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RESEARCH PAPER

Systemic and cerebral exposure to and pharmacokinetics of flavonols and terpene lactones after dosing standardized *Ginkgo biloba* leaf extracts to rats via different routes of administration

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BACKGROUND AND PURPOSE

Flavonols and terpene lactones are putatively responsible for the properties of *Ginkgo biloba* leaf extracts that relate to prevention and treatment of cardiovascular disease and cerebral insufficiency. Here, we characterized rat systemic and cerebral exposure to these ginkgo compounds after dosing, as well as the compounds' pharmacokinetics.

EXPERIMENTAL APPROACH

Rats received single or multiple doses of ShuXueNing injection (prepared from GBE50 for intravenous administration) or GBE50 (a standardized extract of *G. biloba* leaves for oral administration). Brain delivery of the ginkgo compounds was assessed with microdialysis. Various rat samples were analysed using liquid chromatography/mass spectrometry.

KEY RESULTS

Slow terminal elimination features of the flavonols counterbalanced the influence of poor oral bioavailability on their systemic exposure levels, which also resulted in significant accumulation of the compounds in plasma during the subchronic treatment with ShuXueNing injection and GBE50. Unlike the flavonols, the terpene lactones had poor enterohepatic circulation due to their rapid renal excretion and unknown metabolism. The flavonol glycosides occurred as major forms in plasma after dosing with ShuXueNing injection, while the flavonol aglycone conjugates were predominant in plasma after dosing with GBE50. Cerebral exposure was negligible for the flavonols and low for the terpene lactones.

CONCLUSION AND IMPLICATIONS

Unlike the significant systemic exposure levels, the levels of cerebral exposure to the flavonols and terpene lactones are low. The elimination kinetic differences between the two classes of ginkgo compounds influence their relative systemic exposure levels. The information gained is relevant to linking ginkgo administration to the medicinal effects.

Abbreviations

AUC, area under concentration-time curve; B/P ratio, blood to plasma ratio; bECF, brain extracellular fluid; CL_R, renal clearance; $CL_{tot,p}$, total plasma clearance; *F*, bioavailability; f_u, unbound fraction in plasma; i.p., intraperitoneally; i.v., intravenously; K_p, AUC ratio of unbound drug in bECF to that in plasma; MRT, mean residence time; p.o., peroral; PK, pharmacokinetic; V_{SS}, apparent volume of distribution at steady state

Introduction

Products of *Ginkgo biloba* leaf extracts (ginkgo extracts) have become widely used botanical medicines and dietary supplements, especially for the prevention and treatment of cardiovascular disease and cerebral insufficiency (Kleijnen and Knipschild, 1992; Zhou *et al.*, 2004). Impaired blood flow is a common feature of many cardiovascular and cerebrovascular diseases. EGb761, a standardized ginkgo extract, stimulates up-regulation and activation of endothelial nitric oxide synthase in human umbilical vein endothelial cells, which leads to enhancement of nitric oxide generation for vasodilatation (Koltermann *et al.*, 2007). Two-hour intravenous (i.v.) infusion of Ginaton solution, a standardized injectable ginkgo extract, improves coronary blood flow in patients with coronary artery disease (Wu *et al.*, 2007), and oral (p.o.) ingestion of ginkgo extract for 4 weeks increases cerebral blood flow in healthy volunteers (around 60 years of age) (Mashayekh *et al.*, 2011). In addition, EGb761 attenuates oxidized low density lipoprotein-induced functional damage in human umbilical vein endothelial cells (Ou *et al.*, 2009), inhibits endothelial inflammation (Chen *et al.*, 2003; 2011) and reduces lipid accumulation in foam cells (Tsai *et al.*, 2010). The ginkgo extract also decreases blood viscosity and viscoelasticity, reduces erythrocyte malondialdehyde levels and promotes erythrocyte deformability (Huang *et al.*, 2004). The overall therapeutic relevance of these pharmacological properties most likely depends on bioavailability of the bioactive ginkgo ingredients in the systemic circulation. For this reason, it is imperative to investigate the systemic exposure to and plasma pharmacokinetics of ginkgo compounds after administration of the botanical extract.

The neuroprotective effects of ginkgo extracts have been documented in several studies. EGb761 exerts *in vitro* antioxidative effect, inhibition of amyloid-β aggregation and attenuation of mitochondrion-induced apoptosis (Luo *et al.*, 2002; Longpré *et al.*, 2006). Administration of the ginkgo extract via p.o. normalizes reduction of neurogenesis in mouse hippocampus (Tchantchou *et al.*, 2007). The neuroprotective action is dependent on heme oxygenase 1 in ischaemic reperfusion brain injury (Saleem *et al.*, 2008). However, it remains open to question whether the ginkgo constituents or their metabolites reach the brain in concentrations high enough for the neuroprotective effects.

The flavonols and terpene lactones are believed to be responsible for most of the pharmacological effects of ginkgo extract (DeFeudis and Drieu, 2000; Drieu and DeFeudis, 2000; Tchantchou *et al.*, 2009; Yoshitake *et al.*, 2010). Recently, we investigated the intestinal absorption and presystemic elimination of a variety of chemical constituents present in oral GBE50, another standardized extract of *G. biloba* leaves (Li *et al.*, 2012). As a result, many unchanged flavonol glycosides, unchanged terpene lactones and the flavonol aglycone conjugates were detected in the bloodstreams of dosed rats, while the other classes of ginkgo compounds (including biflavones, flavones, isoflavones, flavanols and carboxylic acids) occurred at negligible plasma levels. Full pharmacokinetic (PK) information about systemic and cerebral exposure to ginkgo flavonols and terpene lactones is critically important in establishing a link between administration of the extract and its pharmacological properties. Earlier human and animal PK studies focused on measurement of oral bioavailability (*F*) of the ginkgo terpene lactones (Bhattaram *et al.*, 2002; Biber, 2003; Rossi *et al.*, 2009), while PK assessments of ginkgo flavonols have been relatively scarce (Chen *et al.*, 2010). These PK studies have not fully characterized the pharmacokinetics of the ginkgo compounds. More recently, brain penetration of ginkgo flavonols and terpene lactones has been studied in rats by measurement of the concentrations of these substances in brain tissue homogenate (Rossi *et al.*, 2009; Rangel-Ordóñez *et al.*, 2010) and by microdialysis (Lang *et al.*, 2010). However, the data have been inconclusive. The objective of the current study was to characterize and compare systemic and cerebral exposure to flavonols and terpene lactones and to investigate influence of the route of administration on the pharmacokinetics of these ginkgo compounds. The levels of systemic exposure to flavonols and terpene lactones were found to be significant in rats, but their cerebral exposure levels were not. The two classes of ginkgo compounds had different elimination kinetics.

Methods

A detailed description of experimental procedures and materials is provided in the Supporting Information Appendix S1 Supplemental Methods section, which is available online.

GBE50 and ShuXueNing injection

GBE50, a standardized proprietary extract of *G. biloba* leaves, was a 200:3 solid extract manufactured by Shanghai XingLing Sci. & Tech. Pharmaceuticals (Shanghai, China). ShuXueNing injection was an injectable solution from GBE50 manufactured by Shanghai Asia Pioneer Pharmaceuticals (Shanghai,

Table 1

Content levels of ginkgo compounds in ShuXueNing injection or GBE50

Compound	ShuXueNing injection (μmol·mL^{-1})	GBE50 (μmol·10 mg^{-1})
Bilobalide (**1**)	0.0002	0.954
Ginkgolide A (**2**)	0.289	0.551
Ginkgolide B (**3**)	0.100	0.325
Ginkgolide C (**4**)	0.184	0.316
Ginkgolide J (**5**)	0.091	0.105
Quercetin 3-*O*-dirhamnosyl-glucoside (**10**)	0.072	0.270
Quercetin 3-*O*-(*p*-coumaroyl)-glucosyl-*O*-rhamnoside (**11**)	0.096	0.617
Quercetin 3-*O*-rhamnosyl-*O*-glucoside (**12**)	0.174	0.660
Quercetin 3-*O*-glucosyl-*O*-rhamnoside (**13**)	0.065	0.354
Quercetin (**16**)	0.003	0.088
t-Quercetin	**0.397**	**2.149**
Kaempferol 3-*O*-dirhamnosyl-glucoside (**19**)	0.089	0.425
Kaempferol 3-*O*-(*p*-coumaroyl)-glucosyl-*O*-rhamnoside (**20**)	0.080	0.707
Kaempferol 3-*O*-rhamnosyl-*O*-glucoside (**21**)	0.162	0.655
Kaempferol 3-*O*-glucosyl-*O*-rhamnoside (**22**)	0.072	0.474
Kaempferol (**26**)	0.004	0.095
t-Kaempferol	**0.441**	**2.378**
Isorhamnetin 3-*O*-dirhamnosyl-glucoside (**27**)	0.002	0.018
Isorhamnetin 3-*O*-rhamnosyl-*O*-glucoside (**29**)	0.088	0.559
Isorhamnetin 3-*O*-glucosyl-*O*-rhamnoside (**30**)	0.0003	0.004
Isorhamnetin (**32**)	0.001	0.014
t-Isorhamnetin	**0.127**	**0.665**

The ginkgo compounds are given ID numbers in parentheses, which are consistent with those for GBE50 described in our earlier publication (Li *et al.*, 2012). Hydrochloric acid-based hydrolysis was applied to the sample to convert the flavonol glycosides present into their aglycone forms before measurement of the content levels of t-quercetin, t-kaempferol, and t-isorhamnetin in ShuXueNing injection or GBE50.

China), each millilitre of which was prepared from 3.5 mg of GBE50. Table 1 shows the content levels of flavonols and terpene lactones present in GBE50 and ShuXueNing injection. Figure 1 depicts the chemical structures of flavonols and terpene lactones.

Experimental animals

All rat studies were conducted in compliance with the Guidance for Ethical Treatment of Laboratory Animals (Ministry of Science and Technology of China, 2006; http://www.most.gov.cn/fggw/zfwj/zfwj2006) and the experimental protocols were approved by the Institutional Animal Care and Use Committee at the Shanghai Institute of Materia Medica (Shanghai, China). For blood sampling, the femoral arteries of male Sprague-Dawley rats (260–300 g) were cannulated under pentobarbital anaesthesia. Other rats received bile duct-cannulation surgery for bile collection. After surgery, the femoral-artery-cannulated (FAC) rats and bile-duct-cannulated (BDC) rats were housed individually and allowed to regain the preoperative body weights before use.

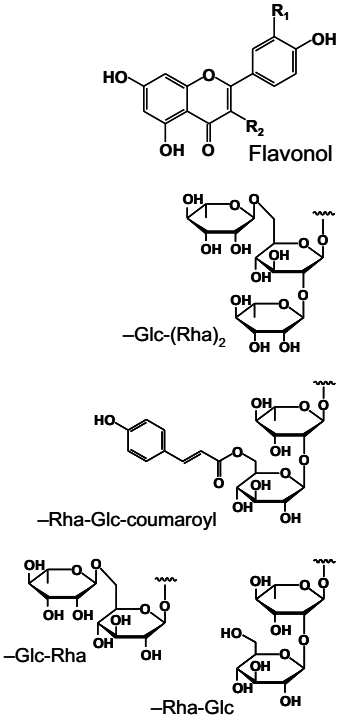
Plasma PK studies

The FAC rats were randomly divided into three groups of four rats each to assess the plasma pharmacokinetics of flavonols and terpene lactones after a 15 min i.v. infusion of ShuXueNing injection at 1, 2 or 4 mL·kg^{-1}. Serial blood samples (~110 μL; 0, 5, 15 and 30 min and 1, 2, 4, 6, 8, 11 and 24 h) were collected and heparinized for plasma preparation. A similar experiment was also performed in FAC rats that received a p.o. dose of GBE50 (suspended in 0.5% w/v sodium carboxymethycellulose) at 10, 30 or 90 mg·kg^{-1} via gavage. In addition, a subchronic PK study was implemented for seven consecutive days. Four FAC rats received ShuXueNing injections at 1 mL·kg^{-1}·day^{-1} and another four FAC rats received GBE50 at 30 mg·kg^{-1}·day^{-1}. Serial blood samples were collected on days 1 and 7.

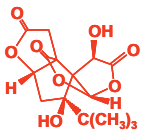
Brain microdialysis

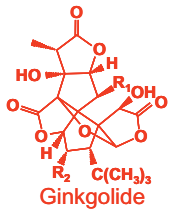
A brain microdialysis study was performed in FAC rats according to a protocol modified from an earlier method (Sun *et al.*, 2009). After a 15 min i.v. infusion of ShuXueNing injection (4 mL·kg^{-1}) or a p.o. dose of GBE50 (90 mg·kg^{-1}), the dialysate samples were collected at 10 min intervals for 6 h. Blood samples were collected at 0, 5, 15 and 30 min and 1, 2, 4 and 6 h after dosing. The brain extracellular fluid (bECF) concentration was calculated using the following equation:

$$C_{bECF} = C_d / R_{in\ vivo} \quad (1)$$



Flavonol	R_1	R_2	Formula	MW
Quercetin 3-*O*-dirhamnosyl-glucoside (**10**)	–OH	–Glc-$(Rha)_2$	$C_{33}H_{40}O_{20}$	757
Quercetin 3-*O*-(*p*-coumaroyl)-glucosyl-*O*-rhamnoside (**11**)	–OH	–Rha-Glc-coumaroyl	$C_{36}H_{36}O_{18}$	757
Quercetin 3-*O*-rhamnosyl-*O*-glucoside (**12**)	–OH	–Glc-Rha	$C_{27}H_{30}O_{16}$	611
Quercetin 3-*O*-glucosyl-*O*-rhamnoside (**13**)	–OH	–Rha-Glc	$C_{27}H_{30}O_{16}$	611
Quercetin (**16**)	–OH	–OH	$C_{15}H_{10}O_7$	302
Kaempferol 3-*O*-dirhamnosyl-glucoside (**19**)	–H	–Glc-$(Rha)_2$	$C_{33}H_{40}O_{19}$	741
Kaempferol 3-*O*-(*p*-coumaroyl)-glucosyl-*O*-rhamnoside (**20**)	–H	–Rha-Glc-coumaroyl	$C_{36}H_{36}O_{17}$	741
Kaempferol 3-*O*-rhamnosyl-*O*-glucoside (**21**)	–H	–Glc-Rha	$C_{27}H_{30}O_{15}$	595
Kaempferol 3-*O*-glucosyl-*O*-rhamnoside (**22**)	–H	–Rha-Glc	$C_{27}H_{30}O_{15}$	595
Kaempferol (**26**)	–H	–OH	$C_{15}H_{10}O_6$	286
Isorhamnetin 3-*O*-dirhamnosyl-glucoside (**27**)	$–OCH_3$	–Rha-Glc-coumaroyl	$C_{34}H_{42}O_{20}$	771
Isorhamnetin 3-*O*-rhamnosyl-*O*-glucoside (**29**)	$–OCH_3$	–Glc-Rha	$C_{28}H_{32}O_{16}$	625
Isorhamnetin 3-*O*-glucosyl-*O*-rhamnoside (**30**)	$–OCH_3$	–Rha-Glc	$C_{28}H_{32}O_{16}$	625
Isorhamnetin (**32**)	$–OCH_3$	–OH	$C_{16}H_{12}O_7$	315





Terpene lactone	R_1	R_2	Formula	MW
Ginkgolide A (**2**)	–H	–H	$C_{20}H_{24}O_9$	408
Ginkgolide B (**3**)	–OH	–H	$C_{20}H_{24}O_{10}$	424
Ginkgolide C (**4**)	–OH	–OH	$C_{20}H_{24}O_{11}$	440
Ginkgolide J (**5**)	–H	–OH	$C_{20}H_{24}O_{10}$	424

Figure 1
Chemical structures of ginkgo flavonols and terpene lactones.

where C_{bECF} is the bECF concentration; C_d is the measured concentration in the dialysate sample. $R_{in\ vivo}$ is the *in vivo* recovery by retrodialysis, which was 16%, 13%, 11%, 9% and 11% for bilobalide, ginkgolides A, B, C and J respectively.

Excretion studies

Rats that had not undergone any surgery were randomly assigned to two groups (four rats/group) and housed singly in rat metabolic cages. Urine and faecal samples were collected before and 0–8, 8–24 and 24–32 h after a 15 min i.v. infusion dose of ShuXueNing injection (4 mL·kg^{-1}) or p.o. dose of GBE50 (90 mg·kg^{-1}).

Three BDC rats received a 15 min i.v. infusion dose of ShuXueNing injection (4 mL·kg^{-1}), and another three BDC rats received a p.o. dose of GBE50 (90 mg·kg^{-1}). Bile was collected before and 0–10, 10–20, 20–40 and 40–50 min and 0.8–1.2, 1.2–1.8, 1.8–2.2, 2.2–3, 3–3.8, 3.8–4.2, 4.2–5.8, 5.8–6.2, 6.2–7.8, 7.8–8.2, 8.2–10.8, 10.8–11.2, 11.2–23.8 and 23.8–24.2 h after dosing.

Tissue distribution study

Rats under isoflurane anaesthesia were killed by bleeding from the abdominal aorta at 0, 15 (i.v. only), 30 min, 1 (i.v. only), 2 (p.o. only), 4 and 8 (p.o. only) h (three rats/time point) after a 15 min i.v. infusion dose of ShuXueNing injection (4 mL·kg^{-1}) or a p.o. dose of GBE50 (90 mg·kg^{-1}). The heart, lungs, brain, liver and kidneys were excised and homogenized in fourfold volumes of ice-cold saline.

Analysis of ginkgo flavonols and terpene lactones in biological samples

A Thermo Fisher TSQ Quantum mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) interfaced via an electrospray ionization probe with an Agilent 1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) was used to measure concentrations of the ginkgo flavonols and terpene lactones in biosamples. Analytical assays were performed as described by Zhao *et al.* (2008). Hydrochloric acid (HCl; 4 M) was used to treat the rat samples to release the flavonol aglycones from the glycosides and conjugated metabolites. Accordingly, the measured flavonol levels are expressed as concentrations of total quercetin (t-quercetin), total kaempferol (t-kaempferol) and total isorhamnetin (t-isorhamnetin). To assess brain penetration, the brain dialysate samples were centrifuged at 16 060× *g* for 10 min. The high-speed, rapid ultrafiltration method described by Guo *et al.* (2006) was used to isolate unbound ginkgo compounds in plasma for measurement of unbound plasma concentration and plasma protein binding. The glycosides and metabolites of ginkgo flavonols in rat plasma, bile and urine were measured and profiled using an AB-SCIEX API 4000 Q Trap mass spectrometer (AB SCIEX, Toronto, Canada) interfaced via a Turbo V ion source with a Waters Acquity UPLC separation module (Waters, Milford, MA, USA).

Assessment of blood to plasma ratios

Rat blood-to-plasma ratios (B/P ratios) were determined for terpene lactones (bilobalide and ginkgolides A, B, C and J),

flavonol glycosides (**10**–**13**, **19**–**22**, **27**, **29** and **30**) and flavonol aglycone conjugates (**M16$_{G-1}$**–**M16$_{G-4}$**, **M16$_{S-3}$**, **M26$_{G-1}$**–**M26$_{G-3}$**, **M32$_{G-1}$**–**M32$_{G-3}$** and **M32$_{S-2}$**). Blood samples were collected from rats that received a 15 min i.v. infusion dose of ShuXueNing injection (4 mL·kg^{-1}) and those that received a p.o. dose of GBE50 (90 mg·kg^{-1}). The blood samples were centrifuged to yield plasma and erythrocyte fractions. The B/P ratio was calculated using the following equation:

$$\text{B/P ratio} = (0.44 \times C_E + 0.56 \times C_P)/C_P \quad (2)$$

where C_E and C_P are erythrocyte and plasma concentrations respectively. The mean hematocrit value of the rats used was 0.44 ± 0.02.

PK data analysis

Plasma PK parameters were estimated by a noncompartmental method using a Thermo Kinetica software package (Thermo Fisher Scientific, Philadelphia, PA, USA). Dose proportionality assessment was conducted by the regression of log-transformed data (the power model) with criteria that were calculated according to the method described by Smith *et al*. (2000). To assess the extent of brain penetration, a K_p value was calculated using the following equation:

$$K_p = AUC_{bECF}/AUC_{u,p} \quad (3)$$

where $AUC_{u,p}$ is the area under the unbound plasma concentration-time curve.

Chemicals and reagents

Pure ginkgo compounds were obtained from the National Institutes for Food and Drug Control (Beijing, China), Tauto Biotech (Shanghai, China) and Extrasynthese (Genay, France). Organic solvents and other reagents were obtained from Sinopharm Chemical Reagent (Shanghai, China). Pentobarbital was obtained from Shanghai Westang Biotechnology (Shanghai, China).

Results

Plasma pharmacokinetics of ginkgo flavonols and terpene lactones after acute or subchronic daily administration of ShuXueNing injection

Total flavonol levels in HCl-hydrolysed rat plasma samples were measured for 24 h after a 15 min i.v. infusion dose of ShuXueNing injection (Figure 2). The plasma concentrations of t-quercetin, t-kaempferol and t-isorhamnetin decreased in a biphasic fashion with first mean half-lives ($t_{1/2-(1)}$) of 1.3–3.1 h and second half-lives ($t_{1/2-(2)}$) of 11.6–30.2 h (Table 2). Significant accumulation of the total flavonols in plasma was observed in the rats that received the subchronic treatment with ShuXueNing injection (1 mL·kg^{-1}·day^{-1}; Figure 3). This indicated that the AUC_{0-24h} values of day 7 were 1.9–3.1 times greater than those of day 1. The mean $CL_{tot,p}$ values were 0.2–0.5 L·h^{-1}·kg^{-1}. Plasma t-quercetin was eliminated ~2-times more rapidly than plasma t-isorhamnetin because, at least in part, of methylation of quercetin compounds into the associated isorhamnetin counterparts (Li *et al*., 2012). The V_{SS} values were 4- to 14-fold greater than the volume of rat total body water (Davies and Morris, 1993).

Plasma ginkgolides A, B, C and J were monitored up to 4 h after dosing (Figure 2), while bilobalide was detected at very low plasma levels because of its low concentration in the injection. The rat plasma concentrations of the ginkgolides decreased rapidly (mean $t_{1/2}$ values, 0.3–0.9 h). No accumulation of plasma ginkgolides was observed in rats during the subchronic treatment with ShuXueNing injection (Figure 3). The mean $CL_{tot,p}$ values of ginkgolides (1.2–3.8 L·h^{-1}·kg^{-1}) were greater than those of the total flavonols (0.2–0.5 L·h^{-1}·kg^{-1}). The V_{SS} values of the terpene lactones (0.8–2.2 L·kg^{-1}; smaller than those of the flavonols) were within the range of the total body water by volume, suggesting that the compounds were roughly evenly distributed throughout the rat blood and tissues. The plasma AUC_{0-24h} of the total flavonols and the AUC_{0-4h} of the ginkgolides increased in a ShuXueNing injection dose-dependent manner, and the results of the assessment of the dose–exposure relationship are summarized in Table 3.

Supplementary assessments were made to foster understanding the metabolic profiles of total flavonols in rats and the distribution of the ginkgo compounds in the blood. The quercetin glycosides **10**, **11**, **12** and **13**, accounting for t-quercetin, were measured predominantly in a pooled rat plasma sample taken 15 min after administration of ShuXueNing injection (Figure 4). The glucuronidated quercetin **M16$_{G-4}$** and the quercetin sulfate **M16$_{S-3}$** were detected in a pooled plasma sample taken 6 h after dosing. t-Kaempferol occurred mainly as glycosides **19**, **20**, **21** and **22**, and t-isorhamnetin occurred mainly as glycosides **27**, **29** and **30** in the 15 min plasma sample. The kaempferol glucuronides **M26$_{G-1}$** and **M26$_{G-2}$**, the isorhamnetin glucuronide **M32$_{G-3}$** and the isorhamnetin sulfate **M32$_{S-2}$** were measured in the 6 h plasma sample. The B/P ratios of these flavonol glycosides and conjugated metabolites were around 0.5, suggesting that these compounds were confined to the plasma. The B/P ratios of ginkgolides C, J, B and A and bilobalide were 0.72, 0.74, 0.93, 1.12 and 1.26 respectively, which were compound membrane permeability dependent. The data suggested that these ginkgo compounds distributed into both the plasma and the erythrocytes. In addition, 36–71% of the flavonol glycosides and aglycone conjugates were bound to rat plasma protein, and the bound terpene lactones in plasma accounted for 67–83%.

Plasma pharmacokinetics of ginkgo flavonols and terpene lactones after acute or subchronic daily ingestion of GBE50

The total ginkgo flavonols were monitored in all the rat plasma samples for 24 h after a p.o. dose of GBE50. The plasma concentration-time curves of total flavonols were bimodal with the first peak concentrations ($C_{max-(1)}$) occurring 15 min and the second ones ($C_{max-(2)}$) appearing 4–6 h after dosing (Figure 2). Oral F values of the total flavonols were low, 0.5–2%, 7–9% and 3–9% for t-quercetin, t-kaempferol and t-isorhamnetin respectively. As with subchronic treatment with ShuXueNing injection, accumulation of the total flavonols in plasma was observed in rats that received subchronic treatment with oral GBE50 (30 mg·kg^{-1}·day^{-1}). This was indicative of the AUC_{0-24h} values of day 7 being 1.8–1.9-times greater than those of day 1 (Figure 3).

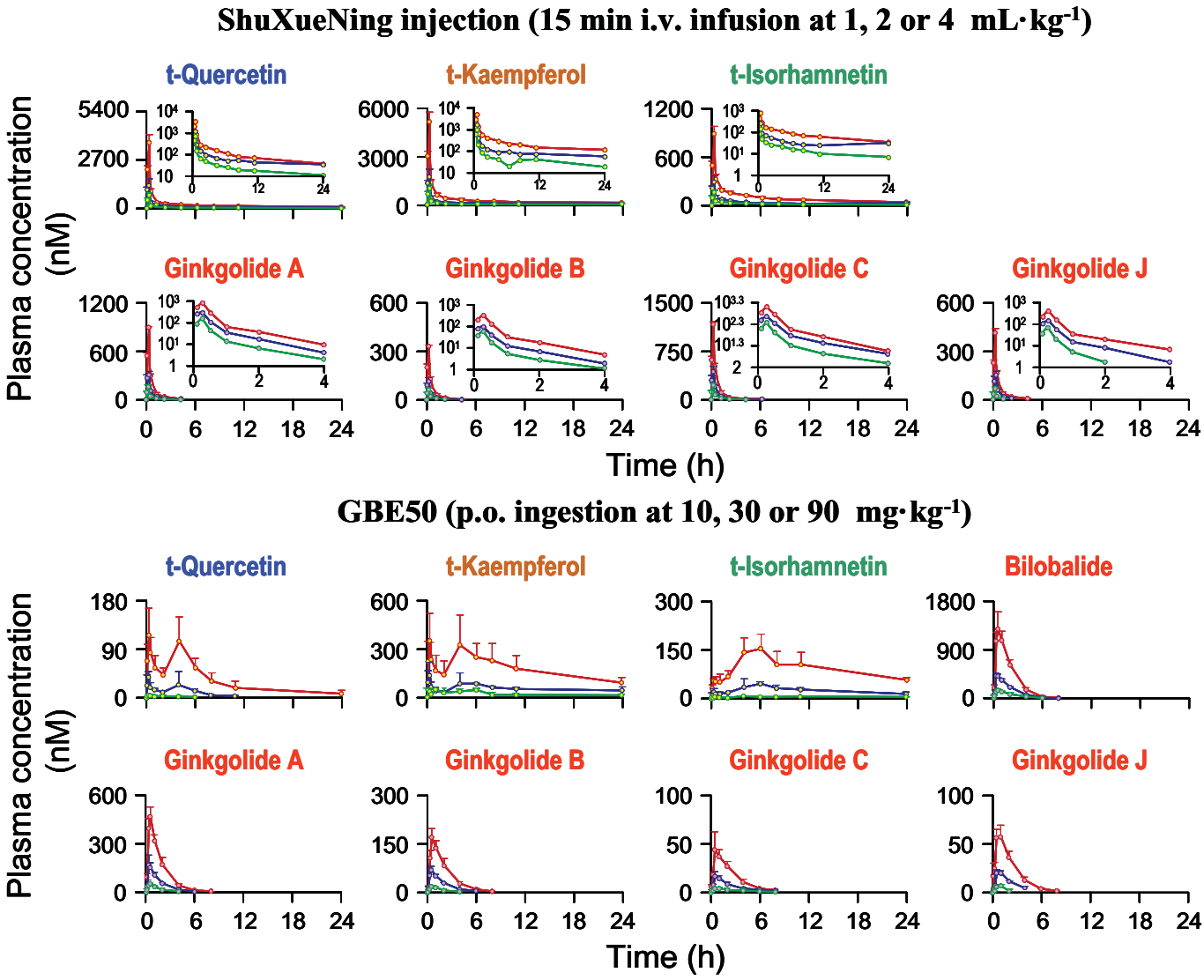


Figure 2

Plasma concentrations of total flavonols (circles filled in yellow) and terpene lactones (circles filled in pink) over time after a 15 min i.v. infusion dose of ShuXueNing injection (upper panel) or a p.o. dose of GBE50 (lower panel) in rats. The test dose levels of ShuXueNing injection included 1 (green curves), 2 (blue curves) and 4 mL·kg^{-1} (red curves), and those of GBE50 were 10 (green curves), 30 (blue curves) and 90 mg·kg^{-1} (red curves). The inserts are the associated semilogarithmic plots of the plasma concentrations of ginkgo compounds over time after administration of ShuXueNing injection.

The terpene lactones were also monitored in the rat plasma samples up to 8 h after dosing (Figure 2). Bilobalide had higher plasma concentrations than the other terpene lactones. Table 4 summarizes the plasma PK data of ginkgo compounds after p.o. administration of GBE50 at 10–90 mg·kg^{-1}. The plasma terpene lactones reached their C_{max} within 0.5–1 h of administration. The mean oral F of all terpene lactones except for ginkgolide C ranged from 33 to 64%. That of ginkgolide C was 5 to 10%. The plasma AUC values of total flavonols and terpene lactones increased with the increased dose of GBE50 (10–90 mg·kg^{-1}). The results of dose–exposure relationship assessment for dosing with GBE50 are summarized in Table 3.

The metabolic profiling analysis of rat plasma samples revealed that the t-quercetin in a pooled plasma sample taken 15 min after administration of GBE50 consisted of the quercetin glucuronides **M16$_{G-1}$**, **M16$_{G-2}$**, **M16$_{G-3}$** and **M16$_{G-4}$** and the absorbed quercetin glycosides **10**, **11**, **12** and **13** (Figure 4). The quercetin glucuronides were most likely derived from the GBE50-containing aglycone quercetin that was absorbed from the small intestine and conjugated in the intestinal epithelia, hepatocytes or both. **M16$_{G-2}$**, **M16$_{G-3}$**, **M16$_{G-4}$** and **M16$_{S-1}$** accounted for the t-quercetin in a pooled plasma sample taken 6 h after dosing. These conjugated metabolites were most likely formed via the colonic microflora-induced deglycosylation of the unabsorbed quercetin glycosides and the subsequent enterohepatic conjugation of the resulting quercetin aglycone. The kaempferol glucuronide **M26$_{G-1}$** was detected in the 15 min plasma sample. This metabolite accounted for the vast majority of

Table 2

Plasma PK parameters of total flavonols and terpene lactones after an i.v. infusion dose of ShuXueNing injection in rats

PK parameter	t-Quercetin	t-Kaempferol	t-Isorhamnetin	Ginkgolide A	Ginkgolide B	Ginkgolide C	Ginkgolide J
ShuXueNing injection (15 min i.v. infusion at 1 mL·kg^{-1})							
C_{15min} (nM)	732 ± 107	1022 ± 186	163 ± 23	161 ± 25	64.5 ± 6.6	223 ± 29	68.4 ± 6.5
$AUC_{0\text{-}t}$ (h·nM)	687 ± 125	1116 ± 192	379 ± 57	72.8 ± 10.9	31.7 ± 4.6	114 ± 11	28.1 ± 4.4
MRT (h)	14.3 ± 4.4	15.0 ± 9.8	22.3 ± 5.2	0.46 ± 0.20	0.63 ± 0.08	0.67 ± 0.09	0.23 ± 0.05
$t_{1/2\text{-}(1)}$ (h)	1.86 ± 0.19	1.34 ± 0.11	2.91 ± 0.46	0.62 ± 0.21	0.77 ± 0.06	0.92 ± 0.07	0.31 ± 0.04
$t_{1/2\text{-}(2)}$ (h)	14.3 ± 3.8	21.5 ± 19.7	18.5 ± 4.2	N.A.	N.A.	N.A.	N.A.
$CL_{tot,p}$ (L·h^{-1}·kg^{-1})	0.46 ± 0.08	0.45 ± 0.24	0.23 ± 0.04	3.85 ± 0.61	2.96 ± 0.42	1.63 ± 0.13	3.40 ± 0.54
V_{SS} (L·kg^{-1})	6.55 ± 1.68	8.42 ± 3.75	4.99 ± 1.20	2.20 ± 0.72	2.22 ± 0.27	1.30 ± 0.17	1.19 ± 0.15
ShuXueNing injection (15 min i.v. infusion at 2 mL·kg^{-1})							
C_{15min} (nM)	1326 ± 275	1785 ± 420	293 ± 73	331 ± 28	120 ± 16	438 ± 72	154 ± 25
$AUC_{0\text{-}t}$ (h·nM)	1660 ± 266	2400 ± 401	927 ± 77	185 ± 17	72.5 ± 7.4	298 ± 16	87.3 ± 11.1
MRT (h)	23.9 ± 11.0	32.6 ± 4.0	25.4 ± 4.0	0.54 ± 0.04	0.62 ± 0.01	0.71 ± 0.10	0.41 ± 0.03
$t_{1/2\text{-}(1)}$ (h)	1.62 ± 0.19	1.86 ± 0.31	2.88 ± 0.45	0.73 ± 0.05	0.69 ± 0.01	0.88 ± 0.07	0.47 ± 0.09
$t_{1/2\text{-}(2)}$ (h)	20.8 ± 10.3	27.4 ± 4.2	18.0 ± 2.6	N.A.	N.A.	N.A.	N.A.
$CL_{tot,p}$ (L·h^{-1}·kg^{-1})	0.32 ± 0.08	0.31 ± 0.08	0.29 ± 0.02	3.05 ± 0.20	2.68 ± 0.10	1.21 ± 0.08	2.09 ± 0.33
V_{SS} (L·kg^{-1})	7.33 ± 2.04	10.1 ± 1.4	2.78 ± 0.21	2.01 ± 0.03	2.00 ± 0.09	1.01 ± 0.15	1.11 ± 0.12
ShuXueNing injection (15 min i.v. infusion at 4 mL·kg^{-1})							
C_{15min} (nM)	3607 ± 426	5113 ± 589	881 ± 86	884 ± 11	330 ± 8	1159 ± 40	407 ± 27
$AUC_{0\text{-}t}$ (h·nM)	3274 ± 482	6275 ± 330	2087 ± 240	429 ± 38	174 ± 20	622 ± 106	211 ± 19
MRT (h)	10.5 ± 4.2	34.7 ± 17.3	19.3 ± 4.6	0.57 ± 0.09	0.60 ± 0.08	0.54 ± 0.03	0.56 ± 0.06
$t_{1/2\text{-}(1)}$ (h)	1.91 ± 0.26	1.80 ± 0.13	3.13 ± 0.60	0.77 ± 0.09	0.66 ± 0.05	0.78 ± 0.07	0.69 ± 0.03
$t_{1/2\text{-}(2)}$ (h)	11.6 ± 3.3	30.2 ± 14.6	16.2 ± 3.7	N.A.	N.A.	N.A.	N.A.
$CL_{tot,p}$ (L·h^{-1}·kg^{-1})	0.44 ± 0.13	0.25 ± 0.08	0.19 ± 0.04	2.75 ± 0.17	2.32 ± 0.19	1.24 ± 0.17	1.78 ± 0.12
V_{SS} (L·kg^{-1})	4.26 ± 0.75	7.84 ± 2.73	3.56 ± 0.44	1.92 ± 0.30	1.68 ± 0.28	0.83 ± 0.07	1.22 ± 0.11

Because of its low content level in ShuXueNing injection (Table 1), a 15 min i.v. infusion dose of bilobalide at 1 mg·kg^{-1} was given to FAC rats to obtain the plasma PK parameters of the compound. The C_{15min}, $AUC_{0\text{-}t}$, MRT, $t_{1/2}$, $CL_{tot,p}$, and V_{SS} value of bilobalide were 2696 ± 154 nM, 1619 ± 35 h·nM, 0.80 ± 0.05 h, 0.87 ± 0.06 h, 1.89 ± 0.04 L·h^{-1}·kg^{-1} and 1.51 ± 0.11 L·kg^{-1} respectively.

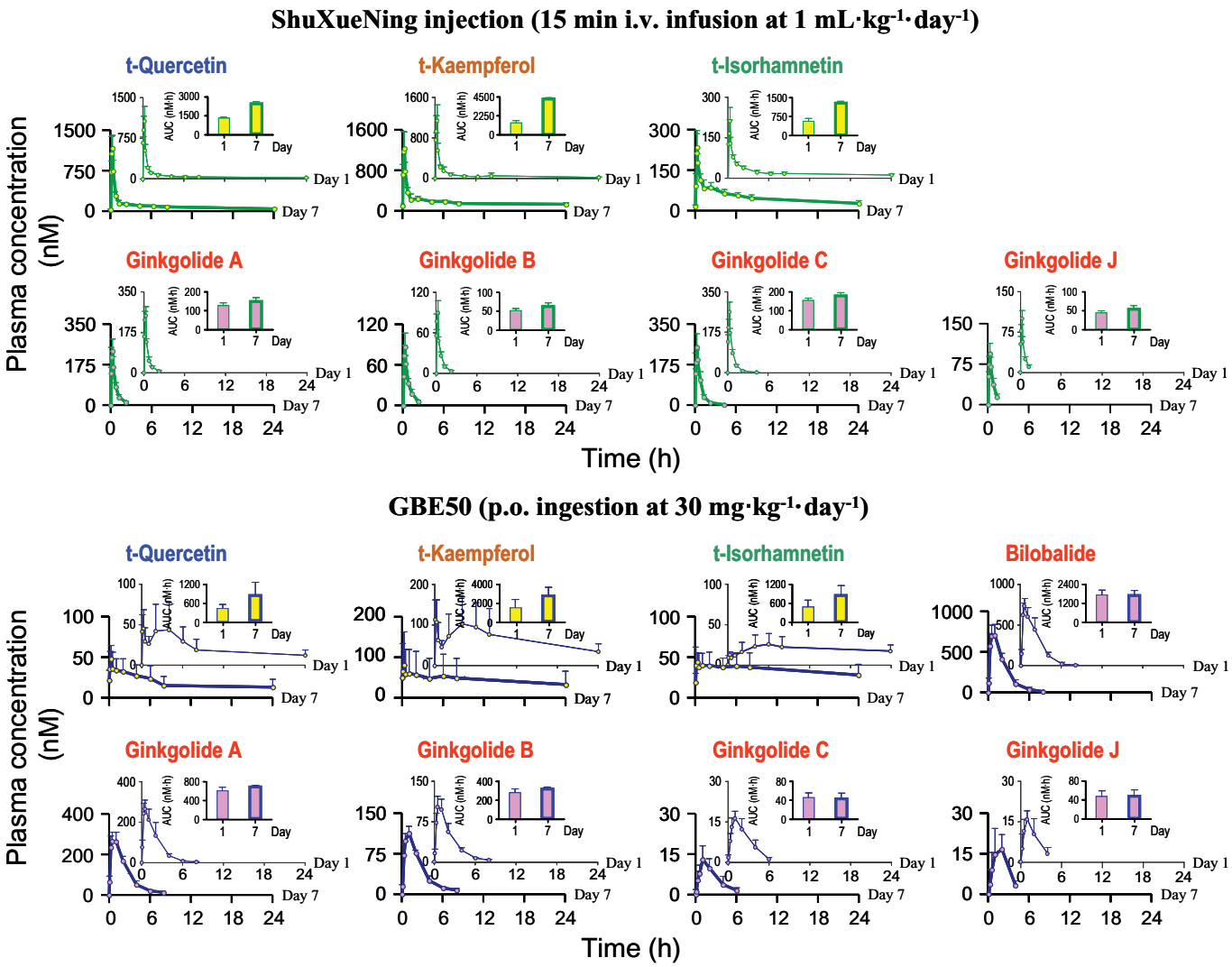


Figure 3

Plasma concentrations of total flavonols (circles filled in yellow) and terpene lactones (circles filled in pink) over time on day 1 (thin green and blue curves) and day 7 (thick green and blue curves) in rats given subchronic treatment with ShuXueNing injection (1 mL·kg^{-1}·day^{-1}; 15 min i.v. infusion; green curves) or GBE50 (30 mg·kg^{-1}·day^{-1}; p.o. ingestion; blue curves). The upper and smaller inserts for the ginkgo compounds show the comparative $AUC_{0\text{-}t}$ values for day1 and day 7.

t-kaempferol. Levels of the kaempferol glycosides **19**, **21** and **22** and the kaempferol glucuronides **M26$_{G\text{-}2}$** and **M26$_{G\text{-}3}$** were also measured. The kaempferol glucuronides **M26$_{G\text{-}1}$**, **M26$_{G\text{-}2}$** and **M26$_{G\text{-}3}$** were observed in the 6 h plasma sample. Levels of the isorhamnetin glycoside **29** and the isorhamnetin conjugates **M32$_{G\text{-}1}$**, **M32$_{G\text{-}2}$**, **M32$_{G\text{-}3}$** and **M32$_{S\text{-}2}$** were measured in the 15 min plasma sample, and the 6 h plasma sample was found to contain **M32$_{G\text{-}3}$**, **M32$_{S\text{-}1}$** and **M32$_{S\text{-}2}$**.

Exposure of brain tissue and other types of tissue to ginkgo flavonols and terpene lactones after administration of ShuXueNing injection or GBE50

After i.v. infusion of ShuXueNing injection (4 mL·kg^{-1}) or p.o. administration of GBE50 (90 mg·kg^{-1}), levels of the terpene lactone bilobalide (p.o. only), ginkgolides A, B and C (i.v. only), and ginkgolide J (i.v. only) were measured in rat bECF (Figure 5), but the ginkgo flavonols were absent. The bECF concentrations of terpene lactones were significantly lower than the associated unbound plasma concentrations; these ginkgo compounds had average K_p values of 0.12–0.29. The results suggested that their ability to enter brain tissue was not very good. The unbound plasma concentrations of terpene lactones and their associated bECF concentrations concomitantly increased up to a maximum and then decreased. Correlations between the unbound plasma concentrations and the associated bECF concentrations were significant for the ginkgo compounds.

After i.v. infusion of ShuXueNing injection (4 mL·kg^{-1}), the total flavonols had the highest concentration in the kidneys, followed by the liver, lungs, plasma and heart, but they

Table 3

Summary of results from dose proportionality assessment

Compound	*r*	*P*	Slope (90% CI)	Conclusion
$AUC_{0\text{-}24h}$ for the total flavonols and $AUC_{0\text{-}6h}$ for the terpene lactones				
Dose range of ShuXueNing injection (15 min i.v. infusion), 1–4 mL·kg^{-1}				
t-Quercetin	0.976	3.04×10^{-7}	1.105 (0.954–1.257)	Inconclusive
t-Kaempferol	0.981	9.60×10^{-8}	1.232 (1.084–1.380)	Inconclusive
t-Isorhamnetin	0.987	1.81×10^{-8}	1.167 (1.051–1.282)	Inconclusive
Ginkgolide A	0.990	4.97×10^{-9}	1.236 (1.130–1.341)	Inconclusive
Ginkgolide B	0.984	1.14×10^{-9}	1.171 (1.086–1.256)	Inconclusive
Ginkgolide C	0.988	1.09×10^{-8}	1.201 (1.089–1.314)	Inconclusive
Ginkgolide J	0.986	2.24×10^{-8}	1.450 (1.302–1.597)	Nonlinear
$AUC_{0\text{-}24h}$ for the total flavonols and $AUC_{0\text{-}8h}$ for the terpene lactones				
Dose range of GBE50 (p.o.), 10–90 mg·kg^{-1}				
t-Quercetin	0.973	4.64×10^{-7}	1.668 (1.428–1.908)	Nonlinear
t-Kaempferol	0.985	1.96×10^{-7}	1.153 (1.022–1.284)	Inconclusive
t-Isorhamnetin	0.988	1.48×10^{-8}	1.582 (1.429–1.735)	Nonlinear
Bilobalide	0.988	1.14×10^{-12}	0.978 (0.945–1.011)	Linear
Ginkgolide A	0.990	6.52×10^{-9}	1.028 (0.938–1.119)	Inconclusive
Ginkgolide B	0.986	2.58×10^{-8}	1.037 (0.930–1.144)	Inconclusive
Ginkgolide C	0.973	5.04×10^{-7}	0.796 (0.680–0.911)	Inconclusive
Ginkgolide J	0.974	4.11×10^{-7}	1.376 (1.181–1.572)	Nonlinear

Critical intervals were 0.839–1.161 for the plasma data with ShuXueNing injection and 0.898–1.102 for those with GBE50. The term '*r*' denotes the correlation coefficient. Correlations were statistically significant with a '*P*' value of <0.05. The term 'linear' was concluded if the 90% confidence interval (90% CI) for slope was contained completely within the critical interval; 'inconclusive' was concluded if the 90% CI lay partly within the critical interval; 'nonlinear' was concluded if the 90% CI was entirely outside the critical interval.

were not detected in the brain (Figure 6). The liver and kidneys showed significantly higher levels of ginkgolide exposure than the plasma, and the levels of exposure to the heart and lungs were comparable with or lower than those of the plasma. Although ginkgolides A, B, C and J were also detected in the brain homogenate samples, the concentrations were low.

After p.o. ingestion of GBE50 (90 mg·kg^{-1}), most tissue concentration-time profiles of the total flavonols were bimodal. The levels of exposure of t-quercetin, t-kaempferol and t-isorhamnetin were ranked as follows: kidneys > liver ≈ plasma > lungs ≈ heart. The flavonols were not measured in the brain homogenate samples. Bilobalide had higher tissue exposure levels than the other terpene lactones. Ginkgolide C had the lowest tissue concentration. The highest tissue exposure levels of terpene lactones from the p.o. ingested GBE50 were observed in the liver and kidneys, followed by the concentrations in heart and lungs. The brain concentrations of bilobalide and ginkgolides A and B were low.

Excretion of ginkgo flavonols and terpene lactones after administration of ShuXueNing injection or GBE50

After i.v. infusion of ShuXueNing injection (4 mL·kg^{-1}), substantial amount of total flavonols was excreted into rat bile, and t-quercetin, t-kaempferol and t-isorhamnetin had $f_{e\text{-}B}$ values of 73, 67, and 321% respectively (Table 5). The renal excretion of total flavonols was relatively low and the $f_{e\text{-}U}$ values were 9, 12 and 26% respectively. The recovery levels of total flavonols in faeces were notably lower than those in bile. Unlike the situation of biliary excretion (CL_B, 0.20–1.41 L·h^{-1}·kg^{-1}), the urinary excretion of total flavonols was slow (CL_R, 0.04–0.06 L·h^{-1}·kg^{-1}). Metabolic profiling revealed that the biliary total flavonols were largely present in the form of the flavonol glycosides, that is **11** and **13** accounting for t-quercetin, **19**, **20**, **21** and **22** accounting for t-kaempferol, and **27**, **29** and **30** accounting for t-isorhamnetin (Figure 4). The glycosides **10**, **12** and **13** accounted for urinary t-quercetin; **19**, **21** and **22** for urinary t-kaempferol; and **29** for urinary t-isorhamnetin. Although the flavonol aglycones quercetin (**16**), kaempferol (**26**) and isorhamnetin (**32**) were not detected in the plasma and bile samples, they were observed in urine despite their low levels. Some of the flavonol aglycone glucuronides and sulfates were also detected in the urine, but they were present at low levels (Figure 4). The urinary and biliary recovery levels of intact ginkgolides were 14–34% and 13–29% respectively, after i.v. infusion of ShuXueNing injection. Although the biliary and urinary excretions are two major elimination pathways, the elimination of ginkgolides may also involve additional major metabolic reactions mediated by an unknown enzyme or enzymes. The biliary excretion of flavonols and ginkgolides described above appeared to involve active transport mechanisms. This is indicative of high AUC_B/AUC_P ratios ranging from 31 to 289. The $CL_R/(f_u \cdot GFR)$ data implied active tubular

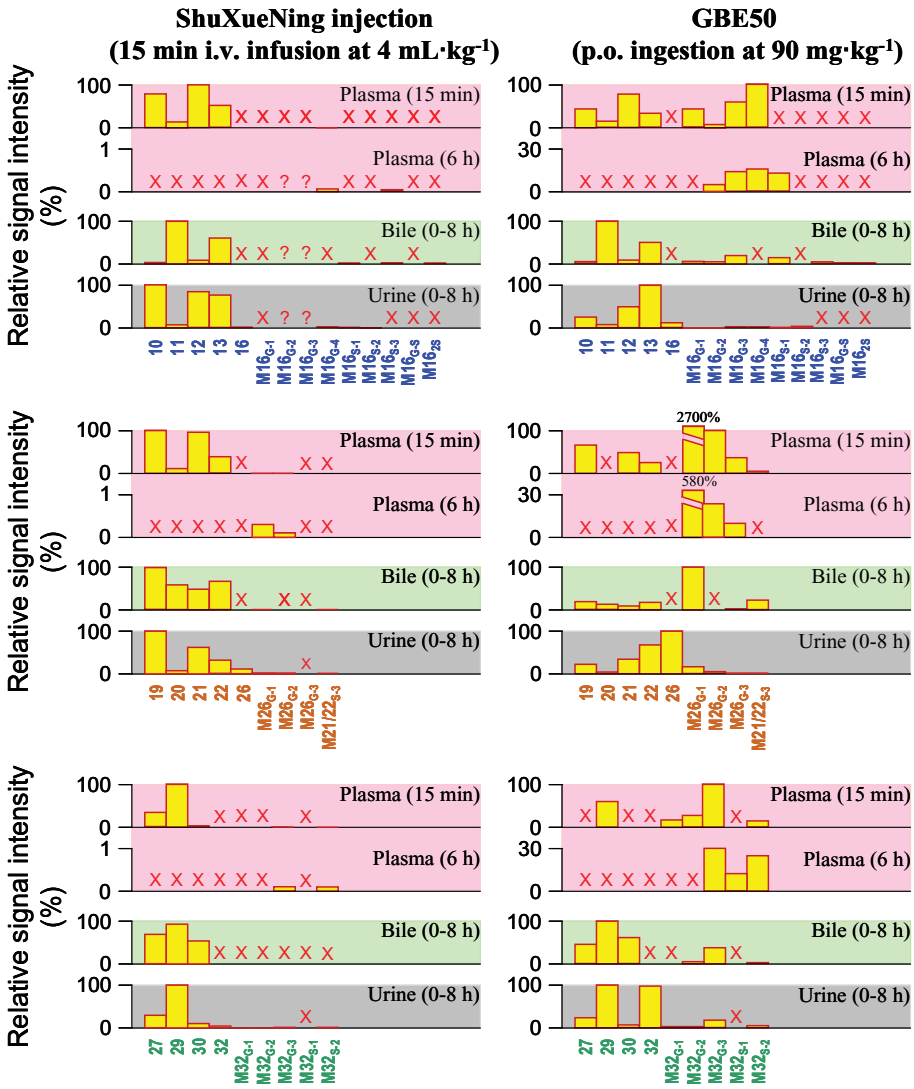


Figure 4

Detection of flavonol glycosides and their metabolites in plasma (pink plots), bile (light green plots), and urine (gray plots) after a 15 min i.v. infusion dose of ShuXueNing injection (4 mL·kg^{-1}) or a p.o. dose of GBE50 (90 mg·kg^{-1}) in rats. The names of the flavonol glycosides **10–13**, **19–22**, **27**, **29** and **30** and of the flavonol aglycones **16**, **26** and **32**, are given in Table 1. The conjugated metabolites **M16$_{G-1}$–M16$_{G-4}$**, **M16$_{S-1}$–M16$_{S-3}$**, **M16$_{G-S}$**, **M16$_{2S}$**, **M26$_{G-1}$–M26$_{G-3}$**, **M21/22$_{S-3}$**, **M32$_{G-1}$–M32$_{G-3}$** and **M32$_{S-1}$/M32$_{S-2}$** were quercetin glucuronide, quercetin sulfate, glucuronidated quercetin sulfate, quercetin disulfate, kaempferol glucuronide, kaempferol 3-*O*-rhamnosyl-*O*-glucoside/kaempferol 3-*O*-glucosyl-*O*-rhamnoside sulfate, isorhamnetin glucuronide and isorhamnetin sulfate respectively. **M16$_{G-1}$** and **M26$_{G-1}$** are quercetin-3-*O*-glucuronide and kaempferol-3-*O*-glucuronide respectively. The symbol '×' (in red) indicates that no substance was detected in the sample. The symbol '?' (in red) indicates that the substance was only detected once.

secretion in the renal excretion of ginkgolides rather than that of flavonols (Table 5).

After p.o. administration of GBE50 (90 mg·kg^{-1}), substantial amounts of the flavonol glycosides **11**, **13**, **19**, **20**, **21**, **22**, **27**, **29** and **30**, the flavonol aglycone conjugates **M16$_{G-1}$**, **M16$_{G-2}$**, **M16$_{G-3}$**, **M16$_{S-1}$**, **M16$_{S-3}$**, **M16$_{G-S}$**, **M16$_{2S}$**, **M26$_{G-1}$** and **M32$_{G-3}$** and the sulfated kaempferol glycoside **M21/22$_{S-3}$** were recovered in rat bile. The flavonol glycosides **10**, **12**, **13**, **19**, **21**, **22**, **27** and **29** and the metabolites **M26$_{G-1}$**, **M26$_{G-2}$** and **M32$_{G-3}$** were recovered in urine. The flavonol aglycones **16**, **26** and **32** were also detected in urine but absent from plasma and bile (Figure 4). Despite the poor intestinal absorption of flavonol glycosides (Li *et al.*, 2012), the faecal recoveries of total flavonols were found to be very low. Around 30% of unchanged bilobalide was excreted via urine, but its biliary excretion was poor ($f_{e\text{-}B}$, 2%). The renal excretion of bilobalide appeared to involve active tubular secretion (Table 5).

Discussion and conclusions

Understanding of the body's exposure to and PK profiles of medicinal ingredients can be extremely beneficial to assessment of the drug responses of a botanical product.

Table 4

Plasma PK parameters of total flavonols and terpene lactones after a p.o. dose of GBE50 in rats

PK parameter	t-Quercetin	t-Kaempferol	t-Isorhamnetin	Bilobalide	Ginkgolide A	Ginkgolide B	Ginkgolide C	Ginkgolide J
GBE50 (p.o., 10 mg·kg^{-1})								
$T_{peak\text{-}(1)}$ (h)	0.25	1.00	0.50	0.25–0.50	0.25–0.50	0.50	0.50–1.00	0.50–1.00
$C_{max\text{-}(1)}$ (nM)	5.24 ± 1.34	49.2 ± 4.1	5.47 ± 1.48	153 ± 10	53.9 ± 5.3	20.1 ± 3.2	6.25 ± 0.85	7.40 ± 1.27
$T_{peak\text{-}(2)}$ (h)	2.00	6.00	4.00	N.A.	N.A.	N.A.	N.A.	N.A.
$C_{max\text{-}(2)}$ (nM)	3.76 ± 1.25	46.6 ± 9.3	9.51 ± 1.94	N.A.	N.A.	N.A.	N.A.	N.A.
$AUC_{0\text{-}t}$ (h·nM)	19.4 ± 3.4	321 ± 39	68.0 ± 10.0	318 ± 26	88.5 ± 15.6	38.7 ± 9.2	20.3 ± 5.0	9.17 ± 1.10
$t_{1/2}$ (h)	–	–	–	0.90 ± 0.11	1.40 ± 0.31	0.93 ± 0.12	2.97 ± 0.32	1.42 ± 0.85
MRT (h)	12.8 ± 7.7	12.3 ± 8.3	8.14 ± 4.32	1.84 ± 0.12	1.86 ± 0.24	1.76 ± 0.07	4.24 ± 0.20	2.35 ± 1.22
F (%)[a]	0.48	6.58	2.62	63.5	51.5	32.9	9.91	32.9
GBE50 (p.o., 30 mg·kg^{-1})								
$T_{peak\text{-}(1)}$ (h)	0.25	0.25	0.25	0.25–0.50	0.25–0.50	0.50	0.50–1.00	0.50–1.00
$C_{max\text{-}(1)}$ (nM)	39.2 ± 11.2	136 ± 82	20.0 ± 9.8	427 ± 34	171 ± 43	69.4 ± 11.7	15.1 ± 3.6	21.0 ± 3.0
$T_{peak\text{-}(2)}$ (h)	4.00	6.00	6.00	N.A.	N.A.	N.A.	N.A.	N.A.
$C_{max\text{-}(2)}$ (nM)	24.5 ± 25.2	83.4 ± 8.8	45.6 ± 4.7	N.A.	N.A.	N.A.	N.A.	N.A.
$AUC_{0\text{-}t}$ (h·nM)	211 ± 103	1286 ± 116	584 ± 113	936 ± 20	304 ± 49	140 ± 19	46.2 ± 5.1	43.5 ± 5.6
$t_{1/2}$ (h)	–	–	–	1.00 ± 0.07	1.23 ± 0.05	1.17 ± 0.33	2.84 ± 1.17	1.37 ± 0.39
MRT (h)	9.78 ± 4.89	20.4 ± 7.3	22.2 ± 7.8	1.91 ± 0.25	1.89 ± 0.31	2.05 ± 0.38	4.15 ± 1.26	2.41 ± 0.52
F (%)	1.75	8.66	7.49	62.3	58.2	39.7	6.72	34.7
GBE50 (p.o., 90 mg·kg^{-1})								
$T_{peak\text{-}(1)}$ (h)	0.25	0.25	0.25	0.50–1.00	0.50	0.50–1.00	0.50–2.00	0.25–0.50
$C_{max\text{-}(1)}$ (nM)	119 ± 50	354 ± 173	54.3 ± 14.6	1356 ± 235	474 ± 63	176 ± 23	47.6 ± 16.0	64.9 ± 6.9
$T_{peak\text{-}(2)}$ (h)	4.00	6.00	6.00	N.A.	N.A.	N.A.	N.A.	N.A.
$C_{max\text{-}(2)}$ (nM)	107 ± 46	251 ± 87	156 ± 46	N.A.	N.A.	N.A.	N.A.	N.A.
$AUC_{0\text{-}t}$ (h·nM)	768 ± 205	4195 ± 1295	2212 ± 427	2724 ± 135	840 ± 78	371 ± 39	115 ± 24	148 ± 17
$t_{1/2}$ (h)	–	–	–	1.05 ± 0.16	1.31 ± 0.22	1.20 ± 0.18	1.63 ± 0.71	1.25 ± 0.15
MRT (h)	10.6 ± 2.8	20.8 ± 6.5	22.8 ± 4.7	1.76 ± 0.09	1.74 ± 0.07	2.07 ± 0.12	2.65 ± 0.43	2.20 ± 0.11
F (%)[a]	2.12	9.46	9.42	60.4	53.4	35.2	5.12	34.1

[a]The oral *F* was calculated using the AUC data of p.o. ingested GBE50 and the associated AUC data of i.v. infused ShuXueNing injection at 2 mL·kg^{-1}.

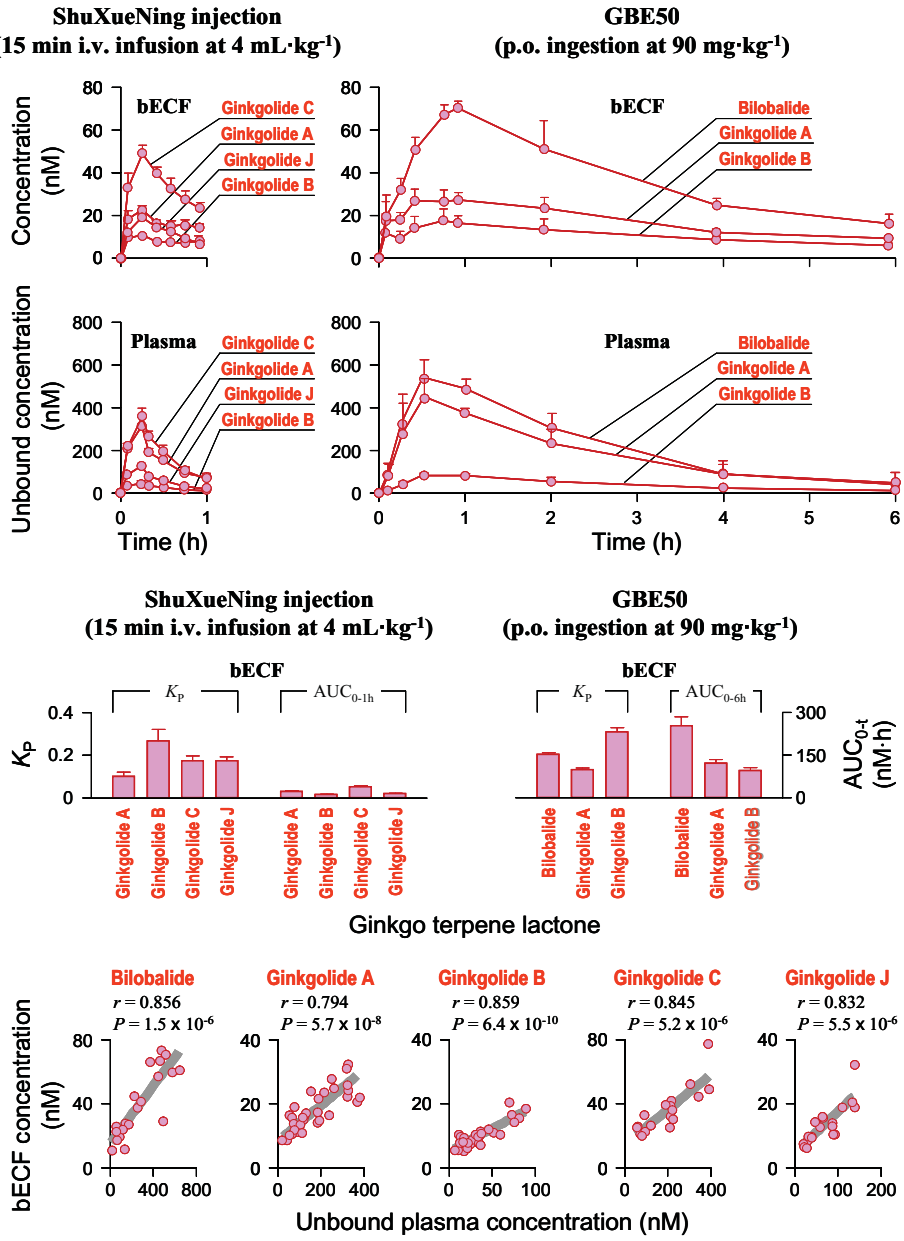


Figure 5

bECF concentrations and unbound plasma concentrations of terpene lactones over time (upper panel), comparative K_P values and comparative bECF AUC values of the ginkgo compounds (middle panel) and correlations between the unbound plasma concentrations and the bECF concentrations (lower panel) after a 15 min i.v. infusion dose of ShuXueNing injection (4 mL·kg^{-1}; red curves) or a p.o. dose of GBE50 (90 mg·kg^{-1}; red curves) in rats. The letter '*r*' represents the correlation coefficient. Correlations were statistically significant, with a *P*-value of <0.05.

In the current study, after an i.v. dose of ShuXueNing injection, the plasma concentrations of t-quercetin, t-kaempferol and t-isorhamnetin at first decreased rapidly ($t_{1/2\text{-}(1)}$, ~1.9 h) and then entered a slow terminal elimination phase ($t_{1/2\text{-}(2)}$, ~15 h). The concentrations of plasma ginkgolides A, B, C and J decreased more rapidly ($t_{1/2}$, ~0.7 h). As depicted in Figure 7, the difference in elimination kinetics led to the mean flavonol-to-terpene lactone ratio in plasma $AUC_{0\text{-}t}$ (7.8; dose, 2 mL·kg^{-1}), which was significantly larger than the associated flavonol-to-terpene lactone ratio in concentration in ShuXueNing injection (1.4). In addition, the plasma total flavonols, rather than the plasma terpene lactones, showed significant accumulation in rats that received multiple doses of ShuXueNing injection (daily dose, 1 mL·kg^{-1}) and the mean flavonol-to-terpene lactone ratio in $AUC_{0\text{-}t}$ increased from 8.4 (day 1) to 16.4 (day 7). The mean flavonol-to-terpene lactone ratio in plasma $AUC_{0\text{-}t}$ (1.4; dose, 30 mg·kg^{-1}) was over one half the flavonol-to-terpene lactone ratio in concentration in GBE50 (2.3), even though the oral *F* values of total flavonols (0.5%–9.5%) were significantly lower than those of terpene lactones (32.9–63.5%; excluding ginkgolide C). These data can be explained by the slow terminal

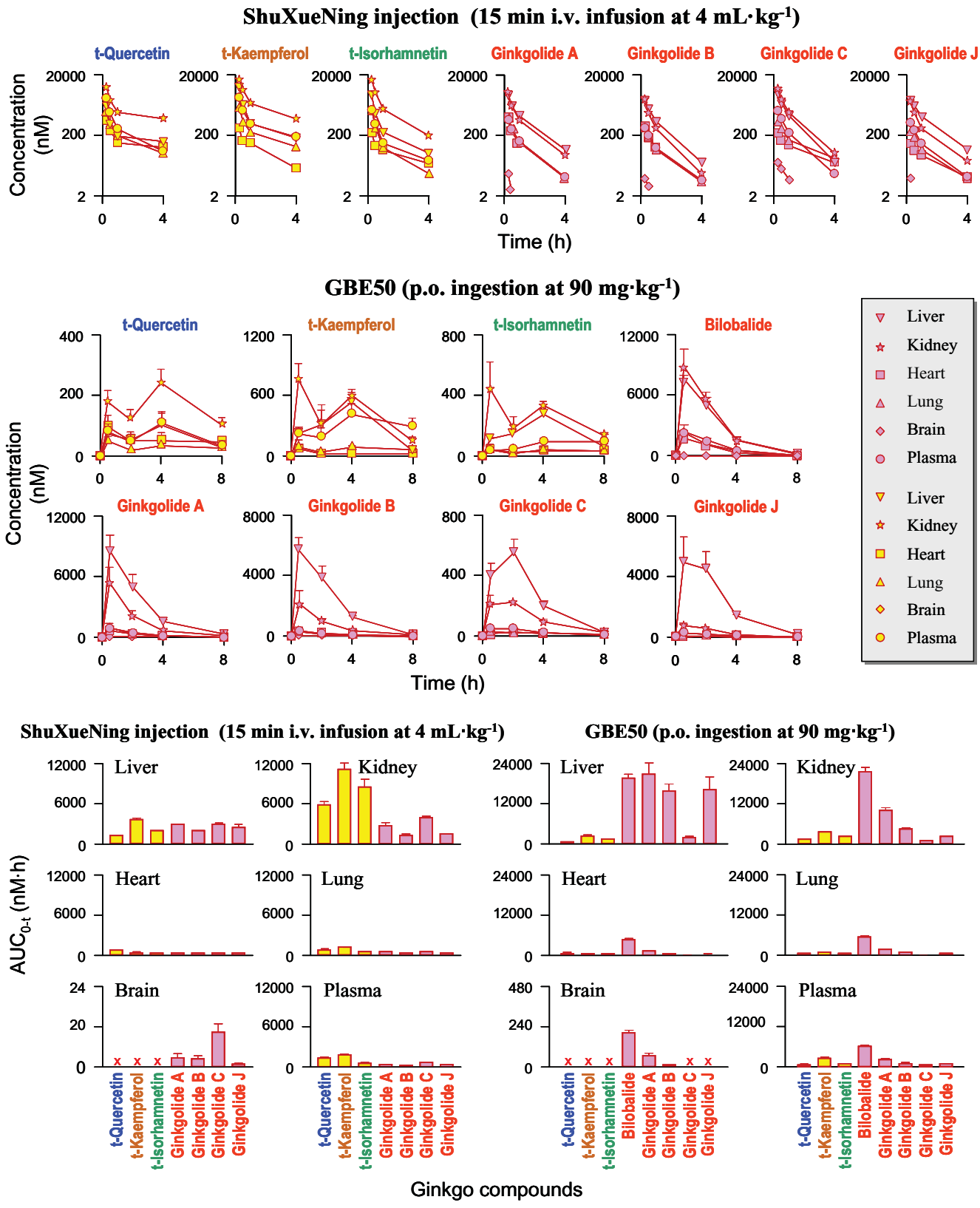


Figure 6

Tissue distribution of total flavonols (markers filled in yellow) and terpene lactones (markers filled in pink) after a 15 min i.v. infusion dose of ShuXueNing injection (4 mL·kg^{-1}; red curve; upper panel) or a p.o. dose of GBE50 (90 mg·kg^{-1}; red curve; middle panel) in rats. Tissue AUC values and the associated plasma AUC values are shown in the lower panel.

Table 5

Excretory data of total flavonols and terpene lactones after an i.v. infusion dose of ShuXueNing injection or a p.o. dose of GBE50 in rats

	Biliary data			Faecal data		Urinary data	
Compound	$f_{e\text{-}B}$ (%)	CL_B (L·h^{-1}·kg^{-1})	AUC_B/AUC_P	$f_{e\text{-}F}$ (%)	$f_{e\text{-}U}$ (%)	CL_R (L·h^{-1}·kg^{-1})	$CL_R/(f_u \cdot GFR)$
ShuXueNing injection (15 min i.v. infusion, 4 mL·kg^{-1})							
t-Quercetin	73.2 ± 8.3	0.36 ± 0.03	70.7 ± 8.9	12.5 ± 4.9	8.68 ± 2.1	0.04 ± 0.01	<1
t-Kaempferol	66.7 ± 16.8	0.20 ± 0.04	41.0 ± 8.3	5.20 ± 1.57	11.8 ± 3.2	0.05 ± 0.01	<1
t-Isorhamnetin	321 ± 54	1.41 ± 0.27	289 ± 58	12.6 ± 4.2	25.6 ± 8.5	0.06 ± 0.02	<1
Ginkgolide A	17.5 ± 6.2	0.20 ± 0.07	36.8 ± 5.8	4.46 ± 0.77	28.9 ± 4.0	0.78 ± 0.14	7.46 ± 1.34
Ginkgolide B	34.2 ± 10.6	0.34 ± 0.12	63.4 ± 16.4	17.7 ± 3.1	28.0 ± 9.2	0.65 ± 0.24	10.7 ± 4.0
Ginkgolide C	17.2 ± 7.6	0.17 ± 0.07	35.6 ± 13.3	12.4 ± 2.0	22.9 ± 3.1	0.27 ± 0.04	3.51 ± 0.52
Ginkgolide J	13.9 ± 6.7	0.17 ± 0.08	31.4 ± 13.4	15.9 ± 3.9	13.2 ± 2.0	0.23 ± 0.04	4.29 ± 0.75
GBE50 (p.o., 90 mg·kg^{-1})							
t-Quercetin	1.1 ± 0.2	0.20 ± 0.05	46.1 ± 14.1	31.0 ± 6.9	0.28 ± 0.14	0.05 ± 0.01	<1
t-Kaempferol	1.7 ± 0.3	0.14 ± 0.07	30.0 ± 12.8	17.7 ± 3.5	0.75 ± 0.21	0.04 ± 0.01	<1
t-Isorhamnetin	7.6 ± 1.7	0.44 ± 0.14	90.9 ± 19.1	3.22 ± 0.91	1.34 ± 0.33	0.05 ± 0.02	<1
Bilobalide	1.9 ± 0.4	0.04 ± 0.00	8.4 ± 1.4	5.35 ± 0.42	30.1 ± 10.1	0.96 ± 0.33	12.5 ± 4.30
Ginkgolide A	14.7 ± 2.5	0.42 ± 0.11	76.8 ± 24.8	22.4 ± 3.9	39.9 ± 3.2	2.04 ± 0.88	19.5 ± 8.4
Ginkgolide B	23.9 ± 3.7	0.84 ± 0.24	155 ± 46	61.4 ± 9.2	27.7 ± 0.9	1.92 ± 0.71	31.7 ± 11.6
Ginkgolide C	6.5 ± 2.7	0.37 ± 0.11	66.3 ± 24.8	84.6 ± 7.8	4.07 ± 1.09	1.01 ± 0.36	13.1 ± 4.7
Ginkgolide J	22.2 ± 10.1	0.35 ± 0.16	69.9 ± 23.9	112 ± 10	23.3 ± 2.3	0.21 ± 0.04	3.92 ± 0.75

elimination features of the flavonols, which counterbalanced their poor oral bioavailability. This resulted in significant accumulation of flavonols during the subchronic treatment with GBE50 (daily dose, 30 mg·kg^{-1}).

This difference between the flavonols and the terpene lactones in elimination kinetics likely resulted from enterohepatic circulation of the flavonols. The terpene lactones did not undergo this type of circulation. Enterohepatic circulation of drugs often occurs by biliary excretion and intestinal reabsorption, which also can involve enterohepatic conjugation and colonic deconjugation. The circulation often leads to prolonged apparent elimination half-lives (Roberts *et al.*, 2002). Most of the major ginkgo flavonol glycosides in ShuXueNing injection had substantial biliary excretion followed by deglycosylation by glycosidases of the colonic microflora. After the resulting aglycones were absorbed, they were immediately conjugated into glucuronides or sulfates. Although the flavonol aglycone conjugates **M16$_{G\text{-}2}$**, **M16$_{G\text{-}3}$**, **M26$_{G\text{-}1}$** and **M32$_{G\text{-}3}$** were excreted into bile, they could re-enter into the systemic circulation by the colonic deconjugation and the intestinal reabsorption and reconjugation, which caused enterohepatic circulation. The aglycone conjugates **M16$_{G\text{-}4}$** and **M26$_{G\text{-}2}$** were poorly excreted in bile and almost all of them appeared to enter the systemic circulation. Because they were also slowly eliminated via renal excretion, these flavonol aglycone conjugates became long-circulating ginkgo metabolites. Although the terpene lactones showed significant biliary excretion and favourable intestinal absorption, their enterohepatic circulation was terminated by substantial elimination from the systemic circulation via the urinary excretion and unknown metabolic reactions (Table 5).

Routes of administration were found to have significant influence on the levels of systemic exposure to the ginkgo flavonols and terpene lactones. In rats given i.v. doses of ShuXueNing injection, the dosed flavonol glycosides were the major forms of flavonols circulating in the bloodstream. In rats given GBE50 by p.o. administration, many flavonol aglycone conjugates were found to be major circulating forms (Figure 4). The total flavonols from p.o. administered GBE50 exhibited bimodal plasma concentration-time profiles (Figure 2). The first concentration peaks were produced by absorption-conjugation of the GBE50-containing aglycones in the small intestine, and the second greater peaks were produced by the colonic deglycosylation-absorption-conjugation of the unabsorbed flavonol glycosides. Unlike that associated with ShuXueNing injection, bilobalide from the p.o. administered GBE50 represented the most abundant terpene lactone measured in plasma. Ginkgolide C from i.v. administered ShuXueNing injection had a higher dose-corrected level of systemic exposure than the other terpene lactones did, but ginkgolide C from p.o. ingestion of GBE50 had a lower dose-corrected level of systemic exposure than the other terpene lactones did.

Drug therapy can fail for PK reasons if too little or too much concentration occurs at the site of action for too short or too long a time. Terpene lactones were found to be eliminated from the body very rapidly (Figure 2 and Tables 2 and 4). It remains open to question whether the reported pharmacological effects of these ginkgo compounds are more closely related to the duration or magnitude of the *in vivo* concentrations produced. The majority of pharmacological assessments have been performed on the flavonol aglycones (Perez-Vizcaino and Duarte, 2010), rather than those ginkgo

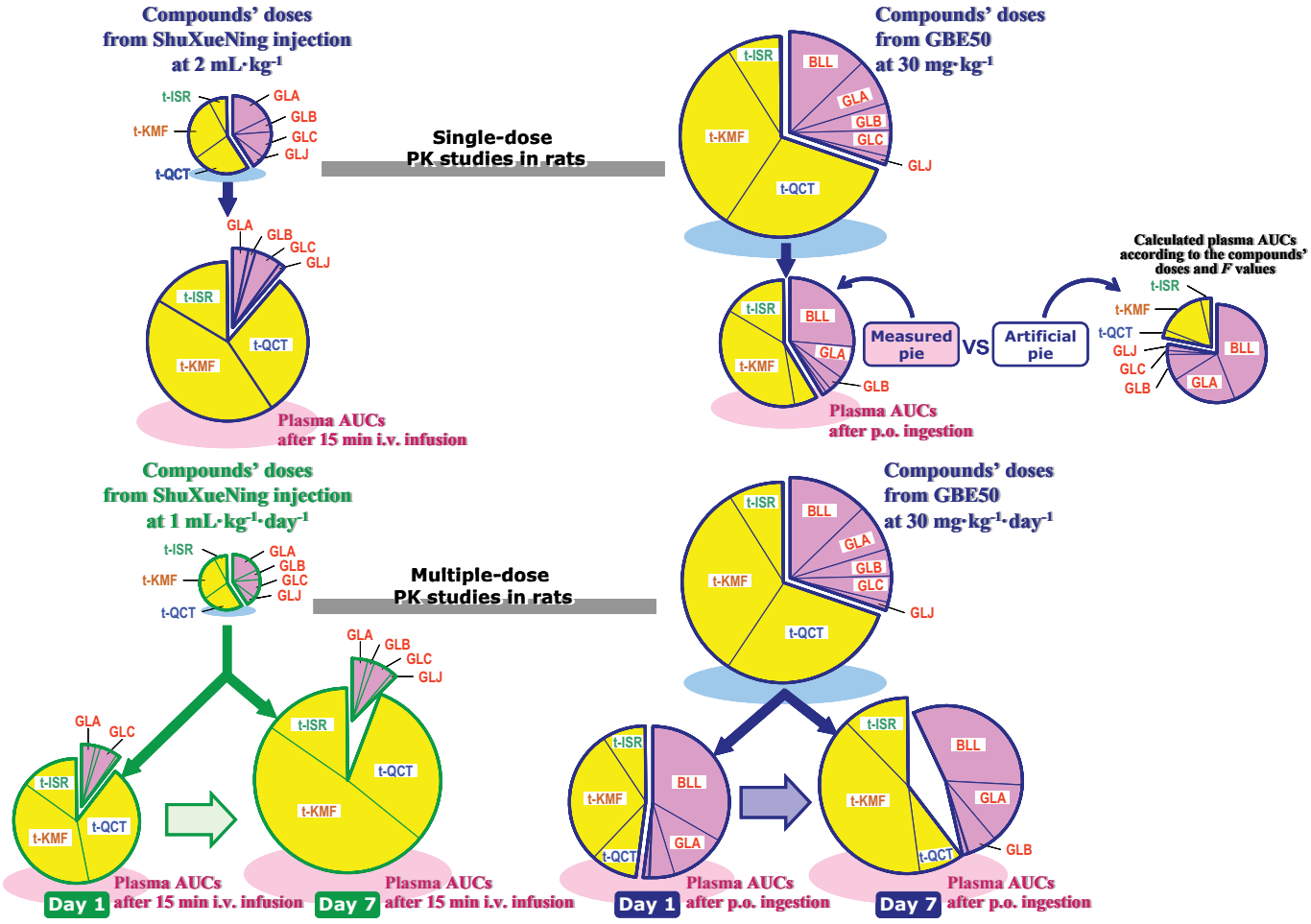


Figure 7

Levels of systemic exposure to total flavonols and terpene lactones after i.v. administration of ShuXueNing injection or p.o. administration of GBE50 in rats and concentrations of these ginkgo compounds in the administered botanical products. The yellow area (ginkgo flavonols) is here compared with the pink area (ginkgo terpene lactones) in the pie chart. The sizes of pies on light blue shadows and the associated sizes of individual sectors for the ginkgo compounds present in ShuXueNing injection or GBE50 were calculated using the content level data shown in Table 1. The sizes of pies on light pink shadows and the associated sizes of the individual sectors for the ginkgo compounds in plasma after administration of ShuXueNing injection or GBE50 were calculated using the AUC data shown in Tables 2 and 4. For comparison purposes, calculations were also made to generate an artificial pie chart for p.o. ingestion of GBE50 at 30 mg·kg^{-1} by considering only the differences in the compound's dose and in compound's oral bioavailability. The differences between the measured pie (on light pink shadow) and the artificial pie (on no shadow) suggested that the slow terminal elimination features of the flavonols counterbalanced the influence of poor oral bioavailability on their levels of systemic exposure. The abbreviations t-QCT, t-KMF, t-ISR, BLL, GLA, GLB, GLC and GLJ denote t-quercetin, t-kaempferol, t-isorhamnetin, bilobalide and ginkgolides A, B, C and J respectively.

flavonol glycosides and aglycone conjugates that substantially detected in plasma after dosing with ginkgo extracts. The flavonol aglycone quercetin, kaempferol and isorhamnetin were not detected in rat plasma after administration of ShuXueNing injection or GBE50. Several recent studies have evaluated the anti-inflammatory and anti-atherogenic activities of quercetin-3-*O*-glucuronide and quercetin-3′-*O*-sulfate from p.o. ingested quercetin or onions (Tribolo *et al.*, 2008; Lodi *et al.*, 2009; Suri *et al.*, 2010; Ishizawa *et al.*, 2011). However, pharmacological evaluations of ginkgo flavonol glycosides and kaempferol and isorhamnetin conjugates have been scarce. Accurate and complete information regarding the pharmacological activity of individual ginkgo flavonol glycosides and aglycone conjugates substantially circulating in the bloodstream after dosing may enhance understanding of the therapeutic effects of ShuXueNing injection and GBE50 and of the differences between them.

The delivery of therapeutic substances to the brain is often limited by the blood–brain barrier, which has often impeded the translation of many promising findings from the laboratory to the clinic (Cecchelli *et al.*, 2007). In the current study, brain exposure to the flavonols and terpene lactones was measured in rats. This is relevant to assessment of the ginkgo's reported neuroprotective properties. Cerebral exposure was found to be negligible for the flavonols and low for the terpene lactones (Figures 5 and 6). Brain penetration of drugs across the blood–brain barrier requires high membrane permeability (Fagerholm, 2008). The flavonol glyco-

sides and aglycone conjugates measured in plasma have poor membrane permeability, and the membrane permeability of terpene lactones, which is intermediate (Li *et al.*, 2012), is probably still too low for brain penetration. Reported concentrations of terpene lactones exerting *in vitro* neuroprotective activities range from 10 to 80 μM (Luo *et al.*, 2002; Ivic *et al.*, 2003; Tchantchou *et al.*, 2009; Shi *et al.*, 2010), which is 2 or 3 orders of magnitude higher than the concentrations measured in the extracellular fluid of the brain in the current study. Given this information, caution should be exercised when translation of the *in vitro* experimental findings of ginkgo compounds' neuroprotective properties to clinical settings. Chronic vascular insufficiency, hypoperfusion, and blood–brain barrier damage are involved in the aetiology of neurodegenerative disorders and vascular alterations may take place at a presymptomatic stage. Insights into cerebrovascular disorders have opened perspectives for new treatment of neurodegenerative diseases by targeting vessels or using angiogenic factors (Storkebaum *et al.*, 2011). Unlike cerebral exposure, the levels of which were very low, systemic exposure to ginkgo flavonols and terpene lactones was significant. Instead of symptomatic treatment of existing dementia, ginkgo extracts might be more effective in preventing of neurodegenerative disorders by slowing down vascular alterations. The nervous, endocrine and immune systems are intimately linked and interdependent. Age-related changes in the neuro-endocrine-immune axis influence both the aging progress and the related diseases, such as neurodegenerative disorders (Vida and De la Fuente, 2013). A link has been drawn between diabetes mellitus and Alzheimer's disease. Drug therapies that increase peripheral insulin sensitivity are showing benefits in Alzheimer's disease patients (Glass *et al.*, 2010). In addition, somatostatin receptors may be pharmacological-target candidates for prevention and treatment of Alzheimer's disease. Intravenous administration of octreotide, which has shown poor brain penetration, can improve memory in Alzheimer's disease patients (Craft *et al.*, 1999; Watson *et al.*, 2009). Enhancement of a patient's innate immunity may represent a novel approach to Alzheimer's disease therapy (Cashman *et al.*, 2008). The naturally occurring compounds curcuminoids selectively enhance Aβ phagocytosis and gene transcription in blood cells of Alzheimer's disease patients (Darvesh *et al.*, 2012). Ginkgo extract has been found to prevent insulin resistance (Zhou *et al.*, 2011). It is unclear whether the ginkgo flavonols and terpene lactones that have poor brain penetration have effects on the neuro-endocrine-immune axis.

PK studies aim to bridge the gaps between phytochemistry and pharmacology and between pharmacology and botanical therapeutics (Li, 2012). Body exposure to the bioactive ingredients of a botanical medicine is a crucial determinant of its drug response and therefore the efficacy and safety. In earlier studies (Lu *et al.*, 2008; Liu *et al.*, 2009; Hu *et al.*, 2013), the compounds that had significant and dose-dependent levels of systemic exposure after dosing with herbal medicines were identified from groups of the known pharmacologically active constituents present in the administered medicines. In the current study, the systemic exposure levels of total flavonols and terpene lactones were found to increase in a dose-dependent manner in rats given ShuXueNing injection or GBE50 (Table 3). These plasma ginkgo compounds could be suitable for use as dose-dependent PK markers to indicate rat exposure to the botanical products. Unlike the plasma terpene lactones, which were detected as single chemical entities, the plasma total flavonols were detected on the basis of the HCl-hydrolysis of multiple flavonol glycosides and aglycone conjugates. The formation of the flavonol aglycone conjugates was affected by the action of the colonic microflora. Because the human colon is significantly more heavily bacterially colonized than the rat colon (Sousa *et al.*, 2008), whether plasma total flavonols are still dose-dependent PK markers in humans remains to be assessed.

In summary, the ginkgo flavonols and terpene lactones have a noted difference in elimination kinetics. The slow terminal elimination features of the flavonols counterbalance the influence of poor oral bioavailability on their levels of systemic exposure. This causes significant accumulation in the bloodstream in response to subchronic treatment with ShuXueNing injection or GBE50. Unlike the flavonols, terpene lactones showed rapid renal excretion and may have undergone unknown metabolic processes, which terminated their enterohepatic circulation. The routes of administration showed significant influences on the systemic exposure to the ginkgo compounds. The flavonol glycosides were detected as major forms in plasma after ShuXueNing injection, but plasma flavonol aglycone conjugates were predominant after dosing with GBE50. Although the levels of systemic exposure to flavonols and terpene lactones were significant and increased in a ShuXueNing injection or GBE50 dose-dependent manner, the levels of cerebral exposure to these ginkgo compounds were very low. Further PK studies are planned to assess the pharmacokinetics and metabolism of the ginkgo compounds in humans and the influence of the colonic microflora on the compounds' exposure in the body.

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Conflict of interest

There are no competing interests to declare.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1 Supplemental Methods.