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Short Communication

Sensitive high-performance liquid chromatographic assay for nifedipine in human plasma utilizing ultraviolet detection

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Abstract

A rapid, simple, sensitive and selective reversed-phase high-performance liquid chromatographic (HPLC) technique is reported for the determination of nifedipine in human plasma. The procedure involves extraction of nifedipine from plasma under alkaline conditions (pH 12), separation via reversed-phase HPLC and ultraviolet detection (350 nm). The peak corresponding to nifedipine was free of interference from its photodegradation products or metabolites. The method was validated over the range 5–250 ng/ml nifedipine using weighted least-squares linear regression analysis. Accuracy and precision were within approximately 10% or less over the concentration range, except for the lowest concentration point which, nonetheless, was acceptable and approached 15%. The minimum quantifiable concentration of nifedipine was determined to be 5 ng/ml. The minimum detectable concentration was in the order of 1 ng/ml. Analysis of plasma samples collected from healthy volunteers demonstrate that this assay is applicable to clinical and pharmacokinetic studies.

1. Introduction

Nifedipine, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine-dicarboxylic acid dimethyl ester (Fig. 1a), is the parent compound of the dihydropyridine class of calcium channel antagonists and is widely used for the treatment of hypertension, angina pectoris and other cardiovascular disorders due to its selective dilation of arteries [1]. In humans, nifedipine is rapidly metabolized by oxidative mechanisms to dehydronifedipine (Fig. 1b), which is further metabolized to more polar compounds [2–10]. Nifedipine is highly sensitive to chemical oxidation forming dehydronifedipine upon ultraviolet

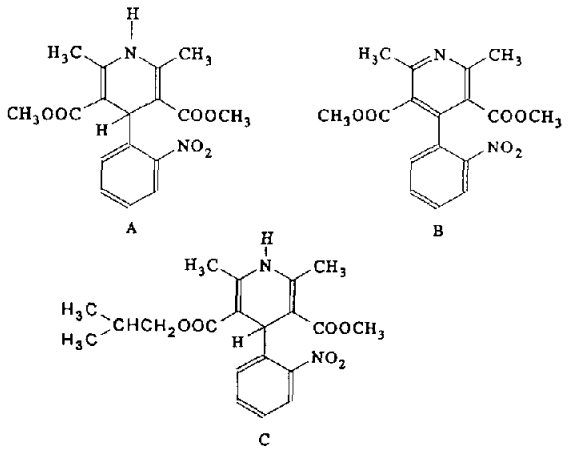

Fig. 1. Structures of (A) Nifedipine, (B) dehydronifedipine, and (C) nisoldipine.

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(UV) exposure, and the nitroso-analogue of dehydronifedipine upon UV-Vis light exposure (*i.e.* daylight) [4–7,10,11]. Analytical methods for detection of nifedipine in various matrices, therefore, must adequately resolve nifedipine from its metabolites or photodegradation products which are devoid of calcium channel blocking activity. Additionally, the assay procedure must be performed under subdued lighting or must utilize sodium lamps [6,7].

To date, most previously reported HPLC methods for determination of nifedipine in biological samples publish the precision of calibration data but usually omit accuracy, especially for nifedipine concentrations below 10 ng/ml [2,3,5–7,10,12–16]. Although detection limits below 10 ng/ml are stated (usually 1–5 ng/ml), the minimum quantifiable concentrations are seldom reported. A summary report on analytical methods validation [17] sponsored by, among others, the U.S. Food and Drug Administration and the Health Protection Branch (Canada), outlines acceptable standards and procedures for development of valid analytical methods. Past nifedipine HPLC assays do not meet all the criteria suggested by the summary report, especially those utilizing UV detection. The study of the pharmacokinetics of nifedipine in humans, however, requires a completely validated assay for concentrations below 10 ng/ml since plasma concentrations of this magnitude are often encountered clinically after immediate or controlled release formulation administration [9,18].

In this paper we report a rapid, sensitive, and selective reversed-phase HPLC assay which meets or exceeds generally accepted criteria for analytical method validation and which is suitable for processing large numbers of nifedipine plasma samples taken during clinical studies.

2. Experimental

2.1. Chemicals

Nifedipine was purchased from Sigma (St. Louis, MO, USA). Internal Standard (I.S., nisoldipine, Fig. 1c) and nifedipine metabolites/photodegradation products were kindly provided by Miles Canada (Etobicoke, Ont., Canada). Methanol and water (HPLC grade) and analytical grade acetic acid and triethylamine (TEA) were obtained from Mallinckrodt (Paris, KY, USA). Isooctane and methyl-*tert.*-butyl ether (MTBE) were purchased from BDH (Toronto, Ont., Canada) and were HPLC grade.

2.2. Chromatography

This assay is a modification of a previously reported nifedipine HPLC assay [14]. Samples were vortex-mixed with a Genie 2 mixer (Fisher Scientific, Edmonton, Canada) and centrifuged with a Dynac II centrifuge (Becton-Dickinson, Parsippany, NJ, USA). Evaporation of solvents utilized a Model SC 100 Savant Speed Vac concentrator–evaporator (Emerston Instruments, Scarborough, Canada). The HPLC system consisted of a Model 600E solvent delivery system, a Model 717 autosampler, a 486 tunable UV-Vis absorbance detector set at 350 nm and a NEC Powermate 486/33i computer with Millennium 2010 chromatography manager v1.1 software (Waters, Mississauga, Ont., Canada). Analytical separation was accomplished using a Nova-Pak 100 mm × 8 mm I.D. radial pack column containing 4 μm C_8 packing material (Waters).

The mobile phase consisted of methanol–water (65:35, v/v) adjusted to approximately pH 4.0 with acetic acid and TEA as 1% and 0.03% final concentration, respectively. The flow-rate was 1.1 ml/min. Sample preparation and analysis were conducted at room temperature under sodium lamps.

2.3. Standard solutions

Stock solutions, 100 μg/ml (as base), of both nifedipine and of I.S. were prepared in methanol. Both solutions were kept protected from light with an aluminum foil covering and stored at 4°C. Both solutions were stable for a period of at least 3 months. A 1 μg/ml solution of

nifedipine in methanol (solution 1) was prepared daily from the nifedipine stock solution. A 100 ng/ml nifedipine solution in methanol (solution 2) was prepared daily from solution 1. A 10 μg/ml solution of I.S. in methanol (solution 3) was prepared daily from the stock I.S. solution.

2.4. Sample preparation

To 1.0 ml of drug-free human plasma, in a disposable glass 125 × 16 mm culture tube, was added nifedipine using either solutions 1 or 2, to yield final concentrations of 5, 10, 15, 25, 50, 100 and 250 ng/ml. After addition of 500 ng of I.S. (solution 3) and 100 μl of 1.0 *M* sodium hydroxide, samples were vortex-mixed for 3 s and 5 ml of MTBE–isooctane (75:25, v/v) was added. The resultant mixture was vortex-mixed for 30 s and centrifuged at 3000 rpm (1800 *g*) for 5 min. The upper organic layer was transferred to a clean dry 100 × 13 mm glass tube and evaporated to dryness (no heat applied) using the Speed Vac concentrator–evaporator. To the resulting residue was added 200 μl of mobile phase and the solution was vortex-mixed for 15 s. Aliquots of 150 μl were injected onto the HPLC column.

2.5. Extraction yield

Solutions 1 or 2 were used to add 10, 50 and 250 ng of nifedipine ($n = 5$ replicates) into disposable glass 125 × 16 mm culture tubes containing 1 ml of blank human plasma. Nifedipine was extracted as previously described except that exactly 3.5 ml of the organic layer was transferred to a clean dry 100 × 13 mm glass tube and evaporated to dryness. To compare extracted *versus* unextracted samples, an equivalent amount of nifedipine (using solutions 1 and 2) was added to another set of glass tubes containing only 5 ml extraction solvent ($n = 5$ replicates); 3.5 ml was transferred to a clean dry 100 × 13 mm glass tube and evaporated to dryness. The peak areas of extracted *versus* unextracted nifedipine samples were compared under identical chromatographic conditions.

2.6. Quantitation

Calibration curves were constructed by plotting the peak-area ratios (nifedipine/I.S.) *versus* their corresponding added plasma concentrations. A weighted least squares regression analysis was performed to generate a best-fit regression line ($1/x$ weighting, where x is the concentration of nifedipine). Data are presented as mean ± S.D.

2.7. Mass spectrometry

To confirm the identity of the nifedipine peak, the corresponding peak was collected from the HPLC eluent and subjected to high-resolution electron impact mass spectral analysis (AEI, MS50, Manchester, UK) via direct insertion probe at 70 eV ionizing potential.

2.8. Other compounds tested

The following nifedipine metabolites/photodegradation products were tested for interference using the same chromatographic conditions as for nifedipine: (1) nitropyridine metabolite (2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester, Bay b 4759); (2) nitrosopyridine photodegradation product (2,6-dimethyl-4-(2-nitrosophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester); and (3) carboxylic acid metabolite (2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid monomethyl ester, Bay o 2820).

3. Results and discussion

Determination of drug concentration and pharmacokinetic parameters in clinical studies requires both precise and accurate data. Most nifedipine assays published to date, report precision of calibration data, however, accuracy is either omitted or reported for nifedipine concentrations ≥10 ng/ml [2,3,5–7,10,12–16]. Failure to report accuracy of low nifedipine concentrations (<10 ng/ml) by these methods may arise from limitations of previous technology (e.g. detector sensitivity), or possibly because

acceptable accuracy was unattainable. In contrast, several published gas chromatographic (GC) assay methods present both accuracy and precision data over the entire concentration range reported [4,9,19]. In a paper by Patrick *et al.* [19], problems encountered developing a sensitive and specific HPLC nifedipine assay (using several existing published methods) were briefly discussed; after several unsuccessful attempts, a GC assay was finally used. It is worth noting that GC methods for nifedipine analysis may have a reduced accuracy as a consequence of thermogradation of nifedipine [7,9,10,19]. Suzuki *et al.* [10] described an HPLC assay that had excellent precision and accuracy using electrochemical detection, but accuracy for concentrations less than 9.6 ng/ml was not reported. Electrochemical detection, however, is recognized to suffer from a lack of durability and inability to detect nifedipine metabolites [7]. In addition to the accuracy and precision considerations, the known metabolites and degradation products of nifedipine must be chromatographed to ensure that none of these products co-elute with nifedipine. Several published nifedipine assays did not test all known major nifedipine metabolites and/or photodegradation products for possible interference [3,7,11,14–16]. Lack of specificity of the assay for nifedipine, therefore, may result in overestimation of actual concentrations of nifedipine in plasma. The present assay has served to chromatograph each of these known products of metabolism and/or degradation while maintaining satisfactory accuracy and precision at low concentrations (<10 ng/ml).

UV detection at 254 nm or less is commonly applied in existing nifedipine assays and is advantageous for optimized detection of nifedipine metabolites and photodegradation products [2,3,5-7,11]. However, significant baseline noise is noticeable in published chromatograms from several of these methods which could compromise accuracy at low nifedipine concentrations [5–7]. Based on our initial results and validation problems using conditions similar to those outlined in several past assay publications, the need for development of a new specific, sensitive and validated assay for nifedipine was determined.

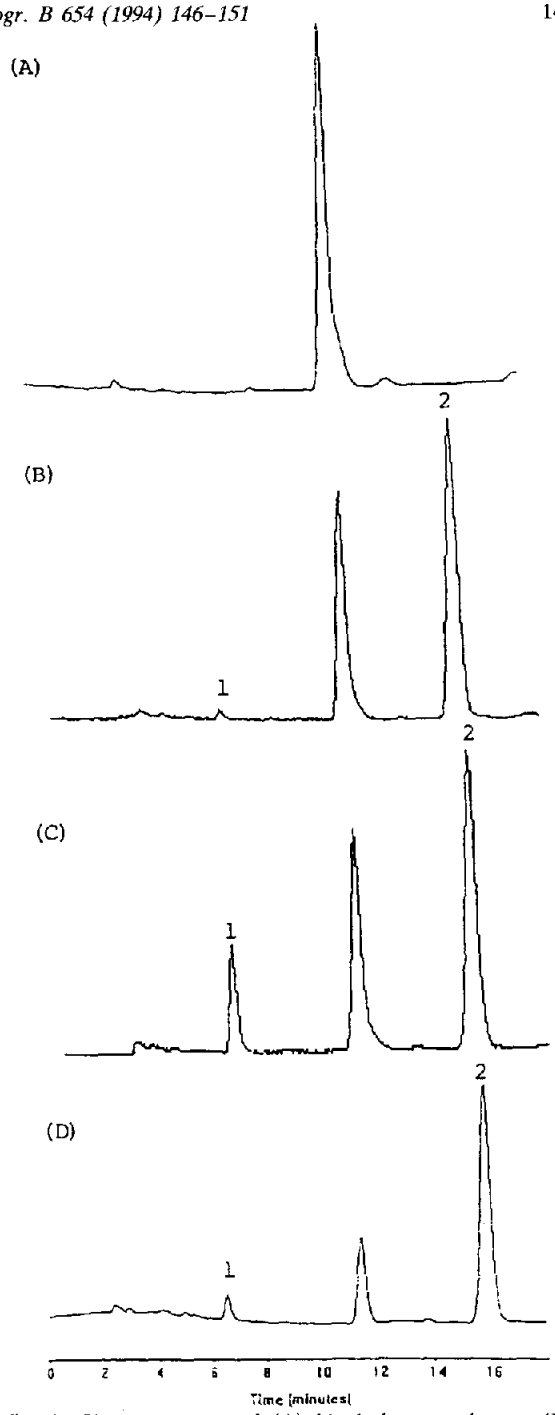

Fig. 2. Chromatograms of (A) blank human plasma, (B) human plasma spiked with 5 ng/ml of nifedipine, (C) human plasma spiked with 100 ng/ml of nifedipine, and (D) human plasma sample taken 24 h after oral administration of a single 30-mg dose of a controlled release nifedipine (osmotic pump). The concentration of nifedipine corresponds to 26 ng/ml. Peak identification: (1) nifedipine; (2) I.S. (nisoldipine).

In this reported method, peaks illustrating nifedipine and I.S. were eluted at approximately 6.5 min and 16 min, respectively (Figs. 2B–D). The chromatograms presented in Fig. 2 were scaled for the highest peaks eluted (either I.S. or an unknown component coextracted from plasma) by the chromatography software and not scaled to optimize illustration of the nifedipine peak. Nifedipine peaks, therefore, appear relatively small, despite a signal-to-noise ratio of greater than 3 observed for even 5 ng/ml nifedipine plasma samples (Fig. 2B). The identity of the nifedipine peak was confirmed using electron impact high resolution mass spectrometry. Blank plasma samples were free of any interfering peaks (Fig. 2A). The minimum quantifiable concentration of nifedipine was determined to be 5 ng/ml. The minimum detectable concentration, however, was in the order of 1 ng/ml.

The calibration curve for nifedipine was typically described by $y = 0.00003 + 0.0025x$ where y corresponds to the peak-area ratio of nifedipine to I.S. and x to added nifedipine concentration. Excellent linearity was observed for all calibration curves ($r^2 > 0.997$). The use of $1/x$ weighting was performed in all calculations of calibration curves. It is necessary to utilize weighting in this analysis to achieve accuracy within 15% of the actual value for the lowest calibration points. Unweighted least-squares regression analysis will tend to bias the upper points on the calibration curve in order to minimize residual error and calculates a line with a best fit for the highest concentrations. The fit of the lower points on the curve may be unsatisfactory since their contribution to residual error is usually small, therefore accuracy of low concentrations may be compromised. The weighting used in this analysis tends to bias low concentrations but still provides acceptable accuracy for the highest concentration on the calibration curve (within ≈10% of the actual value). Application of $1/x^2$ weighting of the calibration data was attempted but unacceptable bias was observed for low calibration points resulting in poor accuracy for high concentrations.

The results in Table 1 describe accuracy (% error) and precision (coefficient of variation, C.V.) of the method. Both accuracy and precision values throughout the concentration range (5–250 ng/ml) were acceptable.

Table 1
Accuracy and precision of the method[a]

Concentration added (ng/ml)	Measured concentration[b] (ng/ml)	Accuracy, error (%)	Precision, C.V. (%)
5	5.72 ± 0.68	+14.40	11.89
10	11.14 ± 1.23	+11.40	11.04
15	15.16 ± 1.10	+1.07	7.26
25	23.62 ± 0.82	−5.52	3.47
50	48.64 ± 5.14	−2.72	10.57
100	92.90 ± 6.18	−7.10	6.65
250	221.46 ± 12.31	−11.42	5.56

[a] $n = 11$ (three sets on day 1, four sets on days 2 and 3).
[b] Reported as mean ± S.D.

The extraction yield of nifedipine from plasma, as indicated by comparing extracted *versus* unextracted samples, appeared to be linear over the calibration range and was 89.87 ± 5.36% (extractions of 10, 50 and 250 ng/ml concentrations, $n = 5$ replicates). Although the extraction was less than 100%, it provided adequate sensitivity to process clinical samples. While examining the efficacy of various extraction solvents, it was decided that a mixture of MTBE and isooctane provided excellent extraction and minimized interfering peaks and baseline noise when compared with numerous other solvents (*e.g.* chloroform and ethyl acetate).

The procedure described here provides the required sensitivity and resolution of nifedipine, without interference from tested metabolites, photodegradation products and I.S. (nisoldipine). Complete resolution of the components from each other and nifedipine was observed, however, the method was not optimized for detection and quantitation of nifedipine metabolites in clinical samples, as known nifedipine metabolites are inactive [1,6].

In our experience with reversed-phase HPLC and UV detection of low concentrations of nifedipine, problems were noted achieving an acceptable signal-to-noise ratio of the nifedipine peak using wavelengths in the 200–280 nm range. As previously stated, significant baseline

noise was apparent in chromatograms from earlier publications utilizing UV detection in this range. Therefore, UV detection at 350 nm was selected for this method which provided improved signal-to-noise ratios for low nifedipine concentrations.

During initial method development, a methanol–water mobile phase without acetic acid and TEA was examined, but problems with peak tailing and short column life were encountered. Although the use of a phosphate buffer, as previously applied by Snedden *et al.* [14], was considered to improve the column life and peak resolution, the current analysis utilized a mobile phase with the pH adjusted to 4 by addition of acetic acid and TEA.

Fig. 2D depicts a representative chromatogram (scaled to show all eluted peaks) of plasma taken from a healthy subject 24 h after oral administration of a nifedipine formulation (30 mg osmotic pump) administered as a single dose. The nifedipine peak in the chromatogram corresponds to 26 ng/ml, which therefore demonstrates the applicability to the analysis of plasma samples from clinical studies.

In conclusion, a rapid, sensitive and selective reversed-phase HPLC analysis of nifedipine is reported. This method improves upon previously reported HPLC techniques for accurate measurement of low nifedipine concentrations. Furthermore, determination of nifedipine in clinical samples was valid and preparation and run times were minimized. The relatively short preparation and analysis time allows for clinical suitability of the method.

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