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Sensitive high-performance liquid chromatographic determination of nifedipine in dog plasma using an automated sample preparation system with laboratory robot

Viola Horváth[a], Andrea Hrabéczy-Páll[a], Zsuzsanna Niegreisz[a], Emilia Kocsi[a], George Horvai[b,*], Lajos Gödörházy[a], Antal Tolokán[a], Imre Klebovich[c], Katalin Balogh-Nemes[c]

[a]*Technical University of Budapest, Institute for General and Analytical Chemistry, H-1111 Budapest, Hungary*
[b]*Division of Chemical Information Technology, Gellért tér 4., H-1111 Budapest, Hungary*
[c]*EGIS Pharmaceuticals Ltd., Department of Pharmacokinetics, P.O.B. 100, H-1475 Budapest, Hungary*

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Abstract

Nifedipine, a calcium-channel blocking drug was analysed in dog plasma after oral dosing with two different formulations. Sample preparation was automated with a laboratory robot. Quantitative determination of the drug was performed on a reversed-phase HPLC system with electrochemical detection (ED) using an internal standard. Validation of the analytical method showed that the system is well suited for pharmacokinetic studies on dogs. The assay was linear in the range 1–50 ng/ml. Inter-day and intra-day variability were between 6.43–18.15% C.V. and 1.57–5.53% C.V., respectively.

Keywords: Nifedipine

1. Introduction

Nifedipine [dimethyl-1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylate] is a calcium entry antagonist selectively dilating arteries with little or no effect on other blood vessels. It is extensively used in the treatment of angina pectoris and arterial hypertension [1,2].

An important feature of nifedipine is its sensitivity to light and oxidants. On exposure to visible light the nitroso–pyridine derivative [dimethyl-4-(2-nitrosophenyl)-2,6-dimethylpyridine 3,5-dicarboxylate] is formed in solution, while under UV light the nitro–pyridine derivative [dimethyl-4-(2-nitrophenyl)-2,6-dimethylpyridine 3,5-dicarboxylate] is formed [3]. In the human body nifedipine is rapidly oxidized enzymatically to its nitropyridine derivative. In further biotransformation, the ester group is hydrolysed and further oxidation takes place at the methyl position [3].

As a consequence of this decomposition, nifedipine is rapidly inactivated in the body to pharmacologically inactive products. The resulting short duration of action creates a problem in the treatment of hypertension which has been overcome using retard nifedipine formulations.

The minimal effective concentration of nifedipine in man is about 15 ng/ml [4]. With conventional

*Corresponding author.

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formulations, plasma concentrations reach 100–200 ng/ml 1–2 h after drug administration, and fall to about 5–10 ng/ml after 6–8 h. With sustained-release formulations, plasma concentrations reach a maximum of 30–60 ng/ml 2–4 h after administration and fall to 5–10 ng/ml in 24 h [4–6]. Therefore, twice daily administration of retard tablets gives a prolonged effect. However, the pharmacokinetic study of sustained-release formulations requires highly sensitive and selective analytical methods because of the low plasma levels.

The determination of nifedipine in biological fluids has involved mainly gas-chromatographic (GC) and high-performance liquid chromatographic (HPLC) methods. In GC methods, electron-capture, [5,7–9], N–P ionization detectors [10], and a mass spectrometer [7,11,12] have been employed. In most HPLC procedures, UV detectors have been used [3,4,6,13–19], but there are some papers in which the application of electrochemical detection is described. This is based on the electrochemical oxidation of nifedipine (oxidation of the dihydropyridine ring to pyridine) at a glassy carbon electrode at 950–1000 mV vs. Ag/AgCl electrode [20–22], or at a carbon fibre electrode at 1400 mV vs. Ag/AgCl [23]. Nifedipine can also be analysed by electrochemical reduction, in which the nitro group is reduced in two steps to the amine [24]. Determination by electrochemical oxidation is superior to reduction in that decomposition products of nifedipine do not interfere in the assay, as they do not contain the dihydropyridine ring.

For plasma clean-up prior to HPLC, extraction with single organic solvents or solvent mixtures such as toluene [19], pentane–dichloromethane [3,4,18], *n*-hexane–dichloromethane [6] or *n*-hexane–ethyl acetate [20] have been employed. In some cases solid-phase extraction has been used for sample clean-up with special extraction columns either in the off-line [13] or in the on-line arrangement [23]. The lower limit of quantification for HPLC determinations of nifedipine in plasma has varied widely with values of 1–2 ng/ml [20], 2 [6,19], 3 [16,18], 5 [13] and 15 ng/ml [23] being claimed.

In the present work a previously developed, highly sensitive method [25] has been further improved for the determination of nifedipine in dog plasma in the concentration range of 1–50 ng/ml. The method has been validated for the purposes of pharmacokinetic and bioavailability studies of retard nifedipine formulations.

In addition to extending the lower detection limit, a new sample preparation system has been designed, coordinated by a laboratory robot. Robots are gaining more and more attention in the analytical laboratory. They find an extending use in the field of pharmaceutical analysis too [26]. Robotic systems carry out tablet content uniformity tests [27–30], drug dissolution tests [31,32] drug analysis and/or sample pretreatment from biological samples [33–40].

The robotic system we built, minimizes the need for human intervention and the whole sample preparation procedure can be conveniently carried out in the dark. An extensive evaluation that comprises a detailed description of the robotic sample preparation system and the optimization of its design is currently in preparation.

2. Experimental

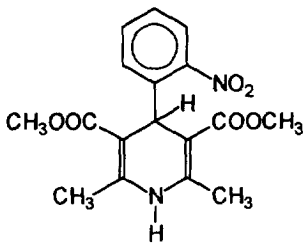
2.1. Materials

Nifedipine and the internal standard [dimethyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate] (see Fig. 1) were supplied by EGIS (Budapest, Hungary). Methanol, *n*-hexane and dichloromethane were purchased from Romil (Loughborough, UK). NaOH was a product of Fluka (Buchs, Switzerland), acetic acid was supplied by Soprelec (Evry Cedex, France). All solutions were prepared with doubly distilled water.

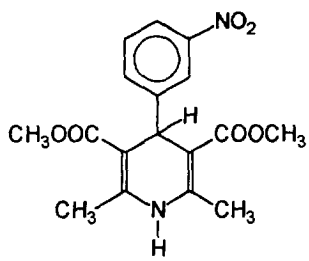
Validation of the assay was carried out using pooled dog plasma from EGIS containing CPD as anticoagulant. CPD was composed of 34.47 g/l anhydrous glucose, 26.3 g/l sodium citrate, 3.27 g/l citric acid and 2.22 g/l NaH_2PO_4. Plasma samples were stored at −20°C.

Cordaflex 10-mg film-coated tablets were supplied by EGIS. The Procardia XL OROS (90 mg) nifedipine formulation is a product of Pfizer (New York, NY, USA).

All commercially purchased chemicals were of analytical grade.



a)



b)

Fig. 1. Structure of nifedipine (a) and the internal standard (b).

2.2. Sample preparation

Stock solutions of nifedipine (0.5 mg/ml) and internal standard (0.25 mg/ml) were prepared in methanol. Both solutions were stored in the refrigerator at +5°C in dark bottles.

The organic solvent mixture used to extract nifedipine from the plasma contained hexane–dichloromethane (70:30, v/v). Nifedipine was extracted from the plasma using an automated sample preparation system controlled by a laboratory robot. The sample preparation procedure involved the following steps: 200 μl 1 *M* NaOH was added to 1 ml sample for pH adjustment; 50 μl internal standard of 2000 ng/ml was pipetted into the sample; after this the mixture was homogenized on a vortex mixer for 10 s; 3 ml extraction solvent mixture was added and the sample was vortexed for efficient extraction for 5 min; samples were centrifuged at 1400 *g* for 15 min; separation of the organic layer was carried out, followed by evaporation of the organic solvent under N_2 atmosphere at 50°C.

All the above steps except for centrifugation were performed by the robot. Batches of maximum 20 samples were prepared by the robot for centrifugation and the same batch was returned to the robot after centrifugation. The final dried sample was dissolved in 250 μl eluent and injected into the chromatographic system manually.

The whole analysis was carried out in a dark room to avoid light-induced decomposition of the samples.

2.3. Apparatus

A Mitsubishi Movemaster RV-M1 type laboratory robot (Mitsubishi, Japan) controlled by a 386 AT IBM compatible computer conducted the sample preparation. The environment of the robot consisted of two automatic burettes (Radelkis OP-930, (Budapest, Hungary) and Metrohm 665-Dosimat (Herisau, Switzerland)) and a Reax 2000 laboratory vortex mixer (Heidolph, Kelheim, Germany). A Sigma 204 laboratory centrifuge (Sigma, Osterode am Harz, Germany) was used to separate the supernatant after liquid–liquid extraction. The organic solvent was evaporated in a P207 test tube thermostat (MTA Kutesz, Budapest, Hungary).

2.4. Chromatographic system

Nifedipine was separated on an ODS Hypersil (Hewlett–Packard, Palo Alto, CA, USA) column (5 μm, 200×4.6 mm I.D.) with a BST ODS Hypersil pre-column (5 μm, 20×4.0 mm I.D., Bio-szeparációs Technikai Kft., Budapest, Hungary). The mobile phase consisted of methanol and 0.01 mol/l acetate buffer pH 4 (75:25, v/v). It was delivered by an LKB 2150 (Pharmacia LKB, Bromma, Sweden) and a Beckman 114 M (Beckman Instruments, Berkeley, CA, USA) HPLC pump. The flow-rate was 0.8 ml/min. The sample was introduced via an automatic injector having a 50-μl loop volume.

Column switching was accomplished with another automatic injector. A BAS LC-3C amperometric detector equipped with a BAS LC-44 thin-layer cell (Bioanalytical Systems, West Lafayette, IN, USA) was used. The working and the reference electrodes were glassy carbon and Ag/AgCl/3 *M* KCl, respectively. The applied potential was 1000 mV. Injector switching, data acquisition and evaluation were controlled by a 486 AT IBM compatible computer using Borwin 1.20 chromatography software (JMBS, Le Fontanil, France). For quantitative evaluation the peak height ratio of nifedipine to internal standard was applied.

Column switching was employed to eliminate long retention time plasma components. After sample introduction nifedipine and the internal standard elute from the pre-column and reach the analytical column in about 1 min. At this stage another valve was switched and the pre-column was disconnected from the main eluent stream. Nifedipine and the internal standard passed with the main stream through the analytical column to the detector. During this time the pre-column was washed clean of strongly retained plasma components using eluent delivered by a second pump. At the end of the chromatographic run the pre-column was sufficiently clean to be switched back into the main stream.

2.5. Statistics

Line fitting with the combinatorial method was carried out in the following way. Straight lines were laid through all possible pairs of measured points. The median of the slopes of these lines is the slope of the fitted straight line and the median of all intercepts is its intercept. This is a robust estimation of the best fitting straight line.

Acceptance range of the mean calibration curve is defined as the mean±20% interval for the measured values.

The lower limit of quantitation is defined as the lowest concentration measured in the calibration, where the C.V. is still less than 20%.

The detection limit of the analytical method is the concentration corresponding to a signal three times higher than the noise.

2.6. Method of validation

2.6.1. Linearity study

Linearity was examined by analysing seven spiked plasma concentrations (1, 2.5, 5, 10, 20, 30 and 50 ng/ml) on six different days. The ratio of the peak heights of nifedipine and the internal standard against plasma concentration was plotted. A straight line was fitted to the calibration points with the combinatorial method.

2.6.2. Precision and accuracy

The determination of inter-day precision and accuracy was accomplished by replicate measurements of spiked plasma samples of concentration 2.5, 5, 10, 20, 30 and 50 ng/ml on six different days. Intra-day precision and accuracy of the analytical method were established by six replicate measurements of spiked plasma samples at three concentrations (5, 20, 50 ng/ml).

2.6.3. Extraction recovery

Percentage recovery from the liquid–liquid extraction was determined by dividing the peak height of nifedipine (or I.S.) obtained for extracted plasma sample by the peak height of a directly injected aqueous standard of the same concentration and multiplying by 100. Recoveries were determined at nifedipine concentrations of 10, 30 and 50 ng/ml. The concentration of the I.S. was 100 ng/ml in each case.

2.6.4. System suitability test

The precision of the chromatographic system (system suitability) was determined based on five replicate analyses of six aqueous standards in the concentration range 2.5–50 ng/ml.

2.6.5. Stability of nifedipine in frozen dog plasma

The stability of nifedipine in dog plasma samples stored in a deep freezer below −20°C was evaluated by spiking dog plasma with nifedipine at two different concentrations (20 and 50 ng/ml) and freezing. After a certain time, a portion from each concentration was thawed, spiked with the I.S. and analyzed. Measurements were made on the 1st, 8th, 16th, 22nd and 35th day.

3. Results and discussion

3.1. Validation

The chromatograms of an aqueous standard, and of extracts of a blank plasma and a spiked plasma sample are shown in Fig. 2. Evidently no other plasma metabolites or endogenous compounds interfere with the measurement of nifedipine and the internal standard. The retention times of nifedipine and the I.S. were 4.7 and 5.3 min respectively. The peak resolution was 2.24. The total HPLC analysis time including the washing of the pre-column was 10 min.

The robotic sample preparation and the HPLC–ED system were validated for the measurement of nifedipine in dog plasma.

The calibration data are plotted in Fig. 3. Excellent linearity was found by fitting a calibration curve with the combinatorial method. The equation of the fitted line and the regression coefficient are also shown in the figure.

Using the data generated in the linearity study, the inter-day precision and accuracy of the method were also calculated. The results at different concentration levels are listed in Table 1.

The inter-day precision of the method is close to the level of acceptance (20%) at the two smallest concentrations but at concentrations >5 ng/ml it is less than 10%. The inter-day accuracy of the method (characterized by the percentage deviation of the average of calculated concentrations from the true value) is always less than 6%.

Intra-day precision and accuracy data are listed in Table 2. Good precision of <6% was obtained with the three different nifedipine concentrations. The intra-day accuracy of the method was less than 2%.

The recovery obtained for nifedipine at 10, 30 and 50 ng/ml was 85%, 90% and 89% respectively. The recovery of the internal standard in the same measurements was 86%, 87% and 85%. More than 85% of the measured compound was recovered from the plasma sample, and there was good agreement in the recovery of nifedipine and the internal standard.

The values obtained for the precision of the chromatographic system are below, or very close to 5% except for the 2.5 ng/ml concentration, but even in this case it does not exceed 8%.

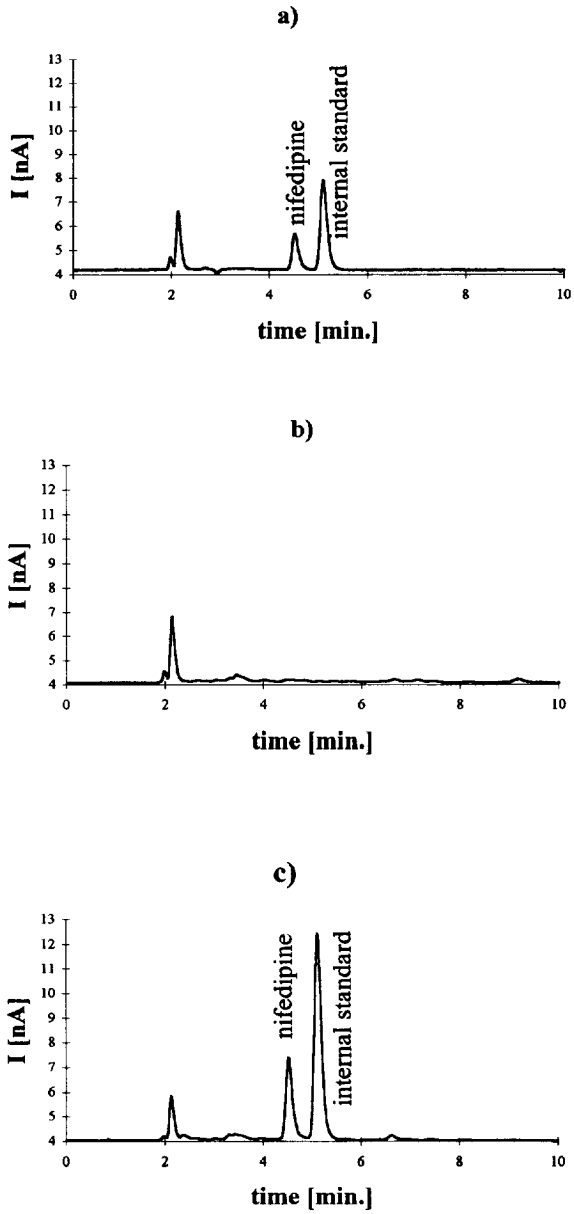


Fig. 2. Chromatogram of 20 ng/ml nifedipine and 100 ng/ml internal standard dissolved in the mobile phase (a), of a blank dog plasma sample (b), and a spiked plasma sample containing 20 ng/ml nifedipine and 100 ng/ml internal standard (c). For chromatographic conditions see Section 2.4

Plasma stability experiments indicate that nifedipine-containing plasma can be stored for 22 days without excessive decomposition of the drug (i.e. less than 20% of the nifedipine decays). By the

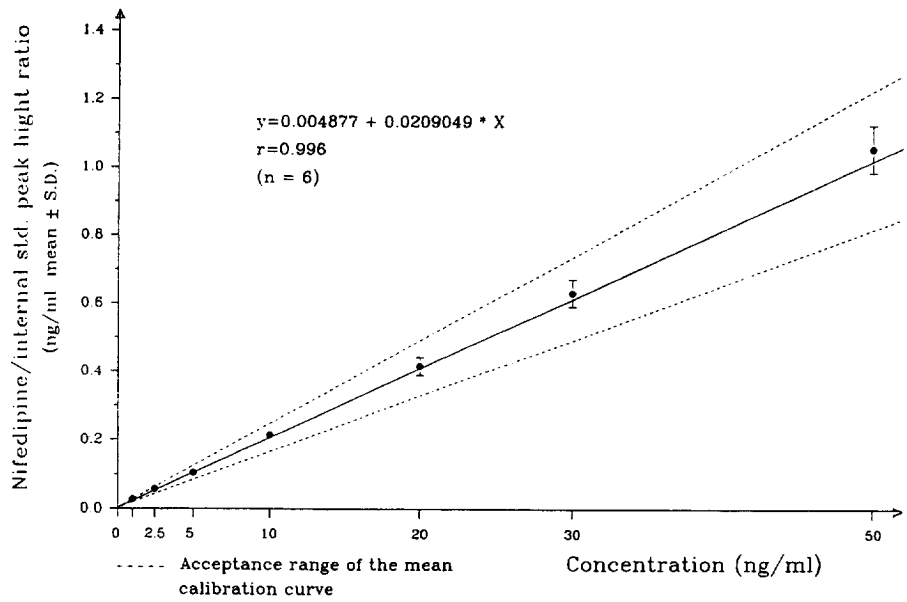


Fig. 3. Calibration in dog plasma on six different days. Calibration points and the fitted line is plotted.

Table 1
Inter-day precision and accuracy of six parallel measurements at different concentration levels

Nominal concentration (ng/ml)	Calculated concentration (ng/ml)	Precision (%)	Accuracy (%)
1	1.06	17.03	5.86
2.5	2.50	18.15	0.00
5	4.76	7.59	−4.72
10	10.00	7.08	0.05
20	19.67	6.48	−1.67
30	30.00	6.43	0.00
50	50.27	6.67	0.54

Table 2
Intra-day precision and accuracy of six parallel measurements at three concentration levels

Nominal concentration (ng/ml)	Calculated concentration (ng/ml)	Precision (%)	Accuracy (%)
5	4.94	5.53	−1.27
20	19.67	2.98	−1.64
50	49.83	1.57	−0.34

35th day however, 78% of the nifedipine decomposed and this is well below the acceptance level.

The detection limit of the analytical method is 0.45 ng/ml. The limit of quantitation is 1 ng/ml nifedipine in plasma.

3.2. Pharmacokinetic studies on dogs

In order to demonstrate the applicability of the analytical method, it was used in pharmacokinetic studies of a conventional film-coated tablet and an

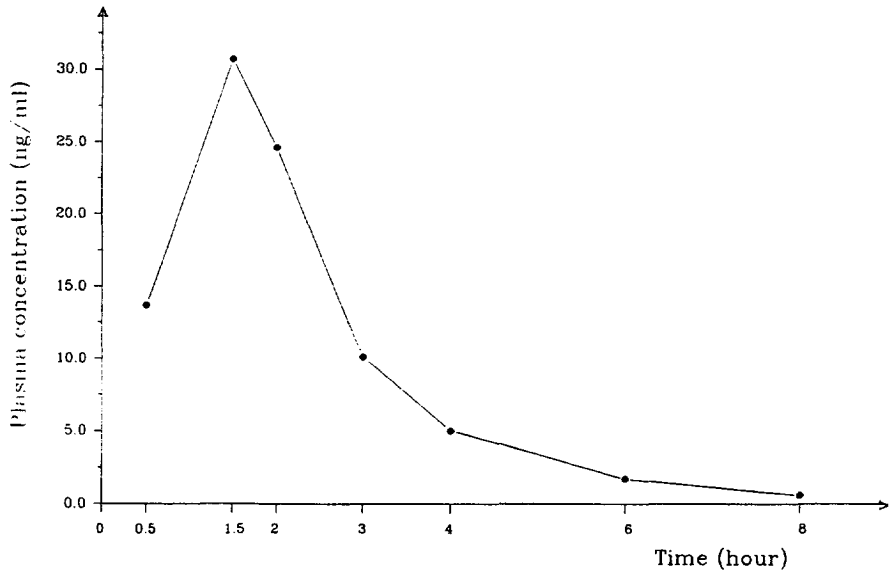


Fig. 4. Pharmacokinetic concentration–time curve of nifedipine in dog plasma after oral administration of 3×10 mg Cordaflex film-coated tablets.

osmotic release retard nifedipine formulation in beagle dogs. After oral treatment with 30 mg Cordaflex (administration of three 10-mg tablets at time zero) and a single 90-mg Procardia XL OROS tablet, blood was taken from dogs at certain times and the samples were treated and analyzed. The nifedipine concentration time plot for the dog plasma is plotted in Fig. 4 and Fig. 5. The nifedipine concentration

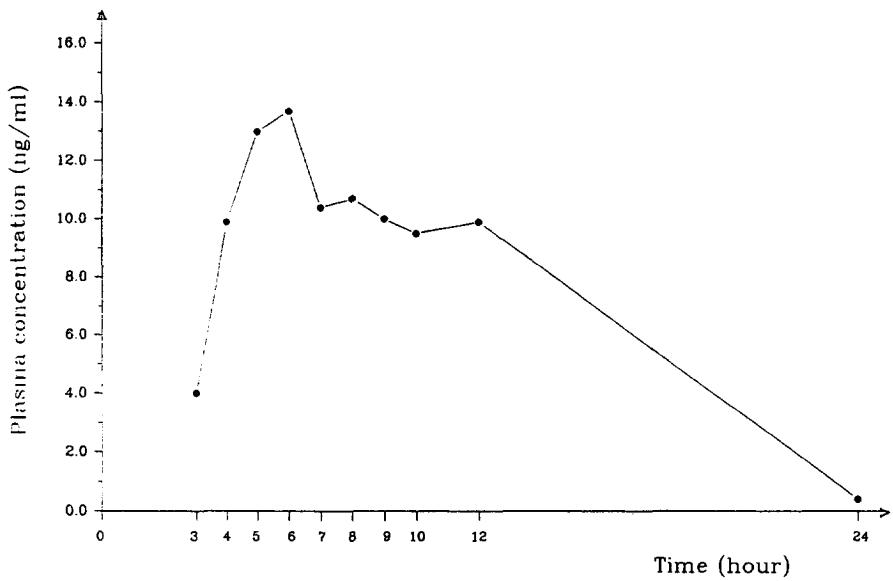


Fig. 5. Pharmacokinetic concentration–time curve of nifedipine in dog plasma after oral administration of a single 90-mg Procardia XL OROS tablet.

profile in the case of the 3×10 mg Cordaflex tablet shows the characteristics of a short action drug (i.e. fast absorption and fast elimination from the body). However, with Procardia the absorption of nifedipine is much slower and the maximum plasma concentration is also less. After the peak a steady-state plasma level can be observed, and the nifedipine concentration can be measured even after 24 h. These features are characteristic of a controlled-release tablet.

These data show that the method presented here is suitable for testing different nifedipine formulations, including retard formulations, that result in very low plasma levels.

3.3. Automation with a robot

A detailed description and analysis of the robotic sample pretreatment system will be presented elsewhere [41]. The work presented in this paper has contributed to the understanding of a robotic system in the pharmacokinetic work. In this field analytical methods are often used near their lower limit of quantitation and the samples are rather complex. Therefore the C.V. of the method is relatively high, i.e. 2–20%. The sources of this variation are not well understood and, at least in this study, were not decreased substantially by replacing human work with the robot. The robot proved to be very useful in other respects, however. Manual sample pretreatment was rather tedious in the present case, because

1. hazardous biological samples had to be handled;
2. operators had to work in subdued light (nifedipine is light sensitive);
3. operators were stressed because a simple mistake in the long pretreatment scheme of any sample might have caused the loss of ten to thirty other analysis results (samples were too small for making duplicates and the loss of one or two crucial points on a pharmacokinetic curve may render the rest of the data of the same curve useless).

It was observed that the introduction of the robot released completely the extreme stress of this work and besides reduced the manpower requirement from three chemists to one chemist and one technician when 30–50 samples (and corresponding number of standards) had to be run in a two-shift working day.

4. Conclusion

An HPLC–ED method for the determination of low levels of nifedipine in dog plasma has been developed by adapting the sample preparation to a laboratory robot. The method was fully validated and is sufficiently sensitive and specific for the quantitation of plasma nifedipine levels and for pharmacokinetic studies of different kinds of nifedipine formulations.

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