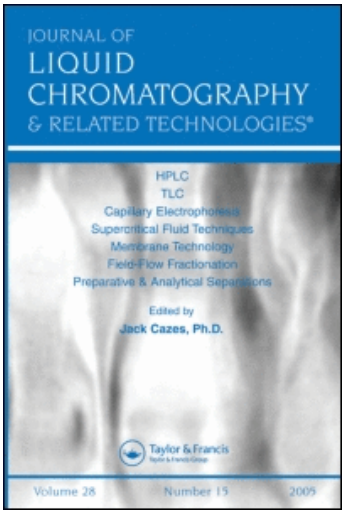

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Preparation and Identification of Scutellarein by HPLC with an Enzymolysis Reaction

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Abstract: An HPLC method was established for preparation of scutellarein from scutellarin using an enzymolysis reaction. The aglycone was identified as scutellarein with its chromatogram, UV, LC-MS, and ^{1}H-NMR spectra. Acid hydrolysis was tried in preliminary experiments, but enzymolysis was adopted after optimization of reaction conditions. High purity scutellarein (content 98.04% $\sim$ 98.36%) was prepared in this way for the first time, which could be used as a working reference substance.

Keywords: Scutellarein, Scutellarin, Preparation, Identification, Enzymolysis, HPLC

INTRODUCTION

Scutellarin, an effective constituent of several species of plants, such as a Chinese herb *Erigeron breviscapus* and a popular western herb *Skullcap*, has been applied to the treatment of occlusive cerebral vascular diseases.[1] Recently, scutellarin was reported to attenuate H_2O_2-induced cytotoxicity, intracellular accumulation of reactive oxygen species (ROS) and Ca^{2+}, lipid peroxidation, and loss of mitochondrial membrane potential (MMP) and DNA, which may represent the cellular mechanisms for its neuroprotective action.[2]

Scutellarein, the aglycone of scutellarin, was found to be an inhibitor of protein kinase C (PKC)[3] and xanthine oxidase activity,[4] which suggested its potential merits in relative fields. Scutellarein was not expediently

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available on the market for its small amount in herbs,[5] and it was found as one of the metabolites of scutellarin, just like other flavonoids such as apigenin.[6] Chemical structures of scutellarin and scutellarein are shown in Figure 1. To be utilized in further researches, such as on its pharmacology and metabolism of scutellarin, some scutellarein was prepared by high performance liquid chromatography (HPLC) after an enzymatic transformation, which turned scutellarin to scutellarein. For the first report, scutellarein (purity 98.04% $\sim$ 98.36%) was acquired in this method. It was more feasible than extraction and purification from the crude herbs.

EXPERIMENTAL

Reagents

Scutellarin and quercetin were provided by National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, PRC). β-glucuronidase from bovine liver (Type B-1, Sigma) catalyzed the enzymatic transformation. HPLC grade methanol and acetonitrile were from Merck (Ger). Acetic acid glacial was HPLC grade from Tedia (USA). Ammonium acetate, ascorbic acid, potassium dihydrogen phosphate, and diethyl ether anhydrous were all AR grade from China Medicine (Group) Shanghai Medical Reagent Corporation. HPLC grade water was prepared using Direct-Q^{TM} water purification system (Millipore, Bedford, MA, USA).

Instruments

A Waters HPLC system, including a 1525EF binary pump and a 2996 PDA detector controlled by the Empower software, was used to prepare scutellarein. An Agilent (USA) 1100 Series LC-UV system, which consisted of a G1311 quaternary pump, a G1313A autosampler, a G1314A DAD detector, a G1316A column oven, and a G1322A degasser was used to monitor the products of acid hydrolysis or enzymolysis. Different from

Figure 1. Structures of scutellarin, scutellarein, and quercetin.

the LC-UV system, an Angilent 1100 Series LC-MS system, which consisted of a G1312A Binary pump, G1315B DAD detector, and a G1946D MS detector was used to identify the scutellarein. A Rotavapor R-200 (Buchi, Switz) and ALPHA 2-4 freeze dryer (Martin Christ, Ger) were used to eliminate solvent. ^{1}H-NMR spectra were measured on Mercury Plus 400 Hz NMR (Varian, USA).

Enzymolysis

In a batch, 3.3 mg scutellarin was dissolved in 3 mL methanol and then diluted by water to 300 mL. 200 mg/mL ascorbic acid solution was freshly prepared. β-glucuronidase was diluted with 0.1 mol/L ammonium acetate buffer (adjusted to pH 5.0 with acetic acid) to 620 units/mL. Procedures, as below, were carried out in parallell in six 250 mL beakers. Scutellarin, 50 mL, 12.5 mL ascorbic acid, 187.5 mL ammonium acetate buffer, and 10 mL β-glucuronidase solutions were mixed and incubated at 37°C[7] overnight, under anaerobic conditions and protected from light.[8] After the procedure, 15 mL, 1 mol/L hydrochloric acid solution was added to each beaker for acidification.

The acidified mixture in the beakers was transferred to 500 mL tap funnels and then partitioned with diethyl ether anhydrous at the volume ratio of 1 : 1. After liquid extraction, the ether layer was evaporated to dryness in a 35°C water bath and redissolved with 3 mL methanol before being injected on to the semipreparative column.

LC-UV Conditions

The same Lunar C_{18} (150 × 4.6 mm, 5 μm) column was used in both monitoring of the acid hydrolysis products and determining the purity of purified scutellarein, but as below, the conditions were somewhat different from each other.

For the former, the column was kept at 40°C and a Phenomenex (4 × 3.0 mm) protective column was used. The mobile phase, a mixture of 0.05% phosphoric acid solution as elution A and methanol as elution B, was delivered at 1.0 mL/min. The elution was adopted using A-B (70 : 30, v/v) at 0 min, after which a step gradient was applied with A-B (30 : 70, v/v) for 15 min, and keeping A-B (30 : 70, v/v) until 18.8 min. The detected wave length was set at 335 nm, and 10 uL was injected.

For the latter, the elution of 40 mmol/L potassium dihydrogen phosphate solution (pH 2.5) and acetonitrile (75 : 25, v/v) was delivered at 1.0 mL/min. UV detection for scutellarein was set at 335 nm. Other conditions are the same as the former.

LC-MS Conditions

An Agilent 1100 Series LC-MS system was used to inject 3 μL aliquot of the processed samples on a Zorbax-Extend C_{18} column (150 × 2.1 mm, 5 μm), obtained from Agilent (USA), which was kept at the temperature of 40°C. A SecurityGuard™ HPLC guard cartridge system from Phenomenex (USA) was used to protect the analytical column. The mobile phase, a mixture of 0.2% acetic acid solution as elution solvent A, and pure acetonitrile as elution solvent B, was delivered at 0.3 mL/min. The elution of the analytes was adopted using A-B (92 : 8, v/v) at 0 min, after which a step gradient was applied with A-B (40 : 60, v/v) until 6 min, then with A-B (10 : 90, v/v) until 10 min, and keeping A-B (10 : 90, v/v) until 14 min; finally, the post time for reequilibrating the column at A-B (92 : 8, v/v) was set to 6 min. MS detection in the positive ion mode was achieved using a mass spectrometer equipped with an electrospray ionization interface (ESI), with 10 L/min drying nitrogen gas flow at 350°C and nebulizer pressure at 0.276 MPa (40 psi). The capillary voltage in the ESI probe was 3500V. In the Selective Ion Monitoring (SIM) mode, the m/z 287.0 ion[9] was detected for scutellarein, the aglycone of scutellarin, and 303.0 for the internal standard quercetin.

Preparative Chromatographic Conditions

A Zorbax Eclipse® XDB-C_{18} semipreparative column (250 × 9.4 mm, 5 μm) was applied for preparation. For separation from the acid hydrolysis products, 0.2% acetic acid solution was changed from 60% to 30% in 10 minutes in a gradient; meanwhile, methanol changed from 40% to 70% when the elution flow rate was 4 mL/min. However, 0.2% acetic acid solution and methanol (7 : 13, v/v) were delivered at 4 mL/min for preparation from the enzymatic products. After 200 μL was injected, a chromatogram at 335 nm and a real time spectrogram were used to monitor the scutellarein to be collected. All the collected samples were mixed and evaporated to near dryness, and a small amount of remnant liquid was eliminated by an ALPHA 2-4 freeze dryer.

RESULTS AND DISCUSSION

Acid Hydrolysis

In plant materials, the flavonoid glycosides are normally hydrolyzed and the resulting aglycones are identified and qualified, because quantitative determination of individual glycosides is difficult due to their large number.[10] Usually, hydrolysis of flavonoid glycosides requires relatively high concentrations of mineral acids under refluxing conditions.[11,12]

Table 1. Summary of acid hydrolysis

No	Scutellarin (mg)	A (mL)	VitC (mg)	Hydrochloric acid	B (mL)	Refluxing (+N_2)	Scutellarein (%)
1	5.0	7	20.8	0.75 mL 12 mol/L	3	80°C 2 h	71.89
2	7.1	7	29.9	1 mL 12 mol/L	3	80°C 2 h	70.96
3	5.0	5	20.6	0.75 mL 12 mol/L	3	80°C 2 h	59.32
4	7.1	7	30.1	1 mL 12 mol/L	3	80°C 1 h	43.41
5	5.3	7	20.6	0.75 mL 12 mol/L	3	70°C 2 h	50.94
6	35.2	35	150.1	5 mL 12 mol/L	15	80°C 2 h	76.30
7	35.2	35	150.4	5 mL 12 mol/L	15	80°C 2 h	76.99
8	35.0	35	150.4	5 mL 12 mol/L	15	80°C 2 h	69.88
9	35.2	35	149.9	5 mL 12 mol/L	15	80°C 2 h	(mixture
10	35.2	35	150.1	5 mL 12 mol/L	15	80°C 2 h	of them)
11	35.1	35	150.2	5 mL 12 mol/L	15	80°C 2 h	71.18

Solution A was prepared by diluting scutellarin in methanol and solution B was a mixture of ascorbic acid (VitC) solution and hydrochloric acid solution. Samples were hydrolyzed under different reflux conditions (see Table 1) under the protection of a nitrogen flow, then evaporated with a Buchi Rotavapor and extracted with anhydrous diethyl ether. The extracts were evaporated and then the residue was redissolved with methanol for injection to an Angilent HPLC. The main peak was identified, primarily, as scutellarein because the retention time and UV spectrogram are identical to the scutellarein prepared from enzymolysis and the remarkable SIM 287 ion in MS positive mode.

As shown in Table 1, No. 1 to 5 were trials on a small scale to select an optimized condition. No 6 to 11 were for the preparation of scutellarein on a Waters HPLC. By comparing the chromatograms, it was not difficult to observe that the impurities in the acid hydrolysis products were much more than those in enzymolysis products. It was possibly, or partially, responsible for unacceptable scutellarein with high purity; a reference substance was

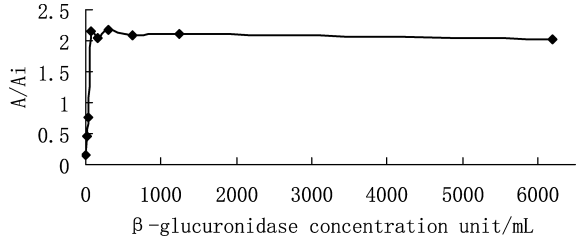

Figure 2. Study of the relationship of β-glucuronidase amount and A/Ai (peak area ratio of scutellarein to quercetin).

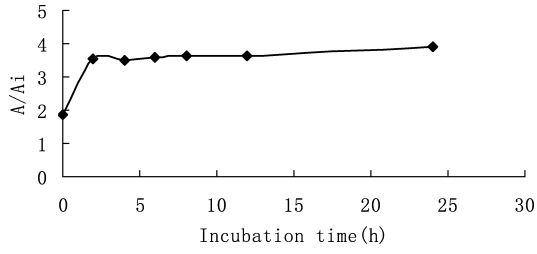

Figure 3. Study of incubation time of the enzymolysis reaction.

prepared by HPLC separation of the acid hydrolysis samples, although the HPLC purification procedure was almost the same as that which was adopted in purification of scutellarein after enzymolysis.

Optimization of Enzymolysis Conditions

To ensure a complete enzymolysis reaction, obtain a high yield, and depress the background, the enzymolysis conditions including the amount of enzyme, incubation time, and the amount of oxidation resistance were investigated on a small scale, which was adopted on a larger scale for preparation of scutellarein. Internal standard quercetin was added in trials for optimization purposes. The optimum condition was indexed by the ratio of chromatographic peaks area of scutellarein to internal standard (As/Ai). First, the amount of enzyme was investigated by varying the concentration of β-glucuronidase from 19.37 units/mL to 6200 units/mL when other conditions were kept unchanged. The result was shown in Figure 2. Incubation time was an important parameter for this reaction, so the different incubation times of 0, 2, 4, 6, 8, 12, and 24 h were investigated. The As/Ai obtained at different incubation times showed that the enzymolysis reaction was complete after 2 h at

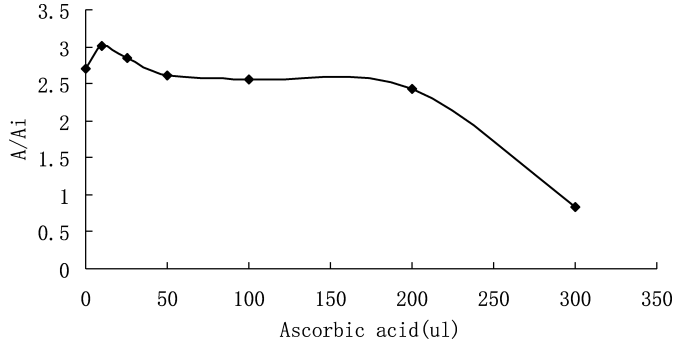

Figure 4. The influence of added amount of ascorbic acid.

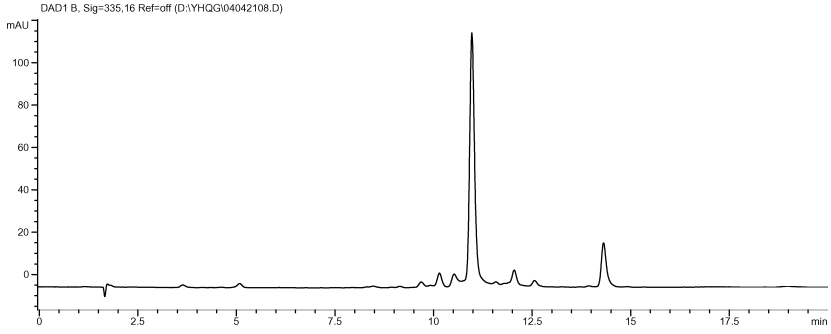

Figure 5. Chromatogram of acid hydrolysis products on the HPLC-UV system.

the incubation temperature 37°C, which was shown in Figure 3, but actually the incubation was overnight, as recommended in other related literature.[8] The effect of the amount of oxidation resistance was carried out by changing the added amount of ascorbic acid (200 mg/mL) from 50 μL to 300 μL. The result was plotted in Figure 4, which showed that the As/Ai values were unvarying when the added amount of ascorbic acid was from 50 to 200 μL, but the As/Ai values were depressed from 200 to 300 μL. Then, 100 μL was chosen to be added in this reaction system.

Chromatographic Conditions

The HPLC-UV conditions were different from those of LC-MS since the HPLC-UV conditions were for monitoring products after acid hydrolysis and determining the purity of scutellarein, and the LC-MS was for identification of scutellarein with the chromatogram, UV, and MS spectra in this research. The content of scutellarein was 98.04% ~ 98.36%, determined by area normalization using a HPLC. Gradient and isocratic elution were respectively adopted for products of acid hydrolysis and purified scutellarein from

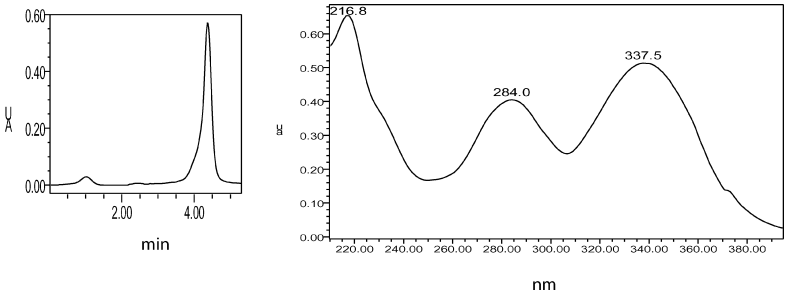

Figure 6. Chromatogram of enzymolysis products on the Waters HPLC system and real time UV spectrogram at 4.45 min.

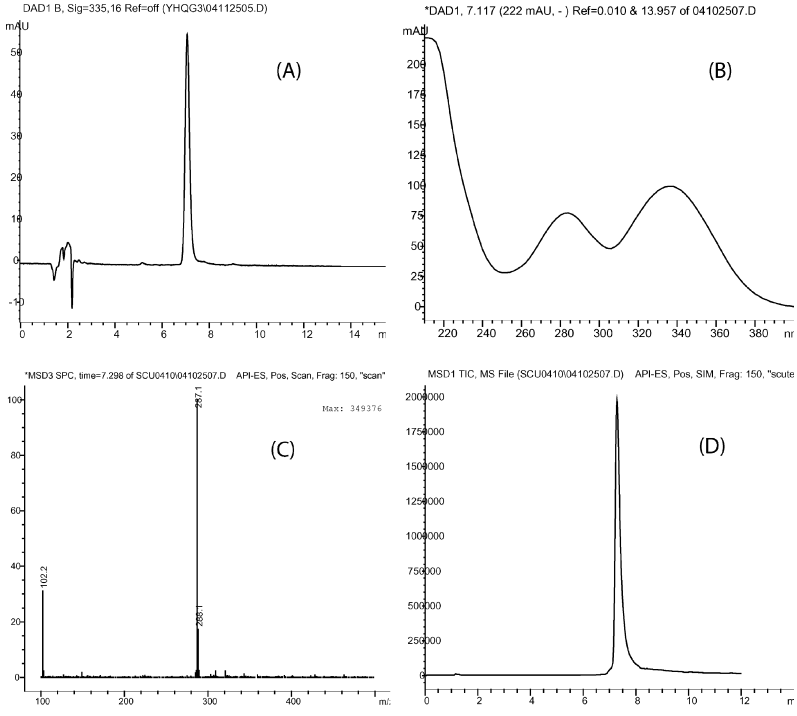

Figure 7. Chromatograms and spectra of purified scutellarein after enzymolysis. (A) Chromatogram, (B) UV spectrum, (C) Mass spectrum in the scan mode, (D) Chromatogram in the SIM mode.

enzymolysis, because there were much more impurities in the former than the latter. Isocratic elution had the advantage of shortening run time and saving mobile phase.

Representative chromatograms and spectrograms were shown in Figures 5–7.

NMR Spectroscopy

Further characterization of the aglycone as scutellarein, was carried out by ^{1}H-NMR spectrometry. The sample was dissolved in acetone-d6. Proton chemical shifts were observed at 7.94 (2H, d, *J*8.61 Hz, 2′, 6′-H), 7.02 (2H, d, *J*8.60 Hz, 3′, 5′-H), 6.65 (1H, s, 8-H), 6.62(1H, s, 3-H).

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