Effect of glutathione depletion on removal of copper from LEC rat livers by tetrathiomolybdate

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a b s t r a c t

Tetrathiomolybdate (TTM) is a powerful and selective copper (Cu) chelator that is used as a therapeutic agent for Wilson disease. TTM is the sole agent that can remove Cu bound to metallothionein (MT) in the livers of Long–Evans rats with a cinnamon-like coat color (LEC rats). However, the administration of excess TTM causes the deposition of Cu and molybdenum (Mo) in the liver. In the present study, the effect of hepatic glutathione (GSH) depletion on the removal of Cu from the livers of LEC rats was evaluated to establish an effective therapy by TTM. Pretreatment with L-buthionine sulfoximine (BSO), a depletor of GSH in vivo, reduced the amounts of Cu and Mo excreted into both the bile and the bloodstream, and increased the amounts of Cu and Mo deposited in the livers of LEC rats in the form of an insoluble complex 4 h after the TTM injection. The results suggest that GSH depletion creates an oxidative environment in the livers of LEC rats, and the oxidative environment facilitates the insolubilization of Cu and Mo in the livers of LEC rats after the TTM injection. Therefore, the effect of TTM on the removal of Cu from the liver was reduced in the oxidized condition. Wilson disease patients and LEC rats develop liver injury caused by oxidative damage. From a clinical viewpoint, increasing in the GSH concentration is expected to enhance the effect of TTM.

1. Introduction

Wilson disease causes the abnormal accumulation of copper (Cu) in the liver as a result of the dysfunction of a Cu-transporting ATPase (ATP7B) [1–3]. The abnormally accumulating Cu in the liver exists in the form bound to metallothionein (MT) to detoxify Cu. However, the accumulation of Cu beyond the amount required for MT synthesis causes fulminant hepatitis as one of the clinical symptoms. Although Wilson disease is an inherited disease, some drug therapies can retard the onset of such a clinical symptom. The oral administration of zinc (Zn) inhibits the absorption of Cu from food and drinking water by inducing MT in the gastrointestinal tract [4]. Treatment with Cu chelating agents also effectively inhibits Cu absorption [5–7]. However, those drugs cannot remove Cu accumulating in the form bound to MT [8] and the chelators also sequester other transition metals, resulting in the disruption of the metabolism of such essential metals as Zn and iron. Tetrathiomolybdate (TTM) acts as an anti-Cu agent in the body [9–11] and is used to suppress the toxicity of Cu in Cu-poisoned animals [12,13]. Recently, TTM is used as an anti-angiogenic agent because Cu is required in angiogenesis during tumor progression [14]. TTMis used to treat neurological presentation ofWilson disease because it is the only agent that can remove Cu bound to MT [15]. Although 50% and 26% of patients treated with penicillamine and trientine which were other clinical Cu chelators showed neurologic deterioration, TTM showed only 4.0% risk of being associated with neurologic worsening [16,17]. However, hepatotoxicity of TTM was commonly observed despite its neurological adverse effects were few [17]. The unique mechanisms underlying the removal of Cu from MT by TTMand the emergence of adverse effects were clearly shown in a complete animal model of Wilson disease, the Long–Evans rat that has a cinnamon-like coat color (LEC rat) [18,19]. TTM forms three complexes with Cu bound to MT depending on the molar ratio of TTM to Cu. First, TTM forms an MT/TTM complex through a sulfur (of TTM)–Cu–sulfur (of cysteine in MT) bridge at a molar ratio less than 1.When themolar ratio is between 1 and 2, TTM forms a soluble Cu/TTM complex when Cu is liberated from MT. This complex is the form of Cu excreted from the liver. Finally, TTM forms an insoluble Cu/TTM complex at the molar ratio greater than 2, and this complex is deposited in the liver [18]. These step-by-step reactions of TTM are specific to Cu and Cu sequestered by thiols, such as MT and Cu chaperons [19]. On the other hand, both chronic and acute adverse effects of TTM have been suggested. The chronic effect is Cu deficiency caused by the continuous administration of TTM. The acute effect of TTM originates from sulfide that is generated by the decomposition (hydrolysis) of TTM in the acidic condition. Therefore, oral and/or consecutive administration is not

recommended to avoid the adverse effects of TTM. Approximately 70% of Cu removed from the liver is excreted into the bile as the soluble Cu/TTM complex and the remaining 30% is excreted into the bloodstream in the same form as that excreted into the bile [20]. Although the Cu/TTM complex in the bile is eventually excreted from the body, the complex in the bloodstream disappears with a long half-life, and is thereafter redistributed to other tissues. Therefore, the bile is a better excretion route than the bloodstream from the viewpoint of clinical efficacy. The soluble Cu/TTM complex in the bile is selectively bound to albumin in vitro. Although the actual binding sites of the complex on albumin are not known yet, disulfide bonds are suggested (unpublished data). In this study, we postulated that the soluble Cu/TTM complex conjugates with glutathione (GSH) in the liver and is then transported by a canalicular multispecific organic anion transporter. The effect of a GSH depletor, L-buthionine sulfoximine (BSO), on the biliary excretion of the soluble Cu/TTM complex was evaluated.

2. Materials and methods

2.1. Reagents and animals

The ammonium salt of TTM was prepared by introducing hydrogen sulfide into a molybdate solution, as already reported elsewhere [19]. L-buthionine-[S,R]-sulfoximine (BSO) and GSH reductase were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Ammonium polymolybdate, 5,5′ -dithiobis(2-nitrobenzoic acid) (DTNB), and other chemicals were purchased from Wako Pure Chemicals Ind. (Osaka, Japan). All reagents were of the highest grade. All animal experiments were carried out according to the "Principles of Laboratory Animal Care" (NIH version, revised 1996) and the Guidelines of the Animal Investigation Committee, Graduate School of Pharmaceutical Sciences, Chiba University, Japan. Male LEC rats were a gift from Professor K. Matsumoto, Tokushima University School of Medicine, Tokushima, Japan. Male Wistar rats (normal rats) were purchased from Clea Japan Co., Tokyo, Japan. The animals were housed in a conventional animal room maintained at 25 ± 1 °C and 55% relative humidity with a 12-h light period, and were fed a standard diet (CE-2; Clea Japan Co.) and tap water ad libitum.

2.2. Animal experiments

Three male LEC rats (8 weeks of age) received an intraperitoneal injection of BSO dissolved in saline at the dose of 4 mmol/kg body weight. Three other rats injected with saline served as control. Bile collection was started 2 h after the BSO injection by bile duct cannulation under ether anesthesia. Both groups of rats received an intravenous injection of TTM at the dose of 10 mg/kg body weight 3 h after the BSO injection. The dose (10 mg/kg body weight) was selected in accordance with our previous studies [18,19,21]. Bile was collected every hour up to 4 h after the TTM injection. Both groups of rats were dissected 4 h after the TTM injection under ether anesthesia, and the livers and serum were collected and stored at −30 °C.

2.3. Sample preparation and determination of concentrations of metals and total GSH

The livers were homogenized in 4 volumes of 50 mM Tris–HCl buffer solution, pH 7.4, which had been bubbled with 99.999% nitrogen, under an atmosphere of nitrogen with a glass-Teflon homogenizer. The homogenate was ultracentrifuged at 105,000 g for 60 min at 2 °C to prepare the supernatants. The bile, liver homogenate and supernatant, and serum were wetashed with a mixture of nitric acid and perchloric acid (4/1, v/v), and then the concentrations of Cu and molybdenum (Mo) were determined with an ICP-MS

(HP 4500; Agilent Technologies, Hachioji, Japan) at m/z=65 and 95, respectively. The concentration of total GSH in the liver supernatant and bile was determined by the enzymatic recycling method according to Owens and Belcher [22].

2.4. HPLC–ICP-MS analysis

A 0.1 ml portion of the serum was subjected to HPLC–ICP-MS analysis on a Shodex Asahipak GS-520 column (7.5×500 mm, Shodex, Tokyo) by elution with 50 mM Tris–HCl buffer solution, pH 7.4, at a flow rate of 1.0 ml/min. This was followed by the direct introduction of the eluate into an ICP-MS. Cu and Mo in the eluate were monitored continuously at m/z=65 and 95, respectively.

2.5. Statistics

The results are presented as the means±standard deviation (S.D.) of three samples. Statistical analysis was performed with the Student's t-test. The significance level was set at pb0.05 (*).

3. Results

3.1. Effects of BSO pretreatment on excretion of Cu from liver to bile in LEC rats

Biliary GSH concentration showed significant decreases on pretreatment with BSO from 1 h before to 4 h after the TTM injection (Fig. 1A). On the other hand, BSO pretreatment did not affect bile flow throughout the experimental period (Fig. 1B). Although the amounts of Cu and Mo in the bile started to increase with time after the TTM injection irrespective of the pretreatment with BSO, the cumulative amounts of Cu and Mo excreted into the bile of rats pretreated with BSO were smaller than those without BSO pretreatment (Fig. 2). This suggests that the BSO pretreatment reduces the amounts of Cu and Mo excreted into the bile. The molar ratio of Cu to Mo in the bile of rats with and without pretreatment with BSO was always unity, suggesting that the Cu and Mo excreted into the bile had an identical form in both groups of rats, i.e., the soluble Cu/TTM complex was excreted into the bile even though LEC rats were pretreated with BSO. The pretreatment with BSO also reduced GSH concentration in the liver supernatants at 4 h after the TTM injection (Fig. 3). It is known that Cu concentration in the whole livers of LEC rats at 8 weeks of age is around 200 μg/g, and most Cu is present in the form bound to MT in the supernatant fraction [23,24]. Cu concentration in the liver at 4 h after the TTM injection was 132.8±16.3 μg/g. This suggests that approximately 33.6% of Cu was removed by the single intravenous injection of TTM without the BSO pretreatment. The remaining Cu in the liver after the TTM injection was recovered in the supernatant fraction (Fig. 4A). On the other hand, the pretreatment with BSO led to less removal of Cu by the TTM injection, reflecting less Cu excretion into the bile (Figs. 1A and 4A). Furthermore, as the Cu concentrations of whole livers and liver supernatants were 189.0±23.9 and 101.1±23.8 μg/g, respectively (Fig. 4A,+BSO), approximately 46.5% of Cu in the liver at 4 h after the TTM injection was insolubilized by the pretreatment with BSO. The concentration of Mo in the whole liver was increased by the pretreatment with BSO at 4 h after the TTM injection (Fig. 4B). However, Mo that increased in the livers of LEC rats treated with BSO and TTM was not recovered in the supernatant fraction (Fig. 4B,+BSO). These results suggest that GSH depletion in the livers of LEC rats facilitates the insolubilization of the soluble Cu/TTM complex, resulting in the decreased excretion of Cu and Mo from the liver.

3.2. Effects of BSO pretreatment on concentrations of Cu and Mo in serum

Cu and Mo were also excreted into the bloodstream at 4 h after the TTM injection, and the molar ratio of Cu to Mo was around unity irrespective of the BSO pretreatment (Fig. 5). However, GSH depletion reduced the amounts of Cu and Mo excreted into the bloodstream (Fig. 5). Cu and Mo excreted into the bloodstream were selectively bound to albumin in both groups of rats, and the amounts of Cu and Mo bound to albumin in the pretreated group were apparently smaller than those

in the non-pretreated one (Fig. 6). The molar ratio of Cu to Mo bound to albumin was unity, i.e., Cu and Mo formed the Cu/TTM complex even if LEC rats were pretreated with BSO. Although the major Cu binding protein in the serum is ceruloplasmin in normal rats, LEC rats secrete an apo-form (containing no Cu) of ceruloplasmin before the onset of hepatitis [25]. Therefore, Cu was not detected at the retention time of 11.0 min, which corresponds to that of ceruloplasmin in LEC rat samples. The minor Mo that was eluted at the retention time of 14.9 min was assigned to molybdate that was generated by the hydrolysis of TTM, as reported previously [18]. These results also support that GSH depletion in the livers of LEC rats enhances the insolubilization of the soluble Cu/TTM complex and then reduces the amount of the complex excreted into both bile and bloodstream.

4. Discussion

We postulated that the soluble Cu/TTM complex is present in the form conjugated with GSH in the liver, and the conjugate is excreted into the bile. Therefore, we predicted that the pretreatment with BSO, which depletes GSH in the liver, reduces the amount of the soluble Cu/TTM complex excreted into the bile. Indeed, the pretreatment with BSO reduced the amount of the complex excreted into the bile. Furthermore, the pretreatment with BSO reduced also the amount of the soluble Cu/TTM complex excreted from the liver into the bloodstream (Figs. 2 and 5). Reflecting these findings, larger amounts of Cu and Mo were recovered in the livers of the BSOpretreated rats than in the livers of the non-pretreated rats (Fig. 4). Therefore, the decreased excretion of the soluble Cu/TTM complex from the liver is thought to be caused by not only inhibiting GSHconjugate formation but also enhancing the polymerization of the Cu/TTM complex, i.e., the insoluble Cu/TTM complex. The spontaneous onset of acute hepatitis (jaundice) occurs at around 15 weeks of age in LEC rats [26]. The onset is caused by oxidative stress induced by excess Cu and MT acting as a pro-oxidant [27]. LEC rats that recovered from jaundice succumbed to chronic hepatitis, i.e., the livers of such LEC rats may be continuously exposed to oxidative stress and become depleted in GSH. In the case of Wilson disease patients, the same oxidative damage as that in LEC rats was observed [28]. After the onset of jaundice, the livers of Wilson disease patients and LEC rats may be in the same situation as the livers of LEC rats treated with BSO. On the other hand, the livers of Wilson disease patients and LEC rats may be in the reduced condition due to MT acting as an anti-oxidant. Therefore, it is concluded that TTM more effectively removes Cu from the livers of Wilson disease patients or LEC rats before the onset of liver injury than after the onset. The results also suggest the possibility of a new therapeutic application of TTM, i.e., the effect of TTM is enhanced when used in combination with such reducing agents as N-acetyl cysteine or glutathione ester [29,30]. It was confirmed that these reducing agents effectively increase the intracellular concentration of GSH in vivo. The combination therapy of TTM and these agents must be explored in future studies.

5. Conclusion

Pretreatment with BSO reduced the amounts of Cu and Mo excreted into both the bile and the bloodstream and increased the amounts of Cu and Mo deposited in the livers of LEC rats in the form of the insoluble complex. These results suggest that GSH depletion creates an oxidative environment in the livers of LEC rats, and the oxidative environment facilitates the insolubilization of Cu and Mo in the livers of LEC rats by TTM injection.

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Figures

Fig. 1. L-Buthionine sulfoximine, a glutathione depletor, reduced biliary excretion of glutathione but not bile flow in LEC rats. LEC rats (3 rats/group) received an intravenous injection of TTM at the dose of 10 mg/kg body weight 3 h after the pretreatment with BSO at the dose of 4 mmol/kg body weight (closed columns) or saline (open columns). The 1 h bile was collected from 1 h before the TTM injection until 4 h after the TTM injection. The total concentration of GSH (A) was determined according to the method of Owens and Belcher [22]. Bile flow (B) is shown as weight per hour. Asterisk (*) indicates the significance level at $p<0.05$.

Fig. 2. L-Buthionine sulfoximine inhibited the excretion of Cu and Mo into bile after TTM injection of LEC rats. The concentrations of Cu (A) and Mo (B) in the same bile as that used in Fig. 1 were determined with an ICP-MS. Closed and open symbols indicate BSO and saline-treated groups, respectively. Asterisk (*) indicates the significance level at $p<0.05$.

Fig. 3. L-Buthionine sulfoximine reduced the concentration of glutathione in the liver supernatants of LEC rats 4 h after TTM injection. The livers were removed from the same rats as those used in Fig. 1, 4 h after the TTM injection, and the total concentration of GSH in the supernatants was determined according to the method of Owens and Belcher [22]. Asterisk (*) indicates the significance level at $p<0.05$.

Fig. 4. Effect of L-buthionine sulfoximine on the concentrations of Cu and Mo in the liver homogenates and supernatants of LEC rats 4 h after TTM injection. The concentrations of Cu (A) and Mo (B) in the homogenates (open columns) and the supernatants (closed columns) of the same livers as those used in Fig. 3 were determined with an ICP-MS.

Fig. 5. Effect of L-buthionine sulfoximine on the concentrations of Cu and Mo in the serum of LEC rats 4 h after TTM injection. The concentrations of Cu (A) and Mo (B) in the serum prepared from the same rats as those used in Fig. 1 were determined with an ICP-MS.

Fig. 6. L-Buthionine sulfoximine reduced the amounts of Cu and Mo selectively bound to albumin as the Cu/TTM complex in the serum of LEC rats 4 h after TTM injection. The serum was collected 4 h after the TTM injection. The serum of Wistar rats without any treatments was also collected to indicate the normal distributions of Cu and Mo in the serum. A 0.1 ml portion of the serum was subjected to HPLC–ICP-MS analysis on a Shodex Asahipak GS-520 column by elution with 50 mM Tris–HCl buffer solution, pH 7.4, at a flow rate of 1.0 ml/min, followed by direct introduction of the

eluate into an ICP-MS. Cu and Mo in the eluate were monitored continuously at m/z=65 and 95, respectively. The vertical bar indicates the detection levels of Cu (left elution profiles) and Mo (right elution profiles).

Fig.1

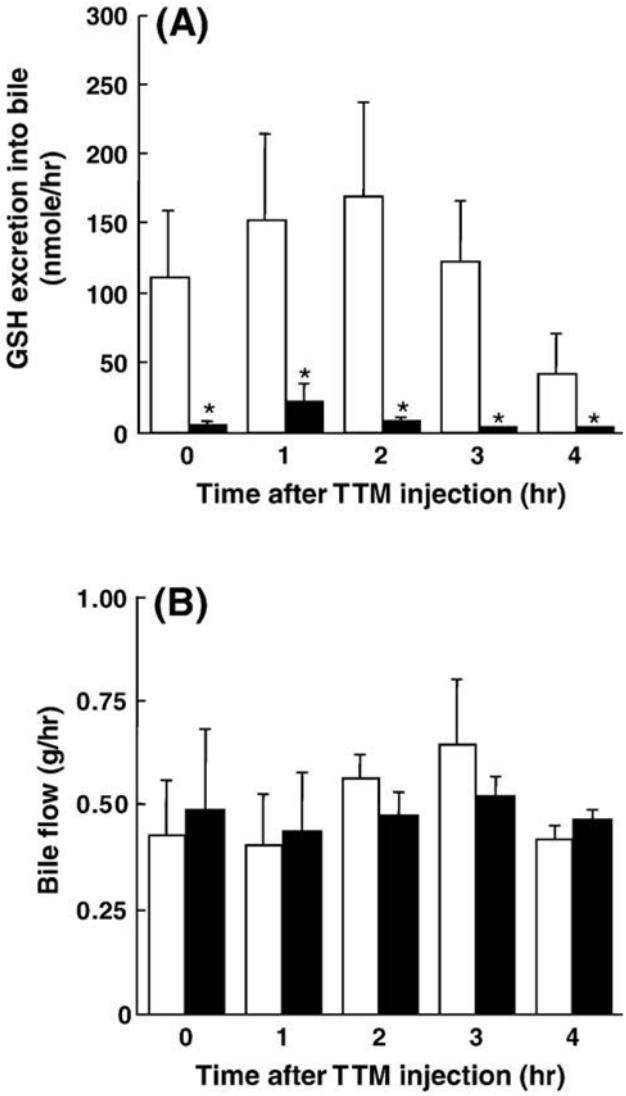

(A)
GSH excretion into bile (nmole/hr)
300
250
200
150
100
50
0
0
1
2
3
4
Time after TTM injection (hr)
(B)
Bile flow (g/hr)
1.00
0.75
0.50
0.25
0
0
1
2
3
4
Time after TTM injection (hr)

Fig.2

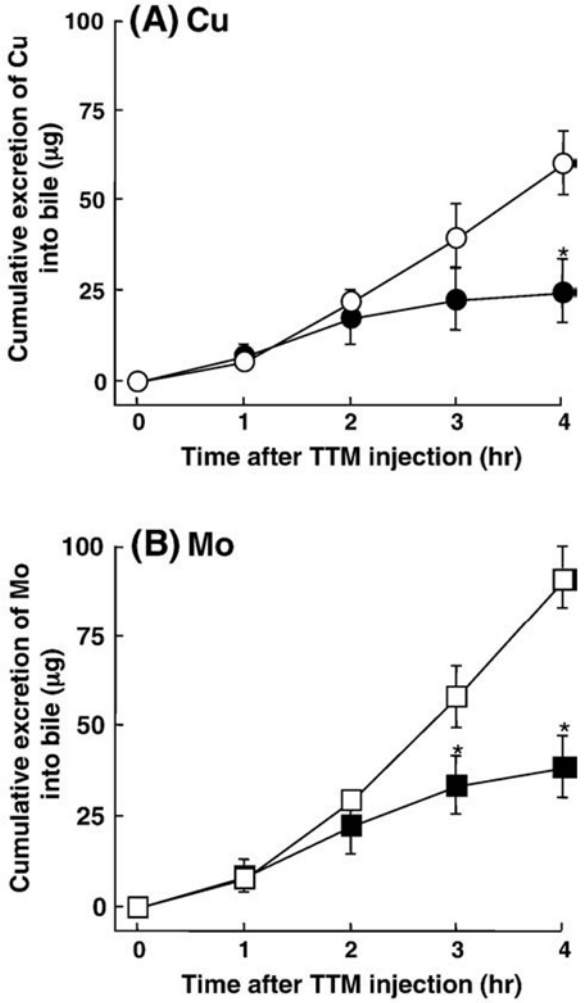

(A) Cu
Cumulative excretion of Cu into bile (μg)
100
75
50
25
0
0
1
2
3
4
Time after TTM injection (hr)
*
(B) Mo
Cumulative excretion of Mo into bile (μg)
100
75
50
25
0
0
1
2
3
4
Time after TTM injection (hr)
*
*

Fig.3

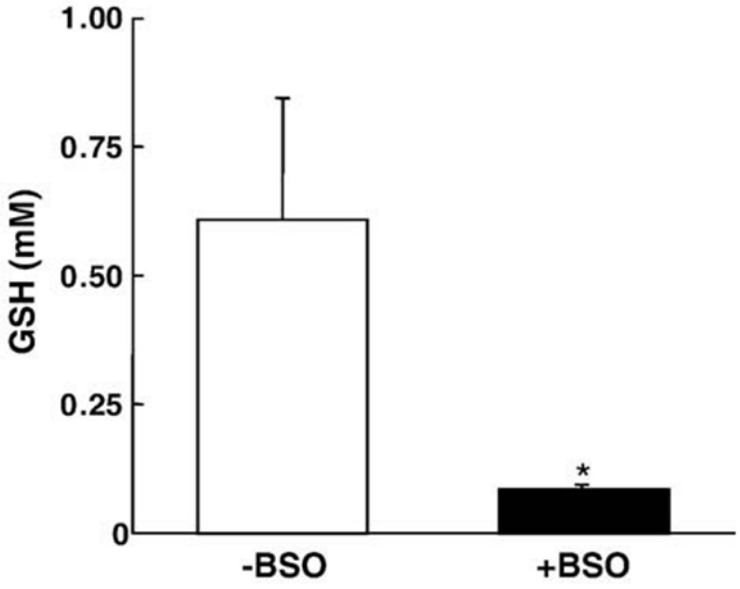


Fig.4

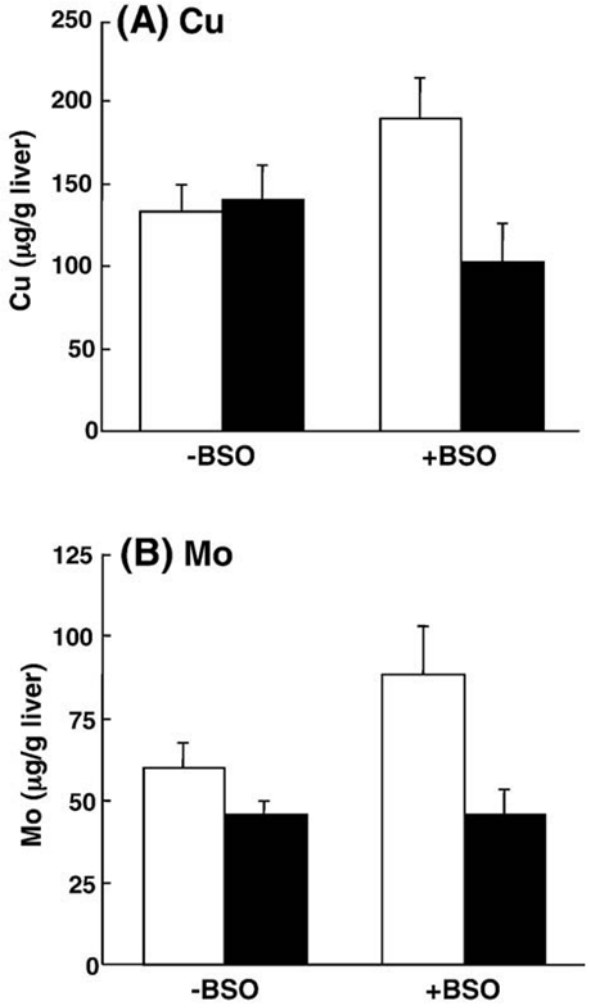

(A) Cu
Cu (μg/g liver)
250
200
150
100
50
0
-BSO
+BSO
(B) Mo
Mo (μg/g liver)
125
100
75
50
25
0
-BSO
+BSO

Fig.5

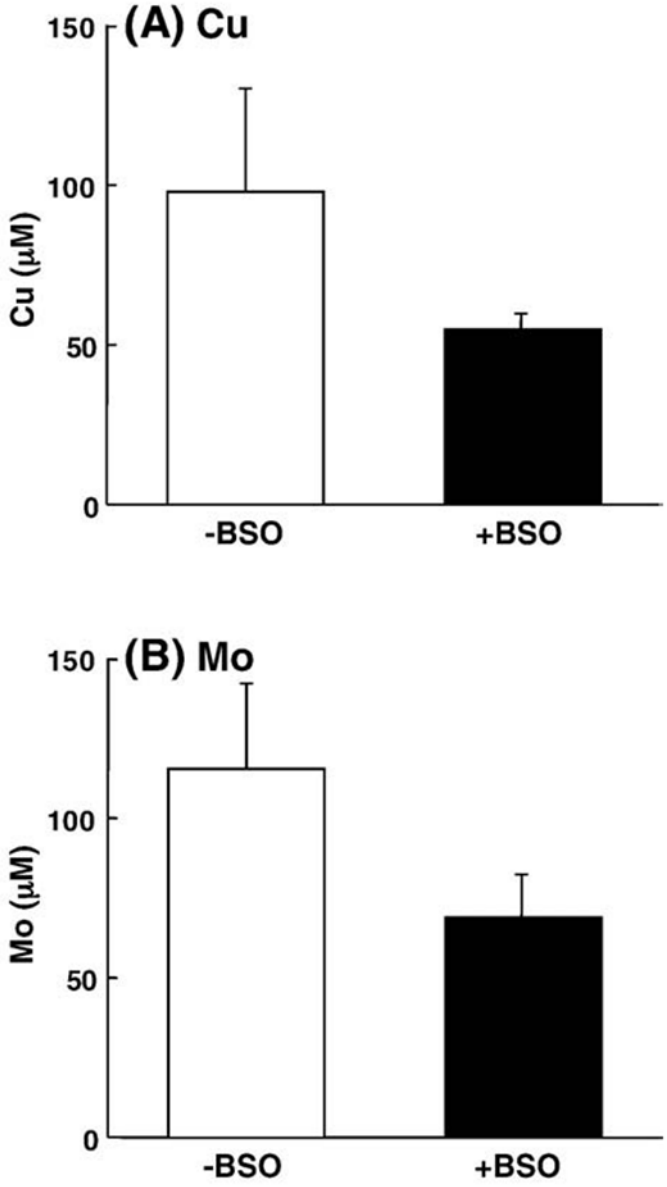
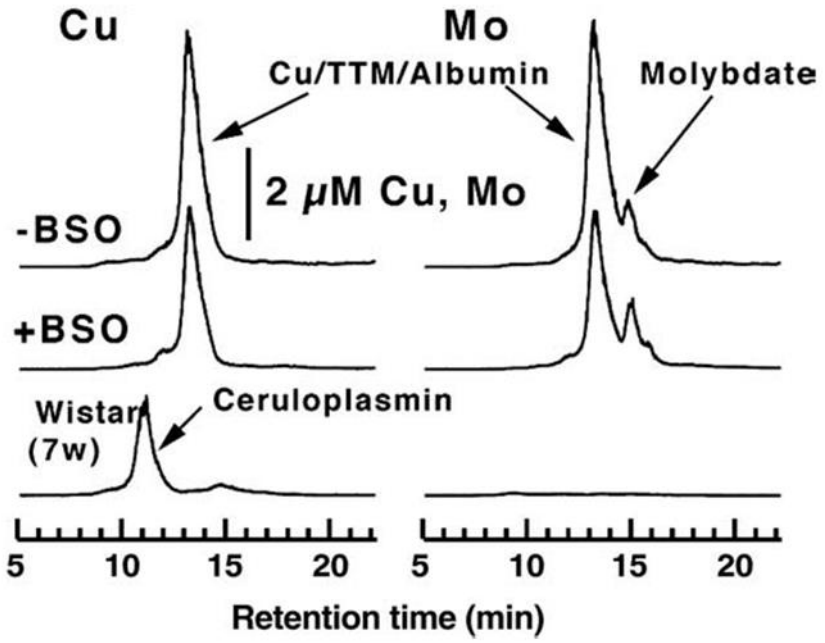


Fig.6


Cu
Mo
Cu/TTM/Albumin
Molybdate
2 μM Cu, Mo
-BSO
+BSO
Wistar
(7w)
Ceruloplasmin
5
10
15
20
5
10
15
20
Retention time (min)