# Determination of Curculigoside in Curculiginis Rhizoma by High Performance Liquid Chromatography[1)]

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**An assay method for the determination of curculigoside in Curculiginis Rhizoma by high performance liquid chromatography (HPLC) was set up. After extraction with ethyl acetate, curculigoside was hydrolyzed in 1 N NaOH, and quantitatively converted to 2,6-dimethoxybenzoic acid (2,6-DA). The determination of curculigoside in Curculiginis Rhizoma was performed indirectly by measuring the content of 2,6-DA by HPLC. The calibration curve for this method exhibited a good linearity with a correlation coefficient of 1.0000 over the concentration range 1.0 to 81.0 μg 2,6-DA/ml (from 3 to 207 μg/ml as curculigoside). Using this method, 7 lots of Curculiginis Rhizoma produced in China contained about 0.2% curculigoside on average. To estimate the reliability of this method, the curculigoside content of Curculiginis Rhizoma was determined by direct assay applying the HPLC method and using curculigoside as the standard. Both methods agreed very well and this method was found to be satisfactory for estimating the contents of curculigoside in Curculiginis Rhizoma.**

**Keywords** Curculiginis Rhizoma; *Curculigo orchioides*; Curculigoside; 2,6-dimethoxybenzoic acid; hydrolysis; HPLC determination

Curculiginis Rhizoma consists of the dried rhizomes of amyryllidaceous *Curculigo orchioides* GAERTNER, and has been used as a tonic for the treatment of declining physical strength in traditional Chinese medicine.[2)] It has been reported to contain free sugars, mucilage, hemicelluloses, polysaccharides,[3)] and glucosides such as curculigoside,[4)] curculigine A[5)] and corchioside A.[6)] Curculigoside (5-hydroxy-2-*O*-β-D-glucopyranosylbenzyl 2,6-dimethoxybenzoate) has been found to be a characteristic constituent of Curculiginis Rhizoma which exerts an immunological and protective effect.[7)] Therefore the determination of curculigoside in Curculiginis Rhizoma would be useful for quality control. However, there are no reports of its assay and curculigoside is not commercially available. Therefore as part of investigations on the analysis of constituents of crude drugs, the assay method for curculigoside in Curculiginis Rhizoma for the purpose of quality control was investigated. As a result, an HPLC method for 2,6-dimethoxybenzoic acid (2,6-DA), the hydrolysis product of curculigoside was developed. This compound is commercially available.

## Experimental

**Materials** Curculiginis Rhizoma came from Tenjin Li Sheng Pharmaceutical Factory, Tenjin, China. 2,6-DA (extra pure reagent) was obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. Methyl *p*-hydroxybenzoate (extra pure reagent) was obtained from Katayama Chemical Industries Co., Ltd., Osaka, Japan. All other chemicals were of special reagent grade.

**Apparatus and HPLC Conditions** The HPLC apparatus consisted of a system controller (SCL-6B, Shimadzu, Kyoto, Japan), auto-injector (SIL-6B, Shimadzu), pump (LC-9A, Shimadzu), reverse-phase column (LiChrospher 100 RP-18(e), 5μm, 250 × 4 mm i.d., E. Merck, Darmstadt, Germany), column heater (CTO-6A, Shimadzu), detector (SPD-6A, Shimadzu) and integrator (C-R5A, Shimadzu). The mobile phase consisted of a mixture of water : acetonitrile : trifluoroacetic acid (800 : 200 : 1), at a flow rate of 1.0 ml/min. The HPLC separation was performed at 40 °C. The detection wavelength chosen was 283 nm where maximum absorption of 2,6-DA occurred and the sample injection volume was 150 μl.

**Sample Preparation** Powdered Curculiginis Rhizoma (0.20 g) was added to 10 ml water and 10 ml diethyl ether for defatting, and shaken vigorously for 10 min. After being centrifuged at 3000 rpm for 10 min, the diethyl ether layer was removed. To the aqueous layer was added 1 g sodium hydrogen carbonate and 10 ml ethyl acetate, and the mixture was again shaken vigorously for 10 min. After centrifugation at 3500 rpm for 10 min, the ethyl acetate layer was removed. After repeating the extraction with ethyl acetate three more times, the ethyl acetate fractions were combined and evaporated under reduced pressure. The ethyl acetate extract was dissolved in 1 ml water, mixed with 1 ml 1 N sodium hydroxide, and heated in a boiling water bath for 90 min. After cooling, the reaction mixture was neutralized with 1 ml 1 N hydrochloric acid, 5 ml of the internal standard solution (0.002% of methyl *p*-hydroxybenzoate in mobile phase) added and the volume made up to 10 ml with mobile phase to give the sample solution.

## Results and Discussion

Determination of curculigoside which is a characteristic constituent of Curculiginis Rhizoma with medicinal properties, can be considered to be a means for controling the quality of Curculiginis Rhizoma. As curculigoside, however, is not commercially available, isolation is necessary. Further, the quantity of curculigoside which can be isolated from 2.8 kg of Curculiginis Rhizoma is only about 660 mg,[4)] and this involves a great deal of labor. We observed that the curculigoside peak and 2,6-DA, the hydrolysis product of curculigoside, were well separated in the chromatogram of the methanol extract of Curculiginis Rhizoma shown in Fig. 1. Thus, the indirect assay of curculigoside was studied under conditions where curculigoside was hydrolyzed and quantitatively converted to 2,6-DA, which is commercially available. The isolated and purified curculigoside and 2,6-DA were measured using NMR, IR, MS and UV. Their structures were confirmed by comparing the data obtained with that of a previous report[4)] for curculigoside and with the data obtained using commercial 2,6-DA.

**Extraction Method and Hydrolysis Conditions** From the results of our examination of the extraction method to obtain curculigoside from Curculiginis Rhizoma, we found that when sodium hydrogen carbonate was used

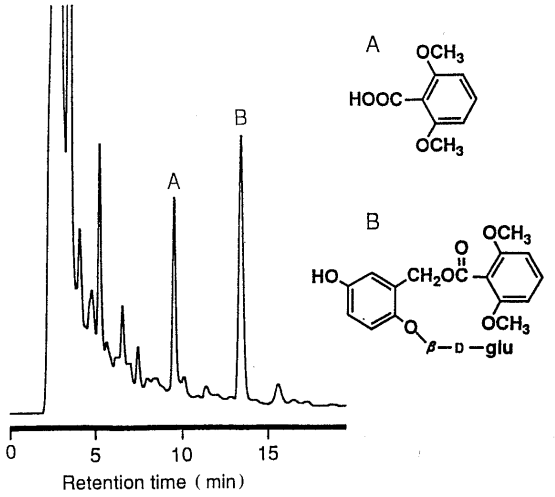


Fig. 1. Chromatogram of Methanol Extract of Curculiginis Rhizoma
A: 2,6-DA. B: Curculigoside.

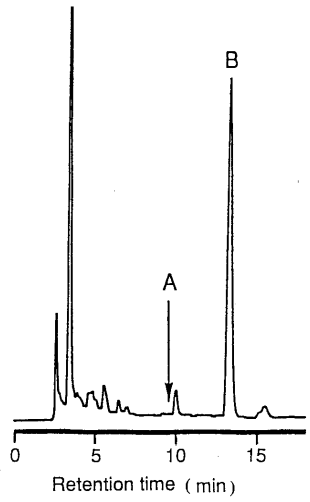


Fig. 2. Chromatogram of the Ethyl Acetate Extract of Curculiginis Rhizoma
A: 2,6-DA. B: Curculigoside.

for salting-out and ethyl acetate the extraction solvent, 2,6-DA in the methanol extract was not extracted while curculigoside was removed selectively as shown in Fig. 2. The removal of curculigoside was complete after 4 extractions and its reproducibility (5 repetitions) was good with a C.V. of 2.08%.

As far as hydrolysis conditions were concerned, satisfactory results were not obtained under acidic conditions, and alkaline conditions were used. As a result, when the ethyl acetate extract was heated in the boiling water bath for 40 min or more, after dissolving in methanol and mixing with 1 N sodium hydroxide, curculigoside hydrolysed and the quantity of 2,6-DA formed became constant, as shown in Fig. 3. The heat stability of 2,6-DA was studied for 3 h under the same conditions described above. It was found that 2,6-DA did not discompose. When various quantities of Curculiginis Rhizoma were hydrolyzed for 90 min, the initial amount was proportional to the quantity (peak area) of 2,6-DA formed from 0.05 to 0.50 g with a regression line of $Y=2565982X+$

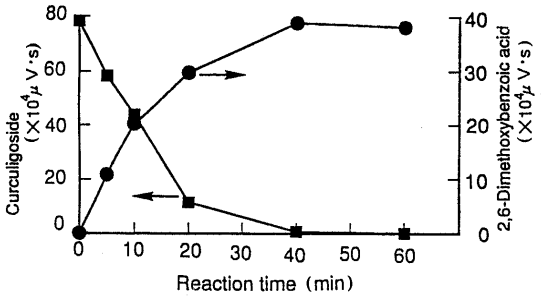


Fig. 3. Effect of Heating Time on Hydrolysis of Curculigoside

TABLE I. Determination of Curculigoside in Curculiginis Rhizoma

| Lot No. | Growing district | | Content of curculigoside (%) |
|---|---|---|---|
| A | Unknown | (China) | 0.155 |
| B | Unknown | (China) | 0.309 |
| C | Unknown | (China) | 0.214 |
| D | Unknown | (China) | 0.178 |
| E | Guang-dong | (広東省) | 0.108 |
| F | Guang-xi | (広西省) | 0.220 |
| G | Si-chuan | (四川省) | 0.162 |
| Average | | | 0.192 |

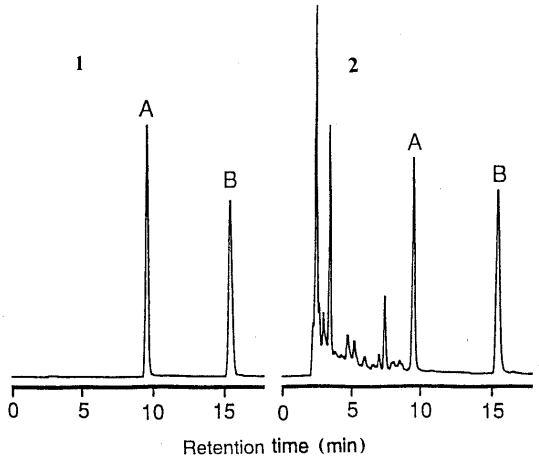


Fig. 4. Typical Chromatograms for the Determination of Curculigoside in Curculiginis Rhizoma
1: Standard solution (16 μg/ml). 2: Sample solution. A: 2,6-DA. B: Internal standard.

8157 and a correlation coefficient of 0.9997, and 2,6-DA formation was found to be quantitative.

**The Internal Standard** A compound which could be separated from 2,6-DA and other impurities was sought. Methyl *p*-hydroxybenzoate was found to be suitable as the internal standard. From the results, the sample preparation as described in the Experimental section.

**Calibration Curve** The peak area ratio of 2,6-DA was proportional to its concentration from 1.0 to 81.0 μg/ml (3−207 μg/ml as curculigoside) under the conditions described with a regression line of $Y=0.0707X+0.0033$ and a correlation coefficient of 1.0000.

**Determination of Curculigoside in Curculiginis Rhizoma** The results of the determination of curculigoside in 7 lots of Curculiginis Rhizoma from China using the new assay

are shown in Table I, and a typical chromatogram is shown in Fig. 4. The curculigoside content of Curculiginis Rhizoma was about 0.2% on a average.

To estimate the reliability of the assay method, the curculigoside content of Curculiginis Rhizoma was determined by the direct assay method applying the HPLC conditions of the new assay method and using curculigoside, isolated from Curculiginis Rhizoma, as a standard. The results showed that the content (average of 5 repetitions) was 0.155% (C.V.=2.22%) by the new assay method and 0.149% (C.V.=2.15%) by the direct method. Hence both assay methods showed good agreement, so the new assay can be used to estimate the curculigoside content of Curculiginis Rhizoma.

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