Simultaneous Determination of Catalpol, Aucubin, and Geniposidic Acid in Different Developmental Stages of Rehmannia glutinosa Leaves by High Performance Liquid Chromatography

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1. Introduction

Rehmannia glutinosa Libosch (dihuang in Chinese) belongs to the family Scrophulariaceae. Its raw or processed roots are the only organ source for clinical uses of traditional Chinese medicine, and the leaves are not utilized at all. However, R. glutinosa leaves have the pharmacological effects and can nourish yin, tonify qi and kidney, and promote blood circulation [1]. In the folk, the fresh leaves are often applied externally to treat malignant sore and tinea manus and pedis [2]. As a perennial herb, R. glutinosa has to be annually cultivated and harvested since its fleshy roots easily decay during the winter dormancy or are consumed when new plantlets come out from them in the next season. Due to its severe continuous cropping obstacle, however, R. glutinosa plants could not grow well on the same land after the first cropping, decreasing the root yield greatly [3]. Thus, exploitation of R. glutinosa leaves not only provides another potential medicinal source for extraction of important bioactive compounds present in its roots but also complements the shortage of R. glutinosa roots due to the limited land and continuous cropping obstacle.

A lot of pharmacologically bioactive secondary metabolites [4, 5] including iridoid glycosides, phenylethanoid glycosides, and polysaccharides have been isolated in R. glutinosa, of which iridoid glycosides are thought to be main bioactive constituents. Till now, more than thirty iridoid glycosides have been separated and identified [4–6], including catalpol, aucubin, geniposidic acid (GPA), rehmanniosides A, B, C, and D, and rehmaglutosides A–K. The pharmacological effects of some of these iridoid glycosides have been extensively studied. For example, as the main active iridoid glycoside in R. glutinosa, catalpol was found to play the important roles in treatment of many diseases including kidney diseases...
[7], neurodegenerative diseases [8], and diabetes [9, 10]. It is revealed that aucubin had pharmacological effects such as antifungals [11], anti-inflammation and antioxidation [12], and hepatoprotection [13]. Similarly, GPA had therapeutic effects in anti-inflammation, liver disorders, and antinociception [14, 15]. Among these three compounds, only catalpol was assayed in detail to reveal its spatiotemporal expression profiling among different developmental stages of R. glutinosa leaves and between leaf and root [16, 17]. It is shown that catalpol content varied among different developmental stages of R. glutinosa leaves and within the whole growth stage. Aucubin and GPA were found to accumulate in roots of R. glutinosa [18, 19], and only the former was quantified and much lower than catalpol in the root [18, 20]. Therefore, in this paper, we aimed to determine the content of these three iridoid glycosides of two R. glutinosa cultivars in different developmental stages of leaves using HPLC-UV method and compared their changing trend.

2. Materials and Methods

2.1. Plant Material. Two primary cultivars of R. glutinosa, Wen 85-5 and Beijing no. 1, were grown in around mid-April in 2014 at the IMPLAD (Institute of Medicinal Plant Development), Beijing, China. The plant interval was 40 cm × 40 cm. Normal field management was applied during the growth period. On 11th September, 2014, the top eight leaves and between leaf and root (4.6 mm × 100 mm, 2.6 µm), waters 2487 dual wavelength detector, and 2707 autosampler (USA). The isocratic mobile phase contained acetonitrile (5%) and 0.1% formic acid in water (95%) and was delivered at 0.4 mL/min. Catalpol and aucubin were monitored at λ 210 nm and geniposidic acid was monitored at λ 240 nm. The presence of the three iridoid glycosides in R. glutinosa leaves was determined by comparing both retention time and spectral data with those of their corresponding authentic standards. Concentrations of three iridoid glycosides in the samples were determined from the below linear standard calibration curves. Their content was shown in dry weight. Since catalpol and other iridoids are not heat-stable, the batch of leaf samples was used to calculate the dry weight of each stage leaf. Three replicates of each stage of leaf sample were measured.

3. Results and Discussion

3.1. Optimization of HPLC-UV Conditions. HPLC-UV method has been successfully applied to quantify catalpol (CA), aucubin (AU), and GPA in medicinal plants [16, 17, 20–22]. The HPLC conditions that researchers used varied in terms of the columns, mobile phase system, and detection wavelengths. Therefore, we optimized HPLC-UV conditions in terms of column type, UV wavelength, mobile phase composition, addition of formic acid in aqueous phase, and ratio of ACN and water in our early work (Table 1). Several UV wavelengths (e.g., 203 nm, 205 nm, 206 nm, and 210 nm) were used to detect the presence and content of catalpol and (or) aucubin. We found that there was no significant difference in detecting these two iridoid glycosides under 203–210 nm and thus chose 210 nm as the detection wavelength, which was used in China pharmacopoeia (2010). There was no much difference of the wavelengths (230–240 nm) in peak shape and separation of GPA except that less miscellaneous peaks and stable baselines were produced at λ 240 nm. Thus, GPA was detected at λ 240 nm in latter experiments. As Ji et al. [16] revealed, adding or not adding formic acid (FA) in the mobile phase did not obviously influence the peak shape and separation of catalpol and aucubin, except for GPA. While investigating the difference of different concentrations of ACN in the mobile phase system on the chromatogram, we found that the elution times of the analytes decreased with ACN concentration increased (Table 2). When ACN was higher than 5%, catalpol peak was overlapped with the solvent peak. However, when less than 5% of ACN was used in the mobile phase, more late AU and GPA were eluted. All the three analytes can be eluted within 10 minutes under 5% of ACN. In order to shorten the running time for compounds behind them, we adopted 5% of ACN in the final mobile phase system. The final HPLC conditions that we adopted to
determine CA, AU, and GPA in our study were summarized in Table 1.

Using the optimized conditions, we further tested our HPLC-UV conditions by checking its linearity, precision, stability, and recovery (Table 3). Figure 1 showed that standards of CA, AU, and GPA were eluted separately at 3.412 min, 4.941 min, and 9.850 min. Their corresponding compounds of CA, AU, and GPA were eluted separately at 3.436 min, 4.941 min, and 9.813 min, respectively. The linear regression equations in the sample extract were detected at 3.436 min, 4.941 min, 4.857 min, and 9.850 min. Their corresponding compounds of CA, AU, and GPA were eluted separately at 3.412 min, 4.941 min, and 9.850 min. The precision estimation, a sample extract was assayed successively for 6 times. The RSD values for CA, AU, and PGA were 1.46%, 2.38%, and 0.43%, respectively, indicating that our sample extract remained stable at least till 24 hours. The extraction recovery was determined by comparing the content of the compound extracted from the samples with the content of compound from nonextracted standard solutions at equivalent concentrations. To test the sample extraction recovery, one mL of the standard solution containing 4.824 mg of CA, 0.225 mg of AU, and 0.0134 mg of GPA, respectively, was added into one gram of leaf powder at the beginning of the extraction. The recovery rates for standards CA, AU, and PGA were 102.59%, 98.29%, and 101.96%, respectively. The calculated LOD (limit of detection) at S/N > 3 was, separately, 1.3333E – 04 mg/mL for CA, 1.8170E – 04 mg/mL for AU, and 4.9926E – 05 mg/mL for GPA.

3.2. The Content of Catalpol, Aucubin, and GPA Changed with the Leaf Development. *R. glutinosa* is a perennial herb with cespitose leaves on its shortened stem. The leaf biomass of *R. glutinosa* cultivars increased greatly from the seedling stage till 120 DAP (days after plantation) and then deceased after mid-September (140 DAP), partially due to ongoing senescence of bottom leaves (Figure 2). However, the top eight positions of leaves still stayed green and varied in leaf size, which can represent the different developmental stages of leaves (Figure 3). The 8th leaves, counted downwards from the uppermost leaves, looked similarly in their size and appearance status as the lower positions of unsenescent leaves. However, their dry weight (dry weight per gram fresh weight) changed dynamically with leaf development (Figure 4). Overall, leaf dry weight decreased with the leaf development and increased for L5 and then decreased in the rest of older leaves. L1–L8 had about 0.14–0.18 grams of dry biomass per gram fresh biomass. In this paper, we just focused on the contents of CA, AU, and GPA in the top eight positions of leaves and investigated their relationship with the leaf developmental stages. Although the contents of CA, AU, and GPA were somehow different between Wen 85-5 and Beijing No. 1, the changing trends of these three iridoid glycosides with the leaf developmental stage were very similar.
Table 3: Validation of the optimized HPLC-UV conditions for three analytes.

| Iridoid   | Calibration curve | Correlation ($R^2$) | Precision (RSD%) | Reproducibility (RSD%) | Stability (RSD%) | Recovery (%) | LLQD (mg/mL) |
|-----------|-------------------|---------------------|-------------------|------------------------|------------------|--------------|--------------|
| Catalpol  | $y = 0.9707x + 6.7723$ | 0.9997              | 0.14              | 0.25                   | 1.46             | 102.59       | $1.333E−04$  |
| Aucubin   | $y = 0.9952x + 6.8458$ | 0.9999              | 2.65              | 3.08                   | 2.38             | 98.29        | $1.8170E−04$ |
| GPA       | $y = 1.0136x + 7.5797$ | 0.9996              | 2.05              | 2.41                   | 0.43             | 101.96       | $4.9926E−05$ |

Figure 1: HPLC-UV chromatographs of standard mixture ((a), (b)) and R. glutinosa extract ((c), (d)).

Figure 2: Changes of fresh weight of R. glutinosa leaves during the growth period.

between the two cultivars, indicating no genetic difference in developmental regulation of these three iridoid glycosides (Figures 5 and 6). Generally, catalpol, a major bioactive iridoid glycoside of R. glutinosa, was higher in younger leaves and decreased with the leaf development, which was consistent with the result of Ji et al. [16]. Likewise in R. glutinosa root [18], aucubin in the leaf was still much lower than catalpol, which was found by Piątczak et al. [23]. The changing trend of aucubin among different developmental stages of R. glutinosa leaves was distinct from catalpol; that is, the older the leaves, the higher the aucubin in them. It was found that aucubin was one of the intermediates for catalpol biosynthesis in Scutellaria albida and Paulownia tomentosa [24]. The opposite metabolic profiling of aucubin and catalpol in R. glutinosa leaf indicated that catalpol might not be synthesized via aucubin; however, this needs to be further investigated. GPA was detected to be present in R. glutinosa leaves and the lowest analyte in all the developmental stages of R. glutinosa leaves. Like aucubin, GPA was increased with the leaf development and higher in older leaves.
4. Conclusion

In this paper, we determined contents of catalpol, aucubin, and geniposidic acid in different developmental stages of *R. glutinosa* leaves using the optimized HPLC-UV conditions and further compared their changing trend with the leaf development. Our results showed that aucubin and GPA in *R. glutinosa* leaves were much lower than catalpol and had the increasing trend with the leaf development, which was different from catalpol. This work provided an important basis for future exploitation of *R. glutinosa* leaves such as isolation of interesting metabolites, for example, the high concentration compound present at 4.16, which was further identified by HPLC-LC-MS method. It also provided a model to study the relationship of aucubin and catalpol metabolisms in *R. glutinosa*.

Competing Interests

The authors declare that there are no competing interests.

Authors’ Contributions

Yanjie Wang and Dengqun Liao contributed equally to this work.

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