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RESEARCH PAPER

Sensitive Determination of Panaxadiol Using Rhodamine B as Sensitizing Derivatization Reagent by Ultrahigh Performance Liquid Chromatography Triple Quadrupole Mass Spectrometry

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Abstract: Commercial Rhodamine B was first reported as a derivatization reagent labeling hydroxyl compound for mass spectrometry sensitization. In virtue of the strategy of ultrasonic-assisted dispersive liquid-liquid microextraction (UA-DLLME) combined with Rhodamine B derivatization, a new method of ultrahigh performance liquid chromatography triple quadrupole mass spectrometry (UHPLC-MS/MS) was developed for the sensitive determination of Panaxadiol (PD). UA-DLLME and derivatization conditions were investigated. PD in cosmetics and ginseng was extracted by UA-DLLME (150 μL chloroform as extraction solvent, 500 μL ethanol as disperser solvent, 3 min). Using Rhodamine B as precolumn derivatization reagent, the stable derivative of PD was obtained in acetonitrile at 70 °C for 30 min under microwave radiation (800 W) with the catalyst of *N*,*N*′-carbonyldiimidazole (CDI) and 4-dimethylaminopyridine (DMAP). And then the PD derivative was separated and detected within 3 min by multiple reaction monitoring (MRM) mode of UHPLC-MS/MS. The LOD was 4.0 ng L^{-1}, and the LOQ was 15.0 ng L^{-1}. This method had perfect linearity, precision and recovery results, and showed obvious advantages such as sensitivity and low matrix effect in comparison with the previously reported methods.

Key Words: Ultrasonic-assisted dispersive liquid-liquid microextraction; Rhodamine B; Derivatization; Mass spectrometry sensitization; Panaxadiol

1 Introduction

Panaxadiol (PD), the common hydrolyzate of protopanoxadiol-type ginsenosides, exhibits some important pharmacological activities. It was widely used as a functional component in various drugs, foods and cosmetics[1]. PD shows poor UV absorption and weak mass spectrometry (MS) ionization efficiency. Therefore, direct detection methods such as spectroscopy[2], chromatography[2,3] and mass spectrometry[4–6] could not meet the requirements of sensitivity and the degree of separation.

Currently, derivatization method for the determination of compounds with hydroxyl groups has attracted much attention[7]. Since these compounds lack of response groups for chromatography or MS, derivatization could greatly enhance the determination sensitivity. Rhodamine B (RB) is a commonly used commercial fluorescent dye which is mainly used in sensors analysis and cell staining for biochemical analysis. Since the RB molecule contains a quaternary ammonium type positive charge, its PD derivative presents positron state which conforms to the mechanism of MS sensitization[8,9]. In this study, an accurate, rapid and sensitive UHPLC-MS/MS method was proposed for the determination of PD in cosmetics and ginsengs by using RB as derivatization reagent and combining with ultrasonic assisted-dispersive liquid-liquid microextraction (UA-DLLME)[10] to greatly

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enhance the determination sensitivity and minimize the matrix effect.

2 Experiment

2.1 Instrument and reagents

Ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis was performed by using an Agilent 1290 series UHPLC system (Agilent, USA) which equipped with a quaternary pump, an online degasser, an autosampler, a thermostated column compartment and an electrospray ionization (ESI) source. Ultrasonic cleaner was purchased from Kunshan Ultrasonic Instrument Co. (Zhejiang, China). Microwave synthesizer (Xianghu Technology Co., China) and rotary evaporator (Yarong Biochemical Instrument Company, China) were also used in this study.

PD standard was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (China). Rhodamine B (99%) was purchased from Aladdin Reagent Company (China). HPLC-grade acetonitrile, methanol and formic acid were all purchased from Sigma (USA). Other reagents were of analytical grade. Water was purified on a Milli-Q system.

2.2 Experimental procedure

2.2.1 Solution preparation

PD and RB standard solutions were prepared in HPLC grade acetonitrile at 1.2×10^{-4} M and 5.0×10^{-3} M, respectively. All further working solutions with different concentrations were prepared by diluting corresponding stock solutions with acetonitrile. *N*,*N*-carbonyldiimidazole (CDI) of 7.5×10^{-2} M and 4-dimethylaminopyridine (DMAP) of 0.16 M were prepared as the same way mentioned above.

2.2.2 Derivatization

According to the reaction mechanism of carboxylic acid reagent labeling amino or hydroxyl[11], a two-step derivatization method was performed using microwave irradiation in this study as shown in Fig.1, and the derivative was produced rapidly.

Firstly, a large amount of derivatization reagent intermediate, 2'-Carbonylimidazole Rhodamine B (CIRB, stable for at least one week with sealed storage) were prepared once. The operation was as follows: 5 mL RB standard solution, 300 μL DMAP and 305 μL CDI standard were orderly added to round-bottom flask, and then heated to 70 °C using microwave irradiation at 800 W for 15 min, and the reactive intermediate (CIRB) was produced. Secondly, suitable amount of PD standard solution or real samples extracts after UA-DLLME was added to a bottle, then 300 μL CIRB solution and 100 μL DMAP solution were orderly added. The bottle was sealed and reacted under microwave irradiation at 800 W and 70 °C for 30 min. The derivatives were filtered through membrane and injected for HPLC-MS analysis.

2.2.3 Real sample preparation

One gram of ginseng powder and 1.0 g of ginseng cosmetic were weighed, respectively. The extraction and acid hydrolysis procedure were performed according to previous report[12]. After protopanoxadiol-type ginsenosides converted to PD, UA-DLLME procedure was then performed as follows: 5 mL of sample solution mentioned above was placed in a conical

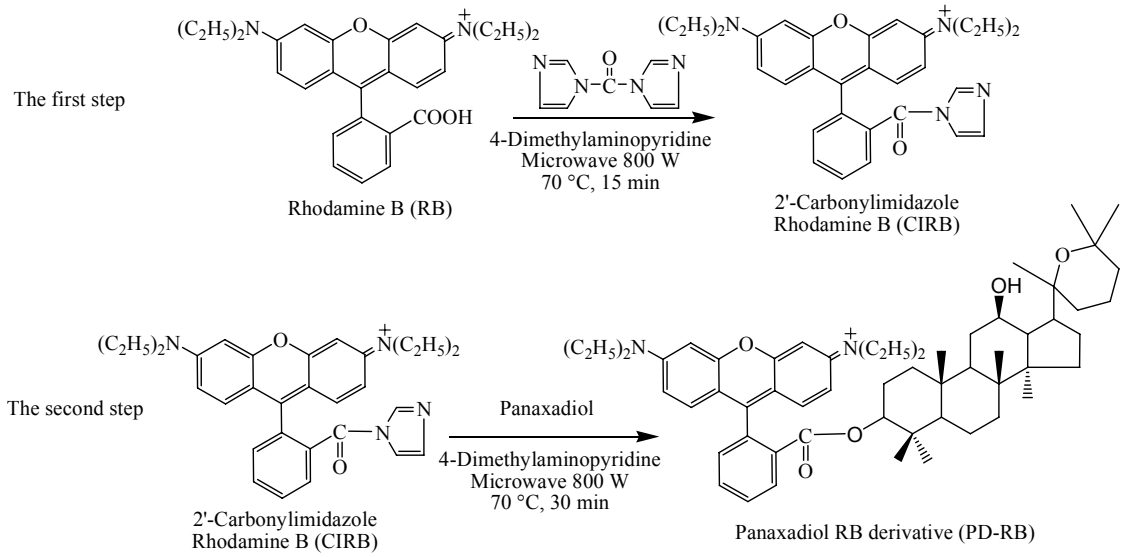

Fig.1 Scheme of derivatization reaction between Rhodamine B (RB) and Panaxadiol (PD)

conical centrifuge tube, and then a mixture of 500 μL of methanol (as disperser solvent) and 150 μL of chloroform (as extraction solvent) was rapidly injected into the sample solution with a syringe. The solution was diluted up to 10 mL with 4.35 mL ultrapure water. Then the solution was ultrasonicated for 3 min to form a homogeneous cloudy solution and then centrifuged at 12000 rpm for 2 min. The sedimented phase was transferred to a vial and dried under nitrogen. The dried sediment was re-dissolved in a certain amount of acetonitrile for derivatization.

2.2.4 UHPLC and MS conditions

Derivatives were separated on a ZORBAX Eclipse C_{18} column (50 mm × 2.1 mm, 1.8 μm, Agilent). The sample injection volume was 5 μL. Eluent A was 5% acetonitrile-water solution containing 0.3% formic acid, and eluent B was acetonitrile containing 0.3% formic acid. The flow rate was constant at 0.2 mL min^{-1} and the column temperature was set at 30 °C. The composition of mobile phases was 25% A and 75% B using a 3 min isocratic elution. Conditions of Agilent 6460 triple quadrupole mass spectrometry with an ESI source were set as follows: drying gas temperature 300 °C, dry gas flow rate of 9 L min^{-1}, nebulizer gas pressure of 40 psi, sheath gas temperature 300 °C, sheath gas flow rate 8 L min^{-1} and capillary voltage of 3.5 kV. The MRM parameters for quantitative transition and qualitative transition were optimized. The optimal fragmentor was 280 V. The optimal collision energy for quantitative transitions (*m/z* 885.4/443.1) and qualitative transitions (*m/z* 885.4/399.1) of PD derivative, were 76 and 82 eV, respectively.

3 Results and discussion

3.1 Optimization of derivatization conditions

In this study, the reaction mechanism (Fig.1) was identical with our previous reported work[11], in which carboxylic acid was used as derivatization reagent to label hydroxyl under the catalysis of CDI and DMAP. The results showed that conventional water bath heating method needed 30 min at 80 °C for the first step and 2 h at 80 °C for the second step, respectively. To accelerate the reaction rate, microwave-assisted method was applied in this study. In the first step, after 15 min at 70 °C and 800 W, the reaction could complete and intermediate CIRB was produced. In the second step, PD could completely react with excess CIRB under microwave irradiation for 30 min at 70 °C and 800 W. In conclusion, microwave-assisted derivatization could greatly enhance the rate of derivatization reaction.

3.2 Optimization of UA-DLLME extraction procedure

The type and volume of extraction and disperser solvents are the most important factors influencing the extraction efficiency. A series of experiments were performed by using dichloromethane, chloroform, trichloroethylene tetrachloroethylene or chlorobenzene as extraction solvent, and acetonitrile, methanol, ethanol, or acetone as disperser solvents. The results indicated that the best extraction efficiency and the highest instrument response were achieved when chloroform was selected as extraction solvent (Fig.2A) and ethanol was selected as disperser solvent (Fig.2C).

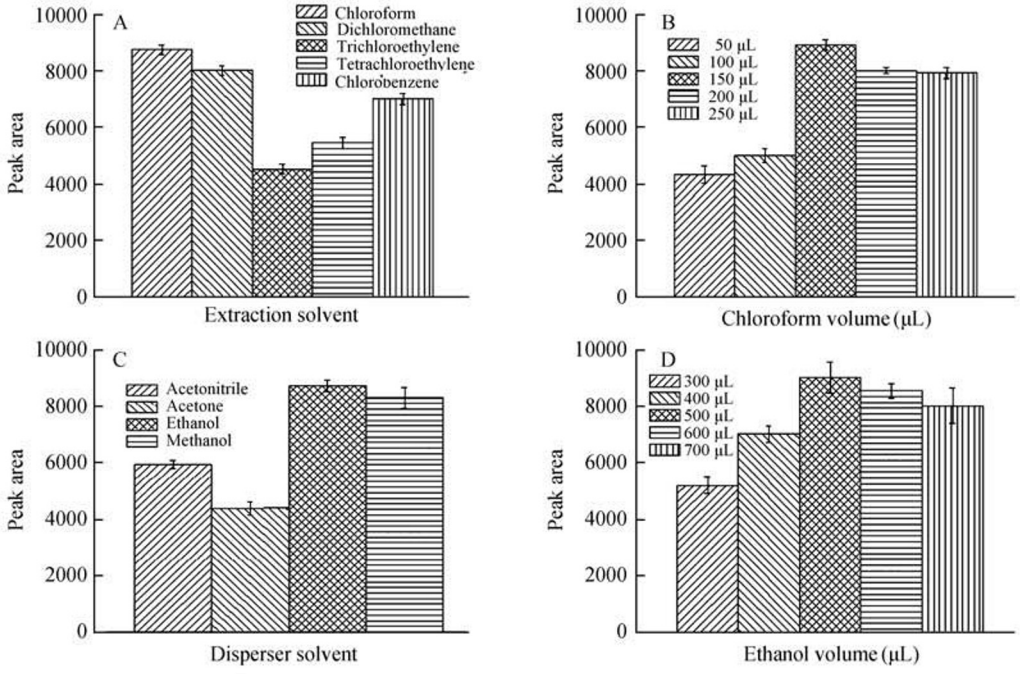

Fig.2 Effect of (A) extraction solvent type, (B) volume of chloroform, (C) disperser solvent type, (D) volume of ethanol on the peak area of panaxadiol derivative

The volumes of extraction and disperser solvents were also investigated. The best extraction efficiency was obtained when 150 μL of chloroform (Fig.2B) and 500 μL of ethanol (Fig.2D) were chosen as the optimum volumes of extraction and disperser solvents. Under the above optimized conditions, the effect of ultrasonication time was optimized. The highest detector response was obtained when ultrasonication time was 2 min at room temperature.

3.3 Method validation

3.3.1 Spectral properties of PD-RB derivative

PD-RB (5.0×10^{-5} M) in 50% acetonitrile/water solution was obtained with semi-preparing liquid chromatography. The maximum visible absorption wavelength of PD-RB was detected at 554 nm, and the molar absorption coefficient (ε) was 6.5×10^5 M^{-1} cm^{-1} at 554 nm. The maximum fluorescence excitation and emission wavelengths were λ_{ex} = 550 nm and λ_{em} = 577 nm, respectively. As shown in Fig.3, in positive ion mode, two specific product ions *m/z* 443.1 and 399.1 were produced at fragmentor 280 V and collision energy 76 eV. Their corresponding chemical structures were RB and RB losing a molecule of carbon dioxide.

3.3.2 Optimization of UHPLC-MS/MS conditions

UHPLC-MS/MS analysis was performed on an Agilent chromatographic column (1.8 μm). Different mobile phases and elution condtions were optimized in details. The results indicated that the mobiles containing 0.3% formic acid were used to improve the peak shape and prevent peak tailing (Fig.3A). This might be because the carboxyl in the position 2' of RB molecule was easily cyclized with the carbon atom on the xanthene ring, which made the molecule lose a positive charge.

As can be seen from above optimized MRM parameters, precursor ion of PD derivative could produce two specific product ions. Their corresponding chemical structures were RB and RB losing a molecule of carbon dioxide (Fig.3B). Because RB molecule has a natural positive charge, this resulted in very high MS ionization efficiency and sensitivity. Using these two specific product ions as the ion channels of MRM detection could greatly improve the analysis selectivity and sensitivity.

3.3.3 Linear regression equation, LOD and LOQ

Calibration curve was constructed by peak areas of the standard solutions versus their concentrations in the range of 20–500 ng L^{-1}. The regression equations was $y = 458.4 + 161.5x$ (y: peak area, x: sample concentration, ng L^{-1}) for PD. Good linearity with correlation coefficient (R^2) of 0.998 was obtained. The limit of detection (S/N = 3) was 4.0 ng L^{-1} and the limit of quantification was 15.0 ng L^{-1}.

3.3.4 Precision and recovery

Under the same experiment conditions, PD derivative solution was analyzed six times in parallel by UHPLC-MS/MS with good precision results. The relative standard deviations (RSDs) for retention times and peak areas were 1.8% and 4.6%, respectively. The ginseng cosmetic and ginseng powder samples were pretreated as above conditions. The recoveries of these samples spiked with PD standard were in the range of 81.3%–114.6%.

3.3.5 Comparison with reported methods

With regard to the derivatization method, compared with previous benzoyl chloride (2 h needed) and 9-methylfluorene formylhydrazine (4 h needed) derivatization method[12–14], the speed of the derivatization reaction in this study was largely increased by assistant microwave. Because derivatization solution of the first step could be largely prepared, and would be stable at least for a week, the derivatization time for vast real samples was decided by the second derivatization step and only 30 min was needed.

About the method sensitivity for the determination of PD in

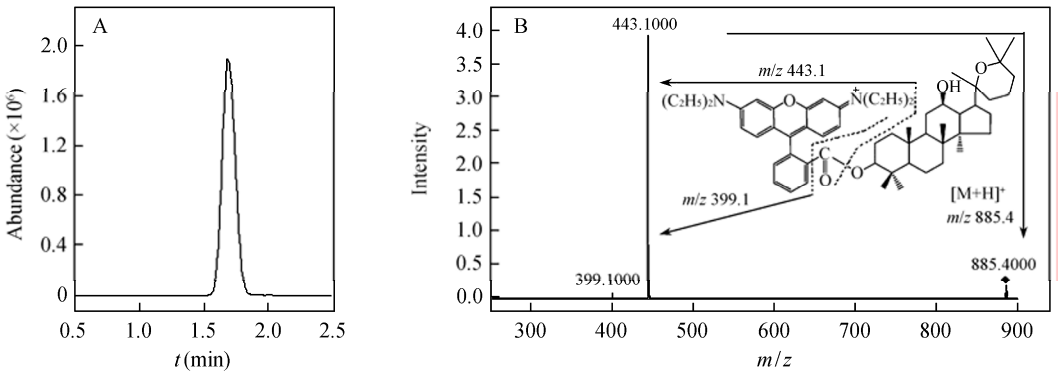

Fig.3 Ion current chromatogram (A) and MS/MS (B) of panaxadiol derivative

ginseng herbs, LOD of PD in this study was 0.004 ng mL^{-1} by a combination of UA-DLLME and derivatization UHPLC-MS/MS (MRM), which was superior to that of HPLC-UV (230 nm) detection (70 ng mL^{-1})[12] and benzoyl chloride derivatization method (2.4 ng mL^{-1})[13]. Furthermore, as reported in previous literature, the LOD of ginsenoside Rg_1 and Rb_1 was 100–200 ng mL^{-1} using 9-methylfluorene formylhydrazine as derivatization reagent[14] with HPLC fluorescence detection, and the LOD of Rg1 was 1.02 ng mL^{-1} without derivatization by direct HPLC-MS/MS detection[15]. In conclusion, this established method was more sensitive at least 250 times than those of reported literatures according to their LODs.

3.4 Analysis of sample

According to the experiment conditions above mentioned, PD content of ginseng herbs (Jinan Tongrentang drugstore, China) and ginseng cosmetics (Shanghai Sinoway Herb Cosmetics Co., China) were determined, respectively. The averaged PD contents of them were 9.2 mg g^{-1} and 35.8 μg g^{-1} (n = 3).

In real samples analysis, RB can also react with panaxatriol (PT), a hydrolysate of panaxtrol saponins. But there is no interference for the PD detection because they have different precursor ions, product ions and retention times in LC-MS analysis. There are two reasons about only PD determination used for the quality control of ginseng herbs: firstly, panaxadiol saponins and panaxtriol saponins produce simultaneously in the growth process of ginseng, each kind can reflect its saponin levels; secondly, shorter analysis time and quicker analysis speed can be obtained by the mere determination of PD, which is an advantage in large amounts of samples analysis. This purpose in this method was identical with that used by Shi *et al*[12], in which only PD determination was used for the quality control for ginseng medicinal materials.

4 Conclusions

In this study, an UHPLC-MS/MS (MRM) method was developed for the determination of panoxadiol with excellent sensitivity, selectivity and accuracy, which provided a powerful technique for the quality control of ginseng medicinal materials and related products.

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