cAMP Radioimmunoassay without Interference from Calcium or EDTA

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Calcium and EDTA interfere with radioimmunoassay of cAMP or acetylated cAMP. Both interferences with the assay of acetylated cAMP can be obviated by including in the buffer 15 mmol of CaCl₂ per liter, which at the same time increases the affinity of the antibody by 89%. As a result, antibody can be decreased by 65%. This method requires no prepurification (extraction or chromatography), and no correction for analytical recovery is necessary. This assay can detect 12 fmol of cAMP per tube, so only microliter quantities of plasma are needed.

**Additional Keyphrases:** reference interval - source of analytical error - parathyroid function assessment

Cyclic AMP (cAMP, adenosine 3',5'-monophosphate) has long been recognized as an important regulator of cell activities and a “second messenger” for many peptide hormones. Of various techniques for measuring cAMP, radioimmunoassay (RIA) after acetylation of the specimen is the most sensitive (1-3). The specificity of the acetylated cAMP RIA reported by Goldberg (1) is so superior that sample prepurification is not required, but we find that calcium or EDTA in specimens interferes with the RIA (1) for acetylated cAMP. We also find that the recommendation of Bruynzeel et al. (4), to assay acetylated cAMP rather than unacetylated cAMP, to prevent EDTA interference, is not sound. We investigated the mechanisms of these interferences.

**Materials and Methods**

**Chemicals**

Calcium chloride dihydrate and disodium ethylenediaminetetraacetate (EDTA) were obtained from Mallinckrodt, Inc., St. Louis, MO 63134. Adenine, adenosine, adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), and guanosine 3',5'-monophosphate (cGMP) were from Sigma Chemical Co., St. Louis, MO 63178. 125I-labeled succinyl cAMP tyrosine methyl ester (tracer) and anti-cAMP antiserum were from Collaborative Research, Inc., Waltham, MA 02154. SQ20,009 (a CAMP phosphodiesterase inhibitor) was a generous gift of Squibb & Sons, Inc., Princeton, NJ 08540.

**Standards**

Cyclic AMP was obtained from Sigma (cat. no. A-4137, lot 39C-7010, free acid). Its purity was checked by liquid chromatography on a μBondapak C18 column with an ultraviolet detector (Waters Associates, Milford, MA 01757). Isocratic elution was as previously described (5). The concentration of the stock standard (0.5 mmol/L) was checked by spectrophotometry (6).

**Plasma Samples Used in Reference Interval Survey**

Venous blood was sampled between 0800 and 0930 hours from apparently healthy volunteers (both sexes, 18–65 years of age) who had been fasting since the previous evening. The blood specimens were collected into Vacutainer Tubes (B/D 4713) containing disodium EDTA (14 mg per tube) and placed in an ice-water bath without delay. Within 30 min of collection, the blood was centrifuged (20 min, 800 X g, 4 °C) and the plasma was divided and stored at -70 °C until analysis.

**Procedures**

**Acetylation.** Each sample was individually acetylated just before the assay, as follows. Pipet 50 μL of plasma into a disposable 12 X 75 mm glass tube, acidify with 50 μL of 0.2 mmol/L HCl, and acetylate with 20 μL of freshly prepared acetylation reagent (one volume of acetic anhydride plus 2.5 volumes of triethylamine). Immediately vortex-mix the contents of the tube for 3 s, keep the tube at room temperature for 3 min, and then place it into an ice-water bath (where it can be stored for up to 1 h). Adjust the volume to 1.0 mL with sodium acetate buffer (50 mmol/L, pH 6.2).

Prepare an aqueous 100 mmol/L cAMP standard from the stock standard, pipet 100 μL of this solution into a disposable glass tube, and acetylate it with 100 μL of freshly prepared acetylation reagent as described above. After vigorous mixing and incubation at room temperature for 3 min, adjust the volume to 2.0 mL with acetate buffer and place the mixture in an ice-water bath. The final cAMP concentration is 5 mmol/L. Prepare all other working standards from this acetylated stock.

**Radioimmunoassay.** Perform all procedures with reagents maintained at 2–4 °C in an ice-water bath. Run samples, standards, and controls (plasma containing SQ 20,009 and an appropriate amount of cAMP) in duplicate. Pipet each acetylated standard (0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 mmol/L), control (see Stability section below), and appropriately diluted sample (100 μL) into numbered tubes. Add 100 μL of tracer and vortex-mix the contents of the tubes. Add antiserum (100 μL, except to the nonspecific tubes, to which add 100 μL of acetate buffer). Add 100 μL of 75 mmol/L CaCl₂ in acetate buffer and sufficient acetate buffer to bring the final volume to 0.5 mL, and vortex-mix. Incubate the tubes at 2–4 °C for 18 to 21 h (short incubations at 37 °C were found to cause degradation of acetylated cAMP in the presence of plasma components), then add 0.5 mL of cold, 20 g/L Staphylococcus aureus cell suspension (IG Sorb; Enzyme Center, Inc., Boston, MA 02111), vortex-mix, incubate at 4 °C for 5 min, and centrifugate (2000 X g, 30 min at 4 °C). Decant the supernates and count the radioactivity in the pellets in a gamma counter.

Calculate the percentage of each standard (Std), control, and sample (Spl) that was bound, according to the following formula, using the average of the counts for each pair of duplicates:

\[ \% \text{B} / \text{B}_0 = \left( \frac{\text{counts(Std) or (Spl)} - \text{counts in nonspecific tubes}}{\text{counts in zero std tubes} - \text{counts in nonspecific tubes}} \right) \times 100 \]
Plot the standard points on logit-log graph paper with % B/B₀ on the ordinate and concentration on the abscissa. The plot should be linear. Read values for samples off this curve directly and multiply by 20 to obtain results in nanomoles per liter.

**Results**

**Interferences by CaCl₂ or EDTA in the Assay for Acetylated cAMP**

Table 1 shows the effect of various compounds on the previously described RIA for acetylated cAMP (1). Clearly, calcium chloride and EDTA interfere substantially with this assay, and certain divalent metal salts such as calcium acetate and magnesium chloride also interfere. A few millimoles of the interfering substances per liter of plasma interfere significantly.

**Mechanism of Interference**

Figure 1 shows the effect of calcium on the antibody affinity for the analyte. The affinity constant (Kₐ) of the antibody was calculated to be 1.68 x 10^10 L/mol and 0.89 x 10^10 L/mol in the presence and absence of calcium, respectively. Thus calcium increased by 89% the affinity of this antibody for the analyte. The affinity constant of the antibody in the presence of 1 mmol of EDTA per liter (0.71 x 10^10 L/mol) was 21% lower than obtained in the same run without EDTA (0.89 x 10^10 L/mol). Therefore, the extent of tracer binding will be lower with a sample containing the usually used amount of EDTA than a sample containing the same amount of cAMP but no EDTA. Thus the apparent cAMP value will be higher for the sample containing EDTA. The interference by calcium can be explained analogously.

**Lack of Interference by CaCl₂ and EDTA in the New Assay**

Searching for ways to eliminate these interferences that do not require prepurification of samples, we found that use of a buffer containing 15 mmol of CaCl₂ per liter appeared to obviate the interferences (Table 1).

**Analytical Variables**

**Reduction of antibody.** Several experiments were performed in which various antibody dilutions were tested in the absence and presence of calcium ions (15 mmol/L final concentration). Because the added calcium increases antibody affinity for cAMP, we reduced our antibody use by 65%.

**Detection limit.** Figure 2 illustrates a typical standard curve for this assay. The detection limit of this improved assay, defined as the point in the linear part of the standard curve where B/B₀ = 90%, was 12 fmol/assay tube, slightly better than the previously described assay (1).

**Specificity.** Cross reactivity of the antibody with cGMP or any adenine nucleotides, nucleoside, and base was negligible (0.005% or less). Furthermore, if plasma samples were left at 37 °C for 3 hr or, if phosphodiesterase (an enzyme that catalyzes the breakdown of cAMP) was added and incubated for 1 hr to destroy endogenous cAMP, no cAMP could be measured.

**Analytical recovery.** On addition and re-assay of cAMP standards to plasma specimens that had been previously assayed, the analytical recovery was found to range from 84% to 114% (average, 96%).

**Parallelism.** When plasma samples were serially diluted and each dilution was assayed, the resulting data gave curves that paralleled the standard curve (Figure 2).

**Precision.** Three cAMP controls were analyzed during an
eight-week period (13 independent determinations). The between-run CV was <9% and within-run CV <5% over the entire reference range.

Stability. Plasma specimens were analyzed at the time of collection (day 0) and again 10 days later after storage at −70 °C. The results indicate that specimens are stable for at least 10 days at −70 °C (Figure 3). Controls prepared with a phosphodiesterase inhibitor (“SQ20,009,” 500 μmol/L), to minimize breakdown of cAMP by phosphodiesterases, were found to be stable for at least eight weeks at −70 °C.

Reference Interval
cAMP concentrations measured in plasma of apparently healthy men (n = 31, 22–65 years of age) and women (n = 32, 18–54 years of age) showed the respective reference intervals to be 14–26 and 13–23 nmol/L. No age-related effect was discernible.

Discussion
EDTA, often used as an anticoagulant for plasma speci-
mens, actually is the preferred anticoagulant in this instance, because it inhibits phosphodiesterase and thus slows down the destruction of cAMP in plasma (1, 2). A plasma specimen from a partly filled Vacutainer Tube containing EDTA may contain twice the concentration of EDTA as other specimens.

Although the calcium concentration in plasma is fairly constant among normal individuals, it can be substantially greater or less in certain disease states.

Therefore, the interferences by calcium or EDTA that we observed cannot be negated by simply preparing cAMP standards in pooled human plasma. Interferences by both calcium and EDTA must be eliminated for plasma cAMP to be reliably measured. Indeed, such a method is a prerequisite if one wishes to measure nephrogenous cAMP for assessing parathyroid function as Brodus et al. (7) suggested. One way to improve the previously described RIA for acetylated cAMP (1) is to pre-purify samples before assay. However, the alternative we have described here is much simpler: it not only minimizes the calcium and EDTA interferences but also increases the affinity of the antibody for the analyte.

References
5. Krstulovic, A. M., Hartwick, R. A., and Brown, P. R., Reversed-phase liquid chromatographic separation of 3',5'-cyclic ribonucleo-