Determination of Serum Concentrations of Glycyrrhizin in Humans by Semi-micro High-Performance Liquid Chromatography after Administration of a Therapeutic Dose

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A simple and sensitive semi-micro high-performance liquid chromatography (HPLC) was established for determining the serum levels of glycyrrhizin (GL) in humans. Butyl p-hydroxybenzoate was used as the internal standard and serum was deproteinized by methanol. The samples were separated on a Capcell Pak C18 UG120 column (150 × 1.5 mm i.d.; particle size, 5 μm). The detection limit of GL in serum was 100 ng/ml, which enables determination of serum levels of GL after administration of a therapeutic dose. The time-course study suggested that the elimination rate of GL differed between subjects for the same administered dose, although the sample was too small to allow a meaningful comment. In clinical practice, GL is used for its antiviral and anti-inflammatory effects. Excessive administration of GL can induce pseudoaldosteronism; however the optimal GL concentration in serum remains to be determined. The determination method reported here is expected to aid in the safe and efficient use of the drug in clinical practice.

Key words glycyrrhizin; serum; semi-micro HPLC; determination

Glycyrrhizin (GL) is a major ingredient of herbal medicines, originating from the roots of licorice, Glycyrrhizae Radix. GL is known to possess antiviral activity. It inhibits HIV plaque formation, adsorption of HIV-1 to T cells and multinucleated giant cell formation in vitro.4 The antiviral activity might be attributable to its ability to induce the generation of γ-interferon, nitric oxide, and extrathymic T cells.3 GL also helps maintain CD4+ cell counts in the peripheral blood of AIDS patients.3 Both GL and glycyrrhetinic acid (GA), the aglycone of GL, also possess anti-inflammatory activity.5 The arachidonate-cascade-related enzymes, such as lipooxygenase and phospholipase A2, which mediate inflammation, are inhibited by GL.5

In humans, orally administered GL is converted to GA by the gut flora prior to absorption and intravenous GL is also quickly converted to GA during enterohepatic circulation. In recent research has revealed that GL and GA possess different biological activities. While GL enhances Fas-mediated apoptosis of T cell lines, GA does not.7 Similarly, only GL inhibits hemolysis of red blood cells induced by gp55 purified from Habu venom.8 The aforementioned studies have shown that GL exerts its activities at relatively low concentrations and discriminative determination of GL and GA is important. In addition, excessive administration of GL may cause pseudoaldosteronism. Therefore, it is clinically important to determine the serum levels of GL. However, there is a paucity of reports regarding a sufficiently sensitive assay method to determine the serum levels of GL in humans. We previously developed a method for determining the serum levels of GL based on a monoclonal antibody-based immunoassay for GA, in which GA was converted to GA by acid hydrolysis.10 Here, we describe a direct semi-micro HPLC for the determination of the serum levels of GL which has sufficient sensitivity to monitor the serum levels of GL after administration of therapeutic doses.

MATERIALS AND METHODS

Reagents Methanol, perchloric acid and butyl p-hydroxybenzoate were purchased from Wako Chemical Industries, Ltd. Acetonitrile of HPLC grade was also obtained from Wako Chemical Industries, Ltd. GL and Stronger Neo Minophagen C (SNMC) were kindly provided by Minophagen Pharmaceuticals Co., Ltd. (Tokyo, Japan). The GL purity was more than 90%, as determined by the HPLC described in the following section.

Chromatography The Nanospace Si-1 HPLC system (Shisecido, Tokyo, Japan), equipped with a Capcell Pak C18 UG120 column (150 × 1.5 mm i.d.; particle size, 5 μm) was used for the semi-micro HPLC. The column was maintained at 40°C and the detection wavelength was set at 254 nm. The mobile phase used was an acetonitrile–water solution (37:63, v/v, pH 2.0, adjusted with perchloric acid) and the flow rate was 100 μl/min. Ten μl of the each sample was injected into the chromatograph throughout the experiments.

Authentic GL Solution GL was dissolved in 50% methanol to give a concentration of 10 mg/ml. Then, the solution was serially diluted with the HPLC mobile phase and subjected to HPLC.

Sample Serum All the serum samples used in this study were collected from 2 healthy volunteers: a 26-year-old male weighing 75 kg and a 32-year-old male weighing 50 kg. These volunteers were given details about the objectives of the study, before obtaining their informed consent. Forty ml SNMC (including 80 mg GL) was injected intravenously and blood was periodically withdrawn from a vein 1, 3, 6, 12, 24, 36 and 48 h after injection. An aliquot of 100 μl serum was added to 200 μl methanol and 30 μl butyl p-hydroxybenzoate (1 μg/ml) as the internal standard and the mixture was vigorously vortexed. After centrifugating the mixture at 15000×g for 5 min, the supernatant was filtered through a membrane filter (pore size, 0.22 μm) and directly injected into the HPLC system.
RESULTS AND DISCUSSION

Previous studies have suggested that GL can exert its biological activities at a serum concentration of 100 ng/ml. Therefore, we attempted to establish a determination method for GL, whose detection limit would be at that level, and established the method described under Experimental. The retention times of authentic GL and butyl p-hydroxybenzoate as the internal standard were 8.2 min and 13.8 min, respectively (Fig. 1A). The detection limit of GL was 0.3 ng per sample injection (S/N=8). The calibration curve was linear between the concentrations of 0.3—300 ng and its correlation coefficient was 1.000. The intra-assay coefficient of variation was 0.63—4.29% for 5 samples each of 1, 10 and 100 ng GL. The inter-assay coefficient of variation was 0.77—2.80% for 5 samples each of 1, 10 and 100 ng GL.

Pretreatment of serum with methanol is all the preparation required and, there is no need for further purification and concentration of the serum, unlike previous methods. The peaks of GL and butyl p-hydroxybenzoate were separated from those of endogenous substances (Fig. 1B). The percentage recovery of GL was 97—104% following the addition of 30, 300 and 3000 ng GL to 100 μl human serum. The detection limit of GL in serum was 100 ng/ml.

After intravenous injection of a therapeutic dose (GL 80 mg) to 2 healthy volunteers, the serum levels of GL were assayed using our method. Serum GL was detected from 1 h to 36 h after administration in one subject and up to 48 h in the other (Fig. 2). Yamamura et al. reported serum levels of GL up to 12 h for the same administered dose and we previously detected it up to 24 h using indirect ELISA. The variation in the serum levels of GL were essentially consistent with these reports. Our data also showed that the serum levels of GL differed between the subjects for the same administered dose, although the sample was too small to allow a meaningful comment. Since there is a paucity of reports regarding the metabolism of GL in humans, this method will be useful for pharmacokinetic and pharmacodynamic studies of GL. In clinical practice, excessive administration of GL can induce pseudoadosteronism, the optimal GL concentration in serum still remains to be determined. This method is expected to aid in the safe and efficient use of the drug in clinical practice.

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REFERENCES