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Short Communication

# High-performance liquid chromatographic determination of cyclic 3′,5′-AMP with fluorescence detection Vasoactive intestinal peptide-induced modification of its concentration in neuroblastoma cells

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**Abstract**

The efficiency of ion-pair reversed-phase HPLC on a Vydac $C_{18}$ column with 50 m*M* ammonium acetate (pH 4.75)–methanol–acetonitrile (88:9:3, v/v/v) as the mobile phase with isocratic separation and fluorescence detection for the determination of cAMP in cellular extracts was evaluated. This method was compared with a radioimmunoassay technique in terms of linearity, reproducibility and sensitivity. No interactions with other nucleotides such as AMP, ADP, ATP and cGMP were observed. Application to the measurement of cAMP modifications was studied in a neuroblastoma cell line: LA-N-2 cells stimulated by a neuropeptide, vasoactive intestinal peptide.

## 1. Introduction

Biological events induced by neurotransmitters often involve stimulation of cyclic AMP (cAMP) production following receptor interaction. In order to study in detail this binding to cell receptor and to design specific antagonistic or agonistic compounds, the evolution of this second messenger has to be investigated.

Vasoactive intestinal peptide (VIP), a 28 amino acid peptide originally isolated from porcine small intestine [1], has been implicated as the nonadrenergic noncholinergic inhibitory neurotransmitter in various tissues. VIP acts through specific binding sites often coupled to adenylate cyclase leading to the activation of a cAMP-dependent protein kinase [2]. Its action on human neuroblastoma cells seems to induce morphological differentiation *in vitro* [3] and to reverse neuroblastoma tumours *in vivo* [4]. This response was found to be related to cAMP production and moreover to potentiate the retinoic acid effect in the differentiation of HL60 and SK-N-SH cells [5].

We describe here an efficient and adaptable method for the rapid and reliable measurement

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of VIP-stimulated cAMP production which could be applied to other cells models.

## 2. Experimental

### 2.1. Chromatographic conditions

A Model 5000 liquid chromatograph (Varian, Orsay, France) equipped with a Jasco FP-210 spectrofluorimeter (excitation at 265 nm, emission at 410 nm) was used throughout. Analyses were performed on a Vydac $C_{18}$ column (200 × 4.6 mm I.D., 300 Å) with a precolumn (20 × 4 mm I.D.) that contained the same stationary phase. The mobile phase was 50 m*M* ammonium acetate (pH 4.75)–methanol–acetonitrile (88:9:3, v/v/v) at a flow-rate of 1.0 ml/min and a pressure of 17 MPa.

### 2.2. Reagents and chemicals

cAMP was purchased from Sigma (Saint-Quentin-Fallavier, France), human VIP from Interchim (Montluçon, France), adenosine deaminase, chloroacetaldehyde and isobutylmethylxanthine (IBMX) from Fluka (Saint-Quentin-Fallavier, France) and culture media from Gibco (Cergy-Pontoise, France). Methanol and acetonitrile were of HPLC grade. Distilled water was purified by passing it through a reverse-osmosis four-filter system (Millipore, Saint-Quentin, France). All other reagents were of the highest available grade and purchased from standard sources.

### 2.3. Sample preparation

*Cell cultures*

The human neuroblastoma cell line LA-N-2 was cultured in Leibovitz L-15 medium supplemented with 10% foetal calf serum, 2 m*M* L-glutamine, 100 UI/ml penicillin and 100 μg/ml streptomycin. Cell cultures were checked monthly for mycoplasma infection using a Mycotect kit (Gibco). Cell protein concentrations were measured by the bicinchoninic acid method (Pierce, Rockford, USA) using bovine serum albumin as a standard.

*Isolation of cAMP from cells*

LA-N-2 cells were seeded in six-well (35 mm) plates at a density of $4.0 \cdot 10^5$ cells per well and cultured without antibiotics until they reached confluence. Cells were rinsed with serum-free medium containing 1% bovine serum albumin and first incubated for 10 min in this medium supplemented with 1 m*M* IBMX. The cells were then exposed to VIP and incubations were terminated by aspiration of the medium and by two washes with ice-cold phosphate-buffered saline. A 1-ml volume of propanol was immediately added to extract cAMP. After a 20-min extraction with propanol at 4°C, the supernatant was evaporated (Speed-Vac concentrator) and the dry residue was resuspended in 20 μl of water. Cell debris was dissolved by addition of 2.0 *M* NaOH for protein determination.

*Derivatization procedure*

Adenosine deaminase (1.0 μl, 1.6 units) was added to the reaction tube and the mixture was vortex mixed, after 10 min of incubation at 37°C, with 7.5 μl of 0.25 *M* $ZnSO_4$ and 7.5 μl of 0.25 *M* $Ba(OH)_2$. The supernatant after centrifugation (12 000 *g* for 4 min) was transferred into a microcentrifuge tube and boiled with 2 μl of 1 *M* sodium acetate (pH 5.0) and 5 μl of chloroacetaldehyde for 20 min (2 × 10 min with intermediate centrifugation). Finally, the reaction mixture was injected into the liquid chromatograph.

## 3. Results and discussion

Fig. 1a shows a typical chromatogram of a derivatized aqueous standard solution containing 1 ng of cAMP. The retention time was 6 min 50 s. The detection limit, defined as the lowest concentration resulting in a signal-to-noise ratio of 3, was 0.2 ng per injection. Interferences from endogenous material were investigated. AMP, ADP, ATP and cGMP were each injected at a concentration of 40 ng/ml and none showed a

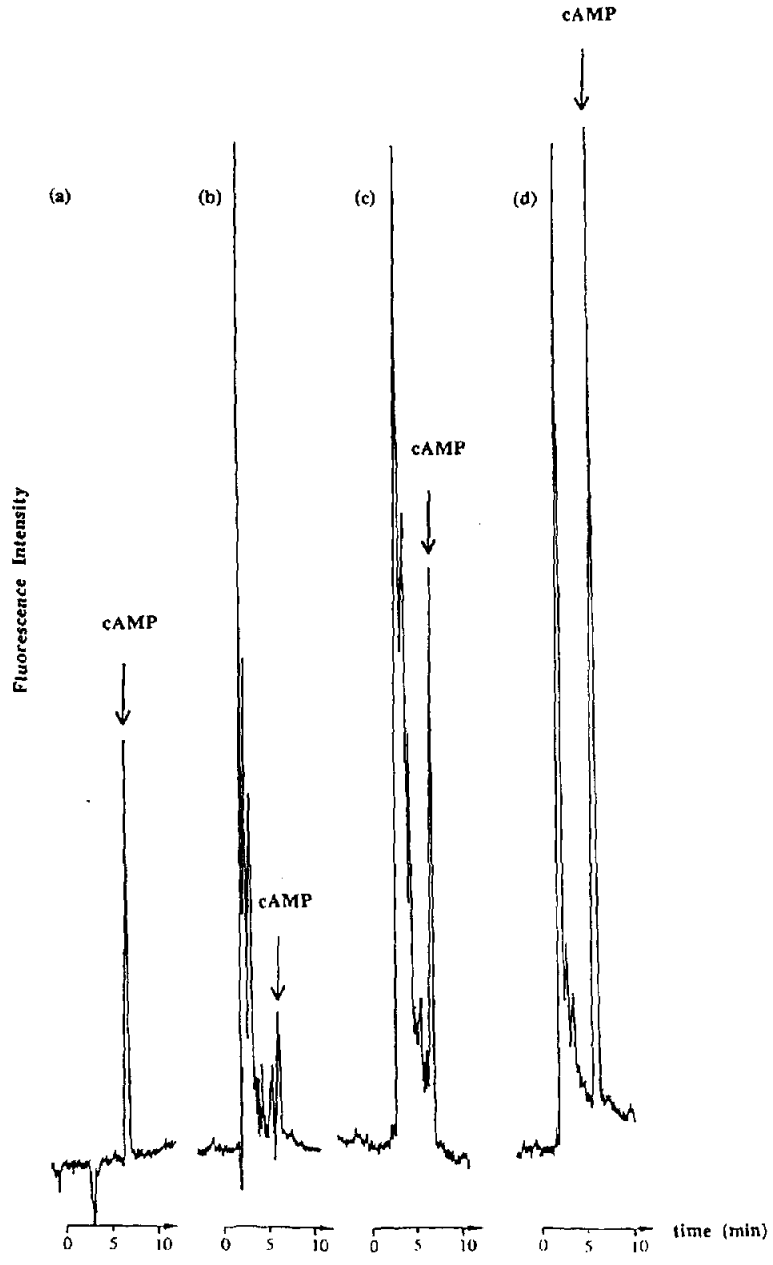


Fig. 1. Chromatogram for a 100-$\mu$l injection of (a) standard compound of cAMP (1 ng) or of a cytosol extract (b) of LA-N-2 cells corresponding to 44 mg of protein, (c) of LA-N-2 cells spiked with 1 ng of cAMP and (d) of LA-N-2 cells spiked with 2 ng of cAMP.

retention time identical with that of cAMP. The calibration graphs showed good linearity between peak area ($y$) and concentration ($x$) from 2 to 160 ng/ml. The equation of the line was $y = -10.01 + 2.31x$ with $r = 0.998$. These results show that the HPLC method has a sensitivity comparable to that of the radioimmunoassay technique ([$^3$H]cAMP assay system; Amersham,

Les Ulis, France), for which the linear range of the graph is from 1.5 to 100 ng/ml.

When a cell extract was injected into the HPLC system, a peak at the same retention time was observed (Fig. 1b), the identity of which was confirmed by adding known amounts of cAMP to a propanol extract (Fig. 1c and d). All the samples tested had levels of cAMP within the HPLC assay linearity range. The intra-assay precision was determined at two concentration levels, 10 and 20 ng/ml ($n = 10$) and the corresponding coefficients of variation (C.V.s) were 7.12 and 6.05%, respectively. For the inter-assay precision ($n = 6$) the C.V.s were 7.43 and 6.82% for the same concentrations. Hence the variations in the cAMP determination results were small.

In LA-N-2 cells, the intracellular level of cAMP was found to be $6.82 \pm 0.51$ ng/mg of protein ($n = 5$). The VIP-induced cAMP concentration increased, in a dose-dependent manner (Fig. 2), up to $57.83 \pm 5.00$ ng/mg of protein on 10-min incubation with the peptide ($10^{-6}$ $M$). To test whether the synthesis of cAMP is dependent on the incubation time, the intracellular content of cAMP was monitored after 1, 2.5, 5, 10, 15 and 20 min (Fig. 3). The amount of cAMP increased up to 10 min and decreased with longer incubation times. Stability experiments performed in frozen supernatants showed no statistically significant differences in the content of cAMP.

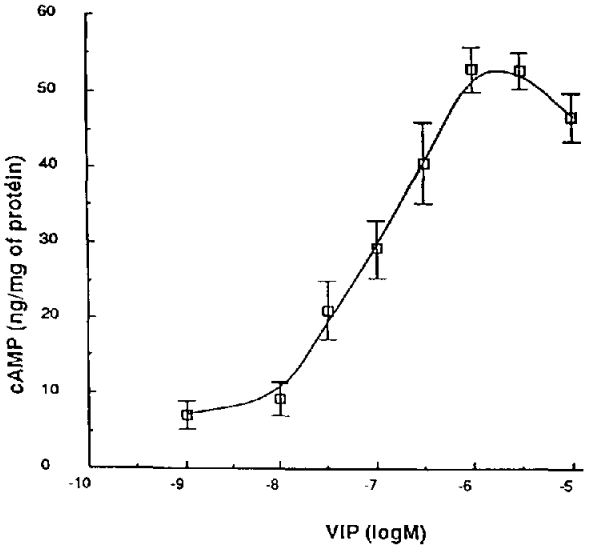


Fig. 2. Dose-dependent effect of VIP on cAMP accumulation in cultured LA-N-2 cells (incubation time 10 min). Individual points are means of three measurements.

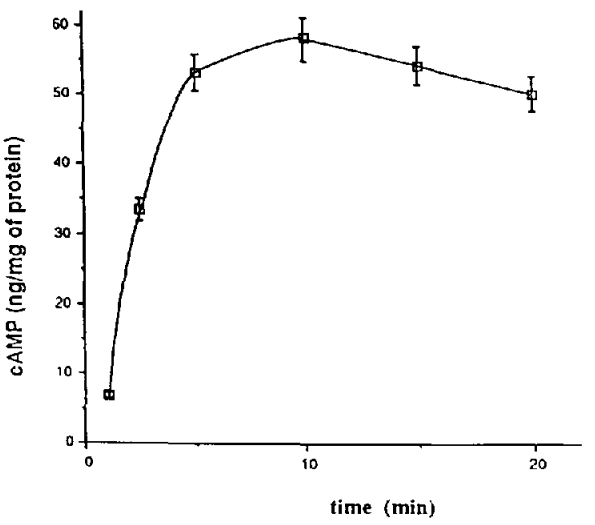


Fig. 3. Time course of cAMP accumulation in LA-N-2 cells in response to VIP ($10^{-6}$ $M$). Individual points are means of four measurements.

The VIP-related cAMP increase was evaluated simultaneously by the HPLC and RIA methods. Fig. 4 shows reliable results with closely related

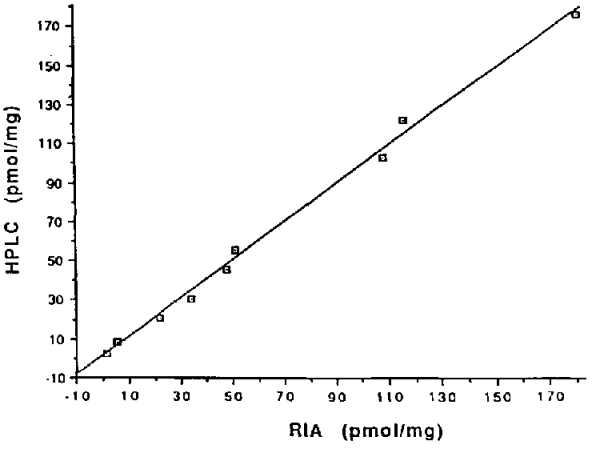


Fig. 4. Correlation between HPLC and RIA determination of cAMP in VIP-treated LA-N-2 cells: $y = 0.92854 + 0.97715x$ ($r^2 = 0.996$).

percentage recoveries between the two determinations.

Methods for determining endogenous levels of cAMP or adenylate cyclase activity have been described by different workers. These procedures used either radiolabelled compounds ([$^{125}$I]cAMP, [$^{3}$H]hypoxanthine, [$\alpha$-$^{32}$P]ATP) as tracers [6–8], or other methods which required enzymatic assay or chromatographic analysis. Radioactive materials are currently used in many laboratories, but although sensitive, this approach is relatively costly and time consuming. An enzymatic assay for adenylate cyclase activity has recently been described using membrane preparations from rabbit hearts with high sensitivity [9]. Other methods include HPLC procedures with UV detection [10–12] which are not competitive in terms of sensitivity with respect to radioactive methods.

Because small amounts of cAMP exist in established cell lines a low detection limit is required. The fluorescence method developed here can be used with whole cells and combines good reproducibility, sensitivity and selectivity. Further, this method is relatively simple and rapid; these features make it very suitable for pharmacological and toxicological studies of all the agents that might affect the synthesis and metabolism of cAMP in several cell types.

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