Journal of Chromatography B, 877 (2009) 513–520

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Development and validation of an LC–MS/MS method for quantification of cyclic guanosine 3′,5′-monophosphate (cGMP) in clinical applications: A comparison with a EIA method

Yanhua Zhang[a,*], Dawn Dufield[b], Jon Klover[b], Wenlin Li[c], Gabriella Szekely-Klepser[d], Christopher Lepsy[a], Nalini Sadagopan[d,**]

[a] Department of Pharmacokinetics, Dynamics & Metabolism, Pfizer Global Research & Development, Groton Laboratories, Eastern Point Road, Groton, CT 06340, United States
[b] Molecular Biology, Pfizer Global Research & Development, 700 Chesterfield Pkwy, Chesterfield, MO 63017, United States
[c] Department of Pharmacokinetics, Dynamics & Metabolism, Pfizer Global Research & Development, 10646 Science Center Drive, San Diego, CA 92121, United States
[d] Department of Pharmacokinetics, Dynamics & Metabolism, Pfizer Global Research & Development, Michigan Laboratories, Ann Arbor, MI 48105, United States

ARTICLE INFO

Article history:
Received 7 October 2008
Accepted 26 December 2008
Available online 6 January 2009

Keywords:
cGMP
Biomarker
Human plasma
LC–MS/MS
EIA
Comparison

ABSTRACT

An LC–MS/MS method was developed and validated to quantify endogenous cyclic guanosine 3′,5′-monophosphate (cGMP) in human plasma. The LC–MS/MS and competitive enzyme immunoassay (EIA) assays were compared. cGMP concentrations of 20 human plasma samples were measured by both methods. For the MS-based assay, plasma samples were subjected to a simple protein precipitation procedure by acetonitrile prior to analysis by electrospray ionization LC–MS/MS. De-protonated analytes generated in negative ionization mode were monitored through multiple reaction monitoring (MRM). A stable isotope-labeled internal standard, $^{13}C_{10}$,$^{15}N_5$-cGMP, which was biosynthesized in-house, was used in the LC–MS/MS method. The competitive EIA was validated using a commercially available cGMP fluorescence assay kit. The intra-assay accuracy and precision for MS-based assay for cGMP were 6–10.1% CV and −3.6% to 7.3% relative error (RE), respectively, while inter-assay precision and accuracy were 5.6–8.1% CV and −2.1% to 6.3% RE, respectively. The intra-assay accuracy and precision for EIA were 17.9–27.1% CV and −4.9% to 24.5% RE, respectively, while inter-assay precision and accuracy were 15.1–39.5% CV and −30.8% to 4.37% RE, respectively. Near the lower limits of detection, there was little correlation between the cGMP concentration values in human plasma generated by these two methods ($R^2 = 0.197$, $P = 0.05$). Overall, the MS-based assay offered better selectivity, recovery, precision and accuracy over a linear range of 0.5–20 ng/mL. The LC–MS/MS method provides an effective tool for the quantitation of cGMP to support clinical mechanistic studies of curative pharmaceuticals.

1. Introduction

The nucleotide cyclic guanosine 3′,5′-monophosphate (cGMP) is an intracellular second messenger that plays a key role in mediating cellular responses to various hormones and neurotransmitters [1–3]. Levels of cGMP are tightly regulated at the level of both its synthesis by guanylate cyclase (GC) and its degradation by cyclic nucleotide phosphodiesterases (PDEs). PDEs consist of 11 families with over 60 isoforms [4,5], and they are regarded as potential targets for new drug development opportunities spanning multiple indications. Inhibition of the PDE isoenzymes causes an increase in intracellular cGMP concentration, which could be potentially linked to therapeutic effects. Hence, cGMP could serve as a mechanistic biomarker for the pharmacological inhibition of PDEs by specific chemical entities [6,7], and the accurate measurement of its concentration could help to understand the underlying mechanisms and possibility of therapeutic interventions.

For a clinical biomarker study, the goal is to apply an analytical method that is capable of generating precise and accurate data for critical decision-making [8,9]. When multiple assays or platforms are available, a suitable bioanalytical method must be identified and selected before clinical studies are undertaken. The method of choice is dependent upon whether it adequately performs with respect to sensitivity, accuracy, precision, selectivity, dynamic range, robustness, and sample integrity in the biological matrix [9]. Based upon our in-house preclinical data, the window of cGMP modulation by PDE inhibitors is known to be narrow, yet statistically significant. Therefore, in order to confidently distinguish cGMP levels between those of a control population and various drug-treated populations, the use of an analytical technique that provides high precision and accuracy is essential.

* Corresponding authors. Tel.: +1 860 686 9301; fax: +1 860 686 1060.
** Corresponding authors.
E-mail address: jenny.y.zhang@Pfizer.com (Y. Zhang).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.12.063

A number of analytical methods, including radioimmunoassay (RIA) [10], non-isotopic immunoassays based on the enzyme-linked immunosorbent assay (ELISA) [11–13], fluorescent detection [14,15], HPLC [16], and mass spectrometry [17,18], have been reported for the measurement of cGMP in various biological matrices. Among them, the ELISA assay in a competitive enzyme immunoassay (EIA) format is the most commonly used quantitative procedure for measurement of cGMP. The method is based on the competition of endogenous cGMP and horseradish peroxidase (HRP)-conjugated cGMP for binding to an anti-cGMP antibody coated onto microtiter plates. EIA methods have demonstrated high sensitivity and throughput capacity for the quantification of cellular antigens with minimum sample handling [12,19]. However, there are limitations associated with them. The cost of an EIA kit is relatively high, making large-scale clinical sample analyses expensive, especially if further extraction or dilution steps are involved in the sample preparation procedures. Furthermore, some EIA kits or methods lack sufficient specificity. Considering that other structurally similar nucleotides exist in human plasma, EIAs may not provide specificity sufficient for the determination of cGMP if immunological cross-reactions occur. Additionally, our preclinical data have demonstrated that EIAs are subject to matrix interference, leading to lower precision and accuracy when measuring cGMP in several biological samples.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is a viable technology for the determination of cGMP due to its specificity, reproducibility and sensitivity. LC–MS/MS is highly specific, thus minimizing interferences from other nucleotides. The greater specificity of LC–MS/MS is derived from analyte specific precursor to product ion mass-to-charge (m/z) values and/or analyte specific retention time. Additionally, the precision and accuracy of the LC–MS/MS assay can be significantly improved if a synthetic stable isotope-labeled internal standard is utilized for the sample preparation procedure [7,20–22]. LC–MS/MS methods have been previously reported to measure cGMP levels in various biological samples [17,18,23]. Sample pre-treatments performed using these methods, however, were excessive compared to the assay described here. The use of tetrabutylammonium bromide as an ion-pairing agent in these methods is less favorable due to its high background contribution and potential source contamination.

The aim of the present study was to develop and validate a rapid, simple, reliable and reproducible LC–MS/MS assay for the determination of cGMP in human plasma, and this method was compared with a commercially available EIA assay that is in routine use for preclinical studies. Endogenous cGMP concentrations in plasma samples of 20 healthy subjects were then analyzed using both methods. The LC–MS/MS method offered better precision and accuracy when compared to the EIA method, making it a better choice for use in clinical settings.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade water, methanol, acetonitrile and formic acid were obtained from Mallinckrodt (Paris, KY). cGMP, dithiothreitol (DTT), HEPES, $MgCl_2$ and bovine serum albumin (BSA) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Stable isotope-labeled guanosine-$^{13}C_{10}$,$^{15}N_5$ 5-triphosphate (GTP) was obtained from Isotec (St. Louis, MO). 3-Ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC_{12}) was obtained from Calbiochem (San Diego, CA). CatchPoint™ Cyclic-cGMP Fluorescent Assay Kits for the EIA method were purchased from Molecular Devices (Sunnyvale, CA). All other solvents were ordered from commercial sources with the highest purity grades available, and used without further processing. Human EDTA plasma was acquired from randomly in-house donors. All subjects were non-smokers and were taking no medications.

2.2. Biosynthesis of $^{13}C_{10}$,$^{15}N_5$-cGMP

Stable isotope-labeled internal standard cGMP was biosynthetically prepared from stable isotope-labeled GTP ($^{13}C_{10}$,$^{15}N_5$-GTP) using soluble guanylate cyclase (sGC) activated by NOC_{12}, a nitric oxide (NO) donor. The synthesis mechanism is shown in Fig. 1. The reaction was performed at 37 °C in a 1.5 mL Eppendorf microcentrifuge tube. Incubated mixtures contained 0.1 M DTT, 1 M HEPES, 0.1 M $MgCl_2$ (pH 7.4), 100 mM $^{13}C_{10}$,$^{15}N_5$-GTP, 20 μL sGC and water. His-tagged sGC was purified from BIIC cells in house (Pfizer Inc., St. Louis). The reactions were initiated by the addition of 20 mM NOC_{12}, followed by brief shaking. After an 8-h incubation, the reaction was terminated by addition of 1 M $NaPO_4$ (pH 4.0). A 15 μL aliquot was taken every hour in order to determine the reaction progress, and the volume taken was replaced with 15 μL of 20 mM NOC_{12} to maintain enzyme activity.

2.3. HPLC methods for determination and purification of $^{13}C_{10}$,$^{15}N_5$-cGMP

$^{13}C_{10}$,$^{15}N_5$-GTP and $^{13}C_{10}$,$^{15}N_5$-cGMP concentrations were monitored by analytical HPLC analysis. The HPLC used was an Agilent HP1100 system consisting of an autosampler, a pump system and a UV detector (Agilent Technologies, Palo Alto, CA). Analytes were separated on a Restek (Bellefonte, PA) Ultra IBD column (4.6 mm × 100 mm, 3 μm particle), using a gradient solvent system

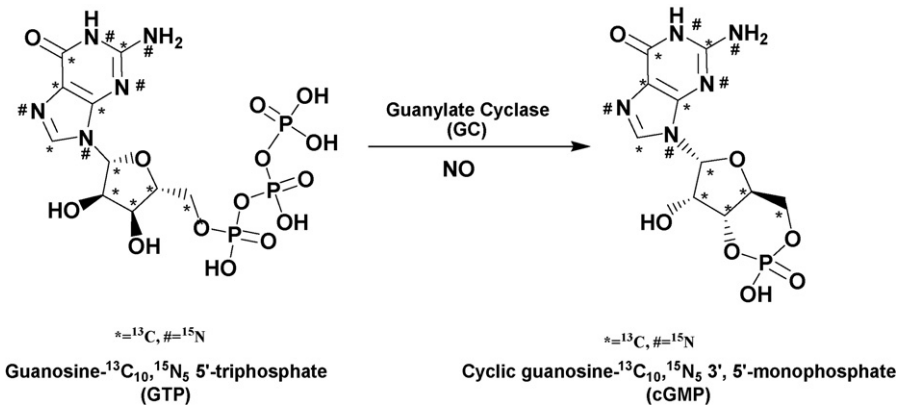

Fig. 1. Biosynthesis of $^{13}C_{10}$,$^{15}N_5$-cGMP.

consisting of solvent A (0.1 M NaPO4/methanol, pH 4.3, 90:10, v/v) and solvent B (methanol). The percentage of solvent B was increased from 10% to 60% over 5 min with the flow rate set at 1.0 mL/min. After 5 min, the percentage of solvent B was increased to 80% within 2 min, before re-equilibrating with the initial mobile phase. Aliquots (10–40 μL) of the reconstituted samples were directly injected onto the column. The wavelength of UV detection was set as 254 nm.

The $^{13}C_{10}$,$^{15}N_5$-cGMP was collected and purified using a preparative HPLC analysis method. The HPLC system consisted of an Agilent HP 1100 system equipped with a UV detector. The UV wavelength was set at both 215 and 254 nm. The chromatographic separation was achieved using a Restek Ultra IBD column (10 mm × 250 mm, 5 μm). The mobile phases consisted of solvent A (0.1 M $NaPO_4$/methanol, pH 4.3, 90:10, v/v) and solvent B (methanol/water, 50:50, v/v). A linear gradient was used to increase % B from 0% to 50% in 12 min. The flow rate was set at 5.0 mL/min. To identify the $^{13}C_{10}$,$^{15}N_5$-cGMP peak and determine the yield, fractions were collected and analyzed using the analytical HPLC method described above. The phosphate salt was converted to a TFA-modified salt by running samples through a Vydac C18 column (Hesperia, CA), 10 mm × 250 mm, at a flow rate of 4 mL/min. The mobile phase composition used for desalting was 0.05% TFA and acetonitrile. The separation was achieved using a gradient of 0–5% acetonitrile over 12 min. Relevant fractions were pooled and lyophilized to dryness.

2.4. Instrumentation

The LC–MS/MS system consisted of a Shimadzu HPLC system (Columbia, MD) and a Sciex API 4000 triple quadropole mass spectrometer (Applied Biosystems, Toronto, Canada). The HPLC system consisted of two Shimadzu LC-10ADvp pumps equipped with a SCL-10Avp controller and an HTS PAL autosampler (LEAP Technologies, Carrboro, NC). Chromatographic separation was achieved using a Metasil AQ C18 column (Varian, Palo Alto, CA), 100 mm × 2.1 mm, 5 μm particle size. Gradient elution with solvent A (0.1% formic acid) and solvent B (100% acetonitrile) at a flow rate of 0.2 mL/min was applied. The initial conditions were 100% A, and a linear gradient was performed with solvent B increasing from 0% to 50% within 1.7 min. This condition was maintained for 2.3 min to remove late-eluting substances from the column (column wash), followed by returning the system to its initial conditions with a 3-min equilibration period. The total analysis time including column wash and equilibration was 7 min. Sample storage in the autosampler and the separation both took place at room temperature.

Quantification of the analytes was performed using electrospray LC–MS/MS technique in multiple reaction monitoring (MRM) mode. A Sciex API 4000 triple quadropole mass spectrometer was operated in negative ionization mode, with Analyst version 1.4 controller software. The source conditions were set as follows: ionspray voltage −4.0 kV, ion source temperature 450 °C, nebulizer gas 45 psi, turbo gas 45 psi, and curtain gas 25 psi. The dwell time for both cGMP and $^{13}C_{10}$,$^{15}N_5$-cGMP was 200 ms. For analytes of interest, precursor-to-product ion transitions were established through direct infusion of neat standard of each compound into the ion source. The following ion transitions were obtained: cGMP m/z 344 → 150 and $^{13}C_{10}$,$^{15}N_5$-cGMP m/z 359 → 160. Sensitivity was optimized for individual compounds by manipulating values of entrance potential (EP), declustering potential (DP), collision energy (CE) and collision exit potential (CXP) in order to achieve the best signals. For this analysis, EP, DP, CE and CXP were as follows: −10 V, −80 V, −35 eV, and −12 V.

The EIA for cGMP measurement was performed using 96-well plate format kits. Plates were read on a Molecular Devices SpectraMax GeminiXS Plate Reader (Molecular Devices, Sunnyvale, CA). SoftMax Pro software was used to control the spectrophotometer and to analyze the absorbance data.

2.5. Standard curve and internal standard preparation

2.5.1. LC–MS/MS

A cGMP stock solution, containing 1 mg/mL cGMP in 50% methanol, was prepared and then serially diluted in water to generate an 8-point standard curve ranging from 0.5 to 20 ng/mL. A 25 ng/mL $^{13}C_{10}$,$^{15}N_5$-cGMP (internal standard) working solution was prepared in acetonitrile.

2.5.2. EIA

For the EIA assay, cGMP standards were prepared from a stock solution of 30 μM cGMP in assay buffer (PBS containing 50 mg/mL BSA) and diluted with the assay buffer to generate standard cGMP solutions with concentrations of 0.05, 0.14, 0.41, 1.28, 3.8, 11.4 and 1150 ng/mL. A 0 ng/mL standard (buffer or extracted BSA buffer only) was also used in the generation of the standard curve as an anchor point. A 40 μL aliquot of each of these eight standards was added in duplicate to the EIA plate in order to obtain an 8-point standard curve.

2.6. QC preparation

Human plasma samples in EDTA from 20 subjects were collected and analyzed to determine the endogenous basal level of cGMP. Equal volumes of plasma samples from seven subjects who had the lowest levels of cGMP were pooled together and used as the QC matrix. For comparison purposes, the same QC matrix was used for both LC–MS/MS and EIA assays. The mean value of endogenous basal levels of pooled samples was used in the calculations for the preparation of each QC level. QC samples at four concentrations ((lower limit quality control (LLQC), low quality control (LQC), medium quality control (MQC), and high quality control (HQC)), which covered the full range of the intended calibration range, were prepared by spiking this pooled matrix with appropriate amounts of cGMP standard solution to yield final concentrations as described herein: (1) for the LC–MS/MS assay, 2-fold water-diluted matrix (LLQC), matrix + 0 ng/mL (LQC), matrix + 5 ng/mL (MQC), and matrix + 15 ng/mL (HQC); and (2) for the EIA assay, 1.5-fold water-diluted matrix (LLQC), matrix + 0 ng/mL (LQC), matrix + 5.18 ng/mL (MQC), and matrix + 15.5 ng/mL (HQC). In order to successfully compare the two methods, the QC levels for LC–MS/MS and EIA assays were selected to be close to one another. Table 1 summarizes the QC preparations for LC–MS/MS and EIA assays.

2.7. Sample preparation

2.7.1. LC–MS/MS

A typical quantitative assay for cGMP consisted of an 8-point standard curve, blank (water), blank spiked with internal standard, matrix blank (QC matrix sample without internal standard), QCs and unknown samples. All the standard and QC samples of 100 μL were transferred into individual wells of a 96-well polypropylene plate. Samples were precipitated with 300 μL of cold acetonitrile

Table 1
Summary of QC preparation for LC–MS/MS and EIA assays.

Sample ID	LC–MS/MS	EIA
LLQC	2-Fold water-diluted matrix	1.5-Fold water-diluted matrix
LQC	Unspiked matrix	Unspiked matrix
MQC	Matrix + 5 ng/mL	Matrix + 5.18 ng/mL
HQC	Matrix + 15 ng/mL	Matrix + 15.5 ng/mL

containing 25 ng/mL $^{13}C_{10}$,$^{15}N_5$-cGMP. The samples were shaken for 10 min and then centrifuged at 4000 rpm for 15 min. A 300 μL aliquot of the supernatant was then transferred to a second 96-well plate and dried for approximately 15 min under nitrogen at 37 °C. Samples were reconstituted by adding 75 μL of water to each well. The samples were shaken for 5 min and then analyzed by LC–MS/MS.

2.7.2. EIA

To remove interfering substances that could cause a significant number of false-positive results, a simple extraction procedure was initially performed. A 300 μL aliquot of acetonitrile was added to 100 μL of unknown samples, standards and controls in a deep 96-well plate. The plate was vortexed for 5 min and then centrifuged at 4500 rpm for 5 min. A 250 μL aliquot of supernatant was removed and evaporated to dryness using a SpeedVac. The lyophilized samples were then resuspended with 125 μL of assay buffer. Then, following the EIA protocol, 40 μL aliquots of unknown samples, standards, and QCs were transferred into individual wells of the EIA plate, followed by adding 40 μL of reconstituted rabbit-anti-cGMP antibody to each of the wells. The microplate was gently agitated on a plate shaker for 5 min, and 40 μL of reconstituted HRP-cGMP conjugate solution was then added to all wells of the microplate. After gently mixing, the plate was incubated for 2 h at room temperature. Upon completion of the incubation period, the plate was washed four times with 300 μL of wash buffer to remove the material that was not bound to the antibody on the plate, and 100 μL of Stoplight Red substrate solution was then added to every well as quickly as possible. The microplate was covered with adhesive seal (protected from light). After a 2-h incubation at room temperature, sample concentrations were determined using a fluorescence plate reader at the fluorescence intensity settings of 530–25 nm for excitation and 590–20 nm for emission.

3. Results and discussion

3.1. LC–MS/MS assay development and validation

3.1.1. Biosynthesis of a stable isotope-labeled internal standard

It is well known that MS-based assays can be affected by matrix-induced ion suppression from biological samples. Ion suppression occurs when an endogenous matrix component eluted from the HPLC column influences the ionization of a coeluting analyte. When developing a biomarker assay, it is especially important to take ion suppression into consideration because the matrices used for standard curve preparation and unknown samples are different (see Section 3.1.3 for details). One approach, known as the isotope-dilution LC–MS/MS method, has been extensively reported to compensate for ion suppression and ensure acceptable recovery and precision of the targeted analyte. In this procedure, a stable isotope-labeled analog, with identical physical and chemical properties as the analyte, is used as an internal standard [7,20–22]. Stable isotope-labeled internal standards enable a more effective reduction of ion suppression compared to a structure analog internal standard, a requirement for bioanalytical methods used in a GLP environment [20,24]. Therefore, the first step in developing a reliable biomarker method is the identification and selection of a suitable stable isotope-labeled internal standard [20]. Considerations should include chemical and isotopic purity, stability, interference with the unlabeled analyte, interference from the unknown matrix, availability, and cost [20]. The stable isotope-labeled internal standard for cGMP was not commercially available and therefore, was synthesized in-house using an isotopically labeled precursor, $^{13}C_{10}$,$^{15}N_5$-GTP, through a biosynthetic pathway, as shown in Fig. 1. The purity of $^{13}C_{10}$,$^{15}N_5$-cGMP was determined by HPLC–UV and LC–MS analysis; the product showed 99% chemical and isotopic purity.

3.1.2. LC–MS/MS optimization

Stock solutions of cGMP and $^{13}C_{10}$,$^{15}N_5$-cGMP were infused into the ion source of the mass spectrometer set in both positive and negative ionization modes. Although positive ion detection yielded higher signal-to-noise ratios for the pseudomolecuar ions of both compounds, it also allowed the introduction of interference from human plasma extracts in the MRM channel for the stable isotope-labeled cGMP (+15 *m*/*z* cGMP) used in this study. In contrast, the channel for $^{13}C_{10}$,$^{15}N_5$-cGMP in negative ionization mode was free of interfering components (Fig. 2). Therefore, the optimization of LC conditions was carried out by the monitoring ion current representing $[M-H]^-$ pseudomolecular ions at *m*/*z* 344 (cGMP) and 359 ($^{13}C_{10}$,$^{15}N_5$-cGMP). The best ionization response was found using a mixture of acetonitrile and 0.1% formic acid buffer (50:50, v/v).

The column selection for the retention of cGMP, due to its hydrophilic nature (CLOGP = −3.36), was challenging. To achieve optimized chromatographic resolution, a variety of LC columns packed with different packing materials, including C18, phenol,

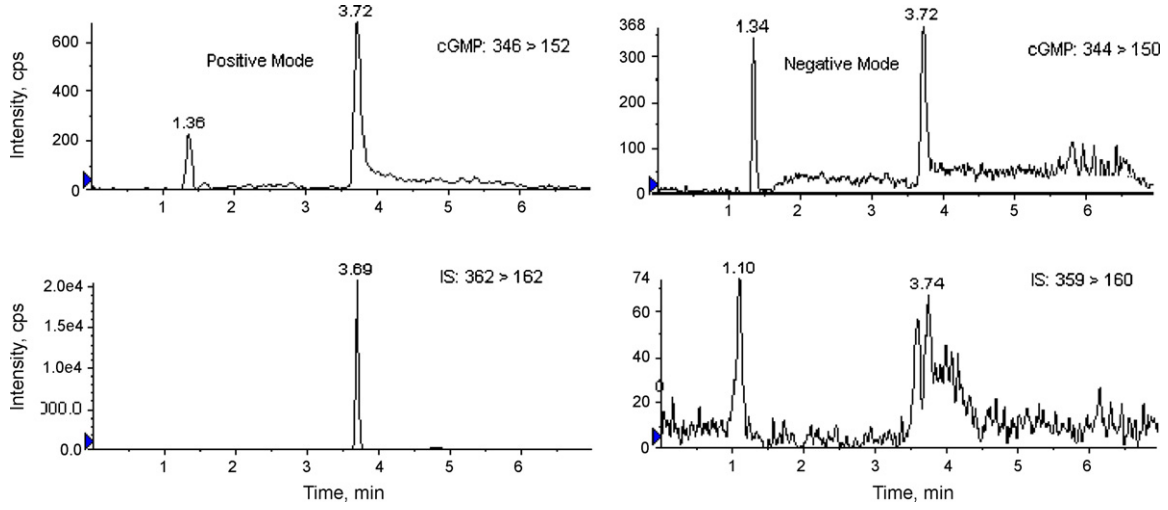

Fig. 2. MRM chromatograms obtained after injecting extracts from unspiked human plasma sample without internal standard in positive (left) or negative (right) ionization mode. The channel for internal standard ($^{13}C_{10}$,$^{15}N_5$-cGMP) in positive ionization mode has interfering components, while the channel in negative ionization mode is free of interfering components.

amide, and HILIC columns, were evaluated. In general, the retention time of cGMP on C18 columns was greater compared to the phenol and amide columns, due to its hydrophilic end-capping groups. The separation performance of a Varian Metasil AQ (5 μM, 100 mm × 2.0 mm) column was similar to that of a Waters Atlantis HILIC Silica (3 μM, 100 mm × 2.1 mm) normal phase column. The retention time of cGMP when using a Varian Metasil AQ column was approximately 10% longer than that obtained using an Atlantis HILIC Silica column under optimized LC gradient conditions with a flow rate of 0.2 mL/min. While both columns were capable of separating analytes from the solvent front, the Varian Metasil AQ was the column of choice since it provided a slightly longer retention time and larger capacity factor with acceptable peak shape. For quantitative endogenous analysis, greater matrix interference is often encountered when compared to PK drug analysis [8], which can be partially addressed through longer chromatographic retention of analytes. The greater capacity factor decreases the likelihood of matrix or ion suppression effects and optimizes elution conditions, facilitating the achievement of excellent and sufficient sensitivity on the mass spectrometer [25].

3.1.3. Selection of the standard curve matrix

Developing biomarker assays and validating them are generally more complicated than those for most PK drug assays [8,9]. Since no blank matrix sample exists that is free of endogenous analyte, the choice of the standard curve matrix for the analysis is challenging [8,26]. Several approaches have been reported to prepare an analyte-free standard curve matrix, such as applying either non-specific or affinity removal of the analyte, using a heterologous matrix from a different species, using a non-matrix buffer solution, or using a surrogate analyte [8,20,21]. The approach taken in our laboratory was to combine an analyte-free substitute matrix and matrix-based QC samples to evaluate performance of the assay [27]. In order to select a suitable standard curve matrix, the standard curve for the cGMP assay was initially prepared and evaluated in three different potential substitute matrices: charcoal stripped human plasma, 10 μg/mL BSA and water. Because trace levels of cGMP were detected in the charcoal stripped human plasma, the calibration curve prepared in this substitute matrix demonstrated poor linearity. The recovery of cGMP from 10 μg/mL BSA was lower than that from water. Water, a non-matrix solution, demonstrated good linearity and recovery and was therefore selected as the standard curve matrix. Ultimately, the assay was transferred to a contract research organization that can support multiple clinical studies with over 4000 clinical samples in multiple batches over the course of more than two years. The robustness of the assay, using water as a substitute matrix, was confirmed for these studies.

3.1.4. Selectivity and recovery

The high intrinsic selectivity and specificity of the LC–MS/MS method was achieved using a combination of different techniques that include protein precipitation, HPLC separation, and mass selective detection [27]. For a typical drug, an exogenous analyte, the assay selectivity is evaluated in several independent sources of blank matrix to demonstrate that no significant level of the targeted analyte is detected [20,27]. For a biomarker, such as an endogenous analyte, assay matrices are complicated due to the presence of often unexpected interference from other endogenous analytes that may be different in different matrices [8]; therefore, the selectivity of the assay was assessed with additional rigor, including evaluating instrument cross-talk, performing precursor ion scans, and examining analyte recovery in the matrix from multiple independent sources [27]. The specificity of this LC–MS/MS method was first evaluated by monitoring ion currents of cGMP in the MRM channel following the injection of five plausibly interfering endogenous analytes. Using the HPLC method described above, the Varian Metasil AQ column was observed to retain endogenous nucleotide analogues of cGMP, 3′,5′-cyclic adenosine monophosphate (cAMP), 3′,5′-cyclic inosine monophosphate (cIMP), adenosine 5′-phosphate (AMP), guanosine 5′-phosphate (GMP), and inosine 5′-phosphate (IMP), but not 3′,5′-cyclic cytidine monophosphate (cCMP), and cytidine 5′-phosphate (CMP). Therefore, cCMP and CMP were less likely to introduce interference caused by analyte cross-talk when compared to cAMP, cIMP, AMP, GMP, and IMP. A cross-talk experiment was performed on these nucleotides to verify the absence of interference with cGMP. A mixture of the nucleotides cAMP, cIMP, AMP, IMP, and GMP, at a concentration of 0.1 mg/mL for each analyte, was monitored with the MRM channel m/z 344 → 150 open (the specific cGMP transition). The channel for cGMP was clear of any interference from other endogenous nucleotides, as indicated by the selected ion chromatogram shown in Fig. 3. It is important to emphasize that no significant interference was observed from cAMP at the selected channel since cAMP and cGMP share the same modulation pathway in humans; furthermore, the level of cAMP can also be modulated by PDE inhibitors. Additionally, potential cross-talk between cGMP and $^{13}C_{10}$,$^{15}N_5$-cGMP was negligible within the concentration range of the assay, as seen in Fig. 4A and D. The Q1 full scan from 200 to 500 m/z was performed for selected blank, standard, and human plasma samples, and the precursor ion of the cGMP was confirmed in the human plasma sample. The recovery in the sample preparation process was also evaluated by comparing the peak response in non-authentic or substitute matrix (water) with that of the authentic matrix (plasma). Nine unique EDTA human plasma samples were prepared in three replicates at three different concentration levels (unspiked matrix, and matrix spiked with 1 and 5 ng/mL cGMP, respectively). Recovery was assessed as the ratio of the measured concentration of spiked sample to the expected concentration of spiked sample (endogenous baseline plus spiked nominal standard concentration) [27]. The mean recovery of cGMP from nine individually processed samples was determined to be in the range of 89.7–104%, suggesting that the recovery was plasma matrix-independent. The selectivity of the assay was confirmed by these high recovery data, suggesting no other endogenous interfering components exist in the human plasma and which can be

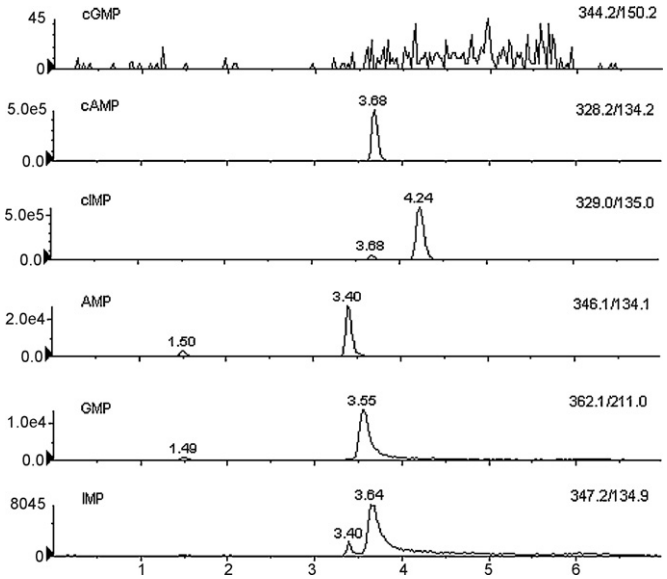

Fig. 3. MRM chromatograms obtained after injecting a mixture of cAMP, cIMP, AMP, GMP, and IMP (excluding cGMP) at a concentration of 0.1 mg/mL for each analyte using the validated LC–MS/MS conditions. MRM transitions and corresponding ion counts are displayed for each analyte. The channel m/z 344 → 150 (cGMP, top chromatogram) is free of any interfering components.

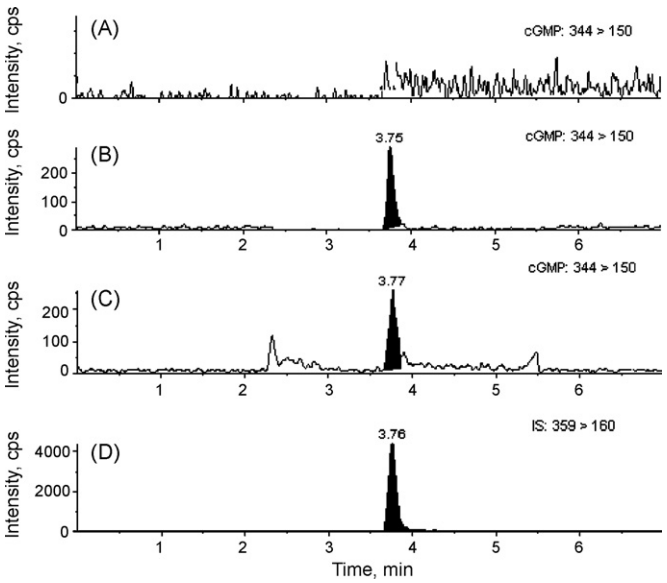

Fig. 4. Representative chromatograms of the solvent blank (A), the LLOQ standard (0.5 ng/mL) (B), EDTA human plasma LLQC sample (1.08 ng/mL) (C), and the stable labeled internal standard (D).

a potential source for variable results between subjects [27]. This also demonstrated that the assay performed in a similar manner in a non-authentic matrix (water) as it did in an authentic sample matrix (plasma). Table 2 shows the recovery data for cGMP in nine independent sources of matrix measured using the LC–MS/MS method.

3.1.5. Linearity and LLOQ

The limit of detection for this assay was 0.1 ng/mL where a signal/noise ratio of 3 was achieved. The LLOQ was 0.5 ng/mL using samples extracted from human plasma. Linearity of the assay was confirmed for the validated range of 0.5–20 ng/mL with a correlation coefficient (R^2) of 0.999. The mean inter-assay accuracy and precision at 0.5 ng/mL were 1% relative error (RE) and 5.4% CV, respectively ($n = 6$). The mean inter-assay accuracy and precision for the rest of the calibration standards were between −3.3% and 2.4% RE and between 1.3% and 6.3% CV, respectively. The assay range was confirmed as appropriate for the measurement of cGMP in human plasma samples collected from multiple clinical studies. Fig. 4B shows a representative chromatogram for the LLOQ standard.

Table 2
Assessment of cGMP recovery in nine independent matrices obtained from LC–MS/MS and EIA methods.

	LC–MS/MS		EIA	
	EB + 1 ng/mL	EB + 5 ng/mL	EB + 2 ng/mL	EB + 10 ng/mL
Source 1	101	96.2	70.7	70.9
Source 2	104	97.1	78.7	77.2
Source 3	101	97.9	77.2	70.0
Source 4	90.0	89.7	93.9	81.6
Source 5	97.3	90.7	82.4	76.5
Source 6	102	98.3	78.3	69.8
Source 7	98.2	91.0	89.1	73.7
Source 8	90.0	94.5	113	60.6
Source 9	95.9	95.4	100	73.9
Mean	97.7	94.5	87.0	72.7
SD	5.1	3.3	12.7	5.9

EB, endogneous basel level.

3.1.6. Precision and accuracy

For a clinical biomarker study, the goal is to find a reproducible assay to generate reliable data that are suitable for strategic decision-making for a drug development program [8,9]. The analytical method must provide the ability to differentiate between normal and disease samples, as well as to evaluate the progression from one state to another [8,9]. In general, assay variability is inversely proportional to differentiation. Most importantly, our experimental results based on preclinical studies indicate that the modulation window for the cGMP by PDE inhibitors is narrow (1- to 2-fold increase), and hence requires an analytical method with the best precision in order to achieve greater differentiation power. The intra-assay precision and accuracy were evaluated using matrix-based QC samples prepared with six replicates at four different concentration levels (LLQC = 0.94 ng/mL, LQC = 1.88 ng/mL, MQC = 6.88 ng/mL, HQC = 16.9 ng/mL), while the inter-assay precision and accuracy were evaluated with three consecutive assay runs. Endogenous cGMP levels in the unspiked undiluted blank plasma QC (LQC) were determined by calculating the mean concentration of all LQCs ($n = 18$) as measured in the three validation batch runs. This value (1.88 ng/mL) was used to calculate the nominal concentrations of the spiked and diluted QCs. The analysis of the plasma-based QC samples demonstrated acceptable precision and accuracy based on validation criteria (±20% CV and RE for LLQC and ±15% CV and RE for the rest of the QCs). The intra-run precision and accuracy were between 6% and 10.1% CV and −3.6% to 7.3% RE, respectively. Inter-run precision and accuracy were in the range of 5.6% and 8.1% CV, and −2.1% and 6.3% RE, respectively. Therefore, the assay was demonstrated to be robust and reproducible. The data also suggest that a water-based standard curve can accurately and precisely quantify the analyte in a biological matrix-based QC sample. Table 3 shows the detailed QC accuracy and precision data. Fig. 4C shows the representative MRM chromatographs obtained from EDTA human plasma LLQC samples (1.08 ng/mL).

3.1.7. Dilution linearity

Due to the relatively high endogenous basal level of cGMP in human plasma matrix (1.88 ng/mL), LLQC samples were prepared by diluting the blank matrix with water. This would allow use of the lower end of the calibration curve if samples were incurred in that concentration range. Such dilution resulted in a difference in the biological matrix content between QC samples and unknown samples. Therefore, an assessment of possible variations in extraction recovery and ionization efficiency at the mass spectrometer source caused by changes in the matrix content, which could affect assay accuracy, was necessary. This was accomplished by determining the dilution linearity of cGMP at concentrations across the range of dilution factors used for the QC samples. Three different sources of EDTA human plasma were diluted with HPLC water, each prepared in four replicates at two concentration levels (0- or 2-fold dilution). Our results demonstrated that the dilution of human plasma was concentration and matrix-independent; the accuracy of the back-calculated concentration was 8.9–10.8% RE, with precision of 4.74–9.15% CV, respectively. Thus, the impact on analyte ionization from sample matrix effects, caused by their differences in biological content, was insignificant for cGMP.

3.1.8. Stability assessment

The stability of cGMP in stock solution, in the biological matrix, and in the final extract was evaluated individually. When prepared in the stock solution at the concentration of 1 mg/mL, the cGMP neat standard was determined to be stable for up to six hours at room temperature for at least one month at −20 °C. The analyte after sample processing was verified as being stable for up to 24 h in a 96-well polypropylene plate stored at room temperature. This was demonstrated by performing a statistical comparison of results generated

Table 3
Accuracy and precision data for the cGMP measurement in human plasma for LC–MS/MS and EIA methods.

QC Levels nominal concentration (ng/mL)	LC–MS/MS method				EIA method			
	LLQC (EB/2) 0.94	LQC (EB + 0 ng/mL) 1.88	MQC (EB + 5 ng/mL) 6.88	HQC (EB + 15 ng/mL) 16.9	LLQC (EB/1.5) 0.76	LQC (EB + 0 ng/mL) 1.14	MQC (EB + 5.18 ng/mL) 6.32	HQC (EB + 15.5 ng/mL) 16.6
Intra-run (n = 6)								
Mean (ng/mL)	1.03	1.89	6.65	15.7	0.58	1.08	4.75	13.4
SD	0.07	0.19	0.4	1.31	0.16	0.22	0.85	2.22
%CV	6.3	10.1	6	8.3	27.1	19.9	17.9	16.5
%RE*	7.3	−1.6	−3.6	−7.1	−24.0	−4.9	−24.8	−19.5
Inter-run (n = 3)								
Mean (ng/mL)	1.02	1.88	6.69	15.8	0.65	1.14	4.38	11.9
SD	0.08	0.15	0.38	1.09	0.26	0.41	1.20	1.79
%CV	7.4	8.1	5.6	6.9	39.5	36.0	27.4	15.1
%RE*	6.3	−2.1	−3	−6.5	−14.4	−0.32	−30.8	−28.4

%CV, percent coefficient of variation; %RE, percent relative error, EB, endogneous basel level; LLQC, lower limit quality control; LQC, low quality control; MQC, medium quality control; HQC, high quality control; STD, standard deviation.
* RE calculations based on mean endogenous back-calculated concentration.

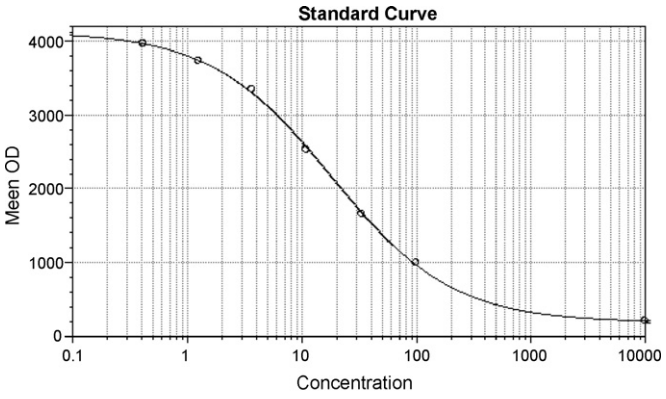

Fig. 5. Standard curve for cGMP in EIA (n = 3).

from the initial injection and subsequent re-injection of one batch in the validation assay. In pooled human plasma, cGMP was confirmed to be stable at room temperature for up to six hours, as well as after three freeze-thaw cycles. Long-term stability of cGMP in human plasma was confirmed at −70 °C for at least 6 months.

3.2. EIA assay validation

For the EIA assay, the limit of quantitation was determined to be 0.05 ng/mL. The calibration curve for cGMP using the EIA is shown in Fig. 5. The calibration curve prepared in the assay buffer was linear, covering the concentration range from 0 to 11.4 ng/mL, where the R^2 value is 0.999 for a 4-parameter curve fit. The mean recovery of cGMP from nine individually processed samples was determined to be between 66.6% and 113%, suggesting that the recovery was plasma matrix-dependent. The intra-assay precision and accuracy was between 17.9% and 27.1% (CV%) and −4.9% to −24.5% (RE%), respectively, while inter-assay precision and accuracy were between 15.1% and 39.5% (CV%), and −30.8% and 4.37% (RE%), respectively. These data suggest that performance of the EIA method was not acceptable across the range 0.05–20 ng/mL based on validation criteria (±20% CV and RE for QCs). Table 2 shows the recovery data for the EIA assay, and Table 3 demonstrates the detailed QC accuracy and precision data for the EIA assay.

3.3. Comparison of performance of LC–MS/MS and EIA methods

Twenty human plasma samples were collected and measured using LC–MS/MS and EIA methods. The mean values of cGMP levels generated from the LC–MS/MS were 1.95 ± 0.81 ng/mL, while cGMP levels for the EIA methods were 0.93 ± 0.41 ng/mL. As can be seen, the EIA produced lower values than the LC–MS/MS method, and there was little correlation between the values produced by these assays in the human plasma samples (R^2 = 0.197, P = 0.05, Fig. 6A). Using another way of graphical representation that helps understand assay concordance, a plot of the difference in concentration values between the methods against the mean concentration values from two methods, we can demonstrate that there was lack of agreement between these two methods for the measurement of cGMP (Fig. 6B) [28]. The differences between these two assays for most samples (18 out of 20) were not significant, while two samples at higher concentrations demonstrated greater disagreement. Overall, the EIA assay appeared to underestimate the cGMP levels, which may partly be explained by the following reasons. First, because the matrix dependence of the isotope-dilution LC–MS/MS assay can be ruled out, it seems that the results produced by the EIA

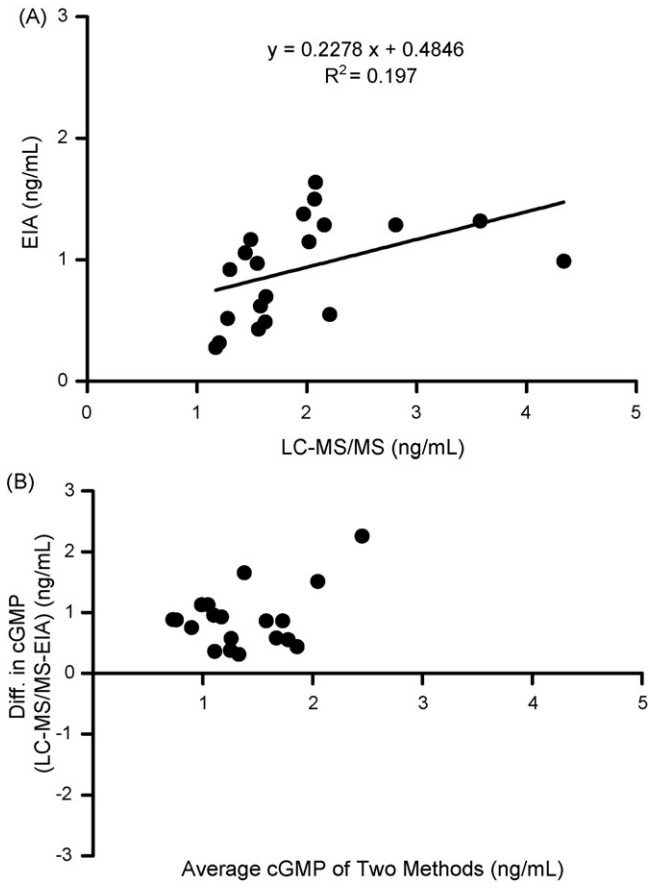

Fig. 6. Comparison of the LC–MS/MS method and EIA for the cGMP measurement in human plasma samples ($n = 20$). (A) Correlation between the LC–MS/MS and EIA methods in human plasma samples ($P = 0.05$). (B) Difference (LC–MS/MS–EIA, ng/mL) against mean for the two methods.

were matrix-dependent. This disadvantage of the EIA has also been reported by Schulze et al. [22] in their publications describing the validation of ELISAs for the measurement of endogenous biomarkers. When the two techniques were compared, the LC–MS/MS assay provided better recovery over the EIA assay, which was evaluated in nine individual human samples at three different concentration levels (unspiked, spiked with 1 and 5 ng/mL cGMP, respectively) (Table 2). The variability in extraction efficiency from different human sample matrices resulting in variable ionization response in the mass spectrometer was corrected in the LC–MS/MS assay using the stable-labeled internal standard, while this was not the case in the EIA assay. Additionally, the calibration for the EIA assay was performed with buffer solution (the supplied calibration samples), which was different from plasma sample. The matrix composition differences between the calibration matrix and plasma samples may cause the offsets of calibration curve. Again this was corrected in the LC–MS/MS assay, which used a water matrix for the calibration curve, with the help of the stable-labeled internal standard. Second, potential non-specific binding of the analyte to the antibodies in the EIA assay can cause low recovery as well; however, the specificity of the EIA assay was demonstrated to be high with little cross-reactivity. Third, this cGMP-EIA kit has been successfully applied to support numerous preclinical cell-based studies with acceptable precision and accuracy. However, in our hands, the reproducibility of the cGMP-EIA kit was poor for the human plasma matrix. The lower reproducibility of the EIA assay suggests that there can be considerable variation in repeated measurements on the same subject [28]. If one method is unable to be continuously repeated, the agreement between the two methods is bound to be poor [28].

Our data also demonstrated that the EIA method had a lower limit of detection (0.05 ng/mL) compared to that of the LC–MS/MS method. However, the LC–MS/MS assay was confirmed to have sufficient sensitivity to support clinical studies, because: (1) analysis of human plasma samples showed the endogenous basal levels of cGMP were above 1 ng/mL; and (2) the modulation of cGMP by PDE inhibitors was expected to be positive.

4. Conclusions

An LC–MS/MS assay was developed for the quantification of cGMP in human plasma, and compared with a commercially available EIA assay. In this assessment, the MS-based assay was determined to be well-suited for the purpose of analyzing clinical samples with better precision, accuracy and recovery, as compared to the EIA assay. For the MS-based assay, the use of an in-house biosynthesized stable isotope-label internal standard was critical to achieving higher precision. In addition to its selectivity and reproducibility, the LC–MS/MS assay can simultaneously monitor the level of cAMP, an alternative biomarker for PDE programs, in the same sample. It is also feasible to measure drug concentrations simultaneously with cyclic nucleotides using this technology. The correlation between these two assays in human plasma appeared to be poor at low, but biological relevant concentrations of cGMP. In conclusion, the LC–MS/MS assay is suitable for clinical investigations in which groups of samples are compared and the endpoint is the shift of the cGMP concentrations in response to an intervention. Currently, this method is being used to support clinical efforts, and has demonstrated its simplicity, robustness and reliability.

References

[1] S.H. Soderling, J.A. Beavo, Curr. Opin. Cell. Biol. 12 (2000) 174.
[2] M. Conti, S.L. Jin, Prog. Nucl. Acid Res. Mol. Biol. 63 (1999) 1.
[3] C. Mehats, C.B. Andersen, M. Filopanti, S.L. Jin, M. Conti, Trends Endocrinol. Metab. 13 (2002) 29.
[4] V.C. Manganiello, E. Degerman, Thromb. Haemost. 82 (1999) 407.
[5] M.D. Houslay, D.R. Adams, Biochem. J. 370 (2003) 1.
[6] D.T. Manallack, R.A. Hughes, P.E. Thompson, J. Med. Chem. 48 (2005) 3449.
[7] E. Stokvis, H. Rosing, J.H. Beijnen, Rapid Commun. Mass Spectrom. 19 (2005) 401.
[8] W.A. Colburn, J.W. Lee, Clin. Pharmacokinet. 42 (2003) 997.
[9] J.W. Lee, V. Devanarayan, Y.C. Barrett, R. Weiner, J. Allinson, S. Fountain, S. Keller, I. Weinryb, M. Green, L. Duan, J.A. Rogers, R. Millham, P.J. O'Brien, J. Sailstad, M. Khan, C. Ray, J.A. Wagner, Pharm. Res. 23 (2006) 312.
[10] A.L. Steiner, A.S. Pagliara, L.R. Chase, D.M. Kipnis, J. Biol. Chem. 247 (1972) 1114.
[11] E. Engvall, K. Jonsson, P. Perlmann, Biochim. Biophys. Acta 251 (1971) 427.
[12] R.A. Dressendorfer, J.M. Heim, R. Gerzer, C.J. Strasburger, J. Immunoassay 16 (1995) 37.
[13] P. Pradelles, J. Grassi, D. Chabardes, N. Guiso, Anal. Chem. 61 (1989) 447.
[14] Y. Umezawa, J. Biotechnol. 82 (2002) 357.
[15] M. Sato, N. Hida, T. Ozawa, Y. Umezawa, Anal. Chem. 72 (2000) 5918.
[16] M.J. Diaz Enrich, J.A. Villamarin, J.I. Ramos Martinez, I. Ibarguren, Anal. Biochem. 285 (2000) 105.
[17] E. Witters, L. Roef, R.P. Newton, W. Van Dongen, E.L. Esmans, H.A. Van Onckelen, Rapid Commun. Mass Spectrom. 10 (1996) 225.
[18] E. Witters, W. Van Dongen, E.L. Esmans, H.A. Van Onckelen, J. Chromatogr. B Biomed. Sci. Appl. 694 (1997) 55.
[19] J.Y. Douillard, T. Hoffman, R.B. Herberman, J. Immunol. Methods 39 (1980) 309.
[20] W. Li, L.H. Cohen, Anal. Chem. 75 (2003) 5854.
[21] M. Jemal, A. Schuster, D.B. Whigan, Rapid Commun. Mass Spectrom. 17 (2003 1723).
[22] F. Schulze, R. Wesemann, E. Schwedhelm, K. Sydow, J. Albsmeier, J.P. Cooke, R.H. Boger, Clin. Chem. Lab Med. 42 (2004) 1377.
[23] R.P. Newton, Biochem. Soc. Trans. 24 (1996) 938.
[24] S. Wang, M. Cyronak, E. Yang, J. Pharm. Biomed. Anal. 43 (2007) 701.
[25] G. Szekely-Klepser, K. Wade, D. Woolson, R. Brown, S. Fountain, E. Kindt, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 826 (2005) 31.
[26] J.A. Wagner, S.A. Williams, C.J. Webster, Clin. Pharmacol. Ther. 81 (2007) 104.
[27] W.W. Li, O. Nemirovskiy, S. Fountain, W. Rodney Mathews, G. Szekely-Klepser, Anal. Biochem. 369 (2007) 41.
[28] J.M. Bland, D.G. Altman, Lancet 1 (1986) 307.