Journal of Pharmaceutical Analysis 11 (2021) 458–464

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

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

Original article

UHPLC-MS/MS analysis of cAMP and cGMP in rat plasma as potential biomarkers of Yin-Yang disharmony in traditional Chinese medicine

Xin Wang [a], Yue Du [a], Cuiting Wu [a], Ming Xu [b], Youping Liu [a], Xin Di [a, *]

[a] *Laboratory of Drug Metabolism and Pharmacokinetics, School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, 110016, PR China*

[b] *Shenyang Analytical Application Center, Shimadzu (China) Co. Ltd., Shenyang, 167 Qingnian Street, Shenyang, 110016, PR China*

ARTICLE INFO

Article history:
Received 20 February 2020
Received in revised form
10 July 2020
Accepted 1 September 2020
Available online 5 September 2020

Keywords:
cAMP
cGMP
Plasma
UHPLC-MS/MS
Yin-Yang disharmony

ABSTRACT

Cyclic 3′,5′-adenosine monophosphate (cAMP) and cyclic 3′,5′-guanosine monophosphate (cGMP) are considered as potential biomarkers for Yin-Yang disharmony in traditional Chinese medicine. However, phosphodiesterase-mediated ex vivo degradation of these molecules in biological samples may result in their underestimation. In the present study, a ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method was developed for determination of cAMP and cGMP in rat plasma, with special consideration of their stability ex vivo. Following precipitation of proteins from plasma samples with 0.4 M perchloric acid, the analytes were chromatographed on a Shimadzu Shim-pack-XR-ODS II column with 2.5 mM ammonium acetate and methanol in gradient mode. The MS/MS detection was performed using multiple reaction monitoring in the positive electrospray ionization mode. The lower limit of quantification was 0.27 ng/mL for cAMP and 0.37 ng/mL for cGMP. The method was used to determine the plasma cAMP and cGMP levels in normal and Yin deficiency diabetic rats treated with or without *Rehmannia glutinosa*. The developed method may be useful for evaluating the regulatory effects of Chinese herbal medicine on the levels of cAMP and cGMP in the body.
© 2020 Xi'an Jiaotong University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Yin and Yang are the two most fundamental concepts in traditional Chinese medicine (TCM). Yin signifies coolness, moisture, stillness, and contraction, whereas Yang signifies heat, dryness, motion, and expansion. Yin and Yang are opposite, complementary, interdependent, and inseparable forces that coexist in the body [1]. In TCM, it is believed that good health comes from the balance of Yin and Yang in the body, whereas disease is caused by an excess or deficiency of Yin or Yang, which is referred to as "Yin-Yang disharmony" [2]. Therefore, TCM treatment lays emphasis on regulating the balance between Yin and Yang in the body through the use of herbal medicines, acupuncture, moxibustion, cupping and scraping [3–5].

The concepts of Yin and Yang in TCM for treatment of diseases are not easily accepted by the Western world. In the 1970s, Goldberg et al. [6,7] and Schuhmacher et al. [8] proposed that antagonistic actions of cyclic 3′,5′-adenosine monophosphate (cAMP) and cyclic 3′,5′-guanosine monophosphate (cGMP) are involved in the regulation of cellular functions, much like the Yin and Yang concept. An appropriate balance of cAMP and cGMP within cells correlates with the balance of Yin and Yang at the cellular level. Evidence has been accumulated that an increase in the cAMP/cGMP ratio in the body corresponds to Yin deficiency and a decrease in this ratio corresponds to Yang deficiency [9–12]. Thus, cAMP and cGMP could be used as potential biomarkers of Yin-Yang disharmony in TCM.

Monitoring of cAMP and cGMP in biological specimens is mostly conducted using commercially available enzyme-linked immunosorbent assay (ELISA) kits owing to their simplicity, high throughput, adequate sensitivity and low cost [13–15]. However, the high incidence of matrix effects caused by cross-reactive or nonspecific reactions with the components of biological matrix may lead to the loss of precision and accuracy in measurements [16]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) combines the excellent separation power of liquid chromatography with the high specificity and sensitivity of mass spectrometry, and allows for rapid qualitative and quantitative analysis

Peer review under responsibility of Xi'an Jiaotong University.
* Corresponding author.
E-mail address: dixin63@hotmail.com (X. Di).

https://doi.org/10.1016/j.jpha.2020.09.001
2095-1779/© 2020 Xi'an Jiaotong University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

of complex samples. There has been an increase in the application of LC-MS/MS for analyzing cAMP and/or cGMP in biological specimens [16–23]. Although LC-MS/MS has the advantages of high specificity, sensitivity, and reproducibility, underestimation of cAMP and cGMP levels may occur without appropriate preanalysis treatment of samples [23]. Because of the presence of enzymatically active phosphodiesterases (PDEs) in biological specimens, ex vivo hydrolysis of cAMP and cGMP can occur during the collection and preparation of samples. To achieve accurate quantification, effective strategies to stabilize cAMP and cGMP in biological samples ex vivo are urgently needed when developing an LC-MS/MS method. Recently, a robust LC-MS/MS assay for the measurement of cAMP and cGMP in rat brain was developed by Chen et al. [23]. Microwave irradiation of rat brain to deactivate PDEs, followed by rapid homogenization with 0.4 M perchloric acid to further stabilize cAMP and cGMP, was used in sample collection. Acceptable frozen storage, freeze-thaw, bench-top, and post-preparation stability data were obtained using this method. This microwave euthanasia method is especially useful for harvesting the brain tissue in rats, but seems unnecessary for body fluids (for which there is no need to kill the animals). Previously, Van Damme et al. [20] reported the use of 3-isobutyl-1-methylxanthine (IBMX), a non-specific inhibitor of PDEs, for the stabilization of cAMP and cGMP in human blood and plasma. However, the bench-top stability of cAMP and cGMP in IBMX-quenched whole blood and plasma was not investigated in their work. Oeckl et al. [21] reported the use of 0.4 M perchloric acid for protein precipitation of mice plasma obtained from ethylene diamine tetraacetic acid (EDTA) treated whole blood and the resulting supernatant was stored at −80 °C until analysis. Unfortunately, no stability data were provided in their study. Without sufficient evidence of stability, as described above, it is uncertain whether cAMP and cGMP are completely stable during the collection of blood and preparation of plasma samples.

In the present study, we aimed to establish an accurate and reliable ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for simultaneous determination of cAMP and cGMP in rat plasma, and perform a detailed investigation of the stability of analytes across pre-analytical procedures. For the first time, we reported the development of overall strategies to prevent the degradation of cAMP and cGMP during the collection of blood and preparation of plasma samples. The method developed in this study was fully validated and applied to the analysis of plasma cAMP and cGMP levels in normal rats and Yin deficiency diabetic rats treated with or without *Rehmannia glutinosa*, a classic nourishing Yin herbal medicine. The feasibility of using the UHPLC-MS/MS method to evaluate the regulatory effects of Chinese herbal medicine on the levels of cAMP and cGMP in the body was demonstrated for the first time.

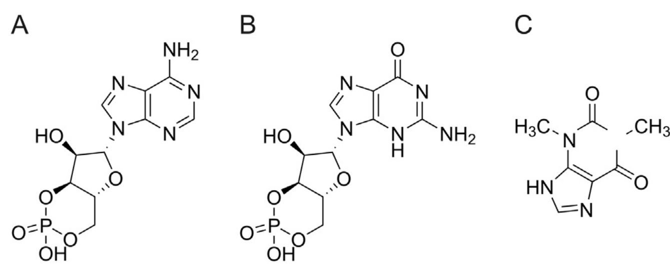

Fig. 1. Chemical structures of (A) cAMP, (B) cGMP and (C) theophylline (IS).

2. Experimental

2.1. Chemicals and materials

cAMP (purity ≥ 99.0%), cGMP (purity ≥ 99.0%), and IBMX were purchased from Anhui Kuer Biological Engineering Co., Ltd. (Hefei, China). Theophylline (purity ≥ 99.0%) was provided by the National Institutes for Food and Drug Control (Beijing, China) and was used as an internal standard (IS). The chemical structures of cAMP, cGMP, and IS are shown in Fig. 1. Streptozotocin (STZ) was obtained from Sigma-Aldrich Inc. (Missouri, USA). Blood glucose Span Diagnostic kit was supplied by Changchun Huili Biotech Co., Ltd. (Changchun, China). Methanol and ammonium acetate (HPLC grade) were obtained from Sigma-Aldrich Inc. (Missouri, USA). All other reagents were of analytical grade or better.

Rehmannia glutinosa was purchased from Beijing Tongrentang Pharmacy (Shenyang, China) and was identified by Professor Jiuzhi Yuan, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University (Shenyang, China).

2.2. Preparation of calibration standards and QC samples

The stock solutions of cAMP (85.3 μg/mL) and cGMP (117.1 μg/mL) were separately prepared in methanol–water (1:9, *V/V*). A series of working standard solutions was prepared by successive dilution of the stock solutions with methanol–water (1:9, *V/V*). Another set of quality control (QC) working solutions was made from separately prepared stock solutions. A stock solution of IS was prepared at 1.001 mg/mL in methanol–water (1:9, *V/V*) and then diluted with methanol–water (1:9, *V/V*) to prepare the IS working solution at 200.2 ng/mL. All solutions were stored at 4 °C.

Blank rat plasma was obtained by aging the plasma containing endogenous cAMP and cGMP at room temperature. Calibration standards were freshly prepared by spiking each working standard solution into blank rat plasma, followed by immediate addition of an equal amount of 0.4 M perchloric acid to stabilize cAMP and cGMP. The final plasma concentrations were 0.27, 0.53, 1.1, 2.1, 4.3, 8.5, 17.1, and 34.1 ng/mL for cAMP and 0.37, 0.73, 1.5, 2.9, 5.9, 11.7, 23.4, and 46.8 ng/mL for cGMP. QC samples were prepared by the same procedure as mentioned above at plasma concentrations of 0.53, 4.3, and 27.3 ng/mL for cAMP, and 0.73, 5.9, and 37.5 ng/mL for cGMP.

2.3. Sample preparation

A 100 μL aliquot of acidified plasma sample was spiked with 20 μL of IS working solution (200.2 ng/mL) and 100 μL of 0.4 M perchloric acid. The mixture was vortexed for 30 s and centrifuged at 12,000 rpm for 4 min. Finally, 10 μL of the supernatant was used for UHPLC-MS/MS analysis.

2.4. UHPLC-MS/MS conditions

UHPLC-MS/MS analyses were performed on an LCMS–8050 mass spectrometer equipped with a Nexera UHPLC and an SIL-30AC autosampler (Shimadzu, Kyoto, Japan). Chromatographic separation was performed using a Shim-pack-XR-ODS II column (75 mm × 3.0 mm, 2.2 μm; Shimadzu, Kyoto, Japan) at 35 °C. The mobile phase consisted of solvent A (2.5 mM ammonium acetate) and solvent B (methanol); a gradient elution was performed as follows: 90% A and 10% B initially, maintained for 0.5 min, increased

to 70% B at 2.0 min, maintained for 2 min and returned to 10% B at 4.1 min. The flow rate was 0.4 mL/min and the injection volume was 10 μL.

The eluates were directed to waste for the first 1.5 min via a divert valve and then introduced into the electrospray ionization (ESI) source, which was operated in the positive mode. The interface voltage was 4.5 kV. The flow rate of drying gas and nebulizing gas was 10.0 and 3.0 L/min, respectively. The desolvation line (DL) temperature was 250 °C and the heat block temperature was 400 °C. The details of the multiple reaction monitoring (MRM) scan parameters are listed in Table 1. Both Q1 and Q3 were set at unit resolution. LabSolutions LC/MS Ver. 5.82 (Shimadzu, Kyoto, Japan) was used for data acquisition and processing.

2.5. Animals and treatments

Male Sprague–Dawley rats (weighing 200–220 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd., (Benxi, China). The rats were bred in a controlled environment at (22 ± 2) °C and (50 ± 10)% relative humidity, and were provided with unlimited access to food and water. All animal studies were carried out following the Guideline of Animal Experimentation of Shenyang Pharmaceutical University, and the experimental procedures were approved by the Animal Ethics Committee of Shenyang Pharmaceutical University, China.

After 7-day acclimatization, eight randomly selected rats were allocated to the normal control group and fed a normal diet, whereas the remaining rats were allocated to the experimental group and fed a high-fat diet. After 2 weeks of feeding, the rats in the experimental group were induced by intraperitoneal injection of STZ (35 mg/kg), whereas the rats in the normal control group were administered an equivalent volume of saline. Blood glucose levels of STZ-induced rats were measured on day 9 after the injection. Rats with fasting blood glucose level of ≥7.8 mmol/L or with nonfasting blood glucose level of ≥11.2 mmol/L were considered to be diabetic.

Sixteen diabetic rats were randomly divided into two groups: model control group ($n = 8$) and *Rehmannia glutinosa* treatment group ($n = 8$). The rats in *Rehmannia glutinosa* treatment group were intragastrically given *Rehmannia glutinosa* extract (hot water extraction followed by alcohol precipitation) at a dose of 8 g/kg for 21 days (twice each day) and the same volume of saline was given to the rats in normal control and model control groups. Whole blood samples were collected into prechilled tubes containing heparin and IBMX (1 mM final concentration) via the oculi chorioideae vein at 2 h after dosing and immediately centrifuged at 3500 rpm for 10 min at 4 °C. Aliquots (100 μL) of plasma were transferred to clean Eppendorf tubes and immediately spiked with 100 μL of 0.4 M perchloric acid. The acidified plasma samples were stored at −20 °C.

2.6. Method validation

The analytical method for simultaneous assay of cAMP and cGMP by UHPLC-MS/MS was validated according to the Food and Drug Administration (FDA) Guidance for Industry on Bioanalytical Method Validation. The selectivity of this method was assessed by analyzing six different batches of blank rat plasma and verifying the presence or absence of interfering peaks from endogenous substances at the retention times of the analytes and IS. The crosstalk effect between coeluting analyte and IS was evaluated by analyzing blank plasma spiked with the highest concentration of individual analytes and IS separately. The carryover effect was evaluated by analyzing a blank plasma sample immediately after an ULOQ sample (calibration standard at the upper limit of quantification) in five consecutive cycles.

The linearity of the method was evaluated by analyzing the calibration standards in duplicate at each concentration level over three consecutive days. Calibration curves were generated by plotting the peak-area ratio of the analytes to IS (y) versus the nominal concentration (x) using $1/x^2$ as a weighting factor. The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve that could be measured with acceptable precision (relative standard deviation, RSD ≤ 20%) and accuracy (relative error, RE within ±20%) using six replicate analyses.

The accuracy and precision of the method were evaluated by analyzing the QC samples at three concentration levels in six replicates on three consecutive days. Acceptable criteria for accuracy (RE) and precision (RSD) were within ±15% and below ±15%, respectively.

The matrix effect was estimated by analyzing six replicates of postextraction spiked plasma samples (prepared from six lots of rat plasma) at three QC concentration levels and comparing the peak area from postextraction spiked samples to that from neat solution at equivalent concentrations. Extraction recovery was determined by comparing the peak areas of the analytes and IS obtained from QC samples with those from postextraction spiked samples.

The stability of the analytes and IS in the stock solution was evaluated by comparing the peak area obtained for freshly prepared stock solution with that obtained for the stock solution stored at room temperature for 24 h and at refrigerated temperature (4 °C) for 30 days. The bench-top stability (2 h at room temperature) and postpreparation stability (24 h at room temperature) of the analytes were investigated by analyzing the QC samples in triplicates at three QC levels.

2.7. Statistical analysis

The cAMP and cGMP concentrations were obtained from calibration curves and expressed as means ± SD. The results were analyzed statistically with independent samples t-test using the SPSS 18.0 software for Windows (SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was regarded as statistically significant.

3. Results and discussion

3.1. Optimization of UHPLC-MS/MS conditions

We tested different columns, including Accucore-150-Amide-HILIC column (100 mm × 2.1 mm, 2.6 μm, Thermo Scientific), Discovery HS F5-3 column (50 mm × 2.1 mm, 3 μm, Sigma-Aldrich),

Table 1
Multiple reaction monitoring scan parameters setting for cAMP, cGMP, and theophylline (IS).

Compound	Precursor ion (m/z)	Product ion (m/z)	Dwell time (ms)	Q1 pre-bias (V)	Collision energy (V)	Q3 pre-bias (V)
cAMP	330.00	136.05	109.0	−26.0	−26.0	−30.0
cGMP	346.00	152.05	109.0	−28.0	−22.0	−17.0
Theophylline	180.90	96.05	100.0	−19.0	−25.0	−17.0

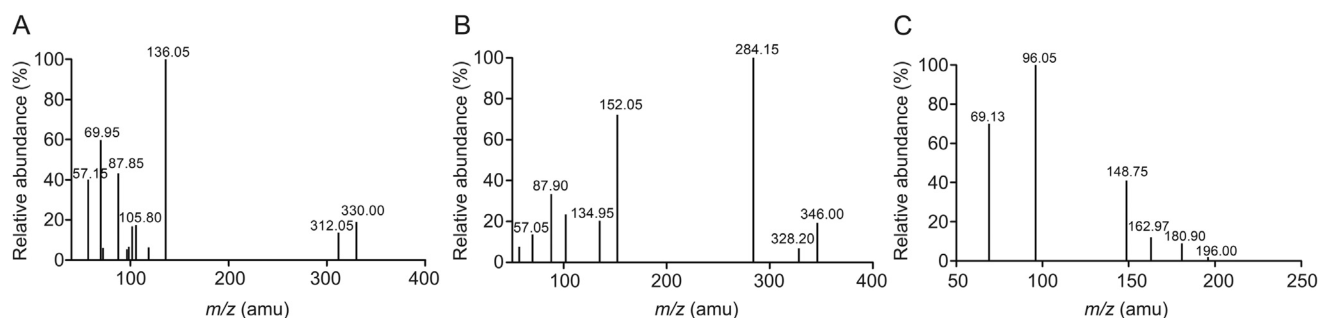

Fig. 2. Full-scan product ion mass spectra of the $[M+H]^+$ ions of (A) cAMP, (B) cGMP and (C) IS.

Shim-pack GISS C_{18} column (50 mm× 2.1 mm, 1.9 μm, Shimadzu), and Shim-pack-XR-ODS II column (75 mm × 3.0 mm, 2.2 μm, Shimadzu). It was found that cAMP and cGMP could not be retained on Accucore-150-Amide-HILIC and Shim-pack GISS C_{18} columns. Adequate retention of cAMP and cGMP was achieved when using low organic concentration (<10%) in the mobile phase, but the ionization efficiency of the analytes was very low. Compared with Discovery HS F5-3 column, Shim-pack-XR-ODS II column provided better retention and detection sensitivity for cAMP and cGMP. Using Shim-pack-XR-ODS II column, different mobile phases consisting of acetonitrile/water or methanol/water mixture were evaluated. Although acetonitrile/water mobile phase gave higher peak signal intensities for cAMP and cGMP, matrix effect was observed because of coelution of the analytes and endogenous components. Thus, methanol/water mobile phase, with the addition a small amount of ammonium acetate to improve peak shapes and increase signal intensity, was optimized. The optimal result was achieved when using methanol/2.5 mM ammonium acetate gradient.

Both positive and negative ionization modes were tested for the analytes using the ESI source. Higher signal intensity of cAMP and cGMP was observed in the positive ion mode than in the negative ion mode. Thus, positive ion mode was used in the present study. MS parameters, such as nebulizing gas flow, DL temperature, heat block temperature, drying gas flow, spray voltage, capillary temperature, and collision energy, were further optimized to obtain maximum sensitivity of MS detection. Fig. 2 displays the full-scan product ion mass spectra of the $[M+H]^+$ ions of cAMP, cGMP, and IS.

Stable isotope-label analog of the analyte is considered as an ideal IS for its ability to correct for the matrix effect and increases the precision and accuracy of LC-MS/MS quantification. However, isotope-labeled IS is very expensive and not always commercially available. To reduce the cost of experiments and to make the method more affordable, non-isotope-labeled ISs, such as metronidazole, tinidazole, voriconazole, theophylline, and diphenhydramine, were tested. Theophylline was selected as the IS in the present study for having similar chromatographic behavior and good stability (in 0.4 M perchloric acid) to the analytes. As a PDE inhibitor, theophylline also played a role in stabilizing cAMP and cGMP during sample preparation.

3.2. Optimization of preanalytical sample handling procedures

cAMP and cGMP were unstable in whole blood containing enzymatically active PDEs. As shown in Fig. 3, after storage of rat whole blood for 15 min at room temperature, the levels of cAMP and cGMP decreased by approximately 50% and 80%, respectively. The addition of IBMX to rat whole blood significantly (but not completely) blocked the degradation of cAMP and cGMP at room temperature. The levels of cAMP and cGMP could be stabilized in rat whole blood kept on ice for 15 min by IBMX (1 mM final concentration). The results indicated that the maximum interval between blood collection and plasma separation to avoid any decrease in cAMP and cGMP levels was 15 min. After centrifugation, freshly separated plasma should be immediately treated with perchloric acid (0.2 M final concentration) to further stabilize cAMP and cGMP. It is worth mentioning that acidified rat plasma is not suitable for long-term storage as slow degradation of cAMP and cGMP was observed in this case. However, cAMP and cGMP were found to be stable in acidified plasma at −20 °C for 24 h.

Because of their high polarity, the extractability of cAMP and cGMP from rat plasma by liquid−liquid extraction is poor. Solid-phase extraction (SPE) of cAMP and cGMP from human plasma based on hydrophilic interaction on silica has been reported [20]. However, the SPE method suffers from the multiple complicated steps that are time and cost consuming. In the present study,

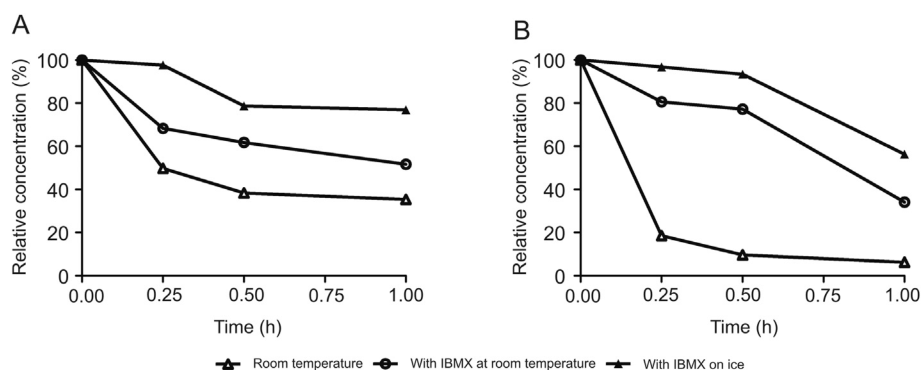

Fig. 3. Ex vivo stability of (A) cAMP and (B) cGMP in rat blood at room temperature, with IBMX at room temperature and with IBMX on ice.

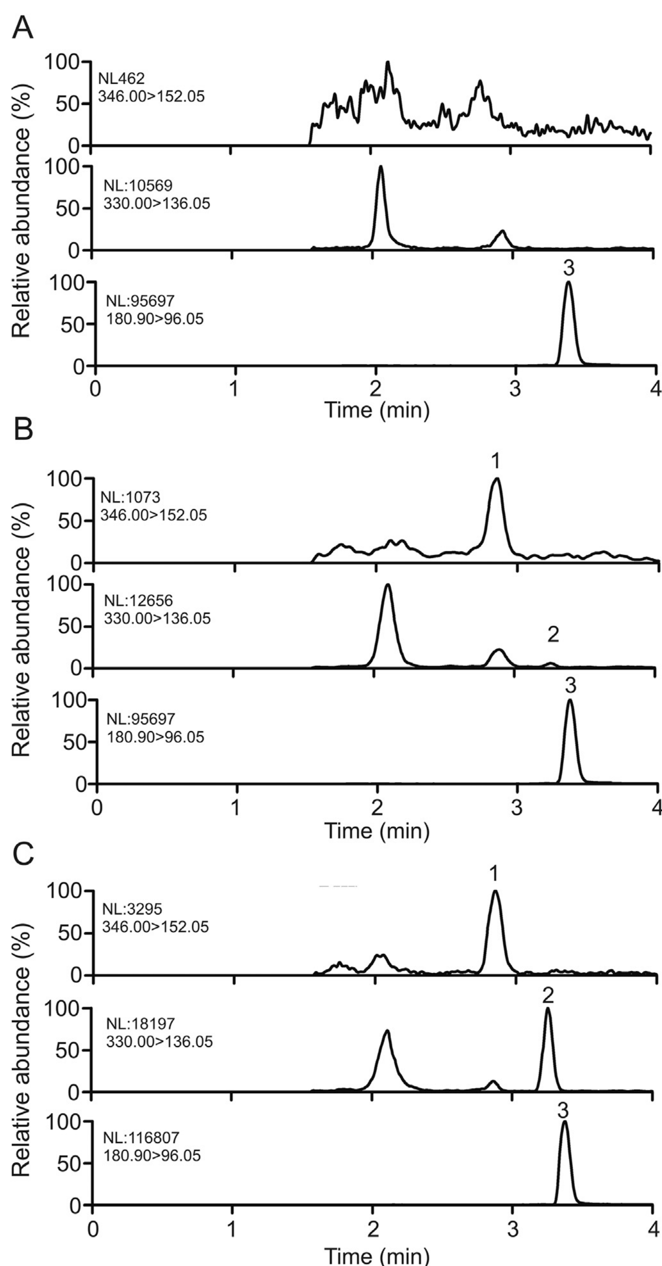

Fig. 4. Representative MRM chromatograms of cAMP, cGMP and IS in rat plasma. (A) Blank plasma; (B) blank plasma spiked with cAMP and cGMP at LLOQ and IS; (C) real plasma sample obtained from a diabetic rat. 1: cGMP; 2: cAMP; 3: theophylline (IS).

protein precipitation was adopted for the preparation of plasma samples because of its simplicity, consistent extraction recovery, and negligible matrix effect. Considering that cAMP and cGMP are relatively stable under the acidic condition, 0.4 M perchloric acid (used to stabilize cAMP and cGMP in rat plasma before) was used instead of methanol and acetonitrile as the protein precipitant.

3.3. Method validation

Representative MRM chromatograms of blank rat plasma, blank rat plasma spiked with the analytes and IS, and rat plasma samples obtained from a diabetic rat are shown in Fig. 4. The retention times for cAMP, cGMP, and IS were 3.258, 2.858, and 3.377 min, respectively. No significant interference from endogenous substances and no crosstalk between coeluting cAMP and IS were observed, which indicated that the developed method was specific for the analysis of cAMP and cGMP in rat plasma. The responses of blank plasma at the retention times of the analytes were less than 20% of the LLOQ sample, indicating that the carryover effect was negligible.

The typical regression equations, correlation coefficients (r), and linear ranges of cAMP and cGMP are listed in Table 2. The calibration curves of the analytes showed good linearity over the specified concentration ranges with $r \geq 0.9963$. The LLOQs for cAMP and cGMP were 0.27 and 0.37 ng/mL, respectively, with RSD $\leq$11.1% and RE within $\pm$2.4%.

The precision and accuracy data for cAMP and cGMP at three QC levels are summarized in Table 3. The intraday and interday precisions did not exceed 14.9%, and the accuracy ranged from −6.3% to 4.5% for cAMP and cGMP. These results indicated that the method was reliable and reproducible for the determination of cAMP and cGMP in rat plasma.

The matrix effects of cAMP and cGMP at the three QC levels were in the range of 91.7%–106.9% and 89.1%–95.7%, respectively. The matrix effect of IS was 111.3%. The extraction recoveries of cAMP and cGMP from rat plasma ranged from 89.2%–97.8% with a maximum RSD of 10.1%. The extraction recovery of IS was 92.1% with an RSD of 7.8%. The results indicated stable extraction recovery and negligible endogenous interference and suitability of the method for determining the levels of cAMP and cGMP in rat plasma.

The stock solutions of cAMP and cGMP as well as of IS were stable at room temperature for 24 h and at 4 °C for 30 days, with an RE between −2.1% and 7.5%. cAMP and cGMP were stable in IBMX-quenched rat whole blood kept on ice for only 15 min. cAMP and cGMP were stable when stored under each of the following conditions: (1) bench top (room temperature) for 2 h, with RSD < 9.8% and RE between 11.0% and 13.2%; (2) freezer (−20 °C) for 24 h, with RSD < 12.7% and RE between −13.2% and 9.4%; (3) autosampler (room temperature) for 24 h, with RSD < 9.7%, and RE between −3.1% and 9.8%.

3.4. Method application

The validated method was applied to the analysis of plasma cAMP and cGMP levels in normal rats and diabetic rats treated with or without *Rehmannia glutinosa*. Diabetes is categorized in TCM as *Xiao Ke* (wasting and thirsting) disease. Its main pathogenesis lies in the consumption of Yin fluid, leading to endogenous dryness-heat in the body, with Yin deficiency being the principal aspect and dryness-heat as the secondary aspect [24]. Therefore, nourishing Yin and reducing fire so as to restore the body's balance between Yin and Yang is the basic principle for the treatment of diabetes in TCM [25]. *Rehmannia glutinosa* is a classic nourishing Yin herbal medicine that has been widely used for the treatment of *Xiao Ke* disease in TCM practice. In the present study, the nourishing Yin effect of *Rehmannia glutinosa* was investigated and verified by the analysis of plasma cAMP and cGMP levels in rats using the developed UHPLC-MS/MS method.

A rat model of type 2 diabetes mellitus (T2DM) induced by a high-fat diet and STZ has been reported to exhibit Yin deficiency syndrome in TCM [26,27]. In addition, the change in the cAMP/cGMP ratio in the blood of the T2DM rat model was reportedly related to Yin deficiency syndrome [26,27]. In this study, the T2DM rat model was successfully established by feeding a high-fat diet for 14 days and administering a single intraperitoneal injection of 35 mg/kg STZ. The plasma glucose levels before modeling in normal, model, and *Rehmannia glutinosa* treatment groups were (4.80 ± 0.56), (4.93 ± 0.52), and (4.53 ± 0.39) mmol/L, respectively, and the plasma glucose levels after modeling in model and *Rehmannia glutinosa* treatment groups were (18.93 ± 8.98) and (14.87 ± 3.87) mmol/L, respectively. The plasma glucose level after

Table 2
Regression equation, linear range, and lower limit of quantification (LLOQ) for the determination of cAMP and cGMP in rat plasma.

Compound	Regression equation	r	Linear range (ng/mL)	LLOQ		
				Nominal concentration (ng/mL)	RSD (%)	RE (%)
cAMP	$y = 0.53849x + 0.00341$	0.9963	0.27–34.1	0.27	11.1	−2.4
cGMP	$y = 1.35120x + 0.00931$	0.9980	0.37–46.8	0.37	8.8	−0.2

RE: relative error; RSD: relative standard error.

Table 3
Precision and accuracy in the determination of cAMP and cGMP in rat plasma by the developed UHPLC-MS/MS method ($n = 6$).

Compounds	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Precision (RSD, %)		Accuracy (RE, %)
			Intraday	Interday	
cAMP	0.53	0.52	6.6	13.8	−6.3
	4.26	4.04	3.7	14.8	−5.2
	27.3	26.8	4.2	14.9	−1.7
cGMP	0.73	0.76	6.0	6.9	4.5
	5.85	5.65	3.1	14.9	−3.5
	37.5	36.2	4.4	14.2	−3.4

RE: relative error; RSD: relative standard error.

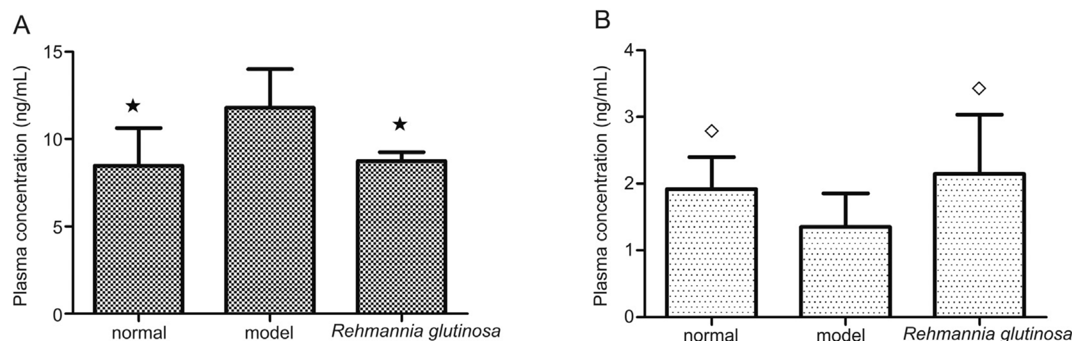

Fig. 5. Plasma concentrations of (A) cAMP and (B) cGMP in normal, model and *Rehmannia glutinosa* treatment groups ($n = 8$). ★ $P < 0.05$ compared with model group; ◇ $P < 0.05$ compared with model group.

administration of *Rehmannia glutinosa* for 21 days in *Rehmannia glutinosa* treatment group was (6.89 ± 0.75) mmol/L and (22.06 ± 6.00) mmol/L in model group. The plasma concentrations of cAMP and cGMP in the rats of normal, model, and *Rehmannia glutinosa* treatment groups were determined by UHPLC-MS/MS (Fig. 5). The levels of cAMP in the model group were significantly higher whereas those of cGMP were significantly lower than those in the normal group ($P < 0.05$). Consistent with the results reported earlier [9–12], Yin deficiency corresponded to an increase in the cAMP/cGMP ratio in the body. Following 21 days of treatment with *Rehmannia glutinosa*, significant reduction in cAMP level as well as significant increase in cGMP level were observed when compared with the model group. The decrease in the cAMP/cGMP ratio indicated the regulatory effect of *Rehmannia glutinosa* on the Yin deficiency symptom.

4. Conclusions

A reliable and accurate UHPLC-MS/MS method was established for simultaneous determination of cAMP and cGMP in rat plasma. Special emphasis was focused on the optimization of preanalytical sample handling procedures. Effective strategies to prevent the degradation of cAMP and cGMP during blood collection and plasma sample preparation were developed. The method was fully validated and successfully applied to the analysis of plasma cAMP and cGMP levels in normal rats and Yin deficiency diabetic rats treated with or without *Rehmannia glutinosa*. For the first time, the applicability of the UHPLC-MS/MS method for accurate quantification of the potential biomarkers for Yin-Yang disharmony in TCM was demonstrated. The present study offers a simple and reliable analytical method for the differentiation of Yin and Yang deficiency syndromes in TCM. Importantly, the method would be a valuable tool for investigating the regulatory effects of TCM on the cAMP and cGMP levels in the body and for evaluating the efficacy of Yin-Yang balancing therapy via the administration of tonifying herbs and formulas.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors acknowledge Prof. Jiuzhi Yuan from Shenyang

Pharmaceutical University for the identification of the plant materials. This work was financially supported by the National Natural Science Foundation of China (No. J1103606).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2020.09.001.

References

[1] T. Haahr, J.S. Jensen, P. Humaidan, Research and business-The Yin and Yang in modern medicine, Reprod. Biomed. Online 40 (2020) 613–616.

[2] Q.N. Hu, T. Yu, J.H. L, et al., End-to-end syndrome differentiation of Yin deficiency and Yang deficiency in traditional Chinese medicine, Comput. Methods Progr. Biomed. 174 (2019) 9–15.

[3] C. Yan, Z. Luo, W. Li, et al., Disturbed Yin-Yang balance: stress increases the susceptibility to primary and recurrent infections of herpes simplex virus type 1, Acta Pharm. Sin. B. 10 (2020) 383–398.

[4] G. Bianco, Fascial neuromodulation: an emerging concept linking acupuncture, fasciology, osteopathy and neuroscience, Eur. J. Transl. Myol. 29 (2019) 195–201.

[5] J.D. Adams, The Effects of Yin, Yang and Qi in the skin on pain, Medicines (Basel) 3 (2016) 1–7.

[6] N.D. Goldberg, M.K. Haddox, S.E. Nicol, et al., Biologic regulation through opposing influences of cyclic GMP and cyclic AMP: the Yin Yang hypothesis, Adv. Cyclic Nucl. Res. 5 (1975) 307–330.

[7] N.D. Goldberg, M.K. Haddox, C.E. Zeiling, et al., Cyclic GMP, cyclic AMP, and the yin yang hypothesis of biologic regulation, J. Invest. Dermatol. 67 (1976) 641–645.

[8] P. Schuhmacher, A. Wallang, Central blood pressure effects of guanylyl-imidodiphosphate and cyclic guanosine-monophosphate, Naunyn-Schmiedeberg's Arch. Pharmacol. 312 (1980) 31–35.

[9] K. Seki, M. Chisaka, M. Eriguchi, et al., An attempt to integrate Western and Chinese medicine: rationale for applying Chinese medicine as chronotherapy against cancer, Biomed. Pharmacother. 59 (2005) S132–S140.

[10] Q. Wang, X.J. Ren, S.L. Yao, et al., Clinical observation on the endocrinal and immune functions in subjects with yin-deficiency constitution, Chin. J. Integr. Med. 16 (2010) 28–32.

[11] Y. Tan, X.R. Liu, C. Lu, et al., Metabolic profiling reveals therapeutic biomarkers of processed *Aconitum Carmichaeli Debx* in treating hydrocortisone induced Kidney–Yang deficiency syndrome rats, J. Ethnopharmacol. 152 (2014) 585–593.

[12] P.Y. Gong, Y.W. He, J. Qi, et al., Synergistic nourishing 'Yin' effect of iridoid and phenylpropanoid glycosides from Radix Scrophulariae in vivo and in vitro, J. Ethnopharmacol. 246 (2020), 112209.

[13] E. Engvall, K. Jonsson, P. Perlmann, Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes, Biochim. Biophys. Acta 251 (1971) 427–434.

[14] P. Pradelled, J. Grassi, D. Chabardes, et al., Enzyme immunoassays of adenosine cyclic 3',5'-monophosphate and guanosine cyclic 3',5'-monophosphate using acetylcholinesterase, Anal. Chem. 61 (1989) 447–453.

[15] M. Tsugawa, K. Moriwaki, S. Iida, et al., An enzyme-linked immunosorbent assay (ELISA) for guanosine 3',5'-cyclic monophosphate (cGMP) in human plasma and urine using monoclonal antibody, J. Immunoassay 12 (1991) 263–276.

[16] Y. Zhang, D. Dufield, J. Klover, et al., 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, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (2009) 513–520.

[17] X. Ren, C. Wang, L. Wu, et al., Bioactivity assay of porcine relaxin based on cAMP accumulation in THP-1 cells quantified by LC–MS/MS, J. Pharmaceut. Biomed. Anal. 111 (2015) 320–323.

[18] W. Goutier, P.A. Spaans, M.A.W. Van der Neut, et al., Development and application of an LC–MS/MS method for measuring the effect of (partial) agonists on cAMP accumulation in vitro, J. Neurosci. Methods 188 (2010) 24–31.

[19] J. Martens-Lobenhoffer, C. Dautz, S.M. Bode-Böger, Improved method for the determination of cyclic guanosine monophosphate (cGMP) in human plasma by LC–MS/MS, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 878 (2010) 487–491.

[20] T. Van Damme, Y. Zhang, F. Lynen, et al., 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, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 909 (2012) 14–21.

[21] P. Oeckl, B. Ferger, Simultaneous LC–MS/MS analysis of the biomarkers cAMP and cGMP in plasma, CSF and brain tissue, J. Neurosci. Methods 203 (2012) 338–343.

[22] H. Bähre, V. Kaever, Measurement of 2',3'-cyclic nucleotides by liquid chromatography–tandem mass spectrometry in cells, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 964 (2014) 208–211.

[23] J. Chen, A. Tabatabaei, D. Zook, et al., A surrogate analyte-based liquid chromatography-tandem mass spectrometry method for the determination of endogenous cyclic nucleotides in rat brain, J. Pharmaceut. Biomed. Anal. 146 (2017) 361–368.

[24] H.Y. Zhang, J. Zhou, L. Zhang, et al., Characteristics of blood glucose excursions in type 2 diabetes mellitus patients with three different Traditional Chinese Medicine syndromes, J. Tradit. Chin. Med. 35 (2015) 537–545.

[25] W. Zheng, G. Wang, Z. Zhang, et al., Research progress on classical traditional Chinese medicine formula Liuwei Dihuang pills in the treatment of type 2 diabetes, Biomed. Pharmacother. 121 (2020), 109564.

[26] W. Yan, J. Han, L.M. Huang, et al., Correlation of syndrome succession and variance of laboratory targets of type 2 diabetic disease syndrome combination rat models, Chin. J. Tradit. Chin. Med. Pharm. 28 (2013) 2726–2729.

[27] Y. Liu, M. Xie, Y. Zhang, Dynamic changes of laboratory parameters of rats with type 2 diabetes and insulin resistance: defining their role in development of traditional Chinese medicine syndrome, Zhong Xi Yi Jie He Xue Bao 10 (2012) 100–108.