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Determination of 18β-glycyrrhetinic acid in biological fluids from humans and rats by solid-phase extraction and high-performance liquid chromatography

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ABSTRACT

Methods have been developed and characterized allowing rapid isolation and quantification of 18β-glycyrrhetinic acid (GRA) in biological fluids from both humans and rats. Sample preparation includes extraction with urea–methanol for plasma samples, and solid-phase extraction (SPE) for urine and bile samples. Hydrolysis of GRA glucuronides in urine and bile was performed by treatment with β-glucuronidase. MGRA, the 3-O-methyl derivative of GRA was synthesized as an internal standard resistant to hydrolysis. High-performance liquid chromatography (HPLC) was performed with an isocratic system using methanol–water–acetic acid (83:16.8:0.2, v/v/v) as solvent on a Lichrocart RP-18 column at 30°C with ultraviolet detection. The methods allowed base line separation of GRA and MGRA from all biological fluids tested, with a detection limit of 0.15 mg/l. Validation of the methods included determination of recovery, accuracy and precision in plasma, bile and urine from humans and rats. The methods were further evaluated by investigating the pharmacokinetics of GRA in normal rats and in rats with a bile fistula. Following an intravenous dose of 10 mg/kg, the plasma concentration–time curve of GRA could be fitted to a one compartment model both in control and bile fistula rats. The elimination half life averaged 15.0 ± 2.2 *versus* 16.8 ± 2.4 min in control and bile fistula rats (difference not significant). Within 90 min following administration of GRA, urinary elimination of GRA and GRA glucuronides was less than 1% in both groups whereas biliary elimination averaged 51.3 ± 3.1%. The results show that the methods developed allow pharmacokinetic studies of GRA in humans and rats.

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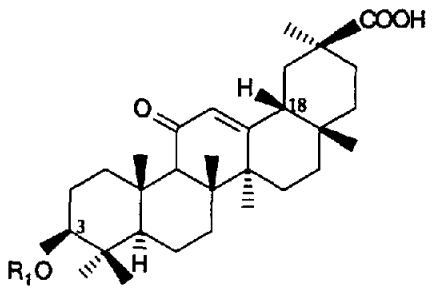
INTRODUCTION

18β-Glycyrrhetinic acid (GRA; Fig. 1) is the aglycone of the saponin glycyrrhizic acid (GZA; Fig. 1), a major constituent of the licorice root (*Glycyrrhiza glabra*) and of licorice [1]. GRA and GZA have recently obtained considerable pharmacological and clinical interest, since these compounds inhibit 11β-hydroxysteroid dehydrogenase, a microsomal enzyme catalyzing the conversion of 11β-hydroxy- to 11-ketosteroids [2–4]. Specifically, this enzyme converts cortisol to the inactive cortison in human tissues, thus regulating the cortisol levels in tissue and protecting the non-selective mineralocorticoid receptor from high cortisol concentrations [5]. The specific inhibition of this enzyme represents the principle mechanism by which excessive ingestion of licorice root or licorice results in a syndrome of "apparent mineralocorticoid excess", including hypertension, hypokalemia, hypernatremia, and suppression of the renin–angiotensin system [3,4]. GRA and GZA possess a variety of additional pharmacological properties, including antiinflammatory [6], antiviral [7] and antioxidative activities [8], and have been used clinically in patients with AIDS [9]. GRA is becoming a useful drug in the study of renal salt excretion and the pathogenesis of hypertension. It may also prove useful to modulate tissue levels of endogenous or exogenous glucocorticoids.

In contrast to the increasing pharmacological and clinical interest attracted by GRA, only few methods have been published characterizing the isolation and quantification of GRA in biological fluids from patients and experimental animals. Therefore, we established and evaluated high-performance liquid chromatographic (HPLC) methods for the determination of GRA in plasma and urine from humans and in plasma, urine and bile from rats. The methods were used for a pharmacokinetic study of GRA in control rats and rats with a bile fistula.



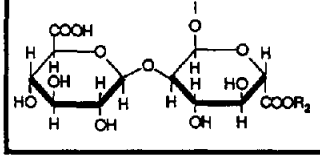
R_1:	R_2		
H	-	18β-Glycyrrhetinic acid	GRA
CH_3	-	3-O-Methyl-18β-glycyrrhetinic acid	MGRA
	H	Glycyrrhizic acid (Glycyrrhizin)	GZA
	NH_4	Glycyrrhizic acid ammonium salt	GZAA

Fig. 1. Chemical structures of 18β-glycyrrhetinic acid (GRA), 3-O-methyl-18β-glycyrrhetinic acid (MGRA), glycyrrhizic acid (GZA) and glycyrrhizic acid ammonium salt (GZAA).

EXPERIMENTAL

Instrumentation

An Aspec automatic sample preparation system combined with a Gilson 401 dilutor (Gilson Medical Electronics, Villiers de Bel, France) was used for solid phase extractions (SPE). The HPLC system consisted of an Altex 420 controller (Kontron, Zurich, Switzerland), two Kontron LC 410 pumps (Kontron) a Varian Marathon autosampler (Varian, Basle, Switzerland), a Kontron Uvicon 720 LC detector and a Hewlett Packard 3396A integrator (Hewlett-Packard, Waldbronn, Germany). Chromatographic separation was achieved using a Lichrocart reversed-phase RP-18 column (125 mm × 4 mm I.D., particle size 5 μm; Merck, Zurich, Switzerland) fitted with a RP-18 guard column (4 mm × 4 mm I.D., particle size 5 μm).

Materials

18β-Glycyrrhetinic acid (GRA), glycyrrhizic acid ammonium salt (GZAA), β-glucuronidase from *Helix pomatia*, tetrabutylammonium dihydrogen phosphate and glacial acetic acid (>99.5% pure) were purchased from Fluka (Buchs, Switzerland). GRA was purified by two-fold recrystallization from ethanol prior to use. All other solvents used were of analytical quality obtained from Merck (Zurich, Switzerland). Double distilled water was used for all purposes. Bakerbond C_{18} SPE cartridge columns (3 ml) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Standard solutions containing GRA or MGRA were prepared in methanol, and solutions containing GZAA in water. The standard solutions were stored at 4°C and were stable for at least 4 months.

Synthesis of 3-O-methyl-glycyrrhetinic acid (MGRA, internal standard)

Recrystallized GRA (1 g) was dissolved in 50 ml of acetone–water (90:10, v/v), and 3 ml of 10% KOH (w/v) were added. Dimethyl sulfate in acetone (10:90, v:v) (3.3 ml) was added slowly to the reaction mixture. During addition of dimethyl sulfate, the reaction vessel was kept on ice to keep the temperature below 40°C. Then, the reaction was allowed to proceed for 2 h at room temperature. After 2 h, the mixture was boiled for 10 min to decompose unreacted dimethyl sulfate, and then poured into 150 ml of cold water to precipitate the reaction product. The precipitate was collected by filtration and purified by two-fold recrystallization from ethanol. As shown by HPLC, acid treatment of the reaction product (10 *M* HCl at 95°C for 60 min) did not result in the appearance of a GRA peak, ruling out formation of the GRA methyl ester (see Fig. 1). The molecular structure of the reaction product was identified as MGRA by ^{13}C NMR spectroscopy (appearance of a methoxy-group signal at 51.76 ppm), distortionless enhancement by polarization transfer (DEPT) (appearance of a peak at 51.83 ppm) and ^{1}H NMR spectroscopy (appearance of a signal at 3.58 ppm). The purity of the synthesized MGRA was estimated to be higher than 98% by HPLC.

Sample preparation

Plasma samples. Plasma samples were prepared based on the methods of Zhang *et al.* [10] and Brown-Thomas *et al.* [11]. To 100 μl of plasma, 15 μg of MGRA (internal standard) in 25 μl of methanol, 150 mg of urea and 1 ml of methanol were added. The resulting suspension was vortex-mixed for 1 min and centrifuged for 10 min at 2000 *g*. A 900-μl sample of the supernatant was removed, membrane-filtered and directly used for HPLC.

Urine and bile samples. To 100 μl of bile or urine samples undergoing enzymatic hydrolysis, 15 μg of MGRA in 25 μl methanol, 100 μl of 0.5 *M* sodium acetate buffer (pH 5.3) and 100 μl β-glucuronidase (3 U/ml) were added. The mixture was vortex-mixed and the covered tubes were incubated at 51°C for 6 h. After incubation, 100 μl of 0.5 *M* NaOH and 200 μl of 0.5 *M* tetrabutylammonium dihydrogen phosphate (TBAP) solution were added, resulting in a pH > 10.5. Isolation of GRA was performed as described below for samples without hydrolysis. To 100 μl of bile or urine samples processed without hydrolysis of GRA glucuronides, 15 μg of

MGRA in 25 μl of methanol, 100 μl of 0.5 *M* NaOH, 200 μl of 0.5 *M* TBAP solution and 200 μl of water were added. The mixture was vortex-mixed and directly subjected to the clean-up procedure. The clean-up of hydrolyzed or non-hydrolyzed samples included SPE with C_{18} cartridges on an Aspec automatic sample system. The C_{18} SPE cartridges were conditioned with 2.5 ml of methanol and 2.5 ml of 0.5 m*M* TBAP. After conditioning, 525 μl of the sample mixture (prepared as described above) were passed through the C_{18} SPE column at a speed of 0.5 ml/min. The columns were dried by passing 3 ml of air and washed with 0.5 ml of methanol–water (40:60, v/v) followed by 6 ml of a 5 m*M* TBAP solution. After drying with 4 ml of air, GRA and MGRA were eluted with 4 ml of tetrahydrofurane at a flow-rate of 0.5 ml/min. Eluates were dried at 50°C under a stream of nitrogen and the residue was dissolved in 1 ml of methanol by sonication for 10 min. The solution was membrane-filtered and used for HPLC.

Chromatographic conditions

Twenty microliter of sample were injected into the HPLC system. Methanol–water–acetic acid (83:16.8:0.2, v/v/v) was used as mobile phase at a flow-rate of 1.0 ml/min and at a temperature of 30°C. The effluent was monitored at 250 nm by flow-through detection.

Standard curves

Standard curves were always run in duplicate at five or more concentrations of GRA or GZAA. Appropriate standard solutions of GRA or GZAA and MGRA (internal standard) were added to blank plasma, bile or urine samples. The preparation of the standards was identical to that of the samples. Standard curves were calculated using the ratio of the peak areas of GRA and MGRA, and were fitted using linear least square regression. The standard curves were linear up to at least 300 mg/l for plasma, 110 mg/l for urine and 1500 mg/l for bile. The detection limit of the method was 0.15 mg/l (corresponding to 4 ng GRA injected) for both samples and standards (signal-to-noise ratio = 5:1).

Recovery of GRA from plasma, urine and bile

Blank plasma, urine or bile samples (100 μl) were spiked with 3 different amounts of GZAA (samples undergoing hydrolysis) or GRA (samples without hydrolysis). The amount of GRA or GZAA added to the samples was chosen in the low, medium and high range of the concentrations expected in samples from humans or animals treated with GRA. The samples were prepared up in triplicate using the methods outlined above. The GRA content in the samples was calculated using a GRA standard curve obtained from standards not subjected to the clean-up procedure. The recovery was calculated as the ratio between the GRA content in the sample and the theoretical content, multiplied by 100.

Precision and accuracy of the GRA determination

Intra-day (n = 3 determinations of the same sample on the same day) and interday variability (sample determined in triplicate at 3 different days within 1 month) were assessed by spiking plasma, urine or bile samples with different amounts of GRA (no hydrolysis) or GZAA (including hydrolysis). Samples and standards were prepared identically using the methods outlined above. Intra-day and inter-day precision are expressed as the ratio between the standard deviation and the mean, multiplied by 100. Intra-day and inter-day accuracy is expressed as the ratio between the GRA content of the sample and the theoretical amount in the sample, multiplied by 100.

GRA pharmacokinetics in control rats and rats with a bile fistula

Male Sprague–Dawley rats (n = 3 control rats and n = 3 bile fistula rats) were anesthetized with pentobarbital i.p. (50 mg/kg). Polyethylene catheters were placed into the left femoral vein and into the urinary bladder. In bile fistula rats, an additional catheter was placed into the common bile duct. GRA (10 mg/kg) was administered intravenously, and blood, urine and bile samples were collected at the time points indicated in the result section. Blood was collected into heparinized tubes and plasma was immediately separat-

ed by centrifugation. Plasma, urine and bile samples were stored at −20°C until analysis. Pilot experiments had shown that GRA caused hemolysis with consecutive renal failure when administered as an ethanolic solution. This could be prevented by mixing the amount of GRA required (contained in a 20-mg/ml ethanolic solution) with 500 μl of a 10% bovine serum albumin solution (w/v). A dose of 10 mg/kg was chosen since pilot experiments had shown that doses over 20 mg/kg were lethal in >50% of the treated rats.

Calculations and statistical analysis

Pharmacokinetic calculations were performed using the PKCALC computer program published by Shumaker [12]. This program calculates the pharmacokinetic parameters according to Perrier and Mayersohn [13] as follows:

$$\text{elimination half-life} = \ln 2/k_e$$
$$V_{ss} = \text{Dose} \cdot \text{AUMC}/\text{AUC}^2 = Cl \cdot \text{MRT}$$
$$Cl = \text{Dose}/\text{AUC}$$
$$\text{MRT} = \text{AUMC}/\text{AUC}$$

with k_e being the elimination rate constant, V_{ss} the steady-state volume of distribution, Cl the total body clearance and MRT the mean residence time. AUC and AUMC were calculated using the trapezoidal rule.

Data are presented as mean ± S.E.M. The means of the pharmacokinetic parameters of control and bile fistula rats were compared by Wilcoxon's signed-rank test. An α-value < 0.05 was considered to be statistically significant.

RESULTS

Plasma samples from rats and humans could be sufficiently cleaned up by the addition of urea and subsequent extraction with methanol. From the representative chromatograms of rat plasma samples displayed in Fig. 2, it can be seen that this fast and simple preparation procedure results in baseline separation of the GRA and MGRA peaks. Similar chromatograms were obtained using human plasma samples (results not shown).

In contrast to plasma samples , bile and urine samples had to be extracted using a different

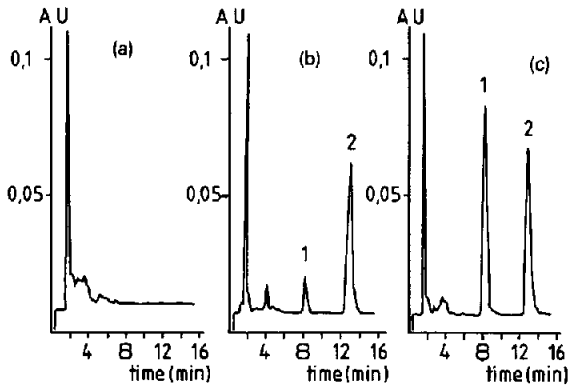


Fig. 2. Chromatograms of GRA and MGRA isolated from rat plasma. (a) Blank plasma, (b) plasma spiked with GRA (2.25 μg/ml), and (c) plasma sample obtained 15 min after intravenous administration of GRA (10 mg/kg). Peaks: 1 = GRA, 2 = MGRA (I.S.).

strategy in order to obtain clean chromatograms. Based on a publication by Brown-Thomas et al. [11], a procedure combining ion-pairing with solid-phase extraction (SPE) using a C_{18} column was adapted and evaluated. Pilot experiments showed that the GRA–TBAP adduct was firmly attached to the C_{18} SPE column and could not be eluted with an aqueous TBAP solution or a limited quantity of methanol. However it could be almost quantitatively eluted with tetrahydrofurane. Extraction procedures using methanol or methanol/phosphate buffer mixtures at different pHs were not successful. Since GRA is almost completely metabolized to the corresponding glucuronides prior to elimination by bile or urine [14,15], a method for the determination of the GRA glucuronides had to be developed. Pilot experiments showed that the GRA esters, which are frequently used as internal standards [11,16], are partially hydrolyzed during hydrolysis of GRA glucuronides, precluding their use as reliable internal standards for procedures involving enzymatic or chemical hydrolysis. The problem was solved by synthesizing the 3-O-methyl derivative of GRA (MGRA) which does not undergo hydrolysis under these conditions and allows reliable quantification of GRA glucuronides. As shown in Figs. 3 and 4, ion-pairing and SPE of bile and urine samples on C_{18} columns resulted

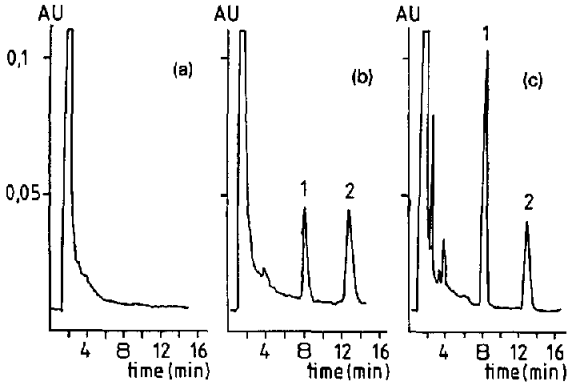


Fig. 3. Chromatograms of GRA and MGRA isolated from rat bile after enzymatic hydrolysis. (a) Blank bile, (b) bile spiked with GZA (26.8 μg/ml, corresponding to 15 μg/ml GRA), and (c) bile sample collected 30 to 45 min after intravenous administration of GRA (10 mg/kg). Peaks: 1 = GRA, 2 = MGRA (I.S.).

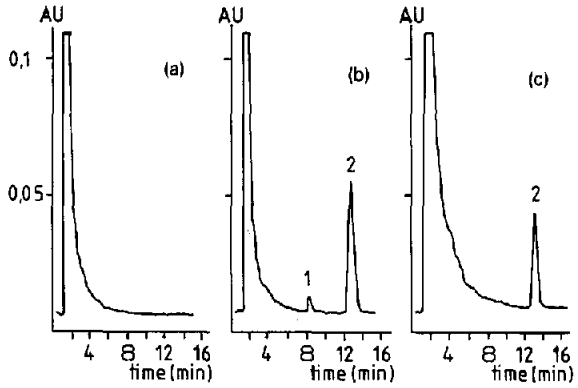


Fig. 4. Chromatograms of GRA and MGRA isolated from rat urine after enzymatic hydrolysis. (a) Blank urine, (b) urine spiked with GZA (1.85 μg/ml, corresponding to 1.05 μg/ml GRA), and (c) a urine sample collected 0 to 90 min after intravenous administration of GRA (10 mg/kg). Peaks: 1 = GRA, 2 = MGRA (I.S.).

in clean chromatograms without interfering peaks, allowing baseline separation of GRA and MGRA. Using this method, similar chromatograms were obtained for plasma and urine from humans (data not shown).

The recoveries of GRA using these procedures were in the range of 86% to 97% for plasma, bile and urine when no hydrolysis was performed (Table I). The recoveries dropped to *ca.* 70% for bile and 85% for urine, when the samples were

TABLE I

RECOVERY OF GRA AND GZAA FROM BIOLOGICAL SAMPLES

Rat plasma, urine or bile samples (0.1 ml) were spiked with GRA or GZAA as indicated in the table. Sample preparation and HPLC conditions are described in Experimental. The work-up included enzymatic hydrolysis for samples spiked with GZAA. Recovery of GRA was calculated as described in Experimental. The amount of GRA retrieved is presented as mean ± S.E.M., the recovery as the mean (n = 3 determinations).

Biological fluid	Spiked amount of GRA (μg)	Spiked amount of GZAA (μg)	Corresponding amount of GRA (μg)	Amount retrieved (mean ± S.E.M.) (μg)	Recovery (mean, n = 3) (%)
Plasma	2.25	–	–	2.15 ± 0.05	94
	9.00	–	–	8.35 ± 0.05	93
	30.0	–	–	28.1 ± 0.5	94
Bile	15.0	–		14.3 ± 0.4	95
	75.0	–	–	70.6 ± 0.3	94
	150	–	–	146 ± 3	97
Bile	–	26.8	15.0	10.3 ± 0.5	69
	–	134	75.0	55.4 ± 5.8	74
	–	268	150	96.2 ± 8.5	64
Urine	1.10	–	–	0.95 ± 0.05	86
	5.50	–	–	4.90 ± 0.30	89
	11.0	–	–	9.60 ± 0.35	87
Urine	–	1.85	1.05	0.90 ± 0.05	86
	–	9.25	5.15	4.65 ± 0.15	90
	–	18.5	10.4	8.50 ± 0.05	82

hydrolyzed prior to GRA isolation. However, as shown in Tables II and III, the intra-day and inter-day accuracy of the methods was mostly 90% or higher with a coefficient of variation of less than 10%, irrespective of the pretreatment of the samples.

For further evaluation of the developed methods, GRA pharmacokinetics were characterized in control rats and rats with a bile fistula. As shown in Fig. 5, the plasma concentration–time curve showed a monophasic decay in both control and bile fistula rats. The calculated pharmacokinetic parameters (AUC, clearance, V_{ss}, MRT and elimination half-life; see Table IV) were the same in control and bile-fistula rats, excluding significant enterohepatic cycling of GRA under the conditions used in the current experiments.

DISCUSSION

Methods were developed and validated that allow rapid isolation and accurate determination of GRA and GRA glucuronides in biological fluids from humans and rats.

The isolation of GRA from plasma or from bile and urine required different techniques. The isolation of GRA from plasma was easily achieved by addition of urea for splitting the protein binding, and by extraction of the lipophilic GRA with methanol. Recoveries from plasma were in the range of 95% for the three concentrations investigated (Table I) with excellent accuracy and precision (Tables II and III). These findings are in agreement with reports in literature, characterizing similar methods used for the de-

TABLE II

INTRA-DAY ACCURACY AND PRECISION OF THE GRA AND GZAA DETERMINATION IN BIOLOGICAL SAMPLES

Rat plasma, urine or bile samples (0.1 ml) were spiked with GRA or GZAA as indicated in the table. Sample preparation and HPLC conditions are described in Experimental. The preparation included enzymatic hydrolysis for samples spiked with GZAA. Accuracy and precision were calculated as described in Experimental and are presented as the mean ± S.E.M. (n = 3 determinations).

Biological fluid	Spiked amount of GRA (μg)	Spiked amount of GZAA (μg)	Corresponding amount of GRA (μg)	Amount determined (mean ± S.E.M.) (μg)	Accuracy (mean, n = 3) (%)	Precision (C.V., mean, n = 3) (%)
Plasma	2.25	–	–	2.20 ± 0.03	97	2.6
	9.00	–	–	8.75 ± 0.06	97	1.0
	30.0	–	–	29.8 ± 0.5	99	3.1
Bile	15.0	–	–	13.4 ± 1.0	89	12.5
	75.0	–	–	79.0 ± 1.6	105	3.6
	150	–	–	148 ± 5	99	5.3
Bile	–	26.8	15.0	14.2 ± 0.7	94	8.1
	–	134	75.0	69.3 ± 0.9	92	2.3
	–	268	150	133 ± 12	89	15.9
Urine	1.10	–	–	1.00 ± 0.06	92	11.4
	5.50	–	–	5.65 ± 0.12	103	3.4
	11.0	–	–	10.95 ± 0.20	100	3.1
Urine	–	1.85	1.05	0.90 ± 0.03	86	5.6
	–	9.25	5.15	5.00 ± 0.17	97	6.1
	–	18.5	10.4	10.3 ± 0.09	99	1.3

TABLE III

INTER-DAY ACCURACY AND PRECISION OF THE GRA AND GZAA DETERMINATION IN BIOLOGICAL SAMPLES

Rat plasma, urine or bile samples (0.1 ml) were spiked with GRA or GZAA as indicated in the table. Sample preparation and HPLC conditions are described in Experimental. The preparation included enzymatic hydrolysis for samples spiked with GZAA. Accuracy and precision were calculated as described in Experimental.

Biological fluid	Spiked amount of GRA (μg)	Spiked amount of GZAA (μg)	Corresponding amount of GRA (μg)	Amount determined (mean ± S.E.M.) (μg)	Accuracy (mean, n = 3) (%)	Precision (C.V., mean, n = 3) (%)
Plasma	30.0	–	–	29.7 ± 0.4	99	2.1
Bile	–	134	75.0	72.0 ± 3.8	96	9.1
Urine	–	9.25	5.15	5.15 ± 0.20	100	5.9

termination of GRA in plasma [17–19]. We were able to show that the method is suitable also for human plasma samples, allowing pharmacokinetic studies of GRA in humans.

Extraction of GRA from urine or bile proofed to be more difficult than from plasma due to the presence of interfering endogenous compounds. Chromatograms with baseline separation of GRA and the internal standard MGRA were obtained by adapting a solid-phase extraction (SPE) method originally developed by Brown-Thomas *et al.* for the determination of GRA in plasma [11]. Similar to the original report, the adapted method allowed mean GRA recoveries of 96% from unhydrolyzed bile and of 88% from unhydrolyzed urine samples (Table II) with acceptable accuracy and precision (Tables II and III).

Since GRA is almost completely glucuronidated prior to renal or biliary elimination [14,15], a method for the determination of GRA glucuronides had to be developed. Due to the large difference in polarity between GRA and GRA glucuronides, the possibility to determine glucuronides and unmetabolized GRA in the same chromatogram was not pursued. Therefore, a method was developed with enzymatic hydrolysis of the GRA glucuronides in urine and bile prior to ex-

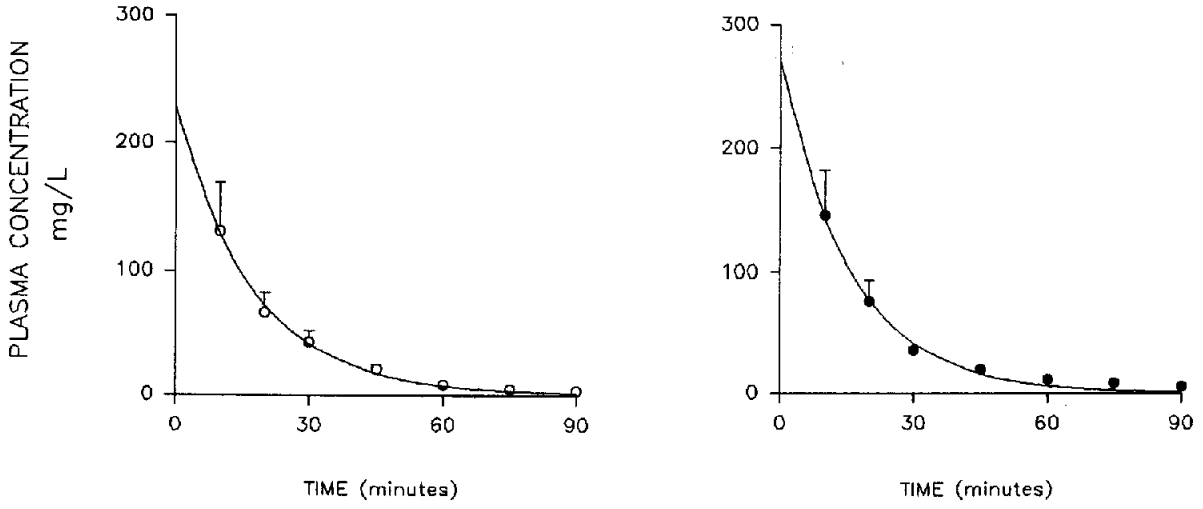


Fig. 5. Plasma concentration–time curves of GRA in control rats (○) and bile fistula rats (●) after intravenous administration of 10 mg GRA.

TABLE IV

PHARMACOKINETICS OF GRA IN CONTROL AND BILE FISTULA RATS

GRA was administered intravenously at a dose of 10 mg/kg. The body weights were 185 ± 15 g for the control and 178 ± 20 g for the bile fistula rats. Urine, blood and bile samples were collected and analyzed as described in Experimental. Pharmacokinetic calculations were performed as described in Experimental. No significant differences could be detected between the two groups. All data are presented as mean ± S.E.M.

Parameter	Control rats (n = 3)	Bile fistula rats (n = 3)
AUC[a] ([mg × min]/ml)	2.02 ± 0.57	2.65 ± 0.32
Clearance (ml/[min × 100 g body weight])	0.47 ± 0.11	0.40 ± 0.05
V_{ss}[b] (ml/100 g body weight)	14.6 ± 3.7	13.1 ± 2.8
Mean residence time (min)	30.3 ± 3.2	32.1 ± 3.7
Elimination half-life (min)	15.0 ± 2.2	16.8 ± 2.4
Elimination in urine		
μg excreted (0–90 min)	0	2.5 ± 1.4
% of dose (0–90 min)	0	0.12 ± 0.06
Elimination in bile		
μg excreted		
0–30 min		368 ± 43
0–60 min		652 ± 60
0–90 min		914 ± 43
% of dose (0–90 min)		51.3 ± 3.1

[a] AUC: area under the plasma concentration–time curve.

[b] V_{ss}: Steady-state volume of distribution.

traction and HPLC. As shown in Table II, enzymatic treatment of urine and bile with β-glucuronidase lowered the recovery from more than 90% without treatment to 70% with hydrolysis. Accuracy and precision were not different from the values obtained for unhydrolyzed samples, however, showing that the recoveries of MGRA and GRA were affected by hydrolysis in similar way. Increasing the amount of tetrahydrofurane did not improve significantly the recovery of GRA, and elution with more lipophilic solvents resulted in the appearance of interfering peaks. In comparison to another published method describing the determination of GRA in urine and bile [17], the current methods have the advantage of avoiding the use of the toxic chloroform for extraction, and they offer the possibility to determine GRA glucuronides with a similar accuracy and precision as the methods developed for GRA determination.

The pharmacokinetic characterization of intravenously administered GRA showed that the elimination of GRA could be described by a monophasic decay both in control rats and in rats carrying a bile fistula (Fig. 5). The calculated pharmacokinetic parameters were not different between control and bile fistula rats (Table IV) and agree well with studies in rats using similar doses of GRA [20]. Higher GRA doses could not be administered, since most of the rats died at doses over 20 mg/kg, possibly due to hemolysis with acute renal failure. Importantly, the concen-

tration–time curves were not different between control and bile fistula rats (Fig. 5), excluding significant enterohepatic cycling under these conditions. This observation differs from pharmacokinetic studies in rats using GZA, where significant enterohepatic cycling has been described [21]. As suspected in an earlier study [17], in rats almost no unmetabolized GRA was excreted and the major metabolites were GRA glucuronides. The current studies thus demonstrate that renal excretion of GRA is not important and that the major portion of the drug is excreted as GRA glucuronides by the bile.

In conclusion, GRA and GRA glucuronides can be isolated and determined quickly, with an acceptable recovery, accuracy and precision from biological fluids using solid phase extraction and quantification by HPLC. The current methods allow pharmacokinetic studies of GRA in experimental animals and in humans.

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