Pharmacokinetics of Isofraxidin in Rat Plasma after Oral Administration of the Extract of *Acanthopanax senticosus* Using HPLC with Solid Phase Extraction Method

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High-performance liquid chromatography coupled with solid phase extraction method was developed for determination of isofraxidin in rat plasma after oral administration of *Acanthopanax senticosus* extract (ASE), and pharmacokinetic parameters of isofraxidin either in ASE or pure compound were measured. The HPLC analysis was performed on a Dikma Diamonsil RP_{18} column (4.6 mm×150 mm, 5 μm) with the isocratic elution of solvent A (acetonitrile) and solvent B (0.1% aqueous phosphoric acid, v/v) (A : B=22 : 78) and the detection wavelength was set at 343 nm. The calibration curve was linear over the range of 0.156—15.625 μg/ml. The limit of detection was 60 ng/ml. The intra-day precision was 5.8%, and the inter-day precision was 6.0%. The recovery was 87.30±1.73%. When the dosage of ASE is equal to pure compound caculated by the amount of isofraxidin, it has been found to have two maximum concentrations in plasma while the pure compound only showed one peak in the plasma concentration–time curve. The determined content of isofraxidin in plasma after oral administration of ASE is the total contents of free isofraxidin and its precursors in ASE *in vitro*. The pharmacokinetic characteristics of ASE showed the priority of the extract and the properities of traditional Chinese medicine.

Key words pharmacokinetics; isofraxidin; *Acanthopanax senticosus*; high-performance liquid chromatography; solid phase extraction

Acanthopanax senticosus widely distributed in the northeastern region of China, Korea *etc.*, being used as an adaptogenic medicine.[1)] The major active constituents are eleutheroside, isofraxidin, acanthoside, daucosterine, β-sitosterol, sesamine and savinine,[2,3)] which have been used clinically to treat stress-induced physiological changes,[4,5)] various allergic conditions,[6)] inflammation,[7)] cancer,[8,9)] chronic bronchitis, hypertension, ischemic heart disease, gastric ulcer[10)] and so on. Isofraxidin as major constituent elicits good anti-fatigue, anti-stress and immuno-accommondating effects, and the biological evaluation of it has been assessed.[11,12)] Li *et al.* have investigated the pharmacokinetics of isofraxidin in the rat after oral administration of the extract of *Acanthopanax senticosus* (ASE),[13)] which partly elucidated the pharmacokinetic properties of isofraxidin, however, because of low recovery of the sample preparation method. Hence the results did not reflect the overall properties of isofraxidin in ASE. Feng *et al.* have investigated the pharmacokinetics of eleutheroside B and eleutheroside E in ASE using solid phase extraction method with a good recovery,[14)] but they investigation did not adress the problem of isofraxidin. In the present studies, the HPLC method coupled with solid phase extraction was developed to determine the pharmacokinetics of isofraxidin (the structure is shown in Fig. 1) in rat plasma after oral administration of either ASE or the pure isofraxidin. The improved recovery (87.30%) made it possible to elucidate the body fate of isofraxidin more comprehensively. Simultaneously, the special priority in dynamic change *in vivo* of the Chinese medicine was much better understood by comparing the main pharmacokinetic parameters of isofraxidin.

Isofraxidin

Fig. 1. Structure of Isofraxidin

Experimental

Materials and Reagents The roots of *Acanthopanax senticosus* were collected from Mishan region (Heilongjiang province, P. R. China), all the crude drugs were authenticated by Prof. Xijun Wang of the Pharmacognosy Department, Heilongjiang University of Chinese Medicine. ASE was prepared in our laboratory according to the method recorded in China Pharmacopia (Edition 2005)[15)] and was dried *in vacuo*. The contents of isofraxidin, caffeic acid, eleutheroside B, chlorogenic acid, and eleutheroside D in ASE was determined by 3D-HPLC (Fig. 2), and the detection wavelength was set at 343, 258, 263, 323, and 269 nm respectively. HPLC grade acetonitrile was purchased from Dikma technology Inc. (Richmond Hill, U.S.A.). Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, U.S.A.). Analytical grade phosphoric acid was purchased from Beijing Reagent Company (Beijing, P. R. China). Isofraxidin was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, P. R. China).

Animals Male Wistar rats, weighing 230—250 g, were purchased from the Animal Center of Heilongjiang University of Chinese Medicine (Harbin, P. R. China). Animals were kept in an environmentally controlled breeding room (temperature: 24±2 °C, humidity: 60±5%, 12 h dark–light cycle) for 1 week before the experiment. The rats were fed standard laboratory chow with water *ad libitum* and all the rats were fasted for 12 h before the experiments but had free access to water. All animal experiments complied with the requirements of the National Act on the Use of Experimental Animals (P. R. China).

Apparatus A Waters 2695 liquid chromatography system equipped with aWaters 600 controller, two Waters 600 pumps, a 2695 autosampler, a Waters 2996 photodiode array detector, and a Waters 2695 column oven was used. The column configuration consisted of a Dikma Diamonsil RP_{18} column (4.6 mm×150 mm, 5 μm) and a Nova-PakC_{18} Guard-Pak™ guard column (2 mm×4 mm, 5 μm). Solid-phase column was Waters Oasis HLB cartridge (3 ml, 60 mg) (Waters, Milford, Massachusetts, U.S.A.).

Chromatographic Conditions The gradient elution was employed for simultaneous determination of the multiconstituents in ASE using solvent A

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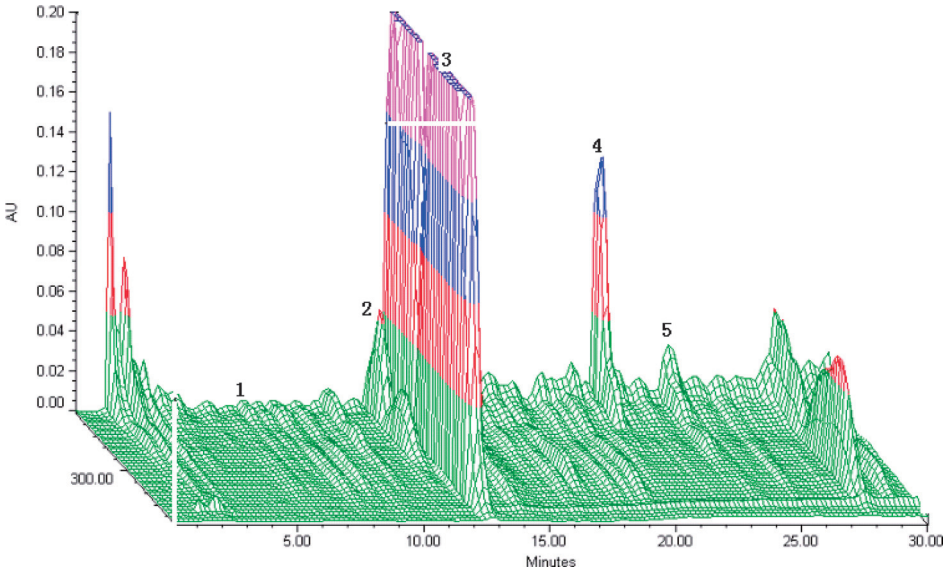


Fig. 2. 3D-Chromatogram of ASE

1, caffeic acid; 2, eleutheroside B; 3, chlorogenic acid; 4, eleutheroside D; 5, isofraxidin. The gradient elution was employed using solvent A (acetonitrile) and solvent B (0.1% aqueous phosphoric acid) at 30 °C; the gradient program used was as follows: initial 0—20 min, linear change from A–B (8 : 92, v/v) to A–B (20 : 80, v/v), from 20 to 35 min, linear change to A–B (43 : 57, v/v); the flow rate was set at 1 ml/min; the injection volume was 10 μl, and the detection wavelength was set at 343, 258, 263, 323, and 269 nm for isofraxidin, caffeic acid, eleutheroside B, chlorogenic acid, and eleutheroside D, respectively.

Table 1. The Contents of Five Marker Constituents in ASE Determined by 3D-HPLC

Compound	Isofraxidin	Caffeic acid	Eleutheroside B	Chlorogenic acid	Eleutheroside D
Contents[a] (mg/g)	0.50±0.01	0.06±0.01	0.43±0.03	5.55±0.65	0.73±0.07

a) Data expressed as mean±S.D. (n=6).

(acetonitrile) and solvent B (0.1% aqueous phosphoric acid, v/v) at 30 °C, as follows: initial 0—20 min, linear change from A–B (8 : 92, v/v) to A–B (20 : 80, v/v), from 20 to 35 min, linear change to A–B (43 : 57, v/v), the flow rate was 1 ml/min, the injection volume was 10 μl.

Isocratic elution was employed for determining the concentration of isofraxidin in rat plasma using solvent A (acetonitrile) and solvent B (0.1% aqueous phosphoric acid, v/v) (A : B=22 : 78) at 30 °C for 12 min, the flow rate was 1 ml/min, the detection wavelength was 343 nm, and the injection volume was 80 μl.

Drug Administration Thirty rats were divided into five groups including two groups treated with ASE in two doses, two groups treated with isofraxidin, and one control group. Dissolving about 1 g of dried powder of ASE and 0.5 mg of isofraxidin in 1 ml of distilled water, the oral administration dose was 15 g/kg and 30 g/kg for ASE, and the same amount of isofraxin included, the same volume of distilled water was administered to the control group according to the dosage.

Plasma Samples Preparation The blood samples were collected under heparin treatment from the caudal vein at 0.083, 0.17, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, 15, 18, 21, and 24 h after oral administration and immediately centrifuged at 7000 rpm for 15 min, and the plasma was separated. The isofraxidin in the plasma was purified and enriched by solid phase extraction method as follows. Waters Oasis HLB C_{18} cartridges (3 ml, 60 mg), previously conditioned using methanol (2 ml) and equilibrated with double deionized water (2 ml), were used. The previously prepared sample (about 0.5 ml of plasma sample acidified with 10 μl of phosphoric acid) was then loaded into the cartridge followed by elution with solvent. When a spiked sample had passed through the cartridge, slight pressure was applied to allow complete passage of the materials through the cartridge, keeping loading at the speed of 2 ml/min, the first wash was performed with 1 ml of 50% methanol then followed with 1 ml of methanol, the elution part of methanol was evaporated to dryness under nitrogen gas at 40 °C, the residue was reconstituted in 0.1 ml of mobile phase, then filtered through 0.45 μm filter membrane. An 80 μl of sample was injected into HPLC.

Calibration Curve Stock solution of standard isofraxidin (0.252 mg/ml) was prepared by dissolving in methanol. Six plasma standards containing isofraxidin 0.156, 0.78, 3.90, 7.81, 11.72, and 15.625 μg/ml were prepared by adding the stock solutions to control plasma, and treated as the above-mentioned solid phase extraction method, then diluting the elution parts with methanol. Plasma calibration curves in the range of 0.156—15.625 μg/ml were constructed by plotting the peak area against concentrations using the least-squares method.

Precision, Accuracy, and Limits of Detection The intra- and inter-day precision of isofraxidin was determined (n=5) at concentrations of 0.3, 1.0, 3.0, 5.0, and 10.0 μg/ml on the same day and on three sequential days, respectively. The accuracy (%Bias) was calculated from the theoretical concentrations (C_{the}) and the mean value of C_{obs} as follows: %Bias=[(C_{obs}−C_{the})/C_{the}]×100. The standard solutions were diluted with methanol to provide appropriate concentration, and the limits of detection for isofraxidin was determined when the ratio of the testing peak signal-to-noise was 3.

Pharmacokinetic Study The pharmacokinetic parameters, elimination rate constant (*Ke* or *k*10), total body clearance (*CL*), absorption half-life ($T_{1/2\alpha}$), and elimination half-life ($T_{1/2\beta}$), were calculated using one or two compartmental methods with 3P87 software (Chinese Society of Mathematical Pharmacology).[16] The area under the curve (*AUC*) was calculated using the trapezoidal method. The half-life ($T_{1/2}$, $T_{1/2\alpha}$ and $T_{1/2\beta}$) values were calculated using the equation: $T_{1/2}$=0.693/K, where K is obtained from the starting or terminal slope of the individual isofraxidin concentration *versus* time curves after logarithmic transformation of the isofraxidin concentration and application of linear regression. The clearance (*CL*) was calculated as: *CL*=dose/*AUC*, *etc.*

Results and Discussion

Validation of Methods The quality of ASE was controlled by simultaneous determination of five marker constituents to keep real composition ratio of the comstituents and to make ASE effective (Table 1). Because the maximal UV absorption wavelengths of five marker substances in ASE are significantly different, photodiode array detector (DAD) was introduced to meet the need of simultaneous detection of constituents with multiple wavelengths.

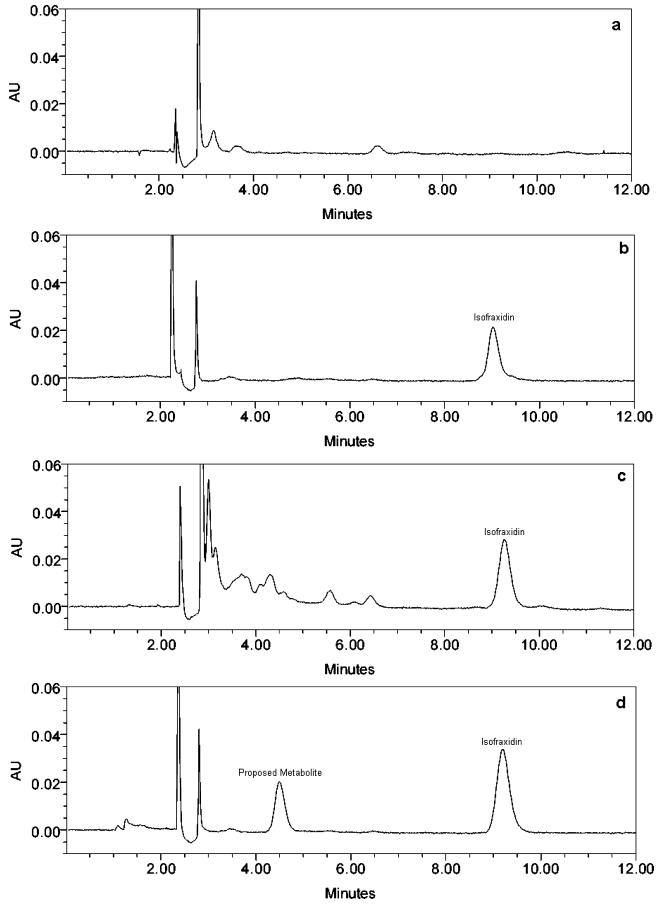


Fig. 3. Typical Chromatograms of Different Sample

(a) Control plasma; (b) standard isofraxidin; (c) plasma after oral administration of ASE; (d) plasma after oral administration of isofraxidin.

The chromatograms for control plasma, standard isofraxidin, and two typical chromatograms of isofraxidin in rat plasma are shown in Fig. 3. The retention time of isofraxidin was approximately 9.31 min. The unidentified peaks (that at retention time about 4.5 min in Figs. 3c, d may be the metabolite of isofraxidin, because it also appears in the plasma after oral administration of pure isofraxidin). These chromatographic conditions revealed no biological substances that would significantly interfere with the accurate determination of isofraxidin. The calibration curve was linear over the range of 0.156—15.625 μg/ml, and the limit of detection was 60 ng/ml. The regression equation obtained by least-squares method was $y=147728x-3929.3$ ($r=0.9996$), where y is the peak area and x is the isofraxidin concentration in plasma (μg/ml). The intra- and inter-day precision and accuracy were determined at five concentrations of plasma solution and the results are shown in Table 2. The relative recovery of the method was accurately calculated by comparing the determined amount and theoretical value of isofraxidin; the recovery was 87.30±1.73% (the average of R.S.D was 1.98%) [shown in Table 3], and not less than 80% meets the demand of the quantity experiment.

Pharmacokinetic Study The plasma concentration–time curves of pure isofraxidin ($n=6$) and ASE ($n=6$) are shown in Figs. 4 and 5. The parameters were calculated using 3P87 software for kinetic analysis. The pharmacokinetic models (one- *vs.* two-compartment) were compared according to Akaike's information criterion (AIC)[17] and the Schwarz criterion (SC),[18] and minimum AIC and SC values were regarded as the best representation of the plasma concentration–time course data. The pharmacokinetic parameters are shown in Tables 4—6. The time course of pure

Table 2. Intra- and Inter-day Precision and Accuracy of the Determination of Isofraxidin

Theoretical concentration (μg/ml)	Observed concentration[a] (μg/ml)	Precision R.S.D. (%)	Accuracy (% Bias)
Intra-day			
0.3	0.30±0.01	3.80	1.47
1.0	1.02±0.06	5.80	2.20
3.0	3.06±0.10	3.26	1.87
5.0	4.99±0.06	1.17	−0.08
10.0	9.99±0.12	1.18	−0.04
Inter-day			
0.3	0.30±0.01	4.91	0.93
1.0	1.02±0.06	6.00	2.20
3.0	3.06±0.10	3.20	2.07
5.0	5.01±0.10	2.00	0.28
10.0	10.03±0.14	1.40	0.38

a) Data expressed as mean±S.D. ($n=5$).

Table 3. Recovery of Isofraxidin in Solid Phase Extraction[a]

Isofraxidin concentration (μg/ml)	Recovery (%)	R.S.D. (%)
1.25	87.20±2.03	2.33
2.50	86.80±1.93	2.23
5.00	86.70±1.35	1.56
10.00	88.50±1.61	1.82
Average	87.30±1.73	1.98

a) Data expressed as mean±S.D. ($n=5$).

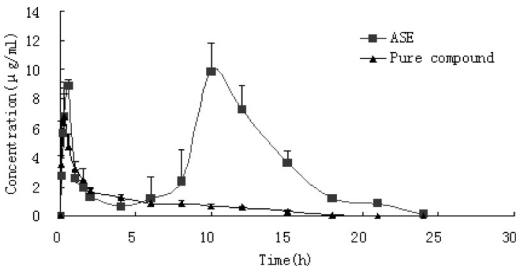


Fig. 4. The Concentration *versus* Time Curve of Isofraxidin in Rat Plasma after Oral Administration of Either ASE (15 g/kg) or Isofraxidin (7.5 mg/kg)

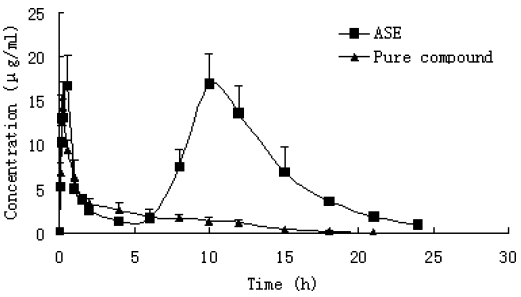


Fig. 5. The Concentration *versus* Time Curve of Isofraxidin in Rat Plasma after Oral Administration of Either ASE (30 g/kg) or Isofraxidin (15 mg/kg)

Table 4. Pharmacokinetic Parameters after Oral Administration of the Pure Isofraxidin

Parameters	Unit	Dose (7.5 mg/kg)	Dose (15 mg/kg)
A	μg/ml	6.39±1.28	12.11±2.07
α	1/h	1.36±0.19	3.61±0,31
B	μg/ml	1.62±0.43	4.59±0.38
β	1/h	0.09±0.01	0.33±0.01
Ka	1/h	22.69±3.22	22.19±4.27
$T_{1/2\alpha}$	h	0.51±0.085	0.57±0.11
$T_{1/2\beta}$	h	7.91±1.03	7.89±0.99
$v/F(C)$	(mg)/(μg/ml)	0.19±0.10	0.30±0.05
$K21$	1/h	0.36±0.06	0.35±0.03
$K10$	1/h	0.33±0.04	0.31±0.03
$K12$	1/h	0.76±0.15	0.76±0.21
AUC	(μg/ml)·h	22.85±4.26	45.14±7.33
$CL_{(s)}$	mg/h/(μg/ml)	0.07±0.01	0.02±0.01
$C_{(max)}$	μg/ml	6.56±0.10	13.80±2.19
$T_{(max)}$	h	0.19±0.03	0.23±0.03

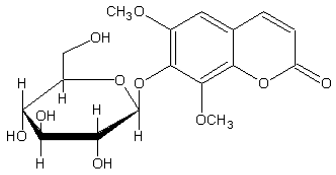
Table 5. Pharmacokinetic Parameters after Oral Administration of ASE (Time: 0—4 h)

Parameters	Unit	Dose (15 g/kg)	Dose (30 g/kg)
A	μg/ml	72.68±9.368	134.81±21.32
α	1/h	1.71±0.41	3.37±0.40
B	μg/ml	2.53±0.30	4.67±0.71
β	1/h	0.33±0.09	0.33±0.08
Ka	1/h	0.04±0.001	0.19±0.69
$T_{1/2\alpha}$	h	0.19±0.02	0.19±0.01
$T_{1/2\beta}$	h	2.08±0.318	2.09±0.33
$v/F(C)$	(mg)/(μg/ml)	0.06±0.01	0.19±0.02
$K21$	1/h	0.81±0.09	0.79±0.08
$K10$	1/h	1.52±0.20	1.51±0.23
$K12$	1/h	1.71±0.31	1.63±0.21
AUC	(μg/ml)·h	10.85±0.11	20.48±4.28
$CL_{(s)}$	mg/h/(μg/ml)	0.69±0.071	0.29±0.03
$C_{(max)}$	μg/ml	7.33±0.71	13.80±2.13
$T_{(max)}$	h	0.31±0.018	0.31±0.02

Table 6. Pharmacokinetic Parameters after Oral Administration of ASE (Time: 4—24 h)

Parameters	Unit	Dose (15 g/kg)	Dose (30 g/kg)
A	μg/ml	155.29±16.35	290.12±40.09
Ke	1/h	0.31±0.01	0.24±0.04
Ka	1/h	0.33±0.03	0.32±0.06
$T_{1/2(ka)}$	h	2.07±0.29	2.14±0.53
$T_{1/2(ke)}$	h	2.21±0.32	2.90±0.41
$T_{(max)}$	h	8.70±0.97	9.37±1.77
$C_{(max)}$	μg/ml	3.62±0.40	10.60±1.93
AUC	(μg/ml)·h	30.35±3.91	104.50±8.37
$CL/F(s)$	μg/ml/h/(μg/ml)	0.03±0.004	0.05±0.01
$V/F(c)$	(μg/ml)/(μg/ml)	0.10±0.02	0.24±0.01

isofraxidin fitted the two compartment models, the absorption was very rapid with the maximum concentration existing approximately 0.19 h which is not significantly different from the published data (T_{max} is about 0.236 h),[13] it was cleared slowly from rat body ($T_{1/2\beta}$=7.91±1.03 h). Contrasively, the plasma concentration of isofraxidin after oral administration of ASE showed two time courses; the first course is from 0 to



Eleutheroside B1

Fig. 6. Structure of Eleutheroside B_1

4 h, the data fitted two compartment models, the pharmacokinetic profile in this period was similar with that of pure compound in the initial 5 h, and the T_{max} and C_{max} values were not statistically different. The behavior of this period was probably the body fate of free isofraxidin as existing in the ASE. The second course of ASE, from 4 to 24 h, is proposed to be the behavior of the precursors of isofraxidin in ASE; the precursors such as eleutheroside B_1 (the structure was shown in Fig. 6)[19,20] was absorbed in the original form at the very beginning, then was metabolized into isofraxidin, therefore, after 4—5 h, the concentration of isofraxidin increased swiftly, and showed the second maximum concentration; the second time course fit the one compartment model. As a whole, the AUC of isofraxidin after oral administration of ASE is much larger than that of the same dosage of pure isofraxidin. The results also show that the elimination of isofraxidin after oral administration of ASE was much longer than that after isofraxidin. Isofraxidin could only be detected in 15 h when pure compound was administered, whereas it could be detected in 24 h when ASE was orally administered; the total CL value was sigificantly different, which demonstrated the priority of Chinese medicine.

Conclusion

By using HPLC coupled with solid phase extraction, the method for analyzing isofraxidin in plasma after oral administration of ASE was improved, therefore the difference of pharmacokinetic properties between isofraxidin and ASE was much more clearly understood. When the dosage of ASE is equal to pure compound calculating by the amount of isofraxidin, the behavior of isofraxidin in the body after oral administration of ASE is significantly different from that after oral administration of pure compound. There are two time courses of isofraxidin after oral administration of ASE. The behavior of the combination of free isofraxidin and its precursors existed in ASE may be the naturally controlling releaser of isofraxidin, since it can show a much larger bioavailability of isofraxidin. It can be concluded that ASE has priority to pure isofraxidin; the pharmacokinetic characteristics of ASE showed the properties of traditional Chinese medicine.

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