Impaired Protein Binding of Chinese Medicine DanShen in Uremic Sera and Sera With Hyperbilirubinemia: Rapid Assessment of Total and Free DanShen Concentrations Using the Fluorescence Polarization Immunoassay for Digoxin

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DanShen is a Chinese medicine that is used to treat cardiovascular disorders. DanShen is moderately to strongly protein bound, mainly to albumin. Because impaired protein binding of albumin-bound drugs in uremia has been reported, we studied protein binding of DanShen by measuring the digoxin-like immunoreactive component of this Chinese medicine. We observed a significantly higher percentage of free fraction of DanShen in uremic sera in vitro. Impaired protein binding of DanShen was also observed in sera from patients with liver disease, who had elevated concentrations of bilirubin. Treating uremic sera with activated charcoal signifi-

cantly improved the protein binding of DanShen, indicating that uremic compounds are responsible for the impaired protein binding of DanShen. On the other hand, when various amounts of bilirubin were added to aliquots of the normal pool supplemented with DanShen, we observed only a modest displacement of DanShen from the protein-binding sites by bilirubin, indicating that hypoalbuminemia may play a major role in impaired protein binding of DanShen in sera with elevated bilirubin concentrations. We conclude that protein binding of DanShen is lower in uremic sera and in sera with elevated bilirubin concentrations. *J. Clin. Lab. Anal.* 17:179–183, 2003. © 2003 Wiley-Liss, Inc.

Key words: DanShen; uremia; protein binding; hyperbilirubinemia

INTRODUCTION

Alternative medicines have become increasingly popular in the U.S. It has been estimated that in 1997 the gross sales of herbal medicines in the U.S. totaled \$3.2 billion (1). DanShen is a traditional Chinese medicine prepared from the root of a Chinese medicinal plant, *Salvia miltiorrhiza*. This drug has been used in China for many years in the treatment of various cardiovascular diseases, including angina pectoris (2). DanShen is also used in the treatment of cerebrovascular diseases and insomnia (3). This herbal product can be purchased from Chinese herbal stores without any prescription.

It has been shown that DanShen can dilate the coronary arteries and slow the heart rate (2). Li et al. (4) demonstrated a dose-dependent hypotensive effect of DanShen with a positive inotropic and negative chronotropic effect. DanShen also protects low-density lipoprotein from copper dichloride-induced lipid peroxidation (5). More than 20 diterpene quinones (tanshinones) have been isolated from DanShen root extract.

These compounds have a common structural feature: the phenanthrene quinone ring (6). We reported previously (7) that DanShen cross-reacts with the polyclonal antibody-based fluorescence polarization immunoassay (FPIA) of digoxin due to the structural similarity between tanshinones and digoxin. Therefore, the FPIA digoxin assay can be used for rapid detection of DanShen in blood in the absence of digoxin (7).

Impaired protein binding of several strongly protein-bound Western medicines has been well documented in the literature (8–10). Because the free fraction of a drug is pharmacologically active, uremic patients are more susceptible to drug toxicity than otherwise healthy individuals, and careful monitoring of, for example,

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free phenytoin rather than total phenytoin is strongly recommended for uremic patients (11). A uremic patient taking Chinese medicine can be as susceptible to drug toxicity as one taking Western medicine. Moreover, while there are clear dosage and toxicity guidelines for Western medicines, no such information is readily available for Chinese medicines, which makes patients taking these medicines even more susceptible to toxicity. Impaired protein binding of strongly protein-bound Western medicines in sera with hyperbilirubinemia has also been reported (12). However, protein binding of DanShen in uremia or liver disease has not been studied to date. Here we report impaired protein binding of DanShen in uremic sera and sera with elevated concentrations of bilirubin.

MATERIALS AND METHODS

DanShen was purchased from a local Chinese herbal medicine store in Chinatown, Houston, Texas. Activated charcoal was purchased from the Aldrich Chemical Company (Milwaukee, WI), and bilirubin was obtained from the Sigma Chemical Company (St. Louis, MO). The fluorescence polarization assay kits for digoxin were obtained from Abbott Laboratories (Abbott Park, IL) and the assays were run on a TDxFLx analyzer (Abbott Laboratories). An ultrafiltration device (Centrifree micropartition system) for preparing protein-free ultrafiltrate of serum was purchased from Amicon Corporation (Danvers, MA). A standard solution of DanShen was prepared in ethyl acetate (25 mg/mL), and a standard solution of bilirubin (10 mg/mL) was prepared in 0.1 N sodium hydroxide.

Uremic serum pools were prepared with samples from patients with uremia. The individual specimens showed high creatinine and BUN concentrations. None of these patients were receiving digoxin. Serum pools were also prepared with specimens from patients with elevated bilirubin concentrations. These serum specimens are routinely submitted to our clinical laboratory for various tests. For this study, we used specimens that were left over after we had performed and reported all of the tests ordered by the clinicians. These specimens would otherwise have been discarded.

We supplemented aliquots of normal, uremic, and hyperbilirubin pools with microliter quantities of ethyl acetate extract of DanShen, and measured total and free apparent digoxin concentrations using the FPIA digoxin assay. In order to avoid a matrix effect due to ethyl acetate, we added ethyl acetate extract of DanShen in a test tube and then evaporated ethyl acetate using nitrogen. Then the dry residue was constituted with an

appropriate amount of serum containing no digoxin or digoxin-like immunoreactive substances (DLIS). After the specimens were supplemented with DanShen, they were incubated in a water bath at 37°C for 1 hr. Total and free apparent digoxin concentrations were then measured using the FPIA digoxin assay. For the total apparent digoxin concentration measurement, 200 μ L of specimen were treated with 200 μ L of sulfosalicylic acid in methanol to precipitate proteins. After the specimen was centrifuged at high speed, the resulting clear supernatant was used for digoxin measurement. To monitor free apparent digoxin concentration, we prepared protein-free ultrafiltrate by centrifuging a specimen in the Centrifree ultrafiltration system for 30 min at 2,500 *g*. Sulfosalicylic acid was added to the protein-free ultrafiltrate in order to preserve the matrix, but no protein precipitation was observed (as expected). The clear specimen was used directly for digoxin measurement. The fluorescence polarization immunoassay for digoxin is linear up to a serum digoxin concentration of 5.0 ng/mL. The detection limit of the assay is 0.20 ng/mL.

To treat the uremic sera with charcoal, we added 50 mg of activated charcoal to each 1 mL of serum aliquot. Then the uremic serum was gently mixed with activated charcoal at room temperature for 1 hr with a rotating mixer. The activated charcoal was then removed from the serum by centrifugation at high speed. We also treated normal serum pools with activated charcoal in order to have controls for comparison. The normal and uremic pools, along with the charcoal-treated normal and uremic pools, were supplemented with DanShen and incubated for 1 hr at 37°C. Total and free apparent digoxin concentrations were then measured using the FPIA digoxin assay.

In the next experiment we supplemented another normal pool with DanShen, and after incubation for 1 hr at 37°C, aliquots of the pool were further supplemented with various concentrations of bilirubin (final concentrations: 5, 10, and 20 mg/dL). After incubation for another 1 hr at 37°C, apparent total and free apparent digoxin concentrations were measured using the FPIA digoxin assay.

We measured total and free apparent digoxin concentrations in triplicate for each specimen in all experiments, and each concentration was expressed as the mean + 1 standard deviation.

Concentrations of albumin, BUN, creatinine, and total bilirubin were measured using a Synchron LX analyzer and appropriate reagent packages supplied by the Beckman Coulter Corporation (Brea, CA). The albumin method, which is based on the reaction between albumin and bromocresol purple to form a complex, monitors the change in absorption at 600 nm.

Statistical analysis was performed with a two-tailed *t*-test. We considered a difference to be statistically significant only at 95% confidence or higher.

RESULTS

The range of albumin concentration in normal pools was 3.6–4.2 gm/dL, while the range was 2.9–3.3 in uremic pools and 3.0–3.6 gm/dL in serum pools with hyperbilirubinemia. The range of creatinine concentration was 0.4–1.0 mg/dL in normal pools, 1.7–4.8 mg/dL in uremic pools, and 0.3–1.2 mg/dL in serum pools with hyperbilirubinemia. The range of total bilirubin concentration was 0.2–0.4 mg/dL in normal pools, 0.3–0.6 mg/dL in uremic pools, and 1.6–4.9 mg/dL in serum pools with elevated bilirubin concentrations. None of the serum pools used in this study showed any apparent digoxin activity by the FPIA, indicating that these sera also had no DLIS.

When we supplemented aliquots of normal, uremic, and serum pools with elevated bilirubin with the same amount of DanShen, and measured total and free apparent digoxin concentrations after 1 hr of incubation, we observed significantly higher apparent free digoxin concentrations in uremic pools and pools with elevated bilirubin concentration compared to the normal pools. For example, the total and free apparent digoxin concentrations in normal pool 1 were 1.42 ng/mL and 0.62 ng/mL, respectively (43.7% free). In contrast, the total and free apparent digoxin concentrations in

uremic pool 1 were 1.36 ng/mL and 0.72 ng/mL (52.9%), respectively (Table 1).

Treatment of uremic serum with activated charcoal is known to correct binding defects of uremic sera for Western medicines (13). Therefore, we explored the possibility of correcting impaired protein binding of DanShen in uremic sera by treating the sera with activated charcoal. After the charcoal treatment experiment, we supplemented both treated and untreated sera with the same amount of DanShen and incubated the specimens for 1 hr at 37°C. Apparent total and free digoxin concentrations were then measured using the FPIA assay for digoxin. For comparison purposes, aliquots of normal serum pools were also treated with charcoal, and both treated and untreated normal pools were supplemented with DanShen. Again, total and free apparent digoxin concentrations were measured after 1 hr of incubation at 37°C. In order to show that the charcoal treatment experiment has no effect on serum albumin concentrations, we measured albumin concentrations before and after the treatment with activated charcoal. We observed no change in albumin concentrations. For example, in one uremic pool the original albumin concentration was 2.9 gm/dL. After charcoal treatment, the observed albumin concentration was also 2.9 gm/dL, indicating that charcoal treatment has no effect on serum albumin concentration.

The apparent total and free digoxin concentrations in the normal serum pool were 1.83 ng/mL and 0.81 ng/mL, respectively (normal pool 4). The corresponding

TABLE 1. Total and free DanShen concentrations (as measured by apparent digoxin concentrations) in normal, uremic, and serum pools with hyperbilirubinemia

Specimen	Apparent Digoxin, ng/ml, Mean (SD), <i>n</i> = 3		% Free
	Total	Free	
Normal pool 1	1.42 (0.06)	0.62 (0.02)	43.7
Normal pool 2	1.49 (0.08)	0.60 (0.04)	40.3
Normal pool 3	1.42 (0.04)	0.58 (0.02)	40.8
Uremic pool 1	1.36 (0.10)	0.72 (0.02)	52.9
Uremic pool 2	1.37 (0.12)	0.68 (0.01)	49.6
Uremic pool 3	1.43 (0.08)	0.76 (0.04)	53.1
Hyperbilirubin pool 1	1.36 (0.10)	0.72 (0.02)	52.9
Hyperbilirubin pool 2	1.46 (0.08)	0.70 (0.02)	47.9
Normal pool 4	1.83 (0.12)	0.81 (0.06)	44.2
Normal pool 4 (charcoal treated)	1.86 (0.28)	0.77 (0.08)	41.4
Uremic pool 4	1.84 (0.08)	0.95 (0.04)	51.6
Uremic pool 4 (charcoal treated)	1.79 (0.09)	0.70 (0.03) ^a	39.1
Normal pool 5	0.78 (0.06)	0.30 (0.02)	38.5
Normal pool 5 (charcoal treated)	0.82 (0.06)	0.23 (0.04)	28.0
Uremic pool 5	0.84 (0.03)	0.41 (0.02)	48.8
Uremic pool 5 (charcoal treated)	0.80 (0.06)	0.21 (0.01) ^a	26.3

^aSignificantly less than the corresponding uremic pool value (control) by *t*-test, two-tailed ($P < 0.05$).

total and free concentrations in the charcoal-treated normal pools were 1.86 ng/mL and 0.77 ng/mL, respectively. The concentrations of total and apparent free digoxin were 1.84 ng/mL and 0.95 ng/mL, respectively, in the uremic pool (uremic pool 4). However, after charcoal treatment, the free apparent digoxin concentration in the same uremic pool was comparable to normal (total: 1.79 ng/mL, free: 0.70 ng/mL, percent free: 39.1). We observed 41.4% free apparent digoxin concentration when the normal pool was treated with activated charcoal (Table 1). The slight decline in percent free concentration (44.2% compared to 41.4%) in the normal pool after the activated-charcoal treatment may be related to the removal of free fatty acids from the serum. Free fatty acids are known to displace strongly protein-bound drugs, such as valproic acid, from the albumin-binding site, causing an elevated concentration of the free form of these drugs (14).

We also explored the possibility of bilirubin displacing DanShen as a mechanism for elevated free concentration in sera with hyperbilirubinemia. For this purpose, aliquots of another normal pool (bilirubin 0.4 mg/dL) were supplemented with various concentrations of bilirubin (5 mg/dL, 10 mg/dL, and 20 mg/dL). After 1 hr of incubation, total and free apparent digoxin concentrations were measured. We observed moderate displacement of DanShen by bilirubin only at high bilirubin concentrations (10 mg/dL and 20 mg/dL). The total apparent digoxin concentration in the pool was 1.68 ng/mL. The free apparent digoxin concentration increased from the control value of 0.76 ng/mL to only 0.79 ng/mL in the presence of 5 mg/dL of bilirubin. This difference is not statistically significant. However, in the presence of 10 mg/dL of bilirubin (measured total bilirubin: 9.3 mg/dL; direct bilirubin: 0.2 mg/dL), the observed free concentration was 0.88 ng/mL, which was statistically significant from the control value of 0.76 ng/mL. In the presence of 20 mg/dL bilirubin (measured: total bilirubin: 18.6 mg/dL; direct bilirubin: 0.3 mg/dL), the observed free concentration was further increased to 0.97 ng/mL, indicating that bilirubin can displace digoxin-like immunoreactive components of DanShen from the protein-binding sites.

DISCUSSION

Elevated DLIS concentrations are found in uremic sera and sera with elevated bilirubin concentrations (15–17). Therefore, we selected only those uremic pools and serum pools with high bilirubin that did not show any measurable DLIS activity as evidenced by a “none detected” level of apparent digoxin in all these pools (apparent digoxin concentrations below the detection limit of 0.20 ng/mL). We were unable to use some pools

with very high bilirubin concentrations (10–20 mg/dL) because they showed measurable apparent digoxin concentrations. Similarly, some uremic pools with very high creatinine also could not be used due to the presence of DLIS. The uremic pools we used had relatively lower levels of creatinine compared to other uremic serum pools. It is possible to observe more impairment of protein binding of DanShen in these pools, but we were unable to conduct such experiments because we used apparent digoxin concentrations as a measure of both total and free DanShen.

The uremic toxins certainly play an important role in impaired protein binding of DanShen. Treatment with activated charcoal significantly improved the protein binding of DanShen in the uremic pools. However, elevated free DanShen concentrations in sera with hyperbilirubinemia may be mostly due to hypoalbuminemia, because bilirubin caused only a modest displacement of DanShen from the protein-binding sites. Albumin is the major binding protein for DanShen. When we prepared albumin solutions in normal saline and supplemented them with DanShen, we observed decreased concentrations of free DanShen with increased concentrations of albumin (data not shown).

In conclusion, our *in vitro* studies indicate that free DanShen concentrations are elevated in sera with hypoalbuminemia. Moreover, decreased protein binding of DanShen was also observed when uremic serum pools were supplemented with DanShen extract. It is possible that these phenomena also occur *in vivo*. Therefore, uremic patients may be at a higher risk of toxicity for DanShen. Fortunately, clinicians are aware of the higher risk of drug toxicity in patients with uremia or liver disease, and they can carefully adjust the dosage of Western medicines, such as phenytoin, to avoid drug toxicity by monitoring free-drug levels. Unfortunately, DanShen can be purchased without a prescription, and the general public may not be aware of the increased potential for toxicity of this Chinese medicine in uremia and liver disease. Moreover, many patients do not inform their physician about their use of complementary or alternative medicines, which presents another serious problem.

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