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Note

Determination of glycyrrhetic acid in human serum by high-performance liquid chromatography with ultraviolet detection

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Glycyrrhizae Radix is prescribed in a great number of "Kampo medicines." Glycyrrhizin (GL) is one of the active ingredients obtained from *Glycyrrhizae Radix*, and is known to be hydrolysed to glycyrrhetic acid (GA, Fig. 1), the aglycone of GL, in the intestine when GL is orally administered [1]. GA shows anti-inflammatory action [2] and an inhibitory effect on the metabolism of steroid hormones *in vitro* [3]. Kiso *et al.* [4] have reported an inhibitory effect of GA on the carbon tetrachloride-induced generation of free radicals by rat microsomes. Recently, Nishino *et al.* [5] have also reported that GA inhibits tumour promotion. Thus, GA is an important compound in studying the pharmacological effects of Kampo medicines.

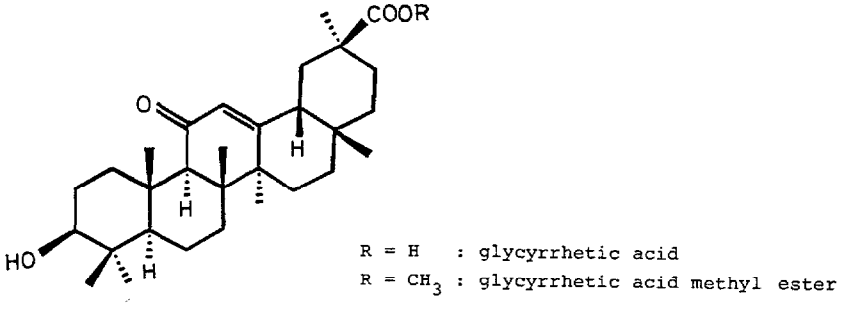

Fig. 1. Structures of glycyrrhetic acid (GA) and its methyl ester (GA-Me)

The analysis of GA in biological fluids has been carried out mainly by high-performance liquid chromatography (HPLC) [6,7] or gas chromatography [8]. In recent years, enzyme immunoassay [9] and radioimmunoassay [10] have also been used. However, these methods lack either sensitivity or specificity for determining

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GA concentrations in human serum after oral administration of Kampo medicines. On the other hand, gas chromatography–mass spectrometry (GC–MS) seems suitable for GA analysis in human plasma [11] but requires expensive apparatus. Therefore, we have attempted to establish a less expensive method for the sensitive determination of GA by HPLC, and to apply it for GA determination in human serum after oral administration of TJ-960 (Shosaiko-to-go-keishi-ka-shakuyaku-to), a Japanese kamp decoction, which is a mixture of nine herbal drugs. *Glycyrrhizae Radix* is one of the components. TJ-960 shows excellent effects on refractory epileptics and inhibits the seizure of amygdaloid kindolled cats and convulsions in the mutant strain El mouse [12].

EXPERIMENTAL

Materials

Glycyrrhetic acid was purchased from Tokyo Kasei (Tokyo, Japan) and recrystallized from ethanol before use. Other reagents were all of analytical grade and purchased from Wako (Tokyo, Japan). Cartridge colums (Bond Elut phenyl, 1 ml, Analytichem, Harbor City, CA, U.S.A.) were used for sample preparation. Glycyrrhetic acid methyl ester (GA-Me) was synthesized in our laboratory and used as an internal standard. In brief, GA (1 g) was dissolved in tetrahydrofuran (20 ml), and excess diazomethane in diethyl ether solution (10 ml) was added. After stirring for 2–3 min, the solution was evaporated to dryness under vacuum at 40°C. The product was recrystallized with chloroform–hexane, and the white powder of GA-Me (0.56 g) was obtained. The molecular structure of GA-Me (Fig. 1) was confirmed by ^{1}H NMR spectroscopy, and the purity was checked by HPLC (>99%).

Apparatus

The HPLC system consisted of an LC-6A pump, a CR-5A recorder, an SPD-6A UV detector and a CTO-6A column oven, all from Shimadzu (Kyoto, Japan). HPLC experiments were performed with a column (150 mm × 4.6 mm I.D.) packed with Inertsil ODS-2 (5 μm particle size, Gaskurokogyo, Japan) at 50°C and at a flow-rate of 1.0 ml/min. The mobile phase was methanol–2% (v/v) acetic acid (4:1, v/v), and the chromatogram was monitored at 254 nm throughout the experiments.

Extraction procedure

The cartridge column was preconditioned with 2 ml of methanol, 1 ml of chloroform and 2 ml of distilled water. A 0.5-ml volume of serum, 0.5 ml of 0.1 *M* acetate buffer (pH 3.0) and an ethanolic solution of the internal standard (200 ng per 25 μl) were introduced onto the cartridge column. After a washing step with 1 ml of water and 0.5 ml of benzene, the GA fraction was eluted with 0.5 ml of acetone. The eluate was evaporated, and then 0.5 ml of 0.1 *M* phosphate buffer

(pH 8.0) and 1 ml of ethyl acetate were added to the fraction. The solution mixture was shaken vigorously for 5 min, then centrifuged at 1500 *g* for 10 min at 4°C. The upper layer (0.8 ml) was collected, and ethyl acetate was evaporated. To the residue were added 200 μl of methanol, and 40 μl of this solution were used for the HPLC analysis.

Calibration curve

The calibration curve was prepared from drug-free serum spiked with GA ranging from 50 to 200 ng/ml. The extraction procedure of the sample was performed as described above.

RESULTS AND DISCUSSION

Chromatograms and linearity

Fig. 2 shows typical chromatograms of GA. We tested various types of HPLC column, including YMC-Pack A 303 (250 mm × 4.6 mm I.D., 5 μm particle size, Yamamura Kagaku, Japan), YMC-Pack A 312 (150 mm × 6.0 mm I.D., 5 μm particle size, Yamamura Kagaku), TSK-Gel 80TM (150 mm × 4.6 mm I.D., 5 μm particle size, Tosoh, Japan) and Inertsil ODS-2 for the GA separation. GA, the internal standard and some interference peaks in human serum were well separated with the Inertsil ODS-2 column and eluted within 20 min. The peak-height ratio of GA to internal standard was found to be linear in the range of

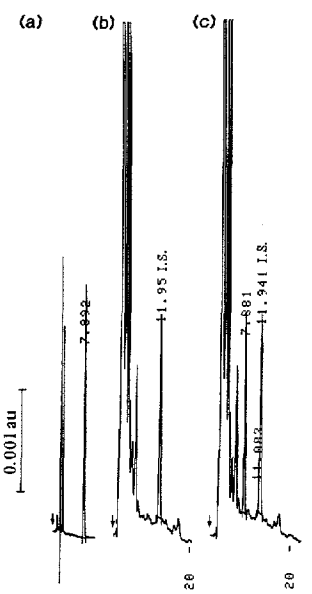

Fig. 2. Chromatograms of (a) a GA standard, (b) a drug-free serum and (c) a serum spiked with 200 ng/ml GA.

TABLE I

INTRA-ASSAY PRECISION AND ACCURACY

Amount added (ng/ml)	n	Amount found (mean ± S.D.) (ng/ml)	Precision (C.V., %)	Accuracy (%)
50	8	49.33 ± 2.95	6.0	98.7
100	8	101.00 ± 6.21	6.2	101.0
200	8	199.67 ± 5.49	2.7	99.8

TABLE II

RECOVERY

Amount added (ng/ml)	n	Recovery (mean ± S.D.) (%)	Mean of recovery (mean ± S.D.) (%)	C.V. (%)
50	8	96.6 ± 12.4	93.6 ± 2.8	12.8
100	8	91.2 ± 3.7		4.1
200	8	92.9 ± 2.6		2.8

measurement, and the following equation was obtained: y (ng/ml) = 177.047x (peak-height ratio)–2.3238 (r = 0.997, n = 8).

Precision and recovery

The intra- and inter-assay precisions were evaluated as coefficients of variation (C.V.) as shown in Table I. The values were all less than 7%.

A number of Japanese foods often contain GL as a food additive or a sweetening agent. Itoh *et al.* [11] reported that the concentration of GA in control plasma was found to be 9.1 ± 6.3 ng/ml by GC–MS. Therefore we prepared

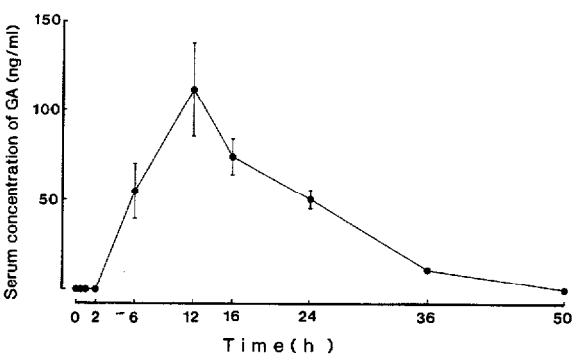

Fig. 3. Time-course of the serum concentration of GA after oral administration of TJ-960 (27.4 mg as GL) in healthy volunteers. Each value represents the mean ± S.E. of five subjects.

drug-free serum from healthy subjects put on a strictly controlled diet for more than 62 h, so that the amount of GA in the serum might be less than 10 ng/ml. The GA derived from food under our experimental conditions could be negligible. The detection limit of GA in this analysis, using 0.5 ml of serum, was estimated to be 10 ng/ml (signal-to-noise ratio of 3:1) and the mean recovery of GA added to human serum through the all extraction steps was 93.6 ± 2.8% (Table II).

Application

TJ-960 (10.5 g containing 27.4 mg of GL) was orally administered to five healthy subjects who fasted for 12 h prior to the ingestion. Blood samples were taken just before the administration and 1, 2, 6, 12, 24, 36 and 50 h after. Around 6 h after the administration GA began to appear in the serum, and then its concentration rapidly rose to a maximum of 111.6 ng/ml, 12 h after administration. GA was gradually eliminated from the serum until 50 h after, as shown in Fig. 3.

The method described in this paper demonstrated both sensitivity and precision, and should be useful in the evaluation of the pharmacokinetic profile of GA following single oral administration of Kampo medicines containing GL.

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