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Rapid Estimation of Glycyrrhizin in Pharmaceutical Preparations by High-Speed Liquid Chromatography

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The method was established to quantitate rapidly, simply and accurately by the technique of high-speed liquid chromatography, glycyrrhizin as the active ingredient of glycyrrhiza contained in kanpo (chinese medical) preparations (in the form of tablet, granule, subtle granule, powder or liquid) which are made of glycyrrhiza together with various other crude drugs.

Glycyrrhizin contained in such preparations was separated in less than 11 minutes in Du Pont LC 830 with a Permaphase AAX column (50 cm × 2.1 mm, i.d.) by gradient elution technique with 1/30 M phosphate buffer (pH 5.2) and 0.3 M $NaClO_4$ solution as the eluants. Quantitative determination was made by this method of glycyrrhizin contained in 27 different formulae with 6 forms of preparation. For tablet form, a relatively satisfactory correlation was found between the glycyrrhiza extract content specified in the prescription and the glycyrrhizin content actually determined. The per cent glycyrrhizin content of preparations containing glycyrrhiza powder (2 granule and 1 powder preparation) was almost identical with the value calculated from the per cent GA content (5.00 per cent) of glycyrrhiza powder referred to in our previous report.[2]

Keywords——pharmaceutical analysis; high-speed liquid chromatography; Permaphase AAX; glycyrrhizin; pharmaceutical preparation

There are few valid methods available at present for the quantitative determination of the active ingredients of crude drugs contained in the great numbers of commercially available kanpo preparations. In order to ensure the constant quality of such products, it is necessary to establish a proper method of quantitating the crude drug ingredients. Glycyrrhiza powder or extract is among the crude drugs commonly present in kanpo products and other preparations. There are a number of reports[3] available as to the procedure of quantitatively determining (chromometry, polarography, paper chromatography, thin-layer chromatography and gas chromatography) the glycyrrhizin content in glycyrrhiza, glycyrrhiza powder, glycyrrhiza extract and glycyrrhiza extract compounded with the extracts of various other crude drugs. In our previous report[2] we also presented high-speed liquid chromatography (HLC) method of determining glycyrrhizin (GA) contained in glycyrrhiza and glycyrrhiza extract. No such procedure is currently available, however, with regard to the GA content of kanpo products in tablet, granular, subtle granular, pulvent or liquid form.

In this study we succeeded in establishing the high-speed liquid chromatographic method for the determination of GA in 27 different preparations: 2 extracts, 2 liquids, 1 powder, 1 subtle granule, 3 granules and 18 tablets.

Experimental

GA——Before use in this study, GA was recrystallized in the same way as reported previously.[2] This served as the reference standard for GA (mp 210° (dec.)).

1) Location: *Yamashiro-cho, 770, Tokushima.*
2) Y. Akada and Y. Tanase, *Yakugaku Zasshi*, **96**, 1035 (1976).
3) L. Fuchs, *Monatsh.*, **81**, 70 (1950); R.H. Cundiff, *Anal. Chem.*, **36**, 1871 (1964); H. Onrust, A.P. Jansen, and B.S.J. Wostman, *Rec. Trav. Chim. Pays-Bas.*, **74**, 1515 (1955); H, Haneda and M. Inagaki, *Yakugaku Zasshi*, **78**, 795 (1956); G. Kurono and M. Sasaki, *Yakugaku Zasshi*, **90**, 497 (1970); S. Ogawa, A. Yoshida, and Y. Mitani, *Yakugaku Zasshi*, **96**, 122 (1976); S. Ogawa, A. Yoshida, and Y. Mitani, *Yakugaku Zasshi*, **96**, 1488 (1976).

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Table I. The Formulae of Kanpo (Chinese Medical) Preparations

No. of preparation	Form	Formula
	Extract	J.P. IX.
1	Soft	
2	Dry	
3	Subtle Granule	GAZ extract 1000 mg in 4.5 g
4	Granule	Caryoph. Flos. Pulv. 10 mg, Foenic. Fruct. Pulv. 20 mg, Cinnam. Cort. Pulv. 74.5 mg, Zingib. Rhiz. Pulv. 24.5 mg, Zanthox. Fruct. Pu Pulv. 1 mg, Copt. Rhiz. Pulv. 15 mg, Phellodend. Cort. Pulv. 35 mg, GAZ Pulv. 118 mg, diastase 50 mg, lactose 10 mg, anhyd. silic. acid 400 mg, sod. bicarb. 300 mg, calc. carb. 225 mg, in 1.5 g.
5		Pulv. Ext. Scopol. 30.0 mg, GAZ Pulv. 50 mg, Zingib. Rhiz. Pulv. 25 mg, Cinnam. Cort. Pulv. 75 mg, inosilin 400 mg, mag. carb. 100 mg, calc. carb. 200 mg, diastase 17 mg, methionine methyl sulf. chlor. 25 mg in 1.4 g.
6		Ext. 2.2 g of GAZ 6 g, Paeon. Rad. 6 g in 9 g.
7	Powder	Caryoph. Flos. Pulv. 7.5 mg, Foenic. Fruct. Pulv. 15 mg, Cinnam. Cort. Pulv. 55.5 mg, Zingib. Rhiz. Pulv. 18 mg, Zanthox. Fruct. Pulv. 0.75 mg, Copt. Rhiz. Pulv. 37.5 mg, GAZ Pulv. 88.5 mg, diastase 150 mg, sod. bicarb. 225 mg, calc. carb. 150 mg, *l*-menthol 1.5 mg, in 1.1 g.
8	Tablet	Ext. 1.1 g of GAZ 2.5 g, Cinnam. Cort. 4.5 g, Zizyph. Fruct. 3.5 g, Paeon. Rad. 6 g, Zingib. Rhiz. 4 g in 9 tables.
9		Ext. 1.3 g of GAZ 2 g, Coic. Sem. 10 g, Ephed. Herb. 4 g, Armen. Sem. 3 g, in 9 tablets.
10		Ext. 1.9 g of GAZ 2.5 g, Zizyph. Fruct. 3 g, Cinnam. Cort. 4 g, Paeon. Rad. 6 g in 9 tables.
11		Ext. 0.9 g of GAZ 2 g, Puerar. Rad. 4 g, Ephed. Herb. 3 g, Zingib. Rhiz. 1 g in 9 tablets.
12		Ext. 1.2 g of GAZ 2 g, Sinomen. Caul. et Rhiz. 5 g, Astrag. Rad. 5 g in 9 tablets.
13		Ext. 1.35 g of GAZ 2.5 g, Pinel. Tub. 5 g, Scutell. Rad. 2.5 g, Gins. Rad. 2.5 g in 9 tablets.
14		Ext. 1.2 g of GAZ 2 g, Bupl. Rad. 4 g, Pinel. Tub. 4 g, Zizyph. Fruct. 3 g in 9 tablets.
15		Ext. 0.9 g of GAZ 2 g, Angel. Rad. 6 g, Rhei. Rhiz. 0.5 g in 9 tablets.
16		Ext. 1.0 g of GAZ 1.5 g, Bupl. Rad. 5 g, Scutell. Rad. 2 g, Gins. Rad. 2 g, Zingib. Rhiz. 1 g, Paeon. Rad. 2 g in 9 tablets.
17		Ext. 1.0 g of GAZ 1.5 g, Schizonep. Herb. 1 g, Copt. Rhiz. 1 g, Menth. Herb. 1 g, Aur. Fruct. Immat. 1.5 g, Garden. Fruct. 2 g in 9 tablets.
18		Ext. 1.3 g of GAZ 1 g, Atractyl. Rhiz. 4 g, Aurant. Nobil. Peric. 2 g, Cimicif. Rhiz. 0.9 g, Zizyph. Fruct. 2 g in 9 tablets.
19		Ext. 1.0 g of GAZ 1 g, Atracyl. Rhiz. 4 g, Magnol. Cort. 3 g, Aurant. Nobil. Peric. 3 g, Zingib. Rhiz. 2 g, Zizyph. Fruct. 2 g in 9 tablets.
20		Ext. 0.5 g of GAZ 3 g, Cinnam. Cort. 3 g, Corydal. Tub. 3 g, Ostr. Test. 4.5 g, Foenic. Furct. 3 g, Amom. Sem. 1.5 g, Alpin. Rhiz. 1.5 g in 9 tablets.
21		Ext. 1.2 g of GAZ 2 g, Atractyl. Rhiz. 2 g, Platycod. Rad. 2 g, Rhei. Rhiz. 1.5 g, Angel. Rad. 1.2 g, Paeon. Rad. 1.2 g, Cnid. Rhiz. 1.2 g, Garden. Fruct. 1.2 g, Forsyth. Fruct. 1.2 g, Menth. Herb. 1.2 g, Ledebour. Rad. 1.2 g, Ephed. Herb. 1.2 g, Schizonep. Herb. 1.2 g, Zingib. Rhiz. 0.4 g in 9 tables.
22		Ext. 1.2 g of GAZ 1 g, Atractyl. Lanc. Rhiz. 2 g, Angel. Rad. 2 g, Platycod. 1 g, Hoel. 2 g, Cnid. Rhiz. 1 g, Cinnam. Cort. 1 g, Zingib. Rhiz. 2 g. Paeon. Rad. 1 g, Zizyph. Fruct. 1 g, Pinel. Tub. 2 g, Magnol. Cort. 1 g, Aur. Fruct. Immat. 1 g, Ephed. Herb. 1 g, Aurant. Nobil. Peric. 2 g, Angel. Dahur. Rad. 1 g, Atractyl. Rhiz. 2 g in 9 tablets.

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No. of preparation	Form	Formula
23		Ext. 1.3 g of GAZ 1 g, Gins. Rad. 4 g, Atractyl. Rhiz. 4 g, Hoel. 4 g, Pinel. Tub. 4 g, Aurant. Nobil. Peric. 2 g, Zizyph. Fruct. 2 g, Zingib. Rhiz. 0.5 g in 9 tablets.
24		Ext. 1.2 g of GAZ 1.5 g, Angel. Rad. 3 g, Paeon. Rad. 3 g, Mout. Cort. 2 g, Garden. Fruct. 2 g, Bupl. Rad. 3 g, Menth. Herb. 1 g in 9 tablets.
25		Ext. of 1.0 g of GAZ 2 g, Cinnam. Cort. 4 g, Paeon. Rad. 4 g, Zizyph. Fruct. 4 g, Zingib. Rhiz. 4 g, Atractyl. Lanc. Rhiz. 4 g, Aconit. Tub. Pulv. 1 g in 9 tablets.
26	Liquid	Ext. 600 mg of GAZ 375 mg, Cinnam. Cort. 750 mg, Foenic. Fruct. 250 mg, Amom. Sem. 250 mg, Atractyl. Rhiz. 750 mg, Hoel. 750 mg. Gins. Rad. 125 mg, Copt. Rhiz. 375 mg in 20 ml.
27		Ext. GAZ 600 mg, Gentian. Rad. 150 mg, Copt. Rhiz. 330 mg, Hoel. 50 mg, Gamb. 40 mg, Cinnam. Cort. 200 mg, Coryoph. Flos. 10 mg in 20 ml.

GAZ: Glycyrrhiza.
Ext: Extract.

Samples——Glycyrrhiza soft and dry extracts, J.P.IX, and other preparations, all in commercially available forms. The formulae of these preparations are shown in Table I.

Conditions of HLC——The apparatus used was Du Pont LC 830 with a Permaphase AAX column (50 cm × 2.1 mm, i.d.) with a UV absorption photometer (wave length 254 nm) as the detector. The mobile phase consisted of phosphate buffer (pH 5.2, composed of 1/30 M KH_2PO_4 and 1/30 M Na_2HPO_4) and 0.3 M $NaClO_4$ with a linear gradient of 2 per cent per min. The chromatography was carried out at an inlet pressure of 300 psi, at room temperature and at a flow rate of 1.1 ml per min.

Method of Determination——Exactly 3—4 mg of GA was taken and dissolved in 10.0 ml of 0.05 N NH_4OH. One to 5.0 μl of the solution was subjected to HLC for the determination of GA (X μg). Then the height (Y cm) of the peak of the chromatogram obtained was determined. From the values for X and Y, a linear regression equation was derived by the method of least squares. The equation obtained was:

$$Y = 3.939X - 0.518 \quad (r = 0.999)$$

Preparations in tablet, fine granular or granular form were pulverized before use. To a definite, exactly weighed amount (mg) of these preparations, powders or extracts, or to a definite volume (μl) of liquids, 5.0 ml of the solvent for extraction (0.05 N NH_4OH) was added and, after shaking for 30 minutes, centrifuged at 3000 rpm for 20 minutes to obtain the supernatant fluid test solution. One to 5.0 μl of the test solution was submitted to HLC to determine the value for Y from the resultant chromatogram from which the GA content of the preparations tested was calculated by means of the linear regression equation shown above.

Evaluation of the Conditions of HLC

1) **Column**——In consideration of GA being a water-soluble acid glycoside, the strongly basic ion exchange resin Permaphase AAX was used as the column in this study.

2) **Mobile Phase**——The mobile phase consisting of water and 0.05 M $NaClO_4$ with a linear gradient of 5 per cent per minute, which was used in our previous study for the estimation of GA in glycyrrhiza extract, proved to be inadequate to separate GA from other ingredients. Further evaluation was then made of different systems as the mobile phase in an attempt to achieve satisfactory separation of GA in preparations which contain a variety of ingredients (*e.g.*, binders, disintegrators, lubricants *etc.*). As a result, a system of phosphate buffer (pH 5.2) and 0.3 M $NaClO_4$ with a linear gradient of 2 per cent per minute was found to be best suited for the purpose.

3) **Inlet Pressure**——Of the different inlet pressure levels examined, ranging from 100—1000 psi, 300 psi proved to be optimal in consideration of the range of the peak and the time required for analysis.

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Calibration Curve and Accuracy of Determination

The amount of GA injected (X μg) was well proportional to the height (Y cm) or area of the peak on the chromatogram. In this study the peak-height (Y cm) was measured in formulating the linear regression equation mentioned in the preceding section. In an attempt to affirm the reproducibility and accuracy of the results obtained from the equation, the values for Y obtained with different amounts of GA injected (X μg) were substituted in the equation to determine the resultant values for X' (X' μg) and further for $(X'/X)\times 100$. It was found that the results thus obtained were highly reproducible with an error of approximately ±2 per cent (Table II).

Table II. The Accuracy of the Qxantitative Determination obtained with Different Amounts of GA[a] by HLC[b] Method

Amount of GA[a] taken (μg)	Amount of GA[a] found (μg)	Per cent of error
0.310	0.317	+2.26
0.600	0.609	+1.50
0.620	0.610	−1.67
0.900	0.909	+1.00
0.900	0.882	−2.00
0.930	0.927	−0.32
1.240	1.245	+0.40

a) Glycyrrhizin.
b) High-speed liquid chromatography.

Table III. GA Amount extracted by Various Solvents in Preparation No. 6

Solvent	Amount of sample taken (μg)	GA amount in sample (μg)	Per cent of GA content
0.05 N NH_4OH	54.54	0.447	0.82
	54.54	0.442	0.81
0.05 N NaOH	59.88	0.485	0.81
	59.88	0.485	0.81
H_2O	102.20	0.644	0.63
	102.20	0.634	0.62
MeOH	99.30	0.497	0.50
	99.30	0.487	0.49
EtOH	110.80	0.255	0.23
	110.80	0.255	0.23

For keys, see in Tables I and II.

Solvents for Extracting GA in Preparations

A comparative assessment was made of 0.05 N NH_4OH, 0.05 N NaOH, H_2O, methanol and ethanol as the solvents for extracting GA from different forms of kanpo preparations including tablet, fine granule, granule, powder and liquid. Table III represents the results obtained with preparation No. 6 as a sample. As is apparent from the table, the amount of GA extracted was the greatest with 0.05 N NH_4OH or 0.05 N NaOH. Similar results were obtained for other preparations. In this study 0.05 N NH_4OH was used as the solvent for extraction of GA, because the use of 0.05 N NaOH as such might involve the risk of GA being hydrolyzed.

Determination of GA after Its Addition to Different Preparations

GA was added in a definite amount to different preparations and then extracted with ammonia solvent to calculate its recovery. The results obtained with preparation No. 6 are

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Table IV. The Determination of GA after Its Addition to Preparation No. 6

Amount of sample taken (μg)	Amount of GA added (μg)	GA amount in sample (μg)	Per cent of total GA (μg)	Recovery per cent of GA added
25.001	none	0.205	0.82	—
25.001	0.233	0.447	1.77	103.86
25.001	0.233	0.439	1.74	100.43
25.001	0.233	0.437	1.73	99.57
25.001	0.233	0.427	1.69	95.28
25.001	0.233	0.451	1.79	105.58
25.001	0.233	0.449	1.78	104.72
25.001	0.233	0.425	1.68	94.42

For keys, see in Tables I and II.

summarized in Table IV. As is seen from the table, GA was recovered at a rate of 94—106 per cent, figures which are essentially comparable to the accuracy of determination of the substance (Table II). This shows that the linear regression equation may be used for the estimation of GA in different preparations. Similar results were obtained for other preparations.

Determination of GA in Different Preparations

The chromatogram obtained with preparation No. 6 is shown in Fig. 1. The peak for GA appeared approximately at 11 minutes, permitting its separation from those for impurities. Other preparations gave a similar chromatographic pattern, enabling solitary separation of GA peak. A thin-layer chromatogram (developing solvents: butanol–3 N NH_4OH–ethanol= 5: 2: 1, v/v: plate: Wacogel B-5; coloring agent: conc. H_2SO_4) of the GA peak showed that it was composed exclusively of GA (Rf=0.28), confirming its homogeneity. It was thus shown that the GA content of different preparations can be directly determined by extracting GA with ammonia solvent and then subjecting a definite amount of the extract to HLC.

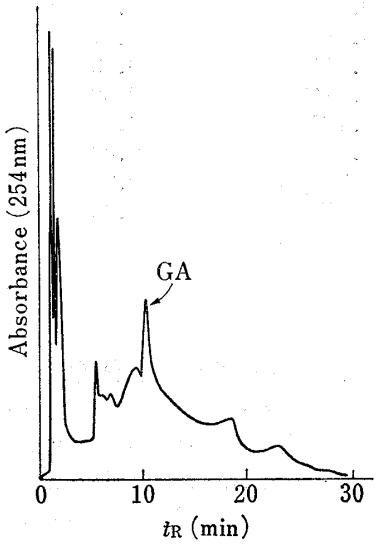

Fig. 1. Chromatogram of GA in Preparation No. 6 by HLC on Permaphase AXX Column using Gradient from 1/30 M Phosphate Buffer (pH 5.2) to 3/10 M $NaClO_4$ at 2%/min as Eluant

For keys, see in Table I and II.

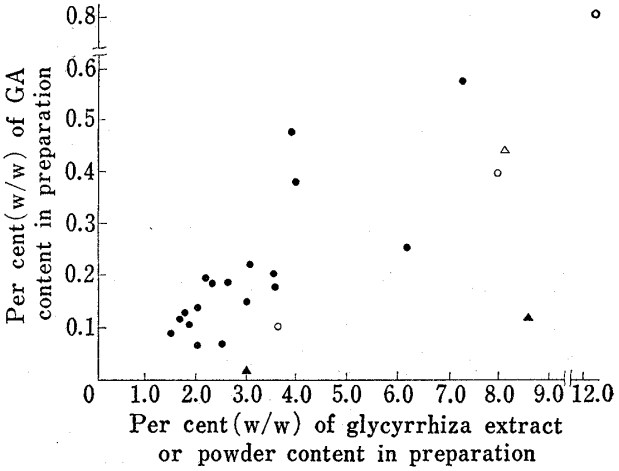

Fig. 2. The Correlationships between Per Cent (w/w) of GA Content and Per Cent (w/w) of Glycyrrhiza Extract or Powder Content in Kanpo Preparations

○: granule, ●: tablet, △: powder, ▲: liquid.
Data of preparation No. 3 that is not represented in Fig. is 9.403 w/w % GA content per 22.222 w/w % glycyrrhiza extract content.

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Table V. GA Contents in Kanpo Preparations

Number of preparation	Amount of sample taken (μg)	GA in sample (μg)	Mean % (w/w) of GA content	% (w/w) of glycyrrhiza extract or powder contained in preparation
1	3.400	0.391	11.647	
	3.400	0.401		
2	1.560	0.906	57.821	
	1.560	0.898		
3	19.990	1.879	9.403	22.222
	19.990	1.880		
4	300.264	1.123	0.402	7.867[a)]
	300.264	1.289		
5	500.200	0.503	0.103	3.571[a)]
	500.200	0.523		
6	100.000	0.818	0.807	12.222
	100.000	0.805		
7	300.132	1.337	0.445	8.045[a)]
	300.132	1.337		
8	100.012	0.378	0.384	3.859
	100.012	0.389		
9	100.020	0.476	0.482	3.803
		0.488		
10	100.050	0.574	0.580	7.191
	100.050	0.586		
11	100.032	0.228	0.224	2.941
	100.032	0.220		
12	100.036	0.215	0.210	3.438
	100.036	0.205		
13	100.024	0.254	0.256	6.080
	100.024	0.259		
14	100.032	0.186	0.179	3.466
	100.032	0.171		
15	100.028	0.146	0.149	2.816
	100.028	0.151		
16	99.990	0.138	0.136	1.895
	99.990	0.133		
17	100.030	0.100	0.100	1.761
	100.030	0.100		
18	100.008	0.116	0.114	1.602
	100.008	0.111		
19	100.056	0.067	0.067	1.866
	100.056	0.067		
20	100.108	0.193	0.196	2.121
	100.108	0.198		
21	100.046	0.073	0.070	2.435
	100.046	0.067		
22	100.020	0.089	0.089	1.377
	100.020	0.089		
23	100.014	0.133	0.130	1.672
	100.014	0.127		
24	100.080	0.187	0.190	2.212
	100.080	0.192		
25	100.054	0.187	0.190	2.523
	100.054	0.193		
26	1.00(μl)	1.257	0.125(w/v)	8.571
	1.00	1.245		
27	1.00(μl)	0.122	0.127(w/v)	3.000
	1.00(μl)	0.131		

a) Glycyrrhiza powder.
For keys, see in Tables I and II.

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Discussion

The GA content of different kanpo preparations as determined by using the linear regression equation set up for standard GA is shown in Table V. Fig. 2 illustrates the correlationship between the per cent GA content (shown in Table V) and glycyrrhiza extract content in w/w % for preparations No. 3, 6 and 8—25, glycyrrhiza powder content in w/w % for preparations No. 4, 5 and 7, and glycyrrhiza extract in w/v % for liquid preparations No. 26 and 27. For tablet preparations, a relatively close correlation was present between the per cent glycyrrhiza extract content and the per cent GA content: the greater the former, the greater the latter. As to preparations with glycyrrhiza powder added, which were limited to 3 in the present study, a calculation was made of the per cent GA content of glycyrrhiza powder added from that of glycyrrhiza powder (5.00 per cent) mentioned in our previous report.[3] The per cent GA content calculated was 0.393, 0.402 and 0.178 respectively for these 3 preparations as compared with that actually determined as 0.402, 0.445 and 0.103 respectively. Thus, somewhat better results were obtained for preparations with glycyrrhiza powder added than those with glycyrrhiza extract added, although these 2 groups of preparations differ in form from one another. No significant results were obtained for the 2 liquid preparations, which were also too limited in number to demonstrate any correlationship in this regard.

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