

## A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY METHOD DEVELOPMENT FOR THE QUANTITATIVE DETERMINATION OF ENALAPRIL MALEATE FROM CACO-2 CELL MONOLAYERS

LILIYA LOGOYDA\*

Department of Pharmaceutical Chemistry, Pharmaceutical Faculty, I. Horbachevsky Ternopil State Medical University, Ternopil City, Ukraine. Email: logojda@tdmu.edu.ua

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### ABSTRACT

**Objective:** A simple, rapid high-performance liquid chromatography-mass spectrometry (HPLC MS/MS) method was developed for the determination of enalapril maleate from confluent Caco-2 monolayers and aqueous solution.

**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] and 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 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, enalapril maleate was eluted at 1.52 min. According to the Caco-2 test results, enalapril showed low permeability. It should be noted that the recovery value for enalapril is 100.62%. Low *in vitro* permeability data for enalapril are in good agreement with the literature data.

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

**Keywords:** High-performance liquid chromatography-mass spectrometry, Enalapril maleate, Caco-2 cells, Recovery, Bioavailability.

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### INTRODUCTION

Enalapril maleate is chemically described as (2S)-1-[[[(2S)-2-[[[(1S)-1-(Ethoxycarbonyl)-3-phenylpropyl] amino] propanoyl] pyrrolidine-2- carboxylic acid (Z)-butenedioate (Fig. 1). Enalapril maleate is a prodrug which metabolized rapidly in the liver to ethyl ester of a long-acting enalaprilat, which inhibits angiotensin-converting enzyme in human subjects and animals. In the contemporary literature, bioanalytical methods were reported for quantification of enalapril [1-4]. However, methods are not developed for examination of enalapril maleate from Caco-2 cell monolayers.

Therefore, the aim of this study was to develop and validate an efficient high-performance liquid chromatography-mass spectrometry (HPLC MS/MS) method for the determination of enalapril maleate from Caco-2 cell monolayers.

### METHODS

#### Chemicals and reagents

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 and Mg (PAA, UK; Cat# H15-002)

Hanks' BSS (1x) without Ca and 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 HPLC, ≥99.9% (Sigma-Aldrich, USA; Cat #34851)

Formic acid for mass spectrometry, ~98% (Fluka, USA; Cat #94318)

Falcon® HTS 24-Multiwell Insert Systems with Media Feeder Tray (BD Biosciences, USA; Prod# 351181)

Falcon® 24 Well TC-Treated Cell PS Permeable Support Companion Plate (BD, Prod# 353504)

Centrifuge Tubes, 50 ml (Santa Cruz, USA; Cat# sc-200251)

Serological Pipettes 5 ml, 10 ml, and 25 ml (Greiner Bio-One)

Disposable pipettor tips (Thermo Scientific, Fisherbrand, and Eppendorf USA)

1.1 ml microtubes in microracks (Thermo Scientific, USA)

Zorbax Eclipse Plus C18 column 2.1×50 mm, 3.5 μm (Agilent Technologies, Inc. USA)

Propranolol hydrochloride  $\geq 99\%$  (TLC), powder (Sigma-Aldrich, USA; Cat # P0884)

Quinidine anhydrous (Sigma-Aldrich, USA; Cat # Q3625 Lot #BCBF1345V)

Atenolol, analytical reference material,  $\geq 98.5\%$  (HPLC) (Sigma-Aldrich, USA; Cat #74827)

The test compound was provided as a dry powder (salt form enalapril maleate) and was dissolved in DMSO at 10 mM to prepare working stocks.

#### Instrumentation and chromatographic conditions

All measurements were performed using Shimadzu VP HPLC system including vacuum degasser, gradient pumps, reverse phase HPLC column, column oven, and autosampler. 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 parameters are listed in Tables 1 and 2. Acquisition and analysis of the data were performed using Analyst 1.5.2 software (PE Sciex). Chromatography was achieved on DiscoveryC18, 50 $\times$ 2.1 mm, and 5  $\mu$ m column. Samples were chromatographed in a gradient mode (eluent A [acetonitrile-water-formic acid, 5:95:0.1 v/v] and 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 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  $\mu$ l.

Caco-2 cells were cultivated in 75 cm<sup>2</sup> flasks to 70–80% of confluence according to the ATCC and Millipore recommendations [5] in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. Cells were detached with Trypsin/EDTA solution and resuspended in the cell culture medium to a final concentration of 2 $\times$ 10<sup>5</sup> cells/ml. 500  $\mu$ l 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

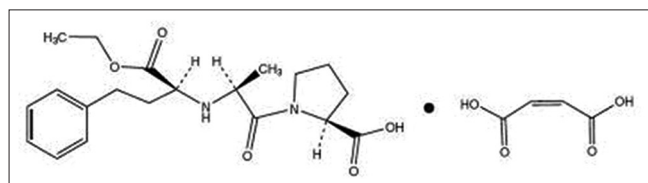


Fig. 1: Chemical structure of enalapril maleate

Table 1: Parameters of ionizer electrospray

S.no	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

Table 2: Multiple reaction monitoring (MRM) parameters

Compound ID	Parent, m/z	Daughter, m/z	Time, ms	DP, V	FP, V	EP, V	CE, V	CXP, V
Bisoprolol	326.435	116.3	50	46	260	11	27	20

DP: Declustering potential, FP: Focusing potential, EP: Entrance potential, CE: Collision energy, CXP: Collision cell exit potential. MRM: Multiple reaction monitoring

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  $\Omega$  $\times$ cm<sup>2</sup>[6] 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  $\mu$ l of the test compound dissolved in transport buffer at 10  $\mu$ M (HBSS, 10 mM HEPES, and pH=7.4) was added into the filter wells; 1000  $\mu$ l of buffer (HBSS, 10 mM HEPES, and 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  $\mu$ l 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 10,000 rpm for 10 min. 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:

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

$V_A$  - volume of transport buffer in acceptor well,

Area - surface area of the insert (equals to effective growth area of the insert - 0.31 cm<sup>2</sup>),

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<sup>-6</sup>cm/s.

The percentage 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 percentage recovery was calculated using the following equation:

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

$V_{acc}$  - volume of compound solution in acceptor well (cm<sup>2</sup>),

$V_d$  - volume of compound solution in donor well (cm<sup>2</sup>),

$C_{acc}$  - concentration of test compound in acceptor well ( $\mu$ M),

$C_{initial,d}$  - initial concentration of test compound in a donor well ( $\mu$ M).

## 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 [7-13]. The resolution of peaks was best achieved with Discovery C18, 50 $\times$ 2.1 mm, and 5  $\mu$ m column. Samples were

Table 3: Optimized chromatographic conditions

Parameter	Chromatographic conditions
Instrument	Shimadzu HT (Shimadzu, Japan) LC system equipped with degasser (DGU-14A), binary pump (LC-20ADXR) along with autosampler (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] and 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 1.01 min returns to the initial 0%
Flow rate	0.4 ml/min
Runtime	2 min
Column temperature	30°C
Volume of injection loop	5 μl

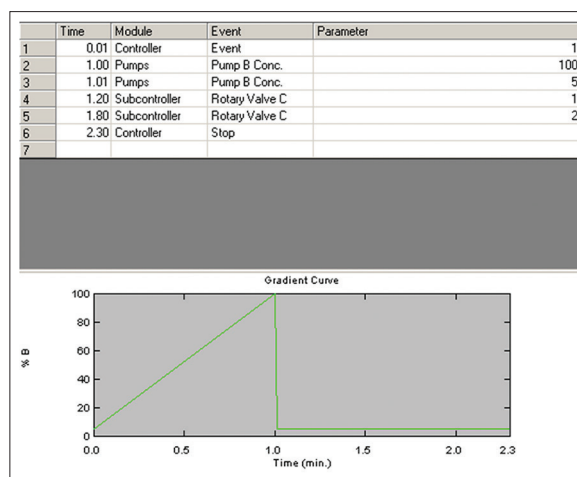


Fig. 2: Gradient curve

Table 4: Data of A-B permeability for the test and reference compounds (at 10 μM)

Compound ID	Permeability (10 <sup>-6</sup> cm/s)				SD (10 <sup>-6</sup> )
	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
Enalapril	0.62	1.39	1.93	1.31	0.66

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

Table 5: Recovery values

Compound ID	% 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
Enalapril	101.73	99.13	107.20	102.69

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

chromatographed in a gradient mode (eluent A [acetonitrile-water-formic acid, 5:95:0.1 v/v] and 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 1.01 min returns to the initial 0%. Gradient curve showed in Fig. 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 3.

Enalapril maleate eluted at ~1.52 min. Typical multiple reaction monitoring chromatograms of enalapril shown in Fig. 3.

A-B permeability data for the test compound of enalapril maleate and three reference compounds are listed in Table 4.

A-B permeability data for all the reference compounds correspond to the literature data, thus validating this study [14-16]. According to the Caco-2 test results, enalapril showed low permeability. It should be noted that the recovery value (Table 5) for enalapril is 100.62%. Low *in vitro* permeability data for enalapril are in good agreement with the literature data (0.79–2.29\*10<sup>-6</sup> cm/s) [17-20].

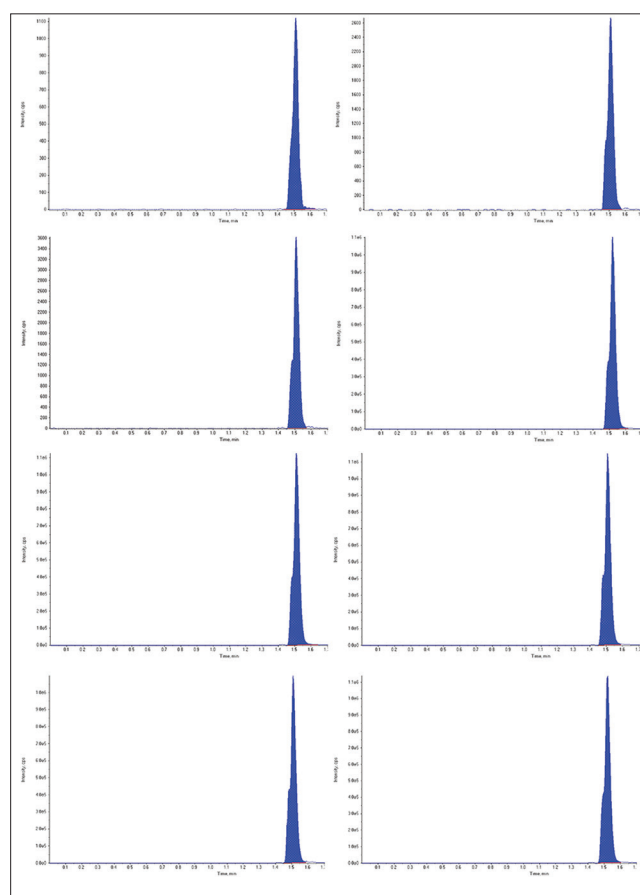


Fig. 3: Typical multiple reaction monitoring chromatograms of enalapril maleate

## CONCLUSION

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

## CONFLICTS OF INTEREST

The authors declared that they have no conflicts of interest.

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