

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

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**ABSTRACT**

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

**Methods:** Chromatography was achieved on discovery C 18, 50 mm×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 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.

**Results:** Under these conditions, bisoprolol was eluted at 1.49 min. According to the Caco-2 test results, bisoprolol appeared to have moderate to high permeability. It should be noted that the recovery value for bisoprolol is 97.69%. Caco-2 permeability values for bisoprolol are in agreement with BCS Class 1 classification for these drugs and their known high bioavailability in humans.

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

**Keywords:** Bisoprolol fumarate, High-performance liquid chromatography–mass spectrometry/mass spectrometry, recovery, Caco-2 cells, Bioavailability.

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**INTRODUCTION**

The Caco-2 monolayers are used as an *in vitro* model to predict drug absorption in humans and to explore mechanism of drug absorption. The Caco-2 cells are derived from a human colon adenocarcinoma and spontaneously differentiate to form confluent monolayer of polarized cells structurally and functionally resembling the small intestinal epithelium [1].

Bisoprolol fumarate can be determined by various methods such as ultraviolet, reverse-phase high-performance liquid chromatography (HPLC), LC–mass spectrometry (MS)/MS, and high-performance thin-layer chromatography. There is an HPLC method described for the determination of bisoprolol (Fig. 1) in pharmaceutical preparations. In addition, there are another methods reported for the determination of bisoprolol in human plasma [2-5].

However, both methods are not developed for the examination of bisoprolol from Caco-2 cell monolayers. Therefore, the aim of this study was to develop and validate an efficient HPLC MS/MS method for the determination of bisoprolol from Caco-2 cell monolayers.

**METHODS****Chemicals and reagents**

Trypsin ethylenediaminetetraacetic acid (EDTA) (10×) 0.5%/0.2% in Dulbecco's phosphate-buffered saline (DPBS) (PAA, UK; Cat# L11-003)

HEPES, high purity grade (Helicon, Am-0485)

DPBS (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)

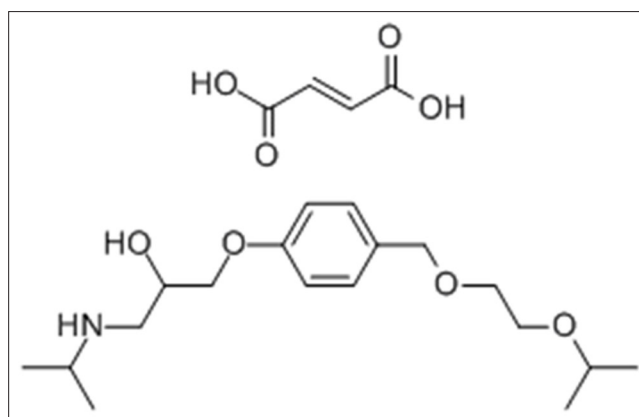


Fig. 1: The chemical structures of bisoprolol fumarate

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)

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, 25 ml (Greiner Bio-One)

Disposable pipette tips (Thermo Scientific, Fisherbrand, 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 ≥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)

Test compound was provided as dry powder (salt form bisoprolol fumarate) and was dissolved in DMSO at 10 mM to prepare working stocks.

**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	Ion Spray voltage (IS)	5000
6	Temperature (TEM)	400
7	TurboIon Spray gas	8
8	Horizontal position	5.3
9	Lateral position	1.3

**Table 2: 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

**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 C 18, 50 mm×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 to 1.01 min returns to the initial 0%
Flow rate	0.400 ml/min
Runtime	5 min
Column temperature	30°C
Volume of injection loop	5 μl

**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 TurboIon Spray ion source was used in both positive and negative ion modes. Parameters of electrospray ionizer and multiple reaction monitoring (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 Discovery C18, 50 mm×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 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.

Caco-2 cells were cultivated in 75 cm<sup>2</sup> flasks to 70–80% of confluence according to the ATCC and Millipore recommendations [6] in 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 2×10<sup>5</sup> 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 of 150–600 Ω×cm<sup>2</sup> [7] 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 two 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.

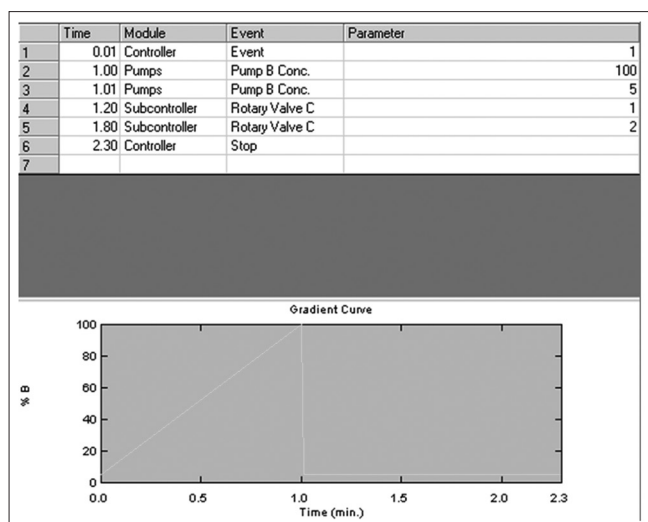


Fig. 2: Gradient curve

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 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 % 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

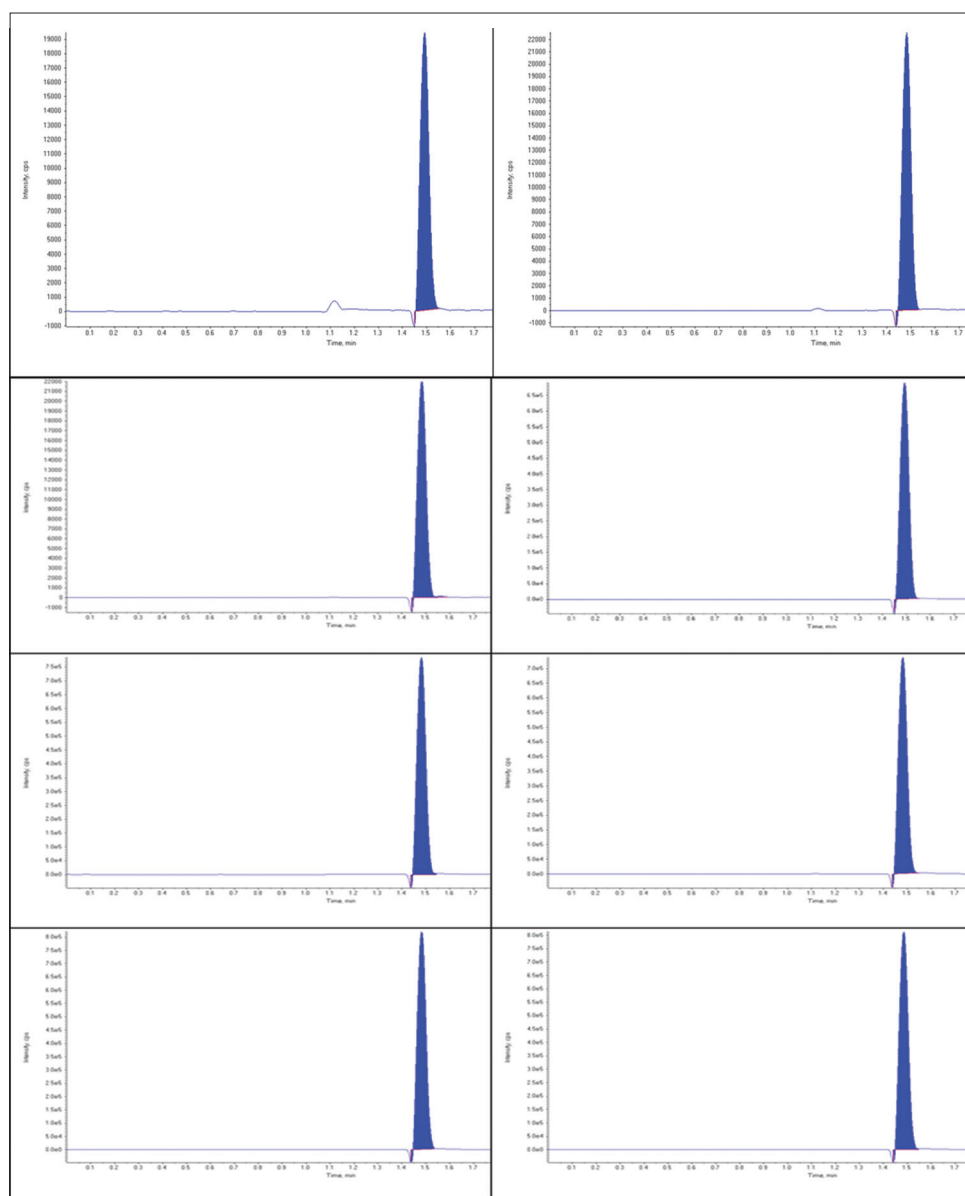


Fig. 3: Typical multiple reaction monitoring chromatograms of bisoprolol

**Table 4: Data of A-B permeability for the test and reference compounds (at 10  $\mu$ M)**

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
Bisoprolol	14.40	16.10	15.70	15.40	0.89

Each value is represented as a mean $\pm$ SD of five 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
Bisoprolol	91.78	104.48	96.82	97.69

by the Caco-2 cells, or accumulation of the compound in the cell monolayer. The % recovery was calculated using the following equation:

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

$V_{\text{acc}}$  – volume of compound solution in acceptor well ( $\text{cm}^2$ ),

$V_{\text{d}}$  – volume of compound solution in donor well ( $\text{cm}^2$ ),

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

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

## RESULTS AND DISCUSSION

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 of interest from the endogenous components, which in turn affect reproducibility and sensitivity of the method [8-10]. The resolution of peaks was best achieved with discovery C 18, 50 mm $\times$ 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] 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 to 1.01 min returns to the initial 0%. Gradient curve was shown 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  $\mu\text{l}$ . The optimum chromatographic conditions and system suitability parameters are tabulated in Table 3.

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

Bisoprolol eluted at  $\sim$ 1.49 min. Typical MRM chromatograms of bisoprolol are shown in Fig. 3. A-B permeability data for the test compound of bisoprolol fumarate 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 [11-13]. According to the Caco-2 test results, bisoprolol appeared to have moderate to high permeability.

It should be noted that the recovery value (Table 5) for bisoprolol is 97.69%. Caco-2 permeability values for bisoprolol are in agreement with BCS Class I classification for these drugs and their known high bioavailability in humans [14-17].

## CONCLUSION

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

## AUTHORS CONTRIBUTION

Liliya Logoyda, Dmytro Korobko conceived and designed the experiments. Liliya Logoyda prepared the samples and performed the experiments. Liliya Logoyda, Dmytro Korobko worked together on the development of the ideas presented in this paper, and contributed to the data analysis and manuscript writing.

## CONFLICT OF INTERESTS

Declared none.

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