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Note

High-performance liquid chromatographic determination of 3′,5′-cyclic adenosine monophosphate in human platelets

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The measurement of 3′,5′-cyclic adenosine monophosphate (cAMP) in biological preparations has traditionally been accomplished by use of a radioactive competitive binding assay or radioimmunoassay [1]. The disadvantages of these assays involve sample preparation time and the use of radioactivity. Reversed-phase high-performance liquid chromatographic (HPLC) separation and measurement of cAMP by UV detection [2] resulted in a rapid and sensitive method of measuring physiologically relevant amounts of cAMP. Further refinements in the sensitivity have been achieved by use of fluorimetric detection. A fluorimetric method has been reported which involves derivatization of cAMP to a highly fluorescent compound, etheno-cAMP [3]. This method is comparable in sensitivity and specificity to the radioactive methods with the added advantage of simple sample preparation and has been validated in a number of animal tissues [4].

The following is a description of a sensitive, selective and reproducible method for the quantitative analysis of cAMP in human platelets. An application of this method is reported, quantifying cAMP produced as a result of prostaglandin E_1 (PGE_1) stimulation of adenylate cyclase in a platelet preparation. This method may also be applicable to other biological extracts.

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EXPERIMENTAL

Apparatus and chromatographic conditions

The HPLC system consisted of an ESA Model 5700 solvent delivery module (Bedford, MA, U.S.A.) with a Rheodyne (Bedford, MA, U.S.A.) 10-μl injection loop. The separation system was a 100 mm$\times$4.6 mm I.D. stainless-steel (3 μm) C_{18} (Rainin, Woburn, MA, U.S.A.) Microsorb column with a 30 mm$\times$4.6 mm I.D. stainless-steel (3 μm) C_{18} guard column and 0.2-μm in-line solvent filter. The detector was an Applied Biosystems (Ramsay, NJ, U.S.A.) 980 programmable fluorescence detector with a 5-μl flow cell utilizing an excitation wavelength of 238 nm and secondary filter with an emission cut-off of 370 nm. Photomultiplier voltage was adjusted to -1030 V with an attenuation of 0.1.

All solvents used for the preparation of the mobile phase were filtered (0.22 μm; Millipore, Bedford, MA, U.S.A.) and degassed under a vacuum. The mobile phase was composed of 0.050 *M* ammonium acetate aqueous buffer titrated to pH 4.75–acetonitrile–methanol (250:8:5). The flow-rate was 1.6 ml/min.

A Perkin-Elmer (Norwalk, CT. U.S.A.) LS3B spectrofluorimeter was employed in obtaining the fluorescence emission spectra of the internal standard and cAMP derivative.

Reagents

Most reagents were HPLC grade and purchased form Sigma (St. Louis, MO, U.S.A.). The phosphodiesterase, 3′,5′-cyclic nucleotide, was prepared from bovine heart and had an activity of 0.20 U/mg. The 5,7-dimethyl-1,2,4-triazolo(1,5-α)pyrimidine (DMTP) and chloroacetaldehyde diethyl acetal were purchased from Aldrich (Milwaukee, WI, U.S.A.).

Hank's balanced salt solution (HBSS) [5] and calcium deficient Eagle's medium [6] were prepared and modified to include 5 m*M* N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid (HEPES) and titrated to pH 7.42.

The adenylate cyclase stimulation experiment used PGE_1 (100 μM) solution in 10 ml of HBSS serially diluted in HBSS to the appropriate concentrations.

Reference standards

Standard solutions of DMTP (200 μM) and cAMP (4.0 m*M*) were prepared in distilled water and frozen in aliquots at -4°C. These were then serially diluted with HBSS at the time of each experiment.

Preparation of samples

Platelets were supplied by the Rhode Island Blood Center (Providence, RI, U.S.A.). These platelets were stored in acid–citrate–dextrose (ACD) [7] at room temperature and were used within one day of the expiration date. Each bag of platelet-rich plasma contained a total of $1\cdot10^{10}$–$5\cdot10^{10}$ platelets sus-

pended in approximately 30 ml of ACD. Calcium-deficient Eagle's medium was added to a final volume of 45 ml and the platelet suspension was spun on a table top centrifuge at 200 g to precipitate erythrocytes. The platelet-rich supernatant was spun at 800 *g* for 10 min at 4°C and the platelet-rich pellet resuspended in 45 ml of Eagle's medium. This platelet suspension was spun at 800 *g* for 10 min at 4°C and the platelet-rich pellet was resuspended in an amount of HBSS calculated to result in a final platelet concentration of approximately $1 \cdot 10^{10}$ platelets per ml. An aliquot of platelet suspension was counted using a hemocytometer. Clumping of platelets was less than 1% of total counted, and erythrocyte contamination was on average 3 cells per high-power field of radius 1.5 mm and depth of 0.1 mm at a magnification of 100×. This suspension was stored at room temperature and used for all subsequent experiments within 60 min of preparation.

For preparation of the standard curve in platelet suspension, a 100-μl aliquot of cell suspension containing approximately $1 \cdot 10^9$ platelets was added to each of fifteen 1.5-ml polyethylene microcentrifuge tubes and immersed in boiling water for 2 min. To each set of three tubes in the series, known amounts of cAMP and HBSS were added corresponding to 0, 8, 16, 32, and 40 p*M*. The final volume prior to addition of the internal standard was adjusted to 0.2 ml with HBSS. Subsequently, 10 μl of DMTP internal standard (200 μM) were added to each tube. The volume prior to deproteinization and addition of derivatization reagents was 0.21 ml.

Derivatization procedure

A 75-μl volume each of 0.25 *M* zinc sulfate and 0.25 *M* barium hydroxide was added to each tube. All tubes were spun on a table top microcentrifuge at 14 000 *g* for 4 min. The resulting supernatant was added to a set of 1.5-ml microcentrifuge tubes, and 20 μl of 1 *M* sodium acetate buffer pH 4.5 and 10 μl of chloroacetaldehyde diethyl acetal were added to each tube. The tubes were capped and immersed in boiling water for 15 min. A 10-μl volume of the resulting supernatant was injected directly into the column.

RESULTS

Evaluation of the method

The maximum total fluorescence of etheno-cAMP was observed at an excitation wavelength of 238 nm through the 370-nm emission cut-off filter used in our system. The fluorescence of 200 μM DMTP in the same system in mobile phase showed two fluorescence peaks at 364 and 470 nm. Neither the peak height nor the fluorescence emission spectrum of the internal standard was affected by the derivatization procedure. When samples of derivatized cAMP in HBSS were stored at room temperature and exposed to ambient fluorescent lighting in 1.5-ml polypropylene microtubes, no change in peak height oc-

curred for up to 24 h. This suggests that the derivative is stable under those conditions. Under the described chromatographic conditions, etheno-cAMP and the internal standard, DMTP, gave well resolved chromatographic peaks (Fig. 1). The identity of the etheno derivative peak was determined by comparing the retention time of the experimental peak with the retention time of an injected standard of the etheno-cAMP. The retention time was identical. The identity of the peak was supported by comparing the chromatograms of two identical samples of the platelet preparation, one of which was treated with phosphodiesterase. The peak in question disappeared in the preparation treated with the enzyme. No underlying peaks were unmasked (Fig. 2). The percentage recovery of this method was determined as follows. A standard curve was generated as outlined above. A second curve was generated by substituting the

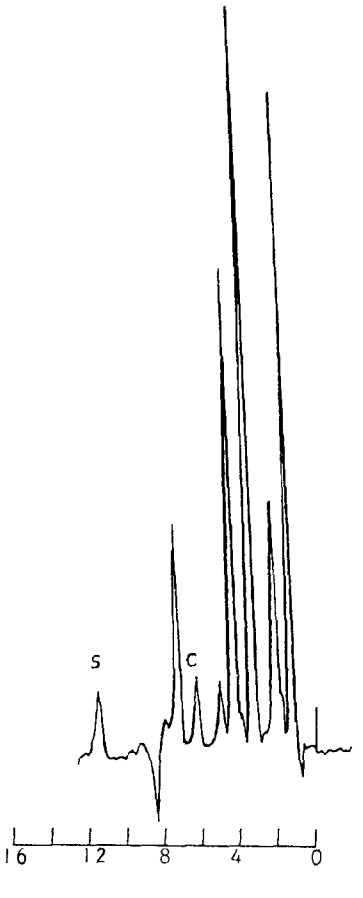

Fig. 1. HPLC profile of 3′,5′-cyclic adenosine monophosphate and the internal standard (DMTP). A 10-μl volume of derivatized supernatant was injected. Peaks: C = cAMP; S = internal standard (DMTP).

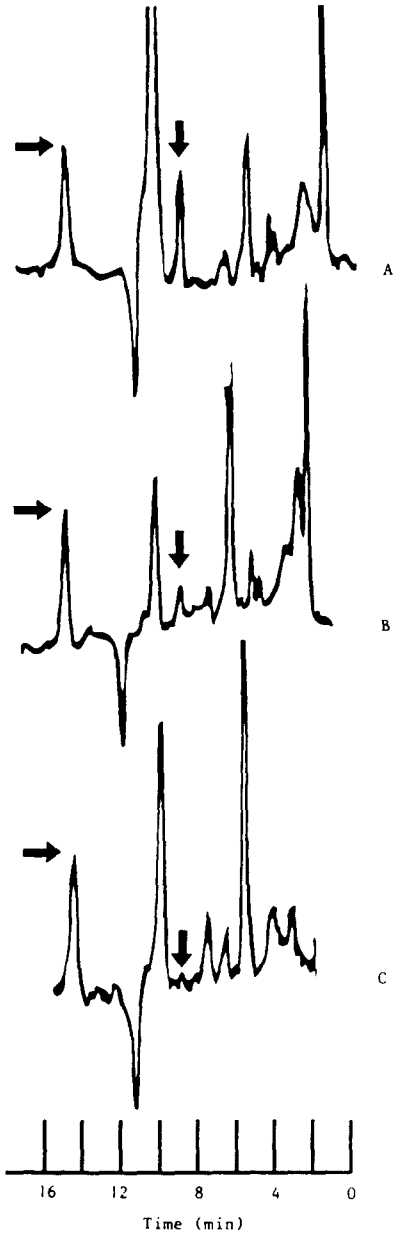

Fig. 2. (A) Chromatogram of a platelet preparation (0.2 ml spiked with 16 p*M* cAMP); volume injected: 10 μl. (B) Chromatogram of a plain platelet preparation showing baseline cAMP content. (C) Chromatogram of a platelet preparation spiked with 16 p*M* cAMP and treated with phosphodiesterase. The vertical arrows represent the cAMP peak and horizontal arrows the internal standard (DMTP). Each vertical line on the scale represents 2 min.

etheno derivative for cAMP. This was added to the tubes after boiling but prior to the deproteinization step. Otherwise, the procedure followed was the same. The peak-height ratios at equal concentrations of cAMP and etheno derivative were compared at each concentration point. This gave a calculated recovery of $54.2 \pm 2.7\%$ at 40, 32, 16, 8 and 0 p*M* added cAMP.

The method has a sensitivity of 0.1 p*M* etheno-cAMP injected. This allows measurement of the amount of basal cAMP contained in 10^8 platelets. This compares with a sensitivity of 0.1 p*M* for regular radioimmunoassay and 3 f*M* for acetylated radioimmunoassay [8]. The method is linear from 0.1 to 1000 p*M* injected. The intercept of the plot of the cAMP concentration versus the peak-height ratio corresponds to a basal level of cAMP in platelets of 44.2 p*M* per $1 \cdot 10^9$ cells. The intercept is not zero because of the basal amount of cAMP contained in the platelet preparation. This value is in good agreement with a level 33.3 p*M* per 10^9 platelets reported using acetylated radioimmunoassay methodology [9]. This intercept occurs at a concentration of cAMP within the sensitivity limits of the method. Between-day variation of the slope of the standard curve ($n=4$) was 0.0245 ± 0.0019 (coefficient of variation = 7.76%). For the linear regressions, r^2 was always > 0.99. Within-day coefficients of variation for added cAMP concentrations were: 0 *M*, 7.6% ($n=3$); 40 n*M*, 7.9% ($n=3$); 80 n*M*, 7.4% ($n=3$); 0.16 μ*M*, 9.6% ($n=3$); 0.20 μ*M*, 9.5% ($n=3$).

Analysis of cAMP in a platelet preparation

The following is a description of the procedure used for the determination of a dose–response curve for PGE_1-mediated adenylate cyclase activation. For each set of three 1.5-ml microtubes, PGE_1 in HBSS in the following concentrations was added: 0, 0.01, 0.10, 1.00, 10 and 100 μ*M*. A 100-μl aliquot containing approximately $1 \cdot 10^9$ platelets was added to each tube to start the reaction. The final reaction volume was 0.20 ml. Each set of tubes containing different concentrations of PGE_1 was incubated in a water bath at 37 °C for 10

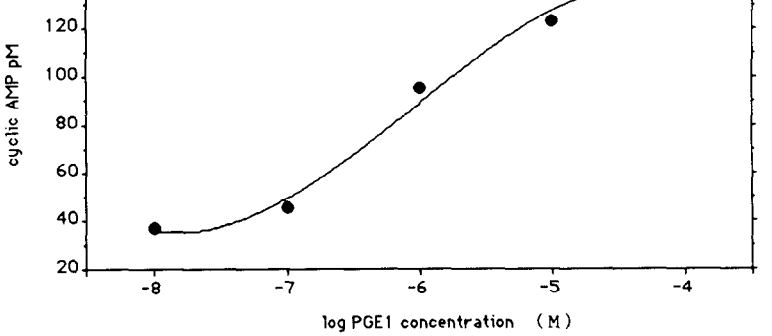

Fig. 3. Plot of the total amount of cAMP present in a volume of 0.2 ml containing approximately $1 \cdot 10^9$ platelets after 10 min of incubation at 37 °C with varying concentrations of PGE_1.

min. A control set of three tubes which did not contain any PGE_1 was immersed in boiling water for 2 min immediately after addition of the cell suspension. The reaction was terminated by immersing the rest of the tubes in boiling water for 2 min and the samples were derivatized as described. The results of the experiment are shown in Fig. 3. The maximal amount of cAMP generated at 10 min at a PGE_1 concentration of 1.00 μM corresponds to about 95 pM per 10^9 cells which is in agreement with the results of other workers using a radioimmunoassay method [10].

CONCLUSION

This method offers a reliable way of measuring cAMP in a platelet preparation. It does not require a platelet sample preparation step, as is needed in methods which rely on the conversion of [^{3}H]adenine to [^{3}H]cAMP [11]. The sensitivity of the method offers the capability of measuring basal platelet cAMP without the use of phosphodiesterase inhibitors. This eliminates the possibility of the interaction of high intracellular levels of cAMP with protein kinases, which may be important in the regulation of hormone receptor activity [12]. Also, because of the specificity of the method, there is no need for post-reaction sample preparation other than a simple deproteinization procedure. This makes this method less technically demanding than methods using a radioactive saturation assay technique [13].

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