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Journal of Chromatography B, 852 (2007) 534–544
JOURNAL OF CHROMATOGRAPHY B
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Rapid and simultaneous determination of nifedipine and dehydronifedipine in human plasma by liquid chromatography–tandem mass spectrometry: Application to a clinical herb–drug interaction study

Xue-Ding Wang [a], Jia-Li Li [a], Yan Lu [a], Xiao Chen [b], Min Huang [a,**], Balram Chowbay [c], Shu-Feng Zhou [d,*]

[a] Institute of Clinical Pharmacology, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, China
[b] Department of Pharmacy, The first affiliated hospital of Sun Yat-sen University, Guangzhou, China
[c] Clinical Pharmacology Lab, Division of Medical Sciences, National Cancer Centre, Singapore 169610, Singapore
[d] Division of Pharmacy, School of Life Sciences, Queensland University of Technology, 2 George Street, Brisbane, Queensland 4001, Australia

Received 28 December 2006; accepted 9 February 2007
Available online 21 February 2007

Abstract

Nifedipine (NIF), a calcium channel antagonist, is metabolized primarily by cytochrome P450 (CYP3A4) to dehydronifedipine (DNIF). As such, NIF is often used as a probe drug for determining CYP3A4 activity in human studies. A rapid and sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was developed and validated to simultaneously determine NIF and DNIF in human plasma using nitrendipine as the internal standard (IS). After extraction of the plasma samples by ether-*n*-hexane (3:1, v/v), NIF, DNIF and the IS were subjected to LC/MS/MS analysis using electro-spray ionization (ESI). Chromatographic separation was performed on a Hypersil BDS C_{18} column (50 mm × 2.1 mm, i.d., 3 μm). The method had a chromatographic running time of approximately 2.5 min and linear calibration curves over the concentrations of 0.5–100 ng/mL for NIF and DNIF. The recoveries of the one-step liquid extraction method were 81.3–89.1% for NIF and 71.6–80.4% for DNIF. The lower limit of quantification (LLOQ) of the analytical method was 0.5 ng/mL for both analytes. The intra- and inter-day precision was less than 15% for all quality control samples at concentrations of 2, 10, and 50 ng/mL. The validated LC/MS/MS method has been successfully used to study pharmacokinetic interactions of NIF with the herbal antidepressant St. John's wort in healthy volunteers. These results indicated that the developed LC/MS/MS method was efficient with a significantly shorter running time (2.5 min) for NIF and DNIF compared to those methods previously reported in the literature. The presented LC/MS/MS method had acceptable accuracy, precision and sensitivity and was used in a clinical pharmacokinetic interaction study of NIF with St. John's wort, a known herbal inducer of CYP3A4. St. John's wort was shown to induce NIF metabolism with increased plasma concentrations of DNIF.
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Keywords: Nifedipine; Dehydronifedipine; Pharmacokinetics; Liquid chromatography/tandem mass spectrometry; St. John's wort

1. Introduction

Nifedipine (dimethyl2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate, NIF, I; Fig. 1), the prototype of the dihydropyridine class of calcium channel blockers, is widely used in the treatment of hypertension, Prinzmetal's angina pectoris and other vascular disorders such as Raynaud's phenomenon [1,2]. It inhibits the influx of extracellular calcium through myocardial and vascular membrane pores by physically plugging the channel, resulting in decreased intracellular calcium levels, inhibition of the contractile processes of smooth muscle cells, dilation of the coronary and systemic arteries, increased oxygen delivery to the myocardial tissue, and decreased total peripheral resistance, systemic blood pressure, and afterload [1,2]. NIF is photo-sensitive and a nitroso-pyridine derivative is formed in solution on exposure to visible light, while a nitro-pyridine derivative is generated under

* Corresponding author. Tel.: +61 7 31381340; fax: +61 7 31381534.
** Corresponding author. Institute of Clinical Pharmacology, School of Pharmaceutical Sciences, Sun Yat-sen University, 74 Zhongshan Road, Section 2, Guangzhou 510080, China. Tel.: +86 20 873 34521; fax: +86 20 873 34718.
E-mail addresses: huangm@gzsums.edu.cn (M. Huang), s4.zhou@qut.edu.au (S.-F. Zhou).

1570-0232/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2007.02.026

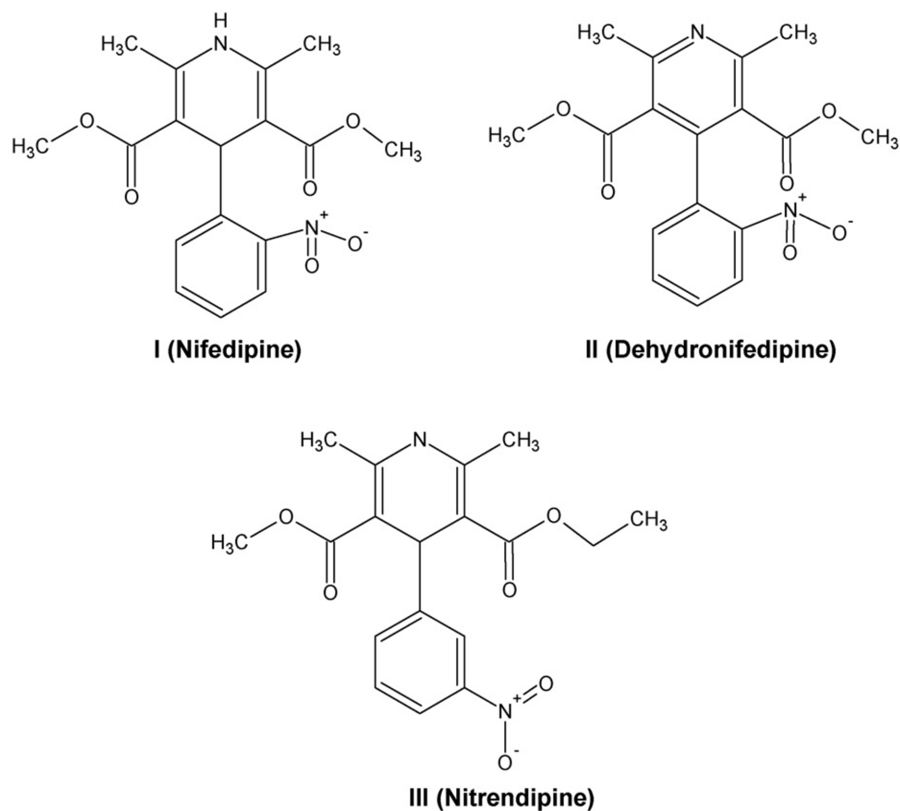

Fig. 1. Chemical structures of nifedipine (I), dehydronifedipine (II), and nitrendipine (III).

ultraviolet light [3]. There is a wide inter-individual variation in the pharmacokinetics and clinical response of NIF. Following an oral 10 mg dose, the maximum concentrations (C_{max}) of NIF range from 17–80 ng/mL with elimination half-lives ($t_{1/2\beta}$) between 1–34 h [4,5]. In humans, NIF is predominantly metabolized by cytochrome P450 (CYP3A4) to its primary pyridine metabolite, dehydronifedipine (DNIF, II; Fig. 1) [6,7]. The C_{max} values of DNIF vary from 8 to 37 ng/mL after an oral dose of 10 mg NIF [4,5]. CYP3A4 is the most abundant CYP enzyme (∼30–40%) in adult liver and metabolizes more than 50% of the clinically used drugs including NIF, cyclosporine, midazolam, and erythromycin [8,9]. CYP3A4 is subjected to inhibition and induction by a number of endogenous and exogenous compounds including many drugs and herbal medicines and regulated at both transcriptional and translational levels [10]. Thus, the quantitative measurement of CYP3A4 activity in humans is important to explore the effects of environmental, physiological, pathological and pharmacogenetic factors on the expression of *CYP3A4* gene. NIF, together with a few other typical CYP3A4 substrates including erythromycin (*N*-demethylation), midazolam (1-hydroxylation), testosterone (6β-hydroxylation), triazolam (1-hydroxylation), cortisol (6β-hydroxylation) and terfenadine (t-butylhydroxylation), have been commonly used as probe drugs when determining phenotypic CYP3A4 activity in humans [6,7,11–17]. The ratio of plasma levels of the resultant oxidized metabolite to those of the parental drug is often used as a reliable indicator of CYP3A4 activity.

To carry out pharmacokinetic and pharmacogenetic studies, sensitive and specific analytical methods are often required for routine analysis of NIF and its metabolite DNIF in biological fluids when NIF is used as a probe drug for exploring CYP3A4 activity. To date, numerous methods for the determination of NIF in biological samples have been reported [18–25]. Most of these reported analytical methods involve the application of gas chromatography (GC) and liquid chromatography (LC). The reported GC methods often utilize electron-capture detection [18,26–30], flame ionization detection [31], nitrogen–phosphorus detection [32], or mass spectrometric (MS) detection [33,34]. Although the sensitivity of GC/MS is high in pharmacokinetic and pharmacogenetic studies, the GC method has several concerning drawbacks, such as thermal degradation of NIF and DNIF due to high temperatures during analysis. Among the LC procedures reported, most of them are the combination of LC separation with UV detection [3,18,23–25,35–39], electrochemical detection [18,40–43], or less frequently, with MS detection [19]. In these reported LC methods, only five of which are able to simultaneously quantitate NIF and DNIF in biological fluids [19,30,35,38,39]. However, these analytical methods without coupling with MS detection all have a lower limit of quantification (LLOQ) of above 50 ng/mL [30,35,38,39], while the LC/MS method reported by Streel et al. [19] gives a significantly improved LLOQ (0.5 ng/mL) for both NIF and DNIF. Yet this LC/MS method developed by Streel et al. [19] requires relatively long running time for each sample (about 15 min) and a complicated and time-consuming sample

preparation procedure including a five-step solid-phase extraction. Thus, it is not suitable for high-throughput analysis when a large number of samples need to be quantitated in therapeutic drug monitoring and pharmacogenetic studies.

Therefore, we developed a rapid and sensitive LC/MS/MS method to simultaneously determine NIF and DNIF in human plasma using a simple one-step liquid-liquid extraction. In this study, we chose electro-spray ionization (ESI) as the ionization source. Our method exhibited excellent performance in terms of high selectivity, robustness and excellent efficiency (2.5 min per sample) with simplicity of sample preparation and minimal matrix effect. This LC/MS/MS method was successfully applied to a pharmacokinetic interaction study of NIF with St. John's wort (*Hypericum perforatum*) in healthy Chinese volunteers. St. John's wort, a commonly used herbal antidepressant, has been shown to induce CYP3A4 and P-glycoprotein and thus alter the pharmacokinetics of a number of clinical important drugs including amitriptyline, cyclosporine, irinotecan, digoxin, fexofenadine, methadone, midazolam, nevirapine, phenprocoumon, simvastatin, tacrolimus, indinavir, theophylline and warfarin [44,45].

2. Experimental

2.1. Chemicals and reagents

Nifedipine (NIF) with a purity of 99.9% and nitrendipine (methylethyl2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate, IS, Fig. 1) with a purity of 97.8% as determined by HPLC with UV detection were synthesized and provided by Baiyunshan Pharmaceutical Inc. (Guangzhou, Guangdong, China). The chemical structures were verified by LC/MS and ^{1}H-nuclear magnetic resonance analysis and compared with reference compounds. Dehydronifedipine (DNIF) with a purity of 99.9% as determined by HPLC with UV detection was obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). Methanol and formic acid of HPLC grade were purchased from Tedia Inc. (Beijing, China). All other reagents were of analytical grade or HPLC grade when appropriate. Blank human plasma from healthy blood donors was obtained from the Central Laboratory of the Blood Transfusion Service of Guangzhou, Guangdong, China. Ultra-pure water was obtained from a Milli Q-plus system (Billerica, MA).

2.2. Preparation of standard and quality control samples

The stock standard solutions of NIF and DNIF were prepared by dissolving accurately weighed individual compounds in methanol–water (50:50, v/v) to give a final concentration of 500 μg/mL in a 10-ml brown flask. The solutions were then serially diluted with methanol–water (50:50, v/v) to obtain working solutions at concentrations over 0.025–5.0 μg/mL. A standard stock solution of nitrendipine (IS) at 500 μg/mL was also prepared in methanol–water solution (50:50, v/v) and then diluted to obtain a working solution at 1 μg/mL. All the solutions were stored at 4 °C and were brought to room temperature before use. The analytical standard and quality control (QC) samples were prepared by spiking blank human plasma (500 μl) with standard working solutions (20 μl) during validation and each experimental run for the pharmacokinetic study. Calibration samples were made at concentrations of 0.5, 1, 2, 5, 10, 25, 50 and 100 ng/mL for both NIF and DNIF. The human plasma volume for each calibration and QC sample was identical (0.5 ml) despite the differential concentrations of NIF and DNIF. This ensured that the same amounts of human plasma proteins were added to each sample. NIF is a highly bound drug in human plasma [46]. Quality control samples were prepared at concentrations of 2, 10, and 50 ng/mL.

2.3. Sample preparation

Standard working solutions (20 μL, each of NIF, DNIF and IS) were added into 0.5 mL blank human plasma sample in 10 ml brown tubes, and the plasma samples were vortexing-mixed and extracted with 2 mL extraction solvent (ether:n-hexane, 3:1, v/v). After vortex-mixing for 2 min and standing at room temperature for 15 min, the mixtures were centrifuged at 2000 × *g* for 10 min. The organic phase was then transferred to a clean brown tube and evaporated to dryness under nitrogen gas at 45 °C. The residues were dissolved in 150 μL methanol/water (50:50, v/v) and an aliquot (10 μL) of the reconstituent was injected onto the LC/MS/MS for analysis. The sample preparation and analysis were carried out in a darkened room to avoid light-induced decomposition of the NIF and its metabolite.

2.4. Liquid chromatographic and mass spectrometric conditions

A Waters 2695 separation module (alliance) (Avondale, CA) was used for solvent and sample delivery. A chromatographic separation was achieved by using a C_{18} column (Hypersil BDS C18, I.D. 2.1 × 50 mm, 3 μm, Elite HPLC Inc., Dalian, China) at room temperature. The mobile phase consisted of methanol–water (containing 1% formic acid) (80:20, v/v), pumped at a flow rate of 200 μL/min. The total running time was 2.5 min for each sample.

A Quattro microTM triple quadrupole mass spectrometer (Micromass, Notre Dame, UK) equipped with an ESI source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive-ion mode (ESI^{+}) and set up in the selected reaction monitoring (SRM) mode. Nitrogen was used as desolvation (500 L/h) and nebuliser (50 L/h) gas. Argon was used as collision gas (0.0033 mbar). The capillary voltage was 3.0 kV for the analytes and the entrance and exit energies of the collision cell were set at −1 and 1, respectively. The source and desolvation temperatures were kept at 110 and 350 °C, respectively. On the basis of the full-scan mass spectra of each analyte, the most abundant ions were selected and the mass spectrometer was set to monitor the transitions of the precursors to the product ions as follows: *m/z* 347.1 → 315.0 for NIF, *m/z* 345.1 → 248.3 for DNIF and *m/z* 361.0 → 315.0 for IS. The dwell time per channel was set to 0.3 s. The optimized ESI^{+}-MS/MS parameters are listed in Table 1. The system

Table 1
Selected ion transitions (m/z values) and optimized mass spectrometric parameters for the LC/MS/MS analysis of nifedipine (NIF), dehydronifedipine (DNIF) and nitrendipine (IS) in selected reaction monitoring (SRM) mode (positive ionisation)

Analyte	Precursor ion $[M+H^+]^+$ (m/z)	Product ion (m/z)	ESI capillary voltage (kV)	Cone voltage (V)	Collision energy (eV)
NIF	347.1	315.0	3.0	20	8
DNIF	345.1	284.3	3.0	25	28
IS	361.0	315.0	3.0	15	12

NIF: nifedipine, DNIF: dehydronifedipine, IS: nitrendipine.

was controlled by Masslynx V 4.0 software (Micromass, Notre Dame, UK).

2.5. Method validation

The method was validated for selectivity, accuracy, precision, recovery, calibration curve and reproducibility according to the FDA guideline for validation of bioanalytical methods [47]. The selectivity was investigated by preparing and analyzing six individual human blank plasma samples at the LLOQ. The LLOQ was defined as the lowest concentration on the calibration curve of the analytes measured with acceptable precision and accuracy (i.e. relative standard deviation (RSD) and relative error < 20%) and with at least five times the response compared to blank response (noise). The limit of detection (LOD) was estimated as the amount of NIF or DNIF, which caused a signal three times to noise. Linearity was assessed by preparing and analyzing NIF and DNIF standard samples over 0.5–100 ng/mL with 8 concentration points in human plasma.

Calibration curves were analyzed by weighted linear regression ($1/X^2$) of the peak area of analyte over that of IS. The $1/X^2$ weighing gave a minimal sum of squares of the residuals and mean absolute errors for the linear regression. Accuracy and precision were assessed by determining QC samples at three concentration levels (five samples for each concentration) on three different validation days. The precision was determined as the RSD or coefficient of variation (CV, %) and the accuracy was expressed as a percentage of the measured concentration over the nominal (theoretical) concentration. The criteria used to assess the suitability of precision and accuracy was as follows: the RSD did not exceed 15% and the accuracy was within 15% of the actual value. The recovery (extraction efficiency) of analytes from human plasma after the extraction procedure was determined by comparing the areas of extracted analytes with those of the non-extracted pure standards that represent 100% recovery. During routine analysis, each analytical run included a blank plasma, a blank plasma spiked with IS, a set of calibration samples, a set of QC samples and unknowns. The stability of analytes was assessed by determining QC samples at three concentrations (2, 10 and 50 ng/mL for both NIF and DNIF) with five samples for each concentration, exposed to different time and temperature conditions. The stability studies included: (a) stability at room temperature for 4 h; (b) stability after three freeze–thaw cycles; (c) stability of the extracted samples at room temperature for 12 h; and (d) the long-term stability after storage at −30 °C for 25 days.

The absolute and relative matrix effect (ME) on the spectral response of NIF and DNIF was assessed as described by Matuszewski et al. [48] with slight modifications as mentioned previously [49]. Since ME is a concern with the fast isocratic system, the co-elution effect and potential ion suppression were evaluated. To assess the co-elution effect, pooled blank plasma was spiked with each analyte or IS, and the corresponding peak area was compared to that from the spiked sample of combined IS and analytes. Triplicate of QC sample at medium concentration were analysed. To assess the 'absolute' ME, i.e. the potential ion suppression due to the matrix components, six different batches of blank plasma were extracted by ether- *n*-hexane (3:1, v/v) and then spiked with NIF or DNIF at medium QC concentration (B) and IS. The corresponding peak areas were then compared to those of the aqueous standards at equivalent concentrations (A). The ratio (B/A × 100) is defined as the ME. A ME value of 100% indicates that the response in the mobile phase and in the plasma extracts was the same and no absolute matrix effect was observed. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression.

2.6. Human pharmacokinetic study

The validated method was applied to a herb–drug interaction study of NIF (10-mg tablet, Batch No. 060101, manufactured by Baiyunshan Pharmaceutical Inc., Guangzhou, China) with St. John's wort. St. John's wort was obtained from Dr.Willmar Schwabe GmbH & Co. AG, Karlsruhe, Gemany. The contents of hypericin and hyperforin, two major active components, in the St. John's wort preprations have been standardized to 0.3% and 5%, respectively, by manufacturer. Analysis using HPLC methods at our laboratory found similar contents of both compounds in the preparations. We recruited six female and six male healthy adult volunteers, from a total of 300 healthy Chinese volunteers who had been screened for the gene encoding nuclear receptor subfamily 1 (NR1I2/PXR) haplotype and *CYP3A4* genotype for this study (the details of the haplotypes and genotypes will be described in another paper). All volunteers completed a thorough medical, biochemical and physical examination. Informed consent was obtained from all subjects after explaining the aims and risks of the study. The study protocol was approved by the Human Investigation Ethical Committee at the School of Pharmaceutical Sciences at Sun Yat-sen University, Guangzhou, China.

The study was conducted before and after a 14-day treatment period with a St. John's wort preparation at 900 mg/day.

This dose is commonly used in human studies, as it can significantly induce the activity and expression of human CYP3A4 and P-glycoprotein [50]. After an overnight fasting (10 h), the volunteers were treated with an oral dose of 10 mg nifedipine with 200 mL of water. Regular standardized low-fat meals were provided until 4 h after dose administration; and water intake was allowed after 2 h of drug administration. Following drug administration, venous blood samples (5 mL) were collected into heparinized brown tubes according to the following schedule: immediately before drug administration and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5, 8, 12 and 24 h after dosing. Blood samples were centrifuged at 1500 × *g* for 10 min at 4 °C to obtain the plasma. The plasma samples were clearly labeled and kept frozen at −30 °C until analysis.

2.7. Pharmacokinetic calculation

The plasma concentration-time profiles of NIF and DNIF were obtained for each individual subject, and non-compartmental pharmacokinetic parameter calculations were performed using the NONMEM Program version 1.1 (GloboMax Inc., Ellicott City, MD). The elimination rate constant (β) was obtained as the slope of the linear regression fit of the log-transformed concentration values versus time data in the terminal phase. The elimination half-life ($t_{1/2\beta}$) was calculated as 0.693/β. The time to peak plasma concentration (T_{max}) and peak plasma concentration (C_{max}) were read directly from the observed concentration versus time profiles. The area under the plasma concentration-time curve to the last measurable concentration (AUC_{0-24h}) was calculated using the linear trapezoidal rule for the observed values from zero time to the last measured time point. The $AUC_{0-\infty}$ (AUC from time zero to infinity) was calculated as following equation:

$$AUC_{0-\infty} = AUC_{0-24h} + \frac{C_t}{\beta} \quad (1)$$

where C_t is the last measurable plasma concentration.

2.8. Statistical analysis

Data are expressed as mean ± SD. Differences between groups for continuous variables on more than one occasion were evaluated with repeated measures analysis of variance (ANOVA). Differences between two groups were analysed using paired Student's *t*-test. A pairwise analysis is the comparison of the outcome of the two measurements, namely, the *before and after* measurements are compared. In the case of means of pharmacokinetic parameters for each individual on the measurement before is subtracted from that after the intervention (i.e. St. John's wort treatment for 14 days). These differences are for all individuals added together producing a mean difference with an associated standard deviation. The null hypothesis is that the average is zero, overall (in net terms) the respondents did not change. Statistical significance was set as $P < 0.05$.

Before start of the present study, we have done a power analysis using the SISA program (http://home.clara.net/sisa). The power of a study refers to the probability of detecting a "true" effect of a factor, the larger the sample size, the higher the power, but the cost of study will also significantly increase. Thus a proper sample size is always needed in clinical pharmacokinetic studies, and the power calculated is the power with which you detect a postulated net change over all individuals. It was 85.9% and 92.5% when assuming α values of 0.05 and 0.1 with a double-sided analysis, respectively. The power was 92.5% and 96.8% if α values of 0.05 and 0.1 with a single-sided analysis were set, respectively.

3. Results and Discussion

3.1. Method development

In this study, ESI was chosen as the ionization source. In the method developed by B Streel et al. [19], the atmospheric pressure chemical ionization (APCI) was used. In APCI, ions are produced in the discharge and extracted into the mass spectrometer. APCI is best suited to relatively polar, semi-volatile samples. Some studies indicate that APCI is much less susceptible to analyte-ion suppression compared with ESI techniques [51,52]. APCI is often used only after ESI has not provided the required sensitivity, as APCI needs strict operating conditions such as high flow rate, high temperature and high voltage, and thus result in incompatibility with thermally labile compounds [51]. It is well known that NIF is thermal- and photo-sensitive, thus ESI ionization source is more suitable for its measurement and adopted in this study.

It was found that the signal intensity of the analytes and IS in human plasma was high using ESI source and the regression curves were linear over 0.5–100 ng/mL. The chemical structures of these components were described in Fig. 1. By using ESI, the analytes and IS formed predominantly protonated quasi-molecular ion $[M+H^+]^+$ in full scan spectra, with an *m/z* 347.1 for NIF, *m/z* 345.0 for DNIF, and *m/z* 361.1 for IS. To determine NIF and DNIF using SRM mode, full scan product-ion spectra of the analytes and IS were investigated. The most abundant ion in the product-ion mass spectrum was at 315.0 for NIF, 284.1 for DNIF and 315.0 for IS. Capillary and cone voltages and collision energies were optimized to obtain the greatest intensity of the most abundant product ion for further MS/MS experiments. The collision behavior of the $[M+H^+]^+$ of these compounds was strongly dependent on the collision energy. For DNIF, an increase in the collision energy caused a marked increase of the fragmentation processes; while an increase in the collision energy caused a marked decrease of the fragmentation processes for NIF and IS. After optimization, the collision behavior was carried out using 8, 12 and 28 V collision energy and 20, 25 and 15 V cone voltages for NIF, IS and DNIF, respectively. The capillary voltage was adjusted to 3.0 kV to obtain a maximum intensity of the product ions. It was found that the source temperature and the desolvation temperature did not significantly influence the MS behavior of these compounds and remained unchanged at the recommended value of 110 °C and 350 °C, respectively. Other MS conditions, including Desolvation/Cone Gas Flow, RF lens voltage and Extractor voltage, were maintained at the auto-tuned (default) values,

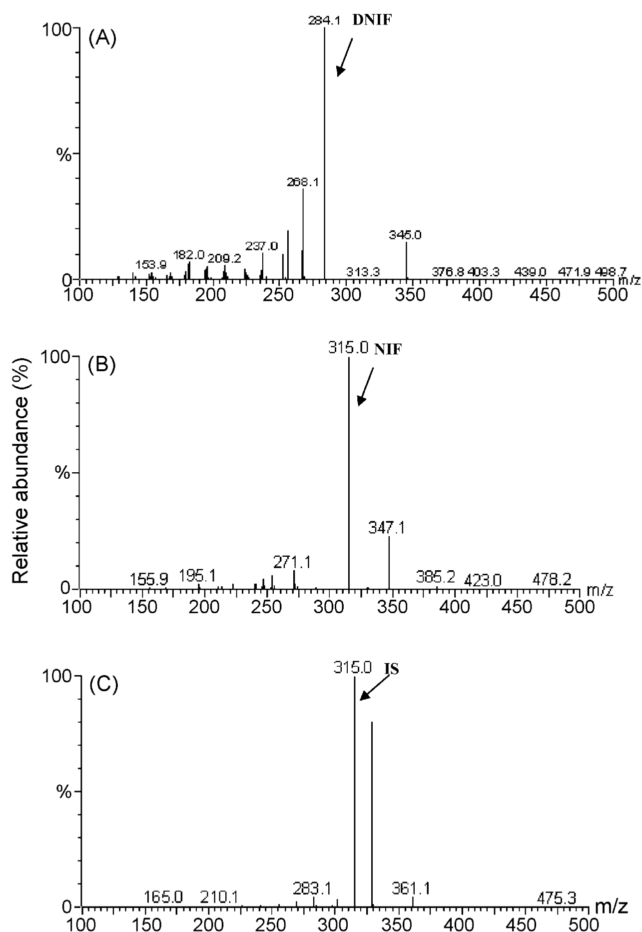

Fig. 2. Representative full-scan production spectra of the protonated molecules of nifedipine (NIF, A), dehydronifedipine (DNIF, B), and nitrendipine (IS, C).

since they did not significantly affect the collision behavior of the analytes. The Analyser parameters were kept in the recommended SRM mode, and the Entrance/Exit were modulated to −1 and +1, respectively. Therefore, the multiple reaction monitoring (MRM) transition of *m/z* 347.1 → 315. 0 for NIF, *m/z* 345.0 → 284.1 for DNIF and *m/z* 361.1 → 315.0 for IS were selected to obtain a maximum sensitivity. The corresponding full-scan ESI$^+$-MS/MS spectra for these target compounds are shown in Fig. 2. NIF and IS (nitrendipine) share a major product ion with an *m/z* of 315.

NIF is highly bound to plasma proteins with a binding percentage of 92–98% [46]. This necessitates the development of the extraction procedure to effectively recover the drug from plasma to exclude or minimize the matrix interferences. The extraction efficiency of different solvent including acetonitrile, ethyl-acetate, toluene, *n*-pentane-dichloromethane (7:3, v/v), *n*-hexane-dichloromethane (7:3, v/v) and ether-*n*-hexane (3:1, v/v) were compared during our method development. Ethyl-acetate, toluene, and *n*-pentane-dichloromethane (7:3, v/v) gave an extraction recovery of 45%, 49% and 51%, respectively, for NIF and DNIF at 10 ng/mL. These three organic solvents also generated a marked decrease (26–35%) in spectral response of NIF and DNIF. Both acetonitrile and ethyl-acetate gave a 42–49% and 51–65% of extraction efficiency for NIF and DNIF, respectively, yet *n*-pentane-dichloromethane and toluene showed an acceptable extraction for NIF (75%) but only moderate extraction efficiency (∼40%) for DNIF and these solvents also increased the matrix effect and caused a marked decrease (>35%) in mass spectral response to the two analytes. The best results were obtained using ether-*n*-hexane (3:1, v/v) as the extraction solvent. Ether-*n*-hexane (3:1, v/v) also showed a moderate matrix effect and decreased the mass spectral response to the analytes by approximately 10–20%, but to a lesser extent compared to other solvents; and the ratio of spectral response of analyte over IS was consistent throughput the analytical process. Thus, ether-*n*-hexane (3:1, v/v) was chosen as the extraction solvent in the study.

Various combinations of methanol and water with varying contents of each component were investigated and compared to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. An acidic modifier (formic acid) in the mobile phase improved peak shape for NIF and DNIF but the peak for IS was slightly skewed, whereas an increase in the water content broadened the peaks of NIF and DNIF. The differential response of NIF and IS peak shape to mobile phase adjustment is probably due to the difference in their LogP values (IS versus NIF: 2.756 versus 2.343). A mobile phase consisting of methanol/water (containing 0.1% formic acid) was finally chosen and the ratio of 80:20 (v/v) was optimal.

The selected transitions are analyte-specific and different from one another (Table 1), and the MS/MS detection of NIF and DNIF in the SRM mode was demonstrated to be specific. Under these conditions, a complete chromatographic separation was not needed, and a mobile phase with a strong polarity (methanol/water, 80:20, v/v) could be used to considerably decrease the total run time. Each chromatographic run was completed within approximately 2.5 min. Fig. 3 illustrates the typical SRM chromatograms of NIF, DNIF and IS.

3.2. Method validation

3.2.1. Selectivity

The LC/MS/MS method demonstrated high specificity because only ions derived from the analytes of interest were monitored. The selectivity toward endogenous plasma matrix was tested in six different batches of human plasma samples by analyzing blanks and spiked samples at LLOQ levels. Endogenous peaks at the retention times of the analytes were not observed for any of the plasma samples evaluated. This indicated no significant interference of the analytes and the IS from the plasma at the expected retention times. Representative chromatograms of blank human plasma and the plasma with added NIF at LLOQ levels are shown in Fig. 3. The retention times for NIF, DNIF and IS were 1.93, 1.84 and 1.95 min, respectively. The method had a significantly shorter total running time (2.5 min) for simultaneous determination of NIF and DNIF compared with those reported in the literature [19,28,30,35,38,39].

3.2.2. Matrix effects

A matrix effect (ME) occurs when a biological sample contains a component that does not give a signal in the SRM channel

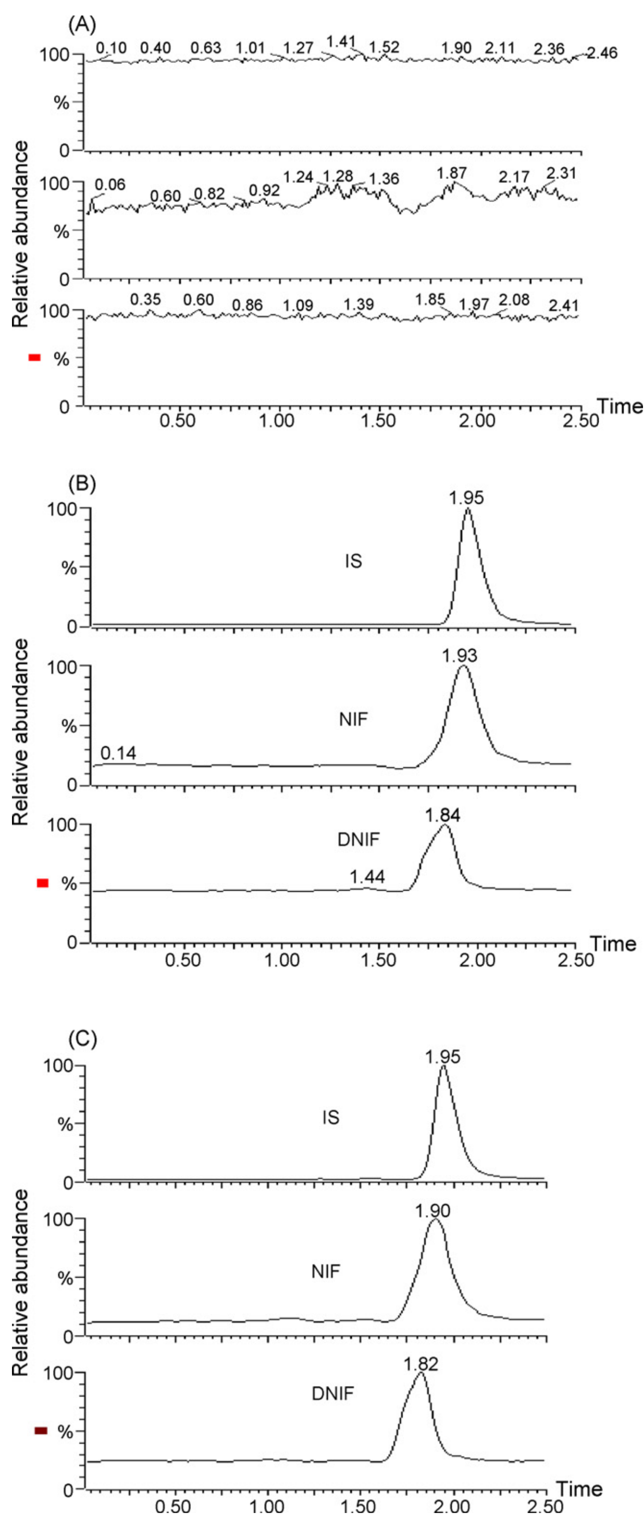

Fig. 3. Representative selected reaction monitoring (SRM) chromatograms of nifedipine (NIF), dehydronifedipine (DNIF) and nitrendipine (IS) in human plasma. (A) a blank plasma sample; (B) a plasma sample with added NIF and DNIF at an LLOQ level (0.5 ng/mL); and (C) a plasma sample from a volunteer 10 h after an oral administration of 10 mg nifedipine tablet.

used for the target analyte but co-elutes with the analyte and affects the spectral response of the analyte [52]. The presence of an ME can decrease or increase the response of the analyte and thus affect the sensitivity of a developed method. Therefore, an assessment of the ME and a minimization of ME are critical for the reliable evaluation of newly developed LC/MS/MS methods. The assessment of the relative ME was made by a direct comparison of the analyte peak area values between different lots (sources) of plasma. The peak area ratios (individual/combined) ranging from 0.95–1.08 showed no co-elution effect and these three co-eluted compounds in the plasma did not cause significant mutual enhancement or suppression of the response. The variability in the values, expressed as RSDs (%), is a measure of the relative ME for the target analyte. The variability was acceptable with RSD values < 8.0% at different concentrations of NIF and DMIF in five different lots of human plasma, indicating that the relative ME for the analyte was minimal in this study.

For the absolute ME, a peak-area comparison showed that there was a 15–22% decrease of the area of these compounds in the post-extraction spiked plasma samples, suggesting a matrix effect on the ionization of these compounds. The result of ME at QC concentrations of NIF and DNIF in five different batches of human plasma shows that there was an ME as indicated by values of <100% (range from 78.1% ∼ 85.2%) in the area of the analyte in spiked plasma samples post-extraction. This indicated that an ionization suppression for NIF and DNIF under the present chromatographic and extraction conditions when an ESI interface was employed. Notably, the ionization suppression for the analytes observed was similar and kept consistent over the QC concentration ranges of the analyte (2 ∼ 50.0 ng/mL) without showing any analyte concentration-dependence as well as for different batches of human plasma. Moreover, such ionization did not affect the slopes and linearity of the established calibration curves throughout the analytical period. Although an ionization suppression was indeed observed for these compounds, a significant change in the ionization response of each analyte was not found, and the ratio of analyte over IS was consistent throughout the analytical period. Thus, despite the matrix effects observed, the present analytical method was considered reliable with a high sensitivity for NIF and DNIF determination in human plasma.

In addition, the “cross-talk” between MS/MS channels used for monitoring analytes was assessed by separately injecting NIF, DNIF and IS (all at 10 ng/mL) and monitoring the response in the other two channels. No “cross-talk” between channels was observed.

3.2.3. Linearity and lower limit of quantification

The slope, the intercept and the correlation coefficient (r) for each standard curve from each analytical run were determined automatically by the Waters Masslynx V 4.0 software program. Table 2 shows the mean slope, intercept and correlation coefficient values for both NIF and DNIF. Typical calibration curves for NIF and DNIF are shown in Fig. 4. The mean squared correlation coefficients (r^2) for the daily calibration curves were all ≥0.995 ($n = 5$) for both NIF and DNIF and the within- and between-run CVs of the response factors for each concentration assayed were <10%. The mean y intercepts were 0.096 and 0.4013 ($n = 5$) for NIF and DNIF, respectively. These values are acceptable for routine analytical methods in clinical settings

Table 2
Slope, intercept and correlation coefficient (r) for the calibration curves for nifedipine and dehydronifedipine ($n=5$)

Compound	Concentration range (ng/mL)	Slope[a]		Intercept[a]		r^2
		Mean ± SD	CV[b] (%)	Mean ± SD	CV[b] (%)	
NIF	0.5–100	0.0542 ± 0.0018	3.5	0.0965 ± 0.0050	5.2	≥ 0.996
DNIF	0.5–100	0.0394 ± 0.0020	5.1	0.4013 ± 0.0382	9.5	≥ 0.995

NIF: nifedipine; DNIF: dehydronifedipine; IS: nitrendipine.
[a] Slope and intercept were determined automatically by Waters Masslynx software.
[b] CV: coefficient of variation = SD/Mean × 100.

although improvements can be made by optimizing further the method. For each point on the calibration curves for the two analytes, the concentrations back-calculated from the equation of the regression analysis were within acceptable limits for accuracy and precision of ≤10%. Both analytes showed excellent linearity over 0.5–100 ng/mL for NIF and DNIF (Table 2). The lowest concentration on the calibration curve of NIF and DNIF was 0.5 ng/mL. The analytes' response at these concentration levels was >5 times of the baseline noise. The precision and accuracy at these concentration levels were acceptable, with <11.5% of the CVs and <10.0% of the relative errors. Thus, the lowest concentration on the calibration curve was accepted as the LLOQ. The limit of detection (LOD) was calculated to be 0.05 ng/mL for NIF and 0.1 ng/mL for DNIF, respectively.

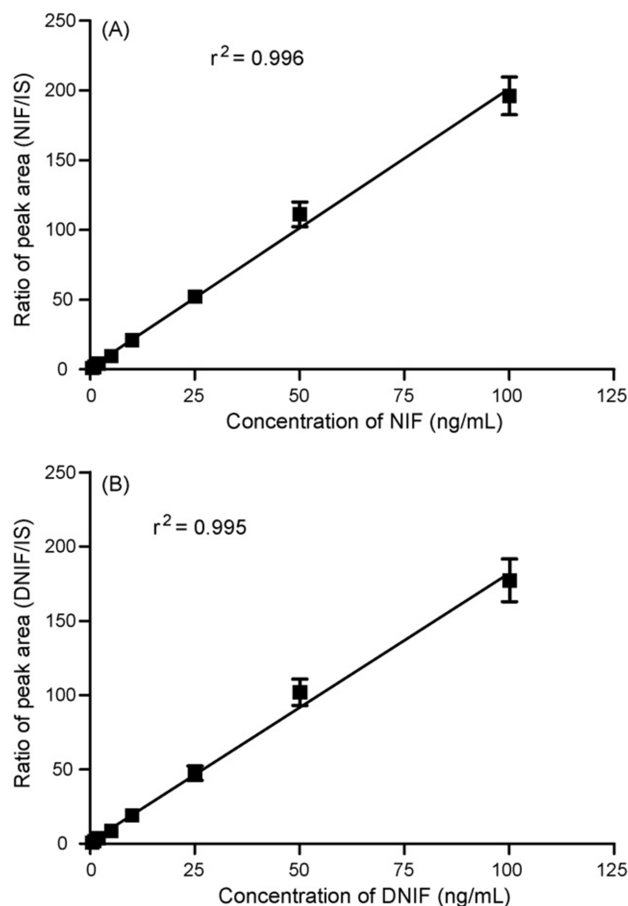

Fig. 4. Representative calibration curves for nifedipine (NIF, plot A) and dehydronifedipine (DNIF, plot B).

3.2.4. Accuracy and precision

The intra- and inter-day precision and accuracy data for NIF and DNIF are summarized in Table 3. All values of accuracy and precision were within recommended limits. Intraday precision ranged between 3.1% and 9.7%, and the inter-day precision was between 3.5% and 11.4%. The mean intra-day errors were between −6.9 and 2.1%, and the mean inter-day errors were between −6.1 and 4.9%.

3.2.5. Recovery and stability

Table 4 shows the recovery (extraction efficiency) of NIF and DNIF from human plasma following ether- n-hexane (3:1, v/v) extraction. The recovery of NIF and DNIF from human plasma ranged over 81.3–89.1% and 71.6–80.4%, respectively; and were similar at all analyte concentrations without significant concentration dependence. This indicated that the extraction efficiency for NIF and DNIF was acceptable.

The stability of NIF and DNIF in human plasma under different storage conditions is presented in Table 5. There was no significant degradation under these conditions described in this study, since their concentrations deviated by no more than 10.5% relative to the reference nominal concentrations. No degradation products were detected under the selected MS conditions. Both NIF and DNIF in human plasma can therefore be stored at room temperature for 4 h, 25 days at −30 °C and after three

Table 3
Intra- and inter-day precision and accuracy data for assays of nifedipine and dehydronifedipine in human plasma ($n=5$)

Compound	Nominal concentration (ng/mL)	Precision		Accuracy (mean relative error, %)
		Mean ± SD	RSD (%)	
Intra-day				
NIF	2	2.1 ± 0.2	7.4	2.1
	10	9.4 ± 0.3	3.1	−6.2
	50	50.9 ± 2.1	4.2	1.8
DNIF	2	2.0 ± 0.2	9.7	1.4
	10	9.6 ± 0.7	7.5	−3.6
	50	46.6 ± 2.0	4.2	−6.9
Inter-day				
NIF	2	1.9 ± 0.1	6.1	−3.6
	10	9.8 ± 0.5	5.2	−1.5
	50	52.4 ± 1.8	3.5	4.9
DNIF	2	2.0 ± 0.2	11.4	0.5
	10	9.4 ± 0.5	4.8	−6.1
	50	47.2 ± 2.6	5.5	−5.7

NIF: nifedipine; DNIF: dehydronifedipine; RSD: relative standard deviation.

Table 4
The recovery (extraction efficiency) for nifedipine (NIF) and dehydronifedipine (DNIF) in human plasma ($n = 5$)

Compound	Nominal concentration (ng/mL)	Recovery[a]	
		Mean ± SD (%)	RSD (%)
NIF	2	89.1 ± 5.0	5.6
	10	81.3 ± 6.1	7.5
	50	82.5 ± 4.9	6.0
DNIF	2	76.3 ± 6.9	9.0
	10	80.4 ± 3.2	4.0
	50	71.6 ± 3.7	5.2

NIF: nifedipine; DNIF: dehydronifedipine; RSD: relative standard deviation.
[a] The recovery (extraction efficiency) of analytes from human plasma after the extraction procedure was determined by comparing the areas of extracted analytes with that of the non-extracted pure standards that represent 100% recovery.

Table 5
Stability of nifedipine (NIF) and dehydronifedipine (DNIF) in human plasma under various storage conditions ($n = 5$)

Storage condition	Compound	Nominal concentration (ng/mL)	Calculated concentration (ng/mL)	
			Mean ± SD (%)	Relative error (%)
−30 °C/25 days				
	NIF	2	2.1 ± 0.1	3.6
		10	9.9 ± 0.6	−0.4
		50	54.2 ± 2.0	8.4
	DNIF	2	2.0 ± 0.1	−0.7
		10	9.7 ± 0.5	−2.4
		50	47.7 ± 2.1	−4.6
−30 °C/3 freeze–thaw cycles				
	NIF	2	2.0 ± 0.1	0.9
		10	9.9 ± 0.6	−1.5
		50	52.9 ± 1.4	5.9
	DNIF	2	2.0 ± 0.1	−0.6
		10	9.6 ± 0.2	−3.8
		50	48.2 ± 1.1	−3.5
4 h at room temperature				
	NIF	2	2.2 ± 0.1	8.4
		10	9.8 ± 0.9	−2.2
		50	55.3 ± 2.4	10.5
	DNIF	2	2.1 ± 0.1	7.1
		10	10.8 ± 0.7	7.6
		50	47.3 ± 3.0	−5.4
12 h at room temperature (extracted samples)				
	NIF	2	1.9 ± 0.1	−5.2
		10	10.6 ± 0.7	5.7
		50	51.3 ± 3.4	2.7
	DNIF	2	2.1 ± 0.1	4.8
		10	10.7 ± 0.8	6.8
		50	46.1 ± 4.6	−7.8

NIF: nifedipine; DNIF: dehydronifedipine.
[a]Relative error: (overall mean assayed concentration − added concentration)/added concentration × 100.

freeze–thaw cycles. Analysis of the QC samples following ether-*n*-hexane (3:1, v/v) extraction procedure showed no significant degradation after 12 h at room temperature. These results indicated that both NIF and DNIF were stable under routine laboratory conditions and no specific procedure (e.g., acidification or addition or organic solvents) was needed to stabilize the compounds for daily clinical drug monitoring and pharmacogenetic study.

3.3. Application to pharmacokinetic study in humans

Before and after the volunteers were treated with St. John's wort at 900 mg/day for 14 days, this method was applied to the analysis of human plasma from healthy subjects after a single oral dose of 10 mg nifedipine. There were two volunteers suffering from cold with administration of antibiotics during the study and they dropped off the experiment. The mean plasma concentration–time curves before and after administration of St. John's wort for NIF and DNIF among the ten volunteers were shown in Fig. 5, and the calculated pharmacokinetic parameters of NIF and DNIF are presented in Table 6. Before administration of St. John's wort, following oral administration of nifedipine at 10 mg, the mean $C_{\max}$ and AUC_{0-24h} for NIF were 50.4 ± 11.9 ng/mL, 303.4 ± 123.4 ng h/mL, and the

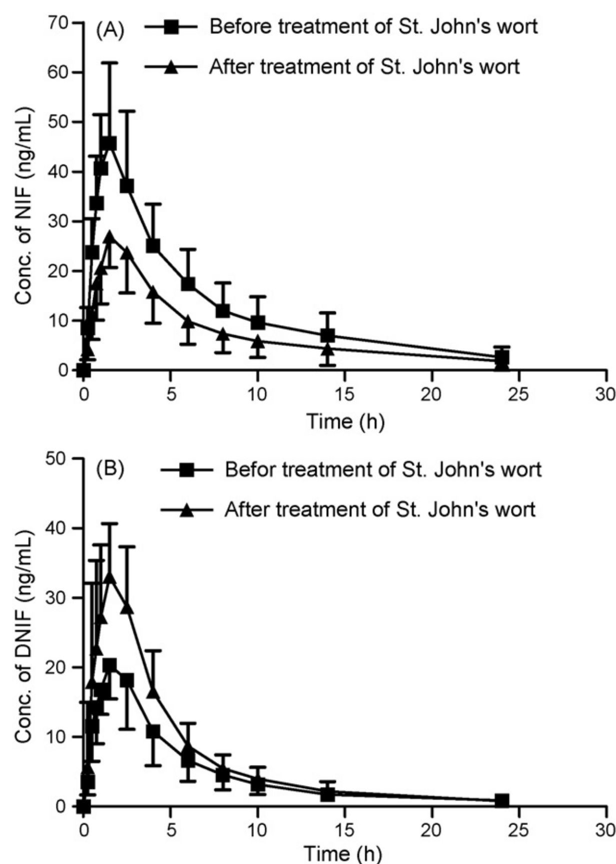

Fig. 5. The mean plasma concentration-time profiles of nifedipine (NIF) and dehydronifedipine (DNIF) before and after oral administration of 14 days of St John's wort in 10 healthy volunteers following oral administration of 10 mg nifedipine tablet.

Table 6
Pharmacokinetic parameters of nifedipine and dehydronifedipine before and after taking oral St John's wort preparation at 900 mg/day for 14 days in healthy Chinese subjects (mean ± SD, $n = 10$)

Parameter	NIF		DNIF	
	Before St. John's wort treatment	After St. John's wort treatment	Before St. John's wort treatment	After St. John's wort treatment
C_{max} (ng/mL)	50.4 ± 11.9	31.0 ± 4.7*	21.3 ± 4.9	30.7 ± 7.6*
T_{max} (h)	1.6 ± 0.6	1.5 ± 0.6	1.6 ± 0.6	1.5 ± 0.4
$t_{1/2\beta}$ (h)	5.5 ± 1.4	5.1 ± 0.6	4.9 ± 1.5	4.7 ± 0.7
AUC_{0-24h} (ng h/mL)	303.4 ± 123.4	167.3 ± 59.1*	103.2 ± 34.4	129.7 ± 59.4*
$AUC_{0-\infty}$ (ng h/mL)	320.1 ± 130.3	175.4 ± 61.9*	108.8 ± 36.9	139.3 ± 59.3*

Data are the mean ± SD. Abbreviation: AUC_{0-24h}, area under the plasma concentration-time curve from time zero to the last measurable time point (24 h); $AUC_{0-\infty}$, total area under the plasma concentration-time curve from time zero to infinity; C_{max}, maximum plasma concentration; DNIF, dehydronifedipine; NIF, nifedipine; T_{max}, maximum time to reach C_{max}; $t_{1/2\beta}$, elimination half-life.
* $P < 0.05$, by unpaired Student's t-test.

mean C_{max} and AUC_{0-24h} for DNIF were 21.3 ± 4.9 ng/mL, and 103.2 ± 34.4 ng h/mL, respectively. The calculated pharmacokinetic parameters of NIF and DNIF in Chinese were comparable to those previously reported in Caucasian, Japanese and Mexican in the literature [5,26,42]. The AUC_{0-24h}, $AUC_{0-\infty}$ and C_{max} of NIF significantly decreased ($P < 0.05$), while the AUC_{0-24h}, $AUC_{0-\infty}$ and C_{max} of DNIF significantly increased after St. John's wort administration for 14 days. After administration of St. John's wort for 14 days, the mean C_{max} and AUC_{0-24h} for NIF were 31.0 ± 4.7 ng/mL, 167.3 ± 59.1 ng h/mL; and the mean C_{max} and AUC_{0-24h} for DNIF were 30.7 ± 7.6 ng/mL and 129.7 ± 59.4 ng h/mL, respectively. However, there was no significant difference in T_{max} for both NIF and DNIF before and after the treatment of St. John's wort. The mean $t_{1/2\beta}$ values of NIF and DNIF after St. John's wort treatment were shorter than those before St. John's wort treatment, but the difference did not achieve statistic significance. The pharmacokinetic parameters for NIF obtained from the healthy Chinese volunteers before administration of St John's wort showed significant inter-individual variation with C_{max} between 34.3 and 73.9 ng/mL. However, the variation became less remarkable with C_{max} varying between 22.9 and 38.5 ng/mL after the treatment of St. John's wort. These results indicated that St. John's wort induced CYP3A4-catalyzed metabolism of NIF to form DNIF in humans.

4. Conclusions

In this study, we reported on a newly developed LC/MS/MS method for the simultaneous determination of NIF and DNIF in human plasma. The sample pretreatment was a single-step liquid–liquid extraction using ether- n-hexane (3:1, v/v). The analytes, NIF, DNIF and the IS were subjected to LC/MS/MS analysis using an ESI technique with satisfactory mass spectral response. A detailed validation following FDA guideline indicated that the developed method had a high sensitivity, reliability, specificity and excellent efficiency with a total running time of 2.5 min per sample, which is suitable for high-throughput pharmacokinetic and pharmacogenetic studies. The method was successfully applied to determine NIF and DNIF plasma concentrations in a pharmacokinetic herb–drug interaction study in human volunteers. The CYP3A4-catalyzed metabolism of NIF was greatly induced by St. John's wort in Chinese volunteers.

Acknowledgements

The authors appreciate the financial support by the National Nature Science Fund of China (No. 30572231), Singapore Cancer Syndicate (Grant SCS PS0023), and the Queensland University of Technology (Brisbane, Queensland, Australia).

References

[1] J.L. Blackshear, C. Orlandi, G.H. Williams, N.K. Hollenberg, J. Cardiovasc. Pharmacol. 8 (1986) 37.
[2] S.R. Hamann, M.T. Piascik, R.G. McAllister Jr., Biopharm. Drug Dispos. 7 (1986) 1.
[3] C.H. Kleinbloesem, J. Van Harten, P. Van Brummelen, D.D. Breimer, J. Chromatogr. 308 (1984) 209.
[4] A. Balogh, S. Gessinger, U. Svarovsky, M. Hippius, U. Mellinger, G. Klinger, A. Hoffmann, M. Oettel, Eur. J. Clin. Pharmacol. 54 (1998) 729.
[5] G. Castaneda-Hernandez, C. Hoyo-Vadillo, J.A. Palma-Aguirre, F.J. Flores-Murrieta, J. Clin. Pharmacol. 33 (1993) 140.
[6] T. Funaki, P.A. Soons, F.P. Guengerich, D.D. Breimer, Biochem. Pharmacol. 38 (1989) 4213.
[7] P.B. Watkins, Pharmacogenetics 4 (1994) 171.
[8] T. Shimada, H. Yamazaki, M. Mimura, Y. Inui, F.P. Guengerich, J. Pharmacol. Exp. Ther. 270 (1994) 414.
[9] S. Rendic, F.J. Di Carlo, Drug Metab. Rev. 29 (1997) 413.
[10] O. Burk, I. Koch, J. Raucy, E. Hustert, M. Eichelbaum, J. Brockmoller, U.M. Zanger, L. Wojnowski, J. Biol. Chem. 279 (2004) 38379.
[11] D.K. Turgeon, A.B. Leichtman, D.S. Blake, R.L. Schmouder, K.S. Lown, T.M. Annesley, P.B. Watkins, Transplantation 57 (1994) 1736.
[12] S.E. Ball, J. Scatina, J. Kao, G.M. Ferron, R. Fruncillo, P. Mayer, I. Weinryb, M. Guida, P.J. Hopkins, N. Warner, J. Hall, Clin. Pharmacol. Ther. 66 (1999) 288.
[13] W.P. Lemahieu, B.D. Maes, Y. Ghoos, P. Rutgeerts, K. Verbeke, Y. Vanrenterghem, Am. J. Physiol. Gastrointest. Liver Physiol. 285 (2003) G470.
[14] A. Galetin, J.B. Houston, J. Pharmacol. Exp. Ther. 318 (2006) 1220.
[15] K.C. Patki, L.L. Von Moltke, D.J. Greenblatt, Drug Metab. Dispos. 31 (2003) 938.
[16] D.M. Stresser, A.P. Blanchard, S.D. Turner, J.C. Erve, A.A. Dandeneau, V.P. Miller, C.L. Crespi, Drug Metab. Dispos. 28 (2000) 1440.
[17] V. Ozdemir, W. Kalowa, B.K. Tang, A.D. Paterson, S.E. Walker, L. Endrenyi, A.D. Kashuba, Pharmacogenetics 10 (2000) 373.
[18] P.A. Soons, J.H. Schellens, M.C. Roosemalen, D.D. Breimer, J. Pharm. Biomed. Anal. 9 (1991) 475.

[19] B. Streel, C. Zimmer, R. Sibenaler, A. Ceccato, J. Chromatogr. B Biomed. Sci. Appl. 720 (1998) 119.
[20] N. Ozaltin, C. Yardimci, I. Suslu, J. Pharm. Biomed. Anal. 30 (2002) 573.
[21] D. Kowalczuk, M.B. Wawrzycka, A. Haratym Maj, J. AOAC Int. 89 (2006) 71.
[22] D. Zendelovska, S. Simeska, O. Sibinovska, E. Kostova, K. Milosevska, K. Jakovski, E. Jovanovska, I. Kikerkov, J. Trojacanec, D. Zafirov, J. Chromatogr. B Analyt. Technol. Biomed Life Sci. 839 (2006) 85.
[23] P. Thongnopnua, K. Viwatwongsa, J. Pharm. Biomed. Anal. 12 (1994) 119.
[24] A. Jankowski, H. Lamparczyk, J. Chromatogr. A 668 (1994) 469.
[25] M.E. Sheridan, G.S. Clarke, M.L. Robinson, J. Pharm. Biomed. Anal. 7 (1989) 519.
[26] G. Pabst, D. Lutz, K.H. Molz, W. Dahmen, H. Jaeger, Arzneimittelforschung 36 (1986) 256.
[27] C. Le Guellec, H. Bun, M. Giocanti, A. Durand, Biomed. Chromatogr. 6 (1992) 20.
[28] J. Dokladalova, J.A. Tykal, S.J. Coco, P.E. Durkee, G.T. Quercia, J.J. Korst, J. Chromatogr. 231 (1982) 451.
[29] P. Jakobsen, O. Lederballe Pedersen, E. Mikkelsen, J. Chromatogr. 162 (1979) 81.
[30] W. Snedden, P.G. Fernandez, C. Nath, Can. J. Physiol. Pharmacol. 64 (1986) 290.
[31] R. Testa, E. Dolfini, C. Reschiotto, C. Secchi, P.A. Biondi, Farmaco [Prat] 34 (1979) 463.
[32] M.T. Rosseel, M.G. Bogaert, J. Chromatogr. 279 (1983) 675.
[33] K.S. Patrick, E.J. Jarvi, A.B. Straughn, M.C. Meyer, J. Chromatogr. 495 (1989) 123.
[34] J. Martens, P. Banditt, F.P. Meyer, J. Chromatogr. B Biomed. Appl. 660 (1994) 297.
[35] M.C. Roosemalen, P.A. Soons, T. Funaki, D.D. Breimer, J. Chromatogr. 565 (1991) 516.
[36] J.S. Grundy, R. Kherani, R.T. Foster, J. Chromatogr. B Biomed. Appl. 654 (1994) 146.
[37] V. Nitsche, H. Schutz, A. Eichinger, J. Chromatogr. 420 (1987) 207.
[38] D.G. Waller, A.G. Renwick, B.S. Gruchy, C.F. George, Br. J. Clin. Pharmacol. 18 (1984) 951.
[39] B.J. Schmid, H.E. Perry, J.R. Idle, J. Chromatogr. 425 (1988) 107.
[40] N.D. Huebert, M. Spedding, K.D. Haegele, J. Chromatogr. 353 (1986) 175.
[41] V. Horvath, A. Hrabeczy-Pall, Z. Niegreisz, E. Kocsi, G. Horvai, L. Godorhazy, A. Tolokan, I. Klebovich, K. Balogh-Nemes, J. Chromatogr. B Biomed. Appl. 686 (1996) 211.
[42] C.H. Kleinbloesem, P. van Brummelen, J.A. van de Linde, P.J. Voogd, D.D. Breimer, Clin. Pharmacol. Ther. 35 (1984) 742.
[43] M. Telting-Diaz, M.T. Kelly, C. Hua, M.R. Smyth, J. Pharm. Biomed. Anal. 9 (1991) 889.
[44] S. Zhou, E. Chan, S.Q. Pan, M. Huang, E.J. Lee, J. Psychopharmacol. 18 (2004) 262.
[45] Z. Hu, X. Yang, P.C. Ho, S.Y. Chan, P.W. Heng, E. Chan, W. Duan, H.L. Koh, S. Zhou, Drugs 65 (2005) 1239.
[46] M. Eichelbaum, H. Echizen, J. Cardiovasc. Pharmacol. 6 (Suppl 7) (1984) S963.
[47] FDA, in, May 2001.
[48] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[49] H.C. Bi, G.P. Zhong, S. Zhou, X. Chen, M. Huang, Rapid Commun. Mass Spectrom. 19 (2005) 2911.
[50] D.L. Whitten, S.P. Myers, J.A. Hawrelak, H. Wohlmuth, Br. J. Clin. Pharmacol. 62 (2006) 512.
[51] C.R. Mallet, Z. Lu, J.R. Mazzeo, Rapid Commun. Mass Spectrom. 18 (2004) 49.
[52] M. Jemal, A. Schuster, D.B. Whigan, Rapid Commun. Mass Spectrom. 17 (2003) 1723.