Short communication

Quantitative determination of fluorinated caffeic acid phenethyl ester derivative from rat blood plasma by liquid chromatography-electrospray ionization tandem mass spectrometry

Xinyu Wang [a,d], Jihai Pang [b], Robert A. Newman [b], Sean M. Kerwin [c],
Phillip D. Bowman [d], Salomon Stavchansky [a,*]

[a] *Pharmaceutics Division, College of Pharmacy, University of Texas at Austin, Austin, TX 78712, United States*
[b] *Department of Experimental Therapeutics, M.D. Anderson Cancer Center, The University of Texas, Houston, TX 77054, United States*
[c] *Division of Medicinal Chemistry and Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX 78712, United States*
[d] *US Army Institute of Surgical Research, San Antonio, TX 78234, USA*

Abstract

The quantitative determination of caffeic acid phenethyl ester (CAPE) and its fluorinated derivative (FCAPE) from rat plasma using ultra-performance liquid chromatography with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) is reported. CAPE and FCAPE were extracted using ethyl acetate in the presence of methyl caffeate (MC) as internal standard. Separation was achieved using a C_{18} column (2.1 mm × 50 mm, 1.7 μm) and gradient elution with water and acetonitrile containing 0.2% and 0.1% formic acid, respectively. A non-linear response over a broad concentration range (1–1000 ng/ml, $r^2 > 0.995$ using a quadratic regression model and 1/concentration weighting) was obtained. The inter-day and intra-day variability for CAPE and FCAPE were found to be less than 14.2% and 9.5%, respectively. Data are presented to illustrate the practicality of the method for the pharmacokinetic evaluation of CAPE and FCAPE after intravenous administration to rats.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Caffeic acid phenethyl ester (CAPE), a plant-derived polyphenolic compound (Fig. 1), is a component of bee propolis. Propolis has been used as a folk medicine remedy at least since 300 B.C. [1]. In recent years, interest in CAPE has increased not only as a potential active pharmacologic agent but also mainly as prospective raw material for pharmaceutical industry as either a starting or intermediate material for the synthesis of closely related compounds. Numerous pharmacological activities have been reported for CAPE including anticancer/tumor [2,3], antiviral [4,5], anti-inflammatory [6,7], and antioxidant [8–10]. Our previous studies have identified a newly synthesized CAPE derivative, FCAPE (Fig. 1), which exhibited similar

cytoprotective effect as CAPE in human endothelial cells against menadione-induced oxidative stress [11] and better stability in Sprague–Dawley rat plasma [12]. Because chemical modifications of CAPE may provide better drug candidates, it was considered useful to develop and validate an analytical assay which could be applied to determination of pharmacokinetic profiles of CAPE and FCAPE in rats.

In spite of the broad interest in CAPE as a therapeutic agent, only a limited number of quantitative analytical methods have been documented. These include an HPLC-UV determination of CAPE from a propolis-containing gel [13], HPLC-ESI-MS measurement of CAPE from crude propolis [14], and HPLC-ESI-MS/MS analysis of CAPE in biological samples [15]. In this paper, we developed a method using ultra-performance liquid chromatography with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) for the determination of FCAPE compared to CAPE. Methyl caffeate (MC, Fig. 1) was used as the internal standard. This method was validated according

---

* Corresponding author. Tel.: +1 512 471 1407.
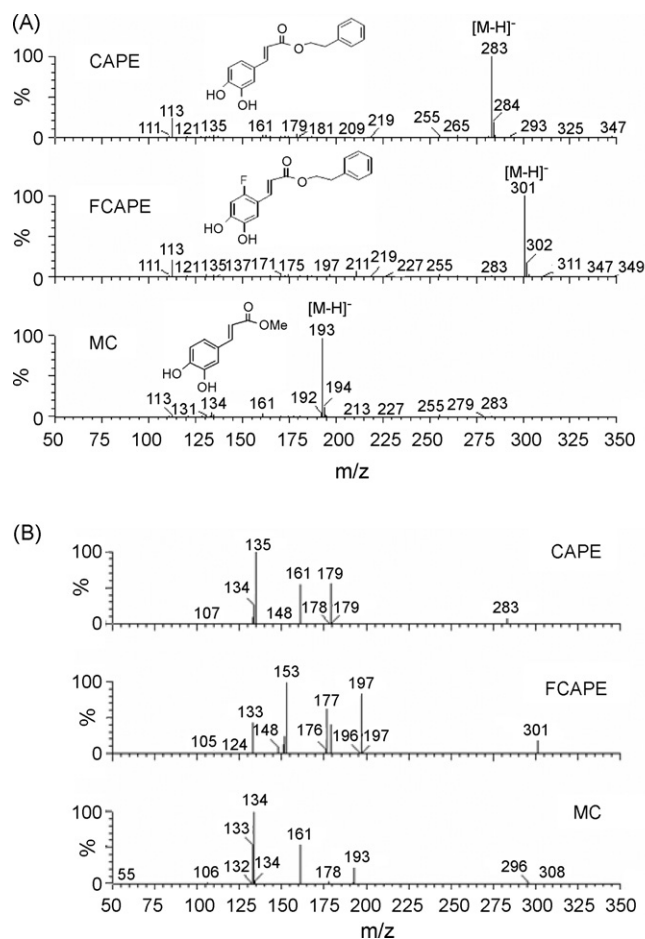 *E-mail address:* stavchansky@mail.utexas.edu (S. Stavchansky).

Fig. 1. Full scan mass spectra for CAPE, FCAPE, and MC (A) and product ion mass spectra where product ions m/z 135, m/z 153, and m/z 134 were monitored for CAPE, FCAPE, and MC, respectively (B). The collision energy for the MS/MS is 20 V.

to the acceptance criteria for bioanalytical method validation described in the FDA guidelines [16]. This assay was applied for the determination of CAPE and FCAPE in rat plasma after intravenous administration with the purpose of establishing the pharmacokinetic profiles of CAPE and FCAPE.

2. Experimental

2.1. Chemical and reagents

CAPE was obtained from Cayman Chemical (Ann Arbor, MI). FCAPE was synthesized and characterized in our laboratories [11]. MC was purchased from LKT Laboratories (St. Paul, MN). Sodium fluoride and formic acid were obtained from Aldrich and Sigma Chemical Co. (St. Louis, MO). Heparinized male Sprague–Dawley rat plasma was obtained from Bioreclamation Inc. (Hicksville, NY). All reagents used were of the highest grade commercially available.

2.2. Instrumentation

The quantitative analysis was performed on a Waters® ACQUITY™ TQD tandem quadrupole UPLC-MS/MS system,

which consists of an ACQUITY Ultra Performance™ liquid chromatography system and an ACQUITY TQ detector (Waters, Milford, MA). This UPLC-MS/MS system was controlled by MassLynx™ 4.1 software.

2.3. UPLC-MS/MS conditions

The UPLC separation was performed on a Waters ACQUITY ethylene-bridged (BEH™) C_{18} column (1.7 μm, 2.1 mm × 50 mm) at 60 °C. The mobile phase consisted of (A) water with 0.2% formic acid and (B) acetonitrile with 0.1% formic acid at a flow rate of 0.4 ml/min. A gradient elution was applied (0–0.12 min, 75% A:25% B; 0.12–0.5 min, 75% A:25% B → 2% A:98% B; 0.5–2 min, 2% A:98% B; 2–2.1 min, 2% A:98% B → 75% A:25% B; 2.1–2.7 min, 75% A:25% B). The sample injection volume was 10 μl. The sample temperature was controlled at 4 °C prior to analysis. The total UPLC run time was 2.7 min.

All MS optimization experiments were performed in MS scan mode and product scan mode. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode using electrospray ionization in negative ion mode. For MRM data collection, the capillary voltage was 2800 V, the cone voltage was 40 V, the source temperature was 125 °C, the desolvation temperature was 350 °C, the cone gas flow was 70 l/h, the desolvation gas flow was 650 l/h, the collision gas pressure was 1.49 mbar, the collision gas flow was 0.15 ml/min, the collision energy was 20 V, the MS inter-scan delay was 0.01 s, the polarity/mode switch inter-scan delay was 0.03 sec, the inter-channel delay was 0.01 s, and the dwell time was 0.1 s. The MRM transitions for the analytes were: m/z 283.00 > m/z 134.90 for CAPE, m/z 301.00 > m/z 152.90 for FCAPE, and m/z 192.90 > m/z 133.80 for MC.

2.4. Preparation of stock solutions, calibration standards, and quality control samples

Stock solutions of CAPE and FCAPE in acetonitrile were prepared at 1 mg/ml for making spiking solutions for the calibration standards. Separate stock solutions of CAPE and FCAPE were used to prepare spiking solutions for the quality control (QC) samples.

A stock solution of MC at 1 mg/ml was prepared to generate an I.S. working solution at a nominal concentration of 20 μg/ml in acetonitrile. Spiking solutions of CAPE and FCAPE were added to Sprague–Dawley rat blank plasma (containing 0.4% sodium fluoride and 0.1 M acetate buffer) to obtain the required concentrations for calibration standards ranging from 10 to 10,000 ng/ml and QC samples at low (25 ng/ml), medium (4500 ng/ml), and high (9000 ng/ml) concentrations for either CAPE or FCAPE.

2.5. Sample extraction procedure

Two hundred microliters of plasma sample in the presence of 0.4% NaF and 0.1 M acetate buffer were transferred to a 1.5-ml centrifuge microtube with the addition of 50 μl of the

I.S. working solution. Six hundred microliters of ethyl acetate were applied twice to extract the plasma sample as previously described [12]. In brief, after 15-min vortexing and 15-min centrifuging at 4 °C, the supernatant from both extractions was pooled and collected in a 2-ml centrifuge microtube. After evaporation to dryness under a nitrogen flow at room temperature, the resulting residues were stored at −80 °C until analyzed. Prior to analysis, the extract residues were reconstituted and diluted in 2 ml water/methanol (50:50, v/v), mixed, and centrifuged. Ten microliters of each sample was injected into the UPLC-MS/MS system.

2.6. Assay validation

Assay validation included determinations of specificity, sensitivity, accuracy, intra- and inter-day precision, concentration–response function (calibration), recovery, and stability. The calibration curve was obtained by plotting the peak area ratios (CAPE/I.S. or FCAPE/I.S.) against the nominal concentrations of the analyte (CAPE or FCAPE) and best fit using a quadratic regression model with $1/X$ weighting. In order to obtain the appropriate MS detection, the calibration curve was developed within the range from 1 to 1000 ng/ml by reconstituting the calibration standards in 10-fold dilution after extraction. The limit of detection (LOD) was determined at a signal-to-noise (S/N) ratio of 3. The lower limit of quantification, the lowest concentration in the calibration curve that can be determined with acceptable accuracy and precision, was measured at a minimum S/N ratio of 10. Accuracy was determined by comparing the mean observed concentration to the theoretical concentration and expressed as the ratio in percentage (%theoretical). Precision was represented as coefficient of variation in percentage (%CV). The inter-day and intra-day accuracy and precision of QC samples were evaluated in three-day core validation runs. Each validation run consisted of calibration standards in triplicate and six replicates of QC samples at three different concentrations plus a minimum of two blank plasma samples without I.S. and two with I.S. (not used in the regression). The recovery of CAPE or FCAPE and I.S. was determined by comparing the peak area of extracted plasma samples to that of the pure standard samples in solvent at three QC concentrations ($n = 3$ for CAPE or FCAPE and $n = 9$ for I.S.). The stability of CAPE or FCAPE in the presence of NaF and acetate buffer was represented as percent recovery and accessed after three freeze and thaw cycles, 24 h at room temperature, and at least one month in −30 °C at three QC concentrations in triplicate. The carry-over effect was evaluated by comparing the level of CAPE or FCAPE at the upper limit of quantification (ULOQ) to a blank sample which followed and represented as a percentage of the ratio of the peak area of the target analyte in the blank sample versus that of the previous ULOQ sample. A carry-over less than 1% was considered to be acceptable.

2.7. Method application

Male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 300 ± 50 g were used for determination of CAPE or FCAPE pharmacokinetics following intravenous administration. The animal study protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin in compliance with US animal laws and policies. CAPE or FCAPE was dissolved in the i.v. solution (ethanol/propylene glycol/water, 15:45:40, v/v/v) and injected through a surgically implanted intravenous catheter in Sprague–Dawley rats at a dose of 10 mg/kg for CAPE and 20 mg/kg for FCAPE. Blood samples were collected in heparinized tubes at 0, 5, 10, 20, 40, 60, 90, 120, and 180 min. Plasma samples were obtained from blood by centrifugation at $10,000 \times g$ for 1 min and then kept frozen at −80 °C with the addition of 0.4% NaF and 0.1 M acetate buffer until analysis. The plasma samples were also diluted 10 times after extraction as were the calibration standards before analysis.

3. Results and discussion

3.1. Method development

A validated method for quantification analysis of CAPE and FCAPE in biological fluid is a prerequisite for investigation of accurate pharmacokinetics. We previously reported an HPLC-UV method useful for determination of the stability of CAPE and FCAPE [12]. Due to the limit of UV detection, a more sensitive method is required for *in vivo* quantification.

We tried an HPLC tandem mass spectrometric method first that was capable of quantitative measurement of CAPE and FCAPE down to a lower limit of 1 ng/ml. However, the carry-over effect was more than 6% which compromised the accuracy of the measurement. The application of an UPLC system and inclusion of one blank between sample injections solved this issue, which minimized the carryover to 0.1% and 0.28% for CAPE and FCAPE, respectively, at ULOQ level.

The tandem MS detector provided the required sensitivity. The full-scan and product ion mass spectra were performed using electrospray negative ionization mode since CAPE, FCAPE, and internal standard MC were all polyphenols and easily lost one proton forming deprotonated $[M − H]^-$ ion peak. The full-scan mass spectra of CAPE, FCAPE, and MC showed abundant deprotonated molecular ion peak at $m/z = 283.00$, 301.00, and 192.90, respectively (Fig. 1A). The consequent product ion mass spectra for CAPE, FCAPE, and I.S. exhibited major fragment ions at $m/z = 134.90$, 152.90, and 133.80, respectively (Fig. 1B).

The chromatographic separation was optimized and achieved in 2.7 min using ACQUITY BEH^{TM} C$_{18}$ column with 1.7 μm particle size and gradient solution. The representative chromatograms for CAPE, FCAPE, and I.S. in rat plasma are shown in Fig. 2. No endogenous interference was found in the area of interest from different sources of rat blank plasma.

Sample clean-up procedure was adopted from our previously described method [12]. The addition of 0.4% NaF and pH adjustment of the blank plasma was necessary to maintain the integrity of CAPE and FCAPE during the preparation of the calibration standards and QC samples. This step was necessary to assure the quality of the data to establish the pharmacokinetic profiles of CAPE and FCAPE.
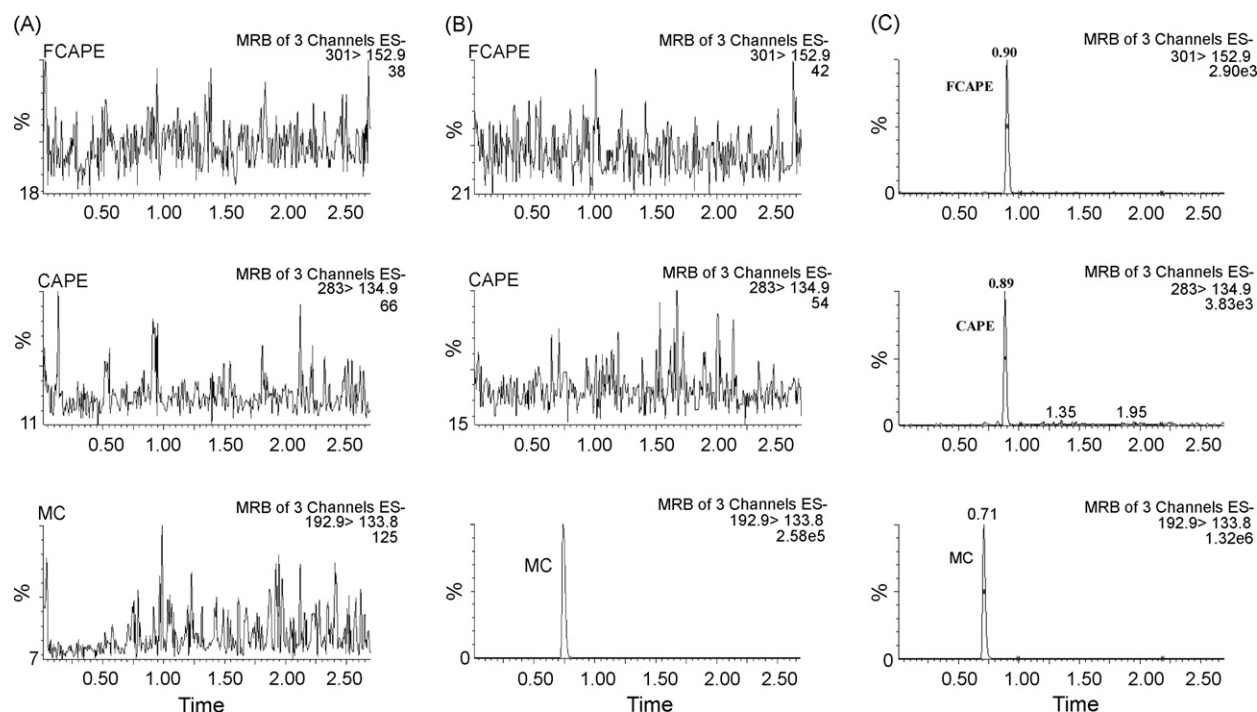
Fig. 2. Typical MRM chromatograms of (A) blank plasma, (B) blank plasma spiked with MC (I.S.), and (C) plasma spiked with CAPE and FCAPE at LLOQ (1 ng/ml).

3.2. Method validation

For calibration purposes, the standard curves were obtained over the concentration range of 1–1000 ng/ml after 10-fold dilution for CAPE or FCAPE by plotting CAPE or FCAPE to I.S. peak area ratios against nominal concentrations with weighted regression analysis. A quadratic regression with $1/X$ weighting gave the best fit for the concentration/detector response relationship for CAPE and FCAPE in rat plasma. The mean quadratic calibration equations for the validation runs were:

$$Y = -0.0007830X^2 + 2.112X + 0.696 \text{ for CAPE and } Y$$
$$= -0.0006568X^2 + 1.769X + 0.509$$

for FCAPE. The mean coefficients of determination (r^2) for the validation runs were 0.9971 for CAPE and 0.9969 for FCAPE. Linear regression of the data was also performed but with a much lower r^2 value (<0.9900) than that obtained using a quadratic regression. For the LC–MS(/MS) analysis, especially with ESI, calibration curves with a dynamic range over 2 orders of magnitude are not always linear possibly due to the concentration-sensitive behavior of ESI. The analyte ion signal can become saturated with the increase of sample concentration [17]. The lower limit of quantification (LLOQ) refers to the lowest calibration standard, which is 1 ng/ml for both CAPE and FCAPE. The precision and accuracy of LLOQ ($n = 9$) were 11.8% and −15.9% for CAPE and 13.3% and −16.0% for FCAPE, respectively. The limit of detection was set at 0.1 ng/ml for both CAPE and FCAPE. The calibration curves for CAPE and FCAPE were acceptable according to the validation criteria as the back-calculated values were within ±15% of the nominal concentrations (±20% at the LLOQ) in the three-day validation.

For the QC samples, the inter-day and intra-day accuracy (% theoretical) of CAPE ranged from 91.6 to 113.0, and the inter-day and intra-day precision (%CV) was less than 14.2 (Table 1); the inter-day and intra-day accuracy (% theoretical) of FCAPE ranged from 92.3 to 110.4, and the inter-day and intra-day precision (%CV) was less than 9.5 (Table 2).

Table 1
Inter-/intraday precision and accuracy for CAPE QC samples

Day	Statistics ($n = 6$)	Intra-day		
		2.5 ng/ml[a]	450 ng/ml[a]	900 ng/ml[a]
1	Mean	2.526	412.3	886.2
	S.D.	0.191	25.9	125.2
	%CV	7.6	6.3	14.1
	%Theoretical[b]	101.0	91.6	98.5
2	Mean	2.629	427.4	836.1
	S.D.	0.127	19.3	23.0
	%CV	4.8	4.5	2.7
	%Theoretical	105.2	95.0	92.9
3	Mean	2.825	436.4	885.1
	S.D.	0.252	11.4	63.3
	%CV	8.9	2.6	7.2
	%Theoretical	113.0	97.0	98.3

Statistics ($n = 18$)	Inter-day		
	2.5 ng/ml	450 ng/ml	900 ng/ml
Mean	2.660	426.8	869.1
S.D.	0.224	21.7	80.7
%CV	8.4	5.1	9.3
%Theoretical	106.4	94.9	96.6

[a] Nominal concentration.
[b] %Theoretical = $\frac{\text{Mean}}{\text{Nominal}} \times 100$.

Table 2
Inter-/intraday precision and accuracy for FCAPE QC samples

Day	Statistics (n = 6)	Intra-day		
		2.5 ng/ml[a]	450 ng/ml[a]	900 ng/ml[a]
1	Mean	2.684	443.5	919.3
	S.D.	0.165	8.7	86.6
	%CV	6.2	2.0	9.4
	%Theoretical[b]	107.4	98.6	102.1
2	Mean	2.759	437.8	849.6
	S.D.	0.177	10.1	42.9
	%CV	6.4	2.3	5.1
	%Theoretical	110.4	97.3	94.4
3	Mean	2.751	415.2	887.3
	S.D.	0.101	35.2	38.8
	%CV	3.7	8.5	4.4
	%Theoretical	110.0	92.3	98.6

Statistics (n = 18)	Inter-day		
	2.5 ng/ml	450 ng/ml	900 ng/ml
Mean	2.731	432.1	885.4
S.D.	0.146	24.0	63.6
%CV	5.3	5.6	7.2
%Theoretical	109.2	96.0	98.4

[a] Nominal concentration.
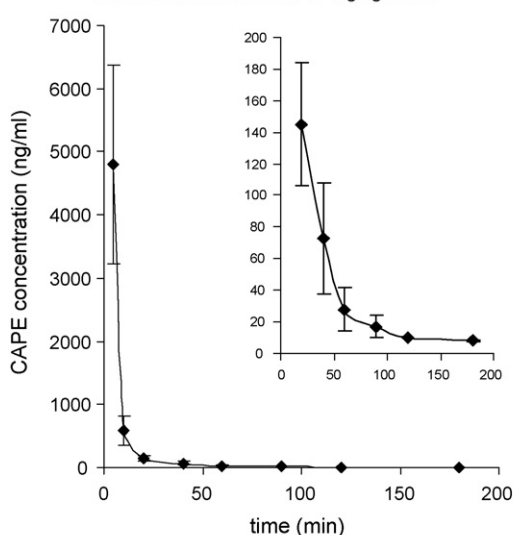[b] %Theoretical = $\frac{\text{Mean}}{\text{Nominal}} \times 100$.

The recovery of CAPE and FCAPE was measured using QC samples by comparing the peak area of pre-extract samples versus neat samples. The mean absolute recovery ranged from 80.2% to 100.7% (%CV < 12.7) for CAPE and 49.9% to 86.3% (%CV < 9.5) for FCAPE, respectively. The mean absolute recovery for I.S. was 98.7% (%CV = 3.1, n = 9). The stability issue of CAPE and FCAPE has been addressed in our previous study [12]. The addition of NaF and pH adjustment prevented degradation of CAPE and FCAPE after three freeze-thaw cycles (−30 °C to 20 °C), 24 h at room temperature (20 °C), and at least one month in −30 °C.

Celli et al. reported an HPLC-MS/MS method for the quantitative determination of CAPE in rat plasma and urine [15]. In his paper, minor carryover effect was observed and reducible with longer column-washing step and one more solvent injection between high concentrated samples. We first tried to establish a similar LC-MS/MS method for the quantification of CAPE and FCAPE in rats. However, the carryover effect became a major issue which could not be minimized to acceptable level even with two additional solvent injections. The application of UPLC reduced the carryover effect to less than 1% for both CAPE and FCAPE. In addition, it improved the sensitivity by LLOQ determination at 1 ng/ml and shortened the run time to 2.7 min compared to the reported method with LLOQ at 5 ng/ml and run time for 14 min.

3.3. Method application

The validated UPLC-ESI-MS/MS method was applied to determination of CAPE and FCAPE in rat plasma after single i.v. dose administration (10 mg/kg for CAPE and 20 mg/kg for FCAPE). The injection sequence for one set of samples (same
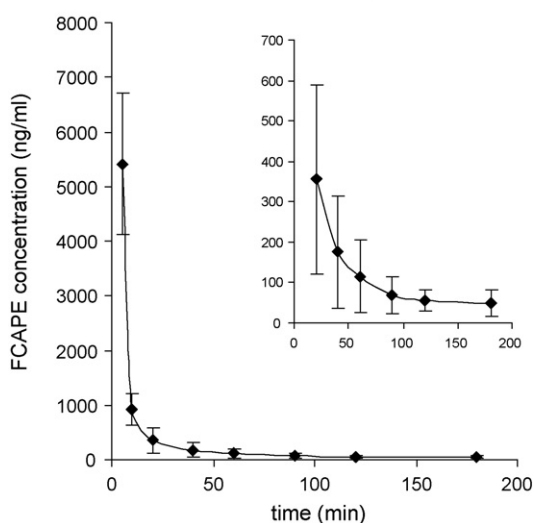

Fig. 3. Plasma concentration–time profiles of CAPE (10 mg/kg, A) and FCAPE (20 mg/kg, B) after i.v. administration to rats (n = 5). Values are reported as mean ± S.D. The inset portion represents the enlargement of plasma concentration–time profile from 20 to 180 min for CAPE and FCAPE.

subject in same day) was arranged in the order of system check samples, blank samples, blank plus I.S. samples, calibration standards, QC samples, test samples, and QC samples. According to the quantitative results, the plasma concentration–time profiles are presented in Fig. 3 for CAPE (A) and FCAPE (B). Both CAPE and FCAPE were rapidly eliminated from the systemic circulation. The method developed was found to be suitable for the determination of CAPE and FCAPE in rat plasma.

4. Conclusions

A rapid and sensitive UPLC-ESI-MS/MS method was developed, validated, and applied to quantitative determination of

X. Wang et al. / J. Chromatogr. B 867 (2008) 138–143

143

CAPE and FCAPE in rat plasma. The chromatographic separation within 3 min allowed a fast sample analysis time and high-throughput capability favoring a high sample load. The calibration curve showed best fit over the concentration range of 1–1000 ng/ml using a quadratic regression with 1/concentration weighting. The intra and inter-day accuracy and precision for QC samples were all within the FDA suggested acceptance criteria. CAPE and FCAPE were stable in rat plasma with the addition of 0.4% NaF and 0.1 M acetate buffer under the storage conditions. These results support an acceptable and reliable method for the establishment of the pharmacokinetic profiles of CAPE and FCAPE in rat blood plasma.

References

[1] A.H. Banskota, Y. Tezuka, J.K. Prasain, K. Matsushige, I. Saiki, S. Kadota, J. Nat. Prod. 61 (1998) 896.

[2] C. Chiao, A.M. Carothers, D. Grunberger, G. Solomon, G.A. Preston, J.C. Barrett, Cancer Res. 55 (1995) 3576.

[3] H.J. Hwang, H.J. Park, H.J. Chung, H.Y. Min, E.J. Park, J.Y. Hong, S.K. Lee, J. Nutr. Biochem. 17 (2006) 356.

[4] M.R. Fesen, K.W. Kohn, F. Leteurtre, Y. Pommier, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 2399.

[5] M.R. Fesen, Y. Pommier, F. Leteurtre, S. Hiroguchi, J. Yung, K.W. Kohn, Biochem. Pharmacol. 48 (1994) 595.

[6] N. Marquez, R. Sancho, A. Macho, M.A. Calzado, B.L. Fiebich, E. Munoz, J. Pharmacol. Exp. Ther. 308 (2004) 993.

[7] M.M. Abdel-Latif, H.J. Windle, B.S. Homasany, K. Sabra, D. Kelleher, Br. J. Pharmacol. 146 (2005) 1139.

[8] J.H. Chen, C.T. Ho, J. Agric. Food Chem. 45 (1997) 2374.

[9] S. Son, B.A. Lewis, J. Agric. Food Chem. 50 (2002) 468.

[10] L.Y. Hsu, C.F. Lin, W.C. Hsu, W.L. Hsu, T.C. Chang, Biol. Pharm. Bull. 28 (2005) 1211.

[11] X. Wang, S. Stavchansky, P.D. Bowman, S.M. Kerwin, Bioorg. Med. Chem. 14 (2006) 4879.

[12] X. Wang, P.D. Bowman, S.M. Kerwin, S. Stavchansky, Biomed. Chromatogr. 21 (2007) 343.

[13] G.C. Ceschel, P. Maffei, A. Sforzini, S. Lombardi Borgia, A. Yasin, C. Ronchi, Fitoterapia 73 (Suppl. 1) (2002) S44.

[14] P. Del Boccio, D. Rotilio, J. Sep. Sci. 27 (2004) 619.

[15] N. Celli, B. Mariani, L.K. Dragani, S. Murzilli, C. Rossi, D. Rotilio, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 810 (2004) 129.

[16] Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), May 2001.

[17] A.P. Bruins, J. Chromatogr. A 794 (1998) 345.