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Effect of Chinese Medicine Danshen and Indian Ayurvedic Medicine Bark of Arjuna Tree on a Relatively New LOCI Digoxin Assay for Application on the Vista 1500 Analyzer

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Background: Danshen is a traditional Chinese medicine and bark of Arjuna tree is an Ayurvedic medicine both indicated as heart tonic. Interference of Danshen in serum digoxin immunoassays has been reported but potential interference of extract of bark of Arjuna tree has not been reported. We studied potential interferences of Danshen and bark of Arjuna tree on a relatively new LOCI digoxin assay for application on the Vista 1500 analyzer (Siemens Diagnostics). *Methods:* Aliquots of drug-free serum were supplemented with ethyl acetate extract of Danshen (two different brands studied) or aqueous or ethyl alcohol extract of bark of Arjuna tree and apparent digoxin concentrations were measured by the LOCI digoxin assay. In another experiment, aliquots of serum pool containing digoxin were further supplemented with Danshen or bark of Arjuna tree extract and digoxin concentrations were measured again using LOCI digoxin assay. *Results:* Little apparent digoxin concentration was observed when aliquots of drug-free serum pools were supplemented with Danshen or bark of Arjuna tree extract. When aliquots of serum digoxin pool were further supplemented with these extract, we observed statistically significant negative interference but such differences may not be clinically significant. *Conclusion:* We conclude that LOCI digoxin assay is virtually free from interferences of Danshen and extract of bark of Arjuna tree. J. Clin. Lab. Anal. 29:263–267, 2015.

Key words: danshen; bark of arjuna tree; LOCI digoxin assay

INTRODUCTION

Danshen, a Chinese medicine prepared from the root of a medicinal plant *Salvia miltiorrhiza* is traditionally used for various cardiovascular diseases including angina pectoris (1). Li et al., demonstrated a dose dependent hypotensive effect of DanShen extract in normotensive rats with positive inotropic and negative chronotropic effects (2). Cheng commented that Danshen is one of the most versatile Chinese herbal medicines because of its properties of improving microcirculation, causing coronary vasodilatation, suppressing the formation of thromboxane, inhibiting platelet aggregation, and protecting effect against myocardial ischemia (3). Recently, scientists of Shanghai Institute of Materia Medica developed *S. miltiorrhiza* depside salt for use as a novel drug for improving blood circulation and treating coronary heart diseases (4). Danshen is also widely available in the United States from Chinese herbal stores and also from online herbal pharmacies. Danshen contains more than 20 diterpene quinones known as "tanshinones." These compounds have the common structural feature of a phenanthrene quinone ring structure (5) and also have structural similarity with digoxin, a cardiac glycoside.

The bark of Arjuna tree (*Terminalia arjuna*) is widely used in Indian Ayurvedic medicine for treating various cardiovascular diseases. The bark of the tree contains

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many bioactive compounds and several clinical studies have reported its efficacy mostly in treating patients with ischemic heart disease, hypertension, and heart failure. The extract of bark of Arjuna tree also has antioxidant, anti-ischemic, antihypertensive, and antihypertrophic effects. However, some of the clinical studies describing efficacy of bark of Arjuna tree extract have poor experimental design and further studies are needed to establish its therapeutic efficacy (6). Oleanane type triterpene glycosides are considered as bioactive cardiac glycosides present in bark of Arjuna tree (7). Upadhyay et al., reported isolation of new triterpene glycoside (arjunetoside) along with oleanolic and arjunic acids from root bark of Arjuna tree (8). These cardiac glycosides also have some structural resemblance with cardiac glycoside digoxin. Both Danshen and extract of bark of Arjuna tree (dried extract in capsule or liquid extract) are readily available from herbal stores in the United States.

Digoxin has a narrow therapeutic range and it is possible that a patient taking digoxin may also take Danshen or bark of Arjuna tree. We reported earlier that Danshen interferes with serum digoxin measurement by the fluorescence polarization immunoassay (FPIA, Abbott Laboratories, Abbott Park, IL) (9). Potential interference of extract of bark of Arjuna tree with digoxin immunoassays has never been reported before. A new homogenous sequential chemiluminescent immunoassay based on luminescent oxygen channeling technology (LOCI) for digoxin (LOCI digoxin Flex® Reagent, now referred as LOCI Digoxin) has been introduced by Siemens Diagnostics (Deerfield, IL) in fall 2010. This assay is an improved digoxin immunoassay with high specificity, but effects of Danshen on this assay has never been studied before. Here, we report our findings on effect of Danshen and extract of bark of Arjuna tree on LOCI digoxin assay.

MATERIALS AND METHODS

Two brands of Danshen were purchased from local Chinese herbal stores in Houston, Texas. The first product (Brand 1) was manufactured by Shanghai Chinese Botanical Works. The second product (Fufang DanShenpian, Brand 2) where Danshen was the major component was also manufactured in Shanghai, but the label of the manufacturing company is written in Chinese. Authentic dried powder of bark of Arjuna tree was obtained from an Ayurvedic store in Kolkata, India.

The LOCI digoxin assay was purchased from Siemens Diagnostics. Digoxin concentrations were measured using the LOCI digoxin immunoassay on the Vista 1500 automated analyzer following manufacturer's recommendations. The LOCI digoxin assay is a homogenous chemiluminescent assay based on the principle of LOCI technology. The reagent includes two synthetic bead reagents and a biotinylated $F(ab')_2$ fragment of anti-digoxin mouse monoclonal antibody. The first bead reagent (Chemibeads) is coated with ouabain, a weaker binding analog of digoxin and also contains a photosensitizer dye. In the first set of reactions, digoxin present in the specimen is allowed to saturate biotinylated $F(ab')_2$ reagent, which is proportional to amount of digoxin present in the specimen. In the second step, Chemibeads are added to form bead/biotinylated $F(ab')_2$ immunocomplexes with nonsaturated fraction of biotinylated $F(ab')_2$. Sensibeads are then added to form bead pair immunocomplexes and illumination of complexes at 680 nm generates singlet oxygen from Sensibeads which diffuses into Chemibeads triggering a chemiluminescent reaction. The resulting signal is measured at 612 nm and intensity of the signal is inversely related to digoxin concentration in the specimen. The assay uses five level calibrators for calibration and the analytical measurement range of this assay is from 0.06 to 5.0 ng/ml.

One drug-free serum pool and one digoxin serum pool were used for this study. Digoxin serum pool was prepared by mixing individual specimens containing digoxin after removing identity of the patients. These specimens are routinely submitted to our clinical laboratory for therapeutic drug monitoring of digoxin and are discarded after 1 week of storage at 4°C. "Leftover" specimens were used for this study after performing all clinical tests, following report of results to clinicians. These specimens were de-identified and combined according to the approved protocol of our institutional IRB. None of the specimens used for the study were more than 7 days old. Usually we combine 12 to 15 individual specimens to prepare a pool

Standard solution of Danshen was prepared in ethyl acetate by adding 25 mg dry Danshen powder (after removing coating of capsule) per 1 ml of ethyl acetate as described earlier (9). After vortex mixing powdered Danshen with solvent, the solution was allowed to stand at 4°C overnight. Then undissolved residue was removed by centrifugation and clear supernatant was used for supplementation. In the first set of experiments, aliquots of drug-free serum pool were supplemented with 5, 10, 25, or 50 μL of extract per milliliter of serum and then apparent digoxin concentrations were measured using the LOCI digoxin assays. In order to ensure that ethyl acetate did not interfere with the measurement of digoxin, ethyl acetate extracts of Danshen were added to dry test tubes followed by evaporation of solvent using nitrogen at room temperature. Then dry residue was reconstituted with 1 ml aliquot of drug-free serum. Each measurement was performed in triplicate and values were expressed as the mean and one standard deviation.

Aqueous or ethanolic extract of dried powder of bark of Arjuna tree was prepared by mixing 25 mg of dry powder per milliliter of water or ethanol. Because in traditional

Ayurvedic medicine practice bark of Arjuna tree is boiled with water and then water extract is taken for treating various heart diseases, aqueous extract was prepared by boiling dried powder with water for 20 min. Then mixture was cooled at room temperature, allowed to stand at 4°C overnight and centrifuged to separate solid undissolved material from the aqueous layer. For preparation of ethanol extract, the mixture was allowed to stand at 4°C overnight followed by centrifugation to separate undissolved material. Then aliquots of drug-free serum were supplemented with 5, 10, 25, or 50 μL of extract (aqueous or ethanol) per milliliter of serum and then apparent digoxin concentrations were measured using the LOCI digoxin assays. In order to ensure ethanol does not interfere with digoxin assay, ethanolic extracts were added to dry test tube first followed by evaporation of ethanol under gentle stream on nitrogen. Then dry residue was reconstructed with drug-free serum. For aqueous extract of bark of Arjuna tree, microliter amounts of extract were added directly to aliquots of drug-free serum pool.

In another set of experiments, aliquots of serum digoxin pool were further supplemented with ethyl acetate extract of Danshen or aqueous or ethanol extract of dried powder of bark of Arjuna tree. Again to avoid interference, extracts were added to dry tubes (except for aqueous extract of bark of the Arjuna tree) followed by evaporation of solvent under nitrogen and finally reconstituting dry residue in aliquots of serum digoxin pool. Serum digoxin concentrations were measured again using LOCI digoxin assay. Each measurement was performed in triplicate and values were expressed as mean and one standard deviation.

Statistical analyses were performed using independent *t*-test, two-tailed. A difference was considered significant at a 95% confidence interval or higher ($P < 0.05$).

RESULTS

When aliquots of drug-free serum pools were supplemented with various amounts of Danshen extract or extract of bark of the Arjuna tree, apparent digoxin concentrations were observed using LOCI digoxin assay but values were relatively low. For example, when 1 ml aliquot of a drug-free serum was supplemented with 50 μL of Danshen extract, the observed apparent digoxin concentration was only 0.18 ng/ml (Table 1).

Because cross-reactivity of a substance should be tested in the presence of the primary analyte (10), aliquots of two different digoxin serum pools were further supplemented with various amount of Danshen or bark of the Arjuna tree extract and serum digoxin concentrations were measured again using three immunoassays. For example, when digoxin pool 1 was supplemented with 25 μl of Danshen (Brand 1) extract per milliliter of serum, the digoxin value was decreased from 1.54 ng/ml (original digoxin value in the pool) to 1.27 ng/ml, a statistically significant decrease but such decrease may not be clinically significant. Interestingly, when digoxin pool 1 was supplemented with 50 μl of Danshen (Brand 1) extract per milliliter of serum, the observed digoxin value was 1.48 ng/ml, which was not significantly different from the digoxin value of the original pool. We observed similar results with the second brand of Danshen (Table 2). Interestingly, we also observed negative interference of bark of Arjuna tree on serum digoxin measurement using LOCI digoxin assay. For example, when an aliquot of the digoxin pool was supplemented with 25 μl of ethanol extract of bark of the Arjuna tree, the digoxin value decreased from 1.54 ng/ml to 1.36 ng/ml, a statistically significant decrease (Table 3).

TABLE 1. Effect of Danshen and bark of Arjuna tree extract on LOCI digoxin assay

Specimen	LOCI digoxin assay Digoxin concentrations, ng/ml, Mean (SD), $n = 3$
Drug-Free Serum Pool 1	None detected
+ 5 μl Danshen (Brand 1)	0.06 (0.002)
+ 10 μl Danshen (Brand 1)	0.08 (0.002)
+ 25 μl Danshen (Brand 1)	0.13 (0.003)
+ 50 μl Danshen (Brand 1)	0.18 (0.011)
+ 5 μl Danshen (Brand 2)	None detected
+ 10 μl Danshen (Brand 2)	0.07 (0.003)
+ 25 μl Danshen (Brand 2)	0.16 (0.014)
+ 50 μl Danshen (Brand 2)	0.19 (0.004)
+ 5 μl Ethanol extract of bark of Arjuna tree	0.06 (0.001)
+ 10 μl Ethanol extract of bark of Arjuna tree	0.07 (0.002)
+ 25 μl Ethanol extract of bark of Arjuna tree	0.11 (0.003)
+ 50 μl Ethanol extract of bark of Arjuna tree	0.14 (0.002)
+ 5 μl Aqueous extract of bark of Arjuna tree	None detected
+ 10 μl Aqueous extract of bark of Arjuna tree	None detected
+ 25 μl Aqueous extract of bark of Arjuna tree	0.09 (0.015)
+ 50 μl Aqueous extract of bark of Arjuna tree	0.12 (0.003)

DISCUSSION

In our previous study, it was demonstrated that ethyl acetate extract of Danshen showed highest cross-reactivity with the FPIA assay (9). Therefore, we used the same study design in the present study. Because both aqueous and ethanol extract of bark of the Arjuna tree were used in previous studies we followed similar approach in this study.

Therapeutic drug monitoring for digoxin is highly recommended for efficacy as well as avoiding digoxin toxicity (11–14). Digoxin toxicity may occur with lower

TABLE 2. Effect of supplementing aliquots of digoxin pool with various amounts of Danshen extract and serum digoxin measurement using LOCI digoxin assay

Specimen	Loci digoxin assay Digoxin concentrations, ng/ml, Mean (SD), $n = 3$
Digoxin serum pool	1.54 (0.02)
1 ml aliquot of digoxin pool	
+ 5 μl Danshen (Brand 1)	1.50 (0.02)
+ 10 μl Danshen (Brand 1)	1.30 (0.01)[a]
+ 25 μl Danshen (Brand 1)	1.27 (0.03)[a]
+ 50 μl Danshen (Brand 1)	1.48 (0.04)
+ 5 μl Danshen (Brand 2)	1.49 (0.03)
+ 10 μl Danshen (Brand 2)	1.41 (0.01)[a]
+ 25 μl Danshen (Brand 2)	1.43 (0.02)[a]
+ 50 μl Danshen (Brand 2)	1.51 (0.02)

[a]Significantly less than the digoxin value in the digoxin pool by independent *t*-test, two tailed.

TABLE 3. Effect of supplementing aliquots of digoxin pool with various amounts of bark of Arjuna tree extract and serum digoxin measurement using LOCI digoxin assay

Specimen	LOCI digoxin assay Digoxin concentrations, ng/ml, Mean (SD), $n = 3$
Digoxin Serum Pool	1.54 (0.02)
+ 5 μl Ethanol extract of bark of Arjuna tree	1.51 (0.03)
+ 10 μl Ethanol extract of bark of Arjuna tree	1.41 (0.01)[a]
+ 25 μl Ethanol extract of bark of Arjuna tree	1.36 (0.01)[a]
+ 50 μl Ethanol extract of bark of Arjuna tree	1.38 (0.04)[a]
+ 5 μl Aqueous extract of bark of Arjuna tree	1.50 (0.03)
+ 10 μl Aqueous extract of bark of Arjuna tree	1.47 (0.05)
+ 25 μl Aqueous extract of bark of Arjuna tree	1.48 (0.04)
+ 50 μl Aqueous extract of bark of Arjuna tree	1.43 (0.01)[a]

[a]Significantly less than the digoxin value in the digoxin pool by independent *t*-test, two tailed.

digoxin level if hypokalemia, hypomagnesemia, or hypothyroidism coexists. Although digoxin concentration in serum or plasma can be detected accurately by sophisticated analytical techniques such as high performance liquid chromatography combined with tandem mass spectrometry (15), in clinical laboratories digoxin immunoassays are the preferred method due to automation and rapid turn around time of results. Therefore, it is important that digoxin immunoassays are relatively free from interferences. In general difference greater than 20% in serum digoxin measurement is considered as clinically significant. The highest bias we observed was 16.9% negative interference with ethyl acetate extract of brand 1 Danshen. Although such negative interference is statistically significant, it may not be clinically significant.

Interestingly, interference of Danshen in LOCI digoxin assay is bidirectional because with lower amount of extract we observed negative interference but with higher amount of extract, observed digoxin values were not different than the original digoxin value of the pool. As Valdes and Jortani pointed out, such bidirectional interference is most likely caused by complex kinetic characteristics of the immunoreaction (16). Under equilibrium conditions, most cross-reactivity results in a positive interference, but due to demand on immunoassay results to be automated and available faster precludes such equilibrium chemistry, making bidirectional interference a possibility in the automated assays governed by nonequilibrium chemistry. In addition, a wash step might have contributed to negative interference by washing off bound interfering substance if present in low concentration during such step, opening up additional bound antibody sites available for label binding, thereby contributing to altered signal causing negative interference. With more interfering substance present such wash off is not feasible contributing to positive interference. We also previously reported bidirectional (positive/negative) interference of spironolactone, potassium canrenoate and their common metabolite canrenone with digoxin immunoassays (17).

Various tanshinones that have structural similarity with digoxin may be responsible for observed apparent digoxin level. Zhong et al. studied tanshinone levels along with other active ingredients present in Danshen by analysis of 74 different samples using ultra-performace liquid chromatography. Typically tanshinone I levels varied from 0.14 to 0.88 mg/gm of sample while another major ingredient tanshinone IIA level varied from 0.83 to 4.92 mg/gm of sample (18). We used 25 mg of Danshen powder and approximate total tanshinone level (tanshinone I and IIA) present in 25 mg sample was estimated to be between 24.3 and 145 μg. Assuming 80% extraction efficiency (typically efficiency if organic extraction 80–90%), total concentration of tanshinone was between 19.4 and 116 μg/ml. Therefore, 5 μl of such solution had between 97 and 580 ng of tanshinones, which was added to 1 ml aliquot of drug-free serum or digoxin pool for this study. However, this is a very approximate estimate because we did not analyze tanshinone content of the Danshen product used for this study using a chromatographic method. No such calculation is possible for bark of Arjuna tree due to lack publication dealing with quantification of active ingredient in commercially available powder of bark of Arjuna tree. We conclude that relatively new LOCI digoxin assay is virtually free from interference of Danshen and bark of the Arjuna tree.

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