Proximity News

SPA

February 1996 - Issue no. 23

High Throughput Screening for cAMP Formation by Scintillation Proximity Radioimmunoassay

An assay has been developed by Amersham International plc for the detection and measurement of cAMP using polyvinyl toluene (PVT)-based Scintillation Proximity Assay (SPA) beads (Amersham™: RPA 556). It is an assay which is simple to perform, requiring minimal manipulations, and so is suitable for high throughput screening applications. SPA has enabled the production of a one-step radioimmunoassay (RIA) in which radioactivity associated with antibody-bound cAMP, can be counted in the presence of the unbound radiolabelled cAMP without the addition of a liquid scintillation cocktail. Existing Amersham cAMP SPA assay systems employ yttrium silicate-based beads. The PVT-based SPA beads employed in this screening assay stay in suspension for longer periods of time, particularly when compared to yttrium silicate-based beads, due to their lower density. This property greatly improves pipetting accuracy and facilitates complete automation of the assay. The method is designed to be carried out on microtitre plates and is optimized for estimating cAMP in large sample numbers. This homogeneous RIA has a working range of 0.2-12.8pmol cAMP/microtitre well (1.32-84.28ng/ml), with a sensitivity of detection of 0.1pmol/well (0.65ng/ml). Studies have demonstrated that the assay is fully amenable to automation, with results comparing very favourably with a manual reference method.

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, and methods developed for measurement of this cyclic nucleotide must contend with high concentrations of interfering non-cyclic nucleotide substances.

Methods for the estimation of cAMP include enzymatic radioisotopic displacement[2], high-pressure liquid chromatography (HPLC)[3], protein kinase activation[4], luciferin-luciferase bioluminescence[5], competitive protein binding[6], and immunoassay techniques[7-10]. This study demonstrates the utility of the scintillation proximity radioimmunoassay technique for cAMP.

Here a rapid, selective and highly sensitive method for estimating cAMP by competitive scintillation proximity radioimmunoassay is described. The method has been designed with automation and high sample throughput in mind.

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[11]. Adenosine 3'5'- cyclic phosphoric acid 2'-O-succinyl-3- [^{125}I]iodo-tyrosine methyl ester was prepared by the method of Horton and Baxendale[12]. In common with conventional heterogeneous radioimmunoassay systems, the assay is based on competition between unlabelled cAMP with a fixed quantity of ^{125}I labelled 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 cAMP SPA screening assay described here eliminates the need to separate antibody bound from free ligand common to heterogeneous radioimmunoassays. In this assay, the antibody bound cAMP reacts with anti-rabbit antibody which is coupled to SPA beads. Any [^{125}I]cAMP that is bound to the primary rabbit antibody will thus generate a light signal. Measurement in a microplate β scintillation counter enables the amount of SPA bead-bound labelled cAMP to be calculated. The concentration of unlabelled cAMP in samples is determined by interpolation from a standard curve.

cAMP in test samples was analyzed by a non-acetylation procedure in microtitre wells. Standards were prepared in assay buffer (0.05M acetate, pH 5.8), with cAMP diluted over the range of 4-256pmol/ml. The zero wells consisted of assay buffer without standard. Diluted sample or standard (50µl, 0.2-12.8pmol/well) was incubated with specific antiserum (50µl), [^{125}I]cAMP (50µl, 20,000-30,000 SPA cpm) and anti-rabbit PVT SPA beads (50µl). Non-specific binding was determined in the absence of specific rabbit antiserum. The plates were sealed and incubated at room temperature (15-30°C) for 15-20 hours, without agitation. The amount of adenosine 3'5'- cyclic phosphoric acid 2`-O-succinyl-3-[^{125}I]iodo-tyrosine methyl ester bound to the SPA beads was determined by counting in either a Packard TopCount™ microplate scintillation counter or a Wallac 1450 MicroBeta™ liquid scintillation counter.

Automated assays were carried out using a Beckman BIOMEK™ 1000 automated laboratory workstation and a Packard MultiPROBE™ 100 robotic system.

Dose-response curves were prepared using different concentrations of [^{125}I]cAMP, specific antiserum 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 2½ hours to 72 hours were examined. Equilibrium was achieved after 24 hours, with little change in binding seen with assays carried out over a longer time period (Figure 1).

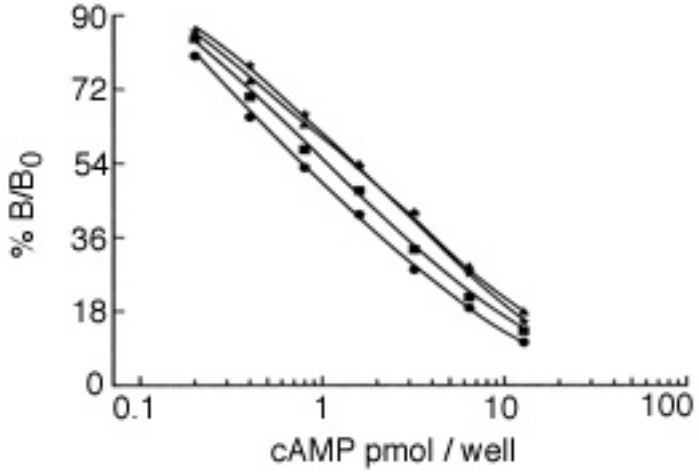

Figure 1. Optimization and equilibrium of the cAMP scintillation proximity radioimmunoassay. The data shows results obtained from assays incubated over a varying time period: 2 ½ h (●), 5h (■), 20h (▲) and 72h (★).

Typical dose-response curves for the cAMP SPA radioimmunoassay system are shown in Figure 2. Assays prepared manually were compared with assays carried out using the BIOMEK workstation and the MultiPROBE robot (Figure 2). No significant differences were observed in curve shape parameters and unknown sample values when assays were performed manually or with either automated system.

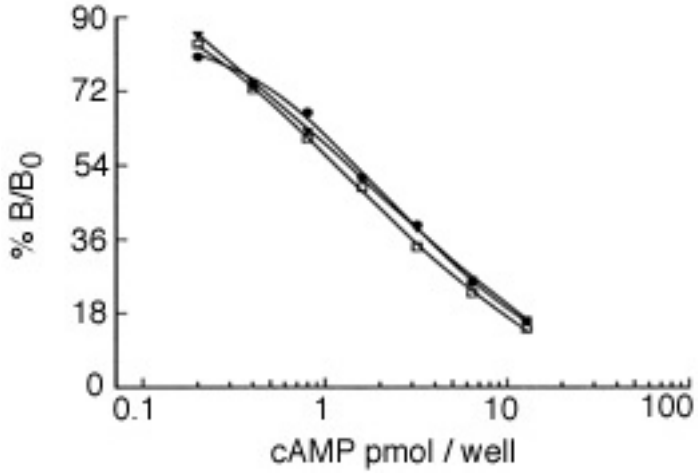

Figure 2. Dose-response curves for the cAMP scintillation proximity radioimmunoassay. The data shows results from assays performed manually (□) and assays carried out using the BIOMEK workstation (●) and the MultiPROBE robot systems (▲).

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 antiserum cross-reactivity with related and other important compounds was determined by the 50% displacement technique[11]. The most important competitors were 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.The specificity of the antiserum is shown in Table 1.

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

An intra-assay precision profile was derived according to the method of Ekins and Edwards[13]. The relationship between the calculated coefficient of variation and the concentration of cAMP is shown in Figure 3.

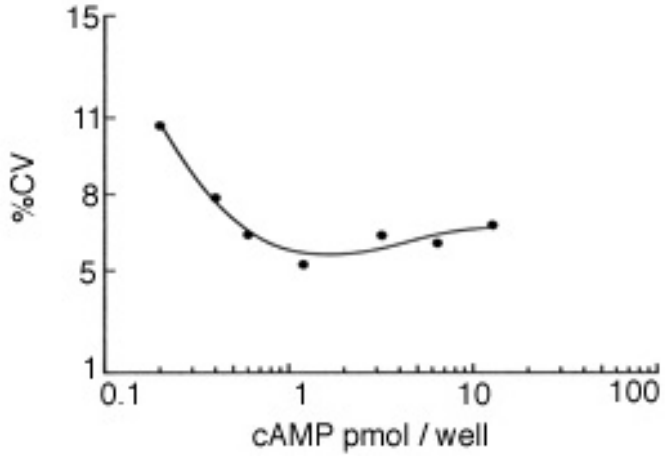

Figure 3. Intra-assay precision profiles for the cAMP SPA screening assay system. Dose-response curves were constructed using twelve replicates of each of the standards. The individual values were interpolated from a composite dose-response curve to give the calculated concentration of cAMP. The coefficient of variation (%CV) in the dose of cAMP was calculated from these values.

A further indicator of the high level of accuracy was the repeated measurement of unknown samples in the same assay (Table 2).

Sample	pmol cAMP/well	% CV
A	0.352 ± 0.040	11.3
B	0.999 ± 0.056	5.7
C	4.189 ± 0.123	2.9

Table 2. Intra-assay precision. The intra-assay precision for duplicate determinations was calculated by measuring unknown samples in the assay. Results are means ± sd (n=20).

Inter assay reproducibility was assessed by repeated analysis of samples in successive assays. The results are shown in table 3.

Sample	pmol cAMP/well	% CV
A	0.331 ± 0.036	10.8
B	0.884 ± 0.099	11.2
C	3.946 ± 0.355	9.0

Table 3. Inter-assay reproducibility. Inter-assay reproducibility was assessed by sequential measurement of samples in different assays. Results are means ± sd (n=20).

The assay, when fully automated, compared very favourably with the manual reference method.

The cAMP SPA screening assay system offers a flexible approach to some complex problems associated with measurement of cAMP levels. Only four pipetting steps are required, thus reducing "hands on" and total assay time. The assay is carried out in single microtitre wells and the addition of liquid scintillant is not required. Its demonstrated use with equipment for automating assays, mean that this cAMP SPA screening assay offers a versatile and rapid assay for determining cAMP concentrations.

References

1. SCHRAM, M. and SELINGER, Z. Message transmission: receptor controlled adenylate cyclase system. *Science* **225**, 1350-1356, (1984).

2. BUTCHER, R.W., HO, R. J., MENG, H. L. and SUTHERLAND, E. W., Adenosine 3' 5'-cyclic monophosphate in biological materials, II. The measurement of adenosine 3' 5'- monophosphate in tissues and the role of the cyclic nucleotides in the lipolytic response of fat to epinephrine. *J. Biol. Chem*. **240**, 4515-4523, (1965).

3. BROOKER, G. High pressure anion exchange chromatography and enzymatic isotopic displacement assays for cyclic AMP and cyclic GMP; in *Advances in Cyclic Nucleotide Research*, **2**, 111-129, (1972).

4. KUO, J. K. and GREENGARD, P. Cyclic nucleotide-dependent protein kinases VIII. An assay method for the measurement of adenosine 3' 5'- monophosphate in various times and a study of agents influencing its level in adipose cells. *J. Biol. Chem*. **245**, 4076-4083, (1970).

5. EBADI, M. S., WEISS, B. and COSTA, E. Microassay of adenosine 3' 5' -monophosphate (cyclic AMP) in brain and other tissues by luciferin- luciferase system. *J. Neurochem.* **18**, 183-192, (1971).

6. TOVEY, K. C., OLDHAM, K. G. and WHELAN, J. A. M. A simple, direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique. *Clin. Chim. Acta* **56**, 221- 234, (1974).

7. HARPER, J. F. and BROOKER, G. Femptomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2' -0-acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide*

Res. **1**, 207-218, (1975).

8. STEINER, A. L. Assay of cyclic nucleotides by radioimmunoassay methods. *Meth. Enzymol.* **38**, 96-105, (1974).

9. CAILLA, H. L., RACINE- WEISBUCH, M. S. and DELAAGE, M. A. Adenosine 3' 5' -cyclic monophosphate at 10^{-15} mole level. *Anal. Biochem*. **56**, 394-407, (1973).

10. TSUGAWA, M., FIDA, S., FUJII, H., MORIWAKI, K., TARUI, S., SUGI, M., YAMANE, M., YAMANE, R. and FUJIMOTO, M. An enzyme-linked immunosorbent assay (ELISA) for adenosine 3' 5' -cyclic monophosphate (cAMP) in human plasma and urine using monoclonal antibody. *J. Immunoassay* **11**, 49-61, (1990).

11. HORTON, J. K., MARTIN, R. C., KALINKA, S., CUSHING, A., KITCHER, J. P., O'SULLIVAN, M. J. and BAXENDALE, P.M. Enzyme immunoassays for the estimation of adenosine 3' 5' -cyclic monophosphate and guanosine 3' 5' -cyclic monophosphate in biological fluids. *J. Immunol. Methods* **155**, 31-40, (1992).

12. HORTON, J. K. and BAXENDALE, P. M. Mass measurements of cyclic AMP formation by radioimmunoassay, enzymeimmunoassay and Scintillation Proximity Assay. *Meth. Mol. Bio*l. **41** 91-105, (1995).

13. EKINS, R. P. and EDWARDS, P. R. The precision profile: its use in assay design, assessment and quality control: In *Immunoassays for Clinical Chemistry* (Hunter, W.M. and Corrie, J.E.T. eds). Churchill Livingstone, Edinburgh, 76-105, (1965).

For further information, contact Amersham Biosciences Technology Support Groups.

Europe
Phone no. +44 (0) 1 494 5 42600
Voicemail +44 (0) 1 494 5 42600
Fax no. +44 (0) 1 494 5 44420
e:mail drug.screening@uk.amershambiosciences.com

North America
Phone no. 1 888 SPA HITS (+1 8 88 77 2 44 87)
Voicemail 1 888 SPA HITS (+1 8 88 77 2 44 87)
Fax no. 1 732 457 8643
e:mail ts-usa@am.amershambiosciences.com

Japan
Phone no. +81 (0) 3 5331 9319
Fax no. +81 (0) 3 5331 9372
e:mail drug.screening@jp.amershambiosciences.com

Amersham Biosciences Amersham Place Little Chalfont Buckinghamshire England HP7 9NA

Amersham Biosciences AB SE-751 84 Uppsala Sweden

Amersham Biosciences Corp 800 Centennial Avenue PO Box 1327 Piscataway NJ 08855 USA

Amersham Biosciences Europe GmbH Munzinger Strasse 9 D-79111 Freiburg Germany

Amersham Biosciences KK Sanken Building 3-25-1 Hyakunincho Shinjuku-ku Tokyo zip 169-0073