Journal of Chromatography B, 878 (2010) 487–491

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Improved method for the determination of cyclic guanosine monophosphate (cGMP) in human plasma by LC–MS/MS

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ARTICLE INFO

Article history:
Received 30 June 2009
Accepted 6 December 2009
Available online 16 December 2009

Keywords:
Cyclic guanosine monophosphate
Weak anion exchanger
Porous graphitic carbon
LC–MS/MS

ABSTRACT

Cyclic guanosine monophosphate (cGMP) is an important second messenger molecule involved in gating ion channels and activating protein kinases. Here, we describe a validated LC–MS/MS method for the quantification of cGMP in human plasma, utilizing a stable isotope labeled analogue of cGMP as I.S. Plasma samples were extracted and concentrated by weak anion exchange solid phase extraction and the extracts were chromatographically separated on a porous graphitic carbon column. The analytes were detected by positive electrospray ionization and tandem mass spectrometry. The calibration function was linear in the range 1–20 nM and the intra- and inter-day precision showed relative standard deviations of better than 2 and 6%, respectively. The accuracy was always better than 4%. Plasma concentrations in healthy human subjects determined with this method were 3.92 ± 1.17 nM ($n = 20$). The method was, due to its isotope labeled I.S., matrix independent.

1. Introduction

Cyclic guanosine-3′,5′-monophosphate (cGMP) is an important second messenger in biological systems and is synthesized from guanosine triphosphate (GTP) by the guanylate cyclase (GC) enzyme family. This family consists of the particulate transmembrane (pGC) class, which is induced by peptide ligands, and the soluble form (sGC), which is induced by nitric oxide and resides in the cytosol. A range of biological functions are triggered by cGMP, such as gating of ion channels or activating protein kinases [1]. The activity of cGMP is regulated by its conversion to guanosine monophosphate (GMP) by the enzyme class of the phosphodiesterases (PDE) [2]. The biochemical pathway of cGMP provides potential drug targets for a range of diseases. For example, currently, PDE-inhibitors such as sildenafil are utilized in clinical practice to treat erectile dysfunction and pulmonary hypertension [3]. To gain more insight into this system and to facilitate clinical research, it is highly desirable to have sensitive and precise methods for the quantification of cGMP in biological fluids.

Besides immunological assays such as radioimmunoassay (RIA) [4] or enzyme immunoassay (EIA) [5], there are a range of published HPLC-based methods for the quantification of cGMP in biological fluids. Utilizing UV-detection of the analytes [6,7] leads to limited sensitivity of the methods, which are therefore restricted to special applications such as enzyme solutions or tissue samples. Derivatization of cGMP with fluorophores and subsequent HPLC with detection by fluorescence [8,9] leads to better sensitivity, but the methods are time-consuming and troublesome and have not been applied to human plasma. Mass spectrometric detection after direct separation of cGMP with HPLC leads to superior selectivity and sensitivity. Two of the published methods [10,11] do not focus solely on cGMP, but profile many different phosphorylated nucleotides simultaneously and need therefore time-consuming chromatographic procedures. Another method based on LC–MS/MS [12] is restricted to enzyme solutions and uses no I.S. Recently, a method quantifying cGMP from human plasma utilizing an isotope labeled analogue of cGMP as I.S. has been published [13]. The authors describe a very straightforward sample preparation and short chromatography, but have to revert to the less sensitive negative ionization electrospray (ESI) mode because of interferences in the positive mode.

In this paper, we present an improved method for the quantification of cGMP in human plasma. It utilizes an isotope labeled internal standard, mixed mode weak anion exchange solid phase extraction (WAX-SPE) for sample preparation and a chromatographic separation on a porous graphitic carbon column. This method is very

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doi:10.1016/j.jchromb.2009.12.009

selective and sensitive and provides very good precision and accuracy.

2. Experimental

2.1. Chemicals

Cyclic guanosine monophosphate sodium salt was obtained from Fluka (Buchs, Switzerland). Guanosine-$^{13}C_{10}$,$^{15}N_5$-5′-triphosphate sodium salt (100 mM solution in water) was purchased from Aldrich (Buchs, Switzerland). Soluble guanylate cyclase (sGC, 10 μg in solution) and 2-(N,N-diethylamino)-diazenolate-2-oxide diethylammonium salt (DEA-NONOate) were obtained from Alexis Biochemicals (Plymouth Meeting, PA, USA). All other chemicals were of analytical grade or better.

2.2. Synthesis of isotope labeled cGMP

The stable isotope labeled I.S. $^{13}C_{10}$-$^{15}N_5$-cGMP was synthesized by the cyclization/dephosphorylation of $^{13}C_{10}$-$^{15}N_5$-GTP by the enzyme sGC. The reaction was carried out according to the procedure described by Sousa et al. [14] with minor modifications. In short, to 1 ml buffer solution (5 mM triethylamine, adjusted to pH 7.6 with 1 M HCl) containing 5 mM $MnCl_2$, 10 μl of the 100 mM $^{13}C_{10}$-$^{15}N_5$-GTP solution were added, resulting in a concentration of 1 mM. To this solution, 4.1 mg DEA-NONOate (resulting in a concentration of 20 mM) and immediately afterwards the solution containing 10 μg sGC were added. This mixture was incubated for 20 min at 37 °C, followed by another addition of 4.1 mg DEA-NONOate and further incubation for 20 min. The final concentration of $^{13}C_{10}$-$^{15}N_5$-cGMP in the reaction mixture was determined by the here-described method against unlabeled cGMP. Subsequently, it was diluted to a concentration of about 200 nM with 0.1 M HCl and was frozen aliquoted at −80 °C until usage.

2.3. Instrumentation

The HPLC part of the analytical apparatus consisted of an Agilent 1100 system (Santa Clara, CA, USA) comprising a binary pump, an autosampler and a thermostatted column compartment. The chromatographic separation took place on a Hypercarb 150 mm × 1 mm 5 μm particle size column (Thermo Scientific Inc., San Jose, CA, USA), protected by a 10 mm × 1 mm precolumn containing the same material. The analytes were detected by a TSQ Discovery Max triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) ion source (Thermo Scientific Inc., San Jose, CA, USA). System control and data handling were carried out by the Thermo Electron Xcalibur software, version 1.2.

2.4. Calibration and quality control samples

A stock solution of cGMP was prepared by dissolving 4.65 mg cGMP sodium salt (according to the quality control sheet, the here used batch contained 21% (w/w) water) in 1 ml water, resulting in a concentration of 10 mM. To prevent erroneous calibration due to additional moisture absorption by the cGMP sodium salt, the concentration of the stock solution was checked by photometry at λ = 252 nm, using a molar extinction coefficient ε of 13,700 M^{-1} cm^{-1} [15]. We found divergences of less than 7% to the expected concentrations, which were regarded as minor. For the preparation of calibration standards, this stock solution was further diluted to 1 μM and 100 nM. Calibration samples were prepared from pooled human plasma or water at the concentrations 0, 1, 2, 4, 8, 15 and 20 nM. Quality control (QC) samples were prepared from pooled human plasma in the spike-concentrations of 1 and 20 nM. The QC-samples were stored at −80 °C until usage.

2.5. Human plasma samples

Human blood samples were drawn from the cubital vein into EDTA plasma sampling tubes. Plasma was obtained by centrifugation at 2000 × *g* for 5 min. Plasma was stored at −80 °C until analysis. All individuals participating in this study gave written informed consent.

2.6. Sample preparation

For plasma sample extraction, OASIS WAX-SPE 1 cm^3 30 mg columns (Waters, Eschborn, Germany) were conditioned by rinsing with 1 ml of methanol, followed by 1 ml of water. To 500 μl of human plasma samples 50 μl of the I.S. solution (200 nM isotopically labeled cGMP in 0.1 M HCl) and 500 μl of 2% (v/v) acetic acid were added and the mixtures were drawn through the SPE columns. The columns were rinsed with 1 ml of 2% (v/v) acetic acid and 1 ml of methanol. After sucking dry for 2 min, the analytes were eluted by 1 ml of 5% ammonia in methanol. The eluates were dried in vacuum and the residues were dissolved in 100 μl of mobile phase A for the HPLC. Other SPE columns used in the development phase of the sample preparation procedure were OASIS HLB, OASIS MAX and OASIS MCX (all from Waters, Eschborn, Germany). The evaluation of their extraction capabilities for cGMP was performed according to the generic procedures described by the manufacturer.

2.7. Chromatography and mass spectrometry

Chromatographic separation was carried out by gradient elution on a porous graphitic carbon column at a constant temperature of 50 °C. The mobile phase A consisted of 1% (v/v) acetic acid and 1% (w/w) ammonium acetate in water. The mobile phase B was acetonitrile. The gradient started with 80% of A and 20% of B for 2 min, then the fraction of B was raised to 50% in 10 min, held for 1 min, and then restored to starting conditions in 0.1 min. The total runtime was 19 min. The flow rate was 0.1 ml/min and injection volume was 25 μl.

The eluate of the analytical column was directed without splitting to the ESI ion source of the mass spectrometer. The ion source was working in the positive mode with a spray voltage of 4200 V, a sheath gas and auxiliary gas flow (both nitrogen) of 23 and 6 arbitrary units, respectively. Capillary temperature and offset voltage were 270 °C and 35 V, respectively. To decluster adduct ions, an in-source collision energy of 9 V was applied. The analytes were detected in the MS/MS single reaction monitoring mode. Both quadrupoles were set to a resolution of 0.7 amu peak full widths at half maximum. Scan width and scan time were 0.1 amu and 0.1 s, respectively, for both ion traces. The observed parent ion masses were *m*/*z* 346 and *m*/*z* 361 and the product ion masses were *m*/*z* 152 and *m*/*z* 162 for cGMP and the I.S., respectively. Collision gas was argon at a pressure of 1.3 mTorr, and collision energy of 20 V for both analytes was applied.

Matrix effects were investigated by comparing the heights of the I.S. peak in aqueous samples and in extracted plasma samples spiked with I.S. after extraction ($n = 5$).

2.8. Validation

The extraction yield was determined by comparing the concentrations of the I.S. in plasma samples spiked with I.S. prior to the extraction with those obtained in plasma samples spiked with the I.S. after the extraction ($n = 5$).

Intra-day ($n = 10$) and inter-day ($n = 5$) precision and accuracy was determined by repeated measurements of the QC-samples. The accuracy of the lowest QC-level was not defined due to the unknown endogenous content of cGMP in the samples. For the other level, accuracy was calculated by comparing the measured concentrations with expected (spike + endogenous) concentrations.

3. Results and discussion

3.1. Synthesis of isotope labeled cGMP

To the best of our knowledge, no isotope labeled cGMP is commercially available. However, stable isotope labeled GTP is obtainable and was converted to cGMP by the enzymatic reaction with sGC. The enzyme sGC needs nitric oxide (NO) for activation and Mn^{2+} (superior to Mg^{2+} [14]) ions as a cofactor to reach full activity. NO was provided by the donor DEA-NONOate. As reported [14], the reaction proceeded for about 20 min at 37 °C. A new addition of DEA-NONOate reactivated the reaction for another 20 min and improved the overall yield from 12 to 17%. Further additions of DEA-NONOate were not effective. After appropriate dilution of the reaction mixture with 0.1 M HCl to reach a concentration of 200 nM, the solution was ready to use without any further cleaning steps.

3.2. Sample extraction

Zhang et al. [13] described a simple protein precipitation as sample preparation. In our experience, such a sample preparation leads to very impure extracts, which can result in reduced chromatographic performance and reduced sensitivity, due to ion suppression effects. Therefore, we opted to use SPE for sample cleanup and concentration. On SPE columns exerting unpolar interactions (OASIS HLB) and on strong cation exchange columns (OASIS MCX), we observed no significant retention of cGMP. On the other hand, by investigating strong anion exchange columns (OASIS MAX), we found that cGMP was retained very strongly and it was virtually impossible to elute it from the columns. However, on weak anion exchange columns (OASIS WAX), it was possible to retain cGMP strongly at acidic pH, while it was eluted at basic pH by neutralizing the ion exchange resin. Applying this procedure, we were able to get sufficiently clean extracts and could concentrate cGMP from the samples by a factor of 5, to achieve improved sensitivity.

The extraction yield was determined using the I.S. as reference, because the unknown endogenous amount of cGMP in plasma samples prevented meaningful calculations. The determination of the extraction yield was carried out by comparing extracted spiked plasma samples with extracted blank plasma samples spiked after the extraction to prevent any matrix effects from impairing the results. Applying this technique, we found an extraction yield of $77.2 \pm 1.8\%$.

3.3. Chromatography and mass spectrometry

As cGMP is a very polar molecule, retention on reversed phase columns is difficult and requires a nearly 100% aqueous mobile phase [12], ion pairing [10] or reversed phase columns with hydrophilic endcapping [13]. On the other hand, porous graphitic carbon columns provide good retention behavior with polar compounds such as cGMP. Using a simple acetic acid/ammonium acetate buffer–acetonitrile gradient as the mobile phase, which favored the ionization efficiency in the ESI ion source, good retention and narrow and symmetric peak shapes could be achieved (Fig. 1). The small diameter of the column applied has the advantage of peak concentration and therefore led to enhanced sensitivity. The drawback is the small flow rate applicable, which in turn led to quite long re-equilibration times and the long overall runtime of 19 min. Under the described conditions, the retention times of cGMP and its I.S. were 9.5 min.

The small flow rate of the chromatographic separation favored the ion forming process in the ESI ion source. The negative charge at the phosphate moiety of cGMP seemed to make negative ESI promising for good ionization efficiency. However, the intensity was found to be about a factor of 5 lower than in positive mode. Nevertheless, in the positive mode, adduct ions were formed between cGMP and ammonium ions from the buffer. By this process, the total intensity of the cGMP signal was dispersed to different masses. The adduct ions could be stripped away by applying an in-source collision energy of 9 V, restoring the full intensity of the quasi-molecular ions with masses of m/z 346 and m/z 362 of cGMP and its I.S., respectively. The parent ions of cGMP and the I.S. were fragmented by collision-induced dissociation (CID) to product ions with masses of m/z 152 and m/z 162, respectively. These fragmentation patterns point to a cleavage of the ribose-guanine bond of cGMP in the CID process, leaving $[guanine+H]^+$ (m/z 152) as the charged fragment. In the case of the I.S., the five ^{13}C and the five ^{15}N atoms in this fragment result in a mass of m/z 162. The other five ^{13}C atoms of the I.S. parent ion remained in the uncharged ribose-phosphate leaving group. The described fragment ions were the most abundant ones and were used for detection and quantification. Due to their low intensities (<10% of the total intensity), no additional fragment ions were used as qualifiers in peak identification. No interferences from endogenous substances were observed (see Fig. 1).

Matrix effects were investigated using the I.S. because of its non-endogenous origin. Two sets of samples were compared: samples spiked with I.S. after extraction (matrix effect, but no extraction loss) and aqueous samples of the I.S. (no matrix effect). We found that plasma samples showed a positive matrix effect of $14.7 \pm 4.2\%$ and therefore had a slightly enhanced intensity. The differences in the matrix effect between various matrix sources were very moderate. In 20 different human plasma samples we found an inter-sample coefficient of variation of 4.3% in the peak areas of the I.S. However, owing to the use of a stable isotope labeled I.S., the quantification was not impaired by any matrix effect in a given sample.

3.4. Validation

In the calibration range between 1 and 20 nM, a linear weighed ($1/x$) least square function could be applied. A calibration from spiked water samples resulted in the parameters slope of 0.0482 ± 0.0006, an insignificant ($p = 0.766$) intercept of 0.0009 ± 0.030 and a correlation coefficient of $R^2 = 0.999$, while the respective parameters of the calibration from plasma samples were a slope of 0.0470 ± 0.0005, an intercept of 0.0832 ± 0.0027 and $R^2 = 1.000$. The significant intercept in the plasma calibration reflected the endogenous amount of 1.77 nM cGMP in these samples. The differences in the slopes between the calibration functions of the two matrices were less than 3% and therefore negligible. In consequence, the calibration from water samples was usable to quantify cGMP in unknown plasma samples. This matrix independence of the method illustrated the advantages of an isotope labeled I.S., which compensates for any matrix effects.

The lower limit of quantification (LOQ) was set to the lowest concentration of the calibration range, i.e. 1 nM. The limit of detection (LOD) was explored with a dilution series of the I.S. in plasma and was found to be about 0.1 nM at a signal-to-noise ratio of 3.

Inter- and intra-day precision and accuracy was tested by repeated quantifications of cGMP in the QC-samples. Furthermore, to explore the applicability of the method at low concentrations, a plasma sample was diluted with water to reach cGMP concentra-

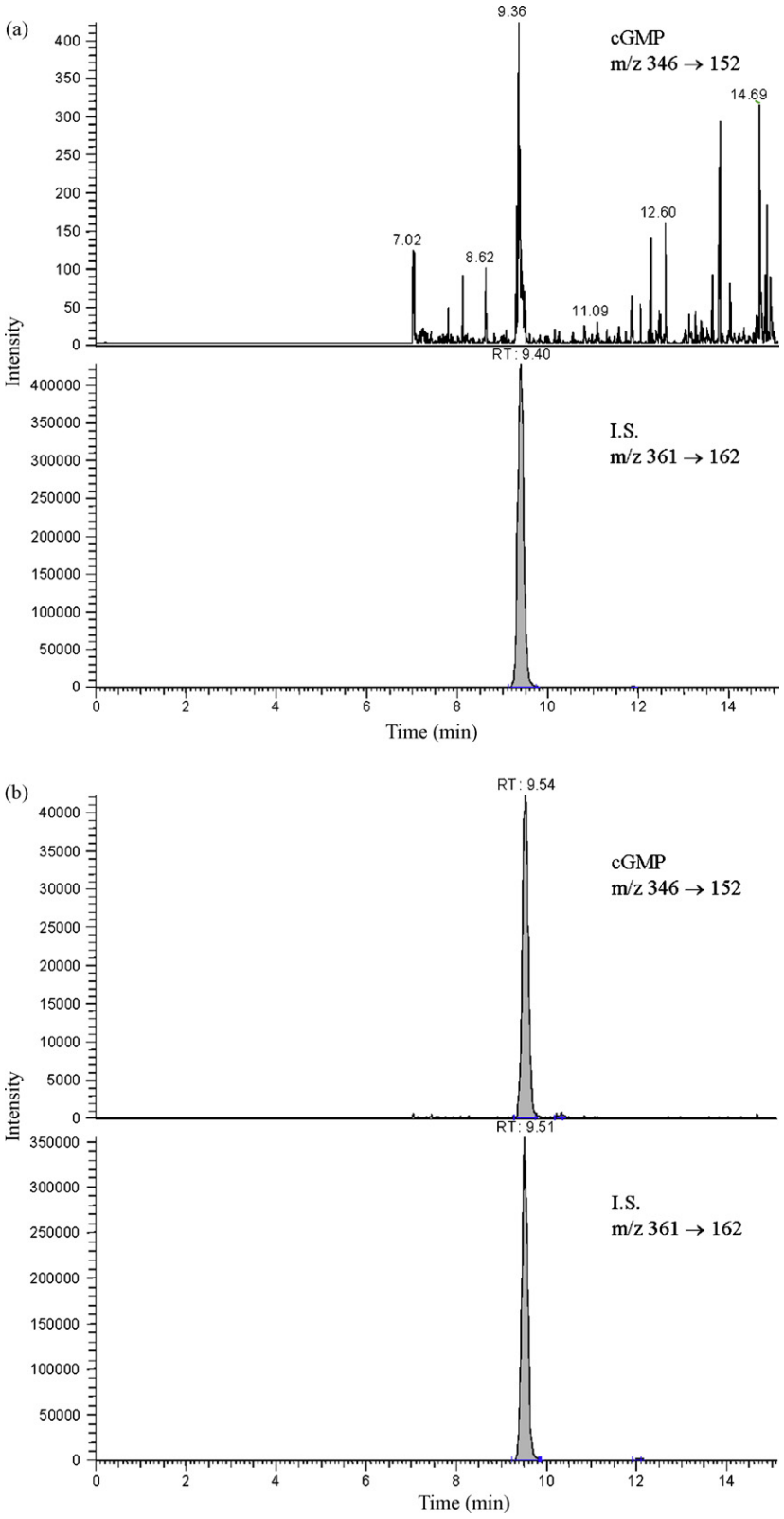

Fig. 1. Typical chromatograms of cGMP (upper panel) and its I.S. (lower panel). (a) Depicts a blank sample from water, containing no cGMP. (b) Depicts an EDTA plasma sample from a healthy volunteer containing 2.59 nM cGMP.

tions in the range of the LOQ, and was repeatedly analyzed ($n = 5$). The results are summarized in Table 1. The low relative standard deviations and good accuracies reflected the advantages of the utilization of an isotope labeled I.S. and are satisfying the requirements for bioanalytical method validation.

3.5. Application of the method

The concentration of cGMP was determined in 20 EDTA plasma samples from apparently healthy human subjects. We found values of 3.92 ± 1.17 nM. This is comparable to results obtained by other

Table 1
Intra- and inter-day precision and accuracy.

Sample	Matrix	Spike concentration (nM)	Expected concentration (nM)	Found concentration (nM)	R.S.D. (%)	Accuracy (%)
Intra-day dilution ($n = 5$)						
Undiluted	Plasma	0	N/A[a]	3.43	1.33	N/A[a]
Dilution factor 2	Plasma:water, 1:1	0	1.72[b]	1.67	3.75	−2.61
Dilution factor 4	Plasma:water, 1:3	0	0.86[b]	0.92	4.57	7.17
Intra-day ($n = 10$)						
Low	Plasma	1	2.77[c]	2.69	1.72	−2.89
High	Plasma	20	21.77[c]	20.93	1.58	−3.86
Inter-day ($n = 5$)						
Low	Plasma	1	2.77[c]	2.67	2.95	−3.61
High	Plasma	20	21.77[c]	22.27	5.61	2.30

[a] Not applicable.
[b] Found endogenous concentration divided by dilution factor.
[c] Sum of endogenous concentration plus spike concentration.

methods such a RIA (3.3 nM, interquartile range 1.1–5.6 nM [16]) or LC–MS/MS (5.65 ± 2.35 nM [13]). The applicability of the method can therefore be regarded as proven.

4. Conclusion

In this work, we present a reliable, precise and accurate method for the determination of cGMP in human plasma samples. The solid phase sample extraction and concentration and the tandem mass spectrometric detection resulted in high sensitivity and selectivity with peak shapes uncompromised by endogenous substances. Compared to a previously published method for the determination of cGMP from human plasma [13], the here-described method showed slightly better sensitivity (limit of quantification 1 nM vs. 1.5 nM) and improved R.S.D.'s (intra-day 1.6–1.7% vs. 6.0–10.1%, inter-day 3.0–5.6% vs. 5.6–8.1%). While the method is well suited to measure cGMP in plasma samples of human subjects, its matrix independence due to the stable isotope labeled I.S. should make it extendable to other biological media, such as urine or tissue samples, without major difficulties. The method provides therefore an important tool for scientific investigations and progress in this field.

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