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Phisit Khemawoot[a], Chiharu Maruyama[b], Hirotaka Tsukada[b], Hiroyo Noda[b], Junko Ishizaki[c], Koichi Yokogawa[a, b] and Ken-ichi Miyamoto[a, b, *]

[a] *Department of Medicinal Informatics, Division of Cardiovascular Medicine, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8640, Japan*

[b] *Department of Hospital Pharmacy, School of Medicine, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan*

[c] *Department of Pharmacy and Health Science, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa, 920-1192, Japan*

*Corresponding author: Ken-ichi Miyamoto, Ph. D., Prof.
Department of Hospital Pharmacy, School of Medicine, Kanazawa University, 13-1, Takara-machi, Kanazawa 920-8641, Japan.
Telephone: (+81) 76-265-2045, FAX: (+81) 76-234-4280
E-Mail: miyaken@pharmacy.m.kanazawa-u.ac.jp

Abstract: We examined the influence of chronic hepatic failure on the disposition kinetics of valproate (VPA) excretion via a phase II reaction in rats treated with carbon tetrachloride (1.0 mg/kg, s.c., 3 times a week) for 2 or 3 months. There was no significant difference in the plasma concentration-time courses of VPA among control and two treated groups up to 120 min after i.v. administration of VPA (75 mg/kg), but subsequently the plasma concentrations of the treated groups declined significantly below the control levels. Expression of Mrp2 mRNA in the liver of the treated groups was significantly lower than in the control group; conversely that in the kidney was significantly higher. The enzyme activity of UGTs in the liver of the treated groups decreased significantly, but UGT1A8 mRNA expression in the duodenum was increased about 3-fold. Cumulative excretion of VPA glucuronide (VPA-G) in bile of the treated groups was significantly reduced, while that in urine was markedly increased. In conclusion, the area under the VPA plasma concentration-time curve is significantly decreased in rats with chronic hepatic failure owing to increased excretion of VPA-G via the kidney as a result of induction of Mrp2, and inhibition of enterohepatic circulation of VPA-G.

Key words: valproate; chronic hepatic failure; glucuronidation; disposition kinetics; UGT; Mrp2

Introduction

To select an appropriate drug therapy for patients with hepatic failure, it is important to understand the changes of drug disposition kinetics in such patients. It is well known that drug metabolism involves phase I and/or phase II reactions. In humans, phase I reactions are mediated predominantly by five families of cytochrome P450 (CYP) species, CYP1A, CYP2C, CYP2D, CYP2E and CYP3A. All the isoforms differ in their patterns of drug-metabolizing activity [1], and the amounts of the isoforms in the liver are altered in conditions involving hepatic failure [2].

We have shown that the degree of increase of serum AST activity can be used to predict the decrease of the total clearance (CL_{tot}) in rats with acute hepatic failure [3, 4]. Further, we showed that serum albumin levels can be used to predict appropriate dosages of hepatically metabolized drugs in patients with chronic hepatic failure [5]. Those reports dealt with the influence of the degree of hepatic failure on phase I reactions involving CYPs.

Phase II reactions include conjugation reactions such as glucuronidation and sulfation, as well as reactions with glutathione, glycine, etc. Glucuronide conjugation is catalyzed by UDP-glucuronosyltransferase (UGT), which has many isoforms, such as UGT1 (for phenolic hydroxyl groups and bilirubin) and UGT2 (for steroid compounds) [6]. Generally, hepatic failure has little influence on the disposition kinetics of drugs metabolized by glucuronidation. However,

there is still little information about the change of disposition kinetics of drugs that are excreted in bile and urine after glucuronidation in patients with hepatic failure. The major metabolic pathway of valproate (VPA) is glucuronidation by UGTs, followed by excretion into bile; β-oxidation and ω-hydroxylation also occur as minor pathways [7].

In this study, we aimed to clarify the influence of chronic hepatic failure on phase II reactions by examining the disposition kinetics of VPA as a model drug in rats with treated with carbon tetrachloride (CCl_4).

Materials and methods

Materials

Sodium valproate, 3,3-dimethylglutaric acid (DMGA) and carbon tetrachloride (CCl_4) were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). N-(*tert*-Butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

Animal experiments

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the

University of Kanazawa. Model rats for chronic hepatic failure were prepared by subcutaneous administration of CCl_4 and used at 48 h after the last administration. A dose of 2 mL/kg of 50% CCl_4 solution in corn oil were administered to male Wistar rats (7 weeks old, Nippon SLC Co., Ltd., Hamamatsu, Japan) 3 times/week (Monday, Wednesday and Friday), for 2 or 3 months. The treated rats for 2 or 3 months were 15 weeks old (body weight 254 ± 16 g, the mean ± sd of three rats) or 17 weeks old (280 ± 15 g), respectively. Untreated control rats received corn oil alone for 3 months were 17 weeks old (433 ± 16 g). A 100 μL aliquot of VPA (75 mg/kg) in normal saline was injected via the femoral vein. Blood samples (300 μl each) were collected at designated time intervals from the jugular vein under light ether anesthesia. Plasma was separated by centrifugation at 3000 x*g* for 10 min and stored at -30°C. The gas chromatography-mass spectrometric (GC-MS) assay of VPA in plasma, bile and urine was carried out using reported methods.

Determination of laboratory data

Measurements of laboratory data for plasma samples were conducted at SRL Co. Ltd. (Tokyo, Japan).

Assay for VPA

Concentrations of VPA in plasma, bile and urine were determined by GC-MS (Model GC-17 system Class 5000, Shimadzu, Kyoto, Japan). The assay for VPA was carried out according to Darius and Meyer [8]. The extraction solvent for VPA was ethyl acetate containing 2.5 µg/mL DMGA as an internal standard.

Aliquots of 100 µl of sample plasma, urine and bile were each mixed with 100 µL of 1 M NaH_2PO_4 buffer (adjusted to pH 5.0) and 1000 µL of ethyl acetate containing the internal standard. The mixture was shaken for 20 min and centrifuged for 5 min at 2800 X *g*. The supernatant organic phase was transferred to another glass tube and preconcentrated to approximately 100 µL under a stream of nitrogen gas at room temperature. Then 40 µL of N-(*tert*-Butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) was added to the residue, and the mixture was shaken vigorously. The sample was transferred to an automated-sampler microvial, and incubated for 3 h at room temperature. An aliquot (1 µL) of the sample was injected into the GC-MS system.

Analyses were carried out in the selected-ion monitoring mode, monitoring ions at *m/z* 201 and m/Z 331 for VPA and DMGA, respectively. Chromatographic separation of VPA was achieved with a methyl siloxane-crosslinked capillary column (HP-1; 25 m x 0.2 mm I.D.; Hewlett-Packard, USA) in a gas chromatograph equipped with a splitless injector. The oven temperature was set at 60°C for 1 min and programmed to 90°C at 30°C/min, to 150°C at 5°C/min and then to 250°C at 40°C/min. The final temperature was maintained for 10 min.

For the estimation of glucuronides, plasma, urine and bile samples were incubated with β-glucuronidase/arylsulfatase at 37°C for 70 min, and VPA was extracted and analyzed as described above. The concentration of the glucuronide of VPA (VPA-G) was obtained by subtracting the concentration of the unconjugated form from that of the hydrolysate.

Reverse transcription-polymerase chain reaction (RT-PCR) assay

Total RNA was isolated from the liver, kidney and duodenum by using an Isogen Kit (Wako Pure Chemicals Co., Ltd). Synthesis of cDNA from the isolated total RNA was carried out using RNase H-reverse transcriptase (GIBCO BRL, Rockville, MD). Reverse transcription (RT) reactions were carried out in a solution of 40 mM KCl, 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 1 mM DTT, 1 mM each of dATP, dCTP, dGTP, and dTTP, 10 units of RNase inhibitor (Promega, Madison, WI), 100 pmoL of random hexamer, total RNA and 200 units of the Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Berlin, Germany) in a final volume of 50 µL at 37°C for 120 min. Polymerase chain reaction (PCR) was carried out in a final volume of 20 µL, containing 1 µL of RT reaction mixture, 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 1 µM each of the mixed oligonucleotide primers, and 1 unit of Taq DNA polymerase (Gibco-BRL). Reported primers were used for rat UGT1A1, rat UGT1A6, rat UGT2B1 [9], rat UGT1A8 [10], rat Mrp2 [11] and rat *ß-actin* [12].

Preparation of liver microsomes

For preparation of microsomes, the liver was homogenized in three volumes of 100 mM Tris-HCl buffer (100 mM KCl, 1 mM EDTA, pH 7.4). Microsomes were prepared as reported previously [3] and stored at -80°C until use. Protein contents were measured according to the method of Lowry et al [13].

Measurement of UGT activity

An aliquot of 100 μL of 1.8 mM 4-nitrophenol, 50 mM $MgCl_2$, 0.5 M Tris-HCl buffer (pH 7.4) and 4 mg/mL of liver microsomes was preincubated in a tube at 37°C for 3 min, then 50 μL of the reaction buffer (containing 20 mM UDP-glucuronic acid) was added. The whole was incubated at 37°C for 10 min, then cooled in an ice bath, and 100 μL of cold acetonitrile was added. The reaction solution was centrifuged at 10000 x*g* for 20 min at 4°C, and the absorbance of the supernatant was measured at 405 nm.

Data analysis

Pharmacokinetics parameters were estimated according to model-independent moment analysis as described by Yamaoka et al. [14]. The data were analyzed by the use of Student's *t*-test to compare the unpaired mean values of two sets of data. The number of determinations is noted in each table and figure. A value of $P < 0.01$ was taken to indicate a significant difference between sets of data. The electrophoresis results were analyzed by using NIH Image software.

Results

Biochemical data in CCl_4-treated rats

Table 1 shows the serum biochemical parameters associated with chronic hepatic failure in rats treated with 1.0 mL/kg CCl_4 3 times/week for 2 or 3 months. The AST, ALT and ALP levels were increased significantly in both treated groups. Total bilirubin increased time-dependently during the administration of CCl_4. In contrast, the level of serum albumin and the A/G ratio were significantly decreased.

Expression of mRNAs and enzyme activity of UGTs

Figure 1 shows the expression of UGT mRNAs (UGT1A1, 1A6, 1A7, 1A8 and 2B1) in liver and duodenum of rats with chronic hepatic failure.

The expression levels of UGT1A7 mRNA in liver of rats with chronic hepatic failure were significantly higher than that in the control rats, while those of UGT1A1 was significantly lower. The expression levels of UGT1A6 mRNA in duodenum of rats with chronic hepatic failure were significantly lower than that in the control rats, whereas those of UGT1A8 mRNA was about 3 times higher.

Figure 2 shows the relative activity of UGTs in the liver of rats with chronic hepatic failure. The activity of UGT_s in the liver of rats treated with CCl_4 for 2 or 3 months was significantly decreased compared with that of the control rats.

Expression of drug transporter mRNA

Figure 3 shows the expression of Mrp2 mRNA in liver and kidney of rats with chronic hepatic failure. The expression levels of Mrp2 in liver of rats with chronic hepatic failure were significantly lower than that in the control rats, while those in kidney were significantly higher.

Plasma concentration-time courses of VPA in rats with chronic hepatic failure

Untreated rats and CCl_4-treated rats at 48 h after the last CCl_4 treatment (for 2 or 3 months) were intravenously injected with VPA (75 mg/kg). As shown in Fig. 4, there was no significant difference in the plasma

concentration-time courses of VPA up to 120 min after the administration among the three groups. However, subsequently the plasma concentrations of VPA in the CCl_4-treated rats decreased significantly compared with that of the control rats. The pharmacokinetic parameters of VPA are summarized in Table 2. The $AUC_{0\text{-}360}$ value of the rats treated for 2 or 3 months was significantly decreased compared with that of the control rats, and the value of total clearance (CL_{tot}) was significantly increased. There was no significant difference in the distribution volume at the steady-state (Vd_{ss}) among the three groups.

Biliary and urinary excretions of VPA and VPA-G

Fig. 5 shows the cumulative excretion levels (percent of dose) of VPA and VPA-G in the bile and urine during 6 h after an i.v. administration of VPA in control rats and rats treated with CCl_4 for 3 months. The cumulative amounts of VPA in the bile and urine of both groups were small (about 1-2 %), but the cumulative amount of VPA-G in the bile of the control group was about two times higher than that in the urine of the control group. The cumulative amount of VPA-G in the bile of the treated group was markedly decreased; conversely the cumulative amount of VPA-G in the urine of the treated group was significantly increased to about twice the normal level.

Discussion

In this study, in order to examine the change in the disposition kinetics of drugs eliminated by phase II metabolic reaction in chronic hepatic failure, we used VPA as a model drug because it is excreted mainly via glucuronidation. VPA is taken up into the liver, where it is converted to the glucuronide conjugate, which is excreted via Mrp2 transporter into the bile and urine.

We examined the enzyme activity of UGTs in the control and CCl_4-treated groups. It is reported that the amounts of UGT mRNA isoforms in liver of rats are variable [15], but the enzyme activity of UGTs is predominantly dependent on the expression of UGT1A1, 1A6 and 1A7 mRNAs [16]. In this study, the mRNA levels of UGT1A6 and 1A7 were significantly increased in the liver of rats with chronic hepatic failure, whereas that of UGT1A1 was significantly decreased (Fig. 1). Daidoji et al. [17] reported that the expression of UGT1A1 mRNA in normal rat liver is higher than that of UGT1A6 mRNA, while UGT1A7 mRNA is only slightly expressed. We also confirmed that the expression of UGT1A1 mRNA in liver is the highest among the three isoforms (data not shown). Therefore, the enzyme activity of UGT_s in the treated groups was decreased compared with that in the control group, owing to the decreased expression of UGT1A1 mRNA, in spite of increased expression of UGT1A7 mRNA (Fig. 2). It is reported that the UGT isoforms catalyzing VPA glucuronidation in humans are UGT1A6, UGT1A9 and UGT2B7 (corresponding in rat UGT2B1), but the activity of UGT1A9 in liver is low

[18]. Therefore, UGT2B1 is the main determinant of the activity level of UGT_s for VPA glucuronidation in rats, and so it appears that VPA glucuronidation is little influenced by chronic hepatic failure, as judged from the unchanged expression of UGT2B1 mRNA.

We found that the expression of UGT1A8 mRNA in the duodenum is increased to about three times the normal level by chronic hepatic failure, whereas that in the liver is unchanged (Fig. 1). UGT1A8 is highly expressed in intestine, where it acts to block enterohepatic recirculation of drugs that have undergone glucuronidation and subsequent hydrolysis of the glucuronide by β-glucuronidase [18]. Therefore, regeneration of VPA from VPA-G in the intestinal tract is likely to be minor in rats with chronic hepatic failure, because UGT1A8 is induced.

From the above results, we might predict that the plasma concentration of VPA after i.v. administration to rats with chronic hepatic failure would not be much changed compared with that of the control rats. However, as shown in Fig. 4, although there was no significant difference in the plasma concentration of VPA at 2 h after administration among the three groups, subsequently, the levels in the CCl_4-treated groups fell to about 1/10 of that in the control group. This result suggests that the disposition kinetics of VPA is only slightly influenced by the glucuronidation activity in the liver, whereas it is considerably influenced by the inhibition of enterohepatic circulation owing to the induction of UGT1A8. This would be consistent with the reported plasma concentration-time courses of VPA after i.v. administration in normal rats with or without bile duct cannulation [19].

Moreover, we examined the influence of chronic hepatic failure on the biliary and urinary excretions of VPA. We found that the VPA was only slightly excreted into the bile and urine, whereas VPA-G was almost entirely excreted (Fig. 5). Yamamura et al. [20] reported that VPA-G is mainly excreted into bile, but we found that VPA-G was excreted into both bile (about 60%) and urine (about 30%) in the control rats. However, in the treated rats, excretion of VPA-G into the bile was greatly decreased compared with the control rats, while that into the urine was greatly increased. This change of the main excretion route appeared to be associated with the change of Mrp2 transporter expression, because Mrp2 mRNA expression in the liver was decreased and that in the kidney was increased in chronic hepatic failure (Fig. 3). Wright et al. [21] clarified that the percentages of recovery of VPA-G in bile and urine of Wistar rats are about 63 and 23%, respectively, whereas those of Mrp2 transporter-deficient rats (TR^{-}rats) are 2 and 50%, respectively. These results suggest that the excretion route of VPA-G changes predominantly from bile to urine as a result of compensatory changes in the expression of Mrp2 in liver and kidney.

Thus, in summary, the $AUC_{0\text{-}360}$ value of VPA was decreased significantly owing to the induction of Mrp2 expression in the kidney and the inhibition of enterohepatic circulation in rats with chronic hepatic failure. However, the change of the disposition kinetics of VPA via this phase II reaction appears to be only slight compared with the changes in the disposition kinetics of drugs metabolized by phase I reactions involving

CYPs.

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Legends

Figure 1. Relative expression of UGT mRNA isoforms of liver (a) and duodenum (b) of untreated rats and rats with chronic hepatic failure. Rats were subcutaneously treated for 2 or 3 months with CCl_4 (1.0 mL/kg, 3 times a week). The mRNA expression levels were determined at 48 h after the last treatment. Each column and bar represents the mean ± sd of three rats.

*,**Significantly different from the control rats at $P<0.05$ and 0.01, respectively.

, no treatment; , CCl_4 treatment for 2 months; , CCl_4 treatment for 3 months

Figure 2. Relative enzyme activity of UGTs in hepatic microsomes of the untrated rats and rats with chronic hepatic failure. Rats were subcutaneously treated for 2 or 3 months with CCl_4 (1.0 mL/kg, 3 times a week). The activities were determined at 48 h after the last treatment. Each column and bar represents the mean ± sd of three rats.

*,**Significantly different from the control rats at $P<0.05$ and 0.01, respectively.

, no treatment; , CCl_4 treatment for 2 months; , CCl_4 treatment for 3 months

Figure 3. Relative expression of Mrp2 mRNA in liver and kidney of untreated rats and rats with chronic hepatic failure.

Rats were subcutaneously treated for 2 or 3 months with CCl_4 (1.0 mL/kg, 3 times a week). The mRNA expression levels were determined at 48 h after the last treatment. Each column and bar represents the mean ± sd of three rats.

**Significantly different from the control rats at P< 0.01.

▨ , no treatment; ▨ , CCl_4 treatment for 2 months; ▩ , CCl_4 treatment for 3 months

Figure 4. Plasma concentration-time courses of VPA after intravenous administration of VPA (75 mg/kg) to control rats (○) and rats treated with CCl_4 for 2 (△) or 3 months (■). Rats were subcutaneously treated for 2 or 3 months with CCl_4 (1.0 mL/kg, 3 times a week). VPA was administered at 48 h after the last treatment. Each symbol and bar represents the mean ± sd of three rats.

**Significantly different from the control rats at P< 0.01.

Figure 5. Cumulative amounts of biliary (a) and urinary (b) excretion of VPA and VPA-G up to 6 h after intravenous administration in untreated rats and rats with chronic hepatic failure.

Rats were subcutaneously treated for 3 months with CCl_4 (1.0 mL/kg, 3 times a week). The rats were given VPA at 48 h after the last treatment. Each column and bar represents the mean ± sd of three rats.

**Significantly different from the control rats at P< 0.01.

▨ , no treatment; ▩ , CCl_4 treatment for 3 months

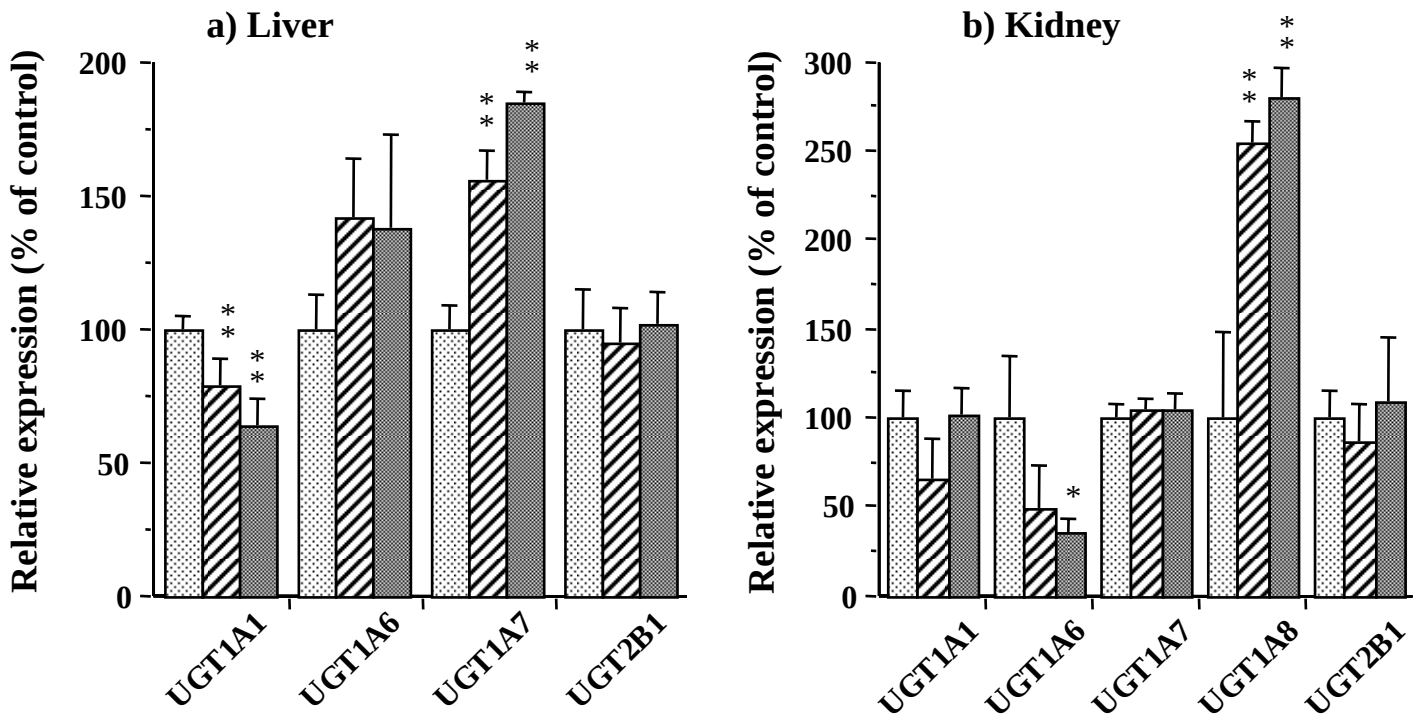
a) Liver
Relative expression (% of control)
200
150
100
50
0
**
**
**
**
UGT1A1
UGT1A6
UGT1A7
UGT2B1
b) Kidney
Relative expression (% of control)
300
250
200
150
100
50
0
*
**
**
UGT1A1
UGT1A6
UGT1A7
UGT1A8
UGT2B1

Fig. 1

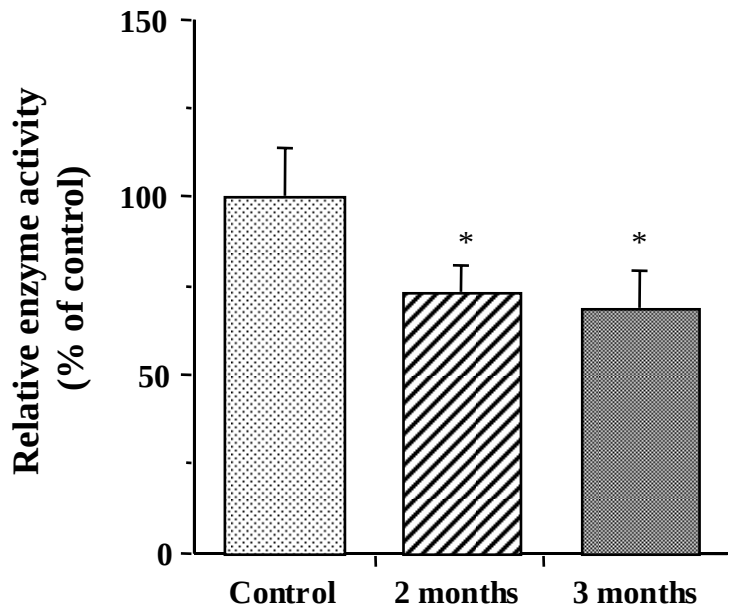
Relative enzyme activity
(% of control)
150
100
50
0
*
*
Control
2 months
3 months

Fig. 2

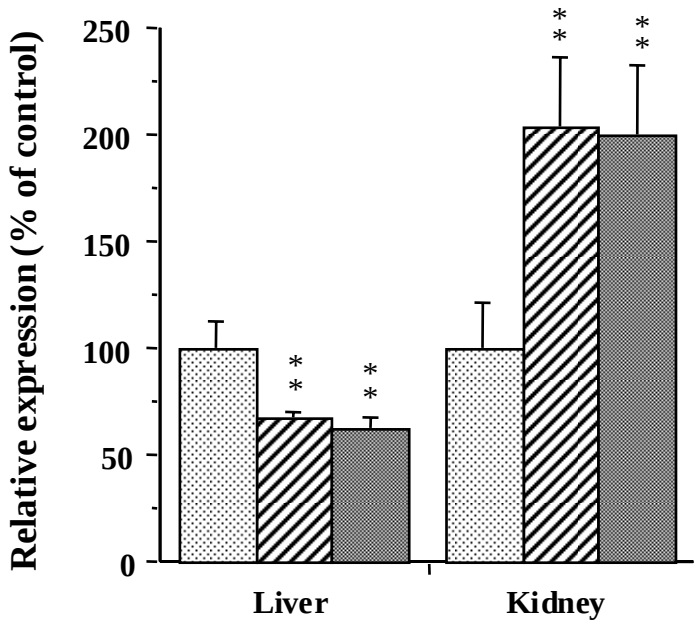
Relative expression (% of control)
250
200
150
100
50
0
**
**
**
**
Liver
Kidney

Fig. 3

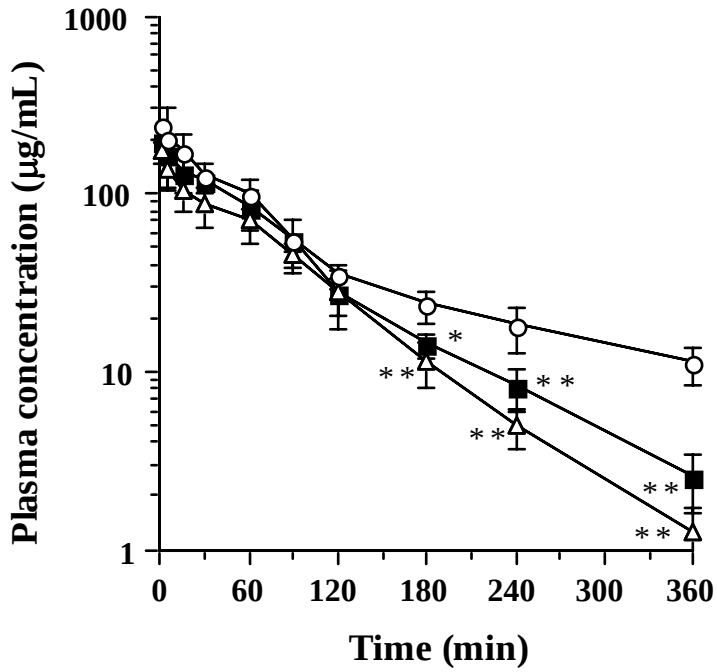
1000
100
10
1
Plasma concentration (μg/mL)
0
60
120
180
240
300
360
Time (min)
*
* *
* *
* *
* *

Fig. 4

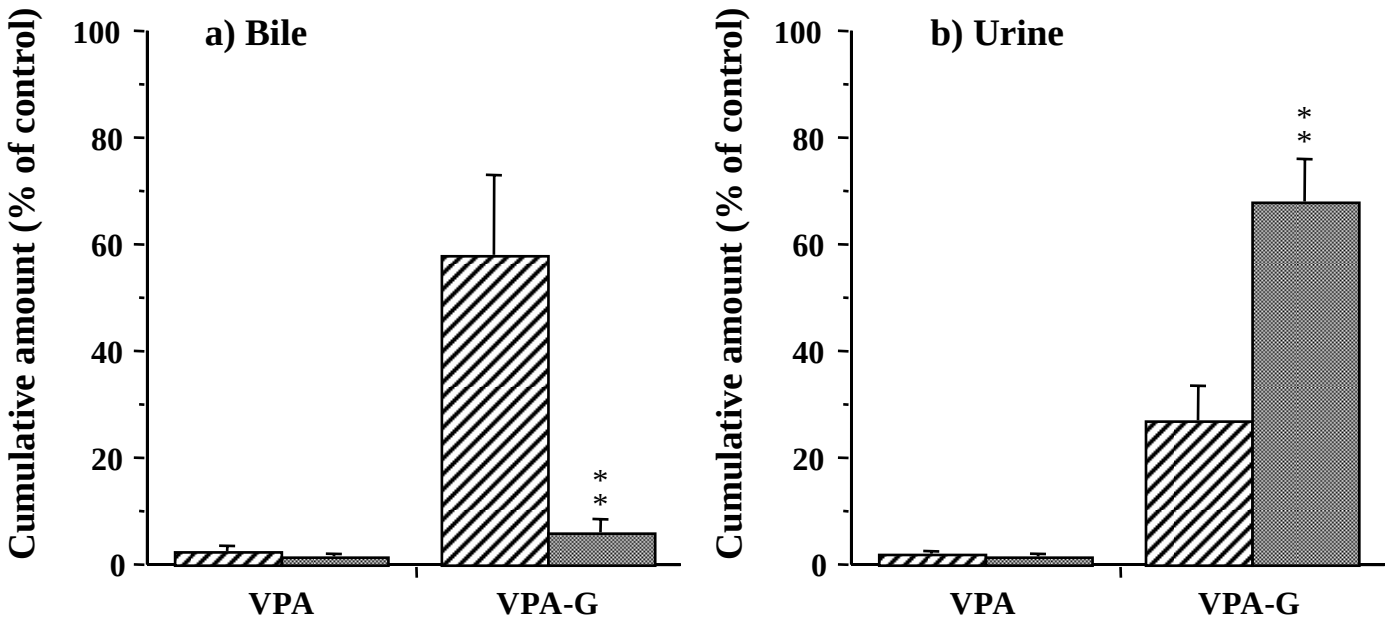
a) Bile
b) Urine
Cumulative amount (% of control)
100
80
60
40
20
0
VPA
VPA-G
**

Fig. 5

Table 1. Biochemical data for CCl_4-treated rats

	Untreated (control)	CCl_4-treated	
		2 months	3 months
AST (IU/L) [a]	79 ± 7	1874 ± 682[f]	1445 ± 845[f]
ALT (IU/L) [b]	42 ± 6	335 ± 95[f]	244 ± 156[f]
ALP (IU/L) [c]	943 ± 168	1687 ± 432[e]	1434 ± 243[e]
Albumin (g/dL)	3.64 ± 0.16	2.64 ± 0.23[f]	2.26 ± 0.62[f]
A/G ratio [d]	2.16 ± 0.24	1.61 ± 0.23[e]	1.36 ± 0.25[e]
Total bilirubin (mg/dL)	0.17 ± 0.06	0.71 ± 0.36[f]	0.89 ± 0.41[f]

Data were determined at 48 h after the last treatment with CCl_4 (1.0 mg/kg, 3 times a week).

Each value represents the mean ± sd of three rats.

[a] asparate aminotransferase; [b] alanine aminotransferase; [c] alkaline phosphatase; [d] albumin/globulin ratio

[e, f] Significantly different from control (untreated) rats at $p<0.05$ and 0.01, respectively.

Table 2. Pharmacokinetic parameters for VPA

Parameter	Untreated rats	CCl_4 for 2 M	CCl_4 for 3 M
$AUC_{0\text{-}360}$ (μg·min/mL) [a)]	16800 ±1030	10500 ±640**	13000 ±720**
MRT (min) [b)]	89.4 ±8.9	68.6 ±6.3*	71.5 ±6.7*
CL_{tot} (mL/min/kg) [c)]	4.46 ±0.27	7.13 ±0.43**	5.75 ±0.32**
Vd_{ss} (mL/kg) [d)]	399 ±58	489 ±68	411 ±55

Rats were intravenously administered VPA (75 mg/kg).
Pharmacokinetic parameters were estimated according to model-independent moment analysis.
Each value represents the mean ±sd of three rats.
[a)] area under plasma concentration-time curve from 0 to 8 h, [b)] mean residence time, [c)] total clearance,
[d)] distribution volume at the steady-state
** Significantly different from the untreated rats at $P<0.05$ and 0.01, respectively.