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Development and validation of an LC–MS/MS method for the determination of cyclocreatine phosphate and its related endogenous biomolecules in rat heart tissues

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Abstract

The cardioprotective drug cyclocreatine phosphate has been awarded Food and Drug Administration-orphan drug designation for the prevention of ischemic injury to enhance cardiac graft recovery and survival in heart transplantation. Cyclocreatine phosphate is the water-soluble derivative of cyclocreatine. Estimating the levels of Cyclocreatine phosphate, Adenosine triphosphate, Creatine Phosphate, Creatine and Cyclocreatine helps us in understanding the energy state as well as evaluating the heart cells' function. The quantification of endogenous compounds imposes a challenging task for analysts because of the absence of a true blank matrix, whose use is required according to international guidelines. Recently, the International Council for Harmonization issued a new guideline that contains guidance on the validation of methods used to quantify endogenous components, such as the background subtraction approach that was employed in our current study. Specifically, we developed and validated a sensitive, reliable and accurate liquid chromatography-tandem mass spectrometry assay to determine simultaneously the levels of mentioned endogenous compounds in rat heart tissue. Tissue samples were prepared by protein precipitation extraction using water: methanol (1:1). Using Ultra Performance Liquid Chromatography, Chromatographic separation was achieved with ZORBAX Eclipse Plus C18 4.6 × 100 mm,3.5 µm column and conditions as following: ammonium acetate (pH 8.5): acetonitrile, 70:30 mobile phase, 0.7 mL/min flow rate and 25 °C temperature. Electrospray ionization mass detector with Multiple reaction monitoring mode was then employed, using both positive and negative modes, Analysis was carried out using 5.00–2000.00 ng/mL linear concentration range within 2 min for each analyte. According to Food and Drug Administration guidelines for bioanalytical methods, validation was carried out. We investigated the matrix effect, recovery efficiency and process efficiency for the analyte in neat solvent, postextraction matrix and tissue. The results stated mean percentage recoveries higher than 99%, accuracy 93.32–111.99%, and Relative Standard Deviation (RSD) below 15% within the

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concentration range of our study which indicated that target analytes' stability in their real matrix is sufficient under the employed experimental conditions.

Highlights

- Development of a validated LC–MS/MS method to quantify levels of CCrP, ATP, CrP, Crt, and CCr, simultaneously.
- LC–MS/MS method is sensitive, simple and suitable for preclinical trials for CCrP quantitation in different biological fluids.
- Validated method with good selectivity, linearity, and stability for CCrP in the rat heart.
- Efficient CCrP quantitation to improve outcomes for heart transplant patients.

Keywords LC-MS/MS, Cyclocreatine phosphate, Cardioprotective, Endogenous biomolecules, Background subtraction

Introduction

Cyclocreatine phosphate (CCrP), the water-soluble derivative of cyclocreatine (CCr), is currently being developed for clinically use during heart transplantation surgery to preserve the hearts of the donors and improve the transplanted hearts' recovery in recipient patients [1–4]. The Food and Drug Administration (FDA) has recently granted CCrP an Orphan drug designation (ODD) for "Prevention of Ischemic Injury to Enhance Cardiac Graft Recovery and Survival in Heart Transplantation (DRU-2015-4951)" [1, 2]. During myocardial ischemia, adenosine triphosphate (ATP) and creatine phosphate (CrP) are decreased quickly. Simultaneous quantification of cardiac markers such as ATP, CrP and creatine (Crt) is required for understanding heart conditions, heart disease maintenance and heart transplantation processes [5–7]. Thus, estimating the levels of CCrP, ATP, CrP, Crt, and CCr helps us understand the energy state as well as evaluating heart cells' function [4, 7]. Additionally, CCrP represents a promising treatment in congenital disorders such as creatine transporter deficiency (CTD), an X-linked congenital disorder with no current available treatment that negatively affects the quality of life for patients as well as caregivers. Thus, there is an urgent need for rapid and cost-effective determination procedures for CCrP in either rodent blood/serum, patient blood/serum, or dietary supplements.

The analytical approaches employed for CCrP monitoring have been reviewed due to its significant role [5, 6]. CCrP is a highly polar, nonelectroactive, nonfluorescent molecule with a weak UV absorption signal which makes its detection using a traditional high-performance liquid chromatography UV detector (HPLC-UV) challenging to be established. Additionally, biological samples are more complex than analytical and validation standpoint in determining the naturally occurring (endogenous) structural analog molecules quantitatively. Further, it is challenging to establish a determination methodology and obtain blank matrices and samples with accurately known analyte concentration of the real biological matrix [8]. A literature survey stated that methods based on hydrophilic interaction liquid chromatography (HILIC) have been only reported to determine CCrP [5, 6].

HPLC represents the most commonly method used to determine compounds in biological samples [7–9]. In addition, due to the background interference of the biological matrix, several LC−MS/MS methods have been used for the analysis of energy-related biomolecules [10, 11]. ATP, CrP, and Crt represent endogenous energy-related biomolecules; thus, quantitative determination in biological samples is highly complicated, either in method development or validation [7, 12]. The main obstacles are the unavailability of blank matrices and obtaining samples with precisely defined analyte concentrations of the real biological matrix [8, 9, 13]. To overcome these obstacles, different ways have been used to address the reference samples' preparation, in addition the validation becomes a real challenge [8, 9, 11, 13].

The advantages of using an internal standard (IS) to reduce the interfering effect of matrix components, minimize the errors of sample processing and the variability of detection, are well known [14, 15]. However, the IS method with endogenous biomolecules is not absolutely needed; sometimes, it could be an obstacle, a source of variability, not always available, and would be extremely expensive to purchase if several metabolites needed to be quantified [14, 15].

Alternatively, a similar molecule is required to serve as a suitable internal standard. Tenofovir was subsequently tried. However, it became obvious after some trials with this potential internal standard that it was not the best choice for method development. With this standard, we did not notice any better variation in the analytical data. As a result, the background subtraction method through the proposed bioanalytical method was employed to overcome the matrix effects without the utilization of an internal standard.

According to the recent ICH guidelines of bioanalytical method validation [16], different approaches have been stated for overcoming unavailability of sample-free

matrices for quantifying endogenous compounds using LC–MS/MS, such as background subtraction [16–20], the standard addition method [21], neat solutions [22], artificial matrices of biological fluids [23], stripped matrices [24], and surrogate analytes [25]. Quantitative analyses of endogenous chemicals can be performed using a variety of approaches. The same biological matrix as the study samples is used in both standard addition and background subtraction procedures for the generation of the calibration standards; thus, both the matrix effect and the recovery of the study samples are equal to those of the calibration standards. Otherwise, the endogenous levels of the analytes constrain the sensitivity of the background subtraction approach. Regarding the standard addition approach, large sample volume is required because calibration curves required to be constructed in each sample individually. On the other hand, the surrogate matrix approach prepares calibration standards using matrices devoid of endogenous analytes such as neat solutions, artificial, and stripped matrices which permits sensitive and direct analytes' quantification. Demonstrating a similar matrix effect and extraction recovery in both the surrogate and original matrices is required [9].

Regarding the background subtraction, The calibration curve is constructed using the subtract result of the endogenous background concentrations of analytes in a pooled/representative matrix from the concentrations of the added standards; subsequently [16]. The calibration curve is constructed by spiking authentic matrix with the compound of interest, in an increasing concentration. The resulting response curve is subsequently corrected for the endogenous (background) signal of the compound of interest in the original matrix [11, 13]. The background subtraction approach has an advantage that the matrix for the calibration curve and the matrix to be analyzed are the same which makes the matrix effect and the recovery significantly close between both calibration curves and samples [9, 11, 13].

Our research group has fabricated a selective MIP membrane for CCrP using electropolymerization of the *o*-PD monomer on a C-SPE substrate which even in the presence of the structural analogs abundant in biological fluids, has satisfactory specificity and selectivity for the template molecule [4]. However, LC–MS/MS is a gold standard technique for bioanalytical measurements that offers high sensitivity and selectivity in the analysis of a CCrP, its structural analogs and related biomarkers in biological matrices with high validity and shortens the analysis time using minimal sample volume, making its application in therapeutic monitoring highly efficient. Thus, in our current study, we report a fully validated analytical LC–MS/MS method for simultaneous measurement of CCrP, ATP, and CrP in the negative mode and Crt and CCr in the positive mode in rat heart tissue using background subtraction approach which is rapid and simple sample preparation and procedure that are compatible with the LC–MS/MS method for endogenous substance. The assay is urgently required for investigating CCrP, its metabolites, and related substances, that helps in further investigating for heart disease treatment.

Background subtraction approach that was employed in our current study.

Experimental procedure

Adult male Wistar rats, aged 6–8 weeks and weighing between 180 and 220 g, were used in this study. The animals were sourced from the Cairo University Research Park's Animal Technology Laboratory (Dokki, Cairo, Egypt). Rats were caged under controlled temperature (20–25 ∘C) and humidity (45–55%) conditions with a twelve-hour light/dark cycle and free access to food and water. Prior to the experiment, animals were housed for a couple of weeks to accommodate them.

All experimental procedures were approved by the Faculty of Pharmacy Ethics Committee at Cairo University (permit number: PT 2733) and conducted in accordance with the US National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH publication no. 85−23, revised in 2011). The animals were euthanized by decapitation under anesthesia using thiopental (50 mg/kg, IP).

Chemicals

CCrP was a gift from Nour Heart, Inc. (USA). ATP, CrP, CCr, and Crt as well as solvents and chemicals (HPLC-grade) were purchased from Sigma Aldrich (USA). For assay development and validation, rat heart tissues and plasma were obtained from Cairo University, Egypt. Ultra-pure water (TOC of less than 5 ppb and resistivity > 18 MΩ.cm^{-1} at 25 °C) was obtained from a Milli-Q UF-Plus system (Millipore, Germany).

Instruments

For chromatographic separation, A Shimadzu UPLC connected to an ExionLC (Sciex) degasser, ZORBAX Eclipse Plus C18 4,6×100 mm, 3,5 µm column (Agilent, USA), pump, autosampler, and column oven, were used. For MS/MS, the AB Sciex Triple QuadTM 5500 detector was used in multiple reaction monitoring (MRM) mode. Electrospray ionization (ESI) mass detector was employed, using the positive mode for Crt and CCr and the negative mode for CCrP, ATP, and CrP. Hardware control and data acquisition were performed using Analyst 1.7.1 software. A vortex mixer (Beijing, China) was used for sample preparation, and centrifugation was performed by Eppendorf AG (Germany).

Standard solutions and calibration curves

Before use, Glassware was washed using methanol then Milli-Q water. Stock solutions of CCrP, ATP, CrP, CCr, and Crt (shown in Fig. 1) at a concentration of 50.00 µg/mL in methanol, were prepared. Four working solutions of concentrations 5.00 and 0.5 µg/mL with serial dilution in methanol, were carried out. Stock and working standard solutions were stored away from direct light at 4 °C. Full validation parameters were developed in rat heart tissue.

The preparation of QC samples, low (QCL), medium (QCM1, QCM2) and high (QCH), was done in methanol at concentrations of 5, 15, 60.00, 600.00 and 1500.00 ng/mL.

For determination of analytes in rat heart tissue, samples were obtained by surgical removal of heart tissue, divided into small portions, then freeze-dried. Aliquots of each tissue were homogenized using an electrical tissue homogenizer with different concentrations of standard analyte solution with precooled methanol-water (v/v, 1:1). Then, homogenates were vortexed and centrifuged at 12,500 × g for 15 min at 4 °C for protein precipitation. The 100 µL of clear supernatant was collected and 5 µL of the supernatant was used for injection into the LC–MS/MS system.

Liquid chromatography and mass spectrometry conditions

Chromatographic separations were done at 25 °C using a ZORBAX Eclipse Plus C18 4,6×100 mm, 3,5 µm column (Agilent, USA) with 0.7 mL/min flow rate. The mobile phase used was ammonium acetate (pH 8.5): acetonitrile (ACN) (70:30) after degassing for 10 min using an ultrasonic bath. The transitions of molecular ions were 221.90>79.00, 210.00>79.00, 144.30>98.10, 132.30>90.00, and 506.10>159.00 for the determination of CCrP, CrP, CCr, Crt and ATP, respectively.

Assay validation

The assay was validated using rat heart tissue according to FDA guidelines for bioanalytical method validation [26], Six different batches of rat blank tissue was used to evaluate the selectivity. The determination of targeted analytes in the current study was developed and validated using the background subtraction method LC–MS/MS. The calibration curves were generated using the subtracted result of the endogenous background concentrations of analytes from the concentrations of the spiked amount [16].

Linearity, LLOQ, accuracy, precision, and stability were determined using the results of analysis [27]. Response linearity in rat heart tissue was calculated and nine fortified samples covered from 5.00 to 2000.00 ng/mL range which are the following concentrations 5, 10, 30, 50, 70, 100, 500, 1000, 2000 ng/mL. Subtracted peak areas of CCrP, CrP, CCr, Crt and ATP were generated against nominal concentrations to evaluate the linearity. LLOQ was practically determined. Moreover, calibration curves for each substance (CCrP, CrP, CCr, Crt and ATP) were plotted in heart matrix and used to predict the analytes' concentration. The accuracy and precision, within run (three replicates on the same day) and between run (three replicates over three consecutive days) were determined through fortified samples' analysis at LLOQ, QCL, QCM1, QCM2, and QCH.

To determine the matrix effect (ME), relative recovery (RE), and process efficiency (PE) values for the targeted molecules, three sets of the standard solutions were prepared at two concentration levels of QCL and QCH as following: Set 1 in neat solvent (mobile phase), Set 2 in

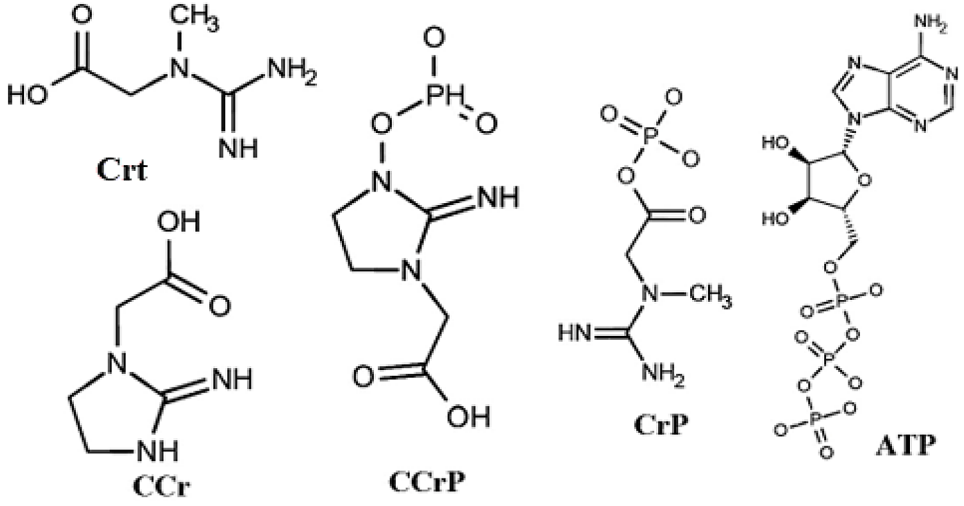

Fig. 1 Chemical structure of cyclocreatine phosphate (CCrP) and its structurally similar compounds in biological fluids: creatine (Crt), cyclocreatine (CCr), creatine phosphate (CrP) and adenosine triphosphate (ATP)

post-extracted heart tissue (fortified after extraction) and Set 3 in pre-extracted heart tissue (fortified before extraction). Then, the absolute peak areas in the three sets were used as the following formulas [28]

$$\mathrm{ME}\ (\%) = \mathrm{Set}\ 2 / \mathrm{Set}\ 1 \times 100 \quad (1)$$

$$\mathrm{RE}\ (\%) = \mathrm{Set}\ 3 / \mathrm{Set}\ 2\,\mathrm{x} \times 100 \quad (2)$$

$$\mathrm{PE}\ (\%) = \mathrm{Set}\ 3 / \mathrm{Set}\ 1 \times 100 \quad (3)$$

In addition, for assessing stability of the drugs in heart tissue, different storage conditions were applied, to be compared with the initial concentrations. If the deviation from those of zero cycle was within ±15%, samples are considered stable. Processed sample stability, Freeze and thaw stability, Long-term stability and Benchtop stability were examined for QCL and QCH samples as following. Processed sample stability is measured for determination if an occasional delay in extracted samples' injection lead to analytes' degradation by storing QC samples in an autosampler at 2–8 °C for 15 h. Freeze and thaw stability is measured by three cycles of storing QC samples in triplicates at −86 °C for 24 h then thawing them unassisted at room temperature, the results were compared to that of the zero cycle. Long-term stability is measured by storing QC samples at −86 °C for a period exceed time from sample collection to processing and analysis (90 days). Benchtop stability is measured by storing QC samples at room temperature for 24 h (time exceeding the samples' preparation time).

Dilution integrity was examined for three spiked samples that were prepared at two different initial concentrations, 3000 µg/mL and 6000.00 µg/mL, to reach the QCH level (1500 ng/mL) through two different dilution factors. Samples were diluted with either 100 µL (1:2) or 300 µL (1:4) of blank tissue, at each concentration level.

Application in rat heart samples

Different groups of rats that were injected with CCrP were coded according to the established protocol for preclinical studies of CCrP (data not shown) [29–32]. The incurred rat heart tissues were obtained by surgical removal of heart tissues, cut into portions, and then frozen at -80˚C. As indicated in Table 6, the described method was applied for the determination of CCrP, CrP, CCr, Crt and ATP in hearts from six groups. These include: saline/control (n=3), CCrP/control (n=3), Isoproterenol (ISO)/saline (85 and 170 mg/kg/day s.c. for 2 consecutive days) (n=3), and ISO/CCrP at 0.8 g/kg/day i.p. (n=5), at 0.4 g/kg/day i.p. (n=2), and at 1.2 g/kg/day i.p. (n=2) administrated 24 h before ISO injection, and then daily for 2 weeks.

Aliquots of each tissue were homogenized using an electrical tissue homogenizer. Then, the tissues were vortexed with precooled methanol-water (v/v, 1:1) for protein precipitation, then centrifugated at 4 °C with speed 12,500×g for 15 min. 100 µL of clear supernatant was diluted with equal amount of water; then, 5 µL was used for injection into LC–MS/MS system.

Results and discussion

Method development

Sample preparation

Considering good water solubility of the analytes, different solvents were evaluated for extraction: methanol, acetonitrile, ethyl acetate, tertiary butyl ether, and dichloromethane. Perchloric acid was avoided due to its incompatibility with the MS system and its further interfering with detection. The recoveries for ethyl acetate, tertiary butyl ether, and dichloromethane varied below 10%. Acetonitrile demonstrated a lower recovery for the target compound, approximately 15%, than methanol. Methanol could extract all drugs with good recovery under similar conditions for extraction from both tissue and plasma samples. With the proportion of water: methanol (1:1), the recoveries for the five target compounds were approximately 75%. Therefore, it was used for protein precipitation with easy preparation and MS-compatibility [7].

LC–MS/MS

Several UPLC−MS trials were conducted while various physicochemical characteristics of the determined compounds and matrix interference affected their separation. In addition, the limited sample volume that could be acquired from rats used in the experimental model necessitated developing a simultaneous assay for investigated compounds. UPLC paired with an MS/MS detector was the optimal method due to its selectivity, specificity, as well as its capacity to assess many analytes with highly varied characteristics in a single run.

Satisfactory results were obtained using a ZORBAX Eclipse Plus C18 4,6×100 mm, 3,5 µm column (Agilent, USA), mobile phase of ammonium acetate (pH 8.5):ACN, 70:30 at 25 °C with 0.7 mL/min flow rate. ESI ion source was selected due to the high polarity of the analytes. For optimizing each compound's mass spectrometric conditions, continuous infusion of corresponding standard solutions with a flow rate of 10 mL/min was done using a syringe infusion pump. Then, for the development of the method, in both positive and negative ionization modes MS parameters were tuned for CCrP, CrP, CCr, Crt and ATP. The precursor ions of CCrP, CrP, CCr, Crt and ATP were detected in full scan mass spectra at 221.90, 210.00, 144.30, 132.30 and 506.10 m/z, respectively. The collision energy was optimized, and MS/MS transitions were

Table 1 Mass spectrometric parameters for the quantification of CCrP, CrP, CCr, Crt and ATP

MS Conditions					
Analyte(s)	**CCrP**	**CrP**	**CCr**	**Crt**	**ATP**
Q1 [a] (m/z)	221.90	210.00	144.30	132.30	506.10
Q3 [b] (m/z)	79.00	79.00	98.10	90.00	159.00
DP [c] (v)	-8.00	-8.00	70.00	45.00	-25.00
EP [f] (v)	-10.00	-10.00	10.00	10.00	-10.00
CE [e] (v)	-15.00	-12.00	21.00	15.00	-33.00
CXP [f] (v)	-8.00	-7.00	8.00	10.00	-11.00
Polarity	Negative	Negative	Positive	Positive	Negative

[a] Q1 Precursor ion

[b] Q3 Product ion

[c] DP Declustering potential

[d] EP Entrance potential

[e] CE Collision Energy

[f] CEP Cell exit potential

selected as following for the determination of CCrP, CrP, CCr, Crt and ATP: 221.90>79.00, 210.00>79.00, 144.30>98.10, 132.30>90.00, and 506.10>159.00, respectively. The mass spectrometer's main working parameters are summarized in.

Table 1 and MS scan of the studied drugs were shown in Fig. 2.

Assay validation

According to FDA guidelines for bioanalytical method validation, the developed method was validated as per [26] as follows:

Selectivity

By analyzing six batches of blank tissue and comparing the results to samples of fortified tissue at the LLOQ. Figure 3 displays representative chromatograms from

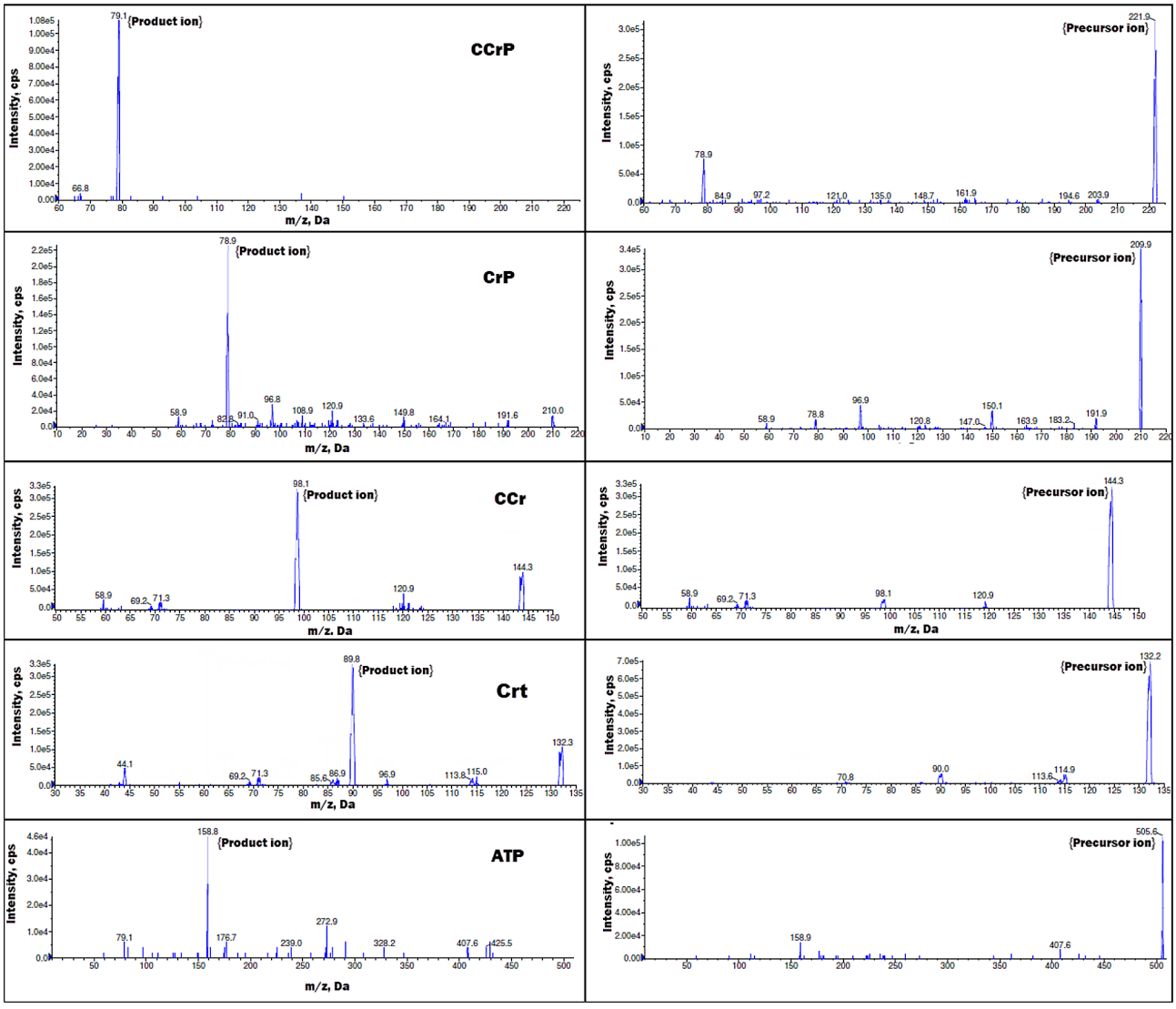

Fig. 2 MS scan of the studied drugs; for CCrP, CrP, CCr, Crt and ATP

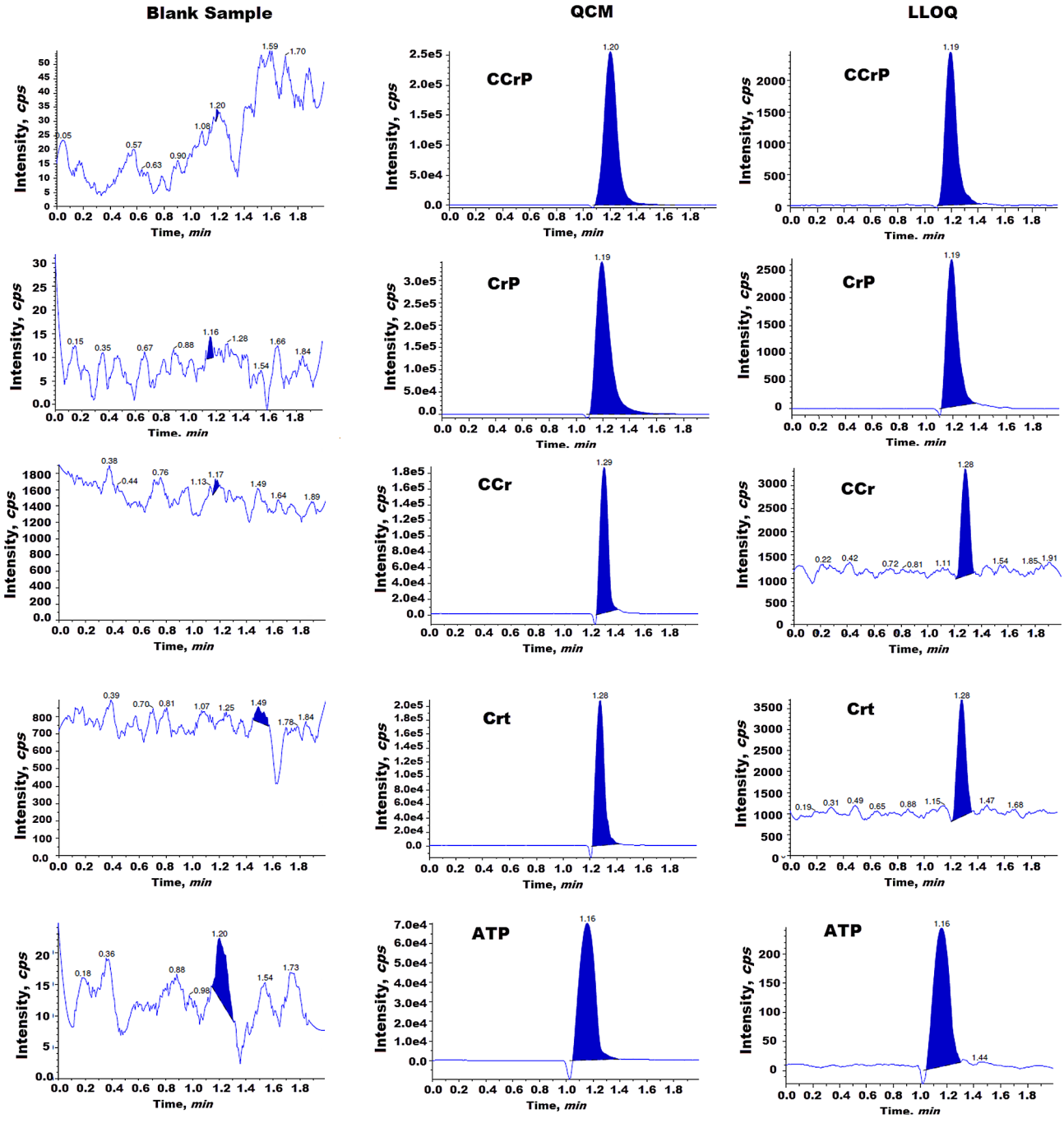

Fig. 3 Mass chromatograms of spiked plasma at QCM2 (600 ng/mL) for CCrP, CrP, CCr, Crt and ATP

analyses of blank tissue and samples at both LLOQ and QCM. These outcomes revealed the method's strong selectivity to the investigated compounds in the presence of matrix constituents (See Fig. 3).

Linearity and lower limit of quantitation

No "real" blank tissue was available as targeted analytes are endogenous substances. Thus, the chromatogram obtained using background subtraction with the blank tissue chromatogram. For linearity determination, the mean of three determinations at nine concentration levels within 5.00–2000.00 ng/mL range of each analyte was used (Figure 4). The regression equations were ($y=2259.61\ x-31.49$, $R^2=0.9997$), ($y=3343.5x$ -4205.95, $R^2=0.9983$), ($y=1102.16\ x+1317.23$, $R^2=0.9973$), ($y=928.7x+2978.9$, $R^2=0.9954$) and ($y=451.79\ x-480.22$, $R^2=0.9942$) for CCrP, CrP, CCr, Crt, ATP, respectively, (y) represents the analytes' subtracted peak

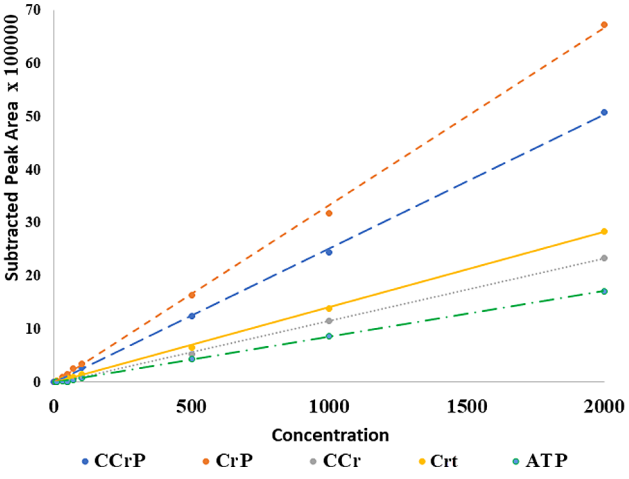

Fig. 4 Calibration curves of peak area ratio vs. concentration of CCrP, CrP, CCr, Crt and ATP in spiked heart samples

area and (x) represents the analyte's concentration in ng/mL. Moreover, the calibration curves for each substance (CCrP, CrP, CCr, Crt and ATP) were plotted and used to predict the concentration of analytes, as shown in Figure 4. For verifying the absence of interference from matrix components, blank and zero samples were included (See Fig. 4).

Accuracy and precision

Through fortified heart samples' analysis at the LLOQ, QCL, QCM, and QCH, the within-run and between-run accuracy and precision results are all within the acceptable range. The results are displayed in Table 2.

Matrix effect

A crucial component of assay validation is the matrix effect's evaluation. In this investigation, subtracted peak area was determined to account for potential endogenous matrix component effects on the quantitative measurement of the study compounds (CCrP, CrP, CCr, Crt and ATP). By comparing peak areas to determine ME%, the effects of suppression or enhancement were determined. Ion suppression was observed for all five compounds at the examined QCL and QCH values, as reported in Table 3: CCrP (89.82±0.07), CrP (95.99±0.01), CCr (92.68±0.01), Crt (96.65±0.01) and ATP (87.95±0.06). As previously reported, the background subtraction overcame the matrix effect with close to 100% recoveries for different analytes with (Table 3) [9]. In addition, in background subtraction, the same matrix as the calibration curve and QC samples is used as well as the variation of any matrix effects is reduced and there are good recovery between calibrators and samples [9, 10].

Recovery

The relative recovery is different than the detector response got for analyte's concentration in neat solvent as the relative recovery is "the detector response obtained from a quantity of the analyte added to and extracted from the biological matrix" [26]. PE is computed through both the matrix effect and recovery to determine the

Table 2 Accuracy and precision for determining CCrP, CrP, CCr, Crt and ATP

Intra-Day (Mean Recoveries ± RSD, *n* = 6)					
	CCrP	**CrP**	**CCr**	**Crt**	**ATP**
LLOQ 5ng/ mL	105.90 ± 8.08	108.28 ± 4.53	107.80 ± 6.27	96.00 ± 8.15	104.85 ± 3.49
QCL 15ng/ mL	101.91 ± 10.7	107.21 ± 2.96	105.50 ± 2.92	108.97 ± 3.66	94.05 ± 2.17
QCM 1 60ng/ mL	94.59 ± 3.01	99.72 ± 2.48	101.67 ± 6.46	104.48 ± 1.33	101.76 ± 6.53
QCM 2 600ng/ mL	103.71 ± 2.01	107.72 ± 2.66	104.11 ± 4.72	103.45 ± 6.54	110.63 ± 2.64
QCH 1500ng/ mL	88.32 ± 1.63	97.65 ± 2.20	108.42 ± 5.25	106.73 ± 5.84	95.17 ± 10.19
Inter-Day (Mean Recoveries ± RSD, *n* = 6)					
	CCrP	**CrP**	**CCr**	**Crt**	**ATP**
LLOQ 5ng/ mL	106.44 ± 8.81	94.31 ± 1.87	95.75 ± 13.72	96.47 ± 13.03	109.22 ± 4.07
QCL 15ng/ mL	94.53 ± 5.05	100.16 ± 5.04	98.68 ± 8.68	98.68 ± 5.36	95.73 ± 7.74
QCM 1 60ng/ mL	98.92 ± 7.54	93.32 ± 6.74	104.51 ± 8.80	102.09 ± 7.03	93.45 ± 5.54
QCM 2 600ng/ mL	111.99 ± 1.54	107.96 ± 4.20	106.68 ± 4.14	106.29 ± 4.89	107.18 ± 2.74
QCH 1500ng/ mL	108.73 ± 3.66	101.73 ± 8.87	104.15 ± 5.88	106.37 ± 4.12	103.34 ± 6.16

Table 3 Recovery and matrix effect for the determination of CCrP, CrP, CCr, Crt, ATP

	Set 1	Set 2	Set 3			
	Neat	**Matrix Matched**	**Tissue**	**ME %***	**RE%****	**PE%*****
	CCrP (Mean Peak Area, *n* = 6)					
QCL	4467.50	3802.20	3808.18	85.11	100.16	85.24
QCH	3646068.33	3446914.83	3446886.67	94.54	100.00	94.54
Mean				89.82	100.08	89.89
RSD				0.07	0.01	0.07
	CrP (Mean Peak Area,*n* = 6)					
QCL	44914.53	43039.98	43078.25	95.83	100.09	95.91
QCH	5345018.33	5139238.67	5138558.33	96.15	99.99	96.14
Mean				95.99	100.04	96.02
RSD				0.01	0.01	0.01
	CCr (Mean Peak Area,*n* = 6)					
QCL	34473.15	31670.37	31631.20	91.87	99.88	91.76
QCH	3130916.67	2927133.33	2930215.00	93.49	100.11	93.59
Mean				92.68	99.99	92.67
RSD				0.01	0.01	0.01
	Crt (Mean Peak Area,*n* = 6)					
QCL	33385.82	32223.48	32079.53	96.52	99.55	96.09
QCH	2707686.67	2620533.33	2617311.67	96.78	99.88	96.66
Mean				96.65	99.72	96.37
RSD				0.01	0.01	0.01
	ATP (Mean Peak Area,*n* = 6)					
QCL	9696.34	8188.33	8178.04	84.45	99.87	84.34
QCH	1606526.67	1469073.33	1468786.67	91.44	99.98	91.43
Mean				87.95	99.93	87.88
RSD				0.06	0.01	0.06

* Matrix effect (ME) expressed as the ratio of the mean peak area of an analyte fortified post extraction (Set 2, Matrix matched) to the mean peak area of the same analyte standards (Set 1, Neat solvent) multiplied by 100

** Recovery (RE) calculated as the ratio of the mean peak area of an analyte fortified before extraction (Set 3, Tissue) to the mean peak area of an analyte fortified post-extraction (Set 2, Matrix matched) multiplied by 100

*** Process efficiency (PE) expressed as the ratio of the mean peak area of an analyte fortified before extraction (Set 3, Tissue) to the mean peak area of the same analyte standards (Set 1, Neat solvent) multiplied by 100

efficiency of the entire procedure. In addition, determining the actual recovery of the analyte unaffected by ME, RE, was calculated, as shown in Table 3.

Methanol was selected as the extraction solvent that consistently produced good drug recovery after testing a number of extraction solvents. The results described show RE% CCrP (100.08±0.01), CrP (100.04±0.01), CCr (99.99±0.01), Crt (99.72±0.01) and ATP (99.93±0.01).

Stability

Some considerations were taken in accordance with ICH guidelines for bioanalytical method validation as following: stability experiments were done to simulate situations that may happen during either sample handling or analysis; preparing analyte samples form freshly prepared stock solution using appropriate biological matrix. By comparing the acquired LC–MS/MS data to the zero cycle data, it was determined that the samples had sufficient stability. First, the stock solution showed stability at 2–8 °C for 15 h (processed sample stability); In addition, the freeze–thaw stability long-term stability and benchtop stability are summarized in Table 4, which reveals that the analytes under study did not degrade.

Dilution integrity

FDA guidelines state that dilution should be performed if the sample concentration is greater than the upper limit of measurement to evaluate if the dilution process change the detected analyte concentration and compromise the designed assay's accuracy and precision (within 15% of nominal concentration represents the acceptable limits). The QCH levels were diluted as previously mentioned. This was crucial since it was anticipated that the quantities of the targeted analytes in the obtained samples would range widely. Using a dilution factor of up to 1:4, the developed method showed that it can be successfully applied within acceptable limits as shown in Table 5.

Application in rat heart samples

The linear relationships among CCrP, CrP, CCr, Crt and ATP in spiked rat tissue ranges and regression equations are shown in Figure 4 for rat hearts. The described

Table 4 The Summary of stability data of CCrP, CrP, CCr, Crt and ATP

	(Mean Recovery ± RSD, *n* = 3)			
	Post Preparative Stability	**Freeze and Thaw**	**Long-term Stability**	**Short Stability 24 h**
	CCrP			
QCL	105.22 ± 2.61	101.27 ± 3.09	107.53 ± 8.24	108.58 ± 2.78
QCH	92.95 ± 3.64	97.11 ± 0.61	93.72 ± 3.79	100.85 ± 0.52
	CrP			
QCL	100.49 ± 2.3	95.79 ± 4.19	95.19 ± 2.3	99.39 ± 7.28
QCH	105.28 ± 4.3	99.14 ± 2.49	86.6 ± 1.06	103.45 ± 6.02
	CCr			
QCL	102.58 ± 4.09	108.51 ± 7.01	104.96 ± 7.73	94.55 ± 7.48
QCH	112.19 ± 2.53	104.33 ± 8.03	102.71 ± 6.41	105.74 ± 7.37
	Crt			
QCL	104.96 ± 13.02	103.68 ± 10.28	104.96 ± 3.04	94.81 ± 4.13
QCH	109.32 ± 5.79	93.27 ± 5.54	111.93 ± 1.28	108.73 ± 3.88
	ATP			
QCL	105.29 ± 5.13	100.29 ± 10.7	102.28 ± 12.08	104.51 ± 5.02
QCH	105.18 ± 5.96	104.88 ± 10.3	110.39 ± 0.52	111.51 ± 3.29

Table 5 Study of dilution integrity of CCrP, CrP, CCr, Crt and ATP

	(Mean Recovery ± RSD, *n* = 6)		
	Analyte	**1:2**	**1:4**
QCH	CCrP	99.32 ± 4.63	96.72 ± 3.8
	CrP	104.86 ± 3.66	101.05 ± 4.31
	CCr	106.54 ± 2.24	96.83 ± 2.82
	Crt	105.01 ± 8.39	98.59 ± 5.59
	ATP	107.14 ± 1.89	105.27 ± 7.16

method was applied for the determination of CCrP, CrP, CCr, Crt and ATP in different rat tissues, as listed in Table 6 for rat hearts. CCrP is a bioenergetic and anti-inflammatory agent that have a promising therapeutic efficacy against myocardial ischemic sequelae, including heart failure, endorsing its clinical appliance to rescue poorly functioning hearts [29–32].

Conclusion

A fast, specific, reliable, accurate and highly sensitive UPLC–MS/MS assay was developed and validated that allowed determination of CCrP, CCr, CrP, Crt and ATP

Table 6 Concentration (ng/mL) of CCrP, CrP, CCr, Crt and ATP obtained from analysis of heart in different groups of rats

Sample Code	CCrP ng/mL	CrP ng/mL	CCr ng/mL	Crt ng/mL	ATP ng/mL
Saline/control 1	< LLOQ*	< LLOQ*	< LLOQ*	2417	428
Saline/control 2	< LLOQ*	< LLOQ*	< LLOQ*	2143	462
Saline/control 3	< LLOQ*	< LLOQ*	< LLOQ*	2251	667
CCrP/control 1	27.22	< LLOQ*	2105.43	2401	678
CCrP/control 2	29.14	< LLOQ*	2496.46	2034	630
CCrP/control 3	33.80	< LLOQ*	2491.09	1747	1005
ISO 1	< LLOQ*	< LLOQ*	< LLOQ*	2409	754
ISO 2	< LLOQ*	< LLOQ*	< LLOQ*	2817	398
ISO 3	< LLOQ*	< LLOQ*	< LLOQ*	2385	724
ISO + CCrP (0.8 mg/kg) 1	178.31	< LLOQ*	4473.22	2455	432
ISO + CCrP (0.8 mg/kg) 2	480.79	< LLOQ*	5348.3	2533	102
ISO + CCrP (0.8 mg/kg) 3	72.18	< LLOQ*	2647.07	2075	216
ISO + CCrP (0.8 mg/kg) 4	68.33	< LLOQ*	5964.89	1928	187
ISO + CCrP (0.8 mg/kg) 5	72.37	< LLOQ*	4724.56	2662	324
ISO + CCrP (0.4 mg/kg) 1	142.88	< LLOQ*	2800.76	2365	565
ISO + CCrP (0.4 mg/kg) 2	112.27	< LLOQ*	704.85	2230	1329
ISO + CCrP (1.2 mg/kg) 1	979	< LLOQ*	5757.35	2610	631
ISO + CCrP (1.2 mg/kg) 2	144.82	< LLOQ*	5600.85	2533	335

< LLOQ* expressed as lower limit of quantification which is below 5ng/mL

simultaneously in rat hearts using protein precipitation for sample preparation and background subtraction approach for quantitation of analytes. The validation results, according to FDA guidelines, stated that the developed assay has the advantages of being selective, precise, reproducible, accurate over low concentration levels comparable with the concentration of the drug in targeted samples with appropriate extraction recovery and avoiding interference with matrix components. The validity of the method was also studied to be applicable in further bioequivalence studies for CCrP determination during the preclinical trial phases and drug development process investigating the influence of CCrP on the levels of related biomarkers and CCrP metabolites. This validated bioanalytical assay will be applicable in several studies, such as those on creatine transporter deficiency, that affects the quality of life for patients and caregivers.

Abbreviations

CCrP	cyclocreatine phosphate
FDA	Food and Drug Administration
CCr	cyclocreatine
ATP	adenosine triphosphate
Crt	creatine
CrP	creatine phosphate
EMA	European Medicines Agency
ICH	International Council for Harmonization
UPLC-MS/MS	ultra-performance liquid chromatography-tandem mass spectrometry
QC	Quality control
QCH	Quality control high
QCL	Quality control low
QCM	Quality control medium
LLOQ	lower limit of quantification
ESI	Electrospray ionization
ODD	Orphan drug designation
CTD	creatine transporter deficiency
HILIC	hydrophilic interaction liquid chromatography
IS	internal standard
NIH	National Institutes of Health
TOC	total organic carbon
MRM	Multiple reaction monitoring
ESI	Electrospray ionization
ME	matrix effect
RE	Recovery efficiency
PE	process efficiency

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Author contributions
Review of literature, Analysis Methodology, Investigation, Bioanalysis validation, Visualization, Writing - original draft were carried out by Ibrahim F. Abo-Elmagd, Amr M. Mahmoud, Medhat A. Al-Ghobashy and Marianne Nebsen. Design of animal model, collecting samples for analysis, Conceptualization, Investigation, Visualization, Writing - review & editing were carried out by Mostafa A Rabie, Ahmed F Mohamed, Lamiaa A Ahmed, Nesrine S. El Sayed, Reem K. Arafa, Robert Todd. Salwa A. Elgebaly contributed the design of animal model, collecting samples for analysis, Visualization, Supervision, funding acquisition, critically revising the manuscript, Project administration and Funding acquisition. All authors reviewed the manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
All experimental procedures were approved by the Ethics Committee at the Faculty of Pharmacy, Cairo University and were conducted in compliance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85 – 23).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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