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Research Article

HPLC MS/MS method development for the quantitative determination of verapamil hydrochloride from Caco-2 cell monolayers

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Abstract

Aim. An understanding of the role that transporters, in particular P-glycoprotein (P-gp), can play in the absorption, distribution, metabolism and excretion (ADME) of candidate drugs, and an assessment of how these processes might impact on toxicity and the potential for drug-drug interactions in the clinic, is required to support drug development and registration. It is therefore necessary to validate preclinical assays for the in vitro evaluation of candidate drugs as substrates or inhibitors of human P-gp. 2. A simple, rapid HPLC MS/MS method was developed for determination of verapamil hydrochloride 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, verapamil hydrochloride was eluted at 1.08 min. A linear response function was established at 1 – 100 ng/mL. The regression equation for the analysis was Y = 0.0162x + 0.00391 with coefficient of correction (R^2) = 0.9992. According to the Caco-2 test results, verapamil showed low permeability. It should be noted that the recovery value for verapamil hydrochloride is 102.69%. The within-run coefficients of variation ranged between 0.336% and 0.617% for verapamil. The within-run percentages of nominal concentrations ranged between 98.82% and 100.62% for verapamil. The between-run coefficients of variation ranged between 0.334% and 0.612% for verapamil. The between-run percentages of nominal concentrations ranged between 98.97% and 101.76% for verapamil. 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 verapamil hydrochloride from confluent Caco-2 monolayers and from aqueous solution. Acquired results demonstrate that proposed strategy can be effortlessly and advantageously applied for examination of verapamil hydrochloride from Caco-2 cell monolayers.

Keywords

HPLC-MS/MS, Verapamil hydrochloride, Caco-2 cells, Recovery, Bioavailability

Introduction

Verapamil is a phenylalkylamine calcium channel blocking agent. Verapamil 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 verapamil is 2-(3,4-dimethoxyphenyl)-5-[2-(3,4-dimethoxyphenyl)ethyl-methylamino]-2-propan-2-ylpentanenitrile. Its structure is shown in Fig. 1.

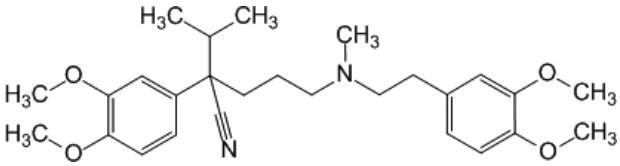

Figure 1. Chemical structure of verapamil.

The State Pharmacopoeia of Ukraine (SPhU) has a monograph on the substance of verapamil hydrochloride and on verapamil hydrochloride tablets (The State Pharmacopeia of Ukraine 2015). To identify the substance of verapamil hydrochloride, SPhU offers UV-spectrophotometry, absorption spectrophotometry in the infrared region, TLC (mobile phase – diethylamine P – cyclohexane (15:85)), quantitative determination – alkalimetry, potentiometric titration. For identification of verapamil hydrochloride in tablets, SPhU proposes UV-spectrophotometry, HPLC/UV (mobile phase – a mixture of heptylamine P^N – acetic acid of ice P – acetonetrile P – solution of 1.36 g/L of sodium acetate P (1:4.7:58:1.37)), qualitative reaction to chlorides. For quantitative determination of verapamil hydrochloride in tablets – UV-spectrophotometry.

The United States Pharmacopoeia regulates the definition of verapamil hydrochloride in substance, tablets and injection solution. In order to identify verapamil hydrochloride in the substance, determination is made by absorption spectrophotometry in the infrared region, HPLC/UV (mobile phase – analogous to SPhU), qualitative reaction to chlorides, for quantitative determination – acidimetry in non-aqueous medium. For the identification of verapamil hydrochloride in tablets, the United States Pharmacopoeia offers the absorption spectrophotometer method in the infrared area and HPLC/UV. The drug Verapamil hydrochloride, tablets are described in the monograph of the United States Pharmacopoeia. According to this article, the HPLC/UV is regulated by the following chromatographic conditions: chromatographic column of category L1 (fixed phase C18) size 4.6 mm × 125 or 150 mm; mobile phase – acetonitrile: 2-aminoheptane: solution A (0.015 M solution of sodium acetate containing 33 ml/l of acetic acid) in ratio (30:0.5:70); wavelength – 278 nm, flow rate – 0.9 ml/min.

The European Pharmacopoeia suggests identifying verapamil hydrochloride UV-spectrophotometry substance, absorption spectrophotometry in the infrared region, TLC (mobile phase – diethylamine P – cyclohexane (15:85)) and qualitative reaction to chlorides, quantitative determination – alkalimetry potentiometric titration (European Pharmacopoeia 2016).

Methods of quantitative determination of verapamil hydrochloride in dosage forms and biological liquids by spectrophotometry, electrochemical method and chromatography methods are described in the scientific literature (Kondratova et al. 2016, 2017; Krynytska and Maruschak 2018; 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 verapamil from Caco-2 cell monolayers (Fujikawa et al. 2005; Gertz et al. 2010). The human intestinal Caco-2 cell line has been extensively used over the last twenty years as a model of the intestinal barrier. An understanding of the role that transporters, in particular P-glycoprotein (P-gp), can play in the absorption, distribution, metabolism and excretion (ADME) of candidate drugs, and an assessment of how these processes might impact on toxicity and the potential for drug-drug interactions in the clinic, is required to support drug development and registration. It is therefore necessary to validate preclinical assays for the in vitro evaluation of candidate drugs as substrates or inhibitors of human P-gp (Gozalbes et al. 2011; Hou et al. 2004). Therefore, the aim of this study was to develop and validate an efficient HPLC MS/MS method for determination of verapamil hydrochloride from Caco-2 cell monolayers.

Materials and methods

Chemicals and reagents

In the present work we were 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 (4.5g/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 (100×) (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).

Verapamil (purity 100.0%) was purchased from Moehs Catalana, S.L., Spain. Test compound was provided as dry powder (salt form verapamil hydrochloride) 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. Paramatrs of electrospray ionizer and MRM parametrs 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 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.

Table 1. Parametrs 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 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; 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 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:

$$\mathbf{P}_{app} = \frac{V_A}{\text{Areaxtime}} \times \frac{[\text{drug}]_{\text{acc}}}{[\text{drug}]_{\text{initial donor}}}$$

$\boldsymbol{V_A}$ – 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:

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

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

$\boldsymbol{V_d}$ – volume of compound solution in donor well (cm^2),

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

$\boldsymbol{C_{initial,d}}$ – 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 co-

	Time	Module	Event	Parameter
1	0.01	Controller	Event	1
2	1.00	Pumps	Pump B Conc.	100
3	1.01	Pumps	Pump B Conc.	5
4	1.20	Subcontroller	Rotary Valve C	1
5	1.80	Subcontroller	Rotary Valve C	2
6	2.30	Controller	Stop	
7				

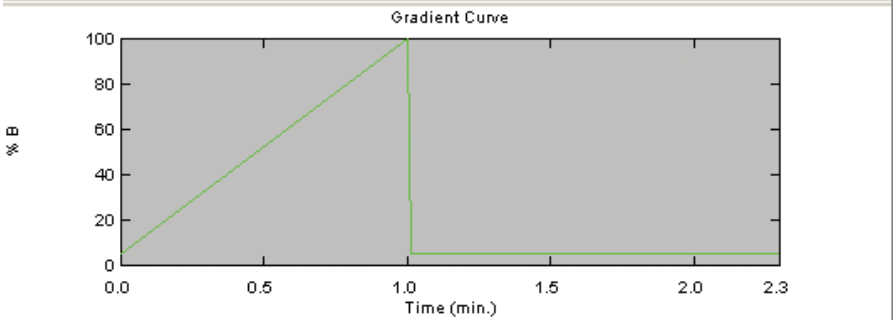

Figure 2. Gradient curve.

lumn 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. 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.

Verapamil hydrochloride eluted at ~1.08 minutes. Typical multiple reaction monitoring chromatograms of verapamil shown in Fig. 3. A-B permeability data for the test compound of verapamil hydrochloride 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, verapamil showed low permeability. It should be noted that the recovery value (Table 4) for verapamil is 102.69%. On the basis of the data presented in Table 3 verapamil 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 verapamil, obtained from a correlation of partition coefficients versus intestinal permeability, also sug-

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
Runtime	2 min
Column temperature	30 °C
Volume of injection loop	5 µl

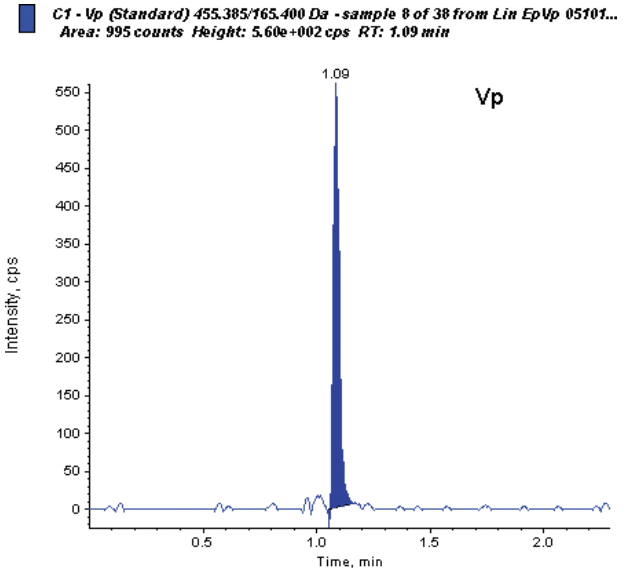

Figure 3. Typical multiple reaction monitoring chromatograms of verapamil.

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**
Verapamil	6.69	7.80	8.31	**7.57**	**0.88**

*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 4. Recovery data.

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**
Verapamil	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.

gest a high permability of verapamil. The observed high permeability of verapamil is in line with the reported oral absorption of about 90%. The presence of an absorption window cannot be ruled out from the literature data reviewed here but the postulated mechanism of the permeability of verapamil, being passive transport, makes such an absorption window unlikely.

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 verapamil hydrochloride of 1 to 100.00 ng/ml with 7 point calibration used for quantification by linear regression, shown in Fig. 4. A linear response function was established at 1 – 100 ng/mL. The regression equation for the analysis was Y = 0.0162x + 0.00391 with coefficient of correction (R^2) = 0.9992.

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

Day	Intra-day precision		Inter-day precision	
	Mean	RSD %	Mean	RSD %
1	98.82	0.378	101.76	0.334
2	100.41	0.617	98.97	0.390
3	100.62	0.336	100.53	0.612

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

The within-run coefficients of variation ranged between 0.336% and 0.617% for verapamil. The within-run percentages of nominal concentrations ranged between 98.82% and 100.62% for verapamil. The between-run coefficients of variation ranged between 0.334% and 0.612% for verapamil. The between-run percentages of nominal concentrations ranged between 98.97% and 101.76% for verapamil. 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.

When the criteria of the Guidances are strictly applied, verapamil hydrochloride is a BCS Class II substance and this API can not be considered a candidate for granting a biowaiver. However, this API is clearly on the borderline, the only problematic area is the insufficient solubility between pH 7.3 and 8.0. *In vivo* the limited solubility in this pH interval will not be problematic. This means that the solubility boundaries for this API should be redefined to for instance 1.0–6.8, as is recently suggested in general. In a provisional classification of the WHO Essential Drugs, verapamil was classified to be BCS Class I.So, from a scientific point of view, verapamil hydrochloride 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 profile comparison criteria as defined in the Guidances, but with a redefined

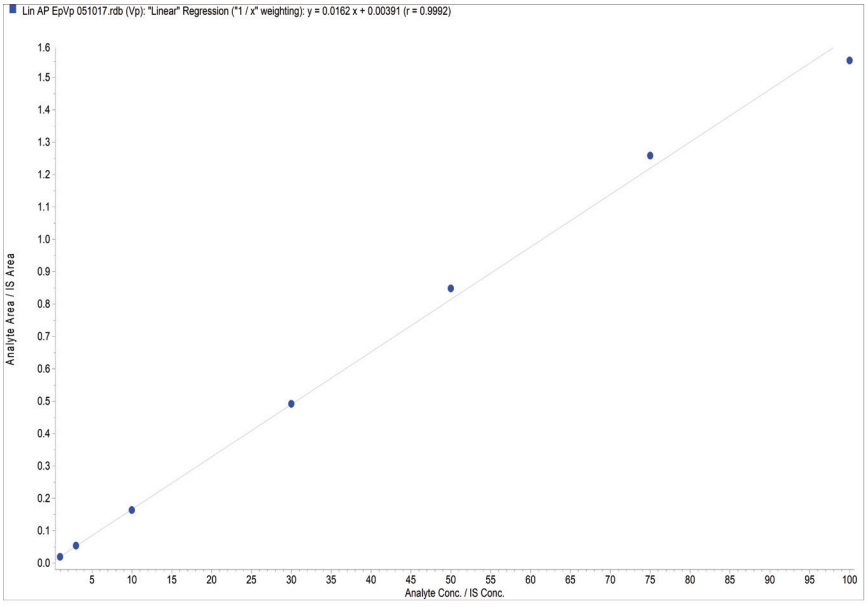

Figure 4. The calibration curve of verapamil hydrochloride in human plasma.

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 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 verapamil.

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

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