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Effects of the aqueous extract from *Salvia miltiorrhiza* Bge on the pharmacokinetics of diazepam and on liver microsomal cytochrome P450 enzyme activity in rats

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Abstract

The aim of this study was to determine the effects of the aqueous extract of *Salvia miltiorrhiza* Bge (danshen in Chinese) on the pharmacokinetics of diazepam and on liver microsomal cytochrome P450 enzyme activity in rats. Rats (n = 5) were pretreated with danshen extract (100 mg kg^{-1} per day, p.o.) for 15 consecutive days. Control rats (n = 5) received saline at the same time. Each rat was then administered a single oral dose of 15 mg kg^{-1} diazepam. The pharmacokinetic parameters of diazepam were significantly different between the two groups. In the danshen pretreated group, the maximum concentration of diazepam and the area under the plasma concentration–time curve were reduced to about 72.7% and 44.4%, respectively, while the total body clearance was markedly increased by 2-fold. To help explain the results, liver microsomal suspensions were obtained from rats that were randomly divided into the control group (n = 10), and the low- (20 mg kg^{-1} for 15 days, p.o., n = 10) and high-dose groups (100 mg kg^{-1} for 15 days, p.o., n = 10) pretreated with danshen extract. Compared with the control rats, the microsomal protein content, cytochrome P450 enzyme level and erythromycin *N*-demethylase activity of pretreated rats were significantly increased. These results indicate that danshen extract can stimulate the activity of cytochrome P450 isoforms, and changes in the pharmacokinetics of diazepam resulting from danshen extract are related to an increase in metabolic activity of cytochrome P450.

Introduction

Traditional Chinese medicine has been widely used in the treatment of many diseases because its therapeutic efficacy is mild and broad, and the incidence of adverse reactions is relatively low in comparison with synthetic drugs (Lee 2000). However, traditional Chinese medicine contains many compounds that influence the activity of cytochrome P450 (CYP) isoforms (Hodek et al 2002), for example, *Angelica dahurica* extract inhibits various CYP isoforms, such as CYP2C, CYP3A and CYP2D1 (Ishihara et al 2000). CYP isoforms contribute to the metabolism of numerous drugs (Wrighton & Stevens 1992; Nishimura et al 1998; Scott & Elmer 2002). Modulation of CYP activity may cause pharmacokinetic changes in other drugs, resulting in a decrease in efficacy or increase in side-effects (Homma et al 1995).

The hot water extract from the danshen root has been reported to have many types of pharmacological action, such as antiplatelet aggregation, promoting blood circulation, relieving blood stasis, clearing heat from the blood, antioxidant activity and antifibrotic activity (Huang & Zhang 1992; Nan et al 2001). Moreover, danshen has been officially listed in the Chinese pharmacopoeia (The Pharmacopoeia Commission of PRC 2000) as a major component in many traditional Chinese medicine preparations such as Compound Danshen Dropping Pills and Danshen Injections, which have been widely used to treat chronic diseases such as hepatitis, cardiovascular disorders and angina pectoris (Luo et al 2001). In clinical practice, principal synthetic or biotechnological drugs are commonly prescribed for these chronic diseases together with traditional Chinese medicine for supplementary purposes. It is therefore important to obtain information about possible pharmacokinetic interactions between danshen and co-administered drugs, as well as its effects on CYP activity.

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In this study, the effects of the aqueous extract of danshen on the pharmacokinetics of diazepam were studied in rats. To further elucidate the results, the effects of danshen aqueous extract on CYP isozyme activity were also studied.

Materials and Methods

Chemicals and reagents

Diazepam, aristolochic acid (the internal standard), erythromycin and bovine serum albumin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). NADPH was obtained from Sigma Chemical Co. (St Louis, USA). Danshen roots were purchased from the market and authenticated as *Salvia miltiorrhiza* Bge by Professor Ma Lin (Institute of Materia Medica, Chinese Academy of Medical Sciences Beijing, China). Acetonitrile was of high-performance liquid chromatography (HPLC) grade from Merck Co. (Darmstade, Germany). All other chemicals used were of the highest purity available.

Animals

Male Wistar rats, 6–7 weeks old, 200–250 g, were obtained from the Institute of Laboratory Animals Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). The rats were randomly divided into the control group, and the low- ($20\,mg\,kg^{-1}$ per day) and high-dose groups ($100\,mg\,kg^{-1}$ per day) pretreated with danshen extract. Rats were maintained in a clean room (Animal Center for Pharmaceutical Research, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College) at a temperature of 20–23 °C, with a 12-h light–dark cycle and 50% relative humidity. Rats were individually housed in metabolic cages with a supply of filtered pathogen-free air. Water was freely available. The Animal Care and Use Committee of the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, approved the animal study protocol.

Danshen aqueous extract was administered orally once daily for 15 consecutive days. The control rats received an equal volume of saline.

Preparation of danshen extract

Danshen extract was prepared by referring to the standard preparation of Danshen Injections (Jin & Cheng-yu 1996). Briefly, danshen roots (600 g) were chopped into small pieces and refluxed three times with 3600 mL of boiling distilled water for 1 h. The extract was concentrated to 1200 mL under reduced pressure. Then, 450 mL of ethanol was added to the concentrated solution, which was kept at 4 °C for 12 h. The solution was then filtered. After removing the ethanol under reduced pressure, the remaining aqueous solution was acidified and extracted three times with 1800 mL ethyl acetate. The solvents were evaporated to dryness under reduced pressure. The extraction yield was approximately 7.8% (w/w). The dried residues were dissolved in 600 mL distilled water and adjusted to pH 6.8 with NaOH (10%). The main active components of this extract include danshensu (3,4-dihydroxyphenllactic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), salvianolic acid A, and salvianolic acid B (Zhou et al 1999).

Pharmacokinetic experiments

On the morning of Day 16, each rat was anaesthetized with ether and the right external jugular vein was cannulated. Animals were then fasted for 12 h but had free access to water. Diazepam ($15\,mg\,kg^{-1}$) was administered orally to the control rats ($n = 5$) and to the rats pretreated with danshen extract ($100\,mg\,kg^{-1}$ daily for 15 days, p.o., $n = 5$).

A blood sample (0.5 mL) was withdrawn into heparinized tubes at 5, 10, 20, 30, 60, 120, 150, 180, 240, 360, 480 and 600 min after dosing. The plasma was immediately separated by centrifugation and stored at −20 °C before HPLC analysis.

The plasma (0.2 mL) was transferred to a tube and 50 μL of the internal standard ($40\,ng\,\mu L^{-1}$) was added and mixed by shaking before adding 3.0 mL of a mixture of ethyl acetate and acetone (10:0.1). The sample was shaken at 100 oscillation min^{-1} for 10 min and centrifuged at 4000 *g* for 10 min. The organic layer was transferred to a test tube and dried under a stream of nitrogen at 45 °C. The residue was reconstituted in 200 μL of mobile phase and a 20-μL sample was analysed by HPLC.

The plasma diazepam concentrations were determined by HPLC. The HPLC system comprised a PU-980 pump, UV-975 detector (Jasco, Japan) and SRI Model 203 PeakSimple Chromatography Data System. A reversed-phase C18 column (YMC, 5 μm, 150 × 3.0 mm i.d.) was used. The mobile phase consisted of acetonitrile, water and acetic acid (48:52:1, v/v) at a flow rate of $0.3\,mL\,min^{-1}$, and the detection wavelength was 254 nm.

The pharmacokinetic parameters were estimated using 3p87 software (Chinese Pharmacological Society, Beijing, China).

Preparation of rat liver microsomes

The livers from the control group ($n = 10$), the low-dose group ($n = 10$) and the high-dose group ($n = 10$), pretreated with the extract, were rinsed with ice-cold sodium chloride solution (0.9%) and homogenized in triple volumes of 1.15% KCl. The homogenate was centrifuged at 9900 *g* for 30 min. The supernatant was then ultracentrifuged at 105 000 *g* for 60 min. Throughout the process, the temperature was kept at 4 °C (Kremers et al 1981). The obtained microsomal pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing glycerin. Each suspension was divided into aliquots, frozen and stored at −70 °C until used.

Assays of CYP activity

The microsomal protein content was measured using the method of Lowry et al (1951), using bovine serum albumin

as a standard. The total CYP in hepatic microsomes was measured according to the method of Omura & Sato (1964).

Erythromycin *N*-demethylase activity in the liver was evaluated by measuring the metabolism velocity of erythromycin in liver microsomal suspensions. The microsomal protein suspension (0.2 mL, 2.5–5 mg protein mL^{-1}) was preincubated at 37 °C for 30 s. $MgCl_2$ (6.25 mM), NADPH (2.5 mM), and erythromycin (1 mM) were dissolved in Tris-HCl buffer (pH 7.4). This mixture (0.8 mL), prewarmed at 37 °C, was added to the microsomal suspension to initiate the metabolic reaction. The enzyme reaction was allowed to continue for 20 min and was terminated by adding 1 mL 5 M KOH solution. Formaldehyde, one of the products of erythromycin, was measured according to the method of Nash (1953).

Statistical analysis

One-way analysis of variance was used to estimate the significance of differences. A difference with $P < 0.05$ was considered statistically significant. Data are expressed as mean ± s.d.

Results

Pharmacokinetics of diazepam

Under the HPLC system condition, the calibration plot for plasma diazepam was linear over the concentration range 50–1250 ng mL^{-1}, the limit of detection was 30 ng mL^{-1}, and the mean recovery was 98.2%. The regression equation was $y = 2.0630 \times 10^{-3}x - 0.0164$, $r = 0.9974$, where y is the peak area ratio of the drug to the internal standard, and x is plasma concentration (ng mL^{-1}) of diazepam. The interday and intraday relative standard deviations were all less than 5%. The retention times of diazepam and the internal standard were 12.30 and 17.50 min, respectively. Matrix impurities did not interfere with diazepam or the internal standard in the typical chromatograms.

The plasma concentration–time curves after a single oral administration of diazepam to rats are presented in Figure 1. The time to reach the maximum concentration was 58.8 min and 72.7 min in the pretreated group and the control group, respectively. The maximum concentration of diazepam was significantly decreased from 297.52 to 216.28 ng mL^{-1} by pretreatment with danshen extract. In the elimination phase, the plasma diazepam concentration tended to be reduced by pretreatment with danshen extract; the plasma diazepam concentration was lower than the limit of detection after 360 min.

The plasma concentration–time curves for both groups were adequately described by a first-order absorption two-compartment open model. The corresponding data are summarized in Table 1. The elimination half-life ($t_{1/2}\beta$), the distribution volume (V/F) and area under the curve (AUC) for diazepam were significantly different between the control group and the group pretreated with danshen extract. The $t_{1/2}\beta$, V/F and AUC were reduced to approximately 39.5%, 28.0% and 44.4%, respectively, by pretreatment with danshen extract, while the clearance was markedly increased by 2-fold. No significant alteration was observed in $t_{1/2}\alpha$ between the two groups.

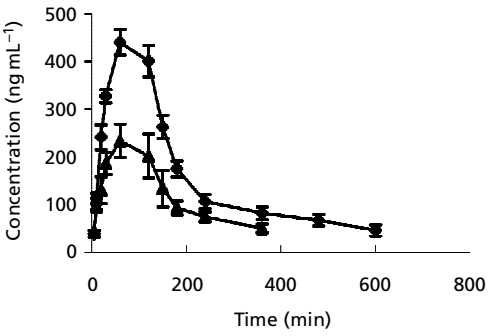

Figure 1 Plasma concentration–time curves of diazepam after a single oral dose of 15.0 mg kg^{-1} diazepam to control rats (n = 5; ♦) and to rats pretreated with danshen extract (100 mg kg^{-1} for 15 days, p.o., n = 5; ▲). Each value represents the mean ± s.d of five animals.

Table 1 Pharmacokinetic parameters of diazepam (15 mg kg^{-1}) after oral administration to control rats (n = 5) and to rats pretreated with danshen extract (n = 5).

Parameter	Control group	Danshen extract pretreated group
$t_{1/2}\alpha$ (min)	54.80 ± 5.09	50.86 ± 9.47
$t_{1/2}\beta$ (min)	602.97 ± 119.90	238.41 ± 76.63*
V/F (L kg^{-1})	105.69 ± 74.50	29.56 ± 6.26*
CL (mL min^{-1} kg^{-1})	81.60 ± 17.95	162.80 ± 15.93*
AUC (mg min L^{-1})	132.14 ± 38.94	58.67 ± 8.55*
t_{max} (min)	72.72 ± 5.94	58.80 ± 4.68*
C_{max} (ng mL^{-1})	297.52 ± 23.64	216.28 ± 30.21*

Values are mean ± s.d. *$P < 0.05$ compared with the control group.

CYP enzyme activity

Table 2 shows the effects of danshen extract on the activity of CYP enzymes in rats. The microsomal protein content increased 1.19-fold and 1.24-fold after pretreatment with low-dose and high-dose danshen extract, respectively. The CYP content and erythromycin *N*-demethylase activity of pretreated groups were significantly higher than in the control group.

Discussion

Increasingly, various preparations of plant sources, often used as folk remedies, are co-administered with allopathic medicines without any knowledge of the potential interactions, desirable or otherwise, that may occur. It is therefore important to obtain information about the

Table 2 Effects of danshen extract on liver microsomal protein content, cytochrome P450 content and erythromycin *N*-demethylase activity in rats.

Group	Protein content (mg (g liver tissue)$^{-1}$)	Cytochrome P450 content (mmol (g protein)$^{-1}$)	Erythromycin *N*-demethylase activity (nmol formaldehyde min^{-1} (mg protein)$^{-1}$)
Control group (n = 10)	20.67 ± 2.97	3.16 ± 0.33	3.58 ± 0.62
Low-dose group (n = 10)	24.50 ± 1.26*	3.84 ± 0.18*	4.32 ± 1.08*
High-dose group (n = 10)	25.61 ± 3.07*	3.86 ± 0.44*	4.80 ± 1.53*

Values are mean ± s.d. *$P < 0.05$ compared with the control group.

possible drug–drug interactions in order to assist in the rational combined use of allopathic and folk medicine.

In this study, we found that the pharmacokinetics of diazepam changed when rats were pretreated with danshen extract, possibly as a result of an increase in CYP isozyme activity. The further experiments in which rat microsomes were prepared and analysed for the protein content, the CYP level and erythromycin *N*-demethylase activity of the control group and pretreated groups indicated that the extract can stimulate the activity of CYP isozymes.

Erythromycin *N*-demethylase activity is recognized as a marker of CYP3A (Yumoto et al 2001). Diazepam is also a useful metabolic probe in both humans and rats, especially of CYP3A (Reilly et al 1990; Neville et al 1993; Zomorod et al 1995; Kenworthy et al 2001). All the results indicated that danshen extract increases the activity of CYP3A.

In summary, danshen extract may stimulate the activity of CYP, especially the activity of CYP3A. Thus, if danshen preparations are administrated with drugs that are substrates of CYP3A, these drugs may be rapidly metabolized.

Further systematic studies are needed to reveal the mechanism of stimulation and to identify the active compounds in the danshen extract that affect the activity of CYP enzymes.

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