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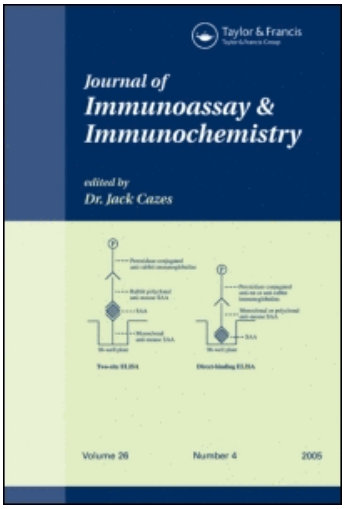

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PREPARATION OF STABLE ^{125}I CYCLIC GMP TYROSINE METHYL ESTER SUITABLE FOR 3',5' CYCLIC GMP RADIOIMMUNOASSAY BY HPLC

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ABSTRACT

Determination of the concentration of cyclic guanosine monophosphate (cGMP) by radioimmunoassay (RIA) depends upon the availability of suitable radiolabeled tracers and antibody to detect the product. Reverse phase chromatographic techniques can easily separate the reaction products of chloramine-T iodination of succinyl cGMP tyrosine methyl ester. The binding characteristics of the iodination reaction products to anti-cGMP antibody have been determined. Purified succinyl cyclic nucleotide ^{125}I-tyrosine methyl ester binds to cGMP antisera identically as commercially available tracer. The tracer is stable for greater than three months.

(KEY WORDS: radioimmunoassay, tracer, HPLC, iodination, cyclic guanosine monophosphate)

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INTRODUCTION

The measurement of cyclic guanosine 3′,5′-monophosphate concentration in biological samples is commonly performed using commercial radioimmunoassay kits that contain sufficient reagents to perform several hundreds of assays. Such kits are convenient but are very expensive on a per assay basis. Much of the expense is associated with preparation and purification of a suitable radiotracer. We developed simple procedures to purify homogeneous, stable iodinated 2′-O-monosuccinyl guanosine 3′,5′-cyclic monophosphate tyrosyl methyl ester (S-cGMP-TME) suitable for the competitive RIA of cGMP.

MATERIALS AND METHODS

Materials:

2′-O-monosuccinylguanosine 3′:5′-cyclic monophosphate tyrosyl methyl ester, sodium salt was obtained from Sigma Chemical Co., St. Louis, MO. Carrier-free Na ^{125}I was obtained from Amersham Corporation, Arlington Heights, IL. Rabbit anti-succinyl cGMP antibody was purchased from Meloy Labs., Inc., Springfield, VA. Guanosine 3′:5′-cyclic monophospate standard was obtained from Sigma Chemical Co., St. Louis, MO. Other buffers and reagents were analytical or HPLC grade.

Methods:

Radioiodination of succinyl cyclic GMP tyrosine methyl ester: The iodination reaction was performed in 0.5 M potassium phosphate

buffer, pH 7.0. The methyl ester was dissolved in distilled water to obtain a concentration of 800 ng/16 μl. Aliquots of this solution were frozen and stored at -80° C for future iodination. These stock solutions are stable for at least 12 months. The Na ^{125}I was diluted in 0.2 M potassium phosphate buffer pH 7.2 to a concentration of approximately 1 mC/20 μl. This reagent was used within two weeks of arrival to minimize breakdown products.

In order of addition, 20 μl Na ^{125}I was added to a small reaction tube followed by 29 μl potassium phosphate reaction buffer, and 16 μl S-cGMP-TME. The contents were gently mixed using a Vortex mixer, then 5 μl of 1 mg/ml chloramine T freshly diluted in potassium phosphate buffer was added to the reaction mix to initiate iodination. The contents were mixed and incubated at room temperature (20-25° C) for 1 minute, then 50 μl of 2.4 mg/ml Na metabisulfite diluted in the reaction buffer was added to the tube to convert the remaining iodine to iodide (total reaction volume, 70 μl; final volume, 120 μl). All operations were performed in an exhaust hood.

Purification of the succinyl cyclic GMP ^{125}I-tyrosine methyl ester ([^{125}I]S-cGMP-TME): Purification of the tracer ligand was performed in two steps, using reverse phase chromatography on C18 disposable cartridges (C18 Sep-Pak, Millipore-Waters, Inc.) followed by analytical gradient separation on high performance liquid chromatography column (HPLC). The procedure is a modification of the method previously reported for purification of

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^{125}I Escherichia coli heat-stable enterotoxin (STa) (1). After termination of the iodination reaction with Na metabisulfite, 880 μl of phosphate buffer was added to the reaction tube and the mixture applied with a 1 ml disposable syringe directly to a C18 Sep-Pak previously primed with 10 ml 100% acetonitrile followed by approximately 20 ml potassium phosphate buffer. After sample application, the Sep-Pak was flushed with 10 ml 10% acetonitrile in 0.01 M ammonium acetate buffer, pH 5.8 to remove unbound free Na ^{125}I. This was followed by 5 ml 60% acetonitrile in 0.01 M ammonium acetate buffer pH 5.8. The iodinated tyrosyl methyl ester products eluted between 0.5 ml and 3.5 ml of the 60% acetonitrile elution buffer, so elution can be into a single tube for further processing. Almost all free iodine eluted with the first 3 ml of 10% acetonitrile wash. To perform HPLC on the mixture, the acetonitrile was gently evaporated under a stream of nitrogen and the remaining aqueous mixture was applied to a Dupont Zorbax ODS 4.6 x 150 mm reverse phase column. The column was previously equilibrated with 12% acetonitrile in 0.01 M ammonium acetate buffer, pH 5.8. After injection of the sample in a volume of approximately 0.5 ml, the chromatogram was developed isocratically for 10 min at a flow rate of 1.0 ml/min. This was followed by gradient elution over 50 min from 12% to 80% acetonitrile at the same flow rate. One ml fractions were collected and 10 μl removed to count in a Packard Auto Gamma

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scintillation counter. Peak fractions were identified and pooled if necessary.

Radioimmunoassay procedure: The competitive RIA for cGMP is a modification of the procedure originally described by Steiner et al. (3) which we have used extensively (4-9). Briefly, the samples, antibody and radio labeled tracer were diluted in 0.05 M sodium acetate buffer, pH 6.2. A reaction volume of 0.4 ml consisting of 0.1 ml antibody dilution, 0.1 ml of [^{125}I]S-cGMP-TME (approximately 10,000 cpm) and 0.2 ml of sample or standard cGMP was incubated overnight in 12 x 75 mm glass tubes at 4° C. The correct antibody dilution for equilibrium conditions was established prior to assay by the antibody titration binding assay described below. Bound and free cyclic nucleotide were separated by ammonium sulfate precipitation. After incubation, 2.5 ml cold 2.08 mol/l ammonium sulfate solution was added to all tubes but controls. The precipitated immune complexes were separated from supernatant by centrifugation at 3000 xg for 20 minutes at 4° C. After decanting the supernatants, the inside walls of the tubes were carefully swabbed with cotton tipped applicators to remove excess moisture. The tubes were counted and the concentration of cGMP was determined from standard curves consisting of graded doses of pure guanosine 3′:5′-cyclic monophosphate. The standard stock solution from which all dilutions of cGMP were made consisted of 2000 pmol pure cGMP/ml in assay buffer. Each experimental point was obtained in duplicate. Antibody titration and cGMP RIA have been performed greater than 10 times.

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RESULTS

Using the iodination conditions described greater than 80% Na^{125} I was reproducibly incorporated into succinyl cGMP tyrosine methyl ester. Separation of iodination products from unbound ^{125}I was virtually complete with the Sep-Pak cartridge chromatography step. The approximate specific activity of the post Sep-Pak pool radioligand was estimated using methods outlined by Bolton (10) to be 480 Ci/mmole.

Figure 1 shows a typical HPLC elution profile of the iodination products eluted from the Sep Pak with 60% acetonitrile buffer. These profiles consistently show at least 4 separated radiolabeled species, called A, B, C, and D. The majority of the isotope incorporation was found in peak A. Occasionally other small peaks were found. The relative amplitude of these components peaks were dependent upon the methyl ester. The C and D peaks are increased and the A and B peaks are reduced when the succinyl methyl ester is stored under suboptimal conditions prior to iodination such as multiple freeze thaw or storage in a frost-free freezer (data not shown).

Each of the iodination products found upon HPLC separation were incubated with different concentrations of anti-cGMP antibody to determine their specific antibody equilibrium binding characteristics. In a typical antibody titration experiment, Figure 2 shows that the pooled Sep-Pak material as well as both peak A and B bind to polyclonal rabbit anti-cGMP in a dose-related

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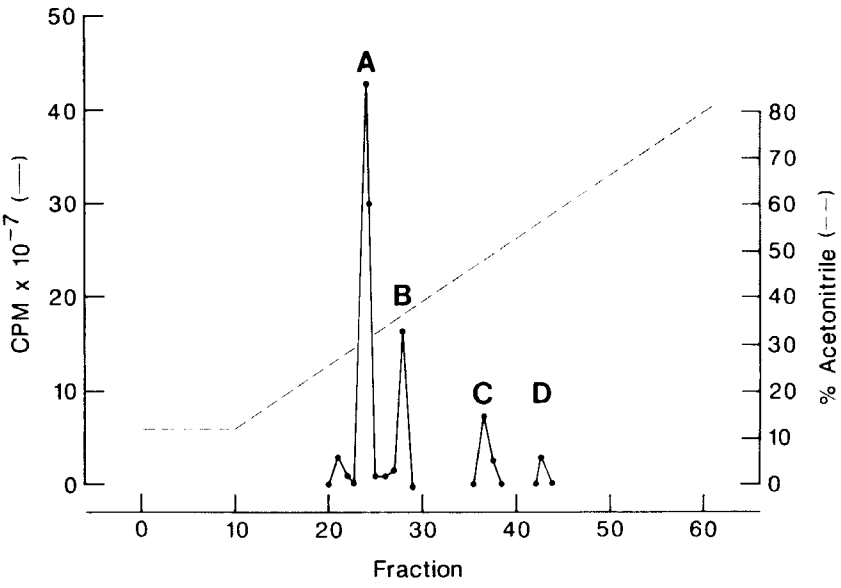

Figure 1 - Gradient elution profile of [^{125}I] succinyl cyclic GMP tyrosine methyl ester iodination derivatives on reverse-phase HPLC. 5 μl of each column eluate fraction was counted; cpm per fraction is shown above.

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manner. Peak A consistently demonstrated the highest specific binding of the three preparations, although the difference in reactivity was 2-fold or less between peaks A and B. Peak A and B may represent mono- and di-iodo derivatives, respectively. The pools corresponding to the peaks C and D demonstrated no antibody binding activity.

The HPLC purified ^{125}I-cGMP products corresponding to peaks and B and the pooled Sep-Pak eluate were tested as radioligands in RIA for cGMP. The resulting standard competition curves are shown

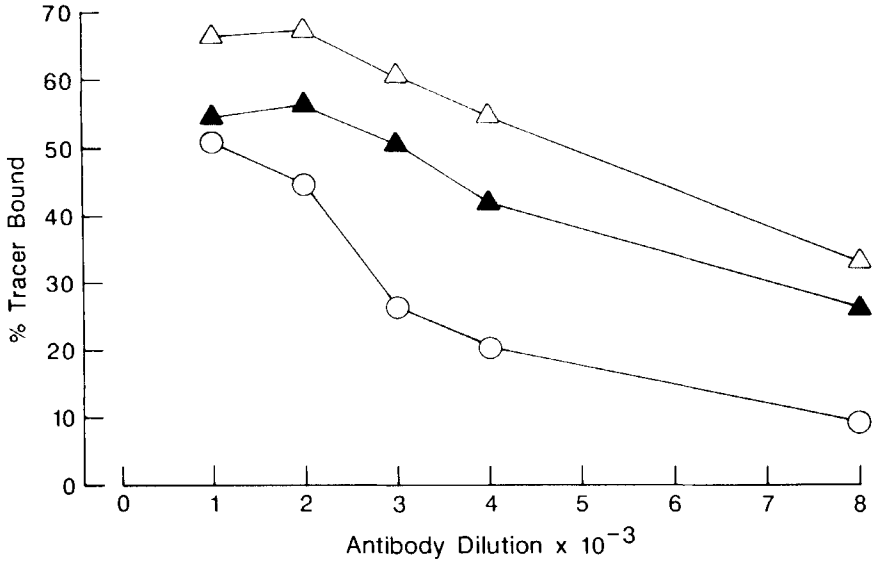

Figure 2 - Antibody titration of post Sep Pak (0), peak A (△), and peak B (▲) purified [^{125}I] S-cGMP-TME products.

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in Figure 3. When tested at dilutions of antibody that yield approximately 40% binding (typically 1:3000 to 1:4000) each of the products yield linear standard competition curves with purified cGMP. The standard curves so generated are virtually superimposable and are virtually identical to those obtained with commercially available radiotracer furnished with cGMP kits (data not shown). Thus when the majority of the radiolabeled products are found in peak A, the pooled Sep-Pak eluates are of sufficient quality to serve as tracer for cGMP detection without further processing. If, however, the proportion of products C or D are

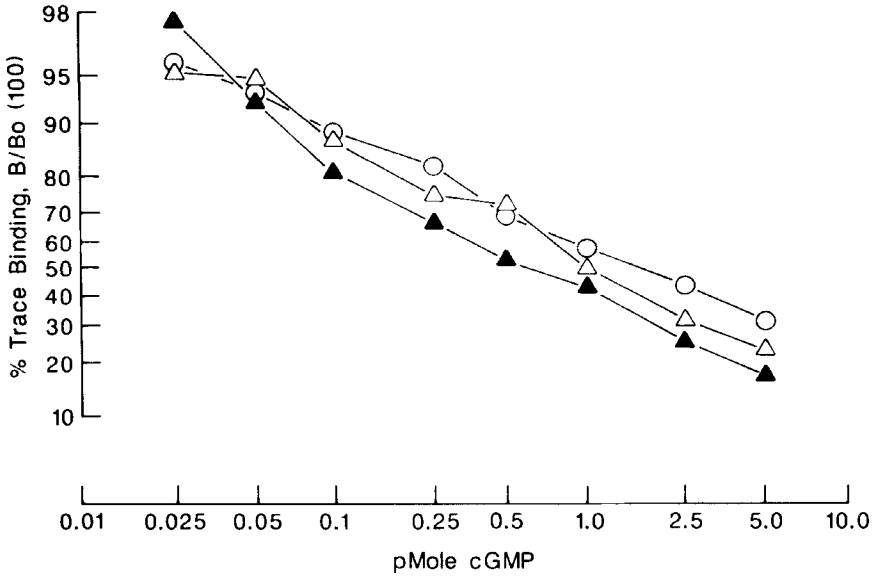

Figure 3 - Radioimmunoassay standard cGMP competition curves using [^{125}I] S-cGMP-TME. Post Sep Pak (O), Peak A (△), Peak B (▲).

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increased due to degradation of the methyl ester, further separation of the reaction products by HPLC is required for homogeneous tracer. Tracer was stable for up to 3 months at 4° C with no change in antibody binding characteristics except for the expected alteration due to radioactive decay. Typical yield of useable tracer from a single iodination is approximately 400 x 10^6 cpm, sufficient for at least 40,000 individual assays with 10,000 cpm per assay tube.

DISCUSSION

The Sep-Pak separation of radiolabeled succinyl cyclic guanosine 3′,5′ monophosphate tyrosine methyl ester GMP tracer from unincorporated ^{125}I is rapid, convenient, and cheap. Brooker et al. (11) and Cailla et al. (12) describe paper and gel filtration chromatographic separation techniques to separate the iodination products of S-cGMP-TME. Unlike these chromatographic separations, the tracer purification procedure offered here is accomplished in a matter of minutes with minimal manipulation, yielding a stable product when stored in the eluting buffer containing acetonitrile. The immunological characteristics of the Sep-Pak purified radioligand are virtually identical to the HPLC purified peak A if the other reaction products are quantitatively minimal. The Sep-Pak product, if shown to be relatively homogeneous by analytical HPLC, is therefore suitable for use as a radio-tracer in the RIA for cGMP.

We have found that storage conditions prior to iodination can alter the iodination characteristics of the methyl ester. Improper storage of the methyl ester will lead to heterogeneous iodination products, some of which are not immunoreactive (e.g. peaks C and D). When this happens, the Sep-Pak eluate is not homogeneous and the specific binding of tracer to antibody is reduced. Therefore, it is imperative to either determine that the Sep-Pak eluate is essentially homogeneous prior to use by performing analytical HPLC on the product, or to routinely

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incorporate HPLC purification for the tracer. We have chosen the latter course using a dedicated reverse phase column.

The separation procedure described yields a similar HPLC profile of reaction products from iodination reactions employing enzymobeads (BioRad, Richmond, CA), although incorporation of ^{125}I into S-cGMP-TME overall is significantly less than found in the chloramine T approach.

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