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Liquid chromatography–mass spectrometry method for the determination of nicardipine in human plasma

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Abstract

A simple and sensitive liquid chromatography–mass spectrometry method is described for the determination of nicardipine in human plasma. Chromatographic separation of the analyte was achieved on a C_{18} column using a mobile phase of methanol, water and formic acid (320:180:0.4, v/v/v). Selected ion monitoring (SIM) in positive mode was used for analyte quantification at *m/z* 480.2 for nicardipine and *m/z* 256.4 for diphenhydramine. The run time was less than 5 min. The linearity over the concentration range of 0.05–20.0 ng/ml for nicardipine was obtained and the lower limit of quantification was 0.05 ng/ml. For each level of QC samples, inter-day and intra-day precisions (R.S.D.) were ≤9.3 and 11.1%, respectively, and accuracy (RE) was ±4.9%. The present LC–MS method was successfully applied in the pharmacokinetic studies of nicardipine hydrochloride delayed-release tablets in two formulations after oral administration to healthy volunteers.

Keywords: Nicardipine; Liquid chromatography–mass spectrometry; Human plasma

1. Introduction

Nicardipine hydrochloride is a second-generation dihydropyridine calcium antagonist that selectively inhibits vascular smooth muscle contraction. It is used as a racemate for the treatment of hypertension, particularly in patients with coexistent coronary artery, cerebrovascular or peripheral vascular disease [1]. The structure of nicardipine hydrochloride is shown in Fig. 1.

For the pharmacokinetic study of nicardipine hydrochloride delayed-release tablets in healthy volunteers, a simple analytical method with high sensitivity was required and liquid chromatography (LC) was preferred. A recent survey revealed that LC methods available for the determination of nicardipine in biological samples were performed with ultraviolet detection [2–8], amperometric detection [9,10] or tandem mass spectrometric detection [11]. Most of the LC methods reported, had a limit of quantification higher than 5 ng/ml, which was not sensitive enough for the pharmacokinetic studies of nicardipine hydrochloride delayed-release tablets in healthy volunteers because the levels of the analyte in human plasma in the initial hours were anticipated to be below or near the limit of quantification of the LC-UV or LC-amperometric detection.

Two formulations of delayed-release tablets were designed and formulated to achieve a delayed-releasing profile in vivo, i.e. significant drug release only occurred at a determined time after oral administration to volunteers. The two candidate formulations of nicardipine hydrochloride delayed-release tablets should be differentiated by pharmacokinetic studies to make a choice between them. Therefore, the analytical method was required to be sensitive enough to determine the low levels of the analyte in plasma in initial hours, which provided the key information for the formulation choice. The formulation displaying a 3 h delayed-release profile after oral administration with lower levels of analyte in the first 3 h and less individual variations was preferred. Therefore, a more sensitive and selective analytical method was needed and liquid chromatography–mass spectrometry (LC–MS) was the method of choice. Compared to UV and amperometric detection, the LC–MS combination provides enhanced sensitivity and selectivity for the analytes in biological samples and already stars in the field of drug development and testing.

This paper describes a liquid chromatography-single quadrupole mass spectrometric method (LC–MS) in positive selected ion monitoring (SIM) mode for the determination of

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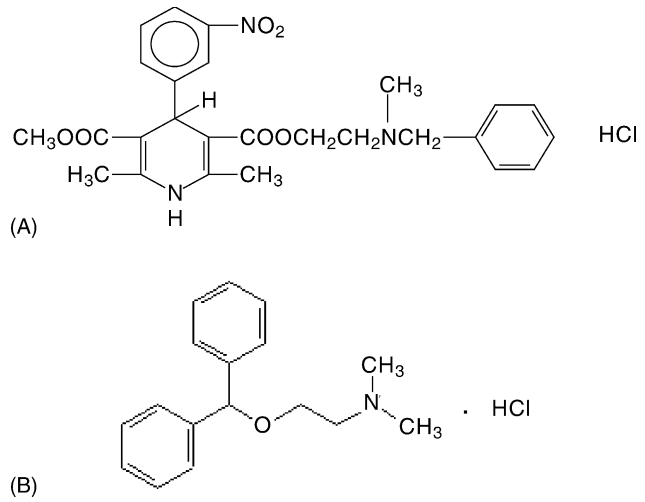

Fig. 1. Chemical structures of nicardipine hydrochloride (A) and diphenhydramine hydrochloride (B).

nicardipine in human plasma. The present method offers a simple sample preparation and higher sensitivity with a lower limit of quantification of 0.05 ng/ml. The described method was validated in terms of selectivity, linearity, lower limit of quantification, accuracy, precision, freeze–thaw cycles and stability of analyte at ambient temperature, and successfully applied in the pharmacokinetic studies of two nicardipine hydrochloride delayed-release tablet formulations in healthy volunteers.

2. Experimental

2.1. Chemicals and materials

Nicardipine hydrochloride reference standard (100.3% purity) was purchased from Nanjing Pharmaceutical Factory. Diphenhydramine hydrochloride (99.0% purity) was a gift from Shenyang Pharmaceutical University. Nicardipine hydrochloride delayed-release tablets (label claim: 20 mg) in formulations 1 and 2 were from Shenyang Pharmtech Institute of Pharmaceuticals (Shenyang, China). Methanol of HPLC grade was purchased from Tianjin Concord Tech Reagent Company (Tianjin, China). All the other reagents were of analytical grade.

2.2. Instrument and LC–MS conditions

HP 1100 series LC/MSD G1946D (Agilent, USA) was used in the present work. Chromatographic separation was performed on a Diamonsil^{TM} C_{18} column (250 mm × 4.6 mm I.D., 5 μm, Dikma, China) at ambient temperature. The mobile phase consisting of a mixture of methanol, water and formic acid (320:180:0.4, v/v/v) was delivered at a flow rate of 0.6 ml/min. The injection volume was 20 μl.

The mass spectrometer was operated in the positive electrospray ionization (ESI) mode. The optimized ionization conditions were: nitrogen flow rate, 8.0 ml/min; gas temperature, 325 °C; nitrogen pressure, 30 psig; capillary current, 24 nA; collision induced dissociation (CID), 170 V for nicardipine and 150 V for diphenhydramine. Selected ion monitoring (SIM) mode was used for the quantification at *m*/*z* 480.2 for nicardipine and *m*/*z* 256.4 for diphenhydramine. The retention times were 4.0 min for nicardipine and 3.6 min for diphenhydramine.

2.3. Preparation of calibration standards and quality control samples

Stock solutions (1 mg/ml) of nicardipine hydrochloride and diphenhydramine hydrochloride (internal standard) were individually prepared in methanol. The stock solution of nicardipine hydrochloride was further diluted with methanol to give a series of standard solutions with concentration of 0.5, 1.0, 5.0, 10.0, 50.0, 100.0 and 200.0 ng/ml. The stock solution of diphenhydramine hydrochloride was further diluted with methanol to give a concentration of 400 ng/ml.

Calibration standards of nicardipine hydrochloride (0.05, 0.1, 0.5, 1.0, 5.0, 10.0 and 20.0 ng/ml) were prepared by spiking appropriate amount of the standard solutions of nicardipine hydrochloride in blank plasma. Quality control (QC) samples were prepared at concentrations of 0.1, 5.0 and 20.0 ng/ml of nicardipine hydrochloride using the pooled plasma. The spiked samples were then treated following the sample preparation procedure as indicated in Section 2.4.

2.4. Sample preparation

One millilitre aliquot of each plasma sample was transferred to a 5-ml centrifuge tube. Hundred microlitres of diphenhydramine hydrochloride in methanol (400 ng/ml), 100 μl of methanol and 50 μl of sodium carbonate aqueous solution (1 mol/l) were added and shaken well. Then 2.5 ml of a *n*-hexane–dichloromethane–isopropyl alcohol mixture (20:10:1, v/v) were added and the contents were mixed by vortexing for 1 min and centrifuged for 10 min to separate the phases. The supernatant was separated and evaporated under a stream of nitrogen at room temperature. The residue was reconstituted with 100 μl of mobile phase and 20 μl was injected onto the LC column.

2.5. Method validation

Validation runs were conducted on three separate days. Each validation run consisted of a set of calibration standards at seven concentrations over the concentration range (each in triplicate) and QC samples at three concentrations ($n = 6$ at each concentration). The results from QC samples in three runs were used to evaluate the accuracy and precision of the method developed. The analyte concentrations in plasma samples were determined by back-calculation of the observed peak area ratios of the analyte and internal standard from the best-fit calibration curve. During routine analysis, each analytical run included a set of calibration standards, a set of QC samples in duplicate and plasma samples to be determined.

The selectivity of the method was investigated by comparing chromatograms of blank plasma, standard plasma sample spiked with nicardipine hydrochloride (0.5 ng/ml) and diphenhydramine hydrochloride (400 ng/ml) and plasma sample after

an oral dose of nicardipine hydrochloride delayed-release tablets (40 mg). An additional test was performed to demonstrate if there was any interference from the plasma matrix. The test was made as follows: standard solutions of nicardipine hydrochloride at 5.0 and 20.0 ng/ml (in triplicate for each concentration) were added in blank human plasma after extraction and determined by the present LC–MS method. Standard solutions of nicardipine hydrochloride at 5.0 and 20.0 ng/ml were directly determined without extraction. Based on the percentage of peak area ratio of the peak area of the analyte added in plasma after extraction relative to that of the analyte without extraction, RE (%) was calculated to evaluate the accuracy of the determination without interferences from the matrix.

The linearity of each calibration curve was determined by plotting the peak area ratio (y) of the analyte to internal standard versus the nominal concentration (x) of the analyte. The calibration curves were obtained by weighted ($1/x^2$) linear regression analysis.

The extraction recoveries of nicardipine were determined at low, medium and high concentrations (0.1, 5.0 and 20.0 ng/ml) by comparing the responses from plasma samples spiked before extraction with the corresponding standard solutions without extraction.

Freeze/thaw stability of nicardipine in plasma was determined at the levels of 0.1, 5.0 and 20.0 ng/ml to measure the accuracy and precision for samples that underwent three freeze–thaw cycles. QC samples at the indicated levels were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen. These freeze–thaw samples were analyzed to see if there is any variation due to thawing of the samples.

Sample stability was determined by analyzing QC samples containing nicardipine hydrochloride of 0.1, 5.0 and 20.0 ng/ml after sample preparation and exposed to ambient temperature over a time period of 12 h.

2.6. Application of the LC–MS method

The LC–MS method was successfully applied in the pharmacokinetic studies of nicardipine hydrochloride delayed-release tablets in 16 healthy male volunteers of 21 ± 1 years in age and 66 ± 7 kg in weight. Each volunteer was judged to be in good health through medical history, physical examination and routine laboratory tests. Written informed consent was obtained from each volunteer after detailed verbal and written information on the objective and the possible risks of the study. Volunteers were excluded if they had a history of smoking or drinking or if they were taking prescription medications within 14 days prior to the start of the study. The study protocol was reviewed and approved by China Drug Evaluation Center.

The volunteers were randomly divided into two groups with eight volunteers in each group. The volunteers were fasted 5 h before the test. Two formulations (formulation 1 and formulation 2) of nicardipine hydrochloride delayed-release tablets were tested in this study. The volunteers in group 1 and group 2 were given single dose of two tablets of formulations 1 and 2 (40 mg nicardipine hydrochloride), respectively, with 250 ml of warm water. Within 10 h after oral administration of the tablets, the volunteers had a standard diet while water intake was free. Smoking and consumption of alcohol and beverages containing caffeine were not allowed during the study. Blood samples (4 ml) were obtained immediately pre-dose and 2.5, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, 14.0, 17.0 and 24.0 h post-dose, which were collected in tubes previously treated with heparin and plasma was separated by centrifugation and kept frozen at −20 °C until analysis. Plasma concentrations of nicardipine were determined by the present LC–MS method.

3. Results and discussion

3.1. Method development

Liquid–liquid extraction was used for the sample preparation. This simple procedure produced a clean chromatogram for a blank plasma sample and yielded satisfactory recoveries of the analytes from the plasma. Generally, compared with the salt form of a drug, its nonionic form can be more easily extracted by organic solvent. Taking into account the pK_a values of the analytes, the medium pH should be 2 pH units higher than the pK_a of nicardipine hydrochloride (pK_a = 8.6, [12]) and diphenhydramine hydrochloride (pK_a = 9.0, [13]) and therefore the proper medium pH should be ≥11. In this work, 50 μl of sodium carbonate aqueous solution (1 mol/l) were added in the process of sample preparation to adjust the medium to about pH 11 and thus to free the drug bases (i.e. nicardipine and diphenhydramine) from their hydrochlorides for the following extraction by organic solvent. In this study, a *n*-hexane–dichloromethane–isopropyl alcohol mixture (20:10:1, v/v/v) was used for extraction.

A Diamonsil^{TM} C_{18} column (250 mm × 4.6 mm I.D., 5 μm) was used. Other chromatographic conditions, especially the composition of mobile phase, were tested to achieve good resolution and symmetric peak shapes of analytes as well as short run time. Internal standard plays an important role in biopharmaceutical analysis and is often required to have similar physical and chemical properties with the analyte such as solubility and acid–base properties (pK_a). On the basis of the above requirements, diphenhydramine hydrochloride was found to be suitable for the present work and finally used as the internal standard. The structure of diphenhydramine hydrochloride is shown in Fig. 1. After liquid–liquid extraction, both analytes are present in drug base forms. Based on the pK_a values of the analytes, the pH value should be below 3.0 to assure the ionized status of the analytes in mobile phase. For the analysis of basic drugs by LC–MS methods, formic acid is the most widely used agent to keep the mobile phase acidic and therefore was used in the present work. It was found that a mixture of methanol, water and formic acid (320:180:0.4, v/v/v) could achieve our purpose and was finally adopted as the mobile phase for the chromatographic separation. The retention times were 4.0 min for nicardipine and 3.6 min for diphenhydramine. The run time was less than 5 min.

Positive ESI source was used. MS parameters involving capillary temperature, vaporizer temperature and flow rate were tested to obtain an optimum ionization yield of the analytes. The fragmentor energy was tested to achieve maximum response of

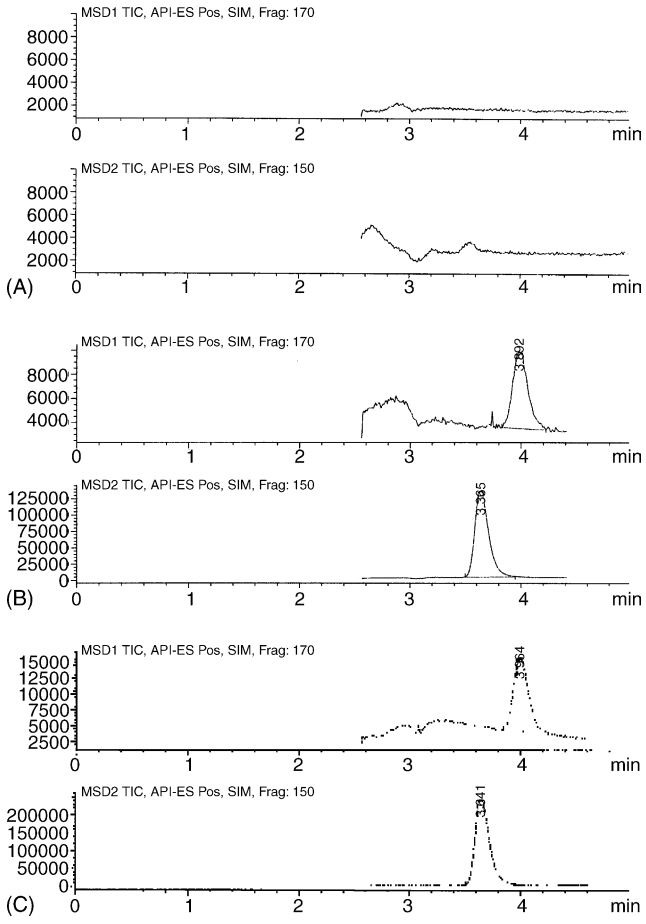

Fig. 2. Representative SIM chromatograms of: (A) blank human plasma sample; (B) blank plasma sample spiked with nicardipine hydrochloride (0.5 ng/ml) and diphenhydramine hydrochloride (400 ng/ml); (C) plasma sample 5 h after an oral dose of nicardipine hydrochloride delayed-release tablets (40 mg) to healthy volunteers with the measured concentration of the analyte at about 2 ng/ml. Two channels were used for the quantification, MSD1 for nicardipine (t_R = 4.0 min) and MSD2 for diphenhydramine (t_R = 3.6 min).

the fragment ion peaks, 170 V for nicardipine and 150 V for the internal standard. Selected ion monitoring (SIM) in positive mode was used for the quantification of nicardipine and the internal standard at *m/z* 480.2 and 256.4, respectively. Two-channel mode was used, channel 1 (MSD1) for nicardipine and channel 2 (MSD2) for the internal standard.

3.2. Selectivity

The results for selectivity are shown in Fig. 2, showing a clean chromatogram from blank plasma sample after sample preparation by liquid–liquid extraction. The results demonstrated the absence of endogenous interferences from the plasma matrix and the satisfying selectivity of the present method for the determination of nicardipine in human plasma.

3.3. Linearity

To evaluate the linearity of the LC–MS method, plasma calibration curves were determined in triplicate on three separate days. Representative regression equation for the calibration curve was $y = 9.55 \times 10^{-2}x + 7.30 \times 10^{-3}$ ($r = 0.9987$, $n = 7$) for nicardipine. Good linearity was observed over the concentration range of 0.05–20.0 ng/ml.

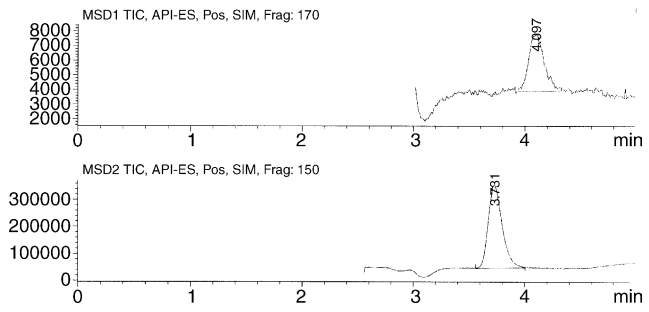

Fig. 3. Representative SIM chromatograms of blank plasma sample spiked with nicardipine hydrochloride (0.05 ng/ml) and diphenhydramine hydrochloride (400 ng/ml). Two channels were used for the quantification, MSD1 for nicardipine (t_R = 4.0 min) and MSD2 for diphenhydramine (t_R = 3.6 min).

3.4. Lower limit of quantification

The lower limit of quantification (LLOQ) is defined as the lowest concentration analyzed with an accuracy of less than 20% and a precision less than 20%. LLOQ for nicardipine was found to be 0.05 ng/ml. A typical SIM chromatogram of plasma sample spiked with the analyte at LLOQ level is shown in Fig. 3. At LLOQ level, the inter- and intra-day imprecision (R.S.D.) were 2.9 and 10.7%, the inaccuracy (RE) was 4.0%.

3.5. Accuracy and precision

The accuracy and precision of the method were evaluated based on the data from QC plasma samples at three concentrations (0.1, 5.0 and 20.0 ng/ml) in three validation runs. The accuracy was determined by calculating the percentage deviation observed in the analysis of QC samples and expressed in the relative error (RE). The intra- and inter-day precision was expressed as the relative standard deviation (R.S.D.). As shown in Table 1, for each QC level of nicardipine, the inter- and intra-day precisions (R.S.D.) were less than 9.3 and 11.1%, and accuracy (RE) was ±5.0%, indicating acceptable accuracy and precision of the present LC–MS method for the determination of nicardipine in human plasma.

3.6. Extraction recovery

The extraction recoveries of nicardipine from human plasma were determined by comparing peak areas from plasma samples spiked before extraction with the corresponding standard solutions without extraction. The results showed that the extraction recoveries from human plasma were 91.2 ± 3.5, 82.0 ± 5.0 and 78.4 ± 3.2% at concentrations of 0.1, 5.0 and 20.0 ng/ml of nicardipine, respectively. It can be found that compared with concentrations of 5.0 and 20.0 ng/ml, about 10% higher recovery was achieved at 0.1 ng/ml. But it was acceptable because the relative deviation at this concentration to 0.05 ng/ml (LLOQ)

Table 1
Accuracy and precision for the determination of nicardipine in human plasma (3 days, six replicates each day)

Calculated *C* (ng/ml)	Found *C* (ng/ml)	Intra-day precision R.S.D. (%)	Inter-day precision R.S.D. (%)	Accuracy RE (%)
0.10	0.10	11.1	9.3	−4.9
5.00	5.07	5.9	6.7	1.5
20.00	19.93	5.0	4.8	−0.4

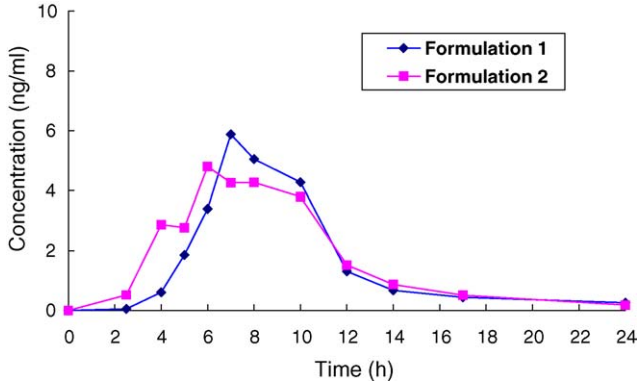

Fig. 4. Mean plasma concentration–time profiles of nicardipine after oral administration of nicardipine hydrochloride delayed-release tablets of formulations 1 and 2 to healthy volunteers.

was within ±20%, which conforms with the guidance for bioanalytical method validation with a concentration near LLOQ.

3.7. Freeze–thaw cycles

The results for three freeze–thaw cycles showed that the analyte was stable in human plasma through three freeze–thaw cycles. For the three levels of the analyte in plasma, the intra-day and inter-day precisions (R.S.D.) ranged from 5.0 to 7.6% and from 9.9 to 11.2%, respectively. The accuracy (RE) ranged from −4.5 to −8.9%.

3.8. Stability

The stability of nicardipine in the supernatant was determined. The analyte was found to be stable for at least 12 h at ambient temperature after sample preparation with an accuracy (RE) ranging from −7.5 to 12.5% at three levels of QC samples.

3.9. Application of the developed LC–MS method

The present LC–MS method achieved satisfactory results for the determination of nicardipine in human plasma and was successfully applied in the pharmacokinetic study of nicardipine hydrochloride delayed-release tablets following oral administration to healthy volunteers. The mean plasma concentration–time profiles for nicardipine are shown in Fig. 4. Based on the mean *c–t* profiles of the two formulations, formulation 1 was finally chosen for the further development due to its satisfying starting time for the drug release (about 3 h) and less individual variations.

4. Conclusions

A sensitive and selective LC–MS method for the determination of nicardipine in human plasma is described. Compared with the methods published, the present LC–MS method features a simple procedure for sample preparation and higher sensitivity with a lower limit of quantification of 0.05 ng/ml, satisfactory selectivity and short run time (less than 5 min). It was successfully applied in pharmacokinetic studies of nicardipine hydrochloride delayed-release tablets in healthy volunteers.

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