An analytical method for simultaneously assessing biological and physical barrie of the rat intestinal mucosa

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Background

The preclinical development of drug candidates often requires laboratory animals to be orally administered a test compound in a solution or suspension containing a high concentration of excipient. However, recent *in vitro* studies suggest that commonly used excipients (e.g., polyethylene glycol) may affect intestinal first-pass elimination mechanisms such as P-gp and cytochrome P450 (CYP) (1-4). To evaluate the effects of excipients on rat intestinal P-gp and CYP, an *in situ* single-pass intestinal perfusion method will be employed using verapamil as a marker of transcellular metabolism/permeation and atenolol as a marker of paracellular permeation.

Atenolol (pKa 9.4 (5); log P = 0.16 (6)) and verapamil (pKa 8.9 (7); logP = 3.8 (6)) will be experimentally administered in a 'cocktail' formulation and therefore simultaneous analysis of the resulting plasma and perfusate samples will be necessary. Both molecules are soluble in water or low concentrations of organic solvent (e.g., methanol), however, based on the greater lipophilicity of verapamil (higher logP) we expect different sample preparation (e.g., extraction) requirements from that of atenolol. Other labs have demonstrated reliable sample preparation methods followed by HPLC-UV and HPLC-MS/MS detection methods for either atenolol or verapmil (8-10). However, we are aware of no published method that describes the simultaneous extraction and quantitative measurement of both these drug molecules. Therefore, it is necessary to develop a suitably sensitive, precise and accurate method that allows for the simultaneous measurement of both molecules from the relevant experimental matrices, rat plasma and buffered lumenal perfusate.

Objectives

- To develop a sample preparation and analytical detection method to simultaneously measure atenolol and verapamil along with the N-demeth-ylated metabolite, norverapamil, in rat plasma and aqueous buffered matrices.
- To use this method in measuring levels of all three analytes in plasma and lumenal perfusate samples following *in situ* rat intestinal perfusion of an atenolol-verapamil drug cocktail formulation.

Methods

MS and MS/MS Scans: Each analyte was solubilized in 50% acetonitrile at a concentration of 20 µg/mL. Solutions were infused (5 µL/min) combined with a mobile phase flow path (50:50 acetonitrile:water, containing 0.1% formic acid) into a triple quadrapole mass spectrometer (QuatroMicro, Waters). Instrument settings include: cone voltage = 40-55V, source temperature = 100° C, desolvation temperature = 300-400° C, cone gas = 60 L/hr. MS and MS/MS spectra were collected and product-ions were selected for subsequent multiple reaction monitoring (MRM) analyses.

Chromatography: Solutions of atenolol, verapamil, norverapamil (Sigma-Aldrich, St. Louis, MO) and metoprolol (internal standard, Sigma-Aldrich) in mobile phase were injected (20 µL) on an Alliance HPLC (Waters, Milford, MA) and chromatographed on a Zorbax Eclipse XDB-C18 column (2.1 x 50 mm, 3.5 mm, Agilent Technologies) using an isocratic acetontrile elution method. Following the testing of various mobile phase compositions, 30% acetontrile (containing 0.1% v/v formic acid), at 0.4 mL/min, was used in subsequent chromatography.

Sample preparation: Rat plasma or buffered perfusate solution were spiked with 1 µM each of atenolol, verapamil and norverapamil (AVN). Samples (200 µL) of each spiked matrix were alkalinized with an equal volume of NaOH (5 M), to which 1 mL of extraction solvent was added. Samples were vigorously vortexed (10 min) and centrifuged (1500 rcf, 5 min). Organic solvent was transferred to clean tubes, dried, reconstituted in mobile phase, chromatographed and detected by MRM analyses (atenolol: 267 → 145 m/z; verapamil: 455 → 165 m/z; norverapamil: 441 → 165 m/z). As a measure of extraction efficiencies, absolute peak areas were compared to those of 1 µM AVN solutions in mobile phase. For subsequent studies matrix standards and experimental gut perfusion samples were extracted using tBME:iso-propanol (4:1) containing metoprolol (268 → 145 m/z) as an internal standard.

Results

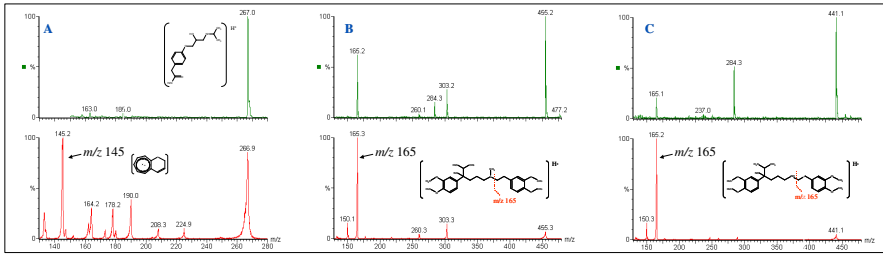

Figure 1. Electrospray ionization (positive ion mode) mass spectra (MS, shown in green) and MS/MS product-ion spectra (shown in red) of (A) atenolol (m/z 266), (B) verapamil (m/z 455) and (C) norverapamil (m/z 441) in 50% acetonitrile containing 0.1% (v/v) formic acid. Each MS/MS scan was performed using the appropriate set mass (parent m/z) resulting in the respective product ion spectra. Arrows indicate the product-ion that was selected in each case for subsequent quantitative (MRM) analyses. Predicted fragment-structure for atenolol was rationalized based on exact mass measurements (145.0653; $C_{10}H_8O$, 4.5 ppm) whereas predicted fragment-structure for verpamil and norverapamil were obtained from the literature (9,10).

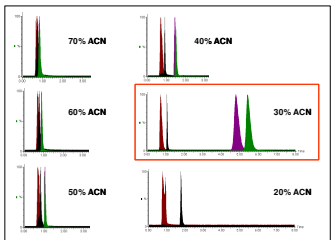

Figure 2. The effect of mobile phase strength (ε) on the retention time of four analytes: atenolol (maroon), metoprolol (black), norverapamil (purple) and verapamil (green).

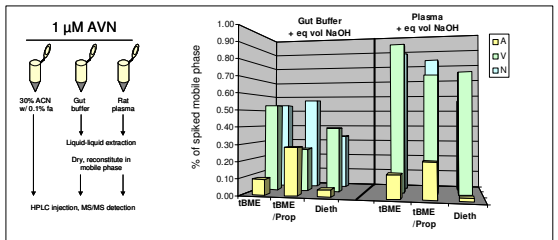

Figure 3. The effect of solvent composition on the liquid-liquid extraction efficiency of atenolol (A), verapamil (V) and norverapamil (N) from two matrices, rat plasma and buffered lumenal perfusate (gut buffer). tBME:iso-propanol (4:1) was selected for subsequent experiments

PLASMA STANDARDS

[Atenolol] (nM)		Averages; n=3	
Nominal (a)	Calc.	Accuracy	CV
50	46	91.6%	10.3%
100	94	94.0%	25.6%
500	477	95.4%	7.7%
1000	1022	102.2%	3.1%

Statistical weighting: None
r^2 = 0.998103

[Verapamil] (nM)		Averages; n=3	
Nominal (b)	Calc.	Accuracy	CV
5	5	100.5%	8.2%
25	24	96.3%	9.0%
50	47	93.1%	1.5%
250	276	110.5%	3.8%
500	546	109.2%	1.8%
2500	2498	99.9%	3.1%
5000	5013	100.3%	1.3%

Statistical weighting: 1/x
Coefficient of Determination: 0.993212

[Norverapamil] (nM)		Averages; n=3	
Nominal (b)	Calc.	Accuracy	CV
5	6	111.1%	23.1%
25	29	114.9%	10.5%
50	51	102.9%	7.3%
250	259	103.8%	8.3%
500	566	113.2%	5.4%
2500	2426	97.1%	4.6%
5000	4631	92.6%	2.2%

Statistical weighting: 1/x
Coefficient of Determination: 0.999100

PERFUSATE STANDARDS

[Atenolol] (nM)		Averages; n=3	
Nominal (c)	Calc.	Accuracy	CV
500	472	94.4%	32.0%
1000	952	95.2%	25.7%
5000	4359	87.2%	33.1%
10000	9228	92.3%	14.7%

Statistical weighting: None
r^2 = 0.996247

[Verapamil] (nM)		Averages; n=3	
Nominal (d)	Calc.	Accuracy	CV
1000	1030	103.0%	7.3%
2000	1922	96.1%	3.6%
10000	7952	79.5%	1.6%
20000	19652	98.3%	3.5%

Statistical weighting: 1/x
Coefficient of Determination: 0.991056

[Norverapamil] (nM)		Averages; n=3	
Nominal (c)	Calc.	Accuracy	CV
500	294	58.8%	3.1%
1000	1108	110.8%	4.2%
5000	5129	102.6%	6.5%
10000	9413	94.1%	5.9%

Statistical weighting: 1/x
Coefficient of Determination: 0.999788

Table 1. Samples of rat plasma or perfusate buffer were spiked with mixtures of atenolol, verapamil and norverapamil (reported as 'nominal' concentration) and subjected to liquid-liquid extraction with tBME:iso-propanol (4:1). Samples were either extracted (a) directly (1x) or diluted (b) 5x, (c) 10x or (d) 20x prior to extraction to obtain signal intensities within the linear range of the instrument. Extracted samples were dried, reconstituted in 30% acetonitrile, 70% water (containing 0.1% formic acid) and analyzed by HPLC-MS/MS according to the methods described here. Calculated concentrations (calc.) from the respective standard curves are reported along with accuracy (calc/nominal) and coefficient of variance (CV; standard deviation of response/average response).

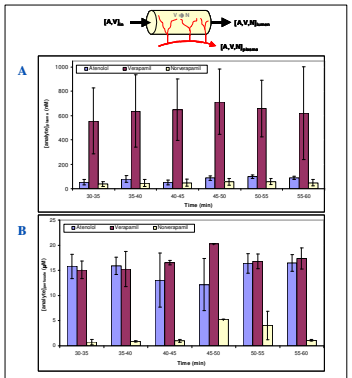

Figure 4. Measured levels of atenolol, verapamil and norverapamil in (A) rat plasma and (B) lumenal perfusate following *in situ* perfusion of rat jejunum with an atenolol/verapamil (each 20 µM) cocktail formulation.

Conclusions

- Atenolol, verapamil and norverapamil were extracted from plasma aqueous buffer matrices using various organic solvents; following which butyl-methyl ether and iso-propanol mixture (4:1) was selected.
- The sample preparation and analytical methods described here are sui efficient, sensitive, precise and accurate to simultaneously measure leve atenolol, verapamil and norverapamil.
- The methods can be used following single-pass *in situ* jejunal perfusion wi atenolol/verapamil drug cocktail to measure drug and metabolite levels i mesenteric plasma as well as lumenal perfusate.

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