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Original Article

Efficacy of urine bile acid as a non-invasive indicator of liver damage in rats

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ABSTRACT — Estimation of liver damage is important in the pathophysiological and toxicological study of liver disease. As a novel, non-invasive marker of liver damage, we studied the efficacy of urine bile acids (UBA) in a rat model of liver disease. Thioacetamide (TAA)-treated rats were used in this study. Single intraperitoneal administration of high-dose TAA induces severe damage to the liver, and thus is used as a model of acute hepatitis. Continuous administration of low-dose TAA yields mild damage to the liver, and induces cirrhosis and hepatic tumors. In this study, it was found that both acute and chronic administration of TAA was associated with a dose-dependent elevation of UBA. The elevation of UBA content correlated with the alteration of blood biochemical indicators, and UBA screening showed a remarkable ability to distinguish liver-damaged rats from healthy rats. In particular, UBA analysis was found to have high sensitivity, specificity, and positive predictive value for the screening of rats with abnormal serum alkaline phosphatase (ALP) activity due to chronic liver damage, which was confirmed to include cholestasis and subsequent cirrhosis by liver histological analysis. In conclusion, we demonstrated that measurement of UBA is a simple, non-invasive and effective method for the screening of cholestasis in TAA-treated rats. We suggest that UBA analysis may have potent applicability for monitoring the progress of liver damage in animal models of chronic liver disease, such as cirrhosis and hepatic encephalopathy.

Key words: Bile acid, Urine, Liver, Cholestasis, Cirrhosis, Thioacetamide

INTRODUCTION

A simple and rapid assay method of evaluating liver damage is needed to examine the toxicity and adverse effects of chemicals in the field of pathophysiology and toxicology. Serum biochemical analyses are used for the evaluation of liver damage in clinical and fundamental research. The serum activities of liver enzymes such as alanine aminotransferase (ALT), and/or the serum content of compounds synthesized in the liver, such as albumin (Alb), show abnormal values according to the extent of liver damage. These are useful indicators for liver damage, but the blood sampling is so stressful and harmful to humans and animals that it is difficult to conduct repetitive analyses in the same individual. This limitation can be a problem in liver disease analysis. For example, in order to study the time course of the toxic or therapeutic effects of a certain compound, a different animal population should be used for each time point in order to prevent repetitive blood sampling that could affect the results. A large number of animals are thus required, and individual differences among them can make analysis difficult. In contrast, urine analysis using a metabolic cage is a non-invasive method that exerts relatively little stress on animals, and that allows longer-term monitoring of liver damage.

Bile acids are potential candidates for urinary markers of liver damage. Bile acids are synthesized from cholesterol in the liver, and secreted to the duodenum via the bile duct (Chiang, 1998; Dawson, 2002). Bile acids induce bile flow and biliary lipid secretion, and promote intestinal absorption of lipophilic compounds such as dietary lipids and fat-soluble vitamins (Hofman, 2007). Most of the secreted bile acids are reabsorbed into the intestine and recirculated into the liver (Dawson, 2002; Hofman, 2007). The enterohepatic circulation of bile acids is such an efficient process that less than 10% of the intestinal bile acids are eliminated in the feces. The secretion of bile acids into

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the systemic circulation is small. The blood concentration of bile acids is less than 10 μmol/l under normal conditions, but is increased in the case of a disorder in the liver or biliary tract (Dawson, 2002). Therefore, the serum bile acid level is a marker of liver disease, although it is not widely used as a routine screening test.

Urine bile acids (UBA) have been detected in patients with hepatic disease since the 1950s (Rudman and Kendall, 1957; Makino *et al*., 1974; Summerfield *et al*., 1976; Almé *et al*., 1977; Simko *et al*., 1987; Batta *et al*., 1989; Shoda *et al*., 1990). Quantification of bile acids has been carried out mainly by gas or liquid chromatography – mass spectrometry (GC-MS, LC-MS) in recent studies. GC-MS and LC-MS can identify various structurally different bile acids precisely, and alteration of the composition of UBA can be analyzed. These methods, however, require complicated sample preparation, a long period of time, and a well-disciplined technician. A more simple and rapid method is needed for routine screening or analysis of a large number of samples. Recently, the direct enzymatic assay of urine sulfated bile acids (USBA) content was developed as a diagnostic tool (Matsui *et al*., 1996), and several reports revealed the clinical importance of USBA analysis (Obatake *et al*., 2002; Shinohara *et al*., 2005; Huang *et al*., 2007). Since a large portion of bile acids are sulfated in human urine (Palmer, 1967; Stiehl, 1974), USBA rather than non-sulfated UBA is useful in clinical application.

UBA has also been analyzed in various animal species, such as hamsters (Galeazzi and Javitt, 1977), chimpanzees (Schweng *et al*., 1978), rabbits (Nakao *et al*., 1980), monkeys (Suzuki *et al*., 1985), and rats (Palmer, 1971; Takita *et al*., 1988; Lee *et al*., 2001). However, these studies focused on the alteration of metabolic ability of the liver and the pathogenic mechanisms of liver disease. UBA content was measured as an index of enzyme activities involved in the metabolism and excretion of bile acids and to reveal the involvement of these enzymes in the pathogenesis of liver disease. Several studies have examined the application of UBA analysis as a screening tool for liver damage. Balkman *et al*. (2003) reported the successful application of UBA analysis in dogs, and Trainor *et al*. (2003) reported its successful application in cats (Balkman *et al*., 2003; Trainor *et al*., 2003). However, there has been no report evaluating the efficacy of UBA analysis for the screening of liver damage in rats, despite the fact that rat models are the most commonly used animal model of liver damage.

In this study, we tried to evaluate the efficacy of UBA measurement as a non-invasive marker of liver damage. Because it is hoped that UBA analysis will be applicable for pathophysiological and toxicological study, we used the experimental animal most frequently used in such studies, i.e., rats. We utilized acute and chronic liver damage models induced by thioacetamide (TAA) administration, measured UBA content in these rats, and analyzed the relationship between UBA content and conventional indicators of liver disease in blood.

MATERIALS AND METHODS

Chemicals

TAA and other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Animals

Six- to 7-week-old male Wistar-Hannover rats (150 – 200 g) were obtained from CLEA Japan Inc. (Tokyo, Japan), and were maintained in an air conditioned room at 24 ± 2°C with a 12/12-hr light/dark cycle (lights on at 07:00). The rats had free access to food and water. Induction of liver damage was performed by single intraperitoneal (ip) administration of TAA or by continuous administration of TAA in the drinking water for 4 – 32 weeks (Mangipudy *et al.*, 1995; Fontana *et al.*, 1996; Haider *et al.*, 2004). At selected time points following treatment, rats were individually kept in a metabolic cage and urine excreted during 24 hr was collected in a tube containing 0.5 ml of 2N HCl. Collected urine was adjusted to pH 7 – 9 with 8N NaOH. Blood was collected within 6 hr after the end of urine collection, and serum and deproteinized blood samples were prepared to measure biochemical indicators. Urine and blood samples were frozen and kept at –80°C until analysis. Animal maintenance and treatments were in accordance with the Helsinki Guidelines for the care and use of laboratory animals. All procedures were approved by the Institutional Animal Care and Use Committee of Josai International University.

Acute liver damage model

Rats were treated with single ip administration of TAA. Rats were divided into three groups: a control group (saline, 10 ml/kg), a low-dose (100 mg/kg in 10 ml/kg saline, TAA100), and high-dose (500 mg/kg in 10ml/kg saline, TAA500) TAA-treatment group. Drugs were injected between 18:00 and 19:00, just before initiation of the dark phase. Two days (36 – 40 hr) after drug treatment, urine was collected for 24 hr in a metabolic cage. Blood was collected within 6 hr after the end of urine collection.

Chronic liver damage model

TAA was added to the drinking water. Rats were divided into three groups: a control group (drinking water without TAA), and a low-dose (0.3 g/l, TAA0.3), and high-dose (0.5 g/l, TAA0.5) TAA treatment group. At 4, 8, 16, and 32 weeks after the initiation of drug administration, urine was collected for 24 hr in a metabolic cage. TAA administration was terminated 24 hr before urine collection. Blood was collected within 6 hr after urine collection.

Liver histology

Liver was perfused with 15 ml of ice-cold phosphate-buffered saline (PBS), and isolated liver samples were fixed in 10% neutral buffered formalin solution. Paraffin-embedded tissues were sectioned at 5 μm. Liver sections were stained with hematoxylin-eosin (H&E) staining.

Blood chemistry

Blood samples were removed from postcaval vein with a 0.70 × 32 mm needle attached to a 5 ml syringe. One ml of blood samples were mixed with sodium tangstate, and were centrifuged at 1,200 × g for 10 min to obtain deproteinized blood samples. Remained blood samples were centrifuged at 1,200 × g for 10 min to obtain serum. Deproteinized blood samples were analyzed for the blood content of ammonia (NH_3), and serum samples were analyzed for the serum activity of alkaline phosphatase (ALP), ALT, aspartate aminotransferase (AST), and γ-glutamyltranspeptidase (γ-GTP), the serum content of Alb and total bilirubin (T-Bil), and the molar ratio of the serum content of branched-amino acids to the serum content of tyrosine (BTR) by the Mitsubishi Chemical Medience Corporation (Tokyo, Japan). Assays were performed according to the standard methods.

UBA assay

UBA content was measured by using an enzyme cycling Total Bile Acid Test Kit (DIAZYME, Hannover, Germany) according to the manufacturer's instructions with slight modification. Two μl of urine sample mixed with 67.5 μl of thio-NAD solution was prewarmed at ambient temperature for 5 min. Thereafter, 22.5 μl of 3-α-hydroxysteroid dehydrogenase (3αHSD) solution was added to the mixture, and the formation of thio-NADH was monitored by the absorbance at 405 nm at ambient temperature. Bile acid content was determined by the change of the absorbance over a period of 5 min. This analysis quantified total amount of non-sulfoconjugated bile acids in urine such as cholic acid and taurocholic acid.

USBA content was measured by the enzyme-linked colorimetric method with UBASTEC (MarukinBio, Kyoto, Japan) according to the manufacturer's instructions. This analysis quantified total amount of sulfoconjugated bile acids in urine such as sulfocholic acid and sulfotaurocholic acid.

Urine creatinine concentration was measured to normalize UBA values by the Jaffé method with a commercially available test kit (Wako, Tokyo, Japan) according to the manufacturer's instructions. Normalized UBA values were expressed in micromoles per gram of creatinine (μmol/g Cre). We selected normalization with creatinine, because this method was generally used in order to normalize the amount of urinary compounds, and several studies revealed that this normalization was suitable for UBA analysis in human, cats, and dogs (Simko *et al.*, 1987; Trainor *et al.*, 2003; Balkman *et al.*, 2003).

Statistical analysis

Data are expressed as the mean ± S.D., or the mean and a range from the minimum to maximum observed values. Comparisons between the control and treatment groups were made by Steel's test. A calculated P value of less than 0.05 was considered statistically significant.

RESULTS

Measurement of UBA

In order to confirm the reliability of UBA quantification, the UBA content of a control rat and a TAA-treated rat were repetitively measured. The intra-assay repeatability (n = 5) of UBA measurement with a single control rat and a single TAA-treated rat yielded 1.01 ± 0.15 and 30.2 ± 0.44 μmol/l with a coefficient of variation (CV) of 14.4% and 1.5%, respectively. The inter-assay repeatability (3 separate assays) with the same samples yielded 1.59 ± 0.51 and 32.7 ± 2.51 μmol/l with a CV of 32.1% and 7.7%, respectively. The CV values of the results from the control rat were relatively high. This may be because the UBA concentration of control rats was near the detection limit. But the UBA content was elevated in TAA-treated rats, and the elevation was greater than the assay variation. We considered that these assay variations were negligible in the following analyses. Finally, we also attempted to detect USBA in rat urine, but failed to do so.

UBA analysis in an acute model

Rats were divided into 3 groups: a control, TAA100, and TAA500 group. Two days after drug treatment, urine and blood were collected and analyzed (Table 1). Rats treated with TAA showed elevated serum activities

of ALP, ALT, and AST, and reduced serum Alb content, indicating liver damage. All control rats showed the same values of serum γ-GTP activity and serum T-Bil content. One rat in the TAA100 group and two rats in the TAA500 group showed elevated serum γ-GTP activity. Four rats in the TAA500 group showed elevated serum T-Bil content. UBA content was also elevated in rats with TAA. UBA content was 1.0 ± 0.6, 14.9 ± 25.6, and 623.1 ± 877.5 μmol/g Cre for the control, TAA100, and TAA500 groups, respectively. Although UBA content showed large individual variation within each group, the results suggested that the UBA content was elevated in a dose-dependent manner.

UBA analysis in a chronic model

Rats were treated with 0.3 g/l or 0.5 g/l of TAA in drinking water. Table 2 shows the results at 8 weeks. In addition to the serum indicators measured in the analysis of an acute model, we measured serum BTR and blood NH_3 content as indicators for hepatic encephalopathy (HE). Serum ALP activity was elevated and serum Alb content was reduced in TAA-treated rats, whereas the serum activities of ALT and AST remained within a normal range. The TAA0.5 group showed higher serum γ-GTP activity than the control rats. All control rats showed the same value, whereas only 3 of 4 rats in the TAA0.5 group showed an elevated level of serum γ-GTP activity. SerumT-Bil content remained unchanged in TAA-treated rats. A reduction of serum BTR and elevation of blood NH_3 content were observed. The elevation of the serum activities of ALP and γ-GTP indicated the induction of cholestasis. The reduction of serum Alb content and serum BTR indicated liver dysfunction. Finally, the reduction of serum BTR and elevation of blood NH_3 content are typical phenomena in HE. In this model, the UBA contents at 8 weeks were 2.1 ± 1.4, 6.5 ± 7.6, and 23.4 ± 16.7 μmol/g Cre for the control, TAA0.3, and TAA0.5 animals, respectively. The UBA content was elevated in TAA-treated rats in a dose-dependent manner.

Urine and blood were collected from rats at 4, 8, 16, 32 weeks from the initiation of TAA administration. As liver damage progressed according to the duration of TAA administration, we could obtain rats with various degrees of liver damage. Fig. 1 shows the change of UBA content over time. UBA content tended to increase for about 10 weeks, then remained at a high level.

In addition, we examined the liver sections stained by H&E in a chronic model. Representative photom-

Table 1. Effects of acute TAA treatment on UBA and blood liver disease indicators

	UBA (μmol/g Cre)	ALP (IU/l)	γ-GTP (IU/l)	ALT (IU/l)	AST (IU/l)	T-Bil (mg/dl)	Alb (mg/dl)
Control (n = 5)	1.0 (0.6 – 2.0)	649.8 (487 – 801)	1	23.1 (6 – 53)	84.7 (10 – 167)	0.1	2.63 (2.5 – 2.7)
TAA100 (n = 6)	14.9* (1.2 – 67.0)	701.8 (568 – 908)	1.17 (1 – 2)	116.7 (5 – 422)	148.3 (58 – 370)	0.1	2.67 (2.4 – 2.8)
TAA500 (n = 6)	623.1* (24.8 – 2281)	1187.0* (759 – 1713)	1.33 (1 – 2)	339.5* (45 – 576)	379.7* (157 – 557)	0.33 (0.1 – 1)	2.28* (2.1 – 2.5)

Data are expressed as the mean and range.
*$p < 0.05$ vs the control by Steel's test

Table 2. Effects of chronic TAA treatment (8 w) on UBA and blood liver disease indicators

	UBA (μmol/g Cre)	ALP (IU/l)	γ-GTP (IU/l)	ALT (IU/l)	AST (IU/l)	T-Bil (mg/dl)	Alb (mg/dl)	BTR	NH3 (μg/dl)
Control (n = 4)	2.1 (1.2 – 4.3)	300.7 (246 – 352)	1	32.7 (28 – 36)	83.3 (74 – 95)	0.1	4.17 (4.0 – 4.4)	3.58 (3.2 – 3.8)	53.0 (46 – 58)
TAA0.3 (n = 4)	6.5 (1.1 – 18.6)	665.6* (481 – 841)	1	31.0 (27 – 35)	85.3 (75 – 99)	0.1	3.60 (3.5 – 3.8)	2.27* (2.0 – 2.6)	56.5 (51 – 64)
TAA0.5 (n = 4)	23.4* (8.8 – 61.9)	993.5* (843 – 1263)	2.25 (1 – 4)	30.5 (25 – 39)	81.3 (65 – 100)	0.1	2.83* (2.1 – 3.2)	2.11* (1.9 – 2.5)	125.5* (94 – 168)

Data are expressed as the mean and range.
*$p < 0.05$ vs the control by Steel's test

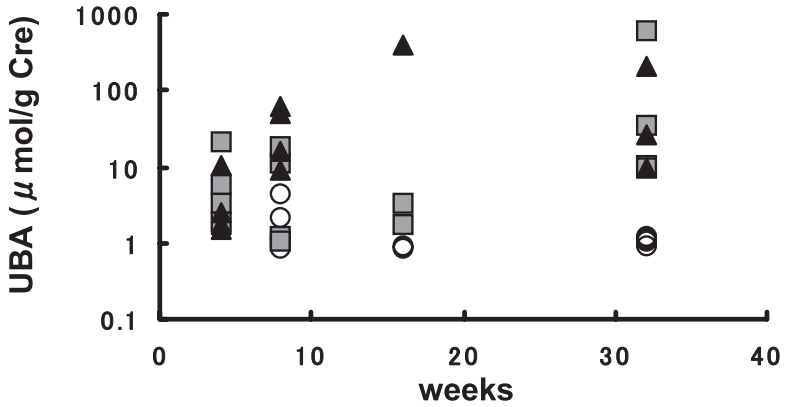

Fig. 1. UBA content in chronic TAA rats. Rats were treated with TAA in drinking water. UBA was quantified at the indicated time points. Open circles, control group; gray squares, TAA0.3 group; closed triangles, TAA0.5 group. One to four rats were analyzed for each group at the respective time. Each plot corresponds to the results for an individual rat.

icrographs of TAA-treated rats over the time-course are shown in Fig. 2. At 8 weeks, the biliary epithelium showed slight proliferation (Figs. 2A and B), indicating the development of cholestasis. After 16 weeks, there was obvious hypertrophy and hyperplasia of the biliary epithelium. Hepatocellular degeneration and necrosis characterized by vacuolization, ballooning, and eosinophilia were also observed (Figs. 2C-F). The TAA0.3 group at 32 weeks (Fig. 2E) and the TAA0.5 group at 16 and 32 weeks (Figs. 2D and F) showed micronodular cirrhosis. The nodules were separated by markedly proliferated fiber, forming pseudonodules. Marked cellular and structural atypia were observed in TAA0.5 at 16 and 32 weeks (Figs. 2D and F). The basement membranes were disordered (Figs. 2D and F). Infiltration of mesenchymal cells and polymorphonuclear cells was observed (Figs. 2D and F). Taken together, the results in Figs. 2D and F indicate the induction of intrahepatic cholangiocarcinoma.

Relationship between UBA and liver damage

We analyzed the relationship between UBA content and liver damage. Each blood marker value obtained from the acute and chronic models is plotted as a function of UBA content in Figs. 3 and 4, respectively. Correlations between UBA content and each blood indicator are shown in Table 3. In the acute model, UBA content showed a high correlation with the serum activities of AST, ALT, and ALP, and the serum content of Alb and T-Bil (Table 3). Fig. 3 shows the correlation between UBA and each indicator. In the chronic model, UBA content showed the highest correlation coefficient with serum Alb content, followed by the serum activities of γ-GTP and ALP (Table 3). Although the correlation coefficients were lower than those in the acute model, Fig. 4 suggested that the UBA content was proportional to the serum ALP activity, serum Alb content, and serum BTR.

We also analyzed the screening ability of UBA analysis to distinguish liver-damaged rats from healthy rats. The normal range for each indicator was determined as the mean ± 95% confidence interval (CI) of control rats. Values outside of this range were considered to be abnormal. The cutoff values for UBA content were 1.52 and 2.38 μmol/g Cre for the acute and chronic models, respectively. Among TAA-treated rats, 11 of 12 rats (92%) and 19 of 25 rats (76%) showed elevated UBA content in the acute and chronic models, respectively. The sensitivity, specificity, and positive predictive value of UBA analysis for the screening of rats that showed abnormal values in each blood indicator are shown in Table 4. In the acute model, the sensitivities were high, but the specificities were low in all cases. In the chronic model, UBA analysis showed high sensitivities and high specificities for serum ALP activity, serum Alb content, and serum BTR. These results indicated that UBA analysis could detect rats showing abnormal values in serum ALP activity, serum Alb content, or serum BTR effectively. The positive predictive values for these indicators were also high.

We defined “liver-damaged” rats as those showing abnormal values in one or more blood indicators. The livers of these rats were damaged to some degree. According to this criterion, 14 of 17 rats in the acute model and 23 of 33 rats in the chronic model were liver-damaged. The screening performances of UBA analysis for these liver-damaged rats are shown in the right-side column in Table 4.

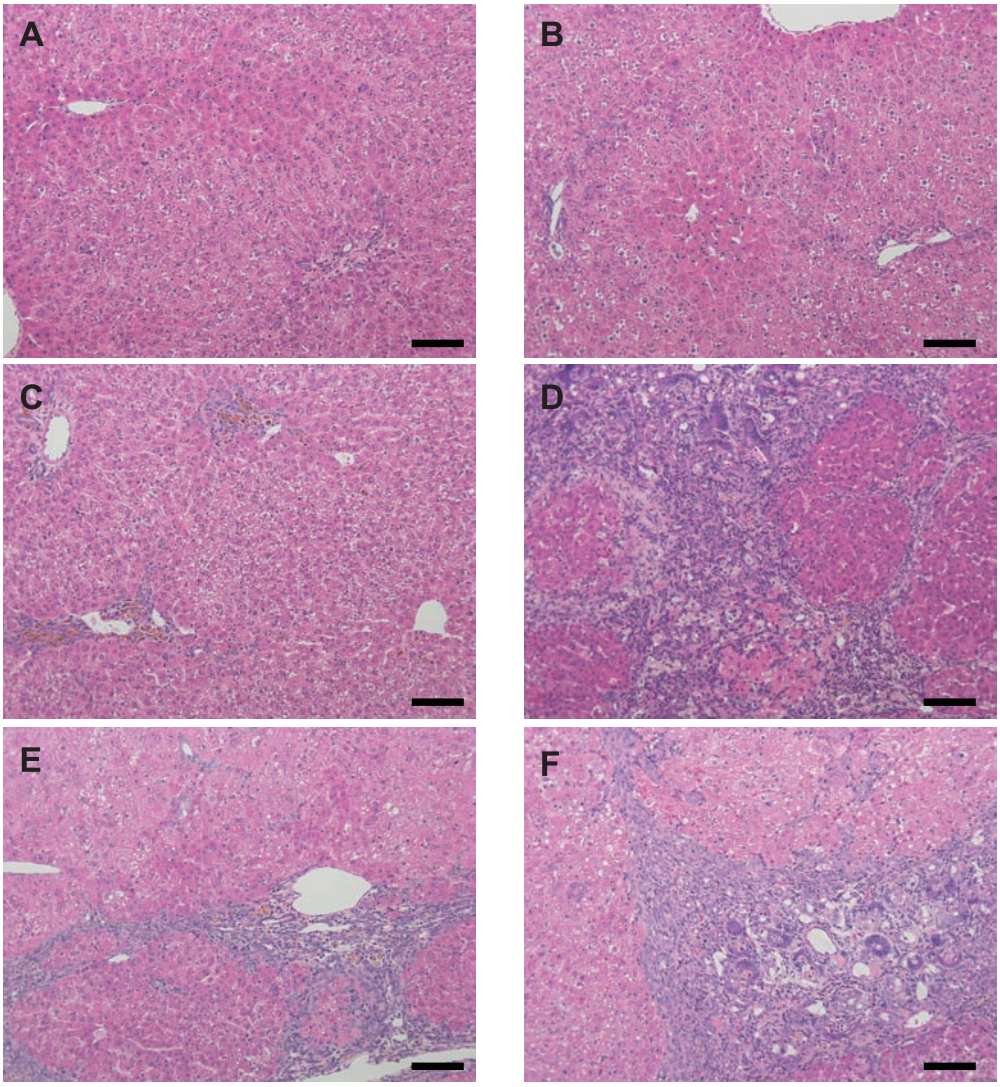

Fig. 2. Liver histology in the chronic TAA-administration model. Samples were collected 8, 16, and 32 weeks after the initiation of TAA administration. (A) TAA0.3, 8 weeks; (B) TAA0.5, 8 weeks; (C) TAA0.3, 16 weeks; (D) TAA0.5, 16 weeks; (E) TAA0.3, 32 weeks; (F) TAA0.5, 32 weeks. Original magnification, A – F ×200. Scale bars indicate 100 μm.

UBA analysis showed 79% sensitivity and 67% specificity in the acute model, and 67% sensitivity and 100% specificity in the chronic model. Positive predictive values were also high in both models.

DISCUSSION

In the present study, we assessed the efficacy of UBA as a non-invasive marker for liver damages in acute and chronic liver disease model rats with TAA treatment. In both the acute and chronic liver disease models, UBA contents were profoundly elevated with TAA treatment in a dose-dependent manner (Tables 1 and 2, Figs. 3 and 4). UBA content was correlated with blood indicators for liver damage (Table 3, Figs. 3 and 4). UBA analysis showed a remarkable ability to screen for liver damage with 79% sensitivity and 67% specificity in the acute model, and 67% sensitivity and 100% specificity in the chronic model (Table 4). These results indicate that, by analyzing excreted urine, we can predict liver damage without blood sampling with 92% and 100% positive predictive value in the acute and chronic model, respectively.

In the acute model, it was more difficult for UBA analysis to predict which blood indicators were abnormal in

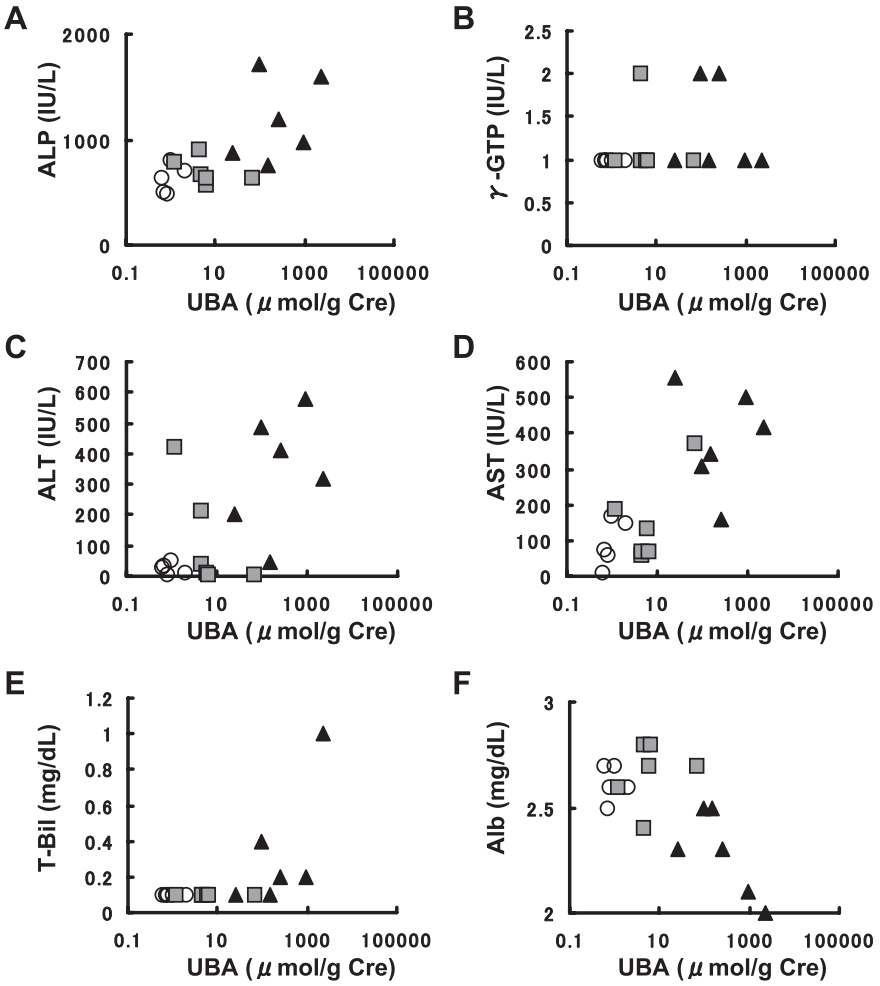

Fig. 3. Relationship between UBA content and blood biochemical markers in the acute TAA-administration model. UBA content, serum ALP activity, serum γ-GTP activity, serum ALT activity, serum AST activity, serum T-Bil content, and serum Alb content were quantified in rats with or without TAA treatment: open circles, saline-treated control group; gray squares, TAA100 group; closed triangles, TAA500 group. UBA content was plotted against serum ALP activity (A), serum γ-GTP activity (B), serum ALT activity (C), serum AST activity (D), serum T-Bil content (E), and serum Alb content (F). Each plot corresponds to the results for an individual rat. UBA content is shown in log scale, and the other contents are shown in linear scale.

rats. Liver damage was so severe in this model that UBA content and all blood indicators showed abnormal values (Table 1, Fig. 3). In the chronic model, however, some indicators showed abnormal values and the other indicators remained normal (Table 2, Fig. 4). UBA analysis showed high correlations with several indicators, especially with serum ALP activity (Tables 3 and 4, Fig. 4). UBA analysis can screen the abnormality of serum ALP activity effectively with 75% sensitivity, 78% specificity, and 90% positive predictive value (Table 4). UBA analysis also showed high screening ability for Alb and BTR with 60 – 90% of sensitivity, specificity, and positive predictive value.

Liver histological analysis revealed that TAA caused injury to the biliary epithelium, which led to cholestasis, cirrhosis, and hepatic tumor (Fig. 2). The elevation of serum activities of ALP and γ-GTP without considerable change in serum activities of ALT and AST supported the induction of cholestasis. The reduction of serum Alb content and serum BTR suggested that liver dysfunction followed cholestasis. Cholestasis seemed to be a major pathogenetic mechanism of the liver damage induced by

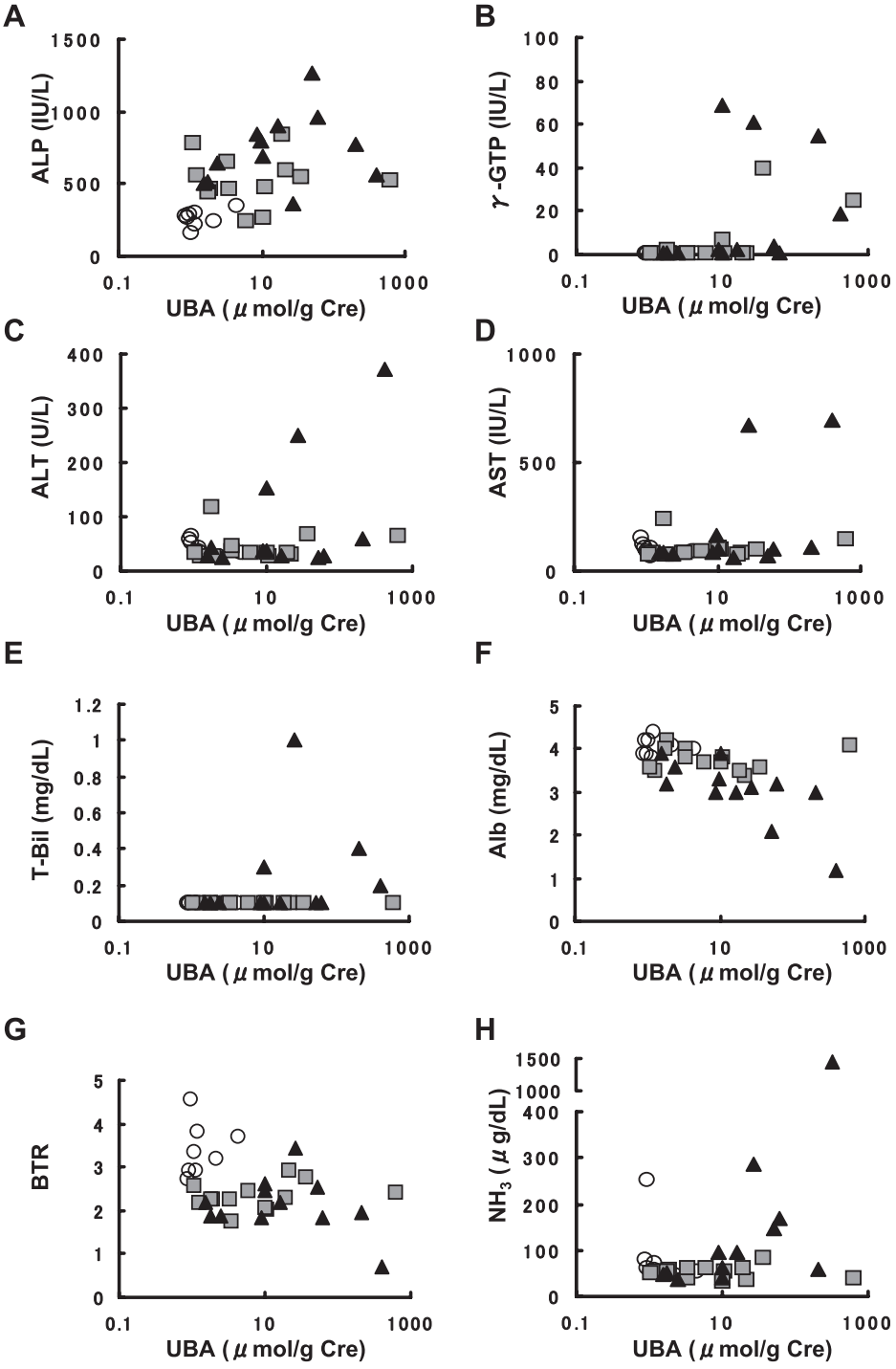

Fig. 4. Relationship between UBA content and blood biochemical markers in the chronic TAA-administration model. UBA content, serum ALP activity, serum γ-GTP activity, serum ALT activity, serum AST activity, serum Alb content, serum BTR, and blood NH_3 content were quantified in rats with or without TAA treatment: open circles, control group; gray squares, TAA0.3 group; closed triangles, TAA0.5 group. UBA content was plotted against serum ALP activity (A), serum γ-GTP activity (B), serum ALT activity (C), serum AST activity (D), serum T-Bil content (E), serum Alb content (F), serum BTR (G), and blood NH_3 content (H). Each plot corresponds to the results for an individual rat. UBA levels are shown in log scale, and the other levels are shown in linear scale.

Table 3. Correlation between $\log_{10}$ [UBA] and blood indicators

	ALP	γ-GTP	ALT	AST	T-Bil	Alb	BTR	NH_3
Acute model (n = 17)	0.692**	0.235	0.586*	0.746**	0.630**	-0.723**	n.d.	n.d.
Chronic model (n = 33)	0.488**	0.504**	0.415*	0.387*	0.286*	-0.613**	-0.424*	0.419*

Correlation coefficients (r) are shown.
n.d., not determined.
*$p < 0.05$, **$p < 0.01$ by correlation analysis.

Table 4. Screening performance (%) of UBA analysis for liver damage

	ALP	γ-GTP	ALT	AST	T-Bil	Alb	BTR	NH_3	liver-damaged†
Acute model (n = 17)									
Sensitivity	78	100	80	82	100	88	n.d.	n.d.	79
Specificity	38	36	43	50	39	44	n.d.	n.d.	67
Positive predictive value	58	25	67	75	33	58	n.d.	n.d.	92
Chronic model (n = 33)									
Sensitivity	75	91	67	57	100	71	67	80	67
Specificity	78	55	42	39	45	67	67	43	100
Positive predictive value	90	50	30	20	20	85	90	20	100

Values were considered normal in the case that they were within the range of the mean ± 95% confidence interval of control rats. Values outside out of this range were considered abnormal.
n.d., not determined.
† Rats were defined as liver-damaged in the case that one or more blood indicators showed an abnormal value.

chronic administration of TAA. These results indicated that UBA content was increased by the induction of cholestasis followed by liver dysfunction. We considered that UBA content appeared to function in a manner similar to serum ALP activity, with both indicating the induction of cholestasis. UBA content may thus be a useful non-invasive marker for cholestasis.

We speculate the mechanism of the elevation of UBA content in cholestasis as follows: Cholestasis caused the inhibition of biliary secretion from the liver, and then bile acids were accumulated in the liver, which led to the elevation of bile acids in systemic circulation. At least a part of the increased bile acids in blood were excreted from urine, resulting in the elevation of UBA content. Since the induction of cholestasis and following liver dysfunction result in the elevation of UBA content and serum ALP activity, and the reduction of serum Alb content and serum BTR, the correlations between UBA content and these three serum indicators are observed in TAA-induced cholestasis.

Although we analyzed only in TAA-treated WistarHannover rats, previous studies reported the elevation of UBA in bile duct ligated rats and the Eisai hyperbilirubinemic rats (Takita *et al.*, 1988; Lee *et al.*, 2001; Chen *et al.*, 2008). These rats are models for extrahepatic cholestasis and intrahepatic cholestasis, respectively. Wistar rats and Sprague-Dawley rats were used in these studies. The elevation of UBA content may be generally observed in rat models of cholestasis with various rat strains.

Serum activities of ALT and AST are indicators for hepatocyte necrosis. UBA analysis showed poor correlation with serum activities of ALT and AST in a chronic model (Tables 3 and 4, Fig. 4), indicating that hepatocyte necrosis does not cause the alteration of UBA content. Although simultaneous elevation of UBA content and serum activity of ALT and/or AST was observed in several rats, serum activity of ALP was also elevated in these rats. UBA content was correlated with serum activity of ALP rather than that of ALT and AST. The elevation of UBA content should be induced by cholestasis rather than hepatocyte necrosis.

The relationship between serum bile acids (SBA) content and UBA content has been reported in several species (Rudman *et al.*, 1957; Trainor *et al.*, 2003;

Balkman *et al.*, 2003; Huang *et al.*, 2007). UBA content reflects abnormally increased SBA content in cats, dogs, and human patient with liver disease. Although we have no data about correlation between SBA content and UBA content in rats, we speculate that UBA content may be correlated with SBA content in rats.

Bile acids consist of various structurally different compounds such as cholic acid and chenodeoxycholic acid. A certain amount of each bile acid is conjugated with amino acid such as glycine and taurine, and/or sulfate. Several studies revealed that the composition of bile acid was altered in human liver disease (van Berge Henegouwen *et al.*, 1976; Shoda *et al.*, 1990). We could not determine the composition of UBA in the present study, because UBA analysis used the enzyme, 3αHSD, that nonspecificically oxidized non-sulfoconjugated bile acids. We could distinguish sulfated- and non-sulfated-bile acids, but could not analyze the precise composition of UBA. This may be disadvantage for the analysis of metabolic pathway of bile acids, but this disadvantage does not affect the usefulness of UBA analysis as a screening method.

We could not detect USBA in rats by enzyme-linked assay, despite the fact that this is a useful marker for diagnosis of human liver disease (Obatake *et al.*, 2002; Shinohara *et al.*, 2005; Huang *et al.*, 2007). Efficiency of sulfoconjugation of bile acids may differ among spiecies. Bile acids are efficiently sulfated in human, hamsters, chimpanzees, and cats (Palmer, 1967; Galeazzi and Javitt, 1977; Schweng *et al.*, 1978; Trainor *et al*., 2003), but sulfation is not so efficient in rats, rabbits, monkeys, and dogs (Palmer, 1971; Nakao *et al.*, 1980; Suzuki *et al.*, 1985; Balkman *et al.*, 2003). USBA analysis may be useful in species that show efficient sulfoconjugation of bile acids. On the other hand, UBA analysis may be useful in species that do not show efficient sulfoconjugation of bile acids. USBA may be detectable in rats by more sensitive methods such as LC-MS, but the enzyme-linked method used in the present study is not available for the screening of liver damage in rats. We consider that UBA analysis is useful in rats, and that UBA analysis in rats is equivalent to USBA analysis in human for the screening of cholestasis.

We used TAA-induced liver damage model in this study, because TAA has been used as an experimental hepatotoxin that induces various types of liver damages in rodents. Single ip administration of TAA causes acute hepatitis (Mangipudy *et al*., 1995; Ramaiah *et al*., 2001). Prolonged administration of a relatively low dose of TAA causes cholestasis, cirrhosis (Müller *et al*., 1988; Fontana *et al*., 1996; Sato *et al*., 2000; Haider *et al*., 2004), and finally, a hepatic tumor (Gupta, 1955; Moreira *et al*., 1995; Yeh *et al*., 2004). The characteristic features of TAA-induced cirrhosis in rats resemble those of human cirrhosis, and TAA-treated rats are used as a suitable model of the pathology of human cirrhosis (Müller *et al*., 1988; Dashti *et al*., 1989; Sato *et al*., 2000).

TAA is metabolized by CYP2E1 to the corresponding sulfoxide and further oxidized form, sulfone, which is an unstable reactive metabolite to initiate hepatic injury by covalently binding to liver macromolecules (Ramaiah *et al*., 2001; Chilakapati *et al*., 2007). Since CYP2E1 activity shows diurnal fluctuation with a peak at dark period (Bruckner *et al*., 2002; Matsunaga *et al*., 2004), TAA toxicity is expected to show circadian rhythmicity with a peak at dark period. We administered TAA in evening, just before the initiation of dark period, in order to obtain liver damaged-rats efficiently. TAA administration in morning might diminish the hepatotoxicity of TAA, but we speculate that the relationships between UBA and blood indicators are not affected, because the mechanism of action is not altered by the timing of TAA administration.

There is a possibility that renal damage causes the elevation of UBA content. But previous studies revealed that TAA showed high specificity for liver as a target organ (Barker and Smuckler, 1974). TAA showed marginal and transient renal injury only at excessively high dose. Although we did not analyze renal injury in this study, we speculated that renal damage contributed little to the elevation of UBA in TAA-induced models.

Application of UBA content as an indicator for liver damage has several advantages over routine serum biochemical analysis. First, UBA analysis is a simple, rapid, and sensitive method. Collected urine can be directly applied to the assay after pH adjustment. Purification, derivatization, and other tedious treatments are not necessary. UBA content is measurable using no more than 5 μl of urine sample. Although we used 24 hr urine in this study, spot urine is applicable for UBA analysis.

Secondly, UBA analysis is not disturbed by diurnal fluctuations of liver function. Since some serum indicators are affected by feeding, blood samples should be collected after a certain postprandial interval in order to obtain reliable diagnostic results. SBA content is increased after feeding (LaRusso *et al*., 1978; Angelin *et al*., 1982; Nakano *et al*., 1990). Therefore, UBA content might also show postprandial elevation. However, we were able to obtain the average UBA content of the day by using 24 hr urine; therefore, postprandial duration does not affect UBA analysis.

Thirdly, UBA analysis is a non-invasive method. Urine collection exerts little stress on the animals, allowing

UBA content to be analyzed repetitively over a long period of time. The progress or reversal of the liver damage could thus be traced by UBA analysis in the same animal over a long-term experiment.

For example, UBA analysis may be useful for the analysis of HE. HE is a neuropsychiatric disorder that occurs in both acute and chronic liver disease (Blei *et al*., 2001; Ferenci *et al*., 2002). Although elevated blood ammonia seems to play an important role, the precise pathophysiologic mechanisms are not understood (Albrecht and Jones, 1999; Hazell and Butterworth, 1999). In the present study, we observed an elevation of blood NH_3 content and a reduction of serum BTR in the chronic TAA model (Table 2), indicating that this model may be a potential rat model of cirrhosis – HE. Recently, Méndez *et al*. (2008) investigated the induction of HE in TAA-induced cirrhotic rats. They conducted several behavioral tests to detect abnormalities, and reported that spatial reference memory was impaired in TAA-induced cirrhotic rats. Simultaneous analysis of these behavioral tests and UBA measurement in TAA-induced cirrhotic rats would clarify the relationship between the progress of liver damage and the induction of encephalopathy.

In conclusion, we demonstrated that UBA content was useful as a simple non-invasive indicator of liver damage in TAA-treated rats. Rats with acute or chronic liver damage were detected effectively by UBA analysis. Furthermore, the elevation of UBA content indicated the elevation of serum ALP activity and the induction of cholestasis in our models of chronic liver damage. UBA analysis may thus be useful in pathophysiological and toxicological investigations to analyze the progress or reversal of liver damage in chronic liver diseases such as cirrhosis and HE.

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