Determination of Sweroside in Rat Plasma and Bile for Oral Bioavailability and Hepatobiliary Excretion

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Sweroside is an active ingredient of iridoid glycoside isolated from the flower buds of *Lonicera japonica* THUNB. A quantitative HPLC-UV method was developed for monitoring sweroside in rat plasma, urine, feces and bile. The method was successfully applied for a basic pharmacokinetic study. The obtained data of pharmacokinetics were applied to evaluate the oral bioavailabilities of sweroside and the active ingredients of purified herbal extracts (IGEs-1). The absolute bioavailability was estimated to $F_{\text{sweroside}}$ 0.31% and $F_{\text{IGEs-1}}$ 0.67%. The majority of sweroside excreted to feces revealed one reason of the low oral bioavailability. The values of $F_{\text{IGEs-1}}$ much higher than that of $F_{\text{sweroside}}$ reveals that ingredients in IGEs-1 such as loganin, secoxyloganin and some phenolic acids may promote the absorption of sweroside. The study of hepatobiliary excretion was achieved by an *in vivo* microdialysis sampling method after intravenous administration of sweroside. The percentage of accumulation of free form sweroside in bile duct was 31.2±7.2% of the total dosage. It may be one reason why sweroside possesses strong hepatoprotective effect.

Key words sweroside; microdialysis; pharmacokinetic; bioavailability; hepatobiliary excretion

Sweroside ($C_{16}H_{22}O_9$, molecular weight 358.344), an iridoid glycoside, belongs to a large group of natural monoterpenoids with a glucose moiety attached to C-1 in the pyrane ring. It widely exists in plants of many families[1—3] and shows a wide variety of biological activities such as hepatoprotective effect, antitumor, antibacterial, antifungal.[4—7] Sweroside is also the main ingredient of purified herbal extracts from the flower buds of *Lonicera japonica* THUNB. (IGEs-1) which were demonstrated to possess perfect hepatoprotective effect in our preliminary experiments. Since sweroside and its correlative plant extracts have exhibited these pharmacological effects, it would be hopeful that sweroside and IGEs-1 to be developed to some promising preparations of herbal medicinal products (HMPs).

Recently, HMPs are getting more common in the main stream in previously skeptical markets such as the United States and United Kingdom.[8] Establishing the pharmacological basis for efficacy of HMPs is a constant challenge. Of particular interest is the question of bioavailability to assess to what degree and how fast compounds are absorbed after administration of HMPs. Of further interest are the elucidation of metabolic pathways, and the assessment of elimination routes and their kinetics. These data become an important issue to link data from pharmacological assays and clinical effects. A better understanding of the pharmacokinetics and bioavailability of HMPs can also help in designing rational dosage regimens. Despite a wide range of research in either sweroside or *L. japonica*,[9,10] to our best knowledge, there is no information available on the pharmacokinetics of sweroside orally and intravenously administered to investigate its oral bioavailability and hepatobiliary excretion. Moreover, no report has been published to elucidate the metabolites and the metabolic pathways of sweroside.

Furthermore, the development of sampling technique has been a focus *in vivo* experiment.[11] Microdialysis system is a sampling technique that can reduce endogenous interference and can be easy to couple with detector for on-line analysis. A series of papers reported the successful application of microdialysis system to pharmacokinetic study.[12—15] However, there is no paper to present the application of microdialysis for the preparation of bile samples of sweroside in rat.

In the present study, a simple, stable and accurate HPLC-UV method was developed to determine sweroside in rat plasma, urine, feces and bile simultaneously. The method was applied to basic pharmacokinetic and oral bioavailability studies after intravenous administration of sweroside and oral administration of sweroside and IGEs-1. A sampling method through a validated bile microdialysis system coupled with this HPLC-UV determination was developed to evaluate the hepatobiliary excretion of sweroside in rat.

Experimental

Preparation of IGEs-1 and Sweroside The flower buds of *Lonicera japonica* THUNB. were collected at the middle of May from Mi county, Henan province, China. The dried and powdered materials were refluxed three times at 85 °C with 70% ethanol (1 : 10, g/ml) after defatted with petroleum. The ethanol extracts were concentrated, and then centrifugated. The supernatant was pushed through a macroreticular resin column, and eluted with 25% ethanol. After concentrated, the fraction was partitioned four times with water-saturated *n*-butanol. The organic layer was evaporated in vacuum, and the residue, named IGEs-1 was obtained. IGEs-1 was further purified by column chromatography over silica gel eluted with chloroform–methanol (85 : 15), and then pure sweroside was obtained. The yield of IGEs-1 could reach over 2% (g/g) and the content of sweroside in IGEs-1 was about 67% (g/g) by HPLC assay. The structure of sweroside was elucidated by comparing the spectral data (^{1}H-, ^{13}C-NMR, and MS) with the literatures.[16,17]

Chemical and Reagent Standard sweroside and gardenoside (as the internal standard, Fig. 1) were purchased from the National Institute for the Control of Pharmaceutical and Biological Product (Beijing, China). Standard sweroside and gardenoside were dissolved in methanol at the concentration of 1 mg/ml and 25 μg/ml as the stock solution respectively, and then stored at 4 °C in a refrigerator. β-Glucuronidase (type H-1 from Helix pomatia, 338000 unit/g) was purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Methanol was of HPLC grade from Merck (Darmstadt, Germany); deionized water was prepared using a Milli-Q purification system (Millipore, Milford, MA, U.S.A.); all the other solvents were analytical grade.

Animals Adult, male Sprague-Dawley rats (280—320 g) were obtained from the Animal Breeding Center of China Pharmaceutical University (Nangjing, China). These animals were specifically pathogen-free and allowed to acclimate to their environmentally controlled quarters (24±1 °C

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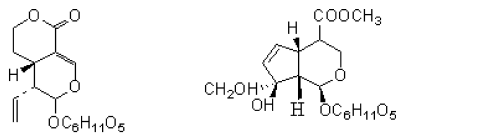
and 12 : 12 h light–dark cycle) for one week. Animals had free access to food and water and fasted overnight before starting the experiments.

Administration and Preparation of Samples The femoral vein was exposed for sweroside injection (1 mg/kg, dissolved in saline). IGEs-1 (800 mg/kg) and sweroside (500 mg/kg) were orally administered, respectively.

Five hundred microlitre blood was withdrawn from the fossa orbitalis into heparin rinsed tubes according to a programmed schedule at 5, 10, 15, 20, 30, 60, 90, 120, 150, 180 and 300 min after dosing. Each blood sample was centrifuged at 2500×***g*** for 15 min at 4 °C. Two hundred microlitre plasma sample was incubated (1 h, 37 °C) with 200 μl of β-glucuronidase enzyme solution (3000 unit/ml). Then, the solution was vortex-mixed with 1.5 ml methanol for 5 min. The denatured protein precipitate was separated by centrifugation at 12000×***g*** for 5 min at 4 °C. The supernatant was evaporated to dryness in a gentle stream of nitrogen at 40 °C and the residue was reconstituted in 100 μl of methanol solution (30 : 70) and then stored at 4 °C in refrigerator for analysis.

Urinary samples and fecal samples were collected within a rational interval by metabolic cages after sweroside administration (500 mg/kg, *per os* (*p.o.*)). A 1.0 ml of rat urine sample was filtered and then transferred into a SPE cartridge (3 ml capacity, 500 mg ODS, Supelclean™, Supelco) preconditioned with 2.0 ml methanol and 2.0 ml double-distilled water. The cartridge was washed with 2.0 ml water followed by elution with 0.5 ml methanol. The eluate was stored at 4 °C in refrigerator for analysis. The weights of fecal sample were recorded and an appropriate volume of methanol (50 ml per gram feces) was added. The mixtures were homogenized by a glass rod and the homogenous slurry was ultrasonically extracted for 20 min. After filtering, 1.0 ml of the resulting solution was diluted with methanol to 100 ml and then stored in refrigerator at 4 °C for analysis.

Bile samples were obtained through a bile microdialysis system in anesthetic rats after injection of sweroside (1 mg/kg, dissolved in saline). Bile microdialysis system consisted of a microinjector, a microinjection pump, an adjustor of microinjection pump (BioanalyticalSystem, Inc., West Lafayette, U.S.A.) and microdialysis probes. The bile duct microdialysis probes were prepared by our laboratory, according to the design originally described by Lunte *et al.*[11] A 5 cm long dialysis membrane (Fresenius Group, Bad Homburg, Germany) was inserted into a polyethylene tubing (PE-60; 0.76 mm i.d.; 1.22 mm o.d.), and the ends of the dialysis membrane and PE-60 were inserted into a silica tubing (40 Am i.d; 140 Am o.d.) and PE-10 (0.28 mm i.d.; 0.61 mm o.d.), respectively. Both the ends of tubing and the union were cemented with epoxy and allowed to dry for a period of 24 h. After the bile duct cannulation, the probe was perfused with saline at a flow rate of 1.5 μl/min. A 30 μl of bile sample was withdrawn from an interval of 20 min and then stored in refrigerator at 4 °C for analysis.



The structure of sweroside (A) The structure of gardenoside (B)

Fig. 1. Chemical Structures of Sweroside (A) and Internal Standard Gardenoside (B)

Chromatographic Conditions HPLC was performed on an Agilent 1100 series LC system (Agilent Technologies Waldbronn, Germany) equipped with a binary pump, an auto plate-sampler, a thermostatically controlled column apartment and a diode array detector. The separation was achieved by an Agilent Zorbax C18 column (150 mm×4.6 mm i.d., 5 μm) connected with a guard column (12.5 mm×4.6 mm i.d., 5 μm). The mobile phase consisted of (A) 0.05% aqueous formic acid (v/v) and (B) methanol using a gradient elution of 5—30% B at 0—10 min, and 30% B at 10—20 min. The flow rate was 1.0 ml/min. The detection wavelength was 240 nm. Every sample spiked with 10 μl of the internal standard (IS) stock solution was centrifuged at 12000×***g*** for 10 min at 4 °C and an aliquot (20 μl) of the supernatant was injected into HPLC.

Method Validation Calibration curves were established using blank matrix spiked with different amounts of sweroside over a concentration range of 0.05—50.00 μg/ml. The peak area ratios (y) of sweroside to the internal standard *versus* the concentrations of sweroside (x) were used to calculate linear regression. Blank matrix spiked with amounts of sweroside at high, mid, low concentration levels (10.0, 1.0, 0.1 μg/ml) as quality control samples (QC samples) were used for evaluation of the intra-day and inter-day precision and accuracy. Five replicates of QC samples on the same day and five consecutive days were determined and the precision was evaluated by the intra-day and inter-day relative standard deviation (R.S.D., %). The accuracy was a measure of the systematic error or bias which was defined as the agreement between the observed concentration and nominal value. The accuracy was calculated from the mean value of the observed concentration (C_{obs}) and the nominal concentration (C_{nom}) as follows: accuracy (bias, %)=$[(C_{obs}-C_{nom})/C_{nom}]\times 100$. The limit of detection (LOD) was defined by the value at signal-to-noise ratio of 3. The lowest concentration of linear regression was defined as the limit of quantification (LOQ). The recoveries from plasma, urine and feces matrices were assessed by QC samples. The samples were processed using the procedures according to the above-mentioned preparation methods and analyzed by HPLC. The extraction recovery was calculated by comparing the peak area ratios to IS of the extracted samples with standard solutions at the same concentration level. Bile recovery test was prepared by a microdialysis probe directly. The *in vivo* recovery across the microdialysis probe was determined by the loss (the extraction ratio) of sweroside, which was calculated from the concentration in the dialysate (C_{out}) relative to the concentration of sweroside in the perfusate (C_{in}). *In vivo* recovery from bile (R_{dial}) was calculated by the following equation, $R_{dial}=1-(C_{out}/C_{in})$.[14,18] The stability of the sample was assessed by determining the QC samples under ambient, frozen and freeze-thaw storage.

Pharmacokinetic Application Pharmacokinetic calculations were performed on each individual set of data using DAS (Drug and Statistics for Windows) software package (PK software version 2.0, Mathematical Pharmacology Professional Committee of China, Shanghai, China) by noncompartmental method. The area under the concentration–time curve (*AUC*) was calculated according to the linear trapezoidal method. The absolute oral bioavailability (F) of the drug was generally measured by comparing the respective *AUC*s after oral and intravenous administration according to the following equation[19]: $F=(AUC_{p.o.}/\mathrm{Dose}_{p.o.})/(AUC_{i.v.}/\mathrm{Dose}_{i.v.})$.

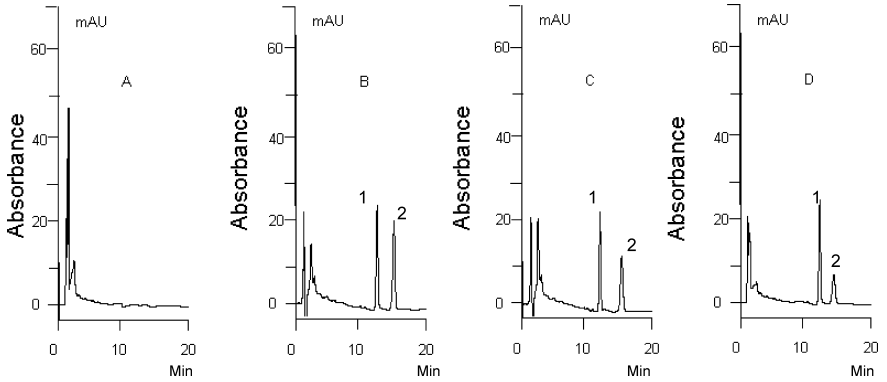


Fig. 2. Typical Chromatograms of (A) Drug-Free Plasma Extract, (B) Blank Plasma Spiked with Standard Sweroside (1 μg/ml), (C) Plasma Containing Sweroside (0.57 μg/ml) Collected from the Rat Plasma 30 min after Sweroside Administration (500 mg/kg *p.o.*), (D) Plasma Containing Sweroside (0.38 μg/ml) Collected from the Rat Plasma 30 min after IGEs-1 Administration (800 mg/kg *p.o.*)

(1) Internal standard (gardenoside) and (2) sweroside.

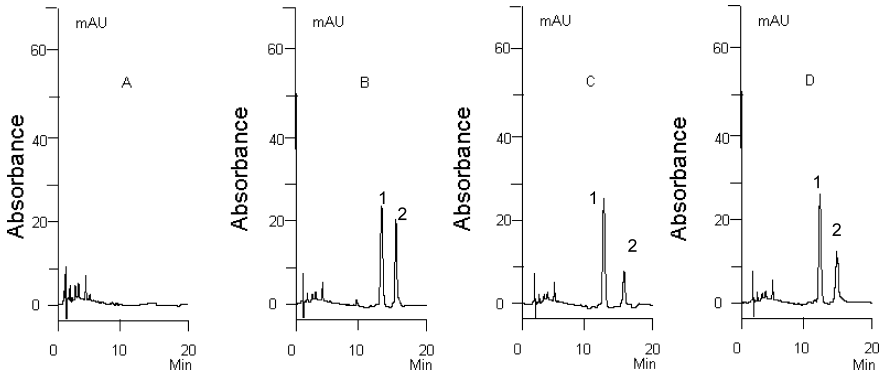


Fig. 3. Typical Chromatograms of (A) a Drug-Free Bile Sample, (B) a Drug-Free Bile Sample Spiked with Standard Sweroside (1.0 μg/ml), Bile Samples Containing (C) Sweroside (0.34 μg/ml) Collected between 40—60 min and (D) Sweroside (0.52 μg/ml) Collected between 80—100 min after Sweroside (1 mg/kg i.v.) Administration

(1) Internal standard (gardenoside) and (2) sweroside.

Results and Discussion

HPLC Determination Figure 2A illustrates the chromatogram of the drug-free plasma extract, showing a clean, stable baseline without interfering endogenous peaks. No carry-over peaks were detected in the chromatograms of plasma samples. Figure 2B shows a chromatogram of sweroside (1.0 μg/ml) spiked in rat plasma. Figures 2C and D are the chromatograms of plasma samples containing sweroside (0.57, 0.38 μg/ml, respectively) collected 30 min after sweroside (1 mg/kg, i.v.) and IGEs-1 administration (800 mg/kg, i.v.). The retention times (t_R) of sweroside and the internal standard were 15.9 and 14.5 min, respectively, and no peak distortion was visible. Figures 3A—D respresent the chromatograms of drug-free bile samples, a drug-free bile sample spiked with sweroside (1.0 μg/ml) and two bile samples containing sweroside (0.34, 0.52 μg/ml) collected between 40—60 min and 80—100 min after sweroside administration (1 mg/kg, i.v.), respectively. In the HPLC assay, no interference from endogenous substance was observed in rat plasma, urine, feces and bile.

Method Validation As shown in Table 1, the intra-day and inter-day precision (R.S.D. %) were all less than 10% in various matrices. Analytical accuracy expressed as the bias variation was ranged from −6.0 to 9.0%. The overall reproducibility of the method was acceptable. Calibration curves, the scope of linearity, the limit of detection (LOD) and the limit of quantitation (LOQ) are presented in Table 2. The extraction recovery from plasma, urine and feces were ranged from 88.7 to 91.2% with S.D. less than 2.4%, which suggested that the pre-treatment method for sweroside was quite efficient. The *in vivo* recovery in bile microdialysis probe was 54.2±0.9%. The value of bile recovery test depended on the sampling efficiency of microdialysis system especially dialysis membrane. As reported in some studies,[14,15,18—20] the variability of R_{dial} was stable (S.D 0.9%) and then the sampling technique was suitable for the application of pharmacokinetics. The stability study showed that QC samples were allowed to stand at room temperature for at least 8 h and −20 °C for at least 2 weeks, and stable after at least three freeze-thaw cycles with the overall bias (%) between the observed concentration and nominal value lower than 7.6%. The validation data were acceptable for sweroside analysis in support of further study of pharmacokinetics.

Table 1. Intra-day and Inter-day Precision (R.S.D., %) and Accuracy (Bias, %) of the HPLC Method for the Determination of Sweroside in Rat Plasma, Urine, Feces and Bile

Matrix	Nominal concentration (μg/ml)		Observed concentration (μg/ml)	Precision (% R.S.D.)	Accuracy (% Bias)
Plasma	Intra-day	0.1	0.097±0.007	7.2	−3.0
	(n=5)	1.0	1.021±0.071	6.9	2.1
		10.0	10.281±0.876	8.5	2.8
	Inter-day	0.1	0.098±0.009	9.1	−2.0
	(n=5)	1.0	1.042±0.054	5.2	4.2
		10.0	10.447±0.656	6.2	4.5
Urine	Intra-day	0.1	0.094±0.007	7.4	−6.0
	(n=5)	1.0	1.067±0.045	4.2	6.7
		10.0	9.959±0.367	3.7	−4.1
	Inter-day	0.1	0.104±0.006	5.8	4.0
	(n=5)	1.0	1.012±0.078	7.7	1.2
		10.0	10.161±0.672	6.6	1.6
Feces	Intra-day	0.1	0.109±0.007	6.4	9.0
	(n=5)	1.0	1.013±0.028	2.8	1.3
		10.0	10.138±0.369	3.6	1.4
	Inter-day	0.1	0.105±0.004	3.8	5.0
	(n=5)	1.0	1.072±0.085	7.9	7.2
		10.0	10.259±0.375	3.6	2.6
Bile	Intra-day	0.1	0.098±0.007	7.1	−2.0
	(n=5)	1.0	1.074±0.025	2.3	3.5
		10.0	10.346±0.867	8.3	1.6
	Inter-day	0.1	0.107±0.003	2.8	7.0
	(n=5)	1.0	0.976±0.078	7.9	−2.4
		10.0	10.085±0.675	6.7	0.8

Pharmacokinetics and Oral Bioavailability The mean concentration–time profiles of sweroside in rat plasma after administration of sweroside (1 mg/kg, i.v.; 500 mg/kg, *p.o.*) and IEG-1 (800 mg/kg, *p.o.*) are shown in Fig. 5. The differences of the kinetic profiles were visible between oral and intravenous administration, whereas the same tendency was presented in the profile of oral administration of sweroside and IEG-1. Because of good water solubility, sweroside could be easily eliminated through renal tubules. As a result, the concentration of sweroside quickly decreased after intravenous administration, and was lower than LOQ after 120 min. Sweroside after oral administration could be also eliminated quickly. However, the dose of oral administration (500 mg/kg, *p.o.*) was much higher than that of intravenous

Table 2. Calibration Curves, the Scope of Linearity, Sensitivity and Recovery

Matrix	Calibration curve	Scope (μg/ml)	r^2	LOQ (μg/ml)	LOD (μg/ml)	Recovery±S.D.
Plasma	$y=3.8787x+0.1314$	0.05—5.00	0.9989	0.05	0.015	90.2±1.8%
Urine	$y=3.9860x+0.0982$	0.05—15.00	0.9991	0.05	0.015	88.7±2.4%
Feces	$y=3.8851x+0.1199$	0.05—5.00	0.9986	0.05	0.012	91.2±1.9%
Bile	$y=3.9539x+0.1363$	0.05—5.00	0.9979	0.05	0.013	54.2±0.9%[a)]

a) *In vivo* microdialysate recovery (%) of sweroside from rat bile.

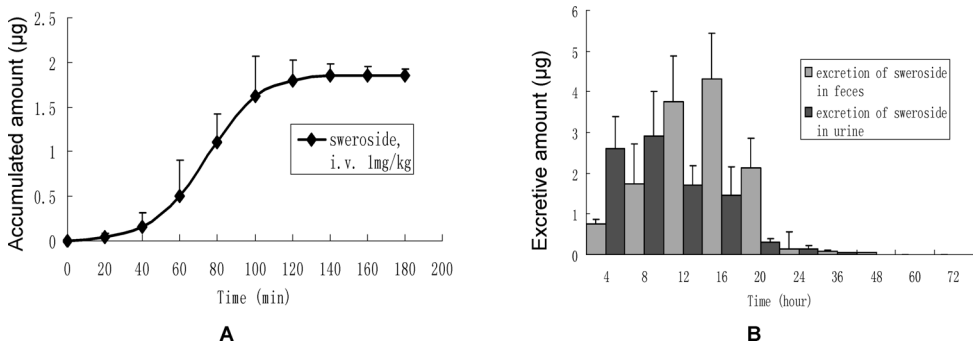


Fig. 4. Excretion of Sweroside in Bile, Urine and Feces

(A) Cumulative excretion of sweroside in rat bile after the administration of sweroside (1 mg/kg i.v.). (B) Excretion of sweroside in rat urine and feces after the administration of sweroside (500 mg/kg *p.o.*).

administration (1 mg/kg, i.v.). Thus, it caused different appearances of elimination (such as $t_{1/2}$, *etc.*) between these two methods of administration. The relevant pharmacokinetic parameters are shown in Table 3. The oral bioavailability of sweroside after administration (500 mg/kg, *p.o.*), and that of IGEs-1 (800 mg/kg, *p.o.*) administration were about 0.31% and 0.67%, respectively. The lower oral bioavailability might be due to the first-pass metabolism and intestinal bacterial biotransformation.[21)] The high hydrophilicity of sweroside caused it difficult to go straight through mesentery. The hepatoprotective effect of sweroside may be encounted hepatic uptake and biliary excretion, which is mainly in feces *via* bile.[22)] The present results indicated that the amounts of sweroside in feces were much higher than in the plasma. Almost 43.38±3.2% of sweroside was cumulated in the feces within 72 h after its administration (500 mg/kg, *p.o.*). It demonstrated that the majority of sweroside was unabsorbed in the gastrointestinal tract and excreted directly. It provided evidence to explain the lower oral bioavailability of sweroside.

Comparing with the values of $F_{\text{sweroside}}$ and $F_{\text{IGEs-1}}$, an interesting fact was found that the value of $F_{\text{IGEs-1}}$ was much higher than that of $F_{\text{sweroside}}$. In other words, swersoide in IGEs-1 was well absorbed in rat than pure sweroside administration. The other ingredients in IGEs-1 such as loganin, secoxyloganin and some phenolic acids might have synergistic effect with sweroside. The other consitituents in IGE-1 might not only promote the absorption of sweroside but also further influence the pharmacological effects of sweroside and its metabolites. Some papers reported the compatibility regulation and interaction of Chinese formula.[23)] Unlike the single chemical compound, extracts from herbal medicines show extremely complicated pharmacological reactions, and constituents in extracts may be substrates, inhibitors, or inducers of CYPs in pharmacokinetics, leading to some synergetic and/or antagonistic reactions between different compounds. Nevertheless, the influence of the other ingredients in IGEs-1 should be complex and more experiment results would be needed to prove the complex interaction. Through evaluation of bioavailability, an initial message of interaction of compounds in IEG-1 would be obtained in order to design rational dosage regimens.

Table 3. Main Pharmacokinetic Parameters in Rat Blood, Urine, Feces and Bile after Sweroside Administration (1 mg/kg, i.v. and 500 mg/kg, *p.o.*) and IGEs-1 Administration (800 mg/kg, *p.o.*) ($n=5$)

Parameters	Sweroside, i.v. 1 mg/kg	Sweroside, *p.o.* 500 mg/kg	IGEs-1, *p.o.* 800 mg/kg
C_{max} (μg/ml)	1.18±0.54	0.32±0.02	0.53±0.02
$t_{1/2}$ (min)	23.27±2.06	64.34±3.21	59.87±2.58
$AUC_{(0—240)}$ (μg ml^{-1} min^{-1})	12.34±0.83	19.09±3.47	41.32±3.47
F		0.31%	0.67%

Hepatobiliary Excretion To evaluation of hepatobiliary excretion of sweroside, the kinetics of the drug in free form was monitored. As shown in Fig. 4A, sweroside was detected during the period of 20—40 min, and its content increased gradually until 120 min when a period of 'plateau' was entered. The accumulated hepatobiliary excretion of sweroside corrected by *in vivo* bile recovery was 1.86 μg. Plentiful unconjugated sweroside was found in rat bile duct 30 min after the administration. The phenomenon revealed that through bile duct was an important route for sweroside. Meanwhile, only free form drug could easily transverse cells or even capillary membranes, and it might provide some evidence to explain the mechanism of hepatoprotective effect of sweroside.

Conclusion

In this study, the HPLC-UV method for determining

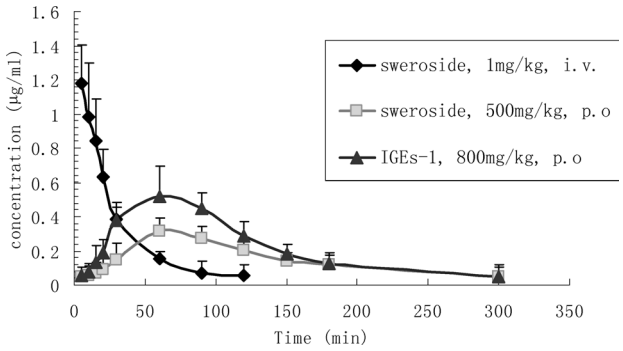


Fig. 5. Concentration–Time Profiles for Sweroside in Rat Plasma after Sweroside (500 mg/kg *p.o.*), Sweroside (1 mg/kg, i.v.) and IGEs-1 (800 mg/kg, *p.o.*) Administration, Respectively.

sweroside in rat plasma, urine, feces and bile was validated to be simple, stable, rapid and accurate. The method was successfully applied for pharmacokinetic studies. The determination of sweroside in rat plasma, urine, feces and bile simultaneously also gave an overall view to elucidate the metabolic paths, elimination routes and the kinetics of sweroside *in vivo* course. Meanwhile, bile microdialysis system was successfully applied to study the hepatobiliary excretion of sweroside in rat. It was proved to be a simple and easy sampling technique without pre-treatment process. Meanwhile, the majority of sweroside detected in the feces revealed the reason for such low oral bioavailability. The results helped to better understand the disposition of sweroside, as well as to design rational dosage regimens of the compound for clinical use as a potential drug.

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