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Note

Separation and quantitative analysis of 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid in Glycyrrhizae Radix by gas–liquid chromatography

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The anti-ulcerative activity of Glycyrrhizae Radix has been demonstrated by Shabana[1] and it is well known for its clinical application to the treatment of stomach ulcers. In Japan and China, Glycyrrhizae Radix also has been used as one of the main components of Kampohozai (Chinese traditional medicine). It has been shown[2,3] that glycyrrhizin and its aglycone, glycyrrhetinic acid, are the active components of Glycyrrhizae Radix. Glycyrrhizae Radix is also an important commercial product used in tobacco and food throughout the world as a sweetener, glycyrrhizin being the sweet-tasting component[4]. Glycyrrhizin or its aglycone has two stereoisomers, 18α-form (*trans*) and 18β-form (*cis*), the structures of which were studied by Ružicka and Jeger[5], Steiner[6] and Beaton and Spring[7]. In our previous paper[8], the anti-inflammatory effects of the stereoisomers, 18α-glycyrrhizin and 18β-glycyrrhizin and their aglycones, 18α-glycyrrhetinic acid (18α-GA) and 18β-glycyrrhetinic acid (18β-GA), were reported. The anti-inflammatory action of 18α-GA was stronger than that of 18β-GA in several experimental models. 18α-GA showed a strong anti-granulomatous action in adrenalectomized rats. These results indicate that the action of 18α-GA is similar to that of glucocorticoid. For the above reason, the existence of 18α-GA in Glycyrrhizae Radix has an important role in evaluating the quality of the latter. Many investigators have reported methods for the determination of glycyrrhizin or glycyrrhetinic acid[9–11], but reports on the separation of the stereoisomers are very rare.

Killachy *et al.*[12] reported the isolation of 18α-GA and 18β-GA, as their silyl ether derivatives, by gas–liquid chromatography (GLC). High-performance liquid chromatography (HPLC) has been reported[13,14] for the determination of the 18β-form. Ion-exchange chromatography with an anion-exchange resin as the stationary phase[13] and ion-pair partition chromatography with octadecyl-silica gel[14] have been applied. For the separation of isomers of glycyrrhizin, a method involving use of a reversed-phase column after hydrolysis[15] and a direct method involving a nitrile-silica gel column[16] were reported. However, the simultaneous determination of iso-

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mers in the extracts of Glycyrrhizae Radix has not been reported. This omission is thought to be because of the difficulty of complete separation of isomers due to the low content of α-form in the extracts. The peak for the α-form readily overlaps with unknown peaks in the chromatogram of GLC or HPLC. In this paper, a method is described for the determination of stereoisomers in the extracts of Glycyrrhizae Radix, collected in five different areas, by GLC after being hydrolysed to 18α-GA and 18β-GA.

EXPERIMENTAL

Materials

Five kinds of Glycyrrhizae Radix (Seihoku-Kanzō, Tōhoku-Kanzō, Shinkyō-Kanzō, Russian-Kanzō and Afghan-Kanzō) were imported to Japan by Maruzen Kasei. The 18α-GA and 18β-GA were obtained, by hydrolysis of their glycosides with 6 *N* hydrochloric acid under reflux for 1 h, from aqueous extracts of Glycyrrhizae Radix. Dehydrocholic acid, used as the internal standard, was purchased from Nakarai Chemical Co.

Apparatus

A GC-6A Shimadzu gas chromatograph was used. The glass column (1 m × 3 mm I.D.) was packed with 3% OV-1 on Chromosorb W AW DMCS (60–80 mesh), the column temperature was 300°C, and the injection port and detector temperature was 320°C. The carrier gas (nitrogen) flow-rate was 30 ml/min.

Preparation of samples

A mixture of 4 g of crushed and pulverised Glycyrrhizae Radix (Seihoku-Kanzō, Tōhoku-Kanzō, Shinkyō-Kanzō, Russian-Kanzō and Afghan-Kanzō) and 60 ml of water was refluxed for 1 h and filtered. After adding 40 ml of 3 *N* hydrochloric acid to 6 ml of the filtrate, the solution was refluxed for 1 h and extracted with three 40-ml portions of chloroform. After washing the organic layer three times with water, sodium sulphate was added and the mixture was allowed to stand for 24 h. After filtration, the organic layer was concentrated *in vacuo*, and the residue was dissolved in 10 ml of chloroform containing 30 mg of dehydrocholic acid (the internal standard).

p-Toluenesulphonyl-N-methyl-N-nitrosoamide (3.6 g), which was purchased from Nakarai Chemical Co., was dissolved in 22 ml of diethyl ether, and the solution was added to a mixture of potasium hydroxide (0.84 g), 96% ethanol (4.2 ml) and water (1.4 ml), drop by drop. Diazomethane (0.5 g) was obtained as a solution in diethyl ether by distilling the mixture under 65°C. After adding the excess diazomethane solution (0.1–0.2 g) to 2 ml of chloroform solution containing glycyrrhetinic acid and the internal standard, the mixed solution was allowed to stand overnight at room temperature. After the methylation, the reaction mixture was concentrated to dryness *in vacuo*. The residue was dissolved in 10 ml of chloroform, and a 2.0–10.0-μl aliquot was subjected to GLC analysis.

Calibration graph

18β-GA (1.0, 1.25, 2.5 and 5.0 mg) and dehydrocholic acid (0.6 mg) were

dissolved in 0.2 ml of chloroform. After methylation according to the method described above, the reaction mixture was concentrated. The residue was dissolved in 10 ml of chloroform, and 10μl aliquot was injected into the GLC column. 18α-GA (0.1, 0.25 and 0.5 mg) was submitted to the same procedure as 18β-GA.

RESULTS AND DISCUSSION

The contents of 18α-GA and 18β-GA in the hydrolysate of aqueous extracts of five kinds of Glycyrrhizae Radix were determined by GLC. Fig. 1 shows the structure of 18α-GA and 18β-GA. Fig. 2 shows the chromatogram of 18α-GA, 18β-GA and dehydrocholic acid (internal standard). The peak for 18α-GA was completely separated from that of 18β-GA.

Fig. 3A–C shows chromatograms of the extracts of Seihoku-Kanzō, Tōhoku-Kanzō, Shinkyō-Kanzō, which were produced in China. The chromatogram of the extracts of Seihoku-Kanzō is similar to that of Tōhoku-Kanzō, but the peak for 18α-GA could not be identified in the chromatogram of the extracts of Shinkyō-Kanzō. Fig. 3 D and E shows chromatograms of the extracts of Russian-Kanzō and Afghan-Kanzō. 18α-GA was not found in Russian-Kanzō, but the content of 18β-GA in Russian-Kanzō was similar to that in Glycyrrhizae Radix produced in China. In Afghan-Kanzō, the content of 18β-GA was *ca.* one-half of that other kinds of Glycyrrhizae Radix, but the content of 18α-GA was similar to that in Seihoku-Kanzō.

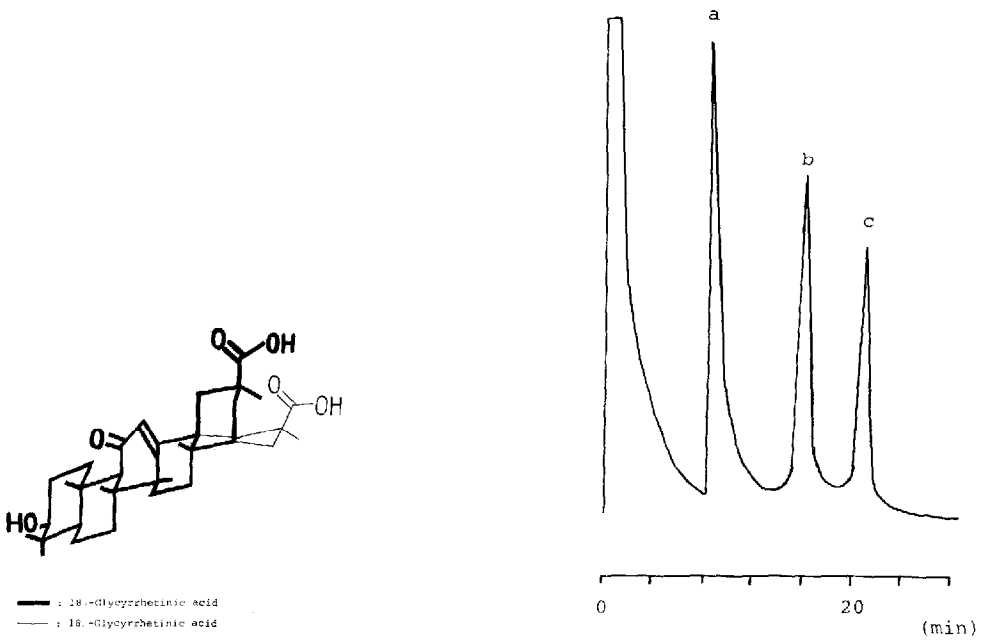

Fig. 1. Structures of 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid.

Fig. 2. Gas chromatograms of 18α-glycyrrhetinic acid, 18β-glycyrrhetinic acid and dehydrocholic acid as internal standard. Peaks: a = dehydrocholic acid (internal standard); b = 18β-glycyrrhetinic acid; c = 18α-glycyrrhetinic acid.

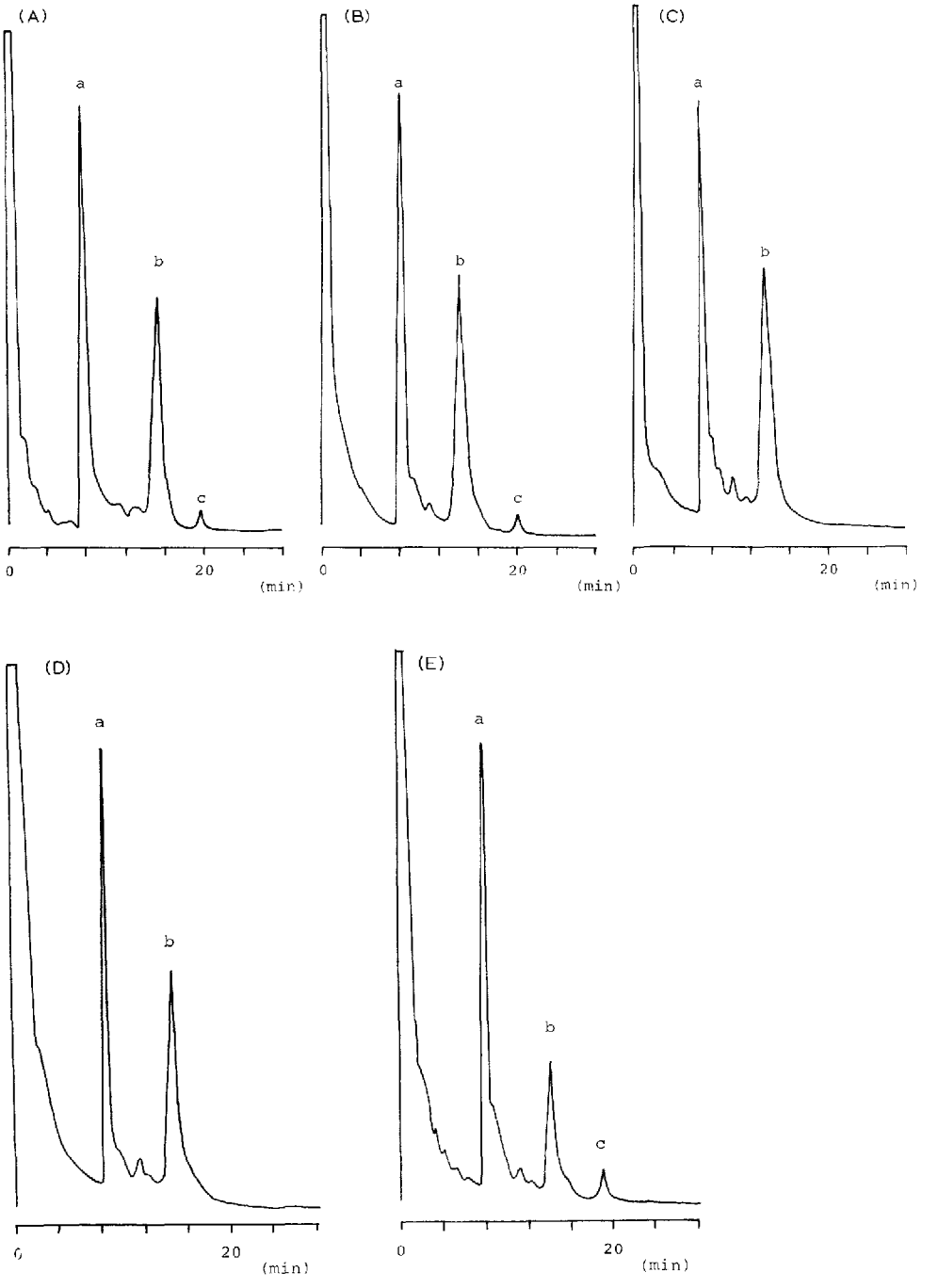

Fig. 3. Gas chromatograms of the extracts from Glycyrrhizae Radix. (A) Seihoku-Kanzō; (B) Tōhoku-Kanzō; (C) Shinkyō-Kanzō; (D) Russian-Kanzō; (E) Afghan-Kanzō. Peaks: a = dehydrocholic acid (internal standard); b = 18β-glycyrrhetinic acid; c = 18α-glycyrrhetinic acid.

TABLE I

DETERMINATION OF 18α-GLYCYRRHETINIC ACID AND 18β-GLYCYRRHETINIC ACID IN GLYCYRRHIZAE RADIX

	18α-Glycyrrhetinic acid (mg/g Glycyrrhizae Radix)	*18β-Glycyrrhetinic acid (mg/g Glycyrrhizae Radix)*	$\frac{18\alpha\text{-GA}}{18\beta\text{-GA}}$ (%)
Seihoku-Kanzō (China)			
lot A	0.23 ± 0.01*	7.03 ± 0.05*	3.3
lot B	0.33 ± 0.02	5.48 ± 0.04	6.0
lot C	0.71 ± 0.09	12.10 ± 0.08	5.9
lot D	0.52 ± 0.07	11.52 ± 0.09	4.5
Tōhoku-Kanzō (China)			
lot A	0.16 ± 0.04	16.80 ± 0.03	1.0
lot B	0.17 ± 0.03	11.81 ± 0.04	1.4
lot C	0.13 ± 0.02	10.05 ± 0.13	1.3
Shinkyo-Kanzō (China)	−**	13.23 ± 0.08	−**
Russian-Kanzō (Russia)	−**	9.52 ± 0.06	−**
Afghan-Kanzō (Afghanistan)	0.52 ± 0.09	3.98 ± 0.02	13.1

* Each value is the mean ± S.E. from three samples.
** Not detected.

Table I summarises the contents of 18α-GA and 18β-GA against the dry weight of crude drugs and the ratio of 18α-GA and 18β-GA. In these five kinds of Glycyrrhizae Radix, the contents of 18α-GA were highest in Seihoku-Kanzō and Afghan-Kanzō. On the other hand, the contents of 18β-GA were about the same in four kinds of Glycyrrhizae Radix, Afghan-Kanzō being the exception. These results indicate that the quality of Seihoku-Kanzō is better than that of other lots of Glycyrrhizae Radix. To evaluate the quality of Glycyrrhizae Radix, which is used as raw material for the extraction of glycyrrhizin and is the most important crude drug in the composition of Kampohozai (Chinese traditional medicine), the quantitative analysis of 18α-GA is necessary.

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