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Determination of CQP propionic acid in rat plasma and study of pharmacokinetics of CQP propionic acid in rats by liquid chromatography

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Abstract

A sensitive method for the determination of CQP propionic acid in rat plasma was developed and validated after solid-phase extraction. Chromatographic separation was achieved on a reversed-phase Alltima C_{18} column with the mobile phase of methanol–0.15% (v/v) phosphoric acid solution (pH 2.5) and step gradient elution resulted in a total run time of about 20 min. The analytes were detected by using UV detector at 345 nm. A good linear relationship was obtained in the concentration range of 50–12,800 ng/mL ($r = 0.9998$). The intra-day RSDs and the inter-day RSDs at the concentration of 200, 800, 6400 and 12,800 ng/mL were less than 7.0% and 11.0%, respectively. The intra-day accuracy ranged from 96.3 to 106.5% and the inter-day accuracy ranged from 98.6 to 113.4%, respectively. Average extraction recoveries ranged from 83.6 to 94.3% in plasma at the concentrations of 200, 800, 6400 and 12,800 ng/mL. This method was successfully applied to the pharmacokinetic studies on rats.

Keywords: CQP propionic acid; Pharmacokinetics; Liquid chromatography

1. Introduction

CQP propionic acid {CQP-PA, [1-(7-chloro-quinoline-4-yl-) piperazine-4-yl]-3-propionic acid, Fig. 1.}, one of the metabolite of piperaquine [1,2], is a new chemical which was isolated from human urine after oral administration of piperaquine. In our following study, it had been proven to have the potent antimalarial activity to be published in other paper.

In the recent years, malaria has been common and life-threatening disease in many tropical and subtropical areas. At present, it is the developing world's most dreaded killer. It kills over two million people per year according to estimates by the World Health Organization [3]. Antimalarial drugs such as chloroquine, piperaquine and artemisinin and its derivatives were mainly used in the clinical therapy. However, chloroquine (CQ) is, today, largely ineffective due to the resistance development [4], also, piperaquine and artemisinin and its derivates have poor bioavailability [5] because of strong lipophilic chemicals [6,7]. CQP-PA may be a very promising reagent for curing *faciparum* malaria because of the potent antimalarial activity. Till date, there is no published method on either assay for the determination of CQP-PA or its pharmacokinetics. Therefore, a simple, sensitive, precise and accurate HPLC method was developed and validated for determination of CQP-PA, which was used for the pharmacokinetic, toxicokinetic and metabolic study of this candidate chemical in rats.

2. Experimental

2.1. Reagents and chemicals

CQP-PA was isolated and purified from the urine of healthy Chinese volunteers after oral administration of piperaquine in our laboratory which was identified by MS, UV, IR, ^{1}H NMR and ^{13}C NMR. Its purity was higher than 98.5% by high-performance liquid chromatography. This project was approved by the local hospital ethics committee for study. Chloroquine

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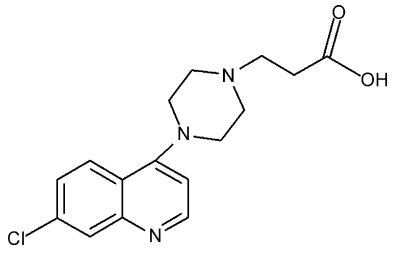

Fig. 1. The structure of CQP-PA.

as an internal standard was obtained from Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). Methanol (HPLC grade) was purchased from Merck Company (Darmstadt, Germany). Deionized water was obtained from a Milli-Q deionized water system (Millipore, Bedford, MA) and all other chemicals and solvents were of analytical grade.

2.2. Instrumentation and chromatographic conditions

Chromatographic separation was performed with a summit chromatographic system (Dionex, USA) equipped with a P680 isocratic pump, TCC-100 column compartment, ASI-100 autosampler and PDA-100 variable UV–vis detector. Separations of CQP-PA were achieved on an Alltima C_{18} column (5-μm, 4.6 mm × 100 mm) with a 5-μm SecurityGuard C_{18} short guard column (Grace, USA). Mobile phase A was methanol, while mobile phase B was 0.15% (v/v) phosphoric acid in water (pH 2.5). All of the mobile phases were filtered through a 0.45 μm membrane and degassed under reduced pressure before use. Linear gradient elution was performed within a 20 min running time and its sequence was as follows: The beginning with 10% of mobile phase A and 90% of mobile phase B, which was maintained for 5 min, then the mobile phase A to B ratio was gradually change to 30:70 at 5 min with steps of increasing A on 4%/min and maintained for 5 min, followed by returning to the initial composition for 5 min. The flow rate was set to 1.0 mL/min and the UV detector was set to 345 nm. The column temperature was set to 20 °C. A volume of 30 μL was injected into the LC system for analysis.

2.3. Preparation of standards and quality control (QC) samples

A concentrated stock solution of CQP-PA (1 mg/mL) was prepared in deionized water. Its working solutions ranging from 5000 to 1,280,000 ng/mL were prepared by serial dilution of the stock solution in deionized water. Working solution of CQP-PA (10 μL) was added to blank rat plasma to yield spiked calibration standards at eight different concentrations ranging from 50 to 12,800 ng/mL (not including blank rat plasma).

The calibration standards were prepared in batches of 2 mL, divided into 100 μL aliquots and stored at −70 °C until analysis. Quality control samples for determination of accuracy and precision in rat plasma were prepared at four concentrations (200, 800, 6400 and 12,800 ng/mL) in batches of 10 mL and divided into 100 μL aliquots. The aliquots were stored at −70 °C until analysis. These samples (200, 800, 6400 and 12,800 ng/mL) were also used to evaluate short-term, long-term and freeze/thaw stability.

2.4. Animal studies

All animal studies were performed in accordance with the experimental protocols approved by the Animal Care Committee of Guangzhou University of Chinese medicine. Ten male Sprague-Dawley rats weighing 280–320 g were supplied by the Guangdong Animal Center (Guangzhou, China). Rats were assigned to receive CQP-PA at concentration of 1 mg/kg i.v. via the tail vein. Blood samples (250 μL) were collected in heparinized tubes from the cervical artery pre-dose and at 0, 2, 4, 5, 7, 10, 15, 20, 25, 30, 45, 60, 75 and 90 min after i.v. via the tail vein administration. Blood samples were immediately centrifuged at 5000 × *g* for 15 min and stored at −70 °C until analysis.

2.5. Sample preparation

Rat plasma samples (100 μL) were thawed for 5 min and diluted with three times volume of phosphoric acid (0.06 mol/L, pH 1.5) followed by addition 30 μL of the CQ concentration of 10 μg/mL. The polypropylene tubes were placed on a vortex mixer for approximately 10 s. Then the mixed samples were loaded to solid-phase extraction (SPE) columns and extraction was carried out using C_{18} SPE cartridges (1 mL, 30 mg, Phenomenex, USA). The cartridges were conditioned with 1 mL methanol followed by 1 mL water. After loading 400 μL of the samples, the SPE cartridges were washed with 1 mL water. The analytes were then eluted with 1 mL methanol. The eluents were then evaporated to dryness at 37 °C under a gentle N_2 stream and reconstituted in 100 μL of methanol–deionized water (3:7, v/v) and a volume of 30 μL were injected for HPLC assay.

2.6. Method validation

2.6.1. Specificity

Blank plasma and CQP-PA in rat plasma after single intravenous push injection from six different rats were analyzed, and their chromatograms were examined for endogenous compounds which not could interfere with the quantitation of CQP-PA.

2.6.2. Accuracy and precision

To validate the quantities of CQP-PA were analyzed four concentrations on the same day. Four different concentrations (200, 800, 6400 and 12,800 ng/mL) were analyzed to cover the entire range of calibration curve. Analysis was being conducted by preparing replicates of five tubes of spiked rat plasma at each concentration level and concentrations were calculated from calibration curves.

The intra-day and inter-day accuracy and precision of the method was evaluated by analysis of five replicates of spiked rat plasma (200, 800, 6400 and 12,800 ng/mL) over 4 days. The procedures were the same as intra-day accuracy and precision.

2.6.3. Recovery

The extraction recovery was determined by (the extracted plasma concentration spiked with CQP-PA/nominal CQP-PA concentration) × 100%. Duplicates of CQP-PA in reconstitution solvent (corresponding to nominal CQP-PA concentrations; at 200, 800, 6400 and 12,800 ng/mL) were directly injected into the LC system. The CQP-PA peak area corresponding to the direct injections were used to build a calibration curve and to predict CQP-PA concentrations. The predicted concentrations of extracted plasma samples were compared with back-calculated concentrations of direct injections to determine recoveries.

2.6.4. Limit of quantification (LOQ) and limit of detection (LOD)

The LOQ was defined as the concentration quantity of the sample, which was quantified with <20% deviation in precision. The LOD was defined as the concentration with a signal-to-baseline noise ratio of 3.

2.6.5. Stability

2.6.5.1. Freeze/thaw stability. Analysis of freeze/thaw stability was conducted on 200, 800, 6400 and 12,800 ng/mL. QC samples which stored at −70 °C for 24 h. Aliquots were naturally thawed at room temperature. When completely thawed, the samples were refrozen for approximately 24 h again at −70 °C. These freeze/thaw samples were analyzed to investigate if there was any variation due to thawing of the samples. The stability data were used to support request for repeat analysis.

2.6.5.2. Storage stability. The stability of CQP-PA in rat plasma was investigated over a time period of 28 days. Spiked samples were prepared with drug free plasma at concentrations of 200, 800, 6400 and 12,800 ng/mL ($n = 5$). Spiked rat plasma samples were stored at −70 °C. Aliquots at each level were thawed and analyzed at 0, 7, 14, 21 and 28 days. A standard calibration curve was freshly prepared on the day of analysis, and concentration levels were predicted on the basis of calibration curve.

2.6.5.3. Autosampler stability. The autosampler stability was carried out for over 48 h by injecting the same extracted plasma sample of CQP-PA at the concentrations of 200, 1600, 6400 and 12,800 ng/mL at intervals of 6 h. The stability in the autosampler, which was set at 4 °C, was carried over a time period of 48 h.

2.7. Pharmacokinetic analysis

All the pharmacokinetic parameters were expressed as mean ± S.D. Pharmacokinetic analyses of CQP-PA were

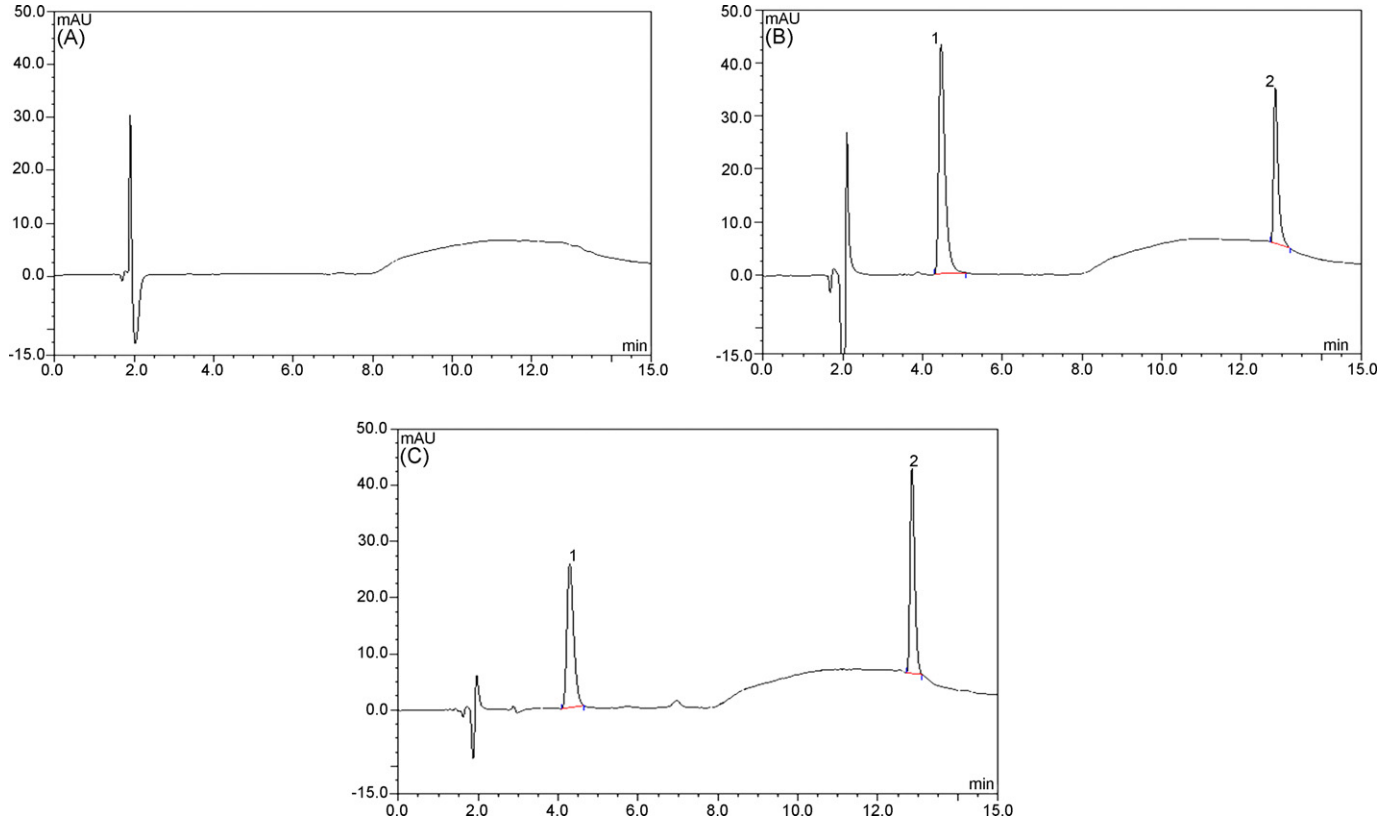

Fig. 2. Typical HPLC chromatograms for CQP-PA and the internal standard (CQ) in rat plasma. (A) Blank plasma sample; (B) blank plasma sample spiked with 10 μg/mL of CQP-PA and 10 μg/mL of the internal standard (CQ); and (C) plasma sample 2 min ($C = 1717.7$ ng/mL) after intravenous administration of 1 mg/kg CQP-PA. Peaks 1 and 2 refer to the CQP-PA and internal standard (CQ), respectively.

performed using PK Solution, version 2.0 (Summit Research Services, USA). The peak plasma concentration (C_0) was obtained directly from the concentration–time data. The elimination rate constant (β) was calculated from the slope of logarithm of plasma concentration versus time using the final three concentration–time data points. The apparent elimination half-life ($t_{1/2(\beta)}$) was calculated as $0.693/\beta$. The area under the plasma concentration–time curve ($AUC_{0\rightarrow\infty}$) and the first moment curve (AUMC) were calculated by trapezoidal rule. Total body clearance was calculated as $C_0/AUC_{0\rightarrow\infty}$. The mean residence time (MRT) after intravenous administration was calculated by $AUMC/AUC_{0\rightarrow\infty}$.

3. Results

3.1. Specificity

CQP-PA and the internal standard CQ eluted at approximately 4.3 and 12.8 min, repetitively. There was no endogenous interference from plasma or at any other point in the chromatogram. Typical HPLC chromatograms for blank plasma, blank plasma spiked with CQP-PA, and CQP-PA in rat plasma 2 min after single i.v. injection are presented in Fig. 2.

3.2. Linearity

There is a good linearity over the range of 50–12,800 ng/mL in the plasma and the calibration curve was $Y = 0.5933x - 0.017$ ($n = 8$) with a correlation coefficient greater than 0.9996.

3.3. Accuracy and precision

The data of precision and accuracy are summarized in Table 1. The intra-day RSDs and the inter-day RSDs at the concentrations of 200, 800, 6400 and 12,800 ng/mL were less than 7.0% and 11.0%, respectively. The intra-day accuracy ranged from 96.3 to 106.5% and the inter-day accuracy ranged from 98.6 to 113.4%.

3.4. Recovery

Percentage recovery of CQP-PA was measured by dividing the ratio of concentration levels to that of controls. The mean recoveries ($n = 5$) for CQP-PA (200, 800, 6400 and 12,800 ng/mL) were 83.6%, 88.2%, 93.4% and 94.3%, respectively. The results are shown in Table 2.

Table 1
The precision and accuracy of the plasma concentration for CQP-PA assay ($n = 5$)

Concentration (ng/mL)	Accuracy (%)		RSD (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
200	106.5	113.4	6.4	10.7
800	99.2	102.1	1.8	4.2
6,400	96.8	101.4	5.3	2.6
12,800	96.3	98.6	4.3	3.8

Table 2
The mean extraction recovery for CQP-PA assay ($n = 5$)

Concentration (ng/mL)	Recovery (%)	RSD (%)
200	83.6	7.2
800	88.2	5.6
6,400	93.4	3.5
12,800	94.3	3.1

Table 3
The freeze/thaw stability for CQP-PA assay ($n = 3$)

Concentration (ng/mL)	Mean concentration (ng/mL)	Recovery (%)	RSD (%)
200	216.9	108.3	9.3
800	822.1	102.6	3.8
6,400	6,296.5	98.3	2.8
12,800	12,486.9	97.4	3.4

3.5. Limit of quantification (LOQ) and limit of detection (LOD)

The LOQ for the method was 40 ng/mL, while the LOD was 25 ng/mL.

3.6. Stability

The freeze/thaw stability of CQP-PA was determined by measuring the recovery and RSD for samples which conducted three freeze/thaw cycles. The results showed that CQP-PA was stable in rat plasma through three freeze/thaw cycles. These data are shown in Table 3.

3.7. Storage stability

The stability of PQP in rat plasma was investigated by analyzing QC samples of 200, 800, 6400 and 12,800 ng/mL stored at −70 °C for 28 days. The RSDs ranged from 2.8 to 6.8% and the recovery ranged from 97.9 to 112.3% for CQP-PA. The results indicated that CQP-PA was found to be stable in rat plasma at −70 °C for at least 28 days. The data of storage stability are summarized in Table 4.

3.8. Autosampler stability

The stability of samples, which were stored in the autosampler at 4 °C, was performed on over a time period of 48 h by injecting the same sample at an interval of 6 h. The RSDs for

Table 4
The storage stability for CQP-PA assay ($n = 5$)

Concentration (ng/mL)	Mean concentration (ng/mL)	Recovery (%)	RSD (%)
200	222.7	112.3	6.8
800	795.6	99.8	4.6
6,400	6,067.1	97.9	3.7
12,800	12,581.2	98.3	2.8

Table 5
The autosampler stability for CQP-PA assay ($n = 9$)

Concentration (ng/mL)	Autosampler stability mean ratio of CQP-PA/CQ over 48 h	RSD (%)
200	0.094 ± 0.007	7.7
1,600	0.735 ± 0.016	2.2
6,400	3.104 ± 0.174	5.6
12,800	6.223 ± 0.258	4.1

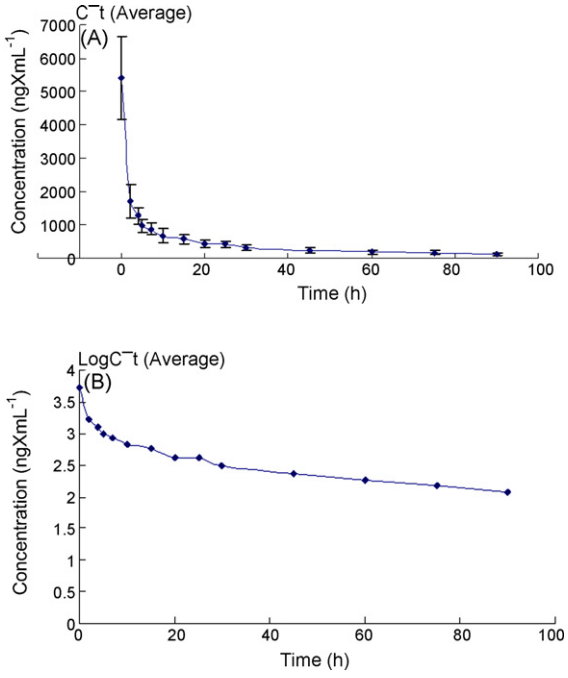

Fig. 3. Mean plasma concentration–time curve after intravenous administrations of 1 mg/kg CQP-PA in rats (each point represents the mean ± S.D. of 10 rats). (A) The concentrations in numeric scale and (B) the concentrations in logarithm scale.

the peak area ratio of CQP-PA at the concentrations of 200, 1600, 6400 and 12,800 ng/mL were less than 8%. This results show that samples of CQP-PA are stable up to 48 h in the autosampler at 4 °C. Results of autosampler stability are shown in Table 5.

3.9. Pharmacokinetic study

The pharmacokinetic property of CQP-PA after single intravenous push injection to rats could be described by an open two-compartment model (PK Solution, version 2.0). The curves of plasma concentration–time were shown in Fig. 3. The present results showed very rapid distribution and elimination of CQP-PA following intravenous push injection in rats.

As for intravenous push injection, the half-lives for the distribution and elimination phase ($t_{1/2(\alpha)}$ and $t_{1/2(\beta)}$) were 5.055 ± 1.648 and 68.23 ± 16.02 min, respectively. The volume of the central compartment (Vc) was 174.09 ± 46.69 mL/kg. The area under the plasma concentration curve ($AUC_{0\rightarrow\infty}$) of CQP-PA after intravenous push injection was 48786.4 ± 8155.9 min × ng/mL. Other pharmacokinetic parameters of this study are shown in Table 6.

Table 6
Pharmacokinetic parameters after intravenous administration of 1 mg/kg CQP-PA to rats ($n = 10$)

Parameter	Values
β (1/min)	0.01233 ± 0.00388
α (1/min)	0.1367 ± 0.0597
C_0 (ng/mL)	5132.45 ± 1040.73
C_l (mL/min/kg)	7.21 ± 2.06
MRT (min)	62.38 ± 28.8
AUMC (min × min × ng/mL)	3215819 ± 1547460
K_{12} (1/min)	0.0761 ± 0.0317
K_{21} (1/min)	0.0437 ± 0.0147
K_{10} (1/min)	0.0424 ± 0.0119

4. Discussion

Several kinds of columns were investigated in this study to select a suitable column for the chromatographic assay. The column (5-μm, 4.6 mm × 100 mm) was found to give sharp, symmetrical peaks and good sensitivity using the gradient elution and a mobile phase consisting of the mixture of phosphoric acid (0.15%) (pH 2.5) and methanol. Various other columns (Zorbax C_{18}, Lura C_{18} and XTerra C_{18}) were also evaluated, but only by using the Alltima C_{18} column, we may obtain the best separation and chromatogram for the CQP-PA and internal standard under the investigated conditions.

Different contents of mobile phase were evaluated for increasing the sensitivity and obtaining better separation and sharper peaks. It was also of importance to investigate whether the pH value has an effect on elution time and sensitivity of the peaks. The pH value of aqueous phase varied from 1 to 7 was evaluated in this study. Poor sensitivity and unsymmetrical peaks between CQP-PA and the internal standard (CQ) were obtained when the pH value range of 4–7. While the pH value of aqueous phase was less than 3, the sensitivity, elution time, symmetrical peaks and good separation on chromatography were acceptable under the conditions mentioned above.

The ultraviolet (UV) spectrum of CQP-PA was the same as or was similar to that of piperaquine [8], it mainly has three intense UV absorption peak at 227, 240 and 345 nm (Fig. 4.).

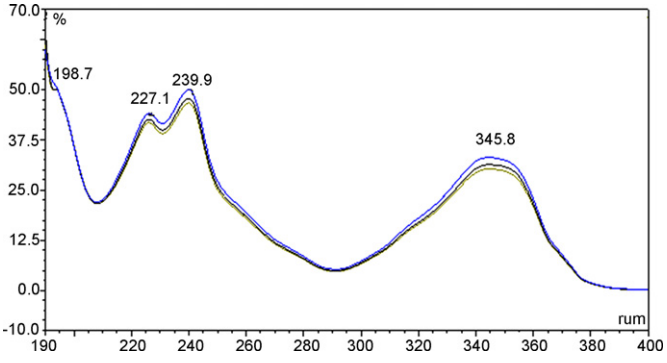

Fig. 4. Wavelength scans of CQP-PA using PAD-detector.

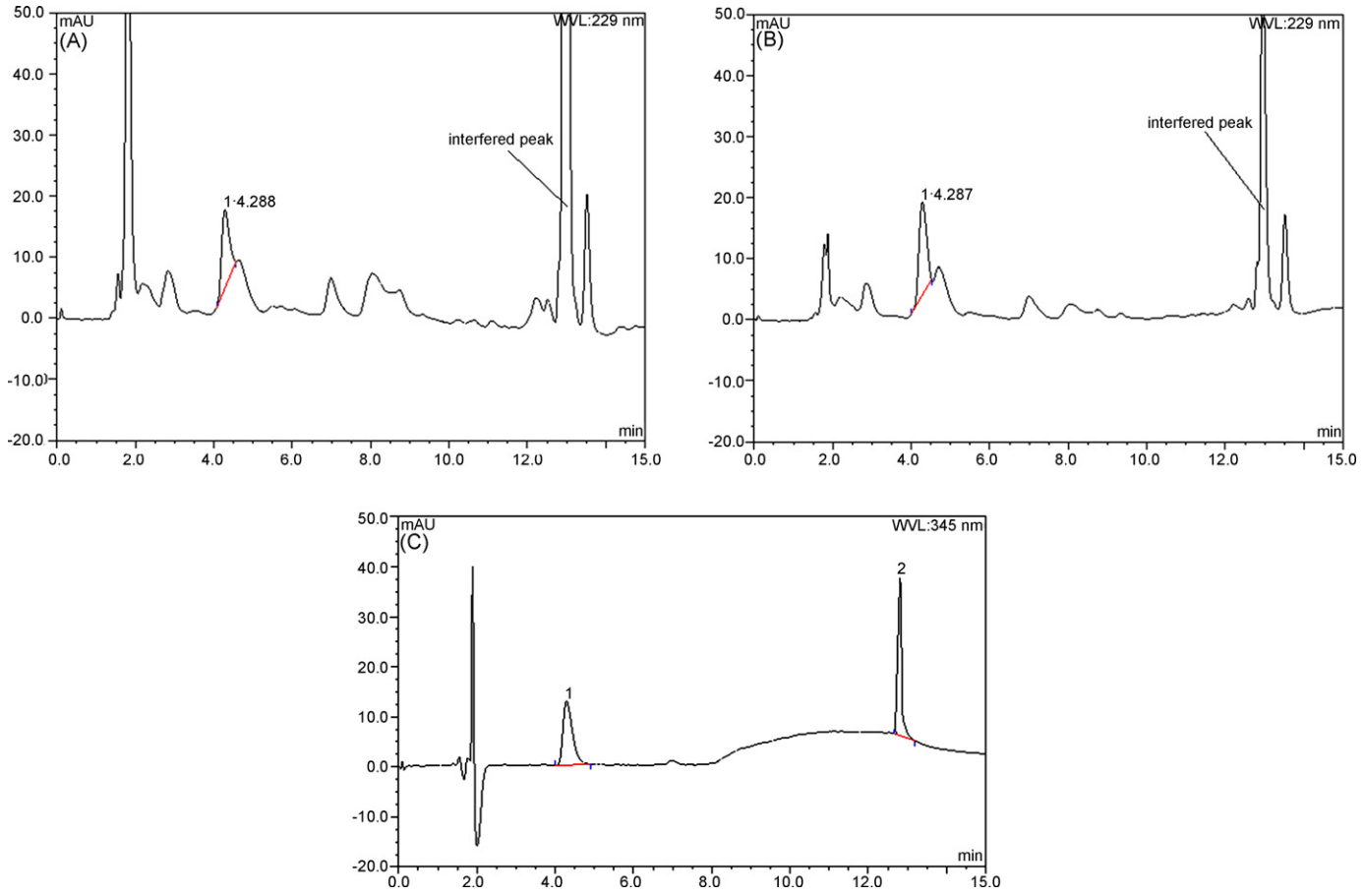

Fig. 5. Typical HPLC chromatograms for CQP-PA (C = 797.3 ng/mL) and the internal standard (CQ) in rat plasma. (A) At 229 nm; (B) at 240 nm; and (C) at 345 nm. Peaks 1 and 2 refer to the CQP-PA and internal standard (CQ), respectively.

Although CQP-PA has a very intense UV absorption peak at 227 and 240 nm, we chose to analyze on the less intense UV absorption peak at 345 nm, because at this wavelength 345 nm, the interference from endogenous substances in plasma was not shown (Fig. 5.), while there were interferences at 227 nm and 240 nm.

Liquid–liquid extraction was tested by using various kinds of organic solvents like dichloromethane, diethyl ether, chloroform, ethyl acetate, petroleum ether, etc. The mixture of these organic solvents at different ratios also had been attempted to extract the CQP-PA in plasma. Poor extraction efficiency was obtained from these organic solvents. The reason for this might be that the CQP-PA is strong hydrophobic properties in nature. It was poorly soluble in the above tested organic solvents and resulted in poor extraction efficiency. However, the application of SPE for extraction of CQP-PA can achieve the higher sensitivity, reproducible operation and extraction efficiency in the chromatographic assay.

The validation of our method consisted of intra- and inter-day accuracy and precision, recovery and stability at three concentration levels of 200, 800, 6400 and 12,800 ng/mL (n = 5). The results showed that the variation in the above mentioned coefficient was within the acceptable range required for validation of an assay [9].

5. Conclusion

A simple, sensitive and specific assay for the determination of CQP-PA in rat plasma has been developed and validated using SPE followed by reversed-phase chromatography. No interfering peaks appeared at the elution times of CQP-PA and internal standard. Adequate specificity, precision, and accuracy of the proposed method were demonstrated over the concentration range of 50–12,800 ng/mL. The method was accurate, reproducible, specific, and applicable to the evaluation of pharmacokinetic study of CQP-PA in rats.

Acknowledgements

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References

[1] J. Taming, T. Singtoroj, A. Annerberg, M. Ashton, Y. Bergqvist, N.J. White, N.P. Day, N. Lindegard, J. Pharm. Biomed. Anal. 41 (2006) 213.
[2] J. Taming, T. Singtoroj, M. Ashton, Y. Bergqvist, N.J. White, N.P. Day, N. Lindegard, Drug Metab. Dispos. 34 (2006) 2011.

[3] J.R. Zucker, C.C. Campbell, Infect. Dis. Clin. North Am. 7 (1993) 547.
[4] A.S. Muula, N. Engl. J. Med. 356 (2007) 868.
[5] Q. Chen, J. Deng, D. Wu, Pharm. Ind. 8 (1979) 19.
[6] T.M. Davis, T.Y. Hung, I.K. Sim, H.A. Karunajeewa, K.E. Ilett, Drugs 65 (2005) 75.
[7] M.E. Sarciron, C. Saccharin, A.F. Petavy, F. Peyron, Am. J. Trop. Med. Hyg. 62 (1) (2000) 73.
[8] N. Lindegardh, M. Ashton, Y. Bergqvist, J. Chromatogr. Sci. 41 (2003) 44.
[9] Guidance for industry, Bioanalytical Method Validation, US Department of Health and Human Services, May 2001.