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Fitoterapia 83 (2012) 1616–1622

Contents lists available at SciVerse ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Pharmacokinetic study of luteolin, apigenin, chrysoeriol and diosmetin after oral administration of Flos Chrysanthemi extract in rats

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ARTICLE INFO

Article history:
Received 14 May 2012
Accepted in revised form 11 September 2012
Available online 20 September 2012

Keywords:
Luteolin
Chrysoeriol
Diosmetin
Pharmacokinetics
Flos Chrysanthemi
Catechol-*O*-methyltransferase (COMT)

ABSTRACT

Flos Chrysanthemi (the flower of *Chrysanthemum morifolium* Ramat.) is widely used in China as a food and traditional Chinese medicine for many diseases. Luteolin and apigenin are two main bioactive components in Flos Chrysanthemi, and chrysoeriol and diosmetin are two methylated metabolites of luteolin *in vivo* by cathechol-*O*-methyltransferase (COMT). However, there was lack of pharmacokinetic information of chrysoeriol and diosmetin after oral administration of Flos Chrysanthemi extract (FCE). The present study aimed to develop an HPLC-UV method for simultaneous determination of rat plasma concentration of luteolin, apigenin, chrysoeriol and diosmetin and utilize it in pharmacokinetic study of the four compounds after orally giving FCE to rats. The method was successfully validated and applied to the pharmacokinetic study when oral administration of FCE to rats with or without co-giving a COMT inhibitor, entacapone. Chrysoeriol and diosmetin were detected in rat plasma after oral administration of FCE and their concentrations were significantly decreased after co-giving entacapone. Furthermore, AUC of luteolin was significantly increased by entacapone, while that of chrysoeriol was decreased by entacapone, which revealed COMT might play an important role in the disposition of luteolin in rats after dosing of FCE. In conclusion, a sensitive, accurate and reproducible HPLC-UV method for simultaneous determination of luteolin, apigenin, chrysoeriol and diosmetin in rat plasma were developed, pharmacokinetics of chrysoeriol and diosmetin combined with luteolin and apigenin were characterized after oral administration of FCE to rats, which gave us more information on pharmacokinetics and potential pharmacological effects of FCE *in vivo*.

1. Introduction

The flower of *Chrysanthemum morifolium* Ramat (Flos Chrysanthemi) is a traditional Chinese medicine, and has been widely used as a healthy food for thousands of years. Many studies reported its healthy benefits, such as antioxidation [1], cardiovascular protection [2], hepato-protective effect [3], antiarrhythmic effect [4] and anticomplementary activity in severe acute respiratory syndrome [5]. A new drug candidate of Flos Chrysanthemi extract (FCE) for treatment of cardiovascular disease had been in clinical trial in China [6]. Luteolin (3′, 4′, 5, 7-tetrahydroxyflavone) and apigenin (4′, 5, 7-trihydroxyflavone) (Fig. 1) are two main bioactive ingredients FCE. These two typical flavonoids also widely distribute in fruits and vegetables, which exhibit as anticancer [7–10] or cancer preventive [11], anti-inflammatory [12,13], neuroprotective [14,15] agents in the past studies.

Luteolin and apigenin mainly underwent conjugation metabolisms by uridine diphospho- glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) *in vivo*. Recently, luteolin had been identified to be a good substrate of catechol-*O*-methyltransferase (COMT), and two methylated metabolites of luteolin, chrysoeriol (4′, 5, 7-trihydroxy-3′-methoxyflavone) and diosmetin (3′, 5, 7-trihydroxy-4′-methoxyflavone), were identified in the rat plasma after hydrolylsis treatment when intravenously given luteolin to rats [16]. Since these flavonoids occurred mainly as glucuronidated and sulfated forms in the blood circulation, and there was lack of standard compounds of these glucuronidated and sulfated flavonoids, the total forms

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http://dx.doi.org/10.1016/j.fitote.2012.09.011

(free, glucuronidated and sulfated) flavonoids were analyzed after a hydrolysis treatment in our previous study [21].

Chrysoeriol and diosmetin not only possessed many biological effects similar to luteolin, such as antioxidant, anti-inflammatory, but also exhibited their own effects such as osteoporosis [17,18]. Furthermore, chrysoeriol and diosmetin were natural prodrugs in cancer prevention which could be converted to luteolin by CYPs [19,20]. Therefore it was crucial to study the pharmacokinetics of chrysoeriol and diosmetin when FCE were orally taken.

On the other hand, luteolin exhibited a much faster elimination rate than apigenin when FCE was orally administrated to rats, COMT was probably an important mechanism causing luteolin a lower exposure [16,21,22]. However it was needed to confirm the role of COMT in the disposition of luteolin when orally dosing FCE to rats.

Therefore, the present study aimed to develop an improved quantitative method to determine luteolin, apigenin, chrysoeriol and diosmetin in rat plasma using HPLC-UV and utilize it to pharmacokinetic study of the four flavonoids in rats after oral administration of FCE with or without a COMT inhibitor, entacapone. The pharmacokinetic data could give us more information on pharmacokinetics and potential pharmacological effects of FCE *in vivo*

2. Materials and methods

2.1. Chemicals

Luteolin and apigenin were purchased from Hangzhou Skyherb technologies Co., Ltd., Hangzhou, China (purity > 99%). Diosmetin was obtained from Shaanxi Huangteng Biotechnology Co., Ltd., Sian, China (purity > 95%). Chrysoeriol was synthesized by Department of Medicinal Chemistry, College of Pharmaceutical Sciences, Zhejiang University (purity > 99%). FCE was provided by the Institute of Material Medica, Zhejiang University, China, containing 6.5% (w/w) luteolin and 5.4% (w/w) apigenin determined by HPLC after hydrolysis with hydrochloric acid. Entacapone was purchased from Jinan Wedo Industrial Co., Ltd., Jinan, China (purity > 99%). Methanol, acetonitrile, formic acid, and water were HPLC-grade. All other chemicals were from standard commercial sources and were of the highest quality available.

2.2. Instrumentation

Analysis was performed using high-performance liquid chromatographic system HP1100 (Agilent Technologies, USA) equipped with an on-line degasser, an auto-sampler, a VWD detector, and a thermostated column compartment.

2.3. Animals

Male Sprague Dawley rats weighing 200 to 250 g were obtained from Experimental Animal Center of Zhejiang Academy of Medical Sciences. All procedures were according to an approved animal use protocol of Zhejiang University. They were housed in cages at 21 ± 1 °C and exposed to a 12:12 h light–dark cycle, free access to food and water. Animals were fasted but free access to water for 12 h before experiment and 2 h after drug administration.

2.4. Chromatographic condition

The HPLC analysis was performed on an Agilent Zorbax SB-C_{18} column (250 mm × 4.6 mm, 5 μm) with a mobile phase

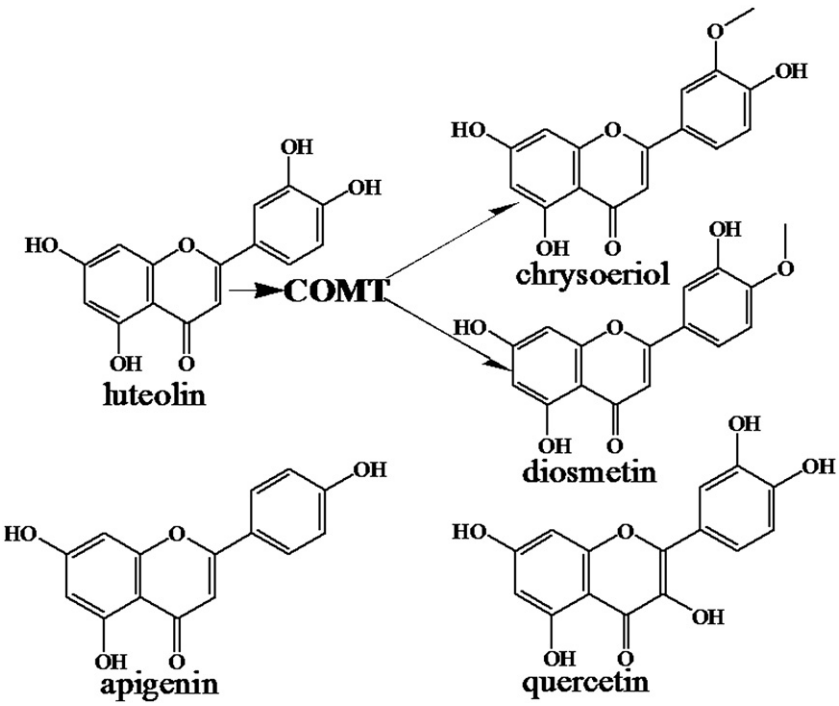

Fig. 1. Chemical structures of luteolin, apigenin, chrysoeriol, diosmetin and quercetin.

consisting of 0.1% formic acid, acetonitrile, and methanol at 30 ± 1 °C with a constant rate of 1 ml/min. The injection volume was 50 μl, and the wavelength was set at 350 nm for quantitative analysis, the proportion of components in the mobile phase was optimized to obtain a well separation of luteolin, apigenin, chrysoeriol, diosmetin and internal standard (quercetin).

2.5. Stocking and working solution

Stock standard solution of luteolin, apigenin, chrysoeriol, diosmetin (approximately 0.5 mg/ml) and the internal standard, quercetin (approximately 1.0 mg/ml), were prepared by dissolving appropriate amounts of these references in methanol, respectively.

A series of working solutions containing luteolin, apigenin, chrysoeriol and diosmetin were prepared by subsequent dilution of the above stock solution with methanol to reach a concentration range of 0.313–125 μg/ml, and the working solution of quercetin (100 μg/ml) was also prepared.

2.6. Sample preparation

Rat plasma samples were prepared according to the method developed in our laboratory [21], and with some modifications. In brief, to detect the total form (free, glucuronidated, sulfated) of flavonoids, a 100 μl plasma was hydrolyzed by hydrochloric acid (2.0 M) at 80 °C for 1.5 h in a 1.5 ml tube, and then 1.0 ml ethyl acetate (containing 0.05 μg/ml quercetin) was added to the mixture. After vortexing for 3 min, the tube was centrifugated for 10 min at 3500 rpm. Then 0.8 ml of the supernatant organic phase was carefully transferred to another 1.5 ml tube and evaporated to dryness under the vacuum desiccator at room temperature. The residue was reconstituted in 100 μl mobile phase, and the concentrations of luteolin, apigenin, and chrysoeriol and diosmetin were determined by the HPLC methods described above.

2.7. Method validation

The method was validated according to the "Guidance for preclinical pharmacokinetic study of chemical drug" recommended by State Food and Drug Administration (SFDA) of China.

2.7.1. Specificity

Blank plasma from at least six rats, blank plasma spiked with four analytes and sample obtained from rat after oral administration of FCE were processed and assayed as described in 2.6. Interference from endogenous or exogenous materials should not occur at the retention time of luteolin, apigenin, chrysoeriol, diosmetin or quercetin.

2.7.2. Linearity and range

The calibration curves ranging from 0.0250 to 10.0 μg/ml were assessed by weighted (1/x) least squares liner regression based on analyte/quercetin peak area ratio prepared in triplicate. The LLOQ was defined as the lowest concentration that could be quantitated with precision and accuracy < 20%.

2.7.3. Precision and accuracy

The standard samples spiked with luteolin, apigenin, chrysoeriol and diosmetin at low, medium and high concentrations (0.05, 1, and 8 μg/ml for all the analytes above) were used for accuracy and precision studies. Five replicates for each concentration were processed and analyzed as described above in 2.6. for accuracy study. The assay recovery and extract recovery were calculated. The intra- and inter-day precisions (relative standard deviations, RSD) were evaluated by analyzing homogeneous samples in five replicates, in 1 or consecutive 3 days. Intra- and inter-day precisions (RSD) were required to be < 15%.

2.7.4. Stability

Freeze–thaw (− 80 °C), short-term (25 °C for 4 h), long-term (− 80 °C for 3 or 7 days), stock solution (25 °C for 24 h or 4 °C for 7 days) and post-preparative stability (25 °C in auto-sampler for 24 h) of luteolin, apignin, chrysoeriol, and diosmetin were evaluated according to FDA guidance (http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf). Low, medium and high concentrations (0.05, 1, and 5 μg/ml for all the analytes above) with triplicates were used in all the stability study except stocking solution stability (only 0.5 mg/ml analytes was used). Data was expressed as remaining(%)of initial determined.

2.8. Pharmacokinetics study

10 male Sprague–Dawley rats were randomly allocated into FCE and (entacapone + FCE) groups with five rats each. Rats were fasted 12 h with free access to water, then a dose of 100 mg/kg FCE combined with or without 20 mg/kg entacapone [dissolved in 20% hydroxypropyl-β-cyclodextrin (w/v)] were orally administered to (entacapone + FCE) and FCE groups, respectively. The blood samples were collected from the orbital venous sinus to heparinized tubes at predose and post-dose at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12 h, and then centrifuged at 2000 *g* for 10 min at 4 °C, the plasma were collected and stored at − 80 °C until analysis.

Pharmacokinetic parameters were calculated with pharmacokinetic software DAS 2.0 (China) and differences between the FCE and (entacapone + FCE) groups were compared with 2-sample t-test (two tails) using Excel 2003 (Microsoft).

3. Results

3.1. HPLC condition and internal standard selection

Chrysoeriol and diosmetin possessed an identical LogP value due to their similar structures (Fig. 1), and they could not be separated well using a mixture of 0.1% formic acid and methanol (or 0.1% formic acid and acetonitrile). Using 0.1% formic acid, acetonitrile and methanol could significantly improved the separation, and after optimization of their combinations, an mobile phase of 0.1% formic acid, acetonitrile and methanol (60:16:24, v/v/v) was obtained to successfully separate analytes (luteolin, apigenin, chrysoeriol and diosmetin) and internal standard (quercetin) (Fig. 2A). Quercetin was selected as an internal standard due to its similar structure with the four analytes, which would exhibit behavior similar to the

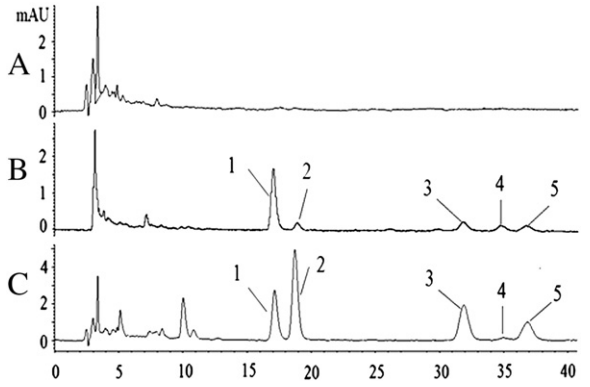

Fig. 2. HPLC chromatograms of luteolin, apigenin, chrysoeriol and diosmetin in rat plasma. A: blank plasma; B: plasma spiked with 0.025 μg/ml of luteolin (2), apigenin (3), chrysoeriol (4), diosmetin (5) and 0.500 μg/ml quercetin (1); C: plasma sample 30 min after oral administration of 100 mg/kg FCE to rats. All samples above were treated with hydrolysis.

analytes during the sample extraction and chromatographic elution.

3.2. Specificity

The specificity of this method to plasma matrix was evaluated with plasma from six rats. The typical chromatograms of (A) a blank plasma sample, (B) a blank plasma sample spiked with 0.05 μg/ml luteolin, apigenin, chrysoeriol and diosmetin and 0.5 μg/ml quercetin (C) a plasma sample from a rat at 30 min after oral administration of 100 mg/kg FCE are shown in Fig. 2. No interferences from endogenous substances in rat plasma was observed at the retention times of interest(All samples were treated with hydrolysis preparation).

3.3. Linearity, range and sensitivity

The peak area ratios of luteolin, apigenin, chrysoeriol and diosmetin to quercetin all displayed a good linear relationship over the range of 0.025–10 μg/ml. The typical regression equations were as follows:

$Y = 3.191X - 0.01150$, $R^2 = 0.9969$, $n = 8$ (luteolin);
$Y = 2.766X + 0.01260$, $R^2 = 0.9919$, $n = 8$ (apigenin);
$Y = 3.195X + 0.01178$, $R^2 = 0.9932$, $n = 8$ (chrysoeriol);
$Y = 2.820X + 0.0003733$, $R^2 = 0.9947$, $n = 8$ (diosmetin).

The LLOQ of all analytes was 0.025 μg/ml.

3.4. Recovery, precision and accuracy

Recovery, precision and accuracy data are presented in Table 1. The extract recoveries for luteolin, apigenin, chrysoeriol, diosmetin were 81.8%–93.3%, 76.94%–96.2%, 84.5%–96.7% and 79.08%–94.1%, respectively, while the assay recoveries of luteolin, apigenin, chrysoeriol, diosmetin were 96.7%–103.4%, 94.5%–104.4%, 93.2%–99.3% and 98.1%–98.3%, respectively. The intra-day precisions (RSDs) for luteolin, apigenin, chrysoeriol, diosmetin were <4.9%, <5.4%, <7.0%, <6.5%, respectively, and the inter-day precisions (RSDs) were <3.5%, <6.4%, <4.2%, <3.2%, respectively. The mean extract recovery of quercetin in samples was 89.5% (detail data not shown).

3.5. Stability

The freeze–thaw, short-term and long-term stability results of luteolin, apigenin, chrysoeriol, diosmetin in rat plasma are shown in Table 2, which revealed the four analytes were relatively stable. In addition, the four analytes in stocking solution at 25 °C for 24 h or at 4 °C for 7 days were stable (remaining of initial determined were >99.1%), and the four analytes and the internal standard (quercetin) during analyzing process in the HPLC auto-sampler (25 °C for 24 h) were stable, too (remaining of initial determined were >96.9%).

3.6. Pharmacokinetic study

The method was successfully applied to analysis of plasma obtained from rats following a single oral dose of 100 mg/kg FCE with or without 20 mg/kg entacapone. Concentration–time profiles for luteolin, apigenin, chrysoeriol, diosmetin after giving FCE with or without 20 mg/kg entacapone were shown in Fig. 3. The plasma concentrations of luteolin in (entacapone + FCE) group were significantly higher than that of the FCE group, and the $AUC_{0\text{-}t}$ and C_{max} of luteolin were significantly increased when entacapone was co-administered with FCE (Table 3), whereas, the plasma concentrations of chrysoeriol diosmetin and the AUC_{0-t} of chrysoeriol were significantly decreased.

Table 1
Precision and accuracy of the method (n = 5).

Analytes	Spiked conc. (μg/ml)	Measured conc.	Assay recovery	Extract recovery	Intra-day	Inter-day
		μg/ml(mean ± SD)	RSD (%)	RSD (%)	RSD (%)	RSD (%)
Luteolin	0.05000	0.05180 ± 0.0017	103.4 ± 2.3	93.3 ± 3.4	2.3	3.3
	1.000	1.009 ± 0.035	97.2 ± 4.8	88.7 ± 3.3	4.9	3.5
	8.00	7.974 ± 0.21	96.7 ± 2.8	81.8 ± 3.0	2.9	2.6
Apigenin	0.05000	0.05130 ± 0.0033	104.4 ± 3.4	96.2 ± 3.1	3.3	6.4
	1.000	0.999 ± 0.040	95.2 ± 5.1	84.5 ± 3.5	5.4	4.0
	8.00	7.878 ± 0.29	94.5 ± 3.1	76.94 ± 2.6	3.2	3.6
Chrysoeriol	0.05000	0.04921 ± 0.0040	99.3 ± 7.0	96.7 ± 8	7.0	0.8
	1.000	0.971 ± 0.040	93.2 ± 6.6	88.7 ± 4.7	7.0	4.2
	8.00	7.929 ± 0.18	96.8 ± 6.4	84.5 ± 3.2	6.6	2.2
Diosmetin	0.05000	0.04870 ± 0.009	98.1 ± 4.0	94.1 ± 4.2	4.1	1.8
	1.000	1.018 ± 0.033	98.3 ± 5.0	86.5 ± 2.1	5.1	3.2
	8.00	7.948 ± 0.079	98.2 ± 6.4	79.08 ± 2.1	6.5	1.0

Table 2
Freeze–thaw, short-term and long-term stability of luteolin, apigenin, chrysoeriol and diosmetin in rat plasma. Data was expressed as remaining (%) of initial determined.

Analyte	(μg/ml)	Freeze–thaw	25 °C(4 h)	−80 °C	
				3 days	7 days
Luteolin	0.05000	94.6	98.8	101.5	95.5
	1.000	97.8	101.5	104.6	96.1
	5.000	98.9	97.4	93.6	94.5
Apigenin	0.05000	93.5	95.2	100.6	91.7
	1.000	95.1	101.5	104.2	91.2
	5.000	99.1	99.5	96.4	94.2
Chrysoeriol	0.05000	94.3	94.8	96.4	93.6
	1.000	95.6	100.6	98.6	92.5
	5.000	99.5	97.4	96.2	94.5
Diosmetin	0.05000	100.0	95.5	99.8	90.5
	1.000	95.5	100.1	100.9	94.1
	5.000	99.1	100.2	95.1	93.2

4. Discussion

In our previous pharmacokinetic studies of FCE in rat, dog and human, we mainly focused on luteolin, apigenin and their glucuronidated and sulfated forms, but methylated metabolites of luteolin, chrysoeriol and diosmetin were never mentioned. Since chrysoeriol and diosmetin were with similar structures, close LogP values and shared the same mass spectrum [16], an optimized chromatographic condition was very important in the present study. In our study, an Agilent Zorbax SB-C_{18} column (250 mm×4.6 mm, 5 μm) was applied to separate these flavonoids. To get a suitable mobile phase condition, the mixtures of 0.1% formic acid and acetonitrile, or 0.1% formic acid and methanol, and the three above were compared, the combination of the three components in the mobile phase was found to make a well separation. After optimizing the proportion of acetonitrile and methanol, a mobile phase of 0.1% formic acid, acetonitrile and methanol (60:16:24, v/v/v) was determined. In addition, chrysoeriol and diosmetin still had free hydroxyl, which could be further conjugated by UGTs or SULTs, therefore a hydrolysis treatment of sample was very important to assay the methylated metabolites of luteolin *in vivo*.

The method was successfully utilized in plasma pharmacokinetics of luteolin, apigenin, chrysoeriol and diosmetin after oral administration of 100 mg/kg FCE co-administrated with or without 20 mg/kg entacapone to rats. Plasma concentration–time profiles of chrysoeriol and diosmetin were obtained, which indicated the two flavonoids would exhibit their biological effects *in vivo* when FCE was taken. And the methylated luteolin were more permeable i*n vivo* due to their relatively higher hydrophobicity than luteolin, so they might be more easily distributed into tissues. In addition, these two methylated luteolin could be demethylated by some CYPs [19,20], so they also could play the biological effects of luteolin *in vivo*.

On the other hand, compared with the control group, the concentrations of luteolin in (entacapone + FCE) group were significantly increased while the methylated metabolites were obviously decreased, which indicated that COMT might play a role in the disposition of luteolin *in vivo*. Because luteolin could be methylated by COMT but not apigenin, so it was speculated that COMT might be a mechanism responsible

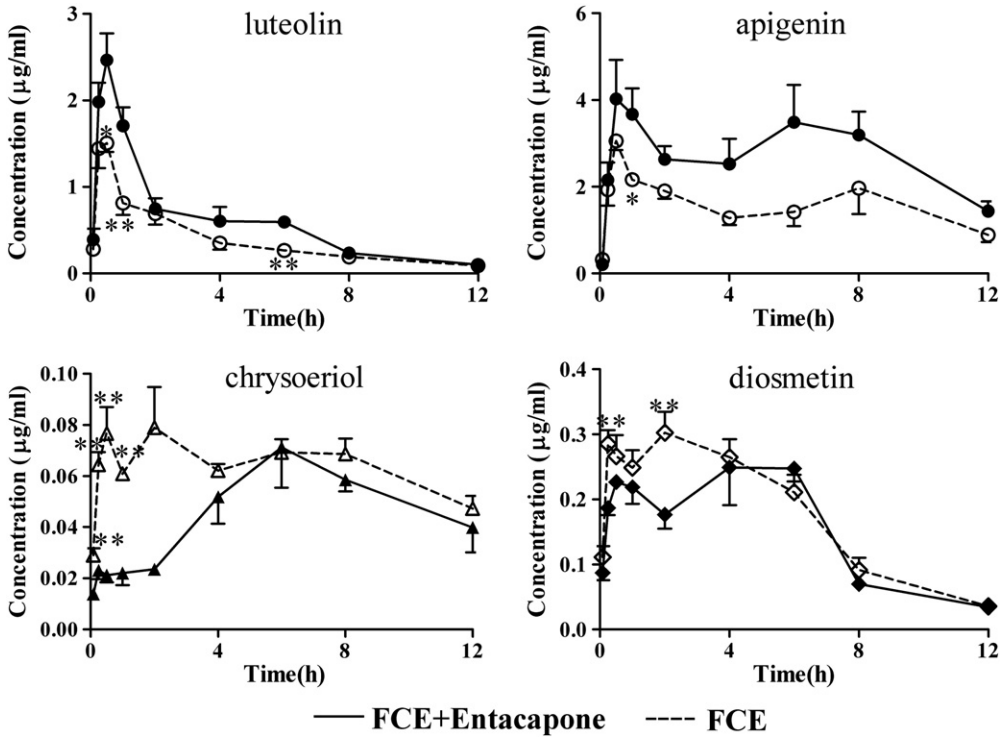

Fig. 3. Mean-plasma concentration–time profiles of luteolin, apigenin, chrysoeriol and diosmetin after oral administration of 100 mg/kg FCE with or without co-giving 20 mg/kg entacapone to rats. Data were expressed as mean ± S.E.M, n = 5. Compared with the entacapone + CME group,*: $P<0.05$, **: $P<0.01$.

Table 3
Main pharmacokinetic parameters of luteolin, apigenin, chrysoeriol and diosmetin in rats after oral administration of 100 mg/kg FCE with or without co-administration of entacapone (20 mg/kg).

Parameters		Apigenin		Luteolin	
		FCE	FCE + entacapone	FCE	FCE + entacapone
AUC (0–12)	mg/l∗h	36.25 ± 16	54.39 ± 24	3.971 ± 1.2	5.862 ± 1.4*
MRT (0–12)	h	9.46 ± 1.1	9.25 ± 0.9	2.589 ± 0.51	2.585 ± 0.52
t1/2z	h	18.44 ± 12	10.98 ± 5.2	2.805 ± 0.9	6.671 ± 7.4
CLz/F	l/h/kg	0.0876 ± 0.060	0.06940 ± 0.046	1.515 ± 0.45	0.818 ± 0.51
Vz/F	l/kg	2.497 ± 1.5	1.305 ± 0.9	5.975 ± 2.1	4.598 ± 2.8
Cmax	mg/l	3.310 ± 0.73	4.567 ± 1.5	1.684 ± 0.21	2.466 ± 0.69
Parameters		Chrysoeriol		Diosmetin	
		FCE	FCE + entacapone	FCE	FCE + entacapone
AUC (0–12)	mg/l∗h	1.468 ± 0.09	1.017 ± 0.17***	2.869 ± 0.74	2.329 ± 0.67
MRT (0–12)	h	10.66 ± 1.1	11.07 ± 0.8	6.568 ± 0.8	6.869 ± 0.39
t1/2z	h	26.24 ± 9	29.80 ± 36	7.359 ± 1.5	7.566 ± 1.6
CLz/F	L/h/kg	1.790 ± 0.60	1.587 ± 0.9	1.773 ± 0.54	2.133 ± 0.50
Vz/F	L/kg	61.84 ± 12	59.26 ± 50	18.37 ± 5.2	23.58 ± 9
Cmax	mg/l	0.0931 ± 0.028	0.06404 ± 0.025	0.3294 ± 0.052	0.3108 ± 0.074

Data are expressed as mean ± SD, n = 5; *: $P<0.05$,**: $P<0.01$ compared with the FCE group.

for the pharmacokinetics differences between luteolin and apigenin when oral FCE to rats [22]. The present study directly evaluated the role of COMT *in vivo* in disposition of luteolin after oral dose of FCE to rats. However, the increase in concentrations of luteolin by COMT inhibition was limited, which could not reverse the pharmacokinetics differences between luteolin and apigenin. It was possible that the inhibition of COMT by entacapone was limited due to the very wide tissue distribution of COMT *in vivo*, and/or other metabolism enzymes, such as UGTs and SULTs, especially in the small intestinal could compete with COMT to decrease the contribution of COMT to disposition of luteolin. The concentrations of apigenin were also increased by entacapone, but the mechanism for this was unclear and needs further investigation.

In conclusion, the present study provided a method for simultaneous determination of luteolin, apigenin, chrysoeriol and diosmetin in rat plasma, which had been successfully utilized in pharmacokinetics study of FCE in rats, and also could be applied in future clinical study of FCE. The pharmacokinetics of the two methylated luteolin *in vivo* could enlarge our knowledge about the disposition profile of the luteolin and pharmacological effects of FCE *in vivo* when oral administration of FCE.

Acknowledgments

This work was supported by the Zhejiang Nature Science Foundation, China (No. Y204379) and the National Major Projects for Science and Technology Development of Ministry Science and Technology of China (2009ZX09304-003).

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