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Selective high-performance liquid chromatographic method for the determination of glycyrrhizin and glycyrrhetic acid-3-O-glucuronide in biological fluids: application of ion-pair extraction and fluorescence labelling agent

YOSHIKAZU YAMAMURA*, JUNICHI KAWAKAMI, TOMOFUMI SANTA, HAJIME KOTAKI, KATSUYOSHI UCHINO, YASUFUMI SAWADA and TATSUJI IGA

Department of Pharmacy, The University of Tokyo Hospital, Faculty of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113 (Japan)

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ABSTRACT

A selective high-performance liquid chromatographic method has been developed for the simultaneous determination of glycyrrhizin and glycyrrhetic acid-3-O-glucuronide in biological fluids of the rat. The procedure is based on the ion-pair formation using tetra-*n*-amylammonium bromide, extraction with ethyl acetate–*n*-heptane from the salt-saturated aqueous phase, labelling with 4-bromomethyl-7-methoxycoumarin, followed by chromatographic separation with fluorescence detection. Glycyrrhizin in plasma, bile and urine could be precisely determined in concentrations as low as 1, 1 and 2.5 μg/ml, respectively, in a 0.1-ml sample. The equivalent values for the glucuronide were 1, 2.5 and 2.5 μg/ml, respectively. The method is applicable in pharmacokinetic studies of glycyrrhizin in small animals.

INTRODUCTION

Glycyrrhizin (G, Fig. 1), the glycoside of glycyrrhetic acid (GA), is a natural compound extracted from the roots of *Glycyrrhiza glabra* L. G is widely used for

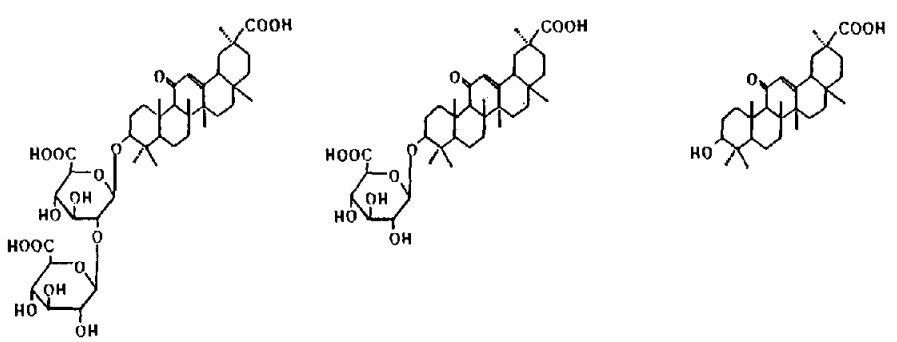

Fig. 1. Structures of glycyrrhizin (G), glycyrrhetic acid-3-O-glucuronide (GA-G) and glycyrrhetic acid (GA).

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the treatment of patients with chronic hepatitis [1], allergic disorder [2] and inflammation [3]. This drug is metabolized to GA [4,5], which is also used to combat inflammation [6], and it has been suggested that GA might be metabolized in part to glycyrrhetic acid-3-O-glucuronide (GA-G, Fig. 1) [7]. Although several studies of the disposition of G involved GA in rats and human have been reported [8–11], it seems that the results are not in agreement. This may be due to differences in analytical methods.

Analytical methods [8–15] for the determination of G in biological samples involve enzyme immunoassay, gas chromatography–mass spectrometry (GC–MS) and high-performance liquid chromatography (HPLC). Although the enzyme immunoassay [8,12] and GC–MS [9] methods were based on the formation of GA by solvolysis with hydrochloric acid, the solvolysis may be incomplete. HPLC methods [10,11,13–15] using UV detection were based on deproteinization with methanol [10,13,14] or with ethanol–ammonium hydroxide [11], and based on the solid-phase extraction using an ODS extraction column [15]. However, it was claimed that G was not extracted in the ethanol phase by deproteinization with that solvent, and also the recovery of G from the Sep-Pak C_{18} column was only 13% [9]. An HPLC method using the ion-pair technique [16] has been reported for the determination of G in *Glycyrrhizin radix*, but this method was applied only to plant products containing G.

In this study, we developed a selective HPLC method for the analysis of G in biological fluids. This method is based on ion-pair extraction and labelling with a fluorescent agent, 4-bromomethyl-7-methoxycoumarin (BrMMC), which is highly reactive towards fatty acids and several organic acids [17]. In addition, in order to examine quantitatively whether GA-G was formed *in vivo* after administration of G or GA to animals, we developed the method for the analysis of GA-G in biological samples.

EXPERIMENTAL

Materials

G and GA used in this study were plant test grade from Nacalai Tesque (Kyoto, Japan). GA-G was synthesized according to the method of Hirooka *et al.* [18], based on the coupling of methyl glycyrrhetate with per-O-acetylated glycosyl bromide of monosaccharide by means of Koenigs–Knorr type condensation followed by hydrolysis. White crystals were obtained with a melting point of 243–244°C. BrMMC was obtained from Wako (Tokyo, Japan). Capric acid as an internal standard for G and GA-G assay was obtained from Tokyo Kasei (Tokyo, Japan). Tetra-*n*-amylammonium bromide (TAA) was obtained from Wako, and tetrabutylammonium bromide (TBA) and tetraheptylammonium bromide (THA) were from Aldrich (Milwaukee, WI, U.S.A.). Glycyrrhetic acid methyl ester (GA-Me), as an internal standard for GA assay, was synthesized by methyl esterification according to the method of Hashimoto *et al.* [19]. All other solvents and reagents used were of reagent grade.

Analytical procedures of G and GA-G

Ion-pair formation and extraction. To 0.1 ml of plasma, bile or urine in a 10-ml glass-stoppered centrifuge tube, were added 0.6 ml of 1 *M* carbonate buffer (pH 10.0) and 1.5 ml of 0.1 *M* TAA as a counter-ion. The aqueous phase was then washed once with 6 ml of benzene (shaken for 10 min). After centrifugation at 1680 *g* for 5 min, 2 ml of the lower aqueous phase were transferred to another tube. To the aqueous phase was added 0.5 g of sodium sulphate (anhydrous), then the sodium sulphate was dissolved by placing the mixture in a water-bath at 50°C. After cooling, 5 ml of ethyl acetate–*n*-heptane (5:1, v/v) were added, and the mixture was shaken for 10 min, then centrifuged at 1680 *g* for 5 min. A 4-ml volume of the upper organic phase was transferred to another tube, and 100 μl of acetone containing 2.8 ng/ml capric acid (the internal standard) was added. The mixture was then evaporated to dryness with a rotary vacuum evaporator at 40°C. The resultant residue was assayed by the procedure described below.

Fluorescent labelling. To the dried residue were added 0.8 ml of acetone containing 2 mg/ml BrMMC as the labelling agent and 0.8 ml of the same solvent containing 30 mg/ml triethylamine as a catalyst. The mixture was allowed to stand for 30 min in a water-bath at 65°C. After reaction, the mixture was evaporated to dryness at room temperature as described above. The residue was dissolved in 100 μl of methanol, then an aliquot (10–20 μl) was injected into the HPLC column.

Apparatus and chromatographic conditions for the assay of G and GA-G

The HPLC apparatus used consisted of a Model 655 liquid chromatograph (Hitachi, Japan) and a Model 650 spectrofluorophotometer. The excitation and emission wavelengths of the detection were set at 335 and 395 nm with a window of 5 and 7 nm, respectively. The column for separation was a stainless-steel tube (150 mm × 6 mm I.D.) packed with Senshu Gel $5C_{18}$ (ODS, 5-μm particles, Senshukagaku, Tokyo, Japan) using a column-packing apparatus (Senshukagaku). The column temperature was maintained at 40°C for the plasma and bile sample analyses, and at 45°C for the urine sample analysis by a column jacket connected to the water-bath. The mobile phase was acetonitrile–0.2% acetic acid solution (65:35, v/v), degassed by sonication before use. The mobile phase was used at a flow-rate of 1.2 ml/min for the plasma and bile sample analyses, and at 0.9 ml/min for the urine sample analysis.

Fast-atom bombardment (FAB) mass spectra of methoxycoumarin (MMC) derivatives of G and GA-G were obtained on a mass spectrometer (Dx303, JEOL, Tokyo, Japan) operated at an ion accelerating voltage of 3 kV. The sample was dissolved in glycerin. FAB ionization was performed with a xenon atom beam.

Calibration curves

G (10 mg) and GA-G (10 mg) were dissolved in water (25 ml) in a glass volumetric flask. Subsequent dilution (1:7) with the same solvent gave a solution

with a concentration of 50 μg/ml. Adequate volumes of this solution were used to make further solutions containing 1, 2.5, 5, 10 and 50 μg/ml each of G and GA-G. To 0.1 ml of each solution was added 0.1 ml of drug-free rat plasma, urine or bile. Calibration curves for G and GA-G were generated by least-squares regression of the peak-height ratio (analyte/internal standard) *versus* a known amount.

Reproducibility

Plasma, urine or bile samples containing G and GA-G of 0.1 ml were prepared and assayed as described above. Reproducibility was determined by repeating the procedure three times for each sample and calculating the standard deviation.

Analytical procedure for GA

Determination of the GA concentration in biological samples was performed by a modification of the method of Sakiya and co-workers [10,13]. GA-Me, the methyl ester derivative of GA, was used as the internal standard. To 0.1 ml of plasma, bile or urine in a centrifuge tube were added 1 ml of 0.1 *M* acetate buffer (pH 5.0) and 5 ml of dichloromethane. The mixture was shaken for 10 min and centrifuged at 1680 *g* for 5 min. A 4-ml volume of the lower organic phase was transferred to another tube, and 100 μl of methanol containing 1 μg/ml GA-Me were added . The mixture was evaporated at 40°C. The residue was dissolved in 50 μl of acetonitrile, then an aliquot (20 μl) was injected into the chromatograph. HPLC conditions were as follows: column, Senshupack $5C_{18}$ (250 mm × 6 mm I.D.); column temperature, 30°C; mobile phase, acetonitrile–0.2% acetic acid (9:1); flow-rate, 1 ml/min; detection wavelength, 254 nm. The retention times of GA and the internal standard were 10 and 17 min, respectively. The coefficients of variation at concentrations of 0.5 and 50 μg/ml of GA in plasma were 3.3 and 2.7% ($n = 4$), respectively.

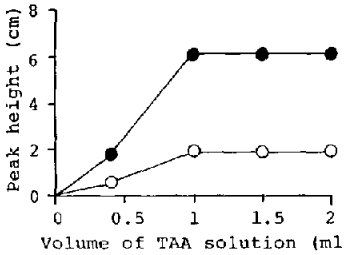
Biomedical application

In a pilot study, bile-fistula rats, weighing 280 g, were intravenously administered a dose of 10 mg/kg for G or 20 mg/kg for GA. Blood samples were drawn from the femoral artery inserted via a polyethylene cannula at 3, 5, 15 and 30 min, and 1, 2, 4, 6, 8 and 10 h after the dose. The blood was transferred to heparinized tubes. Plasma was separated by centrifugation at 1680 *g* for 5 min. Urine and bile were collected at appropriate time intervals up to 24 h. All samples were stored at −20°C until analysis.

RESULTS AND DISCUSSION

Extraction

For the ion-pair extraction of G and GA-G from biological fluids into an organic solvent, we examined various counter ions: TAA, TBA and THA. Extraction solvents examined were dichloromethane, isoamyl alcohol–*n*-heptane



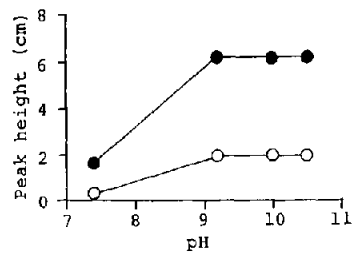


Fig. 2. Effect of the volume of 0.1 *M* tetra-*n*-amylammonium bromide (TAA) solution added to plasma samples. TAA (0.1 *M*) solutions of various volumes were added to 0.1 ml of plasma containing (●) 5 μg of G or (○) 5 μg of GA-G, together with 0.6 ml of 1 *M* carbonate buffer (pH 10.0).

Fig. 3. Effect of pH variation in the aqueous phase on the extraction of G (5 μg, ●) and GA-G (5 μg, ○). The buffer solution (0.6 ml) was 1/15 *M* Sorensen buffer (pH 7.4), and 1 *M* carbonate buffer (pH 9.2, 10.0 and 10.5). The volume of 0.1 *M* TAA solution added was 1.5 ml.

(98.5:1.5, v/v), various mixtures of ethyl acetate and *n*-heptane (3:1, 4:1, 5:1 and 6:1, v/v), and ethyl acetate. THA was not suitable for our purpose, because it was less soluble (*ca.* 0.01 *M* at room temperature) than the other counter ions. When sodium sulphate was added to the sample, both G and GA-G could be extracted with any combination of TAA or TBA and the organic solvents. The combination of TBA and ethyl acetate–*n*-heptane (5:1, v/v) proved to be most suitable, because it produced the highest peaks for G and GA-G, and the smallest endogenous peak on the chromatogram under the HPLC conditions used.

The effect on the extraction of the volume of 0.1 *M* TAA solution added to the plasma sample is shown in Fig. 2. The peak height (cm) was relatively constant in both analytes over the range 1–2 ml of 0.1 *M* TAA solution. The effect on the extraction of pH variation in the aqueous phase is shown in Fig. 3. The peak height was relatively constant in both analytes over the pH range 9.2–10.5. From these findings (Figs. 2 and 3), we selected 1.5 ml as the optimum volume of 0.1 *M* TAA solution, and adjusted the pH to 10.0 in the aqueous phase with 1 *M* carbonated buffer for the extraction of G and GA-G from biological fluids. Capric acid was selected as an internal standard, and this gave sufficiently reproducible results (see below). The reason for the addition of the internal standard after completion of the extraction was that the recovery of the internal standard from the biological fluid samples was low (*ca.* 50%).

Fluorescent labelling

When the HPLC analysis of G and GA-G was performed with UV detection at 254 nm without fluorescent labelling, the quantitative limit of G and GA-G was only 1 μg/ml using 1-ml samples. So, we tried fluorescent labelling of G and GA-G in order to improve the detection limit. BrMMC was suitable for the fluorescent labelling of mixtures of G, GA-G and capric acid (the internal standard).

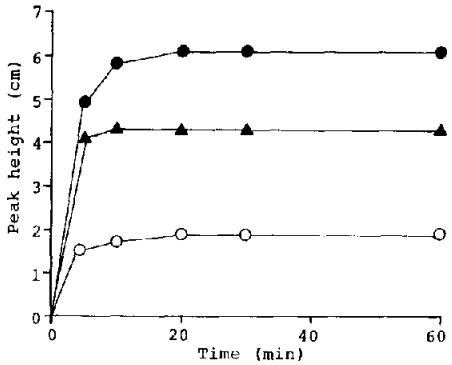


Fig. 4. Effect of reaction time at 65°C on the fluorescent labelling by 0.8 ml of BrMMC (2 mg/ml) in acetone and 0.8 ml of triethylamine (30 mg/ml) in acetone of G (5 μg, ●), GA-G (5 μg, ○) and capric acid (0.28 ng, ▲).

The effect of the reaction time at 65°C on the fluorescent labelling of G, GA-G and the internal standard is shown in Fig. 4. We chose 30 min as the optimum time because the peak heights of all three compounds were at a maximum after 20 min.

These labelled compounds were identified by FAB-MS. The mass spectra of the MMC derivatives of G and GA-G are shown in Fig. 5. The MMC derivative of G gave a molecular ion peak at *m/z* 1197 (M − 1), and fragment peaks at *m/z* 1009 (M − 188) and 821 (M − 376), suggesting the formation of the MMC diester of G. Similarly, the MMC derivative of GA-G gave ion peaks at *m/z* 833 (M − 1) and 645 (M − 188), suggesting the formation of the MMC monoester of GA-G.

Chromatography

Typical chromatograms of G and GA-G in extracts of rat plasma, bile and urine containing these compounds, and blank plasma, bile and urine are shown in Fig. 6. The three peaks of the internal standard, G and GA-G in every chromatogram were well separated from the endogenous peak, and their retention times in plasma and bile were *ca.* 14, 17 and 23 min, respectively, and those in urine were *ca.* 18, 22 and 29 min, respectively.

Calibration curves and reproducibility

The calibration curves for the plasma assay were linear in the range 1–50 μg/ml for both G and GA-G. Those for the bile assay were also linear in the range 1–50 μg/ml for G and 2.5–50 μg/ml for GA, and those for the urine assay in the range 2.5–50 μg/ml for both G and GA-G. The regression equations for G and GA-G in plasma were $y = 0.028x + 0.001$ ($r = 0.999$) and $y = 0.010x + 0.0001$ ($r = 0.998$), respectivley; those in bile were $y = 0.031x - 0.088$ ($r = 0.999$) and $y =$

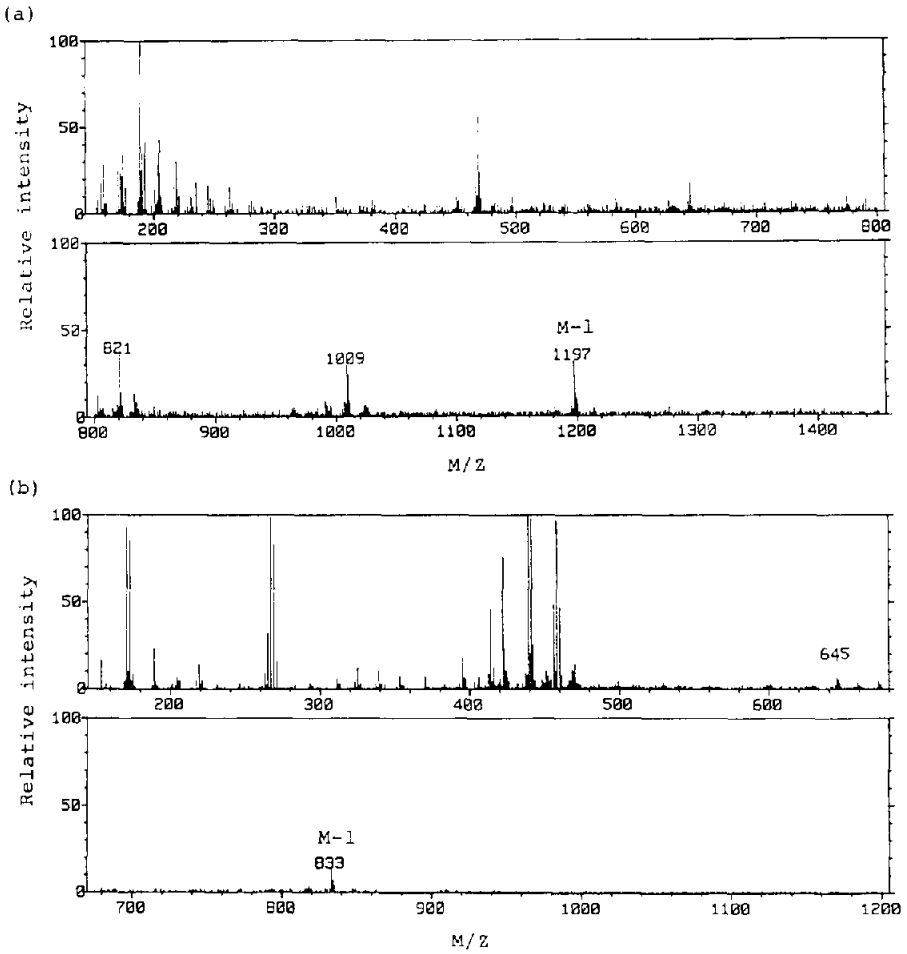


Fig. 5. FAB mass spectra of the methyl methoxycoumarin derivatives of (a) G and (b) GA-G.

$0.011x - 0.001$ ($r = 0.999$); and those in urine were $y = 0.040x + 0.004$ ($r = 0.999$) and $y = 0.011x + 0.001$ ($r = 0.999$).

The reproducibility of the analysis of G and GA-G in plasma, bile and urine was determined at four or five different concentrations of the analytes (1, 2.5, 5, 10 and 50 μg/ml). The coefficients of variation (C.V., $n = 3$) for G were less than 7.1% in plasma, 5.6% in bile and 2.6% in urine, and those for GA-G were less than 8.5% in plasma, 7.6% in bile and 4.5% in urine (Table I). The quantitative limit for G in plasma, bile and urine was 1, 1 and 2.5 μg/ml (signal-to-noise ratio = 10), respectively. The quantitative limit for GA-G in plasma, bile and urine was 1, 2.5 and 2.5 μg/ml (signal-to-noise ratio = 10), respectively.

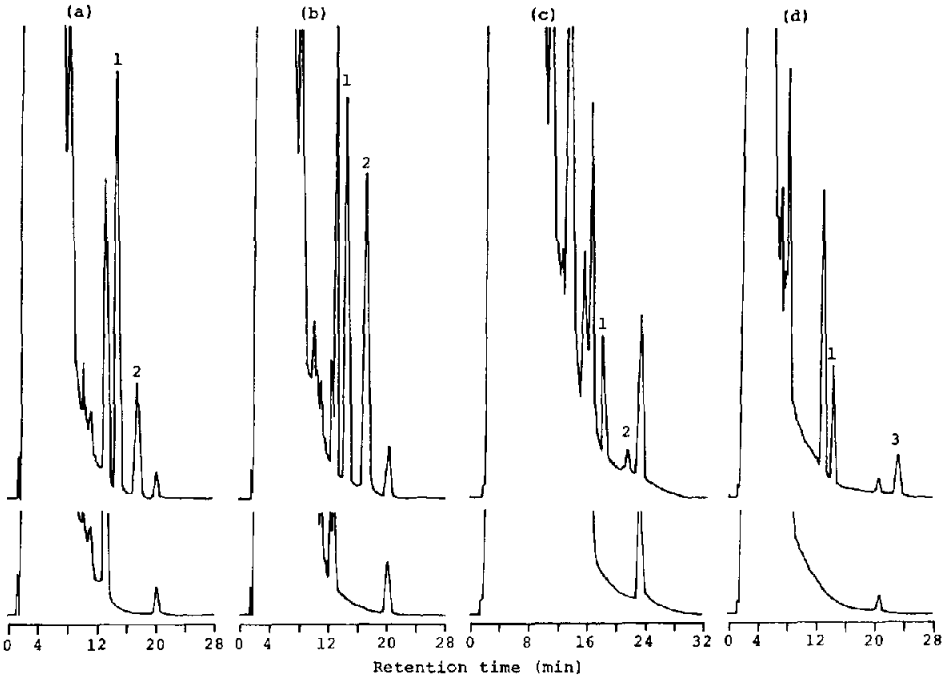


Fig. 6. Chromatograms obtained from: (a) 2-h rat plasma sample after intravenous administration of G (10 mg/kg) (upper trace) and blank rat plasma (lower trace); (b) pooled rat bile sample (3–6 h) after intravenous administration of G (10 mg/kg) (upper trace) and blank rat bile (lower trace): the sample was diluted (1:9) with the blank; (c) pooled rat urine sample (0–24 h) after intravenous administration of G (10 mg/kg) (upper trace) and blank rat urine (lower trace); (d) pooled rat bile sample (0–1 h) after intravenous administration of GA (20 mg/kg) (upper trace) and blank rat bile (lower trace). Peaks: 1 = capric acid (internal standard); 2 = G; 3 = GA-G.

Application to in vivo study in rats

This newly developed method was applied to the analysis of plasma, urine and bile samples after intravenous administration of G or GA to rats. The concentration–time courses of G and GA in plasma after administration of G (10 mg/kg) are shown in Fig. 7a. The plasma concentration of G declined rapidly after administration, whereas that of the metabolite, GA, was very low. In bile and urine, 98.2 and 0.1% of the dose were excreted within 24 h as unchanged G, and 0.1 and 0.03% as GA, respectively. However, GA-G was not detected in any samples. The plasma concentration of GA after intravenous administration of GA (20 mg/kg) also declined rapidly, as shown in Fig. 7b. GA-G was not detected in plasma at any time. In bile and urine, 0.07 and 0.02%, respectively, of the dose were excreted within 24 h as unchanged GA. Although GA-G was detected in bile, the recovery was only 2.0%. These results of the pilot study suggest that GA-G might be one of the minor metabolites of G.

TABLE I

REPRODUCIBILITY OF ANALYSIS OF G AND GA-G IN RAT PLASMA, BILE AND URINE

Sample volume was 0.1 ml; $n = 3$.

Concentration (μg/ml)	G		GA-G	
	Peak-height ratio (mean ± S.D.)	C.V. (%)	Peak-height ratio (mean ± S.D.)	C.V. (%)
Plasma				
1	0.026 ± 0.001	3.8	0.010 ± 0.0006	6.0
2.5	0.069 ± 0.003	4.4	0.022 ± 0.0006	0.7
5	0.140 ± 0.010	7.1	0.047 ± 0.004	8.5
10	0.300 ± 0.006	2.0	0.104 ± 0.006	5.8
50	1.381 ± 0.011	0.8	0.475 ± 0.031	6.5
Bile				
1	0.027 ± 0.002	7.4		
2.5	0.078 ± 0.005	6.4	0.022 ± 0.0006	2.7
5	0.147 ± 0.007	4.8	0.053 ± 0.004	7.6
10	0.281 ± 0.013	4.5	0.090 ± 0.006	6.7
50	1.564 ± 0.059	3.8	0.525 ± 0.013	2.5
Urine				
2.5	0.154 ± 0.004	2.6	0.033 ± 0.001	3.0
5	0.230 ± 0.002	0.9	0.067 ± 0.003	4.5
10	0.408 ± 0.004	1.0	0.116 ± 0.001	0.9
50	2.022 ± 0.032	1.6	0.555 ± 0.021	3.8

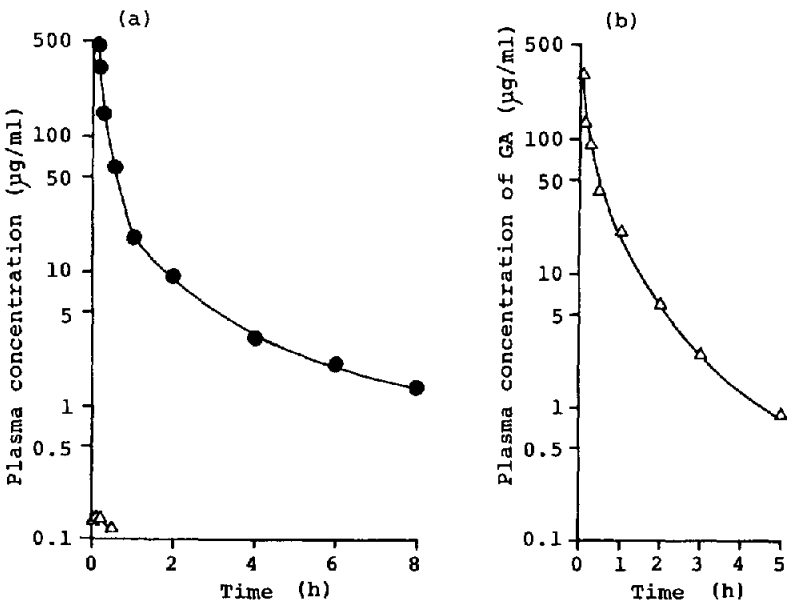


Fig. 7. Plasma concentration–time courses in rats of (a) (●) G and (△) GA after intravenous administration of a 10-mg dose of G, and (b) of (△) GA after intravenous administration of a 20-mg dose of GA.

CONCLUSION

A selective HPLC method for the determination of G and GA-G in plasma, bile and urine analysis was established. This method has been in routine use for over ten months in our laboratory. Judging from the high reproducibility and sensitivity for G and GA-G assay in biological samples, the method will be applicable to pharmacokinetic studies of G in small animals, such as rats.

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