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Extraction and quantification of nicardipine in human plasma

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Abstract

A novel simple method of extraction, separation, identification and quantification of nicardipine in human plasma samples was completely studied. The human plasma samples were initially purified by solid-phase extraction (SPE) using a C_{18} cartridge. The extracted samples were separated and nicardipine present in the samples was quantified by high-performance liquid chromatography (HPLC) on a reversed-phase C_{18} column employing a mobile phase consisting of 60% (v/v) acetonitrile in 0.02 M NaH_2PO_4 with pH of 6.3 and a variable wavelength UV detector set at 254 nm. The recovery of nicardipine from plasma samples using selective SPE was 91±6.0% and had less interfering compounds in the HPLC analysis compared to the use of liquid–liquid (L/L) extraction. In the HPLC analysis, examining the effect of pH values of the mobile phase on the capacity factor (k') of nicardipine revealed a method for selecting a critical k' value of nicardipine to eliminate interfering peaks near the peak specific to the analyte. This method for quantification of nicardipine in human plasma samples was suitable for studying the pharmacokinetic profile of nicardipine administered as an intravenous bolus to cardiac surgical patients.

Keywords: Nicardipine

1. Introduction

Nicardipine, a dihydropyridine calcium channel antagonist, causes coronary and peripheral vasodilatation by blocking the influx of extracellular calcium across cell membranes. It has been reported that the action of nicardipine was arterioselective and effective for the treatment of hypertension, myocardial ischemia, or vasospasm in surgical patients [1,2]. Nicardipine has also been used experimentally as a probe to study the effects of calcium channel antagonists on the role of sympathetic nervous system activity in the development of cardiovascular risk [3].

An accurate and sensitive method for measuring nicardipine concentrations in human plasma was necessary for performing pharmacokinetic and pharmacodynamic studies to assess the therapeutic efficacy and physiological actions of the drug in clinical trials. Previously described methods for measuring nicardipine in plasma samples, such as HPLC [4–6], the combination of HPLC with electrochemical detection [7], and thin-layer chromatography (TLC)–gas chromatography (GC)–mass spectroscopy (MS) [8] were found to be unfeasible for clinical use. The methods employing TLC–GC–MS were too com-

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plex and cumbersome for the routine analysis of large numbers of patient samples. Other methods were not sensitive enough for accurate quantification of low therapeutic concentrations of the drug in clinical specimens. Capillary column GC using electron-capture detection (ECD) has been employed to increase the sensitivity of the assay, but was found to be impractical because the capacity of the capillary column of GC was too small to inject large numbers of crude plasma extracts [9]. Recently, a method for enantioselective determination of nicardipine was reported by Uno et al. [10], but their method required a specialized HPLC precolumn that was prepared in their own laboratory that was not available for commercial use. In addition, a high recovery of nicardipine was not achieved using the reported extraction procedure and the potential for oxidative degradation of nicardipine in the HPLC separation procedure was not tested [10].

In this study, a simple and effective HPLC procedure to measure nicardipine concentrations in human plasma samples was developed employing an available C_{18} column. The procedure retained the simplicity of sample preparation, but also permitted the rapid analysis of large numbers of clinical samples. Specific modifications to influence the retention time and separate nicardipine from interfering compounds were developed to improve the detection limits of the HPLC assay. This novel method for sample preparation, identification, and quantification of nicardipine in human plasma was then applied to characterize the pharmacokinetic and pharmacodynamic profiles of different intravenous bolus doses of nicardipine administered to treat acute intraoperative hypertension in patients undergoing cardiac operations.

2. Experimental

2.1. Reagents and materials

Nicardipine and nimodipine used for the internal standards were obtained from ICN (Irvine, CA, USA). C_{18} SPE cartridges were supplied by Varian (Harbor, CA, USA). SPE column processors were obtained from J.T. Baker (Phillipsburg, NJ, USA). Human plasma was obtained from Sigma (St. Louis, MO, USA). All solvents used were of HPLC grade and all other chemicals used were of analytical grade (Sigma).

2.2. Apparatus

The HPLC system consisted of an LC-600 pump, a variable wavelength UV detector SPD-6AV set at 254 nm, and C-R4A chromatopac (all from Shimadzu, Kyoto, Japan), a Type U6K injector valve equipped with a 250 μl sample loop (Waters Assoc., Milford, MA, USA), and an Ultrasphere ODS analytical column (250 mm×4.6 mm I.D., 5 μm particle size) from Beckman (Palo Alto, CA, USA).

2.3. Clinical plasma samples

According to a protocol approved by the University of Pennsylvania Committee on Studies involving Human Beings, and after obtaining written informed consent, patients undergoing cardiac operation were administered a single intravenous bolus dose of nicardipine 0.25, 0.5, or 1.0 mg in a randomized, double-blinded study protocol immediately after sternal closure if the systolic arterial blood pressure exceeded 120 mmHg. Prior to nicardipine administration (time 0) and at 2, 5, 7, 10, 20, 30, 90, 120, 180, and 240 min after injection of the drug, mixed venous blood samples from the pulmonary artery catheter were obtained for assay in heparinized tubes. Plasma was separated by centrifugation at 3000 *g* for 20 min at 4°C and stored at −30°C until analyzed. Baseline plasma samples obtained prior to nicardipine administration at time 0 served as the blank control for each patient.

2.4. Solid-phase extraction (SPE)

An internal standard consisting of 10 μl of 2.4×10^{-5} *M* nimodipine (100 ng) in ethanol was added to 1 ml of each plasma sample. Plasma samples were then mixed by vortex for 30 s, transferred onto a C_{18} cartridge that was conditioned with 2 ml of ethanol, and washed with water. Residual water was removed from the extraction column under a vacuum with the SPE column processor. The final sample was eluted with 1 ml of methanol containing 1% triethylamine (TEA) then evaporated to dryness under a stream of

nitrogen gas at room temperature. Samples were reconstituted in 200 μl of the mobile phase of the HPLC. A 150-μl aliquot of each sample was injected into the HPLC system for analysis.

2.5. Standard solutions

A stock solution of nicardipine was prepared in ethanol at a concentration of 1 mg/ml and stored at 4°C. On each day the assay was performed, six standard solutions for calibration containing 10, 20, 50, 100, 200, and 600 ng/ml of nicardipine were prepared by serial dilution of the stock solution in the HPLC mobile phase. Calibration curves were constructed by plotting the height of the nicardipine peak against the known concentration of nicardipine in the standards and fitted by linear regression analysis.

Quality control samples with nicardipine concentrations of 5 and 40 ng/ml were prepared by adding 10 and 80 μl of 500 ng/ml nicardipine standard solution, respectively, to 1.0 ml of commercial plasma (Sigma) and stored at −80°C. For each validation run, duplicate quality control samples were thawed, extracted, and assayed using the same procedure employed for the clinical samples.

2.6. Recovery

The recovery of analyte and internal standard from the SPE step were estimated by measuring the amount of compound recovered after the extraction step in standard solutions containing known molar quantities of nicardipine and nimodipine. Standard solutions containing 10, 100, and 500 ng/ml of nicardipine or nimodipine in plasma were used to determine the recovery. Blank samples were prepared by adding the equivalent volume of solvent not containing the drug to 1.0 ml of the control plasma samples and analyzed as controls to verify that no peak appeared near to the retention times of either nicardipine or nimodipine in the final HPLC experiments. The percent recovery of nicardipine and nimodipine were determined by comparing the height of the analyte peak measured in the extracted standards to the height of the analyte peak measured directly in spiked unextracted aqueous samples containing the original amount of the analyte.

2.7. HPLC conditions

All HPLC separations were carried out at ambient temperature at a flow-rate of 1.0 ml/min with a mobile phase of 60:40 (v/v) acetonitrile–0.02 *M* NaH_2PO_4. A variable wavelengh UV detector operated at 254 nm. The effects of altering the pH of the mobile phase on the capacity factor (k') of nicardipine was tested to increase the retention time of nicardipine in order to eliminate interfering peaks. From these preliminary data, an optimal pH of 6.3 was chosen for selecting a proper k' value that produced no interfering peaks near the peak specific to nicardipine.

2.8. Accuracy and precision

Chromatographic peaks for nicardipine and nimodipine were identified by retention time and standard addition protocols [11,12]. All of the detected species were assayed by measuring the chromatographic peak heights and drug concentrations determined from the standard calibration curve that was prepared daily. For each validation run, the recovery of nicardipine in plasma was determined using nimodipine as the internal standard. Quality control samples ($n=6$) with nicardipine concentrations of 300 ng/ml that were in the middle of the standard curve used for calibration were used to determine the inter-assay and intra-assay variability. Variability was expressed as standard error of the mean (±S.E.M.). Coefficients of variation were determined for inter-assay and intra-assay variation. To assess the extent of conversion of nicardipine to the pyridine analogue II during extraction and HPLC analysis, nicardipine was intentionally oxidized to its pyridine metabolite II based on an established protocol [9]. The retention times of nicardipine and its pyridine metabolite II were different in the HPLC separation under the conditions of the assay and their chromatographic peaks were easily distinguished.

3. Results

The mean (±S.E.M.) recoveries of nicardipine and nimodipine after the SPE procedure were 91±6.0% ($n=6$) and 93±5.1% ($n=6$), respectively. The re-

covery of nicardipine and nimodipine using liquid–liquid extraction was the same as that obtained with SPE. In our experiments, SPE eliminated more interfering substances in the HPLC analysis than liquid–liquid extraction, so only results obtained using SPE were reported.

Plasma samples spiked with nicardipine standard resulted in an increased amplitude of the single peak height that was specific for the parent compound, nicardipine (Fig. 2C). The absence of a chromatographic peak specific for the pyridine metabolite II, even in samples with supertherapeutic concentrations of nicardipine, suggested that in vitro oxidation of nicardipine to the pyridine metabolite II did not occur during the assay.

The mean (±S.E.M.) measured nicardipine concentration of six plasma samples containing 300 ng/ml of nicardipine extracted by the SPE procedure and injected into the HPLC for analysis was 280±8.1 ng/ml, indicating that the intra-assay coefficient of variation was 2.9%. The mean (±S.E.M.) measured nicardipine concentration of six plasma samples containing 300 ng/ml of nicardipine assayed on 6 different days using the same method was 276±13.8 ng/ml, indicating that the inter-assay coefficient of variation was 5.0%.

The chromatographic peak height for nicardipine was linearly proportional to the plasma nicardipine concentration over the concentration range of 5–600 ng/ml, and exceeded the range of clinically effective plasma concentrations for the drug. Linear regression analysis for each standard curve used in the assay was significant ($P<0.0001$) with correlation coefficients that exceeded 0.999. The *y*-intercept for the linear regression of each standard curve was less than 2% of the response obtained for a targeted plasma nicardipine concentration in the range of 100–300 ng/ml, and not statistically different from 0.

Increasing the pH of the mobile phase caused an increase in the capacity factor (k') of nicardipine in the HPLC separation (Fig. 1). Based on this finding, a pH of 6.3 was selected for the mobile phase to achieve a clear separation and a distinct chromatographic peak for nicardipine and revealed a clean 'window' in the reagent blank for plasma nicardipine (Fig. 2). Under the conditions of the assay, the signal-to-noise ratio for plasma specimens with

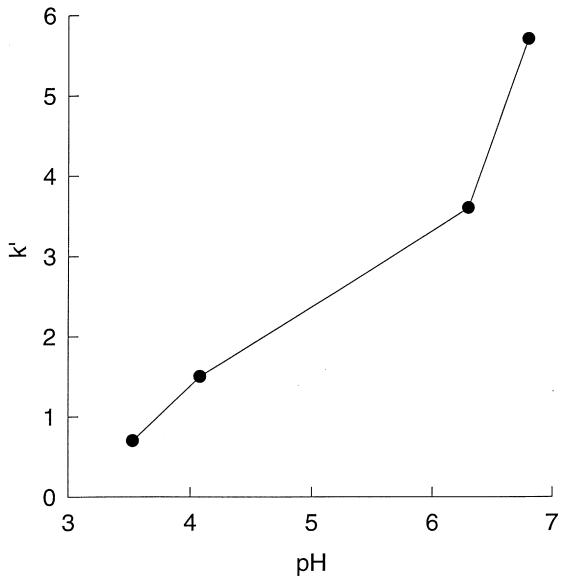


Fig. 1. Increasing the pH value of mobile phase increased the capacity factor k' of nicardipine. Column: Beckmans Ultrasphere ODS column (250×4.6 mm I.D., 5 μm particle size); eluent, 60:40 (v/v) acetonitrile–0.02 *M* NaH_2PO_4 (pH 6.3); flow-rate, 1 ml/min; temperature, ambient.

nicardipine concentrations in the range of 3 ng/ml was 7 (Fig. 2B). Assuming a recovery of 80% after the extraction step and a signal-to-noise ratio of 3 at the detection limit, the assay procedure would have a detection limit of approximately 1.6 ng/ml for nicardipine. A quantitation limit of approximately 5.4 ng/ml for nicardipine was determined by assuming a concentration of the analyte that yielded a signal-to-noise ratio of 10. Because typically only 25% of each sample volume was injected into the HPLC column for measurement, the actual quantification limit for nicardipine that could potentially be achieved was approximately 1.4 ng/ml if the entire sample was injected into the HPLC column. However, nicardipine concentrations less than 5 ng/ml were in the subtherapeutic range for the drug, were not associated with significant changes in arterial pressure, and therefore not clinically important. The initial peak and decline of plasma nicardipine concentrations over time after the intravenous administration of different clinically effective bolus doses of the drug for the treatment of acute intraoperative hypertension were measured using this assay. The results of the assay provided necessary information

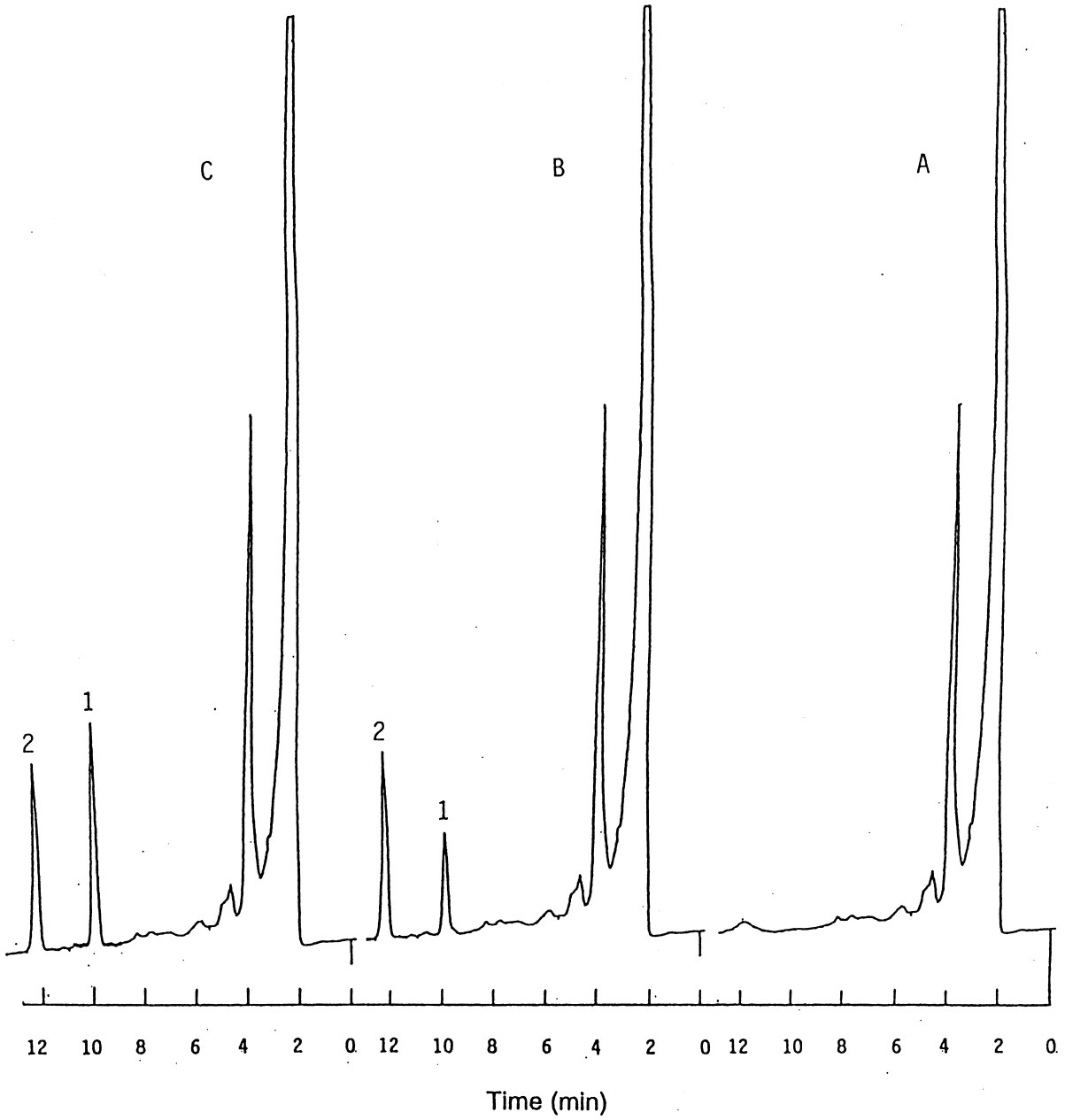


Fig. 2. Typical chromatograms obtained from: (A) extracted patient plasma sample prior to the administration of nicardipine (blank at time 0); (B) extracted patient plasma sample that had a measured nicardipine concentration of 3 ng/ml showing the peak for nicardipine (1) and the peak for the internal standard, nimodipine (2); and (C) same extracted patient plasma sample from (B) that was spiked with 5 ng/ml of nicardipine standard solution resulting only in an increase in the height of the single peak specific to the analyte (1). The signal-to-noise ratio for the nicardipine peak at the concentration of 3 ng/ml was 7. The HPLC conditions were the same as described in Fig. 1.

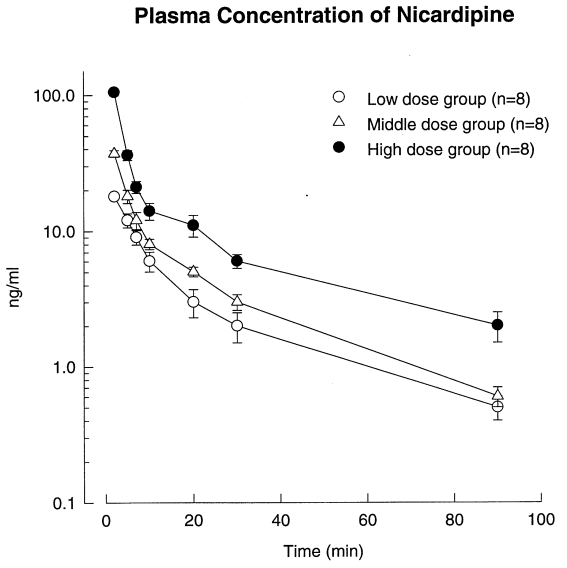


Fig. 3. Mean (±S.E.M.) plasma nicardipine concentrations measured from extracted plasma specimens in patients at different times after the intravenous bolus administration of nicardipine 0.25 mg (low dose group), 0.5 mg (middle dose group), and 1.0 mg (high dose group). The HPLC conditions were the same as described in Fig. 1.

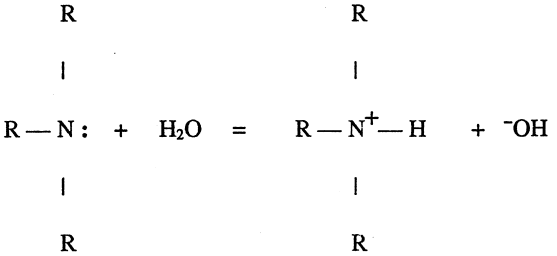
for determining the pharmacokinetic properties of the drug in a clinical setting (Fig. 3).

4. Discussion

A SPE technique was developed and refined to measure plasma nicardipine concentrations in clinical specimens that had a recovery comparable to techniques employing L/L extraction, but eliminated most of the interfering chromatographic peaks in the HPLC separation that were problematic in L/L-extracted samples. Although recent reports suggested that SPE of nicardipine from plasma on C_{18} cartridges yielded a high recovery [10], experiments in our laboratory indicated that recovery of nicardipine using these cartridges were not satisfactory because of incomplete elution by methanol. This problem was overcome successfully by elution using TEA in combination with methanol. Eliminating the interfering chromatographic peaks caused by co-extracted substances present in patient samples permitted the HPLC sensitivity to be improved by operating the detector at a more sensitive attenuation or by using larger sample volumes.

Quantification of nicardipine using packed-column GC was complicated by the oxidation of nicardipine to its pyridine metabolite II during the procedure [4]. As much as 16% of the nicardipine in the original sample was susceptible to oxidative metabolism resulting in the appearance of two distinct chromatographic peaks corresponding to nicardipine and the pyridine metabolite II after samples were injected onto a GC silica column [9]. Silica columns constructed from materials identical to those employed for the GC columns have also been utilized for HPLC analysis of nicardipine concentrations [6]. The exact extent of oxidation of nicardipine in the presence of silica was not predictable, and in vitro oxidation of nicardipine to its pyridine metabolite II may underestimate the actual concentration of the parent drug in the plasma sample. To define whether oxidation of nicardipine occurred in the reversed-phase HPLC assay, a large amount of nicardipine was added to human plasma prior to analysis. No peak associated with the pyridine metabolite II was detected in the procedure, indicating that oxidation of nicardipine to its pyridine metabolite II was negligible when employing a C_{18} column for HPLC separation.

The retention of nicardipine on an unmodified silica column was poor [7]. However, use of an octadecysilicayl gel surface readily provided retention capability because of the polarity of the drug, the character of its hydrophobic side, and the eluotropic strength of the organic solvent of the mobile phase. In addition, adjusting the pH of the mobile phase allowed the retention of nicardipine to be controlled further by altering the function of the tertiary amine on nicardipine based on the following equilibrium reaction:



Increasing the concentration of $[OH]^-$ shifted the

reaction toward the left, decreased the solvation of the amine and its polarity, and allowed its hydrophobic side play a greater role in the distribution of the analyte in the stationary phase, resulting in a longer retention time in HPLC. Therefore, slightly adjusting the pH of the mobile phase permitted the retention behavior of nicardipine to be optimized.

The method described for quantifying nicardipine in plasma samples was tested and applied to measure the plasma concentrations of nicardipine in a clinical trial. The method was sensitive enough to quantify plasma concentrations of physiologically active concentrations of the drug, to permit pharmacokinetic analysis, and to detect differences in peak plasma drug concentrations and clearance rates after different intravenous doses were given for the acute management of hypertension in patients undergoing cardiac operations.

5. Conclusions

A practical method for quantifying therapeutic nicardipine concentrations in plasma specimens that employed SPE and HPLC was developed and tested. The success of the method was attributed to the following: refining the extraction procedure to eliminate interfering chromatographic peaks without compromising recovery; demonstrating that oxidation of nicardipine to its pyridine metabolite II during separation did not occur; and establishing an optimal pH for the mobile phase to control the retention of nicardipine.

Acknowledgements

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