveries were 101.0–106.8% with a larger scatter of results (Table 2).

Adenosine breakdown in blood

Adenosine at micromolar concentrations was broken down by whole blood (at 37°C) of dogs with a half-life of some 3.5 min in agreement with previous reports [23]. However, the immediate cooling of blood in ice, centrifugation and subsequent deproteinization steps in our method were sufficiently rapid (< 2 min) to minimize breakdown of adenosine which, when added in equal quantity at zero time to blood or saline, gave similar values. Whether a pretreatment of blood as described [23] is also required in our method for the determination of adenosine in human blood and that of other species with a rapid uptake and deamination of adenosine remains to be seen.

Sensitivity and precision of adenosine analysis in plasma

Previous analyses of adenosine in plasma and perfusates from contracting heart and skeletal muscle have mainly been by TLC followed by spectrophotometric assay [5, 7, 10]. Adenosine concentrations have been in the range 0.3–1.5×10^{-7} mol l^{-1} in arterial and 1.2–5.5×10^{-7} mol l^{-1} in venous perfusate following ischaemia or contraction. All of these methods require samples of large volumes of blood (10–100 ml) to achieve the required sensitivity (10^{-8}–10^{-7} mol l^{-1}). Our method of extraction and all chromatographic systems (I–IV) achieve this degree of sensitivity (4–1.9×10^{-8} mol l^{-1} respectively) with only 1.5 ml of blood, thus allowing rapid consecutive sampling to ascertain the time course of adenosine release into plasma (Table 2). Therefore, this method can also be used in investigations in which small animals are used.

The present methods are also more precise than previously published ones. For example, in the concentration range 2.8–5.8×10^{-7} mol l^{-1} the coefficient of variation was 42.8% for system I, 27.8% for system II, 22.5% for system III and 7.9% for system IV. At higher concentrations (1.1–7.9×10^{-6} mol l^{-1}) the coefficient of variation was less (23.9%, 15.6% and 2.8% for systems I, III and IV respectively). These are significantly lower than that reported previously at similar concentrations (60% [4] and 40% [9]. There is, therefore, a substantial improvement in precision.

Other methods have achieved better sensitivity than the method reported here using derivatization techniques [34, 35], but these require more complex equipment and more sophisticated chromatography. The detection limit of the present simple and sensitive method (1.8×10^{-8} mol l^{-1}) is below the minimum concentration which is of physiological significance [29].

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