

Rapid Estimation of Glycyrrhizin and Glycyrrhetic Acid in Plasma by High-Speed Liquid Chromatography

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A method was established by which glycyrrhizin and glycyrrhetic acid present in plasma can be extracted with methanol and then separated and determined quantitatively within 10 min by means of high-speed liquid chromatography. Using this method, glycyrrhizin and glycyrrhetic acid added to the plasma were recovered to satisfactory extents. An *in situ* recirculating perfusion technique showed that G is absorbed in rat small intestine in an apparent first-order process. There was no detectable amount of glycyrrhetic acid in the blood after bolus injection of glycyrrhizin into the portal vein, although glycyrrhetic acid was present in a detectable amount in the blood after oral administration. Since it is water-soluble and has a high molecular weight, glycyrrhizin is probably absorbed in the small intestine in the form of glycyrrhetic acid. With the decline of glycyrrhetic acid in the blood, there was a rise in the blood level of a substance which exhibited the same chromatographic behavior as glycyrrhizin. This substance appears to be a glucuronic acid conjugate formed as a metabolite of glycyrrhetic acid, although it is not clear whether it is a mono- or diglucuronic acid conjugate or a mixture of the two. Glycyrrhizin injected into the portal vein was eliminated from the blood only slowly.

Keywords—high-speed liquid chromatography; glycyrrhizin; glycyrrhetic acid; plasma; intestinal absorption; portal vein

Glycyrrhiza or glycyrrhiza extract is contained as a constituent in a number of herbal medicines (*kanpo* preparations). The active ingredient of glycyrrhiza, glycyrrhizin (G), has been reported to have the adverse effect of producing pseudo-aldosteronism when given in massive doses, as described in a notification issued by the Ministry of Welfare in Feb. 1978. It is thus necessary to determine the amount of G contained in various preparations before using them in clinical practice. In a previous study²⁾ we established a simplified method for the determination of G in herbal preparations using the technique of high-speed liquid chromatography (HLC), which requires no more than a simple pretreatment of the sample to permit quantitative determination of the G content in preparations with complex compositions, such as herbal medicines. In the present study as a part of our program to elucidate the bioavailability and *in vivo* behavior of G, an attempt was made to assess the methods available for the separation and quantitative determination of G and its non-saccharide moiety glycyrrhetic acid (GA) to establish a method whereby G and GA in blood plasma and enteric perfusate can be separated and determined in a short period of time. Using the procedure thus established, the plasma level time courses of G and GA were investigated in rats.

Experimental

Materials—Before use in this study, G and Ga were recrystallized from acetic acid and chloroform-petroleum ether, respectively. These served as reference standards for G (mp 210° (dec.)) and GA (mp 296°). Sodium chloride, dibasic sodium phosphate and potassium biphosphate used were of reagent grade and were obtained from commercial sources.

1) Location: Yamashiro-cho, 770, Tokushima.

2) Y. Akada, Y. Sakiya, S. Kawano, and S. Adachi, *Chem. Pharm. Bull.* (Tokyo), 26, 1240 (1978).

Conditions of HLC—The apparatus used was a Du Point LC 830 unit with a Permaphase AAX column (1 m × 2.1 mm, i.d.), using a ultraviolet (UV) absorption photometer (wavelength 245 nm) as the detector. The mobile phase consisted of H₂O and 0.1 M NaClO₄ with a linear gradient of 7 per cent per min. The chromatography was carried out at an inlet pressure of 1000 psi, at 40° and at a flow rate of 0.8 ml per min. One to 5.0 μl of sample solution pretreated as described below for the plasma or the perfusing solution obtained from the experiments in rats was subjected to HLC for the determination of G and GA.

Method of Determination—G and GA were determined from a linear regression equation which was derived from the peak heights of the chromatograms by the method of least squares. To obtain a standard regression line, suitable amounts of G and GA were added to aliquots of 0.5 ml of the plasma or 1 ml of the perfusing solution and then treated by the procedure described above.

Pretreatment of Plasma and Perfusate—(1) One-half ml aliquots of plasma collected at different times in the experiments on rats were shaken for 30 min with 5 ml of methanol and then centrifuged for 30 min at 3000 rpm. The upper layer was filtered through an Amicon membrane filter CF-25. Methanol (5 ml) was added to the residue, and the mixture was treated by the procedure described above. The combined filtrate was evaporated down under reduced pressure. To the residue, 1.0 ml of methanol was added to obtain the sample.

(2) One ml aliquots of the perfusate collected at different times as described below were shaken for 30 min with 5.0 ml of H₂O, 2 g of NaCl, 0.2 ml of conc. HCl and 5.0 ml of ethyl acetate and then centrifuged for 5 min at 3000 rpm. Exactly 4.5 ml of the upper layer was evaporated down under reduced pressure, and 1.0 ml of methanol was added to the residue to obtain the sample.

Animals—Male Wistar rats, weighing 250–260 g, were fasted for about 24 hr prior to the experiments, but water was allowed *ad libitum*.

Preparation of G Solution—G was dissolved in isotonic phosphate buffer (pH 6.4, prepared with 0.123 M Na₂HPO₄ and 0.123 M KH₂PO₄) to obtain perfusing solution containing 16.8 mg or 48 mg of G per 60 ml. For the bolus injection, G was dissolved in water to obtain a solution containing 0.5 g or 1.25 g of G per 50 ml. The latter solution was also used in the experiments on oral administration.

Experimental Procedure in Rats—(1) An *in situ* recirculating method based on that of Schanker *et al.*³⁾ was used with suitable modifications. Animals were anesthetized with ether and maintained under anesthesia for the entire course of the experiment. The animals were laparotomized by a midline incision to expose the small intestine, which was then cannulated with polyvinyl tubing at the proximal end of the duodenum and the distal end of the ileum. After closure of the incision, the tubing was connected to the inflow and outflow cannula, which were placed in a flask containing approximately 100 ml of physiological saline solution at 37°. The small intestine was washed with the saline solution using a perfusion apparatus (type SJ-1210, Mitsumi Science Industries Inc.), with a flow rate from the duodenum to the ileum of 4 ml per min, then the intestinal tract was perfused with 70 ml of the G solution at 37° at a rate of 2.3 ml per min. Two ml samples of the solution were taken from the flask at zero time and four times at intervals of 30 min thereafter. Zero time was defined as the time at which the initial 10 ml of the solution was removed. Phenol red^{4,5)} the intestinal absorption of which may be considered negligible, was dissolved in the G solution as a volume indicator. The G solution in the flask was agitated with a magnetic stirrer throughout the experiment. To 1 ml of each sample, 3 ml of 1 N NaOH was added in order to measure the absorbance of phenol red colorimetrically at 560 nm. The other 1 ml of the sample was subjected to G determination.

(2) A half ml of G solution was administered by bolus injection into the portal vein. Blood samples of about 1 ml were taken through the femoral vein cannula at 0, 10, 30, 60, 90, 120 and 180 min after the injection. Light ether anesthesia was used for the operation.

(3) G solution (5 ml/250 g body weight) was administered orally. Using the method described above, blood samples of about 1 ml were taken at 10, 30, 60, 120, 180 and 240 min after the oral administration. Blood samples collected at different times were centrifuged for 30 min at 3000 rpm and the plasma obtained was subjected to G and GA determination.

Results and Discussion

Evaluation of the HLC Conditions

Since a carboxyl group is present in the chemical structures of both G and GA, the strongly basic ion exchange resin Permaphase AAX was used in this study. Various systems were evaluated as the mobile phase in an attempt to achieve satisfactory separation of G and

3) L.S. Schanker, D.J. Tocco, B.B. Brodie, and C.A.M. Hogben, *J. Pharmacol. Exp. Ther.*, **123**, 81 (1958).

4) K. Kakemi, T. Arita, S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **13**, 861 (1965).

5) K. Kakemi, T. Arita, R. Konishi, *Chem. Pharm. Bull.* (Tokyo), **15**, 1534 (1967).

GA in plasma, which contains a variety of proteins. As a result, a linear gradient system of water and 0.1 M NaClO₄ was found to be best. The optimum addition rate of NaClO₄ was evaluated as 3–10 per cent per min. A lower addition rate of NaClO₄ in this range gives more satisfactory separation of the peaks for G and GA. Conversely, the peak width becomes narrower with increase of the addition rate. In this study, an addition rate of 7 per cent per min was employed for the mobile phase to ensure a relatively narrow peak as well as complete separation of G and GA. As the maximum UV absorption wavelength is within the range of 250–260 nm for both G and GA, these substances could be detected satisfactorily at the standard wavelength, of 254 nm.

Accuracy of Determination of G and GA in Plasma

Methanol, ethanol and ethyl acetate were examined as solvents for the extraction. Methanol gave the best results as regards accuracy, and so was employed in this study. Definite amounts of G and GA were added to 0.5 ml of plasma, and treated by the pretreatment method described above to estimate the accuracy of determination of G and GA. G and GA were determined from a linear regression derived by the method described above. A chromatogram thus obtained is shown in Fig. 1, and the accuracy of measurement is shown in Table I. As can be seen from Fig. 1, G and GA were completely separated from one another, both exhibiting sharp peaks, within approximately 10 min. Table I shows that the yield was satisfactory for both G and GA, with a good mean recovery.

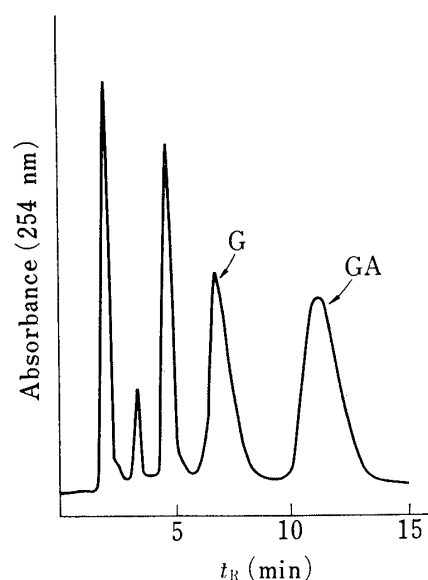


Fig. 1. Chromatogram of G^{a)} and GA^{b)} in Rat Plasma by HLC^{c)} with a Permaphase AAX Column Eluted with a Gradient from H₂O to 0.1 M NaClO₄ at 7%/min

- a) glycyrrhizin.
- b) glycyrrhetic acid.
- c) high-speed liquid chromatography.

TABLE I. The Recoveries of G and GA Added to Rat Plasma^{a)}

Amount of sample added (μ g)		Amount of sample found (μ g)		Recovery (per cent) of sample		
G	GA	G	GA	G	GA	
1.260	0.870	1.256	0.890	99.68	102.30	
1.260	0.870	1.264	0.851	100.32	97.82	
1.260	0.870	1.286	0.860	102.06	98.85	
1.260	0.870	1.237	0.862	98.17	99.08	
0.867	0.533	0.857	0.545	98.85	102.25	
0.867	0.533	0.850	0.543	98.04	101.88	
0.867	0.533	0.848	0.522	97.81	97.94	
				Mean	99.28	100.02

a) G and GA were added to the plasma, which was given standard pretreatment, and the recoveries of these two substances were determined from a linear regression equation. Other details were as in Fig. 1.

Accuracy of Determination of G in the Perfusate

A definite amount of G was added to 1 ml of a buffer solution with the same composition as the enteric perfusate, salted out with NaCl, made acidic with HCl and extracted with ethyl acetate, tetrahydrofuran, butanol or chloroform. Of these solvents, ethyl acetate was

most effective in extracting G. The chromatogram of an extract with this solvent showed no peak that impeded the determination of G. The accuracy of measurement was also satisfactory with this solvent, as in the plasma experiments.

Absorption of G from the Small Intestinal Perfusate

Table II shows the cumulative absorption (per cent) of G from rat small intestine as determined by the *in situ* recirculating perfusion method. The volume of a perfusate will

TABLE II. Cumulative Absorption (Per Cent) of G from Rat Intestine as Determined by the *in Situ* Recirculating Perfusion Technique

Time (min)	Cumulative absorption (per cent)	
	Mean ^{a)}	Mean ^{b)}
30	5.25	4.33
60	7.30	10.58
90	13.70	13.53
120	20.45	19.53

a) The mean of two experiments; perfusate containing 16.8 mg/60 ml of G was used.

b) The mean of four experiments; perfusate containing 48 mg/60 ml of G was used. Other details were as in Fig. 1.

vary as water flows from the small intestine into the blood (positive water flux) or from the blood into the small intestine (negative water flux). Positive water flux invariably occurred in the perfusate used in these experiments, which was a buffer solution isotonic with the body fluids. Phenol red, which is hardly absorbed in the small intestine, was therefore used as a volume indicator in determining the amount of G in samples of the perfusate. The value thus obtained was corrected using the following equation:

$$\text{corrected value of G} = G_t \times A_0/A_t$$

Where G_t denotes the measured value for G in the sample at time t ; and A_0 and A_t the absorbances of phenol red at time 0 and time t , respectively. The corrected value of G was used to calculate the per cent cumulative absorption (Table II). The cumulative absorption of G was essentially the same, regardless of its concentration in the perfusate, being approximately 20 per cent at 120 min. It follows that the amount of G absorbed up to that time was approximately 9.40 mg at the higher concentration and about 3.44 mg at the lower concentration of the substance in the perfusate, which suggests that the rate of absorption may be first-order in the small intestine.

Plasma Concentrations G and GA after Oral Administration of G

Figure 2 shows the mean concentration of G in the blood plasma attained after oral administration. The plasma GA level reached a peak at 30 min, then declined. The plasma level of G was lower than of GA but increased as the plasma GA level was lowered. This suggests that G may be absorbed in the form of GA. The fact that no GA was detected in the plasma following bolus injection of G into the portal vein (Fig. 3) precludes the possibility of absorbed G being converted to GA in the body. It may be, on the other hand, that G is also partly absorbed though at a slower rate than GA. It seems likely that G is not readily absorbed, since it is soluble in water and probably has a molecular weight as large as 823, although this remains to be confirmed. Williams *et al.*^{6,7)} reported that absorbed GA is totally excreted in bile in the form of its metabolites and that about 90–100 per cent of the dose was excreted in 24 hrs. Absorbed GA forms a glucuronic acid conjugate in the

6) D.V. Parke, S. Pollock, and R.T. Williams, *J. Pharm. Pharmacol.*, **15**, 500 (1963).

7) R.T. Williams, P. Millburn, and R.L. Smith, *Ann. N.Y. Acad. Sci.*, **123**, 110 (1965).

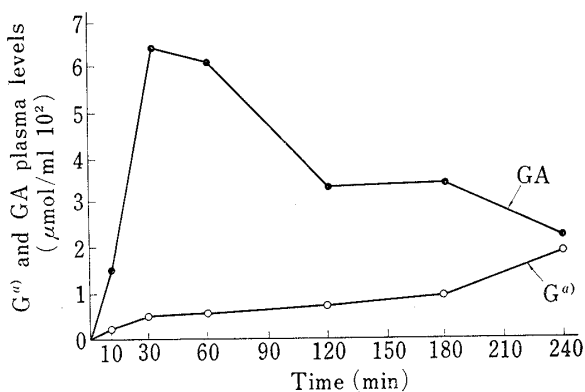


Fig. 2. Mean Plasma Levels of G^{a)} and GA after Oral Administration of G

G (125 mg/5 ml/250 g body weight) was administered orally to rats ($n=4$). Open and closed symbols are mean data.

a) G and its metabolites showed the same behavior the legend to on the chromatogram. For other details, see the legend to Fig. 1.

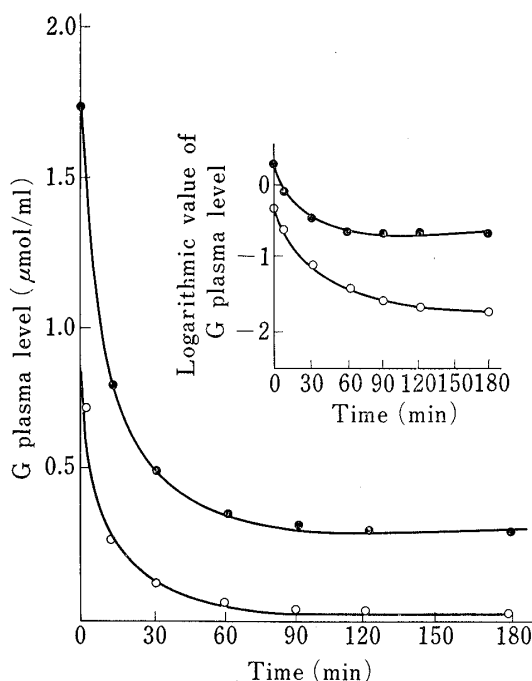


Fig. 3. Time Course of G Plasma Level after Bolus Injection of G into the Portal Vein

Closed and open circles are mean data ($n=4$) after bolus injection of 12.5 mg/0.5 ml and 5 mg/0.5 ml of G, respectively.

The inset shows the same data. Other details were as in Fig. 1.

liver and displays the same chromatographic behavior as G. For this reason, this metabolite is included in estimates of G in this study and is also shown as such in Fig. 2. It is not known whether GA forms a mono or diglucuronic acid conjugate or a mixture of the two. As shown in Fig. 3, the plasma level of G fell rapidly within 30 min after bolus injection into the portal vein but at 60 min or later it fell only slightly further. This suggests that G is distributed in the tissue then eliminated from the blood and excreted only slowly. Figure 3 also shows a logarithmic plot of the time course of the blood concentration of G, which indicates that the mode of elimination of G from the blood cannot be expressed in terms of two exponential processes. Further kinetic studies will be needed to establish the behavior of G in the body using our established method for the separation and quantitative determination of G and GA in the blood.