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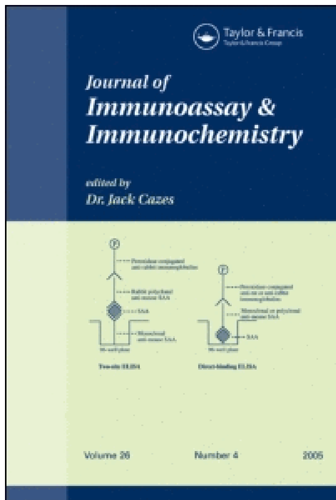

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An Enzyme-Linked Immunosorbent Assay (ELISA) for Guanosine 3',5'-Cyclic Monophosphate (cGMP) in Human Plasma and Urine Using Monoclonal Antibody

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

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ABSTRACT

An ELISA for cGMP in human plasma and urine using a monoclonal antibody is described. The monoclonal antibody was raised against succinyl cGMP conjugated to human serum albumin. The conjugate was 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 cGMP is measurable. Recoveries of added cGMP in plasma and urine were from 97% to 105%. Intra-assay coefficients of variation were less than 7.0% for plasma and 7.1% for urine samples. Inter-assay coefficients of variation for plasma and urine samples were less than 9.9% and 9.5%, respectively. The values obtained by ELISA correlated well with those by radioimmunoassay (RIA) (plasma: r=0.96, n=50; urine: r=0.98, n=60). (KEY WORDS: ELISA, cyclic GMP, monoclonal antibody, plasma, urine and protein A-alkaline phosphatase)

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INTRODUCTION

Recently, plasma and urinary cGMP levels have been reported to be altered in response to various vasodilatators of coronary arteries (1) and atrial natriuretic peptide (2). Therefore, it would be very useful to establish an assay system for measuring human plasma and urinary cGMP. A number of sensitive RIA and enzyme immuno-assays of cGMP have been reported (3-8) and widely used in clinical and experimental studies. Because we have already established an ELISA for adenosine 3',5'-cyclic monophosphate (cAMP) in plasma and urine (9), we applied the same procedure for measurement of cGMP. In an effort to obtain a sensitive and specific assay system, we successfully raised a monoclonal antibody with high specificity and affinity. In this paper, we describe a sensitive and reliable ELISA for cGMP in human plasma and urine using the monoclonal antibody.

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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), cGMP, 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. (CA, 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.

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Production of Monoclonal Antibody

Succinyl cGMP was conjugated with HSA using EDC as previously described (6). The conjugate (10-15 moles succinyl cGMP per one mole of HSA) was dissolved in sterile redistilled water and emulsified with an equal amount of complete Freund's adjuvant (10). An aliquot of emulsion containing 50 μg cGMP-HSA conjugate was injected into a BALB/C mouse intraperitoneally. The mouse was 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. (11). Cross-reactivities of related nucleotides against the antibody were determined and expressed as the amount which causes a 50% displacement of succinyl cGMP binding.

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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 cGMP

Cyclic GMP in test samples was succinylated prior to the assay as described by Yamamoto et al. (6). Briefly, 100 μl of succinylating reagent (90 μl of dioxane containing 4 mg succinic anhydride 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 for cGMP was performed by the procedure previously described for cAMP with slight modifications (9). ELISA microtiter plates were coated with succinyl cGMP-HSA conjugate as follows. The conjugate solution (100 μl) prepared by diluting 30 μl of the stock succinyl cGMP-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, any remaining binding sites 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 cGMP standards or samples were dispensed into each well. One hundred μl of anti-succinyl cGMP antibody (diluted 1:6000 with the assay buffer) was 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) was added to each well. After 2h incubation at 30°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 90 min 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.

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RIA of cGMP

RIA of cGMP was performed as previously described (6) using the same monoclonal antibody.

Preparation of Samples

Peripheral blood and urine were collected from normal individuals and a patient with hyperparathyroidism before and after surgery 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 GMP-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 40 to 800 times by distilled water before succinylation.

RESULTS

Standard Curves

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

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 cGMP (ranging from 1.25 to 10 pmol) to plasma and to urine ranged from 97% to 103% and from 96%

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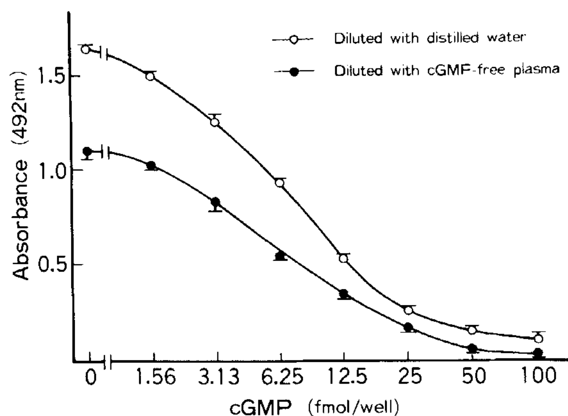

FIGURE 1. Standard curves for cGMP diluted with cGMP-free plasma and distilled water. Values are mean±SD of triplicate determinations.

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TABLE 1.

Cross-Reactivity of Anti-Succinyl cGMP Monoclonal Antibody

Compounds	Cross-Reactivity %
Succinyl cGMP	100
cGMP	10
Succinyl cAMP	0.0001
Succinyl cCMP	0.0001
Succinyl cUMP	0.0001
Succinyl GDP	0.0001
Succinyl GMP	0.0001
Succinyl GTP	0.0001
Succinyl Guanosine	0.002

TABLE 2.

Recovery of cGMP from Plasma and Urine

Samples	Amount Added pmol/ml	Amount Assayed pmol/ml (mean±SD, n=5)	Recovery %
	0	1.71±0.16	-
	1.25	2.98±0.26	101
Plasma	2.50	4.30±0.37	103
	5.0	6.54±0.97	97
	10.0	11.7±1.03	100
	0	1.12±0.19	-
Urine	1.25	2.32±0.25	96
Diluent	2.50	3.52±0.25	96
(1:600)	5.0	6.28±0.49	103
	10.0	11.6±0.58	105

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to 105%, respectively (Table 2). Amounts of cGMP measured in serially diluted samples, in either plasma (1.8 to 7.2 pmol/ml) or urine (1.9 to 30.0 pmol/ml), were in good agreement.

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 3.2-7.0% for plasma cGMP and 2.8-7.1% for urinary cGMP. The inter-assay coefficient of variation within five assays was 4.0-9.9% for plasma cGMP and 2.0-9.5% for urinary cGMP, respectively.

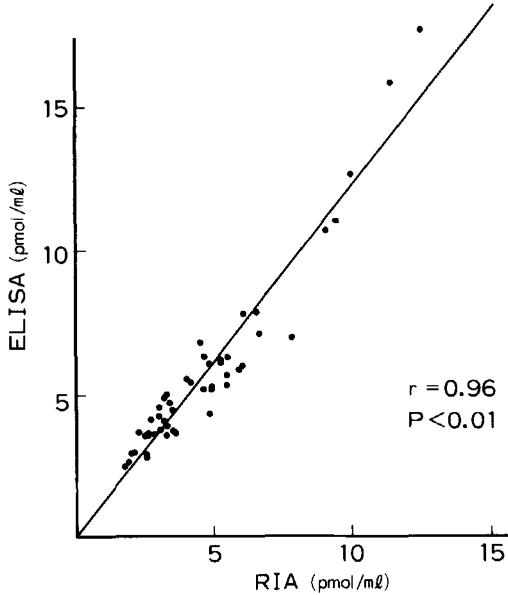

FIGURE 2. Correlation of plasma cGMP concentrations as measured by ELISA or RIA.

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Comparison between ELISA and RIA for cGMP

Levels of cGMP, in plasma or urine, measured by the ELISA and by the RIA are shown in Figure 2 and 3, respectively. There was a good correlation between the values obtained by the ELISA and by the RIA (plasma: Y=1.24X-0.07, r=0.96, n=50, urine: Y=1.08X-0.009, r=0.98, n=60).

Normal Range

Normal concentrations of plasma and urinary cGMP obtained by ELISA were 4.89±1.73 pmol/ml and 1.06±0.74 μmol/g·creatinine (mean±SD, n=43, age; 18-66 y.o.), respectively.

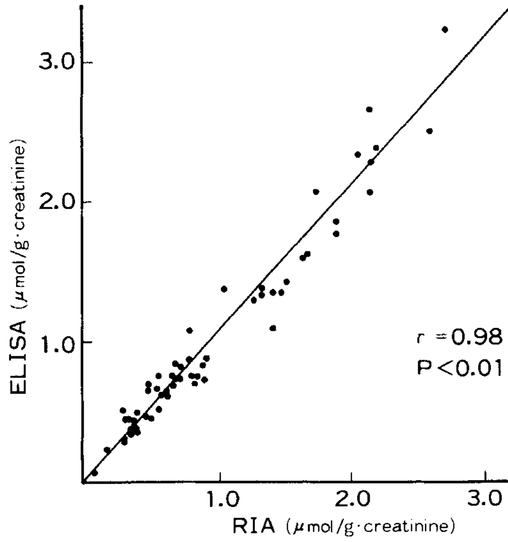

FIGURE 3. Correlation of urinary cGMP concentrations as measured by ELISA or RIA.

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TABLE 3.

Plasma and Urinary cGMP Concentrations in a Patient with Hyperparathyroidism Before and After Removal of Parathyroid Adenomas

Time (Days)	Serum Ca (meq/l)	Plasma cGMP (pmol/ml)	Urine cGMP (μmol/g·creatinine)
Before Surgery			
41	6.1	7.0	0.70
23	6.3	11.0	1.38
16	6.1	17.8	1.83
3	5.8	16.0	0.75
After Surgery			
18	4.3	12.7	0.87
25	4.3	4.1	2.54

Normal range of serum Ca is from 4.2 to 5.0 meq/l.

Plasma and Urinary cGMP in a Patient with Hyperparathyroidism

Plasma and urinary cGMP levels in a patient with hyperparathyroidism before and after removal of parathyroid adenomas are shown in Table 3. Before surgery, hypercalcemia and high plasma cGMP levels were observed. Plasma cGMP levels were decreased after removal of parathyroid adenomas.

DISCUSSION

This paper describes an ELISA for cGMP in human plasma and urine. The method utilizes 96 well microtiter plates, monoclonal antibody raised to succinyl cGMP, succinyl cGMP-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 cGMP in normal human plasma. Because cGMP concentrations in urine are much higher than in plasma, urine samples must be diluted from 40 to 800 times before the assay. The standard curve when plotted as a semilogarithmic function results in a good regression curve. However, the absorbance is lower at each point when cGMP is diluted with cGMP-free plasma. Although the cause of this discrepancy is not clear, the interference might have been caused during the cGMP-antibody reaction because plasma samples applied to the plates are discarded in subsequent steps by repeated washings. Nonetheless, this problem has been

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overcome by diluting cGMP with cGMP-free plasma to make a standard curve when plasma samples are to be measured.

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 cGMP and for urine samples. The recovery has also been shown to be satisfactory in both plasma and urine. Thus, this ELISA has been validated as regards specificity, precision and accuracy, and compares well with the RIA.

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Normal range of plasma cGMP obtained by this method was similar to that reported by Steiner et al. (3) and further supported the validity of this method. Plasma levels of cGMP in a patient with hyperparathyroidism were elevated and returned to normal levels on 25 days after surgery. The finding may suggest that plasma levels of cGMP are related to the parathyroid status, although further observation on a variety of patients is required.

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