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Determination of nifedipine in human plasma and its use in bioequivalence study

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Abstract

A sensitive atmospheric pressure chemical ionization liquid chromatographic–mass spectrometric (APCI–LC–MS) assay with positive ion mode has been developed for the determination of nifedipine in human plasma. In this method, nifedipine was extracted from human plasma using diethyl ether with dimethoxanate as the internal standard. Analysis was achieved on a BDS C_{18} column with methanol–H_2O (66:34, v/v) as the mobile phase. Sustained-release nifedipine tablets from DiSha (test, Weihai, China) and from GuoFeng (reference, Qingdao, China) were evaluated following a single 20 mg oral dose to 20 healthy volunteers. Bioequivalence between the products was determined by calculating 90% confidence intervals (90% CI) for the ratio of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values for the test and reference products, using logarithmic transformed data. The 90% confidence intervals for the ratio of C_{max} (86.6–105.2%), AUC_{0-t} (97.8–110.9%) and $AUC_{0-\infty}$ (96.5–110.4%) values for the test and reference products are within the interval (80.0–125.0% for AUC, and 70–143% for C_{max}), proposed by State of Food and Drug Administration [SFDA, 2005. Guidance for Bioavailability and Bioequivalence Studies for Chemical Drug Products in Human Being. China, p. 19]. It was concluded that the two sustained-release nifedipine tablets are bioequivalent in their rate and extent of absorption and, thus, may be used interchangeably.

Keywords: LC–MS; Nifedipine; Human plasma; Sustained-release tablets; Pharmacokinetic profile

1. Introduction

Nifedipine (1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylic acid dimethyl ester) (Fig. 1) is a dihydropyridine calcium channel blocker used widely in the management of hypertension and angina (Stone et al., 1980; Kleinbloesem et al., 1984). Nifedipine has a very low bioavailability, and it is photosensitive and thermally unstable. These unfavorable pharmacokinetics and physic characteristics make determination of nifedipine in plasma difficult.

Early reported methods for the determination of nifedipine in biological fluids include gas chromatography (GC) (Dai et al., 2001), voltammetric method (Ozaltin et al., 2002), HPLC with UV detection (Kostewlcz et al., 1996; Abou-Auda et al., 2000; Yritia et al., 2000; Niopas and Daftsios, 2003; Li et al., 2005; Vertzoni et al., 2006) or electrochemical detection (ED) (Horvaith et al., 1996) and LC–MS–MS (Streel et al., 1998). However, no report on LC–MS method has been found.

In the present paper, a simple, accurate, selective and sensitive method (APCI–LC–MS) combining a simple liquid–liquid extraction procedure was developed and successfully applied to a bioequivalence study between two sustained-release nifedipine tablets.

2. Experimental

2.1. Chemical and reagents

Nifedipine (purity: >99%) and dimethoxanate (the internal standard, I.S.; purity: 99%) were purchased from Sigma (St. Louis, MO, USA). Methanol was of HPLC grades (Tedia Company, USA). All other chemicals and reagents were of analytical grade. Water was glass-double distilled and further purified for HPLC with a millipore purification system (Nihon Millipore,

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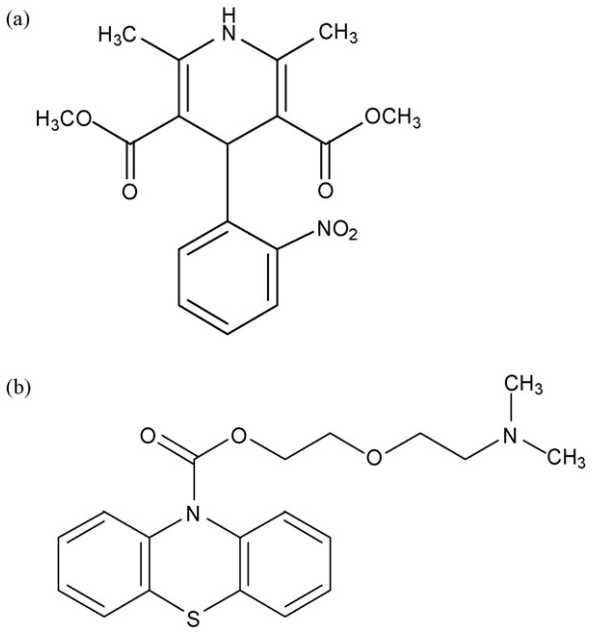

Fig. 1. Chemical structures of nifedipine (a) and dimethoxanate (I.S.) (b).

Tokyo, Japan). Blank human plasma was provided by the central blood bank of Shenyang (Liaoning, China).

2.2. Instrumentation

The LC system consisted of a 10ADvp liquid chromatography equipped with a binary pump, a vacuum degasser, a thermostated column compartment and an autosampler, all from Shimadzu (Japan). Analysis was achieved on a Kromasil BDS C_{18} column (150 mm × 4.6 mm i.d., 5 μm) with a guard column (10 mm × 4.6 mm i.d., 5 μm) (C_{18} column and guard column both were purchased from Dalian Zhonghuida Scientific Instrument Co. Ltd., China) of same packing material, at ambient temperature. The mobile phase consisted of methanol–H_2O (66:34, v/v). The flow-rate was 0.8 ml/min.

Mass spectrometric detection was carried out using a 2010EV single quadrupole instrument (Shimadzu, Japan) equipped with an APCI interface. The following APCI inlet conditions were applied: drying gas (N_2) flow-rate was 2.0 l/min; nebulizer gas (N_2) flow-rate was 2.5 l/min; detector voltage was 1.5 kV; interface temperature was set at 400 °C; CDL temperature was set at 250 °C; heat block temperature was set at 200 °C; positive full scan mode for screening and library-assisted identification; positive SIM mode for quantification.

A computer equipped with a LC–MS Solution 3.0 (Shimadzu, Japan) was used to control the LC–MS system and to collect and treat the data.

2.3. Choice of internal standards

Several substances, such as nitrendipine, nicardipine and dimethoxanate, were tested as internal standards. Among these, dimethoxanate has been chosen as the internal standard in the present analysis because of its stability and the appropriate elution time.

2.4. Extraction optimization

Liquid–liquid extraction was adopted for the isolation of nifedipine from plasma samples. Several extracting solvents including *n*-hexane–dichloromethane (3:2), acetic ether–*n*-hexane (3:2) and diethyl ether were investigated and no significant difference in extraction efficacy was observed. However, diethyl ether which had been employed in this paper could be evaporated to dryness easily and shorten the extraction time.

The concentration of alkalization agent (NaOH) was evaluated and less interference of endogenous substances was determined when 1 M NaOH was used.

2.5. Preparation of calibration standards and quality control samples

The stock solutions of nifedipine (10 μg/ml) and internal standard (dimethoxanate, 100 μg/ml) were prepared in methanol in the dark. Both solutions were stored at −20 °C in dark bottles.

Solutions of nifedipine with concentrations of 1000, 750, 500, 250, 100, 20, 10 ng/ml were prepared by serial dilution of stock solutions with methanol. All solutions of nifedipine were protected from light at 4 °C where they were stable for at least 2 weeks. The internal standard working solution used was 20 μg/ml.

Calibration standard samples were freshly prepared in 1 ml of blank plasma by adding 100 μl of the nifedipine calibration solutions and 100 μl of the internal standard working solution to yield a series of standards for the calibration curve with concentration corresponding to 1, 2, 10, 25, 50 and 100 ng/ml of nifedipine and 2 μg/ml of internal standard, respectively. Quality control (QC) samples were prepared at low (2 ng/ml), medium (25 ng/ml) and high (75 ng/ml) concentrations in the same way as the plasma samples for calibration.

2.6. Sample preparation

Blood samples were collected in tubes containing heparin. After centrifugation at 3000 *g* for 10 min, the separated plasma was collected and stored from light at −20 °C. Before analysis, the plasma samples were thawed at 18 °C.

A 100 μl of the internal standard working solution was added to 1 ml of plasma sample except for the blank plasma sample. The sample was then alkalinized by addition of 100 μl of 1 M NaOH solution, and subjected to liquid–liquid extraction using 4 ml of diethyl ether as extracting solvent. After vortex mixing for 2 min and standing for 8 min, the supernatant organic layer was quantitatively transferred to another 10 ml glass centrifuge tube and evaporated to dryness under a stream of nitrogen at 32 °C. The residue was reconstituted in 100 μl of methanol, vortex-mixed for 1 min, transferred to 0.2 ml Eppendorf tubes and centrifuged at 12,000 *g* for 5 min, a volume of 20 μl was injected onto the column. All operations were performed under the weak red light.

2.7. LC/MS method validation

After separation by LC, nifedipine and dimethoxanate (I.S.) was first directly introduced in mass spectrometry using APCI ionization. The mass spectrometer was set to generate and to select the protonated pseudomolecular ion $[M+H]^+$ (at m/z 347.00 for nifedipine, m/z 359.15 for dimethoxanate) as the target ions (m/z) for quantification in the SIM mode.

The specificity of the assay was evaluated by comparing between the blank plasma samples from six healthy volunteers who did not take nifedipine and blank human plasma sample spiked with nifedipine and the I.S. The efficiency of the extraction procedure was observed at low (2 ng/ml), medium (25 ng/ml) and high (75 ng/ml) concentrations. Recovery was calculated by comparing the respective peak areas of the chromatograms of the extracted samples relative to the untreated standards containing an equivalent amount of the compounds in methanol. Calibration curves were constructed by linear least-squares regression analysis plotting of peak-area ratio (nifedipine/I.S.) versus the drug concentrations. The lower limit of quantification (LLOQ) was defined as the lowest concentration with a coefficient of variation (CV) of less than 20% and accuracy of 80–120%. The accuracy and precision of the method were evaluated with QC samples at concentrations of 2, 25, 75 ng/ml on 3 consecutive days, accompanying by two standard calibration curves on each analytical run. The stability of nifedipine in plasma samples was investigated through three freeze-thaw cycles using the QC samples. The stability of the analyte in plasma and treated sample was investigated at different temperature and time. During the stability study, two standard calibration curves were prepared on each analytical batch.

2.8. Bioequivalence study

Twenty healthy male volunteers, 21–24 years (mean ± S.D., 22 ± 1.1 years), height of 170–180 cm (mean ± S.D., 175.6 ± 3.9 cm), weight of 60–72 kg (mean ± S.D., 68.2 ± 4.8 kg), and within 10% of their ideal body weight, were enrolled. The clinical protocol was approved by the local Ethics Committee and the volunteers given written informed consent to participate in the study. Volunteers were healthy and had no history of heart, kidneys, neurological or metabolic diseases, no history of drug hypersensitivity, also, were not undergoing any pharmacological treatment.

The study was an open, randomized, two-period, two-group crossover trial with an 8-day washout interval. During the first period, volunteers from group A received a single 20 mg dose of sustained-release nifedipine tablets from DiSha (test, Weihai, China), while volunteers from group B received a single 20 mg dose of sustained-release nifedipine tablets from GuoFeng (reference, Qingdao, China). During the second period, the procedure was repeated on the groups in reverse. The tablets were administered to the volunteers in the next morning after an overnight fast, with 250 ml of water. Volunteers received standard lunch and supper, respectively, 4 and 10 h after drug administration. Volunteers did not ingest any alcoholic drink, coffee or other xanthine-containing drinks during the trial. Furthermore, they did not take any other drug, 2 weeks before the study and during the execution.

Blood samples were collected at 0 (pre-dose) and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 15, 24, 36 h post-dose. The samples were centrifuged and the plasma was stored at −20 °C until analyzed. All operations were performed under the weak red light.

2.9. Pharmacokinetics and statistical analysis

The pharmacokinetic parameters, namely: the maximum plasma concentration (C_{max}) and time point of maximum plasma concentration (T_{max}) were obtained directly from the measured data; half-life of drug elimination during the terminal phase ($T_{1/2}$), area under the plasma concentration–time curve from 0 to the last measurable concentration (AUC_{0-t}), area under the plasma concentration–time curve from 0 to infinity ($AUC_{0-\infty}$) and mean residence time (MRT) were computed using DAS Software-Version 2.0 (Chinese Pharmacological Society, China).

Bioequivalence between the products was determined by calculating 90% confidence intervals (90% C.I.) for the ratio of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values for the test and reference products, using logarithmic transformed data. Analysis of variance (ANOVA) obtained from DAS Software-Version 2.0 was used to assess period, group and product effects. The products were considered bioequivalent if the 90% C.I. for AUC and C_{max} fell within 80–125% and 70–143%, respectively.

3. Results

3.1. Bio-analytical method validation

Fig. 2 shows the full-scan mass spectra of nifedipine and dimethoxanate. Typical total ions chromatograms (TICs) of blank plasma, blank plasma spiked with nifedipine (25 ng/ml) and I.S. (2 μg/ml), and plasma sample obtained at 4 h after a single oral dose of 20 mg nifedipine test tablet from a healthy volunteer are presented in Fig. 3. Nifedipine and the I.S. were eluted at about 3.6 and 6.5 min, respectively. No interfering peaks were determined at the retention times of nifedipine and I.S.

The lower limit of quantification (LLOQ) was 1.0 ng/ml with relative standard deviation (R.S.D.) lower than 5.0%. Linearity was observed within the range of 1–100 ng/ml ($Y = (0.0125 \pm 0.00134)X + (0.0187 \pm 0.00124)$, $r = 0.9980$).

The mean extraction recoveries of nifedipine at the three concentrations (2, 25 and 75 ng/ml) were 74.1 ± 8.6%, 71.7 ± 4.2% and 65.6 ± 4.8%, respectively. The mean extraction recovery of the I.S. was 74.0 ± 3.9%. The results of the accuracy and precision of the method are shown in Table 1.

Plasma samples were stable after three freeze-thaw cycles. Nifedipine was shown to be stable for 2 h at room temperature (R.E. < 4.2%) and for at least 18 days at −20 °C (R.E. < 6.2%) in plasma; and to be stable for at least 2 h at 10 °C (R.E. < 7.0%) and for at least 10 h at 4 °C (R.E. < 6.5%) in the reconstitution methanol. These results allow stockpiling of plasma

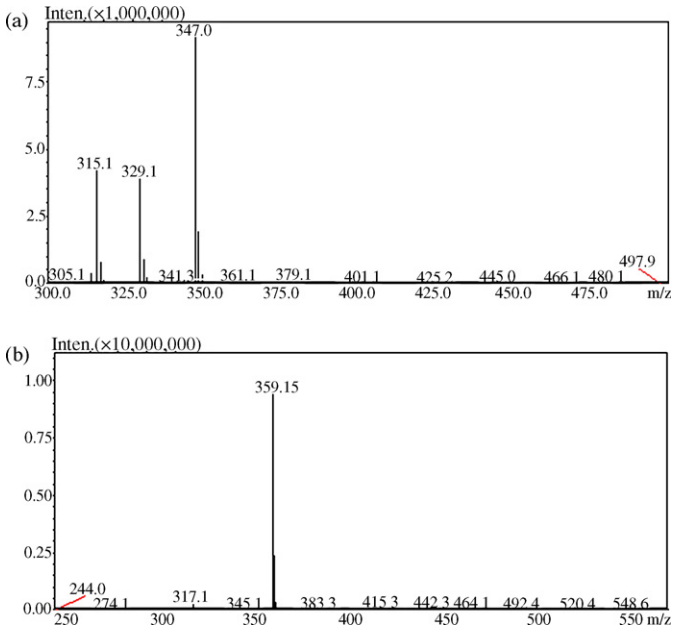

Fig. 2. Mass spectra of nifedipine and dimethoxanate (I.S.) illustrating the base peak ions: *m/z* 347.00 and *m/z* 359.15 as the protonated molecular ions of nifedipine and dimethoxanate (I.S.), respectively. (a) Nifedipine and (b) dimethoxanate (I.S.).

Table 1
The accuracy and precision of the method to determine nifedipine in plasma ($n = 18$)

Concentration (ng/ml)		Relative error (%)	R.S.D. (%)	
Added	Found		Intra-day	Inter-day
2.0	1.96	−2.0	8.2	9.9
25.0	24.1	−3.5	5.7	5.1
75.0	76.6	2.1	4.6	5.6

samples obtained in the bioequivalence study for subsequent analysis.

3.2. Bioequivalence evaluation

The mean (+S.D.) plasma concentration–time profiles and mean pharmacokinetic parameters after a single oral dose of 20 mg test and reference products administration to twenty healthy male subjects are presented in Fig. 4 and Table 2, respectively.

Table 2
The mean pharmacokinetic parameters of nifedipine following a single oral dose of 20 mg sustained-release nifedipine tablet from two companies to 20 healthy male subjects

Parameter	Test	Reference
C_{max} (ng/ml)	32.92 ± 16.7	35.22 ± 18.7
T_{max} (h)	3.5 ± 0.7	3.6 ± 0.9
$T_{1/2}$ (h)	5.3 ± 3.1	5.4 ± 3.7
AUC_{0-t} (ng h/ml)	232.4 ± 68.4	224.0 ± 77.5
$AUC_{0-\infty}$ (ng h/ml)	256.1 ± 78.2	247.6 ± 82.8
MRT (h)	5.9 ± 3.5	5.8 ± 3.6

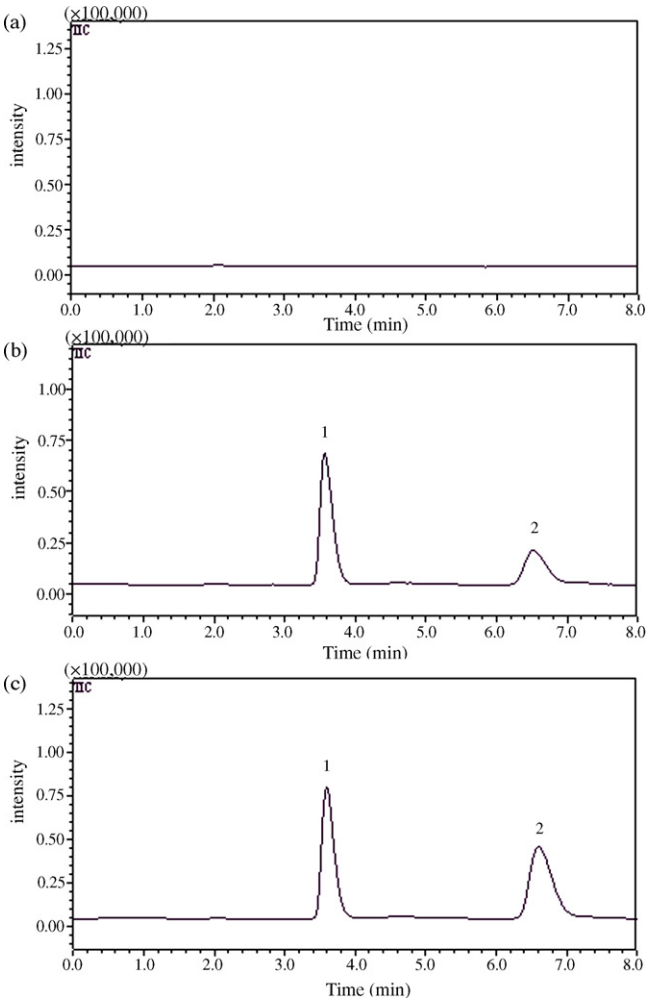

Fig. 3. Representative TICs of blank plasma (a), plasma spiked with I.S. (dimethoxanate, 2 μg/ml) and nifedipine (25 ng/ml) (b), and a plasma sample at 4 h after a single oral dose of 20 mg sustained-release nifedipine tablet (tested preparation) (c). Peak 1, dimethoxanate (I.S.); peak 2, nifedipine.

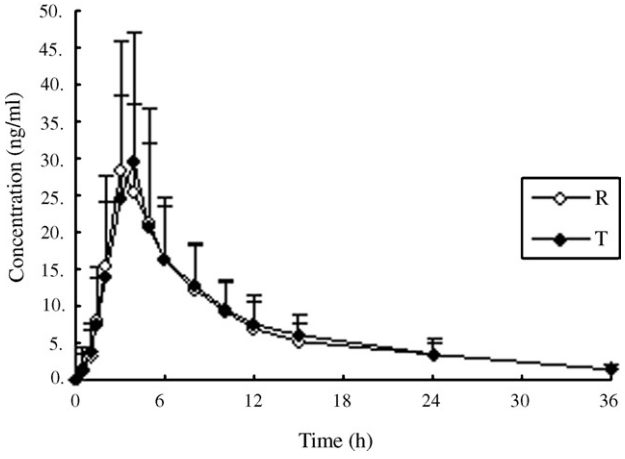

Fig. 4. The mean (+S.D.) plasma concentration–time profile of nifedipine following a single oral dose of 20 mg sustained-release nifedipine tablet from two companies to 20 healthy male subjects.

Table 3
Analysis of variance (ANOVA) for the assessment of the product, period and group effects, statistical power and 90% confidence intervals (90% C.I.) for the ratio of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values for the test and reference products, using logarithmic transformed data, after administration of reference and test products to 20 healthy volunteers ($\alpha = 0.05$)

Pharmacokinetic parameters	ANOVA (p-value), variation source			Statistical power (%)	90% C.I.
	Product	Period	Group		
C_{max}	0.415 ± 0.022	0.440 ± 0.020	0.000 ± 0.535	95.4	86.6–105.2%
AUC_{0-t}	0.277 ± 0.017	0.785 ± 0.001	0.000 ± 0.209	104.2	97.8–110.9%
$AUC_{0-\infty}$	0.421 ± 0.010	0.893 ± 0.000	0.000 ± 0.212	103.3	96.5–110.4%

The results of the analysis of variance (ANOVA) for assessment of product, group and period effects and 90% confidence intervals for the ratio of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values for test and reference products, using logarithmic transformed data, are shown in Table 3.

Power of statistical test was 95.4% for C_{max}, 104.2% for AUC_{0-t} and 103.3% for $AUC_{0-\infty}$.

4. Discussion

The analytical method developed for nifedipine quantification in plasma samples showed good specificity, sensitivity, linearity, precision and accuracy over the entire range of clinically significant and therapeutically achievable plasma concentrations, thereby enabling its use in bioequivalence trials.

The method demonstrated some advantages over other published methods. The method proposed by Dai et al. (2001) is not suitable for the analysis of nifedipine because of the thermal unstable.

Expensive solid-phase extraction procedures have been reported by several authors (Streel et al., 1998; Yritia et al., 2000; Niopas and Daftsios, 2003; Li et al., 2005). However, liquid–liquid extraction using diethyl ether as the extracting solvent is more economic.

Liquid–liquid extractions using the solvent mixture of *n*-hexane and dichloromethane or of acetic ether and *n*-hexane are described by Kostewlcz et al. (1996), Horvaith et al. (1996), Ozaltin et al. (2002) and Vertzoni et al. (2006), but it took more time to evaporate the supernatant to dryness, which is risk for nifedipine because of the thermal unstable. Moreover, the HPLC methods developed by the authors (Kostewlcz et al., 1996; Abou-Auda et al., 2000; Yritia et al., 2000; Li et al., 2005; Vertzoni et al., 2006) require 15 min for each chromatographic analysis. But the method in this paper requires 8 min at most.

Average plasma decay curves (Fig. 4) and pharmacokinetic parameters (Table 2) obtained for the test product were similar to those obtained for the reference product.

The multivariate analysis accomplished through analysis of variance revealed the absence of period and product effects but the existence of group effect. Fig. 5 shows the individual overlays of plasma concentration–time profiles after a single oral dose of 20 mg test or reference product to twenty healthy male subjects. The notable individual differences were displayed, which hint that the sustained-release nifedipine tablets should be administrated with the doctor's advice.

Previous pharmacokinetic data on sustained-release preparation were few and controversial. Examination of two sustained-release nifedipine preparations in humans and in pigs, accomplished by Kostewlcz et al. (1996), showed a mean C_{max} of about 40 ng/ml after single administration of 60 mg sustained-release nifedipine preparation to nine healthy male volunteers during fasting conditions, however, other pharmacokinetic parameters were omitted in the paper. Abou-Auda et al. (2000) reported that the values for C_{max}, $AUC_{0-\infty}$ and T_{max} were 61.35 ng/ml, 552.93 ng h/ml and 1.50 h, respectively, after oral administration of a single dose (20 mg) of sustained-release tablet. While data stated by Dai et al. (2001) revealed

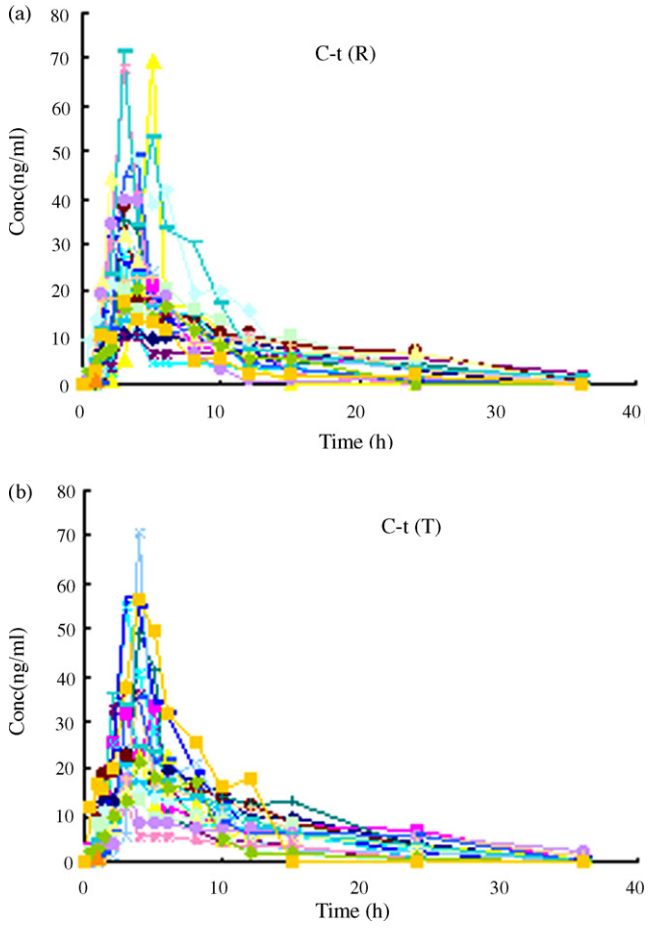

Fig. 5. The individual overlays of plasma concentration–time profiles after a single oral dose of 20 mg reference (a) or test (b) product to 20 healthy male subjects.

the following oral administration of a single dose (30 mg) of sustained-release tablet, the values obtained of C_{max}, $AUC_{0-\infty}$ and T_{max} were 39.38 ± 17.09 ng/ml, 722.59 ± 622.88 ng h/ml and 7.00 ± 5.26 h, respectively, which were close to those in the present study, considering the dose difference. Those differences could be caused by different manufacturers, various mechanisms of slow release or excipients, and different races of subjects.

According to the present study, power of statistical test was 95.4% for C_{max}, 104.2% for AUC_{0-t} and 103.3% for $AUC_{0-\infty}$, and the 90% confidence intervals for the ratio of C_{max} (86.6–105.2%), AUC_{0-t} (97.8–110.9%) and $AUC_{0-\infty}$ (96.5–110.4%) values for the test and reference products are within the interval (80.0–125.0% for AUC, and 70–143% for C_{max}), proposed by SFDA (2005). It was concluded that the test preparetion, sustained-release nifedipine tablets from DiSha (Weihai, China), and reference preparation, sustained-release nifedipine tablets from GuoFeng (Qingdao, China), are bioequivalent and, thus, may be used interchangeably, but it is necessary to administration with the doctor's advice for the group effect.

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