

## Determination of nimodipine in plasma by HPLC-MS/MS and pharmacokinetic application

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To develop and validate a rapid, specific and highly sensitive method to quantify nimodipine in human plasma using dibucaine as the internal standard (IS). The analyte and IS were extracted from plasma samples by liquid-liquid extraction using hexane-ethyl acetate (1:1 v/v). The chromatographic separation was performed on a Varian® Polaris C18 analytical column (3 µm, 50 x 2.0 mm) and pre-column Securityguard™ C18 (4.0 x 3.0 mm) with a mobile phase of Acetonitrile-Ammonium acetate 0.02 ml/L (80:20v/v). The method had a chromatographic run time of 4.5 min and linear calibration curve over the range of 0.1- 40 ng/mL ( $r > 0.9938$ ). The limit of quantification was 100 pg/mL. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. This validated method was successfully applied in determining the pharmacokinetic profile of nimodipine tablets of 30 mg administered to 24 healthy volunteers. The proposed method of analysis provided a sensitive and specific assay for nimodipine determination in human plasma. The time for the determination of one plasma sample was 4.5 min. This method is suitable for the analysis of nimodipine in human plasma samples collected for pharmacokinetic, bioavailability or bioequivalence studies in humans.

**Uniterms:** Nimodipine/determination in plasma. Nimodipine/pharmacokinetic profile. Nimodipine/quantitative analysis. Pharmacokinetics/method validation.

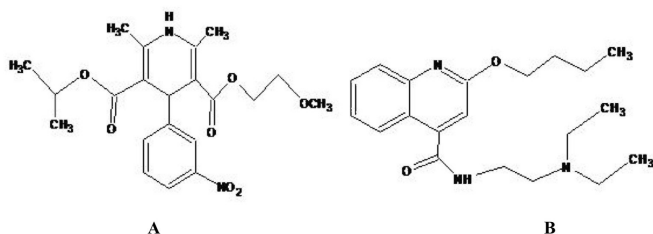
Um método rápido, específico e sensível para quantificar nimodipino em plasma humano usando dibucaína como padrão interno (IS) é descrito. O analito e o IS foram extraídos das amostras de plasma por extração líquido-líquido usando hexano-acetato de etila (1:1 v/v). A separação cromatográfica foi realizada utilizando-se uma coluna analítica C18 Varian® Polaris (3 µm, 50 x 2,0 mm) e uma pré-coluna Securityguard™ C18 (4,0 x 3,0 mm) e acetonitrila-acetato de amônia 0,02 mol/L (80:20 v/v) como fase móvel. O método apresentou tempo total de corrida de 4,5 min e curva de calibração linear com concentrações entre 0,1-40 ng/mL ( $r > 0,9938$ ). O limite de quantificação foi de 100 pg/mL. Os valores de precisão e exatidão foram obtidos por meio da análise das amostras de controle de qualidade. A análise de uma única amostra de plasma foi realizada em 4,5 minutos. A metodologia validada foi aplicada na determinação do perfil farmacocinético do nimodipino, comprimido de 30 mg administrado em 24 voluntários saudáveis. O método para quantificar nimodipino em plasma é adequado para aplicação em estudos farmacocinéticos, biodisponibilidade e bioequivalência em humanos.

**Unitermos:** Nimodipino/determinação em plasma. Nimodipino/perfil farmacocinético. Nimodipino/análise quantitativa. Farmacocinética/validação de método.

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## INTRODUCTION

Nimodipine (Figure 1) is a dihydropyridine calcium channel blocker known for its preferential action on cerebral blood vessels (Kazda, Toward, 1982; Haws *et al.*, 1983), but it is currently used to prevent and treat the ischemic damage caused by cerebral arterial spasm in subarachnoid hemorrhage (Dorhout Mees *et al.*, 2007; Stachura *et al.*, 2002). Nimodipine has also been used in the treatment of ischemic stroke (Svetyi *et al.*, 2002; Sobrado *et al.*, 2003; Fogelholm *et al.*, 2004), multi-infarct dementia (Fischhof *et al.*, 1989; Pantoni *et al.*, 2000) and migraine prophylaxis (Evers, 1999; Victor, Ryan, 2003).



**FIGURE 1** - Chemical structures of nimodipine (A) and dibucaine (B, internal standard).

Several analytical techniques have been applied in the quantification of nimodipine in biological matrices. Gas chromatography (GC) methods with electron-capture (Krol *et al.*, 1984; Jackobsen, *et al.*, 1986) and nitrogen-phosphorus detection (Rosseel, Bogaert, Huyghens, 1990) were used to determine nimodipine in plasma, which can provide a lower limit of quantification (LLOQ) of 5 ng/mL, using 1 mL of plasma. The method described by Krol *et al.* (1984) is sensitive and selective; however, it requires a large amount of plasma and elaborate and lengthy sample preparation.

HPLC with UV detection (Qian, Gallo, 1992; Aymard *et al.*, 1998) and electrochemical detection (López *et al.*, 2000) has a sensitivity of about 1 ng/mL, using 1 mL of plasma, but these methods are time-consuming (more than 10 min). In all the reported methods above, plasma volume requirement was high, chromatographic run time was longer and sensitivity was inadequate for pharmacokinetic studies. The relatively longer run time for nimodipine registered by these analytical methods are not adequate for the analysis of a high number of samples. Fischer *et al.*, 1993 developed a GC-MS method combined with chiral stationary phase HPLC for the separation and determination of nimodipine enantiomers, which had an LLOQ of 0.1 ng/mL, using 0.5 mL of plasma. However, the analytical time was more than 30 min, which is not suitable for the analysis of a large number of biological samples.

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 (Jemal, 2000; Cech, Enke, 2001; Niessen, 2003). This powerful separation and detection technique is widely used for the determination of drugs in biological fluids. Mück (1995) developed an LC-MS-MS method providing an LLOQ of 0.5 ng/mL, using a 1 mL plasma sample. This method was used for the quantification of nimodipine enantiomers. 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.

Nirogi *et al.* (2006) developed an LC-MS-MS method to determine nimodipine in human plasma which had an LLOQ of 200 pg/mL using 0.5 mL plasma. Recently, Qin *et al.* (2008) published a method for quantification of nimodipine in human plasma using ultra performance liquid chromatography–tandem mass spectrometry. The validated method attained a limit of quantification of 0.2 ng/mL using 500 µL plasma. In these methods, nimodipine concentrations were not always detectable in the small plasma samples used (0.5–1 mL). This fact made them unsuitable for time-course analysis in cases where only small plasma volumes are available (pharmacokinetic studies in experimental animals or children).

LC-MS/MS is one of the most common techniques employed for the quantification of drugs in biological matrices, due to its higher levels of specificity and sensitivity. Thus, the present report describes the development and validation of an LC-MS/MS method for the quantification of nimodipine with use of dibucaine as the internal standard (Figure 1) and the method's clinical application in pharmacokinetic and bioavailability studies in human volunteers.

## MATERIAL AND METHODS

### Reference compounds and chemicals

Nimodipine was purchased from ICN Biomedical (Ohio, USA) and dibucaine (IS) was purchased from USP Pharmacopeia (New York, NY, USA). Acetonitrile (ACN) was purchased from EM Science (Gibbstown, NJ, USA); methanol (MeOH), ethyl acetate, hexane and ammonium acetate were purchased from Merck (Rio de Janeiro, RJ, Brazil). The ACN and MeOH used were HPLC grade solvents. All other reagents were analytical grade. A Milli-Q<sup>®</sup> (Millipore, Mosheim, France) water

purification system was used to obtain the purified water for the HPLC analysis.

### Preparation of standard solutions and reagents

A stock solution of nimodipine 100 µg/mL was prepared in ACN. Working standard solutions were prepared from the stock solution by dilution with ACN to produce a final concentration of 10 µg/mL.

A stock solution of dibucaine 100 µg/mL (Figure 1) was prepared in MeOH. Working standard solutions were prepared from the stock solution by dilution with MeOH to produce a final concentration of 50 ng/mL.

### Calibration curves

Calibration standards were prepared by spiking control human plasma with standard solutions containing nimodipine to give standards of 0.1, 0.5, 2.0, 10, 25 and 40 ng/mL. A fixed concentration of dibucaine was added to all assay tubes as an internal standard. Zero concentration plasma samples used in each run were prepared containing internal standard only. In each run, a plasma blank sample (no IS) was also analyzed.

### Quality control samples

Quality control (QC) samples were prepared at three different levels: low level (LQC), three times the lower limit of quantification (LLOQ); middle level (MQC), approximately midway between low quality control and high quality control; and high level (HQC), 75-90% of the upper limit of the quantification (ULOQ). Quality control samples were prepared by spiking different samples of human plasma with 0.3, 15 and 35 ng/mL of nimodipine.

### Sample extraction procedure

To prevent the photodegradation of nimodipine, the whole experiment including plasma collecting, sample preparation and instrumental analyses were performed under feeble yellow light.

A total of 300 µL of plasma were extracted from the appropriate tube after adding 25 µL of internal standard dibucaine (50 ng/mL). The tubes were vortex-mixed for 1 min. A volume of 1000 µL of hexane/ethyl acetate (1:1 v/v) was then added, and the samples were vortex-mixed for 5 min. The tubes were centrifuged at 2000 g for 5 min at 37 °C. The upper organic layer (800 µL) was carefully removed, transferred to new tubes, and evaporated to dryness with a gentle stream of nitrogen in a dry bath at 37 °C.

A 150-µL aliquot of ACN was added to tubes which were then vortex-mixed for 1 min to reconstitute the residue. The solutions were transferred to *microvials* which were capped and placed in an autosampler rack. Aliquots of 10 µL were injected automatically into the liquid chromatography system.

### Mass spectrometry and chromatography conditions

The chromatographic runs were carried out using an HP/Quat Pump Series 1100 analytical pump and an HP/ALS Series 1100 auto sampler. The mobile phase involved a mixture of ACN-0.02 mol/L ammonium acetate (80:20, v/v) pumped at a flow rate of 1.0 mL/min through the analytical column (Varian® Polaris C<sub>18</sub>, 3 µm, 50 x 2.0 mm) and pre-column (Securityguard™ C<sub>18</sub>, 4.0 x 3.0 mm), at 20 °C. A split of the column eluant of approximately 1:10 was included so that only approximately 50 µL/min entered the mass spectrometer. The injection volume was 10 µL and the total run time was 4.5 min.

The mass spectrometer (micromass Quattro LC) equipped with an electrospray source using a crossflow counter electrode was run in positive mode (ES+), and set up in multiple reaction monitoring (MRM), monitoring the transitions 418.6>342.6 and 344.2>271.0 for nimodipine and dibucaine, respectively. The source and desolvation temperature was 100 °C and 300 °C, respectively. The cone and desolvation gas flow (N<sub>2</sub>) was 116 L/h and 533 L/h, respectively. The dwell time was set at 0.5 s, and the values of the capillary voltage, the cone energy and collision energy were 4.2 KV, 15 V, 10 eV for nimodipine and 4.2 KV, 20 V and 21 eV for dibucaine, with a gas pressure (argon) of 2.5 x 10<sup>-3</sup> mbar. Data acquisition and analysis were performed using the software MassLynx (V 3.5, running under Windows NT (v 4.0) on a Pentium PC).

### Method validation

Sample analysis was always carried out in a GLP-compliant manner, and therefore, the LC-MS-MS methods needed to be validated according to currently accepted Food and Drug Administration (FDA) and National Health Surveillance Agency (ANVISA) bioanalytical method validation guidelines (FDA, 2001; ANVISA, 2003). The following parameters were considered.

Linearity (ANVISA, 2003) was determined to assess the performance of the method. A linear least-squares regression with a weighting index of 1/x was performed on the peak areas of nimodipine and IS to nimodipine concentration ratios for the six plasma standards (0.1, 0.5, 2.0,

10, 25 and 40 ng/mL) in triplicate to generate a calibration curve. For the determination of linearity, standard calibration curves of at least six points (non-zero standards) were used. In addition, a blank and zero plasma samples were also analyzed to confirm the absence of interference; these two samples were not used to construct the calibration function. Four out of six non-zero standards, including LLOQ and ULOQ, had to meet the following acceptance criteria: no more than 20% deviation at LLOQ and no more than 15% deviation for standards above the LLOQ. The acceptance criterion for the correlation coefficient was 0.98 or higher (ANVISA, 2003) otherwise, the calibration curve would be rejected. Five replicate analyses were performed.

The method's selectivity was tested by screening six different batches of healthy human plasma. Each blank sample was tested for interference using the proposed extraction procedure and chromatography/mass spectrometry conditions, and compared with those obtained using an aqueous solution of the analyte at a concentration near the LLOQ.

The inter-day precision and accuracy was determined over three days by analyzing 45 QC samples (Hubert *et al.*, 1999). The intra-day precision and accuracy of the assay was measured by analyzing five spiked samples of nimodipine at each QC level (0.3, 15 and 35 ng/mL). The acceptance criteria for precision and accuracy deviation required that values be within 15% of the actual values.

The recoveries were calculated for both nimodipine and dibucaine by comparing peak areas of the extracted samples with the peak areas of unextracted pure authentic standard solutions at three QC levels.

For sensitivity determination, the lowest standard concentration in the calibration curve was considered as the limit of quantification, and had to meet the following criteria: LLOQ response should be five times the response of the blank and the LLOQ response should be identifiable, discrete and reproducible with a precision of 20% and accuracy of 80-120%. The 100 pg/mL concentration was investigated as the lower limit of quantification. Time-dependent precision and accuracy were determined.

Quality control samples were subjected to the following stability tests:

*Short-term stability (autosampler stability)* – Short-term stability was determined by analyzing extracted QC samples kept under autosampler conditions for 10h. Samples were analyzed as mentioned above.

*Freeze and thaw stability* – QC plasma samples containing nimodipine were tested after three freeze (-20 °C) and thaw (room temperature) cycles.

*Long-term stability* – The long-term stability of

nimodipine in human plasma was studied for a period of 61 days employing QC samples at three different levels.

*Standard solution stability* – The stability of standard solutions was evaluated by testing their validity for 6 h at room temperature and after 7 days frozen at -20 °C.

### **Application of the method in a pharmacokinetic study in healthy volunteers**

This method was applied in the analysis of plasma samples after the administration of a single dose of 30 mg nimodipine tablets to healthy volunteers participating in a bioequivalence study. The study was approved by the Federal University of Ceara Ethics Committee. The age of 24 volunteers of both sexes (12 males and 12 females) ranged from 20 to 40 years (mean  $\pm$  SD;  $25.4 \pm 6.19$ ), and subjects had a body mass index (BMI) ranging from 19.7 to 27.6 kg/m<sup>2</sup> ( $22.8 \pm 2.17$ ). All subjects gave their written informed consent, and the Ethics Committee of Federal University of Ceara approved the clinical protocol. The study was conducted in accordance with the provisions of the Declaration of Helsinki (1964), Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (1996) and Edinburgh (2000) revisions.

The study had a single dose, two-way randomized crossover design with a seven days washout period between doses. During each period, the volunteers were hospitalized at 09:00 p.m. and after an overnight fast period they received (at approximately 7:00 a.m.) a single dose of each nimodipine formulation with 200 mL of water. Two hours after drug administration, a light breakfast was provided and a standard lunch and dinner were consumed 5 and 11h after dosing, respectively. No other food was permitted during the "in-house" period, and liquid consumption was allowed ad libitum after lunch (with the exception of xanthine-containing drinks, including tea, coffee, and cola). Concomitant medication was not allowed.

Blood samples (9 mL) from a suitable antecubital vein were collected into heparin-containing tubes before and, 0.167, 0.25, 0.333, 0.5, 0.667, 0.833, 1, 1.25, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36 and 48h after the administration of the nimodipine formulation. The blood samples were centrifuged at 2000 g for 10 min at room temperature and the plasma was removed and stored at -20°C until assayed for its nimodipine content. All samples from a single volunteer were analyzed in the same run in order to avoid inter-assay variations.

Pharmacokinetic parameters were determined from the plasma concentration-time data. The first-order terminal elimination rate constant ( $K_e$ ) was estimated by linear regression from the points describing the elimination pha-

se on a log-linear plot. The elimination half-life ( $t_{1/2}$ ) was derived from this rate constant ( $t_{1/2} = \ln(2)/K_e$ ) where  $t_{1/2}$  is the half-life time,  $\ln(2)$  is the Neperian logarithm and  $K_e$  is the elimination rate constant. The maximum observed plasma concentration ( $C_{max}$ ) and the time taken to achieve this maximum level ( $T_{max}$ ) were obtained directly from the curves. The areas under the curve for nimodipine plasma concentration versus time for 0-48h ( $AUC_{0-48h}$ ) were calculated by applying the linear trapezoidal method. The extrapolation of this area to infinity ( $AUC_{0-\infty}$ ) was done by adding the value  $C_{48}/K_e$  to the calculated  $AUC_{0-48}$  where  $C_{48}$  is the nimodipine plasma concentration at 48h and  $K_e$  is the first-order terminal elimination rate constant.

Bioequivalence (BE) between the reference and test formulations was assessed on the basis of maximum plasma concentration of nimodipine ( $C_{max}$ ) and area under the plasma concentration versus time curve from time 0 to the last sampling time ( $AUC_{0-48h}$ ) by calculating the mean ratios and 90% confidence interval (CIs) on log-transformed data. The inclusion of the 90% CIs for the ratios in the 80% to 125% bioequivalence interval, were analyzed using a parametric test (ANOVA). The variability between the groups (sequence), periods and formulations was evaluated using ANOVA test. The computer programs used to calculate the pharmacokinetic parameters ( $C_{max}$  and AUC) and to carry out statistical analysis were Win-NonlinTM, version 3.1 (Pharsight, Mountain View, CA); EquivTest, version 2.0 (Statistical Solution Ltd., Saugus, MA); and GraphPad Prim, version 2.0 (Software Inc., San Diego, CA).

## RESULTS AND DISCUSSION

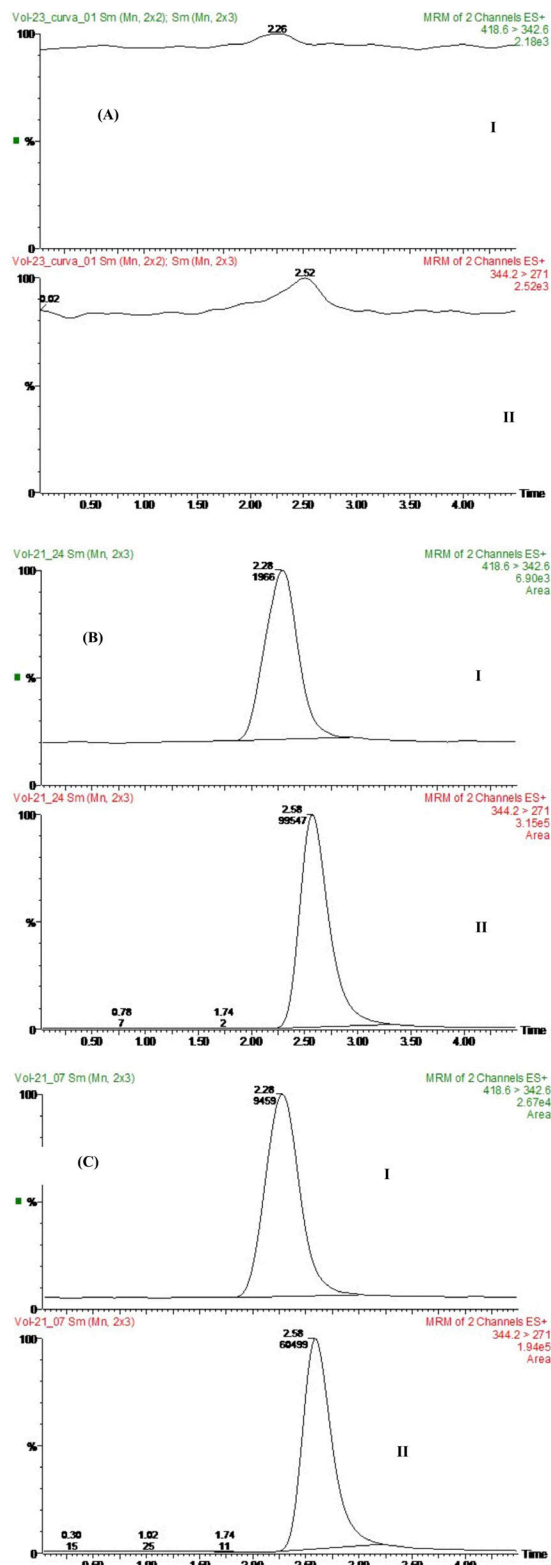
### Method validation

#### Separation and specificity

LC-MS/MS method in multiple reaction monitoring (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.

Nimodipine and IS gave protonated precursor molecular ions  $[MH]^+$  in the MS mode. The major ions observed were  $m/z = 418.6$  for nimodipine and  $m/z = 344.2$  for the IS. The most intense product ions observed in the MS/MS spectra were  $m/z = 342.6$  for nimodipine and  $m/z = 271.0$  for IS.

Figure 2 (A) shows that, due to the high specificity of the MS/MS technique, no endogenous interfering pe-



**FIGURE 2** - Representative MRM chromatograms: (A) a blank plasma sample; (B) a blank plasma sample spiked with nimodipine (15 ng/mL) and dibucaine (50 ng/mL); (C) a volunteer sample 0.5 h after oral administration of nimodipine (30 mg) in healthy volunteer; Peaks I and II refer to nimodipine and dibucaine.

aks appeared at the retention times of the compounds of interest. Figure 2 (B) also shows that under the described chromatographic conditions, the retention times were 2.28 min and 2.58 min for nimodipine and dibucaine, respectively. The total HPLC-MS-MS analysis time was 4.5 min per sample.

Most reported methods require extensive sample work-up and laborious, time-consuming extraction procedures. Although many of these methods offer the advantage of being specific, they do require large volumes of plasma and/or are not economically feasible for routine use in most bioavailability, pharmacokinetic or bioequivalence studies where only small volumes of serum/plasma may be obtained.

The LC-MS-MS method described here provides the necessary level of linearity, accuracy and precision for routine analysis of nimodipine in plasma. In addition, the high selectivity of multiple reaction monitoring (MRM) for different compounds meant that a simple extraction procedure could be used with shorter analysis times.

The combination of HPLC with ESI-MS-MS leads to short retention times and yields both high selectivity and sensitivity. ESI is an ionization technique that produces high mass-to-charge  $[M + 1]^+$  precursor ions with minimal fragmentation of the analyte.

#### *Linearity and lower limit of quantification*

The linear regression of the peak area to concentrations ratios was fitted over the concentration range of 0.10–40 ng/mL, and a typical equation of the calibration curve was as follows:  $Y = 0.0502682 X + 0.00630674$  ( $r = 0.993824$ ), where **Y** is the peak area ratio of nimodipine to dibucaine, and **X** is the concentration of nimodipine, and **r** is the correlation linear coefficient. Results of six representative calibration curves for HPLC-MS-MS determination of nimodipine are given in Table I.

The present LC-MS-MS method offered an LLOQ of 100 pg/mL with a mean accuracy of 7.0% and a mean precision of 103.0% ( $n=5$ ), which is more sensitive than or comparable to reported methods (Krol *et al.*, 1984; Jackobsen *et al.*, 1986; Rosseel Bogaert, Huyghens, 1990; Qian, Gallo, 1992; Aymard *et al.*, 1998; López *et al.*, 2000; Blardi *et al.*, 2002; Mück, 1995; Qiu *et al.*, 2004; Nirogi *et al.*, 2006; Qin *et al.*, 2008). Data for LLOQ are presented in Table II. With the present LLOQ of 100 pg/mL, the nimodipine concentration can be determined in plasma samples up to 48 h after a single oral dose of 30 mg nimodipine, which is sufficiently sensitive to investigate the pharmacokinetic behavior of nimodipine, to establish the relationship between dose and pharmacological effect and to identify doses that produce toxic responses in humans.

The presented method is not entirely different from those described earlier (Gualano *et al.*, 1999; Qiu *et al.*, 2004; Nirogi *et al.*, 2006; Qin *et al.*, 2008) but combines lower plasma volume (300  $\mu$ L), short retention time (2.28 min for nimodipine and 2.58 min for dibucaine), a simple extraction procedure, sensitivity (100 pg/mL), accuracy, precision and linearity.

#### **Precision and accuracy**

The intra-day and inter-day precision and accuracy of the assay were measured by analyzing five spiked samples of nimodipine at each QC level (0.3, 15 and 35 ng/mL). The results for intra-day and inter-day precision and accuracy are summarized in Table II. These results were within the acceptance criteria for precision and accuracy which stipulate that the deviation of values should be within 15% of the actual values (Shah *et al.*, 2000; FDA, 2001; ANVISA, 2003).

#### **Recovery**

The mean extraction recovery of nimodipine, determined at three concentrations of the quality controls, were 71.5 (CV 3.09%), 70.51 (CV 1.64%), 74.02% (CV 4.23%), respectively. The mean extraction recovery of dibucaine was found to be 84.06% (4.78%).

Several kinds of liquid-liquid extraction and solid phase extraction methods have been published for the extraction of nimodipine from plasma samples (Mück, Bode, 1994; López *et al.*, 2000). The solid phase extraction is laborious and requires SPE cartridges, increasing the cost of the analysis. In this experiment, different liquid-liquid extraction conditions were evaluated including different extraction solvents. Four organic extraction solvents (hexane/*n*-butyl alcohol hexane/isoamyl alcohol, diethyl ether/dichloromethane and hexane/ethyl acetate) were evaluated. Among the tested solvents, hexane/ethyl acetate gave the best recovery values for both the drug and internal standard.

#### **Stability studies**

Short-term stability indicated reliable stability behavior under the experimental conditions of the regular runs. Results are given below in Table III. The data that represent the stability of nimodipine plasma samples over three cycles of freezing and thawing are given in Table III. The results indicate that the analyte is stable in human plasma for three freeze-thaw cycles, when stored at  $-20^\circ\text{C}$  and thawed to room temperature.

**TABLE I** - Data of concentrations of nimodipine calibration standards and statistics for precision and accuracy from six representative calibration curves

Nominal Concentration (ng/mL)	Mean $\pm$ SD (ng/mL)	Precision (%)	Accuracy (%)
0.10	0.1 $\pm$ 0.01	10.0	100.0
0.50	0.5 $\pm$ 0.01	2.0	100.0
2.0	1.91 $\pm$ 0.04	2.18	95.33
10.0	9.3 $\pm$ 0.07	0.76	92.97
25.0	26.3 $\pm$ 1.17	4.46	105.20
40.0	42.48 $\pm$ 0.1	0.24	106.21

Equation of the calibration curve:  $Y = 0.0502682 X + 0.00630674$  ( $r = 0.993824$ )

**TABLE II** - Analytical precision and accuracy of nimodipine determination in spiked plasma samples, and of the lower limit of quantification (n=5)

Intra-day	LOQ	LQC	MQC	HQC
<b>Nominal Concentration, ng/mL</b>	0.1	0.3	15	35
Mean $\pm$ SD	0.1 $\pm$ 0.01	0.30 $\pm$ 0.03	16.36 $\pm$ 0.13	38.40 $\pm$ 0.53
Precision (%)	0.0	8.57	0.81	1.37
Accuracy (%)	110	100.67	109.09	109.72
<b>Inter-day</b>				
Mean $\pm$ SD	0.103 $\pm$ 0.01	0.31 $\pm$ 0.02	15.99 $\pm$ 0.66	38.39 $\pm$ 1.29
Precision (%)	7.0	5.46	4.12	3.37
Accuracy (%)	103	102.89	106.60	109.69

Table III summarizes the long-term stability data of nimodipine in plasma samples stored for a period of 61 days at  $-20^{\circ}\text{C}$ . The stability study of nimodipine in human plasma showed good stability behavior as the mean values of the tested samples were within the acceptance criteria of  $\pm 15\%$  of the initial values of the controls. These findings indicate that the storage of nimodipine plasma samples at  $-20^{\circ}\text{C}$  is adequate for up to 61 days, and no stability-related problems would be expected during the routine analysis of samples for pharmacokinetic, bioavailability or bioequivalence studies.

The stability of working solutions was tested and established at room temperature for 6h. Based on the results obtained, working solutions were prepared freshly just spiking samples for the calibration curve and the QC's. The results revealed optimum stability for the prepared working solutions throughout the period intended for their daily use.

### Pharmacokinetic study

Nimodipine was well tolerated at the doses administered and no significant adverse reactions were observed

or reported. No biochemical parameters presented any clinically relevant alterations. There were no drop-outs, and all volunteers who started the study continued to the end and were discharged in good health.

The method was applied to determine the plasma concentration of nimodipine following a single oral administration (30 mg) to 24 healthy volunteers. The mean (SD) concentration-time profiles for the two brands of nimodipine tablets are shown in Figure 3.

The pharmacokinetic parameters for both formulations are shown in Table IV. Mean peak plasma concentration of nimodipine was reached at 9.47 ng/mL (test formulation) and 18.76 ng/mL (reference formulation) at 2.34 and 0.98 h, respectively, after a single oral dose and then levels declined and remained detectable up to 48 h. The extent of absorption is a key characteristic of drug formulation, and therefore, AUC is an important parameter for comparative bioavailability studies (FDA, 2001). However, two other parameters,  $C_{\max}$  and  $T_{\max}$ , are also important features of the therapeutic use of a drug (Chow, Liu, 2000) and hence were also considered in this study.

The statistical comparison of  $AUC_{0-48h}$  and  $C_{\max}$  clearly indicated a significant difference in the two brands of

**TABLE III** - Short-term stability, freeze-thaw stability, standard solution stability and long-term stability tests of nimodipine in human plasma at different QC levels

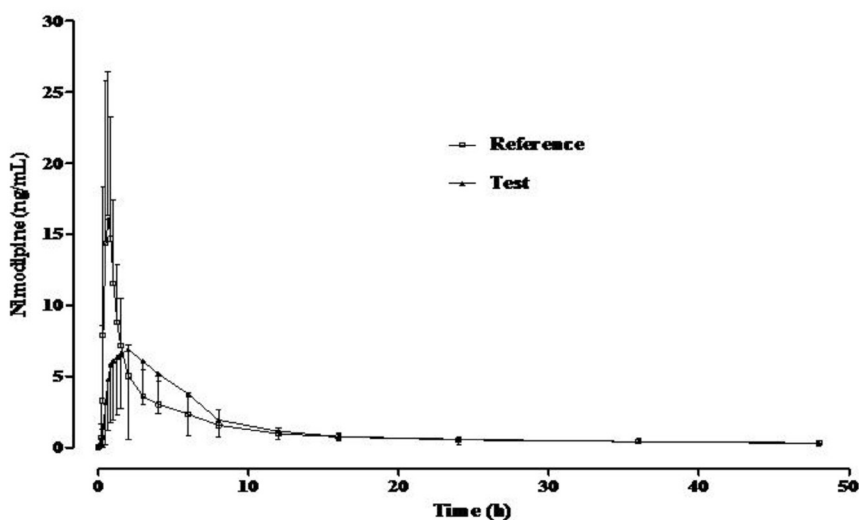
<b>Short-term stability test (values in ng/mL)</b>						
	Reference values: Low sample	Values after 10 h	Reference values: Middle sample	Values after 10 h	Reference values: High sample	Values after 10 h
Mean	0.33	0.32	16.85	16.42	39.15	37.49
Precision (%)	0.0	5.41	0.46	1.07	0.46	0.97
Accuracy (%)	110.0	106.66	112.33	109.48	111.85	107.11
<b>Freeze-thaw stability test (values in ng/mL)</b>						
	Reference values: Low sample	Values after 3 cycles	Reference values: Middle sample	Values after 3 cycles	Reference values: High sample	Values after 3 cycles
Mean	0.32	0.32	16.60	16.49	37.98	39.15
Precision (%)	0.01	0.01	0.21	0.18	1.02	0.33
Accuracy (%)	108.0	106.89	110.69	109.94	108.5	111.86
<b>Standard solution stability test - 6 h room temperature (values in ng/mL)</b>						
	Reference values: Low sample	Values after 6 h	Reference values: Middle sample	Values after 6 h	Reference values: High sample	Values after 6 h
Mean	0.32	0.33	16.60	16.57	37.98	39.53
Precision (%)	2.76	3.50	1.28	2.06	2.70	1.08
Accuracy (%)	108.0	108.67	110.69	110.45	108.5	112.95
<b>Standard solution stability test - 7 days frozen at -20 °C (values in ng/mL)</b>						
	Reference values: Low sample	Values after 7 days	Reference values: Middle sample	Values after 7 days	Reference values: High sample	Values after 7 days
Mean	0.32	0.29	16.60	14.60	37.98	33.87
Precision (%)	2.76	2.44	1.28	1.48	2.70	1.0
Accuracy (%)	108.0	96.67	110.69	97.39	108.5	96.78
<b>Long-term stability test (values in ng/mL)</b>						
	Reference values: Low sample	Values after 61 days	Reference values: Middle sample	Values after 61 days	Reference values: High sample	Values after 61 days
Mean	0.28	0.27	14.80	16.08	35.32	37.63
Precision (%)	1.59	3.26	1.11	3.36	1.14	1.97
Accuracy (%)	94.0	91.33	98.65	107.17	100.9	107.51

nimodipine tablets. The two brands of nimodipine were not bioequivalent (Table V). In Table VI, the results for  $T_{max}$  demonstrated that there was a difference between the mean  $T_{max}$  of the test formulation and the mean  $T_{max}$  of the reference formulation ( $p = 0.02$ ), with a 90% confidence interval.

The variability of the data studied (Tables VII and VIII) shows that for both  $C_{max}$  and  $AUC_{0-48h}$  the effect of sequence is not significant ( $P = 0.1188$  for  $C_{max}$  and  $P = 0.7829$

for  $AUC_{0-48h}$ ). In addition, it was observed that the effect of period is significant ( $P = 0.0164$ ) for  $C_{max}$ , but insignificant for  $AUC_{0-48h}$  ( $P = 0.2226$ ). One possible cause for the period effect is the washout. In this case all the volunteers respected the correct washout period, more than seven half-lives (ANVISA, 2006). In a crossover study, the washout period is the time necessary for the drug received in the first phase of the study to have been completely metabolized and/or eliminated from the body before starting the second study





**FIGURE 3** - Mean plasma nimodipine concentration versus time profile obtained after a single oral administration of 30 mg nimodipine in tablet formulations.

**TABLE IV** - Descriptive statistics of the pharmacokinetic parameters of nimodipine 30mg from reference and test formulation

	AUC <sub>0-∞</sub> (ng*h/mL)		AUC <sub>0-48h</sub> (ng*h/mL)		C <sub>max</sub> (ng/mL)		T <sub>max</sub> (h)		t <sub>1/2</sub> (h)	
	R	T	R	T	R	T	R	T	R	T
<b>Mean</b>	71.62	73.18	57.22	60.66	18.76	9.47	0.98	2.34	32.78	27.83
<b>Geometric Mean</b>	69.44	70.03	54.22	60.27	15.04	8.38	0.78	1.86	29.51	25.04
<b>SD</b>	16.88	23.82	17.76	25.38	10.57	5.80	0.94	1.58	15.58	12.23
<b>Minimum</b>	39.37	41.97	27.49	32.81	3.57	3.77	0.32	0.66	14.52	7.5
<b>Maximum</b>	97.84	147.15	89.66	144.11	34.62	33.51	4	6	71.5	65.36
<b>Median</b>	73.51	67.86	58.03	54.73	19.96	8.42	0.75	2.00	28.33	27.03
<b>CV (%)</b>	95.46	56.33	47.52	31.04	23.57	67.56	61.27	43.94	41.83	32.54

AUC<sub>0-∞</sub> = area under curve for drug concentration versus time from 0 (zero) extrapolated to infinity; AUC<sub>0-48h</sub> = area under the curve for drug concentration versus time from time zero to the time of the last concentration greater than the limit quantification; C<sub>max</sub> = maximum plasma concentration; SD = standard deviation; t<sub>1/2</sub> = plasma elimination half-life; T<sub>max</sub> = time to reach maximum concentration.; T = test formulation; R = reference formulation.

**TABLE V** - Average Bioequivalence Analysis Results for Test/Reference Ratios after Log Transformation for AUC<sub>0-48h</sub> and C<sub>max</sub> Parameters

Test/Reference	Geometric Mean 90% CI	90% CI	Power to test	Percent intra-subject CV%
C <sub>max</sub>	55.73	(44.73-69.42)	23.79	46.59
AUC <sub>0-48h</sub>	104.56	(94.80-115.32)	87.96	19.94

AUC<sub>0-48h</sub> = area under the curve for drug concentration versus time, from time zero to the time of the last concentration greater than the quantification limit; C<sub>max</sub> = maximum plasma concentration.

**TABLE VI** - Analysis of the individual difference of T<sub>max</sub> (test – reference)

Difference of T <sub>max</sub> (test – reference)	90% CI	p-value
1.36	(0.71;2.00)	0.002

T<sub>max</sub> = time to reach maximum concentration; 90% CI= 90% Confidence interval.

**TABLE VII** - Result of analysis of variance (ANOVA) to assess the effects of sequence and period for  $C_{max}$ , log transformed, after administration of the test and reference formulations to 24 healthy volunteers

Source	Degrees of freedom	Sum of Squares	Mean Square	F	P
<b>Inter-Individual</b>					
Sequence	1	1.3058	1.3058	2.6333	0.1188
Residues (inter)	22	10.9093	0.4958	2.5235	0.0174
<b>Intra-Individual</b>					
Drug	1	4.1027	4.1027	20.8794	0.0001
Period	1	1.3241	1.3241	6.7386	0.0164
Residues (intra)	22	4.3229	0.1964		
<b>Total</b>	<b>47</b>	<b>21.9649</b>			

**TABLE VIII** - Result of analysis of variance (ANOVA) to assess the effects of sequence and period for  $AUC_{0-48h}$ , log transformed, after administration of the test and reference formulations to 24 healthy volunteers

Source	Degrees of freedom	Sum of Squares	Mean Square	F	P
<b>Inter-Individual</b>					
Sequence	1	0.0168	0.0168	0.0777	0.7829
Residues (inter)	22	4.7590	0.2163	5.5403	8.391E-05
<b>Intra-Individual</b>					
Drug	1	0.0238	0.0238	0.6113	0.4426
Period	1	0.0614	0.0614	1.5747	0.2226
Residues (intra)	22	0.8589	0.0390		
<b>Total</b>	<b>47</b>	<b>5.7201</b>			

period, i.e. this time must be sufficiently long that no residue is found from one treatment to another. The presence of residual effects influences the statistical inference of bioequivalence between the formulations (ANVISA, 2002).

The observed pharmacokinetics differences can be explained by possible variations in pharmaceutical development during the formulation process, such as the use of different raw materials or distinct amounts of the excipients, size of the particle or the crystalline form of excipient, lipid-soluble or water-soluble, and hydration, among others (Ansel, Popovich, Allen Jr, 2000).

According to the literature, the  $t_{1/2}$  of nimodipine is approximately 8-9h but the initial decline in plasma concentration occurs faster, equivalent to a half-life of 1 to 3h. (Yan, Ding, Liu, 1993; Lian-Quing, Heng-Shan, Gang, 1993; Dollery, 1999; Blardi *et al.*, 2002; Qiu *et al.*, 2004; Zhonggui *et al.*, 2004; Hernandez-Hernandez *et al.*, 2002). Previous studies showed these data, but sampling was concluded between 5 and 10h after dosing, thereby possibly missing a longer elimination phase. Our results demonstrated, however, that the mean half-life of nimodipine for the two formulations tested was 27.83 h (test formulation) and 32.78 h (reference formulation). Such a discrepancy

could be due to the greater sensitivity of our method, with a limit of quantification of 100 pg/mL, while values found in previous studies were in the range 0.24 to 5 ng/mL (Krol *et al.*, 1984; Jackobsen *et al.*, 1986; Rosseel *et al.*, 1990; Qian, Gallo, 1992; Fischer *et al.*, 1993; Yan *et al.*, 1993; Mück, 1995; Aymard *et al.*, 1998; Gualano *et al.*, 1999; López *et al.*, 2000; Blardi *et al.*, 2002; Qiu *et al.*, 2004; Zhonggui *et al.*, 2004; Nirogi *et al.*, 2006). This improved sensitivity enabled the determination of plasma drug concentrations up to 48 h after drug administration, which was not possible in other studies that allowed the measurement of plasma drug levels only up to 10 h after drug administration.

## CONCLUSION

The proposed method of analysis provided a sensitive and specific assay for nimodipine determination in human plasma. The determination of one plasma sample needed only 4.5 min and more than 320 samples could be assayed daily, including sample preparation, data acquisition and processing stages. The method proved superior in sensitivity and speed to other reported methods. It was shown that this method is suitable for the analysis

of nimodipine in human plasma samples collected for pharmacokinetic, bioavailability or bioequivalence studies in humans.

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