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Assay of Nifedipine in the Plasma from Patients with Pulmonary Hypertension

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In order to assay the human plasma concentration of nifedipine in patients with bronchopulmonary dysplasia (BPD) and pulmonary hypertension, a modified high performance liquid chromatography (HPLC) method was applied. The retention times for nifedipine and an internal standard (11-ketoprogesterone) were 10.5 ± 0.41 and 13.1 ± 0.63 min, respectively. Absolute recovery from the plasma was $102.9 \pm 7.07\%$. Reproducibility was excellent and variability between the runs was small. There was a negligible degradation during the assay procedure. The calibration curve shows a good linearity in the range of the desired plasma concentrations of nifedipine. A stability test of nifedipine in the human plasma shows 8 and 13% degradation during the storage of 5 and 9 months, respectively. There were no interferences on the HPLC assay with any possible medications for the BPD. The method has been used to monitor the drug concentrations in a patient. The concentration-time curve of a patient after a single oral dose of 0.3 mg/kg shows a double-peak phenomenon that was quite different from the previous report, suggesting non-bolus administration. However the hemodynamic responses were corresponding to the plasma concentration levels of nifedipine.

Keywords—Capsule, HPLC, Interference, Nifedipine, Stability

Introduction

Nifedipine, 2, 4-nitropheny1-2, 6-dimethy1-3, 5-dicarbomethoxy-1, 4-dihydropyridine, is a calcium channel blocking drug that is a potent vasodilator with little effects on the myocardium or the cardiac conducting system at normal dose levels.[1] Its physiological action is to inhibit the transmembrane influx of extracellular calcium ions across the membranes of myocardial cells and vascular smooth muscle cells, without changing serum calcium levels. It has a more marked peripheral vasodilator effect than verapamil or diltiazem does and it causes some reflex tachycardia. It is an established therapeutic agent in the treatment of essential hypertension, coronary artery spasm, and angina pectoris. Nifedipine is beneficial in the treatment of pulmonary artery hypertension resulting from bronchopulmonary dysplasia (BPD).

Bronchopulmonary dysplasia, a form of chronic pulmonary disease, was first described in the literature in 1967 by Northway *et al.*[2] The cause of this iatrogenic disease is not well understood, but oxygen probably plays a key role. Oxygen must be administered through an endotracheal tube at increased atmospheric pressures to an already damaged lung to cause the changes in lung tissue.[3] This dysplastic changes cause impaired ventilation or perfusion, decreased trachobronchial toilet, cystic and emphysematous changes, and altered pulmonary vascular and lymphatic flow. Clinical management includes mini-

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mizing respiration tract insult, compensating for complications and maintaining nutritional support during recovery. Long term complications of severe BPD include cor pulmonale, sudden infant death, abnormal pulmonary function, and exercise limitations. Cor pulmonale is associated with increased pulmonary artery pressure and usually leads to progressive decline and death of the patient. The long term survival of these patients are questioned, because of limited therapies available. Bronchopulmonary dysplasia causes hypoxia, possible due to vasoconstriction of vascular smooth muscle. Since this constriction may be due to transmembrane extracellular calcium influx, it is theorized that nifedipine, as a calcium channel blocker, may be of benefit in treating the complications of BPD.[1] It is suggested that nifedipine may decrease pulmonary arterial pressure and pulmonary vascular resistance acutely and chronically. In the acute stage, it may be due to a decrease in smooth muscle tone. In the chronic stage, it may be due to a reversal of anatomical changes which brings about a further fall in pulmonary vascular resistance.[3]

From several reports, nifedipine has a large inter-and intra-pharmacokinetic profiles.[4, 5] Nifedipine is rapidly and completely absorbed form the gastrointestinal tract following oral administration.[6] However about 50% of an oral dose as conventional capsules reaches the systemic circulation as an unchanged from since nifedipine is metabolized on the first pass through the liver.[7] Some data indicate that the rate and extent of absorption of nifedipine following a sublingual administration may be decreased substantially with delayed and decreased peak plasma concentration. Oral bioavailability of nifedipine may be increased up to two fold in patients with liver cirrhosis due to decrease in clearance.[1] Nifedipine is extensively (about 96%) bound to plasma proteins. It is metabolized to inactive forms with a half-life of 1.8 hours.[3] Elimination is extensively hepatic, dependent not only on drug metabolizing enzymes but also on hepatic blood flow.

Nifedipine is rapidly oxidized by enzymes to a pyridine metabolite. This pyridine metabolite is also rapidly formed on exposure to the ultraviolet light. A 2-nitroso derivative is formed in normal daylight. Upon biotransformation, hydrolysis to an hydroxy carboxylic acid and further oxidation to a methoxy carboxylic acid derivation occurs. It is known that the metabolites have no activity. The photo-degradation and thermal instability of nifedipine make nifedipine assay difficult in human plasma.[8]

Several gas chromatographic methods with either electron capture detection[4, 9] or selective ion monitoring have been described. These assays have shown a satisfactory detection limit of approximately 2 to 10 ng/ml, but the pyridine derivative is formed in non-reproducible amounts with the high temperatures necessary (230~250 ℃). To overcome this problem, several investigators oxidized the drug prior to analysis. This in turn caused a loss of selectivity. Recently many other investigators tried to achieve better performance using improved gas chromatographic methods.[10] High performance liquid chromatography (HPLC) methods developed in recent years, overcome the problem of thermal instability while maintaining the sensitivity of gas chromatographic assays.[5, 8, 11-16] Even though there are many publications, only a few studies have done in infants or young patients.[17-20] Only a quarter of all the drug approved by United States Food and Drug Administration are labeled as safe and effective in children. That implies pediatric patients as a difficult group to assess for drug treatment. Variations of oral absorption, protein binding, distribution, metabolish, and excretion of drugs exist in the pediatric pupulation. The complexity of variability increases with common congenital anomalies and pathological syndromes. It becomes a challenge to monitor drug therapy in these

complicated patients with little specific published data. Furthermore some assay interferences might possibly exist due to concurrent medications in patients with pulmonary hypertension. The objective of this study is to confirm a modified HPLC method of nifedipine in young patients who were treated with other medications.

Materials and Methods

Materials

Nifedipine and 11-ketoprogesterone were from Sigma, St. Louise, MO. Acetonitrile and methylene chloride were a HPLC grade (Baker). Pentane (Mallinckrodt), potassium phosphate monobasic (Baker), and sodium hydroxide (Baker) were an analytical or HPLC grades.

Nifedipine Assay

Calibration standards of nifedipine (NFP) in acetonitrile were prepared in concentrations of 100~1,000 ng/m*l* and 100 μ*l* of the internal standard (1000 ng/m*l* solution of 11-ketoprogesterone in acetonitrile), the mixture of each tube was evaporated to dryness, 1 m*l* of normal human plasma was added, and each tube was vortexed for 5 sec. One milliliter of 1 N sodium hydroxide solution and 5 m*l* of methylene chloride-pentane (3 : 7 v/v) were added and shaken for 1 min using a Tekmar mixer, and then centrifuged at 2,500 rpm for 5 min. Five milliliters of the organic phase were transferred into a test tube by a pasteur capillary pipette, and reduced to dryness in a Vortex-Evaporator at 45℃ for 30 min. The residue was reconstitued in 100 μ*l* of the mobile phase and 30 μ*l* of the solution were injected into the HPLC system. The peak height ratios of nifedipine to the internal standard were used for the assay. All the works were performed in subdued light and any glassware used throughout the procedure were wrapped in foil. The reported method by Pietta *et al.*[8] was modified for nifedipine assay from the plasma samples. Briefly, HPLC instrumentation consisted of a pump (Kratos Spectroflow 400, Ramsey, NJ), WISP (Model 712, Waters, Milford, MA), UV detector (Kratos Spectroflow 783 or 773), and a reverse phase column (Partisil 10-ODS, Whatmen, Clifton, NJ). The mobile phase consisted of 0.01 M potassium phosphate buffer-acetonitrile (40 : 60 v/v). Before mixing the buffer was brought to pH 6.1 with 1 N NaOH and filtered through a Millipore filter. The mobile phase was degassed by ultrasonification for 15 min. The flow rate was 1.2 m*l*/min. The flow rate was 1.2 m*l*/min. The UV wavelength used was 238 nm at 0.3 aufs.

Stability Test

A certain amount of nifedipine (10 ng, 50 ng and 100 ng) was spiked in 1 m*l* of normal human plasma and stored in the freezer at −20℃ for 5 and 9 months. Remaining nifedipine concentration was determined using the nifedipine assay mentioned above.

Interference Peaks of Other Drugs

Following drugs were examined in terms of any possible interferences with nifedipine during the HPLC assay : chloral hydrate, furosemide, diphenhydramine hydrochloride, digoxin, phenobarbital, spironolactone, chlorothiazide, and acetaminophen. The dosage form of chloral hydrate was liquid in a capsule. The capsule was crushed and the drug was extracted with 10 m*l* of acetonitrile. Its concentration was diluted to 1 mg/m*l*. Dosage form of furosemide, diphenhydramine HCL, digoxin, and phenobarbital was the USP injection. They were diluted with acetonitrile to the concentrations of 20 μg/m*l*, 250 μg/ml, 25 μg/m*l* and 500 μg/m*l*, respectively. Dosage form of spironolactone, chlorothiazide, and acetaminophen was a tablet, that was crushed and extracted with 10 m*l* of acetonitrile. Each concentration was diluted to 250 μg/m*l*. One hundred microliters of each sample solution with proper concentration was added to a test tube and evaporated to the dryness. One milliliter of water was added into each tube. The

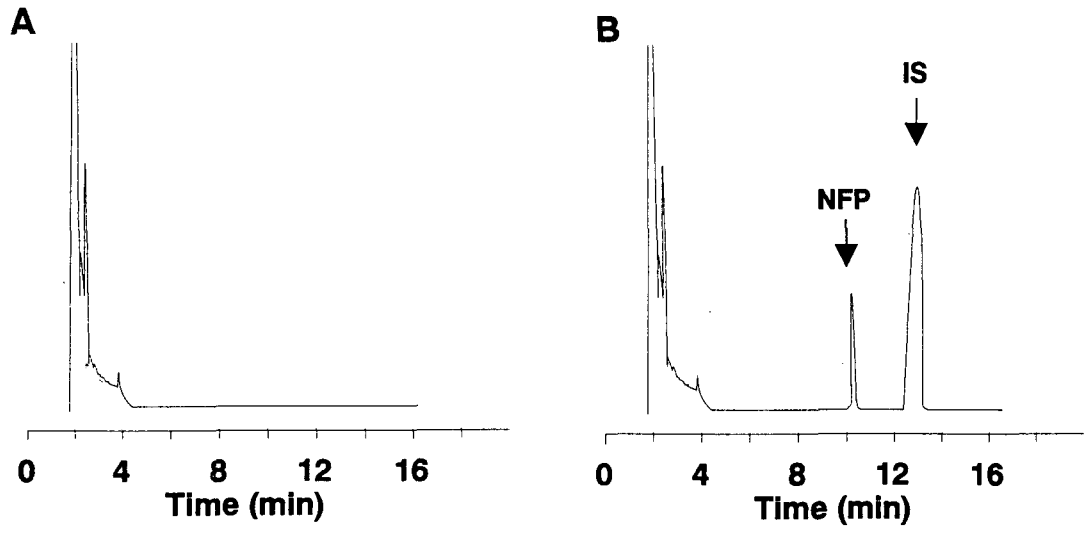

Figure 1—Typical high-performance liquid chromatograms of nifedipine in the human plasma : (A) blank plasma, (B) sample plasma with nifedipine (NFP) and 11-ketoprogesterone (IS).

same procedure was used as in the nifedipine assay and any peaks around the nifedipine peak on the chromatogram were examined.

Pharmacokinetic and pharmacodynamic studies

Nine children, aged 5 months to about 6 years, were studied. The tenth patient was a 18 year old boy (body weight of 65.2 kg) who had the presence of BPD and pulmonary artery hypertension. The patient was maintained on his usual medical regimen with digoxin, spironolactone, and furosemide. However the patient received nothing by mouth, including medications, after midnight of the night before cardiac catheterization. A nifedipine dose of 0.3 mg/kg was administered into the proximal oral cavity. Venous blood samples were obtained for measurement of plasma concentrations at zero, 0.083, 0.167, 0.333, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6 and 8 hr after nifedipine administration. Other sample treatments and hemodynamic measurements were detailed in elsewhere.[17] Hemodynamic values for mean pulmonary artery pressure (mPAP), pulmonary vascular resistance index (PR), cardiac index (CI) and mean arterial pressure (AOP) were measured.[17]

Data Analysis

Nifedipine plasma concentration-time data were analyzed by one-compartment model ana lysis using PCNONLIN (SCI Software, Lexington, Kentucky). The C_{max}, the T_{max} and the area under the concentration-time curve (*AUC*) were obtained directly from the concentration-time data. The estimates of elimination constant (*ke*) were computed from a log-linear regression of nifedipine plasma concentration versus time for points in the elimination phase of drug distribution.

Results and discussion

The HPLC method reported by Pieta *et al.*[8] was a successful approach to separation of several metabolites and photo-degradation products of nifedipine in the plasma. However their minimum detectability of nifedipine was 10 ng. In order to study in the children patients, there might be a problem with availability of sample plasma. Therefore a modified procedure was tried as in the *Materials and Methods* section above. A typical high performance liquid chromatogram of niffedipine in the human plasma was shown in Fig. 1. The retention times for nifedipine and an in-

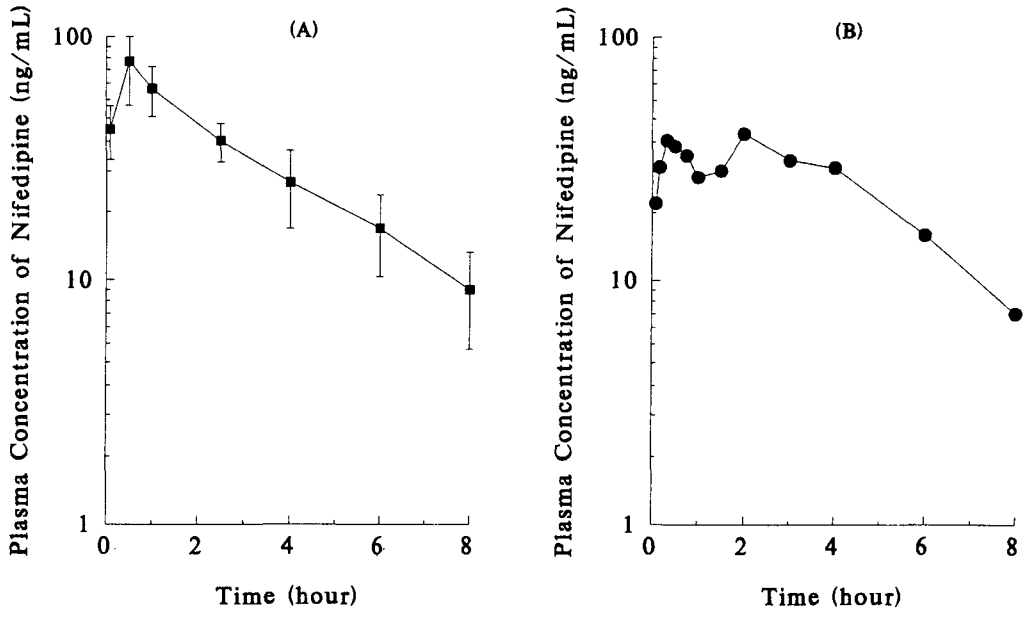

Figure 2—Plasma concentration of nifedipine versus time curve in nine patients (A) and the tenth patient (B) after oral administration of 0.3 mg/kg.

Table I—*Calibration Equations of Nifedipine Obtained from Several Runs of HPLC Assay*

Run	Slope[a]	Intercept[a]	r
1	64.10 ± 2.04	1.81 ± 1.79	0.9979
2	64.71 ± 2.68	1.06 ± 2.55	0.9974
3	61.42 ± 1.54	0.92 ± 1.56	0.9990
4	63.09 ± 0.93	−0.59 ± 0.86	0.9996
5	64.05 ± 1.97	−1.96 ± 1.97	0.9981

[a]Least squares method was used for the calibration curves : (Concentration)=(Slope)*(peak ratio)+(Intercept). Estimate ± Standard error.

ternal standard (11-ketoprogesterone) were 10.5 ± 0.41 and 13.1 ± 0.63 min, respectively. Table I shows several calibration equations of nifedipine obtained from various runs of the HPLC assay. The linearity of the curves was excellent (r^2> 0.997). The limit of quantitation (LOQ) was defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy, precision, and variability.[21] The LOQ of this HPLC assay was 10 ng/ml, even though a peak could be determined down to 4 ng/ml that is slightly better detectability reported than previously.[8] When nifedipine was assayed each time from the plasma, a calibration curve with LOQ of 10 ng/ml was made.

The mean plasma concentrations of nifedipine versus time curve in 9 patients after oral administration of 0.3 mg/kg was shown in Fig. 2 (A). There was no problem appeared until plasma samples from the tenth patient were analyzed. As shown in Fig. 2 (B), there seems to be a double peak phenomenon in the plasma concentration-time curve. According to previous data,[19] C_{max}, T_{max}, and *ke* for nifedipine were 84.2 ng/ml, 1.0 hr and 0.456 $hr,^{-1}$ respectively. In contrast, the tenth patient had 2 peaks, 37.5 ng/ml at 0.33 hr and 40.1 ng/ml at 2 hr. From the terminal line at the elimination phase *ke* was calculated to be 0.342 $hr.^{-1}$

First of all, this difference may come from errors in our analytic method employed. The HPLC method was investigated again. Absolute recovery from plasma using 50 ng/ml of nifedipine was

Table II—*Reproducibility of Nifedipine Assay*

Concentration (ng/ml)	Peak Ratio (NFP/IS) Run 1	Run 2	Run 3
50	0.701	0.745	0.733
	0.813	0.747	0.732
	0.755	0.782	0.797
mean	0.756	0.758	0.754
s.d	0.056	0.021	0.037
%CV[a]	7.4	2.8	4.9
80	1.095	1.242	1.365
	1.200	1.151	1.286
	1.301	1.158	1.228
mean	1.199	1.184	1.293
s.d.	0.103	0.051	0.068
%CV	8.6	4.3	5.3
100	1.667	1.497	1.557
	1.577	1.589	1.614
	1.484	1.604	1.571
mean	1.576	1.563	1.581
s.d.	0.092	0.058	0.030
%CV	5.8	3.7	1.9

[a]Coefficient of variation, %CV=100×(s.d./mean).

Table III—*Stability of Nifedipine in the Human Plasma during the Storage at −20℃*

Nifedipine Concentration (ng/ml)	Percent Remaining Amount (%) After[a] 5 Months	9 Months
10	77.5 ± 3.54	—[b]
50	78.3 ± 2.12	—[b]
100	91.9 ± 1.07	87.0 ± 2.50

[a]Mean ± S.D. (n=3).
[b]Not measured.

102.9 ± 7.07% (n=3). Degradation or loss during the procedure was only 0.69%, that is negligible (data not shown). The reproducibility of nifedipine assay is shown in Table II. Variability during the HPLC run (within-run) was only 1.26 ± 0.013 % (n=3). Relative standard deviations or coefficients of variance (%CV) in various concentrations were low compared to 15% that is one of criteria of acceptance.[21] The stability test suggested some degradation after 5 month storage in the freezer (Table III). However this might not be the case, because the plasma sample were always assayed within a few days after sample collection.

Table IV—*Interference Peaks of Medications with the Nifedipine Peak in the HPLC Assay*

Drugs	Concentration (μg/ml)	Interference[a]
Chloral hydrate	100	No
Furosemide	2	No
Diphenhydramine HCl	25	No
Spironolactone	25	No
Digoxin	2.5	No
Acetaminophen	2.5	No
Chlorothiazide	25	No
Phenobarbital	50	No

[a]All drugs tested did not interfere in the nifedipine assay. For the comparison concentrations of nifedipine and 11-ketoprogesterone used were 0.05 μg/ml and 0.10 μg/ml, respectively.

Other possibility is the interferences with nifedipine during the assay. Some drugs that may be taken frequently by patients were checked if there were any interferences with nifedipine for the assay.[1, 22] Following medications were investigated for the interference peaks around the nifedipine peak in the HPLC assay : chloral hydrate, furosemide, diphenhydramine hydrochloride, digoxin, phenobarbital, spironolactone, chlorothiazide and acetaminophen. The concentrations of possible interference drugs were arbitrary chosen at least 10 times higher concentrations than their normal doses. There were no interference peaks around the nifedipine peak as shown in Table IV. According to our results it can be concluded that the abnormality of plasma concentration-time curve in the tenth patient was not from the errors in HPLC assay itself.

Next possible cause may be from the patient himself. The hemodynamic responses for mean pulmonary artery pressure (mPAP), pulmonary

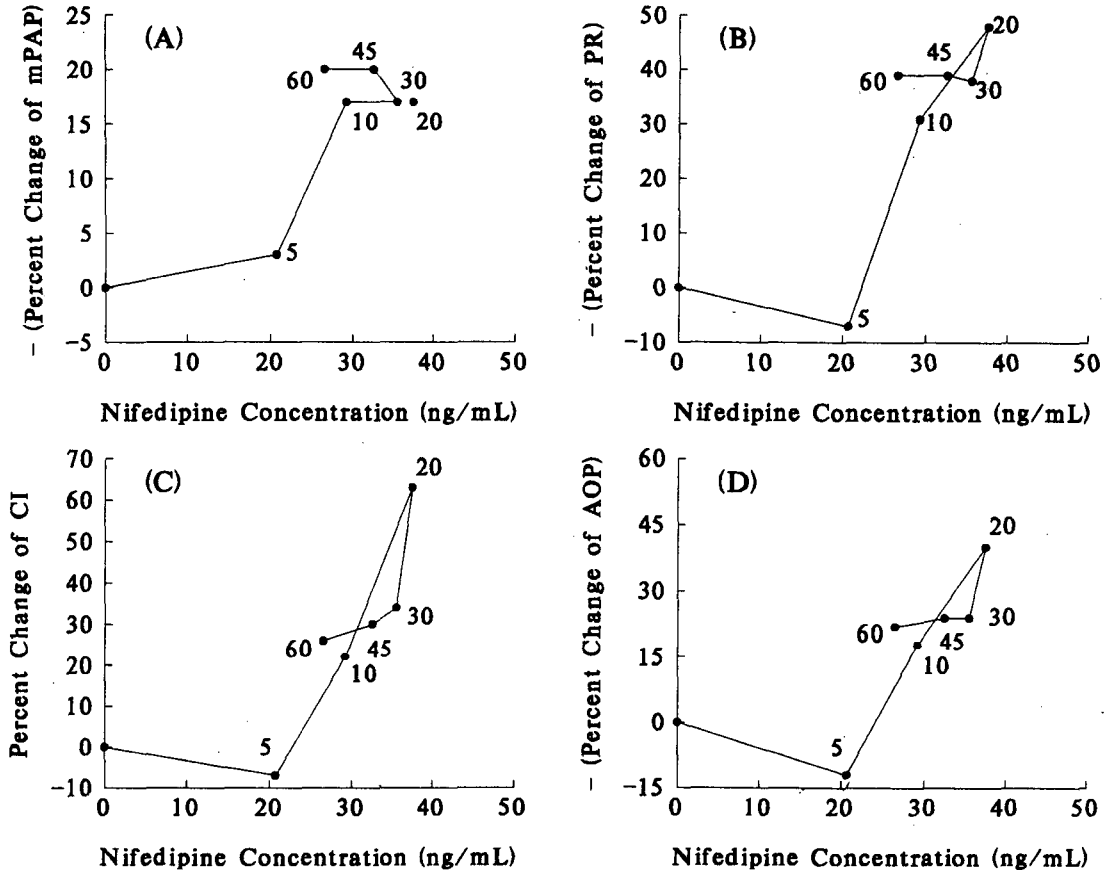

Figure 3—Hysteresis plots of nifedipine concentration versus hemodynamic responses in order of time (min) after an oral dose in the tenth patient : mean pulmonary artery pressure (mPAP; *panel A*), pulmonary vascular resistance index (PR; *panel B*), cardiac index (CI; *panel C*) and mean arterial pressure (AOP; *panel D*).

vascular resistance index (PR), cardiac index (CI), and mean arterial pressure (AOP) were examined. Fig. 3 shows hysteresis plots of nifedipine concentration versus hemodynamic responses in order of time (min) after an oral dose : mPAP, PR, CI and AOP. All hemodynamic responses showed the extended action to the nifedipine concentration in the tenth patient. That is, the double-peak phenomenon in the plasma concentration-time plot may explain this extended responses in the hysteresis plots, since it was known that plasma nifedipine concentrations correlate closely with the hemodynamic responses.[19] It may be caused by slow absorption secondary to the patient being overly sedated and not swallowing the oral liquid all at one time.[23] As a result, the medication was swallowed a little at a time, prolonging the absorption phase. Another clue was the similar *ke* values between patients, suggesting no changes in elimination phase. In conclusion this outlier may be caused by the non-bolus oral administration, but not by the problems in the HPLC method used. No interferences with other medications were possible. However other possibilities couldn't be ruled out such as mistakes in plasma sampling and unknown physiologic differences from the other patients.

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