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Short communication

New method for high-performance liquid chromatographic separation and fluorescence detection of ginsenosides

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Abstract

A novel pre-column derivatization method for the quantitative determination of ginsenosides by HPLC with fluorescence detection was established. The double bond at the C_{24}–C_{25} position of ginsenoside was converted into an aldehyde group by means of ozonolysis. Then the aldehyde group reacts with FMOC-hydrazine forming the ginsenoside FMOC-hydrazone. The derivatized products were separated by RP-HPLC with gradient elution. The detection limits of ginsenosides Rg_1 and Rb_1 were 2.0 ng (about 2.5 pmol) and 1.0 ng (about 0.9 pmol), respectively. This method can be used for all ginsenosides having the C_{24}–C_{25} double bond.

Keywords: Derivatization, LC; Pharmaceutical analysis; Ginsenosides

1. Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae), is one of the best-known Chinese traditional herbal medicines, which has been used as a tonic, sedative, anti-fatigue, or anti-gastric ulcer drug for thousands of years. Ginseng saponins (ginsenosides) (Fig. 1), isolated from ginseng, have been regarded as the principal ingredients responsible for the pharmacological activities of the drug. More than 30 different ginsenosides are known, and can be classified into two groups according to their sapogenins with a dammarane skeleton, namely the protopanaxadiol and protopanaxatriol groups except for ginsenoside R_0. The contents of ginsenoside Rg_1 (protopanaxatriol group) and ginsenoside Rb_1 (protopanaxadiol group) in ginseng radix are relatively higher than that of other ginsenosides [1–3].

There are many methods for analyzing ginsenosides, such as colorimetry, thin-layer chromatography (TLC)–densitometry, gas chromatography (GC), high-performance liquid chromatography (HPLC) [4], and enzyme immunoassay [5,6]. Among these, enzyme immunoassay has the highest sensitivity, but it is suitable for only one ginsenoside and the other ginsenosides if co-existing may interfere with the determination. As for GC, although it will give good sensitivity and resolution, the sample preparation should involve hydrolysis and trimethylsilylation, and only the sapogenins protopanaxadiol and protopanaxatriol can be analyzed [7]. HPLC methods are widely used for analyzing ginsenosides. Since ginsenoside is a poor chromophore, the UV detection is limited to short wavelengths (e.g., 203

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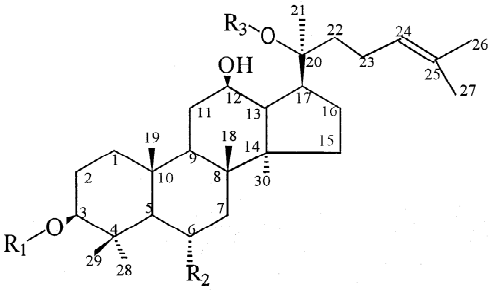

Compounds	R_1	R_2	R_3
Ginsenoside Rb_1	-Glc^2—^{1}Glc	H	-Glc^6—^{1}Glc
Rb_2	-Glc^2—^{1}Glc	H	-Glc^6—^{1}Ara(p)
Rc	-Glc^2—^{1}Glc	H	-Glc^6—^{1}Ara(f)
Rd	-Glc^2—^{1}Glc	H	-Glc
F_2	-Glc	H	-Glc
20(S)-Protopanaxadiol	H	H	H
Ginsenoside Re	H	-O-Glc^2—^{1}Rha	-Glc
Rg_1	H	-O-Glc	-Glc
Rh_1	H	-O-Glc	H
F_1	H	-OH	-Glc
20(S)-Protopanaxatriol	H	-OH	H

Fig. 1. Chemical structures of some ginsenosides and their metabolites. Glc: β-D-glucopyranosyl, Ara (p): α-L-arabinopyranosyl, Ara (f): α-L-arabinofranosyl, Rha: α-L-rhamnopyranosyl.

nm), and many compounds may interfere with the analysis and the detection sensitivity is not ideal. The post-column photoreduction fluorescence detection method has been used for ginsenosides, the limit of detection of ginsenoside Rg_1 is only 35 ng [8]. For the evaporative light scattering detection (ELSD) method, its sensitivity is higher than the UV detection, but the detection limits of ginsenosides Rg_1, Rd and Re reported are 50, 40, 65 ng, respectively [9]. The ion chromatography separation with pulsed amperometric detection (IC–PAD) for ginsenoside has a good sensitivity, the limits of detection for ginsenosides Re and Rg_1 are 0.8 and 1.0 ng, respectively, but for the protopanaxadiol group ginsenoside Rc is only 50 ng [10]. Therefore, it is necessary to develop a sensitive method that can be generally used for all ginsenosides. Recently, the LC–tandem mass spectrometry (MS–MS) method has been developed for the characterization and quantification of ginsenosides contained in the extracts of ginseng, the detection limit for ginsenosides can be at the level of ~2 pg [11], to date, it is the most sensitive method.

A new HPLC method with fluorescence detection is described in this paper. A pre-column derivatization based on the double bond in the C_{24}–C_{25} position of all the ginsenosides was explored. The limit of detection was very much improved.

2. Experimental

2.1. Chemicals and samples

The reference standards ginsenoside Rg_1 and ginsenoside Rb_1 were obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), dimethyl sulfide was from Merck–Schuchardt, Dr. Th. Schuchardt & Co., and trifluoroacetic acid (TFA) was from Sigma. 9-Fluorenylmethoxycarbonyl hydrazine (FMOC-hydrazine) (purity>99%) was synthesized in our laboratory [12]. All other chemicals were of analytical grade and obtained from Beijing Chemical Reagent Factory.

2.2. Preparation of ozone

Ozone was generated by silent discharge of oxygen using a laboratory-made ''ozonator''. The yields of ozone were: $7.84 \cdot 10^{-6}$ mol/min (oxygen flow-rate: 6.0 ml/min), $1.13 \cdot 10^{-5}$ mol/min (oxygen flow-rate: 8 ml/min).

2.3. Derivatization of ginsenosides

A solution of 50 ng–100 μg of ginsenosides in 200 μl of methanol was cooled at −28°C (ice–$CaCl_2$ bath). The ozone–oxygen mixture gas was passed through the solution for 5–10 s at a rate of 6 ml/min. Then, 10 μl of dimethyl sulfide was added. The solution was left to sit at −28°C for 1 h, then at room temperature for another hour to evaporate the excess dimethyl sulfide, and then was dried by nitrogen stream. A 20–150-μl volume of different concentrations of FMOC-hydrazine in acetonitrile, such that the ratio of ginsenoside to FMOC-hydrazine was 1:25, and 20–150 μl of methanol containing 0.5% acetic acid were added to the dried residue, and mixed on the vortex mixer for 5 min. The mixture was heated at 40°C for 4 h in a water bath and then cooled to room temperature. A 10-μl

volume of the derivative solution was injected onto the HPLC system.

2.4. Treatment of serum sample

A 0.5-ml volume of serum containing 5.0 μg or 50 ng ginsenosides was deproteinized with methanol (1.5 ml) and centrifuged at 3000 rpm for 5 min. The supernatant (1.5 ml) was evaporated to dryness under vacuum. The residue was dissolved in 20% methanol aqueous solution (3 ml) and applied to a Sep-Pak C_{18} cartridge prewashed with 2 ml of methanol and 3 ml of distilled water. After washing the column with 3 ml of 30% methanol aqueous solution, the ginsenosides were eluted with 1.5 ml of methanol. The eluate was dried by nitrogen stream. The residue was dissolved in 200 μl of methanol and then treated with the derivatization procedure described above.

2.5. High-performance liquid chromatography

The HPLC system consisted of a Spectra SYSTEM P2000 pump, a Hitachi 204-A fluorescence spectrophotometer (excitation at 270 nm, emission at 310 nm), a WDL-95 chromatography workstation (Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Liaoning, China) The ginsenoside FMOC-hydrazones were separated on a Waters Nova-Pak C_{18} (60 Å, 4 μm, 150×3.9 mm) column. For gradient runs, mobile phase (A) was 50% (v/v) methanol aqueous solution containing 0.1% TFA and (B) was 90% (v/v) methanol aqueous solution containing 0.1% TFA.

3. Results and discussion

3.1. The derivatization reaction

The double bond at the C_{24}–C_{25} position is an invariable group in all ginsenosides, and also in most of the metabolites of ginsenosides [13]. Therefore, it can be used as a site for derivatization and be converted into an aldehyde group at the C_{24} position using ozonolysis. Then the aldehyde group reacts with FMOC-hydrazine forming the ginsenoside FMOC-hydrazone (Fig. 2).

For the series of 20(*S*)-protopanaxadiol saponins (e.g., ginsenosides Rb_1, Rb_2, Rc, Rd and F2), the differences of the compounds exist at the C_3 and C_{20} glycosyl moieties. As to the series of 20(*S*)-protopanaxatriol saponins (e.g., ginsenosides Re, Rg_1, Rh_1 and F_1), the differences are at the C_6 and C_{20} glycosyl moieties. The fluorescence intensity only depends on the FMOC moiety derivatized on the C_{24} position of ginsenosides. So ginsenosides Rb_1 and Rg_1 can be selected as the typical ginsenosides studied in this work. The derivatized products were separated by HPLC. Then the fractions were collected and confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS. The peaks at *m/z* 1033.80 and 1049.78 correspond to $[M+Na]^+$ and $[M+K]^+$ of

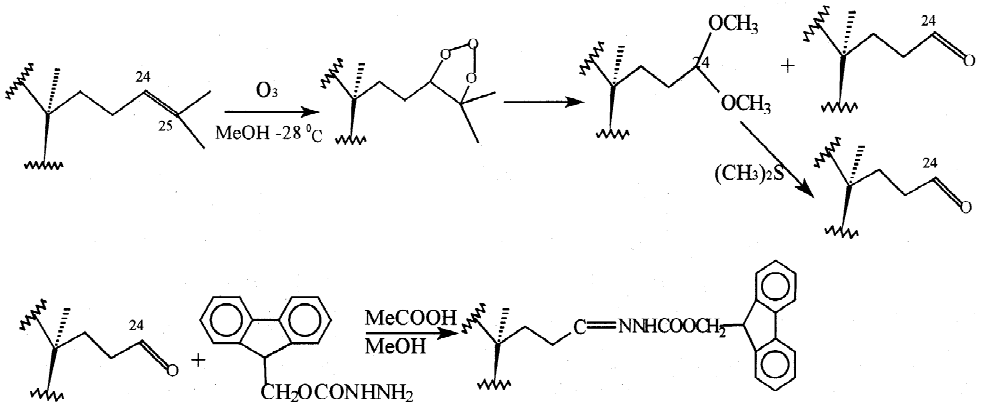

Fig. 2. Procedure of derivatization of ginsenosides with FMOC-hydrazine.

ginsenoside Rg_1 FMOC-hydrazone ($C_{54}H_{78}O_{16}N_2$), the peaks at m/z 1341.90 and 1357.76 correspond to $[M+Na]^+$ and $[M+K]^+$ of ginsenoside Rb_1 FMOC-hydrazone ($C_{66}H_{98}O_{25}N_2$). Dimethyl sulfide as the reducing agent can react with hydroperoxides rapidly at low temperature. It is highly selective, e.g., neither nitro groups nor carbonyl functions are reduced; the reduction is carried out under neutral conditions; and excess dimethyl sulfide is readily removed by evaporation (b.p. 37°C) and the by-products, methanol and dimethyl sulfoxide can be removed easily [14]. Fig. 3 shows the chromatogram of the derivatized products.

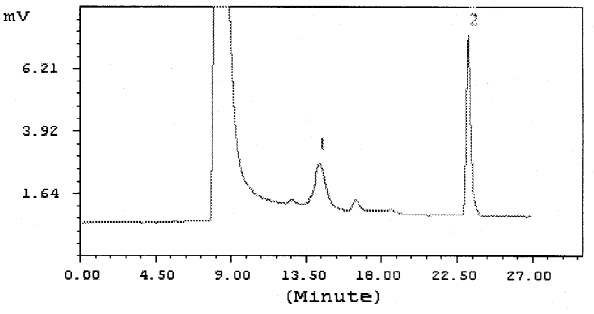

Fig. 3. RP-HPLC of ginsenoside Rg_1 FMOC-hydrazone and ginsenoside Rb_1 FMOC-hydrazone. The separation was achieved with a gradient elution for 26 min at a flow-rate of 0.8 ml/min. Conditions: column Waters Nova-Pak C_{18}, 60 Å, 4 μm 150×3.9 mm; mobile phase (A) 50% (v/v) methanol in 0.1% TFA; (B) 90% (v/v) methanol in 0.1% TFA; the profile of gradient elution: 0–2 min, A→5% B; 2–12 min, 5% B; 12–18 min, 5% B→55% B; 18–22 min, 55% B; 22–24 min, 55% B→70% B. Peaks: 1=ginsenoside Rg_1 FMOC-hydrazone, 2=ginsenoside Rb_1 FMOC-hydrazone. Each peak corresponds to 100 ng of ginsenoside.

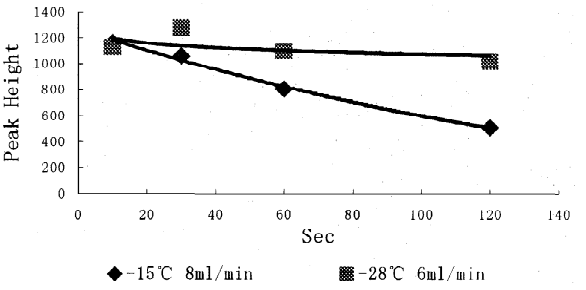

Fig. 4. Effects of temperature and time for introducing ozone on the yield of ginsenoside Rb_1 FMOC-hydrazone. 20 μg of ginsenoside Rb_1 was in 200 μl reaction solution.

3.2. Optimization of derivatization conditions

The effects of temperature and time for ozone introduction on the yield of ginsenoside Rb_1 FMOC-hydrazone was investigated. As shown in Fig. 4, at −15°C, the yield of ginsenoside Rb_1 FMOC-hydrazone decreased with the increasing time for ozone introduction; however, at −28°C, the yield of ginsenoside Rb_1 FMOC-hydrazone did not change obviously. In fact, the molar ratio of ozone to ginsenoside Rb_1 was high enough even if the reaction time was 1 s only. Considering the convenience of operation, the optimum time and temperature of the ozonation should be 5–10 s at −28°C.

In the derivatization, acetic acid was used as a catalyzer of FMOC-hydrazine with the aldehyde group; the acid concentration affects the rate of hydrazone formation and its yield [12]. The effect of 0~5% acetic acid on derivatization of FMOC-hydrazine with ozonized ginsenoside Rb_1 was examined (300 μl of derivative solution contained 20 μg ginsenoside Rb_1). The result showed that when the concentration of acetic acid was above 0.5%, the yield of derivative remained constant. Because the higher concentration of acid would cause the decomposition of ginsenosides [7,15], the optimum concentration of acetic acid should be around 0.5%.

The reaction of FMOC-hydrazine with aldehyde groups is an addition reaction. The addition product then releases water to form a stable product, FMOC-hydrazone. Therefore, increasing the molar ratio of FMOC-hydrazine to ginsenoside would be of benefit to improve the yield of ginsenoside FMOC-hydrazone. The derivative yield was investigated with the molar ratio in the range 2.8:1 to 125:1. The result showed that above the molar ratio 25:1, the derivative yield reached a high level. It reveals that the derivatization was completed at the molar ratio 25:1.

The reaction temperature and time are always the important factors on derivatization yield. From Fig. 5, it can be seen that, at lower temperature (e.g., 30°C), the reaction was slow, while at higher temperature (e.g., 50°C), the reaction reached near completion in 4 h. But at higher temperature, the solvent evaporated quickly so that it was difficult to maintain the concentration of derivatives. At 40°C,

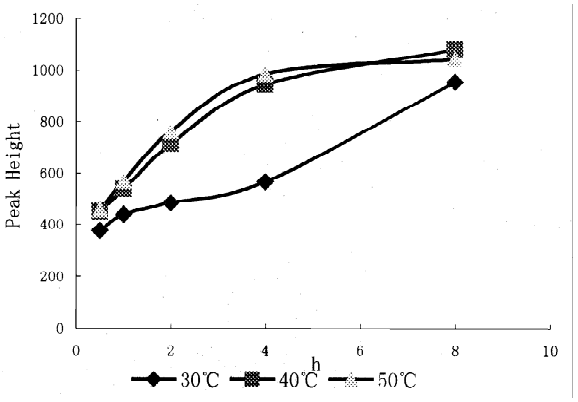

Fig. 5. Effects of reaction time and temperature on the derivation of FMOC-hydrazine with ozonized ginsenoside Rb_1, 20 μg of ginsenoside Rb_1 was in 300 μl reaction solution.

the reaction also reached near completion at 4 h. So the preferred reaction temperature and time should be at 40°C for 4 h.

Three samples derivatized as the described were stored in the derivatized solution at room temperature (24–30°C) for 30 and 100 h, respectively. The percent losses were less than 8.3%. This shows that the derivatives are stable at room temperature. In Fig. 5, it can also be seen that the derivatives are stable during the reaction.

3.3. Regression equation, sensitivity, recovery and reproducibility of the derivatization

The linearity of the detector response was investigated by injection of progressive dilutions of the ginsenoside FMOC-hydrazones. The response was linear in the range from 10^{-6}~10^{-3} g/ml (about 10^{-9}~10^{-6} mol/ml), the injection volume was 10 μl. The regression equations were $y=3008.9x-994$ ($R^2=0.9999$) for ginsenoside Rg_1 and $y=3317.3x+1035$ ($R^2=0.9998$) for ginsenoside Rb_1, where y is the peak area and x is the mass of analyte (ng). The detection limits are 2 ng (about 2.5 pmol) and 1 ng (about 0.9 pmol) with a signal-to-noise ratio of 3 for ginsenosides Rg_1 and Rb_1, respectively.

Ginsenosides Rg_1 (5.0 μg, 50 ng) and Rb1 (3.6 μg, 50 ng) were treated using the described derivatization procedure, then applied to HPLC. In another experiment, 5.0 μg and 50 ng of ginsenosides Rg_1 and Rb_1 were added to 0.5 ml serum, respectively, treated using the previously described procedure for serum sample, then derivatized as described and applied to HPLC. The recovery and the average relative standard deviation (RSD) are shown in Table 1. The reason for the lower recovery in serum would be the loss in the procedure of serum sample treatment.

Table 1
Recovery and average relative standard deviation of ginsenosides using the method

	Concentration (μg/ml)	Recovery (%)	RSD (%) (*n*)
Rg_1	25.0	96	3 (6)
	1.0	94	4 (4)
Rb_1	18.0	97	5 (6)
	1.0	103	5 (6)
Rg_1 in serum	10.0	72	3 (5)
	0.1	72	15 (4)
Rb_1 in serum	10.0	80	7 (6)
	0.1	76	12 (5)

4. Conclusion

The method described in this paper is a novel, reliable and sensitive pre-column derivatization method for fluorometric detection and can be used for all ginsenosides and most of their metabolites that have the C_{24}–C_{25} double bond. The derivatization conditions are mild and the derivatives are stable. The derivatized solution could be injected directly and separated on a C_{18} column with ultraviolet and fluorescence detection. The limits of detection are 2 ng (about 2.5 pmol) and 1 ng (about 0.9 pmol) with a signal-to-noise ratio of 3 for ginsenosides Rg_1 and Rb_1, respectively.

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