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DETERMINATION OF GARDENOSIDE, GENIPOSIDE AND RELATED IRIDOID COMPOUNDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The high-performance liquid chromatographic determination of gardenoside (GAR), geniposide (GEN), deacetylasperulosidic acid methyl ester (DAM), scandoside methyl ester (SSM) and monotropein methyl ester (MTM) is described. GAR, DAM, SSM and MTM were separated on a ODS-Develosil reversed-phase column using acetonitrile–tetrahydrofuran–water (4:1:95, v/v) as the eluent. In the same way, GEN was separated on a ODS-Hypersil reversed-phase column with acetonitrile–tetrahydrofuran–water (8:2:90, v/v) as the eluent. The application of this method to the evaluation of Inchinkoto (traditional Chinese medicine), which contains these compounds, is reported.

INTRODUCTION

Gardenoside (GAR) and geniposide (GEN) are iridoid glycosides found abundantly in Gardeniae Fructus that have choleretic and laxative pharmacological activity[1–5]. Treatment of GAR with hydrochloric acid gives deacetylasperulosidic acid methyl ester (DAM), scandoside methyl ester (SSM) and trace monotropein methyl ester (MTM)[6]. Several high-performance liquid chromatographic (HPLC) methods for iridoid analysis have been described[7–10], but the separation of GAR, DAM, SSM and MTM by HPLC has not so far been reported.

In this paper, the HPLC separation of these compounds and the effects of temperature and pH on their structural transformation are described. In addition, we examined the determination of these compounds in Inchinkoto (traditional Chinese medicine), which includes Gardeniae Fructus as a component.

EXPERIMENTAL

Standards and reagents

GAR and GEN were supplied by Takeda Chemical (Osaka, Japan). DAM, SSM and MTM were obtained by treatment of GAR with hydrochloric acid accord-

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ing to the method reported by Ishiguro *et al.*[6]. Gardeniae Fructus, Artemisiae Capillaris Herba and Rhei Rhizoma were obtained from Daiko Shoyaku (Nagoya, Japan). Specially prepared solvents for HPLC (Kishida Chemical, Osaka, Japan, and Nakarai Chemical, Kyoto, Japan) were used. All other reagents were of special grade.

Apparatus

A Model LC-4A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SPD-2AS variable-wavelength detector was used. Peak heights were measured with a Shimadzu C-R3A computing integrator. A stainless-steel column (250 × 4.6 mm I.D.) packed with ODS-Develosil (5 μm, Nomura Chemical, Aichi, Japan) or ODS-Hypersil (5 μm, Erma Optical Works, Tokyo, Japan) was used. The number of theoretical plates for both columns based on pyrene was 16 000 (flow-rate, 1.0 ml/min; solvent, 60% aqueous acetonitrile).

For the separation of GAR, DAM, SSM and MTM, a column packed with ODS-Develosil and acetonitrile–tetrahydrofuran–water (4:1:95, v/v) as the eluent were used with a flow-rate of 0.6 ml/min and an oven temperature of 40°C. The effluent was monitored at 242 nm and the detector sensitivity was 0.16 a.u.f.s. The chart speed was 2.0 mm/min.

For the separation of GEN, a column packed with ODS-Hypersil was used with acetonitrile–tetrahydrofuran–water (8:2:90, v/v) as the eluent at a flow-rate of 0.6 ml/min and an oven temperature of 40°C. These compounds were detected at 237 nm and the detector attenuation was 0.64 a.u.f.s. The chart speed was 2.0 min/min.

Preparation of samples for HPLC

A mixture of Gardeniae Fructus (3 g), Artemisiae Capillaris Herba (4 g) and Rhei Rhizoma (1 g) was added to 480 ml of distilled water and the volume was reduced to 120 ml. After cooling to room temperature, the pH of a decoction of Inchinkoto was measured with a pH meter (Horiba, Kyoto, Japan). The decoction was filtered through a 0.45-μm membrane filter (Toyokagaku Sangyo, Tokyo, Japan) and subjected to HPLC. Gardeniae Fructus (3 g) was also treated according to the procedure mentioned above and the sample obtained was subjected to HPLC. Further, a mixture of Gardenia Fructus (4 g), Artemisiae Capillaris Herba (4 g) and Rhei Rhizoma (1 g) in 120 ml of water was stood at 25°C or 60°C for 2 h. In the same manner, Gardeniae Fructus (3 g) in 120 ml of water was also stood at 25°C or 60°C. These extracts were subjected to HPLC analysis following filtration through a membrane filter. The volumes injected into the HPLC columns were 5 μl for GEN analysis and 20 μl for GAR, DAM, SSM and MTM analysis.

Calibration graph

Standard samples supplemented with various concentrations of GAR (0.05–0.2 mg/ml), DAM (0.05–0.2 mg/ml), SSM (0.05–0.2 mg/ml) or GEN (0.3–1.2 mg/ml) were prepared for HPLC analysis. The peak heights were calculated in order to construct a calibration graph. Every sample was analysed in triplicate and the results were averaged.

RESULTS

Determination of GAR and its acid reaction products

A chromatogram obtained using acetonitrile–water (5:95, v/v) as the eluent is compared with that obtained using acetonitrile–tetrahydrofuran–water (4:1:95, v/v) in Fig. 1. Both chromatograms showed the successful separation of GAR, DAM, SSM and MTM. The addition of tetrahydrofuran accelerated the elution without giving overlapping peaks. We therefore used the latter eluent for the effective determination of GAR, DAM, SSM and MTM. By linear regression analysis of the calibration graphs for GAR, DAM and SSM in amounts from 1 to 4 μg, a good linear relationship was obtained between peak height and amount (Fig. 2). The detection limit for these compounds was 4 ng, and this sensitivity is high enough for their determination.

GAR, DAM, SSM and MTM in Inchinkoto and the extract of Gardeniae Fructus (3 g) were determined using the above eluent system under various conditions. The chromatogram of Inchinkoto prepared at 100°C is shown in Fig. 3. The three peaks of GAR, DAM and SSM were separated completely even in Inchinkoto. The peak of MTM was not detected in this chromatogram.

Table I shows the changes in the contents of GAR, DAM and SSM at different extraction temperatures (25°C, 60°C and 100°C). The DAM and SSM contents in both extracts increased with increase in the extraction temperatures. The GAR content of both extracts, however, decreased slightly between 60°C and 100°C. Further, greater increases in DAM and SSM and decreases in GAR were observed in the

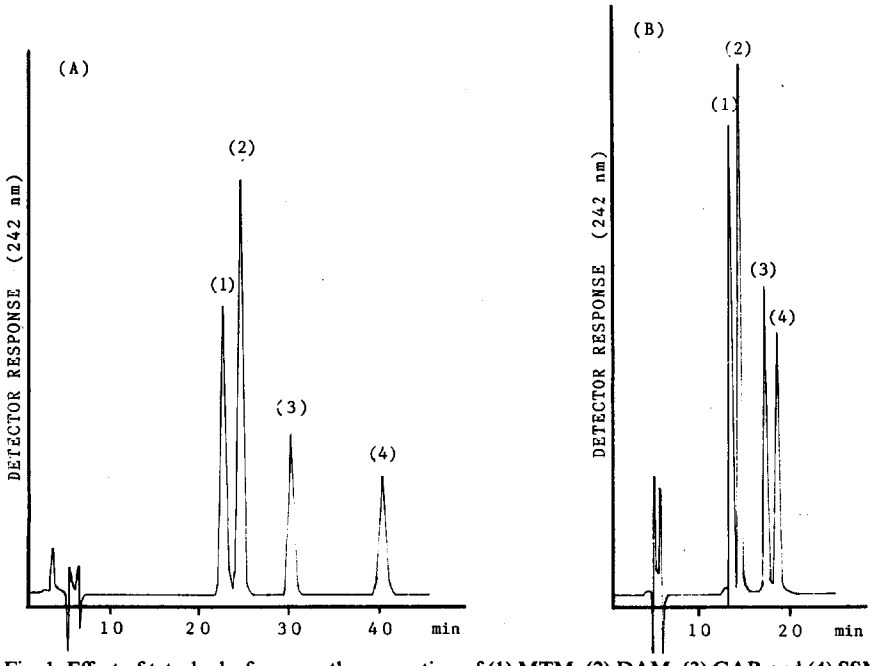

Fig. 1. Effect of tetrahydrofuran on the separation of (1) MTM, (2) DAM, (3) GAR and (4) SSM. Eluent: (A) acetonitrile–water (5:95); (B) acetonitrile–tetrahydrofuran–water (4:1:95).

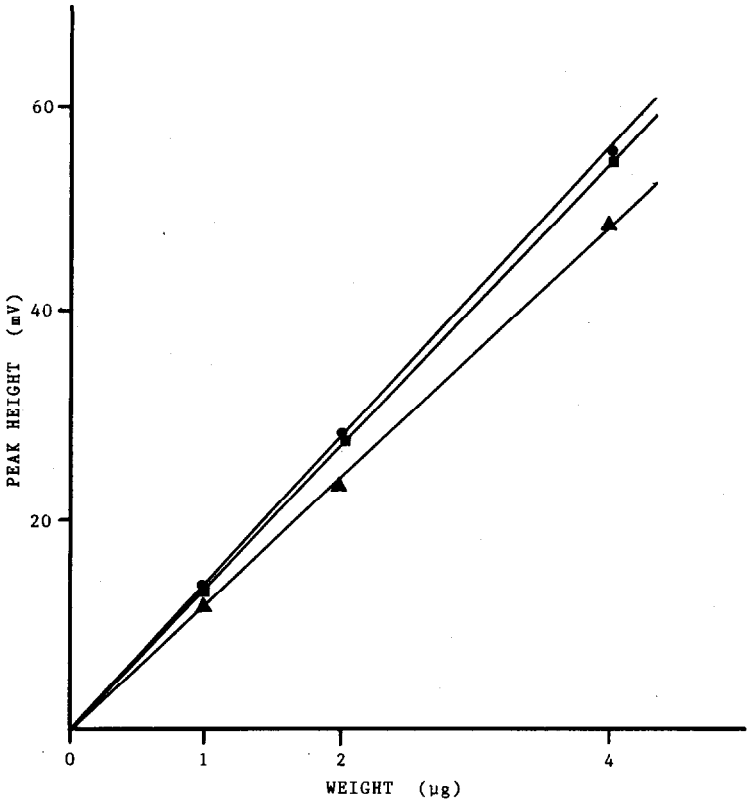

Fig. 2. Calibration graphs for (●) GAR, (■) DAM and (▲) SSM.

extract of Gardeniae Fructus than in that of Inchinkoto. As it is known that iridoid compounds are unstable towards acids, and GAR is easily converted into DAM, SSM and MTM by treatment with hydrochloric acid, the relationship between the pH of these decoctions and the extraction temperature was investigated. The results are shown in Fig. 4. The pH of the extracts of Gardeniae Fructus was lower than that of Inchinkoto at all temperatures. Although the pH of the extract of Gardeniae Fructus is hardly effected by heat, the pH of Inchinkoto increased with increase in the extraction temperatures. This result explains the easier transformation of GAR into DAM and SSM in Gardeniae Fructus than that in Inchinkoto (Table I).

Determination of GEN

As shown in Fig. 5, the separation of GEN from Inchinkoto was achieved by using acetonitrile–tetrahydrofuran–water (8:2:90, v/v) as the eluent. The calibration graph for GEN was linear over the range 1.5–6 μg (Fig. 6), and the limit of detection is 2 ng. GEN showed a single peak under the conditions used. The determination of GEN in Inchinkoto and the extract of Gardeniae Fructus at extraction temperatures of 25°C, 60°C and 100°C is illustrated in Table II. The amount of GEN in each extract increased with increase in the extraction temperature.

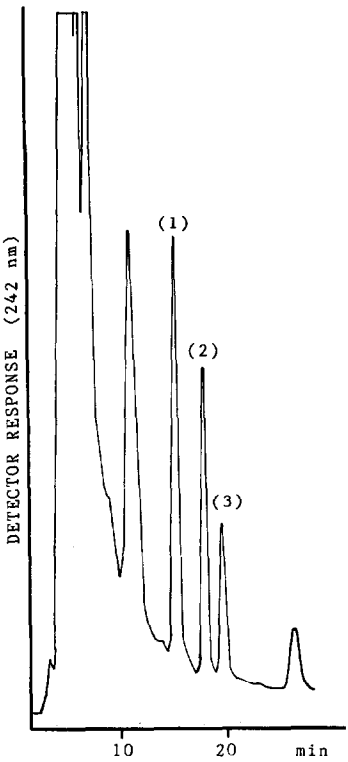

Fig. 3. Chromatogram of (1) DAM, (2) GAR and (3) SSM in Inchinkoto.

DISCUSSION

Recently, traditional Chinese medicines (Kampohozai), decoctions of a mixture of crude drugs, have been used for the treatment of chronic inflammatory diseases in Japan. The Inchinkoto used in this experiment is a decoction of a mixture of Gardeniae Fructus, Artemisiae Capillaris Herba and Rhei Rhizoma, and is applied to the treatment of hepatitis, especially choleresis. The evaluation of traditional Chinese medicines and their component crude drugs is of growing importance owing

TABLE I

DETERMINATION OF GAR, DAM AND SSM IN PREPARED SAMPLES AT VARIOUS TEMPERATURES

Each value represents the mean ± S.D. (mg/decoction) of three determinations.

Compound	*25°C*		*60°C*		*100°C*	
	Inchinkoto	*Gardeniae Fructus*	*Inchinkoto*	*Gardeniae Fructus*	*Inchinkoto*	*Gardeniae Fructus*
GAR	9.14 ± 0.09	9.40 ± 0.04	9.79 ± 0.08	11.33 ± 0.23	9.22 ± 0.32	8.33 ± 0.15
DAM	5.31 ± 0.06	4.48 ± 0.07	6.67 ± 0.27	7.07 ± 0.33	12.62 ± 0.36	16.33 ± 0.28
SSM	4.39 ± 0.03	4.41 ± 0.02	4.69 ± 0.07	5.62 ± 0.17	6.63 ± 0.38	8.36 ± 0.20

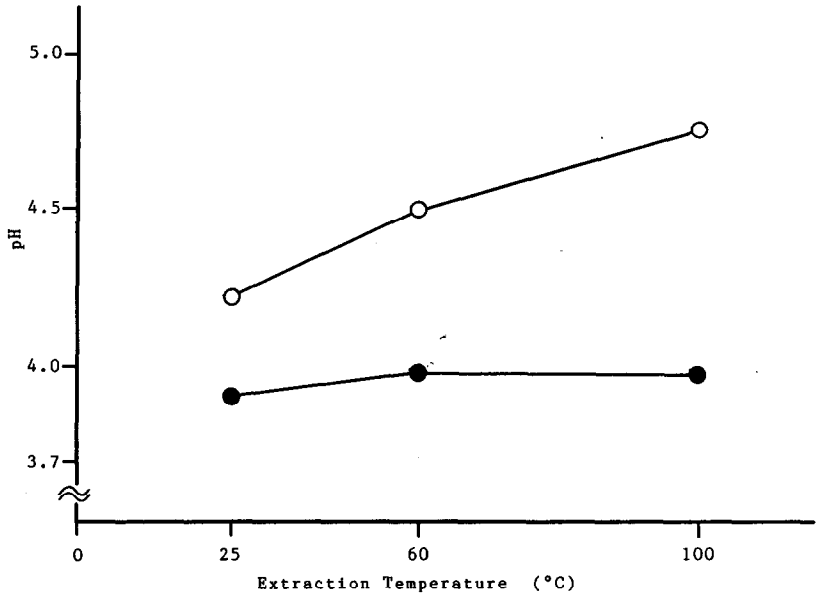

Fig. 4. Relationship between pH of extract and extraction temperature in (○) Inchinkoto and (●) extract of Gardeniae Fructus.

to the variety of cultivation conditions. Recently, gas–liquid chromatography (GLC) and HPLC have been used extensively to determine the main components present in crude drugs. Nevertheless, there have been no reports on the GLC or HPLC analysis of GAR and GEN in Gardeniae Fructus or traditional Chinese medicine because

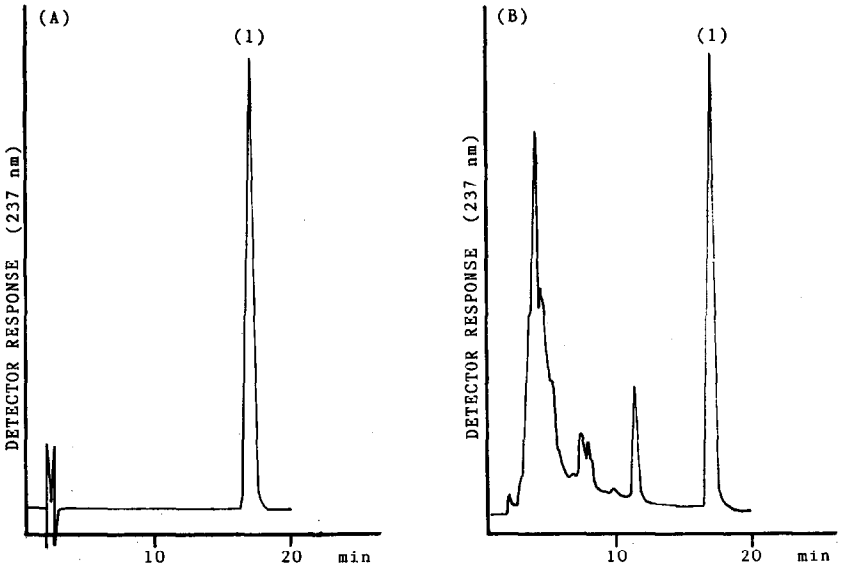

Fig. 5. Chromatograms of GEN (peak 1): (A) standard sample; (B) Inchinkoto.

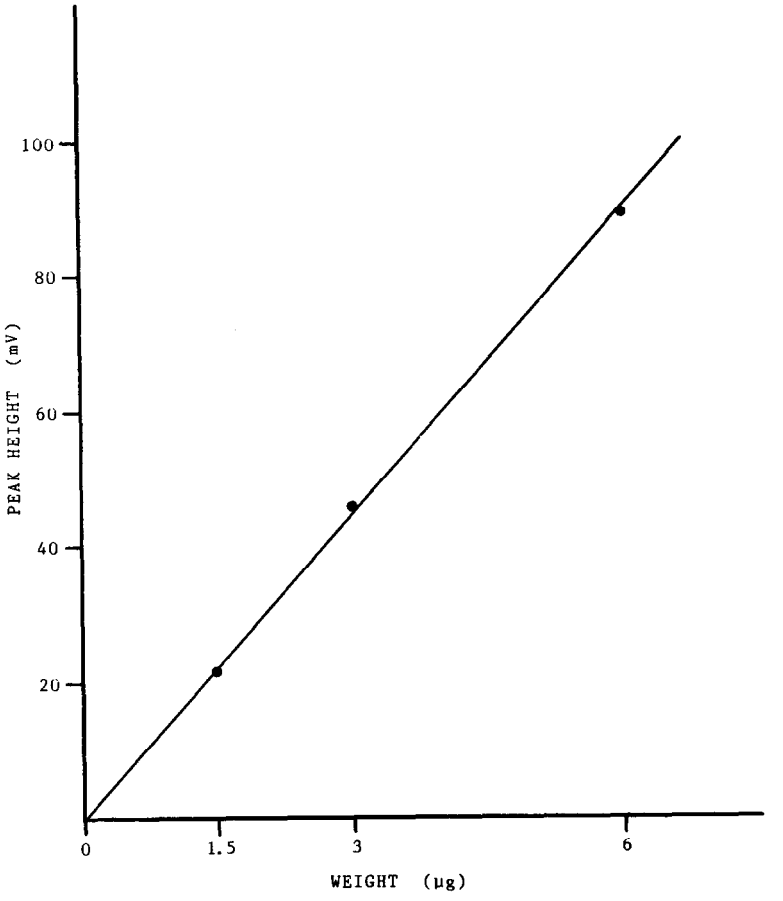

Fig. 6. Calibration graph for GEN.

GAR is unstable towards acids and heat, and it is very difficult to separaste GEN, GAR and related compounds produced from GAR during extraction.

In this study, we were successful in the complete separation of GEN, GAR and the related compounds DAM, SSM and MTM by reversed-phase HPLC. A

TABLE II

DETERMINATION OF GEN IN PREPARED SAMPLES AT VARIOUS TEMPERATURES

Each value represents the mean ± S.D. (mg/decoction) of three determinations.

Temperature (°C)	*Inchinkoto*	*Gardeniae Fructus*
25	111.7 ± 0.36	106.6 ± 0.32
60	114.9 ± 0.38	132.7 ± 0.09
100	128.8 ± 1.15	148.9 ± 0.17

mixture of acetonitrile and water with a small amount of tetrahydrofuran was a suitable eluent, giving complete separastion. GEN was stable at the extraction temperatures (25–100°C), and no structural changes occurred during the extraction. On the other hand, the content of GAR decreased and the content of DAM and SSM, acid reaction products of GAR, increased with increase in the extraction temperatures. During the extraction, the pH of Inchinkoto increased with increase in temperature, but that of Gardeniae Fructus did not change. These results indicate that there is a possibility of alkaline compounds such as alkaloids in Inchinkoto being extracted with increase in the extraction temperature and pH. Consequently, we conclude that the conversion of GAR into DAM and SSM in both extracts is promoted by heating, and that the difference in the contents of those compounds between two extrascts is due to the effect of acidity. MTM, which was derived from GAR by hydrochloric acid treatment, could not be detected under these mild conditions.

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