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Short communication

Validation of an HPLC method for the simultaneous determination of DZ5 and its active metabolite in dog plasma and its pharmacokinetics

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Abstract

A validated HPLC method is described for the simultaneous determination of daidzein 7,4′-di-succinic acid mon-ester-*O*-ethoxy (DZ5) and its active metabolite daidzein 7,4′-dioxy-ethoxy (DZ4) in dog plasma. DZ5 and DZ4 were determined by reversed-phase HPLC (column: Hypersil C18 5 μm silica, 200 mm × 4.6 mm i.d.; eluent: 400 ml water, 500 μl 85% phosphoric acid, 600 ml methanol) and photometric detection (250 nm), with Kaempferol as the internal standard. The calibration curve was linear over the range 0.1–50.0 μg/ml in dog plasma. The average extraction recoveries were 84.6% (DZ5), 82.7% (DZ4) and the within-day and between-day precisions were less than 10.93%. The assay was applied to the analysis of samples from a pharmacokinetic study. Following the oral administration and intravenous administration of DZ5, DZ5 was eliminated rapidly from the plasma and DZ4 was found in plasma. The absolute bioavailability of total DZ5 and DZ4 was 41.5%. The method was demonstrated to be feasible for pharmacokinetic studies of DZ5 in dogs.

Keywords: Daidzein 7,4′-di-succinic acid mon-ester-*O*-ethoxy; Daidzein 7,4′-dioxy-ethoxy; HPLC; Pharmacokinetics

1. Introduction

The isoflavones are phytoestrogens with weak estrogenic activity, which are present in the human diet in soybeans and soy derived products. There are many published studies describing the health benefits of these compounds [1–3]. DZ is one of the major isoflavonoids in soybeans. However, DZ is poorly water-soluble and its bioavailability is very low [4]. In order to improve its solubility and pharmacological efficacy, we modified the chemical structure of daidzein and synthesized a derivative, named daidzein 7,4′-di-succinic acid mon-ester-*O*-ethoxy (DZ5, Fig. 1a). Our pharmacological studies indicated that the protective effects of DZ5 on cerebral ischemia, normabaric hypoxia, histotoxic anoxia and on memory are similar to those of daidzein (data in press).

We found that DZ5 was rapidly hydrolysed in vivo to daidzein 7,4′-dioxy-ethoxy (DZ4, Fig. 1b) by cleavage of the ester group and this was also active. However, two problems are associated with the HPLC analysis of DZ5 and DZ4 in biological fluids. Firstly, it is possible that DZ5 can be hydrolyzed to DZ4 in solvents such as water or methanol, and also in plasma. Therefore, partial degradation of DZ5 may occur after sampling and before injection into the HPLC system. Secondly, a portion of DZ5 may be lost by sublimation during sample treatment when evaporation steps are included. Accordingly, the present paper describes the development of a rapid, simple and sensitive HPLC method for determining DZ5 and DZ4 in biological samples which circumvents these analytical problems. The study also investigated their pharmacokinetic characteristics in dogs following intravenous administration and oral administration of DZ5.

2. Experimental

2.1. Materials

DZ5 and DZ4 were obtained from Shenyang Pharmaceutical University (99.0%, 99.1%, Shenyang, China), the internal standard Kaempferol (99.5%, Fig. 1c) was obtained from the

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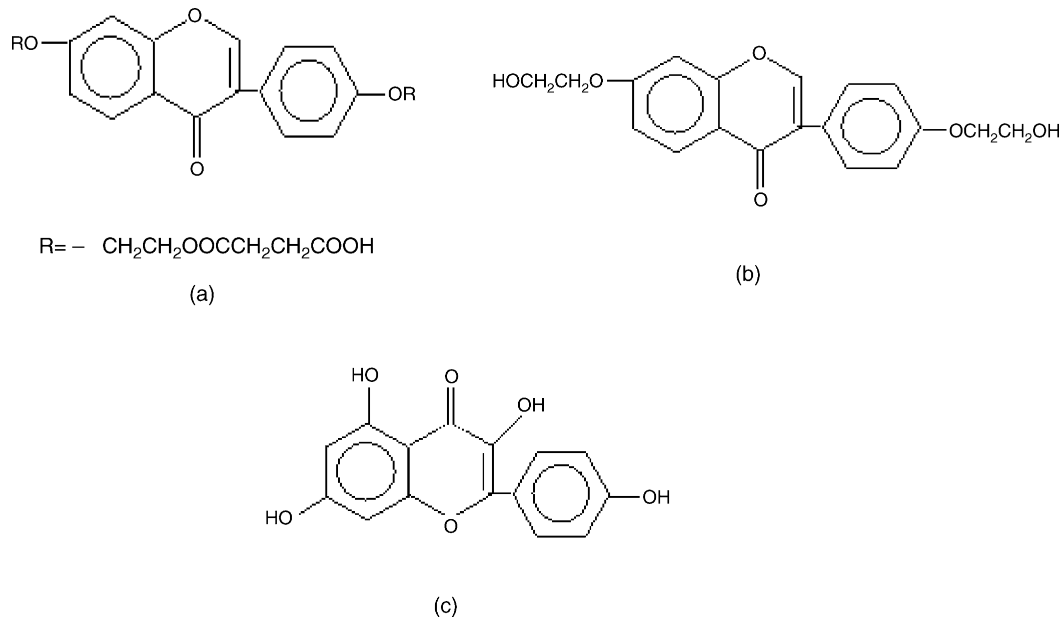

Fig. 1. Chemical structures of DZ5 (a), DZ4 (b) and I.S. (c).

Institute for Drug Control of Liaoning Province. Methanol was of HPLC grade, other reagents of analytical grade were from Yuwang Reagent Co., Ltd. (Shandong, China).

2.2. Apparatus and chromatographic conditions

The Shimadzu HPLC system (Kyoto, Japan) consisted of an LC-10AT pump, and an SPD-10A UV detector set at 250 nm. Separation was performed using a prepacked stainless-steel column (200 mm × 4.6 mm i.d.) with Hypersil C18 5 μm silica. The mobile phase consisted of 400 ml water, 500 μl 85% phosphoric acid, 600 ml methanol and the flow-rate was 1.0 ml/min. The system was operated at room temperature.

2.3. Sample preparation

Venous blood samples from dogs were collected into heparinized plastic tubes. After immediate chilling, the plasma was separated by centrifugation at 4 °C within 15 min. The samples were then shock frozen and stored at −20 °C until the assay. Frozen plasma samples were thawed in an ice-water bath. Then a portion of plasma (200 μl) was added to 50 μl internal standard (I.S.) solution (20 μg/ml) and vortex-mixed for a few seconds. Then 400 μl methanol was added and the solution was mixed again. After 15 min at 4 °C the mixture was centrifuged to separate precipitated proteins. The supernatant was transferred to 1.5 ml tubes containing 100 mg anhydrous sodium sulfate. The suspension was vortex-mixed briefly and incubated at 4 °C for 10 min, centrifuged once again, and the upper organic layer was transferred to the sampler vials, and 20 μl was injected into the HPLC.

The influence of dilution was studied using a blank plasma sample spiked to obtain a concentration that was two-fold higher than the maximal concentration of the calibration curve. It was then diluted to one third with the same plasma.

2.4. Stability of plasma samples

The stock solutions of internal standard, Kaempferol (500 μg/ml), DZ5 and DZ4 (1 mg/ml) were prepared in methanol. All solutions were stored at 4 °C. The stability of DZ5 and DZ4 in plasma was evaluated for 1 month at −20 °C, and for three freeze-thaw cycles. The validation criteria were calculated using commonly accepted statistical procedures.

2.5. Method validation

Kaempferol, as the internal standard, was added to each standard containing known amounts of DZ4 (0.1–50.0 μg/ml) or DZ5 (0.1–50.0 μg/ml). Each sample was prepared in duplicate. The peak-area ratios (DZ4 to Kaempferol, or DZ5 to Kaempferol) were determined in duplicate. The calibration curves were obtained by a least-squares linear fitting of the peak-area ratios versus the amounts of DZ4 or DZ5.

Intra- and inter-run precision was assessed from the results of QCs. The mean values and R.S.D. for QCs at three concentration levels were calculated over three validation runs. Six replicates of each QC level were determined in each run. These data were then used to calculate the intra- and inter-run precision (R.S.D.) by a one-way analysis of variance. The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QCs and expressed as the relative error. The precision and accuracy of each QC value should not exceed a deviation of 15%, except for the QC samples for the limit of quantification (LOQ) where 20% was acceptable.

Extraction recovery was determined using the extraction and analysis of three control levels of analyte, at high, mid and low levels of the calibration range, for each particular assay. Each control level was prepared with an "*n*" of 5.

2.6. Pharmacokinetic study

All animal studies were performed according to the Guidelines for the Care and Use of Laboratory Animals that was approved by the Committee of Ethics of Animal Experimentation of Shenyang Pharmaceutical University.

Beagle dogs (male, 10–13 kg) were provided by the Animal Center of Shenyang Pharmaceutical University (Shenyang, China). Animals were housed in a room with controlled temperature and humidity, and allowed free access to food and water. They were fasted overnight before the experiments. Two groups (six dogs/group) were randomly assigned to be given a single oral dose of 20 mg/kg and a single intravenous dose of 20 mg/kg DZ5, respectively. Blood samples were collected in heparinized tubes at 0, 10, 20, 30, 45, 60, 90, 120, 150, 180, 240, 300 min after intravenous or oral administration. The obtained plasma samples were immediately separated and stored frozen at −20 °C until analysis. All the drug concentration data were analyzed using a non-compartmental method (3p97 software, the Chinese Society of Mathematical Pharmacology) to obtain pharmacokinetic parameters.

3. Results and discussion

3.1. Sample preparation

In our pilot studies, the samples were extracted into organic solvents, and the organic solvents were then evaporated. Methanol was then used to precipitate protein followed by concentration. The loss of DZ5 was found to be over 70%. In the present study, methanol was used to precipitate protein and then sodium sulfate was used to retain the water. The whole process was carried out at low temperature. Little DZ5 was found to be converted to DZ4. Therefore, the method developed in the present study is feasible with respect to the simplicity of sample treatment combined with the stability of DZ5 in the samples before injection.

3.2. Chromatographic separation

The mutual separation of DZ5, DZ4, I.S. and endogenous substances was optimized by using a mobile phase composed of 400 ml water, 500 μl 85% phosphoric acid, and 600 ml methanol. Fig. 2 shows representative chromatograms of blank plasma, plasma spiked with DZ5, DZ4 and I.S., and a dog plasma sample at 90 min after oral administration of DZ5 at a dose of 20 mg/kg. The peaks were well separated and the retention times of DZ4, DZ5, and I.S. were 8, 12 and 22 min. No peaks from endogenous compounds that interfered with DZ4, DZ5 and I.S. were observed in the plasma samples.

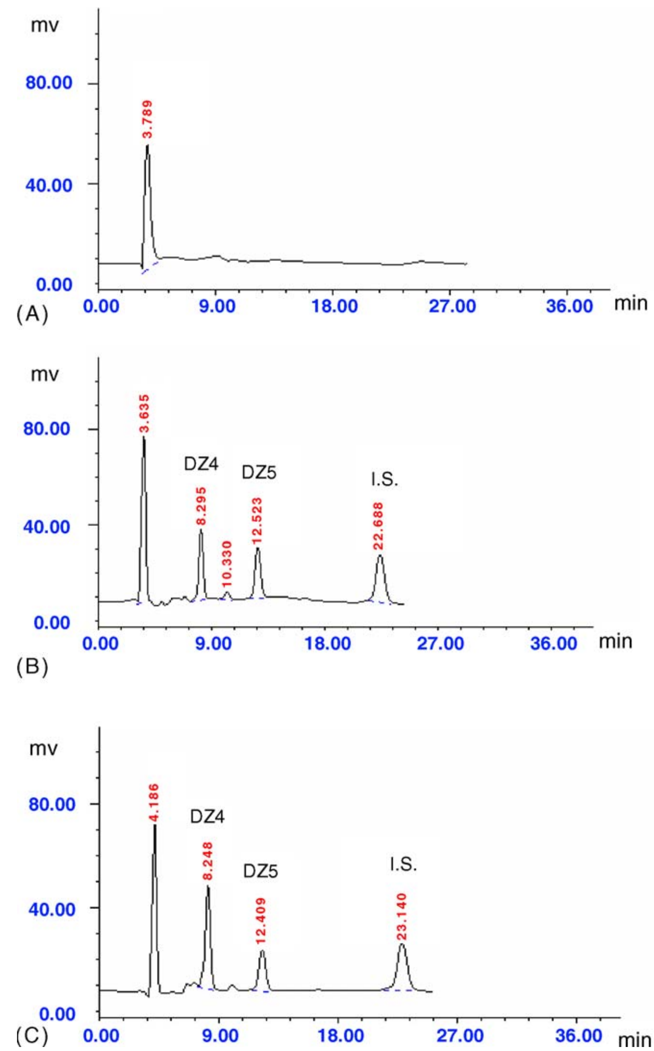

Fig. 2. Chromatograms of blank plasma (A); blank plasma spiked with DZ5 (2 μg/ml), DZ4 (2 μg/ml) and I.S. (10 μg/ml) (B); dog plasma sample at 90 min after oral administration of DZ5 at a dose of 20 mg/kg (C).

3.3. Method validation tests

3.3.1. Linearity and limit of quantification

Calibration curves for the plasma assay developed with a peak-area ratio (y) of DZ5 or DZ4 to I.S. versus drug concentration (C) were found to be linear over the concentration range 0.1–50.0 μg/ml using a weighted least squares method, and the weight was $1/C^2$.The linear regression equations of the calibration curve of DZ5 and DZ4 were $y = 0.1103 \times C - 0.0273$ ($r = 0.9991$), and $y = 0.1654 \times C + 0.0302$ ($r = 0.9994$), respectively. The limit of quantification (LOQ) was 100 ng/ml.

3.3.2. Precision and accuracy

The precision and accuracy of the method were examined by adding known amounts of DZ5 or DZ4 to blank dog plasma. For intra-day precision and accuracy, three replicate quality control samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated on three different days. The results are summarized in Table 1. The intra-day and inter-day precisions were within 15%, which indicated

Table 1
Precision and accuracy of the determination of DZ5 and DZ4 in dog plasma ($n = 3$)

Compound	Added (μg/ml)	Intra-day			Inter-day		
		Measured (μg/ml)	R.S.D. (%)	Accuracy (%)	Measured (μg/ml)	R.S.D. (%)	Accuracy (%)
DZ5	0.2	0.192	6.84	96.0	0.194	9.70	97.0
	5.0	5.163	4.18	103.3	5.033	5.89	100.7
	50.0	49.666	2.41	99.3	50.230	4.35	100.5
DZ4	0.2	0.198	5.59	99.0	0.205	10.93	102.5
	5.0	5.102	6.62	102.1	4.991	5.89	99.8
	50.0	50.802	4.00	101.6	50.112	4.56	100.2

that the method was reproducible. The extraction recoveries of DZ5 and DZ4 were 84.6% and 82.7%, respectively. Therefore, this new method for the analysis of biological samples exhibited good precision and accuracy coupled with a high recovery.

3.4. Effect of dilution

The precision and accuracy of six replicates of diluted plasma were 2.8 (DZ5), 2.4 (DZ4) and 99.4% (DZ5), 99.5% (DZ4), respectively.

3.5. Stability study of DZ5 in plasma

After 4 weeks of storage at −20 °C, QC samples were shown to be stable at three concentrations. No significant degradation of DZ5 was observed after three freeze-thaw cycles. When the stock solutions were stored at 4 °C, decomposition of DZ5 to DZ4 was less than 2% over 1 month. DZ4 and internal standard were stable for at least 1 month (data not shown). Specimens could be stored for 1 day at room temperature and at least 1 week at 4 °C before analysis without decomposition, e.g., hydrolysis of DZ5 to DZ4.

3.6. Pharmacokinetic study

Fig. 3 illustrates the plasma concentrations measured over 5 h after a single i.v. dose of 20 mg/kg DZ5 in dogs. Fig. 4 illustrates the plasma concentrations measured over 5 h after a single oral dose of 20 mg/kg DZ5 in dogs. The main pharmacokinetic parameters of DZ5 and DZ4 in dogs are summarized in Table 2. These results showed that DZ5 was eliminated rapidly from the plasma. Accordingly, plasma concentration of DZ5 was detectable only up to 5 h in dogs. DZ4 was found in plasma

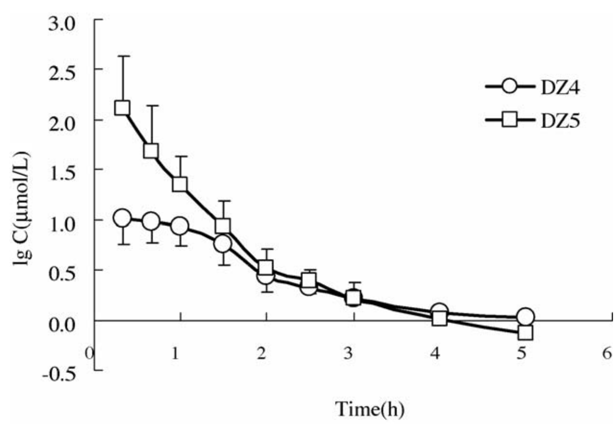

Fig. 3. Mean plasma concentration–time curves of DZ5 and its active metabolite DZ4 in dogs (mean ± S.D., $n = 6$) following intravenous administration of DZ5 20 mg/kg.

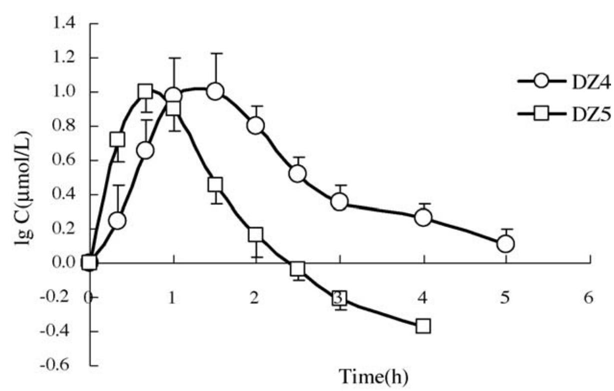

Fig. 4. Mean plasma concentration–time curves of DZ5 and its active metabolite DZ4 in dogs (mean ± S.D., $n = 6$) following oral administration of DZ5 20 mg/kg.

Table 2
The main pharmacokinetic parameters of DZ5 and DZ4 after i.v. or p.o. administration of DZ5 (20 mg/kg) to dogs (mean ± S.D., $n = 6$)

	I.v.		P.o.	
	DZ5	DZ4	DZ5	DZ4
C_{max} (μmol/l)	–	–	10.10 ± 3.83	10.08 ± 4.33
T_{max} (h)	–	–	0.67 ± 0.21	1.50 ± 0.32
K (l/h)	0.48 ± 0.11	0.32 ± 0.14	0.50 ± 0.28	0.34 ± 0.14
AUC_{0-t} (μmol h/l)	55.94 ± 9.05	16.66 ± 5.44	11.72 ± 2.52	18.43 ± 5.91
$AUC_{0-\infty}$ (μmol h/l)	57.46 ± 8.65	20.04 ± 6.36	12.58 ± 2.61	22.19 ± 4.18
Absolute bioavailability (%)			41.5	

after either intravenous or oral administration and 60% of DZ5 was converted into DZ4 following oral administration within 5 h, while only 23% of DZ5 was converted into DZ4 following intravenous administration within 5 h. This is mainly due to the effect of liver esterases on DZ5 following oral administration, in addition to plasma esterases [5]. The absolute bioavailability of the sum of DZ5 and DZ4 was 41.5%, while the absolute bioavailability of DZ was 6.1%, this came from our previous study (data not shown), which indicated that the ester-containing compound DZ5 improved the absorption.

4. Conclusion

In conclusion, the HPLC method presented here is simple and has the necessary precision, sensitivity and accuracy to allow the simultaneous determination of DZ5 and DZ4 in plasma. It has been applied successfully to study the pharmacokinetics in dogs following intravenous or oral administration of DZ5 at doses of 20 mg/kg. The metabolism of DZ5 to DZ4 after oral administration was greater than that after intravenous administration in dogs, and the absolute bioavailability of the sum of DZ5 and DZ4 was 41.5%.

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