DISPOSITION OF GLYCYRRHETIC ACID AND ITS GLYCOSIDES IN HEALTHY SUBJECTS AND PATIENTS WITH PSEUDOALDOSTERONISM

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As a first step to elucidate the disposition of traditional Chinese formulations which contain licorice, the disposition of plain licorice was investigated in humans. Glycyrrhetic acid (GLA) was measured by an enzyme immuno-antibody technique. Glycyrrhetic glycosides (GLA-GS), such as glycyrrhizin, were measured after acid hydrolysis to GLA by the enzyme immuno-antibody assay.

Five normal subjects were orally administered a decoction of licorice containing 133 mg of glycyrrhizin. It was found that the time required for maximum serum concentration of GLA-GS was less than 4 h after the administration. Although there were large individual differences, it was found that GLA-GS was eliminated from the blood for the most part within 72 h. On the other hand, GLA reached maximum serum concentration at about 24 h after administration and in two of the five cases it was still detected in the blood even after 96 h. Urinary excretion of GLA was about 2% of the total dose of glycyrrhizin administered. This suggested that there were great differences among the subjects in the absorption and urinary excretion of GLA-GS.

The serum GLA levels in two clinical cases who presented pseudoaldosteronism by licorice containing formulations were as high as 70–80 ng/ml, with GLA-GS levels being very low. This fact suggests that pseudoaldosteronism develops in association with GLA rather than with GLA-GS.

Keywords — disposition; glycyrrhizin; glycyrrhetic acid; serum level; urinary excretion; pseudoaldosteronism; human being

INTRODUCTION

Licorice, which is a medical plant, has been an important drug in traditional Chinese medicine since ancient times. Since 1976, when this system of medicine was introduced into the medical insurance by the Ministry of Welfare of Japan, about 80 kinds of licorice containing formulations have been widely used in daily practice in our country.

Recent studies have disclosed that licorice and its effective components have anti-inflammatory¹⁾ and Δ^4 - β -reductase suppressing effects.²⁾ Moreover, it has also been known that licorice causes pseudoaldosteronism.^{3,4)} However, no biopharmaceutical study has been made of

decoctions of licorice which are orally administered. The present study was undertaken in an attempt to clarify the disposition of glycyrrhetic acid (GLA) and its glycosides (GLA-GS) in humans by using the enzyme immuno-antibody (EIA) technique.^{5,6)}

MATERIALS AND METHODS

1) *Enzyme Immuno-Antibody Assay* — Measurements were done by the second antibody technique which has been reported by Kanaoka *et al.*⁶⁾ In this study, the technique was partly modified as follows: 18- β GLA (Fluck Co., Ltd.) was mixed with succinic anhydride to obtain glycyrrhetyl succinate which was acetylated at

C-3. This was made into an active ester by condensation with *N*-hydroxy succinimide and then mixed with bovine serum albumin (BSA). After dialysis, it was emulsified with Freund's adjuvant by a conventional method and injected subcutaneously into the backs of rabbits repeatedly, to obtain antiserum.

Glutaric anhydride was added as a spacer with purified 18- β GLA at C-3, which was made into an active ester by condensation with *N*-hydroxy succinimide. By reacting the active ester with β -galactosidase, 18- β GLA-labeled β -galactosidase at C-3 was obtained.

To prepare a standard solution for measurement, commercial GLA (Fluck Co., Ltd.) was used. One mg was dissolved in 1 ml of EtOH and diluted with 0.02 M phosphate buffer (buffer A) containing 0.1 M NaCl, 0.1% NaN₃, 0.01 M MgCl₂, and 0.1% BSA. To construct a standard curve or to assay for GLA in serum or urine samples, the standard solution was mixed with serum or urine and extracted with hydrochloride acidic CHCl₃, which was then evaporated. The standard solution (100–150 μ l) or samples to be assayed for GLA were incubated with 100 μ l of a diluted antiserum ($\times$ 2700) and 50 μ l of β -galactosidase labeled GLA for 2 h at room temperature. Then the reaction mixture was incubated overnight at 4 °C with 50 μ l of anti-rabbit-immunoglobulin G (IgG) goat serum and 20 μ l of normal rabbit serum. One ml of buffer A was added to the reaction mixture, centrifuged at 2500 rpm for 20 min: and the precipitate washed twice and resuspended in buffer A. Next, 150 μ l of 4-methylumbelliferyl β -D-galactoside (10⁻⁴ M) were added to this suspension and the mixture incubated at 37 °C for 30 min to obtain 4-methylumbelliferone, which was measured by fluorometry.

GLA-GS in the samples was measured in the following manner: first, a portion of the sample was measured for GLA to obtain a base line and then another portion from the same sample was hydrolyzed by 4 N HCl to obtain the aglycone of GLA-GS, namely GLA. The amount of GLA-GS in the sample was calculated by sub-

tracting the GLA value obtained from the unhydrolyzed sample from the GLA value obtained from the hydrolyzed sample.

2) *Licorice Administration to Healthy Subjects* — Five grams of licorice from China (Tochimoto-tenkaidô Co., Ltd.) were put in 300 ml of water to make a decoction of 100 ml, which was administered orally. The decoction was analyzed to contain 133 mg of glycyrrhizin by high performance liquid chromatography (HPLC). GLA was not detected in this decoction. The subjects were five male volunteer university students, between the ages of 20 and 24 years. Alcoholic beverages were prohibited during the test period but there were not other restriction on intake of food and participation in sports. Blood samples were taken from the cubitus vein for a 7-d period following administration. Urine was collected and stored, recording the total volume during the collection period.

3) *Clinical Study* — Two cases of pseudoaldosteronism (72-year-old female and 47-year-old female) which had developed after treatment with licorice-containing formulations were investigated. Both GLA and GLA-GS levels in the serum were measured at the time of pseudoaldosteronism and 2 weeks after withdrawal or reduction of the dosage of licorice in the formulations.

RESULTS

I. *Standard Curve and Specificity of GLA Antibody*

Structures of molecules of GLA and glycyrrhizin are shown in Fig. 1, and standard curves of GLA are illustrated in Fig. 2. An aqueous dilution of the standard solution gave a favorable curve between 5 and 500 pg/ml. GLA diluted with serum or urine was measurable in a range of 0.1–100 ng/ml.

GLA-antibody was highly specific and did not cross-react with cholesterol, steroids or glycyrrhizin ammonium. In the present study, however, it was not possible to study in detail any cross-reactive substances in licorice which resemble GLA structure. Hence, the results ob-

tained by EIA should be expressed as GLA-anti-body reactive substances. In order to estimate the amount of GLA and any other GLA-anti-body reactive substances, the same samples were measured by EIA technique and HPLC in a pilot study (Table I).

II. Administration in Normal Subjects

Figure 3 illustrates serum concentration of GLA-GS following oral administration of licorice decoction in the 5 normal subjects.

It was found that there were great individual differences in serum GLA-GS levels, and that the time required for the maximum GLA-GS serum concentration was less than 4 h. GLA-GS levels rapidly decreased after 48 h in 2 cases and showed low levels after 24 h in 3 cases. GLA-GS was not detected after 96 h in all cases.

FIG. 1. Structures of Molecules of GLA and Glycyrrhizin

On the other hand, Fig. 4 shows that the time required for the maximum serum concentration of GLA was about 24 h. The highest serum concentration was 30 ng/ml on the average. The concentration of GLA declined gradually thereafter but was still detected in 4 cases at 72 h and in 2 of the 5 cases at 96 h. Excretory amounts of GLA-GS and GLA in the urine which were collected every 10 h are illustrated in Fig. 5. In a manner similar to the changes found in serum (Figs. 3 and 4), GLA-GS appeared first in urine and then GLA appeared 10 to 20 h later.

As shown in Fig. 3, case 1 showed a rapid decrease in serum GLA-GS concentration during the 24 h period following the administration of licorice decoction. This case showed a peak at 30

FIG. 2. Standard Curves Showing Enzyme-Immuno Assay of GLA in Water, Serum and Urine

△, water; ○, serum; ●, urine.

TABLE I. Serum GLA and GLA-GS Levels Measured by EIA and HPLC

Sample No.	GLA		GLA-GS		Glycyrrhizin
	EIA ng/ml	HPLC ng/ml	EIA ng/ml	HPLC ng/ml	
1) 24 y.o. F.	180	158	176	104	
2) 25 y.o. M.	380	348	—	—	
3) 28 y.o. F.	48	33	222	161	
4) 23 y.o. M.	380	347	174	120	

HPLC, mobile phase for GLA: 0.5% NH_4ClO_4 - 10% CH_3CN in 90% (v/v) MeOH-water, for glycyrrhizin: 0.75% NH_4ClO_4 - 1% CH_3CN in 85% (v/v) MeOH-water. (Shimadzu liquid chromatograph LC-5A).

to 50 h in urinary excretion of GLA. On the other hand, case 2, who showed the highest *AUC* value of GLA-GS in serum, showed 3 peaks at 20, 70 and 110 h for urinary GLA excretion. It was revealed that about 1.7–2.5% of glycyrrhizin was excreted in the urine as GLA or GLA-GS, when calculated on a molar basis.

III. Clinical Study

Figure 6 illustrates serum concentrations of GLA-GS and GLA in two cases of pseudoaldosteronism induced by licorice containing formulations. Case 1 was diagnosed as having atrial fi-

FIG. 3. Changes in Serum GLA-GS Concentrations in 5 Normal Subjects after Oral Administration of Licorice Decoction

Subjects 1 and 2 correspond to cases 1 and 2 in Fig. 5, respectively.

FIG. 4. Changes in Serum GLA Concentration in 5 Normal Subjects after Oral Administration of Licorice Decoction

Subjects 1 and 2 correspond to cases 1 and 2 in Fig. 5, respectively.

brillation and hyperthyroidism and treated with Bukuryo-shigyaku-tô, which contained 3.0 g/d licorice for 4 weeks. Case 2 was treated with Ninjin-tô, which contained 2.0 g/d licorice for 3 weeks and was diagnosed as having chronic pancreatitis. Following 4 weeks and 3 weeks

FIG. 5. Urinary Excretion of GLA and GLA-GS Following Oral Administration of Licorice Decoction

■, GLA; □, GLA-GS.

FIG. 6. Serum GLA and GLA-GS Levels during Pseudoaldosteronism (+), and after Recovery (-)

■, GLA; □, GLA-GS.

after the start of treatment, respectively, the patients showed signs of hypertention, hypokalemia, and edema in the legs. Serum GLA and GLA-GS levels at this time showed high GLA values (case 1: 94 ng/ml, case 2: 70 ng/ml) and very low GLA-GS values (case 1: 5 ng/ml, case 2: 3 ng/ml). The high GLA values noted here correspond to 1.5–2 times the maximum serum concentration of GLA in normal subjects who were given 5 g of licorice.

DISCUSSION

A pharmacokinetic study of glycyrrhizin has been reported by Nakano *et al.*,⁵⁾ but there are no reported investigations using many subjects over a 48 h period. Nakane *et al.*⁵⁾ administered glycyrrhizin intravenously, whereas in our study which was intended to investigate disposition of traditional Chinese formulations, we administered a decoction of licorice orally.

Recently, Kanaoka *et al.*⁶⁾ have reported on EIA technique for GLA, and have asserted that the technique is a suitable analytical tool for quantitative analysis of GLA in the presence of structural and functional analogs in biological fluids. In the present study, plain licorice was administered to determine the presence of any GLA analogs that cross-react with GLA-antibody. The result obtained (Table I) indicates two possibilities, one is that GLA-antibody is highly specific to GLA and second that GLA analogs occur in very low amounts in the serum. In this paper, therefore, until the presence of any cross-reactive analogs is clarified, the term of GLA is used as the putative representative of GLA-antibody reacting substances.

It was found in the present study that the appearance of GLA in the blood occurred a few hours earlier than that in the case of intravenous administration of glycyrrhizin.⁵⁾ A possible explanation for this is that glycyrrhizin contained in the decoction was hydrolyzed by gastric juice, intestinal bacteria, or other enzymatic process in the gastrointestinal wall, and subsequently absorbed in the form of monoglycone or aglycone. This hypothesis is supported by find-

ings in rats.⁷⁾

Nakano *et al.*⁵⁾ have reported that 1–2% of the glycyrrhizin which had been administered intravenously remained in the urinary excretion of GLA and GLA-GS. In the present study where a licorice decoction was orally administered, about 2% of GLA and GLA-GS was found to be excreted in the urine. One conceivable reason for the comparatively low rate of urinary excretion of GLA and GLA-GS is that GLA has a high ability to combine with serum albumin and that GLA is excreted at a high rate in the bile.

The patients who developed pseudoaldosteronism showed high GLA concentrations. The results agree with the hypothesis proposed by previous reports,^{2,8,9)} that GLA plays a main role in the inducement of pseudoaldosteronism. Proposed mechanisms for pseudoaldosteronism induced by GLA are: (1) GLA binds to aldosterone receptors,⁸⁾ (2) GLA potentiates aldosterone effect,⁹⁾ and (3) GLA inhibits aldosterone deactivating enzyme.²⁾ Regardless of the mechanisms, GLA appears to play a more important role in the onset of pseudoaldosteronism than GLA-GS.

From the above findings, it was suggested that the determination of serum GLA concentrations after 2 or 3 weeks of medication is useful to prevent serious pseudoaldosteronism which is induced by the use of formulations of traditional Chinese medicine containing licorice.

The present results also suggested, from a point of view of pharmacokinetics, that the medication of a formulation containing licorice may not be required three times a day, but once or twice a day. However, further investigations of this problem are necessary to resolve this point.

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REFERENCES

- 1) R. S. H. Finney and G. F. Somers: The anti-inflammatory activity of glycyrrhetic acid and derivatives, *J. Pharm. Pharmacol.*, **10**, 613–620 (1958).
- 2) Y. Tamura, T. Nishikawa, K. Yamada, M. Yamamoto and A. Kumagai: Effects of glycyrrhetic acid and its derivatives on Δ^4 -5 α - and 5 β -reductase in rat liver, *Arzneim.-Forsch/ Drug Res.*, **29**, 647–649 (1979).
- 3) H. Shio: A case of pseudoaldosteronism induced by glycyrrhizin and study of all cases in Japan, *Horumon to Rinsho*, **30**, 57–62 (1982).
- 4) R. Takeda, S. Morimoto, K. Uchida, T. Nakai, M. Miyamoto, T. Hashiba, K. Yoshimitsu, K. S. Kim and U. Miwa: Prolonged pseudoaldosteronism induced by glycyrrhizin, *Endocrinol. Jpn.*, **26**, 511–517 (1979).
- 5) N. Nakano, H. Katoh, H. Suzuki, K. Nakao, S. Yano and M. Kanaoka: Enzyme immunoassay of glycyrrhetic acid and glycyrrhizin, *Proc. Symp. WAKAN-YAKU*, **14**, 97–100 (1981).
- 6) K. Kanaoka, S. Yano, H. Katoh and N. Nakano: Glycyrrhetyl amino acids: synthesis and application to enzyme immunoassay for glycyrrhetic acid, *Chem. Pharm. Bull.*, **29**, 1533–1538 (1981).
- 7) K. Yasuda, T. Shibuya, M. Nozaki, K. Tsurumi, H. Fujimura and F. Kaneuchi: Simultaneous determination of glycyrrhizin and glycyrrhetic acid in plasma by high speed liquid chromatography, *Yakugaku Zasshi*, **98**, 1545–1547 (1978).
- 8) A. Ulmann, J. Menard and P. Corvol: Binding of glycyrrhetic acid to kidney mineralocorticoid and glucocorticoid receptors, *Endocrinology*, **97**, 46–51 (1975).
- 9) S. Ishikawa and T. Saito: The effect of glycyrrhetic acid on the action of aldosterone in stimulating sodium transport in frog skin, *Endocrino. Jpn.*, **17**, 697–701 (1980).