ELSEVIER

Journal of Chromatography B, 731 (1999) 403–409

JOURNAL OF CHROMATOGRAPHY B

Short communication

Determination of ginsenoside Rg3 in plasma by solid-phase extraction and high-performance liquid chromatography for pharmacokinetic study

Hailin Wang[a], Hanfa Zou[a,*], Liang Kong[a], Yukui Zhang[a], Huan Pang[b], Chenye Su[b], Guoyou Liu[c], Ming Hui[c], Li Fu[c]

[a]*National Chromatographic R&A Center of China, Dalian Institute of Chemical Physics, Academia Sinica, Dalian 116011, China*
[b]*Department of Clinical Pharmacology, Dalian Medical University, Dalian 116023, China*
[c]*Dalian Tianfu Institute of Natural Medicine, Dalian 116600, China*

Received 26 January 1999; received in revised form 19 May 1999; accepted 27 May 1999

Abstract

A method using high-performance liquid chromatography (HPLC) and solid-phase extraction (SPE) is described for the determination of ginsenoside Rg3 in human plasma. A 2.5-ml volume of plasma was mixed with 2.5 ml 60% methanol aqueous solution, and centrifuged at 1100 *g* for 10 min, the supernatant fluid was further purified by SPE with 200 mg/5 ml 40 μm octadecyl silica and separation was obtained using a reversed-phase column under isocratic conditions with ultraviolet absorbance detection. The intra- and inter-day precision, determined as relative standard deviations, were less than 5.0%, and method recovery was more than 97%. The lower limit of quantitation, based on standards with acceptable RSDs, was 2.5 ng/ml. No endogenous compounds were found to interfere with analyte. A good linear relationship with a regression coefficient of 0.9999 in the range of 2.5 to 200 ng/ml was observed. This method has been demonstrated to be suitable for pharmacokinetic studies in humans. Method development for determination of drug with low UV absorption by SPE and HPLC is also discussed.

Keywords: Ginsenosides

1. Introduction

Ginsenoside Rg3, discovered from Red Panax ginseng, is a new anticancer agent, which shows inhibitive activities of tumor metastasis in mice and in vitro tumor cell invasion [1,2]. A number of methods employing high-performance liquid chromatography (HPLC) to separate ginsenosides for evaluating ginseng Radix and commercial products containing it have been reported [3–8], and a thin-layer chromatography (TLC) method for the pharmacokinetic study of animals with high doses of ginseng saponins (100 mg/kg o.v., or 5 mg/kg i.v.) has also been developed [9]. Karikura et al. [10] have established a highly sensitive analytical method for biological samples with isotope labeling of a ginsenoside, which has been applied to the study of pharmacodynamics of ginsenoside in animals, but it

*Corresponding author. Tel.: +86-411-3693-409; fax: +86-411-3693-407.
E-mail address: zouhfa@pub.dl.lnpta.net.cn (H. Zou)

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00238-8

cannot be applied to humans because of safety. The amount of ginsenoside Rg1 and Rb1 absorbed from the gastrointestinal tract of rat were 1.9% and 0.1% of the dose, respectively, and that of Rb2 was determined to be 3.7% of the dose by using ^{3}H-labeled Rb2 [11]. However, there is no specific HPLC method reported for determining ginsenoside Rg3 in human plasma for pharmacological study with low dose until now, where the concentration of ginsenoside is very low. In this paper, we develop an accurate and sensitive HPLC method to measure ginsenoside Rg3 with ultraviolet detection in human plasma, which was applicable to pharmacological studies with the solid-phase extraction (SPE) as sample preparation.

2. Experimental

2.1. Equipment

The HPLC system consisted of a Shimadzu (Kyoto, Japan) instrument equipped with computer system for acquisition and treatment of data (WDL-95 Chromatography Work Station, National Chromatographic R&A Center of China, Dalian, China), a LC-10AD pump, a Spectra 100 variable-wavelength detector. A vacuum manifold (National Chromatographic R&A Center of China) was used for SPE.

2.2. Reagents

Ginsenoside Rg3 standard was prepared by HPLC with a semi-preparative C_{18} column (15 cm×1.0 cm I.D., 10 μm) and mobile phase of 85% (v/v) methanol aqueous solution in our laboratory, the final preparation was identified by infrared (IR) spectrometry, nuclear magnetic resonance (NMR) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The structure of ginsenoside Rg3 is illustrated in Fig. 1. Its purity analyzed by HPLC is about 99.6%. Acetonitrile was of HPLC grade, all other chemicals were of analytical-reagent grade.

Control (blank) human plasma (collected on sodium heparinate) was obtained from healthy volunteers free of drugs.

2.3. Solid-phase extraction

Venous blood samples (5 ml) were withdrawn to the heparinized Vacutainer tubes, and were at once centrifuged at 800 *g* for 10 min at 4°C. A 2.5-ml volume of plasma was finally obtained, and stored at −20°C until analysis.

The 2.5-ml volume of plasma was mixed with 2.5 ml 60% methanol aqueous solution and shaken for 5 min. After centrifugation at 1000 *g* for 10 min, the supernatant fluid was loaded and drawn through by gravity on an SPE cartridge (5 ml, packed with 200 mg of 40 μm octadecyl silica), which was preconditioned by passing through 5 ml of methanol followed by 5 ml water before loading, and drawn through by gravity. Then, the solid-phase cartridge was washed with 4.0 ml of 60% (v/v) methanol aqueous solution and 2.0 ml of 70% (v/v) methanol aqueous solution in turn, and finally the SPE cartridge was slowly eluted by 1.5 ml of 90% (v/v) methanol aqueous solution. The eluent was evaporated at ambient temperature to dryness under the stream of nitrogen and reconstituted in 150 μl of 60% methanol aqueous solution. A 100-μl aliquot was injected into the HPLC system via GJ605 six-port valve by hand.

2.4. Chromatographic conditions

The mobile phase was acetonitrile–water–isopropanol (43:56:1, v/v), delivered at a flow-rate of 0.8 ml/min. Separation was accomplished at room temperature on a Hypersil ODS2 column (5 μm, 200×4.0 mm I.D.). The UV detector was set to 203 nm.

2.5. Drug standards

Working stock solutions for ginsenoside Rg3 were prepared in 90% methanol aqueous solution at a concentration of 100 μg/ml and stored at 4°C. Plasma standards from the stock solutions were prepared using drug-free plasma from healthy volunteers (2.5, 5, 10, 20, 50, 100, 200 ng/ml).

2.6. Analytical variables

Absolute extraction recoveries of ginsenoside Rg3 from human plasma were estimated using standard

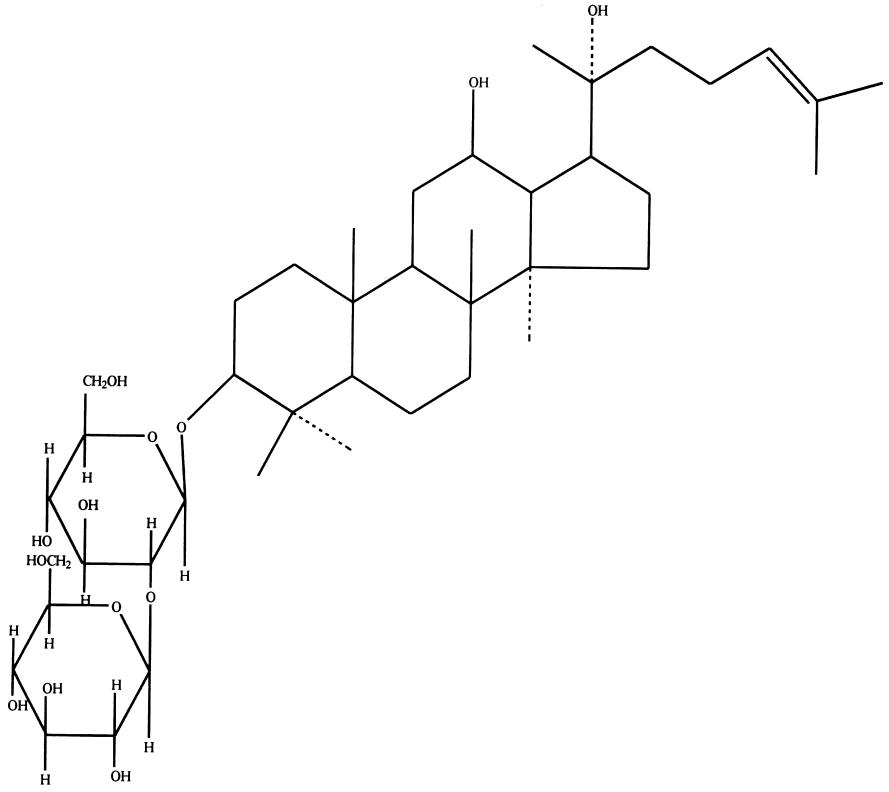

Fig. 1. The structure of ginsenoside Rg3.

samples at concentrations ranging from 10 to 100 ng/ml of ginsenoside Rg3 by comparing the peak areas from processed plasma standard samples to those from a calibration curve prepared from analytes in water. Plasma standard samples (2.5, 5, 10, 20, 50,100, 200 ng/ml) were analyzed in quintuplicate on three separate days during method validation. Linearity of standard curves, intra- and inter-assay precision and recovery were determined from these data.

2.7. Application

The assay has been applied in pharmacokinetic studies. Samples from healthy volunteers were taken prior to the dose and again at 0.25, 0.5, 1.0, 1.5, 2.0, 6.0, 8.0, 10.0, 12.0 and 24 h after drug ingestion. Comparison of peak areas from the unknown samples to those from the calibration curve permitted quantitation of the assayed samples.

3. Results and discussion

3.1. Method development for SPE and HPLC

The UV spectrum of ginsenoside Rg3 is given in Fig. 2, it shows poor UV absorption, and there is no report for derivatization of it to improve its sensitivity, therefore, it must be detected at low UV wavelengths. It was finally detected at 203 nm simultaneously with regard to the background absorption of mobile phase in our experiment. On the other hand, the concentration of ginsenoside Rg3 in plasma is not high, so it requires a high efficiency pretreatment technique adopted with rational method development to remove excessive interferences and the mobile phase for HPLC showing low adsorption background at low UV wavelength and good selectivity to ginsenoside Rg3. The acetonitrile and water system has a lower background and shows better selectivity to ginsenoside Rg3 than the methanol and

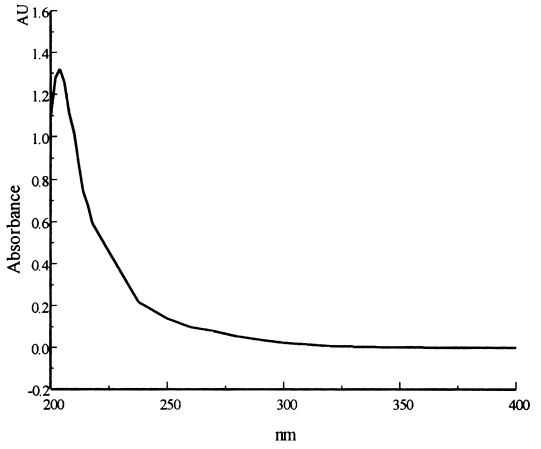

Fig. 2. The ultraviolet spectrum of ginsenoside Rg3.

water system, therefore, the former was chosen for HPLC analysis. SPE is a popular and potential pretreatment method, which shows strong ability to remove excessive interferences and efficiently extract the drug of interest from the plasma sample, and was adopted for extracting ginsenoside Rg3 from human plasma.

We used reversed-phase SPE to extract ginsenoside Rg3 from plasma sample because of its high hydrophobicity, the SPE cartridge was made by packing 200 mg of 40 μm octadecyl silica in one 5-ml polyethylene (PE) barrel, which is enough to extract 10 μg of ginsenoside Rg3 from 2.5 ml plasma.

The method development for SPE consisted of the following steps:

(1) Use high concentrations of standard ginsenoside Rg3 to investigate the type and quantity of packing support in SPE cartridge, and the influence of the type and concentration of organic solvent on the adsorption and elution of the interested drug.

(2) Use 2.5 ml plasma added with standard ginsenoside Rg3 to investigate the influence of drug–protein binding to the adsorption of drug and the type and concentration of organic solvent for washing interferences and elution volume.

(3) Determine all SPE conditions.

During step 1, we found that the recovery of ginsenoside Rg3 was very low, only 40%, even when the SPE cartridge maintained a low pressure. When we let the mobile phase for loading sample flow just by gravity, the recovery went up to above 95%. Therefore, the recovery was seriously influenced by the flow-rate of SPE. This is may be caused by the slow mass transfer process of ginsenoside Rg3 from mobile phase to the intra-surface of the stationary phase because of its high molecular mass (M_r 784). During all SPE steps, the mobile phase must naturally flow by gravity. Ginsenoside Rg3 could be totally adsorbed on the packing support if the ratio of methanol-to-water is below 50:50 (v/v), and could be eluted in 2 ml if it is over 90:10.

During step 2, we found the drug is difficult to be adsorbed on the SPE cartridge if 2.5 ml plasma is directly applied to the SPE cartridge, even diluted plasma with 0.1% salt water, or adjusting plasma pH with acid or base, the recovery is still below 40%. We think there is strong protein-binding between ginsenoside Rg3 and plasma protein, which is non-covalent binding and could be damaged by adding methanol or acetonitrile to plasma. In our work, we found methanol is the best, 30% methanol in plasma solution could thoroughly release the drug from the plasma protein, and allow direct extraction by the stationary phase in the SPE cartridge. Therefore, methanol was required to be added to the plasma sample; up to 30% to damage ginsenoside Rg3 and protein binding before applying the plasma sample to the SPE cartridges. When methanol was added to the plasma sample, only a small protein precipitate appeared, and it was then removed by centrifugation. Most of the interferences are acidic, and their retention behaviors are easy to modify by changing the pH of the mobile phase. However, they can be directly removed by 2.0 ml 70% (v/v) methanol aqueous solution after a 4.0 ml 60% (v/v) methanol aqueous solution wash of the SPE cartridge. Both steps in SPE are necessary to thoroughly remove the interferences in the plasma sample. We have investigated the retention behavior of ginsenoside Rg3 and its interferences in human plasma sample on C_{18} stationary phase with methanol–water and acetonitrile–water as mobile phases, respectively, and found that the retention of ginsenoside Rg3 is more dependent upon the concentration of methanol than its interferences in methanol–water system. When the concentration of methanol is below 75%, the retention of ginsenoside Rg3 is very strong and it is

difficult to elute from the column, most of the interferences are able to be eluted from the column at this concentration, therefore, the methanol–water system is the most suitable mobile phase for use in SPE. We also observed that the latter shows better selection to ginsenoside Rg3, and it is suitable to be adopted as the mobile phase for reversed-phase HPLC analysis as described above.

At the elution process, we applied 90% methanol aqueous solution to the SPE cartridge, and each 0.5-ml fraction was collected for detecting ginsenoside Rg3, we found 1.5 ml of 90% methanol aqueous solution is enough to make most of ginsenoside Rg3 elute from the SPE cartridge (recovery>97%). Fig. 3 illustrates the recovery of ginsenoside Rg3 in each 0.5-ml fraction.

3.2. Performance of HPLC

Fig. 4 illustrates a representative chromatogram of blank plasma (a), plasma with 100 ng/ml of ginsenoside (b) and plasma sample from a volunteer at 1 h after drug oral administration (c). Drug-free pooled human plasma yielded relatively clean chromatograms with no significant interfering peaks. The retention time of ginsenoside Rg3 was 15.9 min.

3.3. Calibration curve

The calibration curves were made on three different days and used to determine the sample concentration. Linearity of the standard curves was found in the range 2.5–200 ng/ml and was statistically confirmed. The determination coefficient (r^2) was of 0.9999, and regression equation was $A=342.69C-50.59$, where A and C represent peak area and concentration, respectively. The limit of detection of ginsenoside was 1.5 ng/ml.

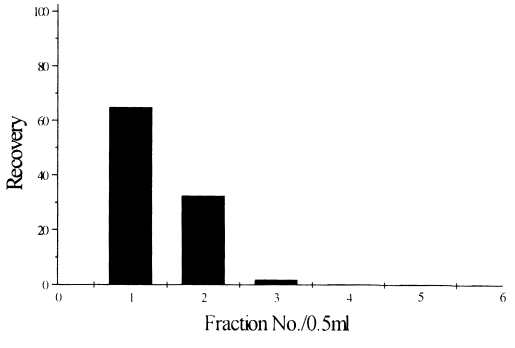

Fig. 3. The concentration distribution of ginsenoside Rg3 in eluent by 90% (v/v) methanol aqueous solution.

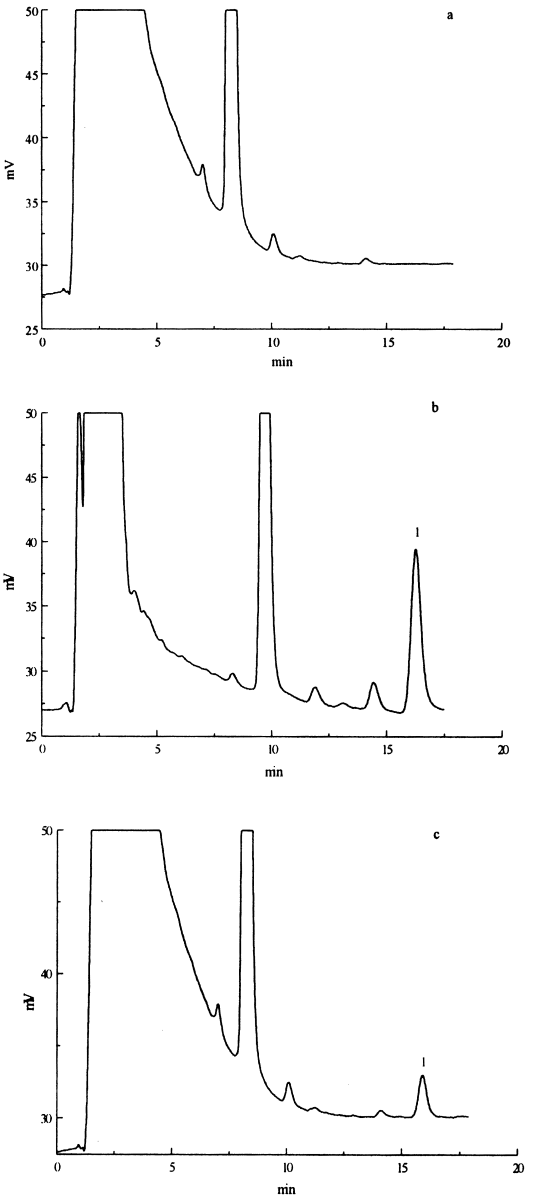

Fig. 4. Chromatogram of blank plasma (a), plasma with 100 ng/ml of ginsenoside (b) and plasma sample from a volunteer at 1 h after drug administration (c). Peak: 1=ginsenoside Rg3.

Table 1
Recovery of the method

Amount added (ng/ml)	Recovery (n=5, %)	Relative standard deviation (n=5, %)
10	99.37	4.83
50	99.46	4.93
100	97.97	4.13

3.4. Precision and recovery

Precision of the assay, calculated as the relative standard deviation (RSD) for intra-assay variability was below 5.0%, and inter-assay validation was below 4.0%. The method was shown good precision and good absolute recovery (above 97%).

Absolute extraction recovery of ginsenoside Rg3 from human plasma was estimated using quintuplicate standard samples at concentrations ranging from 10 ng/ml to 100 ng/ml by comparing the peak areas from processed plasma standard samples to those from a calibration curve prepared directly from analytes in water without any pretreatment. In the range of calibration standards, the mean recovery of ginsenoside Rg3 in human plasma was 98.93% (n=5). The results of method validation and extraction recoveries are summarized in Tables 1 and 2. There is no change observed for ginsenoside Rg3 under storage at −20°C, or during sample preparation process, and this compound is very stable under the present storage and preparation conditions.

3.5. Pharmacokinetic study

The analytical method was applied to a pharmacokinetic study with ginsenoside Rg3. Fig. 4c shows a representative chromatogram from this study. Fig. 5 shows mean plasma concentration–time profiles of ginsenoside Rg3 after oral administration of 3.2 mg/kg in eight healthy volunteers. Ginsenoside Rg3 was not detected in the samples taken at 10 h, 12 h and 24 h. The excellent precision of the method allows the accurate determination of phar-

Table 2
Precision of the method

Amount added (ng/ml)	Intra-day (n=5)		Inter-day (n=5)	
	Amount determined (ng/ml)	RSD (%)	Amount determined (ng/ml)	RSD (%)
10	9.94±0.48	4.83	10.03±0.23	2.30
50	49.73±2.45	4.93	47.86±1.59	3.33
100	97.97±4.05	4.13	100.17±3.27	3.46

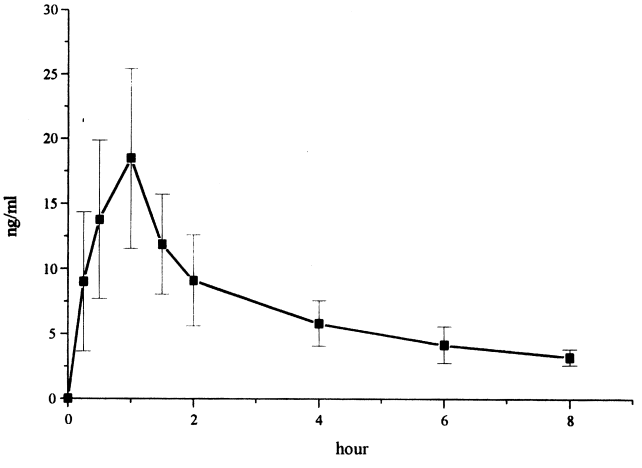

Fig. 5. Mean plasma ginsenoside Rg3 concentration vs. time profiles in eight healthy volunteers after administration of a single 3.2 mg/kg oral dose.

macokinetic parameters from plasma concentration–time data. In our study, the pharmacokinetic parameters of ginsenoside Rg3 are obtained, C_{max} = 15.67±6.14 ng/ml and t_{max} = 0.66±0.01 h.

This paper describes a sensitive, specific, rapid and robust reversed-phase HPLC method with UV detection for the measurement of ginsenoside Rg3 in human plasma. The method has been demonstrated to be suitable for use in pharmacokinetic studies of ginsenoside Rg3 in human.

References

[1] M. Mochizuki, Y.C. Yoo, K. Matsuzawa, I. Saiki, S. Tonooka, K.-I. Samukawa, I. Azuma, Biol. Pharm. Bull. 18 (1995) 1197.
[2] K. Shinkai, H. Akedo, M. Mukai, F. Imamure, A. Isoai, M. Kobayashi, I. Kitagawa, Jpn. J. Cancer Res. 87 (1996) 357–362.
[3] N. Nishimoto, S. Masaki, S. Masaki, S. Hayashi, T. Takemoto, T. Hayashi, N. Tsuji, Shoyakugaku Zasshi 40 (1986) 345.
[4] H. Yamaguchi, R. Kasai, H. Matsuda, O. Tanaka, T. Fuwa, Chem. Pharm. Bull. 36 (1988) 3468.
[5] H. Kanazawa, Y. Nagata, Y. Matsushima, M. Tomoda, N. Takai, J. Chromatogr. 630 (1993) 408.
[6] H. Kanazawa, Y. Nagata, E. Kurosaki, Y. Matsushima, N. Takai, J. Chromatogr. 632 (1993) 79.
[7] G. Gu, L. Zhou, G. Zhao, Se Pu 12 (1994) 40.
[8] K.-I. Samukawa, H. Yamashita, H. Matsuda, M. Kubo, Chem. Pharm. Bull. 43 (1995) 137.
[9] Y. Takino, T. Odani, H. Tanizawa, T. Hayashi, Chem. Pharm. Bull. 32 (1982) 2196.
[10] M. Karikura, H. Tanizawa, T. Hirata, T. Miyase, Y. Takino, Chem. Pharm. Bull. 40 (1992) 2458.
[11] Y. Takino, Yakugaku Zasshi 114 (1994) 550.