

Simultaneous Determination of Glycyrrhizin Metabolites Formed by the Incubation of Glycyrrhizin with Rat Feces by Semi-micro High-Performance Liquid Chromatography

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A method for semi-micro high-performance liquid chromatography (HPLC) has been established for the simultaneous determination of 3 α -hydroxyglycyrrhetic acid and 3-dehydroglycyrrhetic acid together with glycyrrhizin, glycyrrhetic acid and glycyrrhetic acid mono-glucuronide formed by incubation of glycyrrhizin with rat feces. The analysis was accomplished within 25 min with a TSKgel ODS-80TsQA (150 \times 2.0 mm i.d.) column by linear gradient elution using a mobile phase containing aqueous phosphoric acid and acetonitrile at a flow rate of 0.2 ml \cdot min⁻¹, a thermostatic oven at 25 $^{\circ}$ C, and detection at 254 nm. The detection limits of these compounds were 0.2 pmol per injection (5 μ l). The metabolites of glycyrrhizin, by anaerobic or aerobic incubation with rat fecal suspension over 48 h, were determined. Glycyrrhizin was almost completely converted to metabolite glycyrrhetic acid, and metabolites 3 α -hydroxyglycyrrhetic acid and 3-dehydroglycyrrhetic acid in negligible amounts in anaerobic conditions. However, the metabolic time courses of 3-dehydroglycyrrhetic acid when incubated in aerobic conditions revealed that it apparently continued increasing during the whole incubation period.

Key words 3 α -hydroxyglycyrrhetic acid; 3-dehydroglycyrrhetic acid; glycyrrhizin; glycyrrhetic acid; semi-micro HPLC; rat feces

The root of *Glycyrrhiza* sp. (licorice root) is the most common herb used in Kampo medicine. Glycyrrhizin is a saponin, a major effective ingredient of licorice root, which has steroid-like,¹⁾ antiviral²⁾ and interferon-inducing³⁾ effects. It has been used as a medicine in the treatment of chronic hepatitis,⁴⁾ gastric ulcers,⁵⁾ inflammation⁶⁾ and allergic disorder,⁷⁾ and as a sweetener in many foods. However, glycyrrhizin has been reported to produce adverse effects, such as pseudo-aldosteronism, when over consumed.⁸⁾ Glycyrrhizin is known to be hydrolyzed to glycyrrhetic acid, an active metabolite and also a minor ingredient of licorice root, by human intestinal bacteria.⁹⁾ Glycyrrhetic acid is responsible for the adverse reaction of pseudo-aldosteronism after glycyrrhizin intake.¹⁰⁾ The metabolic procession of glycyrrhizin to glycyrrhetic acid by human intestinal bacteria has been considered to follow two pathways: the main pathway directly metabolizing glycyrrhizin to glycyrrhetic acid, and the minor pathway metabolizing glycyrrhizin to glycyrrhetic acid *via* glycyrrhetic acid mono-glucuronide.¹¹⁾ Moreover, glycyrrhetic acid is converted to 3 α -hydroxyglycyrrhetic acid through 3-dehydroglycyrrhetic acid by human and rat intestinal bacteria.¹²⁾ Glycyrrhizin is detected in the plasma when glycyrrhizin is orally administered to rats, but its bioavailability is lower than that of glycyrrhetic acid.¹³⁾ Therefore, quantification of glycyrrhizin and its metabolites is necessary to investigate the biotransformation of glycyrrhizin. Although there have been many reports on the high-performance liquid chromatography (HPLC) determination of glycyrrhizin or glycyrrhetic acid independently, none of these methods were sufficiently sensitive for the simultaneous determination of glycyrrhizin and its metabolites. We have been studying a biotransformation of glycyrrhizin in Kampo medicine and developed a method for semi-micro HPLC for the simultaneous determination of glycyrrhizin, glycyrrhetic acid and glycyrrhetic acid mono-glucuronide in incubation mixtures of rat fecal flora, by the addition of Shakuyaku-kanzo-to decoction (combination of

licorice root and peony root).¹⁴⁾ However, no HPLC method involved in the determination of glycyrrhizin and its metabolites containing 3 α -hydroxyglycyrrhetic acid and 3-dehydroglycyrrhetic acid was reported.

The present study describes a simple, sensitive and reproducible semi-micro HPLC method for the simultaneous determination of glycyrrhizin, glycyrrhetic acid, glycyrrhetic acid mono-glucuronide, 3 α -hydroxyglycyrrhetic acid and 3-dehydroglycyrrhetic acid (Fig. 1). This method was applicable to determine metabolites of glycyrrhizin by anaerobic and aerobic incubations with rat fecal suspensions.

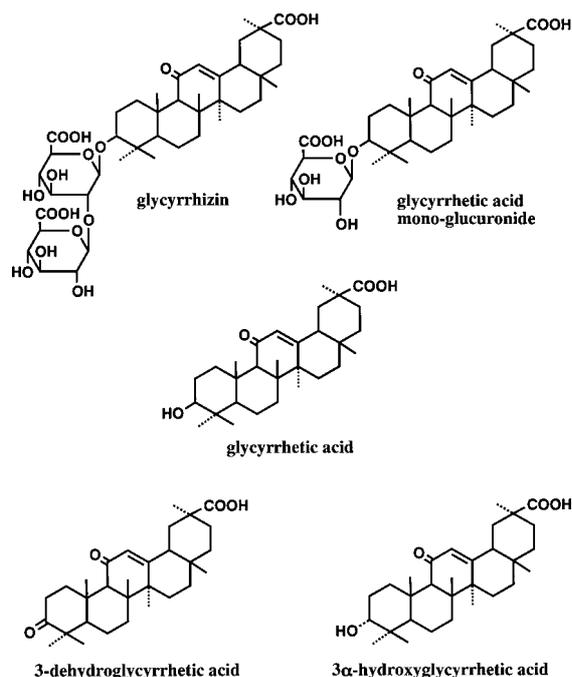


Fig. 1. Structures of the Investigated Compounds

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MATERIALS AND METHODS

Materials Glycyrrhizin, glycyrrhetic acid and glycyrrhetic acid mono-glucuronide were purchased from Wako Pure Chemical Industries (Wako, Osaka, Japan). Glycyrrhizin dipotassium salt was kindly provided by Maruzen Pharmaceuticals Co., Ltd. (Hiroshima, Japan). HPLC grade acetonitrile and analytical reagent grade phosphoric acid (85%) were purchased from Wako. Ultrapure distilled water with a resistivity greater than 18 M Ω was prepared with deionized-distilled water. The organic solvents and other chemical reagents were of analytical reagent grade, and were supplied by Wako.

Instrumentals The HPLC apparatus was an Agilent Technologies 1100 Series system (Waldbronn, Germany) consisting of a binary pump, an autosampler, a thermostated column compartment and a photodiode array detector. All modules and data collection were controlled by Agilent Chemstation software. The column was a TSKgel ODS-80TsQA reversed-phase column, particle size of the packing 5 μ m, 150 \times 2.0 mm i.d. (Tosoh, Tokyo, Japan).

Chromatographic Conditions The mobile phase was a gradient system with 200 μ l \cdot l $^{-1}$ (ca. 0.017%) phosphoric acid in water (A) and acetonitrile (B), which was deaerated by sonication before use. The flow rate was 0.2 ml \cdot min $^{-1}$. The separation was performed for 25 min from 38 to 50% solvent B in 3 min, 50 to 52% solvent B in 7 min, 85 to 90% solvent B in 10 min, and 90% solvent B in 5 min. A re-equilibration period of 12 min was used between individual runs. The injection volume was 5 μ l. The column temperature was 25 $^{\circ}$ C. The detection wavelength was set at 254 nm for determination and in the range of 200 to 500 nm for validation of peak purity.

Incubation of Glycyrrhizin with Rat Fecal Suspension Fresh feces (1.0 g) obtained from male Wistar rats (200 g) were homogenized in 0.1 M potassium phosphate buffer (20 ml, pH 7.4) by bubbling with CO₂ gas to eliminate air, and the sediments were removed by filtration through gauze.¹⁵ The fecal suspension prepared as above was divided into eleven aliquots (1 ml each). These aliquots were mixed with glycyrrhizin dipotassium salt (0.25 ml) in aqueous solution, and sorted into three groups: one control aliquot and two incubation mixtures composed of five aliquots. The concentration resulted in 84 μ M of glycyrrhizin in each sample. To the control mixture, 0.85% phosphoric acid in methanol (1.25 ml) was added immediately. Each incubation mixture was investigated at 37 $^{\circ}$ C for different incubation periods (2, 4, 8, 24, 48 h) in anaerobic and aerobic conditions. Anaerobic procedures were accomplished by using anaerobic jar with an AnaeroPack (Mitsubishi Gas Chemical). The reaction was stopped after 2, 4, 8, 24 or 48 h incubation and mixed immediately with 0.85% phosphoric acid in methanol (1.25 ml). Each mixture was then centrifuged (3000 rpm, 10 min) and the supernatant was passed through a Sartorius membrane (Minisart RC 15, pore size: 0.45 μ m, Japan Sartorius, Tokyo, Japan) and subjected to HPLC.

Synthesis of 3-Dehydroglycyrrhetic Acid The following method was modified from a previous report.⁹ Glycyrrhetic acid (1.0 g) was dissolved in acetone (100 ml), to which 1.0 ml of Johnes reagent¹⁶ was added portionwise while stirring at room temperature. After 20 min, the addition

of excess EtOH stopped the reaction. The reaction mixture was filtered, the filtrate was evaporated *in vacuo*, and the residue was recrystallized from MeOH to afford 3-dehydroglycyrrhetic acid as colorless needles (869 mg), which were identified with ¹H- and ¹³C-NMR spectroscopy.

Synthesis of 3 α -Hydroxyglycyrrhetic Acid A solution of 3-dehydroglycyrrhetic acid (300 mg) in dry tetrahydrofuran (THF) (20 ml), in a nitrogen atmosphere, was cooled in a dry ice-acetone bath (-74° C), and a 1 M solution of K-selectride (15 ml, potassium tri-*sec*-butylborohydride, Aldrich Chemical Company, Milwaukee, WI, U.S.A.) was added with a syringe. After cooling at the same temperature for 2 h, the reaction mixture was left to stand overnight at room temperature. The reaction was quenched with 1 M HCl to pH 2.0 in ice cooling conditions, and extracted with CHCl₃ (50 ml). The extract was washed with brine, dried over anhydrous MgSO₄, and evaporated *in vacuo*.¹⁷ The residue was recrystallized from MeOH to afford 3 α -hydroxyglycyrrhetic acid as colorless needles (176 mg), which were identified with ¹H- and ¹³C-NMR spectroscopy.

Preparation of Calibration Curves An original standard solution was prepared by dissolving 3 α -hydroxyglycyrrhetic acid (2.65 mg) and 3-dehydroglycyrrhetic acid (2.67 mg) in MeOH (2 ml), and diluted to make methanolic standard solutions (0.081–167 μ g \cdot ml $^{-1}$). Calibration curves were generated using ten different concentrations of standard solutions from least-squares regression of peak area in triplicate assays.

Validation of HPLC Analysis A mixture of 3 α -hydroxyglycyrrhetic acid (1.35 mg) and 3-dehydroglycyrrhetic acid (1.85 mg) was dissolved in MeOH (100 ml) containing 0.85% phosphoric acid, and diluted to 5- and 25-fold dilutions with the same solvent. The standard mixture was prepared from a combination of the diluted solution (5.0 ml), fecal suspension (4.0 ml), as above prepared, and water (1.0 ml). The within-day and between-day precision of the method for detection of 3 α -hydroxyglycyrrhetic acid and 3-dehydroglycyrrhetic acid was evaluated by using the standard mixture and the incubation mixture, as above prepared. All mixtures were filtered through a Minisart-RC 15 filter and subjected to HPLC. The within-day precision was examined with ten-replicate assays on the same day. The between-day precision was assessed by analyzing a mixture stored at 4 $^{\circ}$ C on five different days over 2 weeks.

The standard solution was prepared by diluting 3 α -hydroxyglycyrrhetic acid (10.8 μ M) and 3-dehydroglycyrrhetic acid (13.2 μ M) with a mixture (diluent) of water, 0.1 M potassium phosphate buffer (pH 7.4) and MeOH containing 0.85% phosphoric acid (1 : 4 : 5), to 2-, 4-, 8-, 16- and 32-fold dilutions. The test solutions were prepared by the addition of the incubation mixture or diluent (0.5 ml) to the standard solution (0.5 ml). All test solutions were filtered through a Minisart-RC 15 filter and subjected to HPLC. The recoveries were determined from the slope ratios of regression equations, with and without the incubation mixture.

RESULTS AND DISCUSSION

Determination of 3 α -Hydroxyglycyrrhetic Acid and 3-Dehydroglycyrrhetic Acid by HPLC (Fig. 2) In an attempt to conduct biopharmaceutical studies on glycyrrhizin,

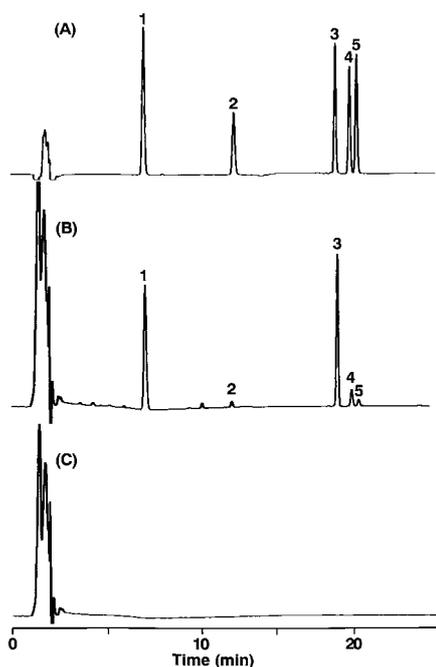


Fig. 2. HPLC Profiles of Standard (A), Glycyrrhizin Metabolites (B) and Blank Incubation Mixtures (C)

For sample preparation and analytical conditions, see the Materials and Methods section. Peaks: 1=glycyrrhizin, 2=glycyrrhetic acid mono-glucuronide, 3=glycyrrhetic acid, 4=3 α -hydroxyglycyrrhetic acid, 5=3-dehydroglycyrrhetic acid.

we have already reported the method for determination of glycyrrhizin, glycyrrhetic acid and glycyrrhetic acid mono-glucuronide by reversed-phase HPLC using an ODS semi-micro column.¹⁴⁾ This method is applicable to the determination of these compounds in an incubation mixture of rat fecal flora by the addition of Shakuyaku-kanzo-to decoction. Glycyrrhetic acid, the main metabolite of glycyrrhizin, was further converted to 3 α -hydroxyglycyrrhetic acid through 3-dehydroglycyrrhetic acid by human and rat intestinal bacteria.¹²⁾ Conventionally, these metabolites have been examined by thin-layer chromatography, and no HPLC method involved in the determination of glycyrrhizin and its metabolites containing 3 α -hydroxyglycyrrhetic acid and 3-dehydroglycyrrhetic acid was reported. However, the simultaneous determination of 3 α -hydroxyglycyrrhetic acid and 3-dehydroglycyrrhetic acid, together with glycyrrhizin, glycyrrhetic acid and glycyrrhetic acid mono-glucuronide, in rat fecal suspension, was adopted at the present HPLC method in which the monitoring time was extended up to 25 min under the final gradient conditions.

The retention times (and capacity factors, k') were 19.93 (3 α -hydroxyglycyrrhetic acid, $k'=13.90$) and 20.35 min (3-dehydroglycyrrhetic acid, $k'=14.21$). The relative standard deviations (RSD, $n=10$) of the retention times were 0.03 and 0.02%. Linearity of these compounds was evaluated over the range of 0.172 to 44.0 μM for 3 α -hydroxyglycyrrhetic acid, and 0.174 to 356 μM for 3-dehydroglycyrrhetic acid. The regression equations were $y=8.82x-0.26$ for 3 α -hydroxyglycyrrhetic acid, and $y=10.26x-0.46$ for 3-dehydroglycyrrhetic acid, with a correlation coefficient of 1.0000, where y is the peak-area and x is the concentration (μM) of these compounds. Both detection limits (signal-to-noise ratio=3) of 3 α -hydroxyglycyrrhetic acid and 3-dehydroglycyrrhetic

Table 1. Within-Day and Day-to-Day Relative Standard Deviations (RSD) for 3 α -Hydroxyglycyrrhetic Acid (1) and 3-Dehydroglycyrrhetic Acid (2) in Standard and Incubation Mixtures

Marker substance	Within-day ^{a)}		Between-day ^{b)}	
	Mean \pm S.D. ^{c)}	RSD (%)	Mean \pm S.D. ^{c)}	RSD (%)
Standard mixture				
1	2.63 \pm 0.003	0.11	2.64 \pm 0.007	0.25
	0.54 \pm 0.003	0.47	0.55 \pm 0.011	2.03
2	3.55 \pm 0.002	0.06	3.55 \pm 0.014	0.38
	0.80 \pm 0.006	0.76	0.91 \pm 0.017	1.90
Incubation mixture				
1	0.98 \pm 0.007	0.70	0.94 \pm 0.016	1.71
2	1.05 \pm 0.007	0.69	1.00 \pm 0.017	1.66

a) $n=10$, b) $n=5$, c) μM .

Table 2. Slopes of Regression Equations of 3 α -Hydroxyglycyrrhetic Acid (1) and 3-Dehydroglycyrrhetic Acid (2) with and without Incubation Mixture

Marker substance	Initial amount of incubation mixture (μM)	Slope of standard solution ^{a)}		Recovery ^{b)} (%)
		With incubation mixture	Without incubation mixture	
1	0.609	9.045	8.754	103.3
2	0.793	10.70	10.21	104.7

a) Concentration of standard (μM): 1=0.170, 0.341, 0.681, 1.36, 2.73, 5.45; 2=0.207, 0.413, 0.826, 1.65, 3.31, 6.61. b) Values are means of three experiments.

acid were 0.2 pmol per injection (5 μl). 3 α -Hydroxyglycyrrhetic acid and 3-dehydroglycyrrhetic acid were clearly separated without any pre-purification and determined within 25 min, without any pre-cleaning of the column (Fig. 2A). Glycyrrhizin was anaerobically incubated with fecal suspensions obtained from fresh rat feces for 4 h. As shown in Fig. 2B, adequate separation of 3 α -hydroxyglycyrrhetic acid and 3-dehydroglycyrrhetic acid was achieved. Distinct elution profiles of these compounds were obtained without any interference from the endogenous peak of the blank incubation mixture (without glycyrrhizin) (Fig. 2C). The identification and purity of the chromatographic peaks were estimated by a photodiode array detector. The UV spectra of the peaks at five different points (up-, top- and down-slope) were compared with those of synthetic specimens.

Validation of HPLC of 3 α -Hydroxyglycyrrhetic Acid and 3-Dehydroglycyrrhetic Acid in the Incubation Mixture (Tables 1, 2) The within-day and between-day precision of the method for 3 α -hydroxyglycyrrhetic acid and 3-dehydroglycyrrhetic acid was evaluated by using the standard mixture and the incubation mixture. The within-day precision was examined with ten-replicate assays per day, and the between-day precision by assays on five different days over the 2 weeks. As shown in Table 1, the within-day and between-day RSDs were within 0.06 to 0.76% and 0.25 to 2.03%, respectively. These compounds were shown to be stable in both the standard and the incubation mixtures for over 2 weeks at 4 $^{\circ}\text{C}$ by the between-day assay. Recovery was investigated using the incubation mixture spiked with the standard solution. The slopes of the regression equations of the standards and the recoveries by this method are shown in Table 2. The recoveries were 103.3% for 3 α -hydroxygly-

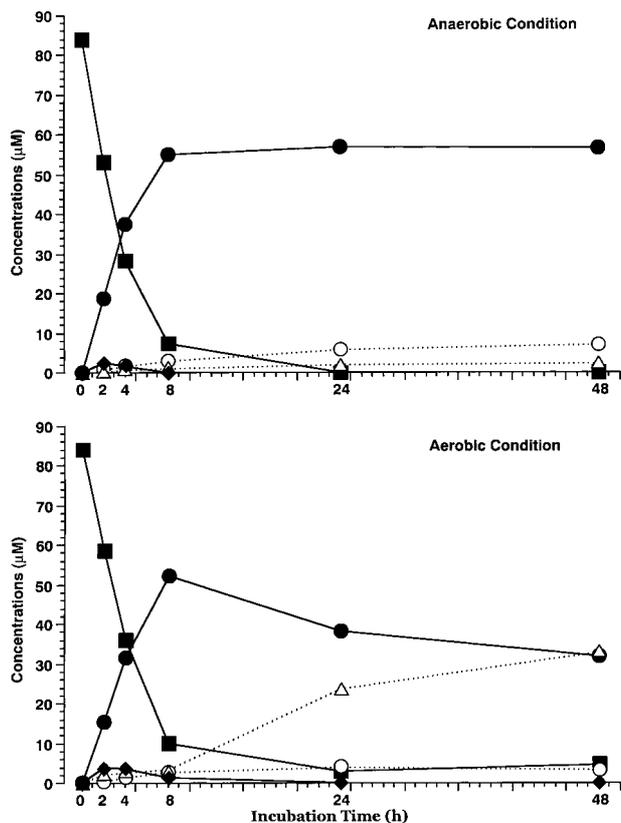


Fig. 3. Metabolism of Glycyrrhizin by Rat Fecal Suspension in Anaerobic and Aerobic Conditions

For sample preparation, see the Materials and Methods section. ■: glycyrrhizin, ●: glycyrrhetic acid, ◆: glycyrrhetic acid mno-glucuronide, ○: 3α-hydroxyglycyrrhetic acid, △: 3-dehydroglycyrrhetic acid.

glycyrrhetic acid and 104.7% for 3-dehydroglycyrrhetic acid.

Metabolism of Glycyrrhizin by Rat Fecal Suspension in Anaerobic and Aerobic Conditions (Fig. 3) Licorice root is a crude drug contained in more than 70% of Kampo medicines certified by the Japanese Ministry of Health and Welfare.¹⁸⁾ Glycyrrhizin, a major ingredient, is also used in the treatment of various diseases. We applied the present procedure to examine the metabolism profiles of glycyrrhizin in rat fecal suspension. Figure 3 depicts the concentration–time profiles of glycyrrhizin in an incubation mixture of rat fecal flora in anaerobic and aerobic conditions. No definitive difference between the two conditions was observed except for 3-dehydroglycyrrhetic acid. Glycyrrhizin was metabolized mainly to glycyrrhetic acid, and negligibly to glycyrrhetic acid mono-glucuronide, 3α-hydroxyglycyrrhetic acid and 3-dehydroglycyrrhetic acid, by anaerobic incubation for 48 h. On the other hand, it was transformed to glycyrrhetic acid and 3-dehydroglycyrrhetic acid in approximate ratios of 1 : 1 by aerobic incubation for 48 h. In particular, glycyrrhetic acid increased during the early phase, but gradually declined thereafter, whereas 3-dehydroglycyrrhetic acid apparently continued increasing during the whole incubation period. Transformation of glycyrrhetic acid to 3-dehydroglycyrrhetic

acid may be explained in terms of the acceleration by aerobic condition.

Oral administration of Kampo medicines in which glycoside ingredients are involved, usually provides the corresponding aglycone after hydrolysis by intestinal bacteria. In fact, glycyrrhizin administered orally is absorbed in the form of glycyrrhetic acid and shows various pharmacological effects as a natural prodrug.¹⁹⁾ Glycyrrhetic acid is further transformed to 3α-hydroxyglycyrrhetic acid via a metabolic intermediate, 3-dehydroglycyrrhetic acid. Since the method described in this report is sufficiently sensitive and reliable to be used for the simultaneous determination of glycyrrhizin and its metabolites, it can be applied for the clinical evaluation of glycyrrhizin in regards to the efficacy and safety of Kampo and herbal medicines containing licorice root.

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