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Original Research Article

Simultaneous Determination of Flavonols and Terpene Lactones in Beagle Dog Plasma by Ultra-Performance Liquid Chromatography-Tandem - Mass Spectrometry: 2. Application to Pharmacokinetic Studies on Ginkgo Leaf Extract

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Abstract

Purpose: *To evaluate the pharmacokinetics of the major compounds in Ginkgo leaf dosage formulations (namely, Yikangning tablets, Ginaton tablets, Aoshi dropping pills and Yinxinke dispersible tablets), commonly used in traditional Chinese medicine.*
Methods: *A randomized 4*4 crossover study with eight beagle dogs was carried out. Plasma samples were collected following oral administration of four different preparations and the effective ingredients, namely, kaempferol, quercetin, isorhamnetin, ginkgolides A, ginkgolides B, ginkgolides C and bilobalide were detected by a validated ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-TMS). Then the pharmacokinetics of these target compounds, of different preparations were studied.*
Results: *The adjusted pharmacokinetic data showed that the area under the concentration-time curve from time-zero to the last measurable concentration (AUC_{0-t}) of kaempferol, quercetin, isorhamnetin, bilobalide, ginkgolides A, ginkgolides B, and ginkgolides C in plasma ranged from 124.37 ± 90.46 to 2261.87 ± 812.35, after administration of Yikangning; 142.28 ± 62.37 to 2529.46 ± 320.48 μg/L•h following administration of Ginaton; 158.52 ± 55.48 to 1987.40 ± 766.21 μg/L•h after Aoshi administration; 160.49 ± 104.66 to 2016.92 ± 1150.92 μg/L•h following Yinxinke administration. The results also indicate that the flavonoids (especially quercetin) in dispersible tablets and dropping pills hexhibited higher AUC than those in conventional tablets. There were no differences between Aoshi (dropping pills) and Yinxinke (dispersible tablets) in terms of the bioavailability of the flavonoids, but the dropping pill flavonoids showed lower t_{max}.*
Conclusion: *The results indicate that UPLC-TMS can used to simultaneously evaluate the plasma pharmacokinetics of Ginko compounds in beagle dogs*

Keywords: *Ginkgo biloba, Beagle dog plasma, Kaempferol, Quercetin, Isorhamnetin, Ginkgolides A, Ginkgolides B, Ginkgolides C, Bilobalide, Pharmacokinetics; Bioavailability*

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INTRODUCTION

Ginkgo probably originated thousands of years ago. *Ginkgo biloba* leaf extracts (GBE) contain 72 ginkgo constituents, including terpene lactones, flavonols, flavones, isoflavones, biflavones, flavanols, and carboxylic acids. The pharmacological activities of GBE are attributed to the synergistic action of terpene lactones and flavonoid glycosides. The products of GBE have become widely used as botanical medicines and dietary supplements, especially for the prevention and treatment of cardiovascular diseases [1] and cerebral insufficiency [2-6].

One of the challenges in clinical application of GBE preparations is the low bioavailability [7] (10 %), poor solubility, poor permeability [8] and the physical problem of delivering a drug across the blood–brain barrier. Hence, many new formulations emerged in the recent years, such as, phospholipid, complexes, tablets [9], solid dispersions [10], and dropping pills. Each formulation has both advantages and disadvantages. For conventional tablets, their technology is sample and auto-operation but they are really hard to take for people who have difficulty swallowing.

Solid dispersible tablets can disintegrate and disperse quickly in the water and in mouth. They have the features of fast acting and high pharmacological effect. However, the solid dispersible tablets must add amount of disintegrants and use micronized raw medicinal material. Dropping pills are prepared by the solid dispersion technology. The process has the advantages of simple equipment, easy control, high efficiency and low cost. However, dropping pills have poor drug loading.

Are new preparations better than conventional tablets in clinical practice? So far, many studies were carried out on preparation methods and *in vitro* dissolution of new preparations, but few *in vivo* studies have been done. According to the Food and Drug Administration of the United States, botanical drug products should be investigated with regard to blood levels of known representative markers, active constituents, and/or major chemical constituents. However, so far, the pharmacokinetic studies of GBE have been carried on mostly in rats, and were deficient in comparisons of different preparations [11-14].

The aim of this study was to evaluate the the pharmacokinetic profiles of four different GBE formulations - Yikangning tablets, Ginaton tablets, Aoshi dropping pills, and Yinxinke dispersible tablets - after a single oral administration in beagle dogs, using a previously developed ultrahigh-performance liquid chromatography coupled with triple quadrupole mass (UPLC–MS/MS) to quantify kaempferol (KMF), quercetin (QCT), isorhamnetin (ISR), ginkgolides A (GA), ginkgolides B (GB), ginkgolides C (GC), and bilobalide (BB) simultaneously.

EXPERIMENTAL

Chemicals and Ginkgo leaf preparations

The standards of kaempferol (KMF), quercetin (QCT), isorhamnetin (ISR), ginkgolides A (GA), ginkgolides B (GB), ginkgolides C (GC) and bilobalide (BB) and domperidone (DPD) (IS) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of QCT and GC was 96.5 and 97.1 %, respectively. The purity of the remaining standards was more than 99 %. LC/MS grades of methanol and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ, USA), HPLC grade of formic acid was supplied by ROE Scientific (Newcastle, USA), and ultrapure water was generated from the Synergy UV water purification system (Millipore Corp, USA).

Yikangning tablets of *G. biloba* leaves extract were made by Yangtze River Pharmaceutical Group Co. Ltd Jiangsu, China. Each tablet contained 80 mg of the extracts. (Batch: 12062621). Ginaton tablets of *G. biloba* leaves extract were made by Willmar Schwabe GmbH & Co. KG (Germany). Each tablet contained 40 mg of the extract. (Batch no. 1941211). Aoshi dropping pills of *G. biloba* leaves extract were made by Zhejiang Jiuxu Pharmaceutical Co., Ltd Zhejiang, China. Each pill contained 10 mg of the extract (Batch no. 20120709). Yinxinke dispersible tablets of *G. biloba* leaves extract were made by Jiangsu Shenlong Pharmaceutical Co., Ltd. (Jiangsu, China). Each tablet contained 40 mg ginkgo extractum (Batch no. 120702).

Instrumentation and chromatographic conditions

The UPLC–MS/MS system used was composed of an Acquity UPLC system and a TQS triple quadrupole tandem mass spectrometer. Chromatographic separation was performed on an ACQUITY UPLC BEH C18 column. The column temperature was maintained at 40 °C and the auto-sampler was conditioned at 4 °C. The mobile phase was composed of 0.1 % formic

acid aqueous solution (A) and acetonitrile (B) at a flow rate of 0.4 mL/min in only 4.0 minutes. Gradient condition of the mobile phase was as follows: 5 % B at 0–1.0 min; 5 → 40 % B at 1.0–1.5 min; 40 % → 43 % B at 1.5–3.0 min, then the system was equilibrated using the initial condition (acetonitrile–water, 5:95, v/v) for 1.0 min.

Assay of the preparations

The four ginkgo leaf preparations (GLP) were analyzed by UPLC–MS/MS. The method can be applied to simultaneous determination of KMF, QCT, ISR, BB, GA, GB and GC. Table 1 shows the levels of KMF, QCT, ISR, BB, GA, GB and GC in the four GLP. Acid hydrolysis was applied to convert flavonoid glycosides into their aglycone forms before measuring the contents of KMF, QCT, and ISR in these preparations.

Pharmacokinetic studies

This study complied with the Guiding Principles for the Care and Use of Laboratory Animal and was approved by the Institutional Animal Care and Use Committee of the Beijing University of Chinese Medicine (SPF animal, certificate no. SCXK (Jing) 2013-0007).

Eight male beagle dogs weighing 11.1 ± 0.3 kg were used. The dogs were housed in a stainless steel cage, with an ambient temperature of 21 – 22 °C and unlimited access to standard laboratory dog diet and water. Except for *ad libitum* water, the animals were fasted for 12 h prior to drug administration.

Then a randomized 4*4 crossover trial with eight beagle dogs was carried out to study the pharmacokinetic characteristics. In the design, the equal numbers of subjects are initially assigned to each sequence. Each subject was randomly assigned to any sequence. The advantage of the randomized 4*4 crossover study is to reduce the number of animals and improve the data reliability by eliminating the error from the experimental period and animals individuals. The design is presented in Table 2.

Eight male beagle dogs were divided randomly into four groups, and each group was treated with a single dose of Yikangning, Ginaton, Aoshi and Yinxinke (take Ginaton as the reference preparation). Each dog received an oral dose equivalent to 640 mg extract under fasting conditions, that is, 8 tablets of Yikangning, 16 tablets of Ginaton, 64 pills of Aoshi, and 16 tablets of Yinxinke. Drugs were put into the epiglottis of beagle dogs, and then the dogs were made to automatically swallow and drink 50 mL water. There was a washout period of 2 weeks between the two adjacent periods, and the order of the drug administration was randomized.

Blood samples were collected for up to 48 h (at 0, 10, 20, 30 and 45 min, then 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24, 36, and 48 h after administration). The blood samples were centrifuged at 5000 rpm for 10 min; then the supernatant plasma were collected into tightly sealed plastic tubes (containing heparin sodium anticoagulant solution), and were finally frozen at - 20 °C until used for analysis.

All the plasma samples were extracted by using liquid–liquid extraction technique as was described. Hydrochloric acid was added into the samples to convert flavonoid glycosides into their aglycone forms. Accordingly, the measured flavonoid levels were expressed as the concentrations of KMF, QCT and ISR. The samples were prepared as following steps: The plasma sample was hydrolyzed for 30 min in a

Table 1: Levels of ginkgo compounds in the four *GLP*

Compound	Yikangning mg/tablet	Ginaton mg/tablet	Aoshi mg/pill	Yinxinke mg/tablet
KMF	3.95	1.36	0.51	3.49
QCT	3.39	1.36	0.45	2.79
ISR	1.59	0.38	0.24	1.25
BB	3.25	1.30	0.47	2.81
GA	1.45	0.34	0.22	1.05
GB	1.03	0.30	0.19	0.70
GC	1.01	0.33	0.08	0.59

Table 2: A four-period crossover design for drug administration

Period	Sequence A	Sequence B	Sequence C	Sequence D
1	Yinxinke	Reference	Yikangning	Aoshi
2	Reference	Yinxinke	Aoshi	Yikangning
3	Yikangning	Aoshi	Reference	Yinxinke
4	Aoshi	Yikangning	Yinxinke	Reference

water bath at 80 °C and extracted with acetidin twice, then analyzed by UPLC-MS/MS.

Data analysis

The pharmacokinetic parameters of different preparations are comparable only if the dogs are treated with the same dosage, but the drug contents (7 effective ingredients) of these preparations were not the same, so a dosage conversion factor was calculated as in Eq 1.

$i = D_r/D_t = (n_r m_r)/(n_t m_t)$…… (1)

where i is the conversion factor, D_r (mg) is the administration dosage of reference preparation, D_t (mg) is the administration dosage of test preparation, n_r (tablet or pill) is the number of reference preparation, m_r (mg/ tablet or mg/ pill) is the content of reference preparation, n_t (tablet or pill) is the number of test preparation, and m_t (mg/ tablet or mg/ pill) is the content of test preparation.

Second, the conversion factor was multiplied by the measured plasma concentration of QCT, KMF, ISR, BB, GA, GB, and GC to calculate the corresponding plasma concentrations when the dogs are treated with the same dose.

The pharmacokinetic parameters, AUC_{0-t} and $T_{1/2}$, were calculated with the software program DAS 3.20 (non-compartmental model). Cmax and Tmax were the actual values. Data were analyzed using SPSS 17.0. AUC_{0-t} and C_{max} were compared via analysis of variance (ANOVA) and multiple comparisons (least significance difference, LSD). T_{max} was measured using nonparametric statistical tests (Wilcoxon).

The relative bioavailability (F_r) was calculated as in Eq 2, using Ginaton as the reference preparation.

$F_r = AUC_t/AUC_r$…… (2)

where AUC_r (μg/L•h) is the area under the concentration–time curve of the reference preparation and AUC_t (μg/L•h) is the area under the concentration–time curve of the test preparation.

RESULTS

The calculation formulas for the total flavone glycol glycosides and the total lactones according to Chinese Pharmacopoeia (2010 version) and USP 35 – NF 30 are as follows: (1) total flavone glycol glycosides (total flavonoids) = 2.51（KMF + QCT + ISR）and (2) total lactones = BB + GA + GB + GC. Figs 1 - 9 show the mean plasma concentration–time curves of the seven sample total flavonoids, and total terpene lactones in beagle dog plasma after administration of four different GLP. Plasma concentration–time data derived from the experiments were analyzed by DAS 3.20 (non-compartmental model), and the main pharmacokinetic parameters were summarized in Table 3–4.

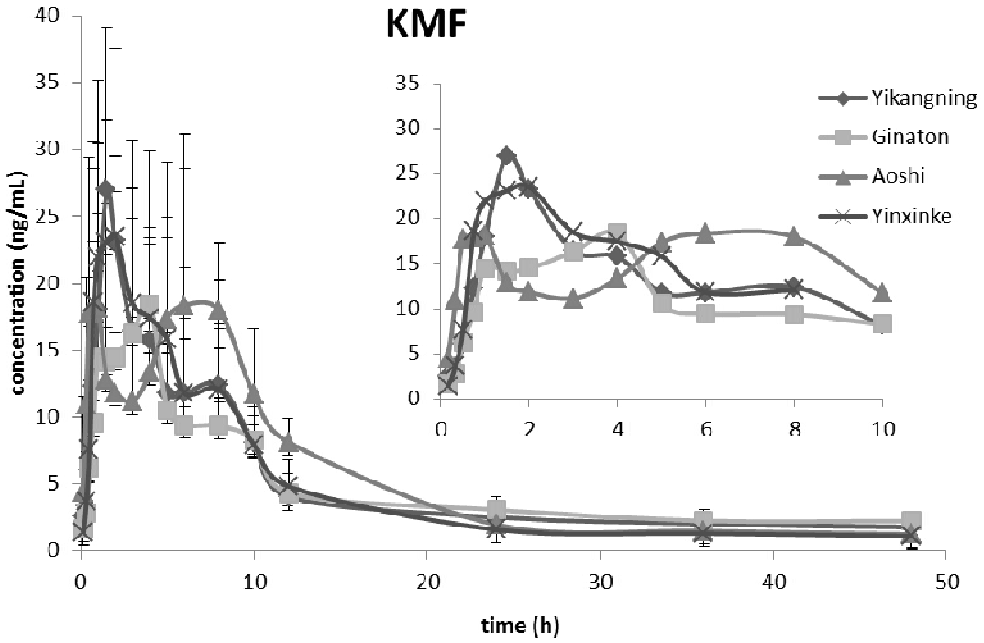

Fig 1: Concentration-time curve of kaempferol (KMF) for different preparations

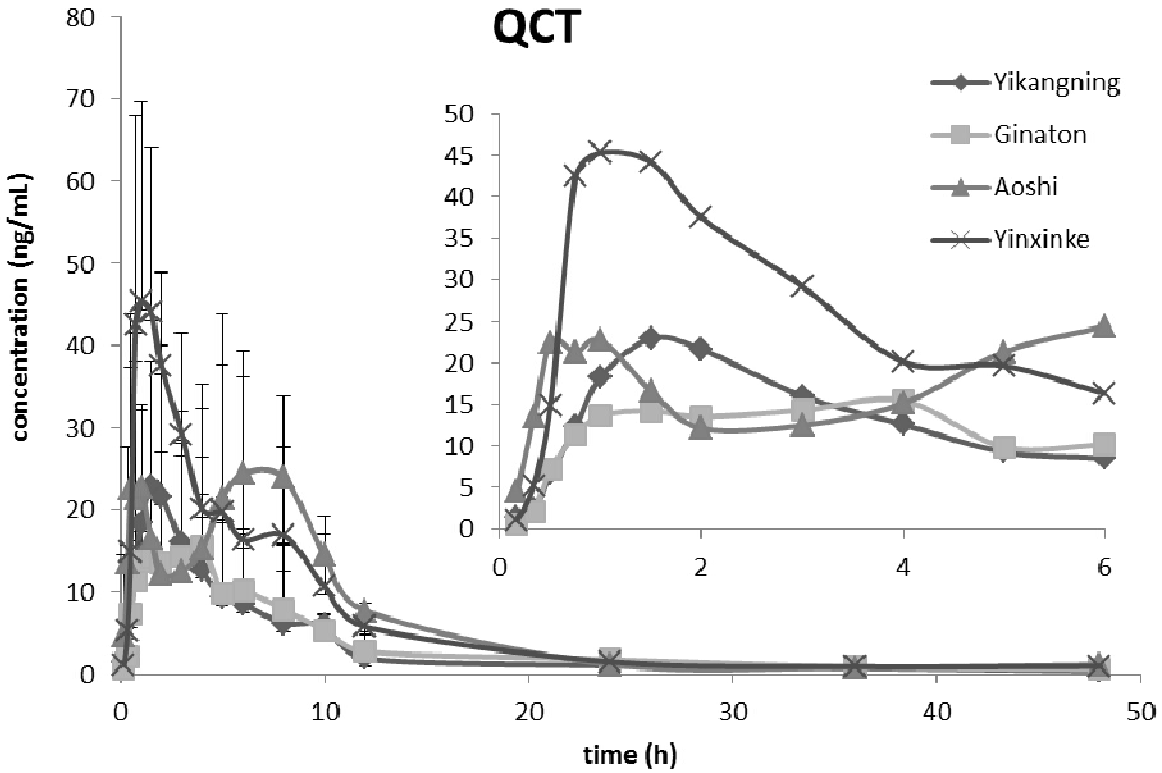

Fig 2: Concentration-time curve of quercetin (QCT) for different preparations

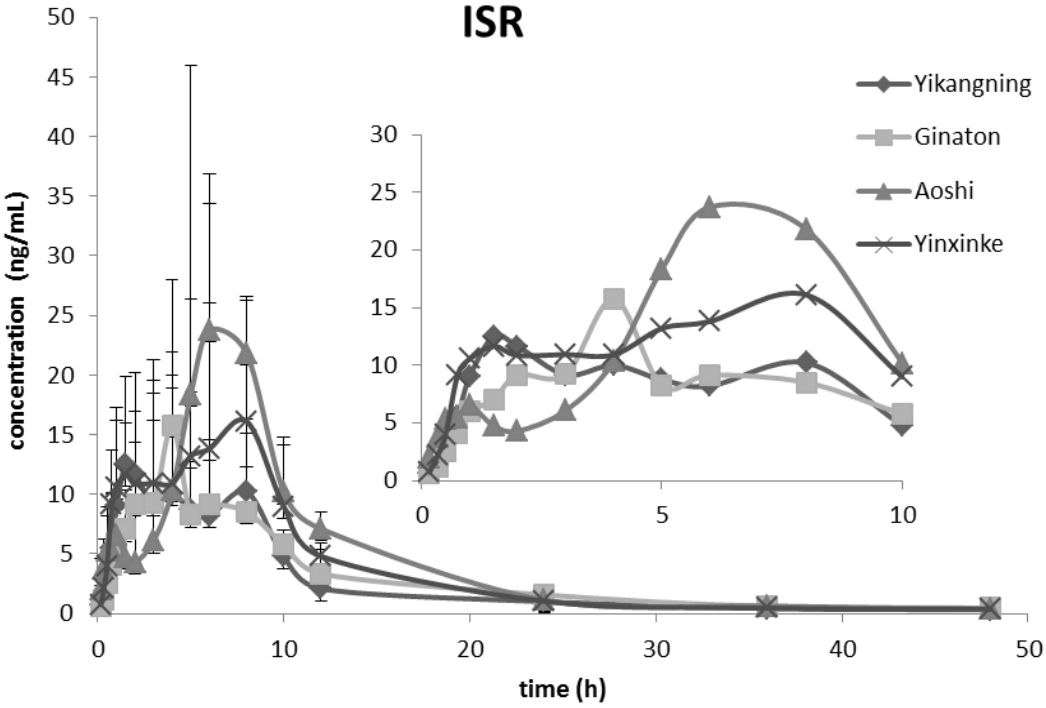

Fig 3: Concentration-time curve of isorhamnetin (ISR) for different preparations

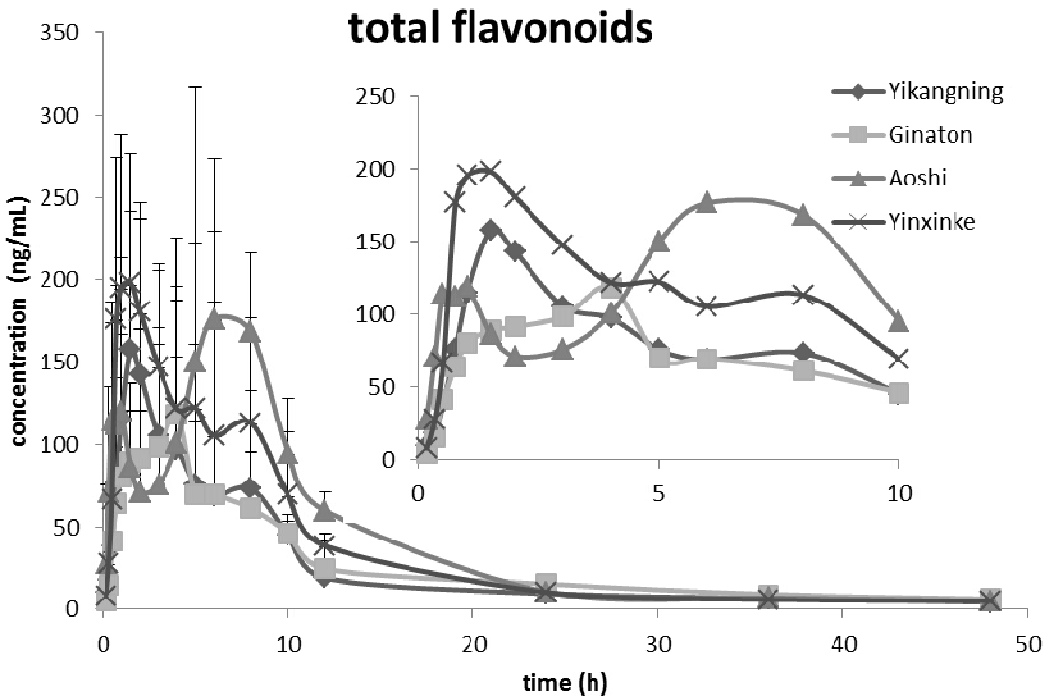

Fig 4: Concentration-time curve of total flavoniods for different preparations

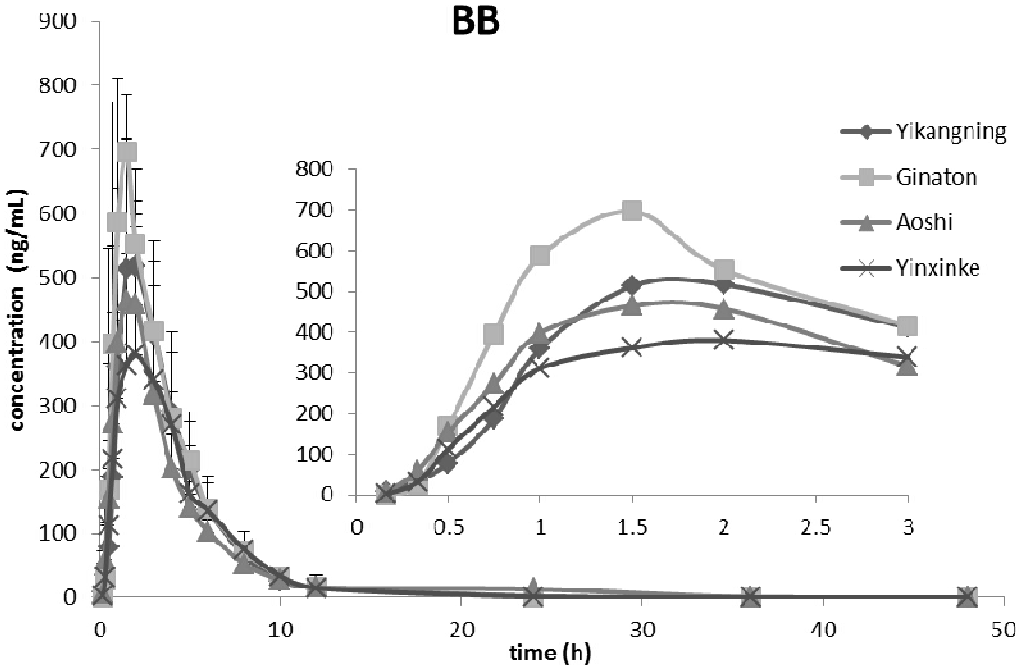

Fig 5: Concentration-time curve of bilobalide (BB) for different preparations

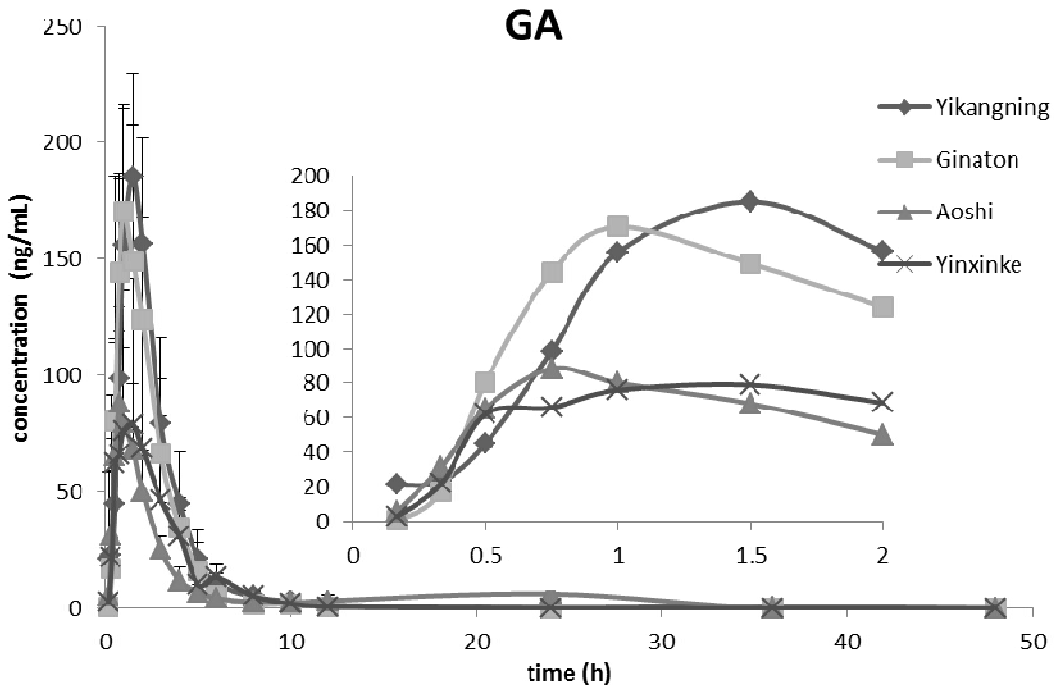

Fig 6: Concentration-time curve of ginkgolides A (GA) for different preparations

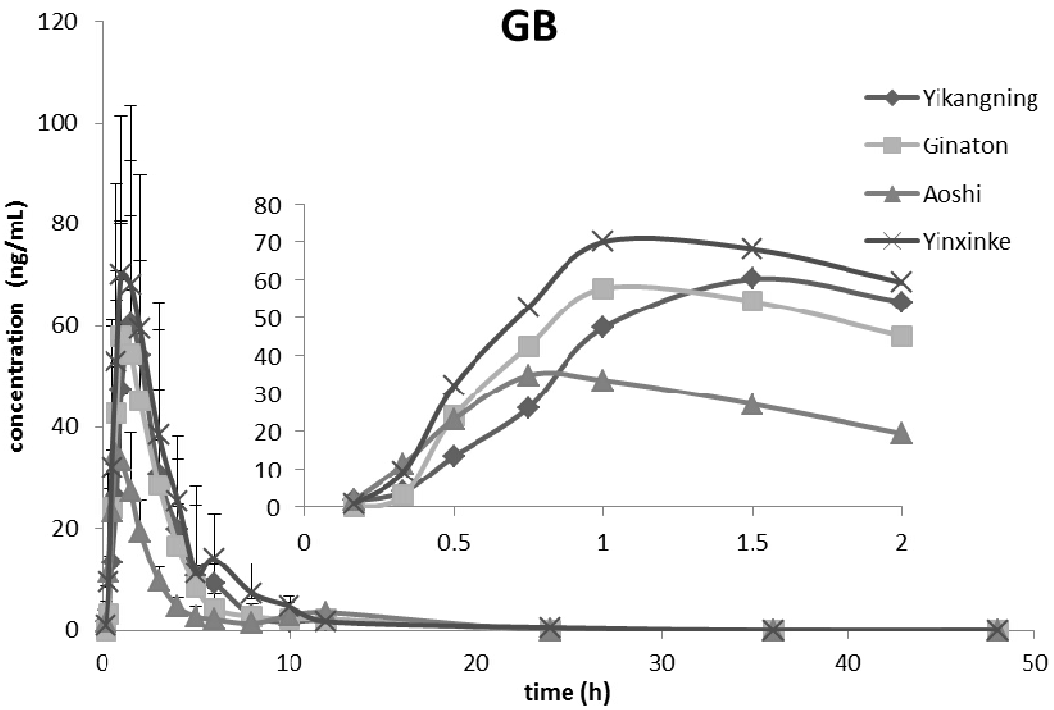

Fig 7: Concentration-time curve of ginkgolides B (GB) for different preparations

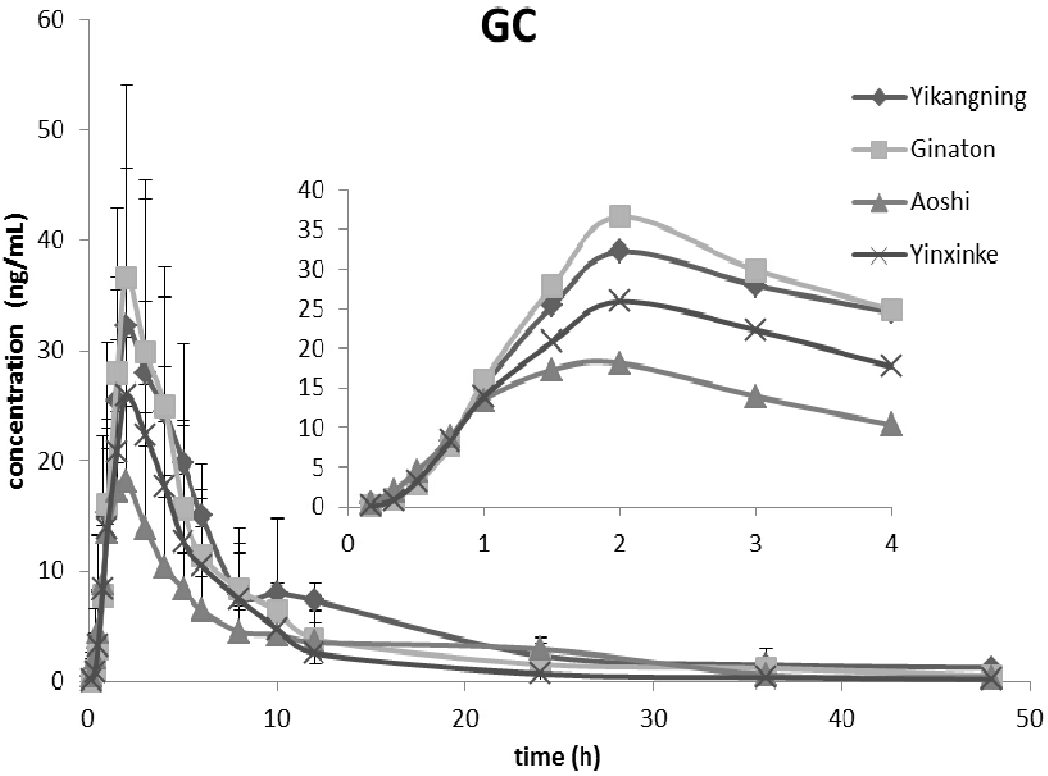

Fig 8: Concentration-time curve of ginkgolides C (GC) for different preparations

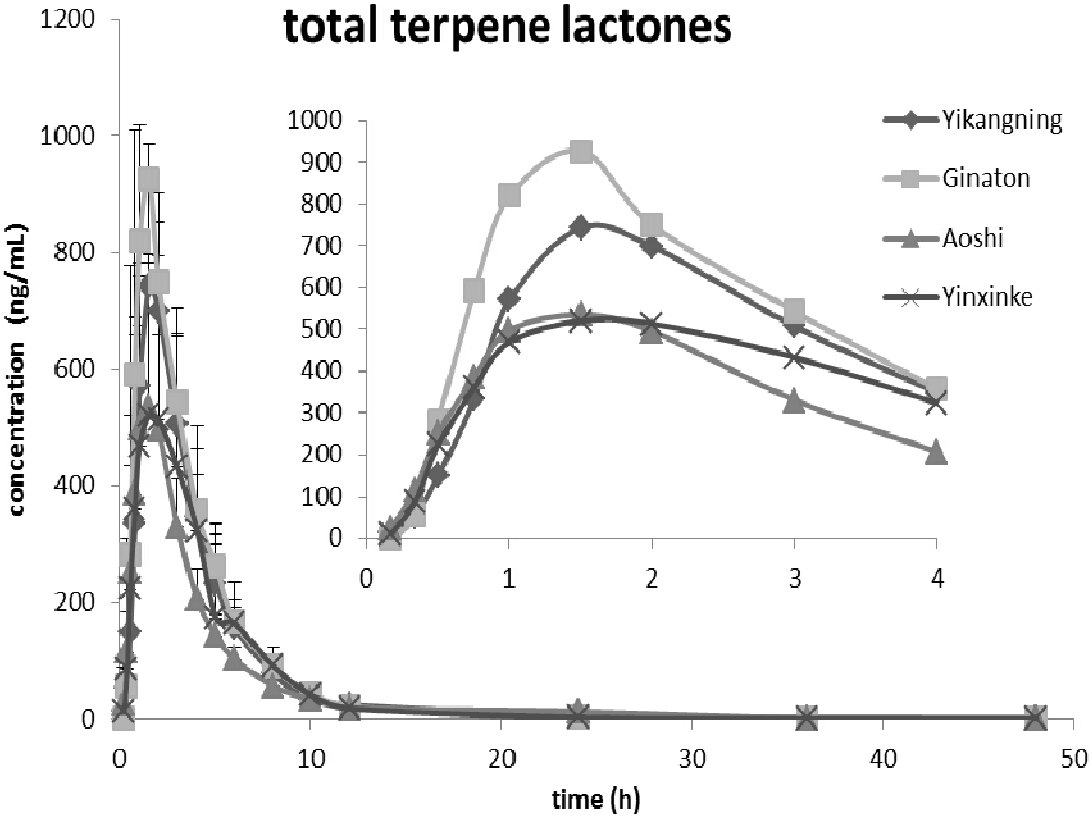

Fig 9: Concentration-time curve of total terpene lactones for different preparations

Table 3: Major pharmacokinetic parameters (non-compartmental model) and relative bioavailability of flavonoids (KMF, QCT, ISR, and total flavonoids) after oral administration in the four GLP

Flavonoid compound	Preparation	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (μg/L)	AUC_{0-t} (μg/L·h)	F_r (%)
KMF	Yikangning	21.76±7.94	1.63±0.64	26.97±17.15	215.38±126.37	97.15
	Ginaton	17.02±7.91	1.63±1.12	14.42±4.96	221.70±80.48	100.00
	Aoshi	11.68±4.57	0.72±0.21	18.15±12.68	267.84±88.99	120.81
	Yinxinke	13.28±7.74	1.34±0.48	23.52±9.10	221.25±95.03	99.80
QCT	Yikangning	14.60±7.99	1.53±0.71	22.97±14.50	151.60±124.33	93.13
	Ginaton	9.82 ±3.61	1.83±1.43	14.22±6.73	162.78±111.66	100.00
	Aoshi	5.24 ±2.31	0.75±0.35	22.49±14.17	275.85±158.87	169.46
	Yinxinke	12.07±6.75	1.34±0.40	45.31±25.47	305.46±178.81	187.65
ISR	Yikangning	10.60±3.91	2.06±1.35	12.54±8.31	124.37±90.46	87.41
	Ginaton	10.97±5.07	2.04±1.25	15.75±12.06	142.28±62.37	100.00
	Aoshi	6.13 ±3.33	0.75±0.19	6.53±4.66	218.57±119.21	153.62
	Yinxinke	8.67 ±3.24	2.22±1.76	11.66±5.64	182.00±160.10	127.92
Total flavonoids	Yikangning	13.20±5.69	1.50±0.76	157.54±98.62	1235.01±864.17	96.53
	Ginaton	10.29±2.90	1.17±1.12	118.31±72.48	1279.36±644.11	100.00
	Aoshi	8.58 ±2.96	0.69±0.22	114.18±72.48	1987.13±937.30	155.32
	Yinxinke	16.73±9.83	1.34±0.40	197.87±92.27	1785.00±1063.65	139.52

Table 4: The main pharmacokinetic parameters (no compartmental) and the relative bioavailability of lactones (BB, GA, GB, GC, and total terpene lactones) after oral administration in the four GLP

Lactone compound	Preparation	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (μg/L)	AUC_{0-t} (μg/L·h)	F_r (%)
BB	Yikangning	3.43 ±1.54	1.88 ±0.52	517.15±202.96	2261.87±812.35	89.42
	Ginaton	2.02 ±0.51	1.67 ±0.75	697.56±224.22	2529.46±320.48	100.00
	Aoshi	2.35 ±0.76	1.44 ±0.42	464.99±241.46	1987.40±766.21	78.57
	Yinxinke	3.54 ±2.04	1.75 ±0.60	380.23±176.66	2016.92±1150.92	79.74
GA	Yikangning	1.60± 1.07	1.50±0.66	185.19±60.37	486.53±120.21	116.08
	Ginaton	1.07±0.45	1.08±0.47	170.44±40.07	419.12±96.72	100.00
	Aoshi	2.19±1.75	1.00±0.33	88.52±48.80	198.65±80.78	47.40
	Yinxinke	1.68±1.26	1.03±0.51	78.96±61.15	271.68±248.38	64.82
GB	Yikangning	1.51± 1.03	1.41 ±0.71	60.31±33.31	186.37±154.12	111.33
	Ginaton	2.14 ±1.24	1.29 ±0.46	57.80±28.23	167.40±96.39	100.00
	Aoshi	2.41 ±1.21	1.06 ±0.29	34.85±19.27	76.61±33.73	45.76
	Yinxinke	2.21 ±1.47	1.22 ±0.41	69.98±35.10	245.23±160.49	146.49
GC	Yikangning	7.97±4.04	2.13±0.35	32.21±11.19	291.73±184.40	137.56
	Ginaton	5.50±2.82	2.17±0.68	36.65±15.03	212.07±124.85	100.00
	Aoshi	5.55±2.19	1.81±0.65	18.14±8.33	158.52±55.48	74.75
	Yinxinke	5.08±2.82	2.06±0.42	25.94±10.15	160.49±104.66	75.68
Total terpene lactones	Yikangning	7.51 ±4.66	1.63 ±0.58	743.4±212.79	3007.64±901.24	88.36
	Ginaton	3.47 ±2.47	1.42 ±0.38	926.71±198.67	3403.77±295.20	100.00
	Aoshi	3.45 ±1.28	1.34 ±0.48	535.87±260.73	2293.22±767.82	67.37
	Yinxinke	3.82 ±1.95	1.50 ±0.76	520.46±205.27	2572.24±1453.58	75.57

To analyze AUC_{0-t}, C_{max}, and T_{max} of flavonoids in the four different GLP with the LSD pairwise comparison methods, which reveal that, for KMF, there was no significant difference for AUC_{0-t} of the four different GLP. For QCT, AUC_{0-t} and C_{max} of Yinxinke had a significant difference compared with Yikangning or Ginaton ($p < 0.05$); for ISR and total flavonoids, there were no significant differences for AUC_{0-t} and C_{max} of the four different GLP. The conclusion suggests that dispersible tablets can increase QCT absorption and bioavailability of QCT *in vivo* compared with traditional tablets. Besides, the T*max* of dropping pill Aoshi was found to be significantly lower among the four preparations.

To analyze AUC_{0-t}, C_{max}, and T_{max} of lactones in the four different GLP with the LSD pairwise comparison methods, which indicate that, for BB, there were no significant differences for AUC_{0-t} of the four different GLP, but Ginaton had a higher C_{max} compared with solid dispersions (Aoshi) and dispersible tablet (Yinxinke, $p < 0.05$). For GA, there were higher AUC_{0-t} and C_{max} when Yikangning and Ginaton were compared with Aoshi and Yinxinke ($p < 0.05$). For GB, Aoshi had lower AUC_{0-t} and C_{max}, when compared with Yikangning and Yinxingke ($p < 0.05$), the C_{max} of Aoshi was also lower than them. For GC, there were higher AUC_{0-t} and C_{max} when Yikangning

was compared with Aoshi ($p < 0.05$); the C_{max} of Ginaton was also higher than Aoshi and Yinxingke ($p < 0.05$). For total lactones, Ginaton had higher AUC_{0-t} and C_{max}, when compared with Aoshi ($p < 0.05$).

DISCUSSION

In this study, a four-period crossover trial was used in pharmacokinetic studies. The crossover design eliminates variability caused by subject (dog) differences in drug absorption, drug clearance, and the volume of drug distribution.

The plasma concentration–time curves of QCT, KMF, and ISR and total flavonoids in beagle dog plasma after administration of the four different GLP show double peaks. The findings are consistent with previously reports. Some researchers [15,16] considered that it might be caused by some factors, such as enterohepatic circulation [7,17], distribution of the drug *in vivo*, and so on. To clarity the reason of double peaks of QCT, KMF, and ISR, a study on the absorption mechanism was performed [15]. The results demonstrated that the first peaks were produced by absorption–conjugation of the GBE-containing aglycones in the small intestine, and the second peaks were produced by the colonic deglycosylation–absorption–conjugation of the unabsorbed flavonoid glycosides.

In this study, the measured plasma concentrations of QCT, KMF, ISR, BB, GA, GB, and GC were multiplied by a conversion factor to calculate the corresponding plasma concentrations of each compound under the same dose. The results suggest that dispersible tablets and dropping pills can increase absorption and bioavailability of flavonoids (especially for QCT) *in vivo* compared with conventional tablet, and this has adverse consequences for lactones. Studies show that the lactones are unstable under acidic conditions, and therefore, preparations with good disintegrating property may cause greater degradation of the drugs [18].

CONCLUSION

Among these preparations, Yinxinke, a dispersible tablet improved the AUC_{0-t} and C_{max} of quercetin, while Aoshi, the dropping pill shortened the T_{max} of all the flavonoids. Good disintegrating property is helpful to the absorption of flavonoids, but may be disadvantageous for lactones compared with conventional preparations.

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