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Quantitative analysis of nifedipine in plasma by high-performance liquid chromatography

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Abstract: A rapid, sensitive and specific high-performance liquid chromatographic assay for the quantification of nifedipine in human plasma was developed, satisfactorily validated and applied to samples of plasma from healthy volunteers. The sample pre-treatment incorporating protein denaturation by urea and ethyl acetate extraction compared favourably in terms of selectivity with previously published methods. The limit of quantitation of this reversed-phase LC method was 7.0 ng ml^{-1} for the analysis of 0.5 ml samples.

Keywords: *Nifedipine; reversed-phase LC; human plasma; stability in plasma.*

Introduction

Nifedipine, a 1,4-dihydropyridine calcium channel blocker, has commonly been used as a potent arterial vasodilator in the management of angina and various cardiovascular diseases [1, 2]. It is extensively biotransformed into the inactive metabolites resulting in only 50% of the oral dose entering into the systemic circulation [2–4]. Following the usual therapeutic dose of nifedipine (10–20 mg), the maximum plasma drug concentrations reported are rarely more than 150 ng ml^{-1} with less than 2 h half-life. In addition, nifedipine is photosensitive and thermally unstable. These unfavourable pharmacokinetics and physical characteristics give rise to difficulties in determining nifedipine in plasma.

Gas chromatographic (GC) [4–12] and liquid chromatographic (LC) [13–22] techniques have been mainly used for the analysis of nifedipine in plasma. Nifedipine was found to partially degrade to the equivalent nitropyridine under GC conditions [6, 9, 16, 23, 24]. Therefore, GC methods are prone to yielding artifactual nifedipine concentrations corresponding to the sum of the nitropyridine, arising as a metabolite and/or a degradation product, and the drug [6, 11]. Hence, LC methods ought to be more suitable for nifedipine analysis.

The various LC methods that have been developed, using either ultraviolet absorbance or electrochemical detection, are not without deficiencies such as chromatographic interferences [13–22]. Some methods require rather large sample volume [13–17, 20–21], while others are time-consuming due to complicated and tedious extraction procedures [13–15] or long elution times per sample [18]. In some methods elevated column temperatures are used. This will require additional apparatus and may lead to the shortening of column life [18].

The aim of this study was to overcome these analytical deficiencies by modifying the extraction procedure and using LC conditions which allowed short elution times.

Experimental

Laboratory precautions

To minimize the photodegradation of nifedipine, all studies were carried out under the illumination of yellow light (Tungsram, Hungary). All containers used were well wrapped with aluminium foil.

Analytical standards and chemicals

Nifedipine was obtained from Siegfried (Zofingen, Switzerland). Butamben (*n*-butyl-*p*-amino benzoate) was received from E. Merck (Darmstadt, Germany). Nitrendipine was generously supplied by Bayer (Thailand).

All chemicals and reagents were used as

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received and were of analytical or HPLC grade. Acetonitrile was purchased from J.T. Baker (Phillipsburgh, NJ, USA). Glacial acetic acid, ammonium acetate and ethylacetate were obtained from E. Merck. Urea was from BDH (Poole, UK).

Solutions

Ammonium acetate buffer (pH 6.1; 0.01 M) was prepared by dissolving approximately 0.8 g ammonium acetate in distilled water. A few drops of glacial acetic acid was added to adjust the pH to 6.1. Distilled water was then added up to 1000 ml.

Urea aqueous solution (8 M) was prepared by dissolving 24.0 g of urea in distilled water and making up to 50 ml in a volumetric flask.

Preparation of calibration solutions

The solutions of nifedipine used for constructing the calibration curves were prepared by serial dilutions of a stock solution containing 500 μg ml^{-1} in methanol. A series of calibration solutions containing nifedipine in concentrations of 0.25, 0.5, 1.0, 2.0, 4.0 and 6.0 μg ml^{-1} were prepared from the stock solution every week. A stock solution of butamben (internal standard, IS) was prepared in methanol (1 mg ml^{-1}) every week and was diluted to a concentration of 12.0 μg ml^{-1} before use. Calibration and IS solutions were kept frozen in foil-wrapped containers.

Chromatographic equipment and conditions

LC was carried out with a CM 4000 multiple solvent delivery system, a SM 4000 programmable wavelength detector and a CI-4100 computing integrator (all from Milton Roy LDC, FL, USA). A Rheodyne (CA, USA) 7100 injector with fixed 20 μl loop was used. A 20 μl aliquot of the extracted sample was injected onto a reversed-phase column (5 μm Spherisorb ODS 2, 250 × 4.6 mm i.d., Phenomenex, California, USA) preceded by a guard column packed with Corasil (Waters, MA, USA). The isocratic mobile phase was methanol–aqueous ammonium acetate buffer (pH 6.1; 0.01 M) (62:38, v/v). The flow rate was 1.0 ml min^{-1}. UV detection was carried out at 247 nm.

Extraction procedure

All frozen plasma samples and blanks were thawed at room temperature in the dark just prior to analysis. The extractions were performed in 115 × 10 mm screwed-cap glass test tubes.

The calibration curves were prepared for each assay run by transferring 20 μl of the calibration solutions containing 3.5–120 ng of nifedipine into a tube containing 0.5 ml of blank human plasma. Similarly, 20 μl of methanol was added to the tubes containing 0.5 ml of plasma taken from human subjects that had received nifedipine. Plasma standards and samples were extracted at the same time. A volume of 0.6 ml 0.1 M potassium hydroxide aqueous solution was added to each tube followed by the addition of 1.0 ml 8.0 M urea solution. The tubes were then gently vortexed. A 20 μl volume of the internal standard working solution was then spiked into each tube with thorough vortex-mixing. An extraction was carried out by adding 4.0 ml of ethylacetate into each tube. The tubes were vortexed for 1 min and then centrifuged at room temperature at 3000 ***g*** for 10 min. The organic layer was transferred to another tube and evaporated under a stream of nitrogen gas. The evaporated residue from each tube was reconstituted with 200 μl of methanol and aliquot of 20 μl was directly injected into the LC system.

Data calculations

A calibration curve was generated for nifedipine by using the least square regression analysis of the peak-height ratio of nifedipine to that of the internal standard against spiked nifedipine concentration. The concentrations of nifedipine in the plasma samples were obtained from interpolation of the calibration curves.

Application to human subjects

The proposed method was utilized to assay nifedipine in plasma samples. The plasma samples were obtained from three healthy volunteers who had received 10 mg of nifedipine orally (Adalat, Bayer, Germany) with 100 ml of water after being fasted for 10–12 h. Blood samples were drawn through a flexible cannula from a forearm vein into the prepared heparinized tubes wrapped with aluminium foils. Blood samples were scheduled to be collected just before the administration of nifedipine and at 10, 20, 30, 45, 60, 90, 120, 180, 240, 300 and 420 min after dosing. The plasma was immediately separated by centrifugation of the blood sample and stored at

−20°C for subsequent analysis within 1 week. During the experiment, the volunteers abstained from food for 2 h after drug administration.

Results and Discussion

Assay development

Nifedipine extraction. Effective nifedipine extraction is the essence of this assay development. Nifedipine was almost completely extracted from the original plasma sample matrix by consecutively adding potassium hydroxide, urea and ethylacetate. Ethylacetate was selected, over various combined solvents studied, to be the extracting solvent and resulted in well-shaped nifedipine LC peaks being obtained without any observed interfering endogenous substance. The use of chloroform or 1:3 v/v mixture of acetonitrile and chloroform, or a 1:3 v/v mixture of acetonitrile and ethylacetate as extracted solvents led to the overlapped peaks of nifedipine and endogenous substance (data not shown).

Basifying plasma samples with potassium hydroxide prior to ethylacetate extraction was found to improve the degree of extraction of nifedipine. Under alkaline medium, nifedipine would be in the unionized form thereby dispersing better into ethylacetate than into the aqueous layer. Also, by adding urea to the initial basified extraction mixture, almost all of endogenous peaks were eliminated along with the nifedipine extraction being improved.

Enhancing the amount of nifedipine extracted was probably caused by the effect of urea of altering plasma protein binding. Nifedipine is highly bound to plasma protein [1] and urea can denature plasma protein without precipitating it [25]. Therefore, the combination of potassium hydroxide, urea and ethylacetate was appropriate for nifedipine in plasma samples and conveniently seemed to have very low extracting power for endogenous interfering substances. The chromatogram shown in Fig. 1(c) clearly demonstrates the improvement of nifedipine extraction and the disappearance of endogenous peaks when compared to Fig. 1(a) and 1(b).

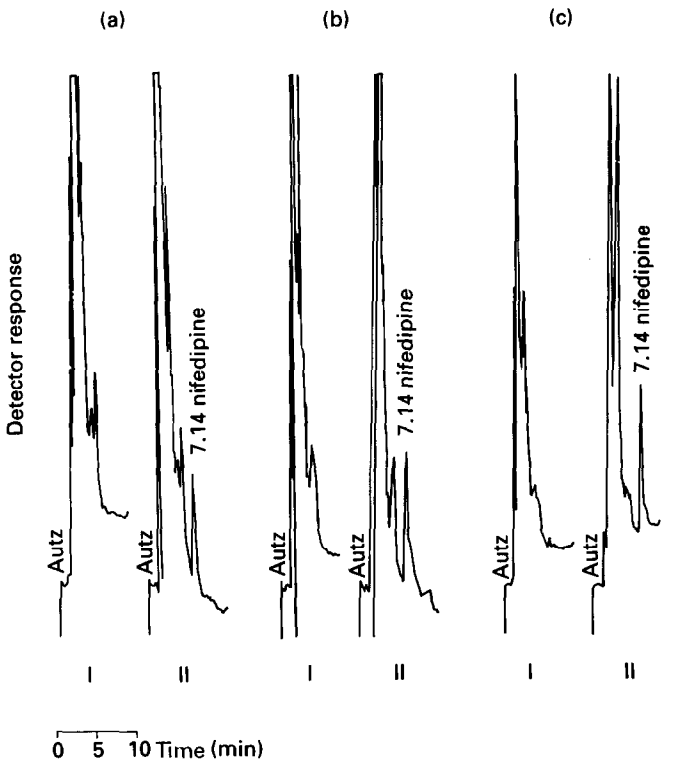

Figure 1
Typical chromatograms of blank human plasma (I) and human plasma spiked with nifedipine [160 ng ml^{-1} (II)] obtained following ethylacetate extraction alone (a); potassium hydroxide and ethylacetate extraction (b); potassium hydroxide, urea and ethylacetate extraction (c).

The technique for denaturing plasma protein before nifedipine extraction to improve the amount of nifedipine extracted and to eliminate most of the endogenous peaks, makes this present study, in common with that of Bach *et al.* [15] distinctive from other published methods. Compared to Bach's study, however, this present study employs a much more economic and simple extraction procedure without the use of disposable extraction columns. Other extraction procedures which use ammonium sulphate or acetonitrile as plasma protein precipitating agents in the studies of Pietta *et al.* [13] and Miyazaki *et al.* [18] seem to achieve less satisfactory results. The majority of other methods using normal extraction of basified plasma samples [16–17, 19–21] give rise to a higher level of endogenous substances and require longer elution time per sample.

Internal standard selection. Butamben, despite its structure, is not similar to nifedipine. However, it was chosen as the internal standard because their physico-chemical properties are alike. It is inexpensive and extractable from plasma under the conditions used to extract nifedipine. Compared to other potential internal standards studied, i.e. diazepam and nitrendipine, butamben showed a shorter retention time with better peak shapes. Butamben may be added either at the same point as the drug or following urea addition without affecting the results. However, adding butamben after urea yields better butamben recoveries, probably due to the denaturation of plasma protein causing more butamben to be extracted by ethylacetate.

Assay characteristics

Specificity and reproducibility. The isocratic reversed-phase LC condition described allowed the separation of nifedipine and the internal standard within a run time of less than 9 min. Typical chromatograms obtained from the analysis of blank human plasma, blank plasma supplemented with nifedipine and internal standard, and plasma sample obtained after oral nifedipine dosing are shown in Fig. 2. Nifedipine and internal standard were resolved with retention times of 7.14 and 8.45 min, respectively. The degree of resolution obtained was considered to be sufficiently large to give reliable quantitation over lengthy volunteer or patient studies. No interfering peaks were observed in these chromatograms.

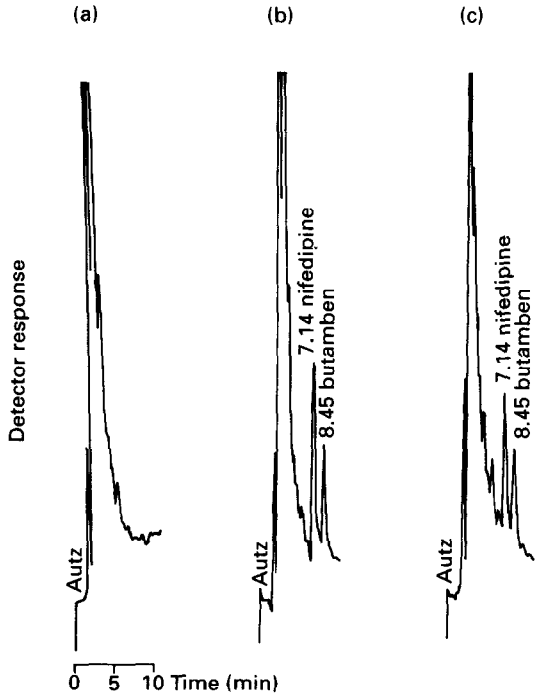

Figure 2
Representative chromatograms obtained following injection of (a) blank human plasma; (b) human plasma containing spiked nifedipine (160 ng ml^{-1}) and butamben (IS) (480 ng ml^{-1}); (c) plasma sample 45 min post dose, from a volunteer subject following administration of 10 mg nifedipine orally, concentration of nifedipine, 127 ng ml^{-1}.

The reproducibility of the retention times of nifedipine and butamben (IS) was determined for 30 consecutive injections during the analysis of a series of nifedipine samples. The relative standard deviations (RSD) were found to be 1.21 and 0.98% for nifedipine and internal standard, respectively.

Limit of quantitation The concentration of nifedipine in plasma that could be detected with a signal to noise ratio of 3.20 ± 0.16 (n = 10) was 7.0 ng ml^{-1} of plasma. Since this concentration of nifedipine in the calibration curve showed acceptable relative standard deviation values in the within-day and day-to-day analysis, it was regarded as the lower limit of quantitation for plasma nifedipine, based on 0.5 ml of plasma sample. It would be possible to increase the limit of quantitation of the assay by increasing the plasma volume used.

Linearity. The peak-height ratios (PHR) of nifedipine and internal standard versus spiked concentration in the range of 7.0–240.0

Table 1
Analytical precision of nifedipine assay in plasma

Conc. (ng ml^{-1})	Within-day (n = 3) Peak-height ratio Mean	(SD)	RSD (%)	Day-to-day (n = 4) Peak height ratio Mean	(SD)	RSD (%)
0.0	0.000		—	0.000		—
7.0	0.152	(0.006)	4.0	0.150	(0.008)	5.0
10.0	0.187	(0.011)	6.1	0.183	(0.002)	1.1
40.0	0.478	(0.039)	8.2	0.467	(0.010)	2.1
80.0	1.01	(0.090)	9.0	0.970	(0.058)	5.9
120.0	1.59	(0.029)	1.8	1.52	(0.036)	2.4
160.0	2.20	(0.143)	6.5	2.02	(0.142)	7.0
240.0	3.04	(0.051)	1.7	3.16	(0.115)	3.6

ng ml^{-1} were fitted to a power function ($y = ax^b$) using least square regression analysis. The equation for the calibration curve in this linear range was

$$\text{PHR} = 0.0249\ (\text{conc.})^{0.8577} \qquad r^2 = 0.999. \tag{1}$$

Precision. The precision of the method was determined by analysing three series of calibration curves on the same day (within-day precision) and four different series of calibration curves on four different days during a period of 30 days (day-to-day precision). The variability in the peak-height ratios at each concentration was determined as the precision of the assay (Table 1). The RSD values from intra-day and inter-day analysis ranged from 1.7 to 9.0 and 1.1 to 7.0%, respectively. Some RSD values in the intra-day were higher than in the inter-day, probably due to some asymmetric error. However, these RSD values are in the acceptable range for the assay [26].

Accuracy. Twelve unknown nifedipine plasma samples were prepared by an independent analyst to simulate the real plasma samples of nifedipine after drug administration. They were analysed by the prescribed method along with the series of calibration solutions. The accuracy of the assay was determined by comparing the measured concentration to its true value (Table 2).

Extraction efficiency. The values of peak-heights measured for nifedipine in the extracted plasma samples over the concentration range of 7.0–240.0 ng ml^{-1} were compared to the peak-height values for unextracted samples. The mean extraction recovery of nifedipine was 93.3 ± 6.0%. The recoveries of nifedipine in the concentration range covered by the calibration curve were independent of concentration (Table 3), indicating the acceptable efficiency of the developed extraction procedure.

Table 2
Accuracy in the analysis of nifedipine unknown samples (n = 12)

Actual conc. (ng ml^{-1})	Measured conc. (ng ml^{-1}) Mean	(SD)	Accuracy* (%)	RSD (%)
10.0	9.8	(0.34)	98.0	3.5
40.0	38.3	(3.06)	95.8	7.9
160.0	157.3	(1.36)	98.3	0.9

$$^*\text{Accuracy} = \frac{\text{measured conc.}}{\text{actual conc.}} \times 100.$$

Table 3
Extraction efficiency of nifedipine from plasma

Conc. (ng ml^{-1})	Percentage recoveries Mean	(SD)	RSD (%)
7.0	84.66	(9.16)	10.8
10.0	90.35	(15.9)	17.6
40.0	88.09	(8.98)	10.2
80.0	96.17	(6.62)	6.9
120.0	98.12	(2.78)	2.8
160.0	93.68	(4.62)	4.9
240.0	101.8	(5.59)	5.5
Mean (SD)	93.27	(5.98)	6.4

Stability. The stability of spiked nifedipine in blank plasma was studied at three different levels of concentration, 40, 120 and 240 ng ml^{-1} plasma. The plasma samples were analysed on the day of preparation as t = 0 and were kept frozen for subsequent analyses at t = 1, 3, 5 and 7 days, respectively. The variations of the peak-height of nifedipine during the period of study are shown in Fig. 3. The results imply that nifedipine plasma

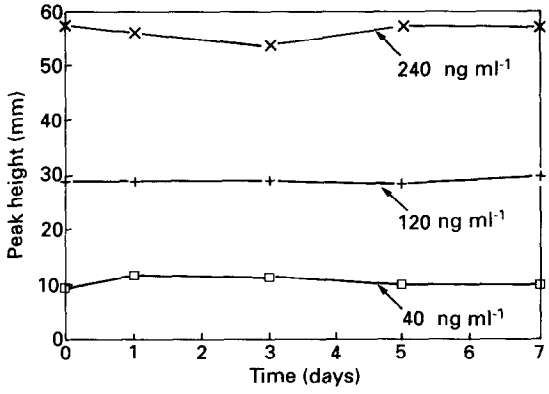

Figure 3
Mean peak height of nifedipine as a function of storage time at −20°C. Plasma nifedipine concentration 40, 120 and 240 ng ml^{-1}; $n = 3$.

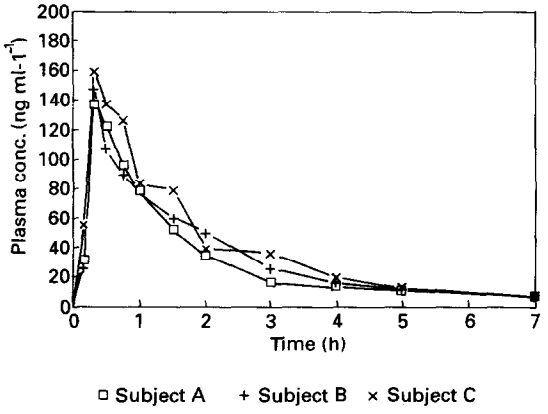

Figure 4
Plasma concentration–time profiles of nifedipine following an oral dosing of 10 mg nifedipine to normal subjects.

samples may be kept frozen for up to 1 week without any deterioration of the samples.

Application of the method

The present method was used to determine nifedipine in plasma samples over a 7 h period from three Thai healthy volunteers who received a single 10-mg oral dose of nifedipine (Adalat-Bayer, Germany). The plasma concentration–time profiles of nifedipine in these three subjects are shown in Fig. 4. The maximum concentrations of nifedipine in plasma samples (C_{max}), as shown in Table 4, were in the range of 137.4–159.5 ng ml^{-1}. These C_{max} values were within the concentration range of the calibration curve (7.0–240.0 ng ml^{-1}). Additionally, the concentration of nifedipine could still be detected up to 7 h after dosing.

Table 4
Pharmacokinetic parameters of nifedipine in Thai healthy subjects following oral administration of 10 mg Adalat

Subjects	T_{max} (min)	C_{max} (ng ml^{-1})	AUC (ng h ml^{-1})	$t_{1/2}$ (h)
A	20	147.6	256.2	2.3
B	20	137.4	247.5	3.5
C	20	159.5	302.4	2.6
Mean	20	148.2	268.8	2.7*
(SD)		(11.1)	(29.5)	(0.08)

*Harmonic mean half-life.
T_{max} = time at maximum nifedipine concentration; C_{max} = maximum nifedipine concentration observed; AUC = area under nifedipine plasma concentration–time curve from zero time to infinity.

Conclusion

The validation data and the outcome of the application of the method to healthy volunteers demonstrate the appropriateness of the method for plasma nifedipine analysis in clinical and pharmacokinetic studies.

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