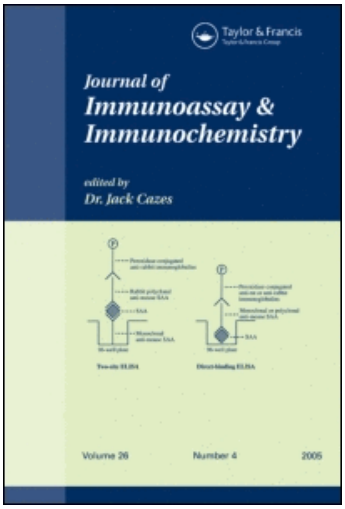

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A Non-Isotopic Immunoassay for Guanosine 3':5'-Cyclic Monophosphate Using a Cyclic GMP-Biotin Conjugate as Tracer

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A NON-ISOTOPIC IMMUNOASSAY FOR GUANOSINE 3':5'-CYCLIC MONOPHOSPHATE USING A CYCLIC GMP-BIOTIN CONJUGATE AS TRACER

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ABSTRACT

2'-*O*-monosuccinylguanosine 3':5'-cyclic monophosphate was coupled to N-biotinyl-1,8-diamino-3,6-dioxaoctane after converting succinyl-cGMP into its N-hydroxysuccinimide active ester. Isolation and purification of the succinyl-cGMP-biotin conjugate was performed with FPLC® using reversed phase chromatography. The synthesis described yielded a conjugate suitable for use as tracer in immunoassays for the cGMP measurement in plasma and urine samples. Employing biotin as the primary probe in a competitive solid phase immunoassay allows for flexible end point determination by means of commercially available labeled streptavidin derivatives. Streptavidin-europium was used in conjunction with the DELFIA®-system for time-resolved fluorometric end point measurement (TR-FIA), streptavidin-horseradish peroxidase was used for colorimetric end point determination (EIA). Both non radioactive immunoassay systems showed excellent correlation with the reference radioimmunoassay, good sensitivity and reproducibility. The succinyl-cGMP-biotin conjugate was shown to be stable for more than two years without any apparent loss of chemical stability or immunological reactivity.

(KEY WORDS: cGMP, labeling procedures, non isotopic immunoassays)

INTRODUCTION

Traditionally, radioactive isotopes such as [^{125}I] or [^{3}H] have been used for the determination of hormones and other physiologically relevant molecules. The drawbacks of radioimmunoassays (RIA), e.g. the half life of [^{125}I]-tracers, radioactive waste and general health considerations have made the use of non-isotopic immunoassay labels desirable.

Since the late 70s the exceptional affinity ($K_A = 10^{15}$ M^{-1}) of the avidin-biotin system has been employed in immunoassay systems [for review see (1)]. Commercially available biotin ester derivatives were coupled to ε-amino groups of proteins (antibodies) or to peptide hormones involved in the immunoassay reaction. The end point was determined colorimetrically (2) or by chemiluminescence (3). Non isotopic immunoassays for the determination of haptens are traditionally performed using hapten-enzyme conjugates (4). There are still few reports about hapten-biotin conjugates for use as tracer in non radioactive immunoassay systems for the determination of steroid hormones (5, 6, 7) or small peptides like gastrin (8).

The second messenger guanosine 3':5'-cyclic monophosphate (cGMP) is synthesized intracellularly after activation of the soluble or the membrane bound guanylyl cyclases, respectively (9, 10). Cyclic GMP has important functions, e.g. in vasodilation (11) or in control ion channels (12). The determination of cGMP levels from plasma or urine of patients is increasingly relevant. As atrial natriuretic peptide (ANP) secretion is stimulated by intravascular hypervolemia (10, 13) and ANP increases plasma cGMP levels, there is an increase in the plasma cGMP levels in patients undergoing chronic haemodialysis. Plasma levels of cGMP are therefore an indicator for fluid overload in

these patients (14). A positive correlation is also found between ANP levels and cGMP levels in patients with cardiac failure, so that plasma cGMP levels are also used as diagnostic tools in cardiology (15, 16, 17).

To the best of the authors' knowledge there are two reports about the determination of cGMP with non-isotopic enzyme immunoassays. One using a cGMP-horseradish peroxidase conjugate as tracer (18) and the other using a cGMP-human serum albumin conjugate immobilized onto microtiterplates (19) thus following the solid-phase-antigen technique (20). We decided to synthesize a low molecular weight cGMP-biotin conjugate for use as tracer in non-isotopic immunoassays exploiting the avidin biotin system and thereby the possibility of flexible end point determination.

MATERIALS AND METHODS

2'-*O*-monosuccinylguanosine 3':5'-cyclic monophosphate, guanosine 3':5'-cyclic monophosphate, D-biotin, dimethylsulfoxide, N-hydroxy-succinimide, dicyclohexylcarbodiimide, urea hydrogen peroxide tablets and 3,3',5,5'-tetramethylbenzidine tablets were purchased from Sigma, Deisenhofen, FRG; N-biotinyl-1,8-diamino-3,6-dioxa-octane was obtained from Boehringer, Mannheim, FRG. Streptavidin-horseradish peroxidase was supplied by Pierce Europe b.v., Oud-Beijerland, NL. Guanosine 3':5'-cyclic phosphoric acid, 2'-*o*-succinyl 3-[^{125}I]iodotyrosine methyl ester was purchased from Amersham, Braunschweig, FRG. Swine anti-rabbit (SaR) immunoglobulins were supplied by DAKO Diagnostika, Hamburg, FRG. The anti-guanosine 3':5' cyclic monophosphate antibody raised in rabbits by immunisation with the 2'-*o*-succinyl-derivative of cGMP was a generous gift of Prof. S. E. Graber, Nashville, TN, USA. All other chemicals were supplied by Merck AG, Darmstadt, FRG and were of analytical grade unless stated otherwise. Microtitre plates (Maxisorb F 96) were

purchased from NUNC, Roskilde, Denmark. FPLC® equipment, reversed phase column (PepRPC® HR 10/10, 15 µm), streptavidin-europium conjugate and enhancement solution for TR-FIA measurement were from Pharmacia, Uppsala, Sweden. Non radioactive immunoassay equipment (washer, dispenser, plate shaker: DELFIA® system) was manufactured by Wallac, Turku, Finland. Time-resolved fluorescence measurements were performed with the DELFIA® 1232 fluorometer, Wallac. The end point of the enzyme immunoassay was determined with a photometer from Anthos, Köln, FRG. Radioimmunoassays were measured in a Zinsser γ-Counter, Frankfurt / M., FRG.

Synthesis Of cGMP-Biotin Conjugate

2'-*O*-monosuccinylguanosine 3':5'-cyclic monophosphate was biotinylated in two steps: 1.) The commercially available succinyl-cGMP was converted into its N-hydroxysuccinimide (NHS) active ester derivative: 4.49 µmol of cGMP were dissolved in 800 µl of dry dimethyl sulfoxide (DMSO) and mixed with 5.39 µmol of NHS and 4.49 µmol of N,N'-dicyclohexylcarbodiimide (DCC) each dissolved in 100 µl of dry DMSO. The reaction was allowed to proceed for 24 h at room temperature. 2.) An equimolar (4.49 µmol) amount of N-biotinyl-1,8-diamino-3,6-dioxaoctane (biotin-dadoo) in 200 µl of DMSO was added to the active ester and left overnight at ambient temperature. The proposed structure of the final product is shown in FIG. 1.

Purification Of The Cyclic GMP-Biotin Conjugate

Purification of the reaction mixture was carried out with a Pharmacia automated FPLC® system on a C_2/C_{18} dual bonded reversed phase

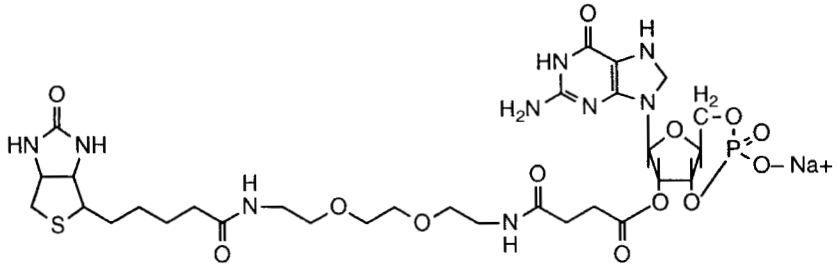

FIGURE 1. Proposed chemical structure of the succinyl-cGMP-biotin tracer conjugate.

column (PepRPC® HR 10/10, 15 µm). The column was equilibrated with a buffer containing 50 mM NaH_2PO_4 (pH 6.5) and 1 mM tetrabutylammoniumhydrogensulfate ($TBA•HSO_4$) as ion pairing reagent. The flow was 0.1 ml/min and fractions of 200 µl were collected. A 25 µl aliquot of the reaction mixture was applied to the column and the elution was monitored at a wavelength of 254 nm. A linear gradient increasing the methanol content in the elution buffer from 10 % to 100 % over a 80 minutes period was applied. A representative chromatogram is displayed in FIG. 2 A.

Characterization Of The Cyclic GMP-Biotin Conjugate

For judging the suitability of the cGMP-conjugate for use as tracer in a competitive immunoassay, we measured the cGMP content of the fractions collected by use of a well established inhouse RIA (21) [FIG. 2 B] as well as the biotin content by use of an inhouse EIA [FIG. 2 C].

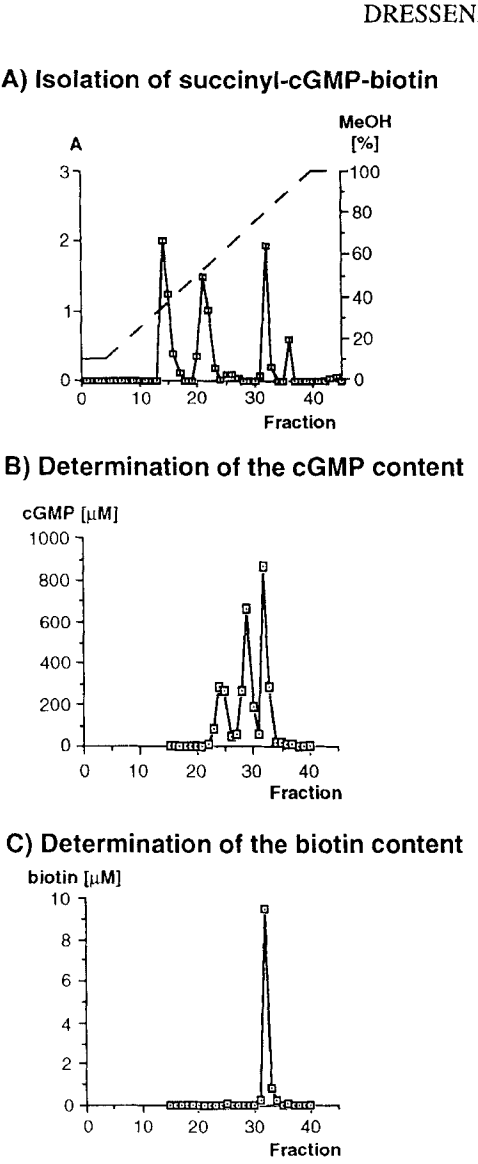

FIGURE 2. Isolation and characterization of the cGMP-biotin molecule. The synthesized succinyl-cGMP-biotin conjugate was purified over a reversed phase column (panel A) using a linear methanol gradient from 10% to 100% within 80 mins. Three main peaks were separeted: excess NHS in fraction #15, the uncoupled succinyl-cGMP in fractions #21-#24. The tracer molecule eluted in fractions #32-#33 at 78 % methanol as confirmed by the determination of its cGMP content (panel B) and its biotin content (panel C).

Non Radioactive Immunoassays

The procedures in the cGMP immunoassay are essentially the same for TR-FIA and EIA end point versions of the assay: Affinity purified swine anti-rabbit (SaR) immunoglobulins (100 ng/well in 50 mM sodium phosphate, pH 9.6) as the capture antibody was adsorbed onto microtiter plates overnight (4 °C). The plates were washed three times (50 mM sodiumphosphate, 0.05% Tween 20, 0.005% azide, pH 7.5) and coated with 200 µl of the rabbit anti-cGMP antibody, diluted 1:200,000 in assay buffer (50 mM Tris-HCl, 0.9% NaCl, 0.01% Tween 40, 0.5% bovine serum albumin, 0.05% bovine γ-globulin and 20 µM DTPA, pH 7.75). The plates sealed with a self-adherent foil, could be stored for at least 3 months at 4 °C without any loss of activity. Prior to the immunoassay procedure the coated plates were washed three times and 100 µl of standard or sample (diluted in assay buffer) was pipetted to the bottom of the microtiterplate wells, followed by 100 µl (0.2 pmol) of cGMP-biotin conjugate in assay buffer. The plates were sealed and incubated overnight at 4 °C. The incubation was terminated by washing the plate three times.

Synthetic cGMP was used as standard in the range between 0.11 nM and 81 nM as well as for controls: a 1 mM stock solution was prepared by dissolving cGMP in double distilled water. The concentration was checked photometrically ($\varepsilon_{254\ nm}$, pH 7 = 12.95; Merck Index, #2702). Standards and controls (1.5 nM and 5.5 nM) were prepared by dilution to the desired concentrations in assay buffer. Aliquots were stored at -80 °C.

End Point Determination

For the TR-FIA, 20 ng of streptavidin-europium was added in 200 µl of assay buffer and incubated for 30 min. The plates were washed six

times, 200 µl of "enhancement solution" (Pharmacia, No. 1244-105) was added in order to trans-chelate the Europium bound to Stav-Eu into a highly fluorescent complex (22). TR-FIA measurement was carried out between 5 and 60 min after the addition of the enhancement solution.

For the EIA, 200 µl of streptavidin-horseradish peroxidase (20 ng streptavidin equivalent) in 200 µl of assay buffer was added and incubated for 30 min. After washing six times, 200 µl of TMB solution [1 tablet of TMB / 20 ml of horseradish peroxidase buffer solution (0.07 M Na_2HPO_4 + 0.07 M citric acid (1+1), pH 5.0)] was added. Substrate development was allowed to proceed at 37 °C for 30 min. The reaction was terminated by addition of 50 µl of sulfuric acid (1 M) and absorbance was measured at 450 nm.

Sample Preparation

Plasma cGMP levels have been determined in ethanol-extracted EDTA-plasma samples as described elsewhere (21). Urine cGMP levels were determined after dilution (1:400 to 1:800) of the samples in RIA-buffer (50 mM sodium acetate, pH 4.0).

RESULTS

Purification Of The cGMP-Biotin Conjugate By Reversed Phase Chromatography

Succinyl-cGMP, succinyl-cGMP active ester and the final product succinyl-cGMP-biotin were run over a reversed phase column in the system described and monitored at a wavelength of 254 nm. Under these chromatographic conditions excess NHS elutes at 38% methanol (fraction #15), excess and uncoupled succinyl-cGMP at

54% methanol (fractions #21 to #24) and the succinyl-cGMP-biotin conjugate elutes 78% methanol (fractions #32 to #33). A representative chromatogram is shown in FIG. 2. All collected fractions were diluted 1:5 with absolute ethanol and stored at -20 °C until further investigation.

Determination Of The cGMP Content And Of The Biotin Content

The fractions from RP chromatography (fraction #15 to #40) of the succinyl-cGMP-biotin conjugate were assayed for their cGMP content with the reference radioimmunoassay. Three peaks were found to contain different concentrations of cGMP: fraction #24 (285 µM), #29 (660 µM) and fraction #32 (870 µM), as displayed in FIG. 2. These concentrations are overestimated as the antibody used in the RIA shows a 100-fold preference for succinyl-cGMP over the unsuccinylated cGMP molecule.

The biotin content of the collected fractions during RP chromatography was determined in the biotin EIA. There was one major peak (see FIG. 2) to be identified in fraction #32 containing 9 µM biotin.

So fraction #32 was identified as the desired succinyl-cGMP-biotin conjugate. Rechromatrography of this fraction showed one single, sharp peak.

Characterization Of Non Radioactive cGMP Immunoassays

Assay protocols for cGMP determination using cGMP-biotin as tracer were established for fluorometric (cGMP TR-FIA) as well as colorimetric (cGMP EIA) end point determination. Both versions follow identical pipetting and processing schemes until the completion of the competitive binding reaction between the sample cGMP and the

cGMP-biotin tracer molecule. Then either Stav-Eu is added for the TR-FIA end point determination or Stav-HRP followed by substrate incubation for colorimetric end point reaction. Representative dose-response curves for fluorometric and colorimetric detection are shown in FIG. 3 in comparison with the reference RIA.

Specificity

The rabbit anti-cGMP antibody showed crossreactivity, as determined in cGMP FIA, of below 0.002% with the GMP derivatives 2':3'-cGMP, 5'-GMP and 5'-GTP and crossreactivity of below 0.0001% with adenosine and its derivatives cAMP, 2':3'-cAMP, 5'-AMP and 5'-ATP at 100% reactivity for cGMP. As the antibody was raised against the 2'-*o*-succinyl-derivative of cGMP, the antiserum showed a 100-fold preference for succinyl-cGMP over cGMP (23).

Sensitivity

The lower detection limits of the cGMP TR-FIA and cGMP EIA were determined by 15-fold repetitive determination of the zero standard. The mean and SD of the signals obtained were calculated and the intercept of the mean –2SD signal (95 % confidence interval) with the standard curve was defined as minimal detectable dose. The lower detection limit was 0.23 nM (= 23 fmol/well) for the TR-FIA and 0.25 nM (= 25 fmol/well) for the EIA version of the method.

Reproducibility

Intra-assay variation was calculated by 16-fold determination of two control samples containing 1.5 nM and 5.5 nM cGMP. For TR-FIA,

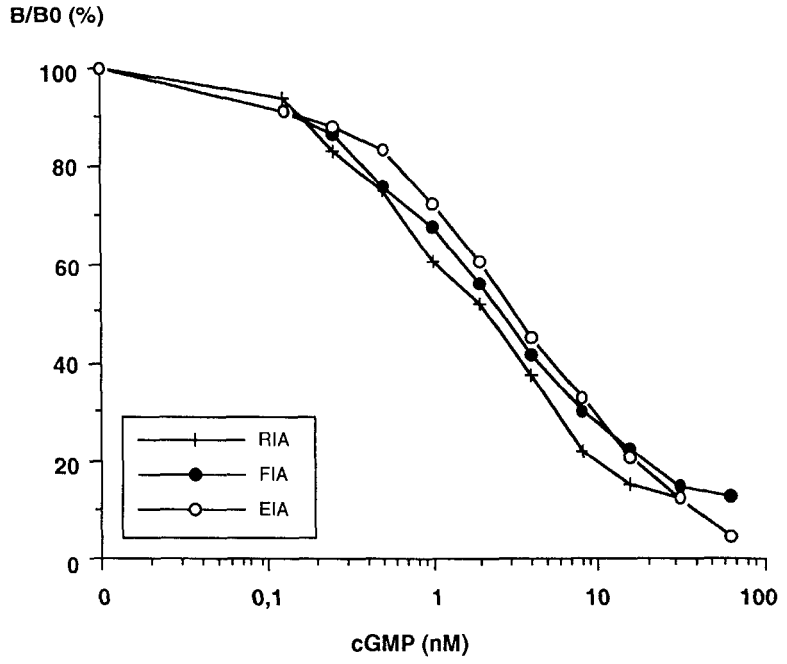

FIGURE 3. Representative displacement curves for cGMP TR-FIA and cGMP EIA in comparison to the reference radioimmunoassay.

the coefficients of variation were 6.3 % and 5.2 %, for EIA 7.9 % and 5.8 %, respectively. The inter-assay variation was determined by repetitive measurement (n=15) of the same 2 samples (1.5 nM and 5.5 nM cGMP). The respective coefficients of variation for the TR-FIA were 8.4 % and 5.8 % and 10.9 % and 7.9 % for the EIA.

Linearity And Recovery

For the investigation of interfering sample matrix effects (linearity of results), four plasma samples were diluted 1:3 and 1:6 in assay buffer (zero standard). The mean result found was 96.4 % of the expected

value. One plasma sample was supplemented 1+1 with different standards used for the displacement curves prior to extraction. The mean recovery in this experiment was 97.7 %.

Regression Analysis Of TR-FIA And EIA Versus The Reference RIA

Forty six plasma samples obtained from the daily laboratory routine (cGMP levels ranging from 3.8 to 42.1 nM) were analysed in both non isotopic assays (cGMP TR-FIA, EIA) and RIA. As the linear measuring range of the RIA is between 2 and 20 nM, the regression analysis was restricted to these values. The correlations found were good with r=0.95 (RIA versus TR-FIA), r=0.94 (RIA versus EIA) and r = 0.99 (TR-FIA versus EIA). The regression lines were $y_{(TR\text{-}FIA)} = 1.25x_{(RIA)} - 0.6$, $y_{(EIA)} = 1.13x_{(RIA)} - 0.16$ and $y_{(EIA)} = 0.96x_{(TR\text{-}FIA)} - 0.05$. The results are displayed in FIG. 4.

The results obtained in the determination of fifty 24-hour urine samples (cGMP levels ranging from 91 to 995 nM) also showed good correlation with r = 0.96 (RIA versus TR-FIA), r = 0.95 (RIA versus EIA) and r = 0.98 (TR-FIA versus EIA). The cGMP TR-FIA as well as the cGMP EIA showed a negative bias in comparison to the RIA method: $y_{(TR\text{-}FIA)} = 1.26x_{(RIA)} - 44.35$, $y_{(EIA)} = 1.18x_{(RIA)} - 63.38$ and $y_{(EIA)} = 0.94x_{(TR\text{-}FIA)} - 24.13$.

DISCUSSION

We have synthesized a cGMP-biotin conjugate by converting succinyl-cGMP into its NHS ester prior to coupling this derivative to the commercially available N-biotinyl-1,8-diamino-3,6-dioxaoctane (biotin-dadoo). Isolation of the conjugate was achieved by reversed phase chromatography, followed by determination of the cGMP as well as the biotin content in the collected fractions. The synthesis of

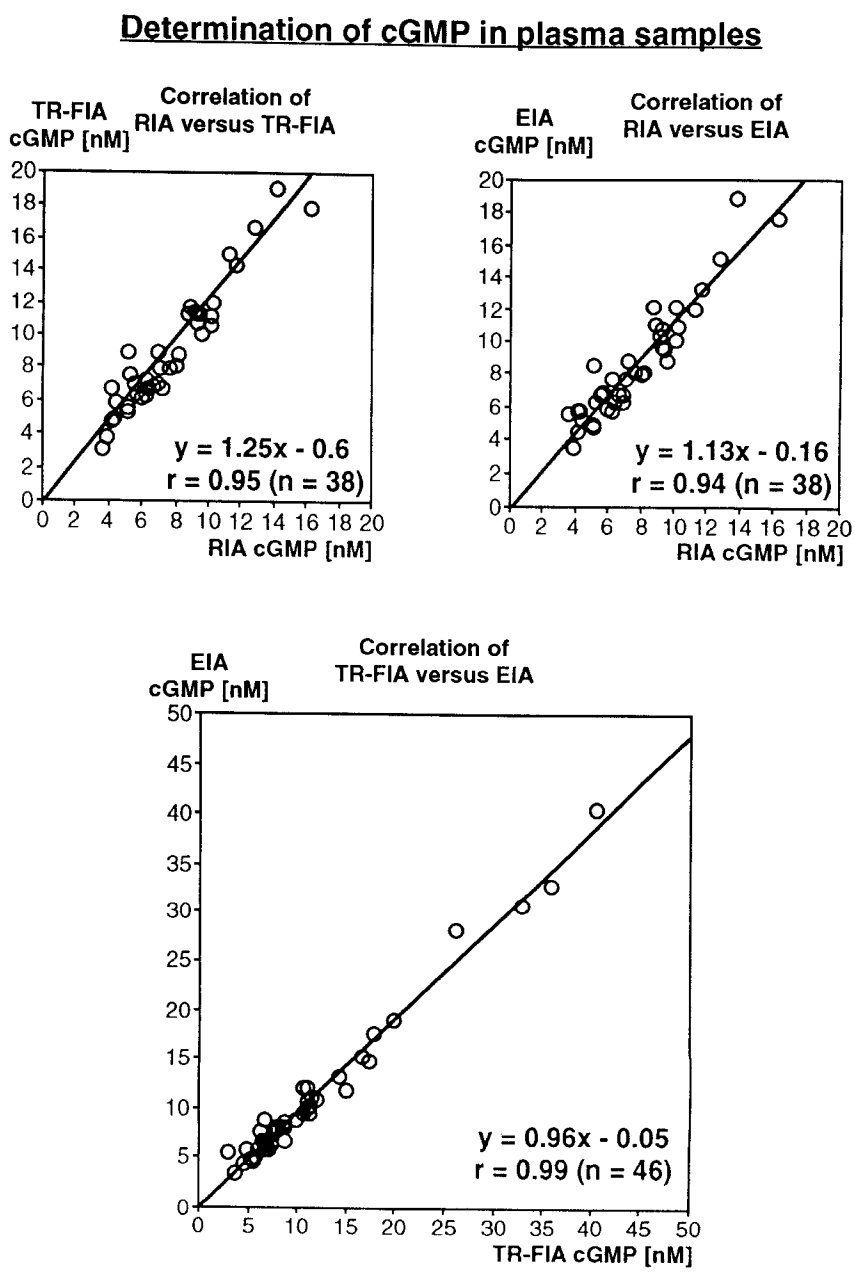

FIGURE 4. Regression analysis and correlation of cGMP concentrations in plasma samples as measured in RIA, TR-FIA and EIA.

the cGMP-biotin conjugate to the best of the authors' knowledge represents the first description of the biotinylation of a cyclic nucleotide for use as a tracer in non isotopic immunoassays.

The use of the biotin-avidin system serves as an amplification of the detectable signal (24). Further advantages are: the molecular weight of the cGMP-biotin conjugate (M_r 801.8) described is more similar to the molecular weight of the native hapten cGMP (M_r 345.2) than cGMP-enzyme conjugates (18) or cGMP-albumin conjugates (19) described previously in the literature, which are about 100-fold larger than the cGMP molecule itself. Chances of interaction with the antibody by molecular movement in this competitive immunoassay are therefore more similar between the tracer and analyte molecule than by use of protein-hapten tracers. The incorporated diamino-dioxaoctane spacer of the cGMP-biotin conjugate allows for binding of the cGMP moiety of the conjugate by a specific antibody and the binding of the ureido ring of biotin by streptavidin without steric interference by the two proteins. The hydrophilic nature of the spacer reduces nonspecific binding during immunoassay procedure.

Hapten-biotin conjugates have proven to be chemically and immunologically stable for more than two years. Re-standardization of the procedure is threfore less frequently needed than in using radioactive tracers. No health considerations nor legal restrictions are to be obeyed in performing these non isotopic immunoassays.

Immunoassays with biotinylated tracers as described here allow for flexible end point determination: it is possible to switch the method of end point determination from fluorometry to absorption measurement just before the incubation step with the signal generating label (streptavidin-europium, streptavidin-enzyme). This modular assay design improves the standardization of immunoassay results between laboratories equipped with EIA readers, TR-FIA fluorometers, potentially with luminometers or conventional γ-counters.

The advantages described for the use of biotinylated tracers as well as the reliability of the performance and the precision of the

results obtained outweigh the disadvantage of the additional incubation step with the labeled streptavidin.

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