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Short communication

Simultaneous quantification of capsaicin and dihydrocapsaicin in rat plasma using HPLC coupled with tandem mass spectrometry

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ABSTRACT

A rapid, simple and sensitive HPLC–ESI–MS/MS method was developed for the simultaneous determination of capsaicin and dihydrocapsaicin in rat plasma. Plasma samples containing capsaicin, dihydrocapsaicin and phenacetin (internal standard) were prepared based on a simple protein precipitation by the addition of two volumes of acetonitrile. The analytes and internal standard were separated on a Zorbax SB-C18 column (3.5 μm, 2.1 mm × 100 mm) with mobile phase of acetonitrile/water (55:45, v/v) containing 0.1% formic acid (v/v) at a flow rate of 0.2 mL/min with an operating temperature of 25 °C. Quantification was performed on a triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) source by selected reaction monitoring (SRM) of the transitions at *m/z* 306–137 for capsaicin, *m/z* 308–137 for dihydrocapsaicin and *m/z* 180–110 for the IS. Linear detection responses were obtained for capsaicin and dihydrocapsaicin ranging from 1 to 500 ng/mL and the lower limits of quantitation (LLOQs) for the two compounds were 1 ng/mL. The intra- and inter-day precisions (R.S.D.%) were within 9.79% for the two analytes, while the deviations of assay accuracies were within ±10.63%. The average recoveries of the analytes were greater than 89.88%. The analytes were proved to be stable during all sample storage, preparation and analytic procedures. The method was successfully applied to the pharmacokinetic studies of capsaicin and dihydrocapsaicin in rats after subcutaneous administration of capsaicin (natural, containing 65% capsaicin and 35% dihydrocapsaicin).

1. Introduction

Capsaicin and dihydrocapsaicin, responsible for up to 90% of pungency of pepper fruits, are the most abundant members of capsaicinoids (capsaicin, dihydrocapsaicin, nordihydrocapsaicin, nonivamide, homocapsaicin, homodihydrocapsaicin, etc.) in chili peppers [1,2]. Capsaicin and dihydrocapsaicin were demonstrated to produce the characteristic sensations and elicit multiple pharmacological actions via activating transient receptor potential channel, vanilloid subfamily member 1(TRPV1) [3]. In addition to food additive uses, capsaicin and dihydrocapsaicin are widely used in the forms of nonprescription or prescription topical analgesics (creams and ointments), self-defense products (pepper spray weapons) and oral herbal supplements.

Previous publications have described the methods for the analyses of capsaicin or/and dihydrocapsaicin in chili peppers, pepper-containing foods as well as pepper spray weapons by HPLC [4–7], GC–MS [2,8], LC–MS [9,10] and LC/MS/MS [11]. In rat blood and tissues, capsaicin and dihydrocapsaicin have been simultaneously determined by Reilly et al. using LC/MS/MS [12], however, the method required sample pretreatment (liquid–liquid extraction), a total 22 min run time and the commercially unavailable octanoyl vanillamide as internal standard. Recently, Beaudry and Vachon have developed a LC/MS/MS method for determination of capsaicin in rat plasma following simple protein precipitation with LLOQ of 10 ng/mL [13]. Considering that the high probability of simultaneous exposure to capsaicin and dihydrocapsaicin and the relatively low plasma concentrations of these two compounds in vivo, we herein developed a sensitive, simple and rapid LC/MS/MS method for simultaneous determination of capsaicin and dihydrocapsaicin with good accuracy at concentrations in rat plasma as low as 1 ng/mL. The total run time of the method per sample was 6.5 min. This method was validated and applied to the pharmacokinetic studies in rats after subcutaneous administration of capsaicin (natural, containing 65% capsaicin and 35% dihydrocapsaicin).

2. Experimental

2.1. Chemicals and reagents

Capsaicin and dihydrocapsaicin (>99% purity) were purchased from the National Institute for the Control of Pharmaceutical and

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Biological Products (Beijing, China). Capsaicin (natural, containing 65% capsaicin and 35% dihydrocapsaicin) and phenacetin were purchased from Sigma–Aldrich. Acetonitrile was of HPLC grade (Fisher, USA). All other chemicals were of analytical reagent grade. Ultrapure water, prepared using a Milli-Q Reagent water system (Millipore, MA, USA), was used throughout the study.

2.2. Instrumentation and analytical conditions

The LC/MS/MS system consists of a Surveyor auto-sampler, a Surveyor LC pump, a TSQ Quantum Access™ triple quadrupole mass spectrometer with an electrospray ionization (ESI) source and Xcalibur 1.4 software for data acquisition and analysis (Thermo Finigan, USA). The analytes and IS were chromatographed by injection of 10 μL sample onto a Zorbax SB-C18 column (3.5 μm, 2.1 mm × 100 mm, Agilent, USA). The mobile phase of acetonitrile/water (55:45, v/v) containing 0.1% formic acid (v/v) was run at a flow rate of 0.2 mL/min with an operating temperature of 25 °C. The temperature of the auto-sampler was set at 4 °C throughout the analyses.

Mass spectrometer equipped with an ESI source run in positive ion mode. The ESI source was operated with a spray voltage of 4500 V. The sheath gas and auxiliary gas were nitrogen delivered at 30 psi and at 10 L/min, respectively. The capillary temperature was set at 350 °C. The collision gas (argon) pressure was 1.0 mTorr. The MS recordings were carried out in SRM mode with specific ion transitions of protonated precursor ion to product ion at *m/z* 306–137 with collision energy (CE) of 23 eV for capsaicin, *m/z* 308–137 with CE of 15 eV for dihydrocapsaicin and *m/z* 180–110 with CE of 20 eV for IS, respectively. The total analytical runtime was 6.5 min.

2.3. Preparation of stocks, calibration standards and quality control samples

The stock solution containing capsaicin and dihydrocapsaicin was prepared in methanol at the same concentrations of 1 mg/mL and appropriate dilutions were made with methanol. A 1 mg/mL solution of phenacetin in methanol was prepared as the internal standard stock solution. All stock solutions were stored at −20 °C prior to use.

Calibration standards were prepared by spiking diluted stock solutions into blank rat plasma, giving final concentrations of 500, 250, 100, 50, 10, 4 and 1 ng/mL for each analyte.

High-, mid- and low-level quality control samples containing 200, 20 and 2 ng/mL of capsaicin and dihydrocapsaicin, were prepared in a manner similar to that used for the preparation of the calibration samples.

2.4. Pretreatment of plasma sample

A 5 μL of IS working solution (1 μg/mL) and 100 μL acetonitrile were added to 50 μL of plasma sample. The mixture was vortex mixed followed by centrifugation at 14,000 rpm for 5 min. A 10 μL aliquot of each supernatant was injected into the LC/MS/MS system for the analysis.

2.5. Method validation

2.5.1. Selectivity

Six different blank rat plasma samples were analyzed to detect the potential interferences co-eluting with the analytes and IS. Chromatographic peaks of analytes and IS were identified on the basis of their retention times and SRM responses.

2.5.2. Matrix effect

The matrix effect was defined as the ion suppression/enhancement on the ionization of analytes, which was evaluated by comparing the responses of the deproteinized samples of blank plasma from six rats spiked QC samples ($n = 5$) with those of the standard samples at equivalent concentrations.

2.5.3. Calibration curve, precision and accuracy

The linearities of the LC/MS/MS method for the determination of capsaicin and dihydrocapsaicin were evaluated by the calibration curves in the range of 1–500 ng/mL. The calibration curves were obtained by plotting the peak area ratios (analyte/internal standard) versus the analytes concentrations prepared. Least squares linear regression analysis was used to determine the slopes, intercepts and correlation coefficients (r^2). The calibration curves require the correlation coefficients of 0.99 or better. To evaluate the precision, at least five QC samples of three different concentrations were processed and injected in a single day (intra-day) and at different days (inter-day). The variability of determination was expressed as the relative standard deviation (R.S.D.%) and the accuracy was expressed as the relative error (R.E.%). The criteria for acceptability of the data included an accuracy within ±15% relative error from the nominal values and a precision of within ±15% relative standard deviation, except for LLOQ, where it should not exceed ±20% of accuracy as well as precision

2.5.4. Recovery

The recoveries of capsaicin and dihydrocapsaicin from rat plasma were determined by comparing peak area ratios from plasma samples with those obtained from the direct injection of pure analytes standard solutions at three QC concentration levels. The recoveries of the two compounds in rat plasma were examined at least five times.

2.5.5. Stability

The stabilities of capsaicin and dihydrocapsaicin in rat plasma were evaluated. Short-term stability was assessed by analyzing QC plasma samples kept at room temperature for 6 h that exceeded the routine preparation time of samples. To evaluate the stability of the treated plasma samples in the auto-sampler, QC samples were prepared and placed at 4 °C for a period of 12 h, and then injected for analyses. Long-term stability was determined by assaying QC plasma samples after storage at −20 °C for 30 days. Freeze–thaw stability was investigated after three freeze (−20 °C)–thaw (room temperature) cycles.

2.6. Pharmacokinetic study

To assess the applicability of present method, we investigated the pharmacokinetics of capsaicin and dihydrocapsaicin in rats after subcutaneous administration of capsaicin (natural). All animal protocols were approved by Institute Animal Care and Welfare Committee. Sprague–Dawley rats (adult male), weighing 180–220 g, were obtained from Beijing Vital River Experimental Animal Co., Ltd. The animals were quarantined for 1 week prior to the study. The rats were maintained on a 12 h light/12 h dark cycle at 22 ± 1 °C and at 60% relative humidity. All animals were weighed daily and observed twice daily, in order to assess their general health. The dosing solutions used for the animal studies were prepared by dissolving the required amounts of capsaicin (nature) in solvent containing 10% ethanol, 10% Tween 80 and 80% normal saline. After subcutaneous injection of capsaicin (nature) at a dose of 10 mg/kg (6.5 mg/kg capsaicin and 3.5 mg/kg dihydrocapsaicin) to rats, approximately 0.2 mL blood samples were collected in heparinized 1.5 mL polythene tubes by orbital bleeding via capillary tubes at 0.08, 0.17, 0.33, 0.5, 0.67, 1, 2, 3, 4, 5, 6, 8 and 12 h.

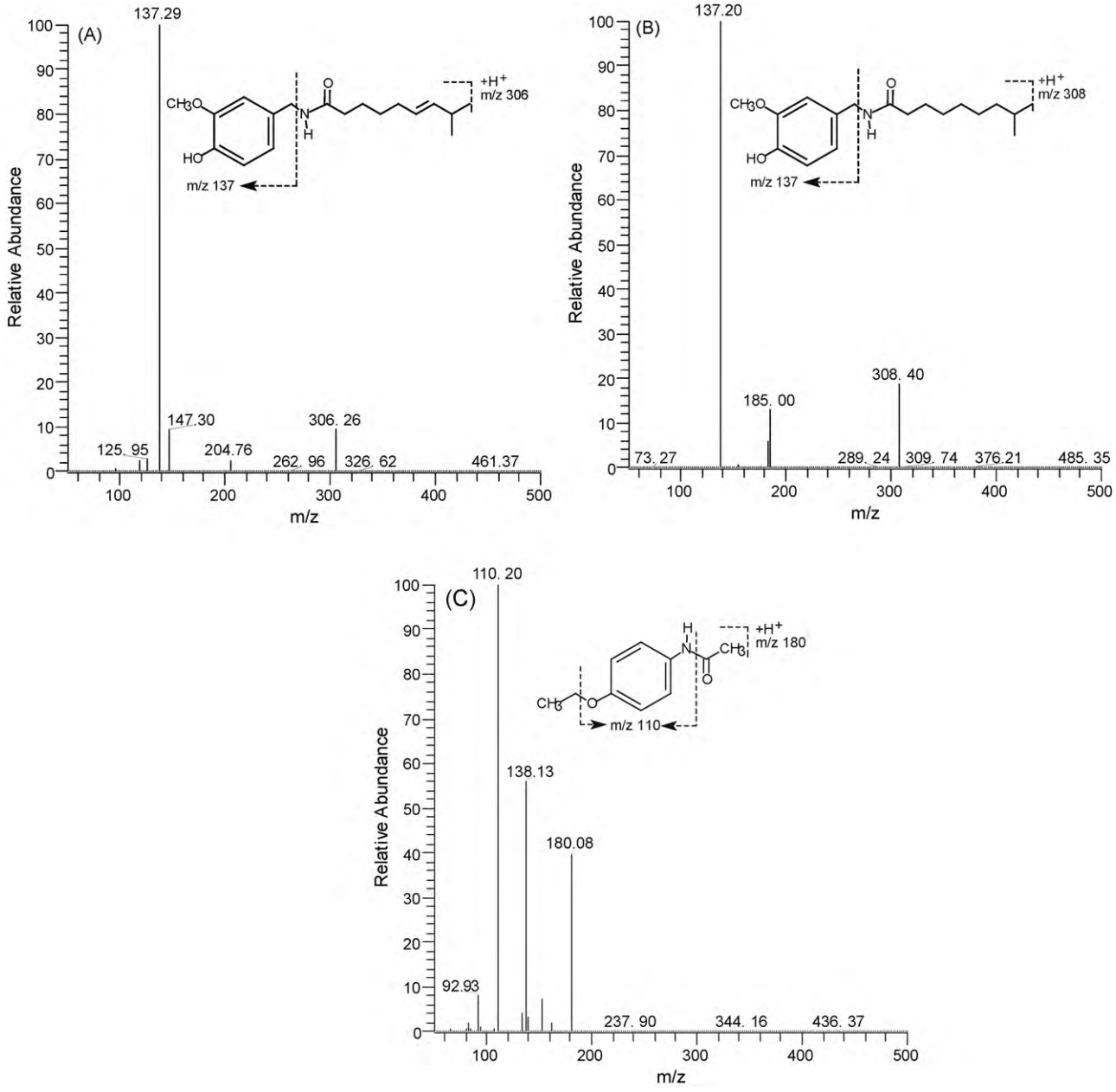

Fig. 1. Product ion mass spectra of $[M+H]^+$ of (A) capsaicin, (B) dihydrocapsaicin and (C) phenacetin (IS).

The blood samples were immediately centrifuged at 5000 rpm for 10 min. The plasma was separated and frozen at −20 °C until analysis. The plasma concentrations of capsaicin and dihydrocapsaicin were expressed as mean ± S.D. and the mean concentration–time curves were plotted. Data fitting and pharmacokinetic parameter estimates were carried out using DAS 2.0 pharmacokinetic program (Chinese Pharmacological Society).

3. Results and discussion

3.1. Chromatographic conditions

To get appropriate retention time, better resolution and sensitivity, the different HPLC parameters including mobile phase, category of column and flow rate of mobile phase were tested and compared. Finally, the mobile phase of acetonitrile/water (55:45, v/v) containing 0.1% formic acid (v/v) at a flow of 0.2 mL/min was found to be suitable for the determination of electrospray responses of capsaicin, dihydrocapsaicin and IS. Zorbax SB-C18 column (3.5 μm, 2.1 mm × 100 mm) was selected for the chromatographic separation. The system provided higher resolution, greater baseline stability and higher ionization efficiency.

3.2. Mass spectrometry

By investigating the full-scan mass spectra of capsaicin, dihydrocapsaicin and IS, we found that the signal intensity in the positive ion mode was much higher than that in the negative ion mode. During a direct infusion experiment, the mass spectra for capsaicin, dihydrocapsaicin and IS revealed peaks at *m/z* 306, 308 and 180, respectively, as protonated molecular ion $[M+H]^+$. As shown in Fig. 1, the most abundant and stable product ions were at *m/z* 137 for both capsaicin and dihydrocapsaicin, and at *m/z* 110 for IS, after fragmentation in the collision cell. The most suitable mass

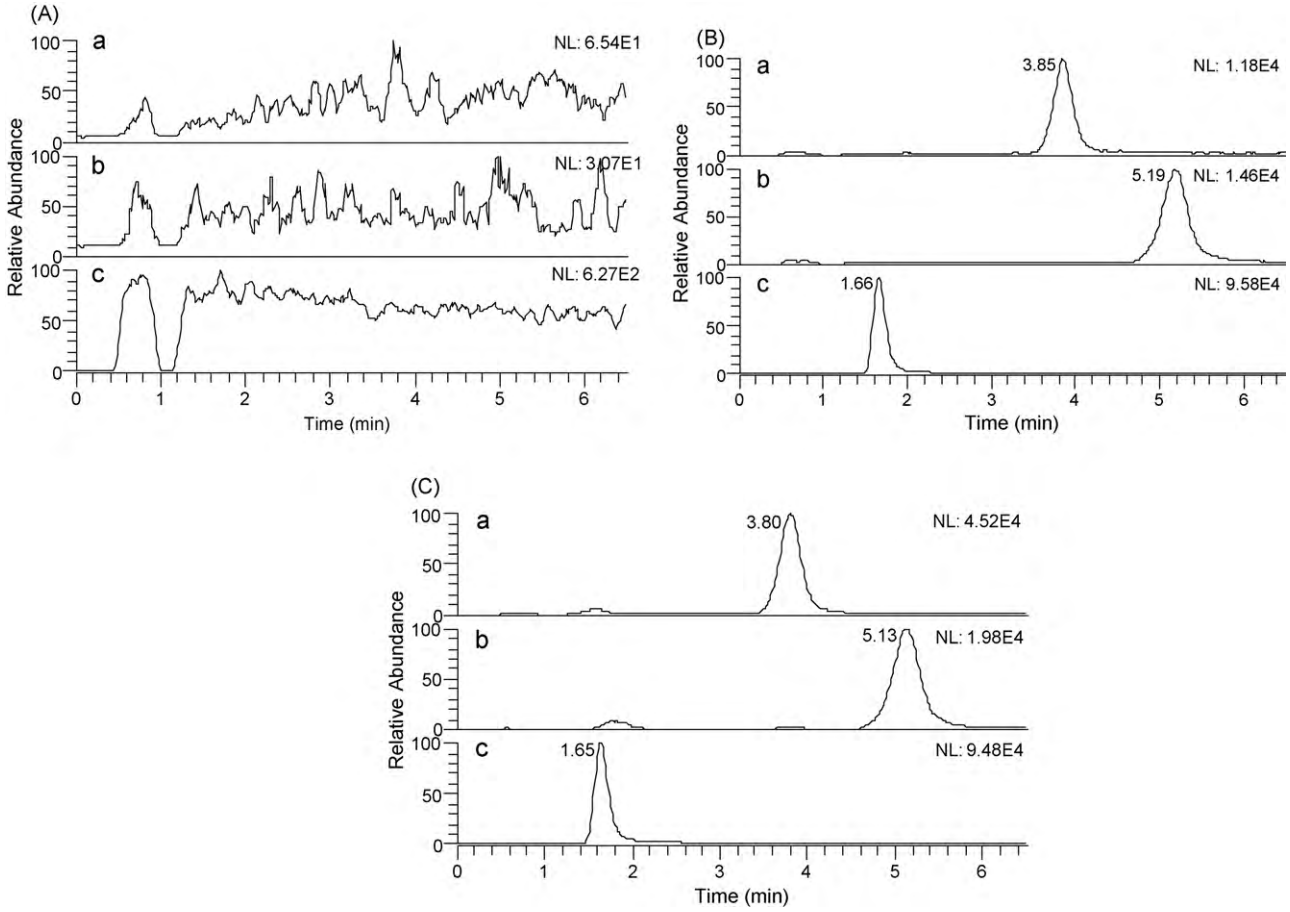

Fig. 2. Typical SRM chromatograms of capsaicin, dihydrocapsaicin and IS: (A) blank rat plasma; (B) blank plasma spiked with capsaicin (10 ng/mL, 3.85 min), dihydrocapsaicin (10 ng/mL, 5.19 min) and IS (1.66 min); and (C) rat plasma sample at 3 h postdose of 10 mg/kg capsaicin (natural) spiked with IS. a: capsaicin (*m/z* 306–137); b: dihydrocapsaicin (*m/z* 308–137); and c: phenacetin (IS) (*m/z* 180–110).

spectrometric conditions were determined by optimizing all the parameters of the mass spectrometer such as collision energy, argon collision gas pressure, sheath gas and auxiliary gas pressure, spray voltage and capillary temperature to obtain more higher and stable signal.

3.3. Method validation

3.3.1. Selectivity

As shown in Fig. 2, there are no significant interferences from rat plasma found at the retention times of the two analytes and IS. The retention times of capsaicin, dihydrocapsaicin and IS are 3.85, 5.19 and 1.66 min, respectively.

3.3.2. Matrix effect

The absolute matrix effect values were 103.94%, 90.09% and 94.07% for capsaicin and 97.24%, 88.35% and 91.52% for dihydrocapsaicin, the relative matrix effect values were 6.4%, 8.5% and 1.4% for capsaicin and 6.2%, 8.4% and 0.44% for dihydrocapsaicin at low, medium and high QC levels, respectively. These results suggested that the matrix effect on the ionization of capsaicin and dihydrocapsaicin was not obvious under our experimental conditions.

3.3.3. Calibration curve

The calibration curves generated from detection of rat plasma containing known amounts of capsaicin and dihydrocapsaicin were linear over the concentration range tested (1–500 ng/mL). The regression equations obtained by least squared regression were $y = (17.941 \pm 2.689) \times 10^{-3}x - (5.616 \pm 2.686) \times 10^{-3}$ ($n = 6$) for capsaicin and $(27.404 \pm 1.163) \times 10^{-3}x - (3.217 \pm 1.271) \times 10^{-3}$ ($n = 6$) for dihydrocapsaicin using weighing factor ($1/X$), where y are the peak area ratios of analytes to IS, and x are the concentrations of analytes. The correlation coefficients (r^2) were $\geq$0.99 for all calibration curves, and the observed deviations were within $\pm$15% for all calibration concentrations. The lowest concentrations in the calibration curves for the two analytes with R.S.D. <20% were taken as LLOQ and were found to be 1 ng/mL which are sufficient for pharmacokinetic studies of capsaicin and dihydrocapsaicin in rats.

3.3.4. Precision and accuracy

The intra- and inter-assay variations were found to be within the accepted limits. The intra- and inter-day precisions of the two analytes at three QC concentrations, as presented in Table 1, were less than 9.79%. Assay accuracies were found to be within $\pm$10.63%. The results indicated that the present method was reliable and reproducible for the simultaneous quantitative analyses of capsaicin and dihydrocapsaicin in rat plasma samples.

3.3.5. Recovery and stability

The recoveries in rat plasma were 92.25–106.56% for capsaicin and 89.88–97.08% for dihydrocapsaicin at three QC concentration levels.

The stability results of capsaicin and dihydrocapsaicin in rat plasma are summarized in Table 2. Capsaicin and dihydrocapsaicin in rat plasma were found to be stable after being placed at room temperature for 6 h, stored at $-20\,^{\circ}$C for 30 days or through three

Table 1
Intra- and inter-day accuracies and precisions of capsaicin and dihydrocapsaicin in rat plasma ($n = 5$).

Sample	Spiked (ng/mL)	Measured (mean ± S.D.)	Accuracy (R.E.%)	Precision (R.S.D.%)
Capsaicin				
Intra-day	2	2.10 ± 0.16	5.4	7.5
	20	21.8 ± 1.43	9.0	6.6
	200	185 ± 11.1	−7.5	6.0
Inter-day	2	2.07 ± 0.16	3.7	7.8
	20	19.2 ± 1.88	−4.1	9.8
	200	191 ± 12.0	−4.4	6.3
Dihydrocapsaicin				
Intra-day	2	2.21 ± 0.09	10.6	4.1
	20	19.6 ± 1.59	−2.3	8.1
	200	191 ± 15.7	−4.3	8.2
Inter-day	2	2.13 ± 0.18	6.3	8.4
	20	18.8 ± 1.12	−6.1	6.0
	200	191 ± 10.9	−4.4	5.7

Table 2
Stability of capsaicin and dihydrocapsaicin in rat plasma ($n = 5$).

Conditions	Spiked (ng/mL)	Capsaicin		Dihydrocapsaicin	
		Measured(mean ± S.D.)	R.E. (%)	Measured(mean ± S.D.)	R.E. (%)
Room temperature (6 h)	2	1.95 ± 0.10	−2.3	1.90 ± 0.14	−5.1
	20	19.3 ± 0.76	−3.6	18.0 ± 1.03	−10.0
	200	207 ± 15.1	3.5	196 ± 14.2	−1.9
Three freeze–thaw cycles	2	2.11 ± 0.16	5.4	2.21 ± 0.16	10.3
	20	20.1 ± 1.97	0.4	19.7 ± 2.00	−1.3
	200	219 ± 8.47	9.6	186 ± 9.09	−7.0
At 4 °C (12 h)	2	1.74 ± 0.04	−12.9	1.77 ± 0.21	−11.3
	20	19.2 ± 1.08	−4.0	19.0 ± 1.06	−4.9
	200	195 ± 10.4	−2.6	196 ± 9.25	−1.9
Stored at −80 °C (1 month)	2	1.91 ± 0.10	−4.4	1.84 ± 0.08	−8.1
	20	18.6 ± 0.95	−6.9	18.2 ± 0.69	−9.1
	200	214 ± 9.53	7.2	206 ± 9.13	3.2

freeze–thaw cycles. Furthermore, samples after treatment were stable at 4 °C in auto-sampler for a period of 12 h, which indicated that a large number of samples could be determined in each analytical run.

3.4. Pharmacokinetic study

The present method was successfully applied to the pharmacokinetic studies of capsaicin and dihydrocapsaicin in Sprague–Dawley rats after subcutaneous injection of 10 mg/kg capsaicin (natural). There are several published literatures about the pharmacokinetic profile of capsaicin or dihydrocapsaicin, however, only one of them involving subcutaneous administration was reported in which the authors determined the concentrations of capsaicin in blood using HPLC method [14]. Moreover, in view of the previous study by Akimoto et al. [15], where capsaicin and dihydrocapsaicin affected the numbers of acquired immunity cells, total WBCs and neutrophils at 3–12 h following a subcutaneous administration of 3 mg/kg capsaicin or dihydrocapsaicin in rats, it is worth to observe the pharmacokinetic profiles of capsaicin and dihydrocapsaicin after subcutaneous administration. As shown in Fig. 3, the maximum plasma concentration (C_{max}) was 104.9 ng/mL for capsaicin and 54.3 ng/mL for dihydrocapsaicin. The time of maximum plasma concentration (T_{max}) was 5 h for capsaicin and 4 h for dihydrocapsaicin. The T_{max} of capsaicin obtained in our study was consistent with previous study [14] that the concentration of capsaicin in blood reached a maximum at 5 h after subcutaneous administration of 50 mg/kg capsaicin in rats. The area under the plasma concentration–time curve from 0 h to the time of last measurable concentration (AUC_{0-t}) was 763.46 and 412.86 μg/L h, the area under the plasma concentration–time curve from 0 h to infinity ($AUC_{0-\infty}$) was 829.83 and 465.78 μg/L h for capsaicin and dihydrocapsaicin, respectively. The half-life of drug elimination at the terminal phase ($t_{1/2}$) was 2.94 h for capsaicin and 3.19 h for dihydrocapsaicin.

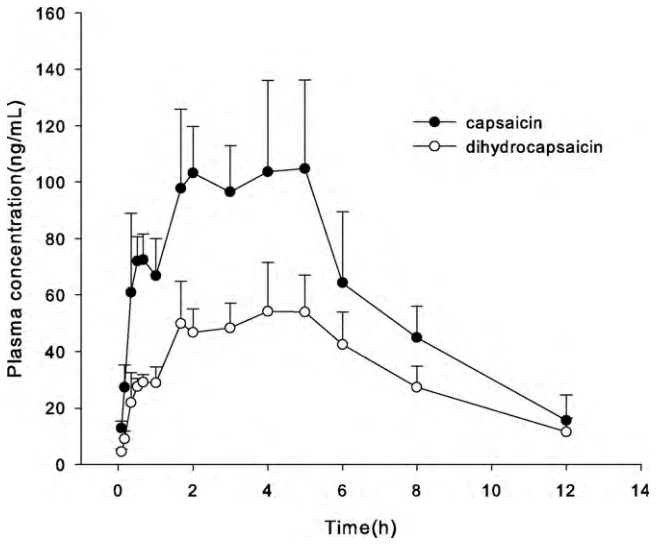

Fig. 3. Mean plasma concentration–time profiles of capsaicin and dihydrocapsaicin after subcutaneous administration of 10 mg/kg capsaicin (natural) in rats. Each point represents mean ± S.D. ($n = 5$).

4. Conclusion

An LC–ESI–MS/MS method with simple protein precipitation for simultaneous determination of capsaicin and dihydrocapsaicin in rat plasma has been developed and validated, which showed excellent sensitivity, good linearity of response, and high precision. The method was successfully applied to the pharmacokinetic studies of capsaicin and dihydrocapsaicin in rats after subcutaneous administration of capsaicin (natural).

References

[1] A. Garces-Claver, R. Gil-Ortega, A. Alvarez-Fernandez, M.S. Arnedo-Andres, J. Agric. Food Chem. 55 (2007) 6951.
[2] A. Pena-Alvarez, E. Ramirez-Maya, L.A. Alvarado-Suarez, J. Chromatogr. A 1216 (2009) 2843.
[3] C.A. Reilly, G.S. Yost, Drug Metab. Rev. 38 (2006) 685.
[4] I. Perucka, W. Oleszek, Food Chem. 71 (2000) 287.
[5] E. Kaale, A. Van Schepdael, E. Roets, J. Hoogmartens, J. Pharm. Biomed. Anal. 30 (2002) 1331.
[6] N. Kozukue, J.S. Han, E. Kozukue, S.J. Lee, J.A. Kim, K.R. Lee, C.E. Levin, M. Friedman, J. Agric. Food Chem. 53 (2005) 9172.
[7] S.H. Choi, B.S. Suh, E. Kozukue, N. Kozukue, C.E. Levin, M. Friedman, J. Agric. Food Chem. 54 (2006) 9024.
[8] W.S. Hawer, J. Ha, J. Hwang, Y. Nam, Food Chem. 49 (1994) 99.
[9] C.A. Reilly, D.J. Crouch, G.S. Yost, J. Forensic Sci. 46 (2001) 502.
[10] A. Garces-Claver, M.S. Arnedo-Andres, J. Abadia, R. Gil-Ortega, A. Alvarez-Fernandez, J. Agric. Food Chem. 54 (2006) 9303.
[11] C.A. Reilly, D.J. Crouc, G.S. Yost, A.A. Fatah, J. Chromatogr. A 912 (2001) 259.
[12] C.A. Reilly, D.J. Crouch, G.S. Yost, A.A. Fatah, J. Anal. Toxicol. 26 (2002) 313.
[13] F. Beaudry, P. Vachon, Biomed. Chromatogr. 23 (2009) 204.
[14] A. Saria, G. Skofitsch, F. Lembeck, J. Pharm. Pharmacol. 34 (1982) 273.
[15] S. Akimoto, J. Tanihata, F. Kawano, S. Sato, Y. Takei, K. Shirato, Y. Someya, S. Nomura, K. Tachiyashiki, K. Imaizumi, J. Nutr. Sci. Vitaminol. (Tokyo) 55 (2009) 282.