Drug–Herb Interactions: Unexpected Suppression of Free Danshen Concentrations by Salicylate

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The general population of the U.S. uses over-the-counter herbal medicines. Danshen is a Chinese herbal product used for the treatment of cardiovascular disease. In a previous study we showed that Danshen has significant digoxin-like immunoreactivity, and we used this parameter to monitor total and free Danshen activities in sera (10). In this report we demonstrated strong protein binding of Danshen (50–70%), and we also identified albumin as the major serum protein that binds Danshen. Because salicylate, which is also strongly bound to albumin, is a widely used over-the-counter medicine in the U.S., we studied Danshen–salicylate interaction in vitro. We observed

no significant change in free Danshen concentrations as measured by free-digoxin-like activity when salicylate concentrations were subtherapeutic ($\leq 100 \mu\text{g/mL}$). With therapeutic concentrations of salicylate ($\geq 150 \mu\text{g/mL}$), the free Danshen concentrations significantly decreased from the control. On the other hand, Danshen can displace salicylate from protein binding, thereby increasing the free salicylate concentration. We conclude that salicylate in therapeutic concentration can significantly decrease free Danshen concentrations, and Danshen can displace salicylate. *J. Clin. Lab. Anal.* 16:290–294, 2002. © 2002 Wiley-Liss, Inc.

Key words: Danshen; salicylate; albumin binding; drug interaction

INTRODUCTION

Alternative medicines have become increasingly popular in the U.S. It has been reported that in 1997 the gross sale of herbal medicines in the U.S. reached an estimated total of 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.

Danshen is capable of dilating coronary arteries and slowing the heart rate (2). Li et al. (4) demonstrated a dose-dependent hypotensive effect of Danshen, with positive inotropic and negative chronotropic effects. 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 all have a phenanthrene quinone ring structure (6).

Because herbal products are widely used in the U.S., interactions between herbal products and Western drugs

are of significant importance in clinical medicine. In rats, Danshen increases elimination of warfarin (7). Decreased serum concentration of cyclosporine due to the use of St. John's wort has been reported (8). We previously described a significant displacement of digitoxin from protein binding sites by the Chinese medicine Chan Su (9).

Salicylate is a widely used over-the-counter, nonsteroidal, antiinflammatory agent. Salicylate is strongly bound to albumin, and several drug–drug interactions have been reported for salicylate. We previously reported digoxin-like immunoreactivity of the Chinese medicine Danshen (10). By taking advantage of this immunoreactivity, we can measure Danshen concentrations in serum- and protein-free ultrafiltrate as a digoxin equivalent using a commercially available fluorescence

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polarization immunoassay digoxin kit (10). In the present work, we identify albumin as the major protein that binds Danshen. Because both Danshen and salicylate are strongly bound to albumin, we studied the interaction between Danshen and salicylate in vitro in human serum pools.

MATERIALS AND METHODS

Danshen was purchased from a Chinese herbal store in Houston, Texas. Salicylate and human albumin were purchased from the Sigma Chemical Company (St. Louis, MO). The fluorescence polarization immunoassay (FPIA) kits for digoxin and salicylate were purchased from Abbott Laboratories (Abbott Park, IL) and both assays were run on a TDx/FLX analyzer. The Centrifree Micropartition System for preparing protein-free ultrafiltrate was obtained from Amicon (Danvers, MA). A standard solution of Danshen (25 mg/mL) was prepared in ethyl acetate, and a standard solution of salicylate (10 mg/mL) was prepared in 0.9% saline. Various amounts of human albumin were dissolved in 0.9% saline in order to study the ability of albumin to bind Danshen.

The FPIA digoxin assay requires pretreatment of serum. For this purpose, 200 μ L of specimen was mixed with 200 μ L of sulfosalicylic acid in 50% methanol. After protein precipitation, the specimen was centrifuged at a high speed and the clear supernatant was used for digoxin measurement. We also treated the protein-free ultrafiltrate in the same way in order to avoid a matrix problem.

In order to study the ability of albumin to bind Danshen, we added equal amounts of Danshen to various concentrations of albumin (10.0–4.0 gm/dL). After incubation at 37°C for 2 hr, we allowed the specimen to reequilibrate at room temperature for 10 min. We measured total and free Danshen activity as a digoxin equivalent. The free activity was measured in the protein-free ultrafiltrate prepared by centrifuging specimens in a Centrifree Micropartition device at 2,500 *g* for 30 min.

Drug-free serum pools were supplemented with Danshen extract, and after incubation for 1 hr, total digoxin activities were measured using the FPIA digoxin assay. Then aliquots of pool already containing Danshen were further supplemented with various amounts of salicylate at therapeutic and above-therapeutic levels. Specimens were further incubated for 2 hr at 37°C. The specimens were then held at room temperature for 10 min. We measured digoxin activity in both the original specimen and the protein-free ultrafiltrates. Each experiment was performed in tripli-

cate, and values were expressed as the mean and one standard deviation (SD).

In a separate experiment, we further supplemented another serum pool containing Danshen with low and therapeutic concentrations of salicylate in order to investigate Danshen–salicylate interaction when salicylate concentrations were low. We also prepared an albumin stock solution and supplemented it with Danshen. Aliquots of albumin solution containing Danshen were then further supplemented with low and therapeutic concentrations of salicylate for further investigation of Danshen–salicylate interactions.

We also prepared a serum pool containing salicylate. Aliquots of the salicylate pool were then supplemented with 10, 25, or 25 μ L of Danshen extract, or received no Danshen (control). After incubation, total and free salicylate concentrations were measured using the FPIA salicylate assay.

Statistical analyses were performed using an independent *t*-test, two-tailed. We considered a difference statistically significant only at a 95% confidence interval or higher.

RESULTS AND DISCUSSION

We observed 68.4% free activity (digoxin equivalent) when we supplemented an albumin stock solution containing 1.0 gm/dL of albumin. The free fraction was 60% when the albumin concentration was 2.0 gm/dL. However, when the concentration of albumin was increased to 4.0 gm/dL, we observed 55.5% free activity, indicating that 44.5% of the Danshen was already bound to albumin. Increasing albumin concentration to 5.0 gm/dL did not significantly improve protein binding (53.2%), indicating that enough binding sites were already available when the albumin concentration was 4.0 gm/dL. Danshen is approximately 50–70% bound to serum proteins, and protein binding depends on the concentration of Danshen. For example, in a serum pool containing Danshen, the total Danshen activity was 1.32 ng/mL digoxin equivalent. The free activity was 0.70 ng/mL digoxin equivalent, indicating that 47% of the Danshen was bound to serum protein. In another pool containing Danshen, the total activity was 0.67 ng/mL, and the free activity was 0.21 ng/mL, indicating that approximately 70% of the Danshen was bound to protein. Thus we identified albumin as one of the major binding proteins for Danshen. Other serum proteins may also bind Danshen.

The concentrations of total and free Danshen in a serum pool 1 were 0.92 and 0.43 digoxin equivalents, respectively, digoxin equivalents. In the presence of 300 μ g/mL of salicylate, the free concentration was significantly reduced to 0.34 ng/mL, while the total

Table 1. Interaction between Danshen and salicylate in normal serum pools

Specimen	Apparent digoxin, ng/ml Mean (SD), n = 3		% Free Activity
	Total Activity	Free Activity	
Serum pool 1	0.92 (0.06)	0.43 (0.02)	46.7%
+ 150 µg/mL salicylate	0.97 (0.05)	0.36 (0.01) ^a	37.1%
+ 300 µg/mL salicylate	0.91 (0.03)	0.33 (0.02) ^a	36.3%
+ 400 µg/mL salicylate	0.94 (0.05)	0.31 (0.02) ^a	32.9%
Serum pool 2	0.89 (0.06)	0.45 (0.02)	50.6%
+ 150 µg/mL salicylate	0.89 (0.03)	0.40 (0.01) ^a	44.9%
+ 300 µg/mL salicylate	0.86 (0.03)	0.34 (0.01) ^a	39.5%
+ 400 µg/mL salicylate	0.84 (0.07)	0.31 (0.04) ^a	36.9%
Serum pool 3	1.32 (0.06)	0.70 (0.04)	53.0%
150 µg/mL salicylate	1.38 (0.06)	0.64 (0.03) ^a	46.3%
+ 300 µg/mL salicylate	1.36 (0.03)	0.61 (0.03) ^a	44.8 %
+ 400 µg/mL salicylate	1.38 (0.07)	0.55 (0.04) ^a	39.9%

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

concentration did not change. In the presence of 400 µg/mL (a concentration above therapeutic), the free concentration was further reduced to 0.31 ng/mL (Table 1). This significant reduction in free Danshen activity is not an artifact from salicylate because total Danshen activity in the presence of salicylate did not change from the control value (where no salicylate was present).

The concentration of free Danshen (as measured by apparent digoxin activity) also decreased significantly in the presence of high salicylate concentration. The therapeutic concentration of salicylate is 150–300 µg/mL. When an aliquot of serum pool already supplemented with Danshen was further supplemented with 800 µg/mL of salicylate, we observed a very significant drop in the free Danshen concentration. The total and free concentrations in the control specimen were 1.03 and 0.31 ng/mL, respectively. The free concentration in the presence of 800 µg/mL of salicylate dropped very significantly below the detection limit of the assay (free concentration = 0.16 ng/mL, detection limit of the assay = 0.20 ng/mL). Therefore, we designated the observed free Danshen concentration in the presence of 800 µg/mL of salicylate as “none detected.” The total concentration in the salicylate-supplemented Danshen was 1.08 ng/mL, which was not statistically different from the control value (no salicylate) of 1.03 ng/mL, as expected.

In a separate experiment, we supplemented another serum pool with Danshen. The aliquots of the pool were then further supplemented with very low to moderate salicylate concentrations (5–150 µg/mL). We observed no significant change in free Danshen activity up to a

salicylate concentration of 100 µg/mL. Of note, we observed a significant drop in free Danshen activity (measured as digoxin equivalent) when salicylate concentration was 150 µg/mL, in agreement with our previous experiments (Fig. 1).

We also added Danshen to an albumin stock solution prepared in 0.9% saline containing 4 gm/dL albumin. Aliquots of the albumin solution were then further supplemented with 100–300 µg/mL of salicylate. After further incubation, the total and free activities were measured. We observed no significant change in free activity from the control when the aliquot was supplemented with 100 µg/mL of salicylate. However, with 300 µg/mL salicylate concentration, we observed a statistically significant reduction in free activity.

We also investigated the ability of Danshen to displace salicylate from the protein-binding site. We added various amounts of Danshen extract to different aliquots of the salicylate pool. After incubation, the total and free salicylate concentrations (in the protein-free ultrafiltrate) were measured. We observed significant increases in free salicylate concentrations in the presence of Danshen extract, indicating that Danshen can displace salicylate. For example, the free salicylate concentration was 43.2 µg/mL in the control, and the free concentration was increased to 55.1 µg/mL in the presence of 10 µL Danshen extract. The free concentration was further increased to 60.2 µg/mL in the presence of 25 µL of Danshen extract, and finally, in the presence of 50 µL of Danshen extract, the free salicylate concentration was 68.6 µg/mL.

The displacement of phenytoin, valproic acid, and carbamazepine by salicylate, leading to increased free concentrations of phenytoin and salicylate, has been reported (11–13). However, our observation of decreased free concentrations of Danshen in the presence of therapeutic and toxic concentrations of salicylate was unexpected. It is possible that with therapeutic salicylate concentrations, some configuration change in the binding sites occurs, making additional binding sites available for Danshen. Other endogenous compounds or serum proteins may also play some role, because when we supplemented the albumin solution with Danshen and salicylate, the reduction of free Danshen concentrations was less remarkable than that observed in serum specimens. Moreover, Danshen can displace salicylate from protein binding, thereby increasing free salicylate concentration. This may also play a role in the reduction of free Danshen activity in the presence of salicylate.

We measured total and free Danshen concentrations in serum as apparent digoxin concentrations, taking advantage of the cross-reactivity of Danshen components with the antidigoxin antibody used in the digoxin immunoassay. Tanshinone I, militrinone, and salvicol,

Fig. 1. Total and free Danshen activities as measured by FPIA digoxin assay in the presence of low and lower-end therapeutic concentrations of salicylate.

Fig. 2. Structure digoxin and some components of Danshen with some structural similarity to digoxin.

which are found in Danshen, have structural similarities to digoxin (Fig. 2). These compounds may be responsible for the digoxin-like immunoreactivity of Danshen. Another limitation of this study is the use of FPIA digoxin immunoassay for measuring Danshen concen-

trations. The binding anomaly of Danshen in the presence of salicylate can also be explained by quenching of the fluorescence by labeled digoxin. Quenching will result in a higher polarization value, implying a lower concentration of free Danshen. However, free Danshen activities were gradually decreased in the presence of increasing salicylate concentrations, and especially in the presence of a very high salicylate concentration of 800 mg/mL. Because total Danshen concentrations did not change in the presence of increasing salicylate, the reductions in free Danshen concentrations in the protein-free ultrafiltrate may not be an artifact, but may represent increased protein binding of Danshen in the presence of salicylate. We recently reported an unexpected suppression of free phenytoin concentration in uremic sera in the presence of salicylate, whereas in the normal sera the free phenytoin concentrations were increased as expected (14). The increased protein binding of phenytoin in uremic sera was probably related to the presence of some uremic toxins that not only block phenytoin-salicylate interactions but also, unexpectedly, improve protein binding of phenytoin in the presence of salicylate. Danshen contains many components, and it is possible that some components (which may or may not show digoxin-like immunoreactivity) can block displacement of strongly protein-bound digoxin-like immunoreactive components of Danshen from displacement by salicylate.

This in vitro study provides some interesting data. However, in vivo experiments are needed to establish the clinical significance of these observations. If these findings are also valid in vivo, such effects could be measured by monitoring free digoxin activity in a clinical laboratory if indicated.

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