<Report>

Rapid and highly accurate assay of glycyrrhizic acid in Glycyrrhizae Radix using high-performance liquid chromatography

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Summary Glycyrrhizae Radix (G. Radix) is an herbal drug with various components for treating liver diseases. The determination of glycyrrhetinic acid (GA) content in G. Radix, despite its volatility, is typically conducted using high-performance liquid chromatography (HPLC) as per the Japanese Pharmacopoeia. This method necessitates rapid measurement due to the GA's propensity for degradation over time. However, this process is long, approximately 15 min, and is insufficiently precise. The HPLC conditions for GA determination in G. Radix are as follows: column, 5 μm, 4.0 × 150 mm octadecylsilyl (ODS); mobile phase: 0.38 w/v% ammonium acetate, 0.50 v/v% acetic acid, 28 v/v% acetonitrile; flow rate:1.0 mL/min. Internal standard is not used. The coefficient of variation of GA in G. Radix using this method is not sufficiently accurate. We improved the HPLC conditions (internal standard, HPLC column, and flow rate). GA determination in G. Radix was as follows: column, 3 μm, 4.0×100 mm ODS; mobile phase:0.38 w/v% ammonium acetate, 0.50 v/v% acetic acid, and 28 v/v% acetonitrile; flow rate:2.0 mL/min. The internal standard, (*E*)-cinnamaldehyde, was suitable for this method. The intraday and interday reproducibilities of GA in G. Radix using this method were 0.127 ± 0.001 g/L (mean ± SD), CV = 0.101%, 0.128 ± 0.001 g/L (mean ± SD), and CV = 0.812%, respectively. The recovery rate for GA was 100.9 ± 0.2% (mean ± SD). The developed method is rapid (within 5.5 min) and highly precise.

Key words: Glycyrrhizae Radix, Glycyrrhizic acid, High-performance liquid chromatography

1. Introduction

Glycyrrhizae Radix et Rhizoma, known as glycyrrhiza or Kanzo in Japan, is the dry rhizome and root of three species: *Glycyrrhiza inflata* Batal. *Glycyrrhiza glabra* L, and *Glycyrrhiza uralensis* Fisch. Kanzo is an herbal drug used to treat liver disease with various components, including licochalcone A, glycycoumarin, isoliquiritigenin, glycyrrhetinic acid, glycyrrhetinic acid (GA), and liquiritigenin. Kanzo has immunoregulatory, anti-cancer, anti-fibrosis, anti-steatosis, anti-oxidative stress, anti-inflammatory and drug-drug interactions[1]. Radix is a commonly used natural medicine that is described in the Japanese Pharmacopoeia as the stolon and root of *Glycyrrhiza*. Prescribed to treat

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many diseases, G. Radix is an effective element in the multidrug preparation of Kampo medicines. Over the last 10 years, extensive research has been conducted on the bioactive elements, biosynthesis, mechanisms of action, and clinical cases of *glycyrrhiza*[2, 3]. The main bioactive secondary metabolites of *glycyrrhiza* include polysaccharides, flavonoids, triterpene saponins, and coumarins[4]. GA is primarily found in G. Radix. GA is a triterpenoid saponin with various pharmacological functions, including anti-allergy[5] and anti-inflammatory[6] effects. Purified GA is also used in the treatment of liver disease[7], and as a non-artificial sweetener[8]. Several reports have shown that long-term or excessive G. Radix in Kampo and GA-alone natural medicine products often lead to pseudoaldosteronism[9], such as hypokalemia[10], peripheral edema[4], hypertension[11], and hypokalemia[10]. These side effects appear to be caused by the inhibiting 11 beta-hydroxysteroid dehydrogenase type 2 in the kidney. As a result, increased sodium retention and potassium excretion occur in the kidneys[12]. The amount of G. Radix used in medicine is important to avoid these side effects. In particular, attention has been paid to OTC medicines, especially regarding the amount of GA in G. Radix contained in the medicines (2.0 g). Currently, it is unknown whether the same amount of GA is present in the G. radix used in Kampo medicine because only the lower limit of GA in G. Radix, but not the upper limit in the Japanese pharmacopeia. The determination of glycyrrhetinic acid (GA) content in G. Radix, despite its volatility, is typically conducted using high-performance liquid chromatography (HPLC) as per the Japanese Pharmacopoeia. This method necessitates rapid measurement due to the GA's propensity for degradation over time, and the quantity of GA in G. Radix must be measured over a short period of time because the cost of the solution to be analyzed is reduced. In the Japanese Pharmacopoeia, the method to determine GA in G. Radix using HPLC, which is a long process that taking approximately 10-30 minutes and is not sufficiently accurate[13]. The aim of this study was to improve the HPLC conditions for long determination times and to improve the accuracy of this method.

2. Materials and Methods

Materials

Glycyrrhizae Radix [*Glycyrrhiza uralensis* Fischer (*Leguminosae*); Uchida Wakan-yaku Co., Ltd., Tokyo, Japan; Lot No. I7C0114] was used in this study, with glycyrrhetinic acid (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) as the standard. (*E*)-Cinnamaldehyde (FUJIFILM Wako Pure Chemical Corporation) was used as the internal standard. HPLC-grade acetonitrile, other solvents, and chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation. Distilled water was prepared using a purification system (Millipore Co. Ltd, Darmstadt, Germany) with a Millipore syringe-driven filter unit (Millex-HP, 0.45 μm pore size, Millipore Co. Ltd.).

Sample preparation

Sample extraction of GA in G. Radix was performed by following the method described in the Japanese Pharmacopoeia. GA was extracted from G. Radix (0.25 g) by shaking 35 mL of 50% ethanol for 15 min (first solution). The supernatant and the precipitate were separated. GA was extracted from the precipitate by shaking in 50% ethanol (12.5 mL) for 15 min (second solution). The first (35 mL) and second (12.5 mL) solutions were then mixed. The mixed solution was filled to a volume of 50 mL by adding 50% ethanol and filtered through a 0.45 μm syringe membrane filter (Minisart RC: Sartorius Co., Ltd.) before injection.

High-performance liquid chromatography (HPLC) analysis

The HPLC system condition [a Shimadzu Prominence system with an LC-20AD pump (Shimadzu, Kyoto, Japan), an SIL-20AC autosampler with an injection volume of 10 μL, an SPD-20 AV UV/VIS detector at a wavelength of 254 nm, the mobile phase (0.38 w/v% ammonium acetate, 0.50 v/v% acetic acid, and 28% v/v acetonitrile), and a column temperature of 40 °C]. A flow rate in the range of 0.5-2.0 mL/min was performed. Separations were carried out in an Inertsil octadecylsilyl (ODS)-4 (5 μm, 4.0 × 150 mm ODS -4; GL Science Co.

Ltd, Tokyo, Japan) or an Inertsil ODS-4 (3 μm, 4.0 × 100 mm ODS-4; GL Science Co. Ltd, Tokyo, Japan). GA content was determined using an absolute calibration standard (0.25 g/dL GA). The standard stock solution was mixed into the samples to determine recovery.

3. Results and discussion

In the Japanese pharmacopoeia, the determination time of GA in G. Radix using HPLC is 15 min, which is not sufficiently precise (Figs. 1(A) and (B), Table 1). The aim of this study was to improve the HPLC conditions and reduce the HPLC time.

Under the 18th Japanese pharmacopoeia, the components of GA in G. Radix were determined using the HPLC system condition with a 5 μm, 4.0 × 150 mm ODS column under flow rates of 1.0, 1.5, and 2.0 mL/min (Fig. 1). Among the three conditions, 2.0 mL/min was optimum because GA and the others were well and rapidly separated under the pressure limit of the column at a flow rate of 2.0 mL/min.

Chromatographers can adjust six basic parameters to control liquid chromatography (LC) separation. These include the mobile phase composition, stationary phase selection, temperature, packing particle size, column size, and flow rate. In this study, flow-rate condition was studied. Figure 2 shows the HPLC pattern for the HPLC system condition with a 3 μm, 4.0 × 100 mm ODS column under flow rates of 1.0, 1.5, or 2.0 mL/min. Among the three tested conditions with flow rates of 1.0 mL/min (analysis time: 8 min), 1.5 mL/min (analysis time: 6 min), and 2.0 mL/min (analysis time: 5 min), it was found that the optimum flow rate was 2.0 mL/min. This flow rate resulted in well-separated peaks for GA and other compounds, and the separation was achieved rapidly without exceeding the pressure limit of the column.

As experimenting of HPLC conditions, flow rate and column, we developed a new HPLC condition (the system HPLC condition with a 3 μm 4.0 × 100 mm ODS column under flow rate of 2.0 mL/min) for quantification of GA in G. Radix using HPLC with (*E*)-cinnamaldehyde as an internal standard in this developed method (Fig. 3). This internal standard was suitable for the developed method. The intraday and interday reproducibility of the developed method was 0.127 ± 0.001 g/L (mean ± SD), CV = 0.101%, and 0.128 ± 0.001 g/L (mean ± SD), CV = 0.812%, respectively. Those of the Japanese pharmacopeia method were 0.122 ± 0.001 g/L (mean ± SD), CV = 0.214%, and 0.125 ± 0.004 g/L (mean ± SD), and CV = 3.02%, respectively (Table 1). In contrast to the Japanese pharmacopeia method, where no internal standard is used, (*E*)-cinnamaldehyde was used as a standard in our method. Consequently, our developed method showed high precision. Using the Japanese pharmacopoeia method, the resolution between galacturonic acid-replaced glycyrrhizin and glycyrrhizic acid was 1.8. Using this developed method, the resolution between galacturonic acid-replaced glycyrrhizin and glycyrrhizic acid using the Japanese pharmacopeia method was 1.5. The Japanese pharmacopeia method had higher resolution than our method. Resolution is a measure of the quality of separation, and a resolution of 1.5 is considered a baseline separation of two peaks. Our developed method is no problem. The developed method is rapid (within 5.5

Table 1 Precision test of assay

	Intraday (n = 6)		Interday (n = 6)	
	Concentration (g/L)	C.V(%)	Concentration (g/L)	C.V(%)
Developed method	0.127 ± 0.001	0.101	0.128 ± 0.001	0.812
Japanese pharmacopoeia method	0.122 ± 0.001	0.214	0.125 ± 0.004	3.02

Concentration data are presented as mean ± standard deviation.

min) and highly precise.

There is a report that shortened the analysis time of GA in G. Radix using HPLC[13], which is a long process requiring approximately 10 min. We developed a new HPLC analysis method for GA in G. Radix based on our previously developed HPLC method[14]. The flow rate, mobile phase, column size, column particle size, and running program were examined. We developed a new HPLC condition (column:3 μm 4.0 × 100 mm ODS, mobile phase:0.38 w/v% ammonium acetate, 0.50 v/v%

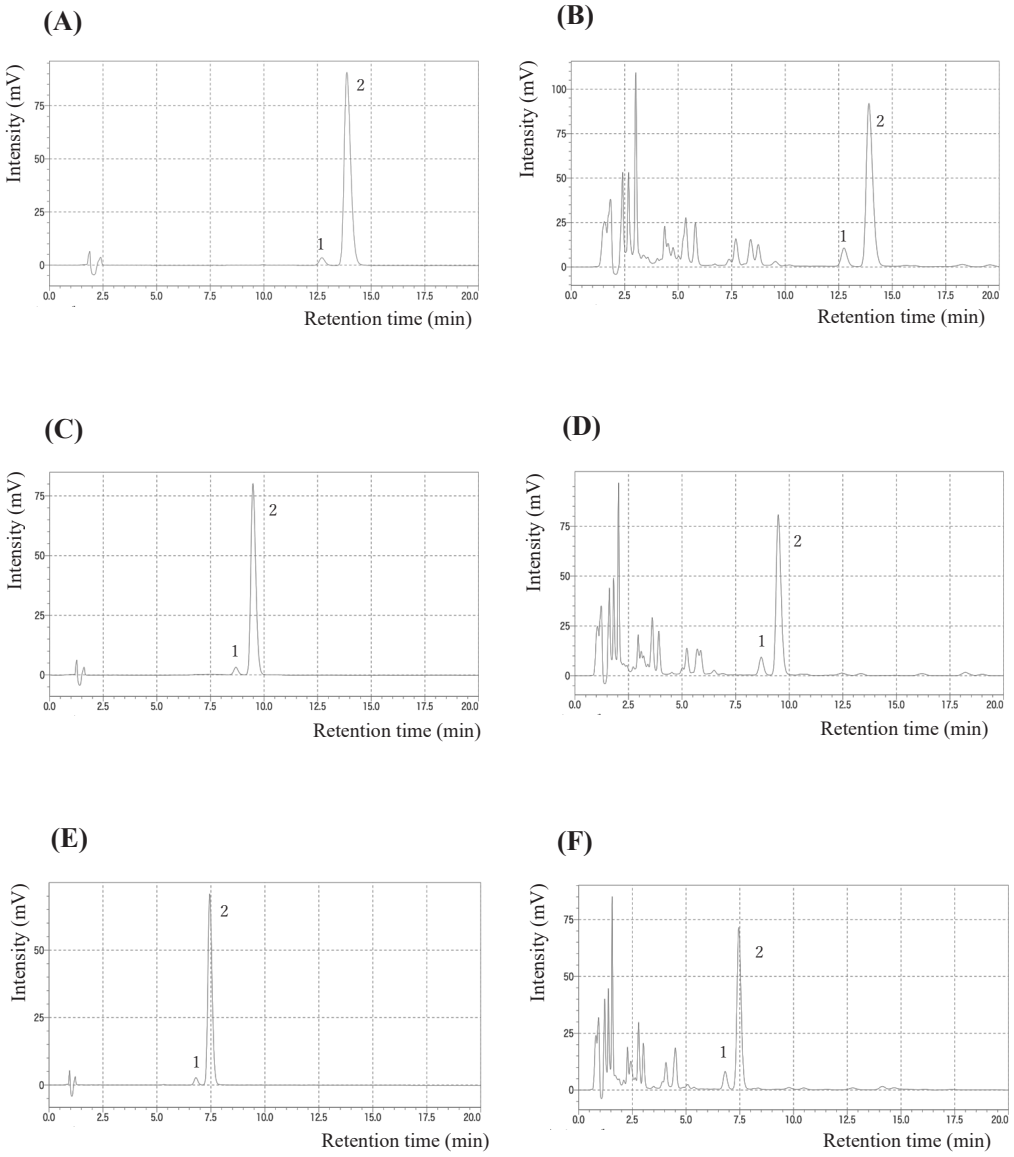

Fig. 1. HPLC chromatograms of three kinds of flow rate (1.0, 1.5, and 2.0 mL/min) under the 18th Japanese pharmacopoeia
(A) 0.25 g/L Glycyrrhizic acid (GA), flow rate: 1.0 ml/min
(B) Glycyrrhizae Radix extract, flow rate: 1.0 ml/min
(C) 0.25 g/L GA, flow rate: 1.5 ml/min
(D) Glycyrrhizae Radix extract, flow rate: 1.5 ml/min
(E) 0.25 g/L GA, flow rate: 2.0 ml/min
(F) Glycyrrhizae Radix extract, flow rate: 2.0 ml/min
HPLC column:5 μm, 4.0 × 150 mm, ODS-4, detection: 254 nm. The mobile phase was0.38 w/w% ammonium acetate, 0.50 v/v% acetic acid, and 28% v/v acetonitrile.
1: galacturonic acid-replaced glycyrrhizin, 2: glycyrrhizic acid

acetic acid, 28 v/v% acetonitrile, flow rate:2.0 mL/min) with quantification of GA in G. Radix using HPLC with (*E*)-cinnamaldehyde as an internal standard. The recovery rate for GA was 100.9 ± 0.2% (mean ± SD) indicates high accuracy of the method. The high precision of the developed method can be attributed to the suitability of the internal standard (*E*)-cinnamaldehyde. The reason for rapid analysis was why a column with a 3 μm, 4.0 × 100 mm ODS, and a flow rate of 2.0 mL/min was suitable.

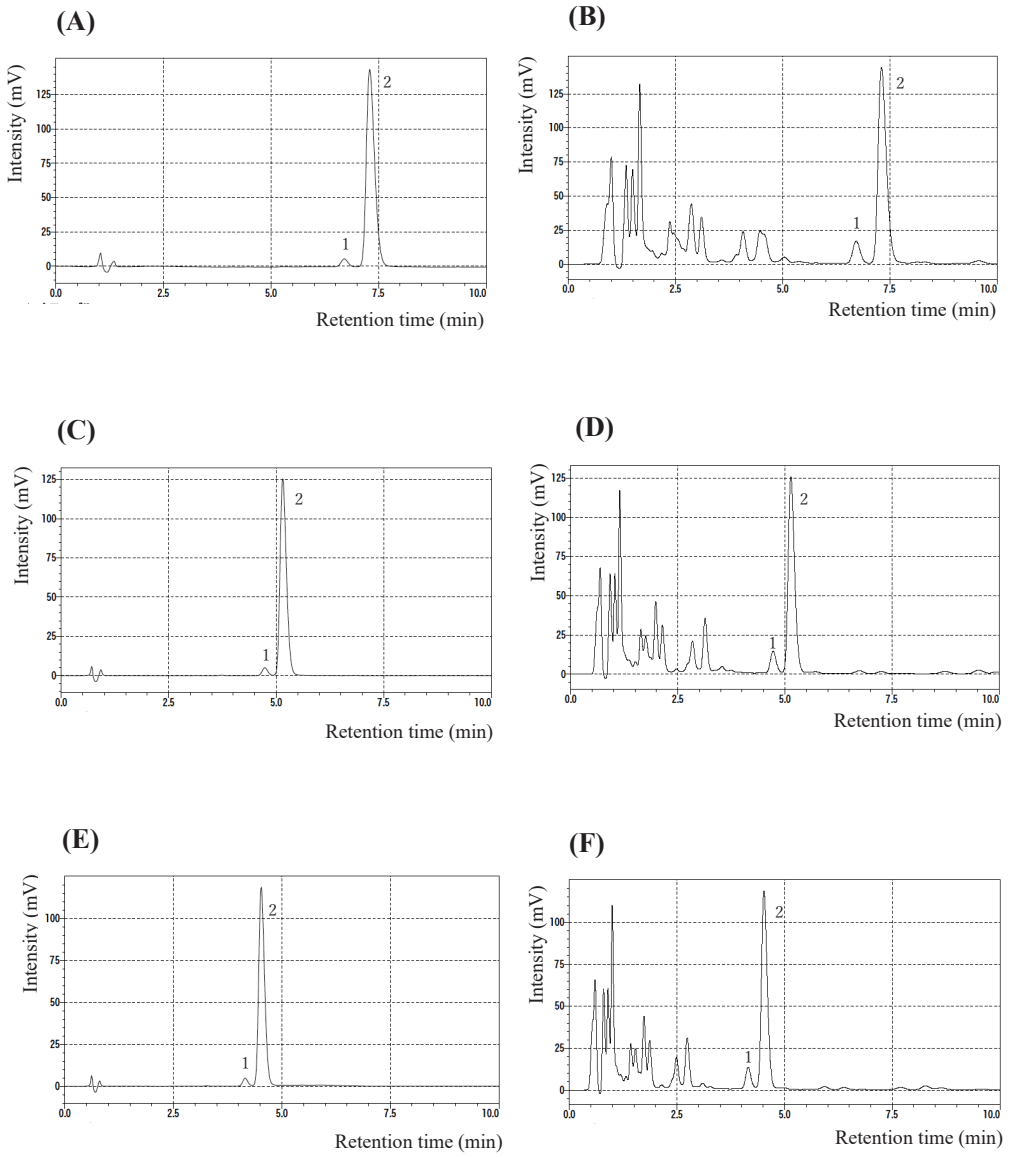

Fig. 2. HPLC chromatograms of three kinds of flow rate (1.0, 1.5, and 2.0 mL/min) under column: 3 μm, 4.0 × 100 mm ODS
(A) 0.25 g/L Glycyrrhizic acid (GA), flow rate: 1.0 ml/min
(B) Glycyrrhizae Radix extract, flow rate: 1.0 ml/min
(C) 0.25 g/L GA, flow rate: 1.5 ml/min
(D) Glycyrrhizae Radix extract, flow rate: 1.5 ml/min
(E) 0.25 g/L GA, flow rate: 2.0 ml/min
(F) Glycyrrhizae Radix extract, flow rate: 2.0 ml/min
HPLC column:3 μm, 4.0 × 100 mm; ODS-4, detection: 254 nm. The mobile phase was 0.38 w/w% ammonium acetate, 0.50 v/v% acetic acid, and 28 v/v% acetonitrile.
1: galacturonic acid-replaced glycyrrhizin, 2: glycyrrhizic acid

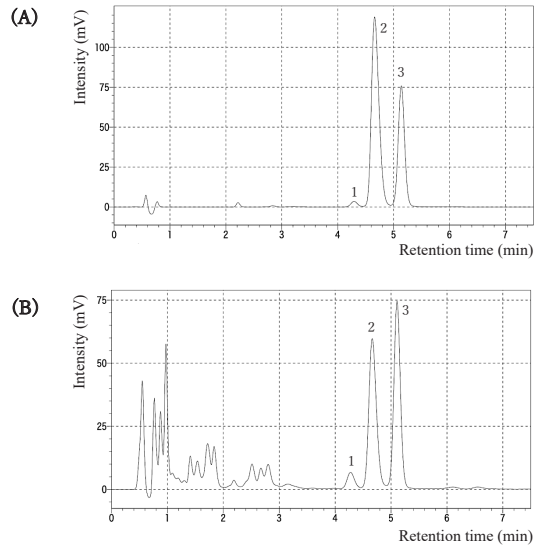

Fig. 3. The HPLC chromatograms with internal standard (A) Glycyrrhizic Acid (GA) + (*E*)-cinnamaldehyde; (B) Glycyrrhizae Radix extract + (*E*)-cinnamaldehyde. HPLC column:3 μm, 4.0 × 100 mm; ODS-4, detection:254 nm. Mobile phase was 0.38 w/v% ammonium acetate, 0.50 v/v% acetic acid, and 28 v/v% acetonitrile. Flow rate: 2.0 mL/min
1: galacturonic acid-replaced glycyrrhizin, 2: glycyrrhizic acid, 3: (*E*)-cinnamaldehyde

Conflict of Interest

The authors declare no conflict of interest.

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