

Zinc sulfate inhibits the enterohepatic cycling of unconjugated bilirubin in subjects with Gilbert's syndrome

Nahum Méndez-Sánchez MD, PhD,¹ Mariana Martínez,¹ Verónica González,¹
Ernesto Roldán-Valadez MD,¹ Miguel A. Flores MD,¹ Misael Uribe MD¹

Abstract

We have previously observed that UCB binds to ZnSO₄ *in vitro*, and suppressed the biliary bilirubin secretion in the hamster. The aim of this study was designed to investigate whether Zn salts might inhibit the enterohepatic cycling of UCB in subjects with Gilbert's syndrome. Fifteen patients with Gilbert's syndrome and 5 normal healthy volunteers were included in this study according to the following criteria: fasting hyperbilirubinemia, no hemolysis, and free of any medication. Patients were randomly assigned to receive acute or chronic treatment. Subjects treated in acute form and normal healthy volunteers were treated with 40 mg of ZnSO₄ in a single dose, whereas patients treated in chronic form received 100 mg ZnSO₄ in a single dose daily for 7 days. The serum UCB levels (mg/dL) decreased from 2.64 ± 1.04 to 2.02 ± 0.87 ($p < 0.001$) and 1.8 ± 0.36 to 1.48 ± 0.32 ($p < 0.005$) in subjects treated in acute and chronic form respectively, but not in the control group. Whereas, the serum Zn levels (g/dL) increased from 96.3 ± 16.8 to 118.8 ± 19.5 ($p < 0.01$) and from 117.6 ± 8.5 to 130.7 ± 6.6 ($p < 0.03$) in subjects treated in acute and chronic form and also in subjects in the control group (98.0 ± 7.3 to 128.0 ± 21.9) $p < 0.03$. This study showed that acute and chronic oral administration of ZnSO₄ decreased serum UCB levels significantly in subjects with Gilbert's syndrome. Most likely by the inhibition of the "normal" enterohepatic cycling of UCB.

Key words: Bilirubin, Zinc, Bile salts, Cholestasis, Gilbert's syndrome, Pigmented gallstones.

¹ Departments of Biomedical Research and Gastroenterology, Medica Sur Clinical & Foundation, Mexico City, Mexico.
Grants support: This work was partly supported by the National Council of Science and Technology of Mexico (CONACYT), Project number 0817P-M9506 (M.U. and N.M-S).

Address for correspondence:

Nahum Méndez-Sánchez, M.D., Ph.D. Departments of Biomedical Research and Gastroenterology, Medica Sur Clinic & Foundation, Puente de Piedra 150, Col. Toriello Guerra, Tlalpan 14050, Mexico City, Mexico. Phone: (+525) 606-6222 ext. 4215; Fax: (+525) 666-4031; E-mail: nmendez@medicasur.org.mx

Introduction

The Gilbert's syndrome is a chronic disease in which unconjugated bilirubin (UCB) is not efficiently conjugated in the liver and it is accumulated in the blood.¹⁻³ Recently, it has been associated with an extra TA in the promoter region of both alleles for bilirubin UDP-glucuronosyltransferase 1 (UGT1A1).⁴⁻⁸ The resultant 65% decrease in transcription of the (TA)7TAA mutant alleles explains the impaired conjugation of bilirubin found in all Gilbert's syndrome subjects.^{9,10}

Under normal conditions conjugation of bilirubin takes place in the hepatic cell, and two or one molecules of glucuronic acid leads to form bilirubin diglucuronides (BDG) and bilirubin monoglucuronides (BMG) respectively.¹¹ In humans, BDG constitutes the major conjugate in bile (about 80%). But in subjects with Gilbert's syndrome the proportion of BDG/BMG is inverted.¹² Interestingly, the BMG are the major conjugate species in mice, sheep, pigs, rabbits, guinea pigs, and hamsters.¹³ According to this information, we believed that both subjects with Gilbert's syndrome and hamsters have an EHC of UCB.

On the other hand, we recently obtained evidence for EHC of UCB in rats following distal, but not proximal, small bowel resection.¹⁴ We proposed that EHC of UCB was secondary to an interruption in the EHC of bile salts. These findings are consistent with results in patients with Crohn's disease involving the ileum,^{15,16} who present with total bilirubin and UCB levels significantly elevated in gallbladder bile.

Furthermore, we recently observed that at physiological pH ZnSO₄ adsorb UCB almost entirely from unsaturated micellar bile salt solution *in vitro*.¹⁷ In addition, in hamsters which have principally BMG in bile,¹³ when we fed them with 1% ZnSO₄ for one week, the biliary bilirubin secretion was suppressed suggesting an inhibition of the EHC of UCB.⁷ The aim of this study was designed to investigate whether Zn salts might inhibit the EHC of UCB in subjects with Gilbert's syndrome.

Methods

Subjects

We recruited subjects from one medical center in Mexico City (Medica Sur Clinic & Foundation). Mexi-

can subjects male and female patients 20-46 years of age with Gilbert's syndrome were included in this study according to the following criteria: a) positive test of fasting hyperbilirubinemia, b) no hemolysis, and c) free of any medication. Normal healthy volunteers acting as controls. Written informed consent was obtained from each subject. The study was approved by the Human Subjects Committee at The Medica Sur Clinic & Foundation as conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

Study design

This was a clinical trial carried out in fifteen patients with Gilbert's syndrome and 5 normal healthy volunteers acting as controls. Subjects who met the entrance criteria were randomly assigned to acute or chronic treatments, subjects treated in acute form and normal healthy volunteers were treated with 40 mg of ZnSO₄ in a single dose. Whereas patients treated in chronic form received 100 mg of ZnSO₄ in a single dose daily for 7 days.

Clinical monitoring

Subjects treated in acute form (n = 10) and normal healthy volunteers B (n = 5) were hospitalized one day before treatment in order to avoid physical activities. After a period of 14 h overnight (baseline) they received a carbohydrate (CHD)-rich breakfast (600 kcal) and two h later a single 40 mg oral dose of ZnSO₄. Subjects treated in chronic form (n = 5) were treated as outpatients with a single 100 mg oral dose of ZnSO₄ daily for 7 days.

Analytical procedures

Serum UCB and Zn levels were monitored by HPLC¹⁸ and atomic absorption spectroscopy at baseline and after oral administration of ZnSO₄, and fecal UCB concentrations were determined before and 24 h after oral administration of ZnSO₄ by HPLC.¹⁸

Statistical analysis

All values are expressed as means $\pm$ SD, the paired "t" Student and Wilcoxon tests were used to compare the serum UCB and Zn levels. The differences were considered as statistically significant when p was < 0.05 .¹⁹

Results

Subjects with Gilbert's syndrome treated in acute form and normal healthy volunteers were directly observed by the investigators, indicated that they had good tolerance of the ZnSO₄. All other subjects treated as out-patients do not referred side effects attributed to ZnSO₄. Clinical characteristics of all groups are given in Table I.

Figure 1 shows the effect of the ZnSO₄ on serum UCB levels (mg/dL) in subjects with Gilbert's syndrome in acute and chronic forms. The UCB levels range from 2.66 ± 0.91 at the baseline to 2.84 ± 0.89 and after administration of ZnSO₄ there was an decrease to 2.34 ± 0.90 ($p < 0.0003$). Whereas serum UCB levels in subjects with Gilbert's syndrome, treated in a chronic form decreased from 1.8 ± 0.36 to 1.48 ± 0.32 ($p < 0.005$). The serum Zn levels (g/dL) in acute and chronic form. The levels increased from 101.51 ± 12.38 to 122.08 ± 20.0 , ($p < 0.04$) and from $117.64 \pm 130.7 \pm 6.6$, ($p < 0.03$) in subjects with Gilbert's syndrome.

Figure 2 shows the effect of ZnSO₄ on serum UCB and Zn levels in the control group. There was no change in UCB levels after administration of ZnSO₄. Whereas Zn levels increased from 98.0 ± 7.3 to 128.0 ± 21.9 ($p < 0.03$). Fecal UCB concentration (g/g dry) was increased after oral administration of ZnSO₄ from 27.25 ± 21.4 to 37.2 ± 23.08 , $p < 0.03$ in subjects treated in acute form, but not in the control group (Table II).

Discussion

The results of this study showed that acute and chronic oral administration of ZnSO₄ decreases serum UCB levels significantly in subjects with Gilbert's syndrome. Most likely by inhibition of the EHC of UCB.

How can we explain the effect of ZnSO₄ on the serum UCB concentration in subjects with Gilbert's syndrome? First, we have shown in our *in vitro* studies that Zn salts at physiological pH and above adsorb UCB essentially completely from unsaturated BS micellar solutions. This indicate that there is a physico-chemical interaction between UCB and Zn salts.¹⁷ The interaction probably is via the carboxyl groups of UCB, as occurs with other divalent cations such as Ca²⁺²⁰⁻²² leading to the formation of calcium bilirubinate [Ca(BH)₂].

Second, we have also found that ZnSO₄ reduced biliary bilirubin secretion in hamsters *in vivo*.¹⁷ One explanation for the reduced secretion of biliary bilirubin observed in the hamsters fed ZnSO₄ is that this is probably the result of the interaction between UCB and ZnSO₄ at the distal

Table I. Characteristics of the three treated groups at entry.

	Acute	Chronic	Control	p Value
Clinical				
No. patients	10	5	5	-
Age (yr)	33.3 ± 5.8	31.8 ± 7.9	25.4 ± 1.5	NS
Sex (M/F)	(8/2)	(4/1)	(4/1)	0.001
BMI	22.6 ± 2.7^a	23.9 ± 2.56	27.4 ± 2.0	0.05
Serum				
UCB (mg/dL)	2.64 ± 1.04^b	2.22 ± 0.8^b	0.9 ± 0.17	0.05

BMI, body mass index UCB, unconjugated bilirubin ^a vs control
^b vs control NS, No significant

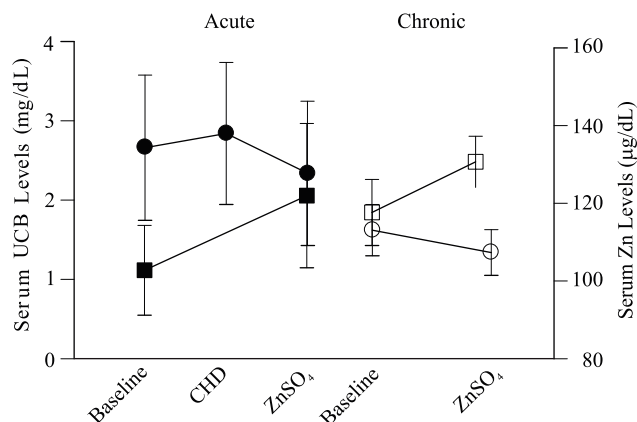


Figure 1. Shows the effect of ZnSO_4 on serum UCB levels in subjects with Gilbert's syndrome, in acute and chronic form. In both forms of treatment there were a significant decrease in serum UCB levels, The UCB levels range from 2.66 ± 0.91 at the baseline to 2.84 ± 0.89 and after administration of ZnSO_4 there was an decrease to 2.34 ± 0.90 ($p < 0.0003$). Whereas serum UCB levels in subjects treated in a chronic form decreased from 1.8 ± 0.36 to 1.48 ± 0.32 ($p < 0.005$). The serum Zn levels ($\mu\text{g/dL}$) in acute an chronic form. The levels increased from 101.51 ± 12.38 to 122.08 ± 20.0 , ($p < 0.04$) and from $117.64 \pm 130.7 \pm 6.6$, ($p < 0.03$) . Values are means $\pm$ SD.

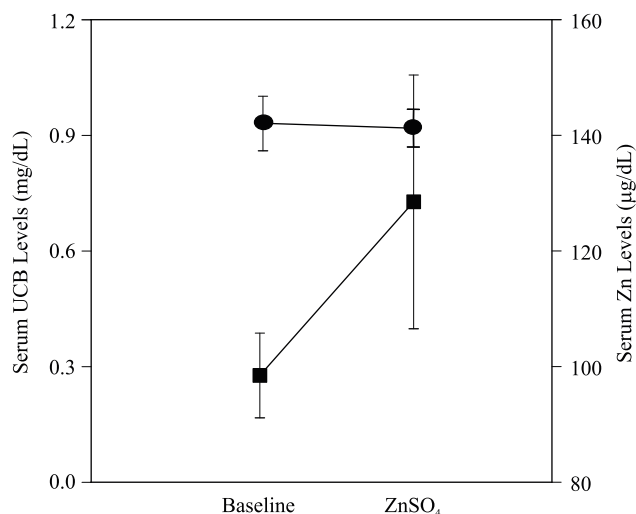


Figure 2. Shows the effect of ZnSO_4 on serum UCB levels in healthy volunteers acting as controls. There was no change in serum UCB levels, after administration of ZnSO_4 . Whereas serum Zn levels ($\mu\text{g/dL}$) increased from 98.0 ± 7.3 to 128.0 ± 21.9 , ($p < 0.03$). Values are means $\pm$ SD.

intestinal lumen where the pH is ~ 8 , and we know from our *in vitro* experiments that at this pH, UCB might interact with ZnSO_4 .¹⁷ Then this interaction leads to formation of a flocculated material, which in turn, may be excreted in the fecal content as we shown in this study. As a result of this interaction, the final event is an interruption of the EHC of UCB by ZnSO_4 .

Third, to look the most potent effect of ZnSO_4 was seen when we used a carbohydrate-rich diet which is known is able to increase the serum UCB levels.^{23,24} In fact, we saw that the effect of ZnSO_4 was more evident after a 14-hour fasting period and a carbohydrate-rich diet. The serum

Table II. Fecal unconjugated bilirubin [UCB ($\mu\text{g/g dry}$)] concentrations before and 24 h after oral administration of ZnSO_4 .

	Before	After	p Value
Control (n = 4)	5.65 ± 1.7	6.05 ± 0.82	NS
Acute (n = 4)	27.25 ± 21.4	37.2 ± 23.08	0.03

NS, Non significant.

UCB levels decreased significantly after the administration of a 40 mg of ZnSO_4 given acutely (see Figure 1), and these findings were resembled after oral administration of 100 mg for seven days. Interestingly, the low dose was probably potent enough to decrease the serum UCB concentration.

Four, serum Zn levels increased 20% and 13% in both acute and chronic administration of ZnSO_4 , suggesting an effect of this salt on serum bilirubin. Whereas fecal UCB concentration was increased after oral administration of ZnSO_4 in subjects treated in acute form, but not in the control group.

On the other hand, we believed that ZnSO_4 might act as insoluble calcium phosphate which has been proposed as a good chelator of UCB from bile salts solutions²⁵ and in fact, oral calcium phosphate/carbonate has been suggested as effective in treating hyperoxaluria in patients with ileal resection.²⁶⁻²⁸ In a recent paper, insoluble calcium salts were shown to suppress serum bilirubin levels in patients with Crigler Najjar Type II syndrome.²⁹ According with the results of the present study Zn which is the least toxic of the trace metals, may be used in the following conditions: a) to inhibit EHC of bilirubin associated with bile salt absorption [distal ileal diseases (Crohn's, AIDS, resection, bypass, and immaturity)], b) to suppress serum bilirubin levels in patients with Crigler Najjar Type II syndrome, c) in patients with chronic cholestasis diseases (primary biliary cirrhosis, primary sclerosing cholangitis), and d) chronic liver diseases (hepatic cirrhosis) interestingly, Dashti et al³⁰ observed in experimental liver cirrhosis produced by thioacetamide that zinc sulphate- treated animals showed a restoration of normal hepatic and plasma zinc and copper levels plasma. Similarly, plasma levels of aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl aminotransferase, and total bilirubin decreased significantly. Light microscopic studies showed that most of the hepatocytes appeared normal in zinc-treated as compared with untreated cirrhotic animals.

In conclusion, this study showed that acute and chronic administration of oral ZnSO_4 decreases serum UCB levels significantly in subjects with Gilbert's syndrome. This observation suggests inhibition of a "normal" EHC of UCB, and strongly support the potential usefulness of ZnSO_4 in the treatment a series of pathological conditions that are characterized by hyperbilirubinemia.

References

1. Berk PD, Martin JF, Blaschke TF, Scharschmidt BF, Plotz PH. Unconjugated hyperbilirubinemia: physiologic evaluation and experimental approaches to therapy. *Ann Intern Med* 1975; 82: 552-570.
2. Powell LW, Hemingway E, Billing BH, Sherlock S. Idiopathic unconjugated hyperbilirubinemia (Gilbert's syndrome): a study of 42 families. *N Engl J Med* 1967; 277: 1108-1112.
3. Foulk WT, Butt HR, Owen CA, Jr., Whitcomb FF, Mason HL. Constitutional hepatic dysfunction (Gilbert's disease): its natural history and related syndromes. *Medicine (Baltimore)* 1959; 38: 25-46.
4. Bosma PJ, Roy Chowdhury J, Bakker CTM, Gantla S, de Boer A, Oostra BA, et al. The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *N Engl J Med* 1995; 333: 1171-1175.
5. Monaghan G, Ryan M, Seddon R, Hume R, Burchell B. Genetic variation in bilirubin UDP-glucuronosyltransferase gene promoter and Gilbert's syndrome. *Lancet* 1996; 347: 578-581.
6. Clarke DJ, Moghrabi N, Monaghan G, Cassidy A, Boxer M, Hume R, et al. Genetic defects of the UDP-glucuronosyltransferase-1 (UGT1) gene that cause familial non-hemolytic unconjugated hyperbilirubinemias. *Clin Chim Acta* 1997; 266: 63-74.
7. Bancroft JD, Kreamer B, Gourley GR. Gilbert's syndrome accelerates development of neonatal jaundice. *J Pediatr* 1998; 132: 656-660.
8. Sampietro M, Lupica L, Perrero L, Romano R, Molteni V, Fiorelli G. TATA-box mutant in the promoter of the uridine diphosphate glucuronosyltransferase gene in Italian patients with Gilbert's syndrome. *Ital J Gastroenterol Hepatol* 1998; 30: 194-198.
9. Black M, Billing BH. Hepatic bilirubin UDP-glucuronyl transferase activity in liver disease and in Gilbert's syndrome. *N Engl J Med* 1969; 280: 1266-1271.
10. Felsher BF, Craig JR, Carpio N. Hepatic bilirubin glucuronidation in Gilbert's syndrome. *J Lab Clin Med* 1973; 81: 829-837.
11. Hauser SC, Gollan J. Bilirubin metabolism and hyperbilirubinemic disorders. In Millward-Sadler GH, Wright R, Arthur MJP. *Wright's liver and biliary disease*. 3rd Ed. London, W.B. Saunders Company, 1992; 317-70.
12. Fevery J, Blanckaert N, Heirwegh KPM, Preaux AM, Berhelot P. Unconjugated bilirubin and an increased proportion of bilirubin monoconjugates in the bile patients with Gilbert's syndrome and Crigler Najjar disease. *J Clin Invest* 1977; 60: 970-979.
13. Spivak W, Carey MC. Reverse-phase h.p.l.c. separation, quantification and preparation of bilirubin and its conjugates from native bile. *Biochem J* 1985; 225: 287-805.
14. Brink AM, Méndez-Sánchez N, Carey MC. 1996. Bilirubin cycles enterohepatically after ileal resection in the rat. *Gastroenterology*. 1996; 110: 1945-1957.
15. Brink MA, Slors JF, Keulemans YC, Mok KS, De Waart DR, Carey MC, et al. Enterohepatic cycling of bilirubin: a putative mechanism for pigment gallstone formation in ileal Crohn's disease. *Gastroenterology* 1999; 116: 1420-7.
16. Dawes LG, Stryler S, Rege R, Nahrwold D. Gallbladder bile composition in Crohn's disease. *Surgical Forum*. 1991; 42: 188-189.
17. Méndez-Sánchez N, Roldán-Valadez E, Flores MA, Cárdenas-Vázquez R, Uribe M. Zinc Salts Precipitate Unconjugated Bilirubin *In Vitro* and Inhibit Enterohepatic Cycling of Bilirubin in Hamsters. *Eur J Clin Invest* 2001; 31: 773-80.
18. Blackaert N. Analysis of bilirubin and bilirubin mono and diconjugates. Determination of their relative amounts in biological samples. *Biochem J* 1980; 185: 115-128.
19. Snedecor GW, Cochran WG. *Statistical methods*. Ames Iowa: The Iowa State University Press 1972.
20. Moore EW. The role of calcium in the pathogenesis of gallstones: Ca++ electrode studies of model bile salt solutions and other biological systems. *Hepatology* 1984; 4: 228S-43S.
21. Sutor DJ, Wilkie LI. Calcium in bile and calcium salts in gallstones. *Clin Chem Acta* 1977; 79: 119-127.
22. Dawes LG, Nahrwold DL, Rege RV. Increased total and free ionized calcium in a canine model of pigment gallstones. *Surgery* 1988; 104: 86-90.
23. Oyama JH. Hyperbilirubinemia in healthy males or acutely restricted dietary intake receiving parenteral nutrition. Abstract. *Am J Clin Nutr* 1972; 25: 459.
24. Gollan JL, Bateman C, Billing BH. Effect of dietary composition on the unconjugated hyperbilirubinemia of Gilbert's syndrome. *Gut* 1976; 17: 335-340.
25. Ostrow JL, Celic L. Bilirubin chemistry, ionization and solubilization by bile salts. *Hepatology* 1984; 4: 38S-45S.
26. Stauffer JQ, Steward RJ, Bertrand G. Acquired hyperoxaluria: relationship to dietary calcium content and severity of steatorrhea. *Gastroenterology* 1974; 66: 783). 122.
27. Earnest DL. Enteric hyperoxaluria. *Adv Intern Med* 1979; 24: 407-27.
28. Caspary WF, Tönissen J, Lankisch PG. "Enteral" Hyperoxaluria. Effect of cholaestryramine, calcium, neomycin, and bile acids on intestinal oxalate absorption in man. *Acta Hepato-Gastroenterol* 1977; 24: 193-200.
29. Van der Veere CN, Jansen PL, Sinaasappel M, Van der Meer R, Van der Sijs H, Rammeloo JA, Goyens P, Van Nieuwkerk CM, Oude Elferink RP. Oral calcium phosphate: a new therapy for Crigler-Najjar disease? *Gastroenterology* 1997; 112: 455-62.
30. Dashti HM, Mathew TC, Jadaon MM, Ashkanani E. Zinc and liver cirrhosis: biochemical and histopathologic assessment. *Nutrition* 1997; 13: 206-12.