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Altered Pharmacokinetics and Hepatic Uptake of TBuMA in Ethynylestradio-Induced Cholestasis

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The objective of this study was to examine the pharmacokinetics of organic cations in intrahepatic cholestatic rats. A pretreatment with 17α-ethynylestradiol was used to induce intrahepatic cholestasis, and tributylmethylammonium (TBuMA) was used as a representative model organic cation. When [^{3}H]TBuMA was intravenously administered, the AUC value for TBuMA was significantly increased by 79% in cholestasis, and its total systemic clearance was consequently decreased by 46%. In addition, the *in vivo* hepatic uptake clearance of TBuMA from the plasma to the liver was decreased by 50% in cholestasis. The concentration of bile salts in plasma was increased by 2.1 fold in cholestatic rats. Since TBuMA forms ion-pair complexes with anionic components such as bile salts, the decreased hepatic uptake of TBuMA in cholestasis may be due to a change in endogenous components, e.g., bile salts in the plasma. In isolated normal hepatocytes, the uptake clearance for TBuMA in the presence of cholestatic plasma was decreased by 20% compared with normal plasma. Therefore, we conclude that the inhibition of the hepatic uptake process by the cholestasis may be in part due to the increased formation of ion-pair complexes of TBuMA with bile salts in the plasma.

Key words: Hepatic uptake, Organic cation, TBuMA, Cholestasis, Ion-pair complex, Hepatocyte

INTRODUCTION

Cholestasis is defined as the stagnation of bile flow and is typically accompanied by increased levels of biliary substances in the blood. Common bile duct stones, sclerosing cholangitis, or cancer of the biliary tree or the pancreas all can obstruct bile flow and produce extrahepatic cholestasis. In contrast, drug-induced 'intrahepatic cholestasis' is apparently due to the biochemical interference of cellular function (James, 1986). It is well known that estrogens cause reversible intrahepatic cholestasis in humans and rodents. Intrahepatic cholestasis occurs in susceptible women during pregnancy, the administration of oral contraceptives and postmenopausal hormone replacement therapy (Shreiber and Simon, 1983). Given these clinical implications, experimental cholestasis induced by the administration of estrogen in rodents, mainly 17α-ethynylestradiol (EE), has been widely used as an experimental model to assess the mechanisms involved in estrogen-induced cholestasis (Crocenzi *et al.*, 2001).

Physiologically, the concentration of bile salts, cholesterol, and bilirubin in plasma was reported to be significantly increased in EE-induced cholestasis (Meng *et al.*, 1997). An abnormality in liver homoestasis by cholestasis is likely to have a significant effect on the pharmacokinetics of xenobiotics. A number of reports have recently reported on changes in the pharmacokinetics of drugs in EE-induced cholestasis (Hung *et al.*, 2005; Schwab *et al.*, 2006). In terms of drug transporters, it has been reported to trigger the expression of Na^+/bile acid (Ntcp) and organic anion transporters (Oatps) in rats (Geier *et al.*, 2003). However, these studies have mainly involved the use of organic anionic drugs. Accordingly, the objective of this study was to investigate the effects of intrahepatic cholestasis on the hepatic pharmacokinetics of organic cations (OCs) in rats.

For this purpose, tributylmethylammonium (TBuMA, Mw: 200) was used as a representative OC, since the hepatic uptake and canalicular excretion of this compound is known to be mediated by a sinusoidal transporter (i.e., an OC transporter 1, OCT1) (Koepsell, 1998; Hong *et al.*,

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2000), and a canalicular transporter (i.e., an ATP-dependent P-glycoprotein, P-gp, system, Song *et al.*, 1999), respectively. TBuMA has advantages over other OCs as a model compound in that it does not bind to proteins either in the plasma or the liver cytosol, and is not metabolized in the body (Neef *et al.*, 1984).

In this study, therefore, the effect of EE-induced cholestasis on the pharmacokinetics of TBuMA was investigated and its *in vivo* hepatic clearance was determined. In addition, the effect of cholestatic plasma on the *in vitro* hepatic clearance of TBuMA was investigated using normal and cholestatic hepatocytes.

MATERIALS AND METHODS

Materials

[^{3}H]TBuMA (specific activity, 1.0 Ci/mmol) was synthesized according to the method of Neef *et al.* (1984) by reacting an excess of the corresponding tertiary amine with [^{3}H]methyliodide (Amersham, Arlington Heights, IL; specific activity, 85 Ci/mmole). The radiochemical purities of synthesized [^{3}H]TBuMA were > 99.0%, as determined by thin-layer chromatography (data not shown). All other reagents employed were of the highest grade commercially available.

Induction of intrahepatic cholestasis by 17α-ethynylestradiol

Male Sprague-Dawley rats, 7 to 8 weeks of age, were used in the study. Cholestasis was induced by the daily subcutaneous injection of 17α-ethynylestradiol (EE) in 1,2-propanediol (5 mg/mL/kg) for 5 consecutive days. For the group of normal rats, 1,2-propanediol was administered only. All studies were performed 24 h after the last administration of EE. To confirm intrahepatic cholestasis, the total amount of bile salts excreted into bile duct for the 3 h period was determined using an enzymatic-fluorometric assay (Choi *et al.*, 2005). Briefly, the bile duct was cannulated with polyethylene tubing (PE-10; Clay Adams, Parsippany, NJ), and bile was collected at 30 min intervals up to 3 h. The enzymatic reaction was quenched by the addition of 3 mL of ice cold water and the fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 465 nm.

In vivo systemic pharmacokinetic study

Under light ether anesthesia, the femoral arteries and veins of normal and cholestatic rats were cannulated with polyethylene tubing (PE-50) for blood sampling and the administration of TBuMA, respectively. After recovery from the surgery, the animals received a bolus dose of 13.3 μmole of [^{3}H]TBuMA (13.2 μCi)/kg via the femoral vein.

Blood samples were collected at appropriate intervals over a 24 h period, and each plasma sample was separated by centrifugation at 3,000 rpm for 10 min. The concentrations of [^{3}H]TBuMA in the plasma were quantified by means of a liquid scintillation counting (LSC System 1409, Wallac). The area under the plasma concentration-time curve from time zero to 24 h (AUC) was calculated by the trapezoidal rule. The total systemic clearance (CL_t) was determined by dividing the administered dose of TBuMA by its AUC. For the determination of the concentration of bile salts in plasma, blank plasma samples were collected before the administration of TBuMA.

Hepatic uptake clearance *in vivo*

For estimating *in vivo* hepatic uptake clearance (CL_{uptake}), [^{3}H]TBuMA (13.3 μmole/kg) was intravenously administered to normal and cholestatic rats, and blood samples were collected at 30 sec intervals. The animals were sacrificed at 3 min after the administration of TBuMA and the liver was immediately dissected, and liver samples were collected and weighed. The samples were solubilized in Soluene-350 (Packard) and counted for radioactivity. The radioactivity in an aliquot of blood and liver sample was determined by liquid scintillation counting. *In vivo* uptake clearance, CL_{uptake}, was estimated by equation 1, where $X_{liver}(t)$ represents the amount of TBuMA in the liver at time t and AUC_t the area under the curve up to time t.

$$CL_{uptake} = X_{liver}(t)/AUC_t \quad (1)$$

Uptake into hepatocytes *in vitro*

Hepatocytes were obtained from normal and cholestatic rats according to the procedure described by Han *et al.* (1999). The cell suspension (2.5×10^6 cells/mL) was pre-incubated in the medium containing 20% of the plasma from normal or cholestatic rats for 5 min at 37°C. [^{3}H]TBuMA (50, 100, 150 μM) was added to the suspension. An aliquot (200 mL) of the suspension was sampled at 120 sec, and placed in a centrifuge tube (0.4 mL) containing KOH (50 μL, 3 M) and silicone/mineral oil (100 μL; density, 1.015). Subsequent centrifugation at 50 g for 5 sec resulted in the sedimentation of hepatocytes through the oil layer into the KOH layer. The bottom KOH layer, containing hepatocytes, was collected, and the radioactivity was determined. The amount of TBuMA in the hepatocytes was expressed in pmol/min/10^6 cells.

RESULTS AND DISCUSSION

Induction of intrahepatic cholestasis by 17α-ethynylestradiol

In order to confirm the induction of cholestasis by the administration of EE, the bile flow rate, total bile salts excreted into bile duct, and the concentration of bile salts

in plasma were determined. As shown in Fig. 1A, the bile flow rate for cholestatic rats was 45.6% that of normal rats (15.27 ± 1.45 μL/min for normal, 6.97 ± 0.89 μL/min for cholestasis). However, the concentration of bile salts was not altered as the result of cholestasis (27.40 mM ± 2.91 for normal, 27.46 ± 2.28 mM for cholestasis, Fig. 1B). As a consequence, total bile salts excreted into bile duct for 24 h were decreased in the case of cholestasis (12555 ± 1713 nmole for normal, 5663 ± 920 nmole for cholestasis, Fig. 1C). On the other hand, the concentration of bile salts in plasma was increased by 2.1 fold in cholestatic rats (27.10 ± 3.47 μM for normal, 57.30 ± 5.55 μM for cholestasis). This clearly indicates that cholestasis was successfully induced by the administration of EE, as reported previously (Crocenzi *et al.*, 2001).

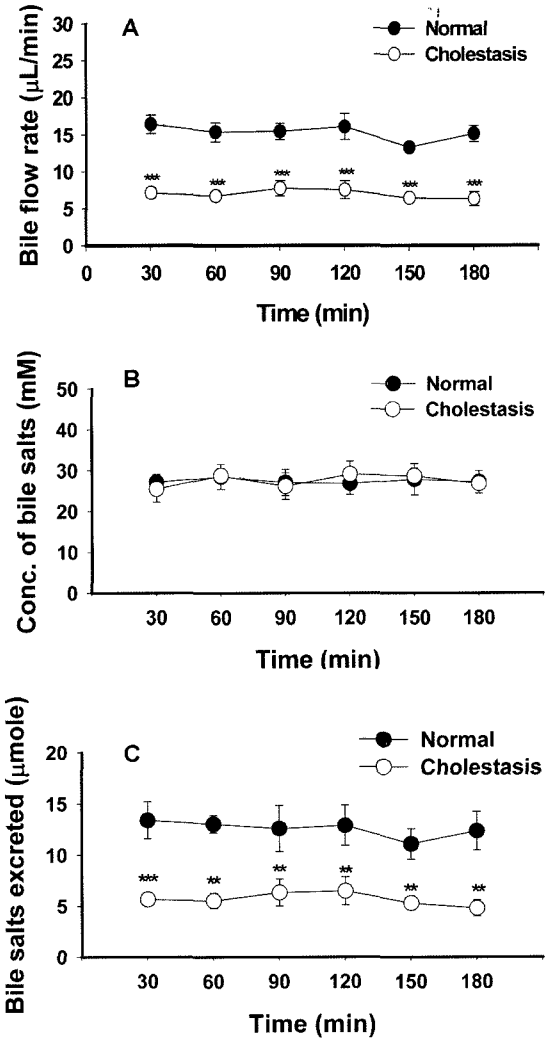

Fig. 1. Induction of intrahepatic cholestasis by 17α-ethynylestradiol. A, bile flow rate; B, concentration of bile salts in the plasma; C, total amount of bile salts excreted into the bile duct for a period of up to 3 h. Each data point represents the mean ± S.D. of four animals (●, normal; ○, cholestatic rats).

In vivo systemic pharmacokinetics of TBuMA

The temporal profiles for the plasma concentration of TBuMA after intravenous administration at a dose of 13.3 μmole/kg in normal and cholestatic rats are shown in Fig. 2, and the relevant pharmacokinetic parameters are listed in Table I. The plasma concentration of TBuMA followed a triexponential decline in both normal and cholestatic rats. The mean half-lives of TBuMA at the terminal phase were extremely long in both normal and cholestatic rats (in excess of 6 h), in agreement with previous studies (Hong *et al.*, 2000; Neef *et al.*, 1984). The mean plasma concentrations were higher in cholestatic rats compared to normal rats, leading to a significant increase (79%) in AUC values (Table I). As a consequence, the total systemic clearance, CL_t, was decreased by about 46% by cholestasis. Since TBuMA is not protein bound in either the plasma or liver cytosol and is not metabolized (Neef *et al.*, 1984), these results suggest that cholestasis interferes with the excretion of TBuMA.

Hepatic uptake clearance *in vivo* & *in vitro*

In our previous studies using rats with the experimental hepatic injury induced by carbon tetrachloride (CCl_4), the

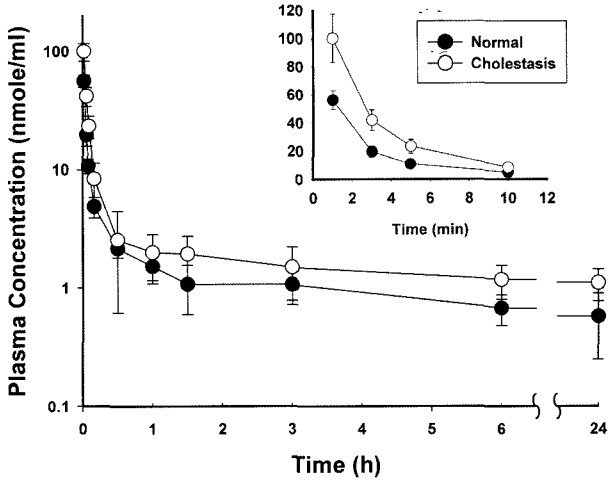

Fig. 2. Temporal profiles for the plasma concentration of TBuMA in normal (●) and cholestatic rats (○). Each data point represents the mean ± S.D. of four animals.

Table I. The effect of 17α-ethynylestradiol-induced cholestasis on the pharmacokinetics of TBuMA after i.v. administration (13.3 μmole /kg)

Pharmacokinetic parameters	Normal	Cholestasis
AUC (μM×min)	1235 ± 370	2214 ± 405 *
CL_t (mL/min/kg)	11.22 ± 2.24	6.15 ± 1.11*

[a] Each data point is expressed as the mean ± S. E. of four separate experiments.
[b] Calculated up to 24 h
* $p<0.01$ for the normal group by the unpaired student's t-test.

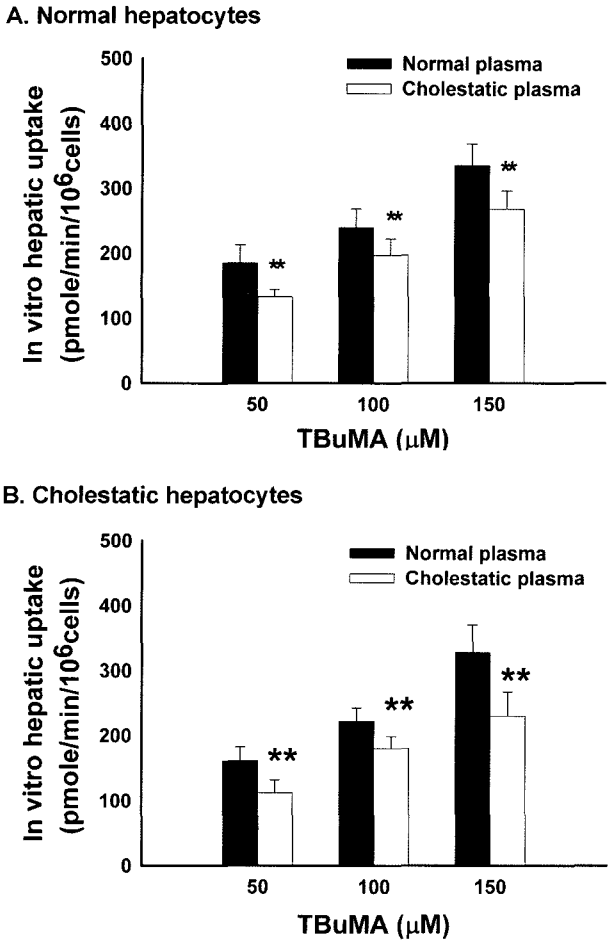

Fig. 3. *In vitro* hepatic uptake clearance of TBuMA in the isolated hepatocytes from normal (A) and cholestatic (B) rats in the presence of normal (solid bars) and cholestatic plasma (open bars). Each data point represents the mean ± S.D. of three different preparations. **; statistically significant ($p < 0.05$).

AUC of TBuMA was increased and its systemic clearance was decreased because of the selective inhibition of transporters mediating the transport of OCs across the sinusoidal membrane in the liver (Hong *et al.*, 2000). It also has been reported that TEA shares the same transporter (OCT1) with TBuMA in the process of hepatic uptake, and its plasma concentrations were increased by cholestasis (Denk *et al.*, 2004). These data suggest that the decreased systemic clearance may be due to the decreased transport across the sinusoidal membrane from the blood to the liver in cholestasis, and prompted us to compare the uptake clearance of TBuMA *in vivo* and *in vitro* between normal and cholestatic rats. When [^{3}H]TBuMA (13.3 μmole/kg) was intravenously administrated to normal and cholestatic rats, its CL_{uptake}, estimated from the concentration in the liver divided by the area under the curve up to the initial time (3 min), was decreased by 50% in cholestasis (0.52 ± 0.08 mL/min/g for normal, 0.26 ± 0.04 ml/min/g for cholestasis).

It is a well known fact that TBuMA, an OC, forms ion-pair complexes with anionic components such as bile salts, thus increasing its lipophilicity (Song *et al.*, 2001). It should also be noted that the OCT1 is responsible for the hepatic uptake of TBuMA across the sinusoidal membrane in the liver, and shows higher affinity to hydrophilic substrates (Neef *et al*, 1984), suggesting that the ion-pair complexes may not be a good substrate for the transporter. On the other hand, the amount of bile salts in plasma was increased by EE-induced cholestasis, and may be associated with the decrease in the hepatic uptake of TBuMA in cholestasis. To verify this hypothesis, hepatic uptake *in vitro* was determined. In the presence of plasma of cholestatic rats the *in vitro* hepatic uptake clearance of TBuMA was decreased by 20% compared with that of normal rat plasma (Fig. 3A). This was confirmed at three different concentrations (50, 100, 150 μM) as well as in isolated cholestatic hepatocytes (Fig. 3B). We speculate that the affinity of TBuMA for the hepatic uptake transporter (OCT1) decreased with the ion-pair complexation with certain endogenous compounds (Song *et al.*, 2001), resulting in a decrease in hepatic uptake clearance and increase in AUC.

CONCLUSION

In systemic pharmacokinetic studies, the area under the curve (AUC) for TBuMA was increased and its systemic clearance (CL_t) was decreased. In addition, the *in vivo* and *in vitro* hepatic uptake clearances of TBuMA across the sinusoidal membrane were decreased in 17α-ethynylestradiol (EE)-induced cholestasis. Therefore, we conclude that the inhibition of the hepatic uptake process by the cholestasis may be in part due to the increased formation of ion-pair complexes of TBuMA with bile salts in the plasma.

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