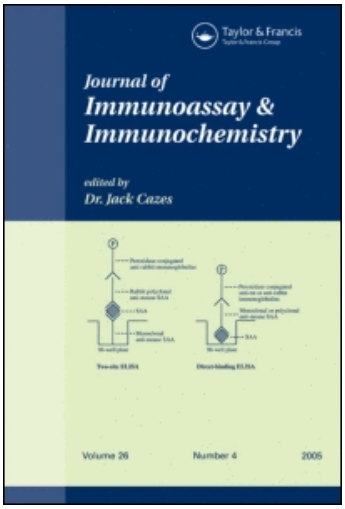

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A Simple and Sensitive Radioimmunoassay for Adenosine

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A SIMPLE AND SENSITIVE RADIOIMMUNOASSAY FOR ADENOSINE

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ABSTRACT

We developed a simple and sensitive radioimmunoassay (RIA) for adenosine. The RIA is based on the double antibody method with adenosine 2',3'-O-disuccinyl-3-[^{125}I]-iodotyrosine methyl ester (diSc-adenosine-[^{125}I]-TME) as a tracer. Anti-adenosine antiserum for the RIA was raised in rabbits immunized with diSc-adenosine conjugated to human serum albumin (diSc-adenosine-HSA). All samples and standards were succinylated prior to assay. The present immunoassay allows detection of 6.25-400 pmol/ml of adenosine in sample. Values obtained by the RIA and by a HPLC analysis showed a high correlation with correlation coefficient of 0.997.

In order to determine adenosine in plasmas, blood cells must be separated in the presence of 6 mM EDTA, 0.006% dipyridamole (Dip) and 23 μM 2'-deoxycoformycin (dCF) at 2°C. Adenosine in plasma could be accurately determined by the proposed method even

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without any pretreatments by deproteinizing. The adenosine levels with or without EDTA-treated normal human plasmas determined were 26.2 ± 7.26 and 100 ± 3.62 pmol/ml (Mean ± SEM), respectively.
(KEY WORDS: adenosine, radioimmunoassay (RIA), dipyridamole, plasma adenosine level, EDTA, deoxycoformycin)

INTRODUCTION

One of prime nucleosides, adenosine, is presumed to be related to physiological roles in the regulation of immune response (1 and 2), coronary vasodilation (3-7), neurotransmission (8), hormone secretion (9) and cyclic nucleotide formation (10-16). In order to determine the physiological level of adenosine, several quantitative assay methods including enzymatic assay (17 and 18), binding protein assay (19) and HPLC (20) have been established. However, they have not been used widely, because they do not have enough sensitivity and/or specificity to detect low levels of adenosine in various biological fluids without adequate purification and/or concentration. To eliminate these problems, radioimmunoassays (RIA) with ^{3}H-labeled adenosine as a tracer have been developed (21 and 22). However, they are plagued with quenching caused either by impurities of samples or by fluorescent materials contained in biological samples. Moreover, their assay procedures for plasma samples require any complicated pretreatments for deproteinizing. Furthermore, it has been known that the endogenous adenosine level in whole blood is regulated by the adenosine transporters and intra- and extra- cellular enzymes,

such as adenosine deaminase (ADA), ecto-nucleotidase (23), and adenosine kinase (24).

In an attempt to overcome these problems, we have developed a simple and sensitive RIA system for adenosine with ^{125}I-labeled adenosine disuccinyl-tyrosine-methyl ester (diSc-adenosine-[^{125}I]-TME) as a tracer. We also established a method applicable to biological fluids, such as peripheral blood.

MATERIALS AND METHODS

Reagents

Adenosine and its related compounds, adenine, 2'-deoxy-adenosine, AMP, ADP, ATP, cyclic AMP, inosine, FAD, and dCF (ADA competitive inhibitor) (25 and 26) were prepared by Yamasa Shoyu Co. Ltd. (Chiba, Japan). Human serum albumin (HSA) was obtained from Sigma Chemical Co. (MO., U.S.A.); bovine serum albumin (BSA) from Chiba Chikusan (Chiba, Japan); 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC) from Nakarai Tesque Inc. (Kyoto, Japan); and ^{125}I-iodine from ICN Biomedical Inc. (CA, U.S.A.). All other chemicals were from commercial sources and of reagent grade quality.

Rabbits

Japanese male albino rabbits, ICL; JW (about 2 kg of body weight), for immunization were obtained from Ichikawaya (Chiba, Japan).

Synthesis of 2',3'-Disuccinyladenosine (DiSc-Adenosine)

Adenosine was succinylated according to the method of Sato et al. (21): Adenosine (20 mmol), succinic anhydride (200 mmol), and triethylamine (58 mmol) were mixed in 400 ml of 50% (v/v) 1,4-dioxane on ice. After being kept for 10 min under vigorous shaking, the reaction mixture was filtrated through a paper filter (Toyo#2:Toyo-roshi, Tokyo, Japan) to remove insoluble materials. The filtrate was evaporated at 40°C under reduced pressure and then redissolved in 1ℓ of a solvent composed of distilled water, ethanol and 1,4-dioxane (4:3:3, v/v/v). The lyophilization procedure was repeated three times to attain 100% purity, as revealed by HPLC. The yield of the pure product was 55-60% of total applied amount. NMR study of this compound showed that the succinyl substitution was at both 2'- and 3'- positions. Adenosine was quantitatively converted by mild hydrolysis of the product with 0.1 M NaOH.

Preparation of Immunogen

DiSc-adenosine (200 mg) was mixed with 100 mg of HSA and 100 mg of EDC in 30 ml of 50 mM sodium acetate buffer, pH 5.5. After incubation at 25°C for 20 hr in the dark, the reaction mixture was dialyzed against 0.9% NaCl flowing at a rate of 400 ml/hr for 48 hr to separate diSc-adenosine-HSA from dialyzable molecules. The number of disuccinyladenosine residues introduced into each HSA molecule was estimated to be 17-20 moles on the assumption

that the molar extinction coefficient (ε) of adenosine is 1.51 x 10^4 at 260 nm.

Preparation of Anti-Adenosine Antiserum

Antibody was produced in rabbits by repeated immunizations. A diSc-adenosine-HSA solution was emulsified with an equal volume of complete Freund's adjuvant. An emulsion containing 0.8-1.2 mg of the immunogen was injected intradermally (i.d.) on their backs. All animals received additional i.d. injections several times in the same manner at two month-intervals and they were bled from the carotid artery on day 7 after the last injection. The separated sera were stored in small aliquots at -20°C.

Preparation of DiSc-Adenosine-Tyrosine Methyl Ester (diSc-Adenosine-TME)

DiSc-adenosine was conjugated with tyrosine methyl ester (TME) in the presence of N,N'-dicyclohexylcarbodiimide (DCC): diSc-adenosine (2 mmol), TME (10 mmol) were reacted in 30 ml of N,N-dimethylformamide containing 2 mmol of DCC at 4°C for 60 min in the dark. After the incubation, the reaction mixture was evaporated under reduced pressure condition and redissolved in absolute methanol. DiSc-adenosine-TME was isolated by column chromatography on a Prep-Pack column (500/C18; Waters Associates, Mass., U.S.A.), which had previously been equilibrated with absolute methanol.

Iodination of DiSc-Adenosine-TME

DiSc-adenosine-TME (1 mg) was dissolved in 100 µl of N,N-dimethylformamide and then mixed with 5 ml of 0.2 M sodium phosphate buffer, pH 7.5. Chloramine T (5 mg) was dissolved in 2 ml of 0.2 M sodium phosphate buffer, pH 7.5. Iodination of diSc-adenosine-TME was performed by the chloramine T method (27) with small modifications: Briefly, 40 µl of diSc-adenosine-TME solution (12 nmol), 5 µl of $Na^{125}I$ solution (18.5 MBq, 0.23 nmol) and 5 µl of chloramine T solution (44 nmol) were mixed and stirred at room temperature for 30 seconds. To terminate the reaction, 5 µl of sodium pyrosulfate (66 nmol), 100 µl of potassium iodide (3 µmol) and 100 µl of methanol were subsequently added to the reaction mixture. The iodinated material was purified by HPLC equipped with a O.D.S. reversed-phase column (4ϕ x 250 mm), with a solvent composed of methanol and 50 mM sodium acetate buffer, pH 6.5 (2:1, v/v).

Preparation of Second Antibody Solution

A solution containing goat anti-rabbit IgG was prepared as follows ; 100 µl of goat antiserum against rabbit IgG was dropped with gentle stirring into 50 ml of 0.1 M sodium phosphate buffer, pH 6.0 containing 0.5% BSA and 3% (w/v) of polyethylene glycol 6000.

Preparation of Plasma Samples

Blood was collected into a commercially available vacuum tube (VACUTAINER®) containing heparin with or without EDTA. One

milliliter of the collected blood was immediately mixed with 20 µl of 500 mM EDTA, pH 7.4, 500 µl of 0.02% Dip and 100 µl of 374 µM dCF and kept at 2°C. Plasma was immediately separated by centrifugation at 2500 X g at 4°C for 10 min.

Assay Procedure

Prior to RIA, adenosine in test samples was succinylated as described by Sato et al. (21): Briefly, 100 µl of succinyl reagent (a mixture of 4 mg of succinic anhydride, 10 µl of distilled triethylamine and 90 µl of 1,4-dioxane) was added to an equal volume of sample or standard solutions. After vortex mixing, the mixture was allowed to stand at room temperature for 10 min. Eight hundred microliters of 0.1 M sodium phosphate buffer, pH 6.0 was added to 200 µl of the succinylated sample. For the competitive reaction, the assay tube containing 100 µl of succinylated sample or standard solution, 100 µl of diSc-adenosine-[^{125}I]-TME (0.5 pmol) and 100 µl of 2000-fold diluted anti-adenosine antiserum in 50 mM sodium acetate buffer, pH 6.4 was incubated at 4°C for 18 hr. To the solution, 500 µl of the second antibody solution consisting of goat anti-rabbit IgG antiserum was added. After another incubation at 4°C for 1 hr, unreacted materials were removed by centrifugaion at 3000 rpm at 4°C for 20 min. The remaining radioactivity in the tube was counted by a gamma counter.

RESULTS

Adenosine Antibody

Antibody specific for adenosine was generated in all 40 rabbits immunized with diSc-adenosine conjugated to HSA. The antiserum was capable of binding more than 40% of diSc-adenosine-[^{125}I]-TME (0.5 pmol) at a dilution of 1:2000 after 18 hr incubation.

Sensitivity and Specificity of the RIA

A dose-responce curve of the RIA for adenosine, which was constructed for authentic diSc-adenosine-[^{125}I]-TME with triplicate incubations, showed a sigmoidal response curve when plotted as a semilogarithmic paper from 6.25 to 400 pmol/ml (Figure 1). The intercept at 50% of B/Bo in dose-response curve showed 30 pmol/ml in the succinylation method from 11,000 pmol/ml of non-succinylation method. The sensitivity of this succinylation method was calculated about 360-fold higher than that of the method without succinylation. Therefore, all samples were succinylated prior the assay. The minimum detectable level of adenosine was as low as 5 pmol/ml.

The specificity of the antiserum was determined by means of displacement of binding of diSc-adenosine-[^{125}I]-TME to the antibody with various amounts of the structurally related compounds; Sc-AMP, Sc-ADP, Sc-ATP, Sc-adenosine, Sc-2'-deoxyadenosine, Sc-FAD, and Sc-inosine (Figure 2). The results

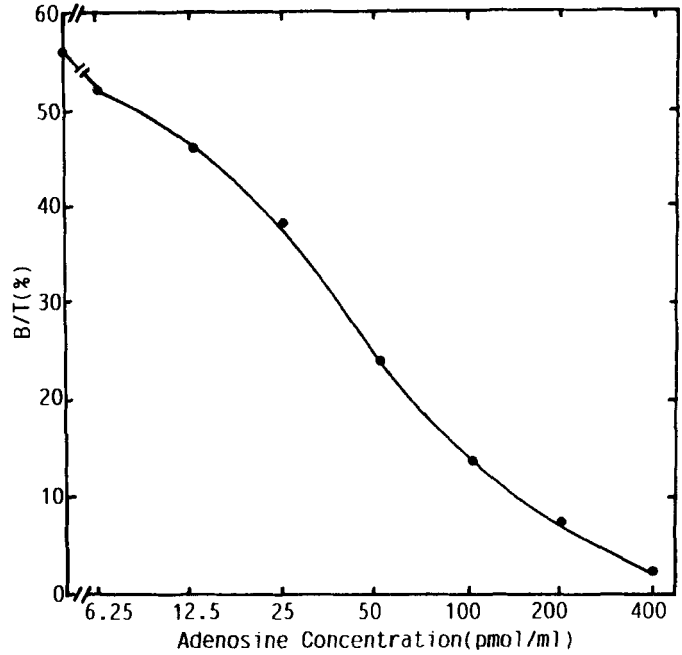

FIGURE 1. Displacement Curve for Adenosine in the RIA. The RIA was carried out by the standard procedure described in Materials and Methods.

show that the antibody was highly specific to adenosine since none of the adenosine-related compounds except 2'-deoxyadenosine caused any significant interference. Even Sc-2'-deoxyadenosine competed with the tracer binding only at a concentration 50 times as high as that of adenosine. Since the concentration of 2'-deoxyadenosine in biological fluids is estimated to be relatively much lower than adenosine, its cross-reactivity must be practically negligible.

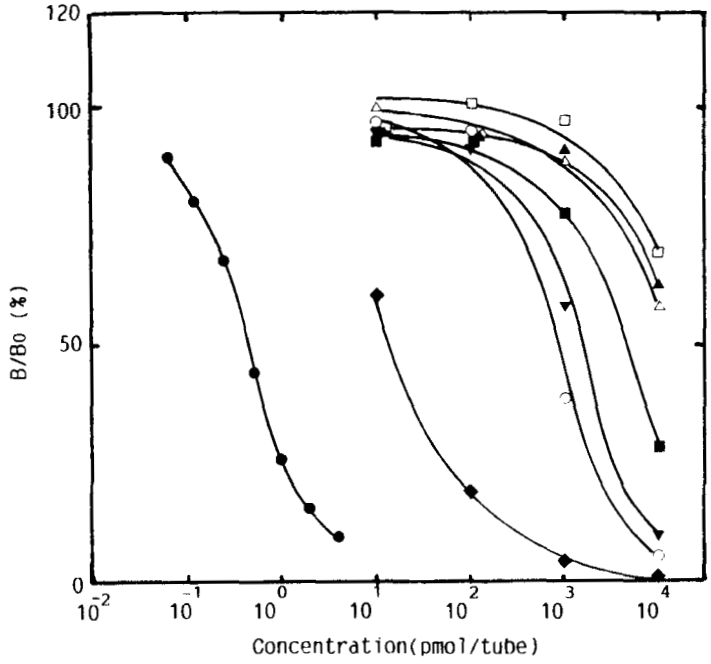

FIGURE 2. Cross-Reactivity of Adenosine and its Related Compounds with DiSc-Adenosine-TME for the Adenosine Antiserum.
Each compound was succinylated and its binding affinity was measured by the RIA. (1)adenosine(●—●), (2)deoxyadenosine (◆—◆), (3)AMP(○—○), (4)ADP(△—△), (5)ATP(□—□), (6)adenine (▲—▲), (7)FAD(▼—▼) and (8)inosine(■—■).

Reproducibility of the Assay

Reproducibilities in intra- and inter-assays were studied with three human plasmas. The coefficients of variations of intra-assay and inter-assay were observed to be 1.9-3.1% and 4.1-9.4%, respectively (Table 1).

TABLE 1.

Intra- and Inter-assay Variances

		Adenosine (pmol/ml)		
		High	Middle	Low
Intra-assay Variance	Mean	77.3	34.0	16.0
	S.D.	1.5	0.9	0.5
	C.V.(%)	1.9	2.6	3.1
Inter-assay Variance	Mean	78.4	34.4	17.0
	S.D.	3.2	1.9	1.6
	C.V.(%)	4.1	5.5	9.4

For the evaluation of intra-assay variance five assays were performed at same time with each plasma. The inter-assay variance was evaluated by measuring each plasma on five different days.

Recovery Test

In the recovery test with human plasma, recoveries of the added adenosine were 86-112% (Table 2). These results demonstrate that there were no inhibitory or interfering substances in the plasma samples.

Dilution Test

In the dilution test after succinylation with human plasma the value of measured adenosine was directly proportional to the concentration of plasma (Figure 3).

TABLE 2.

Recovery Test

Plasma	Adenosine (pmol/ml)			Recovery
Sample	Added	Found	Calculated	(%)
	0	16.2		
A	40	60.3	44.1	110
	80	101.3	85.1	106
	160	172.7	156.5	98
	0	10.6		
B	40	45.0	34.4	86
	80	94.5	83.9	105
	160	158.0	147.4	92
	0	9.5		
C	40	52.7	43.2	108
	80	99.3	89.8	112
	160	156.1	146.6	92

Adenosine of the plasma to which authentic adenosine was exogenously added was measured by the RIA.

Plasma Level of Adenosine in Human Subjects

The assay was applied to the determination of adenosine in human plasma.

In order to examine the effect of incubation period after blood collection on the plasma adenosine level, heparin-treated blood was incubated for various periods up to 100 min before addition of the stopping solution (i.e. metabolic inhibitors of adenosine). As shown in Figure 4, adenosine concentrations determined in four plasmas from normal subjects were about 100 pmol/ml and almost constant regardless of incubation period.

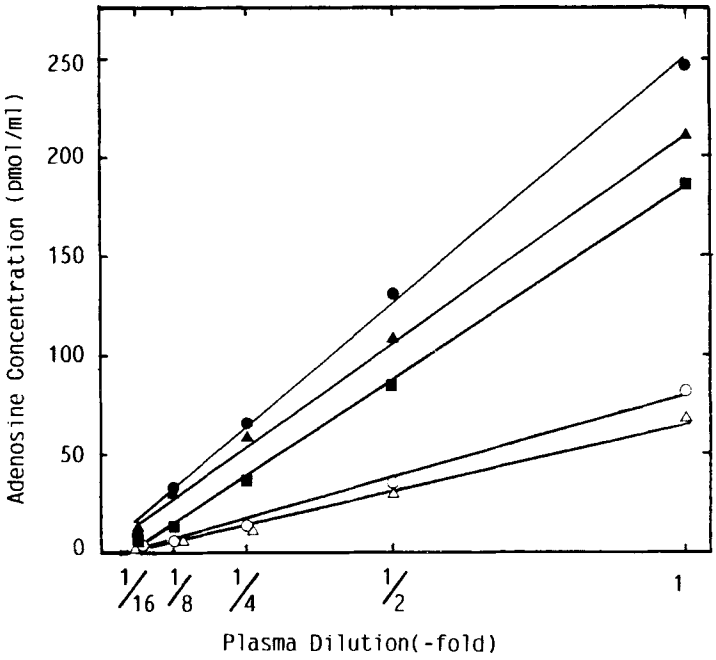

FIGURE 3. Dilution Study
Plasma samples were succinylated as described in "Materials and Method" and then they were diluted with the dilution buffer consisting of succinyl reagent, distilled water, 0.1 M sodium phosphate buffer (1:1:8, v/v/v), and their adenosine concentrations were assayed by the RIA.

Effect of haemolysis on the adenosine level in plasma was examined under hypotonic conditions. Adenosine level in plasma was estimated artificially high by the RIA at the condition with 0.6% or more of haemolysis (data not shown).

Effect of anticoagulants in blood collection on the adenosine level in for plasmas from normal blood donors were also examined. The adenosine levels in plasmas without EDTA (100 ±

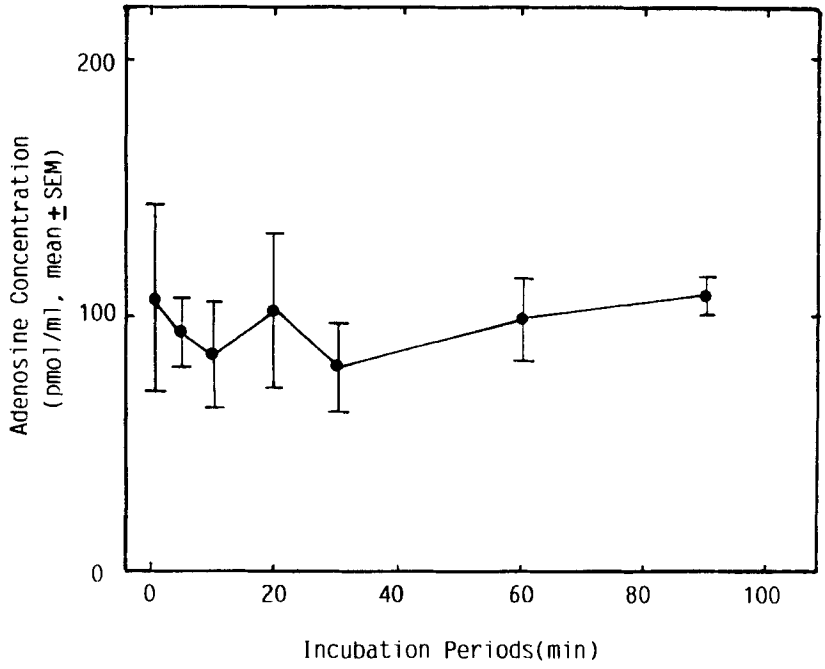

FIGURE 4. Adenosine Levels in Peripheral Venous Blood. Heparin-treated blood from normal subjects(n=4) were incubated for periods indicated above. After addition of the stopping solution and centrifugation, the adenosine concentration in plasma was measured by the RIA.

3.62 pmol/ml) were higher than those in plasmas with EDTA (26.2 ± 7.26 pmol/ml).

Correlation between Adenosine Concentration measured by the RIA and HPLC

Adenosine in 25-individual dogs, plasmas deproteinized by 6% (w/v) perchloic acid were determined by the RIA and HPLC according to the method of Jacobson et al. (20) with small

modifications. There was a good correlation with correlation coefficient of 0.997 between the values obtained by these two methods.

DISCUSSION

The purposes of the present study were 1) to establish a simple and sensitive immunoassay method for adenosine and 2) to establish a method of preparing biological fluids, especially peripheral blood, for determination of physiological adenosine levels by the assay.

In order to obtain a high specific antibody for adenosine, we used a diSc-adenosine-HSA conjugate as an immunogen for rabbits. As the result, we were able to prepare an antiserum with a high specificity which clearly differentiated adenosine from 2'-deoxyadenosine (ca. 50-fold) and the other related compounds (Figure 2). The RIA with the specific antibody was sensitive enough to detect as low as 5 pmol/ml of adenosine. It is therefore now possible to use small quantities of plasma to detect physiological changes in adenosine concentration. The precision and accuracy of the RIA was satisfactory. There was a good correlation between the adenosine levels determined by the RIA and those by the HPLC. The RIA procedure is extremely simple and does not require any complicated pretreatments such as deproteinizing.

We observed that the adenosine levels in plasmas containing heparin, dCF and Dip, with or without EDTA were 26.2 ± 7.26 and 100 ± 3.62 pmol/ml (mean ± SEM), respectively. Further, recent studies (28 and 29) showed that the plasma levels of adenosine from arterial and venous blood from different species were in the range of 50-250 nM under physiological conditions.

The extracellular adenosine concentration in whole blood might be influenced by the following factors; enzymatic degradation of adenosine by adenosine deaminase, nucleosidase, or phosphorylase, production of adenosine from adenosine nucleotides including ATP by Mg^{2+} and Ca^{2+}-dependent ecto-nucleotidases (30 and 31), uptake of adenosine by blood cells and phosphorylation of adenosine by kinase. The addition of EDTA, dCF, and Dip to blood samples made it possible to prevent any metabolic changes of adenosine and to obtain reliable values for the plasma. Gewirtz et al. also reported the effect of EDTA addition that inhibited the production of adenosine by hemolysis (29).

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