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Research Article

Method development for the quantitative determination of captopril from Caco-2 cell monolayers by using LC-MS/MS

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Abstract

Aim. Caco-2 cells are a human colon epithelial cancer cell line used as a model of human intestinal absorption of drugs and other compounds. Although compounds were used in the original Caco-2 cells monolayer assays, compounds have been replaced in most laboratories by the use of liquid chromatography-mass spectrometry (LC-MS) and LC-tandem mass spectrometry (LC-MS/MS). Mass spectrometry not only eliminates the need for compounds, but permits the simultaneous measurement of multiple compounds. The measurement of multiple compounds per assay reduces the number of incubations that need to be carried out, thereby increasing the throughput of the experiments. Furthermore, LC-MS and LC-MS-MS add another dimension to Caco-2 assays by facilitating the investigation of the metabolism of compounds by Caco-2 cells. A simple, rapid LC-MS/MS method has been developed for determination of captopril from confluent Caco-2 monolayers and from aqueous solution.

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, captopril was eluted at 1.42 min. A linear response function was established at 2 – 200 ng/mL. The regression equation for the analysis was y =0.0187x+0.000248 with coefficient of correction (r^2) = 0.9993. According to the Caco-2 test results, captopril showed low permeability. It should be noted that the recovery value is 103.20%. The within-run coefficients of variation ranged between 0.321% and 0.541%. The within-run percentages of nominal concentrations ranged between 99.13% and 101.12%. The between-run coefficients of variation ranged between 0.314% and 0.663%. The between-run percentages of nominal concentrations ranged between 99.17% and 101.03%.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 captopril from confluent Caco-2 monolayers and from aqueous solution. Acquired results demonstrate that proposed strategy can be effortlessly and advantageously applied for examination of captopril from Caco-2 cell monolayers.

Keywords

Caco-2 cells, Captopril, LC-MS/MS, Permeability, Recovery

Introduction

Captopril is an angiotensin-converting enzyme (ACE) inhibitor used in the therapy of hypertension and heart failure. Captopril is associated with a low rate of transient serum aminotransferase elevations and has been linked to rare instances of acute liver injury. Chemical name of captopril is (2S)-1-[(2S)-2- methyl-3- sulfanylpropanoyl] pyrrolidine -2- carboxylic acid (Fig. 1).

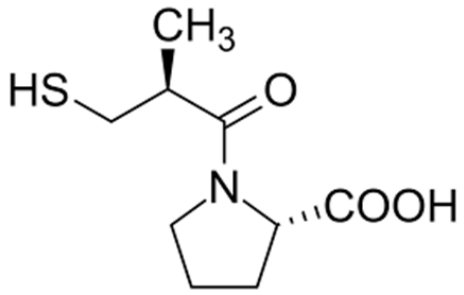

Figure 1. Chemical structure of captopril.

The State Pharmacopoeia of Ukraine (SPhU) has the monograph on the substance of captopril and on tablets of captopril (The State Pharmacopeia of Ukraine 2015). For identification of captopril substance, the SPhU proposes to determine the specific optical rotation and the method of absorption spectrophotometry in the infrared region, the quantitative determination – iodometry potentiometric titration using the combined platinum electrode. For identification of captopril in tablets, the SPhU proposes TLC (mobile phase – a mixture of methanol P, ice acetic acid P, toluene P (1:25:75). For quantitative determination of captopril in tablets – HPLC/UV (mobile phase – a mixture of phosphoric acid of concentrated P, water P and methanol P (0.5:450:550), mobile phase rate – 1.0 mL/min, detection of wavelength 220 nm).

The United States Pharmacopeia regulates the definition of captopril in substances, tablets and combined tablets with hydrochlorothiazide. For identification of captopril in the substance, the method of absorption spectrophotometry in the infrared region and the determination of specific optical rotation is proposed, for quantitative determination – iodatometry. For the identification of captopril in tablets, the United States Pharmacopoeia offers TLC (mobile phase – analogue to the SPhU), for quantification – HPLC/UV. According to this monograph, the following chromatographic conditions are used: chromatographic column of category L1 (with fixed phase C18) size 4.6 × 250 mm; mobile phase – methanol P: water P: phosphoric acid P (550:450:0.5); wavelength – 220 nm, flow rate – 1.0 mL/min.

The European Pharmacopoeia suggests for identification of captopril substance to determine the specific optical rotation and the method of absorption spectrophotometry in the infrared region, the quantitative determination – iodometry, potentiometric titration with the use of a combined platinum electrode (European Pharmacopoeia 2016).

Methods of quantitative determination of captopril in dosage forms and biological liquids by spectrophotometry, electrochemical method and chromatography methods are described in the scientific literature (Kondratova et al. 2016; Logoyda 2018abc?, 2018abc?; Logoyda et al. 2018abc?, 2018abc?; Logoyda 2019ab?; Mykhalkiv et al. 2018ab?; Polyauk et al. 2017). However, methods are not developed for examination of captopril from Caco-2 cell monolayers (Fujikawa et al. 2005; Gertz et al. 2010; Krynytska et al. 2018). Caco-2 cells are a human colon epithelial cancer cell line used as a model of human intestinal absorption of drugs and other compounds. When cultured as a monolayer, Caco-2 cells differentiate to form tight junctions between cells to serve as a model of paracellular movement of compounds across the monolayer. In addition, Caco-2 cells express transporter proteins, efflux proteins, and Phase II conjugation enzymes to model a variety of transcellular pathways as well as metabolic transformation of test substances. In many respects, the Caco-2 cell monolayer mimics the human intestinal epithelium. Although compounds were used in the original Caco-2 cells monolayer assays, compounds have been replaced in most laboratories by the use of liquid chromatography-mass spectrometry (LC-MS) and LC-tandem mass spectrometry (LC-MS/MS). Mass spectrometry not only eliminates the need for compounds, but permits the simultaneous measurement of multiple compounds. The measurement of multiple compounds per assay reduces the number of incubations that need to be carried out, thereby increasing the throughput of the experiments. Furthermore, LC-MS and LC-MS-MS add another dimension to Caco-2 assays by facilitating the investigation of the metabolism of compounds by Caco-2 cells (Gozalbes et al. 2011; Hou et al. 2004; Logoyda 2018abc?, 2018abc?; Logoyda et al. 2018abc?). Therefore, the aim of this study was to develop and validate an efficient LC-MS/MS method for determination of captopril from Caco-2 cell monolayers.

Materials and methods

Chemicals and reagents

In the present work we were used Trypsin EDTA (10x) 0.5% / 0.2% in DPBS (PAA, UK; Cat L11-003), HEPES,

High Purity Grade (Helicon, Am-0485), Dulbecco's PBS (1x) without Ca & Mg (PAA, UK; Cat H15-002), Hanks' BSS (1x) without Ca & Mg without Phenol Red (PAA, UK; Cat H15-009), DMSO Chromasolv Plus, HPLC grade, ≥99.7% (Sigma-Aldrich, USA; Cat 34869), DMEM (4.5g/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 (100x) (PAA, UK; Cat P11-010), Acetonitrile Chromasolv gradient grade for HLC (>99.9%) (Sigma-Aldrich, USA; Cat 34851), Formic acid for mass sectrometry 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).

Captopril (purity 99.9%) was purchased from Moehs Catalana, S.L., Spain. Test compound was provided as dry powder and was dissolved in DMSO at 10 mM to prepare working stocks.

Chromatographic system

All measurements were performed using Shimadzu VP HPLC system including vacuum degasser, gradient pumps, reverse phase HPLC column, column oven and autosampler (Logoyda 2019ab?). The LC system was coupled with tandem mass spectrometer API 3000 (PE Sciex). The TurboIonSpray ion source was used in both positive and negative ion modes. Paramaters of electrospray ionizer and MRM parameters are listed in Table 1. Acquisition and analysis of the data were performed using Analyst 1.5.2 software (PE Sciex).

Table 1. Parameters of ionizer electrospray.

	Parameter	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

Chromatographic conditions

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 0.400 mL/min into the mass spectrometer ESI chamber. The sample volume was 5 µl.

Sample preparation

Caco-2 cells were cultivated in 75 cm^2 flasks to 70–80% of confluence according to the ATCC and Millipore recommendations in humidified atmosphere at 37 °C and 5% CO_2. Cells were detached with Trypsin/EDTA solution and resuspended in the cell culture medium to a final concentration of 2×10^5 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×cm^2 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; 1000µL 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 coupled 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:

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

$\boldsymbol{V_A}$ – volume of transport buffer in acceptor well,
Area – surface area of the insert (equals to effective growth area of the insert – 0.31 cm^2),
Time – time of the assay,
$\boldsymbol{[drug]_{acc}}$ – concentration of test compound in acceptor well,
$\boldsymbol{[drug]_{initial,d}}$ – initial concentration of test compound in a donor well.
$\boldsymbol{P_{app}}$ 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:

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

V_{acc} – volume of compound solution in acceptor well (cm^2),
V_d – volume of compound solution in donor well (cm^2),
C_{acc} – concentration of test compound in acceptor well (mM),
$C_{initial,d}$ – initial concentration of test compound in a donor well (mM).

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 Discovery C18, 50 × 2.1 mm, 5 µm column. Discovery C18 column has many advantages (excellent reproducibility, exceptional peak shape for basic and acidic analytes, stable, low-bleed LC-MS separations, separation of peptides and small proteins, lower hydrophobicity than many comparable C18 columns, providing faster analysis). 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 Figure 2. The mobile phase was delivered at a flow rate of 0.400 mL/min into the mass spectrometer ESI chamber. The injection volume was 5 µl.The optimum chromatographic conditions and system suitability parameters are tabulated in Table 2.

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

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	Discovery C18, 50 × 2.1 mm, 5 µm
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
Run time	2 min
Column temperature	30 °C
Volume of injection loop	5 µl

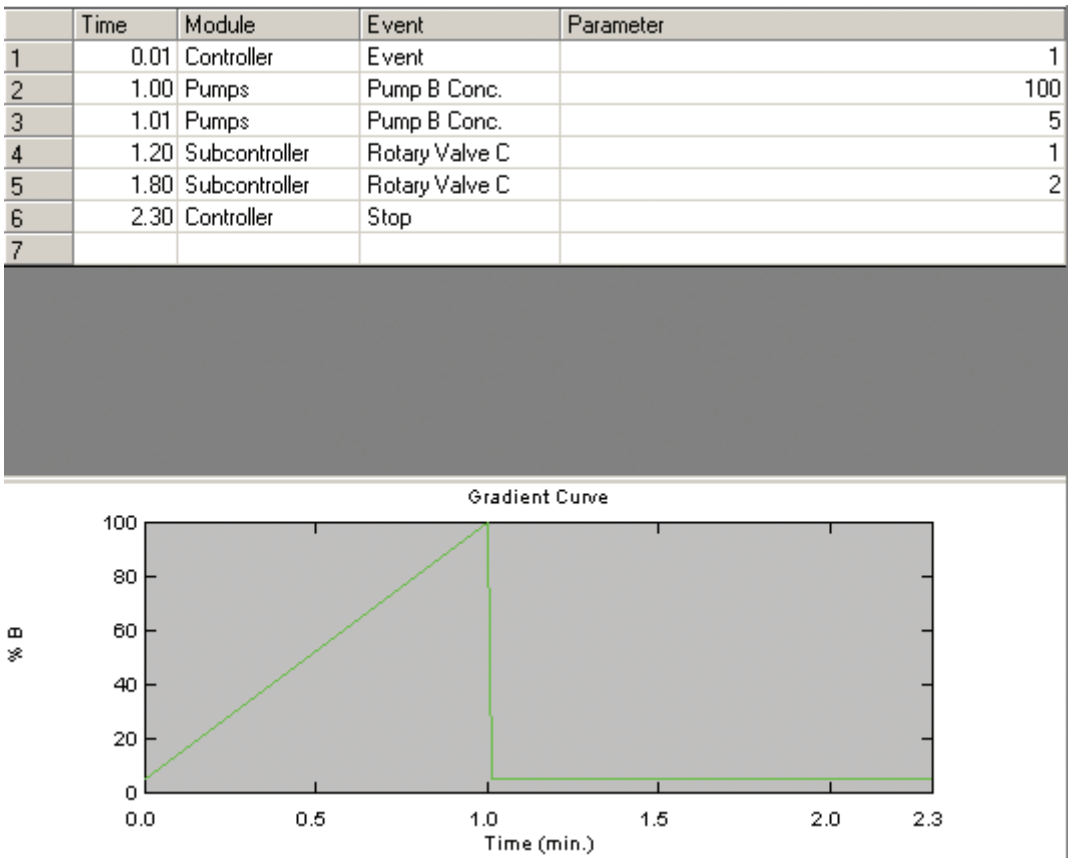

Figure 2. Gradient curve.

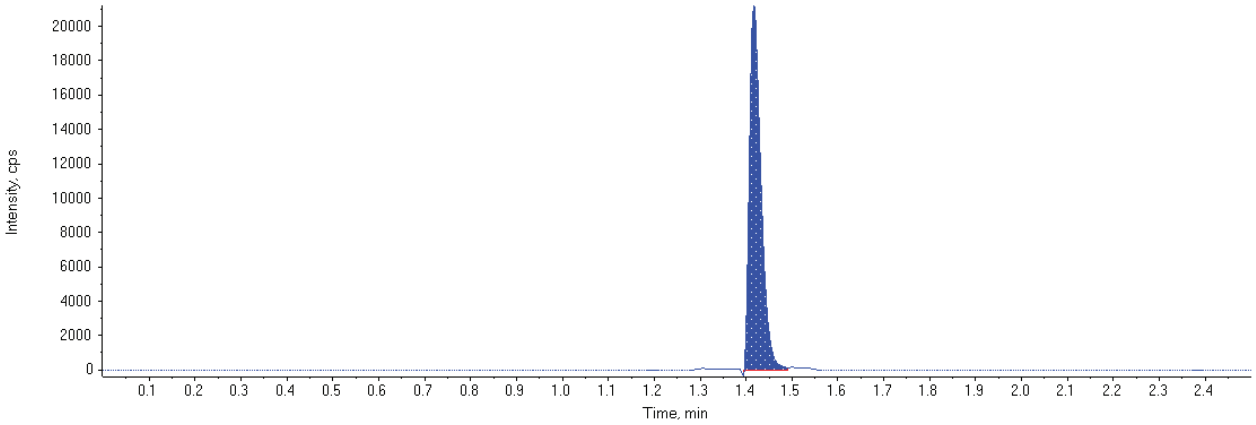

Figure 3. Typical multiple reaction monitoring chromatograms of captopril.

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

Compound ID	Permeability (10^{-6} cm/s)				SD (10^{-6})
	1	2	3	Mean	
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**
Captopril	0.62	1.39	1.93	**1.31**	**0.66**

Table 4. Recovery data.

Name of compound	% recovery			
	1	2	3	Mean
Atenolol	109.61	99.70	101.90	**103.74**
Propranolol	112.78	97.86	97.50	**102.71**
Quinidine	96.49	102.59	97.68	**98.92**
Captopril	100.01	99.81	109.78	**103.20**

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

Captopril eluted at ~1.42 minutes. Typical multiple reaction monitoring chromatograms of captopril shown in Fig. 3. A-B permeability data for the test compound of captopril 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, captopril showed low permeability. It should be noted that the recovery value (Table 4) for captopril is 103.20%. The permeability class boundary is based indirectly on the extent of absorption of a drug substance in humans and directly on measurements of the rate of mass transfer across human intestinal membrane. Alternatively, nonhuman systems capable of predicting the extent of drug absorption in humans can be used (e.g., in vitro epithelial cell culture methods). According to US FDA Biopharmaceutics Classification System (BCS) guidance in the absence of evidence suggesting instability in the GI tract, a drug substance is considered to be highly permeable when the extent of absorption in humans is determined to be 90% or more of an administered dose based on a mass balance determination or in comparison to an intravenous reference dose. According to WHO guidance an API is considered highly permeable when the extent of absorption in humans is 85% or more based on a mass balance determination or in comparison with an intravenous comparator dose. The initial recommendation in the BCS Guidance suggested an absorption value of 90% as a prerequisite for classification as highly permeable. However, successive scientific discussions and scientific publications have suggested relaxing the criterion to 85% absorption for classifying an API as highly permeable. An acceptable alternative test method for permeability determination of the API could be in vivo intestinal perfusion in humans. When this method is used for permeation studies, suitability of the methodology should be demonstrated, including determination of permeability relative to that of a reference compound whose fraction of dose absorbed has been documented to be at least 85%, as well as use of a negative control. According to EMEA BCS guidance if a drug substance has linear and complete absorption then it is considered highly permeable. BCS is a scientific framework for classifying drug substances based on their aqueous solubility and intestinal permeability. When the criteria of the Guidances are strictly applied, captopril is a BCS Class III substance and this API can be considered a candidate for granting a biowaiver. In the case of class III drugs (low permeability, high solubility), the extent of absorption is limited by the permeation rate across intestinal epithelium. These drugs exhibit a high variation in the rate and extent of absorption.

Calibration curve in bioanalytical method is a linear relationship between concentration (independent variable) and response (dependent variable) using a least squares method. The calibration standard curves had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve (peak area ratio Vs Concentration) was linear over working range for captopril of 2 to 200.00 ng/mL with 7 point calibration used for quantification by linear regression, shown in Fig. 4. A linear response function was established at 2–200 ng/mL. The regression equation for the analysis was y =0.0187x+0.000248 with coefficient of correction (r^2) = 0.9993.

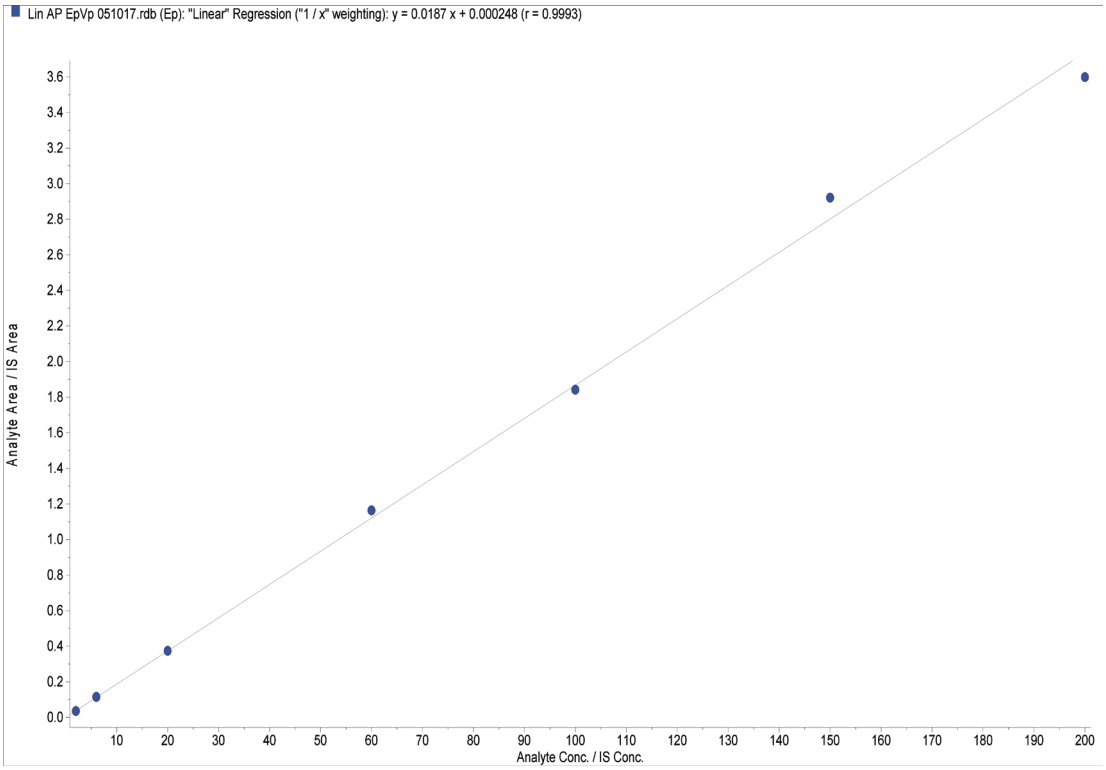

Figure 4. The calibration curve of captopril.

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

Day	Intra-day precision		Inter-day precision	
	Mean	RSD %	Mean	RSD %
1	99.13	0.321	100.77	0.314
2	100.31	0.541	99.17	0.490
3	101.12	0.396	101.03	0.663

The within-run coefficients of variation ranged between 0.321% and 0.541%. The within-run percentages of nominal concentrations ranged between 99.13% and 101.12%. The between-run coefficients of variation ranged between 0.314% and 0.663% . The between-run percentages of nominal concentrations ranged between 99.17% and 101.03%. 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.

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

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

In the case of class III drugs (low permeability, high solubility), the extent of absorption is limited by the permeation rate across intestinal epithelium. These drugs exhibit a high variation in the rate and extent of absorption. This will be our future perspective for *in vitro* and *in vivo* evaluation of different solid dosage forms containing captopril.

Conclusion

Chromatographic separation 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)). Statistical analysis proves that the method is reproducible and selective for the simultaneous estimation of captopril.

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

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