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Simultaneous quantification of GMP, AMP, cyclic GMP and cyclic AMP by liquid chromatography coupled to tandem mass spectrometry

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Abstract

Phosphodiesterases are drug targets for treating various diseases. Inhibition of these can increase cAMP and cGMP levels, which can affect a variety of physiological responses. Here we report a new method for determining PDE activity by combining high-performance liquid chromatography and tandem mass spectrometry. Characteristic peaks of the substrates, cGMP or cAMP and products, GMP or AMP, were identified in positive-ion electrospray ionization using multiple reaction monitoring. The method can be applied to determine activity of PDE inhibitors. Our results showed that this new method was fast, sensitive and highly reproducible.

Keywords: Phosphodiesterases; Mass spectrometry; Sildenafil; cGMP; cAMP

1. Introduction

Cyclic nucleotides (cGMP and cAMP) are intracellular second messengers playing a key role in many physiological processes, including metabolism, contractility, motility, and transcription in virtually all cell types [1]. Phosphodiesterase (PDE) catalyzes the hydrolysis of cyclic nucleotides to inactive 5′-nucleotides (GMP and AMP) [2]. At present, mammalian PDE enzymes are classified into 11 distinct enzyme families differing by amino acid sequence, overall domain structure, and catalytic and regulatory considerations [3].

Phosphodiesterases are drug targets for the treatment of various diseases, including heart failure, retinal degeneration, depression, asthma, inflammation and erectile dysfunction [4]. In order to screen the activity of candidate compounds, simple and sensitive methods for the measurement of PDE activity are required to better estimate the blocking effect of the inhibitors.

In recent years, two major approaches for determining PDE activity have been developed. The method of Thompson and Appleman [5] has been widely used for more than three decades, using tritium-labeled cAMP or cGMP as the substrate, while the PDE reaction is coupled to the 5′-nucleotidase activity of snake venom. Recently, the throughput of this analysis has improved by the use of 96-well plates [6].

The other approach uses selective precipitation, where either [^{3}H]cAMP in the supernatant or precipitated [^{3}H]AMP is measured [7]. The PDE assay has been adapted to the scintillation proximity assay (SPA) format, representing one of the most widely used contemporary methods [8,9].

The described methods for the quantitative analysis of these cyclic nucleotides require special radioactive substrates such as [8-^{3}H]-cGMP [10], fluorogenic substrates such as 2′-*O*-anthraniloyl-cGMP and 2′-*O*-methylathraniloyl-cGMP [11] or fluorescence derivatization reagent, such as (3,4-dimethoxyphenyl)glyoxal (DMPG) [12]. Although sensitive, both methods require time consuming analysis and allow for determining the phosphodiesterase activity of only one compound at time.

Mass spectrometric detection of intact polar compounds has now become possible as the result of the development of soft ionization techniques. Ionization methods commonly used such as fast atom bombardment (FAB) [13,14] matrix-assisted laser desorption ionization (MALDI) [15] and electrospray ionization (ESI) [16]. In the field of nucleotide chemistry, static fast atom bombardment mass spectrometry (FAB-MS) was a monumental step forward, and its potency for the identification of cyclic

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nucleotides was demonstrated by Newton et al. [13,17,18]. Witters et al. [19] showed that cyclic nucleotides (among cAMP and cGMP), can be identified using specific collision-activated dissociation (CAD) fragments as diagnostic ions.

Here we report a new method for determination of PDE activity by combined high-performance liquid chromatography (HPLC) to tandem mass spectrometry (MS) with positive-ion electrospray ionization using multiple reaction monitoring (MRM). The following nucleoside cyclic monophosphates were analyzed for PDE activity: cyclic guanosine monophosphate (cGMP), guanosine monophosphate (GMP), cyclic adenosine monophosphate (cAMP) and adenosine monophosphate (cAMP).

2. Experimental

2.1. Chemicals and solvents

The nucleotides cGMP, cAMP, guanosine 5′-monophosphate (GMP) and adenosine 5′-monophosphate (AMP) were purchased from Sigma Chemical Co. (St. Louis, USA). The compound 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pyrazolo [4,3-*d*]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine (sildenafil) was synthesized by Laboratorios Cristalia (Itapira, SP, Brazil). Acetonitrile (HPLC grade) were obtained from Mallinckrodt (Mallinckrodt Chemicals, USA), formic acid, analytical grade, was purchased from Merck (Rio de Janeiro, Brazil). Water was purified, using the Milli-Q or Elga UHQ systems, prior to use. DMSO was purchased from Sigma (St. Louis, MO, USA). All other reagents used were of commercially available grade.

2.2. Preparation of human platelet PDE

Human platelet PDE sample were prepared according to the method of Radomski et al. [20]. Samples of blood from healthy donors were collected in 0.1 vol of 3.13% sodium citrate and centrifuged 200 × *g* for 15 min at 10 °C. Platelet-rich plasma (PRP) was collected, and 10 mM EDTA was added to avoid platelet activation during washing, and PRP was then centrifuged at 900 × *g* for 15 min at 10 °C. The platelet pellets were washed in Krebs buffer (118 mM NaCl, 25 mM $NaHCO_3$, 5.6 mM glucose, 4.7 mM KCl, 1.2 mM KH_2PO_4 and 1.17 mM $MgSO_4{\cdot}7H_2O$, pH 7.4) containing 10 mM EDTA. Plateletes were resuspended in Krebs buffer with 0.32 M sucrose to a concentration of 1×10^9 cells/ml. Cell extract was prepared by sonication for 10 s, threefold at 15 kHz. The protein concentration was determined by Protein Assay kit (BioRad) using bovine serum albumin as standard. Platelet extract was used as a source of crude phosphodiesterases and was stored frozen at −80 °C until used.

2.3. Determination of PDE activity

The standard enzymatic reaction mixture (total volume of 200 μl) contained 50 mM Tris–HCl (pH 8.0), 100 mM $MgCl_2$ and platelet extract (final protein concentration 0.075 mg/ml). Reaction was initiated by addition of the substrate cGMP or cAMP (20 μM) and incubated at 35 °C for 30 min. The reaction was stopped by putting the sample in boiling water for 2 min. The protein precipitate was removed by centrifugation, and supernatant was stored at −20 °C until analysis. Phosphodiesterase activity was determined as a percentage of the peak area of GMP or AMP with respect to a DMSO control (where the control without any inhibitor present indicates maximal GMP or AMP production under the assay conditions).

2.4. Effects of PDE inhibitor

A solution of sildenafil dissolved in DMSO was prepared at increasing concentration (0.005–1.0 μM) and added to the reaction mixture at room temperature 5 min before addition of cGMP or cAMP. Samples of blood from four healthy donors were prepared in five concentration of sildenafil all assays were completed at least in triplicate totalizing 60 samples analyzed. Data is presented as the mean ± S.E. The IC50 is defined as the concentration of inhibitor where 50% of the GMP or AMP production has been inhibited as compared to the DMSO control.

2.5. LC–MS/MS analysis

The LC–MS/MS system consisted of LC ADVp Liquid Chromatograph Shimadzu System (Shimadzu Corporation, Japan) coupled to a PE Sciex API 4000 triple quadrupole mass spectrometer (PE Sciex, Toronto, Canada) operating in electrospray positive-ionization mode. Samples were injected into the system by means of a CTC HTS PAL autoinjector.

The chromatography separation was performed at room temperature using a C18 (100 mm × 2.1 mm, 4 μm) Jones® connected to a C18 (1 cm × 4 mm, 4 μm) Jones® pre-column. The mobile phase was: A (water containing 10 mM formic acid) and B (acetonitrile containing 10 mM formic acid). The gradient elution started with an isocratic step elution (100% eluent A) for 2 min followed by a 5 min gradient to 60% eluent B at a flow rate of 0.35 ml/min.

The fractionated compounds were introduced directly in the electrospray source, ionized and monitored by multiple reaction monitoring (MRM). The source block temperature was set to 500 °C and the electrospray capillary voltage to 4.5 kV. The dwell time for each fragmentation pathway was 200 ms. Nitrogen was used as collision gas. Pressure of the collision gas (CAD) was 49.0 kPa. The injection volume was 40 μl of each sample, previously diluted 500-fold with water. The total run-time was 10 min.

2.6. Calibration curve and limit of quantification

A calibration curve was prepared with blank samples, same conditions at the enzyme reaction with time reaction zero min, which were fortified with a standard solution mixture (cGMP, cAMP, GMP and AMP at 100 ng/ml), to analyte final concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 and 15.0 ng/ml, prepared in duplicate.

For determining the limit of quantification, eight blank samples of enzymatic reaction buffer were fortified with a standard solution mixture (cGMP, cAMP, GMP and AMP at 100 ng/ml) to a final concentration of 0.5 ng/ml. Another eight blank samples were fortified to a final concentration of 0.25 ng/ml (total of 16 samples).

3. Results and discussion

Cyclic nucleotides with a significant regulatory role are characterized by the presence of a 3′,5′-phosphodiester bond in the β-D-ribofuranosyl moiety. Some of these cyclic nucleotides have weak basic characteristics in their heterocyclic moiety (purine- or pyrimidine ring). Since they can behave as zwitterions, both positive- and negative-ES ionization could potentially be used for their analysis. Inherent to their structure, the separation of nucleotides is usually performed by anion-exchange chromatography, though the mobile phase compositions are highly incompatible with LC–MS. Consistent with this study, the nucleotides already had been analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC), with or without an ion-pairing reagent [19,21]. In this study, ion-pairing reagents were not used because of their substantial background contribution and source pollution [22,23]; however, the nucleotides were eluted far from the column void volume although there was no difference in the retention times between nucleotides.

A recently reported method HPLC–MS [24,25] analyzed only a nucleotide or cyclic nucleotide. This method has more advantages than other methods because of simultaneous analysis of a mixture of nucleotide and cyclic nucleotide (GMP, AMP, cGMP and cAMP) regulated by PDE activity by HPLC–MS/MS.

Negative-ion mode of ESI–MS seems to be a logical starting point for nucleotide analysis because of the presence of one or more negatively charged phosphate groups in the molecules [19]. However other methods using positive-ion ESI-MS have shown their fast, specific, and sensitive advantages. Claire [26] reported the detection of a unique and abundant product ion from a nucleotide analog by LC–MS/MS with positive-ion mode. Cai [27] reported the selection of the most abundant MS/MS quantitative ions of six ribo- and ribodeoxynucleotides by using ion-pairing LC coupled with positive-ion ESI–MS/MS. Fung et al. [28] discussed the advantage of positive-ion mode compared to negative-ion mode for simultaneous determination of nucleoside drug analogs and their corresponding nucleotides when dimethylhexylamine (DMHA) was used as the ion-pairing agent. Cai et al. [29] recently reported that the detection of formed adduct ion greatly enhanced the sensitivity when using positive-ion ESI for the analysis of nucleotides such as ATP. Although an ion-pairing agent was not used, preventing source pollution, in this method, the positive-ion mode ESI ionization was more sensitive than negative-ion mode for nucleotides. Figs. 1 and 2 contain representative mass spectra from parent ions and major fragment ions, used in MRM, for the different nucleotides analyzed.

HPLC coupled with UV detection is a commonly used technique for nucleotide separation, needing chromatographic peak

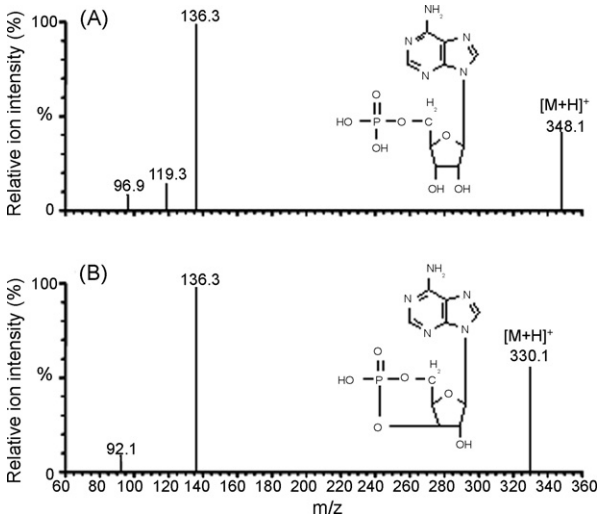

Fig. 1. Positive-ion spectra and chemical structure of AMP (A) and cAMP (B). The spectra are recorded by selecting the pseudo molecular ion ($[M+H]^+$) in the first quadrupole (Q1). After collision activation of the selected ions in the collision cell, the daughter ion spectra are recorded by scanning the last quadrupole (Q3).

separation for the identification of nucleotides. This drawback increases the time needed for sample analysis. Methods such as RIA and EIA are advantageous because of their low detection limits (femtomole); however, they are specific for only one nucleotide detection. Detection of all four nucleotides was carried out using HPLC coupled with mass spectrometry in MRM mode using the transition ions listed in Table 1. The chromatogram of all four cyclic nucleotides (Fig. 3) showed that chromatographic separation is not a critical parameter because all four nucleotides (cAMP, cGMP, AMP and GMP) can be simultaneously detected even at similar retention times. This is one of the advantages of MRM analysis compared to other methods for nucleotides identification, as well as quantification

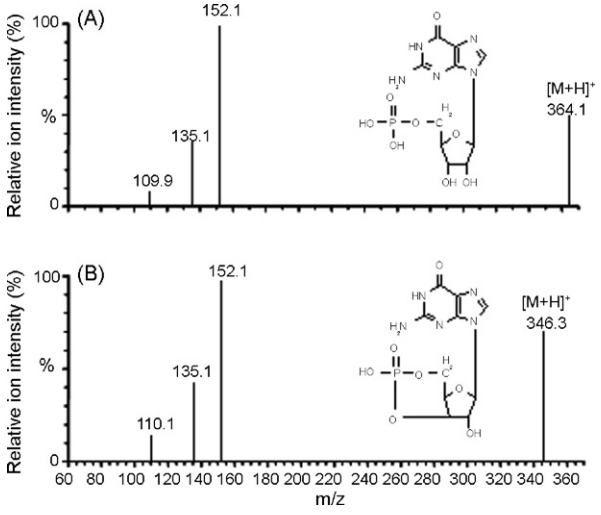

Fig. 2. Positive-ion spectra and chemical structure of GMP (A) and cGMP (B). The spectra are recorded by selecting the pseudo molecular ion ($[M+H]^+$) in the first quadrupole (Q1). After collision activation of the selected ions in the collision cell, the daughter ion spectra are recorded by scanning the last quadrupole (Q3).

Table 1
Monitored ions, MRM, ESI, positive-ionization mode

Compound	Parent (*m/z*)	Daughter (*m/z*)	Collision exit potential (V)	Collision energy (eV)
AMP	348.10	136.30	24	27
cAMP	330.10	136.30	24	33
GMP	364.10	152.10	28	21
cGMP	346.30	152.10	12	27

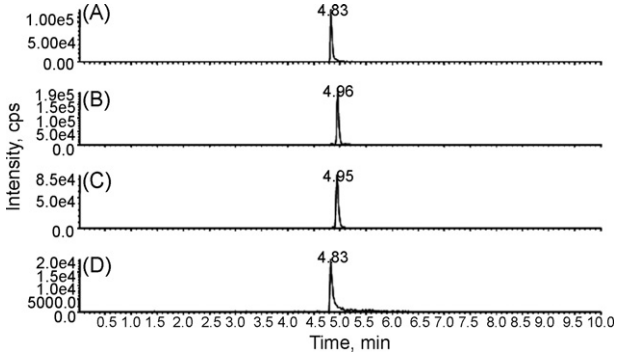

Fig. 3. HPLC–MS/MS chromatograms (standard solution, 10 ng/ml). Each chromatogram is divided into four time windows. (A) Time window 1 shows the 348.1/136.3 transitions corresponding to AMP with retention times were 4.83 min. (B) Time window 2 shows the 330.1/136.3 transitions corresponding to cAMP with retention times of 4.93 min. (C) Time window 3 shows the 346.3/152.1 transitions corresponding to cGMP with retention times of 4.95 min. (D) Time window 4 shows the 364.1/152.1 transitions corresponding to GMP with retention times of 4.83 min.

of PDE5 activity described in the literature [7,9,11,12,30]. Although the GMP peak (Fig. 3) revealed badly tailing, it did not have integration difficulty, and it was confirmed with a good correlation coefficient to the nucleotide.

The obtained correlation coefficients were approximately 0.999 for cGMP and AMP and approximately 0.997 for GMP and cAMP, showed good linearity of the method. The limit of detection of nucleotides was 0.25 ng/ml, with S/N = 25.3 to AMP, S/N = 20.6 to cAMP, S/N = 21.8 to cGMP and S/N = 14.4 to GMP (Fig. 4). This method can have a better lower limit; however, this value was enough for four nucleotides when drug inhibitory activity was assayed *in vitro*. Accuracy between

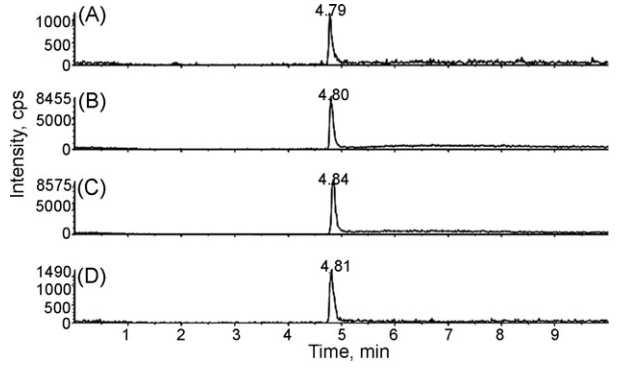

Fig. 4. HPLC–MS/MS chromatograms (standard solution, 0.25 ng/ml). Each chromatogram is divided into four time windows: (A) AMP, (B) cAMP, (C) cGMP and (D) GMP.

Table 2
Limit of quantification validation

	Mean	S.D.	Accuracy (%)	CV (%)
0.25 ng/ml (nominal concentration)				
cGMP	0.251	0.012	100.3	4.7
cAMP	0.252	0.007	100.6	2.8
GMP	0.258	0.013	103.0	4.9
AMP	0.256	0.012	102.4	4.5
0.5 ng/ml (nominal concentration)				
cGMP	0.506	0.017	101.2	3.3
cAMP	0.503	0.034	100.5	6.8
GMP	0.479	0.042	95.9	8.7
AMP	0.510	0.035	102.1	6.8

Eight blank samples were fortified with cGMP, cAMP, GMP and AMP to a final concentration of 0.25 ng/ml and another eight to a final concentration of 0.5 ng/ml (total of 16 samples). These samples were analyzed according to the proposed method.

70–130% and a coefficient of variability (CV) lower than 20% were found for all assays (Table 2), indicating that the method is repeatable and reproducible, according to internationally accepted guidelines [31]. Considering that this method is mainly applied to determine the ratio cyclic nucleotide/inactive nucleotide, less restrictions are required for its validation. The matrix effects were similar in all samples.

The amount of GMP increased linearly with protein concentration up to 0.75 μg/ml (Fig. 5). Hydrolysis of cGMP by platelet PDE and formation of GMP was inhibited pre-incubation with the PDE5 inhibitor sildenafil. Dose–response analysis of sildenafil showed IC50 values of 20 nM (Fig. 6). The inhibition of PDE5 by sildenafil was observed through the monitoring of cyclic nucleotide and inactive nucleotide.

In summary, we have described a method that has wide-ranging potential use in biomedical and kinetic studies. This method is fast, sensitive and has a high reproducibility for qualitative and quantitative analysis of a nucleotide or cyclic nucleotide of PDE activity *in vitro*. The high selectivity of this method can be applied to study different PDE activities

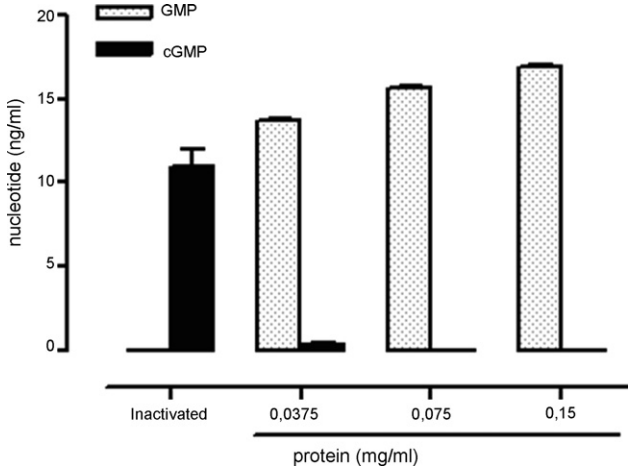

Fig. 5. Effect of protein concentration on the enzymatic hydrolysis of nucleotides cGMP *in vitro*. Results are the mean ± S.D. of three determinations.

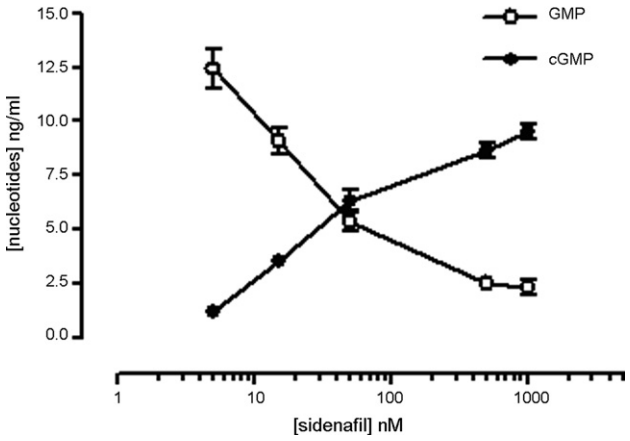

Fig. 6. Effect of sildenafil on cGMP hydrolysis inhibition by PDE5 ($n = 4$, mean ± S.D.).

simultaneously, such as those specific for cGMP and cAMP. The method has been successfully applied to determine IC50 of PDE inhibitors, also to quantify both nucleotide and product of enzymatic reaction of PDEs and can be used for the simultaneous detection of a complex mixture of nucleotides (cGMP, cAMP, GMP and AMP), which is the main advantage of this HPLC–MS/MS system.

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