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Development of an on-line extraction turbulent flow chromatography tandem mass spectrometry method for cassette analysis of Caco-2 cell based bi-directional assay samples

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Abstract

Caco-2 cells are frequently used for screening compounds for their permeability characteristics and P-glycoprotein (P-gp) interaction potential. Bi-directional permeability studies performed on Caco-2 cells followed by analysis by HPLC-UV or LC–MS method constitutes the "method of choice" for the functional assessment of efflux characteristics of a test compound. A high throughput LC–MS/MS method has been developed using on-line extraction turbulent flow chromatography coupled to tandem mass spectrometric detection to analyze multiple compounds present in Hanks balanced salt solution in a single analytical run. All standard curves (P-gp substrates: quinidine, etoposide, rhodamine 123, dexamethasone, and verapamil and non-substrates: metoprolol, sulfasalazine, propranolol, nadolol, and furosemide) were prepared in a cassette mode (ten-in-one) while Caco-2 cell incubations were performed both in discreet mode and in cassette mode. The standard curve range for most compounds was 10–2500 nM with regression coefficients (R^2) greater than 0.99 for all compounds. The applicability and reliability of the analysis method was evaluated by successful demonstration of efflux ratio greater than 1 for the P-gp substrates studied in the Caco-2 cell model. The use of cassette mode analysis through selected reaction monitoring mass spectrometry presents an attractive option to increase the throughput, sensitivity, selectivity, and efficiency of the model over discreet mode UV detection.

Keywords: Turbulent flow chromatography; Tandem mass spectrometry; Caco-2; LC–MS/MS analysis; On-line extraction

1. Introduction

Amongst the biopharmaceutical properties that need to be considered in early discovery, assessment of intrinsic permeability and P-glycoprotein (P-gp) interaction potential is critical in determining the fate of a test compound. Transport of drug substances across the intestinal membrane is a complex and dynamic process. In addition to influx, various efflux mechanisms (via carriers and transporters) are also functional that limit the systemic availability of compounds. Drug efflux transporters such as P-gp are well established to be a major determinant of absorption, distribution and elimination of a wide variety of marketed drugs [1–3]. P-gp is known to limit the oral absorption of drugs such as cyclosporine and taxol; it can limit entry of drugs such as HIV protease inhibitors into brain and central nervous system; and it can actively facilitate excretion of drugs via biliary and urinary routes [4–7]. Since P-gp interactions of a drug can play such a pivotal role in dictating its pharmacokinetics and eventually its distribution into target organs, increasing efforts are being made in early discovery and development to identify compounds that can potentially interact with P-gp.

Caco-2 cell based bi-directional permeability assay is currently accepted as the "method of choice" for P-gp substrate identification in drug discovery labs [8–10]. However, the current approach for high-throughput Caco-2 cell analysis is one in which a single compound is incubated per well in a 24-well Caco-2 cell plate. The analytical method used for detection is either an HPLC-UV or a LC–MS method performed in discreet mode. A different approach using LC–MS/MS for detection has been evaluated for a series of literature based biopharmaceutics classification system (BCS) compounds and P-gp substrates with known permeability values. These compounds included metoprolol, sulfasalazine, etoposide, propranolol, rhodamine 123, nadolol, dexamethasone, furosemide,

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quinidine, and verapamil. Both positive and negative P-gp substrates were chosen for comparison. All compounds were commercial available.

A major obstacle for LC–MS/MS-based analysis is the presence of non-volatile buffers and saturated salts that are typically used in Caco-2 cell experiments, such as primarily Hanks balanced salt solution (HBSS). These buffers and salts must be diverted or removed prior to the introduction of the samples into the mass spectrometry source to avoid signal suppression and source contamination [11–13]. The diversion of buffers from the mass spectrometer source can be simply accomplished with the use of a divert value, preferable controlled through a software link. Turbulent flow also diverts flow away from the source but adds additional in-line retention of analyte and provides a further sample clean-up step. Comparison of data using a divert value and a divert value with a turbulent flow chromatography (TFC) cartridge was not explored in these experiments. The commercial availability of complete turbulent flow chromatographic systems that can interface with the mass spectrometer has eliminated the need to assemble these complicated systems in "contact closure" configurations. The use of turbulent flow chromatography not only diverts the sample onto an on-line trap column, but also removes polar interferences with a high aqueous flow rate thereby essentially fractionating multiple component analytes [14–20]. The trapped analytes are back-eluted from the on-line trap column and further chromatographically resolved on a second analytical C18 column. Combining the TFC technique with the specificity of a mass spectrometer operating in the selected reaction monitoring mass spectrometry (SRM) mode permits Caco-2 cell analysis in a cassette N-in-1 mode [21–24]. Several different researchers have published papers for utilizing cassette mode approach. In this strategy, throughput can increase substantially as multiple compounds are incubated in a single well. LC–MS/MS also provides detection at much lower concentrations as compared to LC-UV detection. Initial concentrations of 3 μM represent a more realistic physiological concentration of drug exposure in vivo as compared to 50 μM needed for LC-UV assay.

2. Experimental

2.1. Reagents and chemicals

Caco-2 cells (passage # 17) were obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium, nonessential amino acids and Antibiotics were purchased from JHR Biosciences (Lenexa, KS). Fetal bovine serum was obtained from Hyclone Lab. Inc. (Logan, Utah). HTS-Transwell® inserts (surface area: 0.33 cm^2 with a polycarbonate membrane (0.4 μm pore size) were purchased from Costar (Cambridge, MA). Hank's balanced salt solution (HBSS), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 4-morpholineethnesulfonic acid (MES) were purchased from Sigma Chemical Co. (St. Louis, MO). The following compounds were purchased commercially from Sigma-Aldrich Chemie (Steinheim, Germany): metoprolol, sulfasalazine, propranolol, rhodamine 123, dexamethasone, and furosemide (Fig. 1). Nadolol and verapamil were purchased from Fluka (Switzerland). Etoposide and quinidine were purchased from Alexis (CA, USA). Acetonitrile (HPLC grade), methanol (HPLC grade), formic acid, and dimethlysulfoxide were purchased from EMD Chemicals (Gibbstown, NJ, USA). Deionized water was further purified with a Purelab plus filter system from US Filter (Lowell, MA, USA). 96-well plates (Costar), plate seals, and plate sealer were purchased from Corning Inc. (NY, USA).

2.2. Caco-2 cell culturing and bi-directional permeability procedure

Caco-2 cells were seeded onto 24-well polycarbonate filter membrane at a density of 36,000 cells/well. The cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, 100 U/mL penicillin-G, and 100 μg/mL streptomycin. The culture medium was replaced every 2 days and the cells were maintained at 37 °C, 95% relative humidity, and 5% CO_2. Permeability studies were conducted with the monolayers cultured for approximately 21 days with the cell passage numbers between 20 and 40. Physiologically and morphologically well developed Caco-2 cell monolayers with TEER values greater than 400 Ω cm^2 were used. The transport medium used for the bi-directional studies was HBSS buffer containing 10 mM HEPES. The pH of both the apical compartment and the basolateral compartment was maintained at 7.4. Prior to all experiments, each monolayer was washed twice with buffer and TEER was measured to ensure the integrity of the monolayers. The concentration of test compounds was 3 μM in this assay. The bi-directional permeability studies were initiated by adding an appropriate volume of buffer containing test compound (either alone or in cassette mode, $N = 3$) to either the apical (for apical to basolateral transport; A to B) or basolateral (for basolateral to apical transport; B to A) side of the monolayer. Volume of the apical and basolateral compartment was maintained at 0.2 ml and 0.6 ml, respectively. The monolayers were then placed in an incubator for 2 h at 37 °C. Samples were taken from both the apical and basolateral compartment at the end of the 2-h period and the concentrations of test compound were analyzed by a LC–MS/MS method as described earlier.

Permeability coefficient (Pc) was calculated according to the following equation:

$$Pc = \frac{\mathrm{d}A}{\mathrm{d}t\, SC_0}$$

where $\mathrm{d}A/\mathrm{d}t$ is the flux of the test compound across the monolayer (nmol/s), S the surface area of the cell monolayer (0.33 cm^2), and C_0 is the initial concentration (3 μM) in the donor compartment. The Pc values were expressed as nm/s. Discrete and cassette mode Caco-2 bi-directional studies were performed with marketed compounds characteristic of low (nadolol, furosemide, sulfasalazine) and high (metoprolol and propronolol) permeability classes. Classical P-gp substrates, such as etoposide, verapamil, rhodamine123, dexamethasone

and quinidine, were also included in the studies. These compounds were selected to provide the structural and physicochemical diversity needed to ensure the utility of the model. Since the assay applies to bi-directional permeability in Caco-2 cells, common P-gp substrates that are known to demonstrate vectorial transport (i.e. efflux ratio higher than 2) were used as positive controls.

2.3. Instrumentation

The LC system used for all analysis was an Aria TX-2 TurboFlow® (Cohesive Technologies, Franklin, MA, USA). This LC system consists of eight Shimadzu 10ADvp pumps and two Shimadzu 10ADvp controllers. The auto sampler was a dual injection arm from CTC Analytics and the software version was Aria 1.4.1. The Aria was configured in the Quick Elute Mode and was capable of alternating injections using two injection arms and two injection ports. The liquid handlers used to transfer samples and standards prior to analysis consisted of a Quadra 96 Model 196-320 (Tomtec, Hamden, CT, USA) and a Packard multiPROBE II Plus (Perkin–Elmer, Shelton, CT, USA). The mass spectrometer used for quantitative analysis was a MDS Sciex API-3000 triple quadrupole equipped with a turbo ion spray source (Toronto, Canada). The software version operating the Sciex was Analyst 1.2.

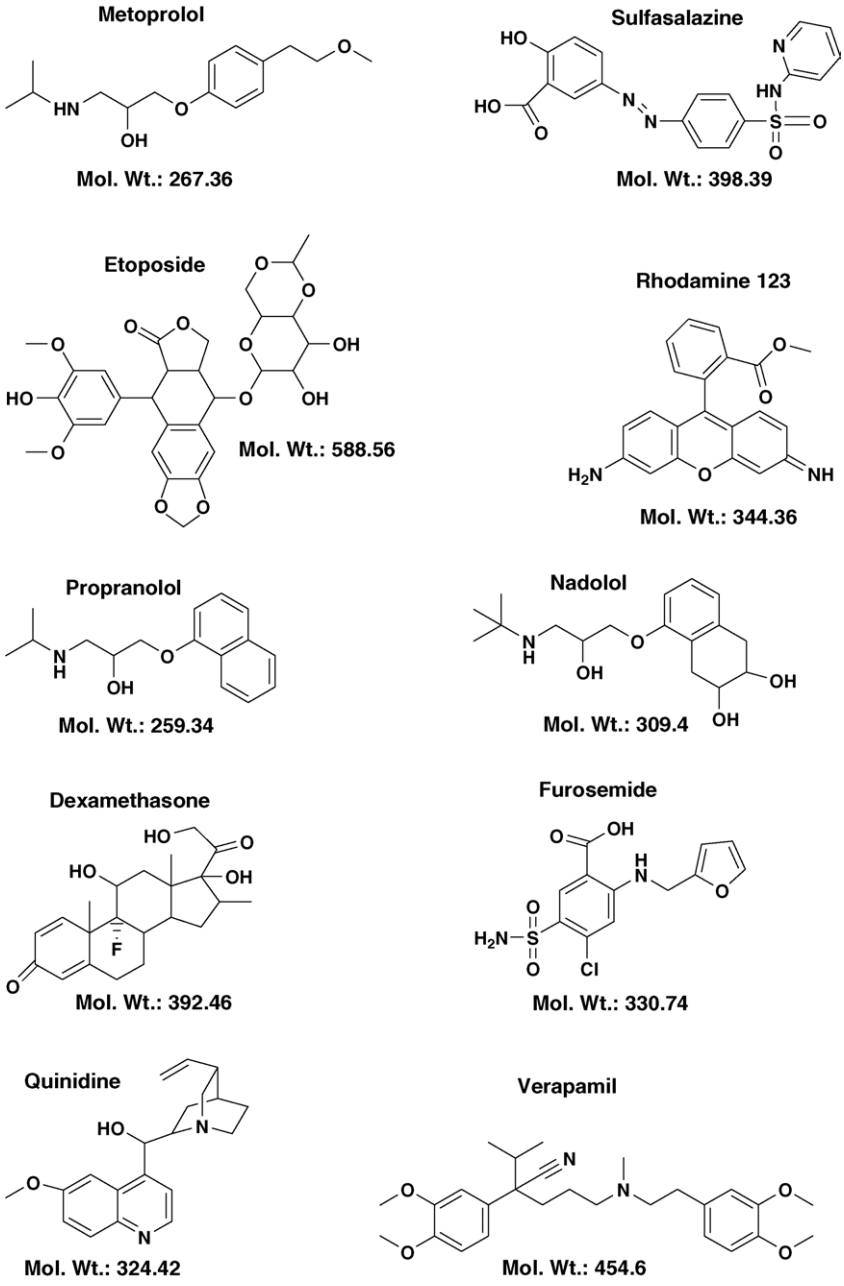

Fig. 1. Compounds evaluated and their corresponding molecular weights (Mol. Wt.).

2.4. Chromatographic conditions

Chromatography was conducted using two different types of columns: high TurboFlow® liquid chromatography (HTLC) Cyclone trap column 0.5 mm × 50 mm (Cohesive Technology, Franklin, MA, USA). The Cyclone was composed of mixed porous material at approximately 50 μm pore size. The Cyclone column has the properties of an on-line reverse phase trap column for small molecular weight molecules and a size exclusion fractionation column under high aqueous flow rates (1.8 mL/min) creating turbulent flow conditions. The second column used in-line was an analytical C18 column (Atlantis 2.1 × 50 mm, 5 μm pore size) from the Waters Corp. (Ireland). The flow rate of the Atlantis column was kept at 0.3 mL/min. All columns remained at room temperature during data collection. The compounds evaluated in these experiments were injected in alternating multiplex system with two HTLC columns and two analytical C18 columns.

The Aria TX-2 was configured in a Quick Elute Mode. This configuration includes six divert values connected in series that were controlled by the Aria software. A total of four pumps per channel include: two loading pumps and two eluting pumps. The loading pumps and eluting pumps share the same aqueous solvent reservoir using water with 0.1% formic acid and the same organic solvent reservoir using acetonitrile with 0.1% formic acid.

In the loading step, the HTLC on-line trap Cyclone column was maintained at 100% aqueous for 1.5 min at a flow rate of 1.8 mL/min, after the sample was injected. The eluting pumps were maintained at 50% acetonitrile at a flow rate of 0.3 mL/min. In the eluting step, the loading pumps for the HTLC trap column uses 100% acetonitrile to rinse and wash, followed by 100% aqueous to re-equilibrate at a flow rate of 1.8 mL/min prior to the next injection. At the same time, the flow from the eluting pumps was diverted to back-elute all trapped analytes during the transfer step from the HTLC on-line trap column onto the C18 analytical Atlantis column at a flow rate of 0.3 mL/min. A 1-min gradient was run on the eluting pumps up to 95% acetonitrile and was held for 1 min after which the eluting pumps re-equilibrates the C18 column back to 50% acetonitrile. The MS acquisition time was 3 min for every injection and the total run time was 6 and a 1/2 min.

2.5. Standard solution preparation

Individual 10 mM stock solutions of all compounds were first prepared in dimethlysulfoxide. Next, a set of working stock solutions containing all 10 compounds were prepared in 1:1 acetonitrile/water at 25 μM, 10 μM, 5 μM, 1 μM, 0.5 μM, 0.25 μM, and 0.1 μM by serial dilution from each 10 mM stock. Each of the standard curve solutions were diluted directly from the working stocks by adding 20 μL of working stock and 180 μL of HBSS. The final standard calibration concentrations were 2500 nM, 1000 nM, 500 nM, 100 nM, 50 nM, 25 nM, and 10 nM. Preparing working stock solutions using 50% organic/aqueous solvent mixture helped to reduce and or eliminate any possible non-specific binding due to absorbance in most cases.

2.6. Sample processing

Standard curve solutions were prepared manually in HBSS and transferred into 96-well plates containing Caco-2 cell incubations to be analyzed. The Tomtec or the Packard multiPROBE II plus was used to transfer 50 μL of each standard into a new 96-well plate. Next, an additional 400 μL of methanol was added to each well to improve the overall solubility and to dilute samples such that the signal response remained linear in the mass spectrometer. The injection volume was 10 μL.

2.7. Mass spectrometry settings

The mass spectrometer used for all experiments was an AB-Sciex API-3000 triple quadrupole with turbo ion spray source (TIS). The temperature of the source was maintained at 400 °C. Nitrogen was used as the nebulizer gas, setting 6, curtain gas, setting 8, and collision gas, setting 7 for all analysis. For positive electrospray (ESI), ion spray voltage was 4500 V and the EP and CXP ion optics was set at 10 V. Both positive and negative ionization was used in SRM mode. Negative ESI was performed with the same gas settings. Negative ESI, ion spray voltage was set to −4500 V and the EP and CXP ion optics was set at −10 V. Each compound was optimized for quadrupole 1 (Q1) transition and quadrupole 3 (Q3) fragmentation including declustering potential (DP), focusing potential (FP), and collision energy (CE)

Table 1
Compound dependent Sciex API-3000 mass spectrometer voltage settings including MS/MS transitions, declustering potential (DP), focusing potential (FP), and collision energy (CE) for all compounds evaluated

Compound	Ionization mode	Q1/Q3 transitions *m/z*, precursor ion > product ion	DP (V)	FP (V)	CE (V)
Metoprolol	ESI+	268.1 > 116.2	40	190	25
Sulfasalazine	ESI+	399.0 > 381.0	40	190	25
Etoposide	ESI+	589.3 > 229.0	50	170	25
Propranolol	ESI+	260.1 > 116.1	40	130	25
Rhoadamine 123	ESI+	345.1 > 285.2	80	260	60
Nadolol	ESI+	310.2 > 254.2	35	150	25
Dexamethasone	ESI+	393.2 > 373.1	45	210	12
Furosemide	ESI−	328.7 > 284.9	−35	−175	−20
Quinidine	ESI+	325.2 > 184.0	60	220	40
Verapamil	ESI+	455.3 > 165.0	65	230	35

voltages as shown in Table 1. Product ions were selected by ramping the collision energy in MRM scan mode during direct infusion to the source of the mass spectrometer. The most prominent product ions were chosen based on the highest response corresponding to the strongest transmission through the collision cell. The MS/MS transitions reported in Table 1 were the direct results obtained from optimizing each compound individually on the Sciex API-3000 and may be different from transitions reported in other literature references. Electrospray ionization is a "soft" ionization technique that can vary between different manufactures of tandem mass spectrometer platforms. The dwell time was 50 ms for all SRM transitions. All the data was obtained with each quadrupole set at unit resolution. Positive ESI Q1 spectra was dominated by the $[M+H]^+$ ions for each of the compounds. The only compound detected as a negative ion was furosemide which generated a negative ESI Q1 spectra that was dominated by the $[M-H]^-$ ions. Fig. 1 shows each compound and their corresponding molecular weights.

Table 2
Accuracy and precision data determined from the standard curve calibration concentration from four replicates using LC–MS/MS turbulent flow chromatography

Compound	Concentration (nM)						
	10.0	25.0	50.0	100.0	500.0	1000.0	2500.0
Metoprolol, $n=4$							
Mean	9.8	24.9	53.3	107.6	495.2	985.1	2266.5
%CV	14.7	9.0	4.0	2.5	5.7	0.5	4.1
%Accuracy	98.2	99.4	106.5	107.6	99.0	98.5	90.7
Sulfasalazine, $n=4$							
Mean	10.3	23.8	48.2	96.6	508.9	1034.4	2591.7
%CV	12.6	10.0	12.6	2.3	2.3	2.4	2.8
%Accuracy	102.9	95.1	96.4	96.6	101.8	103.4	103.7
Etoposide, $n=4$							
Mean	10.5	23.0	45.2	98.6	521.1	1010.6	2712.8
%CV	6.7	11.1	6.4	16.9	11.3	6.5	11.2
%Accuracy	105.1	92.1	90.5	98.6	104.2	101.1	108.5
Propranolol, $n=4$							
Mean	10.2	23.9	48.8	102.5	510.5	1003.5	2493.8
%CV	11.5	3.1	8.7	5.3	6.4	2.6	3.4
%Accuracy	101.9	95.7	97.7	102.5	102.1	100.4	99.8
Rhodamine 123, $n=4$							
Mean	10.5	23.9	43.4	88.2	511.5	1115.6	2753.3
%CV	10.0	8.2	8.8	13.5	4.1	1.8	6.5
%Accuracy	105.4	95.6	86.8	88.2	102.3	111.6	110.1
Nadolol, $n=4$							
Mean	9.6	26.2	51.9	110.8	502.4	966.2	2179.9
%CV	13.4	8.6	3.2	2.9	1.9	1.6	1.4
%Accuracy	96.3	104.7	103.9	110.8	100.5	96.6	87.2
Dexamethasone, $n=4$							
Mean	10.2	24.1	47.3	104.8	525.5	977.2	2484.6
%CV	17.2	8.8	4.7	8.0	9.1	3.5	7.0
%Accuracy	102.0	96.3	94.7	104.8	105.1	97.7	99.4
Furosemide, $n=4$							
Mean	10.0	24.3	50.4	107.8	518.2	982.5	2300.8
%CV	7.7	8.7	4.2	2.7	7.4	4.9	1.7
%Accuracy	100.1	97.4	100.8	107.8	103.6	98.3	92.0
Quinidine, $n=4$							
Mean	NA	26.2	45.6	97.8	496.2	1021.0	2619.2
%CV	NA	9.8	10.1	8.9	2.5	7.8	3.4
%Accuracy	NA	104.9	91.2	97.8	99.2	102.1	104.8
Verapamil, $n=4$							
Mean	10.6	23.3	43.6	88.5	521.4	1148.2	2649.1
%CV	6.8	12.2	9.1	7.1	9.6	4.8	4.8
%Accuracy	106.2	93.1	87.1	88.8	104.3	114.8	106.0

The accuracy was defined as the percent difference from the nominal concentration. The mean was determined by averaging the calculated concentrations for each calibration standard weighted $1/x^2$ linear regression model. The intra-day precision was expressed as the percent coefficient of variation (CV) calculated as the standard deviation divided by the mean concentration.

3. Results and discussion

3.1. Method validation

3.1.1. Standard curves

Seven point standard curves in replicates of four were used to evaluate the overall accuracy and precision of the bioanalytical method. The standard curve ranged from 10 nM to 2500 nM for all compounds studied except quinidine (25–2500 nM). All standard curves were analyzed as a simultaneous ten-in-one or cassette approach. The seven-point standard curves used to generate this validation data was also intended to fully explore the response and linearity at the low nM ranges using a turbulent flow chromatography approach coupled to the selectivity of tandem mass spectrometry. All standard curves were evaluated with a weighted $1/x^2$ linear regression model. The regression was based on an external plot of area cps counts. An internal standard (IS) was not used in these assays due to the unavailability of a suitable IS for such a structurally diverse set of compounds. The use of internal standards in MS is to help reduce injection to injection variability. This method was proven accurate and precise in the absence of an IS demonstrating robustness needed in high throughput screening.

Individual compound summaries are presented in Table 2. Analysis was performed with $n=4$ replicates. All regression coefficients (R^2) were greater than 0.99 (data not shown). The accuracy was defined as the percent difference from the nominal concentration. The mean was determined by averaging the calculated concentrations for each calibration standard. The intra-day precision was expressed as the percent coefficient of variation (CV) calculated as the standard deviation divided by the mean concentration. The overall accuracy for all compounds was ±15% of the nominal concentration. These results indicate multiple component analysis does not adversely affect accuracy and precision under SRM mass spectrometric detection. A high throughput screening model based on MS detection using SRM scanning could be set up for cassette mode Caco-2 cell incubation screening. This MS based model would increase the number of compounds and turnaround time associated with analysis over a discreet mode incubation using UV detection.

3.1.2. Accuracy and precision

The accuracy and precision were determined from the standard curve calibration concentration response difference from the nominal concentration. Table 2 shows this data. All

Table 3
Efflux ratios from P-gp substrates etoposide, dexamethasone, and quinidine in the Caco-2 cell bi-directional assay and measured nM concentrations after incubations at 3 μM concentrations

Compound	Study type	A–B (mean)	A–B (SD)	B–A (mean)	B–A (SD)	Efflux ratio	A–B top nM (mean)	A–B bottom nM (mean)	B–A top nM (mean)	B–A bottom nM (mean)
Etoposide	Cassette	7.4	0.6	86	17	11.6	4785	22	770	6853
Etoposide	Discreet	4	0.8	86	7	21.5	6167	11	718	8475
Dexamethasone	Cassette	153	8	320	20	2.1	2227	165	1039	2536
Dexamethasone	Discreet	150	16	269	7	1.8	2135	178	961	2611
Quinidine	Cassette	146	21	509	25	3.5	5097	436	4508	5120
Quinidine	Discreet	183	32	632	51	3.5	2831	194	2035	2688

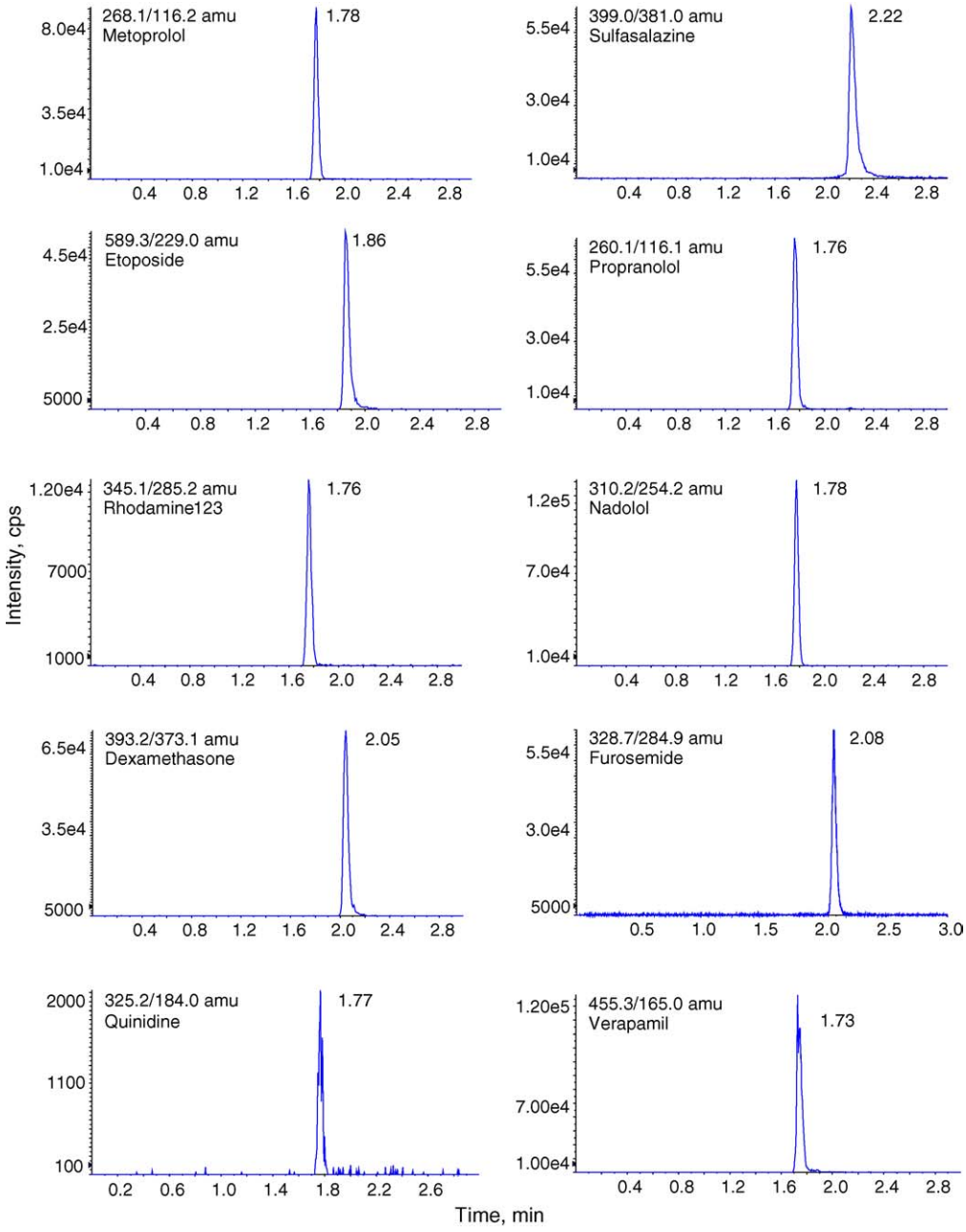

Fig. 2. Representative extracted ion chromatograms (XIC) of all compounds evaluated at standard curve concentration 500 nM scanned by SRM.

compounds yielded a linear response within the dynamic range of the curve. In this data set 10 compounds were analyzed simultaneously in each of the standard curve concentrations. The overall percent accuracy for all of the compounds evaluated was within ±15% of the nominal concentration. The %CV was also within ±20 for all compounds evaluated. Mass spectrometry detection has a distinct advantage over UV detection based upon the selectivity of the mass filters thus allowing multiple components to co-elute without inference by the other compounds present. Cassette incubations of Caco-2 cells can increase the productivity of the screening process while reducing the resources of equipment and personnel.

This validation method was used to determine actual efflux ratios from P-gp substrates etoposide, dexamethasone, and quinidine in the Caco-2 cell bi-directional assay. Data for these compounds is shown in Table 3. Efflux ratios greater than 1 were observed for these P-gp substrates. The absolute nM concentration values were comparable between discreet and cassette mode incubations. Interactions between compounds may occur when a cassette approach is used as a tool for screening. The use of a reference compound in the cassette mode may prove to be useful to validate each assay experiment. This option was not explored in the scope of the data presented in this research. Balancing the risk associated by the need for increased sample analysis and possible drug interactions has to be weighed by each laboratory utilizing the cassette approach.

3.1.3. Specificity in cassette mode

Triple quadrupole mass spectrometry is the "gold standard" for quantitative analysis. Scanning in SRM mode permits multiple analytes in one injection to be differentiated by unique mass fragmentation. Specificity was examined from the extracted ion chromatograms (XIC) in SRM mode. Fig. 2 shows the XIC for all compounds studied at the 500 nM concentration. There was no interference detected from MS/MS fragmentation between compounds. All compounds showed good peak shape which lead to consistent area integration by Analyst software. Turbulent flow chromatography adds a concentration step during the on-line extraction process which can yield sharp peaks as shown in Fig. 2.

4. Conclusions

A bioanalytical method was validated on an Aria TX-2 TurboFlow® system from Cohesive Technologies, using turbulent flow chromatography and SRM mass spectrometry. The use of TFC not only diverts the sample onto an on-line trap column, but also removes polar interferences with a high aqueous flow rate thereby essentially fractionating multiple component analytes. TFC as a sample preparation technique was automated and took place following sample injection; thereby it does not require any additional time to prepare samples off-line. The run time was 6 and a 1/2 min per sample with a 3 min MS acquisition window. The Aria was equipped with a dual injection arm autosampler, dual injection ports, and multiplexed LCs capable of alternating injections reducing the overall run time to 3 and a 1/2 min. The mass spectrometer continues to acquire data from one injection while the columns are re-equilibrating from the previous injection. This process optimizes MS function by reducing stand-by idle time and is very appropriate for large scale screening of discovery compounds. The standard curves were prepared in a cassette mode (ten-in-one) and detected by SRM. Accuracy and precision was determined by the results obtained from four separate standard curve analysis. The overall percent accuracy for all of the compounds evaluated was with ±15% of the nominal concentration. The %CV was also within ±20 for all compounds evaluated. The validation parameters (sensitivity, accuracy, precision, specificity, repeatability, etc.) obtained by the method was comparable to other established analytical techniques.

Analysis of Caco-2 cell incubations using LC–MS/MS detection can lower the initial concentration of compound to 3 μM due to the improved sensitivity of detection by triple quadrupole mass spectrometers. Mass spectrometry offers increased selectivity reducing the potential of possible interference from metabolites, chemical impurities, and or degradations of the parent compound. Scanning in SRM mode can easily accommodate quantitation of multiple analytes simultaneously without interference by co-eluting compounds. Coupling TFC on-line extraction to divert the phosphate buffer used in these types of experiments away from the ESI source produced a consistent response across a 96-well plate. This method is amendable to a high throughput assay performed in 96-well plates to support Discovery screening. The Caco-2 cell based bi-directional assay presents an efficient model to identify P-gp substrates in early Discovery. However, in spite of its in vitro nature that makes it amenable to higher throughput, it often becomes the bottleneck during drug discovery screening. The ability to perform cassette mode P-gp assay without compromising its quality and predictability can lead to a significant improvement in the efficiency of the model. This cassette mode P-gp assay presents an improved screening paradigm for fast and reliable identification of potential P-gp substrates in early discovery.

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