Quantitative determination of corosolic acid in rat plasma by LC-MS/MS-ESI: Application to a pharmacokinetic study

Baohong Li[1*], Jindong Wu[1] and Xianglu Li[2]
[1]School of Pharmacy, Guangdong Medical College, Dongguan, PR China
[2]Nature's & US Limited Liability Corporation, St. Louis, USA

Abstract: A sensitive and robust liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the determination of corosolic acid (CRA) in rat plasma. The plasma samples were prepared by a simple protein precipitation treatment. Chromatographic separation was performed on an Agela MG-C_{18} column (50 mm × 4.6 mm; i.d., 5 µm) at 30°C. The mobile phase consisting of methanol-10 mM ammonium acetate–formic acid (80:20:0.1, *v/v/v;* pH=3.8) was delivered with a flow rate of 0.6 ml/min and a running time of 2.5 min. Quantification was performed with positive electrospray ionization (ESI) in the multiple-reaction monitoring (MRM) mode at *m/z* 490 → *m/z* 205 for CRA, and *m/z* 471 → *m/z* 203 for TEOA (internal standard, IS), respectively. Linearity was evaluated over the concentration range of 1.00-1000 ng/ml with a lower limit of quantification (LLOQ) of 1.00 ng/ml. The validated method was successfully applied to the pharmacokinetic study of CRA after the drug was orally administered in Sprague-Dawley rats.

Keywords: Corosolic acid, LC-MS/MS-ESI, rat plasma, pharmacokinetics.

INTRODUCTION

Corosolic acid (CRA, fig. 1), or 2α-hydroxyursolic acid, is reportedly present in several Chinese medicinal herbs, including the *Lagerstroemia speciosa* (Fukushima *et al.,* 2006), banaba leaves (Yamaguchi *et al.,* 2006), *Weigela subsessilis* (Lee *et al.,* 2010a) and *Eriobotrya japonica* (Hu *et al.,* 2006), among others. In recent years, studies have focused on the pharmacological activities of CRA, such its anti-diabetes (Shi *et al.,* 2008; Miura *et al.,* 2004 and 2006), anti-inflammation (Yamaguchi *et al.,* 2006; Banno *et al.,* 2004), antiproliferation (Fujiwara *et al.,* 2011; Lee *et al.,* 2010b) and protein kinase C inhibition activities (Ahn *et al.,* 1998; Lee *et al.,* 2010b). The anti-diabetic activity of CRA has attracted special attention, and several *in vivo* experiments involving the compound have been carried out in animals and human (Stohs *et al.,* 2012; Yamada *et al.,* 2008).

CRA effectively lowers glucose levels in human subjects after oral administration (Fukushima *et al.,* 2006). Furthermore, plant extracts (commercially named Glucosol™) containing the primary active principle, CRA, have been marketed for reducing blood glucose levels and weight-loss in Japan and the United States (Judy *et al.,* 2003). To date, however, limited literature on the determination of CRA in raw materials using TLC (Zong *et al.,* 2007), HPLC-DAD (Yang *et al.,* 2011) or HPLC-UV (Hu *et al.,* 2006) is available. To the best of our knowledge, only one study on the detection of CRA in selective ion monitoring mode by liquid chromatography-mass spectrometry (LC-MS) in biological fluids has been published (Liu *et al.,* 2011). Unfortunately, this method provided a CRA quantification limit of 20.0 ng/ml, which is inadequate for CRA detection in pharmacokinetic studies on rat plasma because the low concentration of the compound in this substrate.

In the present study, we present the development and validation of a highly sensitive and selective liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for determining CRA in biological fluids under multiple-reaction monitoring (MRM) mode. The proposed method has several advantages over an earlier published LC-MS method (Liu *et al.,* 2011), including lower plasma volume (75 µl), shorter run time (2.5 min), higher sensitivity (1.00 ng/ml), and higher selectivity. The validated method was successfully applied to quantitate CRA levels in rat plasma in a pharmacokinetic study.

MATERIALS AND METHODS

Chemicals and reagents

The reference standard of CRA (>98.0%) was purchased from Shenyang Longpower Technology Co., Ltd. (Shenyang, China) and 2α, 3α, 24-trihydroxyurs-12-en-28-oic acid (TEOA, >98.0%) as the internal standard (IS) was isolated in our research group. Their structures were characterized by MS and NMR analysis (data not shown). HPLC-grade methanol and acetonitrile were purchased from Sigma (St Louis, MO, USA); analytical grade ammonium acetate and formic acid were from Tedia (Fairfield, OH, USA). Deionized water was prepared through a Milli-Q water purification system (Millipore, Bedford, MA, USA).

**Corresponding author:* e-mail: baohong_li@163.com

Animal study

All the animal study was approved by the Animal Welfare and Ethics Committee of Guangdong Medical College (Dongguan, China). Male Six Sprague–Dawley rats (200~220 g) were purchased from the Experimental Animal Center of Guangdong Medical College (Dongguan, China). The rats were fasted overnight with water *ad libitum* before the experiment, and orally administered at a dose of 100 mg/kg CRA, which was homogeneously suspended in 0.5% carboxymethyl cellulose sodium (CMC-Na) aqueous solution. 200 µl of blood samples were collected from the orbital vein under aether anesthesia into a heparinized microcentrifuge tube with the saline containing 100 IU/ml heparin before administration and 0.083, 0.25, 0.50, 1.0, 1.5, 2.0, 3.0, 5.0, 8.0, 12.0 and 24.0 h. Plasma was harvested by centrifuging blood samples at 3000 rpm/min for 8 min and then frozen at –80°C until LC-MS/MS analysis.

LC-MS/MS conditions

An Agilent 6410 triple quadrupole LC-MS system with positive electrospray ion (ESI) source was applied for analysis. The chromatographic separation was performed on an Agela MG-C_{18} analytical column (50 mm × 4.6 mm, i.d., 5µm). The column temperature was kept at 30°C and the injection volume was 5µl. Methanol-10 mM ammonium acetate-formic acid (80:20:0.1, *v/v/v*; pH=3.8) was used as a mobile phase at a flow rate of 0.6 ml/min. Agilent MassHunter B 01.03 software was used for data analysis.

Quantification was performed using MRM mode with the transitions of *m/z* 490→205 for CRA and *m/z* 471→203 for TEOA (IS). The main instrument parameters were shown: capillary voltage, 4500 V; gas temperature, 325°C; gas flow, 10 l/min; nebulizer pressure, 30 psi.

Calibration standards and quality control samples

Individual stock solutions of CRA (510 µg/ml) and TEOA (IS, 1.00 mg/ml) were prepared in methanol. Working solutions for calibration and quality-control (QC) were prepared from the stock solution by dilution using acetonitrile, respectively. A 100 ng/ml working solution of IS was prepared by diluting IS stock solution with acetonitrile.

Calibration standards were prepared by spiking blank rat plasma with appropriate amounts of the CRA working solutions to obtain the concentration range 1.00-1000 ng/ml for CRA in rat plasma (1.00, 3.00, 10.0, 30.0, 100, 300 and 1000 ng/ml). QC samples were prepared in the same way at three different plasma concentrations (low, 3.00 ng/ml; medium, 30.0 ng/ml; and high, 900 ng/ml). All the samples were stored at-80°C until analysis.

Plasma sample preparation

75 µl of thawed plasma samples were accurately pipetted and then mixed with 75 µl of the IS working solution (TEOA, 100 ng/ml). After 75 µl of acetonitrile as precipitation reagent was added, the plasma samples were vortexed for 60 s and then centrifuged for 5 min at 11000 rpm/min. About 100 µl of aliquots were transferred to autosampler vials for LC-MS/MS analysis.

Method validation

A total validation process for the assay in rat plasma was carried out according to the US-FDA guidelines (US DHHS *et al.*, 2001).

Calibration curves were constructed by plotting the peak area ratio of CRA:IS against the nominal concentration of calibration standards in control rat plasma. Linearity was evaluated at concentration ranges of 1.00-1000 ng/ml for CRA in rat plasma. Sensitivity was determined in terms of limit of detection (LOD) and lower limit of quantification (LLOQ) at signal-to-noise ratio of 3:1 and 10:1, respectively. The LOD and LLOQ values were estimated to be 0.300 ng/ml and 1.00 ng/ml, respectively. Accuracy and precision were determined for low, medium, and high QC samples, respectively. The accuracy was expressed as relative error (RE), which was calculated by (Found conc. -Added conc.)/(Added conc.) × 100%. The precision was evaluated as relative standard deviation (RSD). Accuracy and precision values should be within 20% for LLOQ and less than 15% for QC samples. Recoveries and matrix effects of the analyte were determined at the three QC levels (3.00, 30.0 and 900 ng/ml) in triplicate, whereas those of IS were evaluated at a single concentration of 100 ng/ml. The stability of the analyte in rat plasma was investigated including three freeze-thaw cycle stability (–80°C ↔ 25°C), short-term stability (2 h of storage at room temperature), post-preparative stability (12 h in the autosampler tray), and long-term stability (23 days at-80°C) at low and high concentration levels of QC samples, respectively.

Pharmacokinetic analysis

Pharmacokinetic parameters of CRA in rats were calculated by noncompartmental methods using DAS software (version 2.0, China State Drug Administration), including maximum plasma concentration (C_{max}), observed time to C_{max} (T_{max}), terminal or elimination half-life ($T_{1/2}$), area under the plasma concentration–time curve from time 0 to the last measured concentration (AUC_{0-t}), and area under the plasma concentration–time curve from time 0 to infinite ($AUC_{0-\infty}$).

RESULTS

Mass spectrometric condition

ESI-MS/MS was applied to measure the analytes. The full-scan and product ion spectra of the analyte and IS were investigated to quantify the analytes in the MRM scan mode. Given that CRA has one carboxyl group in its structure, the feasibility of using ESI in positive/negative

ion detection mode was first evaluated. No characteristic product ions of the deprotonated molecule $[M-H]^-$ at *m/z* 471 for CRA were observed under negative-ion detection mode, although the signal intensity of the deprotonated molecule $[M-H]^-$ at *m/z* 471 was high. By contrast, under positive-ion detection mode, the mass spectra of CRA revealed a prominent peak at *m/z* 490 (higher than the peaks of the protonated molecule $[M + H]^+$ at *m/z* 473 and the adduct ion $[M + Na]^+$ at *m/z* 495), which was assigned as the ammonium ion $[M + NH_4]^+$. The product ion mass spectra of the ammonium ion $[M + NH_4]^+$ at *m/z* 490 showed the formation of characteristic product ions at *m/z* 189, 205, 249, 313, 409 and 437 (fig. 2). Thus, $[M + NH_4]^+$ ions were identified as the precursor ions to acquire the product ions for MRM analysis. The monitored transitions *m/z* 490 → *m/z* 205 for CRA were selected for MRM analysis.

In the full scan spectra of the IS, the most abundant ions observed at *m/z* 471 were dehydrated protonated molecule $[M + H - H_2O]^+$. The MS^2 scan showed strong product-ion of IS at *m/z* 203 (fig. 2).

Specificity and selectivity

Representative MRM chromatographic profiles of blank plasma, blank plasma spiked with CRA at the LLOQ, and rat plasma sample at 2.0 h after oral administration of CRA are shown in fig. 3. The retention times of CRA and IS are 1.68 and 0.84 min, respectively. No endogenous interference was observed at their retention times of CRA and IS.

Linearity and LLOQ

The plasma calibration curves were constructed over the concentration range of 1.00-1000 ng/ml in duplicates in plasma on three consecutive days. A typical equation was: y=0.0024 x + 0.0114 (r^2=0.9967), where y represents the peak-area ratio of CRA to IS, and x the plasma concentration of CRA. The LLOQ was 1.00 ng/ml, at which the accuracy (RE%) was within 4.9%, and the precision (RSD%) was 10.3% in rat plasma.

Accuracy and precision

The intra- and inter-day precision and accuracy are listed in table 1. Intra- and inter-day precision ranged from 2.0 to 4.2%; intra- and inter-day accuracy from-1.8 to 3.4%. The values on both occasions were all within the accepted variable limits.

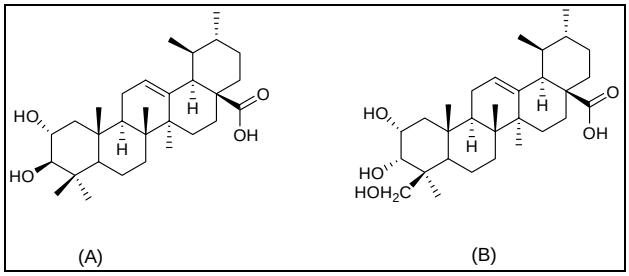

Fig. 1: The chemical structure of CRA (A) and TEOA (B, IS).

Recovery and matrix effect

The extraction recoveries of CRA and IS in rat plasma were over the range of 95.7-98.9%, showing that protein precipitation could offer excellent extraction efficiency for the analyte in biological matrices. Matrix effects of CRA and IS were found acceptable in different matrices and between different sources (80-120%), indicating that

Table 1: Precision and accuracy of CRA in rat plasma (intra-day, n = 6; inter-day, n = 18, 3 days)

Nominal concentration (ng/ml)	Intra-day (n=6)			Intra-day (n=18)		
	Determined concentration (ng/ml)	Accuracy (%RE)	Precision (%RSD)	Determined concentration (ng/ml)	Accuracy (%RE)	Precision (%RSD)
3.00	2.94±0.12	-2.1	4.2	2.96±0.16	-1.2	2.0
30.0	29.45±1.22	-1.8	4.1	29.45±1.46	-1.8	2.2
900	930.20±20.36	3.4	2.2	918.67±27.34	2.1	3.1

Table 2: Stability of CRA in rat plasma under different conditions (n = 6)

Conditions	Determined concentration (ng/ml)	Accuracy (%RE)	Precision (%RSD)
3.00 ng/ml oh	2.88±0.04	-3.9	1.5
Room temperature 2h	2.96±0.16	-1.3	5.5
Autosampler for 12h	3.02±0.25	0.8	8.2
Three freeze-thaw cycles	2.82±0.01	-6.0	0.3
Long-term stability at-80°C 23 days	3.05±0.21	1.6	6.8
900 ng/ml oh	926.76±5.29	3.0	0.6
Room temperature 2h	933.14±12.00	3.7	1.3
Autosampler for 12h	929.35±27.31	3.3	2.9
Three freeze-thaw cycles	904.35±34.16	0.5	3.8
Long-term stability at-80°C 23 days	902.68±63.84	0.3	7.1

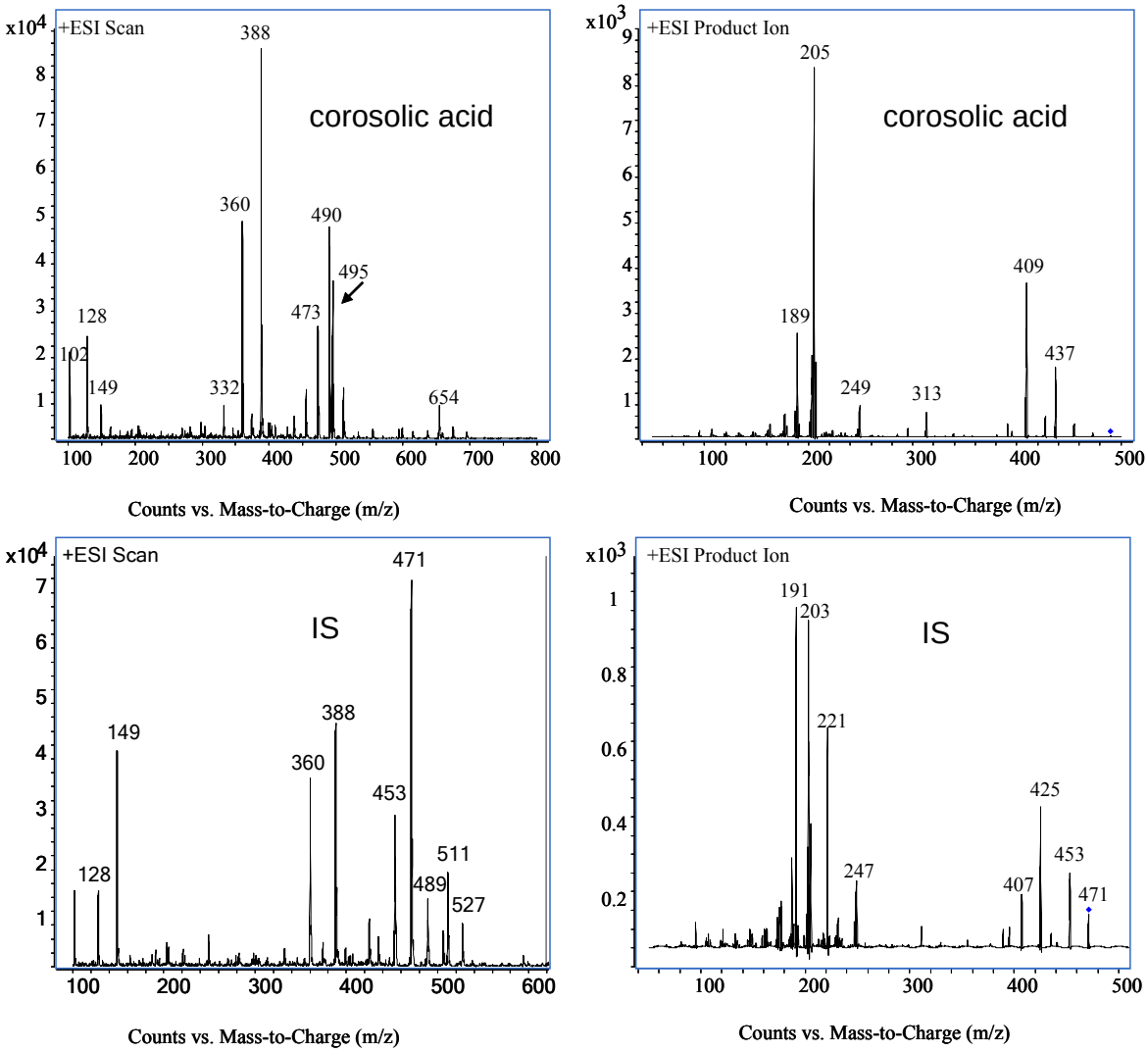

Fig. 2: MS and MS/MS spectra of CRA and of TEOA (IS).

the matrix had little coeluting endogenous substances that could influence the ionization of the analyte and IS.

Stability

The analyte was found to be stable under a variety of storage and process conditions, and the ranges of the accuracy for the analyte are between -3.9% and 3.7% (table 2).

Pharmacokinetic study

The validated LC-MS/MS method was successfully applied to the pharmacokinetic study of CRA in rat plasma. Following a single dose of 100 mg/kg CRA in Sprague–Dawley rats, the mean plasma concentration *vs* time profiles are shown in fig. 4 and the main pharmacokinetic parameters of CRA are presented in table 3. The mean maximum CRA plasma concentration was 60.77±11.75 ng/ml, T_{max} was 1.58±0.38 h, $T_{1/2}$ was 5.45±0.93 h, AUC_{0-t} was 422.66±48.53 ng h/ml, and $AUC_{0-\infty}$ was 445.88±59.81 ng h/ml.

DISCUSSION

Various chromatographic conditions were employed to estimate the selectivity, sensitivity, and peak shape of the proposed method. Methanol or acetonitrile containing different buffers (e.g., ammonium acetate, formic acid) and various HPLC analytical columns [Dikma RP-C_{18} column (150 mm × 4.6 mm, i.d., 5 µm), Shiseido Capcell UG120-C_{18} column (100 mm × 4.6 mm, i.d., 5 µm), and Agela MG-C_{18} column (50 mm × 4.6 mm, i.d., 5 µm) were tested for complete chromatographic resolution of CRA and IS (data not shown). Good resolution of the peaks was achieved with methanol-10 mM ammonium acetate-formic acid (80:20:0.1, *v/v/v*; pH=3.8) at a flow rate of 0.6 ml/min on an Agela MG-C_{18} column (50 mm × 4.6 mm, i.d., 5 µm), which indicates the suitability of these conditions for the determination of CRA and the IS. Formic acid and ammonium acetate as buffers can significantly increase protonation and improve the resultant peak shapes.

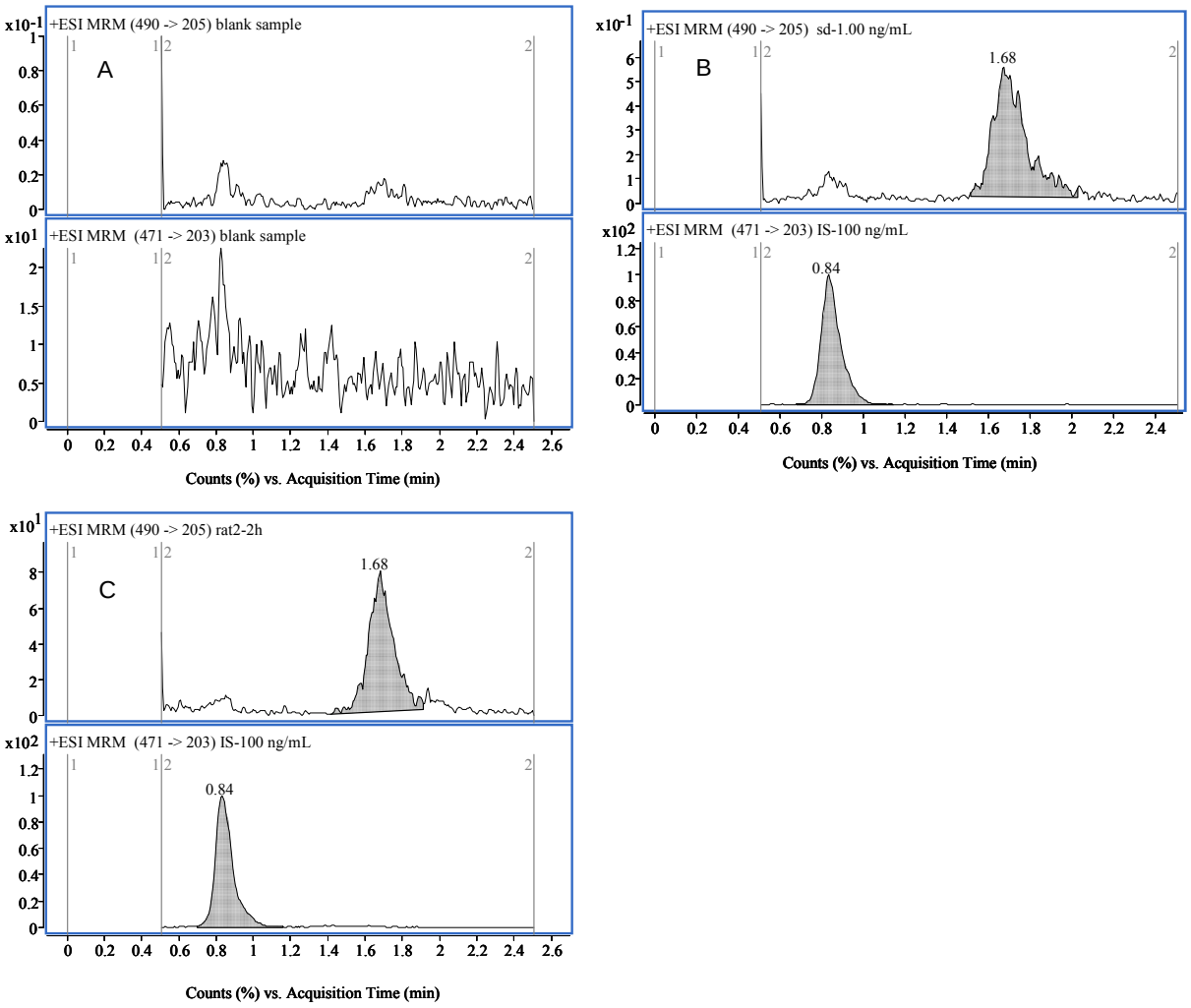

Fig. 3: MRM chromatograms of CRA and IS in rat plasma. (A) Blank plasma sample; (B) plasma sample spiked with CRA at 1.00 ng/ml and IS at 100 ng/ml; (C) plasma sample collected at 2.0 h after an oral dose of 100 mg/kg CRA.

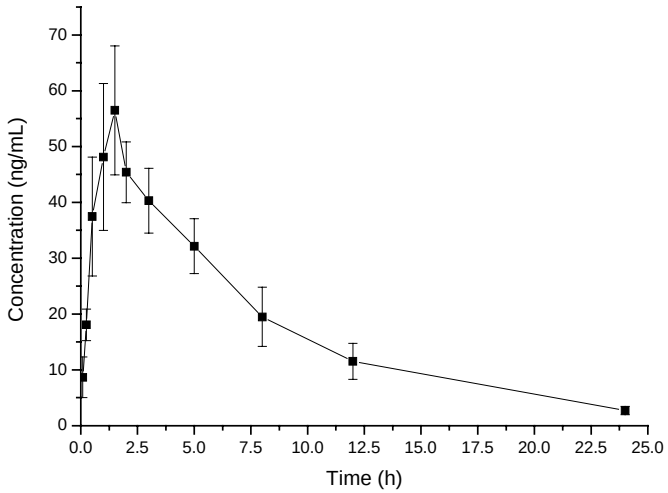

Fig. 4: Mean plasma concentrations *vs* time profiles of CRA after oral administration of 100mg/kg to rats. Each point represents mean ± SD (n = 6).

CONCLUSION

In summary, a sensitive and robust LC-MS/MS method was developed and validated to measure CRA in rat plasma. The method employed a simple protein precipitation treatment for sample preparation with satisfactory recovery and no significant matrix effects. This method gave adequate sensitivity with an LLOQ of 1.00 ng/ml for CRA and further successfully applied to the pharmacokinetic study in rats.

ACKNOWLEDGMENTS

The work was financially supported by the Natural Science Foundation of Guangdong Province (Nos. 9151008018000003).

REFERENCES

Ahn KS, Hahm MS, Park EJ, Lee HK and Kim IH (1998). Corosolic acid isolated from the fruit of *Crataegus pinnatifida* var. psilosa is a protein kinase C inhibitor as well as a cytotoxic agent. *Planta Med.,* **64**(5): 468-470.

Banno N, Akihisa T, Tokuda H, Yasukawa K, Higashihara H, Ukiya M, Watanabe K, Kimura Y, Hasegawa J and Nishino H (2004). Triterpene acids from the leaves of *Perilla frutescens* and their anti-inflammatory and anti-tumor promoting effects. *Biosci. Biotechnol. Biochem.,* **68**(1): 85-90.

Fukushima M, Matsuyama F, Ueda N, Eqawa K, Takemoto J, Kajimoto Y, Yonaha N, Miura T, Kaneko T, Nishi Y, Mitsui R, Fujita Y, Yamada Y and Seino Y (2006). Effect of corosolic acid on postchallenge plasma glucose levels. *Diabetes Res. Clin. Pract.,* **73**(2): 174-177.

Fujiwara Y, Komohara Y, Ikeda T and Takeya M (2011). Corosolic acid inhibits glioblastoma cell proliferation by suppressing the activation of signal transducer and activator of transcription-3 and nuclear-factor kappa B in tumor cells and tumor-associated macrophages. *Cancer Sci.,* **102**(1): 206-211.

Hu CP, Chen LS, Xin Y and Cai QX. Determination of corosolic acid in *Eriobotrya japonica* leaves by reversed-phase high performance liquid chromatography (2006). *Chin. J. Chromatogr.,* **24**(5): 492-494.

Judy WV, Hari SP, Stogsdill WW, Judy JS, Naguib YM and Passwater R (2003). Antidiabetic activity of a standardized extract (Glucosol™) from *Lagerstroemia speciosa* leaves in type II diabetics: A dose-dependence study. *J. Ethnopharmacol.,* **87**(1): 115-117.

Lee MS, Lee CM, Cha EY, Thuong PT, Bae KH, Song IS, Noh SM and Sul JY (2010a). Activation of AMP-activated protein kinase on human gastric cancer cells by apoptosis induced by corosolic acid isolated from *Weigela subsessilis. Phytother. Res.,* **24**(12): 1857-1861.

Lee MS, Cha EY, Thuong PT, Kim JY, Ahn MS and Sul JY (2010b). Down-regulation of human epidermal growth factor receptor 2/neu oncogene by corosolic acid induces cell cycle arrest and apoptosis in NCI-N87 human gastric cancer cells. *Biol. Pharm. Bull.,* **33**(6): 931-937.

Liu QW, Zhao D, Chen XJ, Li ZB, Li N, Han D, Talbi A and Biwoele T (2011). Determination of corosolic acid, a natural potential anti-diabetes compound, in rat plasma by high-performance liquid chromatography-mass spectrometry and its application to pharmacokinetic and bioavailability studies. *Planta Med.,* **77**(15): 1707-1711.

Miura T, Itoh Y, Kaneko T, Ueda N, Ishida T, Fukushima M, Matsuyama F and Seino Y (2004). Corosolic acid induces GLUT4 translocation in genetically type 2 diabetic mice. *Biol. Pharm. Bull.,* **27**(7): 1103-1105.

Miura T, Ueda N, Yamada K, Fukushima M, Ishida T, Kaneko T, Matsuyama F and Seino Y (2006). Antidiabetic effects of corosolic acid in KK-Ay diabetic mice. *Biol. Pharm. Bull.,* **29**(3): 585-587.

Shi L, Zhang W, Zhou YY, Zhang YN, Li JY, Hu LH and Li J (2008). Corosolic acid stimulates glucose uptake via enhancing insulin receptor phosphorylation. *Eur. J. Pharmacol.,* **584**(1): 21-29.

Stohs SJ, Miller H and Kaats GR (2012). A review of the efficacy and safety of banaba (*Lagerstroemia speciosa* L.) and corosolic acid. *Phytother. Res.,* **26**(3): 317-324.

US DHHS, FDA and CDER (2006). *Guidance for Industry: Bioequivalence Guidance.* US Department of Health and Human Services, Food and Drug Administration, Center for Veterinary Medicine.

Yamada K, Hosokawa M, Fujimoto S, Fujiwara H, Fujita Y, Harada N, Yamada C, Fukushima M, Ueda N, Kaneko T, Matsuyama F, Yamada Y, Seino Y and Inagaki N (2008). Effect of corosolic acid on gluconeogenesis in rat liver. *Diabetes Res. Clin. Pract.,* **80**(1): 48-55.

Yamaguchi Y, Yamada K, Yoshikawa N, Nakamura K, Haginaka J and Kunitomo M (2006). Corosolic acid prevents oxidative stress, inflammation and hypertension in SHR/NDmcr-cp rats, a model of metabolic syndrome. *Life Sci.,* **79**(26): 2474-2479.

Yang J, Wen XD, Jia BX, Mao Q, Wang Q and Lai MX (2011). Quality evaluation of *Potentilla discolor* by High-performance liquid chromatography coupled with diode array detection and electrospray ionisation tandem mass spectrometry. *Phytochem. Anal.,* **22**(6): 547-554.

Zong W, Xia W and Cui B (2007). Determination of corosolic and maslinic acids in *Lagerstroemia speciosa* leaves by TLC/HPLC method. *Pharm. Chem. J.,* **41**(4): 43-45.