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Liquid chromatographic – electrospray tandem mass spectrometric method for the quantification of nimodipine in human plasma

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A simple, sensitive and rapid liquid chromatography/electrospray ionization tandem mass spectrometry (LC-MS/MS) method was developed and validated for the quantification of nimodipine, a calcium channel blocker, in human plasma. Following liquid-liquid extraction, the analytes were separated using an isocratic mobile phase on a reverse phase C_{18} column and analyzed by MS in the multiple reaction monitoring mode using the respective $[M + H]^+$ ions, m/z 419/343 for nimodipine and m/z 409/228 for the IS. The assay exhibited a linear dynamic range of 0.2–50 ng/mL for nimodipine in human plasma. The lower limit of quantification was 200 pg/mL with a relative standard deviation of less than 8%. Acceptable precision and accuracy were obtained for concentrations over the standard curve range. A run time of 3 min for each sample made it possible to analyze more than 250 human plasma samples per day. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability or bioequivalence studies.

1. Introduction

Nimodipine, is a dihydropyridine calcium antagonist with a preferential action on cerebral actions (Langley and Sorkin 1989), used in clinical practice for many years. Because of its special selectivity for brain blood vessels, it is used mainly in the prevention and treatment of the delayed ischaemic neurological deficits that frequently occur in patients with subarachnoid haemorrhages. Nimodipine may be effective in the prevention and treatment of other neurological conditions (Allen et al. 1983; Bailey et al. 1991; Gelmers et al. 1988; Morich et al. 1996).

Several methods for the quantification of nimodipine in biological fluids have been described, such as gas chromatography (GC) with electron-capture (Jakobsen et al. 1986; Krol et al. 1984) or nitrogen-phosphorus detection (Rosseel et al. 1990), liquid chromatography (LC) with UV detection (Aymard et al. 1998; Blardi et al. 2002; Qian and Gallo 1992) and LC with electrochemical detection (Lopez et al. 2000). In all the reported methods, plasma volume requirement was high, chromatographic run time was longer and sensitivity was inadequate for pharmacokinetic studies. Fischer et al. (1993) reported a GC-MS method combined with a chiral stationary phase HPLC for the separation and quantification of nimodipine enantiomers with a lower limit of quantification (LLOQ) per enantiomer 0.1 ng/mL using 0.5 mL serum, but the chromatographic run time was more than 30 min which was not appropriate for the analyses of large number of biological samples.

The advent of the atmospheric pressure ionization (API) source was a breakthrough that allowed the efficient coupling of LC and MS, which leads to a more specific technique. The usefulness of liquid chromatography/tandem mass spectrometry (LC-MS/MS) has been demonstrated for a wide range of applications in the bioanalytical, environmental and pharmaceutical fields (Cech and Enke 2001; Jemal 2000; Nirogi et al. 2005; Niessen 2003; Ramakrishna et al. 2005a–c). This powerful separation and detection technique is widely used for the determination of drugs in biological fluids. Mück et al. (1995) reported an LC-MS/MS method using a deuterium-labeled internal standard, providing an LLOQ of 0.5 ng/mL in 1 mL plasma sample, but the method was also used for the quantification of nimodipine enantiomers. The mobile phase consisted of ethanol-n-heptane (20 : 80, v/v) including 2 mM ammonium acetate. Therefore, the potential hazard by using flammable eluents and the occurrence of discharge at the tip of the sprayer has to be carefully addressed. Gualano et al. (1999) and Qiu et al. (2004) reported an LC-MS/MS method with an atmospheric pressure chemical ionization (APCI) source in selected reaction monitoring mode for the determination of nimodipine in human plasma with an LLOQ of 0.25 and 0.24 ng/mL, respectively.

The purpose of this investigation was to explore the high selectivity and sensitivity of a triple-quadrupole MS system with an electrospray interface for the development and validation of a robust reversed phase LC-MS/MS method in multiple reaction monitoring (MRM) mode for the quantification of nimodipine in human plasma. It was essential to establish an assay capable of quantifying nimodipine at concentrations down to 200 pg/mL. At the same time, it was expected that this method would be efficient in analyzing large numbers of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of nimodipine.

2. Investigations, results and discussion

2.1. Mass spectrometry

In order to develop a method with the desired LLOQ (200 pg/mL), it was necessary to use MS-MS detection, as MS-MS methods provide improved limit of detection for trace-mixture analysis. The inherent selectivity of MS-MS detection was also expected to be beneficial in developing a selective and sensitive method. The product ion mass spectrum of nimodipine and the IS are shown in Fig. 1a, b. $[M+H]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain the product ion spectra. The most sensitive mass transition was from m/z 419 to 343 for the nimodipine and from m/z 409 to 228 for the IS. A proposed fragmentation pattern is also shown in the Scheme. The main fragmentation occurs through the loss of the alcohol parts of carboxyl groups, with formation of substituted ketene ions. In nimodipine analysis the loss of 2-methoxy-ethanol is energetically favored in relation to the 1-methyl-ethanol.

LC-MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table 1 are the result of this optimization.

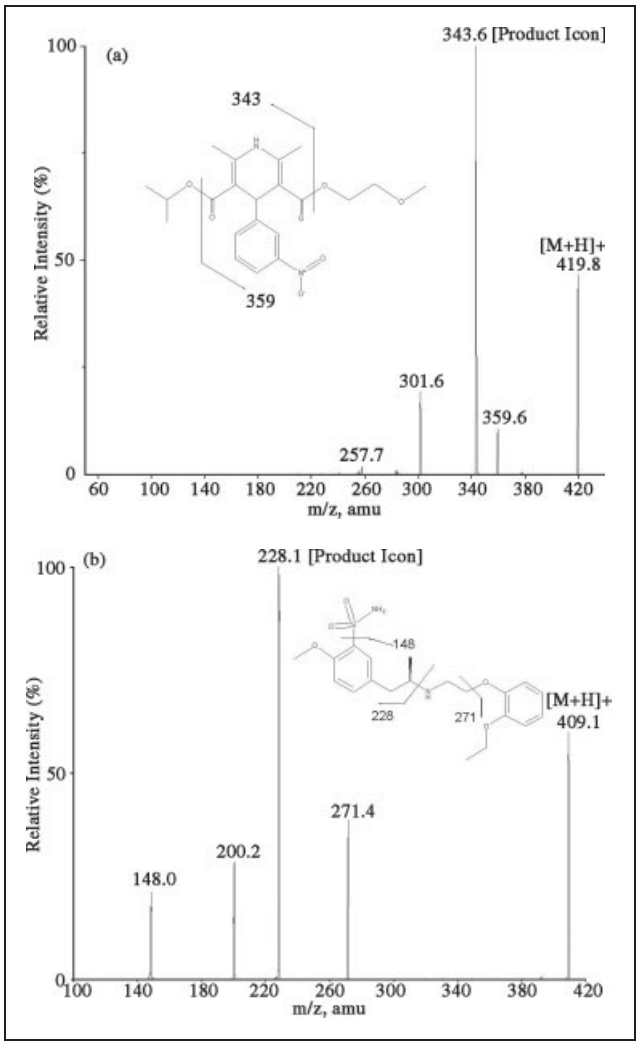

Fig. 1: Full scan positive ion turbolonspray product ion mass spectra and the proposed patterns of fragmentation (Scheme) of (a) nimodipine and (b) tamsulosin (internal standard). The protonated molecules were used as precursor ions for MS-MS

2.2. Method development

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. It was found that a mixture of 0.03% formic acid – acetonitrile (20 : 80, v/v) could achieve this purpose and was finally adopted as the mobile phase. Moreover, it was necessary to reconstitute the residues with the mobile phase to produce the expected peak shapes of the analyte. The high proportion of organic solvent eluted the analyte and the IS at retention times of 2.46 and 0.95 min, respectively. A flow rate of 1 mL/min produced good peak shapes and permitted a run time of 3.0 min.

Liquid-liquid extraction (LLE) was used for the sample preparation in this work. LLE can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC-MS/MS analyses. Six organic solvents, diethyl ether, ethyl acetate, hexane, dichloromethane, chloroform and butyl *tert*-methyl ether, and their mixtures in different combinations and ratios were evaluated. Finally, a mixture of butyl *tert*-methyl ether and dichloromethane (8 : 2, v/v) was found to be optimal, which can produce a clean chromatogram for a blank plasma sample and yield the highest recovery for the analyte from the plasma.

For a LC-MS/MS analysis, utilization of stable isotope-labeled drugs as internal standards proves to be helpful when a significant matrix effect is possible. An isotope labeled analyte was not obtainable to serve as IS, so, in the initial stages of this work, several compounds were investigated to find a suitable IS, and finally tamsulosin was found to be suitable for the present purpose. Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components of the sample matrix. These potential matrix effects were evaluated by spiking blank plasma extracts (after LLE treatment as described above) at the low and high QC levels. The resulting chromatograms were compared with those obtained for clean standard solutions at the same concentrations. Five independent plasma lots were used, with five samples from each lot. The results (data not shown) showed that there was no significant difference between peak responses for spiked plasma extracts and clean solutions. This result most likely reflects the efficacy of the sample clean-up with LLE. In any event, the use of matrix-matched calibration standards would have minimized any such effects on the quantification.

2.3. Assay performance and validation

The eight-point calibration curve was linear over the concentration range 0.2–50 ng/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighing factors ($1/x$, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$

Scheme

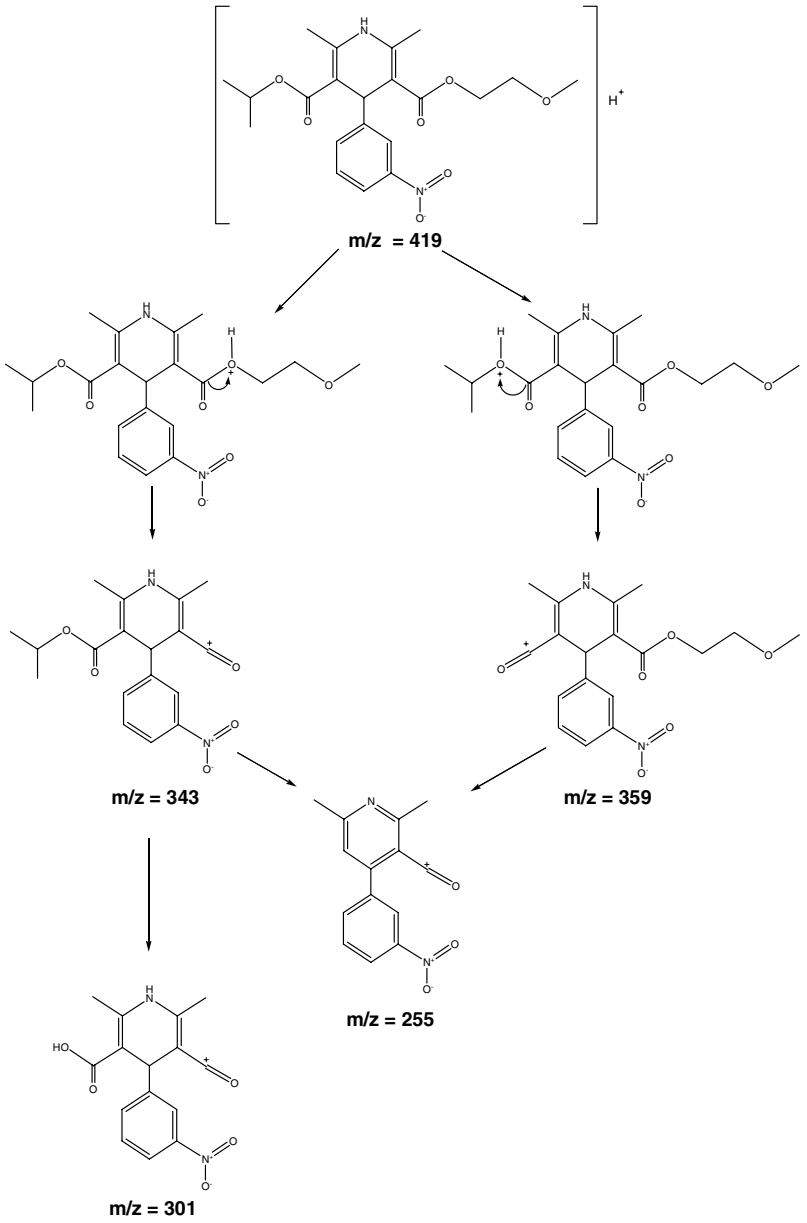

Table 1: Tandem mass spectrometer main working parameters

Parameter	Value
Source temperature (°C)	150
Dwell time per transition (msec)	200
Ion Source gas 1 (psi)	20
Ion Source gas 2 (psi)	20
Curtain gas (psi)	12
Collision gas (psi)	4
Ion spray voltage (V)	5800
Entrance potential (V)	10
Declustering potential (V)	60 (Analyte) and 85 (IS)
Collision energy (V)	10 (Analyte) and 33 (IS)
Collision cell exit potential (V)	9 (Analyte) and 5.5 (IS)
Resolution	Unit
Mode of analysis	Positive
Ion transition for nimodipine (m/z)	419.4 ± 0.5/343.5 ± 0.5
Ion transition for tamsulosin (m/z)	409.1 ± 0.5/22.8 ± 0.5

weighing factor, giving a mean linear regression equation for the calibration curve of:

$$y = 0.3628(\pm 0.0328)x + 0.0011(\pm 0.0006)$$

where y is the peak area ratio of the analyte to the IS and x is the concentration ratio of the analyte to the IS. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.999; Table 2 summarizes the calibration curve results.

The selectivity of the method was examined by analyzing (n = 5) blank human plasma extract (Fig. 2a) and an extract spiked only with the IS (Fig. 2b). As shown in Fig. 2a, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. Similarly, Fig. 2b shows the absence of direct interference from the IS to the MRM channel of the analyte. Fig. 2c depicts a representative ion-chromatogram for the LLOQ (200 pg/mL). Excellent sensitivity was observed for a 25-μL injection volume; the LLOQ corresponds to ca. 5 pg on-column.

Table 2: Precision and accuracy data for back-calculated concentrations of calibration samples for nimodipine in human plasma

Concentration added (ng/mL)	Concentration found (mean ± SD, n = 5; ng/mL)	Precision (%)	Accuracy (%)
0.2	0.20 ± 0.02	10.1	102.0
0.4	0.41 ± 0.02	4.1	103.5
1	0.99 ± 0.08	7.7	98.5
2	2.02 ± 0.11	5.2	101.2
5	5.04 ± 0.13	2.5	100.7
10	9.90 ± 0.90	9.0	99.0
20	20.80 ± 0.73	3.5	104.0
50	50.30 ± 1.31	2.6	100.6

The MRM chromatograms obtained for an extracted plasma sample of a healthy subject who participated in a bioequivalence study conducted on 18 subjects are depicted in Fig. 3. Nimodipine was identified and was quantified as 2.1 ng/mL.

2.4. Validation parameters at the Lower Limit of Quantification (LLOQ)

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision, and was found to be 200 pg/mL in human plasma. The mean response for the analyte peak at the assay sensitivity limit (200 pg/mL) was ~18-fold greater than the mean response for the peak in five blank human plasma samples at the retention time of the analyte. The between-batch precision at the LLOQ was 6.5%, and the between-batch accuracy was 107.1% (Table 3). The within-batch precision was 7.8% and the accuracy was 98.4% for nimodipine.

2.5. Validation parameters at the middle and upper concentrations

The middle and upper quantification levels of nimodipine ranged from 0.6–40 ng/mL in human plasma. For the between-batch experiments the precision ranged from 6.3 to 8.1% and the accuracy from 101.4 to 104.5% (Table 3). For the within-batch experiments the precision and accuracy for the analyte met the acceptance criteria (<±15%). The average absolute recoveries for nimodipine at three different concentrations (low, medium and high QC samples) are shown in Table 4. The recovery of the analyte was high (54.2 ± 0.9%). The recovery of the IS was 31.1 ± 0.4% at the concentration used in the assay (100 ng/mL). Recovery of the analyte and the IS were low, but it was consistent, precise and reproducible. Therefore the assay has proved to be robust in high throughput bioanalysis.

2.6. Stability studies

The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated as follows. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions (−50 °C), and to freeze-thaw stability studies. All the stability studies were conducted at two concentration levels (0.6 and 40 ng/mL as low and high values) with five determinations for each.

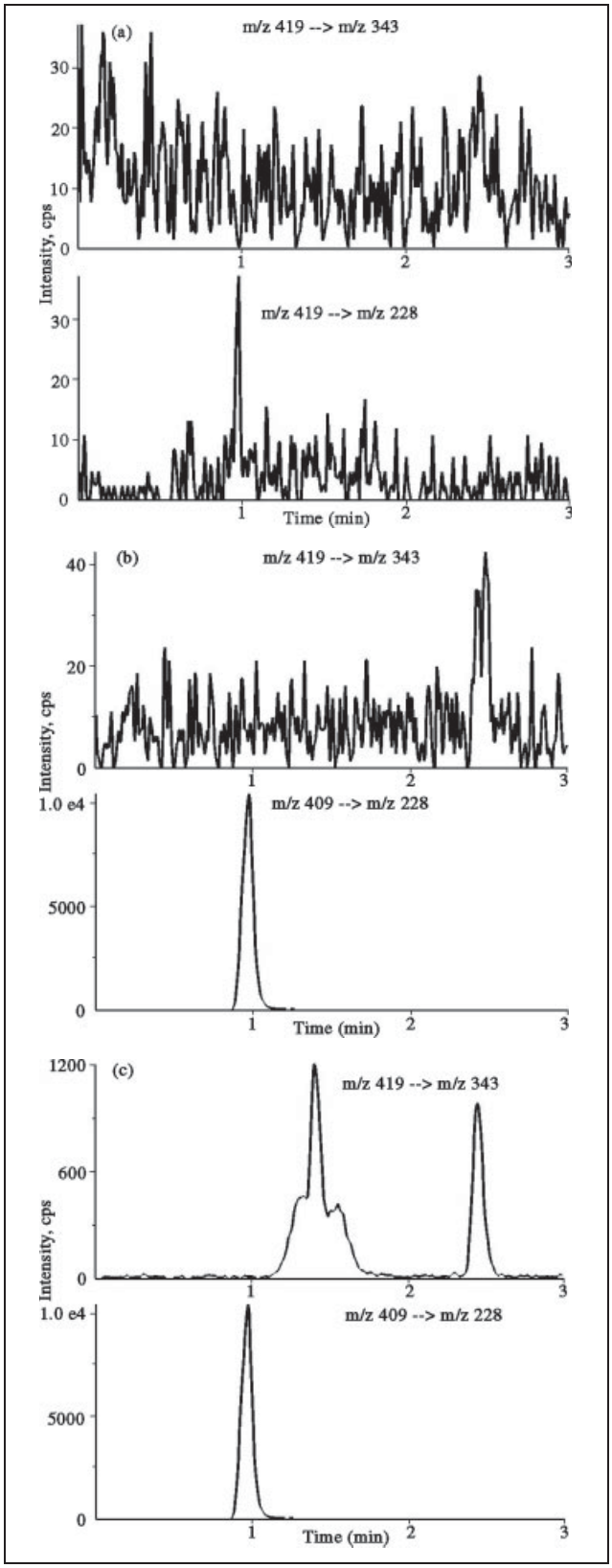

Fig. 2: MRM chromatograms for nimodipine and the IS resulting from analysis of: (a) blank (drug and IS free) human plasma; (b) blank (drug-free spiked with IS) human plasma; (c) 200 pg/mL (LLOQ) of nimodipine spiked with the IS

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 24 h). Samples

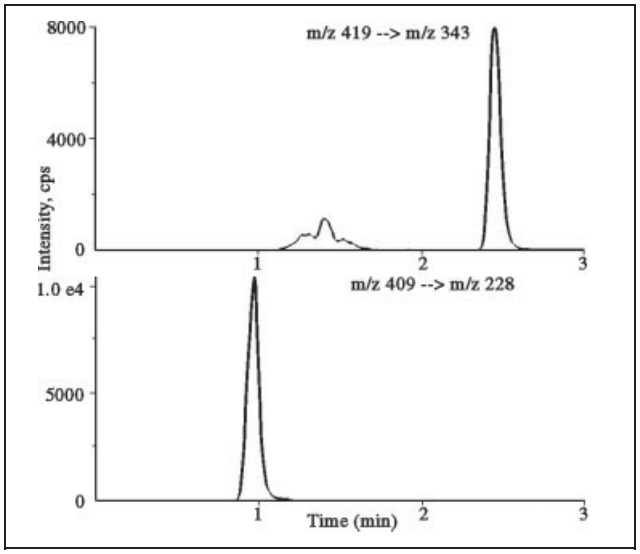

Fig. 3: MRM chromatograms resulting from the analysis of a subject plasma sample after the administration of a 30 mg oral single dose of nimodipine. The sample concentration was determined to be 2.1 ng/mL

were extracted and analyzed as described above, and the results are given in Table 5. These results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in the autosampler for 24 h was also assessed. The results indicate that solutions of nimodipine and the IS can remain in the autosampler for at least 24 h without showing significant loss in the quantified values, indicating that samples should be processed within this period of time (Table 5).

The data representing the stability of nimodipine in plasma at two QC levels over three freeze/thaw cycles are given in Table 5. These tests indicate that the analyte is stable in human plasma for three freeze/thaw cycles, when stored at below $-50\,^{\circ}C$ and thawed to room temperature.

Table 5 also summarizes the long-term stability data for nimodipine in plasma samples stored for a period of 21 days at below $-50\,^{\circ}C$. The stability study of nimodipine in human plasma showed reliable stability behavior, as the mean of the results of the tested samples were within the acceptance criteria of $\pm 15\%$ of the initial values of the controls. These findings indicate that storage of nimodipine in plasma samples at below $-50\,^{\circ}C$ is adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic, bioavailability or bioequivalence studies.

The stability of the stock solutions was tested and established at room temperature for 2 h, 24 h, and under refrigeration ($\sim 4\,^{\circ}C$) for 3 months. The recoveries for nimodipine and IS were 103.2 (coefficient of variance (CV) 1.2%), 99.6 (CV 2.5%), 104.3 (CV 1.8%) and 100.4 (CV 0.8%), 101.5 (CV 1.3%), 99.1 (CV 0.5%) respectively.

Table 4: Absolute recoveries of nimodipine from human plasma

Sample concentration (ng/mL)	Absolute recovery (%) (mean ± SD, n = 5)
0.6	53.2 ± 2.1
25	54.6 ± 2.6
40	54.9 ± 1.4

Table 5: Stability of nimodipine in human plasma

Sample concentration (ng/mL; n = 5)	Concentration found (ng/mL)	Precision (%)	Accuracy (%)
Short-term stability for 24 h in plasma			
0.6	0.65	7.9	107.5
40	43.50	7.8	107.4
Three freeze-thaw cycles			
0.6	0.65	12.2	108.6
40	44.03	6.1	108.7
Autosampler stability for 24 h			
0.6	0.61	6.7	101.3
40	38.75	5.1	95.7
Stability for 21 days at $< -50\,^{\circ}C$			
0.6	0.58	6.2	96.6
40	37.21	7.5	93.0

The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

2.7. Application

The validated method has been successfully used to quantify nimodipine concentrations in human plasma samples after the administration of a single 30 mg oral dose of nimodipine. The representative mean concentration versus time profiles for 6 subjects, each receiving a single dose, is presented in Fig. 4.

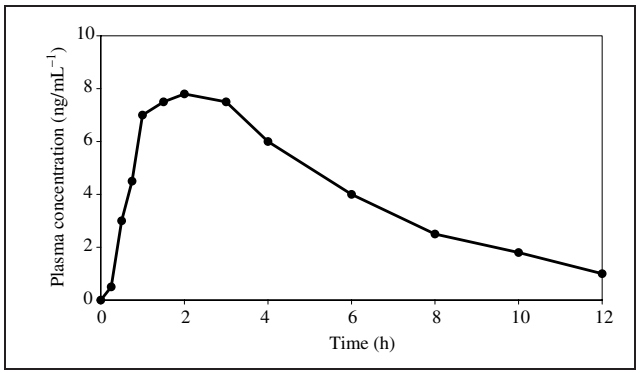

Fig. 4: Representative mean data showing plasma concentration-time profiles of six healthy subjects after the administration of an oral single dose of 30 mg of nimodipine

Table 3: Precision and accuracy of the LC-MS-MS method for determining nimodipine concentrations in plasma samples

Concentration added (ng/mL)	Within-batch precision (n = 5)			Between-batch precision (n = 3)		
	Concentration found mean ± SD; (ng/mL)	Precision (%)	Accuracy (%)	Concentration found mean ± SD; (ng/mL)	Precision (%)	Accuracy (%)
0.2	0.20 ± 0.02	7.8	98.4	0.22 ± 0.01	6.5	107.1
0.6	0.56 ± 0.02	4.0	92.4	0.63 ± 0.05	7.5	104.5
25	23.30 ± 2.07	8.9	92.1	25.86 ± 2.09	8.1	102.2
40	38.60 ± 2.86	7.4	95.4	41.04 ± 2.58	6.3	101.4

3. Experimental

3.1. Chemicals

Nimodipine drug substance was obtained from Vimta Labs (Hyderabad, India), and tamsulosin (internal standard, IS) was from the R & D Department of this institute (Hyderabad, India). Drug-free human plasma was obtained from the Usha Mullapudi Cardiac Center (Hyderabad, India). HPLC-grade LiChrosolv methanol and LiChrosolv acetonitrile were purchased from Merck (Darmstadt, Germany). Methyl *tert*-butyl ether, dichloromethane and formic acid were purchased from Merck (Worli, Mumbai, India). HPLC grade water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

3.2. LC-MS/MS instrument and conditions

The 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany) is equipped with a G1312A binary pump, a G1379A degasser, a G1367A autosampler equipped with a G1330B thermostat, a G1316A thermostatted column compartment and a G1323B control module. The chromatographic separation was on a Waters symmetry® C_{18} column (5.0 μm, 150 × 4.6 mm i.d.) at 30 °C. The isocratic mobile phase composition was a mixture of 0.03% formic acid/acetonitrile (20/80, v/v), which was pumped at a flow rate of 1.0 mL/min with a split ratio of 50 : 50. As nimodipine is sensitive to photodegradation, sample preparation and instrumental analyses were performed under feeble yellow light.

Mass spectrometric detection was performed using an API 4000 triple quadrupole instrument (MDS-SCIEX, Toronto, Canada) using MRM. A turbo-electrospray interface in positive ionization mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed using Analyst 1.4.1 software package (SCIEX).

3.3. Sample preparation

A plasma sample (0.5 mL) was transferred to a 15-mL glass test tube, then 50 μL of IS working solution (100 ng/mL) was added. After vortex mixing for 10 s, 4-mL aliquot of extraction mixture, methyl *tert*-butyl ether/dichloromethane (8/2, v/v), was added and the sample was vortex-mixed for 5 min using a multi-tube vortexer. The organic layer (3 mL) was transferred to a 5-mL glass tube and evaporated to dryness using an evaporator at 40 °C under a stream of nitrogen. Then the dried extract was reconstituted in 250 μL of mobile phase and a 25-μL aliquot was injected into the chromatographic system.

3.4. Bioanalytical method validation

Standard stock solutions of nimodipine (1 mg/mL) and the IS (1 mg/mL) were separately prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in water/methanol (50 : 50, v/v; diluent). The IS working solution (100 ng/mL) was prepared by diluting its stock solution with diluent. Working solutions (0.2 mL) were added to drug-free human plasma (9.8 mL) as a bulk, to obtain nimodipine concentration levels of 0.2, 0.4, 1, 2, 5, 10, 20 and 50 ng/mL as a single batch at each concentration. Quality control (QC) samples were also prepared as a bulk on an independent weighing of standard drug, at concentrations of 0.2 (LLOQ), 0.6 (low), 25 (medium) and 40 ng/mL (high) as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in micro centrifuge tubes (Tarson, 2 mL) and stored in the freezer at below −50 °C until analyses.

A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS) and eight non-zero samples covering the total range 0.2–50 ng/mL, including the LLOQ. The calibration curves were generated using the analyte to IS peak area ratios by weighed ($1/x^2$) least-squares linear regression on five consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification.

The within-batch precision and accuracy were determined by analyzing five sets of QC samples in a batch. The between-batch precision and accuracy were determined by analyzing five sets of QC samples on three different batches. The QC samples were randomized daily, processed and analyzed in a position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for the other concentrations.

Recovery of nimodipine from the extraction procedure was determined by a comparison of the peak area of nimodipine in spiked plasma samples (five each of low, medium and high QCs) with the peak area of nimodipine in samples prepared by spiking extracted drug-free plasma samples with the same amounts of nimodipine at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted QC samples (n = 5) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography.

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