METABOLISM OF TANSHINOL BORNEOL ESTER IN RAT AND HUMAN LIVER MICROSONMES

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Non-standard Abbreviations:

DBZ, tanshinol borneol ester; RLM, rat liver microsome; HLM, human liver microsome; TCM, traditional Chinese medicine; DME, drug metabolizing enzyme; CYP, cytochrome P450; FMO, flavin-containing monoxygenase; UGT, uridine 5’-triphosphate glucuronosyltransferase; LC-MS, liquid chromatography-mass spectrometry; β-NADP, β-nicotinamide adenine dinucleotide phosphate; G-6-P, D-glucose 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; NADPH, reduced form of β-nicotinamide adenine dinucleotide phosphate; EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; CLint, intrinsic clearance; t1/2, half-life; HPLC-UV, high-performance liquid chromatography-ultraviolet; tR, retention time; Km, Michaelis-Menten constants; Vmax, maximum velocity; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry.
ABSTRACT

Tanshinol borneol ester (DBZ) is an experimental drug that exhibits efficacious anti-ischemic activity in rats. Although the specific metabolic properties of DBZ are still unknown, previous studies in rats have strongly suggested that DBZ is extensively metabolized after administration, and thus likely acts as a prodrug. Since the enzymes involved in drug metabolism differ between humans and rats in isoform composition, expression and catalytic activity, the pharmacokinetics of the same drug in the two species may also differ. Establishing the differences between DBZ metabolism in human and rat liver microsomes can help to predict DBZ pharmacokinetics in humans and aid in the assessment of its potential efficacy, toxicity and mechanism of action. In this work, the microsomal stabilities and metabolic kinetics of DBZ in rat and human liver microsomes were compared, and the DBZ metabolites generated in human liver microsomes (HLMs) identified. The results suggested that DBZ is more stable in HLMs than in rat liver microsomes (RLMs). The intrinsic clearance of DBZ in HLMs was 10-17 fold lower than in RLMs, which indicates lower DBZ clearance in humans. Metabolite analysis suggested that DBZ is hydroxylated by liver microsomal enzymes, resulting in the production of five metabolites. Although the kinetics of metabolite formation in HLMs and RLMs were different, the same metabolites were generated, indicating that the same metabolic pathway is present in both species. The results obtained from this work suggest the potential for DBZ to act as a prodrug with anti-ischemic activity in humans.
Introduction

Tanshinol borneol ester \((1,7,7\text{-trimethylbicyclo[2.2.1]heptan-2-yl})3-(3,4\text{-dihydroxyphenyl})-2\text{-hydroxy-propanoate, abbreviated to DBZ})\) is an experimental drug that exhibits efficacious anti-ischemic activity in rats. DBZ is synthesized by the dehydration reaction of two natural compounds, danshensu \((3, 4\text{-dihydroxyphenyllactic acid})\) and borneol \((\text{endo-1,7,7\text{-trimethyl-bicyclo[2.2.1]heptan-2-ol}})\). (Fig 1) Danshensu is the major bioactive component in Radix \textit{Salvia miltiorrhiza}, an herb commonly used in traditional Chinese medicine (TCM) for the treatment of cardiovascular disease (Luo et al., 2001; Cao et al., 2008; Cao et al., 2009). The ability of \textit{Salvia miltiorrhiza} to "thin" the blood and reduce blood clotting is well documented (Sun et al., 2005; Ling et al., 2009). Borneol is a compound derived from \textit{Dryobalanops aromatica}, and is a commonly used adjuvant component in many traditional Chinese herbal medicines to facilitate the delivery of other components (Dharmananda, 1998). Many studies have reported that borneol can enhance the absorption, distribution, permeability and efficacy of other drugs (Mai et al., 2003; Xiao et al., 2007; Cai et al., 2008; Yang et al., 2009).

Unlike chemical drugs, Chinese herbal medicines usually consist of several types of medicinal herbs or minerals that may work by different mechanisms but act in combination to achieve their therapeutic effect (Lu et al., 2008; Wang et al., 2008). In such combination therapy, it is believed that one of the herbs is the active component, while others serve as adjuvants to enhance the efficacy or facilitate the delivery of the active component (Anonymous, 1979, 2003). An herbal medicine composed of Radix \textit{Salvia miltiorrhiza}, borneol and Radix \textit{Notoginseng} has been successfully used and recognized in the official Chinese Pharmacopoeia for the prevention and management of cardiovascular disease (The State Pharmacopoeia Commission of China, 2005).
The design of DBZ, a chemical combination of danshensu and borneol, was inspired by the successful use of this traditional Chinese herbal medicine containing a natural source of danshensu and borneol. Interestingly, in rats, DBZ exhibits cardiovascular effects similar to the herbal medicine, although the exact mechanisms of DBZ's activity are not well understood.

Pharmacokinetic studies in rats have indicated that DBZ is extensively metabolized after injection, as evidenced by the inability to detect DBZ in rat plasma or urine. However, several structurally-related compounds, in particular danshensu, were detected, and these compounds most likely are the metabolites of DBZ. This observation, together with the knowledge that bioactive components in Radix *Salvia miltiorrhiza* exhibit cardiovascular benefits, suggested that DBZ may be a prodrug which is active following its biotransformation to danshensu. Understanding DBZ's metabolism and identifying its metabolites could be helpful for elucidating the mechanism of action of this drug.

Animal models, and particularly rats, are commonly used to predict the metabolism and toxicity of new human drug candidates. However, it is important to realize that humans differ from animals in isoform composition, expression and catalytic activities of enzymes involved in drug metabolism. (Sharer et al., 1995; Godin et al., 2006; Tong et al., 2009) In fact, even small changes in the amino acid sequences of these enzymes can give rise to profound differences in substrate specificity and catalytic activity. The differences between species in expression of the most important family of drug metabolizing enzymes (DMEs), the cytochrome P450s (CYPs), are a major cause of species differences in drug metabolism (Martignoni et al., 2006). To assess these metabolic differences *in vitro*, liver microsomes are commonly used because they contain many important DMEs, including all CYPs, flavin-containing monoxygenases (FMOs) and uridine
5'-triphosphate glucuronosyltransferases (UGTs). Comparative studies of the metabolism of drugs in RLMs and HLMs can help to understand the species differences in drug metabolism between rats and humans and predict the drugs’ hepatic clearances in humans (Naritomi et al., 2001).

In this work, the metabolism of DBZ in both RLMs and HLMs was studied. The microsomal stabilities of DBZ, DBZ metabolites, and the metabolic kinetics in both species of liver microsomes were compared to determine the species differences in DBZ metabolism. DBZ was found to be cleared faster in RLMs than in HLMs, while the metabolites generated in both microsomes were the same. Furthermore, the DBZ metabolites generated in HLMs were identified by liquid chromatography-mass spectrometry (LC-MS) and their chemical structures deduced. These findings provide useful information for understanding DBZ’s mechanism of action and for predicting DBZ metabolism in human.
Materials and Methods

Materials. DBZ was synthesized in our lab and verified by LC-MS and nuclear magnetic resonance spectroscopy. β-nicotinamide adenine dinucleotide phosphate (β-NADP), D-glucose 6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Sigma (St. Louis, MO). All other reagents and organic solvents for HPLC were of the highest grade from commercial sources.

Rat liver tissues were obtained from male Sprague-Dawley rats (90 days old) weighing 350 to 400 g. Human liver tissues were provided by Taizhou University’s school of medicine, as surgical specimens of human liver.

Microsome Preparation. Pooled rat and human liver microsomes were prepared by differential centrifugation according to methods previously described (Pearce et al., 1996). Liver tissues were homogenized in 4x volume of microsome buffer (0.1 M potassium phosphate, 10% sucrose, 0.1 mM EDTA, 2 mM DTT, and 1 mM PMSF, pH 7.4), then centrifuged (9,000 ×g, 30 min, 4°C). The pellet was discarded and the supernatant was then centrifuged (100,000 ×g, 60 min, 4°C). The microsomal pellet was washed by resuspension in fresh microsome buffer and centrifuged again (100,000×g, 60 min, 4°C). Microsomes were resuspended in microsome buffer and stored frozen in 0.5 mL aliquots at -80°C. Microsomal protein concentration was quantified by Bradford assay (Bradford, 1976).

Microsomal Incubation. DBZ was incubated with pooled liver microsomes in 0.4 mL of 0.1 M phosphate buffer (pH 7.4). Incubations were initiated by the addition of NADPH-regenerating buffer (β-NADP at 1.3 mM, G-6-P and MgCl₂ at 3.3 mM, and G6PDH at 0.4 U/mL), and samples were maintained at 37°C for a given time. Stop-buffer (0.5 μg of nitrendipine (as internal standard)
in 0.15 mL of ice-cold acetonitrile) was added to terminate the incubation. Incubation mixtures were extracted by adding 0.8 mL of ethyl acetate and 0.1 mL of 1 M NaCl. After vortexing, samples were centrifuged at 4°C, 12000 ×g for 10 min. The organic phase was pipetted into a new tube for evaporation, and then the residue was dissolved in 50 μL of HPLC mobile phase buffer for analysis.

**DBZ Microsomal Stability.** Intrinsic clearance (CL$_{int}$) of DBZ was estimated from the in vitro half-life of substrate depletion (t$_{1/2}$), incubation volume (V), and mass of microsomal proteins in the incubation mixture (P), according to the following equation: CL$_{int}$ = (0.693/t$_{1/2}$) × (V/P). (Obach et al., 1997) For t$_{1/2}$ determination, DBZ at 3 μM was incubated with 0.1 mg/mL RLMs for 0, 0.5, 1, 1.5, 2, 3, 5 and 8 min, and with 0.5 mg/mL HLMs for 0, 2, 4, 8, 12, 15, 20, 30 and 45 min. After extraction, DBZ in samples was analyzed by HPLC-UV detection as described below. The percent of DBZ remaining was plotted versus incubation time. The t$_{1/2}$ of DBZ was calculated by regression analysis of semi-logarithmic plots.

**DBZ Kinetics.** The incubations were carried out using conditions that had previously been determined to be within the linear range for metabolite formation with respect to time and microsome concentration. DBZ was added in serial 2-fold dilutions from 320 to 8 μM. DBZ at each concentration was incubated with RLMs (0.2 mg/mL) and HLMs (0.5mg/mL) for 10 and 30 min, respectively. After extraction, metabolites were analyzed by HPLC-UV detection as described below. The metabolite formation rates were plotted versus DBZ concentrations to obtain the Michaelis-Menten constant (K$_m$) and maximum velocity (V$_{max}$) values of the reactions.

**HPLC-UV Analysis.** 40 μL of each sample was injected into an Agilent 1200 Series RRLC system (Agilent Technologies, Inc.; Santa Clara, CA). Components were separated on an Agilent
TC-C18 column (150×4.6 mm, 5 μm) and detected by UV\textsubscript{280} nm. Column temperature was maintained at 30°C. For DBZ analysis, the mobile phase, at a flow rate of 0.8 mL/min, contained 40% solvent A (0.2% formic acid in water) and 60% solvent B (methanol). For metabolite analysis, the methanol in the mobile phase was lowered to 45% to enhance separation.

**LC-MS/MS Analysis.** DBZ (100 μM) was incubated with HLMs (0.5 mg/mL) in 0.5 mL of 0.1 M phosphate buffer (pH 7.4) for 60 min in order to generate enough metabolites for detection. The reaction was terminated by adding 0.15 mL of ice-cold acetonitrile.

LC-MS/MS analysis was carried out using an Agilent 1200 Series RRLC system coupled to an Agilent 6510 Accurate-Mass Q-TOF spectrometer. An Agilent Plus C18 column (2.1×100 mm, 1.8 μm) was employed for separation. Column temperature was maintained at 30°C. The mobile phase flow rate was 0.3 mL/min. The gradient of methanol in the mobile phase was raised from 35% to 70% over 12 min, and from 70% to 95% over the following 6 min (12~18 min), and then cycled back to the initial conditions.

Accurate mass measurements were performed using an Agilent 6510 Accurate-Mass Q-TOF instrument by flow injection of 5 μL of the sample. The detection by LC-MS was accomplished using electrospray ionization in the negative mode, with the scanning range from 100 to 1100 m/z (4GHz), drying gas flow 9 L/min, nebulizer pressure 40 psi, dry gas temperature 300°C, ion spray voltage 4000 V, fragmentor voltage 130 V. The mass scanning range for LC-MS/MS was from 50 to 1100 m/z (4GHz) with the reference mass 1033.9881. The data collection was performed using Agilent MassHunter WorkStation software.

**Data Analysis.** \(K_m\) and \(V_{\text{max}}\) were determined by non-linear regression fitting using Prism® Version 4.03 (GraphPad Software, Inc.; San Diego, CA). The Student’s \(t\) test was employed for
statistical analysis. The $p$ value of < 0.05 was considered statistically significant.
Results

DBZ Microsomal Stability: RLMs versus HLMs. The metabolism of a particular drug can differ from species to species. To address the differences in metabolic clearance of DBZ in RLMs and HLMs, we incubated DBZ with liver microsomes from each species, and determined the half-life ($t_{1/2}$) of DBZ depletion. The $t_{1/2}$ of DBZ in HLMs was 9.28 ± 1.59 min (mean ± S.E., n=4), which was 3-fold longer than in RLMs. (Fig. 2) The DBZ clearance (CL_{int}) in HLMs, as calculated from the $t_{1/2}$ values, was about 17-fold lower than in RLMs. These results suggested that DBZ is more stable in HLMs than in RLMs.

Comparison of DBZ Metabolites. To determine which metabolites of DBZ were generated by RLMs and HLMs, we incubated DBZ with liver microsomes from each species and analyzed the metabolites by HPLC-UV detection. Compared with the negative control, DBZ incubated with either RLMs or HLMs resulted in the generation of five new peaks on the chromatograms, which indicated the formation of five metabolites. These metabolites were designated M1 to M5 according to their retention times ($t_R$s). (Fig. 3) The chromatographic patterns of metabolites generated by HLMs and RLMs were similar. The most abundant metabolite generated by RLMs was M4, while for HLMs the most abundant metabolite was M3. (Fig. 3. a, c) This result suggested that the five metabolites generated by RLMs and HLMs are the same, although the most abundant metabolite is different.

Kinetics of Metabolites Formation: RLMs versus HLMs. To further determine the differences in DBZ metabolism between RLMs and HLMs, we examined the kinetics of metabolite formation in RLMs and HLMs. The kinetic parameters are summarized in Table 1. The $K_m$ values for metabolite formation for all five metabolites generated by RLMs were about twice as high as for
HLMs. The $V_{\text{max}}$ values for metabolite formation by RLMs were significantly higher than for HLMs. For example, the $V_{\text{max}}$ for M4-formation by RLMs was 44-fold higher than for HLMs ($p < 0.01$). The intrinsic clearance ($CL_{\text{int}}$) for formation of each DBZ metabolite was calculated from the ratio of $V_{\text{max}}/K_m$. For RLMs, the highest $CL_{\text{int}}$ of DBZ-metabolite formation was for M4 formation. In contrast, for HLMs, the highest $CL_{\text{int}}$ was for M3 formation. The total of the $CL_{\text{int}}$s for formation of all metabolites in RLMs reflects the total $CL_{\text{int}}$ of DBZ in RLMs, which is 10-fold higher than in HLMs. These results suggested that DBZ is cleared faster in RLMs than in HLMs, and also that the major DBZ metabolite formed in RLMs is M4, while in HLMs it is M3.

**Metabolite Profiling.** To identify the DBZ metabolites generated by HLMs, both DBZ and its metabolites were analyzed by LC-MS/MS. On the negative ion spectrum of DBZ (the parent compound), an abundance of ion was seen at $m/z$ 333.1715 (ion formula C$_{19}$H$_{25}$O$_5$; calc. $m/z$ 333.1710; mass accuracy, 1.25 ppm) (Fig. 4 f). The five metabolites, M1 to M5, showed an abundance of ions at $m/z$ 349.1670, 349.1669, 349.1659, 349.1664, and 349.1658, respectively (Fig. 4 a-e). All ions of the five metabolites had a calculated ion formula of C$_{19}$H$_{25}$O$_6$ (calc. $m/z$ 349.1654; mass accuracy, < 1.5 ppm), representing the difference of a single oxygen atom from the ion formula of DBZ. This result indicated that DBZ is hydroxylated in HLMs, resulting in the production of five different metabolites with an additional oxygen atom.

The fragment ions of M1, M2 and M3 were similar to each other not only in category but also in percent abundance, which indicated that these three metabolites have a very similar structure (Fig. 4 a-c). The fragment ions of M4 and M5 were almost identical to each other, but they differed from those of the other three metabolites in the abundance of ion at $m/z$ 197 (Fig. 4 d, e). This suggested a structural similarity between M4 and M5, which is not shared by the metabolites.
M1, M2 and M3.

To obtain further insight into the chemical structures of the DBZ metabolites, the structures of the fragment ions on the MS/MS spectra of DBZ and its metabolites were comparatively analyzed (Fig. 5). The MS/MS spectrum of M3 is shown in Fig. 5 b as a representative spectrum of the first three metabolites, which exhibit a similar spectrum. Likewise, the spectrum of M4 is shown in Fig. 5 c to represent the spectrum of the last two metabolites.

The major abundant fragment ions of DBZ shown on the MS/MS spectrum were ions at \( m/z \) 123, 151 and 179. The chemical structures of these three ions were deduced from their formulae calculated from the accurate mass values. They all contained an aromatic group, which was derived from the danshensu moiety of DBZ (Fig. 5 a). These three ions, which were abundant on the MS/MS spectrum of DBZ, also appeared in abundance on the MS/MS spectra of all five metabolites. This indicated that the metabolites shared with DBZ a structure that could generate ions at \( m/z \) 123, 151 and 179, which is the danshensu moiety of DBZ. This result also suggested that the hydroxylation site of the metabolite is not on the danshensu moiety.

One difference between DBZ and its metabolites was the abundance of ions at \( m/z \) 211 on the spectrum of DBZ, which was not found on the spectra of any of the metabolites. By contrast, an ion at \( m/z \) 227 was found on the spectrum of M3 but not on that of DBZ (Fig. 5 a, b). These two ions have a mass value discrepancy of sixteen, and they were derived from the borneol moiety of DBZ according to their deduced chemical structures (Fig. 5 b). This result suggested an oxygen atom was added to the borneol moiety of DBZ in the production of the first three metabolites. Although the ion at \( m/z \) 227 was not found on the spectra of M4 and M5, the abundance of the ion at \( m/z \) 197 indicated that the hydroxylation sites in M4 and M5 were not on the danshensu moiety.
but could only be on the borneol moiety of DBZ. The deduced chemical structures of the ion at
m/z 197 revealed that the danshensu moiety of M4 had no additional oxygen atom (Fig. 5 c).
These results confirmed that the hydroxylation of DBZ takes place on the borneol moiety of DBZ,
and that the danshensu moiety is not modified by enzymes present in HLMs.
Discussion

In the present study, the metabolic clearance of DBZ in rat and human liver microsomes was compared. The results predict different DBZ pharmacokinetics in rats and humans, since the metabolic clearance of DBZ in HLMs was much lower than in RLMs. DBZ is also cleared more extensively in RLMs than in HLMs. The half-life ($t_{1/2}$) of DBZ depletion in RLMs was about 2 min, which is 3-fold faster than in HLMs (Fig 2), and the DBZ clearance in RLMs was 17-fold higher than in HLMs. The fast and extensive metabolism of DBZ in RLMs is consistent with the results of pharmacokinetic studies in rats, which found that no DBZ is detectable in rat plasma and urine immediately after injection (data not shown). This is likely because DBZ had already been cleared during the time the blood and urine samples were collected, since the $t_{1/2}$ of DBZ in RLMs is much shorter than the time required for sample preparation.

The Michaelis-Menten kinetic studies for metabolite formation in RLMs and HLMs confirmed that DBZ metabolism in RLMs is more extensive than in HLMs. The $CL_{int}$ for the formation of all five DBZ metabolites in RLMs were significantly higher than in HLMs (Table 1). This result also indicated that the enzyme affinities for DBZ in HLMs are higher than in RLMs (reflected by lower $K_m$ values), which supports the idea that the enzymes responsible for DBZ metabolism in RLMs and HLMs are different. It is reasonable that the DBZ metabolic kinetics in RLMs and HLMs differ, because the different enzymes would have distinct capacities for drug metabolism (Sharer et al., 1995; Guengerich, 1997). The in vitro kinetic data for DBZ metabolism in rat and human liver microsomes, when correlated with the pharmacokinetic observations in rats, should help to predict the pharmacokinetics in humans (Obach, 1999; Naritomi et al., 2001).

Although the kinetics for DBZ metabolite formation in RLMs and HLMs are different, the
specific metabolites generated in both liver microsomes are the same (Fig. 3). Some drug-metabolizing enzymes found in liver microsomes, such as the CYP superfamily, have well-conserved structures among different species (Mestres, 2005; Poulos and Johnson, 2005), and thus have similar abilities to generate the same metabolites. Determining whether the DBZ metabolites generated in HLMs are the same as in RLMs is crucial to predicting the efficacy of DBZ in humans, because DBZ’s anti-ischemic effects in rat most likely result from DBZ metabolites. The five metabolites generated by HLMs have the same retention times ($t_R$) as the corresponding metabolites generated by RLMs, according to HPLC analysis (Fig. 3). When the HPLC conditions were varied, such as by lowering the column’s temperature or switching the ratio of mobile phase solvents, the $t_R$s for metabolites generated by both liver microsomes remained unchanged (data not shown). This confirms that the metabolites generated in both species of microsomes are the same, which indicates the presence of the same pathway for DBZ metabolism in rats and humans.

The LC-MS analyses demonstrated that the mass value of each of the five DBZ metabolites is sixteen higher than the mass value of DBZ, which indicates that a single oxygen atom was added (Fig. 4). Comparing the MS/MS spectra of DBZ with its metabolites, the oxygen atom is likely added to the borneol moiety of the DBZ structure, resulting in the hydroxylation of DBZ (Fig. 5). There are six candidate sites that are likely to be hydroxylated in the borneol structure: three methyl (–CH$_3$) and three methylene (=CH$_2$) groups (Fig. 1). Hydroxylation of different candidate sites apparently resulted in the five different metabolites observed. The hydroxylation sites in M4 and M5 might result in the molecule being more susceptible to cleavage at the bond linking danshensu and borneol to generate an ion at m/z 197. Therefore, the abundance of this ion
on the MS/MS spectra of M4 and M5 was higher than for the other three metabolites (Fig. 4 d, e).

The particular group that was hydroxylated to generate each metabolite cannot be determined from the mass spectra. However, these results demonstrated that the danshensu moiety of DBZ is not modified by enzymes in liver microsomes.

Monooxygenase activity is a feature of phase I enzymes that are embedded in liver microsomes, typically CYPs and FMO (McManus et al., 1987; Waxman et al., 1988). Their activities usually result in the hydroxylation of substrates, which makes these substrates more easily cleared by downstream metabolic processes, such as conjunction reactions catalyzed by phase II enzymes. The hydroxylation site of the metabolites generated by phase I enzymes usually is also the site recognized by phase II enzymes for conjunction reactions (Sheweita, 2000; Guengerich, 2006). For the DBZ metabolites, all five hydroxylation sites were located in the borneol moiety of the compound, and thus only this part, and not the danshensu moiety of DBZ, would likely be modified by phase II enzymes. This may be the reason why compounds structurally related to borneol are not found in rat plasma and urine after DBZ administration, while danshensu is found.

Identifying the metabolites of DBZ can help in the understanding of DBZ’s mechanism of action. DBZ is designed by combining danshensu and borneol in a single molecule for the purpose of enhancing the efficacy of danshensu. This drug exhibits the expected cardiovascular effects in rats, similar to the herbal medicine containing Salvia miltiorrhiza and borneol. Pharmacokinetic studies in rats strongly suggested that DBZ acts as a prodrug, because it is extensively biotransformed in rats and the major metabolite in the plasma and urine is danshensu, the bioactive component of Salvia miltiorrhiza. Although the in vitro metabolite analysis could not
directly prove that danshensu is the final metabolite of DBZ in humans, the results demonstrated that the hydroxylated metabolites of DBZ generated in HLMs and RLMs are the same, and thus the metabolic pathways in humans and rats may both ultimately lead to the production of danshensu. The metabolite analysis also demonstrated that the danshensu moiety of DBZ was not modified by HLM enzymes, which also suggested that danshensu could be the final metabolite in humans. These in vitro DBZ metabolism studies support the hypothesis that DBZ acts as a prodrug with effects similar to danshensu. However, additional studies, such as phase II metabolism and pharmacodynamic studies, should be conducted to further understand DBZ’s mechanism of action.

In conclusion, the metabolic kinetics of DBZ in HLMs and RLMs were shown to differ, with DBZ being metabolized more extensively and quickly in RLMs than in HLMs. The kinetic parameters from the in vitro assays suggest that a different DBZ pharmacokinetic profile will be observed in humans, although the metabolites generated by both species of liver microsomes are the same. The structural analyses of the metabolites suggested that DBZ is hydroxylated by enzymes embedded in liver microsomes, and that the hydroxylation sites are on the borneol but not on the danshensu moiety of DBZ. These results support the prediction that danshensu is the active metabolite of DBZ in humans, and suggest that DBZ may be effective as a prodrug for the clinical treatment of ischemia.
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References


Sun J, Huang SH, Tan BK, Whiteman M, Zhu YC, Wu YJ, Ng Y, Duan W, and Zhu YZ (2005) Effects...


Footnotes

D.L. and X.Z. contributed equally to this work.

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Legends for figures

**Figure 1.** Chemical structures of danshensu, borneol and DBZ, with the six candidate sites for hydroxylation numbered.

**Figure 2.** *In vitro* half-life of DBZ incubated with liver microsomes. (a) with RLMs, $t_{1/2} = 2.35$ min. (b) with HLMs, $t_{1/2} = 9.12$ min. DBZ at 3 μM. Data points represent means ± S.E. obtained from four independent experiments. Dotted line represents 50% of DBZ remaining.

**Figure 3.** Chromatograms of samples obtained by incubating DBZ (200 μM) with liver microsomes (0.5 mg/mL). (a) RLMs, +NADPH, (b) RLMs, -NADPH; (c) HLMs, +NADPH, (d) HLMs, -NADPH. Incubation time for RLMs is 30min, for HLMs is 120min. Metabolite peaks were designated M1 to M5 according their retention times. Conditions: column (150×4.6 mm, 5 μm); mobile phase: H$_2$O (0.2% formic acid)/MeOH, 55/45, 0.8mL/min; 30°C.

**Figure 4.** Comparison of the MS/MS spectra of DBZ and its metabolites detected in the negative-ion-mode ESI-MS analysis of samples incubated with HLMs. (a) M1, (b) M2, (c) M3, (d) M4, (e) M5 and (f) DBZ. Spectra were recorded using an Accurate-Mass 6510 Q-TOF instrument.

**Figure 5.** Structures of the major fragment ions detected on the MS/MS spectra of (a) DBZ, and the major metabolites, (b) M3 and (c) M4. Structures of the fragment ions were deduced from their formulae calculated according to the accurate mass values.
TABLE 1

Comparison of kinetic parameters for DBZ metabolites formed in RLMs and HLMs.

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<td>12.9 ± 0.3</td>
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<td>18.7 ± 1.9</td>
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<td>24.1 ± 0.8*</td>
<td>41.9 ± 2.6</td>
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<td>M5</td>
<td>31.5 ± 1.6</td>
<td>23.2 ± 0.5*</td>
<td>7.6 ± 0.5</td>
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a. The quantity of each metabolite was represented by its area under the curve (AUC) on the chromatogram, since calibration was unnecessary for the purpose of this comparison; Values are means ± S.E. obtained from three independent experiments; * p <0.05, ** p <0.01 versus the RLM values.
Figure 2

% of DBZ remaining vs. incubation time (min).

(a). RLM

(b). HLM