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Determination of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in human plasma and animal tissues by solid phase extraction on silica and liquid chromatography–triple quadrupole mass spectrometry

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ABSTRACT

3′,5′-Cyclic guanosine monophosphate (cGMP) and 3′,5′-cyclic adenosine monophosphate (cAMP) are essential second messenger molecules. They are involved in signal transduction within cells, in physiological functions such as neurotransmission and in the modulation of cell growth and differentiation of organisms, respectively. A quantitative solid phase extraction method (SPE) based on hydrophilic interaction on silica was developed and applied to both plasma and tissue samples. The stable isotope-labeled internal standards $^{2}D_{1}$,$^{15}N_{3}$-3′,5′-cGMP and $^{13}C_{10}$,$^{15}N_{5}$-3′,5′-cAMP were added prior to the sample preparation to ensure high precision and accuracy. The samples were analyzed by reversed-phase liquid chromatography (RP-LC). Negative electrospray (ESI)-MS/MS was used to selectively monitor several transitions of each metabolite. The method for the analysis of 3′,5′-cAMP and 3′,5′-cGMP in plasma was validated in the range of 0.15–20 ng/mL (R^2 = 0.9996 and 0.9994 for 3′,5′-cAMP and 3′,5′-cGMP, respectively). Basal plasma concentrations for fifteen healthy human patients determined with this method varied between 4.66–9.20 ng/mL for 3′,5′-cAMP and between 0.30–1.20 ng/mL for 3′,5′-cGMP, with precisions better than 9.1%. 3′,5′-cGMP and 3′,5′-cAMP together with their 2′,3′-isomers were also determined in a semi quantitative way in animal tissues. The structures of the isomers were confirmed by analysis with LC-high resolution time-of-flight MS and subsequently by comparison of retention times with standards.

1. Introduction

Second messengers like 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic guanosine monophosphate (cGMP) play an important role in signal transduction within cells [1–4]. Eukaryotes, like humans, are organism with cells that contain complex structures enclosed within membranes. Signal transduction is consequently very important to moderate information through the cell membrane. They amplify the signal coming from hormones or neurotransmitters outside the cell and cause a change in the activity of the cell. Thus second messengers can rapidly propagate a signal from outside the cell to the appropriate molecules within the cell. These small cyclic nucleotides function as second messengers and can bind to cyclic nucleotide-gated ion channels [1,5] and to target proteins like protein kinases, e.g. protein kinase A and G [6,7]. Cyclic nucleotide-gated channels are important in phototransduction [1,8] and other sensory transduction processes like olfaction [9,10] and taste [11,12]. Protein kinase G (cGMP-dependent protein kinase or PKG) plays a role in cell division and smooth muscle relaxation (vasodilation) [13,14]. Protein kinases A (cAMP-dependent protein kinase or PKA) are present in a variety of cells and act on different substrates, so PKA and cAMP regulation are involved in many different pathways, including regulation of lipid metabolism [15,16].

3′,5′-cGMP is synthesized by guanylate cyclase (GC) and it degrades by cyclic nucleotide phosphodiesterases (PDEs) that break the phosphodiester bonds. More specific, the drug sildefanil (Viagra) is a selective and potent inhibitor of cGMP-specific phosphodiesterase type 5 (PDE5) that degrades cGMP in the corpus cavernosum. Consequently, an increase in cGMP concentration and smooth muscle relaxation (vasodilation) is the result, leading to an increase of blood flow in the tissues of the penis, causing an erection [17–19]. Therefore cGMP could serve as a mechanistic biomarker for the pharmaceutical inhibition of PDE's by specific chemical entities [20,21]. 3′,5′-Cyclic adenosine monophosphate (cAMP) is also an important second messenger and modulates cell growth and differentiation in organisms and regulates a number of physiological processes like neuronal signaling and cellular proliferation [2,22].

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It is one of the most prominent nucleotides in eukaryotic cells and is present in almost all cell types of animals. 3′,5′-cAMP is synthesized by adenylate cyclase (GC) and it degrades by nucleotide phosphodiesterases (PDEs). Note that the presence of naturally occurring 2′,3′-isomers of these cyclic nucleotides in animal tissues has also been described [23–27]. In vivo, 2′,3′-cAMP is converted to 2′-AMP/3′-AMP, and these AMPs are metabolized to adenosine. This pathway exists endogenously in both mice and human. One of the conclusions found in the literature is that the conversion of 2′,3′-cAMP to adenosine by the extracellular 2′,3′-cAMP-adenosine pathway would protect tissues by reducing a pro-death factor (2′,3′-cAMP) while increasing a retaliatory metabolite (adenosine).

Until 1970, concentrations of cyclic nucleotides in biological samples were measured with complex experimental approaches [28]. Radioimmunoassay replaced these older approaches with a significant increase in detection limit [29,30]. Other techniques for analyzing these cyclic nucleotides were developed, including liquid chromatography (LC) with fluorescence [31] and photo diode array detection (DAD) [32]. Also ion-pair LC with DAD detection after fractionation of the sample on an anion exchange column was reported [33]. The low sensitivity and time-consuming purification procedures of these methods, limited the use of LC. Presently the enzyme-linked immunosorbent assay (ELISA) is the most commonly used quantitative method for this type of nucleotides [34–37]. ELISA has been used extensively for the quantification of cellular antigens and numerous molecules in biological fluids. Although the specificity and the cost of ELISA analysis thereby involved are adequate, the approach is increasingly hindered by its single solute approach. As more and more metabolites require monitoring, sensitive, specific and rugged LC–MS/MS methods are developed. LC–MS/MS has been applied for the determination of cyclic nucleotides in biological samples [38–40]. Martens-Lobenhoffer et al. described the development of a weak anion exchange solid phase extraction (IEX SPE) method in combination with positive electrospray LC–MS/MS for the quantification of 3′,5′-cGMP in human plasma [41]. Very recently Oeckl and Ferger described an LC–MS/MS method for the analysis of both 3′,5′-cAMP and 3′,5′-cGMP [42]. The sample preparation included protein precipitation. 3′,5′-cAMP and 3′,5′-cGMP were chromatographically not resolved.

In order to determine cAMP and cGMP in hundreds of blood and tissue samples, a method was developed for relative small sample quantities, i.e. 200 μL plasma and 150 mg of tissue and, moreover, that did not suffer from ion suppression in MS. In this respect, sample preparation was of utmost importance. Solid phase extraction on silica, exploiting the mechanism of hydrophilic interaction liquid chromatography (HILIC), was very effective in enrichment of the target solutes and in fractionation of interferences.

2. Experimental

2.1. Chemicals, reagents and materials

3′,5′-cGMP, 3′,5′-cAMP, 2′,3′-cAMP and IBMX (3-isobutyl-1-methylxanthine) were purchased from Sigma–Aldrich (Bornem, Belgium). 2′,3′-cGMP was obtained from BIOLOG Life Science Institute (Bremen, Germany). The stable isotope-labeled $^{2}D_{1}$,$^{15}N_{3}$-3′,5′-cGMP and $^{13}C_{10}$,$^{15}N_{5}$-3′,5′-cAMP were synthesized in house (Pfizer Global Research & Development, Groton, CT, USA). Stock solutions of all standards (1 mg/mL) were prepared in 0.25% acetic acid in water in glass vials and stored at −20 °C. Working solutions were prepared each day by dilution in LC–MS water. LC–MS-grade water, acetonitrile, methanol, acetic acid and formic acid were obtained from Biosolve (Valkenswaard, The Netherlands). HyperSep Silica SPE cartridges (500 mg/3 mL) were purchased from Thermo Fisher Scientific (Erembodegem, Belgium). Sample grinding kits (1.5 mL microcentrifuge tubes, grinding resin, and disposable pestles) were obtained from GE Healthcare Europe GmbH (Diegem, Belgium). PVDF 0.45 μm micro filters from Grace Division (Lokeren, Belgium) were used.

2.2. Human plasma and animal tissues

Heparinized human blood was taken from fifteen volunteers in the laboratory. IBMX (phosphodiesterase inhibitor) was immediately added (10 μL of 100 mM IBMX solution to each mL of blood; 1 mM final concentration) to stabilize cAMP and cGMP that quickly degrade in human blood at room temperature through enzymatic processes. Plasma was obtained by cold centrifugation for 10 min at 2500 rpm and stored at −80 °C until analysis. Animal tissues were kindly donated by the Faculty of Veterinary Medicine of the Ghent University, Belgium. The samples were stored at −80 °C.

2.3. Sample preparation

Plasma samples were thawed to room temperature and 200 μL of plasma was spiked with 5 ng/mL of the labeled internal standards in 0.25% acetic acid in water. 600 μL of 2% acetic acid in water, 200 μL of 5/95 100 mM ammoniumformate/acetonitrile and 3.2 mL of acetonitrile was added. The samples were then vortexed for 2 min and centrifuged for 5 min at 3000 rpm. The complete supernatant was transferred to the silica SPE cartridge that was previously conditioned with 5 mL of water under vacuum and equilibrated with 5 mL of 5/95 100 mM ammoniumformate/acetonitrile. During loading the supernatant was slowly drawn through the SPE cartridge by vacuum. Subsequently the cartridge was washed with 1 mL of 10/90 water/acetonitrile and the analytes were eluted with 2 mL of water in eppendorf tubes and filtered. Samples were dried under nitrogen, reconstituted in 60 μL of water and vortexed for 2 min. 50 μL was injected into the LC–MS/MS system for analysis.

Frozen animal tissues were thawed to room temperature and cut into smaller pieces. 150 mg of animal tissue was transferred to a sample grinding tube and 600 μL of 2% acetic acid in water was added together with 6 μL of 100 mM IBMX stabilizer. The sample was grinded manually with a pestle and centrifuged for 5 min at 7000 rpm. The supernatant was transferred in a vial and 200 μL water, 200 μL 5/95 100 mM ammoniumformate/acetonitrile and 3.2 mL acetonitrile was added. Extracts were then further treated as described for the plasma samples.

2.4. Preparation of calibration standards and quality control (QC) samples

Calibration curve standards were prepared by dilution of 3′,5′-cAMP and 3′,5′-cGMP stock solutions in water down to 0.15, 0.5, 1, 2, 5, 10 and 20 ng/mL concentrations. These samples for calibration were also spiked with 5 ng/mL $^{13}C_{10}$,$^{15}N_{5}$-3′,5′-cAMP and $^{2}D_{1}$,$^{15}N_{3}$-3′,5′-cGMP. Quality control (QC) samples at four concentrations were prepared by spiking the pooled cAMP and cGMP free plasma with the appropriate amounts of cyclic nucleotide standard solutions. cAMP and cGMP free plasma was obtained by aging the plasma at room temperature and regular LC–MS/MS monitoring of residue concentrations. 10 μL of 100 mM IBMX was subsequently added therein together with the 3′,5′-cAMP and 3′,5′-cGMP standards. These QC samples covered the full range of the intended calibration range: lower limit quality control (LLQC), low quality control (LQC), medium quality control (MQC), and high quality control (HQC). The corresponding concentrations were respectively matrix + 0.15 ng/mL (LLQC), matrix + 0.5 ng/mL (LQC), matrix + 5 ng/mL (MQC), and matrix + 15 ng/mL (HQC). In case of the

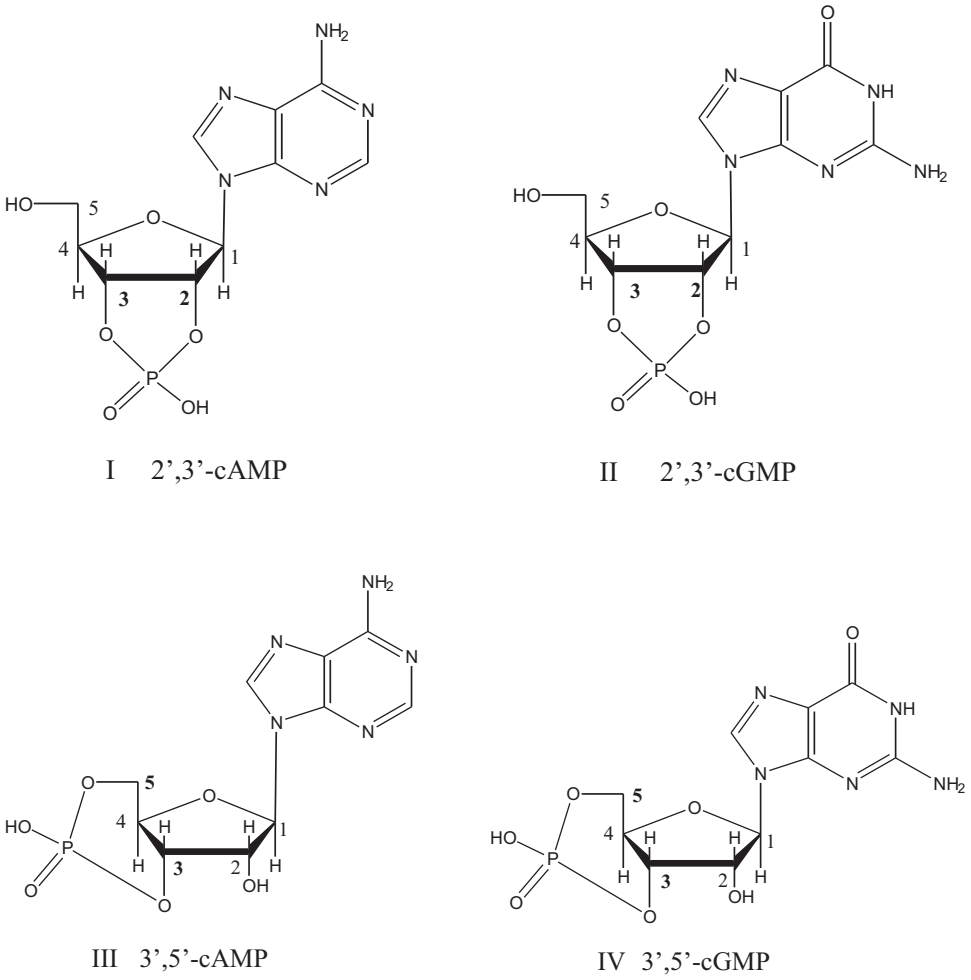

Fig. 1. Four cyclic nucleotides detected in plasma and/or animal tissue samples.

LLQC sample, the 0.15 ng/mL added to the matrix was close to the highest possible LOQ of 0.125 ng/mL (for cGMP), measurable with the used triple quadropole instrument.

2.5. Instrumentation

For LC–MS/MS analysis an Alliance 2690 (Waters, Milford, MA, USA) equipped with autosampler, column oven and binary pump was coupled to an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Toronto, Canada) with Turbo Ion Spray (electrospray) source. The MS was operated in the negative-ion multiple reaction monitoring mode (MRM). Data acquisition was performed with Analyst 1.4.1 software.

Separations were carried out on a Zorbax SB-C18 column 150 mm × 3.0 mm i.d., 3.5 μm particle size (Agilent Technologies, Brussels, Belgium), coupled to a C18 Security Guard column 4 mm × 3.0 mm i.d. (Phenomenex, Torrance, CA, USA). Solvent A was 0.1% formic acid and solvent B acetonitrile/methanol/water in volume ratio 1/2/4. The flow rate was 0.35 mL/min. A linear gradient was used with initial conditions 100% A and increasing solvent B from 0% to 50% in 10 min. Solvent B then went to 100% B in 1 min to clean the column and maintained for 1 min, followed by column regeneration for 10 min. Total analysis time was 22 min. The column temperature was 25 °C and the autosampler temperature was set at 10 °C.

MS instrument parameters were optimized for maximum sensitivity in MRM mode. Negative voltage and temperature were respectively set on −4200 V and 470 °C. Nebulizer gas was set at 15, curtain gas at 6 and collision gas at 3. The dwell time was 150 ms. The Q1 resolution was set on unit, the Q3 resolution was set on low. The precursor to product ion transition (parent ion → daughter ion) was measured through direct infusion of each compound into the ion source and the most abundant product ion was selected. Solute identity was confirmed in all cases by various transitions. An Infinity 1290 LC coupled to a 6230 TOF LC/MS (Agilent Technologies, Waldbronn, Germany) with Jet Stream technology was used to record high resolution spectra of 2′,3′-cAMP, 2′,3′-cGMP and the 3′,5′-analogs.

3. Results and discussion

Most important considerations in developing a method that can be routinely applied for the determination of the four cyclic nucleotides (Fig. 1) in biological samples were: (i) method should be highly selective and specific, (ii) sample sizes should be small and (iii) matrix effects resulting in ion suppression should be absent. To realize this, both the analytical (LC–MS/MS) and sample preparation steps were optimized.

3.1. Optimization of LC–MS/MS

The cyclic nucleotides can, in principle, be analyzed by different LC modes, e.g. RP-LC, HILIC, and ion-pair LC. RP-LC is preferred in the pharmaceutical industry because of its high robustness compared to the other LC modes and has also been applied for the

Table 1
MRM transitions and compound dependent parameters. Quantifier ions are marked in bold.

Nr.	Compound	Parent ion (*m*/*z*)	Daughter ion (*m*/*z*)	Declustering potential (V)	Focusing potential (V)	Collision energy (V)	Collision cell exit potential (V)
			134.0			−22	−7
I	2′,3′-cAMP	**328.0**	107.2	−61	−50	−62	−7
			92.2			−70	−5
II	2′,3′-cGMP	343.9	66.0	−96	−270	−88	−5
III	3′,5′-cAMP	**328.0**	**134.2**	−81	−230	−36	−7
			149.9			−34	−7
IV	3′,5′-cGMP	**344.0**	133.1	−91	−215	−52	−7
			79.0			−74	−5
V	IS 3′,5′-cAMP	342.9	144.1	−61	−190	−34	−7
VI	IS 3′,5′-cGMP	348.0	154.1	−86	−290	−32	−7

determination of 3′,5′-cAMP and 3′,5′-cGMP by LC–MS in biological samples [38–41]. Several RP-LC columns were tested in this study. Zorbax SB-C18 was chosen for its long-term stability and reproducibility and because of the excellent retention, peak symmetry and efficiency which was measured for the cGMP and cAMP standards. A 3 mm i.d. column was the best compromise for MS (flow rate of 0.35 mL/min, no flow splitting) and volume loadability (50 μL water injection volume). Concerning the mobile phase composition, high amounts of water are required to give the very polar cyclic nucleotides sufficient retention. Therefore the gradient started at 100% aqueous and increased to only 20% organic at the end of the gradient. Moreover, methanol was added in excess to the organic phase (2 compared to 1 for acetonitrile) to increase retention further [40].

Both positive and negative ESI voltage could be applied for sensitive detection of the cyclic nucleotides. Although positive ion detection yielded somewhat higher signal-to-noise ratios for the pseudo molecular ions of both compounds, it also introduced interferences from human plasma extracts in the MRM channel for several ion transitions used in this study as well as the formation of instable adduct ratios. In contrast, MRM transitions in negative ionization mode were free of interfering components. Consequently the negative mode was selected. Optimization of the MRM conditions was established through direct infusion of the neat standards of each compound solubilized in the initial mobile phase. MS parameters were tuned and selected for the individual compounds to achieve maximum signal intensity. The MRM transitions for the individual compounds were investigated and the most abundant daughter ion was selected as quantifier ion (Table 1).

One quantifier daughter ion (corresponding to the nucleotide base and the most intense daughter ion) and two validation daughter ions for 3′,5′-cGMP were added to ensure the identification and quantification of this cyclic nucleotide because of its lower signal intensity and limit of detection, compared to 3′,5′-cAMP. An MRM chromatogram with the specific ion transitions of the four described cyclic molecules is shown in Fig. 2.

Isotope dilution MS is by far the best method to provide accurate and precise data for the cyclic nucleotides. Stable isotope-labeled internal standards, with identical chemical and physical properties as the analytes, were used. Isotope-labeled standards of 3′,5′-cGMP

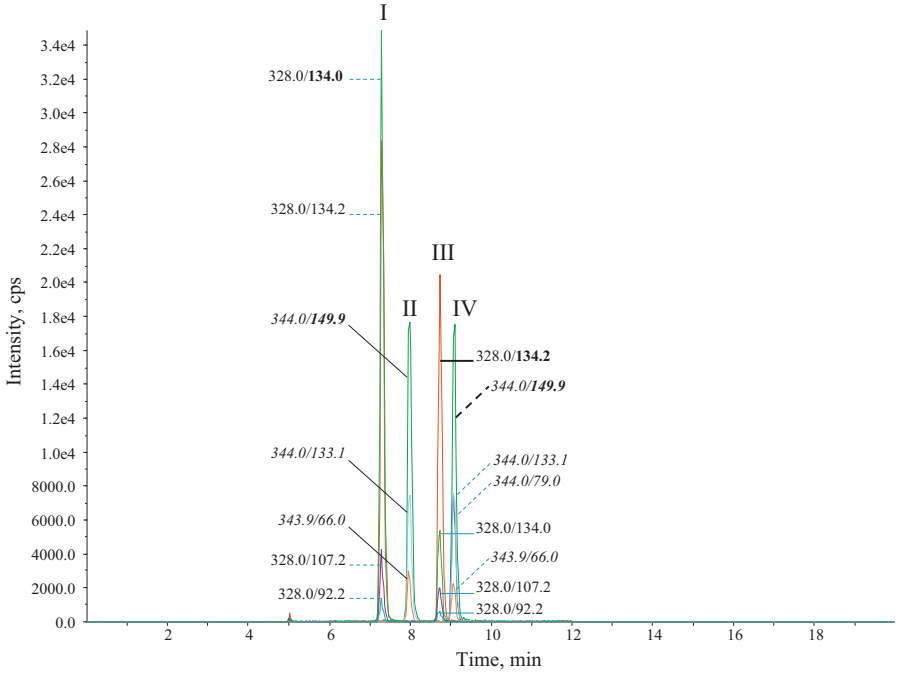

Fig. 2. MRM chromatogram of a mixture of: I. 2′,3′-cAMP, II. 2′,3′-cGMP, III. 3′,5′-cAMP and IV. 3′,5′-cGMP at a concentration of 10 ng/mL for each analyte using the validated LC–MS/MS method. Multiple MRM transitions for the same molecule (quantification- and validation daughter ions) are shown. Quantification daughter ions are marked in bold.

Table 2
Precision and accuracy data for the 3′,5′-cGMP and 3′,5′-cAMP measurement in human plasma by LC–MS/MS.

	Spiked (ng/mL)	Intra-run precision and accuracy			Inter-run precision and accuracy		
		Measured (mean ± SD)	Precision (RSD in %)	Accuracy (%)	Measured (mean ± SD)	Precision (RSD in %)	Accuracy (%)
3′,5′-cGMP	0.15	0.159 ± 0.016	10.1	106.1	0.161 ± 0.019	12.1	107.6
	0.50	0.491 ± 0.032	6.5	98.3	0.495 ± 0.050	10.3	99.1
	5.0	5.21 ± 0.29	5.7	104.2	5.17 ± 0.30	5.9	103.4
	15.0	14.88 ± 0.46	3.1	99.2	14.84 ± 0.68	4.6	98.9
3′,5′-cAMP	0.15	0.160 ± 0.018	11.3	106.7	0.162 ± 0.020	12.4	108.2
	0.50	0.517 ± 0.027	5.2	103.4	0.501 ± 0.034	6.8	100.2
	5.0	5.17 ± 0.32	6.1	103.3	5.23 ± 0.27	5.2	104.6
	15.0	14.84 ± 0.55	3.7	98.9	14.90 ± 0.51	3.4	99.3

and 3′,5′-cAMP are not commercially available but the synthesis using an isotopically labeled precursor has been described [40]. The precision of the LC–MS/MS assay was significantly improved by using labeled internal standards.

3.2. Sample preparation

In bio-analytical LC–MS/MS, ion suppression is a well-known phenomenon. Matrix effects from endogenous solutes in plasma can be linked to three main contributors: salts, proteins and phospholipids [43,44]. Ion suppression due to these solutes can be addressed by adequate sample preparation methods that include de-proteination and de-lipidation. In recent years, we conducted a lot of research in the development of high throughput sample preparation techniques for pharmacokinetic studies of biological samples by LC–MS/MS. The data will be published elsewhere [45]. HILIC SPE is, in this respect, a relatively unexploited but very powerful tool that was also applied in this work. In the sample preparation procedures described in Section 2.3, the vast majority of the proteins are removed by precipitation (and centrifugation) in the 90% acetonitrile solution. The supernatant is subsequently transferred to the HILIC SPE cartridge, which acts as an additional trap for residual protein content. Salts elute early in the RPLC analysis and are therefore of no concern as the solutes of interest are well retained. Phospholipids can experience some partial breakthrough during the SPE process (elution with 100% water), but they are subsequently completely retained on the (C18) guard column (which is replaced after 50 analyses) under the highly aqueous mobile conditions used. In this way, ion suppression was effectively avoided as ascertained by a constant response of the labeled standards during the complete study.

3.3. Figures of merit

In order to investigate the figures of merit of the methodology for quantitative analysis of 3′,5′-cGMP and 3′,5′-cAMP in plasma, recovery, selectivity, sensitivity, linearity and precision were determined by using blank and spiked plasma. The recovery of the sample preparation method was evaluated by comparing the MS response of analytes in plasma samples spiked before and after SPE on silica. Three samples of the same plasma were spiked with 10 ng/mL of 3′,5′-cGMP and 3′,5′-cAMP and with 20 ng/mL of IS $^{2}D_{1}$,$^{15}N_{3}$-3′,5′-cGMP and IS $^{13}C_{10}$,$^{15}N_{5}$-3′,5′-cAMP. Each sample was then analyzed in triplicate. Recovery values of 103.2% (RSD 6.4%) and 105.6% (RSD 5.7%) for respectively 3′,5′-cAMP and 3′,5′-cGMP demonstrated the effectiveness of the sample preparation method while the instrumental RSD was below 2%. Selectivity is excellent due to precise retention times (±0.1 min), specific MRM transitions and the ratio of the different MRM transitions.

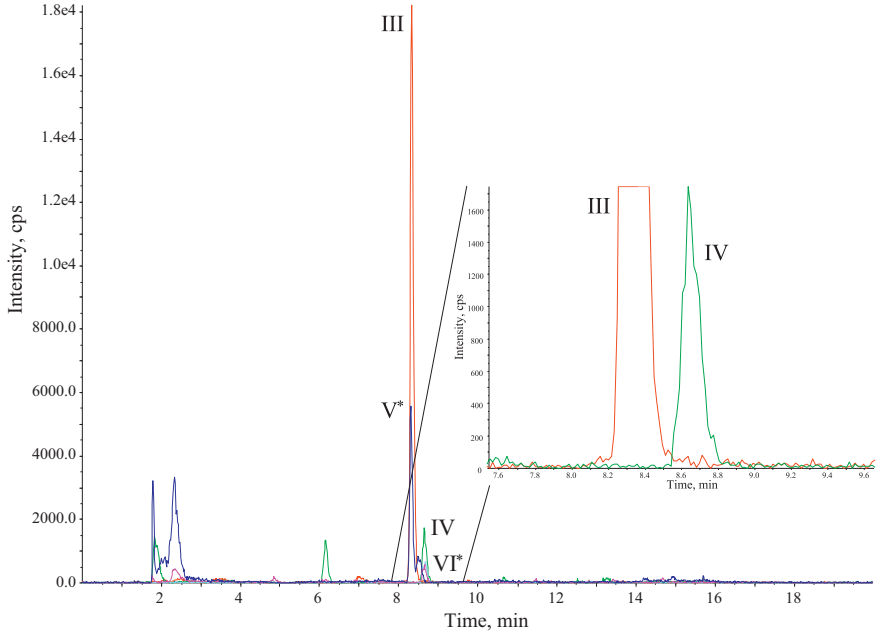

Fig. 3. Representative MRM chromatogram of a human plasma sample (200 μL) after SPE on silica and analysis by LC–MS/MS. III. 3′,5′-cAMP and IV. 3′,5′-cGMP. Spiked with 5 ng/mL internal standard of 3′,5′-cAMP (V*) and 3′,5′-cGMP (VI*). Measured concentrations are 6.4 ng/mL and 0.6 ng/mL for respectively 3′,5′-cAMP and 3′,5′-cGMP. Also a zoom of the two quantifier daughter ions for 3′,5′-cAMP and 3′,5′-cGMP is displayed.

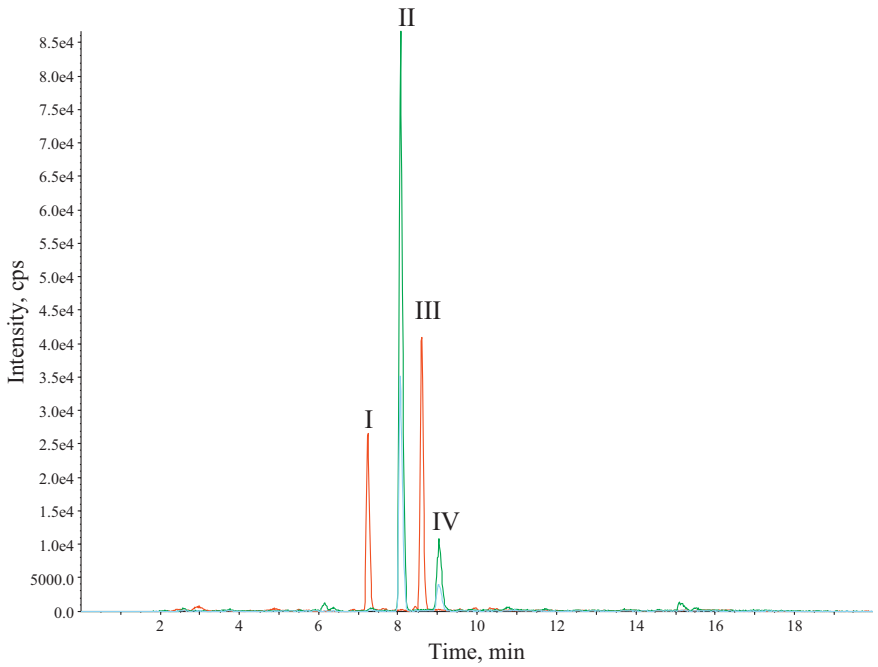

Fig. 4. MRM chromatogram of an extract of rabbit pancreas (150 mg) after SPE on silica LC–MS/MS: I. 2′,3′-cAMP, II. 2′,3′-cGMP, III. 3′,5′-cAMP and IV. 3′,5′-cGMP.

The limit of detection (LOD) for the total assay was 0.023 ng/mL for 3′,5′-cAMP and 0.038 ng/mL for 3′,5′-cGMP (signal to noise ratio 3). The limit of quantification (LOQ) was 0.076 ng/mL for 3′,5′-cAMP and 0.125 ng/mL for 3′,5′-cGMP (signal to noise ratio 10). Linearity of the method was studied in the range of 0.15–20 ng/mL for both cyclic nucleotides and correlation coefficients (R^2) of 0.9996 and 0.9994 for, respectively, 3′,5′-cAMP and 3′,5′-cGMP were calculated. This broad calibration range was chosen to anticipate for possible outliers with a concentration outside this range. Note that the lowest point in the calibration curve (0.15 ng/mL) is close (but above) the lowest LOQ of this method, i.e. 0.125 ng/mL for 3′,5′-cGMP. 3′,5′-cGMP and the tenfold higher concentrated 3′,5′-cAMP were calibrated separately.

Precision and accuracy data, represented in Table 2, was validated using the European Medicines Agency (EMA) guidelines for bioanalytical method validation (21 July 2011) and of a representative manuscript following those procedures [46]. The intra-day precision and accuracy were determined by evaluating matrix-based QC samples prepared with six replicates at four different concentration levels (LLQC, LQC, MQC, HQC), while the inter-day precision and accuracy were evaluated over three different days. Precision was expressed as the relative standard deviation of the determined concentrations. Accuracy was calculated by dividing the mean measured concentration by the mean nominal concentration times 100. The results of the analysis of the plasma-based QC samples demonstrated acceptable precision and accuracy based on validation criteria (precision with RSD less than 20% and accuracy within 80–120% for LLQC and precision with RSD less than 15% and accuracy within 85–115% for the rest of the QCs).

The 3′,5′-cGMP, 3′,5′-cAMP and IS stock solutions (IS $^{2}D_{1}$,$^{15}N_{3}$-3′,5′-cGMP and IS $^{13}C_{10}$,$^{15}N_{5}$-3′,5′-cAMP) were determined to be stable for a least 24 h and for one month at room temperature and at −20 °C, respectively. When 1 mM IBMX was added to the plasma samples prior to analysis, these solutes (including IS) after sample processing were found stable for up to 152 h at room temperature. Long term stability of 3′,5′-cGMP and 3′,5′-cAMP in IBMX quenched human plasma was confirmed at −80 °C for at least 6 months [40].

Table 3
Measured concentrations of 3′,5′-cAMP and 3′,5′-cGMP in human plasma of fifteen healthy patients.

Plasma sample	Conc 3′,5′-cAMP (ng/mL) ± SD (n = 3)	Precision (RSD %)	Conc 3′,5′-cGMP (ng/mL) ± SD (n = 3)	Precision (RSD %)
1	5.34 ± 0.18	3.36	0.30 ± 0.02	5.67
2	4.83 ± 0.10	2.07	0.40 ± 0.03	6.39
3	4.80 ± 0.16	3.43	0.74 ± 0.02	2.44
4	6.81 ± 0.24	3.53	0.96 ± 0.05	4.82
5	4.66 ± 0.29	6.12	0.39 ± 0.02	4.46
6	7.17 ± 0.49	6.79	1.06 ± 0.10	9.06
7	6.35 ± 0.13	1.98	0.68 ± 0.03	4.12
8	6.14 ± 0.32	5.17	0.43 ± 0.03	5.79
9	9.20 ± 0.39	4.27	1.20 ± 0.02	1.51
10	5.42 ± 0.11	2.07	1.03 ± 0.03	2.83
11	7.12 ± 0.14	1.96	0.87 ± 0.03	3.17
12	5.73 ± 0.11	1.85	0.80 ± 0.01	1.78
13	6.46 ± 0.17	2.70	0.85 ± 0.07	8.12
14	8.09 ± 0.18	2.17	5.39 ± 0.16	2.94
15	6.80 ± 0.28	4.18	0.80 ± 0.04	4.47

Table 4
Difference between the measured mass and the calculated mass from the correct formula for a TOF-MS experiment with pancreas of a rabbit.

Compound	Formula	Mass (Da)	Calculated mass (Da)	Difference (ppm)
3′,5′-cGMP	$C_{10}H_{12}N_5O_7P$	345.04715	345.04743	0.81
3′,5′-cAMP	$C_{10}H_{12}N_5O_6P$	329.05321	329.05252	2.09
2′,3′-cGMP	$C_{10}H_{12}N_5O_7P$	345.04685	345.04743	1.68
2′,3′-cAMP	$C_{10}H_{12}N_5O_6P$	329.05221	329.05252	1.25

Table 5
Measured concentrations of cAMP and cGMP in animal tissue samples by LC–MS/MS.

Animal tissue sample	Conc 2′,3′-cAMP (pg/mg)	Conc 2′,3′-cGMP (pg/mg)	Conc 3′,5′-cAMP (pg/mg)	Conc 3′,5′-cGMP (pg/mg)
Pancreas of rabbit	7.6	30.7	11.8	5.2
Kidney of rabbit	4.5	19.4	1.3	–

3.4. Applications

The concentration of 3′,5′-cGMP and 3′,5′-cAMP in human plasma was measured for fifteen healthy volunteers. A representative MRM chromatogram is shown in Fig. 3.

Samples were measured in triplicate and standard deviations (SD) and relative standard deviations (RSD) were calculated. Results are shown in Table 3. 3′,5′-cAMP concentrations varied between 4.66–9.20 ng/mL and 3′,5′-cGMP between 0.30–1.20 ng/mL which is in excellent agreement with literature data [40–42]. Plasma sample number 14 was an outlier for 3′,5′-cGMP and needs further biological investigation. It was not taken into account to calculate the mean value.

Quantifying cGMP and cAMP in animal tissues is becoming more and more relevant in drug discovery and several samples were investigated: the pancreas, the kidney and heart of rabbits and the pancreas of rats. Two representative samples are discussed, i.e. the pancreas and kidney of rabbits. Analysis of the pancreas of a rabbit with the SPE on silica LC–MS/MS method using the same MRMs showed four peaks (Fig. 4).

Two peaks could be elucidated to 3′,5′-cGMP and 3′,5′-cAMP by their retention times and specific MRM transitions. However, two additional signals depicting the same MRM transitions as the 3′,5′-cyclic nucleotides were detected at 1.5 min and 1 min earlier. Response on the same MRMs suggested isomers and indeed 2′,3′-isomers of the 3′,5′-cyclic nucleotides have been reported to occur in animal tissues [23–27]. High resolution MS was used to confirm the elemental composition of the "at that time" unknown structures (Table 4). The identity and retention times corresponding to the 2′,3′-isomers was subsequently confirmed by purchasing these naturally occurring isomers of the 3′,5′-cyclic nucleotides.

Corresponding quantitative data are included in Table 5. It should be mentioned that the method for the analysis of the tissue samples could not yet be fully validated because of limited sample availability. Therefore semi quantitative results were included to inform the reader about the interesting possibilities that HILIC-SPE HPLC-MS/MS can offer for animal tissue analyses.

4. Conclusion

An accurate and sensitive method was developed for the determination of the highly relevant second messenger molecules 3′,5′-cAMP and 3′,5′-cGMP and its naturally occurring 2′,3′-isomers in human plasma and animal tissues. SPE on silica was used as sample preparation method for the enrichment of the targets and the removal of interferences leading to MS ion suppression. Samples were then analyzed by RP-LC combined with MS/MS operated in the negative ESI mode. 3′,5′-cAMP and 3′,5′-cGMP concentrations in human plasma are 4.66–9.20 ng/mL and 0.30–1.20 ng/mL. Two isomers of 3′,5′-cAMP and 3′,5′-cGMP namely 2′,3′-cAMP and 2′,3′-cGMP were detected in tissue samples.

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