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Novel generic UPLC/MS/MS method for high throughput analysis applied to permeability assessment in early Drug Discovery

Jurgen Mensch [a,*], Mark Noppe [a], Jef Adriaensen [a], Anouche Melis [a], Claire Mackie [b], Patrick Augustijns [c], Marcus E Brewster [a]

[a] *Pharmaceutical Sciences, Johnson & Johnson Pharmaceutical Research & Development, A Division of Janssen Pharmaceutica N.V., Turnhoutseweg 30, 2340 Beerse, Belgium*
[b] *ADME-Tox, Johnson & Johnson Pharmaceutical Research & Development, A Division of Janssen Pharmaceutica N.V., Turnhoutseweg 30, 2340 Beerse, Belgium*
[c] *Laboratory for Pharmacotechnology and Biopharmacy, Katholieke Universiteit Leuven, O&N, Gasthuisberg, 3000 Leuven, Belgium*

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Abstract

A novel generic ultra performance liquid chromatography-tandem mass spectrometric (UPLC/MS/MS) method for the high throughput quantification of samples generated during permeability assessment (PAMPA) has been developed and validated. The novel UPLC/MS/MS methodology consists of two stages. Firstly, running a 1.5 min isocratic method, compound-specific multiple reaction monitoring (MRM) methods were automatically prepared. In a second stage, samples were analyzed by a 1.5 min generic gradient UPLC method on a BEH C18 column (50 mm × 2.1 mm). Compounds were detected with a Waters Micromass Quattro Premier mass spectrometer operating in positive electrospray ionization using the compound-specific MRM methods. The linearity for the validation compounds (caffeine, propranolol, ampicillin, atenolol, griseofulvin and carbamazepine) typically ranges from 3.05 nM to 12,500 nM and the limits of detection for all generically developed methods are in the range between 0.61 nM and 12 nM in an aqueous buffer. The novel generic methodology was successfully introduced within early Drug Discovery and resulted in a four-fold increase of throughput as well as a significant increase in sensitivity compared to other in-house generic LC/MS methods.
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1. Introduction

Various cell-based *in vitro* methods for the assessment of permeability have been developed [1–6] in order to predict drug transport properties in humans. Traditional methods include different cell cultures mimicking for instance the gastrointestinal tract (Caco-2), the blood brain barrier (BBMEC, co-cultures) or the bronchial epithelium (Calu-3). In addition to cell cultures, traditional methods include tissue-based ex vivo experiments in which animal and human tissue (e.g. intestine, colon, skin) is mounted in side by side diffusion chambers. The methods mentioned so far are time-consuming, labor-intensive, relatively expensive and limited in throughput. As a result, many new procedures have been developed to estimate the drug permeability in early Drug Discovery in a fast and cost efficient way.

One such method is the parallel artificial permeability assay (PAMPA). PAMPA was developed as an alternative for the low throughput Caco-2 assay as well as a high throughput assay for the prediction of blood brain barrier permeability [7]. PAMPA measures permeability of an immobilized lipid membrane and can be automated resulting in efficient processing of many compounds in parallel. Together with the rapid development of combinatorial chemistry and the increasing number of compounds with poor aqueous solubility, the analysis of samples from PAMPA studies has become the ‘bottleneck’ of this drug screening assay. Therefore, an essential aspect of the Drug Discovery process is to dramatically increase the throughput of sample analysis for this assay.

* Corresponding author. Tel.: +32 14 60 6320; fax: +32 14 60 7083.
E-mail address: jmensch@prdbe.jnj.com (J. Mensch).

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For the analysis of samples from *in vitro* permeability tests [8–12], high performance liquid chromatography (HPLC) coupled with mass spectrometric detection has been widely used. LC/MS/MS offers the advantages of increased sensitivity and specificity [13]. Unfortunately, this approach is rather slow. The analysis with LC/MS/MS is significantly improved by the use of ultra performance liquid chromatography (UPLC) [14–22]. UPLC is a relatively new technique using analytical Bridged Ethane Hybrid (BEH) C18 columns packed with 1.7 μm particles, which offers the advantages of increased speed, improved sensitivity, selectivity and specificity compared to HPLC analysis [14–22]. As speed and sensitivity are of great importance for the analysis of samples obtained from high throughput *in vitro* applications in general, UPLC/MS/MS can play an important role in the quantification of samples from the PAMPA assay specifically.

The aim of this study was the development of a novel generic UPLC/MS/MS method with automated and compound-specific optimization of the MS/MS parameters to increase the throughput, sensitivity and specificity for the quantification of compound levels in the Discovery PAMPA assay.

2. Experimental

2.1. Chemicals (materials and reagents)

Reference standards, caffeine (99.9%), carbamazepine (≥99%), atenolol (≥99%), griseofulvin (≥95%), acycloguanisine (99.6%), propranolol (99%), ampicillin (≥98%) and verapamil (≥99.9%) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Itraconazole (≥98.75%), flunarizine (≥99%) and cisapride (≥99%) were synthesized in-house. Working standard solutions/mixtures were prepared in methanol and pIOn buffer (exact composition not known). Methanol, acetonitrile and formic acid, all MS grade, were purchased from Biosolve (Valkenswaard, The Netherlands). Buffer solutions and lipid mixtures for the PAMPA assay were purchased from pION Inc. (Woburn, MA, USA).

2.2. Equipment and experimental conditions

UPLC analyses were performed with a Waters Acquity Ultra Performance LC system (Waters, Milford, MA, USA). UPLC separation was achieved on an Acquity UPLC BEH C18 column (50 mm × 2.1 mm, i.d., 1.7 μm particle size, Waters) maintained at 55 °C and the mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The compounds were injected in the mobile phase with an injection volume of 2.0 μl (full-loop injection). The separated compounds were detected with a Waters Micromass Quattro Premier triple quadrupole mass spectrometer (Waters). The mass spectrometer was operated with an electrospray source in positive ionization mode. The ionization source conditions were: capillary voltage of 3.0 kV, source temperature of 120 °C and desolvation temperature of 350 °C. The cone and desolvation gas flows were 100 l/h and 800 l/h, respectively and were obtained from an in-house nitrogen source. Argon was used as collision gas and was regulated at 0.35 ml/h and the multipliers were set to 650 V. With these UPLC/MS/MS conditions the compounds were analyzed by multiple reaction monitoring (MRM).

The novel UPLC/MS/MS method consists of two stages. In a first stage, for each compound the cone voltage and collision energy was automatically optimized by separate loop injections (no column connected). To perform these loop injections, an additional switching valve (Waters) was installed. An isocratic UPLC method was applied with a flow rate of 0.2 ml/min resulting in a constant pressure of 7 bar and a total run time of 1.5 min. The isocratic condition of the mobile phase was: 50% solvent A and 50% solvent B. The compound was first injected in the mobile phase and MS spectra were acquired with a total cycle time of 0.5 s and a maximum dwell time of 0.1 s. With these settings of total cycle time and maximum dwell time, mass spectra were acquired over a range of different cone voltages (10–50 V) in steps of 6 V. The cone voltage was evaluated for best response of the parent ion and the optimal cone voltage was automatically transferred to an MRM method for the particular compound. As MRM methods are used for analysis of PAMPA samples, a second injection was made to optimize the collision energy. The parent ion was transmitted into the collision cell, fragmented, and daughter ion spectra were acquired at different collision energies ranging from 10 eV to 50 eV in steps of 10 eV. The collision energy was evaluated for best response of the most intense daughter ion. The optimal collision energy was also automatically transferred to the MRM. This optimization process was repeated for each compound and the MRM conditions were stored before sample acquisition.

In a second stage, full-loop column injections of PAMPA samples were performed and the compounds were separated with a generic gradient UPLC method with a flow rate of 0.6 ml/min and a run time of 1.5 min. With this flow rate, the Acquity UPLC operating pressure was 400 bar at the initial gradient condition. The gradient condition of the mobile phase was: 0 min 35% solvent B, 1.0 min 100% solvent B, 1.4 min 100% solvent B, 1.42 min 35% solvent B and 1.5 min 35% solvent B. With this gradient method so far all injected compounds were eluted within the 1.5 min run time and detected in the mass spectrometer with the earlier prepared compound-specific MRM method. The resulting chromatographic peaks were integrated (area under the concentration versus time curve). All aspects of automatic optimization, data acquisition and data processing were controlled using Masslynx^{TM} 4.0 and QuanLynx^{TM} (Waters).

2.3. PAMPA assay and sample collection

The parallel artificial permeability assay was performed in a 96-well plate format similar to that described in the literature [2,4,7,23]. All compounds were tested in triplicate and at different pH values (pH 4.0 and pH 7.4) in order to judge the possible effect of ionisation on the permeability coefficient. The membranes of a 96-well filter plate (Millipore, Bedford, MA, USA) were coated with 4 μl of a 2% (w/v) dodecane

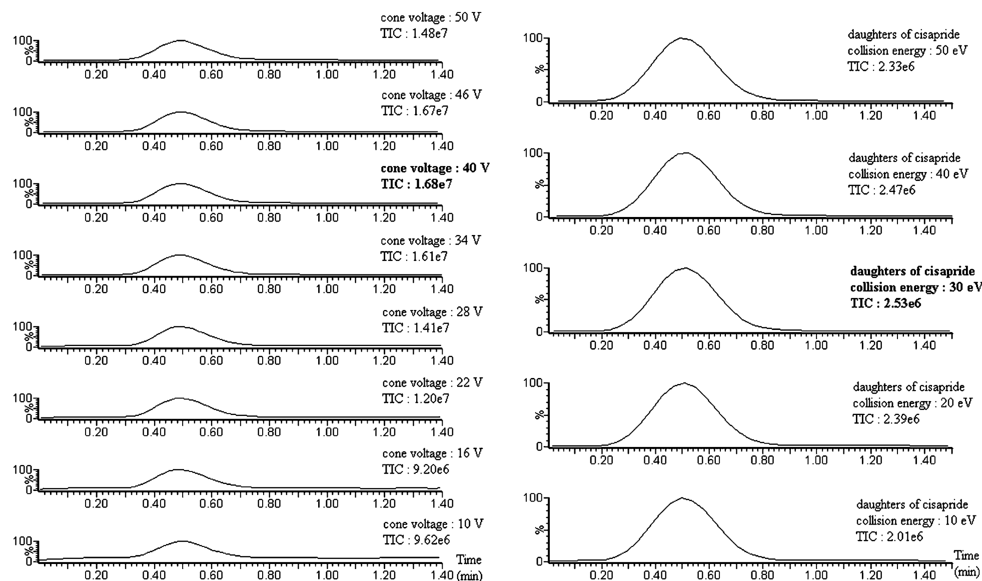

Fig. 1. The cisapride chromatograms obtained in positive electrospray mode during the automatic optimization of cone voltage and collision energy. On the left hand side, eight chromatograms at different cone voltages ranging from 10 to 50 V in relation to their intensity are shown. The cone voltage of 40 V resulted in the most intense cisapride parent ion. On the right hand side the chromatograms of the collision energy optimization are plotted at the fixed optimal cone voltage of 40 V for cisapride. Finally the collision energy of 30 eV showed the best response for the daughter ion of ciaspride with m/z = 184.

solution of dioleoylphosphatidylcholine; subsequently, the filter plate was placed on top of a 96-well microtiter plate. A "sandwich" was created in which two compartments (bottom = donor compartment, top = acceptor compartment) were separated by the coated filter. All acceptor wells were filled with 200 μl of pION buffer solution adjusted to pH 7.4 or pH 4.0. The donor wells were filled with 200 μl of the test compound solution in pION buffer (pH 4.0 or pH 7.4). These test compound solutions were prepared by dilution of a 5 mM stock solution in DMSO.

The created "sandwich" was then incubated at 20–22 °C for approximately 18 h. Afterwards the sandwich was disassembled and the donor and acceptor solutions were transferred to a disposable UV-transparent plate (pION Inc.). UV absorption spectra between 250 nm and 500 nm were obtained with a SPECTRAMax 190 micro-plate spectrophotometer (Molecular Device Corporation, Sunnyvale, CA, USA). Previous studies (results not reported here) indicate that about 40% of the tested compounds are UV-undetectable, therefore the samples of the PAMPA experiments were also transferred to the Acquity UPLC/MS/MS system and were analyzed using the novel generic UPLC/MS/MS method. The PAMPA Evolution version 2.2 (pION Inc.) was used to calculate the effective permeability taking into account iso-pH conditions and membrane retention of the drug molecule: $P_{\mathrm{eff}} = -(2.303\,V_{\mathrm{d}}/(A(t-\tau_{\mathrm{lag}})))(1/(1+r_{\mathrm{v}}))\,(\log_{10}[(1-(1+r_{\mathrm{v}}^{-1})/(1-R))(C_{\mathrm{a}}(t)/C_{\mathrm{d}}(0))])$ [24] where the aqueous compartment volume ratio, $r_{\mathrm{v}} = V_{\mathrm{d}}/V_{\mathrm{a}}$ (in our case $r_{\mathrm{v}} = 1$). Note that V_{d} = volume of donor well, V_{a} = volume in acceptor well, A = filter area, t = permeation time, τ_{lag} = time needed to saturate the membrane, R = mole fraction of solute lost to the membrane, C_{d} and C_{a} = concentration in donor and acceptor well.

3. Results and discussion

3.1. Generic and automatic optimization of the MS/MS parameters

The MS/MS parameters, cone voltage and collision energy, of the compounds were evaluated for best response of parent ion and daughter ion, respectively in positive electrospray ionisation mode and were transferred into a MRM method. With this automatic optimization process, each compound-specific MRM method was created in 3 min, and more then 15 data points were acquired for each chromatographic peak obtained at any cone voltage (8 different cone voltages) and collision energy (5 different collision energies). Fig. 1 shows representative chromatograms for the automatic cone voltage and collision energy optimization for cisapride.

The intra- and inter-day reproducibility of the automatic optimization method was evaluated by optimizing the cone voltage and collision energy of several chemically diverse compounds (caffeine, carbamazepine, griseofulvin, acycloguanisine, atenolol). All compounds were automatically optimized 10 times on day 1 and once on days 7, 14 and 35. The optimization results were compared and the resulting mass transitions, corresponding cone voltages and collision energies for the five compounds are reported in Table 1. The results showed good inter- and intra-day reproducibility for all compounds. In all experiments, one transition was obtained for caffeine (195 *m/z* > 138 *m/z*), carbamazepine (237 *m/z* > 194 *m/z*) and acycloguanisine (226 *m/z* > 152 *m/z*) together with one specific cone voltage and one specific collision energy. The generic optimization method selected two optimal cone voltages with best response of the parent ions for griseofulvin and atenolol

Table 1
Mass transitions, cone voltages (CV) and collision energy (CE) obtained for the five compounds at days 1, 7, 14 and 35

	Day 1 ($n=10$)			Day 7 ($n=1$)			Day 14 ($n=1$)			Day 35 ($n=1$)		
	Mass transitions	CV	CE	Mass transition	CV	CE	Mass transition	CV	CE	Mass transition	CV	CE
Caffeine	195 > 138	40	20	195 > 138	40	20	195 > 138	40	20	195 > 138	40	20
Carbamazepine	237 > 194	40	20	237 > 194	40	20	237 > 194	40	20	237 > 194	40	20
Griseofulvin	353 > 165	40	20	353 > 165	34	20	353 > 69	40	30	353 > 165	40	20
	353 > 69	46	30									
Acycloguanisine	226 > 152	22	10	226 > 152	22	10	226 > 152	22	10	226 > 152	22	10
Atenolol	267 > 145	34	20	267 > 145	34	20	267 > 145	40	20	267 > 145	34	20
	267 > 74	40										

Table 2
Effective permeability values of griseofulvin obtained with different mass transitions

	Mass transitions	P_{eff} ($\times 10^{-6}$ cm/s)	
		Average ($n=6$)	S.D. ($n=6$)
Griseofulvin	353 > 165	14.50	4.80
	353 > 69	15.18	5.07

No statistically significant difference was found between the mean P_{eff} values of griseofulvin at both transitions.

because the intensities of the parent ions were equal over a large range of cone voltages. For the same reason also two daughter ions were selected for both compounds. As the intensities of parent ions and daughter ions at the different MS/MS parameters were very similar, the analysis of samples from PAMPA with both transitions did not affect the resulting effective permeability values (P_{eff}). Typical results of mean P_{eff} values obtained at different mass transitions are shown in Table 2 and a comparison-of-means statistical analysis was performed. The comparison-of-means test constructs confidence intervals for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from −5.67 to 7.04. As the interval contains the value 0.0, there is no statistically significant difference at the 95% confidence interval between the mean P_{eff} values obtained at the different mass transitions.

3.2. Performance of the generic Acquity UPLC/MS/MS method

The limit of detection (LOD) of the generic UPLC/MS/MS method for the selected compounds was assessed based on signal-to-noise ratio (S/N) determination. The signal-to-noise ratio was determined by comparing measured signals from samples with known low concentrations of compound with the baseline. The minimum concentration at which the compound could be reliably detected was at a S/N = 2/1. The limits of detection of the method for the selected compounds are presented in Table 3. The detection limits of the generic UPLC/MS/MS method were less then 6.1 nM for all compounds except for ampicillin (LOD = 12.21 nM).

The reproducibility and linearity of the generic UPLC/MS/MS method were evaluated by triplicate injections of calibration curve samples of several compounds. The calibration curve samples were prepared in pION buffer adjusted to pH 7.4, the same buffer that was used with PAMPA experiments. Calibration curves were constructed by plotting the peak area (AUC) of the compounds against the concentration. Linearity of the generic UPLC/MS/MS method was investigated from the limit of quantitation (LOQ; S/N = 10/1) for the different compounds up to 25,000 nM. The calibration curves for all compounds were linear over a large range (Table 3) varying from the smallest range of 97.7–25,000 nM for ampicillin to the largest range for propranolol of 3.05–12,500 nM. As shown in Table 3, the correlation coefficient (R^2) for all tested compounds was higher than 0.993 and the relative standard deviation (R.S.D.) of the individual peak areas was less than 15% at the limit of quantitation and less than 5% at the highest linear concentration.

The intra-day repeatability of the generic UPLC/MS/MS method was assessed by obtaining the peak area of 10 samples in triplicate with concentrations in the linear range of the method for the specific compounds. The intra-day repeatability was determined based on peak areas because the permeability

Table 3
Performance results obtained with the novel generic Acquity UPLC/MS/MS methodology

Compound	Linearity (nM)	Correlation coefficient (R^2)	LOD (nM)	Intra-day repeatability %R.S.D. for peak area (conc. nM)		
Caffeine	12.21–6250	0.993	3.05	12.32 (6.1)	3.16 (781.3)	3.03 (6250)
Propranolol	3.05–12500	0.999	0.76	11.87 (3.05)	8.97 (781.3)	1.15 (12500)
Ampicillin	97.7–25000	0.999	12.21	5.44 (97.7)	6.94 (781.3)	1.46 (25000)
Atenolol	24.41–12500	0.996	6.10	5.64 (24.24)	3.62 (781.3)	2.11 (12500)
Griseofulvin	6.1–25000	0.998	0.61	6.3 (12.21)	1.60 (781.3)	1.20 (25000)
Carbamazepine	6.1–25000	0.996	0.61	3.8 (12.21)	0.30 (781.3)	0.20 (25000)

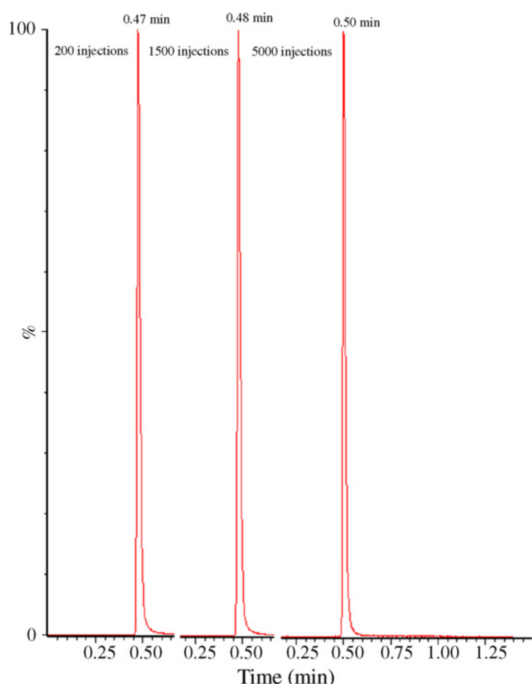

Fig. 2. MRM chromatograms of carbamazepine after 200 injections ($r_t = 0.47$), 1500 injections ($r_t = 0.48$) and 5000 injections ($r_t = 0.50$) on the same Acquity UPLC BEH C18 column (50 mm × 2.1 mm, i.d., 1.7 μm particle size).

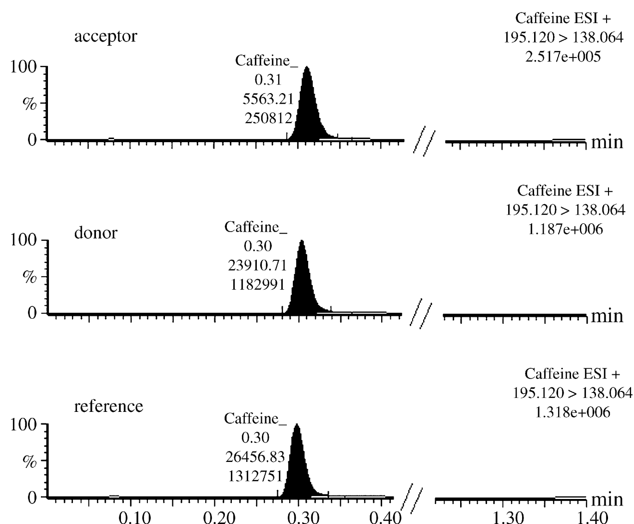

Fig. 3. The MRM chromatograms for Caffeine of acceptor, donor and reference samples obtained from the PAMPA assay. The chromatograms are based on the transition settings of caffeine as reported in Table 1 and the conditions described in the text.

is calculated in a relative way (based on the peak areas of the acceptor and donor samples for each compound). As all samples from the same PAMPA assay were analyzed within 20 h because of the short run time of the generic UPLC/MS/MS method (1.5 min), only the intra-day repeatability was assessed. The intra-day R.S.D. values for ampicillin, atenolol, griseofulvin and carbamazepine were less than 7% for all concentrations tested while caffeine and propranolol showed R.S.D. values ranging from 0.7% to 12.32% (Table 3).

The Acquity UPLC BEH C18 column (50 mm × 2.1 mm, i.d., 1.7 μm particle size) demonstrated excellent stability as shown by very stable retention times and unchanged peak shapes. Even after about 5000 injections of PAMPA samples, the retention time of carbamazepine changed from 0.47 min to 0.50 min and no peak broadening was observed (Fig. 2). The average peak width of all compounds analyzed with the novel generic UPLC/MS/MS method was about 6 s at the base.

3.3. The novel generic Acquity UPLC/MS/MS method and its application for the PAMPA assay

The permeability of the artificial membrane for a drug is determined by the ratio of the amount of drug at the acceptor side to the amount at the donor side. For this reason, no calibration curve is necessary for the analysis of the PAMPA samples. The inclusion of standards to construct calibration curves for all compounds would dramatically decrease the throughput of the assay. Of course to obtain accurate results the peak areas must be linear with respect to the concentration. The experimental data indicated that the obtained peak areas of acceptor and donor samples were in the linear range for all compounds tested. Fig. 3 shows the typical MRM chromatograms of the acceptor, donor and reference samples of caffeine evaluated in the PAMPA assay. The resulting effective permeability (P_{eff}) for caffeine evaluated at pH 7.4 is $3.8 \pm 0.57 \times 10^{-6}$ cm/s ($n = 20$).

Over the years many thousands of compounds have been evaluated for their gastrointestinal passive permeability properties with the PAMPA assay. With UV detection, no permeability coefficients could be determined for 40% of the tested compounds mainly ascribed to poor solubility properties of the compounds or low UV response. Using the new generic UPLC/MS/MS method with automatic optimization of cone voltage and collision energy, the estimation of the permeability coefficients has risen to 97% of all compounds assessed with the PAMPA assay. As new as the improved sensitivity and specificity, the generic UPLC/MS/MS method has substantially increased the throughput of the analysis compared to the more traditional LC/MS(/MS) methods. The automatic and compound-specific optimization of cone voltage and collision energy, the generation of the MRM method and the analysis of the PAMPA samples (acceptor, donor, reference) at two pH values in triplicate results in 20 injections/compound. These analyses are now performed in less then 37 min, resulting in a four-fold increase in throughput compared to the previously used generic LC/MS method.

3.4. Cassette dosing

Another way of increasing throughput is cassette dosing analysis (sample pooling). Fig. 4 shows the chromatogram of a mixture of five compounds obtained with the novel generic UPLC/MS/MS method. The generic UPLC/MS/MS method is well suited for the analysis of mixtures. However, some precautions should be taken when cassette dosing analysis is used for low permeability compounds or compounds from the same chemical class. Sample pooling of low permeability compounds

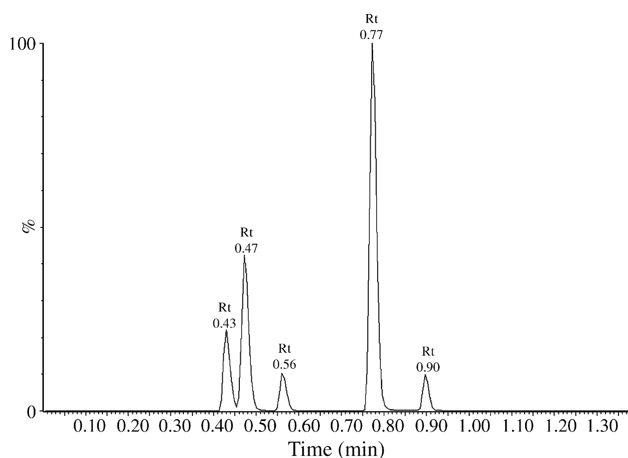

Fig. 4. The MRM chromatogram of a mixture of propranolol ($R_t = 0.43$), cisapride ($R_t = 0.47$), verapamil ($R_t = 0.56$), flunarizine ($R_t = 0.77$) and itraconazole($R_t = 0.90$). The chromatogram contains 5 MRM transitions obtained in positive electrospray mode (propranolol: 260 > 116; cisapride: 466 > 184; verapamil: 455 > 165; flunarizine: 405 > 203; itraconazole: 705 > 392).

might result in dilutions to concentrations below the LOQ and compounds that are not chemical diverse could co-elute and be fragmented to the same daughter ion.

4. Conclusion

The novel generic Acquity UPLC/MS/MS method with automatic optimization resulted in an analysis time of 1.5 min/sample and has shown to be suitable for >97% of the tested Johnson & Johnson Pharmaceutical Research and Development compounds. Using the Acquity UPLC technology combined with the automated MS/MS analysis, a four-fold increase in throughput (1.5 min versus 6.5 min run time), as well as a significant increase in sensitivity (>20×), was obtained compared to the traditional in-house generic LC/MS methodology [13]. It was shown that UPLC/MS/MS could be a useful tool for the analysis of samples from permeability ranking experiments. Due to the improved throughput and sensitivity, all PAMPA samples are now routinely analyzed using the Acquity UPLC/MS/MS technology and efforts are on going to further decrease the run time and to analyze pooled samples.

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