ORIGINAL RESEARCH

A simple method for nicardipine hydrochloride quantification in plasma using solid-phase extraction and reversed-phase high-performance liquid chromatography

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ABSTRACT: A simple and sensitive reversed-phase liquid chromatography method was developed and validated for the determination of nicardipine hydrochloride (NC) in rabbit plasma. Nicardipine hydrochloride and nimodipine, used as internal standard, were initially extracted from plasma by a rapid solid-phase extraction using C_{18} cartridges. After extraction, nicardipine hydrochloride was separated by HPLC on a C_{18} column and quantified by ultraviolet detection at 254 nm. A mixture of acetonitrile–0.02 M sodium phosphate buffer–methanol (45:40:15) with 0.2% of triethylamine of pH of 6.1 was used as mobile phase. The mean ($\pm$SD) extraction efficiency of NC was 77.56 ± 5.4, 84.23 ± 4.32 and $83.94 \pm 3.87\%$ for drug concentrations of 5, 25 and 100 ng/mL, respectively. The method proved to be linear in the range of 5–100 ng/mL with a regression coefficient of 0.9993. The relative standard deviations of intra- and inter-day analysis for NC in plasma were 3.26–6.52% ($n = 5$) and 4.71–9.38% ($n = 5$), respectively. The differences of the mean value measured from the concentration prepared, expressed in percentages (bias percentage), were only -5.2, 0.4 and 0.8% at NC 5, 25 and 50 ng/mL, which confirmed the accuracy of the method. The analytical technique was used to determine NC plasma concentration after drug oral administration to rabbits. The results inferred that NC is rapidly absorbed in rabbits and has a short half-life ($t_{1/2} = 1.34$ h). Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: Nicardipine Hydrochloride; reversed-phase HPLC; solid-phase extraction

INTRODUCTION

Nicardipine hydrochloride (NC) is a calcium-channel blocking agent, which is widely used in the management of mild to moderate hypertension, angina pectoris and cerebrovascular disease due to its potent vasodilating activity (Iwanami *et al.*, 1979; Sorkin and Clissold, 1987; Terai *et al.*, 1981). This drug, like other dihydropyridine derivatives, undergoes rapid absorption and extensive biotransformation in the liver, after oral administration to humans and laboratory animals, which display relatively low plasma concentrations (Higuchi and Shrobara, 1980; Sorkin and Clissold, 1987). For this reason, a sensitive and specific method is required for NC quantification in plasma samples.

Several methods for NC determination in human or laboratory animals plasma have been reported, including gas chromatography (GC) (Watari *et al.*, 1990), GC with electron-capture detection (Higuchi *et al.*, 1975), GC-mass spectrometry (Marciniec and Kujawa, 1995),

combination of thin-layer chromatography with GC and mass spectrometric detection (Higuchi and Kawamura, 1981) and voltammetry (Obendorf and Stubauer, 1995). However, some of these methods are too complex and cumbersome for the routine analysis of large numbers of samples and involve unconventional, specialized and expansive equipment (Higuchi and Kawamura, 1981) and others are not sensitive enough for accurate quantification of low therapeutic concentrations of NC (Obendorf and Stubauer, 1995), which limits their application. In addition, some of these methods (eg GC techniques) are relatively non-specific because require NC oxidation prior to the analysis.

In recent years, high-performance liquid chromatography (HPLC) either with ultraviolet (Greiner *et al.*, 1988; Kobayashi, 1987; Li *et al.*, 1998; Uno *et al.*, 1997) or electrochemical detection (Eastwood *et al.*, 1990; Telting-Diaz *et al.*, 1991) has overcome some of these difficulties and has been extensively used to determine NC in biological samples. Nevertheless, almost all the published methods of HPLC for the measurement of NC in plasma, involve relatively laborious and tedious liquid–liquid extraction procedures prior to HPLC analysis, which limit their application for the analysis of large number of samples resulting from clinical or experimental research. The HPLC method described here

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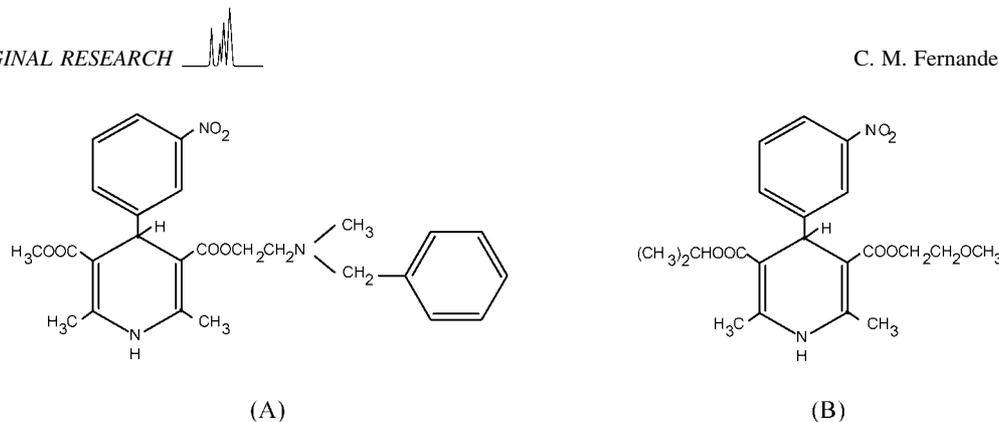**Abbreviations used:** NC, nicardipine hydrochloride; NM, nimodipine.

Figure 1. Chemical structures of nicardipine (A) and nimodipine (B).

overcomes this question by means of a rapid solid phase extraction (SPE) procedure followed by the direct injection of the extract into the chromatographic system.

A simple, rapid and precise method for the determination of NC in plasma, involving solid phase extraction for sample preparation, followed by a reversed-phase HPLC technique, was developed, optimized and validated. Further, the analytical technique was applied to characterize the pharmacokinetic profile of NC after oral administration to rabbits.

EXPERIMENTAL

Materials and reagents. NC (100.37%) and nimodipine (NM) (98.7%), used as internal standard, were purchased from Effechem SRL (Milan, Italy) and Sigma-Aldrich (Steinheim, Germany), respectively. The chemical structures of both drugs are shown in Fig. 1. Acetonitrile, methanol, ethanol and chloroform were of HPLC grade (Merck). All other reagents and chemicals were of analytical grade (Merck and Sigma-Aldrich) and water was obtained by a Millipore Elix 5 system.

The Spe-ed C_{18} cartridges (100 mg of sorbent amount) used for the solid phase extraction were obtained from Applied Separations (Allentown, USA). The vacuum manifold column processor for SPE and the corresponding drying attachment were purchased from Lida (Kenosha, USA).

Since NC and NM are light sensitive, almost all experiments were carried out in a darkroom under yellow light (Philips Powertone SON E27), in order to avoid photodecomposition. When this photoprotection was impossible to achieve, all samples were protected from light by wrapping the vials with aluminum foil.

Apparatus and chromatographic conditions. The HPLC system used consisted of a Hewlett Packard (Waldbronn, Germany) model 1050 quaternary solvent-delivery pump, equipped with a HP1050 multiple wavelength UV detector operated at 254 nm and an injector with a 20 μL loop.

The reversed-phase chromatographic separations were carried out on a stainless-steel column (250 × 4 mm i.d.) packed with Licrospher® 100 RP$_{18}$ (mean particle size 5 μm, Merck) maintained at 30°C. A guard column similarly packed (Licrospher® 100 RP$_{18}$, 5 μm, Merck) was used before the analytical column.

A mixture of acetonitrile–aqueous 0.02 M sodium phosphate buffer-methanol (45:40:15, v/v) containing 0.2% (v/v) of triethylamine with pH of 6.1 was used as mobile phase. In previous studies, various mobile phases were tested with different organic solvents contents and pH. The pH of the mobile phase was adjusted using either 1% (v/v) phosphoric acid and 0.1 N sodium hydroxide solution. The final eluting system was selected in terms of optimum resolution and peak shapes of NC with a short retention time. The HPLC eluent was filtered through 0.45 μm membrane filters (PVDF, Tracer) and was degassed by sonication or with a vigorous stream of helium. The mobile phase was delivered isocratically at a flow rate of 1.2 mL/min.

Chromatographic peak heights were measured using a Hewlett Packard model 3396A recording integrator.

Preparation of standard solutions. Stock standard solutions of NC and NM were prepared in absolute ethanol at a concentration of 3.4 and 2.5 μg/mL, respectively, by diluting the corresponding 1 mg/mL ethanolic solution. All the stock solutions were kept at 4°C in glass wrapped with aluminum foil.

For each validation run, a 100 ng/mL solution of NC in plasma was freshly prepared by appropriate dilution of the 3.4 μg/mL ethanolic solution with NC-free rabbit plasma.

Procedure for sample preparation. The internal standard (20 μL of the 2.5 μg/mL ethanolic solution) and 30 μL of 0.1 M NaOH solution were added to 800 μL of plasma sample. After 30 s of vortex mixing, the mixture was applied to a Spe-ed C_{18} cartridge that had previously been conditioned with 3 mL of ethanol and 3 mL of water. After the complete aspiration of the sample solution through the cartridge, the extraction column was air dried under vacuum for 2 min with a SPE column processor. The cartridge was then washed with 3 mL of water and the residual water was removed from the extraction column under vacuum for 5 min. The desired fraction was further eluted with four portions of 200 μL of chloroform. Among various organic solvents tested for the elution procedure, chloroform proved to be the most efficient and selective. The eluate was evaporated to dryness under a stream of nitrogen in vacuum and at room temperature. The residue was dissolved in 80 μL of the HPLC eluent. After 1 min of vortex mixing, 50 μL of the sample solution were injected into the HPLC system for analysis.

Validation of the method. The linearity of the method was tested between 5 and 100 ng/mL. The spiked plasma standards

containing 5, 10, 25, 50 and 100 ng/mL of NC were prepared by dilution from the 100 ng/mL plasma solution freshly prepared. The different spiked plasma samples were processed according to the extraction procedure described above. A calibration curve was constructed by plotting the NC/NM peak-height ratios against the corresponding NC concentrations in plasma. The plot was subject to a linear regression analysis and the unknown NC concentrations were determined from the regression equation. The data obtained were used for the subsequent study of the validation parameters.

NC standard plasma solutions covering different concentration levels (5, 25 and 100 ng/mL), prepared by diluting the 100 ng/mL plasma solution, were used to determine the precision and the accuracy of the method. For the inter-day assay the standards were prepared over a 5-day period, while the intra-day assay was performed for 1 day by analyzing each concentration five times. The NC standard plasma solutions were also processed by the extraction technique previously reported.

To establish the extraction efficiency, NC plasma standard solutions were prepared by adding aliquots of the 100 ng/mL plasma solution to drug-free plasma, at three different concentration levels (5, 25 and 100 ng/mL). The recovery percentage of NC was determined by comparing the drug/internal standard peak height ratios after extraction of the spiked samples with the corresponding ratios obtained after direct injection of non-extracted NC standard solutions in mobile phase, at same concentrations. The direct injection analysis was considered as the 100% of recovery. The mean extraction efficiency was obtained by assaying five replicates for each concentration of NC.

In a preliminary study performed during a period of 4 months, NC plasma samples proved to be stable when stored at -80 or $-25°C$.

Drug administration and sampling. Six male rabbits with an average weight of 4 kg were kept under fasting conditions for at least 12 h before dosing. NC (15 mg/kg) was orally administered, in gelatin capsules, to six animals. Blood samples (1.5 mL) were collected in tubes with EDTA, prior to NC administration and at 0.5, 1, 2, 3, 4, 6, 8 and 10 h after the drug administration. Plasma samples were separated by centrifugation at 3000 rpm for 10 min and stored at $-80°C$ until analysis. Baseline plasma samples obtained prior to NC administration at time 0 served as the blank control for each animal.

RESULTS AND DISCUSSION

Procedure for sample preparation

According to preliminary solubility studies, chloroform, acetic acid, ethanol, methanol and acetonitrile (with or without 1% of triethylamine) were tested as elution solvents in the SPE of NC and NM. Some of these solvents caused a poor NC extraction (eg ethanol) and others promoted the coextraction of various constituents of plasma or components of the cartridges (eg methanol and triethylamine), interfering with the HPLC analysis of NC. These results were not in agreement with studies previously reported, which used methanol (Uno *et al.*, 1997) and methanol with 1% of
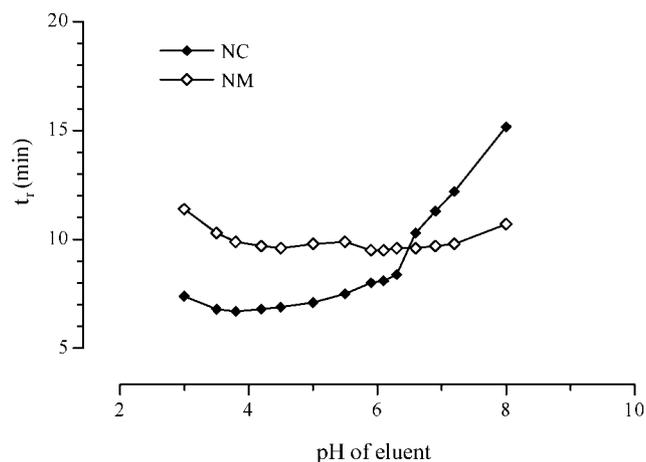

Figure 2. Influence of eluent pH on the retention time of NC and NM.

triethylamine (Meng *et al.*, 1998) as elution solvents. In the present study, chloroform proved to be the most selective and efficient eluting solvent since it greatly reduced the interfering peaks in the plasma chromatograms and gave the highest recovery rates for NC and NM. In addition, to achieve higher extraction efficiency, NaOH should be added to the initial plasma sample, to assure that the whole NC is in the molecular form, by adjusting the pH above the drug pK_a ($pK_a = 7.2$). This procedure will reduce the analyte solubility in the sample solution and improve the retention by the sorbent bed of the cartridge.

Optimization of the chromatographic method

Although the UV absorption maximum of NC and NM was at 238 nm, in this study the absorption at 254 nm was used for quantification. The use of the absorption at 254 nm reduces the theoretical sensitivity to some extent, but has the enormous advantage of providing cleaner chromatograms for plasma samples.

The separation of the analytes, peak shapes and retention times were optimized by modifying the mobile phase constitution and the flow rate until attaining a good resolution in a relatively short run time for each injection (12 min).

The effect of the mobile phase composition on the chromatographic separation was firstly investigated. The results showed that NC and NM retention times were obviously prolonged with decreasing the acetonitrile and/or methanol content or increasing the buffer content. However, the responses of peak height changed only slight with varying composition of the mobile phase. The presence of methanol was found to be necessary to ensure a good separation between NC, NM and plasma interfering peaks. Further, the experimental results showed that the addition of triethylamine in the mobile
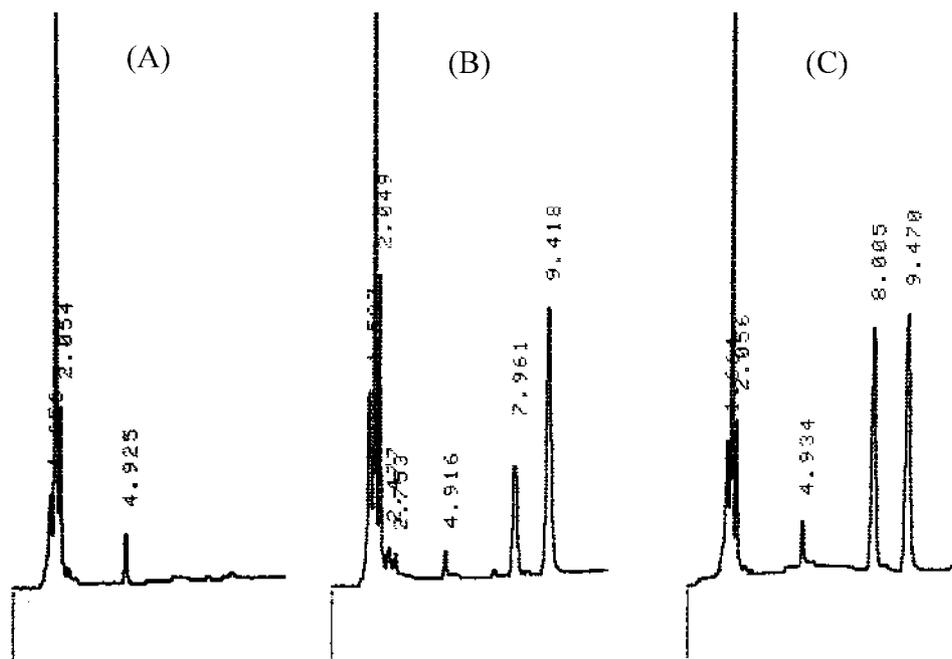
Figure 3. Typical chromatograms of extracted rabbit plasma: (A) rabbit drug-free plasma; (B) rabbit plasma spiked with NC ($t_r = 7.961$ min) and NM ($t_r = 9.418$ min) and (C) plasma sample from an animal treated with 15 mg/kg of NC. t_r = retention time.

phase increased the selectivity and sensitivity of NC, because it changed the retention time and sharpened the drug peak. To maintain the elution order (NC before its internal standard) and the quality of the separation, it was also necessary to adjust the pH of the mobile phase. As can be seen in Fig. 2, the chromatographic behavior of NM, a neutral substance, was not strongly affected by the variation of pH of the HPLC eluent. Nevertheless, this factor played an important role in the retention time of NC, a basic drug. At low values of pH, NC eluted at shorter time, because the high degree of protonation of its tertiary amine function hinders the interactions with the lipophilic stationary phase. An optimal pH of 6.1 was chosen for selecting a proper retention time value that produced no interfering peaks near the specific peak of NC and because is at least 1 pH unit different from the NC pKa, which shifts the equilibrium so that 99% of NC will be in one form. Analytes may sometimes appear as broad or tailing peaks when the mobile phase pH is at, or near, their pK_a values.

From these preliminary studies, we decided to use a mixture of acetonitrile–aqueous 0.02 M sodium phosphate buffer–methanol (45:40:15, v/v) containing 0.2 % (v/v) of triethylamine with pH of 6.1 as mobile phase at a flow rate of 1.2 mL/min.

Typical chromatograms of a standard mixture of NC and NM in rabbit plasma samples, is shown in Fig. 3. Under the chromatographic conditions described above, the observed mean retention times for NC and NM were

8.0 and 9.5 min, respectively. The lack of response in blank plasma was demonstrated.

Validation of the method

As it can be seen in Fig. 3, a good separation of NC with minimal interference with internal standard or with endogenous components of plasma was obtained by the present extraction and chromatographic techniques. It can be concluded that the proposed method is selective for NC.

The calibration curve of NC/NM peak-height vs NC concentration in plasma was linear in the concentration range of 5–100 ng/mL with a correlation coefficient of 0.9993 ($n = 5$), which proved excellent linearity in this concentration range.

The accuracy and precision (intra-day precision or repeatability and inter-day or intermediate precision) of this method, evaluated by assaying five spiked plasma samples containing different concentrations of NC, are summarized in Table 1. The differences of the mean value measured from the concentration prepared, expressed in percentages (bias percentage), were only − 5.2, 0.4 and 0.8% at 5, 25 and 50 ng/mL, which confirmed the accuracy of the method. The range of percentage of relative standard deviation (RSD) was 3.26–6.52% and 4.71–9.38% for within-day and between-day analyses, respectively. The RSD values

Table 1. Accuracy and precision of the method for the determination of NC in rabbit plasma, expressed as bias (%) and relative standard deviation (RSD), respectively ($n = 5$)

Concentration prepared (ng/mL)	Accuracy Bias (%)	Intra-day precision RSD (%)	Inter-day precision RSD (%)
5	−5.20	6.52	9.38
25	0.43	3.26	5.32
100	0.78	4.07	4.71
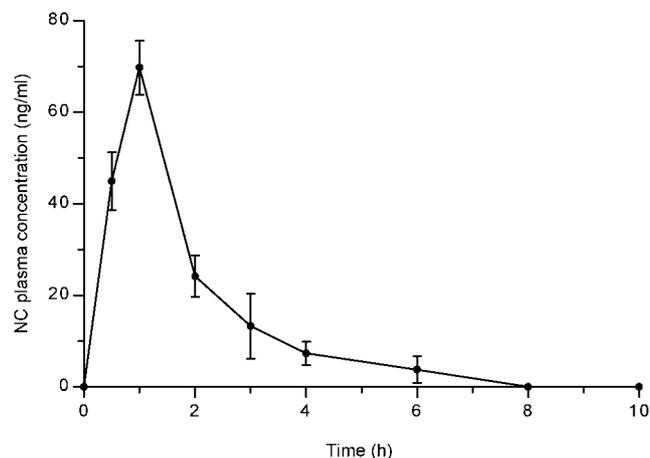
Figure 4. Mean (±SEM) drug plasma concentrations after oral administration of a single dose of 15 mg/kg of NC to six rabbits.

obtained allow us to conclude that the method has acceptable precision, although a slightly greater variation has been found at 5 ng/mL.

The mean (±SD) extraction efficiency for NC from plasma was found to be consistent over the evaluated concentrations: 77.56 ± 5.4, 84.23 ± 4.32 and $83.94 \pm 3.87\%$, for the drug concentrations 5, 25 and 100 ng/mL, respectively, when compared with non-extracted samples containing NC in the same concentration ($n = 5$). The extraction recovery of the internal standard from plasma was 86.6 ± 5.9 ($n = 5$).

Application to pharmacokinetic studies in rabbits

Representative plasma concentration-time profile of NC after administration of 15 mg/kg of drug to rabbits is illustrated in Fig. 4, as evidence of the usefulness of this method. The mean pharmacokinetic parameters were calculated for the six animals. The absorption of NC in rabbits was very rapid, with a time to reach the maximum plasma level (69.5 ng/mL) of 1 h. NC presented a short elimination half-life (1.34 h) and the area under the NC plasma concentration–time curve up to 10 h (AUC_{0-10h}) was estimated as 132 ng h/mL.

CONCLUSION

The present analytical technique consisted of a simple solid-phase extraction procedure and a subsequent reversed-phase HPLC analysis with UV detection, which could be easy reproduced in conventional equipments available in most laboratories. The present method proved to be, according to the validation parameters, selective precise and accurate and also simple, useful and appropriate for studying the pharmacokinetic profile of NC administered to laboratory animals, such as rabbits.

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