

HPLC-MS/MS method development for the quantitative determination of nifedipine for Caco-2 permeability assay

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Abstract

Aim. Poorly water-soluble drugs such as nifedipine offer challenging problems in drug formulation as poor solubility is generally associated with poor dissolution characteristics and thus with poor oral bioavailability (BCS class II drugs). Methods of quantitative determination of nifedipine by methods of spectrophotometry and chromatography are described in the scientific literature. However, methods are not developed for examination of nifedipine from Caco-2 cell monolayers. Caco-2 cell monolayers have been extensively used for years as a tool to test permeability, assess the oral absorption potential and study the absorption mechanism of compounds. Therefore, the aim of this study was to develop and validate an efficient HPLC MS/MS method for determination of nifedipine from Caco-2 cell monolayers.

Materials and methods. Chromatography was achieved on Discovery C18, 50 × 2.1 mm, 5 μm column. Samples were chromatographed in a gradient mode (eluent A (acetonitrile – water – formic acid, 5 : 95 : 0.1 v/v), eluent B (acetonitrile – formic acid, 100 : 0.1 v/v)). The initial content of the eluent B is 0%, which increases linearly by 1.0 min to 100% and to 1.01 min returns to the initial 0%. The mobile phase was delivered at a flow rate of 0.4 mL/min into the mass spectrometer ESI chamber. The sample volume was 5 μL.

Results. Under these conditions, nifedipine was eluted at 1.83 min. A linear response function was established at 1 – 100 ng/mL. The regression equation for the analysis was $Y = 0.0323x - 0.00121$ with coefficient of correction (R^2) = 0.9987. According to the Caco-2 test results, nifedipine showed high permeability. The within-run coefficients of variation ranged between 0.331% and 0.619% for nifedipine. The within-run percentages of nominal concentrations ranged between 98.80% and 100.63% for nifedipine. The between-run coefficients of variation ranged between 0.332% and 0.615% for nifedipine. The between-run percentages of nominal concentrations ranged between 98.98% and 101.71% for nifedipine. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted limits.

Conclusion. From results of analysis, it can be concluded that developed method is simple and rapid for determination of nifedipine from confluent Caco-2 monolayers and from aqueous solution. Acquired results demonstrate that proposed strategy can be effortlessly and advantageously applied for examination of nifedipine from Caco-2 cell monolayers.

Keywords

LC-MS/MS, Nifedipine, Caco-2 cells, Recovery, Bioavailability

Introduction

Poorly water-soluble drugs such as nifedipine (~ 20 pg/mL) offer challenging problems in drug formulation as poor solubility is generally associated with poor dissolution characteristics and thus with poor oral bioavailability (BCS class II drugs). Nifedipine is a dihydropyridine calcium channel blocking agent. Nifedipine inhibits the transmembrane influx of extracellular calcium ions into myocardial and vascular smooth muscle cells, causing dilatation of the main coronary and systemic arteries and decreasing myocardial contractility. Chemical name of nifedipine is dimethyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (Fig. 1).

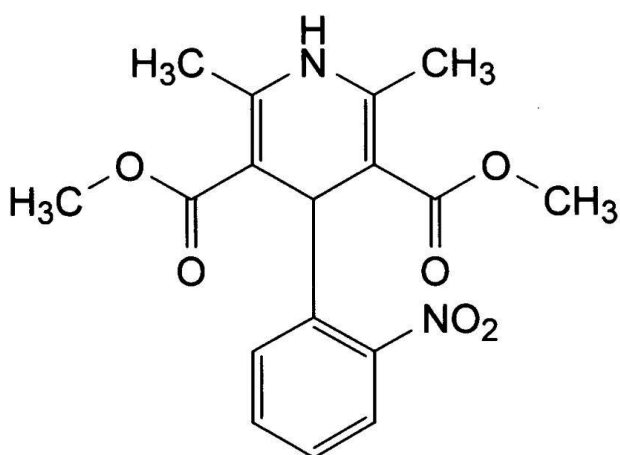


Figure 1. The chemical structure of nifedipine.

The State Pharmacopoeia of Ukraine (SPhU) (The State Pharmacopoeia of Ukraine 2015) has a monograph on the substance of nifedipine. For identification of the substance of nifedipine, the SPhU offers the determination of melting point, absorption spectrophotometry in the infrared region, TLC (mobile phase – ethyl acetate P – cyclohexane (40:60)) and qualitative reaction of formation of azo dye after the previous restoration of nitro group to amino group, quantitative determination – cerimetry. The United States Pharmacopoeia regulates the definition of nifedipine in substances and tablets. For identification, the definition of absorption spectrophotometry in the infrared region and UV-spectrophotometry is proposed. For quantitative determination of nifedipine in tablets – HPLC/UV method. In accordance with this article, the following chromatographic conditions are used: chromatographic column of category L1 (with fixed phase C18) with size 4.6 mm x 250 mm; mobile phase – acetonitrile: methanol: water (25:25:50); solvent – methanol, wavelength – 235 nm, flow rate – 1.0 ml/min.

The European Pharmacopoeia has (European Pharmacopoeia 2016) a monograph on the substance of nifedipine. For identification, it is proposed to determine the melting point, absorption spectrophotometry in the infrared region, TLC (mobile phase – a mixture of ethyl acetate P and cyclohexane P (40:60 V/V) and qualitative reaction to

the primary aromatic amino group – reaction of formation of azo dye (after preliminary reduction of nitro group to amino group). For the quantitative determination of nifedipine – method of cerimetry.

Methods of quantitative determination of nifedipine by methods of spectrophotometry and chromatography are described in the scientific literature (Kondratova et al. 2016, 2017; Liliya et al. 2016; Logoyda 2018a, b, c, 2019; Logoyda et al. 2017a, b, c, 2018a, b, c; Mykhalkiv et al. 2018a, b). However, methods are not developed for examination of nifedipine from Caco-2 cell monolayers (Fujikawa et al. 2005; Gertz et al. 2010). Caco-2 cell monolayers have been extensively used for years as a tool to test permeability, assess the oral absorption potential and study the absorption mechanism of compounds (Gozalbes et al. 2011; Hou et al. 2004). Therefore, the aim of this study was to develop and validate an efficient LC MS/MS method for determination of nifedipine from Caco-2 cell monolayers.

Materials and methods

Chemicals and reagents

In the present work we used Trypsin EDTA (10×) 0.5% / 0.2% in DPBS (PAA, UK; Cat L11-003), HEPES, High Purity Grade (Helicon, Am-0485), Dulbecco's PBS (1×) without Ca & Mg (PAA, UK; Cat H15-002), Hanks' BSS (1×) without Ca & Mg without Phenol Red (PAA, UK; Cat H15-009), DMSO Chromasolv Plus, HPLC grade, ≥ 99.7% (Sigma-Aldrich, USA; Cat 34869), DMEM (4.5 g/l) liquid without L-Glutamine (PAA, UK; Cat E15-009), L-Glutamine (200 mM) (PAA, UK; Cat M11-004), Fetal Bovine Serum «GOLD» EU approved (PAA, UK; Cat A15-151), Penicillin/Streptomycin (100×) (PAA, UK; Cat P11-010), Acetonitrile Chromasolv gradient grade for HPLC (> 99.9%) (Sigma-Aldrich, USA; Cat 34851), Formic acid for mass spectrometry 98% (Fluka, USA; Cat 94318), Propranolol hydrochloride ≥ 99% (TLC), powder (Sigma-Aldrich, USA; Cat P0884), Quinidine anhydrous (Sigma-Aldrich, USA; Cat Q3625 Lot BCBF1345V), Atenolol, analytical reference material, ≥ 98.5% (HPLC) (Sigma-Aldrich, USA; Cat 74827).

Nifedipine (purity 99.98%) was purchased from Moehs Catalana, S.L., Spain. Test compound was provided as dry powder (nifedipine) and was dissolved in DMSO at 10 mM to prepare working stocks.

Instrumentation and chromatographic conditions

In the present study, optimization and critical evaluation of mobile phase composition (gradient), flow rate and analytical column were important to obtain good resolution of peaks, which in turn affect reproducibility and sensitivity of the method. Selection of chromatographic conditions for the proposed method was optimized to suit the preclinical pharmacokinetic studies. Initial feasibility

experiments of a various mixture(s) of solvents such as acetonitrile, methanol and formic acid along with altered flow rates (in the range of 0.1–0.6 ml/min) were performed to optimize an effective chromatographic resolution of nifedipine. Various analytical columns were tested to obtained good and reproducible response within short run time. The HPLC system was coupled with tandem mass spectrometer API 3000 (PE Sciex). The TurboIonSpray ion source was used in both positive and negative ion modes. Parameters of electrospray ionizer and MRM characteristics are listed in Table 1. Acquisition and analysis of the data were performed using Analyst 1.5.2 software (PE Sciex). Chromatography was achieved on DiscoveryC18, 50 × 2.1 mm, 5 μm column. Samples were chromatographed in a gradient mode (eluent A (acetonitrile – water – formic acid, 5 : 95 : 0.1 v/v), eluent B (acetonitrile – formic acid, 100 : 0.1 v/v)). The initial content of the eluent B is 0%, which increases linearly by 1.0 min to 100% and to 1.01 min returns to the initial 0%. The mobile phase was delivered at a flow rate of 0.400 ml/min into the mass spectrometer ESI chamber. The sample volume was 5 μl.

Table 1. Parameters of ionizer electrospray.

Parametr	Value
1 Polarity	Positive
2 Nebulizer Gas (NEB, Gas 1)	15
3 Curtain Gas (CUR)	8
4 Collision Gas (CAD)	4
5 IonSpray Voltage (IS)	5000
6 Temperature (TEM)	400
7 Turbo IonSpray Gas	8
8 Horizontal Position	5.3
9 Lateral Position	1.3

Caco-2 cells were cultivated in 75 cm² flasks to 70–80% of confluence according to the ATCC and Millipore recommendations in humidified atmosphere at 37 °C and 5% CO₂. Cells were detached with Trypsin/EDTA solution and resuspended in the cell culture medium to a final concentration of 2 × 10⁵ cells/ml. 500 μl of the cell suspension was added to each well of HTS 24-Multiwell Insert System and 35 ml of prewarmed complete medium was added to the feeder tray. Caco-2 cells were incubated in Multiwell Insert System for 21 days before the transport experiments. The medium in filter plate and feeder tray was changed every other day. After 21 days of cell growth, the integrity of the monolayer was verified by measuring the transepithelial electrical resistance (TEER) for every well using the Millicell-ERS system ohm meter. The final TEER values were within the range 150–600 Ω × cm² as required for the assay conditions. 24-well insert plate was removed from its feeder plate and placed in a new sterile 24-well transport analysis plate. The medium was aspirated and inserts washed with PBS twice.

To determine the rate of compounds transport in apical (A) to basolateral (B) direction, 300 μL of the test compound dissolved in transport buffer at 10 μM (HBSS, 10 mM HEPES, pH = 7.4) was added into the filter wells; 1000 μL of buffer (HBSS, 10 mM HEPES, pH = 7.4) was

added to transport analysis plate wells. The plates were incubated for 90 min at 37 °C with shaking at 100 RPM. 75 μL aliquots were taken from the donor and receiver compartments for LC-MS/MS analysis. All samples were mixed with 2 volumes of acetonitrile with following protein sedimentation by centrifuging at 10000 rpm for 10 minutes. Supernatants were analyzed using the HPLC system with tandem mass spectrometer.

Propranolol (high permeability), Atenolol (low permeability) and Quinidine (moderate permeability) were used as reference compounds.

The apparent permeability (P_{app}) was calculated for Caco-2 permeability assay using the following equation:

$$P_{app} = \frac{V_A}{Area \times time} \times \frac{[drug]_{acc}}{[drug]_{initial,donor}}$$

V_A – volume of transport buffer in acceptor well,

$Area$ – surface area of the insert (equals to effective growth area of the insert – 0.31 cm²),

$Time$ – time of the assay,

$[drug]_{acc}$ – concentration of test compound in acceptor well,

$[drug]_{initial,d}$ – initial concentration of test compound in a donor well.

P_{app} is expressed in 10⁻⁶ cm/sec.

The % recovery can be useful in interpreting the Caco-2 data. If the recovery is very low, this may indicate problems with poor solubility, binding of the compound to the test plate materials, metabolism by the Caco-2 cells or accumulation of the compound in the cell monolayer. The % recovery was calculated using the following equation:

$$\% \text{ recovery} = \frac{C_{acc} \times V_{acc} + C_d \times V_d}{C_{initial,d} \times V_d} \times 100,$$

V_{acc} – volume of compound solution in acceptor well (cm²),

V_d – volume of compound solution in donor well (cm²),

C_{acc} – concentration of test compound in acceptor well (μM),

$C_{initial,d}$ – initial concentration of test compound in a donor well (μM).

Results and discussion

In the present study, optimization and critical evaluation of mobile phase composition, flow rate, and analytical column were important to obtain good resolution of peaks of interest from the endogenous components, which in turn affect reproducibility and sensitivity of the method. The resolution of peaks was best achieved with DiscoveryC18, 50 × 2.1 mm, 5 μm column. Samples were chromatographed in a gradient mode (eluent A (acetonitrile – water – formic acid, 5 : 95 : 0.1 v/v), eluent B (acetonitrile – formic acid, 100 : 0.1 v/v)). The initial content of the eluent B is 0%, which increases linearly by 1.0 min to 100% and to 1.01 min returns to the initial 0%. Gradient curve shown in Fig. 2. The mobile phase was delivered at a flow rate of

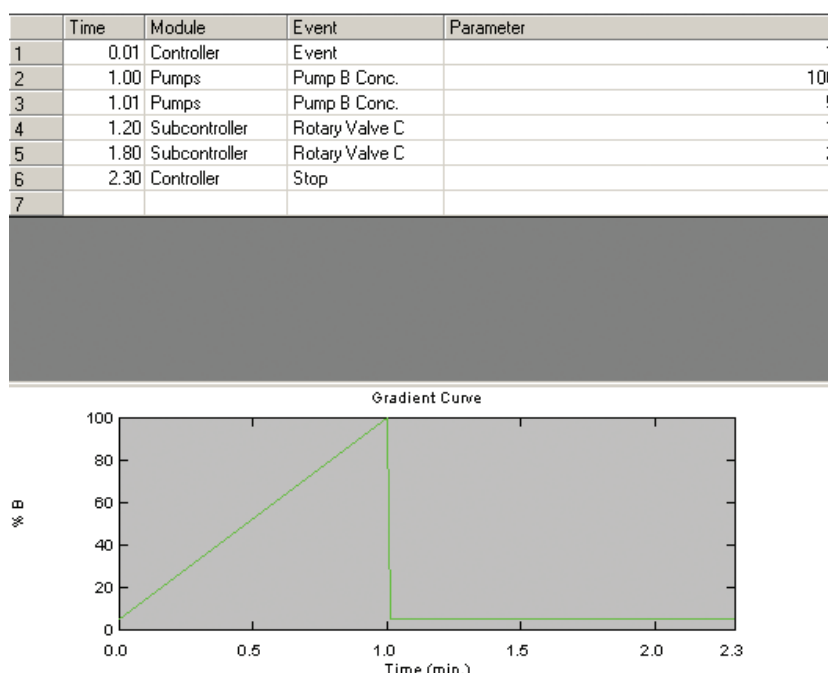


Figure 2. Gradient curve.

0.400 mL/min into the mass spectrometer ESI chamber. The injection volume was 5 μ L. The optimum chromatographic conditions and system suitability parameters are tabulated in Table 2.

Nifedipine eluted at ~1.83 minutes. Typical multiple reaction monitoring chromatograms of nifedipine are shown in Fig. 3. A-B permeability data for the test compound of nifedipine and 3 reference compounds are listed in the Table 3. A-B permeability data for all the reference compounds correspond to the literature data, thus validating this study. According to the Caco-2 test results, nifedipine showed high permeability. It should be noted that the recovery value (Table 4) for nifedipine is 103.74%. On the basis of the data presented in Tables 3, 4 nifedipine can be considered as a highly permeable drug substance. Permeability values obtained *in vivo* by the intestinal perfusion technique were comparable with the P_{eff} obtained by Caco-2 cell line studies. Permeability values of nifedipine, obtained from a correlation of partition coefficients versus intestinal permeability, also suggest a high permeability of nifedipine.

Table 2. Optimized chromatographic conditions.

Parameter	Chromatographic conditions
Instrument	Shimadzu HT (Shimadzu, Japan) LC system equipped with degasser (DGU-14A), binary pump (LC-20ADXR) along with auto-sampler (SIL-20ACXR)
Column	DiscoveryC18, 50 \times 2.1 mm, 5 μ m
Mobile phase	Gradient mode (eluent A (acetonitrile – water – formic acid, 5 : 95 : 0.1 v/v), eluent B (acetonitrile – formic acid, 100 : 0.1 v/v)). The initial content of the eluent B is 0%, which increases linearly by 1.0 min to 100% and to 1.01 min returns to the initial 0%
Flow rate	0.4 mL/min
Runtime	2 min
Column temperature	30 $^{\circ}$ C
Volume of injection loop	5 μ L

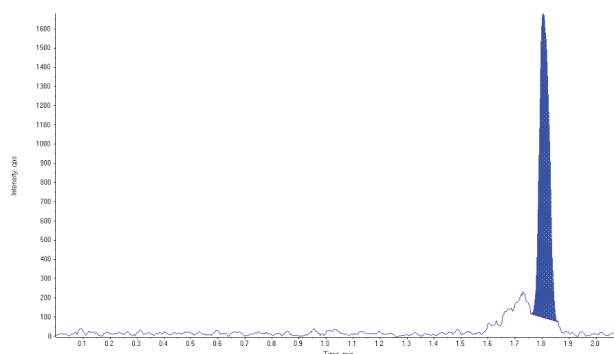


Figure 3. Typical multiple reaction monitoring chromatograms of nifedipine.

Table 3. Data of A-B permeability for the test and reference compounds (at 10 μ M).

Compound ID	Permeability (10^{-6} cm/s)			Mean	SD (10^{-6})
	1	2	3		
Atenolol	1.83	1.99	1.48	1.77	0.26
Propranolol	37.50	35.20	35.70	36.13	1.21
Quinidine	16.50	23.80	20.00	20.10	3.65
Nifedipine	8.31	7.80	6.69	7.58	0.89

*Each value is represented as a mean \pm SD of 5 observations (n = 5), SD: Standard Deviation, RSD: Relative Standard Deviation, Acceptance criteria < 2.0.

Table 4. Recovery data.

Compound ID	% recovery			Mean
	1	2	3	
Atenolol	101.73	99.13	107.20	102.69
Propranolol	112.78	97.86	97.50	102.71
Quinidine	96.49	102.59	97.68	98.92
Nifedipine	109.61	99.70	101.90	103.74

*Each value is represented as a mean \pm SD of 5 observations (n = 5), SD: Standard Deviation, RSD: Relative Standard Deviation, Acceptance criteria < 2.0.

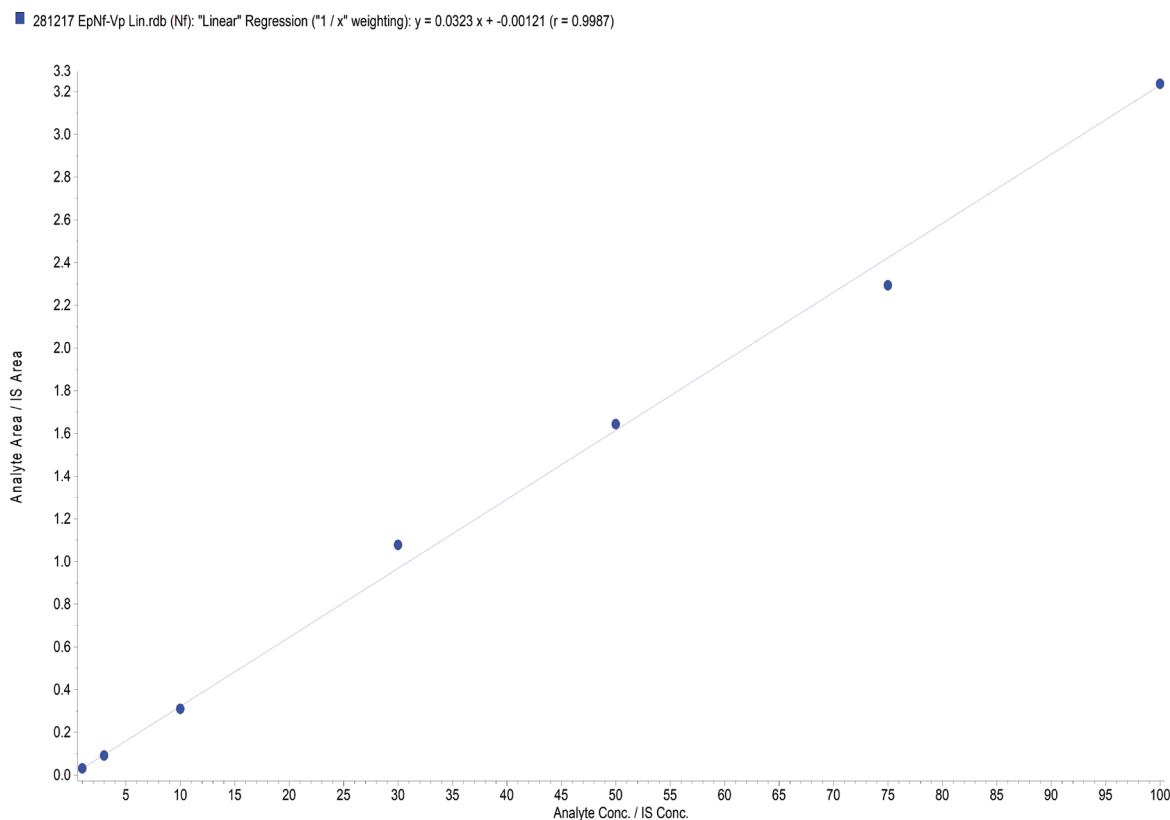


Figure 4. The calibration curve of nifedipine in human plasma.

The calibration curve (Fig. 4) (peak area ratio Vs Concentration) was linear over working range for nifedipine of 1 to 100.00 ng/ml with 7 point calibration used for quantification by linear regression. A linear response function was established at 1 – 100 ng/mL. The regression equation for the analysis was $Y = 0.0323x - 0.00121$ with coefficient of correction (R^2) = 0.9987.

The within-run coefficients of variation ranged between 0.331% and 0.619% for nifedipine. The within-run percentages of nominal concentrations ranged between 98.80% and 100.63% for nifedipine. The between-run coefficients of variation ranged between 0.332% and 0.615% for nifedipine. The between-run percentages of nominal concentrations ranged between 98.98% and 101.71% for nifedipine. Results are presented in Table 5. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted limits.

The results were found to be within the assay variability limits during the entire process.

We selected nifedipine as a CYP3A4 test drug because *in vitro* results were obtained by the closely related nifedipine derivative denitronifedipine. Nifedipine undergoes extensive CYP3A4-dependent biotransformation both in the gut wall and the liver. When the criteria of the Guidances are strictly applied, nifedipine is a BCS Class II substance and this API can not be considered a candidate for granting a biowaiver. From a scientific point of view, nifedipine is a candidate for granting a biowaiver when the tablets are formulated with well-known excipients, show rapid *in vitro* dissolution, and meet the dissolution

Table 5. Intra-day and inter-day precision data of nifedipine.

Day	Intra-day precision		Inter-day precision	
	Mean	RSD %	Mean	RSD %
1	98.80	0.378	101.71	0.332
2	100.41	0.619	98.98	0.390
3	100.63	0.331	100.53	0.615

*Each value is represented as a mean \pm SD of observations, SD: Standard Deviation, RSD: Relative Standard Deviation, #Acceptance criteria < 2.0

profile comparison criteria as defined in the Guidances, but with a redefined upper boundary for the pH of 6.8. The USP criteria and method are suitable to assure batch to batch consistency.

Conclusion

Chromatographic separation achieved on DiscoveryC18, 50×2.1 mm, $5 \mu\text{m}$ column. Samples were chromatographed in a gradient mode (eluent A (acetonitrile – water – formic acid, 5 : 95 : 0.1 v/v), eluent B (acetonitrile – formic acid, 100 : 0.1 v/v)). Statistical analysis proves that the method is reproducible and selective for the simultaneous estimation of nifedipine.

In summary it can be concluded that developed method is simple and rapid for determination of nifedipine from confluent Caco-2 monolayers and from aqueous solution. Acquired results demonstrate that proposed strategy can be effortlessly and advantageously applied for examination of nifedipine from Caco-2 cell monolayers.

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