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Journal of Chromatography B, 854 (2007) 260–267
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Development of a quantitative LC–MS/MS analytical method coupled with turbulent flow chromatography for digoxin for the *in vitro* P-gp inhibition assay

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Received 26 February 2007; accepted 23 April 2007
Available online 1 May 2007

Abstract

Caco-2 cells, the human colon carcinoma cells, are typically used for screening compounds for their permeability characteristics and P-glycoprotein (P-gp) interaction potential during discovery and development. The P-gp inhibition of test compounds is assessed by performing bi-directional permeability studies with digoxin, a well established P-gp substrate probe. Studies performed with digoxin alone as well as digoxin in presence of test compounds as putative inhibitors constitute the P-gp inhibition assay used to assess the potential liability of discovery compounds. Radiolabeled ^{3}H-digoxin is commonly used in such studies followed by liquid scintillation counting. This manuscript describes the development of a sensitive, accurate, and reproducible LC–MS/MS method for analysis of digoxin and its internal standard digitoxin using an on-line extraction turbulent flow chromatography coupled to tandem mass spectrometric detection that is amendable to high throughput with use of 96-well plates. The standard curve for digoxin was linear between 10 nM and 5000 nM with regression coefficient (R^2) of 0.99. The applicability and reliability of the analysis method was evaluated by successful demonstration of efflux ratio (permeability B to A over permeability A to B) greater than 10 for digoxin in Caco-2 cells. Additional evaluations were performed on 13 marketed compounds by conducting inhibition studies in Caco-2 cells using classical P-gp inhibitors (ketoconazole, cyclosporin, verapamil, quinidine, saquinavir etc.) and comparing the results to historical data with ^{3}H-digoxin studies. Similarly, P-gp inhibition studies with LC–MS/MS analytical method for digoxin were also performed for 21 additional test compounds classified as negative, moderate, and potent P-gp inhibitors spanning multiple chemo types and results compared with the historical P-gp inhibition data from the ^{3}H-digoxin studies. A very good correlation coefficient (R^2) of 0.89 between the results from the two analytical methods affords an attractive LC–MS/MS analytical option for labs that need to conduct the P-gp inhibition assay without using radiolabeled compounds.

Keywords: Caco-2; LC–MS/MS; Turbulent flow chromatography; P-glycoprotein inhibition; Digoxin; Permeability

1. Introduction

P-glycoprotein (P-gp) is an ATP-dependent efflux transporter protein that is strategically located in several key tissues such as the small intestine, liver, kidney and blood–brain barrier. P-gp, by its ubiquitous expression coupled with the capacity to interact with a wide-spectrum of substrates, is known to play a prominent role in dictating the pharmacokinetics and pharmacodynamics of several drugs. It is widely recognized to be a major determinant of absorption, distribution and elimination of a wide array of marketed drugs [1].

In drug development, P-gp interaction potential of compounds is a factor in determining whether a test compound will be selected for further development. P-gp interaction could result from either the compound being a substrate or inhibitor for this important transporter. Caco-2 cell based digoxin transport inhibition assay is a well documented model to establish the drug candidate's potential to be a P-gp inhibitor [2,3]. Radiolabeled ^{3}H-Digoxin is typically used to perform the P-gp inhibition studies for test compounds. Current approaches to quantitate digoxin

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doi:10.1016/j.jchromb.2007.04.030

involve using radioactive ^{3}H-digoxin in conjunction with liquid scintillation counting [4–11]. There are several recurring costs associated with handling radioactive samples such as personnel trainings, spot checks, waste disposal etc. that can be a significant financial burden. The generation, storage and disposal of these radioactive samples present a formidable challenge to discovery organizations where this assay is being pushed upstream with more and more compounds being tested for their P-gp inhibition potential.

This manuscript presents efforts dedicated towards development of an LC–MS/MS detection method for digoxin using a turbulent flow chromatography technique to assay Caco-2 cell based bi-directional samples from the P-gp inhibition assay. This research was a follow-up from our earlier application of this analytical technique towards analysis of samples from Caco-2 cell based P-gp substrate assay [1].

Turbulent flow chromatography (TFC) interfaced with tandem mass spectrometry has been in use for the last decade. TFC provides an on-line extraction sample clean up that is automated and takes place in real time during the course of an injection thus eliminating manual sample preparation techniques such as liquid–liquid extraction, off-line solid phase extraction, and protein precipitation [12–21]. Many recent publications utilizing TFC and the combined specificity of selected reaction monitoring through use of triple quadrupole mass spectrometry offers proof that this analytical approach is well accepted [22–28]. The development of a LC–MS/MS analytical method for digoxin for the P-gp inhibition assay has several other advantages: potential ease-of-transfer of the assay for higher throughput applications, only digoxin is being monitored thus the LC–MS/MS assay can be run continuously without optimizing for new compounds, there are no analytical issues associated with metabolism (i.e. degradation of digoxin) or tritium–water exchange. Thus, the specificity of MS/MS detection using tandem mass spectrometry affords an attractive alternative to eliminate the use of radioactive isotopes in this P-gp inhibition assay while providing high sensitivity.

2. Experimental

2.1. Reagents and chemicals

Caco-2 cells 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): digoxin, digitoxin, metoprolol, cyclosporinol, rhodamine 123, dexamethasone, ketoconazole, saquinavir, verapamil, vinblastine, vincristine, etoposide, quinidine, indinavir and caffeine. Acetonitrile (HPLC grade), formic acid, and dimethlysulfoxide were purchased from EMD Chemicals (Gibbstown, NJ, USA). De-ionized 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 P-gp inhibition study protocol

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 ohm 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 10 μM in this assay while the concentration of digoxin was 5 μM. Control studies were performed to assess the apical to basolateral (A to B) and basolateral to apical (B to A) transport of digoxin (5 μM). P-gp inhibition potential of test compound was assessed by co-incubating the test compound along with digoxin and measuring the permeability value of digoxin in both directions. Permeability studies were initiated by adding an appropriate volume ($A = 0.2$ mL, $B = 0.6$ mL) of buffer containing digoxin (either alone or with the test compound) to the apical compartment (for A to B transport) or to the basolateral compartment (for B to A transport) of the transwell. Test compound was added at a concentration of 10 μM in both compartments. Caco-2 cells were maintained for 2 h at 37 °C inside an incubator. Samples were taken from both the apical and basolateral compartments at the end of the 2-h period and analyzed by a LC–MS/MS method as described earlier.

2.2.1. Data analysis

The A to B as well as B to A permeability of digoxin was calculated in the presence and absence of the test compound.

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

$$\mathrm{Pc} = \frac{\mathrm{d}A}{(\mathrm{d}t S C_{\mathrm{o}})}$$

where dA/dt is the flux of the digoxin across the monolayer (nmol/s), S is the surface area of the cell monolayer (0.33 cm^2), and C_{o} is the initial concentration (5 μM) in the donor compartment. The Pc values were expressed as nm/s.

The P-gp inhibition (%) by test compound was calculated as listed below.

$$\text{P- gp inhibition}\,(\%) = \left\{1 - \left(\frac{(\text{BA}_\text{i} - \text{AB}_\text{i})}{(\text{BA} - \text{AB})}\right)\right\} \times 100$$

where AB and BA are the A to B and B to A permeability of digoxin alone and AB_i and BA_i are the A to B and B to A permeability of digoxin in presence of the test compound.

Recovery (mass balance) from each well was calculated as listed below.

$$\text{Recovery}(\%) = 100 \times \left\{\frac{[(V_\text{r} \times C_\text{r}) + (V_\text{d} \times C_\text{d})]}{[V_\text{d} \times C_\text{o}]}\right\}$$

C_o = initial concentration in the donor compartment; V_r = volume of the receiver compartment; V_d = volume of the donor compartment; C_r = concentration of the receiver compartment at the end of 2 h; C_d = concentration of the donor compartment at the end of 2 h.

2.3. Instrumentation

The LC system used for all analysis was an Aria TX-2 TurboFlow® (Thermo Scientific, Franklin, MA, USA). This LC system consists of eight Shimadzu LC-10ADvp pumps and two Shimadzu SCL-10Avp controllers. The autosampler was a dual injection arm from CTC Analytics and the control software was Aria v1.5.1. The Aria was configured in the Quick Elute Mode. The liquid handler used to transfer samples and standards prior to analysis consisted of 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 operating the Sciex was Analyst v1.4.

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 (Thermo Scientific, Franklin, MA, USA). The Cyclone was composed of mixed porous material at approximately 50 μm particle size. This trap column serves as the de-salting step to flush any polar salts and phosphate buffers in the media under high turbulent flow conditions to waste. The second column used in-line was an analytical C18 column (Atlantis 2.1 mm × 50 mm, 5 μm particle size) from the Waters Corp. (Ireland). All columns remained at room temperature during data collection.

Samples were first injected onto the HTLC on-line trap Cyclone column maintained at 100% aqueous (0.1% formic acid) for 0.5 min at a flow rate of 1.8 mL/min. The C18 Atlantis analytical column was maintained at 50% acetonitrile (0.1% formic acid) and 50% aqueous (0.1% formic acid) at a flow rate of 0.3 mL/min. At 0.5–1 min the analytes were back-eluted off the on-line trap Cyclone column onto the C18 Atlantis analytical column. A 1-min gradient was run on the pumps up to 95% acetonitrile (0.1% formic acid) and was held for 1 min after which the pumps re-equilibrates the C18 column back to 50% acetonitrile (0.1% formic acid) and 50% aqueous (0.1% formic acid). At 1–3 min the on-line trap Cyclone column was washed with 100% acetonitrile (0.1% formic acid) then re-equilibrated with 100% aqueous (0.1% formic acid) for an additional 2.5 min. The MS acquisition window was 3 min for every injection and the total run time was five and a half minutes. Plates were run in a staggered injection mode across a dual injection port CTC autosampler.

2.5. Standard solution preparation

Standard curves were prepared in HBSS using digoxin at 5000 nM, 2000 nM, 1000 nM, 160 nM, 50 nM, and 10 nM by serial dilution diluted from a 1 mM stock. One set of standard curve was prepared for each 96-well plate of samples to be analyzed.

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 for P-gp digoxin inhibition assay. In all cases, only digoxin is being quantitated in the absence and presence of different compounds. The Packard MultiPROBE II plus was used to transfer 50 μL of each standard and sample into a new 96-well plate. Next, an additional 50 μL of acetonitrile containing the internal standard (IS), digitoxin at a concentration of 200 nM was added to each well. The injection volume was 20 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 8, curtain gas, setting 7, and collision gas, setting 6 for all analysis. Negative ionization was used in selected reaction monitoring (SRM) scan mode under electrospray (ESI), ionspray voltage was −4000 V, entrance potential (EP) was set to −10 V, and collision cell exit potential (CXP) was set at −15 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) voltages. The SRM transitions for digoxin were 779 m/z > 649 m/z, DP equal to −158 V, FP equal to −300 V, and CE equal to −46 V. The SRM transitions for the internal standard digitoxin were 763 m/z > 503 m/z, DP equal to −160 V, FP equal to −258 V, and CE equal to −55 V. The dwell time was 50 ms for all SRM transitions. All the data were obtained with each quadrupole set at low resolution. Negative ESI Q1 spectrum was dominated by the $[M–H]^-$ ions for each of the compounds. Fig. 1 shows each compound and their corresponding molecular weights as well as the proposed mass fragments scanned for in SRM mode. Representative chromatograms of a blank buffer sample with IS and a 10 nM standard of digoxin with IS are shown side by side in Fig. 2. Both chromatograms are extracted from the SRM channel for

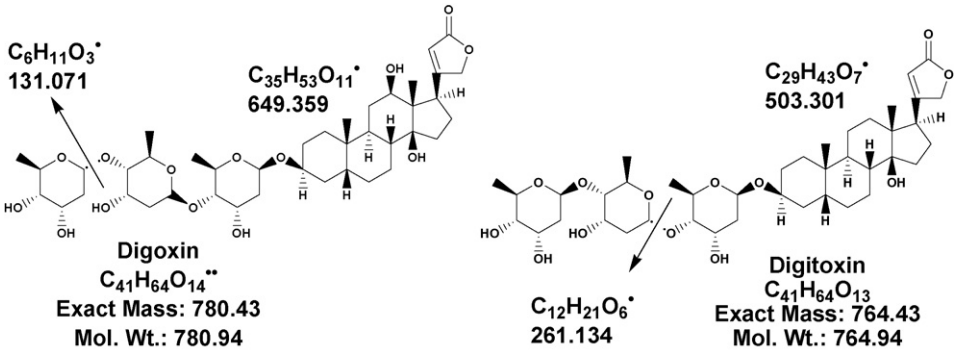

Fig. 1. Compound structures, molecular weights, and MS/MS mass fragments.

digoxin 779 $m/z > 649$ m/z. The internal standard is not shown in Fig. 2. The retention time of digoxin is indicated by an arrow at 1.18 min.

Digoxin is not a highly sensitive molecule for mass spectrometry as it is very neutral and difficult to charge under electrospray. The limit of detection was determined to be 10 nM with a signal-to-noise ratio of approximately 3 calculated by peak height. Several internal standards were evaluated such as propranolol, sulfasalazine, and omeprazole. Although somewhat successful these molecules vary too much in structure and molecular weights to be a good internal standard. A search of the Sigma–Aldrich database yielded a similar analog of digoxin as digitoxin. Digoxin and digitoxin are secondary cardiac glycosides and share a great deal of similarity in both structure and molecular weights. Use of a stable label isotope of digoxin as an internal standard is recommended if available.

3. Results and discussion

3.1. Method evaluation and pilot study

3.1.1. Standard curves, raw data, accuracy, and precision

Eight 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 to 5000 nM for digoxin prepared in HBSS. This method development stage work was not incubated in Caco-2 cell lines and was only used to evaluate the linearity, sensitivity, and limits of quantitation for digoxin. 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 variance (CV) calculated as the standard deviation divided by the mean concentration. The overall accuracy and %CV for digoxin was ±20% of the nominal concentration (data not shown).

This assay was next evaluated using digoxin incubated in Caco-2 cell based bi-directional assay samples. The first part of the pilot study was to determine if digoxin incubated in the absence of other compounds quantitated by LC–MS/MS method would be in agreement with the historical ^{3}H-digoxin %inhibition efflux ratios. Fig. 3 shows the data from four unique studies using Caco-2 cell lines incubated with digoxin in triplicate in the absence of any other compounds. Digoxin shows appropriate vectorial transport with A to B permeability value <5 nm/s and B to A permeability >130 nm/s. Efflux ratio of >10 was consistently observed for digoxin using this analytical technique. These standard curves were prepared in single replicates and show similar response as the initial evaluation without Caco-2 cells with accuracy ±25% CV and R^2 equal to 0.98 (data not shown).

Table 1 shows raw mass spectrometry data with digoxin alone and in the presence of four different test compounds. Calculated values in nM units are derived from a plot of the standard curve regression using Analyst software. The initial nominal concentration of digoxin was 5 μM in the presence of test compounds (not quantitated) at 10 μM. LC–MS/MS quantitation of digoxin before Caco-2 cell incubation ranged from 2.8 to 6.6 μM which was in the expected range. The measured concentration of digoxin after Caco-2 cell based bi-directional incubations in wells A to B top, A to B bottom, B to A top, and B to A bottom range from 5.5 μM to less than 1 nM. Peak area counts for digoxin, IS, and area ratio are also shown in Table 1. In each of these experiments, digoxin showed the expected efflux ratio when incubated alone and in the presence of these test compounds. The mass spectrometer was able to quantitate accurately over the range of 10–5000 nM. Concentrations of digoxin quantitated below 10 nM are considered to be not accurate and are estimated by the software by extending the regression line past the lowest standard curve point. The overall accuracy for the standard curve for digoxin was within ±25% CV and R^2 equal to 0.99 (not shown).

3.1.2. P-gp Inhibition comparison for classical P-gp inhibitors: radiolabeled versus LC–MS/MS method

The next evaluation of the ruggedness and accuracy of this method was to compare the historical database of marketed compounds inhibition permeability values determined by radiolabeled ^{3}H-digoxin to cold digoxin determined by LC–MS/MS. Table 2 shows the % P-gp inhibition values for 13 different known positive and negative P-gp inhibitors. The %P-gp inhibition observed using radiolabeled digoxin (historical database results) was compared to the recent inhibition obtained using LC–MS/MS method. A very good correlation R^2 equal to 0.92 (data not shown) was observed between a plot of the historical data using radiolabeled digoxin and the LC–MS/MS

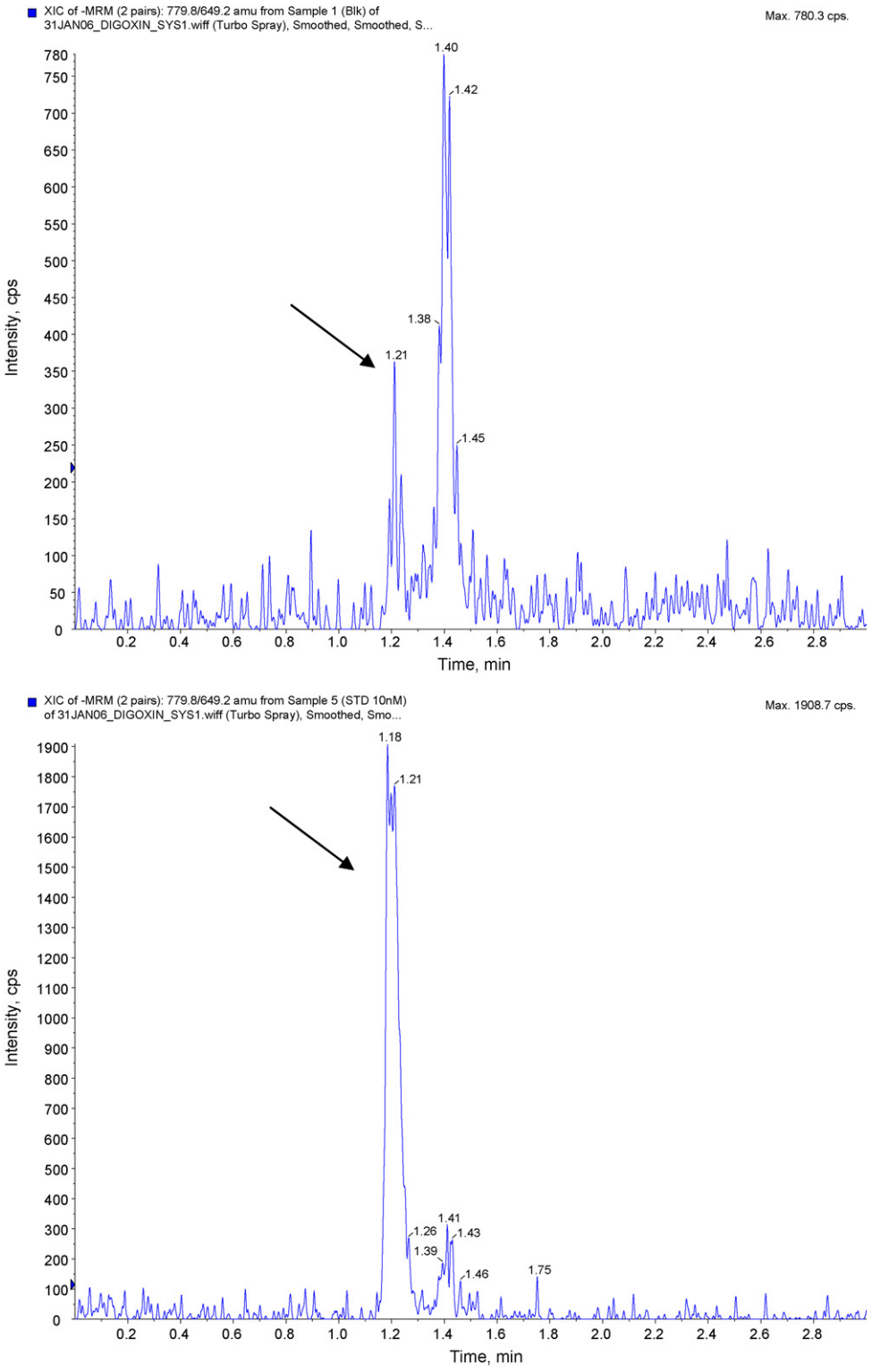

Fig. 2. LC–MS/MS extracted ion chromatogram for blank buffer with internal standard (top) and a 10 nM standard for digoxin (bottom). Blank with IS: SRM 779 *m/z* > 649 *m/z*; 10 nM digoxin: SRM 779 *m/z* > 649 *m/z*; arrows indicate retention time of digoxin at 1.18 min. SRM represents selected reaction monitoring of Q1 and Q3 fragmentation. Internal standard is not shown here.

Table 1
Raw mass spectrometry data for digoxin alone and in the presence of four different test compounds

Sample name	Digoxin (nM)	Digoxin peak area	Area ratio	IS peak area
Digoxin 5 μM alone	4449.5	1,984,698	12.1	163,630
A to B top	4888.7	2,185,713	13.3	164,028
B to A top	979.6	417,977	2.7	155,981
A to B bottom	2.3	2,865	0.0	155,400
B to A bottom	4539.1	2,054,420	12.4	166,036
Digoxin 5 μM + compound-1	6696.3	2,889,928	18.2	158,372
A to B top	5588.4	2,385,198	15.2	156,604
B to A top	713.1	327,235	2.0	167,459
A to B bottom	26.2	18,046	0.1	216,624
B to A bottom	4513.7	2,777,049	12.3	225,703
Digoxin 5 μM + compound-2	5329.2	3,136,711	14.5	215,955
A to B top	4623.1	2,746,250	12.6	217,921
B to A top	622.2	384,318	1.7	225,201
A to B bottom	22.8	17,480	0.1	235,824
B to A bottom	4667.3	2,882,534	12.7	226,573
Digoxin 5 μM + compound-3	3871.8	2,831,238	10.6	268,211
A to B top	3425.6	2,433,155	9.3	260,483
B to A top	453.6	333,467	1.2	267,340
A to B bottom	32.1	24,559	0.1	247,200
B to A bottom	3878.0	2,637,596	10.6	249,471
Digoxin 5 μM + compound-4	2821.6	1,687,791	7.7	219,308
A to B top	3072.4	1,790,854	8.4	213,730
B to A top	454.0	274,283	1.2	219,705
A to B bottom	<1	2,907	0.0	227,046
B to A bottom	3128.8	1,886,811	8.5	221,132

method. Compounds such as cyclosporin and ketoconazole that are known to be very potent P-gp inhibitors consistently demonstrated % P-gp inhibition value greater than 80% via both analytical techniques. Moderate P-gp inhibitors such as verapamil, saquinavir and quinidine had % P-gp inhibition between 40% and 60% via both methods. Negative controls (i.e. non-inhibitors such as metoprolol and caffeine) had no inhibition in either method. All the data generated by LC–MS/MS is essentially similar to the data generated by the radiolabeled digoxin method providing proof-of-principle regarding the utility of LC–MS/MS analytical method for digoxin.

3.1.3. P-gp Inhibition comparison for 21 additional test compounds: radiolabeled versus LC–MS/MS method

Further evaluation of the LC–MS/MS method was performed using 21 additional test compounds known to be classified as negative, moderate, and potent P-gp inhibitors spanning multiple chemo types. Table 3 shows %P-gp inhibition values for all 21 additional test compounds via the two analytical methods. A very good correlation R^2 equal to 0.89 (data not shown) was observed between a plot of the results obtained via LC–MS/MS method versus the historical radiolabeled ^{3}H-digoxin method. Fig. 4 combines data from both Tables 2 and 3 to demonstrate the agreement of the two analytical techniques (LC–MS/MS versus radiolabeled) for digoxin for all compounds studied in this manuscript. Five compounds show identical % inhibition comparison via ^{3}H-digoxin versus LC–MS/MS determination

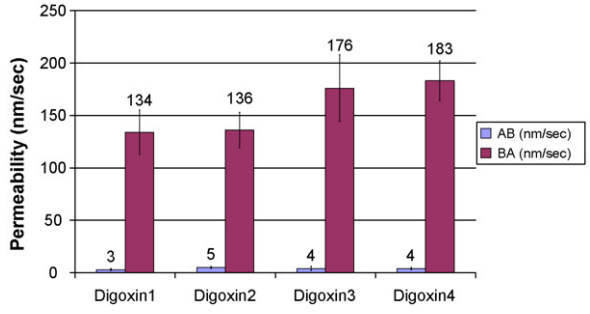

Fig. 3. Evaluation of digoxin using LC–MS/MS quantitation. Efflux ratio >10 from four different studies with digoxin incubated in the absence of other compounds with Caco-2 cell lines analyzed in triplicate by LC–MS/MS.

Table 2
P-gp inhibition comparison of marketed compounds from radiolabeled ^{3}H-digoxin vs. LC–MS/MS digoxin quantitation

Compound	%P-gp inhibition using radiolabeled digoxin	%P-gp inhibition using digoxin by LC–MS/MS
Digoxin + cyclosporin	81	98
Digoxin + ketoconazole	81	95
Digoxin + indinavir	4	25
Digoxin + saquinavir	57	61
Digoxin + rhodamine 123	23	41
Digoxin + verapamil	47	61
Digoxin + quinidine	44	70
Digoxin + vincristine	5	0
Digoxin + vinblastine	4	5
Digoxin + metoprolol	0	0
Digoxin + dexamethasone	5	0
Digoxin + etoposide	7	11
Digoxin + caffeine	0	0

Table 3
P-gp inhibition comparison of 21 additional test compounds from radiolabeled ^{3}H-digoxin vs. LC–MS/MS digoxin quantitation

Compound	%P-gp inhibition using radiolabeled digoxin	%P-gp inhibition using digoxin by LC–MS/MS
Digoxin + Cmpd-1	15	35
Digoxin + Cmpd-2	17	21
Digoxin + Cmpd-3	46	58
Digoxin + Cmpd-4	14	18
Digoxin + Cmpd-5	68	69
Digoxin + Cmpd-6	5	1
Digoxin + Cmpd-7	4	17
Digoxin + Cmpd-8	26	14
Digoxin + Cmpd-9	13	7
Digoxin + Cmpd-10	52	58
Digoxin + Cmpd-11	76	71
Digoxin + Cmpd-12	90	82
Digoxin + Cmpd-13	6	14
Digoxin + Cmpd-14	39	66
Digoxin + Cmpd-15	1	6
Digoxin + Cmpd-16	1	1
Digoxin + Cmpd-17	1	1
Digoxin + Cmpd-18	1	1
Digoxin + Cmpd-19	5	1
Digoxin + Cmpd-20	22	25
Digoxin + Cmpd-21	68	60

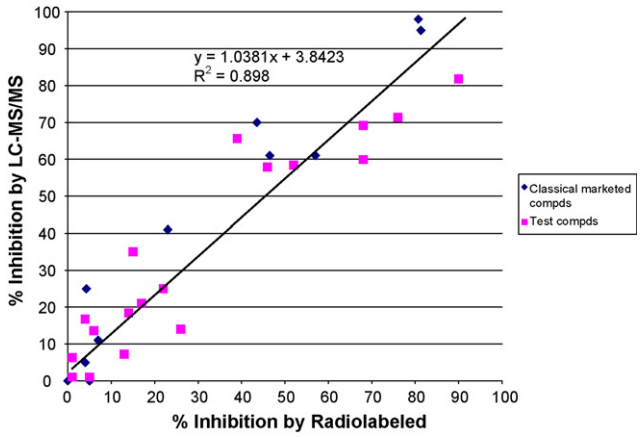

Fig. 4. Comparison of %P-gp inhibition from historical radiolabeled ^{3}H-digoxin vs. LC–MS/MS digoxin quantitation.

with values of either 0 and 0 or 1 and 1, respectively. Two compounds show % inhibition comparison values of 5 and 0 and two compounds show % inhibition values of 5 and 1 for ^{3}H-digoxin versus LC–MS/MS determination. A very good correlation R^2 equal to 0.90 was observed. In all cases, the compounds were placed in the same bin by LC–MS/MS as they were placed by the initial radiolabeled digoxin assay.

4. Discussion and conclusions

A bioanalytical method was evaluated in a pilot study of 34 different compounds on an Aria TX-2 TurboFlow® system from Thermo Scientific, using turbulent flow chromatography and tandem mass spectrometry. The use of TFC is very effective in de-salting samples with high levels of buffers and chelating agents. 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 five and a half minutes 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 min when running in staggered injection mode. The compounds studied included known marketed compounds and classical P-gp inhibitors such as ketoconazole, cyclosporin, verapamil, quinidine, saquinavir as well as negative controls such as caffeine and metoprolol. The standard curves were prepared in HBSS buffer and detected by SRM. The standard curve range was 10–5000 nM for digoxin based upon a linear $1/x^2$ regression. The test compounds were incubated at 10 μM while digoxin was co-incubated with test compounds at 5 μM concentrations. There was no observed interference of the test compounds with digoxin quantitation. The limit of detection for digoxin at 10 nM represents 0.2% of the initial nominal concentration of 5 μM thus providing accurate efflux ratios for compounds with very low P-gp inhibition. The correlation coefficients (R^2) were 0.93 from a plot of efflux ratios from direct comparison of radiolabeled digoxin to the LC–MS/MS permeability values obtained for these marketed test compounds. Further studies of 21 additional test compounds yield a regression coefficient of 0.89. A very good linear relationship was established for comparison of historical radiolabeled digoxin to the detection of cold digoxin using LC–MS/MS. An internal standard digitoxin was used for quantitation at a concentration of 200 nM. The internal standard tracked the analyte very well and shares a high level of structural similarity. The evaluation parameters (sensitivity, accuracy, precision, specificity, repeatability etc.) obtained by the method was comparable to ^{3}H-digoxin established analytical technique.

The Caco-2 cell based bi-directional assay for digoxin inhibition presents an efficient model to identify P-gp inhibitors in early Discovery. However, in spite of its *in vitro* nature that makes it amenable to higher throughput, this assay often becomes the bottleneck during drug discovery screening. The LC–MS/MS assay method described in this work provided a fast, accurate and sensitive analytical method for analyzing samples generated from the P-gp inhibition assay which is a key assay performed in early drug discovery stage.

References

[1] J. Smalley, P. Kadiyala, B. Xin, P. Balimane, T. Olah, J. Chromatogr. B 830 (2006) 270.
[2] P. Balimane, K. Patel, A. Marino, S. Chong, Eur. J. Pharm. Biopharm. 58 (2004) 99.
[3] J. Keogh, J. Kunta, Eur. J. Pharm. Sci. 27 (2006) 543.
[4] M. Yao, H. Zhang, S. Chong, M. Zhu, R. Morrison, J. Pharm. Biomed. Anal. 32 (2003) 1189.
[5] C. Huang, G. Wang, H. Li, H. Xie, J. Sun, H. Lv, T. Lv, J. Pharm. Biomed. Anal. 40 (2006) 778.
[6] N. Yoshida, M. Koizume, I. Adachi, J. Kawakami, Food Chem. Toxicol. 44 (2006) 2033.
[7] A. Collett, J. Tanianis-Hughes, G. Carlson, M. Harwood, G. Warhurst, Eur. J. Pharm. Sci. 26 (2005) 386.

[8] A. Jedlicka, T. Grafnetterova, V. Miller, J. Pharm. Biomed. Anal. 33 (2003) 109.
[9] J. Lin, Adv. Drug Deliv. Rev. 55 (2003) 53.
[10] M. Sababi, O. Borga, U. Hultkvist-Bengtsson, Eur. J. Pharm. Sci. 14 (2001) 21.
[11] K. Kelly, B. Kimball, J. Jonston, J. Chromatogr. A 711 (1995) 289.
[12] D. Zimmer, V. Pickard, W. Czembor, C. Muller, J. Chromatogr. A 854 (1999) 23.
[13] H.Z. Bu, M. Poglod, R. Micetich, J. Khan, Rapid Commun. Mass Spectrom. 14 (2000) 523.
[14] M. Jemal, Z. Ouyang, M. Powell, J. Pharm. Biomed. Anal. 23 (2000) 323.
[15] A. Asperger, J. Efer, T. Koal, W. Engewald, J. Chromatogr. A 960 (2002) 109.
[16] J.L. Herman, Rapid Commun. Mass Spectrom. 16 (2002) 421.
[17] R. Grant, C. Cameron, S. Mackenzie-McMurter, Rapid Commun. Mass Spectrom. 16 (2002) 1785.
[18] L. Ynddal, S. Hansen, J. Chromatogr. A 1020 (2003) 59.
[19] Y.J. Xue, K. Turner, J. Meeker, J. Pursley, M. Arnold, S. Unger, J. Chromatogr. B 795 (2003) 215.
[20] S. Calderoli, E. Frigerio, C.A. James, Chromatographia 59 (2004) S183.
[21] S. Wu, J. Xing, A. Apedo, D. Wang-Iverson, T. Olah, A. Tymiak, N. Zhao, Rapid Commun. Mass Spectrom. 18 (2004) 2531.
[22] X. Xu, K. Han, H. Song, M.-W. Lo, J. Chromatogr. B 814 (2005) 29.
[23] C. Chassaing, H. Stafford, J. Luckwell, A. Wright, A. Edgington, Chromatographia 62 (2005) 17.
[24] S. Zhou, H. Zhou, M. Larson, D. Miller, D. Mao, X. Jiang, W. Naidong, Rapid Commun. Mass Spectrom. 19 (2005) 2144.
[25] A. Vintiloiu, W. Mullett, R. Papp, D. Lubda, E. Kwong, J. Chromatogr. A 1082 (2005) 150.
[26] J.L. Herman, Rapid Commun. Mass Spectrom. 19 (2005) 696.
[27] C. Kousoulos, Y. Dotsikas, Y. Loukas, Talanta 72 (2007) 360.
[28] Y. Mao, M.-Q. Huang, Y.-Q. Xia, M. Jemal, J. Pharm. Biomed. Anal. 43 (2007) 1808.