

TopCount *Topics*

TCA-021

High Throughput Screening for cAMP Formation by Scintillation Proximity Radioimmunoassay

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Abstract

The introduction of an SPA (Scintillation Proximity Assay, Amersham International plc) reagent has enabled the production of a one-step RIA in which radioactivity associated with antibody-bound cAMP can be counted in the presence of unbound radiolabeled cAMP without the addition of liquid scintillation cocktail.

The development of second generation, low density, polyvinyl toluene (PVT)-based fluomicrospheres employed in the screening assay described here, remain in suspension for long periods of time, particularly when compared with yttrium silicate-based beads. This property greatly improves pipetting accuracy and facilitates complete automation using the MultiPROBE[®] Automated Liquid Handling System and the TopCount[™] Microplate Scintillation and Luminescence Counter (Packard Instrument Company). PVT beads are not only ideally suited for high throughput screening applications, they also have the flexibility to meet the complex technical demands of cAMP assays. Studies described in this paper demonstrate that the assay is fully amenable to automation with results equivalent to a manual reference method.

Introduction

Adenosine 3'5'-cyclic monophosphate (cAMP) is involved in a myriad of normal and pathological processes. Indeed, this cyclic nucleotide serves as a second messenger for the action of endogenous and exogenous agents in organisms ranging from bacteria to humans.¹ The ubiquitous nature of cAMP has made its measurement essential for the study of numerous hormones, local mediators, neurotransmitters, drugs and toxins. The role of cAMP in cellular metabolism has made it increasingly desirable to measure this nucleotide in biological specimens with ease and dependability. cAMP is present in extremely low concentrations in tissues and cell culture supernatants. Methods developed for measurement of this cyclic nucleotide must contend with

high concentrations of interfering non-cyclic nucleotide substances.

Here we describe a rapid, selective and highly sensitive method for estimating cAMP by competitive scintillation proximity radioimmunoassay. Antibodies to the cyclic nucleotide were raised in rabbits after immunization with antigen, in which a 2'-O-succinyl derivative of the cyclic nucleotide had been conjugated to protein. The label is an iodinated derivative of cAMP. In common with conventional heterogeneous radioimmunoassay systems, the assay is based on competition between unlabeled cAMP with a fixed quantity of [¹²⁵I]-labeled cAMP for a limited number of binding sites on a cAMP specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand. The Biotrak[™] cAMP SPA Screening Assay System from Amersham[™] eliminates the need to separate antibody bound ligand from free ligand common to heterogeneous radioimmunoassays. In the assay, the antibody bound cAMP reacts with the SPA reagent, which contains anti-rabbit second antibody bound to fluomicrospheres. Any [¹²⁵I] cAMP that is bound to the primary rabbit antibody will be immobilized on the fluomicrospheres and will generate light. The homogeneous RIA has a working range of 0.2-12.8 pmol/microplate well (1.32-84.28ng/ml), with a sensitivity of detection of 0.1 pmol/well (0.65ng/ml). Measurement in the TopCount enables the amount of fluomicrosphere-bound labeled cAMP to be calculated. The concentration of unlabeled cAMP in samples is determined by interpolation from a standard curve. The method uses a second generation of fluomicrospheres (polyvinyl toluene, PVT-based beads). The PVT fluomicrospheres are prepared at low density resulting in beads remaining in suspension for relatively long periods. This greatly improves pipetting accuracy and facilitates complete automation of the assay using the Packard Multi-



PROBE. The method is designed to be carried out in microplates and is optimum for estimating cAMP in large sample numbers.

The simplicity of the SPA assay is greatly enhanced by advances in instrumentation. The MultiPROBE allows accurate, unattended pipetting of all assay samples, standards, and reagents. Four tips with variable spacing allow reagents to be pipetted from reagent troughs, test tubes or other containers, and dispensed into microplates. If required, disposable tips can be used to eliminate any contamination from sample carry-over. An eight-tip system is available for rapid dispensing and dilutions. When ready for analysis, the samples can be measured directly in the same microplate on the multidetector TopCount. Up to 12-wells can be measured simultaneously and a stacker allows the unattended analysis of thousands of samples. To keep track of the large amounts of data being generated, bar code identified data can be stored and analyzed on the TopCount or networked according to individual laboratory requirements.

Assay technology and instrumentation are becoming more closely linked and more interdependent. The technical advances in Amersham's SPA and Packard's TopCount and MultiPROBE demonstrate a combined approach to provide optimum solutions to biological assays.

Method

A MultiPROBE 104 Automated Liquid Handling System was used for the automated pipetting steps. Control assays were performed using hand held pipettes. Ninety-six well microplates were analyzed in a TopCount model which measures 12 wells simultaneously.

Preparation of Immunogens

Human serum albumin (HSA):cAMP conjugates were prepared by the method of Horton *et al.*² Briefly, HSA was dissolved in redistilled water and a triethylamine (TEA): dimethylformamide (DMF) mixture, followed by a cAMP reaction mixture. This latter mixture was prepared by dissolving 2'-O-monosuccinyladenosine 3',5'-cyclic monophosphate in DMF. The TEA:DMF mixture was added to this solution and followed by an ethylchloroformate:DMF mixture. This was then incubated at room temperature, before addition of the HSA solution, prepared as above, for 30 minutes. Low molecular weight components of the reaction mixture were removed by gel filtration on a short Sephadex™ G-25 column, equilibrated and eluted with 0.01M sodium chloride.

Immunization

Antibodies were raised in rabbits by repeated immunizations. Succinyl cyclic nucleotide-HSA conjugates were dissolved in sterile redistilled water and emulsified with an equal volume of complete

Freund's adjuvant. An emulsion containing immunogen was injected intracutaneously at multiple sites on the animal's back. The rabbits were boosted once every four weeks for two months with immunogen in incomplete Freund's adjuvant. Bleeds were taken from the carotid artery seven to ten days after the last injection. The separated sera were aliquoted, lyophilized and stored at -70 °C.

Standards

Adenosine 3',5'-cyclic monophosphate used for standards was checked for purity by chromatography. The concentration of the stock standards was confirmed by spectrophotometric measurement.

cAMP Radioiodination

Adenosine 3',5'-cyclic phosphoric acid 2'-O-succinyl-3-[¹²⁵I]iodo-tyrosine methyl ester was prepared by the method of Horton and Baxendale,³ whereby 2'-O-monosuccinyl adenosine 3',5'-cyclic monophosphoric acid tyrosine methyl ester was reacted with sodium [¹²⁵I]iodide and chloramine-T to give a specific activity of ~2000 Ci/mmol. The product was purified by HPLC.

Assay Procedure

cAMP in test samples was analyzed by a non-acetylation procedure in microplate wells. Standards were prepared in assay buffer (0.05M acetate, pH 5.8), with cAMP diluted over the range of 4-256 pmol/ml. The zero wells consisted of assay buffer without standard. Diluted sample or standard (50 µL, 0.2-12.8 pmol/well) was incubated with specific antisera (50 µL), [¹²⁵I]cAMP (50 µL, 20,000-30,000 CPM) and SPA anti-rabbit reagent (50 µL; Amersham). Nonspecific binding was determined in the absence of specific rabbit antisera. The plates were sealed and incubated at room temperature (15-30°C) for 15-20 hours, without agitation. The amount of [¹²⁵I] cAMP bound to the fluomicrospheres was determined by counting for two minutes in the TopCount.

Results

Assay Conditions and Equilibrium

Dose-response curves were prepared using different concentrations of [¹²⁵I]cAMP, specific antisera and SPA beads to achieve the widest working range and sensitivity for the detection of cAMP in biological samples. A number of incubation times from 2.5 hours to 72 hours were examined. Equilibrium was achieved after 20 hours, with little change in binding with assays carried out over a longer time period (Figure 1).

Curve Parameters and Cross-Reactivities

Typical dose-response curves for the cAMP SPA radioimmunoassay system are shown in Figure 2. Curves and unknown sample values prepared manually were compared with assays carried out using the

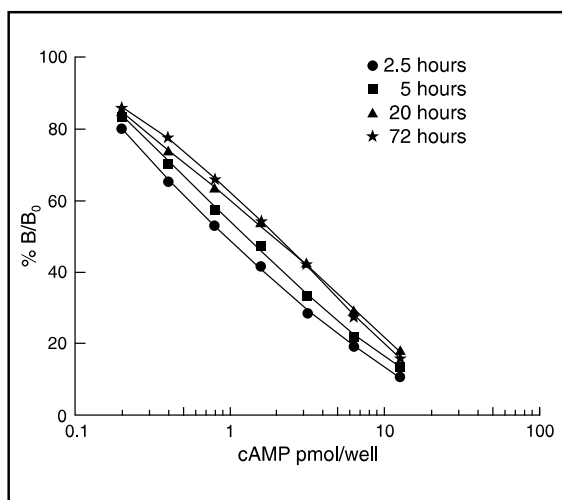


Figure 1.

Optimization and equilibrium of the cAMP scintillation proximity radioimmunoassay. The data shows results obtained from assays incubated over a varying time period: 2.5 hours, 5 hours, 20 hours and 72 hours.

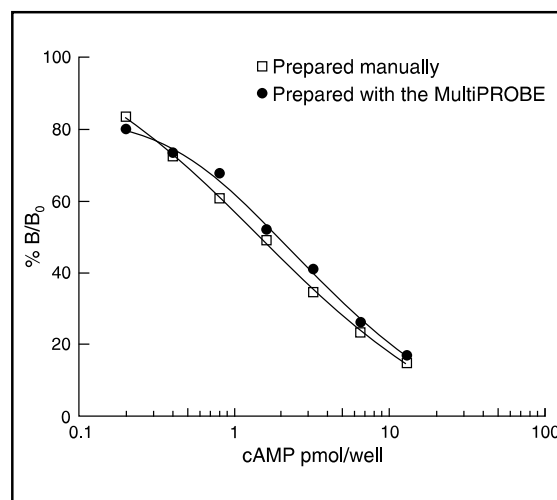


Figure 2.

Dose-response curves for the cAMP scintillation proximity radioimmunoassay. The data shows results from assays prepared manually, and assays carried out using the Packard MultiPROBE Automated Liquid Handling System.

Packard MultiPROBE 104 Automated Liquid Handling System. No significant differences were observed in curve shape parameters and unknown sample values when assays were performed manually or with the automated system.

Specificity

The specificity of the antiserum is shown in Table 1. The antiserum cross-reactivity with related and other important compounds was determined by the 50% displacement technique. The most important competitors are cGMP and ATP, both of which exhibited very low cross-reactivities. Other potential cross-reactants, including AMP, ADP, cIMP, cTMP and cCMP, also showed extremely low cross-reactivities in the assay.

Sensitivity

A measure of the sensitivity of detection of the assay was obtained by measuring 20 replicates of the zero standard. At two standard deviations below the mean, the relevant figure was 0.1 pmol/well (0.65 ng/ml).

Precision

A within assay precision profile was derived according to the method of Ekins and Edwards.⁴ The relationship between the calculated coefficient of variation and the concentration of cAMP is shown in Figure 3. A further indicator of the high level of accuracy was the repeated measurement of three unknown samples in the same assay (Table 2). Between assay reproducibility was assessed by repeated analysis of samples in successive assays. The results are shown in Table 3.

Discussion

A novel technique is described here for the accurate detection and measurement of cAMP in biological

Compound	Percent Cross-Reactivity
cAMP	100
cIMP	0.4
cGMP	0.0004
cCMP	0.00005
cTMP	0.0001
AMP	0.0002
ADP	0.0001
ATP	0.00002
EDTA	0.0000001
Theophylline	0.000002
Iso-butyl-methyl-xanthine	0.000008

Table 1.

Specificity of the rabbit anti-cAMP sera. The cAMP antisera cross-reactivity with related and other important compounds were determined by the 50% displacement technique.

samples. The method involves radiolabeling succinyl adenosine 3'5'-cyclic phosphoric acid tyrosine methyl ester. The assay is simple, sensitive, reliable and compared with other published assays for cAMP, is highly convenient. Other methods for the estimation of cAMP include enzymatic radioisotopic displacement,⁵ high pressure liquid chromatography (HPLC),⁶ protein kinase activation,⁷ luciferin-luciferase bioluminescence,⁸ competitive protein binding,⁹ and immunoassay techniques.¹⁰⁻¹³ This study demonstrates the utility of the scintillation proximity radioimmunoassay technique for cAMP. The assay, when fully automated, compares very favorably with the manual reference method. SPA technology greatly simplifies the ease of performing radioimmunoassays. Only four

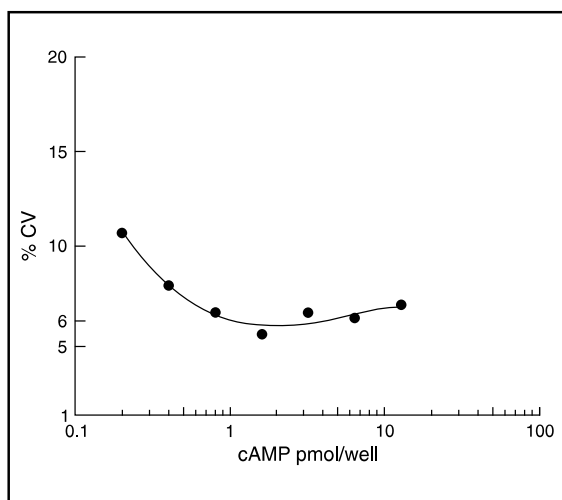


Figure 3.

Intra-assay precision profiles for the cAMP SPA screening assay system. Dose-response curves were constructed using twelve replicates of each of the standards. The individual values were interpolated from a composite dose-response curve to give the calculated concentration of cAMP. The coefficient of variation (%CV) in the dose of cAMP was calculated from these values.

Sample	pmol cAMP/well +/- ISD	%CV	n
A	0.352 +/- 0.040	11.3	20
B	0.999 +/- 0.056	5.7	20
C	4.189 +/- 0.123	2.9	20

Table 2.

The within-assay precision for duplicate determinations was calculated by measuring unknown samples in the assay.

Sample	pmol cAMP/well +/- ISD	%CV	n
A	0.331 +/- 0.036	10.8	20
B	0.884 +/- 0.099	11.2	20
C	3.946 +/- 0.355	9	20

Table 3.

Between-assay reproducibility was assessed by sequential measurement of samples in different assays. The assays were performed in duplicate as described in the text.

pipetting steps are required, thus reducing "hands on" and total assay time. The assay is carried out in single microplate wells, and the addition of liquid scintillant is not required.

An important benefit of homogeneous assays such as the cAMP SPA system is its suitability for automation in high throughput screening programs. This is difficult to achieve with most of the traditional assay methods described above. Almost all aspects of the assay can be automated by the use of the MultiPROBE Automated Liquid Handling System and the TopCount Microplate Scintillation and Luminescence Counter. The four simple pipetting steps required to prepare the samples can be done automatically on the MultiPROBE. This reduces "hands-on" time and day-to-day variability. The use of the multidetector TopCount with automatic plate chang-

ing capabilities allows unattended operation for improved assay throughput. By linking the MultiPROBE and TopCount with a robotic arm, SPA assays can be completely automated to run overnight with no technician intervention.

The cAMP SPA screening assay system offers a flexible approach to some complex problems associated with measurement of cAMP levels. The homogeneous nature of the assay eliminates the need for time consuming separation of bound from free radioactivity. These benefits, plus its demonstrated use with equipment for automating assays, mean that this cAMP SPA screening assay system offers a versatile and rapid assay for determining cAMP concentrations, whether by individual research scientists or high throughput screening laboratories.

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