[Chem. Pharm. Bull. 32(9)3734—3738(1984)]

High-Performance Liquid Chromatographic Determination of Glycyrrhizin and Glycyrrhetinic Acid in Biological Materials

TSUTOMU ICHIKAWA, SHIRO ISHIDA, YOKO SAKIYA,* and YOSHINOBU AKADA

Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima-shi 770, Japan

(Received February 10, 1984)

High-performance liquid chromatographic methods were developed for the determination of glycyrrhizin (G) and glycyrrhetinic acid (GA) in blood, plasma, bile, urine, and visceral tissues of rats. The detection limit for G and GA was 0.125 μg/ml or /g wet weight. The precision and sensitivity of the assay appear to be satisfactory for determination of the levels of these compounds in biological materials. These determination methods were developed to study plasma disposition, tissue distribution, and biliary and urinary excretions of G and GA after a bolus *i.v.* administration of G or GA to rats. Plasma disposition followed a two-compartment model for both G and GA with β-elimination half-lives of approximately 50 and 80 min, respectively. The cumulative amounts of G and GA excreted in bile during 24 h after an *i.v.* dose were 88.5 and 0.36% of the dose, respectively, and those excreted in urine were, respectively, only 4.8 and 0.03% of the dose. These compounds were distributed in visceral tissues at lower concentrations than in blood.

Keywords——glycyrrhizin; glycyrrhetinic acid; determination; high-performance liquid chromatography; rat; bio-materials; disposition; excretion

The active ingredients of glycyrrhiza, glycyrrhizin (G) and its aglycone, glycyrrhetinic acid (GA), are contained in a great number of commercial preparations. There are a number of reports[1-5] on quantitative determination of G content in glycyrrhiza, glycyrrhiza powder, and glycyrrhiza extract. However, there are few valid methods available at present for the quantitative determinations of G and GA in biological materials, *e.g.*, visceral tissues, bile, and urine. In a previous report[6] we presented a high-performance liquid chromatographic (HPLC) determination method for G and GA in rat plasma using a strongly basic ion exchange resin (Permaphase AAX) column and a linear gradient system of water and 0.1 M $NaClO_4$ solution as a mobile phase. However, in this method, G and GA in visceral tissues were not well separated from biological constituents. In this study, we present quantitative determination methods for G and GA in blood, plasma, bile, urine, and visceral tissues of rats by HPLC with an adsorption chromatographic column. These determination methods were applied to study plasma disposition, tissue distribution, and biliary and urinary excretions of G and GA after an *i.v.* dose in rats.

Experimental

Materials——G and GA reagents were purchased from Tokyo Kasei Co., Ltd. MeOH and CH_3CN were of liquid chromatographic reagent grade (Wako Pure Chemicals Co.). AcOEt and $CHCl_3$ were distilled before use. All other reagents were commercial products of analytical grade.

HPLC Conditions——The apparatus used was a Hitachi liquid chromatograph, model 655, with a Hitachi gel

3011 column (250 × 4 mm i.d.) and a ultraviolet (UV) detector operating at 252 nm. The mobile phases consisted of (1) 0.75% NH_4ClO_4–1% CH_3CN in 85% (v/v) MeOH–water solution for G in plasma, (2) 0.25% NH_4ClO_4–3% CH_3CN in 82.5% (v/v) MeOH–water solution for G in blood, bile, urine, and visceral tissues, (3) 0.5% NH_4ClO_4–10% CH_3CN in 90% (v/v) MeOH–water solution for GA in blood, plasma, bile, and urine, and (4) 1% NH_4ClO_4–0.025% NH_3 in 90% (v/v) MeOH–water solution for GA in visceral tissues. The column was maintained at room temperature and the mobile phase flow rate was 1.0 ml/min. For quantitative calculations, a reporting integrator (Reporting Integrator 3390A, Yokogawa-Hewlett Packard Co.) was employed.

Animal Experiments——Adult male Wistar rats weighing 250 ± 10 g were used. Under light ether anesthesia, the femoral artery, bile fistula, and urinary bladder were each cannulated with polyethylene tubing. After the cannulation, a bolus injection of G (100 mg/kg) or GA (60 mg/kg) was made into the femoral vein. (1) Blood samples were drawn through the cannula at appropriate time intervals and then were centrifuged to separate the plasma. (2) Bile and urine samples were collected through the cannulas during 24 h after administration of G (100 mg/kg) or GA (60 mg/kg) to the cannulated rats. (3) At 1 h after the *i.v.* dose of G (100 mg/kg) or GA (60 mg/kg), the cannulated rat was sacrificed by an injection of saturated KCl solution into the carotid artery. The bled visceral tissues were frozen until analysis. Each tissue was homogenized with two volumes of physiological saline in an ice bath. (4) Blood, plasma, bile, urine, and visceral tissue samples were prepared by the same method as described above, except for the administration of G and GA, and known amounts of G or GA (200.0, 150.0, 100.0, 25.0, 10.0, 5.0, 1.0, 0.5, and 0.0125 μg/ml or /g of wet weight) were added to each sample. Visceral tissues were homogenized after the addition of G or GA.

Sample Analysis——Samples of 300 μl for bile, urine, and homogenate, and 100 μl for blood and plasma were used for the determination of G and GA. (1) Extraction of G. A sample was shaken vigorously with 5 ml of MeOH for 20 min and then was centrifuged for 20 min at 15000 rpm. This extraction was repeated twice, and the combined MeOH extracts containing G were evaporated to dryness at 40 °C, transferred to a 10-ml test tube by washing with MeOH, and dried under a nitrogen stream. The residue was dissolved in 200 μl of MeOH, and 5—10 μl of this solution was injected into the liquid chromatograph. (2) Extraction of GA. Five ml of saturated $(NH_4)_2CO_3$ solution and 10 ml of $CHCl_3$ were added to a sample, and 1 ml of 5% CCl_3COOH solution was added in the case of samples other than bile and urine. The sample solution was shaken vigorously for 20 min and then centrifuged for 20 min at 3000 rpm. This extraction was repeated twice, and the combined $CHCl_3$ extracts containing GA were dried by the same method as described above. The residue was dissolved in 200 μl of MeOH, and 5—10 μl of this solution was injected into the liquid chromatograph.

Results and Discussion

G and GA purified from commercial reagents were used in this study because these reagents contain unknown impurities. Purified G and GA showed mp 209—211 °C (dec.), $[\alpha]_D^{20}$ +49.5° (c=1.5, ethanol), mp 295—297 °C and $[\alpha]_D^{20}$ +166° (c=0.1, chloroform), respectively. These values were similar to the literature values,[7] *i.e.*, G: mp 212—217 °C (dec.), $[\alpha]_D^{20}$ +46.9° (c=1.5, ethanol), GA: mp 296 °C, $[\alpha]_D^{20}$ +163° (c=0.1, chloroform). Since different biological constituents exist among blood, bile, urine, and visceral tissues, various HPLC conditions, *e.g.*, an adsorption or ion exchange column and various mobile phase systems, were evaluated in an attempt to achieve satisfactory separations of G and GA in each bio-materials, and the HPLC conditions described here were found to be best. Known amounts of G or GA were added to the samples of blood, plasma, bile, urine, and visceral tissues, and were analyzed. Figure 1 shows the HPLC separations of these compounds extracted from plasma and liver. The retention times of G and GA were less than 20 min, and both compounds were well separated from biological constituents in all cases. As shown in Table I, the recoveries of G and GA from each bio-material were quantitative, and the detection limit for both compounds was 0.125 μg/ml or /g wet weight. From these results, the precision and sensitivity of the assay appeared to be satisfactory for determination of the levels of the compounds in bio-materials. Accordingly, these determination methods were applied to study plasma disposition, tissue distribution, and billiary and urinary excretions of G and GA after *i.v.* administration in rats. Figure 2 shows the time courses of G and GA plasma concentrations following a bolus *i.v.* administration of G or GA to rats with bile fistula and urinary bladder cannulations. Plasma G and GA concentrations declined bi-exponentially, indicating a two-compartment disposition. Pharmacokinetic parameters (A, α,

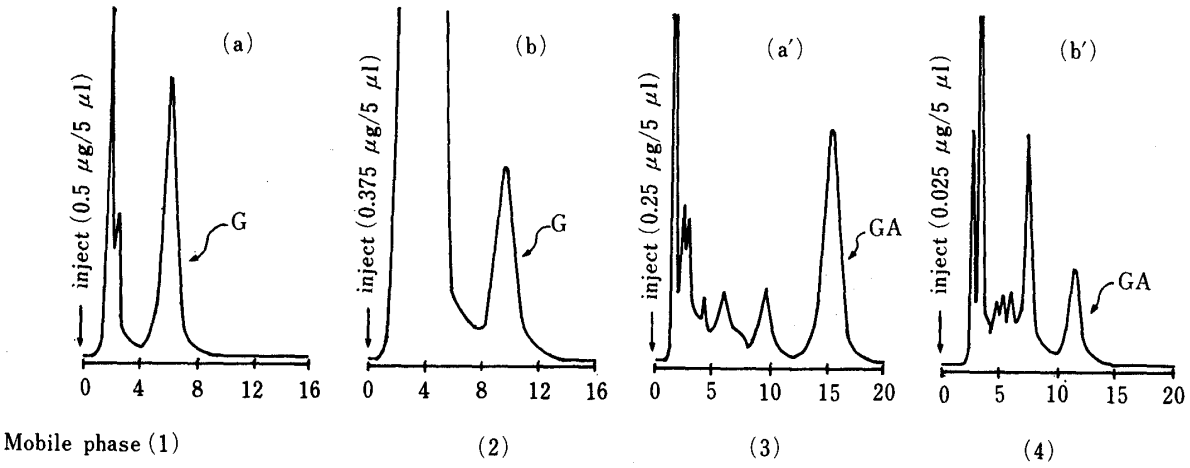

Fig. 1. High-Performance Liquid Chromatograms Showing the Separation of Glycyrrhizin and Glycyrrhetinic Acid Extracted from Plasma and Liver of Rats

Apparatus: Hitachi HLC 655.

Conditions: column, Hitachi gel 3011 (250 × 4 mm i.d.); column temp., room temp.; flow rate, 1.0 ml/min; detector, UV 252 nm; mobile phase, (1) 0.75% NH_4ClO_4–1% CH_3CN in 85% (v/v) MeOH–water solution, (2) 0.25% NH_4ClO_4–3% CH_3CN in 82.5% (v/v) MeOH–water solution, (3) 0.5% NH_4ClO_4–10% CH_3CN in 90% (v/v) MeOH–water solution, (4) 1% NH_4ClO_4–0.025% NH_3 in 90% (v/v) MeOH–water solution.

(a) and (a'): plasma. (b) and (b'): liver homogenate. G: glycyrrhizin. GA: glycyrrhetinic acid. G and GA were extracted from each sample after addition of (a): 20 μg/100 μl, (b): 15 μg/300 μl, (a'): 10 μg/100 μl, and (b'): 1 μg/300 μl, respectively. For the preparation of plasma and liver homogenate samples, see the text.

TABLE I. Recoveries of Glycyrrhizin and Glycyrrhetinic Acid from Biological Materials

Tissue	Recovery,[a] %	
	Glycyrrhizin	Glycyrrhetinic acid
Saline	100.7±1.5	100.5±1.2
Blood	105.5±0.4	95.5±5.5
Plasma	100.2±0.5	104.4±3.7
Lung	100.7±0.8	101.5±0.3
Brain	99.4±0.6	108.7±1.3
Heart	100.2±0.5	102.4±0.9
Liver	104.3±2.7	101.9±0.6
Pancreas	101.4±0.7	103.8±4.9
Spleen	99.7±1.0	102.8±0.8
Kidney	98.0±1.1	101.7±0.4
Muscle	100.2±1.3	100.6±1.5
Skin	101.9±1.6	105.9±0.2
Adipose	100.0±0.3	98.1±2.2
Stomach	102.1±0.3	102.5±3.9
Small intestine	104.0±1.5	102.6±0.6
Bile	98.5±4.8	100.7±0.9
Urine	99.7±1.0	100.3±0.8

a) Each value represents the mean±S.E. of nine determinations for the particular amount added. Amounts added were 200.0, 150.0, 100.0, 25.0, 10.0, 5.0, 1.0, 0.5, and 0.125 μg/ml or /g wet weight. The reproducibilities (mean±S.E.) for G and GA were 101.5—98.9±1.5—2.1% and 100.7—99.4±1.2—2.0%, respectively. For other details, see the legend to Fig. 1.

B, β) were computed by a non-linear itrative least-squares method,[8] and are listed in Table II. Elimination half-life ($t_{0.5}^{\beta}$) and distribution volume ($V_{d\beta}$) were calculated from these parameters (Table II). The elimination half-lives were approximately 50 and 80 min in G and GA,

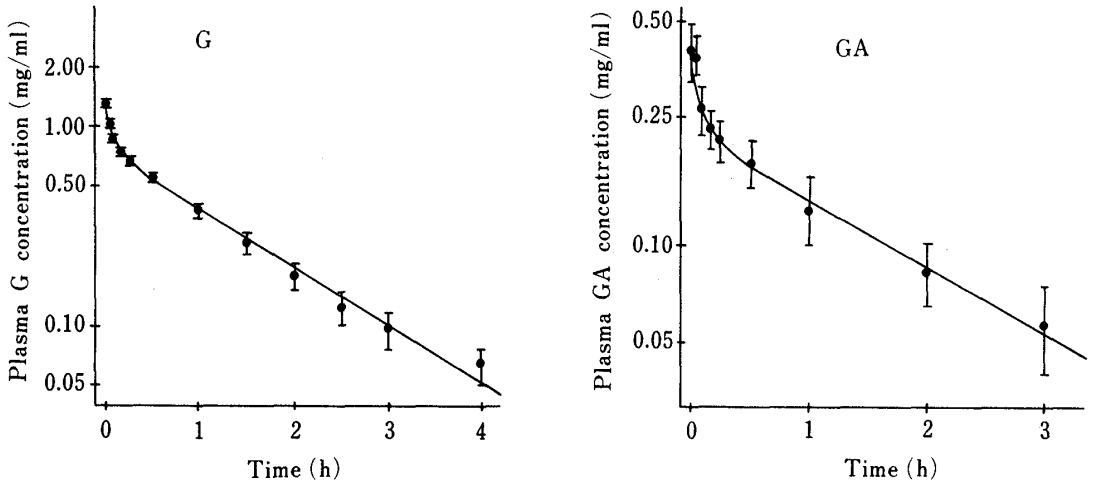

Fig. 2. Plasma Disappearance Curves of Glycyrrhizin (G) and Glycyrrhetinic Acid (GA) after Bolus *i.v.* Administration of G (100 mg/kg) or GA (60 mg/kg) to Rats with Bile Fistula and Urinary Bladder Cannulations

Each point and vertical bar represent mean ± S.E. for five rats. Curves were calculated by the least-squares method using a digital computer (see the text).

TABLE II. Pharmacokinetic Parameters of Glycyrrhizin (G) and Glycyrrhetinic Acid (GA) in Rats with Bile Fistula and Urinary Bladder Cannulations

Parameter	Dose	
	G, 100 mg/kg Mean ± S.E.	GA, 60 mg/kg Mean ± S.E.
A, mg/ml	0.82 0.14	0.17 0.05
α, min^{-1}	0.42 0.05	0.24 0.06
B, mg/ml	0.83 0.03	0.24 0.04
$\beta \times 10^2$, min^{-1}	1.34 0.11	0.90 0.11
$t^{\beta}_{0.5}$, min^{-1}	54.31 4.85	82.20 9.80
$V_{d\beta}$, ml	29.44 0.91	67.66 10.55

Each value was obtained from five rats. $t^{\beta}_{0.5} = 0.693/\beta$, $V_{d\beta} = [\text{dose}/(A/\alpha + B/\beta)]/\beta$.

respectively. A comparatively small distribution volume was seen for both compounds. Table III shows the concentrations of G and GA in blood, plasma, and each tissue in the terminal phases of G and GA at 1 h after an *i.v.* dose to the cannulated rats. G and GA concentrations in each tissue were lower than those in blood, indicating that the distribution volume is small for both compounds. This is supported by the $V_{d\beta}$ values of G and GA shown in Table II. G concentration in the liver was highest among the tissues and organs, while low values were found in the pancreas and small intestine, and a negligible amount in the brain. On the other hand, GA was found in the brain, but at low concentration, High GA concentrations were seen in the lung, liver, heart, skin, and kidney. As shown in Table IV, the cumulative amounts of G and GA excreted in bile during 24 h after *i.v.* administration to cannulated rats were 88.5 and 0.36%, respectively, of the doses, while those excreted in urine were only 4.8 and 0.03%, respectively, of the doses. The results obtained from *in vivo* studies in rats suggest that G and GA are largely bound to plasma protein, an enterohepatic circulation is seen in the case of G, and almost all of the GA is metabolized. The determination methods for G and GA presented here should be useful for further studies of these problems.

TABLE III. Glycyrrhizin (G) and Glycyrrhetinic Acid (GA) Concentrations in Tissues at the Terminal Phase (60 minutes) after *i.v.* Administration of G (100 mg/kg) or GA (60 mg/kg) in Rats with Bile Fistula and Urinary Bladder Cannulations

Tissue	G conc. μg/ml Mean±S.E.	GA conc. μg/ml Mean±S.E.
Lung	26.80 2.70	20.61 1.53
Brain	0.00	3.66 0.35
Heart	13.65 1.51	11.05 1.18
Liver	131.07 4.75	13.33 0.84
Pancreas	2.50 0.52	0.69 0.42
Spleen	12.63 3.65	6.88 1.35
Stomach	16.73 2.39	5.72 0.76
Small intestine	6.23 0.79	5.78 0.45
Kidney	59.45 6.63	11.75 1.98
Muscle	12.87 0.53	9.61 0.75
Skin	35.97 5.36	14.82 0.74
Adipose	15.08 3.07	4.27 0.39
Plasma	416.23 20.61	92.43 6.07
Blood	205.69 9.47	40.06 6.27

Each value was obtained from five rats.

TABLE IV. Cumulative Amounts of Glycyrrhizin (G) and Glycyrrhetinic Acid (GA) Excreted in Bile and Urine during 24 h after *i.v.* Administration of G (100 mg/kg) or GA (60 mg/kg) to Rats

	Cumulative amount, % per dose	
	Bile	Urine
G	88.53±4.54	4.83±2.12
GA	0.36±0.07	0.39±0.07

Each value represents the mean±S.E. of five rats.

References

1) G. Kurono and M. Sasaki, *Yakugaku Zasshi*, **90**, 497 (1970).
2) S. Ogawa, A. Yoshida, and Y. Mitani, *Yakugaku Zasshi*, **96**, 1488 (1976).
3) K. Yasuda, T. Shibuya, M. Nozaki, K. Tsurumi, H. Fujita, and F. Kaneuchi, *Yakugaku Zasshi*, **98**, 1545 (1978).
4) Y. Kitada, K. Tamase, M. Sasaki, and Y. Nishikawa, *J. Food Hyg. Soc. Jpn.*, **21**, 354 (1980).
5) Y. Akada, Y. Sakiya, S. Kawano, and S. Adachi, *Chem. Pharm. Bull.*, **26**, 1240 (1978).
6) Y. Sakiya, Y. Akada, S. Kawano, and Y. Miyauchi, *Chem. Pharm. Bull.*, **27**, 1125 (1979).
7) The Merck Index, 9th edition.
8) T. Nakagawa, Y. Koyanagi, and H. Togawa, *University of Tokyo Computer Center, Center News*, **11**(4), 70 (1979).