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High-performance liquid chromatographic determination of 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid in rat plasma: application to pharmacokinetic study

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ABSTRACT

A high-performance liquid chromatographic method for the separation and determination of 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid has been developed. Indomethancin was used as an internal standard. The drugs were separated on a reversed-phase column and detected by UV detection at a wavelength of 254 nm. Methanol–water–perchloric acid–ammonia (80:20:0.4:0.4, v/v) was used as the mobile phase at pH of 7.0–7.5. The detection limit of both compounds was 0.1 μg/ml in rat plasma. The method was applied to pharmacokinetic studies of glycyrrhetinic acids in rats. The results suggest that the pharmacokinetics appeared to be non-linear in nature.

INTRODUCTION

Glycyrrhizin and its aglycone, 18β-glycyrrhetinic acid (18β-GA), are two principal constituents of licorice (*Glycyrrhizae radix*, Chinese name: Gancao), which is a well known and very important herbal drug in traditional Chinese medicine. They are widely used as antiulcer [1], antiinflammatory [2–5], and antihepatotoxic [6,7] agent. As reported by Amagaya *et al.* [5] the antiinflammatory activity of the 18β-GA stereoisomer, 18α-glycyrrhetinic acid (18α-GA), also obtained from licorice, was found to be more active than 18β-GA against carrageenan-induced edema in mice. Though licorice has a long history as a traditional drug, relatively few methods [8–15] are available at present for the quantitative determination in plasma of 18β-GA and glycyrrhizin, and very little information on its pharmacokinetics is available.

This paper describes the separation and determination of 18α-GA and 18β-GA in rat plasma by high-performance liquid chromatography (HPLC) with a reversed-phase (RP-18) chromatographic column. This determination method was also applied to the study of the plasma disposition or pharmacokinetics of 18α-GA and 18β-GA.

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EXPERIMENTAL

Chemicals

18α-GA (Fig. 1), 18β-GA (Fig. 1) and glycyrrhizin were purchased from Sigma (St. Louis, MO, U.S.A.). Indomethacin, perchloric acid (70%), methanol, acetonitrile and ammonia solution (32%) were obtained from Merck (Darmstadt, Germany).

The stock standard solutions of 18α-GA or 18β-GA were prepared by dissolving 10 mg of 18α-GA or 18β-GA in 100 ml of methanol, and indomethacin was dissolved in acetonitrile at a concentration of 5 μg/ml.

Chromatographic system

The HPLC system consisted of a Waters U6K injector, a Waters 441 detector, a Waters 510 chromatographic pump (Milford, MA, U.S.A.). Separation was achieved on a reversed-phase column (125 mm × 4 mm I.D., 5 μm, RP-18, Merck) fitted with a guard-column (4 mm × 4 mm I.D., 5 μm, RP-18, Merck). The mobile phase was methanol–water–ammonia solution–perchloric acid (80:20:0.4:0.4. v/v), and the flow-rate was 1.0 ml/min. The chromatogram was monitored at a wavelength of 254 nm throughout the experiments. The system was used at room temperature (25°C). Data-processing was handled by a Sic Chromatocorder 11 integrator (Tokyo, Japan).

Animals

Male Sprague–Dawley rats (250–300 g) and male New Zealand white rabbits (2.0–2.5 kg) were obtained from the Laboratory Animal Center at the National Yang-Ming Medical College and the National Taiwan University, respectively. These animals were kept in our own environmentally controlled quarters, with the temperature maintained at 24 ± 1°C and a light–dark cycle of 7:00–19:00 for at least one week before use. Water and standard laboratory chow were given *ad libitum* until 18 h before the experiments, at which time food only was withdrawn.

Blood sampling and treatment

Blood samples (0.3 ml) were directly withdrawn from rats via heart puncture under pentobarbital anaesthesia (30 mg/kg intraperitoneally), whereas 0.5 ml was

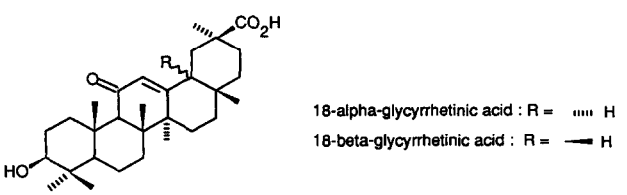

Fig. 1. Structures of 18α-glycyrrhetinic acid (18α-GA) and 18β-glycyrrhetinic acid (18β-GA).

directly withdrawn from the ear vein of conscious rabbits minimally restrained in a rabbit holder. Each sample was transferred to a heparinized microfuge tube and centrifuged at 8000 *g* for 5 min (Sigma 202-MK). The resulting plasma (0.1 ml) was mixed with 0.2 ml of acetonitrile, which contained 5 μg/ml the internal standard (indomethacin). The denatured protein precipitate was separated by centrifugation again at 8000 *g* for 5 min. The supernatant (10 μl) was directly injected into the HPLC column.

Blood samples were collected at time intervals of 2.5, 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360 and 480 min after intravenous administration of glycyrrhetinic acids.

Recovery

Plasma samples were spiked with standards in the range 5–200 ng/μl. Following the denaturation of proteins, fixed amounts of the internal standard were added for normalization. The values were compared with corresponding values of standards and internal standard carried in vehicles to yield the recovery values.

Precision

Standard curves were constructed from three concentrations of standards (5, 100 and 1000 μg/ml) spiked in rat plasma to determine the intra-day and inter-day variations of the method. Intra-day determinations were carried out four times at different times of the day. Means, standard deviations (S.D.) and coefficients of variation (C.V.) were calculated from these values.

Pharmacokinetic studies

All data were processed by the program of Pharmaco (Regents of University of California). The plasma concentration after intravenous administration of 18α-GA or 18β-GA in rat or rabbit was analysed by using the open one- and two-compartment model, respectively. The data for the area under the plasma concentration–time curves (AUC) were calculated by the trapezoidal method and extrapolated to infinite time.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

To optimize the HPLC conditions for the separation of 18α-GA and 18β-GA, the effect of the mobile phase composition and the pH were investigated, with the proportions of ammonia solution and perchloric acid both fixed at 0.5 and the pH at 7.5. The ratio of methanol to water was changed from 100:0 to 65:35, and the effect on the separation of GA stereoisomers was studied. It appeared that the two stereoisomers, 18α-GA and 18β-GA, were better separated as the amount of water increased. However, the two compounds were satisfactorily resolved by methanol–water (80:20, v/v) without undue prolongation of the retention times.

The pH value was a very important factor in the separation. At pH 4.5, the peaks of the two acids overlapped and the retention time was undesirably long at 16.4 min. The resolution was zero. When the pH value was increased to 7.5 by the addition of ammonia solution to the mobile phase, well resolved chromatographic peaks were produced with relatively short retention times of 7.5 min for 18α-GA and 8.7 min for 18β-GA. Thus, the separation was improved and the retention time was shortened as the pH increased. However, further improvement above pH 7.0 was minimal.

The amounts of perchloric acid and ammonia solution also affected the separation. The resolution was satisfactory when the proportions of perchloric acid and ammonia solution both reached 0.4. The retention times of 18α-GA and 18β-GA were 7.7 and 8.8 min, respectively. Further addition of perchloric acid and ammonia solution appeared to have little effect.

Fig. 2 summarizes the effect of the composition of the mobile phase. It appeared that conditions were optimal at a methanol–water–perchloric acid–ammonia solution compsition of 80:20:0.4:0.4 (v/v) at pH 7.0–7.5. These conditions were adopted for subsequent assays.

Recovery

Table I shows the recovery from rat plasma of 18α-GA and 18β-GA. Over the range 5–200 ng/μl, the recoveries were from 95.35 to 99.11% for 18α-GA and from 87.90 to 92.18% for 18β-GA.

Linearity, detection limit and reproducibility

Rat plasma samples, spiked at six different concentrations of 18α-GA and 18β-GA, were analysed. The peak-area ratios (18α-GA or 18β-GA to indometha-

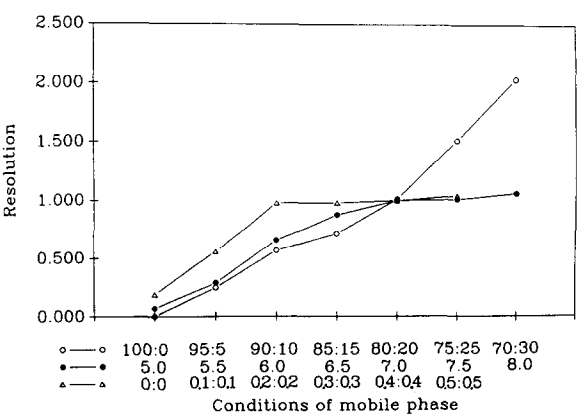

Fig. 2. Effects of the mobile phase composition and pH on the resolution of 18α-GA and 18β-GA. (○) Methanol–water ratio; (●) pH value; (△) perchloric acid and ammonia solution proportion in mobile phase.

TABLE I

RECOVERY OF 18α-GA AND 18β-GA FROM RAT PLASMA

Spiked concentration (ng/μl)	Recovery (%)	
	18α-GA	18β-GA
5	96.93	87.90
50	95.35	92.18
200	99.11	91.97

cin, internal standard) were linearly related to the concentration for both drugs over the range 2–200 μg/ml. The equation for the straight lines were $y = 0.0181 + 0.0424x$ ($r = 0.999$) for 18α-GA and $y = 0.0102 + 0.0524x$ ($r = 0.999$) for 18β-GA.

The detection limit for both drugs, at a signal-to-noise ratio of 4, was found to be 0.1 μg/ml in rat or rabbit plasma.

The reproducibility of the method can be expressed as both the intra-day variability and the inter-day variability. The intra-day C.V. for 18α-GA and 18β-GA in the concentration range 5–200 ng/μl were 4.18–1.47 and 4.68–2.50%, respectively (Table II). Inter-day C.V. for 18α-GA and 18β-GA in the same concentration range were 4.61–0.28 and 9.25–0.50%, respectively (Table III).

Plasma stability

At 37°C. To 10, 50 or 100 μl of standard solutions containing 1 μg/μl of the standards, 990, 950 or 900 μl of rat plasma were added to give final concentrations of 10, 50 and 100 ng/μl. Aliquots were withdrawn at 0, 1, 2, 4, 8, 12, 24, 36 and 48 h for assay by HPLC. For the concentrations (10, 50 and 100 ng/μl) tested,

TABLE II

INTRA-DAY PRECISION FOR 18α-GA AND 18β-GA DETERMINATION IN RAT PLASMA

Data are expressed as mean ± S.D. ($n = 4$).

Compound	Spiked concentration (ng/μl)	Measured concentration (ng/μl)	C.V. (%)
18α-GA	5	4.80 ± 0.20	4.18
	50	49.59 ± 0.88	1.78
	200	204.81 ± 3.00	1.47
18β-GA	5	5.29 ± 0.25	4.68
	50	49.19 ± 0.71	1.45
	200	204.30 ± 5.10	2.50

TABLE III

INTER-DAY PRECISION FOR 18α-GA AND 18β-GA DETERMINATION IN RAT PLASMA

Data are expressed as mean ± S.D. (n = 4).

Compound	Spiked concentration (ng/μl)	Measured concentration (ng/μl)	C.V. (%)
18α-GA	5	4.81 ± 0.22	4.61
	50	49.02 ± 0.49	1.01
	200	199.66 ± 0.55	0.28
18β-GA	5	4.94 ± 0.46	9.25
	50	49.19 ± 0.71	1.45
	200	199.66 ± 0.99	0.50

the mean detected values (ng/μl) were 9.31, 47.76 and 95.63, and the corresponding C.V. for 18α-GA were 5.94, 3.62 and 3.99%, respectively. For 18β-GA, the mean detected values were 9.78, 51.41 and 99.18 ng/μl and the corresponding C.V. were 6.82, 4.02 and 4.01% respectively.

At −20°C. Rat plasma containing the standards at concentrations of 10, 50

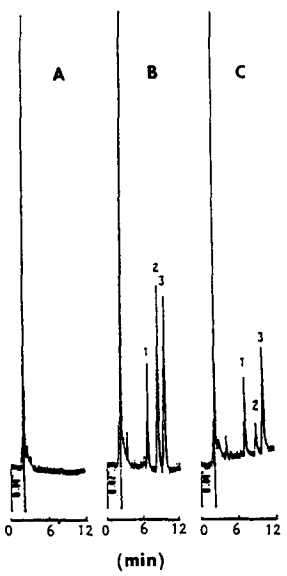

Fig. 3. Chromatograms of 18α-GA and 18β-GA in rabbit plasma. (A) Blank plasma; (B) blank plasma spiked with progesterone, 18α-GA (0.5 μg/ml) and 18β-GA (0.5 μg/ml); (C) plasma sample obtained after 18α-GA and 18β-GA intravenous coadministration, then spiked with the internal standard, progesterone. Peaks correspond to 0.1 and 0.3 μg/ml, respectively. Peaks: 1 = progesterone; 2 = 18α-GA; 3 = 18β-GA.

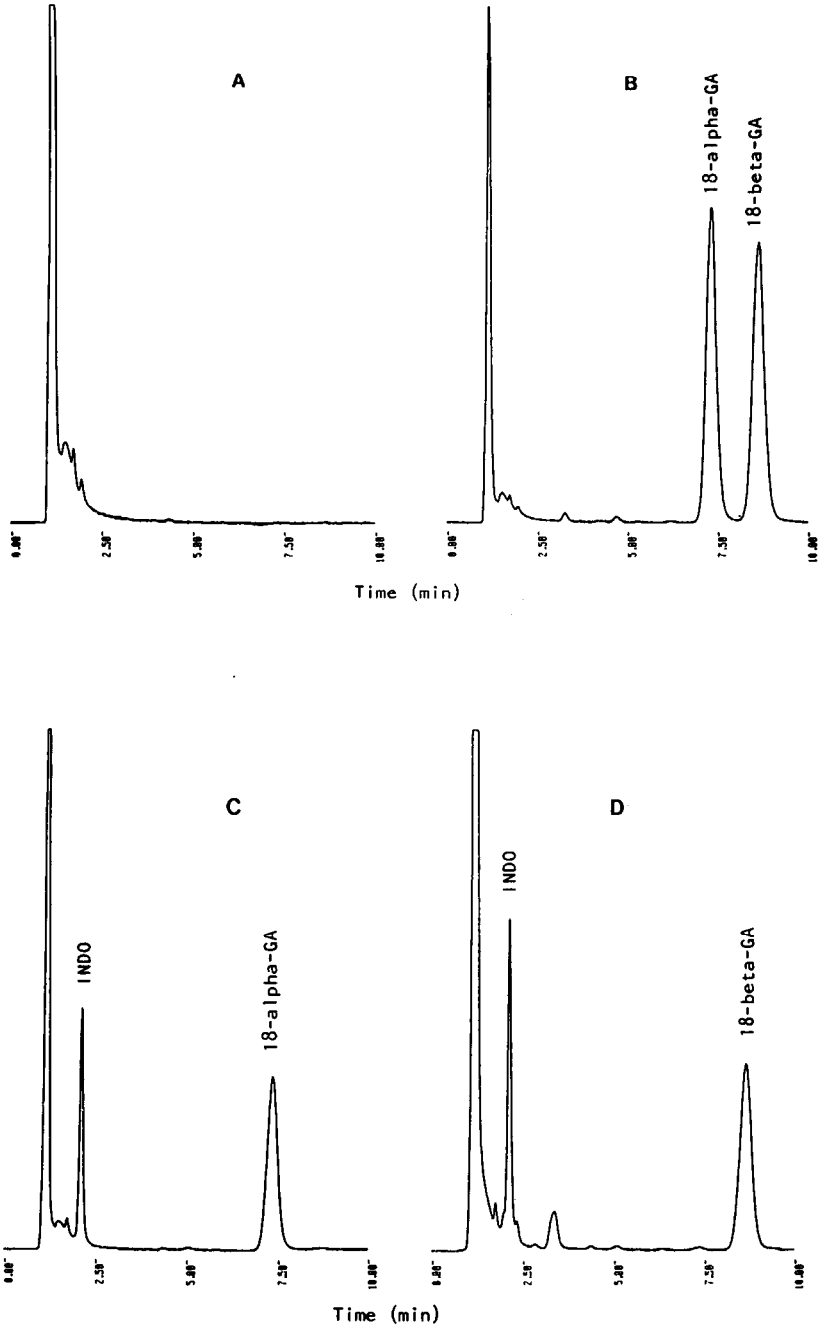

Fig. 4. Chromatograms of 18α-GA and 18β-GA in rat plasma. (A) Blank plasma; (B) blank plasma spiked with 18α-GA and 18β-GA; (C) sample obtained after a 20 mg/kg intravenous dose of 18α-GA and spiked with the internal standard, indomethacin (INDO); (D) sample obtained after a 20 mg/kg intravenous dose of 18β-GA and spiked with the internal standard, indomethacin.

and 100 ng/μl was prepared as described above and stored at −20°C. HPLC determinations were carried out at 0, 1, 2, 3, 5 and 7 days. Over the range tested (10, 50 and 100 ng/μl) the mean detected values were 9.52, 47.51 and 101.69 ng/μl, and the corresponding C.V. for 18α-GA were 4.60, 2.96 and 8.32%, respectively. For 18β-GA, the mean detected values were 9.54, 47.61 and 95.13 ng/μl, and the corresponding C.V. were 5.85, 4.18 and 3.50%, respectively.

Application to pharmacokinetic studies

The method can be used to quantify 18α-GA and 18β-GA in rabbit plasma via a simple procedure and an isocratic HPLC system. Blank samples did not show any interfering peaks in the analytical region of the chromatograms (Fig. 3A), nor did plasma spiked with progesterone (internal standard), 18α-GA or 18β-GA (Fig. 3B). None of these compounds was found in normal rabbit plasma but, when added, they were clearly recoverable and separable. In addition, the compounds were detectable when given intravenously through one ear vein and sampled from the contralateral ear vein (Fig. 3C). Similarly, as shown in Fig. 4, the isomers, and indomethacin (another suitable internal standard), can be detected in rat plasma.

Until recently, no data concerning the pharmacokinetics of 18α-GA had been reported. Fig. 5 shows the blood levels of 18α-GA or 18β-GA in a male rabbit following bolus intravenous administration (20 mg/kg). Table IV shows the pharmacokinetic parameters of 18β-GA as computed by the Pharmaco program. The plasma concentration of 18α-GA (20 mg/kg intravenously) fell below the detectable level so quickly that no pharmacokinetic parameters could be determined. However, all six rats died after receiving 18α-GA (20 mg/kg intravenously). In contrast, the same dose of 18β-GA did not induce fatality in treated rats. Thus 18α-GA is more toxic than 18β-GA.

A pharmacokinetic study of 18β-GA in male rats was carried out using two doses (5 or 10 mg/kg); the plasma concentration–time curves are shown in Fig. 6.

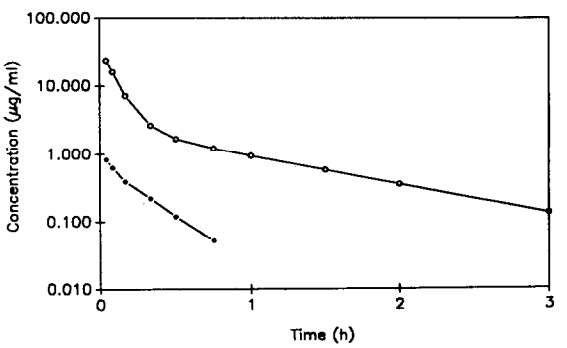

Fig. 5. Concentration–time plot for (●) 18α-GA and (○) 18β-GA in a male rabbit, following 20 mg/kg intravenous doses.

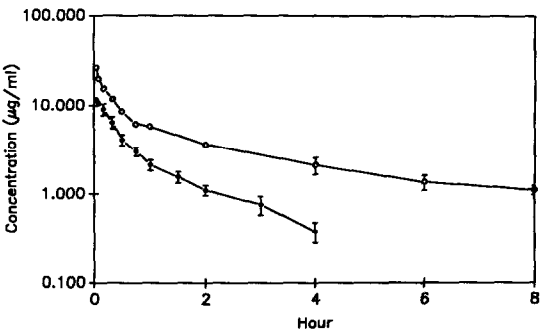

Fig. 6. Concentration–time plot for 18β-GA in a male rat, following intravenous doses of (○) 10 mg/kg and (●) 5 mg/kg.

The pharmacokinetic parameters of 18β-GA are listed in Table IV. The elimination half-life became greater as the dose was increased. However, the area under the curve was not proportional to the amount of bioavailable drug. These characteristics of pharmacokinetic parameters suggest that the pharmacokinetic behaviour of 18β-GA is non-linear (dose-dependent).

TABLE IV

PHARMACOKINETIC PARAMETERS OF 18β-GA AFTER ADMINISTRATION IN (A) RABBIT (20 mg/kg), (B) RAT (5 mg/kg) AND (C) RAT (10 mg/kg).

Data are expressed as mean ± S.E.M. (n = 4).

Parameter[a]	A	B	C
A (μg/ml)	31.44 ± 3.52	9.93 ± 0.83	19.41 ± 2.10
B (μg/ml)	2.05 ± 0.48	3.96 ± 0.48	6.64 ± 0.39
a (1/h)	10.10 ± 1.44	4.32 ± 0.47	3.96 ± 0.21
b (1/h)	0.88 ± 0.08	0.58 ± 0.04	0.25 ± 0.02
K_{10} (1/h)	6.55 ± 1.26	1.53 ± 0.11	0.84 ± 0.10
K_{12} (1/h)	3.01 ± 0.27	1.75 ± 0.34	2.14 ± 0.14
K_{21} (1/h)	1.42 ± 0.18	1.61 ± 0.12	1.22 ± 0.09
V_d (l/kg)	5.68 ± 0.94	1.05 ± 0.13	1.33 ± 0.06
V_c (l/kg)	82.35 ± 15.25	0.37 ± 0.02	0.40 ± 0.03
V_t (l/kg)	0.82 ± 0.09	0.40 ± 0.08	0.69 ± 0.03
$Cl_{(t)}$ (ml/min/kg)	4.49 ± 0.59	10.05 ± 1.15	5.55 ± 0.55
$T_{1/2}$ (h)	1.22 ± 0.08	1.22 ± 0.08	2.85 ± 0.26
$AUC_{0-inf.}$ (μg h/ml kg)	8.93 ± 1.14	8.93 ± 1.14	31.19 ± 3.11

[a] A and B = intercepts for compartments 1 and 2; a and b = rate constants for compartments 1 and 2; K_{10} = excretion rate constant; K_{12} = transfer rate constant (from the central to the tissue compartment); K_{21} = transfer rate constant (from the tissue to the central compartment); V_d = volume of distribution; V_c = volume of central compartment; V_t = volume of tissue compartment; $Cl_{(t)}$ = total body clearance; $T_{1/2}$ = elimination half-life; AUC_{0-inf} = area under the concentration–time curve.

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