Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 243–249
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Simultaneous determination of vitexin-4″-*O*-glucoside and vitexin-2″-*O*-rhamnoside from hawthorn leaves flavonoids in rat plasma by HPLC method and its application to pharmacokinetic studies

Guo Ma, Xue-Hua Jiang *, Zhuo Chen, Jing Ren, Chen-Rui Li, Tai-Ming Liu

Department of Clinical Pharmacy, West China School of Pharmacy, Sichuan University, Key Laboratory of Drug Targeting of Ministry of Education, Chengdu 610041, PR China

Received 1 September 2006; received in revised form 12 January 2007; accepted 17 January 2007
Available online 30 January 2007

Abstract

The present study was to investigate the pharmacokinetics of the two similar flavonoid glycosides, vitexin-4″-*O*-glucoside (VGL) and vitexin-2″-*O*-rhamnoside (VRH) in rats after intravenous administration of hawthorn leaves flavonoids (HLF). Blood samples were collected via tail vein at time intervals after drug administration and the plasma concentrations of the studied ingredients were analyzed by HPLC after the plasma protein was precipitated directly with methanol. VGL and VRH were successfully separated using a C_{18} column with a UV detection at 330 nm and a mobile phase of methanol–acetonitrile–tetrahydrofuran–0.5% acetic acid (1:1:19.4:78.6, v/v/v/v). The assay linearities of VGL and VRH were confirmed over the range 0.23–138.42 and 0.36–218.49 μg/ml, respectively. The accuracy and precision of the two analytes at high, medium and low concentration were within the range of −3.13% to 3.51% and below 4%, the mean assay recoveries of them ($n = 5$) ranged from 96.87% to 101.75% and 96.88% to 103.51% for intra- and inter-day assays and the mean extraction recoveries of them ($n = 5$) varied from 92.68% to 95.74% for VGL and 93.45% to 99.26% for VRH, respectively. After intravenous administration of HLF to rats over the doses range of 10–40 mg/kg, the plasma concentration–time curves of VGL and VRH were both conformed to the three-compartment open pharmacokinetic model and linear pharmacokinetic characteristics.

Keywords: Hawthorn leave flavonoids; Vitexin-4″-*O*-glucoside; Vitexin-2″-*O*-rhamnoside; Pharmacokinetics

1. Introduction

Hawthorn (*Crataegus*) is widely used as a medicinal plant for a long time both in folk and official medicine. It is widely distributed in northern temperate zones, primarily in East Asia, Europe and North America [1]. There are about 16 species in China, where *Crataegus Pinnatifida* Bge. *Var Major* N. E. Br and *Crataegus Pinnatifida* Bge. are the two major species, named as *Shanlihong* and *Shanzha*, respectively. Their fruits, leaves and flowers are considered the mainly medicinal parts of the plant. Hawthorn leaves are cheap and in great abundance in nature. Hawthorn, hawthorn leaves and its extract have confirmed by various studies to possess a wide range of pharmacological action, especially in the prevention and treatment of cardiovascular diseases, such as a protective effect against arrhythmia [2], an inhibitory action against thrombogenesis [3], an increase of coronary vessel flow [4], a treatment of chronic cardiac insufficiency and congestive heart failure [5,6], a decrease of blood pressure [7], an effective inhibitor of lipid oxidation [8] and a protect against ischemia/reperfusion brain damage as an antioxidant [9]. At the same time, it shows mild pharmacological action, safety and few side effects [10], even at very high doses. Past and ongoing studies suggest these beneficial effects come from the active component flavonoids [11].

Hawthorn leaves flavonoids (HLF), i.e. hawthorn leaves extract, are the major bioactive constituents of hawthorn leaves. The flavonoids exist copiously in hawthorn leaves extract, contents of which exceed 80%(w/w). HLF include many kinds of flavanoids, e.g. vitexin-4″-*O*-glucoside (VGL), vitexin-2″-*O*-rhamnoside (VRH), vitexin, vitexin-6″-*O*-acetyl, rutin, hyperoside, quercitrin, quercetin, etc. [12–15]. Hawthorn, hawthorn leaves, hawthorn leaves extract and its preparation-Yixintong tablets have been recorded in the Chinese Pharmacopoeia [16],

* Corresponding author. Tel.: +86 28 85501370; fax: +86 28 85503024.
E-mail address: jxh1013@vip.163.com (X.-H. Jiang).

0731-7085/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2007.01.033

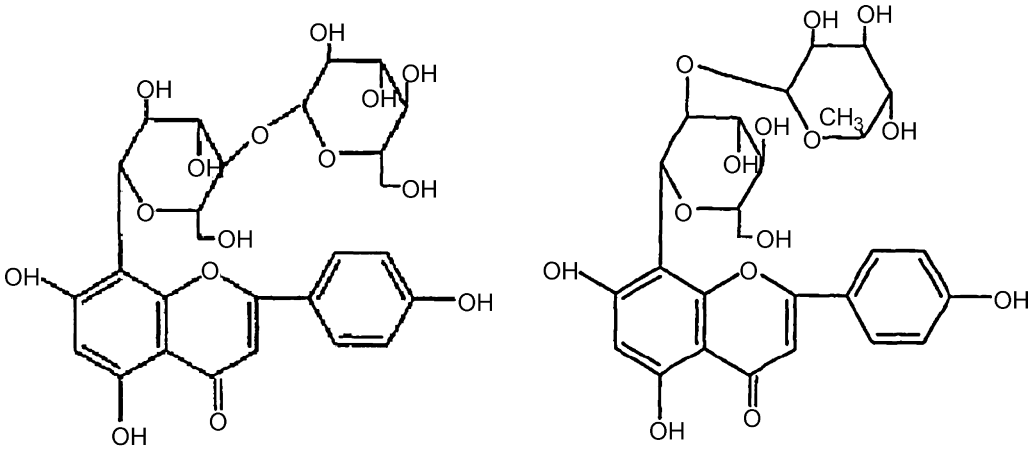

Fig. 1. Chemical structures of vitexin-4″-*O*-glucoside and vitexin-2″-*O*-rhamnoside.

and they are among the most popular herbal medicinal products in China. The two similar flavonoid glycosides, VGL and VRH (Fig. 1.), are the representative and specific ingredients of hawthorn leaves and its extract. The contents of VGL and VRH are the secondary and the highest in HLF, respectively. So VGL and VRH are chose as markers in the quality control of HLF. VGL and VRH both dissolve in water, methanol and alcohol.

Pharmacokinetic studies of active ingredients in natural product and Chinese herbs are important so as to illustrate their action mechanism for the development of traditional Chinese medicine. There were some preliminary researches about quantifying the drug in plant tissue, natural product and preparations [17–19]. Chang et al. [20,21] reported the determination of four active polyphenol components of hawthorn: chlorogenic acid, epicatechin, hyperoside and isoquercitrin in rat plasma and the comparison of pharmacokinetics of the four active ingredients in extract versus individual pure compound in male Sprague–Dawley rats. However, up to now, there are no investigation concerning the determination of VGL and VRH from HLF or hawthorn leaves extract in biological fluids and their pharmacokinetic studies, even though they are the two supreme active components of hawthorn leaves. It is necessary to develop an analytical method for their determination in biological samples, e.g. plasma. In this paper, a sensitive, simple and accurate method was developed to simultaneously determine the plasma levels of VGL and VRH from HLF and has been successfully applied to pharmacokinetic investigation after the intravenous administration of HLF to rats.

2. Materials and methods

2.1. Chemicals and reagent

Vitexin-4″-*O*-glucoside was isolated from HLF in our laboratory and its purity was tested and found to be better than 99.6% by HPLC. Vitexin-2″-*O*-rhamnoside was purchased from National Institute for the Control of Pharmaceutical and Biological Products (100%, Beijing, China). HLF was provided by Jincheng Zhongjin Medicine Co., Ltd. (total flavoniod >93.3%, Jincheng, China). The test formulation of HLF solution (The contents of VGL and VRH in HLF were 6.13% and 13.98%, respectively.) was prepared in our laboratory. The drug free rat plasma samples were provided by the Laboratory Animal Service Center of Sichuan University. Tetrahydrofuran, methanol and acetonitrile (all of HPLC grade) were provided by Fisher Scientific International, Inc. (Fair Lawn, NJ, USA). Acetic acid (analytical grade) was obtained from Kelong Chemical Industrial Reagents Factory (Chengdu, China). Purified water was prepared in a water purification system (Jinteng Corp., Tianjin, China). All other reagents were of analytical grade at least.

2.2. Chromatographic conditions

The Shimadzu HPLC system (Kyoto, Japan) was equipped with a Shimadzu LC-10AT pump, a Shimadzu SPD-10A VP UV–vis spectrophotometric detector, a Shimadzu SCL-10A VP system controller and a ThermaSphere^{TM} TS-130 HPLC Column Temperature Controller (Phenomenex, Inc., Germany). System control and data analyses were carried out using a AnaStar chromatogram data workstation (Version 5.3, Autoscience, Tianjin, China). The HPLC column (reversed-phase Diamonsil^{TM} C_{18} column, 200 mm × 4.6 mm, i.d., 5 μm particle size, Dikma) connected with a guard column (EasyGuard C_{18} Kit, 8 mm × 4 mm, Dikma); Vortex WH-3 (Anting Scientific Instrument, Shanghai, China).

The mobile phase for HPLC analysis consisted of methanol–acetonitrile–tetrahydrofuran–0.5% acetic acid (1:1:19.4:78.6, v/v/v/v), were filtered under reduced pressure and degassed by ultrasonic, prior to use. The HPLC analysis was performed with a flow rate of 0.8 ml/min and a UV detection at 330 nm. The HPLC column temperature was 30 °C. The sample injection volume was 20 μl.

2.3. Preparation of stock solutions

A stock solution containing VGL and VRH was prepared by dissolving in methanol to yield concentrations of

13.842 and 21.849 mg/ml (Sartorius AG electronic balance, CP 225D, $d = 0.01$ mg, Germany), respectively. Working standard solutions were freshly prepared by mixing and diluting the above stock solution with 50% methanol at appropriate ratios to yield concentrations of 1.15, 2.85, 7.10, 17.70, 44.25, 110.70, 276.90, 692.10 μg/ml of VGL and 1.80, 4.45, 11.15, 27.90, 69.90, 174.75, 436.95, 1092.45 μg/ml of VRH, respectively. The stock solution and working standard solutions consisted of VGL and VRH were stored at −20 and 25 °C, respectively.

2.4. Extraction procedure

A 300 μl methanol was added to plasma sample (100 μl) in a silylated centrifuge tube. The mixture was vortexed for 3 min and centrifuged at 12,000 rpm for 5 min to separate the protein from the organic phase. The supernatant was collected and centrifuged at 12,000 rpm for 5 min again. After centrifuging, 20 μl of the supernatant was injected into the HPLC system for analysis.

2.5. HPLC validation procedures

2.5.1. Calibration curves and linearity

The samples for plasma standard calibration curves were prepared by spiking the blank rat plasma (80 μl) with 20 μl of the appropriate working solution and mixing them to yield the following concentrations of VGL: 0.23, 0.57, 1.42, 3.54, 8.85, 22.14, 55.38 and 138.42 μg/ml and VRH: 0.36, 0.89, 2.23, 5.58, 13.98, 34.95, 87.39 and 218.49 μg/ml, respectively. The standard calibration samples (100 μl) were mixed with 300 μl methanol and then extracted as described in Section 2.4. The peak areas of the analytes were plotted against standard concentrations to establish calibration curves for VGL and VRH and statistical software DAS 2.01 were used for least squares linear regression analysis.

2.5.2. Precision, accuracy and recovery

The intra-day accuracy and precision were determined within one day by analyzing five replicates quality control samples at concentrations of 0.32, 3.72 and 62.03 μg/ml for VGL and 0.57, 5.86 and 97.88 μg/ml for VRH. The inter-day accuracy and precision were determined on five separate days within 1 week for the quality control samples. The relative standard deviation (%, R.S.D.) of each concentration was calculated to determine the precision of the method.

The recovery included extraction recovery and assay recovery. The extraction recovery was determined in sets of five replicates quality control samples by measuring the amount of each compound recovered after extraction and calculated by comparing the peak areas of the extracted samples to that of the unextracted standard solutions containing an equivalent amount of the analytes. The assay recovery was determined in the same method, but calculated by comparing the concentrations of the analytes from the calibration curves with that in the prepared sample. The mean assay recovery value (%, mean ± S.D.) represented the accuracy of the method.

2.5.3. Stability

Investigation of stability contained the stability of stock solution, working standard solutions and quality control plasma sample in the study. Storage conditions of the stock and standard solutions see Section 2.3. The stability of quality control plasma sample was evaluated in two ways: (1) the stability of the analytes in the plasma sample before extraction stored at 25, −20 °C and during three freeze–thaw cycles; (2) the stability of the analytes after extraction from plasma stored at 25, 4 and −20 °C. The quality control plasma samples in triplicates at low, medium and high concentration were used, which were prepared by spiking the blank rat plasma (8 ml) with 2 ml of the appropriate HLF solution and mixing them to yield the following concentrations: 4, 40, 400 μg/ml of HLF (equivalent to 0.245, 2.45, 24.50 μg/ml of VGL and 0.559, 5.59, 55.90 μg/ml of VRH, respectively).

The rat plasma samples containing VGL and VRH were stored at 25 °C for 2 h, −20 °C for 60 days, as well as three freeze–thaw cycles, and the processed samples at 25 °C for 24 h, 4 °C for 48 h and −20 °C for 72 h, respectively. Freeze–thaw stability of the plasma samples was evaluated by exposing them to three freeze–thaw (−20 °C for 24 h, −20 °C for 12 h, −20 °C for 12 h) (25 °C for 30 min per time, 3 times) cycles before sample preparation.

2.6. Applicability of the method in pharmacokinetic studies

2.6.1. Preparation of HLF solution for injection

The dosing HLF was dissolved in liquor natrii chloridi isotonicus containing 10% propylene glycol, agitatied, filtered by 0.2 μm micropore film. The HLF solution for injection were freshly prepared at concentrations of 10, 20, 40 mg/ml, respectively.

2.6.2. Animals

Sprague–Dawley rats (male and female, 250 ± 20 g) were supplied by the Laboratory Animal Service Center of Sichuan University (Animal study protocol number: CSDGZ-10). All animal studies were performed according to the requirement of the National Act on the Use of Experimental Animal (China) that was approved by the Committee of Ethics of Animal Experimentation of Sichuan University.

The rats ($n = 18$) used in this study were assigned randomly into three groups. Each group includes six rats. All these animals were housed in an air-conditioned room (temperature, 25 °C; relative humidity, 60%), and allowed to freely access to food and water. The rats were fasted for 12 h before dosing.

2.6.3. Pharmacokinetics

The three rat groups (six rats per group) studied, were assigned to receive HLF solution via tail vein injection at a single dose of 10, 20, 40 mg/kg of HLF (equivalent to 0.61, 1.22, 2.45 mg/kg of VGL and 1.40, 2.80, 5.60 mg/kg of VRH, respectively), respectively. Serial blood samples (0.25 ml) were obtained via the rats tail vein at 0 (predose), 0.083, 0.17, 0.25, 0.33, 0.42, 0.50, 0.67, 0.83, 1.0, 1.5, 2, 3, 4, 5, 6, 8 h

after administration and collected into heparinized centrifuge tubes. The blood samples were immediately centrifuged at 8000 rpm for 5 min at room temperature. The plasma sample was stored at −20 °C until analysis. The data analysis of plasma concentration of VGL and VRH versus time and calculation of pharmacokinetic parameters in rats were executed by the statistics software DAS 2.0.1. The pharmacokinetic parameters were estimated using compartmental and statistical moment (i.e. non-compartmental) analysis methods. Dose-linear pharmacokinetics of the compounds were carried out using least squares regression analysis. Using SPSS 12.0, one-way ANOVA analysis was used to compare pharmacokinetic parameters of VGL and VRH.

3. Results and discussion

3.1. Chromatographic procedure

The purpose of this study was to develop a simple and rapid HPLC method for the simultaneous determination of active components of HLF in biological fluid. The two analytes, VGL and VRH, have similar chemical structures; i.e., 4″-*O*-glucoside and 2″-*O*-rhamnoside of vitexin, respectively. Because of the extraordinary similarity in polarity and chemical structures, it was very difficult to separate them completely when various proportional solvent system, e.g. methanol (acetonitrile)–water, methanol (acetonitrile)–phosphoric acid (acetic acid)–water, whether a isocratic elution or gradient elution, were chosen. After trial and error, a isocratic elution of methanol–acetonitrile–tetrahydrofuran–0.5% acetic acid (1:1:19.4:78.6, v/v/v/v) was finally used to achieve complete separation of VGL and VRH.

The representative chromatograms of blank plasma, plasma samples spiked with VGL and VRH, and a plasma sample obtained from a rat following intravenous administration of HLF (40 mg/kg) are shown in Fig. 2. The peaks of the analytes in the plasma were identified by comparing their retention time with that of the standard. The HPLC method achieved good baseline separation of VGL and VRH at retention times of 9.2 min for VGL and 12.3 min for VRH. In this study, the retention times of VGL and VRH varied from 9.183 to 9.187 min and from 12.308 to 12.325 min, respectively. There were no interfering peaks of endogenous compounds in the chromatographic determinations.

In this study, several organic solvents were tested for precipitation of protein and extraction of the two analytes from rat plasma. They included methanol, ethanol, acetonitrile and methanol-ethyl acetate (1:2, v/v) and the volume of them were one to four times that of plasma sample. The result showed that the effect of precipitating protein and extraction recovery were best when methanol was chosen. The extraction recoveries of the two analytes were 97.54 ± 0.63%, 96.43 ± 1.04% for VGL ($n = 5$) and 98.70 ± 0.71%, 99.20 ± 1.22% for VRH ($n = 5$) when plasma sample-methanol were 1:3 and 1:4 (v/v), respectively. According to student's *t*-test, the recovery of VGL and VRH did not show a significant difference ($p > 0.05$). Considering detection sensitivity, the methanol volume added was three times that of plasma sample. In addition, 10% salicylsulfonic acid was used to precipitate protein in the experiment. It has a conspicuous effect on precipitating protein, but defects of which showed a bad peak shape and baseline drift in chromatograms.

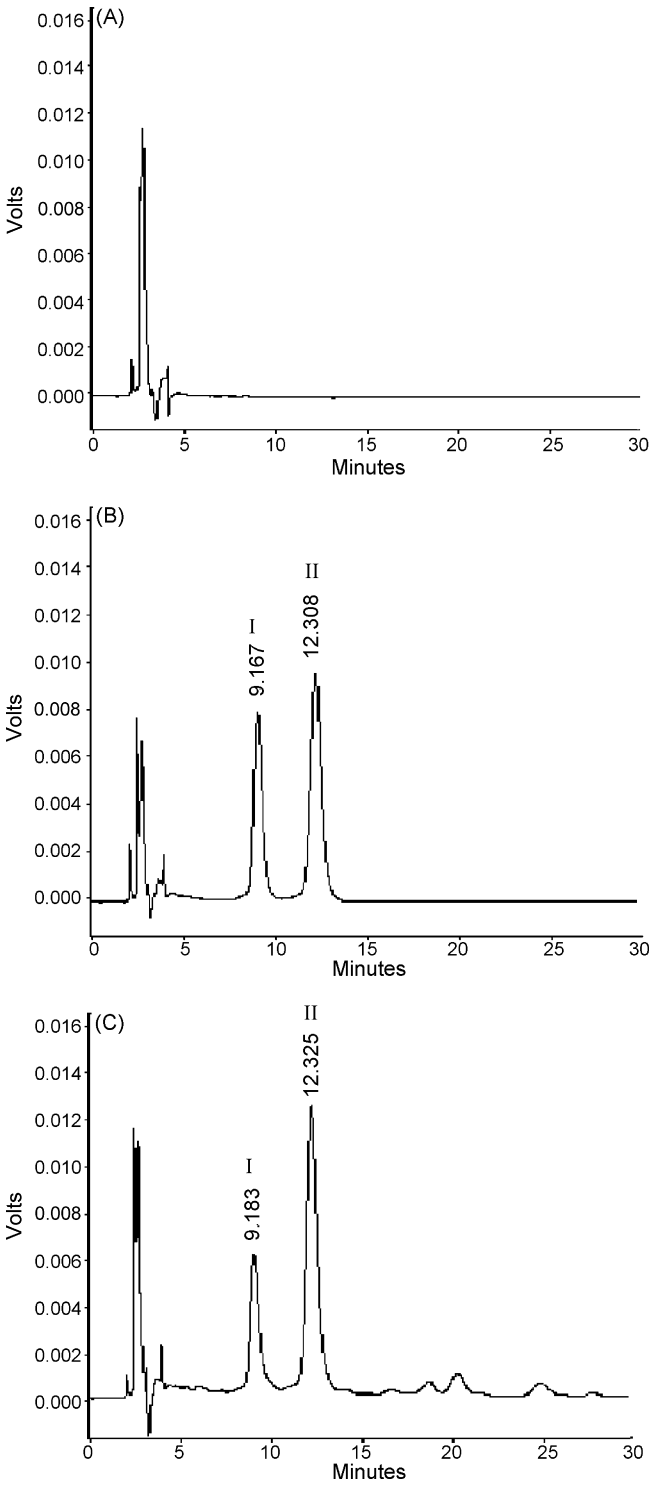

Fig. 2. Chromatograms of rat plasma samples. (A) Blank plasma; (B) blank plasma spiked with both VGL (I) and VRH (II) at the concentration of 22.14 and 34.95 μg/ml; (C) plasma sample obtained 25 min after intravenous administration of HLF at a dose of 40 mg/kg to a rat.

Table 1
Intra- and inter-day (5 separate days) precision, accuracy and recovery for the determination of VGL and VRH in rat plasma

Conc. added (μg/ml) ($n=5$)	Assay recovery ($n=5$)						Extraction recovery ($n=5$)	
	Intra-day			Inter-day			Recovery mean ± S.D. (%)	R.S.D. (%)
	Conc. assayed mean ± S.D. (μg/ml)	Recovery mean ± S.D. (%)	R.S.D. (%)	Conc. assayed mean ± S.D. (μg/ml)	Recovery mean ± S.D. (%)	R.S.D. (%)		
VGL								
0.32	0.31 ± 0.010	96.87 ± 3.20	3.25	0.31 ± 0.010	96.88 ± 3.13	3.23	95.57 ± 2.87	3.02
3.72	3.67 ± 0.051	98.66 ± 1.37	1.39	3.73 ± 0.052	100.27 ± 1.40	1.39	95.74 ± 1.19	1.24
62.03	62.01 ± 0.471	99.97 ± 0.76	0.76	63.05 ± 0.989	101.64 ± 1.59	1.57	92.68 ± 2.19	2.36
VRH								
0.57	0.58 ± 0.012	101.75 ± 2.15	2.12	0.59 ± 0.010	103.51 ± 1.75	1.69	99.26 ± 2.85	2.87
5.86	5.86 ± 0.056	100.00 ± 0.95	0.95	5.99 ± 0.111	102.16 ± 1.90	1.86	97.74 ± 1.32	1.35
97.88	96.32 ± 0.701	98.41 ± 0.72	0.73	97.81 ± 1.485	99.93 ± 1.52	1.52	93.45 ± 1.85	1.98

Conc.: Concentration; R.S.D.: relative standard deviation.

3.2. Linearity

The calibration curves of VGL and VRH were linear over the range 0.23–138.42 and 0.36–218.49 μg/ml, respectively. The regression equations of calibration curves were $Y=-1771.543+11599.386X$ ($r=1.000$) and $Y=-5189.671+11722.273X$ ($r=1.000$) for VGL and VRH, respectively. The weight were both 1.0. The model (linear) is adequate to describe the observed data for linearity, which show no significant curvature (ANOVA, lack of fit, $p>0.05$). Their correlation coefficients ($r>0.9999$) showed good linearities. This result showed the usefulness of the present HPLC method in the assays of VGL and VRH from low to high plasma levels.

The limit of quantitation (LOQ) was achieved as the lowest point on the standard curve, 0.23 μg/ml for VGL with R.S.D. of 2.15% ($n=5$) and 0.36 μg/ml for VRH with the R.S.D. of 2.36% ($n=5$). The limit of detection (LOD) was defined as the lowest concentration of the drug resulting in a signal-to-noise ratio of 3:1. The limits of detection (LOD) of the analytes in plasma was determined to be 0.030 μg/ml for VGL and 0.048 μg/ml for VRH.

3.3. Precision, accuracy and recovery

Quality control samples ($n=5$) representing low, medium and high concentration contained 0.32, 3.72 and 62.03 μg/ml for VGL and 0.57, 5.86 and 97.88 μg/ml for VRH, respectively. The maximum intra- and inter-day precision was 3.25% for VGL and 2.12% for VRH (Table 1). The assay showed that the intra- and inter-day precisions for VGL and VRH with a R.S.D. of less than 20% were obtained at the lowest concentrations. Typically, the LOQ is defined as the concentration where the R.S.D. is 20%. The assay showed that the intra- and inter-day precisions for VGL and VRH with a R.S.D. of less than 15% were obtained, which comply with the requirements of CDER FDA. Data on accuracies about the recoveries of VGL and VRH were 96.87 ± 3.20% ($n=5$) and 103.51 ± 1.75% ($n=5$), at the lowest concentration of 0.32 μg/ml for VGL and 0.57 μg/ml for VRH in spiked rat plasma samples (Table 1).

The mean extraction recoveries of the two analytes ($n=5$) from spiked rat plasma (Table 1) were satisfactory at low, medium and high concentrations, which varied from 92.68 ± 2.19% to 95.74 ± 1.19% for VGL and 93.45 ± 1.85% to 99.26 ± 2.85% for VRH. High recovery of VGL and VRH from rat plasma suggested that there was negligible loss during drug extraction.

3.4. Stability

The stability experiment indicated the stock solution stored at −20 °C was stable for at least 6 months. The working standard solutions stored at 25 °C were stable for at least 24 h.

The stabilities of VGL and VRH in rat plasma sample and plasma extract ($n=3$) at different temperatures are shown in Table 2. The two analytes in rat plasma sample were stable stored at 25 °C for 2 h, −20 °C for at least 60 days at low, medium and high concentration, respectively. Therefore, the plasma sample should be processed within 2 h at 25 °C or 60 days at −20 °C. The freeze–thaw consisted of storing at −20 °C and then thawing at a temperature of 25 °C. After three freeze–thaw cycles, the two analytes were stable and the recoveries from frozen and thawed plasma samples were 98.15 ± 2.12% at 0.245 μg/ml for VGL and 103.05 ± 2.05% at 0.559 μg/ml for VRH (Table 2). The two analytes in plasma extract were stable stored at 25 °C for 24 h, 4 °C for 48 h, −20 °C for at least 72 h at low, medium and high concentration, respectively, which indicated the plasma extract waiting to be analyzed should be injected into HPLC system within 24 h at 25 °C, 48 h at 4 °C or 72 h at −20 °C.

3.5. Application of the HPLC method for pharmacokinetic studies

The validated methods have been successfully applied for pharmacokinetic studies analysing VGL and VRH in rat plasma after intravenous administration of a HLF solution. The mean plasma concentration time profiles of VGL and VRH are shown in Fig. 3. The pharmacokinetic parameters are listed in Table 3.

Table 2
Stability of VGL and VRH in a rat plasma sample and plasma extract (mean ± S.D., $n = 3$)

Storage conditions	Conc. added (μg/ml)	VGL			VRH		
		0.245	2.45	24.50	0.559	5.59	55.90
Plasma sample							
25 °C × 2 h	Conc. assayed (μg/ml)	0.235 ± 0.013	2.573 ± 0.11	25.414 ± 0.598	0.558 ± 0.030	6.054 ± 0.274	58.511 ± 2.544
	Recovery (%)	95.97 ± 5.47	105.01 ± 4.50	103.73 ± 2.44	99.74 ± 5.34	108.30 ± 4.90	104.67 ± 4.55
−20 °C × 60 days	Conc. assayed (μg/ml)	0.236 ± 0.014	2.421 ± 0.03	25.465 ± 0.951	0.564 ± 0.011	5.405 ± 0.182	56.33 ± 1.369
	Recovery (%)	96.18 ± 5.83	98.83 ± 1.16	103.94 ± 3.74	100.92 ± 1.98	96.69 ± 3.01	100.77 ± 2.34
Three freeze–thaw	Conc. assayed (μg/ml)	0.241 ± 0.005	2.471 ± 0.026	24.598 ± 0.176	0.576 ± 0.011	5.580 ± 0.015	55.978 ± 0.281
	Recovery (%)	98.15 ± 2.12	100.85 ± 1.02	100.40 ± 0.694	103.05 ± 2.05	99.82 ± 0.24	100.14 ± 0.48
Plasma extract							
25 °C × 24 h	Conc. assayed (μg/ml)	0.239 ± 0.004	2.443 ± 0.034	26.058 ± 0.92	0.558 ± 0.014	5.847 ± 0.103	58.947 ± 1.492
	Recovery (%)	97.44 ± 1.56	99.72 ± 1.33	106.36 ± 3.62	99.87 ± 2.53	104.59 ± 1.70	105.45 ± 2.55
4 °C × 48 h	Conc. assayed (μg/ml)	0.236 ± 0.010	2.491 ± 0.106	24.608 ± 0.290	0.522 ± 0.027	5.931 ± 0.159	57.611 ± 1.276
	Recovery (%)	96.33 ± 4.29	101.67 ± 4.11	100.44 ± 1.14	93.41 ± 4.94	106.10 ± 2.63	103.06 ± 2.18
−20 °C × 72 h	Conc. assayed (μg/ml)	0.235 ± 0.012	2.428 ± 0.046	24.164 ± 0.163	0.541 ± 0.015	5.625 ± 0.195	55.145 ± 0.427
	Recovery (%)	95.97 ± 5.14	99.11 ± 1.79	98.63 ± 0.64	96.84 ± 2.76	100.62 ± 3.22	98.65 ± 0.73

The "Conc. added" indicates the plasma concentration (μg/ml, mean ± S.D.) at initial time point and the "Conc. assayed" and "recovery" indicate the plasma concentration (μg/ml, mean ± S.D.) and recovery (%, mean ± S.D.) at the last time point under different storage conditions ($n = 3$), respectively.

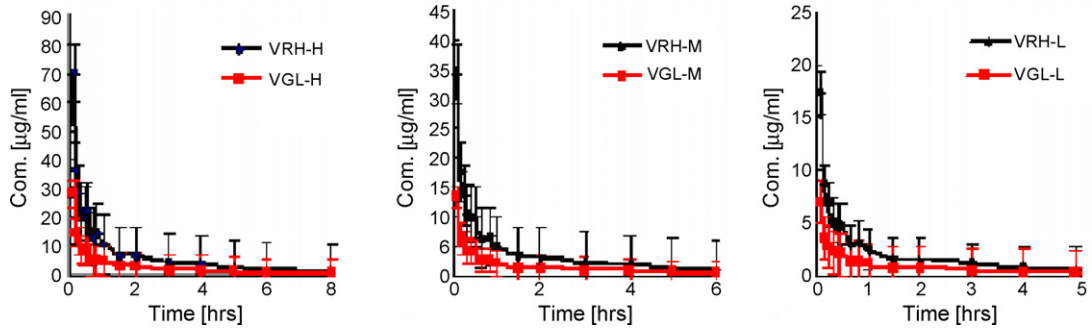

Fig. 3. Mean plasma concentration–time curves of VGL and VRH in rats ($n = 6$) after intravenous administration of HLF at a single dose of 40, 20 and 10 mg/kg, respectively, equivalent to 2.45 (■, VGL-H), 1.22 (■, VGL-M), 0.61 (■, VGL-L) mg/kg of VGL and 5.60 (♦, VRH-H), 2.80 (♦, VRH-M), 1.40 (♦, VRH-L) mg/kg of VRH, respectively. These sampling points, which could not be detected or plasma concentration of which were lower than LOQ, didn't included in the mean plasma concentration–time curves.

With minimum akaike information criterion (AIC) values, a three-compartment open pharmacokinetic model was proposed and validated through the DAS 2.0.1. VGL and VRH appeared to be distributed most rapidly into a highly perfused central compartment (first compartment), less rapidly into a second compartment (shallow tissue compartment) and slowly into a third compartment (deep tissue compartment). The triphasic phenomenon with a rapid distribution and a relatively slow dis-

Table 3
Pharmacokinetic parameters for VGL and VRH in SD rats (mean ± S.D., $n = 6$) after a single intravenous administration of HLF

Pharmacokinetic parameter	VGL			VRH		
	0.61 mg/kg	1.22 mg/kg	2.45 mg/kg	1.40 mg/kg	2.80 mg/kg	5.60 mg/kg
$t_{1/2\alpha}$ (h)	0.028 ± 0.002	0.029 ± 0.006	0.031 ± 0.003	0.029 ± 0.002	0.03 ± 0.005	0.033 ± 0.004
$t_{1/2\beta}$ (h)	0.251 ± 0.004	0.25 ± 0.037	0.265 ± 0.006	0.25 ± 0.01	0.246 ± 0.035	0.26 ± 0.006
$t_{1/2\gamma}$ (h)	2.671 ± 0.087	2.416 ± 0.137	2.373 ± 0.068	2.325 ± 0.163	2.273 ± 0.095	2.176 ± 0.051
V_1 (l/kg)	0.021 ± 0.004	0.024 ± 0.01	0.024 ± 0.005	0.020 ± 0.006	0.022 ± 0.008	0.022 ± 0.003
CL (l/h/kg)	0.096 ± 0.013	0.101 ± 0.014	0.097 ± 0.010	0.094 ± 0.01	0.0932 ± 0.013	0.089 ± 0.01
$AUC_{(0-t)}$ (mg h/l)	4.492 ± 0.54	9.337 ± 1.215	20.891 ± 2.489	10.892 ± 1.168	23.766 ± 3.447	51.71 ± 6.504
$AUC_{(0-\infty)}$ (mg h/l)	5.60 ± 0.778	10.578 ± 1.507	21.605 ± 2.459	12.664 ± 1.387	26.324 ± 4.238	53.984 ± 6.615
$MRT_{(0-t)}$ (h)	1.268 ± 0.013	1.478 ± 0.032	1.73 ± 0.018	1.212 ± 0.016	1.45 ± 0.05	1.668 ± 0.012
$MRT_{(0-\infty)}$ (h)	2.543 ± 0.225	2.269 ± 0.096	2.18 ± 0.054	2.123 ± 0.215	2.142 ± 0.108	2.11 ± 0.071

$t_{1/2\alpha}$: Half-life of rapid distribution phase; $t_{1/2\beta}$: half-life of slow distribution phase; $t_{1/2\gamma}$: half-life of elimination phase; V_1: apparent volumes of distribution of the central compartments; CL: total body clearance; AUC: area under the plasma concentration vs. time curve; MRT: mean residence time.

tribution followed by a slower elimination phase was observed from the compartment model parameters $t_{1/2\alpha}$, $t_{1/2\beta}$ and $t_{1/2\gamma}$. The values of $t_{1/2\alpha}$, $t_{1/2\beta}$, $t_{1/2\gamma}$, V_1, CL and $MRT_{(0-\infty)}$ of VGL and VRH were about 0.028–0.033, 0.24–0.27, 2.18–2.67 h, 0.020–0.024 l/kg, 0.089–0.101 l/h/kg and 2.11–2.54 h at three different doses, respectively. The plasma concentrations and AUC of VGL and VRH in rat plasma were proportional to the administrated doses. Dose-linear pharmacokinetics of VGL and VRH was shown using least squares regression analysis in rats after intravenous administration of HLF at doses of 10–40 mg/kg.

In addition, the pharmacokinetic behaviors of VGL and VRH in rats after intravenous administration of HLF were similar owing to their similar chemical structures. It was confirmed by their plasma concentration–time curves and some pharmacokinetic parameters (i.e. $t_{1/2\alpha}$, $t_{1/2\beta}$, $t_{1/2\gamma}$, V_1, CL and MRT) of the two active ingredients. Using SPSS 12.0, one-way ANOVA analysis, these pharmacokinetic parameters did not show significantly different ($p > 0.05$) over the doses range studied.

4. Conclusions

A simple HPLC assay method has been developed for simultaneous determination of VGL and VRH in rat plasma without an internal standard. Treatment with methanol for VGL and VRH results in chromatograms free of interference. The procedures of both VGL and VRH have been validated, and the results demonstrate that the standard curve is linear over the concentration of 0.23–138.42 μg/ml VGL and 0.36–218.49 μg/ml VRH. The analysis requires 100 μl plasma for both compounds. The LOQs are 0.23 μg/ml for VGL and 0.36 μg/ml for VRH and the R.S.D.s for both compounds are less than 20%. The method is suitable for pharmacokinetic studies of VGL and VRH in rat after intravenous administration.

Acknowledgments

This project was supported by National Natural Science Foundation of China (nos. 30070935 and 30271614).

The DAS (Drug and Statistics) 2.0.1 software was offered by Professor RY, Sun, Clinical Research Center of Drug, Shanghai University of T.C.M.

References

[1] J.M. Rigelsky, B.V. Sweet, Am. J. Health Syst. Pharm. 59 (2002) 417–422.
[2] M. Veveris, E. Koch, S.S. Chatterjee, Life Sci. 74 (2004) 1945–1955.
[3] W.J. Lan, Y.K. Ge, X.X. Zheng, Hangtian Yixue Yu Yixue Gongcheng 18 (2005) 157–160.
[4] P. Bohinc, Medicinski Razgledi 26 (1987) 53.
[5] M.H. Pittler, K. Schmidt, E. Ernst, Pharm Unserer Zeit 34 (2005) 66–71.
[6] M.H. Pittler, E. Ernst, Am. J. Med. 114 (2003) 665–674.
[7] A.F. Walker, G. Marakis, A.P. Morris, P.A. Robinson, Phytother. Res. 16 (2002) 48–54.
[8] C. Quettier-Deleu, G. Voiselle, J.C. Fruchart, P. Duriez, E. Teissier, et al., Pharmazie 58 (2003) 577–581.
[9] D.L. Zhang, Y.T. Zhang, J.J. Yin, B.L. Zhao, J. Neurochem. 90 (2004) 211–219.
[10] H.P.T. Ammon, M. Haendel, Planta Med. 43 (1981) 105–120.
[11] W.T. Chang, D. Jenny, Z.H. Shao, Am. J. Chin. Med. 33 (2005) 1–10.
[12] P.C. Zhang, S.X. Xu, J. Asian Nat. Prod. Res. 5 (2003) 131–136.
[13] X.B. Ding, Y.Q. Jiang, Y. Zhong, C.X. Zuo, Zhongguo Zhongyao Zazhi 15 (1990) 295–297.
[14] G. Melikoglu, F. Mericli, A.H. Mericl, Boll. Chim. Farm. 138 (1999) 351–352.
[15] A. Rehwald, B. Meier, O. Sticher, J. Chromatogr. A 677 (1994) 25–33.
[16] China Pharmacopoeia Committee, The China Pharmacopoeia, vol. 1, Chemical Industry Press, Beijing, 2005.
[17] R.H. Liu, B.Y. Yu, S.X. Qiu, D. Zheng, Chin. J. Nat. Med. 3 (2005) 162–167.
[18] A. Urbonaviciute, V. Jakstas, O. Kornysova, V. Janulis, A. Maruska, J. Chromatogr. A 1112 (2006) 339–344.
[19] J. Su, W.D. Zhang, Y. Zhou, J. Zhou, Z.B. Gu, Zhongguo Zhongyao Zazhi 29 (2004) 525–527.
[20] Q. Chang, M. Zhu, Z. Zuo, M. Chow, W.K. Ho, J. Chromatogr. B Biomed. Sci. Appl. 760 (2001) 227–235.
[21] Q. Chang, Z. Zuo, W.K. Ho, M.S. Chow, J. Clin. Pharmacol. 45 (2005) 106–112.