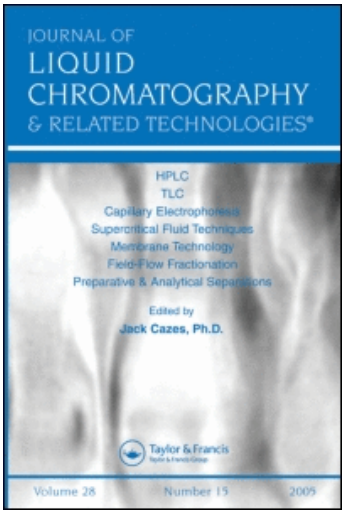

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A SIMPLE HPLC METHOD WITH SPECTROPHOTOMETRIC DETECTION FOR THE SIMULTANEOUS ASSAY OF NIFEDIPINE AND VERAPAMIL IN RAT PLASMA

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A SIMPLE HPLC METHOD WITH SPECTROPHOTOMETRIC DETECTION FOR THE SIMULTANEOUS ASSAY OF NIFEDIPINE AND VERAPAMIL IN RAT PLASMA

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ABSTRACT

A simple, specific, and accurate HPLC assay method is presented for the simultaneous measurement of the calcium antagonists nifedipine and verapamil in rat plasma. The plasma sample was deproteinized by treatment with an equivalent volume of an internal standard solution (nicardipine hydrochloride in acetonitrile containing 0.1% of perchloric acid), followed by brief centrifugation. A 50 μL aliquot of the clear supernatant was analyzed on a Microsorb-MV C18, 5 μm, column using a mixture of acetonitrile-methanol-0.01 M phosphate buffer pH 5.2 (55:15:30) as the mobile phase. At a flow rate of 1 mL/min and detection at 235 nm, nifedipine, verapamil, and nicardipine were observed to elute at about 3.4 min, 6.4 min, and 9.8 min, respectively. Detector responses were linearly related to concentrations of nifedipine in the range 0.2-1 μg/mL, and of verapamil between 0.4-2 μg/mL. As little as 0.05 μg/mL of nifedipine and 0.08 μg/mL of verapamil were accurately measured by the proposed method. Mean drug recoveries from rat plasma samples spiked with 0.25-1 μg/mL of nifedipine and 0.5-2 μg/mL of verapamil was in all cases >95% (range 97.9-98.3%, n = 5, for nifedipine; 95.7-97.1%, n = 5, for verapamil). The method was found to be well suited for assessing the plasma pharmacokinetics of the title drugs following their oral and intraperitoneal coadministration to rats.

INTRODUCTION

Nifedipine (a dihydropyridine), verapamil (a phenylalkylamine), and diltiazem (a benzothiazepin) are the prototypes of drugs pharmacologically classified as calcium antagonists, i.e., capable of inhibiting the inward flux of extracellular calcium ions across specific channels in cell membranes of cardiac and vascular smooth muscles.[1,2] As a result, these drugs can uncouple the process of calcium-mediated excitation-contraction at target cells and bring about peripheral and coronary vasodilation and depression of the cardiac activity.[3] In this respect, whereas verapamil and diltiazem are about equipotent in their vasodilating and cardiac depressant actions, nifedipine is significantly more active on vascular than on cardiac smooth muscles.[4,5] Regardless of their intrinsic pharmacological differences, all of these agents have been found useful to control attacks of angina pectoris and to treat essential hypertension and certain forms of arrhythmia.[5-7] Furthermore, in those instances where therapy with a single calcium antagonist has failed to provide effective control of the hypertensive state or was the cause of significant side effects, the addition of a second drug that decreases blood pressure by a different mechanism of action may result in a more satisfactory therapeutic response.[5,8-13] For this reason, diltiazem, nifedipine, or verapamil has been used in conjunction with either a β-adrenoceptor blocker,[5,8-10] with a thiazide diuretic,[8,11] or with an ACE inhibitor.[5,8] Alternatively, two calcium antagonists possessing different pharmacological specificities and adverse reaction profiles have been given in combination with each other.[12,13] However, because of the lesser similarity in pharmacological activity between nifedipine and verapamil than between diltiazem and verapamil, the former drug combination is considered to be a therapeutically more appropriate than the latter one.[13]

Because of the increasing clinical use of combinations of calcium antagonists in the treatment of hypertension, this laboratory is, at present, evaluating the pharmacokinetic profile of a number of these pharmacological agents in laboratory animals. In support of this work, an HPLC method with photometric detection that simultaneously measures nifedipine and verapamil in rat plasma was developed. The details of this method, representing an advantageous alternative to available HPLC methods for measuring individual calcium antagonists,[14-37] constitute the subject of the present report.

EXPERIMENTAL

Materials and Reagents

The samples of nifedipine, nicardipine hydrochloride, and verapamil hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO, and used as received. Analytical reagent grade dibasic sodium phosphate (anhydrous), perchloric acid, and phosphoric acid were from J. T. Baker, Phillipsburg,

NJ. HPLC grade acetonitrile, methanol, and water were from E.M. Science, Gibbstown, NJ.

Instrument

Analyses were conducted isocratically using a modular liquid chromatograph consisting of model M-45 solvent delivery system (Waters Associates, Milford, MA), Spectroflow 783 programmable absorbance detector (ABI Analytical/Kratos Division, Ramsey, NJ), and model HP 3394A recording integrator (Hewlett-Packard, Avondale, PA). Samples were injected by means of a WISP model 710B autosampler (Waters).

Chromatographic Conditions

The drugs were separated on a Microsorb-MV C18, 25 cm x 4.6 mm i.d., 5 μm, column (Rainin Instrument Co., Woburn, MA). The mobile phase consisted of a mixture of acetonitrile-methanol-0.01 M disodium hydrogen phosphate, adjusted to pH 5.2 with 50% phosphoric acid (55:15:30), filtered through a 0.45 μm membrane filter, and degassed by sonication prior to use. The flow rate was 1 mL/min, and the detection wavelength 235 nm. An internal standard solution was prepared by dissolving nicardipine hydrochloride in 0.1% of perchloric acid in acetonitrile to a concentration of 2 μg/mL.

Method Validation

To investigate the linearity of the method, varying volumes of stock solutions of nifedipine (50 μg/mL) in methanol and of verapamil hydrochloride (100 μg/mL) in distilled water were mixed with each other in individual volumetric flasks, and each mixture diluted with drug-free rat plasma to obtain concentrations of nifedipine in the range 0.2-1 μg/mL and of verapamil in the range 0.4-2 μg/mL. Then, the dilutions were put through the proposed assay method. To evaluate the accuracy of the method, the same drug dilutions were prepared in duplicate but using water as the diluent. In all cases, detector responses were assessed on the basis of the peak height ratios of each analyte to that of the internal standard, and used to construct a five-points calibration curve for each drug by linear regression analysis based on the results for duplicate samples.

Within-day reproducibility was established by analyzing triplicate sets of plasma samples spiked with 0.25, 0.50 and 1 μg/mL of each analyte on three different occasions. Interday reproducibility was studied by analyzing the same plasma samples once a day for three consecutive days. To minimize the photodegradation of nifedipine, all manipulations with this compound were carried

out under a yellow lamp and all its solutions were stored in screw-capped amber glass containers and at 4°C.

Animal Studies

Pharmacokinetic studies were conducted using male Sprague-Dawley rats, 225-250 g in weight (Taconic Farms, Germantown, NY), assigned to groups of 3 rats each, and fasted overnight for 14 hr prior to an experiment. The treatment solution was prepared by dissolving nifedipine and verapamil hydrochloride in pyrogen-free distilled water to which a few drops of ethanol were added to remove any turbidity. One group of rats received the treatment solution by the oral route from a syringe fitted with a curved oral feeding needle; the other group received the same solution by the intraperitoneal route from a tuberculin syringe fitted with a 25-gauge needle. The dose of nifedipine was 2 mg/kg and that of verapamil hydrochloride 6 mg/kg. Blood samples were collected by the orbital sinus technique at 15, 30, 60, 90, 120, 150, and 180 min, into Microtainer® tubes containing EDTA potassium (Becton Dickinson and Co., Franklin Lakes, NJ). After vortex mixing and centrifugation at 3,500 rpm for 10 min, the corresponding plasma samples were immediately transferred to clean test tubes and stored at -30°C pending their analysis.

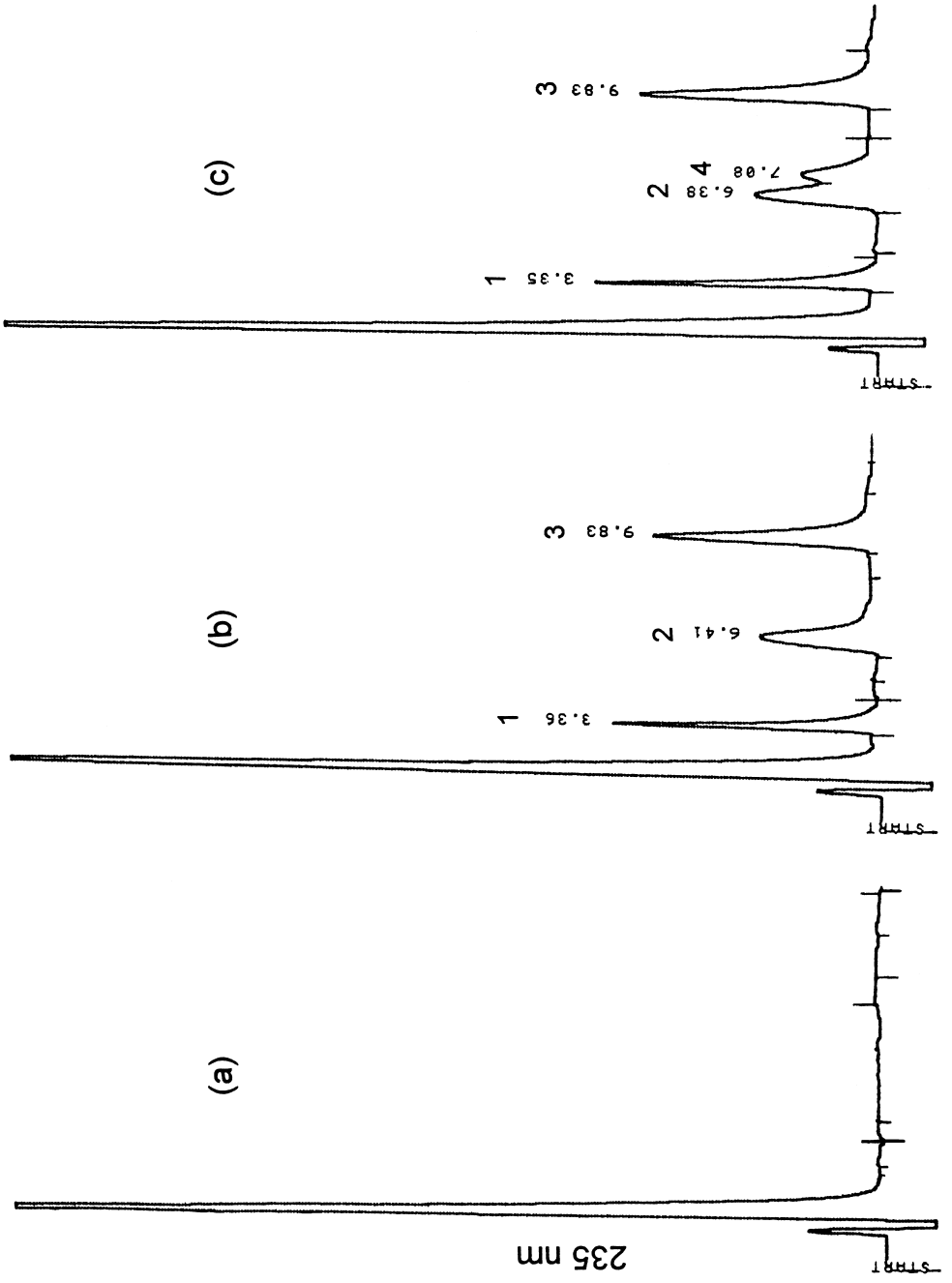
Assay Method

Into a 1.5 mL polyethylene tube with a snap cap, 200 µL of a plasma sample and 200 µL of the internal standard solution were added in succession. After capping, the tube contents were vortex mixed for 30 sec, and next centrifuged at 5,000 rpm for 10 min. A 50 µL aliquot of the clear supernatant was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Typical chromatograms of a drug-free rat plasma and of plasma that contained nifedipine, verapamil, and nicardipine, the internal standard, are shown in Figures 1(a) and 1(b), respectively.

Figure 1. Representative chromatograms of: (a) a drug-free rat plasma; (b) a plasma sample from a rat that had received both nifedipine and verapamil; and (c) a plasma sample from a rat that had received both nifedipine and verapamil and which had been spiked with diltiazem. Peaks: 1, nifedipine; 2, verapamil; 3, nicardipine (the internal standard); 4, diltiazem.



A resolution (R) >2.0 was attained between any two chromatographic peaks. The approximate retention times for nifedipine, verapamil, and nicardipine were 3.4, 6.4, and 9.8 min, respectively. A chromatographic run could be completed in about 12 min. Nicardipine was chosen as the internal standard because of its structural similarity to nifedipine, good solubility properties in the deproteinizing-extracting medium, and strong detector response. In addition, it was well resolved from the peaks of quantitative interest. Since nifedipine is therapeutically used in combination with either atenolol or diltiazem, the potential utility of the recommended chromatographic conditions to analyze these mixtures was also evaluated. In the case of atenolol, it was found that this drug eluted in too close proximity to peaks representing endogenous plasma components to be quantified accurately. Changes in the ratio of the various components of the mobile phase failed to improve the separation. On the other hand, under the same experimental conditions, diltiazem (retention time = 7.1 min) eluted right after verapamil (retention time = 6.4 min) and as a chromatographic peak that was only partially resolved from that of verapamil (Figure 1(c)). Preliminary experiments with mobile phases containing only acetonitrile-methanol-water, and acidified with different concentrations of phosphoric acid resulted in nifedipine, verapamil, and nicardipine eluting as sharp and symmetrical peaks, especially verapamil; however; the degree of chromatographic resolution was suboptimal. This problem was readily corrected upon the addition of a buffer to the mobile phase.

Numerous procedures have been described for the individual isolation of nifedipine and verapamil from plasma or serum samples prior to their analysis by HPLC. For the most part, these procedures have entailed the extraction of the drug from an alkaline medium with an immiscible organic solvent, followed by evaporation and reconstitution of the residue in the mobile phase[14-18] or a suitable organic solvent.[19-21] Variations of this approach have consisted of a preliminary liquid-liquid extraction followed by a back extraction into an acidic aqueous solution[22-24] or by a second back extraction from an aqueous into an organic phase,[25-27] deproteinization with an inorganic divalent salt prior to liquid-liquid extraction,[28] and the addition of urea prior to extraction to minimize drug binding to plasma proteins.[29] Alternatively, methods relying on the use of solid-phase extraction have also been suggested.[26,30-33] In comparison to these multistep sample preparation procedures, the one used in the present study is much simpler in execution since the deproteinization and extraction of the sample was carried out by simply treating a volume of plasma sample with an equal volume of 0.1% solution of perchloric acid in acetonitrile followed by brief centrifugation. The extract thus obtained was cleaner than one obtained by using an equivalent volume of pure acetonitrile[34,35] or methanol[36] and, hence, amenable to direct injection into the liquid chromatograph.

Detector responses (peak heights) were found to be linearly related to concentrations of nifedipine in the range 0.2-1.0 μg/mL and of verapamil in the

Table 1

Recovery Values for Nifedipine and Verapamil from Rat Plasma

Amount Added (μg/mL)	n	Nifedipine Mean ± SD (%)	Nifedipine RSD (%)	Verapamil Mean ± SD (%)	Verapamil RSD (%)
0.25	5	93.3 ± 0.02	0.02	97.1 ± 0.03	0.03
0.50	5	97.9 ± 0.03	0.03	96.5 ± 0.04	0.04
1.00	5	98.1 ± 0.01	0.01	95.7 ± 0.04	0.04

range 0.4-2.0 μg/mL. The line equations for the calibration curves of nifedipine and verapamil were $y = 0.59x - 0.042$ and $y = 0.251x + 0.0061$, respectively, and in which y is the peak height ratio of the analyte to the internal standard and x is the amount of analyte added. Overall, the degree of linearity for both curves was excellent ($r^2 > 0.998$ for both curves). As an assessment of accuracy, the levels of drug measured in plasma were related to those measured in aqueous solutions by the equation % recovery = [peak height ratio for the drug in plasma/peak height ratio for the drug in water] x 100. The results of this study, as summarized in Table 1, were derived by treating a plasma sample with an equal volume of deproteinizing internal standard solution that contained 0.1% of perchloric acid. The mean recovery of nifedipine was >97% (range = 97.9-98.3%) and that of verapamil >95% (range = 95.7-97.1%). By using a plasma sample to deproteinizing internal standard solution ratio of 4:1 and a higher concentration of perchloric acid (1%), a mean recovery of verapamil of 89.9% (range = 85.6-93.0 %) was previously reported by this laboratory.[37] This difference in drug recovery is probably a reflection of the lower level of verapamil that can remain bound to a looser pellet of precipitated protein than to a more compact pellet and, at the same time, agrees with the known high tendency of this drug to bind to plasma proteins.[38,39] The same type of result would be expected from nifedipine since this drug also shows a high degree of binding to plasma proteins.[40,41] By using a 200 μL volume of plasma sample, the lowest quantifiable levels of nifedipine and verapamil by the proposed HPLC methods were 0.05 ng/mL and 0.08 ng/mL, respectively, at a signal to noise ratio of 2 to 1.

As a verification of within-day reproducibility, the proposed method was used to analyze three identical sets of drug-free rat plasma samples spiked with 0.25-1.00 μg/mL each of nifedipine and verapamil at different times on the same day. To evaluate day-to-day reproducibility, the same spiked plasma samples were assayed once a day over three consecutive days. The intraday RSD for nifedipine ranged from 1.48-5.78% and that of verapamil from 7.1-10.0% (Table 2). The corresponding interday RSD values ranged from 2.05-4.34% for nifedipine, and from 4.78-9.25% for verapamil (Table 3).

Table 2

Results of the Intraday Variability Study on the Proposed HPLC Method[a]

Concentration Measured (μg/mL)	n	Nifedipine Mean ± SD (%)	RSD (%)	Verapamil Mean ± SD (%)	RSD (%)
0.25	3	0.13 ± 0.01	3.07	0.06 ± 0.01	10.00
0.50	3	0.27 ± 0.01	1.48	0.14 ± 0.01	7.10
1.00	3	0.57 ± 0.03	0.03	0.26 ± 0.02	8.40

[a] Based on peak height ratio.

The proposed method was applied to the simultaneous analysis of nifedipine and verapamil in the plasma of rats that had received these drugs concurrently either by the intraperitoneal or by the oral route of administration. Figures 2 and 3 show the mean plasma concentration-time curves by each route for groups of 3 rats each. The pharmacokinetic data derived from these plasma values are summarized in Table 4.

In conclusion, the HPLC method described in this report represents a simple, rapid, and accurate analytical approach for the concurrent assay of the calcium antagonists nifedipine and verapamil in plasma. Because of its small sample requirement, extreme simplicity, good accuracy and reproducibility, and

Table 3

Results of the Interday Variability Study on the Proposed HPLC Method[a]

Concentration Measured (μg/mL)	n	Nifedipine Mean ± SD (%)	RSD (%)	Verapamil Mean ± SD (%)	RSD (%)
0.25	3	0.14 ± 0.01	4.34	0.06 ± 0.01	9.25
0.50	3	0.29 ± 0.01	2.05	0.12 ± 0.01	7.75
1.00	3	0.58 ± 0.01	2.41	0.23 ± 0.01	4.78

[a] Based on peak height ratios.

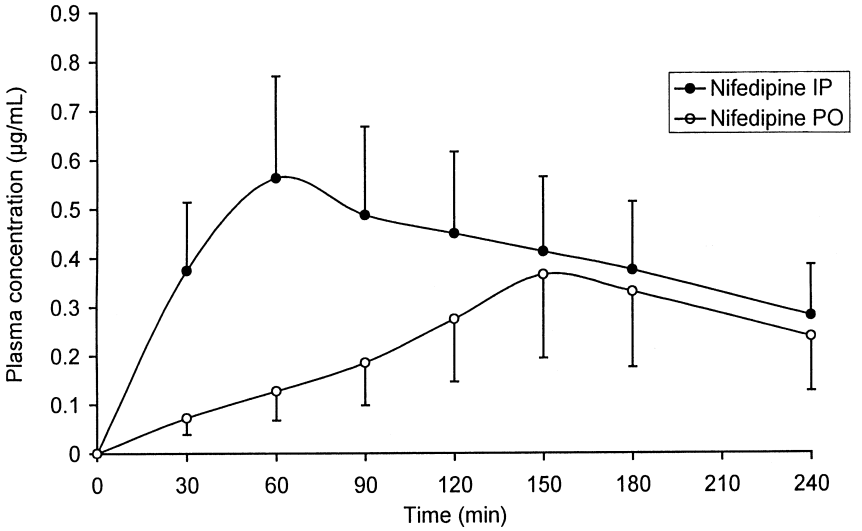


Figure 2. Temporal changes in the mean plasma levels of nifedipine from rats that had received the drug (2 mg/kg) by the intraperitoneal and oral routes. Vertical lines represent the S.D. for n = 3 rats.

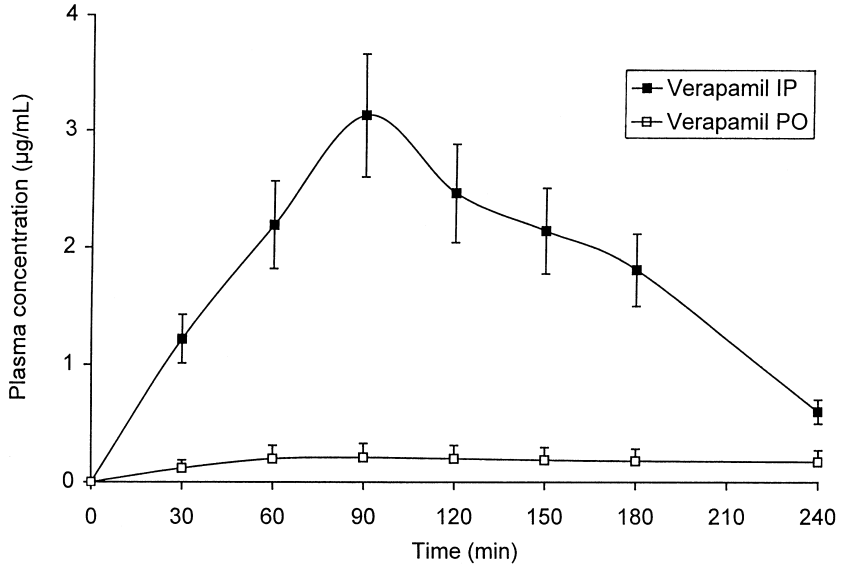


Figure 3. Temporal changes in the mean plasma levels of verapamil from rats that had received the drug (6 mg/kg) by the intraperitoneal and oral routes. Vertical lines represent the S.D. for n = 3 rats.

Table 4

Pharmacokinetic Data for Plasma Nifedipine and Verapamil Following Their Concurrent Intraperitoneal and Oral Administration to Rats[a]

Drug	Route	$t_{1/2}$ (min)	K_e (min^{-1})	C_{max} (μg/mL)	t_{max} (min)	$AUC_{0-\infty}$ (μg/mL)
Nifedipine	interperitoneal	188.00±9.29	0.0037±0.0002	0.563±0.003	60.00±7.00	164.76±4.09
Nifedipine	oral	134.00±7.55	0.0052±0.0002	0.415±0.004	150.00±15.00	103.49±7.54
Verapamil	interperitoneal	66.00±4.58	0.0105±0.0042	3.13±0.1200	90.0±13.00	473.87±13.95
Verapamil	oral	43.00±28.57	0.0013±0.0003	0.21±0.0600	90.0±11.00	176.00±6.51

[a] Doses: nifedipine, 2 mg/mL; verapamil, 6 mg/kg. Values are the mean ± SD, n = 3.

reasonable sensitivity, this method is well suited for studying the pharmacokinetics of these drugs in a small animal model such as the rat.

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