Pharmaceutical Research, Vol. 26, No. 8, August 2009 (© 2009)
DOI: 10.1007/s11095-009-9899-x

Research Paper

Mechanism-Based Pharmacokinetic–Pharmacodynamic Modeling of Bidirectional Effect of Danshensu on Plasma Homocysteine in Rats

Yuancheng Chen,[1] Yanguang Cao,[1] Jia Zhou,[1] and Xiaoquan Liu[1,2]

Received December 24, 2008; accepted April 14, 2009; published online May 7, 2009

Purpose. To develop a mechanism-based pharmacokinetic–pharmacodynamic (PK-PD) model to characterize and predict the bidirectional effect of danshensu on plasma total homocysteine (tHcy) in rats described in our previous paper.
Methods. The effect of danshensu on tHcy was assessed in rats after simultaneously methionine loading. Danshensu, its methylated metabolite and tHcy were all quantified after single intravenous injection of 20 mg/kg danshensu. The bidirectional effect, of which, elevated by danshensu methylation and decreased via transsulfuration promotion, was characterized by a PK-PD model, where direct stimulatory sigmoidal function and time-dependent transduction function were introduced for the two effects description, respectively.
Results. Modeling and simulations reveals that: (1) the elevated effect by methylation occurs before the decreased effect via transsulfuration promotion, and the decreased effect is more profoundly dose-dependent than the elevated effect; (2) two steps are simplified to describe the delayed stimulatory effect on the transsulfuration in the model; (3) long term administration of danshensu dose not affect tHcy in normal rats, while it significantly reduces tHcy in rats treated with methionine. This is in consistent with previous report.
Conclusions. The profiles were well-described by our PK-PD model, which constitutes a basis for the future development of mechanism-based model for polyphenols on Hcy in this paradigm.

KEY WORDS: bidirectional effect; danshensu; homocysteine; modeling; pharmacokinetic–pharmacodynamic.

INTRODUCTION

Integration of pharmacokinetic–pharmacodynamic (PK-PD) concepts is a potential tool to reduce the cost and enhance the efficiency of the decision making process in the drug development (1). PK-PD models characterize time course of the effect intensity resulted from certain dosing regimen, thus supporting identification and evaluation of drug response determinants, especially mechanism-based models. In recent years, mechanism-based models gained rapid progress due to their insights into how drugs exert their effects on specific process, thus improving properties for

Yuancheng Chen, Yanguang Cao contributed equally to this work.

[1] Center of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing City, 210009, People's Republic of China.
[2] To whom correspondence should be addressed. (e-mail: lxq_cpu@yahoo.com.cn)

ABBREVIATIONS: A_c, amount of danshensu in central compartment ($A_{c,0}$, initial value); A_m, amount of methylated metabolite of danshensu ($A_{m,0}$, initial value); A_p, amount of danshensu in peripheral compartment ($A_{p,0}$, initial value); C, concentration of danshensu in central compartment; C_m, concentration of methylated metabolite of danshensu; COMT, catechol-O-methyltransferase; DSS, danshensu; D_T, changed value of T_2 level; f_{DSS_kp}, transfer function describing the stimulation of danshensu (C) on k_p; f_{DSS_kt}, transfer function describing the induction of danshensu (C) on the transduction pathway (k_t); f_{T_kr}, transfer function characterizing the promotion of transit compartment T_2 on k_r; Hcy, homocysteine; k_0, zero-order rate constant of methionine production; k_{12}, first-order rate constant of danshensu from central to peripheral compartment; k_{21}, first-order rate constant of danshensu from peripheral to central compartment; k_e, first-order rate constant of danshensu elimination from central compartment; k_{em}, first-order rate constant of elimination of the metabolite; k_m, first-order rate constant of methylation process; k_p, first-order rate constant of transformation from methionine to Hcy; k_r, first-order rate constant of Hcy elimination; k_t, turnover rate constant of transduction; Met, methionine; Met-Loading rats, a general term for (−,M) rats and (D,M) rats; n_{DSS_kp}, Hill coefficient for danshensu stimulation on Hcy; n_{Met}, ratio between P_0' and P_0 in Met-Loading rats; Normal rats, a general term for (−,−) rats and (D,−) rats; P, plasma concentration of methionine (P_0 and P_0', initial concentration in normal rats and Met-Loading rats, respectively); PD, pharmacodynamics; PK, pharmacokinetics; R, plasma concentration of tHcy (R_0, initial concentration); SAM, S-adenosylmethionine; $SC_{50\_DSS\_kp}$, concentration of danshensu producing 50% of $S_{max\_DSS\_kp}$; $SC_{50\_DSS\_kt}$, concentration of danshensu producing 50% $S_{max\_DSS\_kt}$; $S_{max\_DSS\_kp}$, capacity factor for danshensu stimulation on Hcy; $S_{max\_DSS\_kt}$, capacity factor for danshensu stimulation on k_t; $S_{max\_T\_kr}$, capacity factor for transduction-induced stimulation on k_r; T_1 and T_2, first and second transduction compartment ($T_{1,0}$ and $T_{2,0}$, initial values); T_{50}, D_T level producing 50% of $S_{max\_T\_kr}$; tHcy, total homocysteine; SAH, S-adenosyl-l-homocysteine; V_d, apparent distribution volume of danshensu and the metabolite; $V_{d,Met}$, apparent distribution volume of methionine; (−,−) group, rats with normal saline; (D,−) group, rats with danshensu; (−,M) group, rats with methionine loading; (D,M) group, rats with both methionine and danshensu.

extrapolation and predictions (2,3). Some mechanism-based models have been successfully developed for drugs including monoclonal antibodies (4), antiviral agents (5), corticosteroids (6) and narcotic analgesics (7). To date, there are still limited mechanism-based models for the cardiovascular drugs. One reason may be the complexity of the cardiovascular disease and its association with other diseases such as inflammation, endocrine disturbance and diabetes (2).

Homocysteine (Hcy) is regarded as an independent risk factor in the cardiovascular diseases such as arteriosclerosis and arterial thrombosis (8,9). There are several mathematical models describing the kinetics of Hcy in the methionine cycle, which all placed emphasis on the complex regulatory mechanism within methionine cycle and considered genetic abnormality and dietary deficiency in short term (10–12). However, until now, there are still no models for the exogenous agent intervention on the methionine cycle. Thus, the aim of this paper is to develop a model to characterize the intervention process of danshensu on the plasma tHcy level within methionine cycle.

Danshensu is an active ingredient from *Salvia Miltiorrhiza* that has been widely used in many countries for the treatment of cardiovascular and cerebrovascular diseases (13). Danshensu, an active polyphenol, was recently indicated for regulation of cardiovascular disturbances and also significant protection against Hcy-induced endothelial dysfunction (14,15). Recently, we found the bidirectional effect of danshensu on Hcy in rats (16). In detail, the methylation metabolism of danshensu induced slight elevation of tHcy, which was in similar with other polyphenols (17,18). In contrast, danshensu decreased the tHcy-elevation effect induced by methionine via transsulfuration promotion. Actually, there is no universal conclusion about the tHcy influence of polyphenols ingestion. Here we gave the first evaluation on the association between polyphenols ingestion and tHcy metabolism, considering the body state and homeostasis in the model. In our opinion, PK-PD models, especially mechanism-based models, can give comprehensive description of these factors in the complex system and help us to deeply understand the kinetic relationship between polyphenols and tHcy. Therefore, the purpose of this paper is to develop a mechanism-based PK-PD model to constitute a basis for the future investigation of the effect of polyphenols on Hcy level in the preclinical research with potential impact for clinical studies.

MATERIALS AND METHODS

Chemicals

Danshensu (3-(3,4-dihydroxy-phenyl) lactic acid, purity 99%) and methionine were purchased from QingZe Co. Ltd (Nanjing, China) and WanQing Chemical Glassware & Instrument Co. Ltd (Nanjing, China), respectively. Other chemicals and solvents were of analytical grade and purchased from Nanjing Chemical Reagent Co. Ltd (Nanjing, China).

Animals

Male Sprague Dawley rats weighing 180–250 g were used throughout this study. They were housed at 22±5°C with a 12-h dark/light cycle, and were provided with water and standard chow *ad libitum* for 1 week before experiment. The experiments were performed under a license granted by Jiangsu Science and Technology Office (China), with approval from Animal Ethics Committee of China Pharmaceutical University and Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985). Every effort was made to minimize stress to the animals.

Experimental Design

Pharmacodynamic experiment investigating the effect of danshensu on plasma tHcy level in normal rats and methionine loading rats has been performed and described in our previous paper (16). Briefly, danshensu and methionine were dissolved in normal saline before single intravenous administration. Rats were fasted overnight, and then were lightly anaesthetized using diethyl ether (19). Surgery was conducted to expose one side femoral vein quickly, then isotonic drug solution was administered through femoral vein by syringe within 10 s (5 mL kg^{-1}), time began when the drug entered into the body completely.

Rats were randomly assigned into four groups (n=5): normal rats were given danshensu (20 mg kg^{-1}) [(D,−) group] or same volume of normal saline [(−,−) group]; Met-Loading rats were given methionine (0.8 mmol kg^{-1}) plus danshensu (20 mg kg^{-1}) [(D,M) group] or normal saline [(−,M) group] simultaneously. Blood was collected before and after 5, 15, 30, 60, 120 and 240 min of drug administration. At each time point, blood was taken from orbital sinus after light ethyl ether anesthesia. In the initial period (5 min and 15 min), 300 μL blood was collected due to the relative higher concentration of danshensu and its methylated metabolite. Approximate 400 μL blood was taken for the rest points.

Blood samples were immediately processed by centrifugation (5,000 g×3 min, 4°C), the supernatant was transferred and divided into equal duplicate for the analysis of tHcy and danshensu, respectively.

Plasma tHcy concentration was determined as described in our previous paper (16). Plasma concentration of danshensu and the metabolite, which is mainly 4-hydroxy-3-methyloxyphenyl lactic acid (20), were quantified as previously described (21).

Pharmacokinetic–Pharmacodynamic Modeling

Fig. 1 shows a general schematic for the entire PK-PD model. Pharmacokinetics was described by a linear two-compartment model with first-order elimination and metabolism for danshensu and one-compartment model for the methylated metabolite. Equations and initial conditions describing the amounts of danshensu and the metabolite are described as follows:

$$\frac{dA_c}{dt} = -(k_m + k_e + k_{12})A_c + k_{21}A_p; A_{c,0} = \mathrm{Dose}_{DSS} \quad (1)$$

$$\frac{dA_p}{dt} = k_{12}A_c - k_{21}A_p; A_{p,0} = 0 \quad (2)$$

$$\frac{dA_m}{dt} = k_m A_c - k_{em}A_m; A_{m,0} = 0 \quad (3)$$

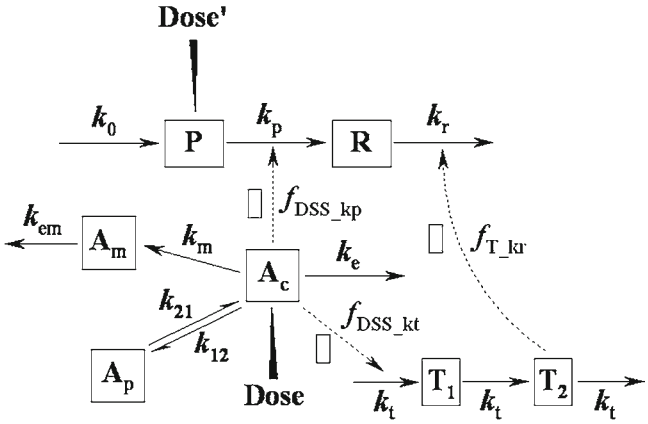

Fig. 1. Schematic presentation of the bidirectional effects of danshensu on plasma tHcy level. *Parameters and symbols* are described in the abbreviations and text. *Lines with arrows* indicate elimination or distribution of the pharmacokinetic indices, or indicate the conversion of the responses. *Dashed lines with arrows and open boxes* mean stimulatory effect being exerted by the connected factors. *Long closed triangles* symbol denotes the administration site for danshensu and methionine, respectively.

Where $A_{c,0}$, $A_{p,0}$ are the initial amount of danshensu in the central compartment (A_c) and peripheral compartment (A_p), respectively. $A_{m,0}$ denotes the initial amount of methylated metabolite of danshensu (A_m). Other symbols are defined in the abbreviation section. $Dose_{DSS}$ is the dose of danshensu (20 mg kg^{-1}).

Since preliminary modeling indicated that the distribution volume of danshensu and the metabolite had similar estimates, a single volume variable (V_d) was used in the final pharmacokinetic modeling for model parsimony. Thus, the concentration of danshensu (C) and the metabolite (C_m) are defined by $C = A_c/V_d$ and $C_m = A_m/V_d$, respectively. In addition, we tried to describe the metabolic process using Michaelis–Menton equation in the preliminary simulation, the result indicated that the goodness of fit do not show any good improvement. Therefore, k_m was assumed to be the first-order rate constant governing the methylation metabolism of danshensu.

A precursor-dependent indirect response model proposed by Sharma *et al.* (22) was adapted and applied to describe the tHcy kinetics. The equation and initial conditions describing methionine level and Hcy turnover are described as follows:

$$\frac{dP}{dt} = k_0 - k_p \cdot P \cdot f_{DSS\_k_p}; P_0 = \frac{k_0}{k_p} \tag{4}$$

$$\frac{dR}{dt} = k_p \cdot P \cdot f_{DSS\_k_p} - k_r \cdot R \cdot f_{T\_k_r}; R_0 = \frac{k_0}{k_r} \tag{5}$$

Where P_0 and R_0 are the initial plasma concentration of methionine (P) and tHcy (R), respectively. The value of P_0 (91.7 μmol L^{-1}) was derived from the literature (23). R_0 was fixed as the global mean of tHcy concentration of (−,−) rats (9.71 μmol L^{-1}). Other symbols are defined in the Abbreviations. The rate constant k_p summarizes the conversion process from methionine to Hcy including two intermediates, *i.e.* S-adenosylmethionine (SAM) and S-adenosyl-l-homocysteine (SAH). The rate constant k_r describes the first-order loss for Hcy, which represents the transsulfuration pathway in the methionine cycle.

Due to the impact of methionine loading, P_0 changed into P_0' for (−,M) and (D,M) rats, which could be described as follows:

$$P_0' = n_{Met} \cdot \frac{k_0}{k_p} \tag{6}$$

Where n_{Met} indicates the ratio of methionine concentration in plasma compared to the baseline (P_0) at time-zero after methionine loading. Thus, we can calculate apparent distribution volume of methionine: $V_{d,Met} = Dose_{Met}/(n_{Met} \cdot P_0)$, $Dose_{Met}$ is the loading dose of methionine (0.8 mmol kg^{-1}).

As a typical catechol-containing compound, methylation of danshensu is an important pathway of metabolism (24), which is mediated by catechol-O-methyltransferase (COMT). Interestingly, COMT enzyme could also promote the conversion from SAM to SAH, which raise tHcy level finally. Thus, elevation of plasma tHcy stimulated by the danshensu methylation metabolism can be described using transfer function f_{DSS_kp} as follows:

$$f_{DSS\_k_p} = 1 + \frac{S_{max\_DSS\_k_p} \cdot C^{n_{DSS\_k_p}}}{SC_{50\_DSS\_k_p}^{n_{DSS\_k_p}} + C^{n_{DSS\_k_p}}} \tag{7}$$

Where the parameter symbols are defined in the abbreviations section.

It has been reported that danshensu can reduce plasma tHcy level through activation of transsulfuration pathway acting as direct activator or inducer to up-regulate related enzymes in (D,M) rats (16). We use transduction models adapted from Sun and Jusko *et al.* (24) to conceptualize this stimulatory effect defined as follows:

$$\frac{dT_1}{dt} = k_t \cdot (f_{DSS\_k_t} - T_1); T_{1,0} = 1 \tag{8}$$

$$\frac{dT_2}{dt} = k_t \cdot (T_1 - T_2); T_{2,0} = 1 \tag{9}$$

$$f_{DSS\_k_t} = 1 + \frac{S_{max\_DSS\_k_t} \cdot C}{SC_{50\_DSS\_k_t} + C} \tag{10}$$

$$f_{T\_k_r} = 1 + \frac{S_{max\_T\_k_r} \cdot (R - R_0) \cdot D_T}{T_{50} + D_T} \tag{11}$$

$$D_T = T_2 - T_{2,0} = T_2 - 1 \tag{12}$$

Where T_1 and T_2 indicate the transit compartment relevant for the series of events that take place before the transsulfuration promotion, $T_{1,0}$ and $T_{2,0}$ are the corresponding initial values. f_{DSS_kt} is the transfer function characterizing the stimulation of danshensu on the transduction pathway. f_{T_kr} indicates the promotion of transit compartment T_2 on the elimination of tHcy (k_r) induced by danshensu indirectly. Other symbols are defined in the Abbreviations.

For (D,−) rats, stimulatory effect of transduction on the elimination of Hcy was assumed very weak, thus transfer

function f_{T_kr} was set equal to 1. Exposure of danshensu would cause alteration of T_1 and T_2, but could not promote the transsulfuration through the f_{T_kr} pathway. In this case, the unknown parameters remained to be estimated are relevant to the precursor-dependent indirect response model and the stimulation of tHcy by danshensu methylation through f_{DSS_kp}, *i.e.*, k_0, k_p, k_r, $S_{max\_DSS\_kp}$, $SC_{50\_DSS\_kp}$ and n_{DSS_kp}.

Pharmacokinetic and Pharmacodynamic Modeling and Simulations

The model was constructed in two phases: (1) fitting of pharmacokinetic data on the concentration–time profiles of danshensu and its methylated metabolite; (2) fitting of pharmacodynamic data (concentration–time profiles of plasma tHcy). Non-compartmental analysis was first performed for initial estimates of the pharmacokinetic models using Excel software (Microsoft, Redmond, WA). Plasma concentration data of danshensu and its methylated metabolite were incorporated to obtain the PK parameter estimates simultaneously. Data from (−,M) group together with (D,M) group or (D,−) group were incorporated to obtain the pharmacodynamic parameter estimates for Met-Loading rats or normal rats, respectively. All parameter estimates were achieved by fitting the mean data. Model fittings were performed by nonlinear regression analysis using the maximum likelihood algorithm in MATLAB (The Mathworks Inc., USA). ODE functions were used for solving differential equations. Model selection was based on the Akaike Information Criterion (AIC). Goodness of fit for both PK and PD were assessed by the objective function (*fval*%: mean residual ratio) and by visual inspection of various diagnostic plots.

In order to obtain more accurate parameter estimates, nonparametric bootstrap analysis was performed as an internal model evaluation technique for the PK-PD model. In detail, a new replication of the original dataset (a bootstrap sample) was obtained by N random draws of individual data (with replacement) from the original dataset (25). The final PK-PD model was refitted to the average values of each new dataset, and this process was repeated 200 times with different random draws. The stability of the final model was evaluated by visual inspection of the distribution of the model parameter estimates from the average values of new datasets and compared with that obtained from the fit of the average values of original dataset.

All results were expressed as the Mean ± SD. After applying the Bartlett's test for non-homogeneity of variances, statistical analysis including two-tailed Student's t-test, or the nonparametric Kruskal–Wallis test was used. The acceptable level of significance was established at $P<0.05$ and $P<0.01$.

Model simulations were conducted using mean estimates obtained from PK-PD modeling to observe the effect of danshensu on plasma tHcy level in these cases:

(a) Effect of different doses of danshensu (10, 20, 40 mg kg^{-1}, *i.v.*) on plasma tHcy level in normal rats (*i.v.*, normal saline) and Met-Loading rats (*i.v.*, 0.8 mmol kg^{-1} methionine).
(b) Effect of different doses of methionine (0.2, 0.8, 3.2 mmol kg^{-1}) on plasma tHcy level and the regulatory effect of danshensu in Met-Loading rats.
(c) Effect of danshensu on plasma tHcy level after long term administration of danshensu with or without methionine loading (7 days).
(d) Preventive role of danshensu on plasma tHcy level after long term administration of danshensu (20 mg kg^{-1}, *i.p.*, 7 days), then single dose of methionine (0.8 mmol kg^{-1}, *i.v.*) was given at the last day (8th day).
(e) Illustration of bidirectional effect, considering the case of elevation of plasma tHcy level induced by danshensu methylation alone, and the case of reduction of plasma tHcy concentration activated by the delayed transduction events alone.

RESULTS

PK and PD Profiles in Normal and Met-Loading Rats

Compared to the normal rats, the great effect of high methionine level on the pharmacokinetics of danshensu was clearly observed in Met-Loading rats (Fig. 2A; Table I). Compared to the (D,−) group, the terminal half-life $T_{1/2}$ significantly increased from 0.24 to 0.94 h (*i.e.*, 2.9 fold increase) in (D,M) group ($P<0.05$), which implied the reduction of the elimination rate of danshensu. Meanwhile, clearance was markedly reduced by approximate 46% (from 1.93 to 1.06 L kg^{-1} h^{-1}) and MRT was elevated nearly one-fold (from

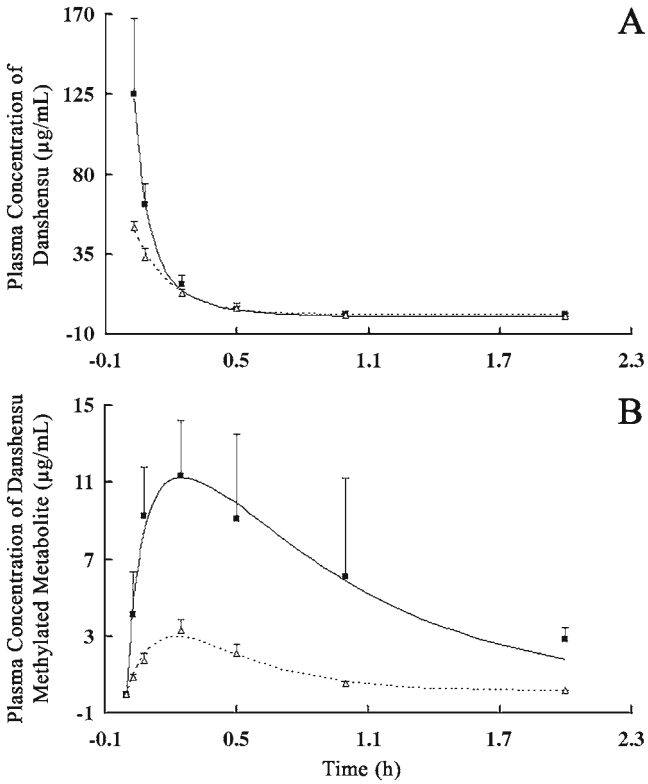

Fig. 2. Time profiles of danshensu (**A**) and its methylated metabolite (**B**) following single intravenous administration of danshensu (20 mg kg^{-1}) in rats. *Open triangles* and *closed squares* indicate measured values in (D,−) group and (D,M) group, respectively. Model fittings are presented as *dot line* for (D,−) group, and *solid line* for (D,M) group, respectively. Observations are reported as Mean ± 1 SD ($n=5$ each group).

Table I. Noncompartmental Parameters of Danshensu, Its Methylated Metabolite, and Plasma tHcy in Rats

	Danshensu		Methylated metabolite		tHcy		
	(D,−) rats[a]	(D,M) rats[b]	(D,−) rats[a]	(D,M) rats[b]	(D,−) rats[a]	(D,M) rats[b]	(−,M) rats[c]
Pharmacokinetics							
$T_{1/2}$ (h)	0.24±0.06	0.94±0.40$^{\#}$	0.52±0.27	0.96±0.94			
AUC_{0-t} (μg h mL^{-1})	9.26±1.02	17.6±2.68$^{\#\#}$	1.93±0.39	12.7±5.80$^{\#}$			
$AUC_{0-\infty}$ (μg h mL^{-1})	9.43±0.96	19.3±2.91$^{\#\#}$	2.20±0.48	16.8±7.48$^{\#}$			
MRT (h)	0.24±0.02	0.55±0.14$^{\#}$	0.69±0.21	1.40±1.21			
CL (L kg^{-1} h^{-1})	1.93±0.20	1.06±0.16$^{\#\#}$	\	\			
C_{max} (μg mL^{-1})	\	\	3.29±0.57	14.0±1.19$^{\#\#}$			
T_{max} (h)	\	\	0.25±0.00	0.27±0.15			
Pharmacodynamics							
AUEC[d] (μmol h L^{-1})					25.9±34.4	55.6±14.6*	90.7±19.4$^{\#\#}$
C_{max} (μmol L^{-1})					24.3±15.9	34.0±3.00**	48.9±7.20$^{\#}$
T_{max} (h)					0.55±0.45	1.30±0.67	1.60±0.55$^{\#}$

Values are reported as the Mean±1 SD. of individual estimates (n=5 each group)
$T_{1/2}$ terminal elimination half-life, AUC_{0-t}, area under measured plasma concentration–time curve, $AUC_{0-\infty}$, area under plasma concentration–time curve extrapolated to infinity, CL total systemic clearance, MRT mean residence time, C_{max} and T_{max} time and value of maximum plasma concentration of the methylated metabolite or tHcy
*P<0.05; **P<0.01 compared to the (−,M) group (based on Student t test). $^{\#}P$<0.05; $^{\#\#}P$<0.01 compared with corresponding (D,−) group (based on Student t test)
[a] Rats were administered with single dose of danshensu (20 mg kg^{-1})
[b] Rats were administered with single dose of danshensu (20 mg kg^{-1}) and methionine (0.8 mmol kg^{-1}) simultaneously
[c] Rats were administered with single dose of methionine (0.8 mmol kg^{-1})
[d] AUEC = $AUC_{\_Treatment\ Group} - AUC_{\_(-,-)\ Group}$. AUC: area under measured plasma concentration–time curve of tHcy. 'Treatment Group' in '$AUC_{\_Treatment\ Group}$': (−,M) Group or (D,−) Group or (D,M) Group

0.24 to 0.55 h). $AUC_{0-\infty}$ also increased about one-fold (from 9.43 to 19.25 μg h mL^{-1}) in (D,M) group, indicating the great impact of methionine on the PK behavior of danshensu.

Compared to danshensu, methionine gave greater influence on the pharmacokinetic behavior of the methylated metabolite (Fig. 2B; and Table I). As shown in Table I, methionine enhanced $AUC_{0-\infty}$ and C_{max} of methylated metabolite by nearly 6.6 fold (from 2.2 to 16.78 μg h mL^{-1}) and 3.3 fold (from 3.29 to 14.02 μg mL^{-1}), implying the promotion of danshensu methylation by methionine. T_{max} was constant in all groups.

As Fig. 3 indicated, plasma tHcy profiles followed a time-dependent bidirectional pattern. The influence directions of danshensu on plasma tHcy were different between (D,M) rats and (D,−) rats, which was in line with our previous report (16). In detail, danshensu sharply raised tHcy level in the initial period (0~30 min) and then decreased tHcy level greatly in the following period. In (D,M) group, AUEC of tHcy was notably reduced from 90.7 to 55.6 μmol h L^{-1} (*i.e.*, 38.7% reduction) by danshensu (P<0.05). As shown in Table I, C_{max} also reduced from 48.9 to 34.0 μmol·L^{-1} (*i.e.*, 30.5% decline) significantly (P<0.01). Therefore, we can conclude that danshensu can inhibit Hcy-elevating effect by methionine.

PK-PD Modeling

Pharmacokinetic profiles were reasonably described using a linear two-compartmental model for danshensu and one-compartment model for the methylated metabolite. Methylation of danshensu could be well described using first-order process (Figs. 1 and 2). For the metabolite, simulation results quite matched with the measured values, suggesting that first-order metabolism model is enough to describe the methylation reaction of danshensu.

Additionally, a single volume variable (V_d) was used to describe the distribution of danshensu and the metabolite, which allowed for the reduction in the number of model parameters and improved identifiability and estimation pre-

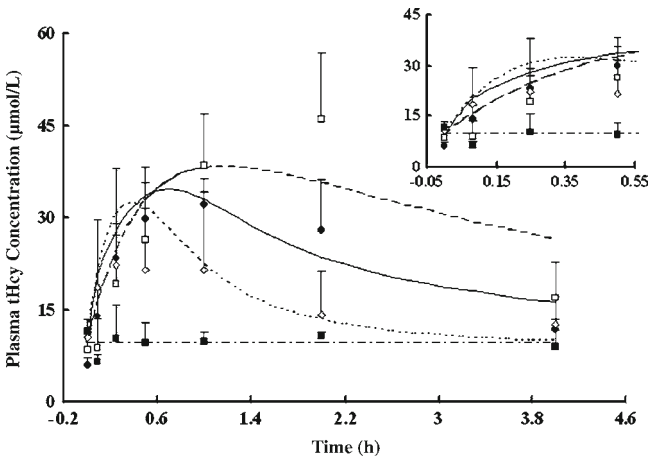

Fig. 3. Time profile of plasma tHcy concentration in rats. Measured values and model fittings are shown in *symbols and lines*, respectively. Model control (methionine loading, 0.8 mmol kg^{-1}, *i.v.*) are shown in *open squares* and *dash line*. Regulative effect of danshensu following single *i.v.* dose of 20 mg kg^{-1} are shown in *closed circles* with *solid line* in (D,M) group, and *open diamonds* with *dot line* in (D,−) group, respectively. *Closed squares* with horizontal *dash dot line* indicate baseline level of plasma tHcy in (−,−) group (normal saline, *i.v.*). *Inset graph* showed the effect of danshensu on the concentration of Hcy in the first 30 min. All observations are reported as Mean ± 1 SD (n=5 each group).

cision. Effect of high methionine level on the pharmacokinetic behavior of danshensu and its methylated metabolite could be observed, especially the distribution volume (V_d) (Table II). In (D,M) group, V_d was only one third (0.10 L kg^{-1}) of that in (D,−) group (0.31 L kg^{-1}) ($P<0.01$), suggesting that the disposition level of danshensu and the metabolite were notably restricted by high methionine concentration. Due to this, both the initial concentrations of danshensu and C_{max} of methylated metabolite were increased by more than two fold.

Model fittings of tHcy level were in consistent with measured values using precursor-dependent model (Fig. 3). An overshoot area existed in the tHcy curve of (D,M) group compared to that of the (−,M) group, suggesting the elevation of tHcy by the methylation of danshensu. Subsequently, marked reduction of tHcy could be obviously observed, which implied that delayed promotion of the transsulfuration appeared after rapid methylation of danshensu. This was also reflected in the difference of AUEC during the overshoot period between the (D,−) rats and the (D,M) rats, in which, the latter was smaller than the former (Table I). This indicated that the transsulfuration promotion had begun since methionine loading, however, this effect was weak compared to the elevation effect induced by danshensu methylation at the beginning time.

In addition, n_{Met} were obtained according to the relationship between $V_{d,Met}$ and P_0' at time-zero after methionine loading (Eq. 6), *i.e.*, 4.83±0.22, which meant that approximately five fold of baseline plasma concentration of methionine was achieved soon after the loading.

From the 200 bootstrap replicates of the model, 8.5% and 21.5% failed to minimize successfully in the pharmacokinetic and pharmacodynamic modeling for Met-Loading rats, respectively. For normal rats, 27% failed to converge in the pharmacodynamic modeling. As shown in Table II, parameter estimates obtained from the fit of original mean data were similar to the mean estimates of the bootstrap replicates. The results suggest the absence of bias and a reasonable precision in the parameter estimates for the final PK-PD model.

PK-PD Model Simulations

Based on mean parameter estimates of the integrated PK-PD model, simulations were performed to predict the kinetics of plasma tHcy (Fig. 4). The differences of plasma tHcy kinetics were also compared between normal rats and Met-Loading rats. As shown in Fig. 4A, the tHcy level stimulated by the danshensu methylation was sharply elevated and C_{max} of tHcy increased dose-dependently. T_{max} of tHcy were nearly the same in all dosage groups. The increase of AUEC was not proportional to the danshensu dose, suggesting that the elevation of tHcy by danshensu methyl-

Table II. Estimated Pharmacokinetic and Pharmacodynamic Parameters of Danshensu in Rats

	Normal rats[a]			Met-Loading rats[b]		
		Bootstrap dataset[d]			Bootstrap dataset[d]	
Parameter (units)	Original dataset[c]	Mean ± SD	%CV	Original dataset[c]	Mean ± SD	%CV
Pharmacokinetics						
k_e (h^{-1})	3.92	3.54±0.43	12	10.4	7.85±3.46	44
k_m (h^{-1})	0.61	0.62±0.04	6.5	0.93	0.97±0.15	15
k_{em} (h^{-1})	3.20	3.18±0.22	6.9	1.19	1.48±0.47	32
V_d (L kg^{-1})	0.31	0.31±0.01	3.2	0.10	0.10±0.02*	20
k_{12} (h^{-1})	1.33	1.76±0.31	18	3.96	5.95±3.06	51
k_{21} (h^{-1})	0.33	0.25±0.10	40	8.40	4.91±4.27*	87
Pharmacodynamics						
$S_{max\_DSS\_kp}$	13.1	16.6±7.67	46	0.64	0.67±0.33	49
$SC_{50\_DSS\_kp}$ (μg mL^{-1})	105	105±0.39	0.4	71.1	70.1±0.90*	1.3
n_{DSS_kp}	0.38	0.36±0.22	61	7.73	6.67±3.18	48
$S_{max\_T\_kr}$	\	\	\	20.3	20.3±0.05	0.2
T_{50} (10^{-3})	\	\	\	3.58	3.58±0.17	4.7
$S_{max\_DSS\_kt}$	\	\	\	19.3	19.3±0.03	0.2
$SC_{50\_DSS\_kt}$ (μg mL^{-1})	\	\	\	15.9	12.1±6.29	52
k_t (h^{-1} 10^{-3})	\	\	\	0.76	0.65±0.22	34
k_0 (μmol h^{-1} L^{-1})	22.0	21.5±1.94	9.0	20.8	20.9±1.92	9.2
k_p[e] (h^{-1})	0.24	0.23±0.02	8.7	0.23	0.23±0.02	8.7
k_r[f] (h^{-1})	2.27	2.21±0.20	9.0	2.14	2.15±0.20	9.2
$V_{d,Met}$ (L kg^{-1})	1.78	1.77±0.08	4.5	1.79	1.80±0.09	5.0

*$P<0.01$ compared with Normal rats (based on Student t test)
[a] Rats were administered with single dose of danshensu (20 mg·kg^{-1}) or normal saline (*i.v.*)
[b] Rats were given methionine (0.8 mmol kg^{-1}) plus danshensu (20 mg kg^{-1}) or normal saline simultaneously (*i.v.*)
[c] Estimates from the fit of original mean data ($n=5$ each group)
[d] Values are reported as the Mean±1 SD of individual estimates ($N=200$)
[e] Calculation according to: $k_p = k_0/P_0$, P_0 is the methionine baseline value (91.7 μmol L^{-1}) derived from literature (23)
[f] Calculation according to: $k_r = k_0/R_0$, R_0 was fixed as the global mean of plasma tHcy concentration of (−,−) rats (9.71 μmol L^{-1})

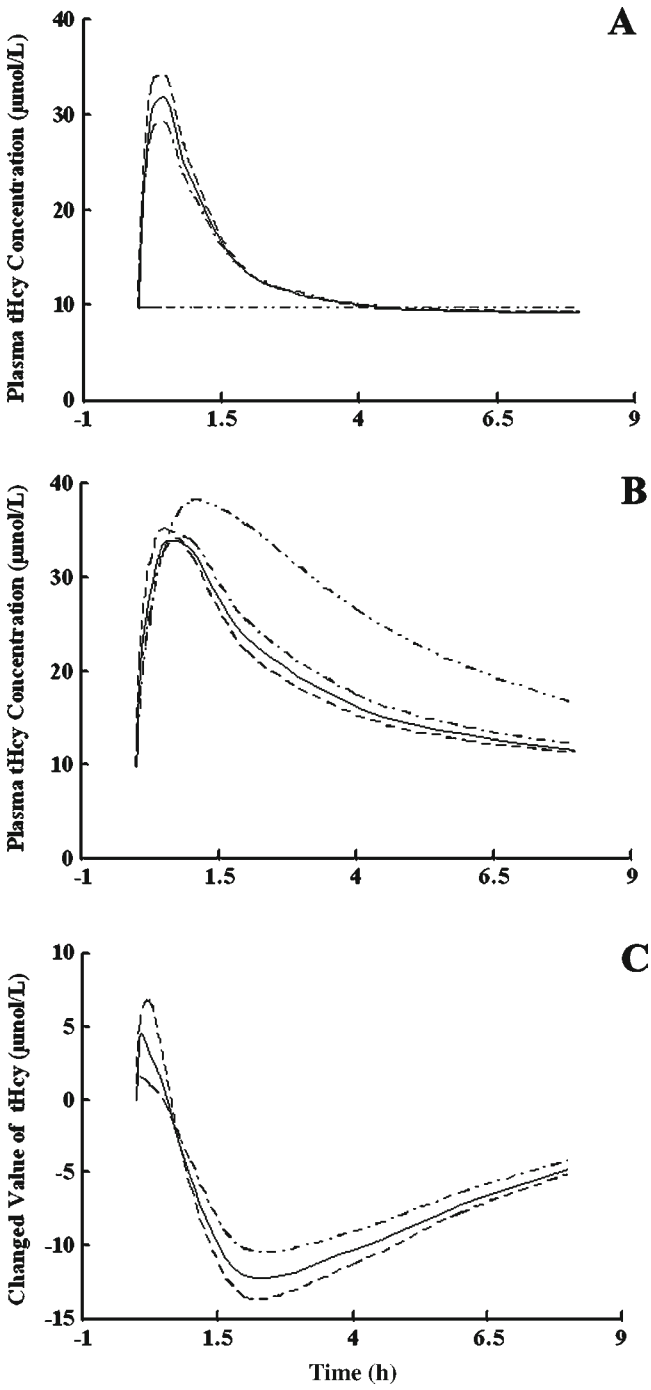

Fig. 4. Simulated plasma tHcy concentration–time profiles after different dosing regimens of danshensu in normal rats (**A**) and Met-Loading rats (**B**). A single 10, 20, 40 mg kg^{-1} *i.v.* bolus dosage of danshensu is shown by *dash dot lines*, *solid lines* and *long dash lines*, respectively. Baseline level of plasma tHcy level (R_0) is shown by *horizontal line* (**A**). (−,M) group (0.8 mmol kg^{-1} methionine, *i.v.*) is shown by *dash dot dot lines* (**B**). **C** The regulatory effect of danshensu on plasma tHcy level [(D,M) group − (−,M) group]. *All lines* are depicted using mean estimates of PK and PD parameters of danshensu in normal rats for panel **A** and Met-Loading rats for Panel **B** and **C**, respectively.

ation was not linear with dose after administration of high dose of danshensu.

In Met-Loading rats treated by danshensu, plasma tHcy profiles were strikingly different to the normal rats (Fig. 4B). Both the initial elevation and following decrease of tHcy was shown in a dose-dependent manner (Fig. 4C). As the Fig. 4C indicated, decreased effect via transsulfuration promotion was more profound than the elevation effect induced by danshensu methylation. Compared to 20 and 40 mg kg^{-1} groups, initial elevation of plasma tHcy was slightly smaller in the 10 mg kg^{-1} group. During the period of transsulfuration promotion, AUEC (relative changed area under plasma concentration time profile of tHcy compared to the (−,M) group) was not proportional to the dose, which was similar to that in normal rats, suggesting that the tHcy-lowering effect was limited when high dose of danshensu was given to rats.

The tHcy-lowering effect by danshensu exhibited more obvious after higher dose of methionine loading (Fig. 5), indicating that the lowering effect of danshensu on plasma tHcy is closely related to the loading dose of methionine. T_{min} of changed value of plasma tHcy was shorter when higher dose of methionine was given to Met-Loading rats, which implied the decreased effect of danshensu was more significant after administration of higher dose of methionine.

Plasma tHcy profile displayed different pattern after long term administration of danshensu in Met-Loading rats (Fig. 6A) and normal rats (Fig. 6B). When the plasma tHcy level stayed steady state after 2nd administration of methionine in the (−,M) group, C_{max} of tHcy showed gradually decline in the (D,M) group. This indicated the increasing tHcy-lowering effect of danshensu would be obtained after long term administration. In contrast, the influence of danshensu on plasma tHcy level showed transient increase in (D,−) group (Fig. 6B), and this magnitude was weak compared to the profile shown in Fig. 6A. Thus, combined with Fig. 5, we conclude that danshensu would not affect plasma tHcy level in normal rats.

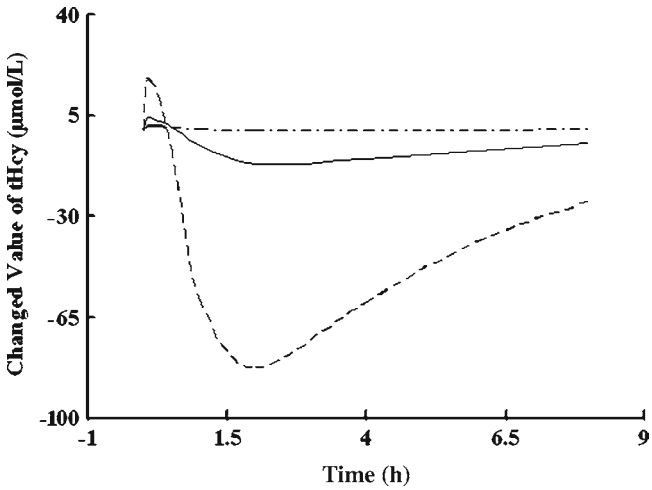

Fig. 5. Effect of danshensu on plasma tHcy level in rats treated with different dose of methionine simultaneously. *Dash dot line*, *solid line* and *dash line* showed the regulatory effect of danshensu (20 mg kg^{-1}) when 0.2, 0.8 and 3.2 mmol kg^{-1} methionine was given to rats through intravenous administration. Changed value of tHcy = (D,M) group − (−,M) group. *All lines* are depicted using the mean estimates of PK and PD parameters in Met-Loading rats.

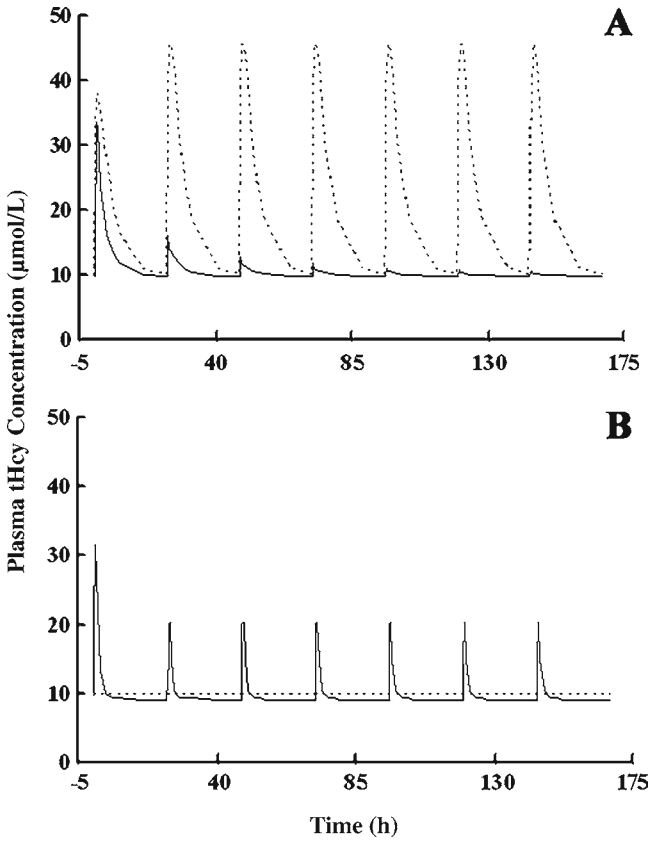

Fig. 6. Effect of danshensu on the plasma tHcy level after long term administration of danshensu with or without methionine in rats. *Dot line* and *solid line* in the panel **A** showed the concentration of tHcy in the (−,M) group (0.8 mmol kg^{-1} methionine, *i.p.*) and (D,M) group (0.8 mmol kg^{-1} methionine+20 mg kg^{-1} danshensu, *i.p.*), respectively. *Dot line* and *solid line* in the panel **B** indicated the plasma tHcy level in the (−,−) group (normal saline, *i.p.*) and (D,−) group (20 mg kg^{-1} danshensu, *i.p.*), respectively. *All lines* are depicted using mean estimates of PK and PD parameters of danshensu in Met-Loading rats for panel **A** and normal rats for panel **B**, respectively.

Danshensu exerted obvious preventive effect on the plasma tHcy level after long term administration of danshensu in normal rats (Fig. 7). As shown in Fig. 7, compared to the model control group, plasma tHcy concentration showed a minor increase after methionine loading at the last day. Meanwhile, the elevation magnitude of plasma tHcy induced by methionine loading was weak compared to the ones during the prevention period. From the modeling point of view, this is due to the accumulative elevation of transit compartments T_1 and T_2 after danshensu induction. The structure of cascade connection of transit compartments made these transduction events occur more slowly than the danshensu methylation during the prevention period. Therefore, after methionine loading at the last day, obvious tHcy-lowering effect could be observed resulted from accumulative level of transit compartment T_2 through f_{T_kr} pathway. Taken together, we conclude that danshensu exhibits its beneficial effect to reduce plasma tHcy level after long term pretreatment, thus can it reduce potential cardiovascular risk.

Fig. 8 illustrated how the bidirectional regulation of danshensu on the plasma tHcy level developed. As shown

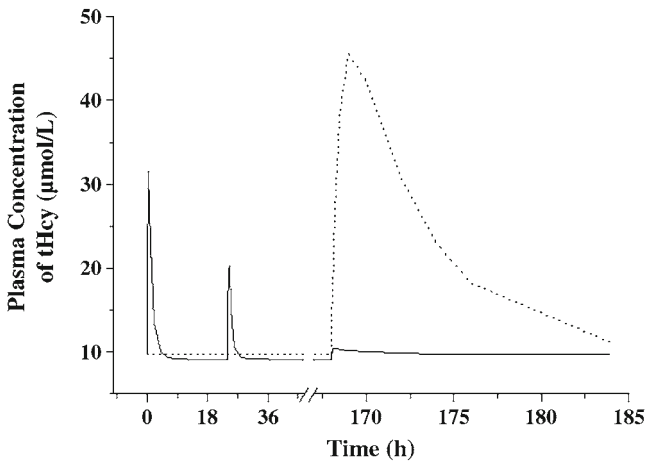

Fig. 7. Preventive role of danshensu on the level of plasma tHcy after long term administration of danshensu (20 mg kg^{-1}, *i.p.*, 7 days) and methionine loading (0.8 mmol kg^{-1}, *i.v.*) at the last day (8th day) in rats. *Solid line* showed the impact of danshensu on the plasma concentration of tHcy, *dot line* showed the tHcy level in the control group. *All lines* are depicted using the mean estimates of PK and PD parameters of danshensu in rats.

from the Fig. 8, the elevation of plasma tHcy induced by danshensu methylation and the reduction of tHcy level via the transsulfuration promotion showed different time-dependent pattern. The methylation-induced curve rose rapidly and up to the peak value (~4 μmol L^{-1}) in a short time (approximately 15 min), and then declined slowly to the baseline in 1 h. For the transsulfuration-induced curve, a delayed onset was observed, which lasted about 0.5 h, and then the tHcy-lowering effect gradually exhibited (nadir: −12 μmol L^{-1}, ~2.5 h). The 'synthesis' of these two curves brought about the bidirectional regulation curve, as shown in *solid line* in the Fig. 8. In addition, simulation results showed

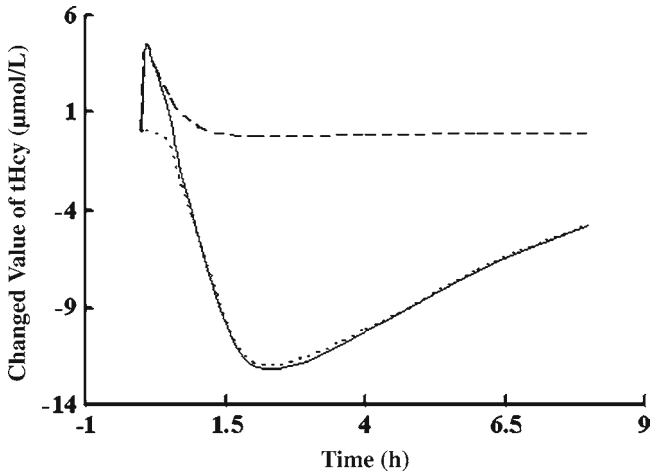

Fig. 8. Graphic presentation of the bidirectional regulation of danshensu on plasma tHcy level (shown in *solid line*) compared to the (−,M) group. *Dot line* reflects the case of stimulation on transsulfuration pathway resulted from transduction events induced by danshensu. *Dash line* indicates the case of elevation of plasma tHcy level induced by methylation metabolism of danshensu. *All lines* are depicted using the mean estimates of PK and PD parameters of danshensu in (D,M) group (20 mg kg^{-1} danshensu+0.8 mmol kg^{-1} methionine).

that the reduction period of plasma tHcy lasted about 19.5 h, which was significant longer than the elevation period (~0.5 h), suggesting that the decreased effect of tHcy via the transsulfuration promotion was more profound than the elevation of tHcy by danshensu methylation.

DISCUSSION

In this report, we developed an integrated mathematical model to characterize the relationship of time profiles of plasma tHcy and danshensu. Bidirectional effect of danshensu on tHcy was an attractive phenomenon because different mechanisms were included as described in the text, *i.e.*, methylation and transsulfuration activation. It has been well established that there is an intrinsic link between methylation, Hcy and transsulfuration, thus can it permit a mechanistic framework to give comprehensive understanding for the effect of drug intervention on the methionine cycle.

Initially a stimulatory E_{max} function was employed to model the methylation process, but $SC_{50\_DSS\_kp}$ was larger than the measured concentration of danshensu ($SC_{50\_DSS\_kp}$> 200 μg mL^{-1}). Thus, Hill coefficient n_{DSS_kp} was introduced to describe the extent of methylation reaction. This model improvement explained the difference between danshensu methylation and methionine metabolism, and the values of $SC_{50\_DSS\_kp}$ were achieved in a reasonable range. For (D,M) rats, significantly smaller $SC_{50\_DSS\_kp}$ reflected obvious synergism from the combination of danshensu methylation and methionine metabolism. In addition, $SC_{50\_DSS\_kp}$ was larger than the highest concentration of danshensu in (D,−) group, indicating that the intensity of danshensu methylation was weaker than that in (D,M) group. n_{DSS_kp} in (D,M) group was much higher than that in (D,−) group, suggesting the impact of methionine mainly contributed to the elevation of plasma tHcy at the beginning time. Estimated value of n_{DSS_kp} below 1 in (D,−) group, indicating that the methylation of danshensu was week, which was consistent with the tendency of $SC_{50\_DSS\_kp}$.

Our preliminary pharmacodynamic model for tHcy was a simple indirect response model, which could not distinguish the factors for the elevation of tHcy resulted from danshensu methylation or methionine loading. Thus, precursor-dependent model was used to describe the dynamics of methionine and tHcy simultaneously, which could reflect the effect of different doses of methionine on tHcy, as shown in Fig. 5. Simulation results showed that danshensu exhibited more obvious tHcy-lowering effect when higher dose of methionine was given, which was in consistent with the results of Sekiya *et al.* (26).

Effect of danshensu on the transsulfuration pathway was initially modeled by a stimulatory E_{max} function directly linked to the dissipate term of tHcy (*i.e.*, k_r). However, there was an obvious time-lag to the onset of transsulfuration in the measured values, implying a delayed and indirect mechanism mediated the activation of transsulfuration. Transduction models were introduced to address the problem. Different numbers of compartments were tested to capture the number of compartments required to account for the delay of onset of transsulfuration. This interpretation is more true to the underlying physiology than a delay or onset time that is also more numerically difficult to compute. Additionally, onset times do not provide the smooth reduction observed early in the onset of transsulfuration. Similarly, the concept of utilizing cascade transduction models as a pharmacodynamic driving function has been described for modeling the onset of rheumatoid arthritis induced by collagen (27). Other applications of this approach include the PK-PD modeling of the parasympathomimetic activity of scopolamine and atropine in rats (28) and the chemotherapeutic effects of methotrexate (29).

Various numbers of transit-compartments were tried to link the pharmacokinetics of danshensu and the onset of transsulfuration. Extremely low $SC_{50\_DSS\_kt}$ and high n_{DSS_kp} were achieved when using one-compartment transduction model, which demonstrated that it was not sufficient to account for the delay. No significant improvement of model fitting criteria was gained in the three-compartment transduction model. Thus, two-compartment transduction model was employed to quantify the delay of transsulfuration onset considering the simplicity. In addition, we tested whether the model structure for (D,M) rats was appropriate for (D,−) rats. Significant low value of k_t ($<10^{-5}$) and high T_{50} (1.34) were obtained, indicating that the influence of danshensu on the transsulfuration was very weak under normal condition.

Model simulations revealed that danshensu exhibited preventive role after long term administration in normal rats, which was in line with our previous result (16). This mainly contributes to the proposed assumption mentioned in the "MATERIALS AND METHODS" section, *i.e.*, transfer function f_{T_kr} will be activated after administration of methionine. It has been demonstrated that cystathionine β-synthase (CBS) could be activated by SAM and SAH, thus increasing the elimination of Hcy (10,30). Levels of SAM and SAH will rise after methionine loading, which in turn elevate Hcy level, and decrease Hcy level via transsulfuration through the CBS activation with a time delay. Therefore, long term administration of danshensu possibly increase transsulfuration-related enzymes activity, and this effect will be implemented via the activation of CBS after methionine loading, so the reduction of plasma tHcy could be observed eventually.

Model simulation results showed that long term co-administration of danshensu and methionine obviously reduced tHcy level (Fig. 6A), which was in agreement with the experimental result in mice treated by other polyphenols (31). Meanwhile, a clinical study showed that black tea did not influence plasma tHcy concentration in normal subjects for 4 weeks (17), which could also be delineated using our PK-PD model (Fig. 6B). Large variation of $SC_{50\_DSS\_kt}$ existed in (D,M) group, which may be due to the individual differences in the promotion of transsulfuration of tHcy induced by danshensu.

The model does not consider the impact of high methionine level on the pharmacokinetics of danshensu and its methylated metabolite after loading. That attempt of modeling the influence of methionine on the pharmacokinetics of danshensu was unsuccessful due to the complexity and unknown interaction mechanism. In fact, significantly lower V_d and clearance indicated greater exposure of danshensu in (D,M) group than that in (D,−) group (Tables I and II). From this point, danshensu exerts better regulatory function on tHcy in the hyperhomocysteinemia induced by chronic administration of methionine.

Recent clinical studies showed that plasma tHcy-lowering therapy with vitamins administration did not reduce the

cardiovascular risk during a mean follow-up period of five years (32). An important reason for this failure results from the adverse effect induced by high dose administration, such as angiogenesis inhibition, restenosis rate elevation (33). Just from this point, it is urgent need to investigate new strategy for Hcy-lowering. Danshensu is an active ingredient from Danshen that has been shown wide cardiovascular benefit, which may exclude the problems of vitamin therapy. Of course, the clinical cardiovascular benefits about danshensu for Hcy-lowering still need further validation.

CONCLUSIONS

To our knowledge, this is the first PK-PD model characterizing the bidirectional regulation of polyphenols on the Hcy plasma levels *in vivo*. Pharmacokinetics was described using two-compartment model for danshensu and one-compartment model for the methylated metabolite with linear metabolic process. Stimulatory sigmoid E_{max} function and two-compartment transduction function were used to describe the tHcy-elevation effect driven by the methylation and the tHcy-lowering effect resulted from the transsulfuration activation. The proposed PK-PD model characterized the effect of danshensu on plasma tHcy well both in normal and Met-Loading rats following single dose administration of danshensu. The different effects of danshensu on plasma tHcy between the healthy and pharmacological settings described in this report illustrate the importance of conducting PK-PD studies in both settings.

ACKNOWLEDGMENTS

We would like to thank Xiao-Jia Wang, Yue-Miao Yin, Chun-Hua Xu, Ping Wang for technical help with animal and bioanalytical aspects of the studies. This research was supported by the National Natural Science Foundation of the People's Republic of China (No. 30772609).

REFERENCES

1. Meibohm B, Derendorf H. Pharmacokinetic/pharmacodynamic studies in drug product development. J Pharm Sci 2002;91:18–31. doi:10.1002/jps.1167.
2. Rajman I. PK/PD modelling and simulations: utility in drug development. Drug Discov Today 2008;13:341–6. doi:10.1016/j.drudis.2008.01.003.
3. Lalonde RL, Kowalski KG, Hutmacher MM, Ewy W, Nichols DJ, Milligan PA, *et al*. Model-based drug development. Clin Pharmacol Ther 2007;82:21–32. doi:10.1038/sj.clpt.6100235.
4. Hayashi N, Tsukamoto Y, Sallas WM. A mechanism-based binding model for the population pharmacokinetics and pharmacodynamics of omalizumab. Br J Clin Pharmacol 2007;63:548–61. doi:10.1111/j.1365-2125.2006.02803.x.
5. Agoram B, Marino R, George S. Development of a pharmacokinetic pharmacodynamic (PKPD) model to predict onset of hemolytic anemia in (PEG) interferon/ribavirin treated hepatitis-C virus (HCV)-infected patients. Clin Pharmacol Ther 2004;75:78. doi:10.1016/j.clpt.2003.11.296.
6. Hazra A, Pyszczynski N, Dubois DC. Modeling receptor/gene-mediated effects of corticosteroids on hepatic tyrosine aminotransferase dynamics in rats: dual regulation by endogenous and exogenous corticosteroids. J Pharmacokinet Pharmacodyn 2007;34:643–67. doi:10.1007/s10928-007-9063-3.
7. Yassen A, Olofsen E, Romberg R. Mechanism-based PK/PD modeling of the respiratory depressant effect of buprenorphine and fentanyl in healthy volunteers. Clin Pharmacol Ther 2007;81:50–8. doi:10.1038/sj.clpt.6100025.
8. Nygard O, Nordrehaug JE, Refsum H, Ueland PM, Farstad M, Vollset SE. Plasma homocysteine levels and mortality in patients with coronary artery disease. N Engl J Med 1997;337:230–6. doi:10.1056/NEJM199707243370403.
9. Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. BMJ 2002;325:1202. doi:10.1136/bmj.325.7374.1202.
10. Reed MC, Nijhout HF, Sparks R, Ulrich CM. A mathematical model of the methionine cycle. J Theor Biol 2004;226:33–43. doi:10.1016/j.jtbi.2003.08.001.
11. Martinov MV, Vitvitsky VM, Mosharov EV, Banerjee R, Ataullakhanov FI. A substrate switch: a new mode of regulation in the methionine metabolic pathway. J Theor Biol 2000;204:521–32. doi:10.1006/jtbi.2000.2035.
12. Prudova A, Martinov MV, Vitvitsky VM, Ataullakhanov FI, Banerjee R. Analysis of pathological defects in methionine metabolism using a simple mathematical model. Biochim Biophys Acta 2005;1741:331–8. doi:10.1016/j.bbadis.2005.04.008.
13. Zhou L, Zuo Z, Chow MS. Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. J Clin Pharmacol 2005;45:1345–59. doi:10.1177/0091270005282630.
14. Wu L, Qiao H, Li Y, Li L. Cardioprotective effects of the combined use of puerarin and Danshensu on acute ischemic myocardial injury in rats. Phytother Res 2007;21:751–6. doi:10.1002/ptr.2157.
15. Chan K, Chui SH, Wong DY, Ha WY, Chan CL, Wong RN. Protective effects of Danshensu from the aqueous extract of Salvia miltiorrhiza (Danshen) against homocysteine-induced endothelial dysfunction. Life Sci 2004;75:3157–71. doi:10.1016/j.lfs.2004.06.010.
16. Cao YG, Chai JG, Chen YC, Zhao J, Zhou J, Shao JP, et al. Beneficial effects of danshensu, an active component of *Salvia miltiorrhiza*, on homocysteine metabolism via the trans-sulphuration pathway in rats. Br J Pharmacol 2009; doi:10.1111/j.1476-5381.2009.00179.x.
17. Hodgson JM, Burke V, Beilin LJ, Croft KD, Puddey IB. Can black tea influence plasma total homocysteine concentrations? Am J Clin Nutr 2003;77:907–11.
18. de Bree A, Verschuren WM, Blom HJ, Kromhout D. Lifestyle factors and plasma homocysteine concentrations in a general population sample. Am J Epidemiol 2001;154:150–4. doi:10.1093/aje/154.2.150.
19. Plate AY, Crankshaw DL, Gallaher DD. The effect of anesthesia by diethyl ether or isoflurane on activity of cytochrome P450 2E1 and P450 reductases in rat liver. Anesth Analg 2005;101:1063–4. doi:10.1213/01.ane.0000166791.30963.ef table of contents.
20. Zhang ZC, Xu M, Sun SF, Qiao X, Wang BR, Han J, *et al*. Metabolic analysis of four phenolic acids in rat by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2008;871:7–14. doi:10.1016/j.jchromb.2008.06.019.
21. Li X, Li X, Wang L, Li Y, Xu Y, Xue M. Simultaneous determination of danshensu, ferulic acid, cryptotanshinone and tanshinone IIA in rabbit plasma by HPLC and their pharmacokinetic application in danxiongfang. J Pharm Biomed Anal 2007;44:1106–12. doi:10.1016/j.jpba.2007.04.033.
22. Sharma A, Ebling WF, Jusko WJ. Precursor-dependent indirect pharmacodynamic response model for tolerance and rebound phenomena. J Pharm Sci 1998;87:1577–84. doi:10.1021/js980171q.
23. Chen XL, Cai DL, Li Y, Hu TJ. The impact of high methionine diet on the growth and the amino acid metabolism of rats (Article in Chinese). Chin J Clin Nutr 2002;10:248–51.
24. Sun YN, Jusko WJ. Transit compartments *versus* gamma distribution function to model signal transduction processes in pharmacodynamics. J Pharm Sci 1998;87:732–7. doi:10.1021/js970414z.
25. Perez-Ruixo JJ, Krzyzanski W, Hing J. Pharmacodynamic analysis of recombinant human erythropoietin effect on reticulocyte production rate and age distribution in healthy subjects. Clin Pharmacokinet 2008;47:399–415. doi:10.2165/00003088-200847060-00004.

26. Sekiya A, Fukada S, Morita T, Kawagishi H, Sugiyama K. Suppression of methionine-induced hyperhomocysteinemia by dietary eritadenine in rats. Biosci Biotechnol Biochem 2006;70:1987–91. doi:10.1271/bbb.60075.
27. Earp JC, Dubois DC, Molano DS, Pyszczynski NA, Keller CE, Almon RR, *et al.* Modeling corticosteroid effects in a rat model of rheumatoid arthritis I: mechanistic disease progression model for the time course of collagen-induced arthritis in Lewis rats. J Pharmacol Exp Ther 2008;326:532–45. doi:10.1124/jpet.108.137372.
28. Perlstein I, Stepensky D, Krzyzanski W, Hoffman A. A signal transduction pharmacodynamic model of the kinetics of the parasympathomimetic activity of low-dose scopolamine and atropine in rats. J Pharm Sci 2002;91:2500–10. doi:10.1002/jps.10243.
29. Lobo ED, Balthasar JP. Pharmacodynamic modeling of chemotherapeutic effects: application of a transit compartment model to characterize methotrexate effects *in vitro*. AAPS PharmSci 2002;4:E42. doi:10.1208/ps040442.
30. Nijhout HF, Reed MC, Anderson DF, Mattingly JC, James SJ, Ulrich CM. Long-range allosteric interactions between the folate and methionine cycles stabilize DNA methylation reaction rate. Epigenetics 2006;1:81–7.
31. Hamelet J, Demuth K, Dairou J, Ledru A, Paul JL, Dupret JM, *et al*. Effects of catechin on homocysteine metabolism in hyperhomocysteinemic mice. Biochem Biophys Res Commun 2007;355:221–7. doi:10.1016/j.bbrc.2007.01.142.
32. Ciaccio M, Bivona G, Bellia C. Therapeutical approach to plasma homocysteine and cardiovascular risk reduction. Ther Clin Risk Manag 2008;4:219–24.
33. Bonaa KH, Njolstad I, Ueland PM, Schirmer H, Tverdal A, Steigen T, *et al*. Homocysteine lowering and cardiovascular events after acute myocardial infarction. N Engl J Med 2006;354:1578–88. doi:10.1056/NEJMoa055227.