Identification of Confusable Herbal Medicines by Mapping of Partial Degradation Products from Herbal Medicine Polysaccharides

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Over the years, herbal medicines (HMs) have been widely used in clinical applications worldwide. In order to guarantee their clinical curative effects, it is necessary to effectively and accurately identify HMs, especially for confusable or relative species.1 Confusable HMs are usually those medicines with similar characteristics, similar name or mixed clinical prescription due to traditional use customs with different pharmacological actions. These HMs should be distinguished explicitly.2 At present, in addition to the traditional methods for HM identification, such as macroscopic and microscopic identification,3 thin layer chromatography (TLC) identification,4 infrared radiation (IR) identification,5 and ultraviolet (UV) and fluorescent light (FL) identification,6 molecular identification using DNA barcode7 and fingerprint profiling of small molecular polysaccharides is not only a major challenge, but usually reveals little feature information of the target. However, the degradation products of polysaccharides are likely to show the unique feature of the polysaccharides. Wang et al.12 described a significant correlation between cold-hot natures and polysaccharides, which was based on the HPLC data of monosaccharides from 60 kinds of traditional Chinese medicine (TCM) polysaccharides. Wang et al.13 employed ion chromatography fingerprint analysis to identify and quantify the monosaccharide of complete hydrolysate of tea polysaccharides from different sources. Jing et al.14 applied the HPLC fingerprints of complete and partial hydrolyzed products of Flammulina velutipes polysaccharide to evaluate the polysaccharide quality from various sources.

However, the bond types and interior structures of the polysaccharide that significantly affected their physiological activity were damaged in case of the complete hydrolysis.15 As a result, partial degradation products (PDPs) are the more suitable analytes to be mapped. Di et al.16 demonstrated the feasibility of employing the high performance thin layer chromatography (HPTLC) peak fingerprints of acid hydrolysis carbohydrates for the screening of various Ganoderma species/products. Morlock et al.17 characterized and differentiated biopolymers based on their characteristic fingerprints of methanolysis products of polysaccharides by HPTLC. Sun et al.18 developed a fingerprint analysis method to characterize and distinguish the polysaccharides from different Ganoderma by their HPLC profiles of partly hydrolyzed products. Wu et al.19 studied oligosaccharides from wild and artificial cultivation of cordyceps polysaccharides by HPTLC, and the results revealed that the oligosaccharide profiles of wild and...
artificial ones were significantly similar, and the artificial cultivation ones were expected to become the alternatives of wild *cordyceps*. Wang et al. studied the partial acid hydrolysis-hydrophilic interaction liquid chromatography (HILIC) fingerprints of *Astragalus membranaceus* polysaccharides and developed a comprehensive quality evaluation method for *Astragalus membranaceus*. The applications of profiles of PDPs from HMs have achieved valuable results in many areas.

Radix Glehniae and Radix Adenophorae come from different families and genera and have different nourishing yin efficacy. Radix Sophorae Tonkinensis has a significant anti-tumor effect, whereas Rhizoma Menispermi has an obvious anti-inflammatory effect, though they have the similar effect of heat-clearing and detoxifying. The supplementary action of Radix Achyranthis Bidentatae is better than that of Radix Cyathulae, and Radix Cyathulae is more biased in the effect of activating blood circulation and stimulating meridians, and they all belong to Amaranthaceae plants and often are confused in clinical treatment. However, these HMs are all rich in bioactive polysaccharides. In this paper, we attempt to discriminate the above three groups of confusable HMs by profiling of PDPs from their polysaccharides and further investigate the feasibility of the method for the identification of confusable HMs by expanding its application.

Materials and Methods

Reagents and materials

The compound 1-phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Rizhao Lideshi Chemical Co., Ltd. (China), and β-cyclodextrin (β-CD) was purchased from Beijing Shuangxuan Microbe Culture Medium Products Factory (China). Arabinose, galactose, mannose, galacturonic acid and glucuronic acid were purchased from Beijing Solarbio Technology Co., Ltd. (China). Xylose was purchased from Baoding Second Chemical Plant (China). Rhamnose was purchased from Hefei Hiomi Biotechnology Co., Ltd. (China). Glucose, methanol and acetonitrile were from Tianjin Kernel Chemical Reagent Co., Ltd. (China), HPLC grade. All other chemicals that we used were of analytical grade and obtained commercially. Deionized water was used to prepare all solutions. The mobile phase was prepared with 0.3 M hydrochloric acid solution (0.3 M). Finally, 1 mL chloroform was added and shaken thoroughly. The mixture was left to react at 70°C for 0.5 h and then the solution was stored at 4°C. The precipitate was collected after being centrifuged and reconstituted with 40 mL deionized water. The solution was completely deproteinated with 8 mL Sevag (chloroform/butylalcohol 4:1, v/v) several times. Next, ethanol was added to a final concentration of 80%, and the solution was stored at 4°C for 8 h again, and then the precipitate was collected and subsequently dried at 55°C to a constant weight, and the HM crude polysaccharides were obtained.

Partial degradation with hydrochloric acid solution

An aliquot of 0.01 g crude polysaccharide was hydrolyzed with 0.4 mL hydrochloric acid solution (0.3 M) at 85°C for 3 h (samples 1 - 16) in a sealed glass vial. Then, 0.4 mL sodium hydroxide solution (0.3 M) was added to neutralize the hydrolyzed solution.

Derivatization with PMP

A sample of 200 μL polysaccharide hydrolyzate was mixed with 100 μL sodium hydroxide solution (0.3 M), followed by adding 100 μL PMP methanol solution (0.5 M), and oscillated sufficiently. The mixture was left to react at 70°C for 0.5 h and then was neutralized with 100 μL hydrochloric acid solution (0.3 M). Finally, 1 mL chloroform was added and shaken sufficiently to remove the excessive PMP. The supernatant was obtained after centrifugation at 4000 r/min for 3 min and filtered through a 0.45-μm membrane filter, and then the HPLC analysis was performed.

Optimization of chromatographic conditions

First, 0.01 g of β-CD was hydrolyzed with hydrochloric acid solution (2 mL, 0.3 M) under 90°C for 2 h, and then glucooligosaccharides were obtained. The other procedures followed were just the same as the above operations. The analysis of the glucooligosaccharides was performed with an HPLC method, and the optimization of the chromatographic conditions was carried out according to the resolution and retention times of the oligosaccharides.

Results and Discussion

Optimization of the HPLC chromatographic conditions for the separation of oligosaccharides

It has been reported that the degree of polymerization (DP) of active oligosaccharides is generally less than 8. β-CD is a cyclic oligosaccharide composed of 7 glucose, so we used the conditions were expected to become the alternatives of wild *cordyceps*. Wang et al. studied the partial acid hydrolysis-hydrophilic interaction liquid chromatography (HILIC) fingerprints of *Astragalus membranaceus* polysaccharides and developed a comprehensive quality evaluation method for *Astragalus membranaceus*. The applications of profiles of PDPs from HMs have achieved valuable results in many areas. Radix Glehniae and Radix Adenophorae come from different families and genera and have different nourishing yin efficacy. Radix Sophorae Tonkinensis has a significant anti-tumor effect, whereas Rhizoma Menispermi has an obvious anti-inflammatory effect, though they have the similar effect of heat-clearing and detoxifying. The supplementary action of Radix Achyranthis Bidentatae is better than that of Radix Cyathulae, and Radix Cyathulae is more biased in the effect of activating blood circulation and stimulating meridians, and they all belong to Amaranthaceae plants and often are confused in clinical treatment. However, these HMs are all rich in bioactive polysaccharides. In this paper, we attempt to discriminate the above three groups of confusable HMs by profiling of PDPs from their polysaccharides and further investigate the feasibility of the method for the identification of confusable HMs by expanding its application.

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partial degraded β-CD as the reference of mixture glucooligosaccharides to optimize the chromatographic conditions. The chromatogram of the glucooligosaccharides is shown in Fig. 1. We confirmed glucose and maltose peaks by adding the corresponding standards into glucooligosaccharides. Furthermore, the longer the oligosaccharide chain, the greater the polarity, so its interaction with C18 is weaker, and the retention time becomes shorter. Thus, it can be concluded that the peaks were maltoheptaose, maltohexaose, maltopentaose, maltotetraose, maltotriose, maltose and glucose successively.

In pre-tests, it was found that the acetonitrile proportion, buffer concentration and pH value of the mobile phase showed a great effect on the resolution between maltohexaose and maltoheptaose ($R_s$), and the final elution time ($T$). Thus, comprehensive evaluation of $R_s$ and $T$ was used for condition optimization. The results are shown in Figs. 2 – 4.

Four proportions of acetonitrile in mobile phase (19, 21, 23, and 25%, v/v) were investigated in the study. The $T$ was longer than 1 h when the acetonitrile proportion was 19%. We can see from Fig. 2 that when the proportion was 23%, it achieved the desired resolution ($R_s > 1.5$), and the $T$ was relatively shorter, so the acetonitrile proportion of 23% was selected.

Six buffer concentrations (0, 10, 25, 50, 75, and 100 mM) of ammonium acetate were also studied, and the results are shown in Fig. 3. When the buffer concentration was 0 mM, all the oligosaccharide peaks did not achieve the desired separation. Within 50 mM, both $T$ and $R_s$ increased accordingly with the increase of buffer concentrations. This could be due to the solubility of the solutes decreased in the mobile phase by the salting-out effect, which enhanced its retention on the stationary phase, and then a better separation of oligosaccharides was finally achieved. When the concentration exceeded 50 mM, the $R_s$ were much the same ($\geq 1.5$). Ultimately, we selected the concentration of 50 mM.

Finally, the pH value was studied, and the results are shown in Fig. 4. When the pH value increased, the $T$ was shortened. Due to the increasing of pH values, the enol group of PMP was easier to dissociate, and the ketone group’s transformation into enol group would also increase the polarity of the derivatives. In addition, the effects of the column temperature and flow rate were also investigated, and the results showed that they had little effect on the separation. For shortening the cycle of run, a flow rate of 1.3 mL/min and column temperature of 25°C were chosen under the premise of suited column pressure.

The optimized separation conditions were as follows: the mobile phase was 50 mM buffer of ammonium acetate (pH 5.5) with 23% acetonitrile; the flow rate was 1.3 mL/min; and the column temperature was 25°C. Among them, acetonitrile proportion and pH value had a more significant impact on the
separation of PDPs, while that of the buffer concentration higher than 50 mM was weaker.

Methodology validation

**Precision of intra-day and inter-day measurements.** According to the above sample preparation process and chromatographic conditions, after the polysaccharides extracted from Radix Glehniae were partially degraded and derived with PMP, the sample was continuously injected for five times to measure the precision of intra-day recordings, and repeated the analysis within 3 days to measure the precision of inter-day results. The RSDs of the peak areas (mainly the peaks 2 – 7 in Fig. 5A) and their retention times (RTs) were calculated. The intra-day RSDs were between 1.23 – 2.06% for peak areas and 0.63 – 0.69% for RTs, respectively, and the inter-day RSDs were between 4.15 – 5.00% for peak areas and 0.68 – 1.42% for RTs, respectively, which indicated that the method had good precision.

**Repeatability and stability tests.** According to the above sample preparation process and chromatographic conditions, five Radix Glehniae polysaccharide samples were parallelly prepared and analyzed. The RSDs of repeatability tests were between 2.34 - 4.66% for peak areas and 0.42 - 0.78% for RTs, respectively. Besides, the same sample was analyzed at 0, 2, 6, 12, 24 h, respectively, and the RSDs of the stability tests were between 3.54 - 4.92% for peak areas. The results showed that the method had good reproducibility and the sample had good stability within 24 h.

**Determination of HM PDPs fingerprints.**

PDPs from six kinds of HM polysaccharides were analyzed under the HPLC chromatographic conditions above (A, B and C in Fig. 5). Notably, the PDPs were all analyzed under the same condition.

In these figures, there were obvious differences in the PDP fingerprints of different HM polysaccharides, such as peak numbers and their areas. In addition, the fingerprints of the same kind of HM from different sources were fundamentally the same, except for some minor differences in their contents that may be caused by the different origins or storage conditions. Therefore, each HM had its characteristic PDP fingerprint. In addition, in the case of the same family and genus, the fingerprints (Fig. 5C) of Radix Achyranthis Bidentatae and Radix Cyathulae was similar in peak numbers and areas. It indicated that HMs from the same family and genus might contain the similar or the same type of polysaccharides. The above results showed that the developed method could not only be applied to HM identification, but also provide plenty of structure information about HM polysaccharides.

**Identifying confusable HMs assisted with cluster analysis**

The CA was performed by Minitab 16 software. According to the differences in the characteristic peaks and peak areas, the confusable herbal medicines can be distinguished. The PMP peak in HPLC fingerprint was used as the reference peak to locate the positions of saccharide peaks. The peak areas were input in order in the software. According to the levels of
took glucooligosaccharides (DP 1 – 7, partially degraded some monosaccharides more or less. In this experiment, we found that the end of the glycosidic bond was easier to break under acidic conditions. Thus, the obtained PDPs could contain fragments such as (DP3); 6, disaccharide (DP2); 7, glucose; Gal, galactose; Ara, arabinose.

Radix Adenophorae and Radix Sophorae Tokinensis PDPs by MALDI-TOF MS, results showed that most of the PDPs were oligosaccharides, and only a few belonged to monosaccharides, and the molecular weight distribution of these samples was consistent with that of the corresponding glucooligosaccharides. The peaks of DP greater than 7 were small or not detectable on HPLC. Further investigation of oligosaccharide identification is needed.

**Conclusions**

In this paper, a targeted and effective method was introduced to separate PDPs from confusable HM polysaccharides. The obtained characteristic PDP fingerprints by HPLC can be used to identify the confusable HMs effectively, assisted with CA. HPLC takes a longer time, but it possesses good separation ability and better reproducibility compared with capillary electrophoresis method. The method has promising applications in the identification of confusable HMs, especially for those with low amounts of characteristic small molecular compounds. This method can assist the standardization of quality control and planting of HM.

In addition, HMs from the same family and genus may well contain similar PDP segments with the same pharmacological effects, and the exploration of medicine sources of oligosaccharides can be expanded.

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