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Determination and Pharmacokinetics of 2''-O-Rhamnosylswertisin in Rat Plasma by RP-LC

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A simple, rapid and sensitive HPLC-UV method for determining 2''-O-rhamnosylswertisin in rat plasma was developed. Sample preparations were carried out by protein precipitation with acetonitrile, followed by the evaporation of the acetonitrile to dryness. The analyses were carried out on a Kromasil C_{18} (250 mm × 4.6 mm, 5 µm) column kept at room temperature (25 ± 3 ºC) using a mobile phase composed of acetonitrile-water-glacial acetic acid (19.9:80:0.1, v/v/v). The flow rate was 1 mL min^{-1}. The recovery, stability, inter- and intra-day precisions and accuracy for all samples were satisfactory. The method was applied to the pharmacokinetics study of 2''-O-rhamnosylswertisin after oral administration of 13 g kg^{-1} *Belamcanda chinensis* leaves extract to Wistar rats. Pharmacokinetics after intravenous administration of 10 mg kg^{-1} of 2''-O-rhamnosylswertisin and the absolute bioavailability was also studied.

Key Words: Column liquid chromatography, 2''-O-Rhamnosylswertisin, Belamcanda chinensis leaves extract, Pharmacokinetics, Absolute bioavailability.

INTRODUCTION

Belamcanda chinensis rhizome is widely used in traditional Chinese medicine[1-7]. But few studies had reported about the leaves of *Belamcanda chinensis*. The leaves extract showed good hypoglycemic activity in pharmacologic experiments. The total flavonoids extract of the leaves of *Belamcanda chinensis* had conformed as the effective components [not published]. 2''-O-Rhamnosylswertisin (ORS) is one of the main compounds of the extract which shows hypoglycemic activity in adipocyte model screening[8]. This compound exist in many plants[9-11] and have potent antinociceptive activity[12]. The pharmacokinetics study of 2''-O-rhamnosylswertisin is important for the potential clinical application of *Belamcanda chinensis* extract.

A sensitive HPLC method for the quantitative for determination of 2''-O-rhamnosylswertisin in rat plasma was developed for the first time. The method was applied in the pharmacokinetics study of 2''-O-rhamnosylswertisin after intravenous administration and after oral administration of *Belamcanda chinensis* extract. Absolute bioavailability of 2''-O-rhamnosylswertisin was also studied.

EXPERIMENTAL

2''-O-Rhamnosylswertisin (99.0 % purity) and *Belamcanda chinensis* extract were provided by Beijing BIT & GY Pharmaceutical R & D Co. Ltd. (Beijing, China). HPLC analysis showed that *Belamcanda chinensis* extract contained 3 % 2''-O-rhamnosylswertisin. Ferulic acid which used as the internal standard (I.S.) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Their structures were shown in Fig. 1. Glacial acetic acid (analytical grade) was of analytical grade was produced by Beijing Xingzhi Chemical Factory (Beijing, China). Acetonitrile was of chromatographic grade from Fisher Scientific (New Jersey, USA). Distilled water, prepared by a Milli-Q water purification system (Bedford, USA) was used throughout the study.

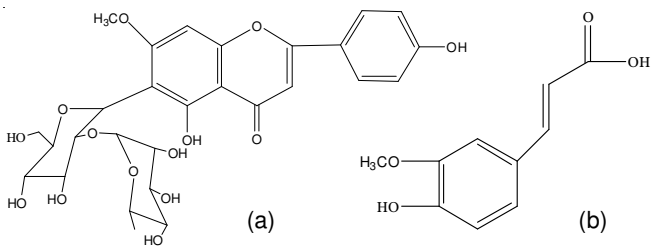

Fig. 1. Chemical structures of (a) 2''-O-rhamnosylswertisin and (b) ferulic acid

Animals: Male Wistar rats (body weight: 200 ± 20 g each) were purchased from Department of Laboratory Animal Science, Peking University Health Science Center (Beijing, China). All animals were kept in an environmentally controlled

breeding room at 20 ± 2 ºC, humidity: 60 ± 5 % and 12 h dark to light cycle for 1 week before the experiment. All the rats were fasted for 12 h before the experiment and had free access to water.

HPLC conditions: A LC-20A Series chromatographic system (Shimadzu, Kyoto, Japan) equipped with a SPD-20A (Shimadzu, Kyoto, Japan) UV detector was used for 2''-O-rhamnosylswertisin assays. The detector wavelength was set at 338 nm. The analyses were carried out on a Kromasil C_{18} (250 mm × 4.6 mm, 5 µm) column (Elite, Dalian, China) kept at room temperature (25 ± 3 ºC) using a mobile phase composed of acetonitrile-water-glacial acetic acid (19.9:80:0.1, v/v/v). The flow rate was 1 mL min^{-1}. And a LC-solution workstation was used for acquisition of data.

Preparation of stock and working solutions: The standard stock solution was prepared by dissolving of 10.3 mg 2''-O-rhamnosylswertisin in 25 mL methanol to obtain a nominal concentration of 0.412 mg mL^{-1}. The internal standard solution was prepared at 0.093 mg mL^{-1} and then diluted 30 folds to obtain a nominal concentration of 31 ng mL^{-1}. All the stock solutions were kept at 4 ºC and brought to 20 ºC before use. Working solutions were prepared by diluting the stock solution with methanol to different concentrations.

Preparation of calibration standards and quality control samples: Calibration standards in plasma at concentrations of 0.206, 2.065, 4.130, 8.260, 16.520, 41.300 and 82.600 µg mL^{-1} were prepared by spiking control rat plasma with appropriate amounts of the standard stock solution. Quality control (QC) samples to determine the recovery, accuracy and precision of the method were independently prepared at low (0.206 µg mL^{-1}), medium (20.650 µg mL^{-1}) and high (82.600 µg mL^{-1}) concentrations in the same way as the rat plasma samples for calibration.

Drug administration and sampling: Six rats were dosed with 13 g kg^{-1} of *Belamcanda chinensis* extract. Serial blood samples (0.5 mL) were collected at 0, 5, 15, 30, 45, 60, 120, 240, 360, 480 and 720 min post-dose. 2''-O-rhamnosylswertisin was dissolved in alcohol and diluted in 40 folds 0.9 % physiological saline to a final concentration of 1 mg/mL and then dozed at 10 mg kg^{-1}. Serial blood samples (0.5 mL) were collected at 0, 2, 5, 10, 15, 30, 45, 60, 120, 240 min post-dose. All blood samples were stored in heparinized tubes and then centrifuged at 3500 rpm for 15 min at 4 ºC, plasma was stored at -20 ºC until analysis.

Sample preparation: To 200 µL plasma 20 µL of I.S. solution (31 ng mL^{-1}) and 400 µL acetonitrile were added. Each tube was mixed by vortexing for 90 s. After centrifugation at 3500 rpm for 15 min, the supernatant was transferred into labelled clean blank test tubes and evaporated to dryness at 40 ºC under a stream of nitrogen. The residues were dissolved in 100 µL of mobile phase and then vortexing for 90 s. A 10 µL aliquot was injected onto the chromatographic column after centrifugation at 3500 rpm for 15 min.

Selectivity: Selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma with the plasma spiked 2''-O-rhamnosylswertisin and the plasma after administration of *Belamcanda chinensis* extract.

Calibration curves, limit of quantitation, limit of detection: 20 µL I.S. was added to each standard sample just prior to sample processing. The procedure was prepared in triplicate for each point. Working standard solutions were prepared freshly every day. Plasma samples were quantified using the ratio of the peak area of 2''-O-rhamnosylswertisin to that of I.S. as the assay response. The peak area ratio (y) and concentration of 2''-O-rhamnosylswertisin (x) were subjected to a weighted ($1/x^2$) least squares linear regression analysis to calculate calibration equation and correlation coefficients.

Limit of quantification (LOQ) and limit of detection (LOD) were calculated according to USPXXVIII[13]. As the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the baseline noise, respectively.

Precision and accuracy: Accuracy and precision were also assessed by analysis of quality control samples using six replicate preparations of plasma samples at three different concentrations (0.206, 20.650 and 82.600 µg mL^{-1}) for 2''-O-rhamnosylswertisin on three validation days. Accuracy was expressed by relative error (RE) and precision by relative standard deviation (RSD).

Recovery: The recoveries of 2''-O-rhamnosylswertisin was calculated by comparing the analytical results of extracted quality control samples obtained by spiking extracted blank rat plasma according to the calibration curves with the known quality control samples concentrations.

Stability: Stability of 2''-O-rhamnosylswertisin in control plasma samples was tested by making three consecutive injections of the same quality control samples at three concentrations (0.206, 20.650 and 82.600 µg mL^{-1}) after placed at room temperature, three freeze-thaw cycles and 30 days stored at -20 ºC. The results were compared with results for freshly prepared quality control samples and the percentage concentration deviation was calculated.

Pharmacokinetics analysis and absolute bioavailability: The pharmacokinetic parameters 2''-O-rhamnosylswertisin were analyzed by non-compartmental analysis. The area under the plasma concentration-time curves (AUC) was calculated by the trapezoidal method. The maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (t_{max}) were obtained by visual inspection of the experimental data. The terminal half-life ($t_{1/2}$) was calculated as 0.693/k and k was the slope of the terminal regression line. The absolute bioavailability (F) of 2''-O-rhamnosylswertisin after oral administration of *Belamcanda chinensis* extract was calculated by the following equation:

$$F = \frac{oralAUC_{0-\infty}}{ivAUC_{0-\infty}} \times \frac{ivDose}{oralDose} \times 100$$

where, $AUC_{0-\infty}$ is the AUC from time 0 to infinity.

RESULTS AND DISCUSSION

Choice of chromatographic conditions: In previous research of our team, an HPLC method was used for 2''-O-rhamnosylswertisin detection using a mobile phase composed of acetonitrile-water (20:80, v/v) and a Kromasil C_{18} (250 mm × 4.6 mm, 5 µm) reversed phase column. Under this condition, main components in *Belamcanda chinensis* extract can be separated. However the peak of internal standard showed as a leading peak. After changed the mobile phase to the mixture acetonitrile-water-glacial acetic acid (19.9:80:0.1, v/v/v), the

new method provided a good separation of the analyte from the I.S. peak in an acceptable run time (<15 min), coupled with a good selectivity and peak shape.

2''-O-rhamnosylswertisin possesses four strong absorption wavelengths (215, 232, 269 and 338 nm). A detection wavelength of 338 nm was chosen for the higher sensitivity and a lower potential for interference. Several compounds (vanillin, quercetin, astragaloside, sinomin, ferulic acid, hyperin, rutin) were tested as possible internal standards while ferulic acid was selected because of its proper retention time.

During development of the method, ethyl acetate was tested to extract 2''-O-rhamnosylswertisin in rat plasma, however, the recovery was unsatisfactory (< 30 %). The low recovery of 2''-O-rhamnosylswertisin should contribute to its high aqueous solubility. Li *et al.*[14] studied the determination of spinosin in rat plasma. The method of deproteinization by acetonitrile was chosen after compared with other methods. The construction of 2''-O-rhamnosylswertisin is similar to spinosin which is also flavone-C-glycosides. This method was investigated in this study and the result showed deproteinization by acetonitrile supplied good resolution and a high recovery.

Selectivity: No interfering peaks were found at the retention times of analytes. The retention times of 2''-O-rhamnosylswertisin and the internal standard were 8.7 min and 14.8 min, respectively. The total run time was less than 15 min. Typical chromatograms of blank plasma, blank plasma spiked with 2''-O-rhamnosylswertisin and internal standard and rat plasma sample after administration of *Belamcanda chinensis* extract extraction were presented in Fig. 2.

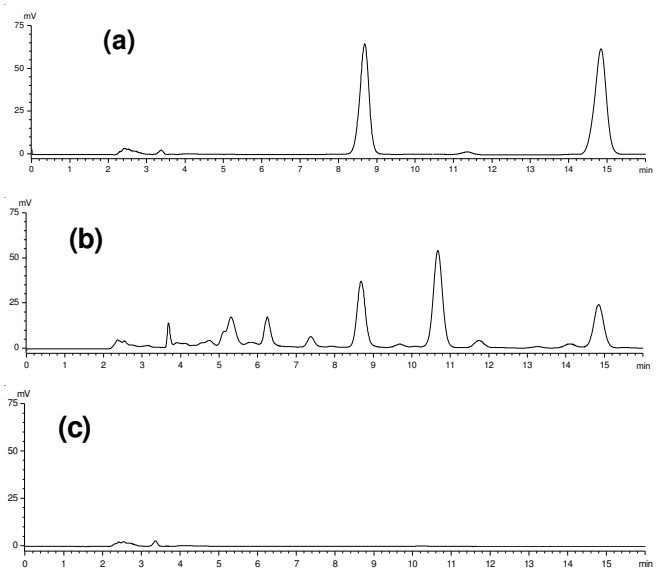

Fig. 2. HPLC chromatograms of (a) blank plasma, (b) blank plasma spiked with 2''-O-rhamnosylswertisin and internal standard and (c) plasma sample (15 min) after oral administration of *Belamcanda chinensis* extract at a dose of 13 g kg^{-1}

Linearity of calibration curves: Calibration curve was set up for 2''-O-rhamnosylswertisin and good linearity (R^2 = 0.9979) was found in the 0.206-80.600 μg mL^{-1} concentration range. The equation of calibration curve is: y = 0.0129 + 0.2351x.

The limit of quantitation in rat plasma was 0.206 μg mL^{-1}, while the limit of detection was 0.062 μg mL^{-1}.

Precision and accuracy: The accuracy and precision at low, medium and high concentration of 2''-O-rhamnosylswertisin are summarized in Table-1. The intra day precision ranged from 0.8 to 5.1 % and the inter day precision ranged from 0.3 to 7.2 %. The results showed that both the precision and accuracy were satisfactory.

TABLE-1
PRECISION AND ACCURACY OF HPLC METHOD TO DETERMINE 2''-O-RHAMNOSYL-SWERTISIN IN RAT PLASMA (n = 6)

Added value (μg mL^{-1})	Detected value (μg mL^{-1})	Between-day RSD (%)	Within-day RSD (%)	RE (%)
0.206	0.205	7.2	5.1	-0.2
20.65	20.615	0.3	0.8	-0.1
82.6	80.407	1.5	1.8	-2.1

Recovery: The recovery results were shown in Table-2. Mean recoveries for 2''-O-rhamnosylswertisin at low, medium and high concentration of 2''-O-rhamnosylswertisin were 99.7, 99.9 and 97.9 %, respectively.

TABLE-2
RECOVERY OF HPLC METHOD TO DETERMINE 2''-O-RHAMNOSYL-SWERTISIN IN RAT PLASMA SAMPLES (n = 6)

Added value (μg mL^{-1})	Detected value (μg mL^{-1})	Recovery (%)	RSD (%)
0.206	0.021	99.7	5.1
20.65	20.615	99.9	0.8
82.6	80.407	97.9	1.8

Stability: Results of stability experiments were presented in Table-3. Results of the stability study demonstrated that 2''-O-rhamnosylswertisin was stable in plasma occurred under experimental conditions.

TABLE-3
STABILITY OF 2''-O-RHAMNOSYL-SWERTISIN IN RATS PLASMA IN DIFFERENT CONDITIONS (n = 6)

Conditions	Added value (μg mL^{-1})	Detected value (μg mL^{-1})	RE (%)
At 20 ºC for 8 h	0.206	0.199	-3.65
	20.650	20.609	-0.20
	82.600	79.918	-3.25
4 Weeks at –20 ºC	0.206	0.211	2.40
	20.650	20.598	-0.25
	82.600	80.406	-2.66
Three freeze-thaw cycles	0.206	0.206	-0.21
	20.650	20.639	-0.05
	82.600	80.899	-2.06

Pharmacokinetics and absolute bioavailability of 2''-O-rhamnosylswertisin: The pharmacokinetic profiles of 2''-O-rhamnosylswertisin after intravenous administration and 2''-O-rhamnosylswertisin after orally administration of *Belamcanda chinensis* extract are shown in Figs. 3 and 4. The results of non-compartmental pharmacokinetic analysis are summarized in Table-4. The rat intravenous administration studies indicated that 2''-O-rhamnosylswertisin exhibit low volume of distribution in rats. The low volume of distribution showed 2''-O-

rhamnosylswertisin was mainly contributed in plasma. T_{max} of 2''-O-rhamnosylswertisin after intravenous administration of *Belamcanda chinensis* extract was 15 min which revealed 2''-O-rhamnosylswertisin was rapid absorbed after orally dosed *Belamcanda chinensis* extract. There were two peaks in plasma concentration-time curve. Re-entry peak appeared at 1 h after oral administration, which indicated 2''-O-rhamnosylswertisin is re-absorbed by enterohepatic cycling or from the distal parts of the small intestine or the colon[15]. The absolute bioavailability of 2''-O-rhamnosylswertisin after oral administration of *Belamcanda chinensis* extract was 2.9 %.

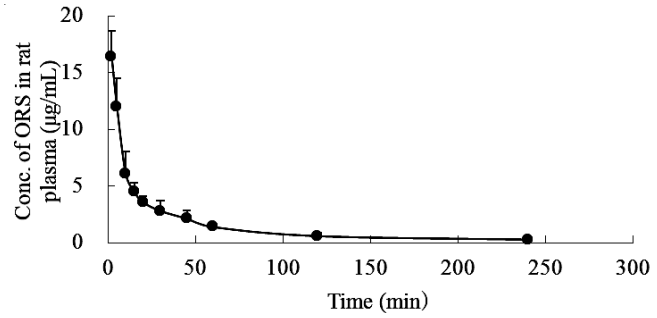

Fig. 3. Mean plasma concentration-time curves of 2''-O-rhamnosylswertisin (10 mg kg^{-1}) in rats following intravenous administration of 2''-O-rhamnosylswertisin (n = 6)

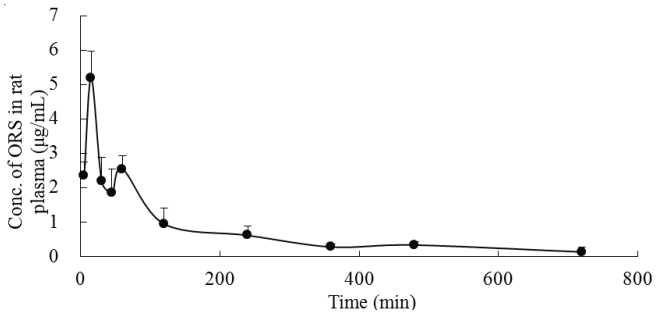

Fig. 4. Mean plasma concentration-time curves of 2''-O-rhamnosylswertisin in rats following oral administration of *Belamcanda chinensis* extract (13 g kg^{-1}) (n = 6)

TABLE-4
PHARMACOKINETIC PARAMETERS OF 2"-O-RHAMNOSYLSWERTISIN (ORS) IN RAT PLASMA AFTER INTRAVENOUS ADMINISTRATION OF 2''-O-RHAMNOSYLSWERTISIN (n = 6) AND AFTER ORAL ADMINISTRATION OF *Belamcanda chinensis* EXTRACT (n = 6)

Parameters	Intravenous administration of 2"-O-rhamnosylswertisin (Mean ± SD)	Oral administration of *Belamcanda chinensis* extract (Mean ± SD)
$AUC_{0-\infty}$ (mg h mL^{-1})	6.41 ± 1.30	8.50 ± 2.55
$AUC_{0-\infty}$ (mg h mL^{-1})	7.06 ± 1.50	9.39 ± 2.66
$T_{0.5}$ (h)	1.18 ± 0.46	3.61 ± 1.62
CLs (L h^{-1})	1.46 ± 0.31	47.42 ± 11.92
Vc (L Kg^{-1})	2.42 ± 1.10	246.56 ± 148.29
ke(h^{-1})	0.70 ± 0.39	0.23 ± 0.11

Conclusion

An HPLC method with UV-detection for determination of 2''-O-rhamnosylswertisin in rat plasma has been successfully established for the first time. The developed method showed acceptable linearity, precision, accuracy and stability. The method was applied in the pharmacokinetics of 2''-O-rhamnosylswertisin. The pharmacokinetics of 2''-O-rhamnosylswertisin after oral administration of *Belamcanda chinensis* extract and 2''-O-rhamnosylswertisin after intravenous administration were studied. The pharmacokinetic parameters of 2''-O-rhamnosylswertisin after intravenous administration showed low volume of distribution in rats. 2''-O-rhamnosylswertisin was absorbed very fast after oral administration of *Belamcanda chinensis* extract that arrived to max concentration in plasma at 15 min. The concentration-time curve of 2''-O-rhamnosylswertisin after oral administration of *Belamcanda chinensis* extract exhibits double peaks. Reentry peak appeared at 1 h after oral dozed. The double peaks phenomenon needs for further research. The bioavailability of 2''-O-rhamnosylswertisin after oral administration of *Belamcanda chinensis* extract was as poor as 2.9 %, which might limit the clinical application of *Belamcanda chinensis* extract.

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