

# Pharmacokinetics and Bioequivalence Study of Simvastatin Orally Disintegrating Tablets in Chinese Healthy Volunteers by LC-ESI-MS/MS

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## Abstract

A simple, rapid and sensitive liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) assay for the determination of simvastatin in human plasma using lovastatin as internal standard (IS) was established. After being extracted by methyl tert-butyl ether, solutes were separated on a C<sub>18</sub> column with a mobile phase consisting of methanol-water-5M ammonium acetate (90:10:0.1, v/v/v). The quantification of target compounds was carried out by using multiple reaction monitoring (MRM) mode: m/z 419.2 → 199.1 and 405.1 → 285.1 for simvastatin and IS, respectively. The method had a run time of 3.3 min and a linear calibration curve in the range of 0.1-20 ng/ml. The lower limit of quantification (LOQ) was about 0.1 ng/ml. The mean extraction recovery of simvastatin was over 92.48%. Intra- and inter-day variability values were less than 10.5% and 9.30%, respectively. This method offered good precision and accuracy and was successfully applied for a bioequivalence studies of 20 mg of simvastatin orally disintegrating tablets in 20 Chinese healthy volunteers.

**Keywords:** Simvastatin; LC-ESI-MS/MS; Pharmacokinetics; Orally disintegrating tablets

## Introduction

Simvastatin lowers plasma cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase. It is widely used in the treatment of hypercholesterolemia. Plasma levels of simvastatin following therapeutic oral doses are reported to be very low [1-3]. Therefore, to monitor the low therapeutic drug level, it is urgent to develop sensitive and selective methods.

There have been several reported methods for determining simvastatin [4-6]. GC-MS methods are highly sensitive and selective to analyze the therapeutic plasma level of simvastatin, but the operation and clean-up procedure are complicated [7]. HPLC with fluorescence detection is also a highly sensitive method, but it is inconvenient and time-consuming as samples need complex derivatization before analysis. The LC-MS methods [8-10] addressed concerns on inter-conversion in different degrees and also presented improvements in chromatography or MS/MS detection. This paper describes a simple, rapid and sensitive LC-ESI-MS/MS method for direct quantification of simvastatin in human plasma. The method was validated to meet the acceptance criteria industrial guidance for the bioanalytical method validation. The method has been successfully applied to a bioequivalence study of Chinese healthy volunteers.

## Experimental

### Chemicals and reagents

Simvastatin standard reference (lot no. 100601-200502, Figure 1A) and the internal standard (lovastatin, lot no. 100600-200502, Figure 1B) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPB, Beijing, China); The reference preparations (shujiangzhi<sup>®</sup> tablet, 20 mg/tablet) were obtained from Merck Sharp & Dohme Ltd. U.K (Hangzhou) and the test preparations (simvastatin orally disintegrating tablets, 10 mg/tablet) were provided by Zhejiang CONBA Pharmaceutical Co., Ltd. (Hangzhou). HPLC grade methanol was purchased from Tedia Company (lot no. 901901, USA). Ultra-pure water was obtained from Wahaha Group Co., Ltd. (Hangzhou, East China) and was used to

prepare all aqueous solutions. Methyl tert-butyl ether was purchased from Sinopharm Chemical Reagent Co., Ltd (lot no.T20090326, AR). Ammonium acetate was purchased from Shanghai Chemical Reagent Co., Ltd (lot no.T040426, AR). Drug-free and drug-containing plasma were taken from the volunteers. Plasma was stored at -70 until analysis.

### Instrumentation

An Agilent 1200SL Series liquid chromatographic system interfaced to an Agilent 6410B Triple Quad LC-ESI-MS/MS (MassHunter Data Acquisition, Qualitation and Quantitation soft-ware, USA) was equipped with an Agilent XDB-C<sub>18</sub> column (1.8 μm, 4.6×50 mm) at a column temperature of 40. Other instruments used included a WH-3 Micro Whirlpool mixer and a Feige TGL-16C centrifuges.

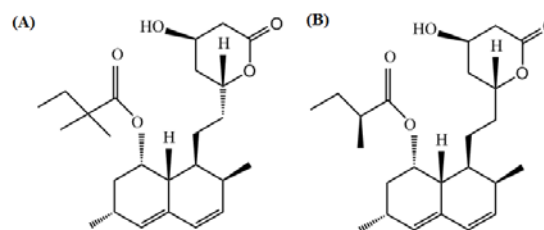


Figure 1: Structure of (A) simvastatin and (B) lovastatin (IS).

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## Chromatographic conditions

The mobile phase was constituted of methanol-water-5 M ammonium acetate (90:10:0.1, v/v/v) isocratically at a flow rate of 0.4 ml/min. The column temperature was 40, and the injection volume was 10  $\mu$ l.

## Mass-spectrometric conditions

The mass spectrometer, equipped with an ESI source which runs in positive mode, was set up in MRM mode to monitor the transitions 419.2  $\rightarrow$  199.1 and 405.1  $\rightarrow$  285.1 for simvastatin and IS, respectively. Figure 2 shows the production spectra obtained from simvastatin (Figure 2A) and lovastatin (Figure 2B). In order to optimize all the MS parameters, a standard solution of simvastatin and lovastatin was infused into the mass spectrometer. The following optimized parameters were obtained: the fragmentor and the collision energy were 60 V / 8 eV and 40 V / 10 eV for simvastatin and lovastatin, respectively. The drying-gas ( $N_2$ ) flow rate was 10 l/min. The capillary voltage was set at 4.0 kV. The desolvation temperature was 300. Figure 3 shows the proposed fragmentation pathways for simvastatin (Figure 3A) and lovastatin (Figure 3B).

## Preparation of stock solution, calibration standard and quality control samples

Stock solutions of simvastatin and lovastatin (IS) were prepared in methanol (1 mg/ml) and were further individually diluted with methanol to obtain the desired concentrations. The stock solutions were kept refrigerated and restored to room temperature before use.

The stock solution was appropriately diluted with methanol to obtain IS (100 ng/ml) solution and solutions for calibration at 5, 10, 25, 50, 100, 250, 500 and 1000 ng/ml of simvastatin. A 0.5 ml volume of drug-free plasma was placed in a 5 ml centrifuge tube, then 10  $\mu$ l of serial simvastatin stock solution and 10  $\mu$ l of IS solution were added. The rest of the procedure was the same as that of "Sample preparation"

The final simvastatin concentrations (in plasma) were 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 ng/ml. Quantity control (QC) samples were prepared in drug-free plasma at concentrations of 0.1, 0.2, 1.0 and 10.0 ng/ml for simvastatin in the same manner according to calibration curves.

## Sample preparation

Frozen human plasma samples were thawed at ambient temperature. A 0.5 ml volume of plasma was placed in a 5 ml centrifuge tube and 10  $\mu$ l of IS solution was added. After a thorough vortex mixing for 30 s, mixtures were extracted with 2 ml of methyl tert-butyl ether, vortex-mixed for 3 min, and centrifuged at 4000 r/min for 10 min. 1.5 ml of organic layer was removed to another centrifuge tube and evaporated under a stream of nitrogen gas in the thermostatically controlled water-bath maintained at 40 until completely dry. The dried residue obtained was dissolved in 100  $\mu$ l of methanol, vortex-mixed for 2 min, centrifuged at 12000 r/min for 10 min, and 10  $\mu$ l of the supernatant liquid was injected into the LC-ESI-MS/MS system.

## Validation

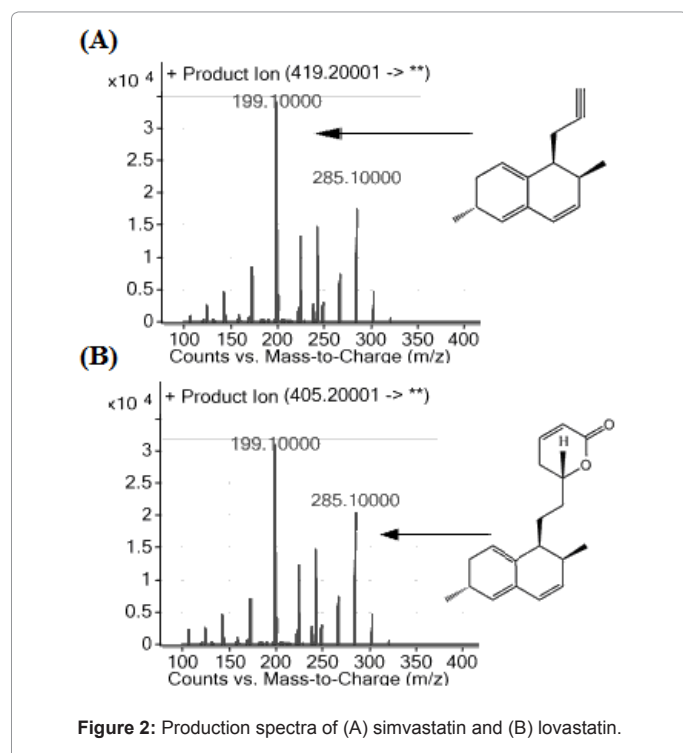
**Specificity and selectivity:** The specificity and selectivity of the method were checked by comparing the chromatogram of six randomly selected batches of blank human plasma with the corresponding spiked plasma. Each blank plasma sample was tested using the proposed extraction procedure and LC-ESI-MS/MS conditions to ensure no interference of simvastatin and IS from plasma. The chromatograms of blank human plasma (Figure 4A) had no significant endogenous peaks at the retention time of simvastatin or IS. The chromatograms of blank plasma spiked with simvastatin and IS were shown in Figure 4B. Volunteer's plasma taken after 0.5 h of a single oral administration of 20 mg simvastatin spiked with IS were shown in Figure 4C.

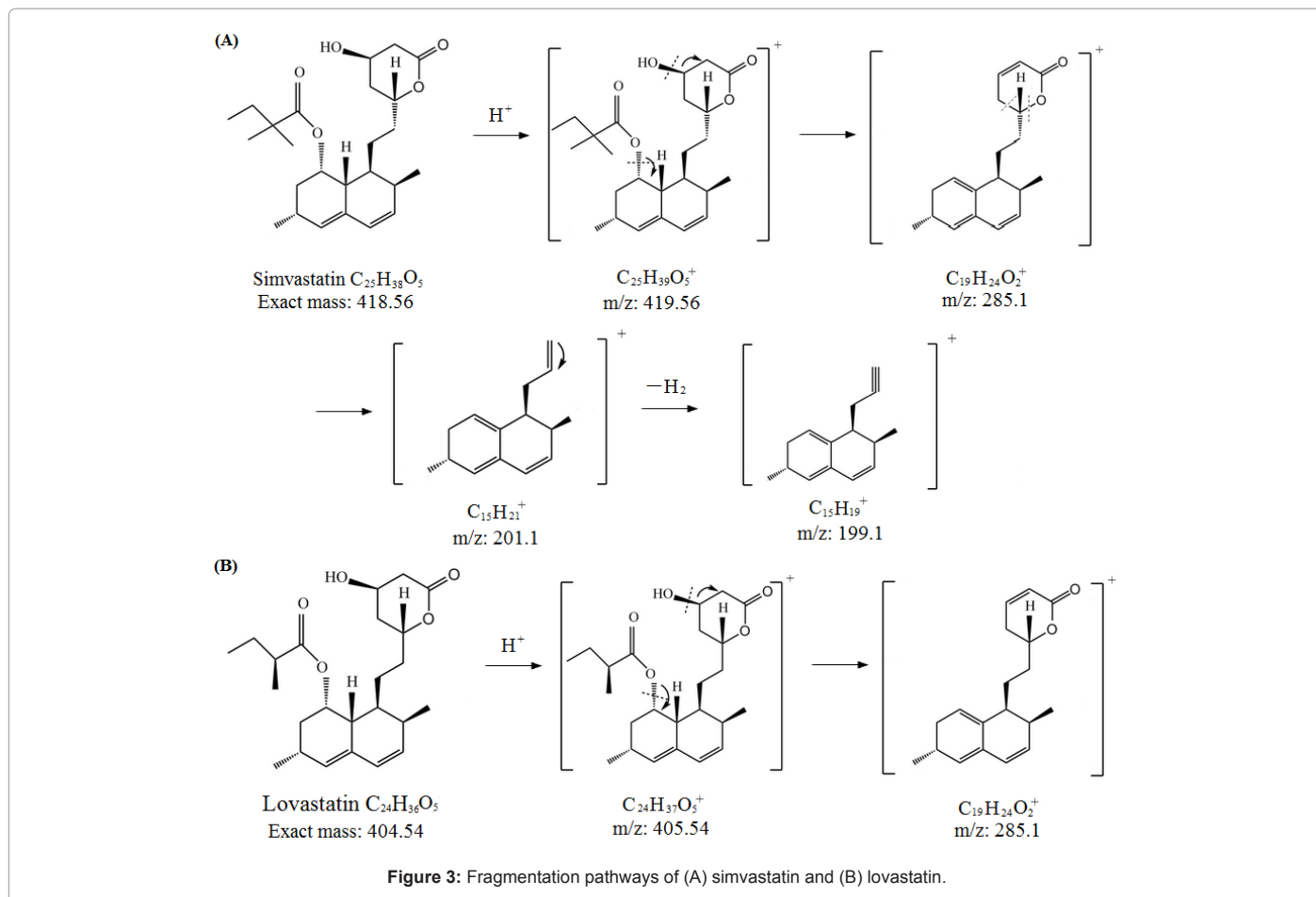
**Linearity:** Linearity was evaluated using freshly prepared spiked plasma samples at the concentration range of 0.1-20 ng/ml. Each calibration curve consisted of a drug free human plasma sample at eight calibrator concentrations. Five such linearity curves were analyzed. In the plasma, simvastatin standard curves were calculated by the equation:  $y = aC + b$  using weighted ( $1/C^2$ ) least square regression. A correlation of more than 0.99 was desirable for all the calibration curves (Figure 5).

**Precision and accuracy:** The accuracy and precision of the assay were determined by calculating the intra-batch and inter-batch variation for LOQ, low, medium, and high QC plasma samples (0.1, 0.2, 1.0, and 10.0 ng/ml, n=5) on the same day and on three different days. The evaluation of precision was based on the criteria that the deviation of each concentration level should not be more than  $\pm 15.0\%$  from the nominal concentration except for the LOQ, for which the deviation should not be more than  $\pm 20.0\%$ . Similarly, for accuracy, the mean value should not deviate by  $\pm 15.0\%$  of the nominal concentration except for the LOQ where it should not deviate by more than  $\pm 20.0\%$  of the nominal concentration.

**Extraction recovery and matrix effects:** The extraction recoveries of simvastatin were determined at LOQ, low, medium and high concentrations (0.1, 0.2, 1.0 and 10.0 ng/ml) by comparing the responses of simvastatin extracted from plasma samples with standard solutions without extraction.

The matrix effects were defined as the direct or indirect interference corresponding to the presence of unintended or other interfering substances in the samples. This was evaluated by comparing the peak-area ratios of simvastatin and IS in the blank plasma samples





after extraction and reconstitution (A) with those for the compounds dissolved in mobile phase (B). Four different concentration levels (0.1, 0.2, 1.0 and 10.0 ng/ml) of simvastatin were evaluated by analyzing five samples at each level. Matrix Effects% =  $100\% \times (A/B1)$ , if the ratio of A to B is less than 85% or more than 115%, and the matrix effect will not be accepted. So a negative result indicates suppression, and a positive result indicates enhancement of the simvastatin signal.

**Stability:** To determine the long-term stability of simvastatin in human serum, five aliquots of LOQ, low, medium and high concentrations samples (0.1, 0.2, 1.0 and 10.0 ng/ml) were kept in a deep freezer at -70 for 17 days. The short-term temperature stability was assessed by analyzing these four concentration samples that were kept at ambient temperature (25) for 12 h. Repeated freeze-thaw stability (-70 in plasma) was checked through three cycles in one day. The concentrations determined were compared with the actual values of QC samples, the deviation of LOQ should be within  $\pm 20.0\%$ , others  $\pm 15\%$ . The stability experiments were aimed at testing all possible conditions that the samples might experience after collection and prior to the analysis.

### Pharmacokinetics and bioequivalence study in healthy volunteers

An open-randomized, balanced, two-period crossover experiment was used for the assessment of the pharmacokinetics and bioequivalence in healthy volunteers with a 1-week washout period. A total of 20 Chinese healthy volunteers, whose mean age was 23.3 years (range, 19-

29) and mean BMI was  $21.9 \text{ kg/m}^2$  (range, 19.6-24.0), participated in the study after signing the consent form.

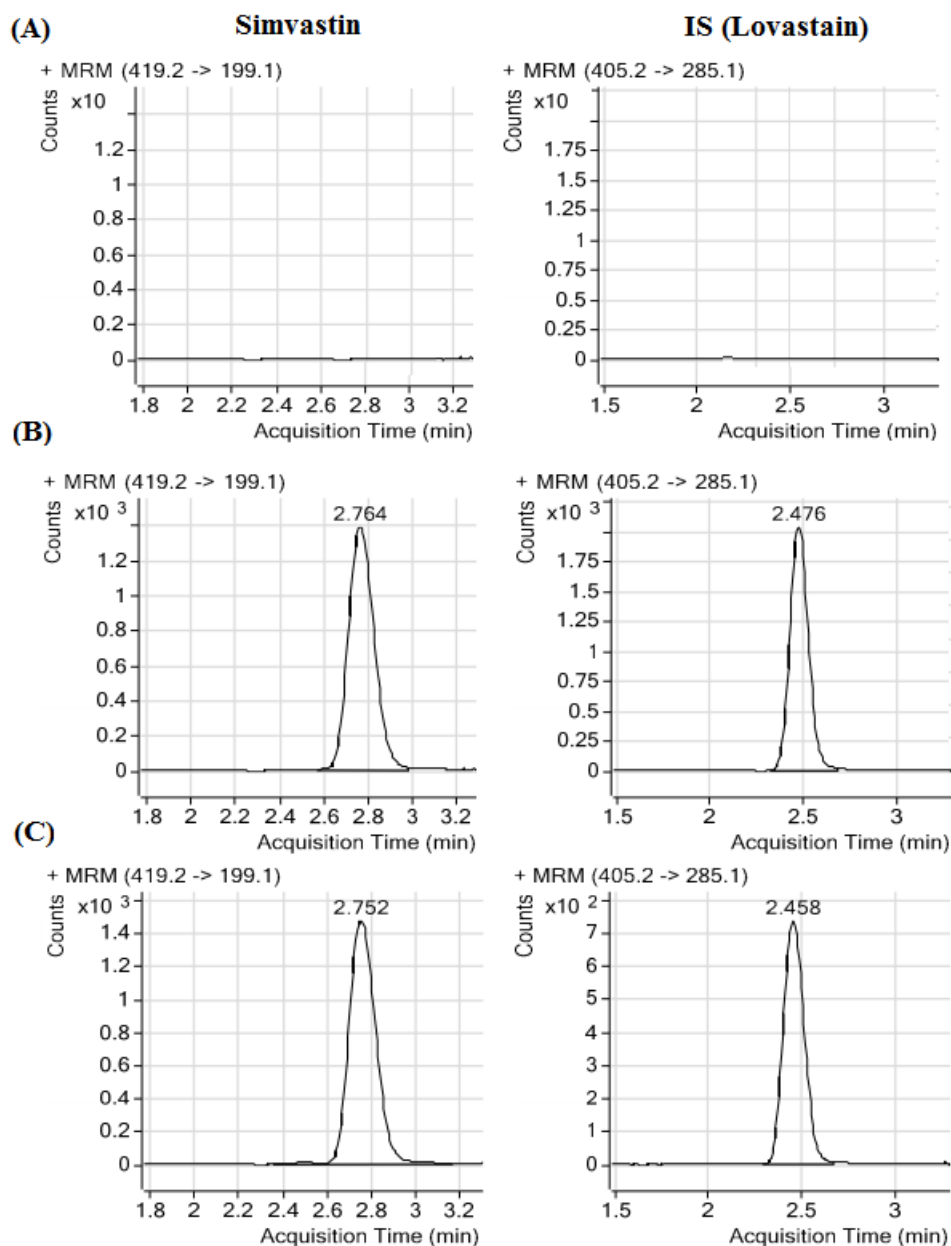
Each volunteer was administered 20 mg of simvastatin (by way of a simvastatin orally disintegrating tablets (Zhejiang CONBA Pharmaceutical Co., Ltd.) as test or a shujiangzhi<sup>®</sup> tablet (Merck Sharp & Dohme Ltd. U.K.) as reference drug). A crossover study was followed by a washout period of 1 week. During the test period, all subjects remained under close medical supervision and were supplied uniform diets. Venous blood samples (3 ml) were withdrawn by an indwelling catheter into heparin-containing tubes from a suitable antecubital vein at 0.00, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 9.0, 12.0 and 24.0 h after drug administration. Blood samples were centrifuged immediately at 4000 r/min for 15 min. Plasma was separated and put into tubes and stored at -70 until analysis.

## Results and Discussion

### Method development

**Selection of IS:** For a proper internal standard, its structure or chemical properties should be similar to the analyte, and it should also have retention and solubility which are similar to the analyte. Lovastatin was chosen as the internal standard for the assay because of the similarity of structure, retention and fragmentation pathways between it and the analyte.

**Conditions of chromatography:** In this paper, we chose 40 as the column temperature, because it can reduce the viscosity of the



**Figure 4:** Representative HPLC chromatogram of (A) blank plasma, (B) blank plasma spiked with simvastatin and IS, and (C) 0.5 h after administration (the IS retention time is about 2.4 min, simvastatin retention time is about 2.7 min).

mobile phase and improve the peak shapes. In order to get the shortest analysis time and avoid the matrix effects, different percentages of methanol in the mobile phase were tested, and the results showed that methanol-water (90:10, v/v) at 0.4 ml/min can avoid the interference of endogenous substances and get a short analysis time. With the purpose of achieving the maximum signal response under the MS conditions, 5 M ammonium acetate (0.1%) was added into the mobile phase to improve the sensitivity. The chromatographic conditions were: methanol-water-5 M ammonium acetate (90:10:0.1, v/v/v), which was operated isocratically in 0.4 ml/min at a column temperature of 40. Blank human plasma had no significant endogenous peaks at the retention time of simvastatin or IS.

## Validation

**Linearity and precision:** The standard calibration curves ( $y = aC + b$ ,  $n=5$  at each concentration) showed good linearity within the range of 0.1 to 20 ng/ml of simvastatin in human plasma. This was obtained by plotting the peak-area ratios ( $y$ ) of simvastatin to IS versus drug concentration ( $C$ , ng/ml) and performing a  $1/C^2$  weighted linear regression. The linearity was also assessed for three consecutive days for the calibration curve standard solutions. Intra- and inter-day precisions and accuracies were determined by analyzing QC samples against a calibration curve, on the same day and on different days. As shown in Table 1, this method allowed good precision and accuracy.

The LOQ of simvastatin was 0.1 ng/ml. The LOQ was defined as 10 times the S/N (signal- to-noise ratio).

**Stability:** Simvastatin was found to be stable for 17 days at -70 and for 12.0 h in human plasma when kept in the autosampler. The RSD was 4.60, 13.35, 3.12 and 5.76%, respectively. Plasma samples of simvastatin were found to be stable after subjecting them to three freeze–thaw cycles. The RSD was 13.34, 12.17, 9.57 and 1.15%, respectively (Table 2).

**Extraction recoveries and matrix effects:** The extraction recoveries and matrix effects of simvastatin in human plasma ranged from 92.48 to 104.4% and –6.59 to 3.45%, respectively (Table 3).

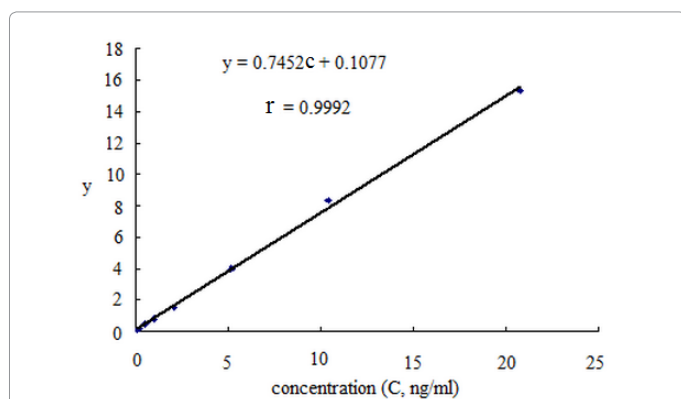


Figure 5: The calibration curves for simvastatin in the range of 0.1-20 ng/ml.

Content	Added concentration (ng/ml)	Mean found concentration (ng/ml)	Precision RSD (%)	Mean accuracy (%)
Intra-day	0.1037	0.09616	7.91	92.7
	0.2074	0.2140	9.84	103.2
	1.037	1.026	11.0	98.8
	10.37	10.56	3.35	101.3
Inter-day	0.1037	0.1033	7.43	95.4
	0.2074	0.2033	8.16	105.7
	1.037	0.9837	9.30	100.8
	10.37	10.55	6.36	98.6

Table 1: Precision and accuracy for analysis of simvastatin in human plasma.

Content	Added concentration (ng/ml)	Mean found concentration (ng/ml)	RSD (%)
Long-term stability	0.1037	0.108	5.17
	0.2074	0.2025	10.58
	1.037	0.9931	8.19
	10.37	10.43	1.12
Short-term stability	0.1037	0.1105	4.60
	0.2074	0.2196	13.35
	1.037	0.9984	3.12
	10.37	11.05	5.76
Repeated freeze–thaw stability	0.1037	0.09990	13.34
	0.2074	0.2075	12.17
	1.037	1.035	9.57
	10.37	10.63	1.15

Table 2: Stability of simvastatin in human plasma.

Concentration (ng/ml)	Mean recovery (%)	Matrix effect (%)
0.1037	104.4±5.62	-3.65±5.69
0.2074	97.03±4.91	-3.42±4.15
1.037	92.48±10.5	-6.59±2.82
10.37	96.44±6.35	3.45±2.29

Table 3: Extraction recovery and matrix effect of simvastatin in human plasma, n = 5.

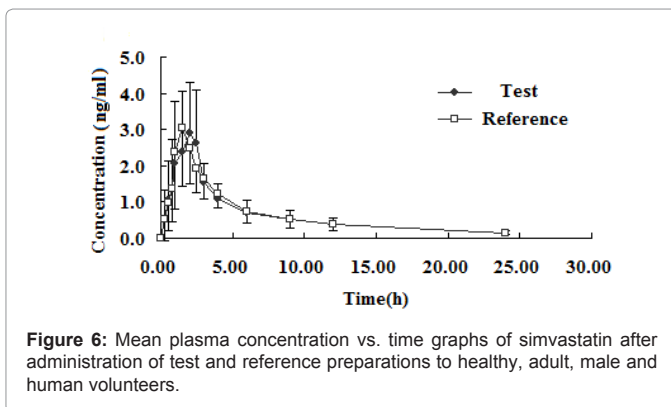


Figure 6: Mean plasma concentration vs. time graphs of simvastatin after administration of test and reference preparations to healthy, adult, male and human volunteers.

Pharmacokinetic parameters	Reference formulation (mean ± S.D.)	Test formulation (mean ± S.D.)	90% Confidence interval (%)
T <sub>max</sub> (h)	1.8±0.5	1.3±0.4	
C <sub>max</sub> (ng/ml)	4.01±1.74	3.75±1.51	85.03% ~ 104.76%
AUC <sub>0-24</sub> (ng h/ml)	15.43±4.33	14.0±3.44	95.88% ~ 108.37%
AUC <sub>0-∞</sub> (ng h/ml)	16.75±4.58	16.96±4.25	
t <sub>1/2</sub> (h)	5.74±0.79	5.77±0.89	
MRT	8.68±1.32	8.38±1.32	

Table 4: Bioequivalence parameters obtained after oral administration of test and reference formulations at the simvastatin dose of 20 mg in 20 healthy volunteers.

### Pharmacokinetics and bioequivalence study

The proposed validated method was successfully used for a pharmacokinetic study in 20 Chinese healthy volunteers for reference and test formulations of 20 mg simvastatin. The mean plasma concentration-time curve was shown in Figure 6.

By using statistics software of bioequivalence (BAPP 3.1, Chinese) analysis, the pharmacokinetic comparison between the two formulations was made in terms of extent (AUC<sub>0-24</sub> and AUC<sub>0-∞</sub>) and rate (C<sub>max</sub> and T<sub>max</sub>) of absorption. The mean pharmacokinetic parameters for the test and reference formulation were presented in Table 4. In all subjects, the ratio AUC<sub>0-24</sub>/AUC<sub>0-∞</sub> was above 88% (mean value 92%) which indicates the suitability of the analytical method for bioequivalence studies and a proper study design. These values are close to those obtained in other studies of the pharmacokinetics of simvastatin (Zhang et al., 2004). Since the 90% CI (Confidence interval) for C<sub>max</sub> and AUCs ratios were all inside the interval, it was concluded that simvastatin orally disintegrating tablets produced by Zhejiang CONBA Pharmaceutical Co., Ltd. China are bioequivalent to shuijiangzhi® tablet as to the extent of absorption. Its relative bioavailability is 103.2±16.6%.

## Conclusion

In this study of Chinese healthy adult male volunteers, a single 20-mg dose of the orally disintegrating tablets formulation (test) of simvastatin met the regulatory criteria for bioequivalence to a single 20 mg dose of the established tablet formulation (reference) based on the extent of absorption. The mean  $T_{max}$  of test formulation is shorter than reference formulation, proving that the absorption of simvastatin orally disintegrating tablets is quicker than that attained by commonly prescribed simvastatin tablets. This is in line with the characteristics of the tested drug formulations.

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