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Short communication

Microsomal metabolism of calycosin, formononetin and drug–drug interactions by dynamic microdialysis sampling and HPLC–DAD–MS analysis

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ABSTRACT

A dynamic microdialysis sampling method with liquid chromatography–diode array detection and time-of-flight mass spectrometry (LC–DAD–TOF/MS) analysis was developed to investigate rat microsomal metabolisms of calycosin and formononetin, and their drug–drug interactions. Two hydroxylated metabolites from calycosin, and three hydroxylated or 4′-*O*-demethylated derivatives from formononetin were detected and identified after co-incubation with microsomes. Calibration curves offered linear ranges of two orders of magnitude with $r^2 > 0.999$ for calycosin, formononetin and daidzein. The quantitative LC method provides a range of 0.028–0.034 μg/mL for limits of detection, overall precision less than 5% and accuracy less than 3% by RSD. Besides, calycosin and formononetin were found to produce the depressive effect on the CYP450 enzyme reaction, and inhibit phase I enzyme reaction of each other when they are concurrent. Dynamic microdialysis sampling with LC–DAD–TOF/MS analysis developed in this work is a powerful tool for *in vitro* metabolism studies of drugs and metabolic interactions.

1. Introduction

Drug-metabolism studies have key roles in medicinal chemistry for lead optimization, detection of potentially toxic metabolites, and identifying the route and rate of drug clearance from the body. Because of the sensitivity, speed of analysis and ease of use, the preferred analytical strategy for metabolite identification and quantitative studies is liquid chromatography coupled with mass spectrometry (LC/MS) [1]. Traditional sample pretreatment methods include protein removal by precipitation with an acid or an organic solvent followed by centrifugation. Most analytes require extraction from the supernatant into an organic phase after protein removal. Microdialysis is a powerful sampling technique based on passive diffusion of analyte across a semipermeable dialysis membrane with a defined certain molecular weight cut-off [2]. Since proteins are excluded from the sample by the dialysis membrane, microdialysis sampling seems to be ideal for metabolism studies [3–8]. LC/MS and microdialysis that enable to recover an analyte continuously have been reported as a new analytical technique [9,10].

Unlike the single chemical compound, herbal medicines show extremely complicated pharmacological reactions, and constituents in herbal medicines may be substrates, inhibitors, or inducers of CYPs in pharmacokinetics, leading to some synergetic and/or antagonistic reactions between different compounds [11]. One sort of components contained in herbal medicines attracting more and more public attention is the isoflavones, which exist as prevalent groups of phenolic compounds in nature, displaying a wide range of biological and pharmacological properties. Calycosin and formononetin, two isoflavones widely distributed in Leguminosae [12,13], are the major active components in some herbal medicines, such as Radix Astragali and Trifolium. They possess beneficial effects including antioxidation, neuroprotective roles and protecting endothelial cells [14–17].

Investigations on the metabolism of isoflavone compounds and the determination of their metabolic pathways are very limited, and the knowledge about their CYPs-mediated drug–drug interactions (DDIs) is lack. In this paper, a dynamic microdialysis sampling and sensitive LC–diode array detection (DAD) and time-of-flight mass spectrometry (TOF/MS) analysis method has been developed to study the microsomal metabolites of calycosin and formononetin, and the metabolism-based DDIs between the two isoflavones were also investigated.

2. Experimental

2.1. Chemicals and reagents

Daidzein was obtained from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Calycosin and formononetin were isolated previously

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from the dried medicinal plant *A. membranaceus* var. *mongholicus* (Bge.) Hsiao (family Leguminosae) in the authors' laboratory. The two isoflavone compounds were isolated by repeated silica gel, Sephadex LH-20, and Rp-18 silica gel column chromatography. And their structures were elucidated by IR, MS, ^{1}H and ^{13}C NMR in comparison with the data in Refs. [18–20]. The purity of each reference compound was determined to be more than 98% by normalization of the peak areas detected by HPLC–MS. Glucose 6-phosphate, NADP and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical (St. Louis, MO, USA). Acetonitrile was of HPLC grade from Merck (Darmstadt, Germany), and 96% formic acid was of HPLC grade (Tedia, USA); deionized water (18.2 MΩ) was purified using a Milli-Q system (Millipore, Milford, MA, USA).

2.2. Apparatus and chromatographic conditions

Chromatographic analysis was performed on an Agilent 1100 series LC system (Agilent, Germany) equipped with a binary pump and a thermostatically controlled column apartment. Chromatographic separation was carried out at 25 °C on a ZORBAX C-18 column (250 mm × 4.6 mm, 5 μm) and a ZORBAXC-18 guard column (12.5 mm × 4.6 mm, 5 μm). The mobile phase consisted of 1% formic acid water and acetonitrile using an isocratic elution of 40% acetonitrile, and the flow rate was set at 0.8 mL/min. The detection wavelength was set at 260 nm. Detections were performed by an orthogonal DAD–TOF/MS (Agilent Corp, Santa Clara, CA, USA) equipped with an ESI source. The mass range was set at m/z 100–1500. The conditions of ESI source were as follows: drying gas (N_2) flow rate, 9 L/min; drying gas temperature, 320 °C; nebulizer, 35 psig; fragment, 100 V; capillary, 3500 V. All the acquisition and analysis of data were controlled by Agilent LC–MS/TOF Software Ver. A.01.00 (Agilent Technologies, USA).

2.3. Microdialysis system and probe recovery

Microdialysis sampling was performed with a 1 mL Bee syringe and 3-syringe bracket microdialysis Pumps (Bioanalytical System Inc., West Lafayette, IN, USA). Loop microdialysis probes with a 2-cm 20 kDa molecular weight cutoff polyether sulfone tubular membrane (Fresenius Medical Care AG, Wendel, German) were used. The magnetic stirring apparatus was applied with slow rotation speed to provide homogeneous reaction concentration without cutting off current on the surface of probe membrane. In this study, microdialysis conditions for calycosin and formononetin, such as probe membrane, probe-to-probe performance variability, perfusion flow rates, and probe recovery, were evaluated. Sample collection intervals and substrate concentrations were adjusted to the enzyme reaction rate as well as the LC/MS assay detection limit.

The in vitro recoveries of the probe for calycosin, formononetin and daidzein were determined prior to actual metabolism studies. Each probe was soaked in distilled deionized water at least 15 min prior to flowing any solution, then it was flushed with distilled deionized water for 1 h followed by Tris–HCl buffer solution for another 30 min. The probes were immersed in the solution of standard references and then perfused with 0.05 M Tris–HCl buffer solution (pH 7.4) for 2 h at a flow rate of 2 μL/min. The samples were collected every 20 min for a 2-h period. Dialysate samples were collected offline and stored at −80 °C until analysis on LC–DAD–TOF/MS. The relative recovery for each analyte was obtained from the equation: $\%R = 100 \times (C_{dialysate}/C_{sample})$, where $C_{dialysate}$ is the concentration of the dialysate and the C_{sample} is the concentration of the analyte in the vial. The probes were thoroughly washed with water to remove any adsorbed analyte before they were used in metabolism studies.

2.4. Rat liver microsomal incubation and metabolism studies

Microsomes were prepared from livers of overnight fasted male Sprague–Dawley rats (200–230 g), using fractional centrifugation [21]. Microsomal protein contents were determined according to the method by Lowry et al. [22]. Stock liver microsomes (16 mg protein/mL, as obtained) were stored at −80 °C until use. The isoflavone compounds with a concentration of 0.05 mg/mL in dimethyl sulfoxide were incubated in a 3-mL glass vessel with 1 mg of microsomes in 0.05 M/L Tris–HCl buffer solution (pH 7.4) at 37 °C with magnetic stirring. The microdialysis probe was placed in this solution and perfused with the same buffer as in the incubation mixture. After collecting 20 min blank dialysates, the reaction was initiated by adding an NADPH generating system consisting of 10 mM glucose-6-phosphate, 1 mM $NADP^+$, 2U glucose-6-phosphate dehydrogenase and 5 mM/L $MgCl_2$. Discontinuous sampling was applied and the dialysates of reaction mixture were collected at every 20 min. Since samples collected by microdialysis are protein-free, a 2-μL microdialysates were directly injected into LC–DAD/TOF–MS system for further analyses without fore treatment.

2.5. Validation of the method

Stock solution of the mixture of the daidzein, calycosin and formononetin was prepared by dissolving accurately weighted portions of the standards in Tris–HCl buffer solution (containing 5% DMSO), transferring it to a 5-mL volumetric flask, and then adding Tris–HCl buffer solution to make up the volume, the concentration of each compound is, 0.28 (daidzein), 0.33 (formononetin) and 0.26 (calycosin) mg/mL, respectively. The stock solution was further diluted to make different concentration ranges. The calibration curves were constructed with at least six concentrations by plotting the value of peak areas versus the value of concentrations of each analyte. The limit of detection (LOD) and limit of quantification (LOQ) was considered as the final concentration producing a signal-to-noise ratio of 3 and 10, respectively.

Stock solution of reference compounds were spiked into Tris–HCl buffer solution to give quality control (QC) samples of low, middle, and high concentrations. The precision and accuracy of method were assessed by performing replicate analyses of QC samples. The precision was determined from inter-day and intra-day using five determinations of three concentrations and expressed as relative standard deviation (R.S.D.). The stability of analytes was evaluated by analyzing QC samples at two concentrations exposed to different time and temperature conditions: modeling five freeze/thaw cycles (−80 °C to ambient temperature), 12 h storage at room temperature and frozen at −80 °C for 1 week.

3. Results and discussion

3.1. Validation of the method

The relative *in vitro* recoveries (mean ± S.D., $n = 5$) of the dialysis probe for calycosin, formononetin and daidzein are 70.24% ± 4.75, 73.27% ± 4.52 and 71.12% ± 4.89, respectively. The *in vitro* recovery or dialysis efficiency can be affected by some factors such as temperature and perfusion rate. Thus, each probe must be calibrated prior to use and all physical components must be kept constant.

The linear regression of daidzein ($y = 2560.6x + 9.1235$), formononetin ($y = 2305.6x + 9.8716$) and calycosin ($y = 12448x + 11.706$) in dialysate displayed good linear relationship with $r^2 > 0.999$ over the range of 0.22–55 μg/mL, 0.26–65 μg/mL and 0.2–52 μg/mL, respec-

Table 1
Precision and accuracy for quantification of calycosin, formononetin and daidzein in microdialysis samples.

Analyte	Spiked concentration (ng/mL)	Measured concentration (ng/mL) (mean ± S.D.)	Precision (R.S.D.%)	Accuracy percent error (%)
Calycosin	Intra-day			
	0.5	0.49 ± 0.019	3.81	−2.0
	5	5.06 ± 0.182	3.59	1.2
	50	49.58 ± 1.62	3.26	−0.8
	Inter-day			
	0.5	0.49 ± 0.018	3.73	−2.8
	5	5.01 ± 0.167	3.34	0.20
	50	49.62 ± 1.161	2.34	−0.76
Formononetin	Intra-day			
	0.5	0.48 ± 0.015	3.12	−2.8
	5	5.06 ± 0.175	3.45	1.28
	50	50.58 ± 1.915	3.79	1.16
	Inter-day			
	0.5	0.51 ± 0.0217	4.27	1.60
	5	5.05 ± 0.122	2.43	1.00
	50	50.70 ± 1.440	2.84	1.40
Daidzein	Intra-day			
	0.5	0.51 ± 0.019	3.85	1.20
	5	5.10 ± 0.07	1.39	2.00
	50	50.88 ± 2.01	3.96	1.76
	Inter-day			
	0.5	0.51 ± 0.025	4.88	2.8
	5	5.07 ± 0.156	3.08	1.4
	50	51.10 ± 1.294	2.53	2.2

Table 2
Stability of the calycosin formononetin daidzein in dialysate ($n = 5$).

Analyte	Spiked concentration (μg/mL)	Measured concentration (μg/mL) (mean ± S.D.)		
		Freeze/thaw cycles	Room temperature	At −80 °C for 1 week
Calycosin	0.5	0.50 ± 0.019	0.49 ± 0.013	0.49 ± 0.022
	5	5.10 ± 0.187	5.03 ± 0.172	5.05 ± 0.187
	50	49.88 ± 1.94	50.02 ± 1.88	49.78 ± 1.42
Formononetin	0.5	0.48 ± 0.019	0.51 ± 0.021	0.51 ± 0.021
	5	5.10 ± 0.179	5.09 ± 0.134	5.11 ± 0.160
	50	51.08 ± 2.19	50.0 ± 2.21	51.2 ± 1.75
Daidzein	0.5	0.51 ± 0.017	0.52 ± 0.022	0.52 ± 0.026
	5	5.04 ± 0.152	5.09 ± 0.152	5.11 ± 0.159
	50	50.68 ± 1.92	51.5 ± 1.54	50.6 ± 1.92

tively. The LOD and LOQ for the three compounds fell in the range of 0.028–0.034 μg/mL and 0.064–0.093 μg/mL. As can be seen from Table 1, the overall mean precision ranged from 1.39 to 4.88% in dialysate, while the accuracy (bias %) varied from −0.28 to 2.8%. Stability data are summarized in Table 2 and indicated that the calycosin, formononetin and daidzein were all stable in dialysate for five freeze/thaw cycles, 24 h at room temperature. Moreover, the results of the stability showed that all the analytes were stable for at least 1 week when kept frozen at −80 °C. These results are within the acceptable criteria for precision and accuracy, indicating the developed method is accurate and reliable.

3.2. Microsomal metabolism of calycosin and formononetin

3.2.1. Metabolites identification

Using microdialysis sampling system, the dialysates from reaction system were conveniently collected at different times, and then directly analyzed by LC–DAD/TOF–MS without complicated sample pretreatment. The biotransformation of calycosin and formononetin was investigated by incubating each isoflavone with rat liver microsomes. Fig. 1(A) shows the metabolic fingerprinting chromatogram of calycosin at 260 nm. Two metabolites named C-M1 and C-M2 were detected. Calycosin, the parent compound

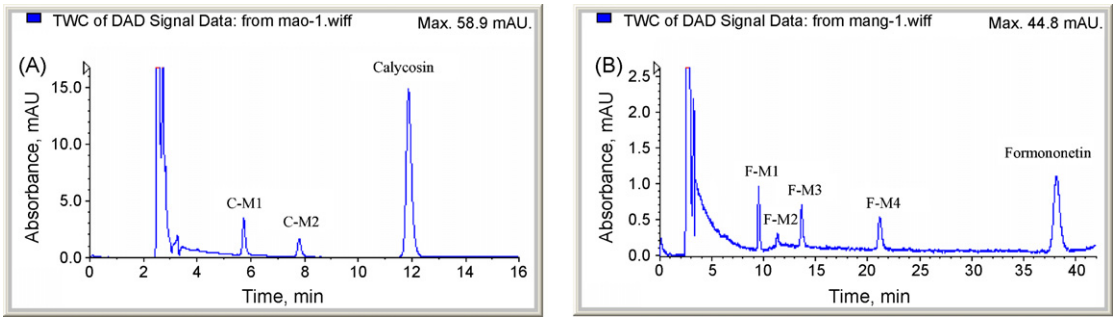

Fig. 1. Representative metabolic chromatogram of a dialysate sample from calycosin (A) and formononetin (B) incubation. Rat liver microsomes (1 mg/mL) were incubated with 0.05 mg/mL calycosin or formononetin at 37 °C for 1 h. Chromatographic conditions are described in Section 2.2.

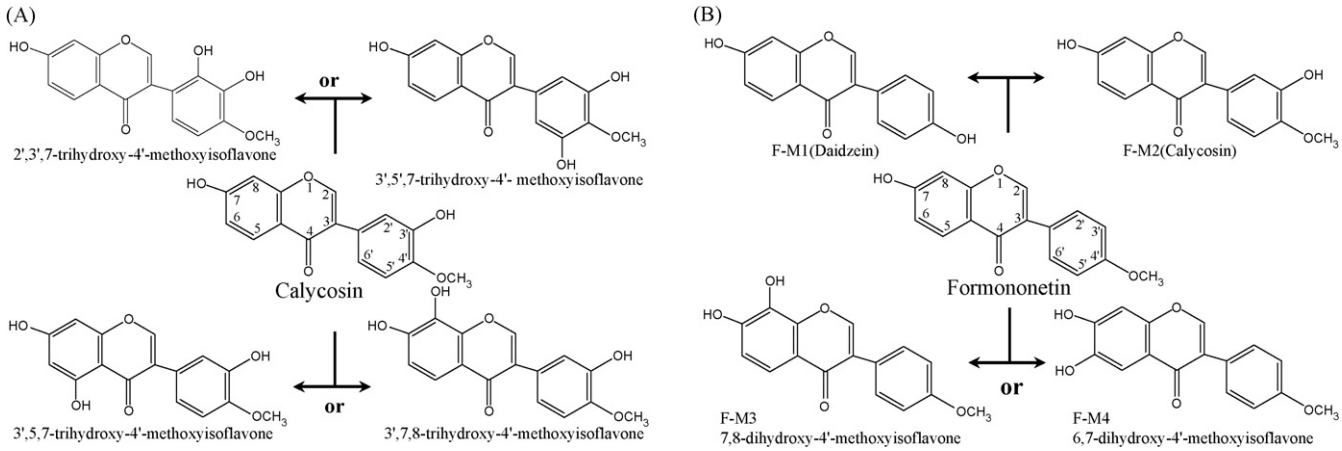

Fig. 2. Proposed metabolic pathways of calycosin (A) and formononetin (B) in incubations with rat liver microsomes.

with the retention time at 12.01 min, was easily elucidated by comparison of retention time and MS data with a standard. In positive ion mode, both C-M1 (t_R at 5.79) and C-M2 (t_R at 7.83) produced a dominant protonated ion $[M+H]^+$ at m/z 301.0713 $C_{16}H_{13}O_6$, less than 3 ppm), a sodic adduct ion $[M+Na]^+$ at m/z 323.0536, and a kalium adduct ion $[M+K]^+$ at m/z 339.0276. It can be found that the molecular ions of the metabolites are 16 Da more than those of the parent compound, demonstrating that C-M1 and C-M2 are mono-hydroxyl metabolites of calycosin. Previous studies showed that the B-ring is the preferred position to undergoing biotransformation by rat liver microsomes for flavonoids, and then the A-ring [14]. Based on biosynthesis pathway of isoflavones and known compounds from The Combined Chemical Dictionary [23], calycosin is possible to be hydroxylated at the 2′ or 5′ position at B-ring, or 5 or 8 position at A-ring. The two metabolites were tentatively assigned as 2′,3′,7-trihydroxy-4′-methoxyisoflavone (Koparin) or 3′,5′,7-trihydroxy-4′-methoxyisoflavone (Gliricidin) or 3′,5,7-trihydroxy-4′-methoxyisoflavone (Pratensein) or 3′,7,8-trihydroxy-4′-methoxyisoflavone. Their absolute configurations need further NMR investigations.

The chemical structure of formononetin is similar with that of calycosin, differing in the substituent group at position 3′ of ring B: a hydrogen for formononetin while an hydroxyl group for calycosin). Fig. 1(B) shows the DAD metabolic fingerprinting chromatogram of formononetin at 260 nm. Four metabolites named F-M1, F-M2, F-M3 and F-M4 were observed. The parent compound of formononetin with t_R 38.30 was easily identified in comparison with a standard. In positive ion mode, F-M2 (t_R at 11.33), F-M3 (t_R at 13.65), and F-M4 (t_R at 21.24) all yielded identical protonated molecular ion $[M+H]^+$ at m/z 285.0754 ($C_{16}H_{13}O_5$, less than 5 ppm), sodic adduct ion $[M+Na]^+$ at m/z 307.0586, and kalium adduct ion $[M+K]^+$ at m/z 323.0331, 16 Da more than those of the parent compound. Similarly, F-M1, F-M2 and F-M3 are mono-hydroxyl metabolites of formononetin. F-M2 was further identified as calycosin in comparison with a standard. Formononetin is presumable to be hydroxylated in the 2′-position at B-ring, or 6/8-position at A-ring. Tolleson et al. studied the metabolism of formononetin by human liver microsomes, and found that 6,7-dihydroxy-4′-methoxyisoflavone (texasin) and 7,8-dihydroxy-4′-methoxyisoflavone (retusin) are two major hydroxylated metabolites of metabolites [24]. According to their differences in polarity and corresponding retention times, F-M3 and F-M4 were tentatively identified as retusin and texasin, respectively. In some exceptional examples, F-M1 (t_R at 9.53) generated a protonated molecular ion $[M+H]^+$ at m/z 255.0650 ($C_{15}H_{11}O_4$, less than 5 ppm), a sodic adduct ion $[M+Na]^+$ at m/z 277.0463, and a kalium adduct ion $[M+K]^+$ at m/z 293.0219, 14 Da less than those of the parent compound, indicating a effective demethylation reaction. F-M1 was further confirmed as daidzein in comparison with a standard. The presumed biotransformation route of calycosin and formononetin were presented in Fig. 2(A) and (B). The data presented here demonstrate for the first time that rat liver microsomal enzymes are capable of catalyzing hydroxylation and 4′-*O*-demethylation reactions of calycosin and formononetin.

3.2.2. Analysis of metabolic profiles

A comparison of the extent of metabolism of the investigated isoflavones in microsomes indicated that the number of free hydroxy groups is particularly important for the degree of metabolism [14]. Generally the less polar flavonoids were metabolized faster and more extensively. It was observed that the CYPs biotransformation of formononetin was markedly faster and more completely than that of the corresponding monohydroxylated compound calycosin (Fig. 3(A)).

From the time-course curves of enzymatic reactions of C-M1 and C-M2 catalyzed by rat liver microsomes shown in Fig. 3(B), we can see that the two metabolites of calycosin could be both detected during 20–40 min, and the content of C-M1 measured with peak area is higher than that of CM2; both contents increased gradually till after 140 min when they enter a period of 'plateau'. As the content of C-M1 is always higher than C-M2 during whole reaction process, it was suggested that calycosin in liver microsome incubation system was inclined to produce one certain replacing location.

The metabolic profiles of F-M1 and M2 shown in Fig. 3(C), F-M3 and M4 shown in Fig. 3(D) are somewhat different from those of metabolites derived from calycosin. All metabolites except calycosin (F-M2) were gradually increasing in the content during the period of 20–40 min, and then up to its plateau stage during 140–180 min. The profiles of F-M3 and F-M4 were quite similar. It was interesting that the content of calycosin (F-M2) was rapidly up to its highest amount within 20 min, and decreased slowly, until to the plateau stage during 140–180 min. This result suggested that the hydroxylation of formononetin at 3′-position was the fastest biotransformation in liver microsome incubation system compared with that at other sites.

3.3. Effects of metabolism-based DDIs of calycosin with formononetin

According to the above results, both calycosin and formononetin were the reactive substrates of liver microsome CYPs. Therefore, the depressive effect might occur due to substrate competition when they exist together. We investigated the metabolism-based DDIs of calycosin with formononetin on their respective microso-

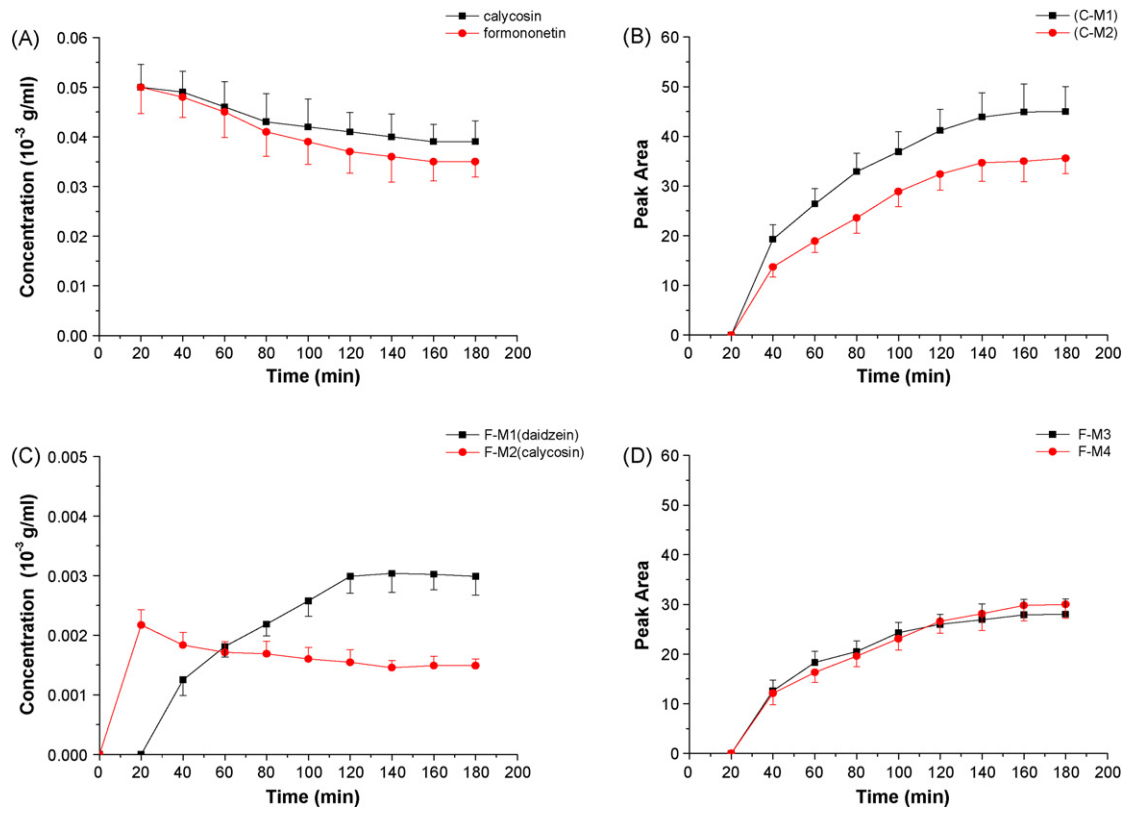

Fig. 3. Metabolic profiles of calycosin and formononetin. (A) Metabolic profiles of calycosin and formononetin; (B) metabolic profiles of calycosin metabolites: C-M1 and C-M2; (C) metabolic profiles of formononetin metabolites: F-M1 (daidzein) and F-M2 (calycosin); (D) metabolic profiles of formononetin metabolites: F-M3 and F-M4.

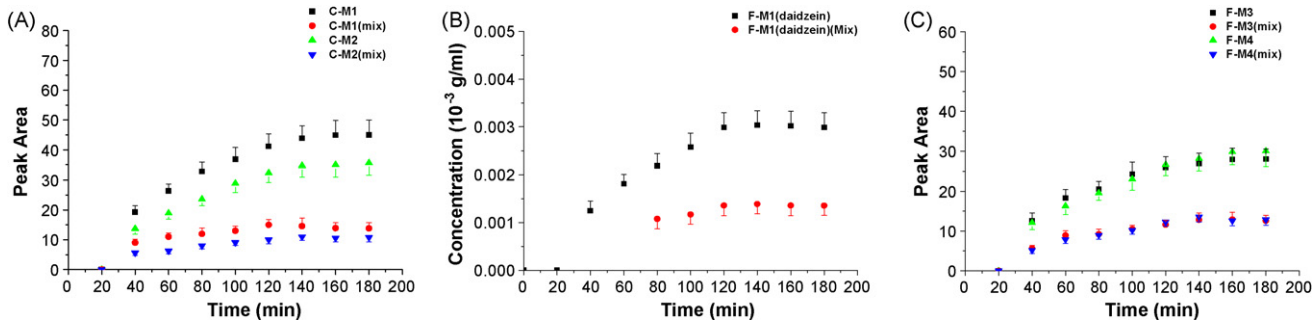

Fig. 4. Metabolism-based drug–drug interactions of calycosin with formononetin in microsome reaction system. (A) Drug–drug interactions effects on metabolic profiles of calycosin metabolites: C-M1 and C-M2; (B) effects on metabolic profiles of formononetin metabolites: F-M1 (daidzein); (C) effects on metabolic profiles of formononetin metabolites: F-M3 and F-M4.

mal metabolites. Fig. 4 showed the time-change contents of C-M1 and C-M2 (A), F-M1 (B), F-M3 and F-M4 (C) when calycosin or formononetin were metabolized as a single compound or metabolized in mixtures under the same conditions. Obvious competitive inhibitions were observed between calycosin and formononetin, leading to the significant decrease of some metabolites from single compound to the mixture.

Metabolic interaction might strengthen some pharmacological action, or by contrast weaken others. For example, calycosin and formononetin have pharmacological effects including antioxidant, anti-ischemia, and estrogenic activities, while daidzein, the metabolite of formononetin, has obvious higher estrogenic activity than its parent compound [15,16,25]. The experimental results showed that calycosin and formononetin would produce the depressive effect on the CYP450 enzyme reaction and inhibit their phase I enzyme reaction, correspondingly producing antagonistic effects on estrogenic activity, while producing synergistic effects on antioxidation and anti-ischemia.

4. Conclusion

The present study is the first investigation on the metabolism of isoflavone aglycones by rat microsomal enzymes, and the DDIs of natural products. This assay demonstrates that microdialysis sampling and LC–DAD/TOF–MS is a powerful tool with great flexibility and high throughput for *in vitro* metabolism studies of drugs. Compared with conventional methods, its continuous, real-time monitoring sampling and simple sample preparation are very attractive. Further studies are needed to systematically investigate the DDIs, and clarify whether the metabolic pathways of isoflavones found in rat liver microsomes are identical to those in rat and man.

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