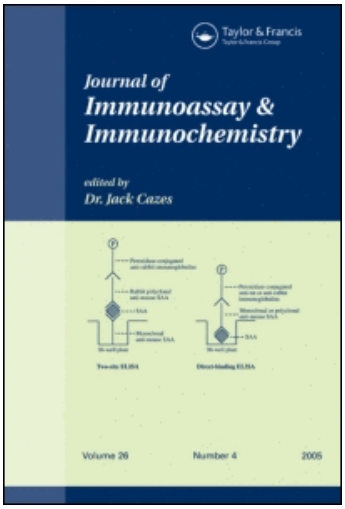

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An Enzyme-Linked Immunosorbent Assay (ELISA) For Adenosine 3', 5'-Cyclic Monophosphate (cAMP) In Human Plasma and Urine Using Monoclonal Antibody

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AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE (cAMP) IN HUMAN PLASMA AND URINE USING MONOCLONAL ANTIBODY

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ABSTRACT

A reliable and sensitive ELISA for cAMP in human plasma and urine is described, using a monoclonal antibody and a 96 well microtiter plate. Succinyl cAMP is conjugated to human serum albumin and adsorbed to the ELISA plate, giving an immobilized antigen approach which simplifies subsequent assay procedures. As low as 1.56 fmol/well of both plasma and urinary cAMP is measurable. Recoveries of added cAMP in plasma and urine were from 99% to 109%. Intra-assay coefficients of variation were less than 6.1% for plasma and 7.0% for urine samples. Inter-assay coefficients of variation for plasma and urine samples were less than 8.9% and 9.5%, respectively. There was a good correlation between the values obtained by ELISA and radioimmunoassay (RIA)

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(plasma: r=0.94, n=66; urine: r=0.98, n=64; nephrogenous cAMP: r=0.96, n=51). (KEY WORDS: ELISA, cyclic AMP, monoclonal antibody, protein A-alkaline phosphatase and nephrogenous cAMP)

INTRODUCTION

A number of sensitive RIA and enzyme immuno-assays of cAMP have been reported (1,2,3,4,5 and 6) and widely used in clinical and experimental studies. One of the clinical applications for cAMP determinations in plasma and urine is to measure nephrogenous cAMP. Nephrogenous cAMP can be calculated from plasma and urinary cAMP concentrations, which is a good index for the diagnosis of parathyroid disease. In an effort to obtain a sensitive and specific assay system for measurement of cAMP in plasma and urine, we successfully raised a monoclonal antibody with high specificity and affinity. In this paper, we describe a sensitive and reliable ELISA for measurements of cAMP in human plasma and urine using the monoclonal antibody.

MATERIALS AND METHODS

Materials

ELISA microtiter plates (MS-3596F/H plates, lot #830011) were purchased from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan). Human serum albumin (crystallized and lyophilized)(HSA), bovine serum albumin (fraction V)(BSA), cAMP, ethylenediamine tetraacetic acid (EDTA), and 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) were purchased from Sigma Chemical Co. (Mo., U. S. A.); succinic anhydride and triethylamine, from Tokyo Kasei (Tokyo, Japan);

dioxane, from Ishizu pharmaceutical Co., Ltd. (Osaka, Japan); Freund's complete adjuvant, from Difco Lab. (Mich., U.S.A.); protein A-alkaline phosphatase (ALP), from Zymed Lab., Inc. (CS, U.S.A.); and ALP assay kits consisting of a ALP substrate solution, containing phenylphosphoric acid and 4-amino-antipyrine, and ALP color reagent, containing periodic potassium, were from Sanko Junyaku Co., Ltd. (Tokyo, Japan). All other chemicals and solvents from commercial sources were of reagent grade quality.

Production of Monoclonal Antibody

Succinyl cAMP was conjugated with HSA using EDC as previously described (6). The conjugate (10-15 moles succinyl cAMP per one mole of HSA) was dissolved in sterile redistilled water and emulsified with an equal amount of complete Freund's adjuvant (7). An aliquot of emulsion containing 50 μg cAMP-HSA conjugate was injected into a BALB/C mouse intraperitoneally. The mice were boosted with the immunogen once every 2 weeks for 2 months. Three days after the final immunization, spleens were removed and fused with mouse myeloma cells (P3U1). Hybridomas producing an antibody with high binding capacity and affinity were obtained as described by Galfre et al. (8). Cross-reactivities of related nucleotides against the antibody were determined and expressed as the amount which causes a 50% displacement of cAMP binding.

Buffers for ELISA

Phosphate buffered saline (PBS) containing 0.05% Tween 20 (v/v), pH 7.4 was used for washing the microtiter plates. PBS

containing 0.05% Tween 20 (v/v) and 0.1% BSA (w/v) was used as the assay buffer.

Succinylation of cAMP

Cyclic AMP in test samples were succinylated prior to the assay as described by Yamamoto et al. (6). Briefly, 100 µl of succinylating reagent (90 µl of succinic anhydride in dioxane and 10 µl of triethylamine) was added to equal volumes of plasma, urine or standard solution. After vortexing, the mixture was allowed to stand at room temperature for 10 min and diluted with 800 µl of 0.3 M imidazole buffer (pH 6.5) to stop the reaction.

ELISA Procedure

ELISA microtiter plates were coated with succinyl cAMP-HSA conjugate as follows. The conjugate solution (100 µl) prepared by diluting 30 µl of the stock succinyl cAMP-HSA conjugate (20 µg/ml) to 20 ml with PBS was transfered to each well and the plate was kept at 4°C overnight. Unadsorbed conjugate was removed by washing three times. After washing, the plates were blocked by the addition of the assay buffer (200 µl/well) for 30 min at 37°C.

After blocking, 25 µl aliquots of succinyl cAMP standards or samples were dispensed into each well. One hundred µl of anti-succinyl cAMP antibody (diluted 1:2000 with the assay buffer) were added into each well and the plates were incubated overnight at 4°C. After the incubation, the plate was washed three times and 100 µl of protein A-ALP solution (enzyme activity 300 mU/ml) were added to each well. After 1h-incubation at 37°C, the plates were

finally washed three times and the enzyme substrate solution (100 µl) was added. The enzyme reaction was allowed to proceed at 37°C for 1h and terminated by the addition of 100 µl color reagent. Absorbance at 492 nm was read on a 2-wave length microplate photometer (MTP-12, Corona Electric Co.). All measurements for standard and samples were made in triplicate and the results were interpolated from the standard curve obtained in the same plate.

RIA of cAMP

RIA of cAMP was carried out by using the same monoclonal antibody. The procedure was performed as previously described (4).

Preparation of Samples

Peripheral blood was taken and urine was collected from human subjects in the morning. Whole blood was immediately mixed with EDTA-2Na (1.5 mg/ml blood) and plasma was obtained by centrifugation at 2000 rpm for 10 min at 4°C. Plasma and urine samples were kept at -20°C until assayed. Cyclic AMP-free plasma was obtained by treating normal plasma with 5% charcoal and 0.5% dextran for 30 min at room temperature. Urine samples were diluted 500 to 2,000 times by distilled water before succinylation.

Nephrogenous cAMP was calculated using the formula of Broadus et al. (9). Ellsworth-Howard test was done in a patient with hypoparathyroidism.

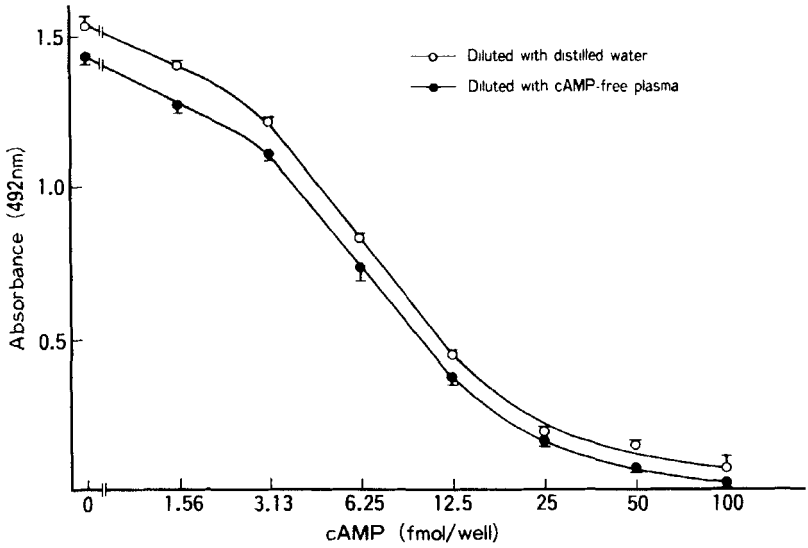

FIGURE 1. Standard curves for plasma cAMP and urinary cAMP. Values are mean±SD of triplicate determinations.

RESULTS

Standard Curves

Typical standard curves for plasma cAMP and urinary cAMP are shown in Figure 1. The minimum detectable concentration was 0.6 pmol/ml. The absorbance at any concentration of cAMP when dilution was made with distilled water was higher than those diluted with cAMP-free plasma. Therefore, for the measurement of plasma concentrations of cAMP, the standard solutions were diluted with cAMP-free plasma in the following experiments. The standard solutions for urinary cAMP measurement was made up with water.

TABLE 1.

Cross-Reactivity of Anti-succinyl cAMP Monoclonal Antibody

Compounds	Cross-reactivity %
cAMP	100
cCMP	0.0014
cIMP	0.00088
cGMP	0.00028
ATP	< 0.00005
ADP	< 0.00005
AMP	< 0.00005
CMP	< 0.00005
IMP	< 0.00005
UMP	< 0.00005
Adenosine	< 0.0005
Adenine	< 0.0005
GMP	< 0.00005
GDP	< 0.00005
GTP	< 0.00005
Guanosine	< 0.0005
Guanine	< 0.0005

Specificity of Monoclonal Antibody

The cross-reactivities of the antibody to various nucleotides, nucleosides and bases in this assay are shown in Table 1. Cross-reactivities of all the compounds were negligible.

Recovery and Dilution Test

Recovery of exogenously added cAMP (ranging from 1.25 to 10 pmol) to plasma or urine ranged from 99% to 108% in plasma and 99% to 109% in urine. Amounts of cAMP measured in serially diluted samples, in either plasma or urine, were in good agreement (Table 2).

TABLE 2.

Dilution Study

I. Plasma

Dilution	as Read from Standard Curve (pmol/ml, mean±SD, n=5)	cAMP (pmol/ml)
1: 5	5.36±0.08	26.8
1:10	2.68±0.36	26.8
1:20	1.34±0.15	26.8

II. Urine

Dilution	as Read from Standard Curve (pmol/ml, mean±SD, n=5)	cAMP (pmol/ml)
1: 400	11.95±0.29	4780
1: 800	5.95±0.24	4760
1:1600	2.98±0.14	4770

Diluted with cAMP-free plasma. Diluted with distilled water.

Precision

The precision was determined by analyses of 4 pools of plasma and urine samples. The intra-assay coefficient of variation with ten determinations was 5.2-6.1% for plasma cAMP and 3.3-7.0% for urinary cAMP. The inter-assay coefficient of variation within five assays was 6.7-8.9% for plasma cAMP and 5.1-9.5% for urinary cAMP, respectively.

Comparison between by ELISA and by RIA for cAMP

Levels of cAMP, in plasma or urine, measured by the ELISA and by the RIA are shown in Figure 2 and 3, respectively. The

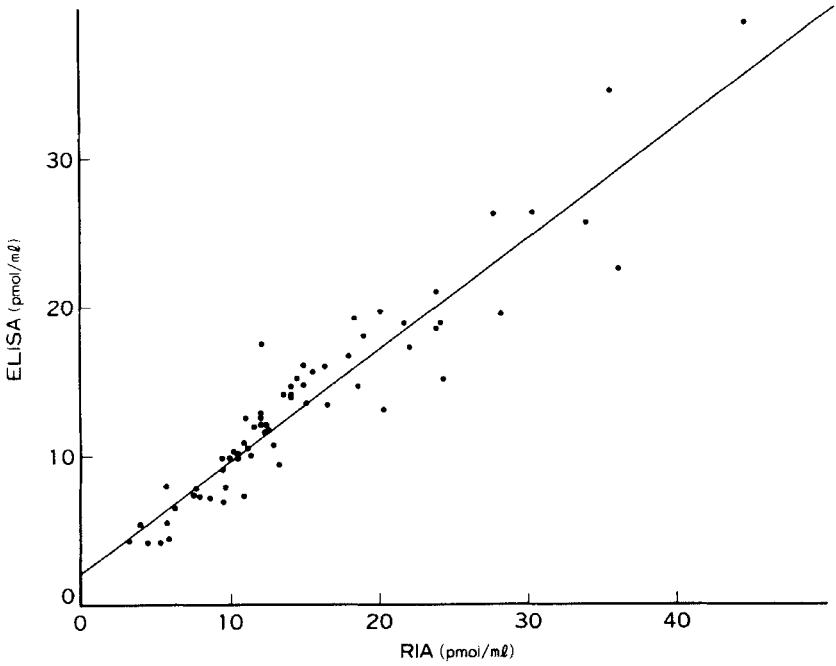

FIGURE 2. Correlation of plasma cAMP concentrations as measured by ELISA or RIA.

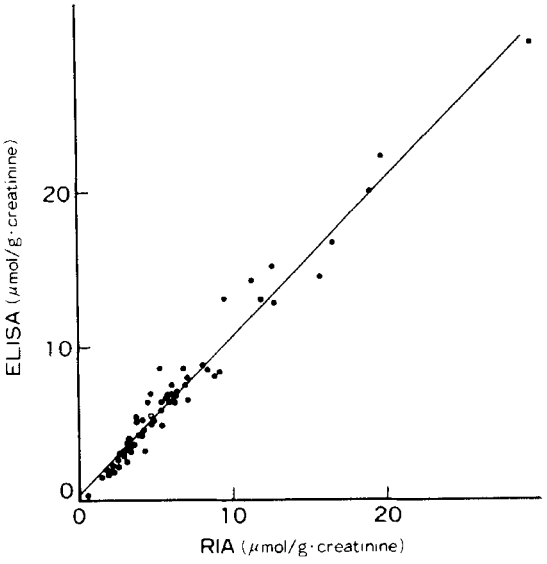

FIGURE 3. Correlation of urinary cAMP concentrations as measured by ELISA or RIA.

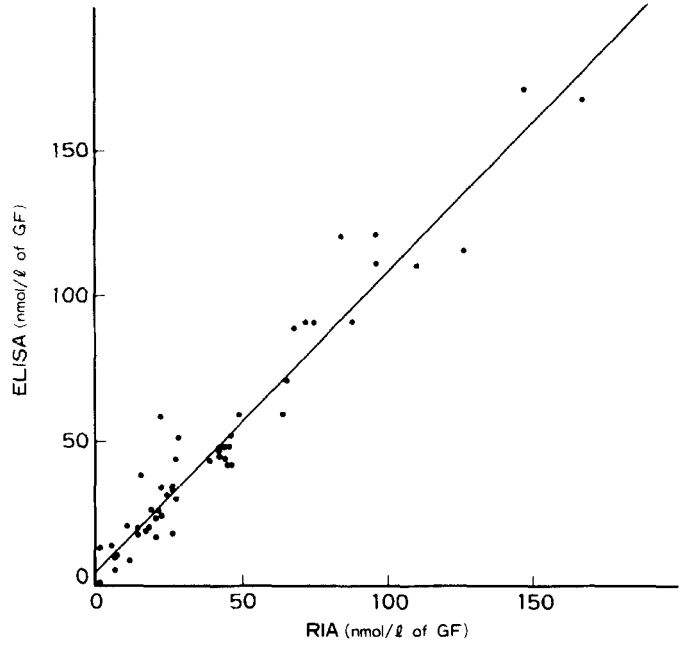

FIGURE 4. Correlation of nephrogenous cAMP concentrations as measured by ELISA or RIA.

amount of nephrogenous cAMP calculated from the cAMP values of plasma and urine, measured by either the ELISA or the RIA, are shown in Figure 4. There was a good correlation between the values obtained by the ELISA and by the RIA (plasma: $Y=0.76X+2.12$, $r=0.94$, $n=66$, urine: $Y=1.04X+0.36$, $r=0.98$, $n=64$ and nephrogenous cAMP: $Y=1.04X+5.25$, $r=0.96$, $n=51$). Ellsworth-Howard test was conducted on a patient with hypoparathyroidism. Patient's urinary cAMP levels were increased after an intravenous injection of parathyroid hormone (PTH). There was also a good

TABLE 3.

Ellsworth-Howard Test

Time (h)	RIA (μmol/g creatinine)	ELISA
-2	3.29	3.29
-1	2.87	3.07
0	2.97	2.54
1	19.00	20.00
2	4.70	5.45

PTH was injected just after collecting urine at 0 time.

correlation in urinary cAMP excretion as measured by both methods (Table 3).

DISCUSSION

This paper describes an ELISA for cAMP in human plasma and urine. The method utilizes 96 well microtiter plates, monoclonal antibody raised to succinyl cAMP, succinyl cAMP-HSA conjugate on the plates as immobilized antigen and protein A conjugated with ALP. The detection limit (1.56 fmol/well or 0.6 pmol/ml) of the ELISA is comparable to that of RIA, allowing the measurement of cAMP in normal human plasma. Because cAMP concentrations in urine are much higher than in plasma, urine samples must be diluted from 500 to 2,000 times before the assay. The standard curve when plotted as a semilogarithmic function results in a good regression curve. However, the absorbance is slightly lower

at each point when cAMP is diluted with cAMP-free plasma. Although the cause of this discrepancy is not clear, the interference might have been caused during the cAMP-antibody reaction because plasma samples applied to the plates are discarded in subsequent steps by repeated washings. Nonetheless, this problem has been overcome by diluting cAMP with cAMP-free plasma to make a standard curve when plasma samples are to be measured.

Dilution curves of both plasma and urine samples show a good parallelism. A high degree of precision and accuracy has been observed for plasma samples containing various concentrations of cAMP (3.7 to 20.5 pmol/ml) and for urine samples (2.7 to 16.1 pmol/ml). The recovery has also been shown to be satisfactory in both plasma and urine. Thus, the ELISA has been validated as regards specificity, precision and accuracy, and compares well with the RIA.

Nephrogenous cAMP, an index for renal handling of cAMP, measured by the ELISA and the RIA was shown to correlate very closely with each other. An increase in urinary excretion of cAMP following PTH administration in a patient with hypoparathyroidism has been demonstrated. These data indicate that the ELISA described here offers a simple, sensitive and specific method for cAMP determinations in plasma and urine and that the method is applicable for clinical studies to assess the renal handling of cAMP in patients with parathyroid diseases.

Acknowledgments

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