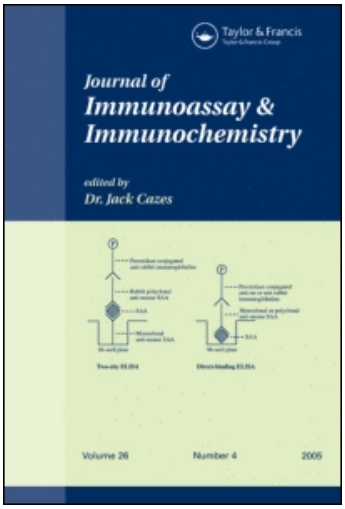

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A Rapid and Economical Method of Preparing Radioiodinated Cyclic Nucleotide Derivatives for use in Radioimmunoassays

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A RAPID AND ECONOMICAL METHOD OF PREPARING RADIOIODINATED CYCLIC NUCLEOTIDE DERIVATIVES FOR USE IN RADIOIMMUNOASSAYS

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ABSTRACT

2'-O-succinyladenosine 3':5'-cyclic monophosphate tyrosyl methyl ester (ScAMP-TME) and 2'-O-succinylguanosine 3':5'-cyclic monophosphate tyrosyl methyl ester (ScGMP-TME) were radioiodinated using chloramine T and $Na^{125}I$. The resulting radiolabeled cyclic nucleotide derivatives, ScAMP-^{125}I-TME and ScGMP-^{125}I-TME, were subsequently purified by reverse-phase chromatography on Sep-Pak C18 cartridges (Waters Associates, Milford, MA) and tested as tracers in sensitive radioimmunoassays for cAMP and cGMP, respectively. Purified ScAMP-^{125}I-TME and ScGMP-^{125}I-TME functioned in the respective radioimmunoassays for up to 12 weeks when suspended in a 1:1 (v:v) mixture of n-propanol and 20 mM sodium acetate, pH 6.0. Thus, this purification method enables rapid and economical preparation of tracers for cyclic nucleotide radioimmunoassays. Furthermore, our findings suggest that reverse-phase chromatography may be applicable to the purification of other small polar molecules to which tyrosyl groups have been added for the purpose of radioiodination.
(KEY WORDS: cyclic nucleotides, radioimmunoassay, reverse-phase chromatography, radioiodination)

INTRODUCTION

The cyclic nucleotides adenosine 3':5'-cyclic monophosphate (cAMP) and guanosine 3':5'-cyclic monophosphate (cGMP) serve as second messengers for the actions of a variety of endogenous and exogenous agents in organisms ranging from bacteria to humans

(1,2). The ubiquitous nature of these second messengers has made their measurement essential to the study of the actions of numerous hormones, local mediators, neurotransmitters, drugs and toxins. Since the early 1970's, the method of choice for quantitation of cyclic nucleotides has been radioimmunoassay (3). The most sensitive radioimmunoassays for cAMP and cGMP utilize a radioiodinated derivative of the parent compound, commonly the 2'-O-succinyl cyclic nucleotide iodotyrosyl methyl ester, as the tracer, and require acetylation of the samples prior to measurement (4). The radioiodinated tracer can be obtained commercially or can be generated in the laboratory using standard radioiodination procedures. Although from a cost standpoint synthesizing one's own tracer is preferable to purchasing from commercial sources, certain difficulties do attend this procedure. One of these is the separation of unincorporated ^{125}I species from ^{125}I-labeled cyclic nucleotide derivatives. To date, the most common means of achieving this separation have been paper chromatography, thin layer chromatography, and gel filtration (3,4). Despite their effectiveness, we feel that these techniques are unnecessarily slow and/or laborious. Recently, however, a reverse-phase high performance liquid chromatographic (RP-HPLC) method that results in quick and efficient resolution of iodinated cyclic nucleotide derivatives has been reported (5). Unfortunately, not all laboratories interested in measuring cyclic nucleotides by radioimmunoassay possess an HPLC system and a reverse-phase column which can be

dedicated to this separation. For these reasons, we sought to develop an alternative separation method based on reverse-phase chromatography on a Sep-Pak C18 cartridge (Waters Associates, Milford, MA). Here we describe this rapid, simple, and economical means of preparing radioiodinated 2-O-succinyl cyclic nucleotide iodotyrosyl methyl esters and demonstrate their utility in radioimmunoassays for cAMP and cGMP.

MATERIALS AND METHODS

Radioiodination of 2'-O-succinyl cyclic nucleotide tyrosyl methyl esters

2'-O-succinyladenosine 3':5'-cyclic monophosphate tyrosyl methyl ester (ScAMP-TME) and 2'-O-succinylguanosine 3':5'-cyclic monophosphate tyrosyl methyl ester (ScGMP-TME) obtained from Sigma (St. Louis, MO) were radioiodinated using chloramine T (Sigma) as previously described (3). Briefly, 2 ug of either ScAMP-TME or ScGMP-TME and 0.5 mCi of $Na^{125}I$ (Amersham, Arlington Hts., IL) were added to 300 ul of 100 mM sodium phosphate buffer, pH 7.5, in a 1.5 ml plastic Eppendorf tube. The radiodination was initiated by addition of 100 ul of a 0.5 mg/ml solution of chloramine T in the sodium phosphate buffer. The reaction was allowed to proceed for 45 seconds before termination by the addition of 600 ul of a solution of sodium metabisulfite (0.33 mg/ml) and NaI (0.17 mg/ml) in the sodium phosphate buffer.

Separation of unincorporated ^{125}I from ScAMP-^{125}I-TME or ScGMP-^{125}I-TME

Immediately after termination of the radioiodination reaction by addition of sodium metabisulfite, the 1 ml reaction mixture was

withdrawn from the plastic Eppendorf tube using a disposable syringe and loaded onto a Sep-Pak C18 cartridge (Waters Associates, Milford, MA). Prior to loading, the Sep-Pak had been prepared by successive washes with 5 ml of methanol, 5 ml of 40% acetonitrile in 0.1% trifluoroacetic acid (TFA), and 5 ml of 5% acetonitrile in 0.1% TFA. Following loading of the reaction mixture, the Sep-Pak was subjected to successive washes with 5 ml of 5% acetonitrile in 0.1% TFA and 3 ml of 40% acetonitrile in 0.1% TFA. The majority of the radioactivity corresponding to the iodinated cyclic nucleotide derivatives eluted in the second ml of the 3 ml wash with 40% acetonitrile in 0.1% TFA. This fraction was retained and either dried under a stream of nitrogen or lyophilized prior to resuspension for use in the respective radioimmunoassays. The resuspension solution was either 5 mM sodium acetate, pH 4.75, or a mixture of n-propanol:20 mM sodium acetate, pH 6.0, (1:1, v:v).

Radioimmunoassays for cAMP and cGMP

Radioimmunoassays for cAMP and cGMP were performed essentially as previously described (4). cAMP and cGMP obtained from Sigma (St. Louis, MO) were used to prepare standards. The standards were acetylated in 5mM sodium acetate, pH 4.75, by addition of a 2:1 (v:v) mixture of triethylamine and acetic anhydride. Antibodies to cAMP and cGMP, generated in rabbits, were used at final dilutions of 1:10,000 and 1:90,000, respectively. For comparison with tracer prepared by the method described above, ScAMP-^{125}I-TME was purchased from New England

Nuclear (Boston, MA). Dextran/charcoal was used to separate bound from free in the cAMP radioimmunoassay; whereas, a double antibody method was used in the cGMP radioimmunoassay, the second antibody being goat anti-rabbit IgG (Linco, St. Louis, MO).

Determination of the Specific Activities of ScAMP-^{125}I-TME and ScGMP-^{125}I-TME

The specific activities of the iodinated cyclic nucleotide derivatives were determined by a previously described self-displacement technique (6) under essentially the same conditions as the standard radioimmunoassays for cAMP and cGMP (described above).

RESULTS

Separation of unincorportated ^{125}I from ScAMP-^{125}I-TME or ScGMP-^{125}I-TME on a Sep-Pak C18 Cartridge

Following radioiodination of the respective 2'-O-succinyl cyclic nucleotide tyrosyl methyl esters, unincorporated ^{125}I was rapidly separated from ScAMP-^{125}I-TME or ScGMP-^{125}I-TME by subjecting the respective reaction mixtures to reverse-phase chromatography on Sep-Pak C18 cartridges. A representative profile of the radioactivity eluted from a Sep-Pak C18 cartridge loaded with a ScGMP-TME radioiodination reaction mixture is shown in figure 1. Unincorporated ^{125}I did not adsorb to Sep-Pak and, consequently, was present in the initial wash with 5% acetonitrile in 0.1% TFA. The peak of radioactivity eluting in the subsequent wash with 40% acetonitrile in 0.1% TFA corresponded to

ScGMP-^{125}I-TME as evidenced by its utility as a tracer in the cGMP radioimmunoassay (see figure 2). Analogous radioactivity elution profiles were obtained following applications of ScAMP-TME radioiodination reaction mixtures to Sep-Pak C18 cartridges (data not shown).

Radioimmunoassays for cAMP and cGMP

Representative standard curves from radioimmunoassays for cAMP and cGMP utilizing ScAMP-^{125}I-TME and ScGMP-^{125}I-TME prepared as in Materials and Methods are shown in figure 2. For comparison purposes, a curve generated using ScAMP-^{125}I-TME purchased from New England Nuclear (Boston, MA) is also shown. Commercially available ScAMP-^{125}I-TME and that prepared by our method proved indistinguishable in the cAMP radioimmunoassay. An analogous comparison was not attempted for ScGMP-^{125}I-TME. We were able to consistently detect 25-30 fmoles/tube of cAMP and 10-15 fmoles/tube of cGMP in the respective radioimmunoassays. We experienced no problems with either assay for at least 6 weeks after preparation of the tracers. Beyond 6 weeks, however, the background counts in our radioimmunoassays began to rise slightly, and by 8 weeks the tracers were no longer usable. Interestingly, we found that this time-dependent increase in background counts could be delayed considerably by suspending the tracers in mixtures of n-propanol: 20 mM sodium acetate, pH 6.0, (1:1, v:v) as opposed to 5mM sodium acetate pH 4.75. Tracers stored in the n-propanol:sodium acetate mixture functioned in the respective radioimmunoassays for up to 12 weeks.

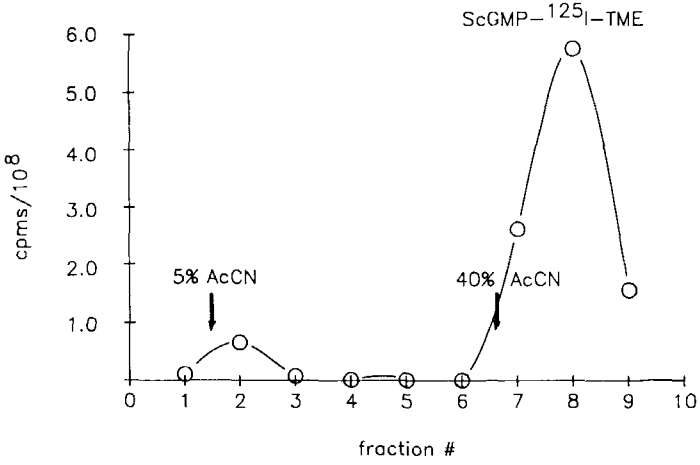

FIGURE 1. Profile of radioactivity eluting from a Sep-Pak C18 cartridge following application of a ScGMP-TME radioiodination reaction mixture. The major peak eluting in 40% acetonitrile (AcCN) with 0.1% TFA corresponded to ScGMP-125I-TME as evidenced by its utility as a tracer in the cyclic GMP radioimmunoassay.

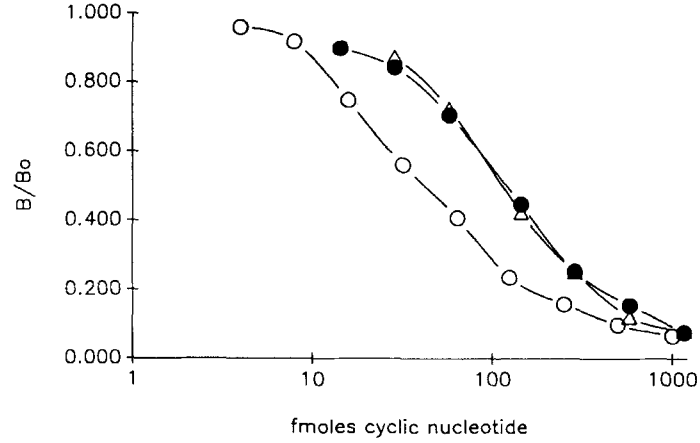

FIGURE 2. Representative standard curves from radioimmunoassays for: O-O, cGMP using tracer prepared by our protocol; ●-●, cAMP using tracer prepared by our protocol; and △-△ , cAMP using tracer obtained from New England Nuclear.

Specific Activities of ScAMP-^{125}I-TME and ScGMP-^{125}I-TME

The specific activities of ScAMP-^{125}I-TME and ScGMP-^{125}I-TME prepared by our protocol were found to range from 400 to 700 Ci/mmol. Commercial sources of these tracers claim the theoretically maximal specific activities of 2200 Ci/mmol.

DISCUSSION

We have described a rapid, simple, and economical method for preparing radioiodinated cyclic nucleotide derivatives for use in cyclic nucleotide radioimmunoassays. Our method involves purification of these derivatives by reverse-phase chromatography on Sep-Pak C18 cartridges. The applicability of reverse-phase chromatography to the purification of 2'-O-succinyl cyclic nucleotide tyrosyl methyl esters has been demonstrated previously using an HPLC system (5). Because not all investigators desiring to measure cyclic nucleotides by radioimmunoassay possess such a system, we believe our method provides an attractive alternative. Interestingly, the success of either method probably can be attributed to the succinyl tyrosyl methyl ester moiety conferring sufficient hydrophobicity upon the derivatized cyclic nucleotide to allow separation from unincorporated ^{125}I by reverse-phase chromatography. This conjecture is supported by the fact that ^{3}H-cAMP is not retained by Sep-Pak C18 cartridges under the conditions used to purify its 2'-O-succinyl iodotyrosyl methyl ester derivative (data not shown).

ScAMP-^{125}I-TME and ScGMP-^{125}I-TME prepared by our method functioned in sensitive radioimmunoassays for 8-12 weeks, with

ScAMP-^{125}I-TME being indistinguishable from that obtained commercially. The useful lifetime of these tracers was found to be dependent upon the solvent in which they were suspended and stored; suspension in a 1:1 (v:v) mixture of n-propanol and 20 mM sodium acetate, pH 6.0, resulted in a useful lifetime of 10-12 weeks as opposed to the 8-10 weeks obtained from suspension in 5 mM sodium acetate, pH 4.75. Therefore, the separation procedure described in this study should provide a simple, convenient, and economical method for the purification of radioiodinated cyclic nucleotide derivatives. Moreover, this work provides a basis for utilizing reverse-phase chromatography for the purification of other low molecular weight polar molecules to which tyrosyl groups have been added for the purpose of radioiodination.

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