Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 8–16
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

A novel high-throughput automated chip-based nanoelectrospray tandem mass spectrometric method for PAMPA sample analysis

Praveen V. Balimane [a,*], Ellen Pace [b], Saeho Chong [a], Mingshe Zhu [a], Mohammed Jemal [a], Colleen K. Van Pelt [b]

[a] *Bristol-Myers Squibb, P.O. Box No. 4000, Mailstop: F.13-07, Route 206 & Province Line Road, Princeton, NJ 08543, USA*
[b] *Advion Biosciences, Ithaca, NY, USA*

Received 16 December 2004; received in revised form 17 March 2005; accepted 20 March 2005
Available online 22 June 2005

Abstract

Parallel artificial membrane permeability assay (PAMPA) has recently gained popularity as a novel, high-throughput assay capable of rapidly screening compounds for their permeability characteristics in early drug discovery. The analytical techniques typically used for PAMPA sample analysis are HPLC-UV, LC/MS or more recently UV-plate reader. The LC techniques, though sturdy and accurate, are often labor and time intensive and are not ideal for high-throughput. On the other hand, UV-plate reader technique is amenable to high-throughput but is not sensitive enough to detect the lower concentrations that are often encountered in early drug discovery work. This article investigates a novel analytical method, a chip-based automated nanoelectrospray mass spectrometric method for its ability to rapidly analyze PAMPA permeability samples. The utility and advantages of this novel analytical method is demonstrated by comparing PAMPA permeability values obtained from nanoelectrospray to those from conventional analytical methods. Ten marketed drugs having a broad range of structural space, physico-chemical properties and extent of intestinal absorption were selected as test compounds for this investigation. PAMPA permeability and recovery experiments were conducted with model compounds followed by analysis by UV-plate reader, UV-HPLC as well as the automated nanoelectrospray technique (nanoESI-MS/MS). There was a very good correlation ($r^2 > 0.9$) between the results obtained using nanoelectrospray and the other analytical techniques tested. Moreover, the nanoelectrospray approach presented several advantages over the standard techniques such as higher sensitivity and ability to detect individual compounds in cassette studies, making it an attractive high-throughput analytical technique. Thus, it has been demonstrated that nanoelectrospray analysis provides a highly efficient and accurate analytical methodology to analyze PAMPA samples generated in early drug discovery.

Keywords: Permeability-screening; Nanoelectrospray analysis; PAMPA permeability; Drug discovery; High-throughput screening; HPLC; LC/MS

1. Introduction

Despite tremendous innovations in the field of drug delivery, the oral route still remains the most preferred route of administration for most new chemical entities (NCEs) and marketed drugs. The oral route is preferred by virtue of its convenience, low costs and high patient compliance compared to alternate routes. However, compounds intended for oral administration must have adequate aqueous solubility and intestinal permeability, in order to achieve therapeutic concentrations. Due to the remarkable progress in the field of genomics and combinatorial chemistry, synthesizing a large number of potential drug candidates is no longer a bottleneck in the drug discovery process. Instead, the task of screening compounds simultaneously for biological activity and biopharmaceutical properties (e.g., solubility,

Abbreviations: ADME, absorption, distribution, metabolism, elimination; DMSO, di-methyl sulphoxide; IAM, immobilized artificial membrane; LC/MS, liquid chromatography/mass spectrometry; HPLC, high pressure liquid chromatography; MDCK, mardin darby canine kidney cells; NCE, new chemical entity; PAMPA, parallel artificial membrane permeability assay; Pc, permeability in nm/s; P-gp, P-glycoprotein; S.D., standard deviation

* Corresponding author. Tel.: +1 609 252 4401; fax: +1 609 252 6802.
E-mail address: praveen.balimane@bms.com (P.V. Balimane).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.03.043

permeability/absorption, stability, etc.) has become the major challenge. This has provided a great impetus within the pharmaceutical industry to implement appropriate screening models that are high capacity, cost-effective and highly predictive of in vivo permeability and absorption [1–8]

Transport of drug substances across the intestinal membrane is a complex and dynamic process. It includes the passage of compounds across various functional pathways in parallel. Passive permeability occurs through the cell membrane of enterocytes (transcellular) or via the tight junctions between the enterocytes (paracellular). Carrier-mediated transport occurs by the transporter proteins present in the lipid membranes. Various influx (peptide transporters) and efflux mechanisms (P-gp) are also known to be functional in human intestine. To evaluate permeability/absorption of drug candidates during the drug candidate selection process, drug discovery scientists currently employ various techniques [1,2,6,9,10]. The need to keep up with the compounds coming from the combinatorial chemistry labs have resulted in advancements such as in silico approaches, automation, miniaturization and other concerted attempts to increase the throughput of existing models. The most pervasive preclinical methodologies currently used across the industry are: in vitro methods (animal tissue based ussing chamber or membrane vesicles, cell-based Caco-2, Mardin-darby canine kidney MDCK, artificial lipid based parallel artificial membrane permeability assay (PAMPA or IAM); in situ methods (single pass perfusion); and in vivo methods (whole animal PK studies). Recently, Kansy et al. [11] pioneered the utility and predictability of the PAMPA as a high-throughput permeability-screening tool in early drug discovery. Coating a hydrophobic filter material with a mixture of lecithin and an inert organic solvent creates an artificial lipid membrane. The extent of permeation through the membrane is measured and compared to known extent of drug absorption in humans. An excellent correlation was demonstrated between the flux across the PAMPA system and the extent of absorption of a diverse set of well-characterized drugs in humans. This technique is much less labor intensive than cell culture or in vivo methods, but appears to have similar predictive power.

However, the utility of any of these methods eventually depends on successfully integrating it with a versatile analytical technique that can handle the samples generated in an efficient, accurate and cost-effective manner. Caco-2 and PAMPA are, by far, the most popular techniques used for permeability assessment and there is a very high level of automation incorporated in their operation [12–14]. It is not uncommon to have 100s and even 1000s of compounds assessed every week. Even though the experimental front-end is extremely streamlined, it is the analytical back-end that sometimes becomes the bottleneck. HPLC and LC/MS methods have been modified and made amenable to high-throughput, but individual runs are still often multiple minutes in duration [15,16]. This leads to the loss of precious discovery time and slows down the capacity of these assays. Any advancement in analytical techniques that can provide reliable, accurate and reproducible data has the potential to significantly improve the throughput of these permeability assays.

Nanoelectrospray was first developed by Wilm and Mann [17,18]. The purpose of this study was to demonstrate the use of an automated chip-based nanoelectrospray method (NanoMate 100^{TM}, Advion BioSciences) for analysis of samples generated from PAMPA. This technique has been used for a variety of different studies [19], including Caco-2 [20], however, there is no published material demonstrating the application of this new technology to PAMPA permeability measurements. The chip-based automated nanoelectrospray system includes a NanoMate robot that holds a 96-well sample plate, a rack of 96 pipette tips and an ESI Chip. This chip consists of a 10 × 10 array of nanoelectrospray emitters or nozzles. Sample analysis is achieved by a liquid delivery mandrel picking up a pipette tip, aspirating sample and then delivering the sample to a nozzle on the ESI Chip^{TM}. The nanoelectrospray technique presents a novel analytical method that is highly suited for sample analysis in early discovery stage. It is efficient, sensitive and reproducible but at the same time amenable to high-throughput, making it a very attractive tool for integration into the drug discovery cycle.

2. Experimental

2.1. Materials and chemicals

PAMPA Explorer^{TM} permeability analysis kit was obtained from pION Inc. (Woburn, MA). The 96-well filter plates, transport buffers, lipid solution and consumable were also supplied by pION Inc. The 10 model marketed compounds (antipyrine, ketoprofen, carbamazepine, propranolol, ranitidine, verapamil, metoprolol, hydrochlorothiazide, norfloxacin and sulfasalazine) were purchased from Sigma (St. Louis, MO). All other chemicals and organic solvents were also obtained from Sigma and other established suppliers.

2.2. Permeability studies

The permeability method used in these studies was carried out in a 96-well format, a modification of the PAMPA method described in the literature [11,21–23]. A 96-well microtiter plate and a 96-well filter plate (Millipore, Bedford, MA, USA) were assembled into a “sandwich” such that each composite well was separated by a 125 μm micro-filter disc (0.45 μm pores). The hydrophobic filter material of the 96-well filter plate was coated with 5 μL of the pION lipid solution and gently shaken to ensure uniform coating. Subsequently, the filter plate was placed on the microtiter plate containing 200 μL of 100 μM test compound solution (containing 1% DMSO), which constituted the donor compartment. The test solution was prepared by dilution (×100-fold) from a 10 mM stock solution in DMSO using the pION buffer solution at pH 7.4, followed by filtration through a 0.20 μm polyvinylidene fluoride (PVDF) 96-well filter plate (Corning

Costar, Corning, NY, USA). The acceptor wells (i.e. the top of the wells) of the sandwich were hydrated with 200 μL of the specialized ionic buffer solution. The system was then incubated at room temperature for 4 h. At the end of the incubation time, samples were removed from the receiver and donor compartment and analyzed by UV-plate reader, HPLC-UV as well as nanoelectrospray technology. All the permeability studies were performed in triplicates (i.e. three wells per compound).

The apparent permeability (P) was estimated using the equation:

$$P = \frac{V \times dC}{A \times C_0 \times dT}$$

Where P is the permeability in nm/s, V is the volume of the receiver compartment (0.2 mL), A is the surface area (0.3 cm^2), Co is the starting concentration in the donor compartment in μM or ng/mL (100 μM), and dC/dT is the rate of change of compound concentration, in μM/s or ng/mL/s, in the receiver compartment with time.

2.3. Single and cassette mode PAMPA studies

Ten marketed compounds were selected to compare the different analytical techniques following the PAMPA experiment. Great care was taken to ensure that these compounds had broad structural diversity, differing physico-chemical properties and human absorption values from low (∼10%) to high (>90%) (Table 1). PAMPA permeability studies were performed with the marketed compounds being present either singly (i.e. one compound per well) or in cassette mode (i.e. multiple compounds per well). For the single mode analysis, the 10 test compounds were incubated at 100 μM concentration on the donor side. For cassette studies, three compounds per well were used. Mixture 1 included sulfasalzine, antipyrine and metoprolol. Out of the three compounds in this set, sulfasalazine and antipyrine were known to have low/medium permeability and metoprolol was known to have high permeability. Similarly, mixture 2 contained ranitidine, ketoprofen and verapamil. Rantidine and ketoprofen were known to have low/medium permeability and verapamil was known to have high permeability. When present as drug mixtures, the individual starting donor concentration of each compound was 100 μM (similar to the concentration used in the single compound study).

2.4. UV-plate reader analysis

PAMPA sample analysis by UV-plate reader was performed immediately at the end of the 4 h incubation. Following the disassembly of the sandwich plate, exactly 150 μL the solution from the acceptor well and the donor wells were transferred to disposable UV-transparent plates (supplied by pION Inc., Woburn, MA, USA). UV absorption (for the donor plate, acceptor plate and the reference plate containing 100 μM test compound) was measured with a SPECTRAMax 190 microplate spectrometer (Molecular Device Corporation, Sunnyvale, CA, USA) at absorption wavelengths spanning 190–500 nm. This was done to localize the λmax for the individual compounds being monitored. After the UV absorption was measured using the plate reader, the samples from the donor, acceptor and reference wells were split into two sets and used for analysis by HPLC-UV and nanoelectrospray.

2.5. HPLC-UV analysis

PAMPA samples were analyzed using a modified version of a generic HLPC method used for the analysis of Caco-2 samples [24]. The HPLC system consisted of the 2690 Waters separation module and a Waters 996 photodiode array detector (Waters, Milliford, MA). The column used was YMC ODA-AQ 4.6 mm × 150 mm, 3 μm particle, maintained at 25 °C and a flow rate of 1 ml/min. The mobile phases used were: (A) 95% water, 5% acetonitrile, 0.115% tri-fluoroacetic acid; and (B) 5% water, 95% acetonitrile, 0.115% tri-fluroacetic acid. The initial mobile phase composition was 100% A. After injection the composition was

Table 1
Marketed compounds studied

Compound	Human absorption (%)	MW	clog P[a]	PSA	Passive (P), active (A), efflux (E), paracellular (R)	Caco-2[b] Pc (nm/s)
Sulfasalazine	12	398.4	3.2	133.05	P, E	16.0
Norfloxacin	35	319.3	1.5	73.81	P	45.0
Ranitidine	55	314.4	1.3	79.72	P, R	35.0
Hydrochlorothiazide	76	297.7	−0.1	131.19	P	16.0
Ketoprofen	76	254.3	2.8	54.92	P	n/a
Carbamazepine	90	236.3	2.7	44.27	P	128.0
Propranolol	90	259.4	3.1	43.69	P	133.0
Metoprolol	95	267.4	1.8	55.17	P	120.0
Verapamil	95	454.6	5.0	63.51	P, E	148.0
Antipyrine	97	188.2	0.3	24.30	P, A	n/a

MW, molecular weight; PSA, polar surface area; n/a, not available.

[a] From ACD labs.

[b] The Caco-2 permeability values were obtained from the internal Bristol-Myers Squibb research lab studies. The studies were performed using ATCC acquired Caco-2 cells cultured on 24-well transwell plates for ∼21 days. Permeability studies were performed using HBSS buffer at 37 °C, with apical pH of 6.5 and basolateral pH of 7.4 and incubation time of 2 h.

changed to 95% A and 5% B over 1 min, followed by the change to 5% A and 95% B over the next 6 min. The system was maintained at this composition for an additional 7 min, followed by reverting back to 100% A and equilibrating for 5 min. The total analysis time was ∼13 min. Standard curves were generated by injecting concentrations 0.1–50 μM.

2.6. Nanoelectrospray analysis

The NanoMate is an automated nanoelectrospray system that is compatible with a variety of mass spectrometers. The combination autosampler and ionization source is shown in an earlier publication [20]. The microchips are manufactured from silicon wafers using deep reactive ion etching processes [25]. The chip consists of nozzles or spray emitters etched into the planar surface. Each nozzle has an inner diameter of 7 μm, an outer diameter of 28 μm and a height of 60 μm. On the planar surface, opposite to that of the nozzles, are inlets. A sample delivery pressure (0.1–0.3 psi) is applied to deliver the sample to the end of the tip, into the inlet, and through the through-chip channel. A high voltage is also applied to generate the nanoelectrospray plume. Each sample is analyzed with a dedicated pipette tip and a dedicated nozzle, so there is no possibility of cross-contamination between samples.

For each of the 10 model compounds studied, a four-point standard curve was prepared. The donor standards were prepared in donor buffer at concentrations of 25, 50, 75, and 100 ng/mL. The receiver standards were prepared in receiver buffer at concentrations of 0.5, 5, 20, and 50 ng/mL. The standard curves were analyzed in parallel with the corresponding PAMPA samples, prepared in triplicate, for a total of seven donor samples and seven receiver samples per compound. The reference samples and the cassette screening samples were also analyzed in triplicate.

Ten microlitres of PAMPA sample was added to 40 μL of cold acetonitrile with 0.1% formic acid. The samples were first vortexed and then centrifuged at 13,000 rpm for 20 min. Twenty microlitres was transferred from each sample into a 96-well plate. The samples were then analyzed using a NanoMate (Advion BioSciences, Ithaca, NY). Two microlitres of sample was aspirated from the 96-well plate and delivered to the ESI Chip. For donor PAMPA samples, the delivery pressure was 0.3 psi and the spray voltage was 1.4 kV. For receiver samples the delivery pressure was 0.3 psi and the spray voltage was 1.6 kV. The total analysis time was 49 s per sample, making it amenable to high-throughput permeability studies.

All compounds were analyzed in positive ionization mode, except for ketoprofen and hydrochlorothiazide, which were analyzed in negative mode. The collision energies ranged from 25 to 40 eV for positive ionization and −11 to −27 eV for negative ionization. A selected reaction monitoring experiment was performed on the samples with a dwell time of 200 ms and the following transitions were monitored for each: carbamazepine, *m*/*z* 237.1–194.0; verapamil, *m*/*z* 455.3–303.1; metoprolol, *m*/*z* 268.1–191.0; antipyrine, *m*/*z* 189.0–104.0; ranitidine, *m*/*z* 315.1–175.9; hydrochlorothiazide, *m*/*z* 295.8–268.7; norfloxacin, *m*/*z* 320.1–276.0; sulfasalazine, *m*/*z* 399.0–222.9; ketoprofen, *m*/*z* 252.9–208.8; propranolol, *m*/*z* 260.1–183.0.

3. Results and discussion

For the nanoelectrospray analysis, a four-point standard curve was prepared for each of the 10 compounds to verify that quantitative responses could be obtained from the dilution preparation. For each compound, the donor and receiver buffers were spiked with various concentrations of analyte. Representative ion current profiles are shown for propranolol in Fig. 1A. The top, middle and bottom rows in Fig. 1A show ion current profiles for the propranolol reference (0 h) sample, receiver (4 h) sample, and donor (4 h) sample, respectively. The area underneath these profiles was used to calculate permeabilities for the samples and to construct calibration curves for the standards. The donor curve consisted of four standards ranging from 25 to 100 ng/ml. A representative calibration curve obtained for the donor ranitidine samples is shown in Fig. 1B. The receiver curves consisted of four standards ranging from 0.5 to 50 ng/ml. The calibration curve obtained for the receiver ranitidine samples is also shown in Fig. 1B. The curves obtained for the remaining nine compounds were similar. The percent accuracy for all the standards analyzed for both donor and receiver samples for all 10 compounds ranged from 81.1 to 119%. If one of the four standards was a gross outlier, it was eliminated. These preliminary data show that the nanoelectrospray approach provides linear, quantitative responses. Day to day repeatability and reproducibility has not been discussed in this paper but was demonstrated earlier by the authors [20].

The comparison of permeability values for the 10 marketed drugs using the three analytical techniques (UV-plate reader, HPLC-UV and nanoelectrospray) are shown in Table 2. The compounds were selected so as to present a spectrum of human intestinal absorption values (sulfasalazine and norfloxaxin with low absorption; ranitidine, hydrochlorothiazide and ketoprofen with medium absorption; and the remaining compounds with high absorption). From the table it is evident that the three analytical techniques demonstrated similar trends in permeability values for the compounds from these absorption sets (low, medium and high). As expected, sulfasalazine and norfloxacin had very low permeability values (<20 nm/s) via all analytical techniques. Amongst the high absorption category, all compounds except antipyrine had very high PAMPA permeability values (>100 nm/s) by all three techniques. The reason for the under-prediction of antipyrine permeability might be due to the tentative role of transporters as well as the interplay between membrane composition and fluidity, leading to its increased in vivo absorption [26]. Since PAMPA lacks any active transporters and accounts just for passive permeability, antipyrine gets underpredicted. Similarly, the compounds with medium human absorption values (ranitidine and hydrochlorothiazide) were

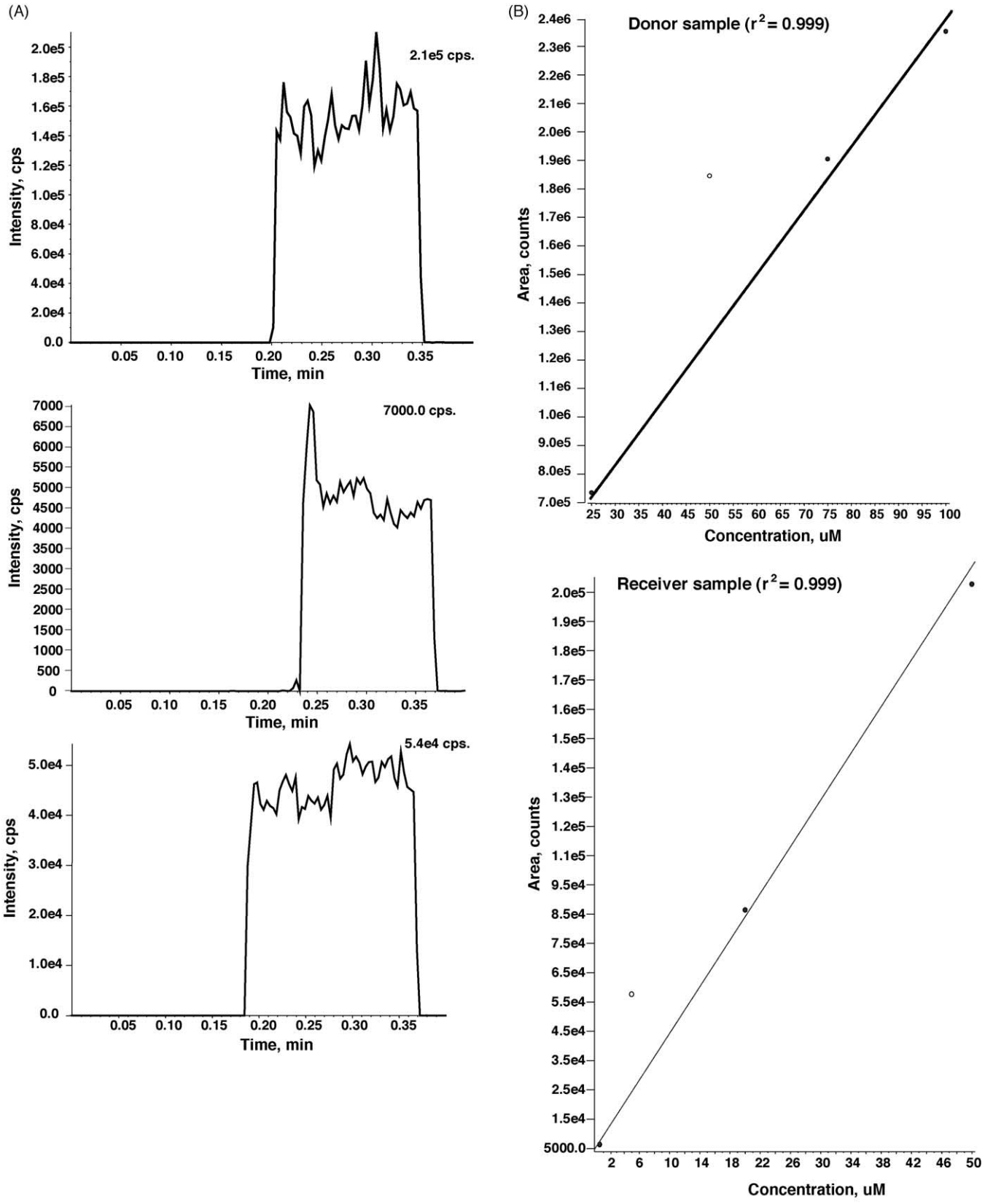

Fig. 1. (A) This figure shows the representative ion current profiles for propranolol. The top row shows ion current profile for the propranolol reference (0 h) sample. The middle row shows ion current profile for the propranolol receiver (4 h) sample. The bottom row shows ion current profile for the propranolol donor (4 h) sample. (B) Shows the calibration curves for both ranitidine donor and ranitidine receiver samples. Both curves use a linear regression and $1/x^2$ weighting. The open dot represents an outlier that was excluded from the generation of the calibration curve.

Table 2
Permeability values (mean ± S.D.) for the test compounds using the three analytical methods

Compound	Human absorption (%)	Permeability by UV-plate reader	Permeability by HPLC-UV	Permeability by nanoelectrospray
Sulfasalazine	12	No detection	0.9 ± 0.8	2.0 ± 0.5
Norfloxacin	35	2.0 ± 1.0	13.0 ± 8.0	7.5 ± 1.6
Ranitidine	55	3.0 ± 1.0	19.0 ± 5.0	2.0 ± 0.5
Hydrochlorothiazide	76	2.0 ± 0.5	7.0 ± 8.0	2.0 ± 0.5
Ketoprofen	76	13.1 ± 6.8	21.0 ± 0.5	15.1 ± 1.8
Carbamazepine	90	283.5 ± 9.8	166.0 ± 8.0	114.4 ± 13
Propranolol	90	588.6 ± 172	190.0 ± 18	249.1 ± 40
Metoprolol	95	388.8 ± 27	266.0 ± 18	213.3 ± 38
Verapamil	95	371.6 ± 137	211.0 ± 60	244.0 ± 32
Antipyrine	97	2.0 ± 1.8	2.0 ± 0.5	14.3 ± 6.4

consistently under-predicted, which is not uncommon in any in vitro permeability assay. Both these compounds have been reported to interact with transporter proteins [27–30]. However, as shown in Fig. 2A and B, there was a very good correlation ($r^2 > 0.9$) for the permeability values obtained by nanoelectrospray and those obtained by UV-plate reader and HPLC. This indicates a very good agreement between the novel nanoelectrospray technique and the conventional analytical techniques, such as UV-plate reader and HPLC, when dealing with samples from PAMPA experiments.

Though similar permeability values were obtained independently by these unique analytical techniques, UV-plate reader obviously allows for much more rapid sample analysis, as compared to the HPLC or nanoelectrospray approaches. However, as shown in the Table 2, the UV-plate reader was not able to derive the permeability value for sulfasalazine. Very low amounts of sulfasalazine transport across the lipid bilayer and make it into the acceptor well, making it undetectable using the UV-plate reader. Both HPLC and nanoelectrospray, being more sensitive techniques, detect minuscule amounts of sulfasalazine in the acceptor well, thus associating a very low permeability value to it. With regards to the remaining compounds, permeability values were consistent across the analytical techniques. For the four high permeability compounds (carbamazepine, propranolol, metoprolol and verapamil), even though the permeability values were greater than 100 nm/s by all three methods, the variability was significantly higher by the UV-plate reader method. In case of UV-plate reader, the permeability values for high absorption compounds was 284–589 nm/s (wide dynamic range). For the nanoelectrospray technique, the permeability values for high absorption compounds was 114–249 nm/s (narrower dynamic range) and had much lower variability between the wells. Mass balance recovery calculations demonstrated that greater than 85% of the mass was recovered for all the model test compounds after the PAMPA study.

The recently developed PAMPA permeability model, in addition to the Caco-2 model, is gaining acceptance as a tool for predicting the human absorption of test compounds [5,6,21,22,31]. To test the predictability of the PAMPA, the permeability values (using the nanoelectrospray technique) obtained for the model set of compounds was correlated with their human absorption values. Fig. 3 demonstrates that even

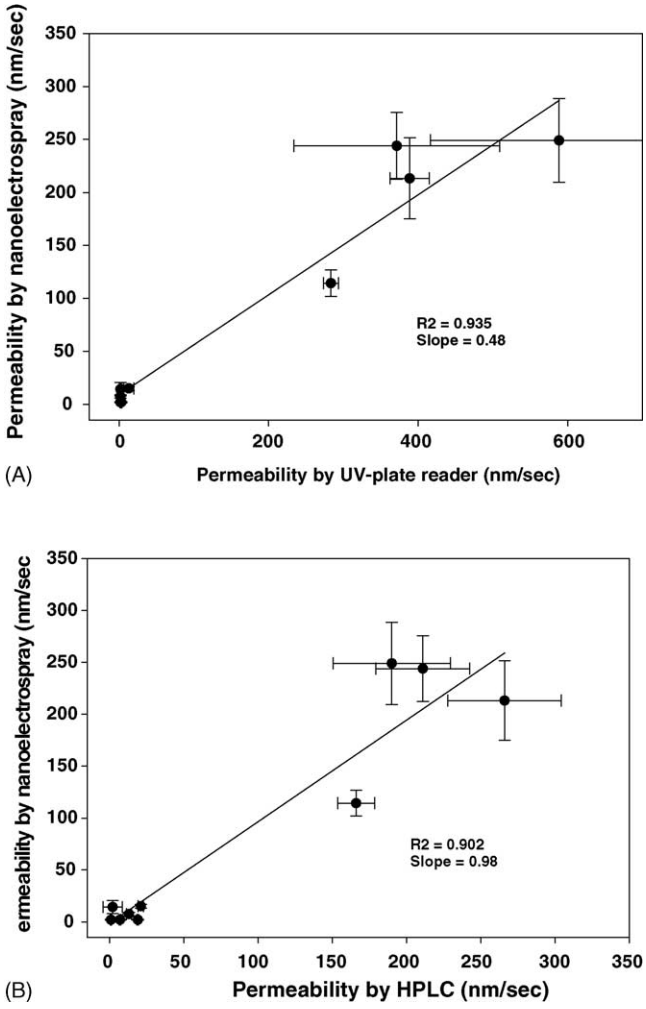

Fig. 2. (A) Correlation of PAMPA permeability values obtained for the 10 marketed compounds using the nanoelectrospray vs. the UV-plate reader technique. Permeability studies were conducted at 100 μM concentration at 25 °C for 4 h. Each data point represents mean ± S.D. of three repeats. (B) Correlation of PAMPA permeability values obtained for the 10 marketed compounds using the nanoelectrospray vs. the HPLC technique. Permeability studies were conducted at 100 μM concentration at 25 °C for 4 h. Each data point represents mean ± S.D. of three repeats.

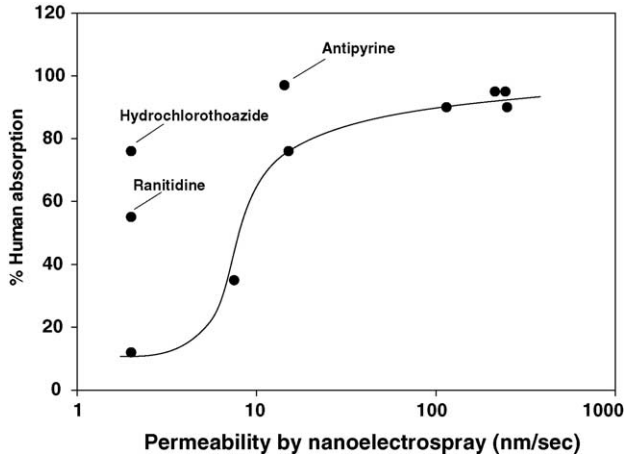

Fig. 3. Predictability of human absorption values from the PAMPA permeability values obtained using the nanoelectrospray analytical technique. Permeability studies were conducted at 100 μM concentration at 25 °C for 4 h. Each data point represents mean permeability values of three repeats.

for the limited set of compounds, there was good predictability of human absorption from the PAMPA permeability values. For the high and low absorption compounds the predictability was sturdier compared to the medium absorption compounds, where the steepness of the slope makes the estimation of the human absorption more difficult. Since, Caco-2 is the gold standard for cell-based permeability models for prediction of human absorption values, the PAMPA permeability values (obtained from the nanoelectrospray technique) were plotted against the historical Caco-2 data from our lab. Fig. 4 demonstrates that there was very strong agreement between the permeability values obtained by the two distinct in vitro models. Correlation (r^2) value of 0.88 was observed between the two methodologies.

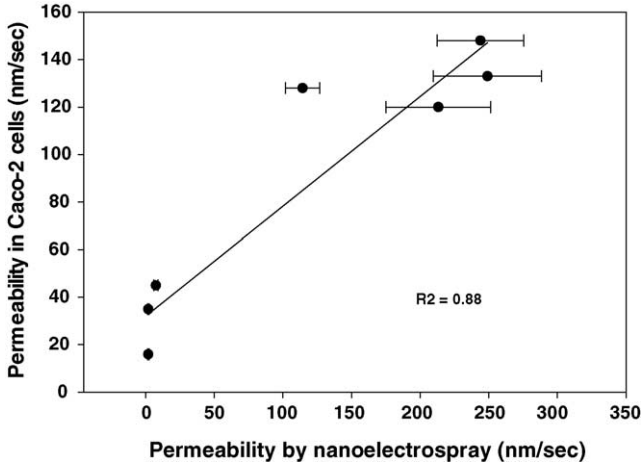

Fig. 4. Correlation of historical Caco-2 data for the 10 marketed compounds with the PAMPA data obtained using the nanoelectrospray analytical technique. Caco-2 studies were conducted at 200 μM concentration at 37 °C for 2 h, whereas PAMPA permeability studies were conducted at 100 μM concentration at 25 °C for 4 h. Each data point represents mean ± S.D. of three repeats (only mean for Caco-2 cells).

To accelerate drug discovery pharmaceutical profiling, another recent development has been the use of "cassette" mode studies [32–34]. This involves the co-incubation of multiple compounds in a study, either at the experimental end or the analytical end, so as to increase the throughput of an assay. PAMPA model, by it very nature (a pure passive transcellular permeability model devoid of any transporter proteins or paracellular pores), presents an ideal system for cassette mode permeability studies. Cassette mode studies were performed using a three-in-one strategy (i.e. three compounds per well). Mixture 1 included a mixture of sulfasalazine, antipyrine and metoprolol. Similarly, mixture 2 contained ranitidine, ketoprofen and verapamil. When present as drug mixtures, the individual starting donor concentration of each compound was 100 μM (similar to the concentration used in the single compound study). As is evident from Table 3, when mixtures of compounds were studied together, UV-plate reader was not able to distinguish unique peaks for each compound in either the reference plate (where the three compounds were present at 100 μM) or the acceptor plate, thus leading to an error in the results. The UV-plate reader analytical method depends on the identification of a "unique" compound peak in each sample for the calculation of permeability values. Therefore, any time more than one compound is co-incubated the technique would generate an error and be unable to provide permeability values. HPLC-UV method, though a more sensitive method, also suffers from the same drawback. Identification of a unique peak for each compound in any given sample is critical for the permeability calculations. Using the generic HPLC method used for discovery profiling within the organization, we could successfully generate unique (and non co-eluting) peaks for a few of the compounds used in cassette mode studies. Among the compounds included in mixture 1, metoprolol and antipyrine had co-eluting peaks (retention times 3.0 and 2.9 min, respectively) with sulfasalazine having a unique peak at 4.2 min. Similarly, among the compounds included in mixture 2, ketoprofen and verapamil had co-eluting peaks (retention times 4.0 and 3.9 min, respectively) with ranitidine having a unique peak at 1.9 min. Ketoprofen and verapamil had some peak overlap, but with careful software manipulation, some handle on peak areas could be achieved for these two compounds. As shown in the Table 3, out of the three compounds in mixture 1, only sulfasalazine permeability values could be accurately estimated using HPLC. As in the single study, sulfasalazine was demonstrated to have low permeability (<20 nm/s) when studied in cassette mode. Due to the lack of unique peaks for metoprolol and antipyrine, their permeability values could not be estimated. Similarly, in mixture 2, ranitidine was determined to have a low permeability in cassette mode (consistent with its permeability in single mode). Verapamil, despite of a potentially overlapping peak with ketoprofen, could be identified as a high permeability (>100 nm/s) compound. The permeability value for ketoprofen could not be reliably estimated in the cassette mode. Thus, out of the six compounds tested in cassette mode, only three could be accurately

Table 3
Comparison of permeability values (mean ± S.D.) obtained from single vs. cassette mode studies

Compound	Single mode	Cassette mode		
	Permeability by nanoelectrospray	Permeability by UV-plate reader	Permeability by HPLC-UV	Permeability by nanoelectrospray
Sulfasalazine	2.0 ± 0.5	Error	1.5 ± 0.4	2.0 ± 0.5
Antipyrine	14.3 ± 6.4	Error	Error	2.0 ± 0.5
Metoprolol	213.3 ± 38	Error	Error	360.2 ± 22
Ranitidine	2.0 ± 0.5	Error	9.2 ± 1.1	4.1 ± 0.8
Ketoprofen	15.1 ± 1.8	Error	Error	n/a
Verapamil	244.0 ± 32	Error	308.1 ± 43	583.9 ± 151

n/a, not available due to sample requirement for negative ionization mode. Cassette mode samples were analyzed in positive ionization mode only.

predicted using HPLC-UV. It is entirely possible that with appropriate method development, HPLC might be able to isolate individual unique peaks for test compounds and thus be capable of handling drug mixtures. But in discovery stage applications, where one might have compound mixtures with similar physico-chemical properties, it is possible that liquid chromatographic separation of individual compounds might become difficult or even impossible, making the analytical method inappropriate for handling cassette studies.

The nanoelectrospray mass spectrometry method offers several benefits over HPLC-UV and UV-plate reader. Most importantly, mass spectrometry offers a means to measure analytes based on their mass and thus has the potential to discriminate all analytes that are not isobaric. This is a significant advantage over the HPLC or UV-plate reader techniques, since it allows for identification and quantitation of the parent compound independent of its metabolites or other impurities. In Caco-2 and PAMPA permeability assays, often times the instability of the test compound could lead to multiple peaks that would confound the analysis if it is not mass-based. This mass-based analysis is also, particularly, useful for PAMPA screening studies, as it provides the opportunity of cassette screening. Here we have demonstrated that the permeability of three compounds could be determined from a single sample. This would not be possible with either the HPLC-UV or UV-plate reader approach.

Most often mass spectrometry is used in combination with HPLC. HPLC/MS is the industry standard for analyzing complex mixtures. However, PAMPA samples are present in a relatively simple matrix that does not necessitate HPLC separation prior to the MS analysis. A simple infusion of samples into the mass spectrometer is all that is needed analytically; however, it is highly desirable that the infusions be performed in an automated manner. Moreover the use of nanoelectrospray ionization versus electrospray is desirable for this PAMPA analysis due to improved ionization efficiency and less matrix suppression, which are both advantages inherent to the technique of nanoelectrospray [35]. Reduced matrix suppression is, particularly, desirable as dilution and centrifugation were the only sample preparation procedures performed. Therefore, chip-based automated nanoelectrospray mass spectrometry was the analytical technique selected. The Advion NanoMate provided automated nanoelectrospray infusion and it was coupled to a Sciex API 3000 mass spectrometer, which provided the additional benefits of selectivity. Nanoelectropsray and LC/MS/MS technique both have their advantages and disadvantages. The primary disadvantage of the LC/MS/MS approach is the time-consuming method development involved. It would be impossible to develop a single, generic LC method capable of analyzing all compounds. Specific columns, solvent systems and gradients would need to be identified for compound classes.

As with all analytical techniques, this nanoelectrospray approach also has some disadvantages. Drawbacks to the nanoelectrospray method include cost and the need for the sample to be present in a relatively simple matrix. This is an infusion approach and without the LC separation, complex matrices such as blood and plasma cannot be analyzed directly.

There was very good agreement between the permeability values obtained for the compounds by single mode versus cassette mode (nanoelectrospray technique), with sulfasalazine identified as a low permeability compound and metoprolol and verapamil identified as unequivocal high permeability compounds by both strategies. We conducted three-in-one studies, however, it is conceivable that the nanoelectrospray methodology might be capable of handling much higher number of compounds together in the PAMPA studies. Thus, the nanoelectrospray technique presents a highly sensitive but at the same time a very versatile analytical tool that can have a real impact in handling drug discovery samples. Successful integration of nanoelectrospray technique in the various screening models presents a potential to enhance the productivity and efficiency of drug discovery organizations.

4. Conclusions

This research work presents the novel nanoelectrospray technique as a viable analytical tool for analyzing the experimental samples obtained from PAMPA permeability studies. Successful drug discovery depends on rapid selection of potential compounds from the plethora of chemicals coming from combinatorial chemistry labs. Analytical methods that can analyze large number of compounds rapidly with superior sensitivity and accuracy play a pivotal role in selecting the right compounds for development. Nanoelectrospray has been demonstrated to have the key characteristics that

an analytical method needs to have at the drug discovery stage: efficient, reproducible, accurate and most importantly amenable to high-throughput. Successful integration of such an analytical tool can improve the chances of better drug discovery in the future.

Acknowledgements

pION Inc., is acknowledged for their support with the supplies needed for the PAMPA experiment. Karishma Patel (Bristol-Myers Squibb) is thanked for technical and experimental support.

References

[1] P.V. Balimane, S. Chong, R.A. Morrison, J. Pharmacol. Toxicol. Methods 44 (2000) 301–312.
[2] I. Hidalgo, Curr. Topics Med. Chem. 1 (2001) 385–401.
[3] E. Kerns, J. Pharm. Sci. 90 (2001) 1838–1858.
[4] C. Lipinski, F. Lombardo, B. Dominy, P. Feeney, Adv. Drug Deliv. Rev. 46 (2001) 3–26.
[5] A. Avdeef, Curr. Topics Med. Chem. 1 (2001) 277–351.
[6] S. Miret, L. Abrahamse, E. de Groene, J. Biomol. Screen. 9 (2004) 598–606.
[7] N. Parrot, T. Lave, Eur. J. Pharm. Sci. 17 (2002) 51–61.
[8] P. Artursson, Crit. Rev. Ther. Drug Carrier Syst. 8 (1991) 305–330.
[9] J. Tukker, in: J. Dressman, H. Lennernas (Eds.), Oral Drug Absorption: Prediction and Assessment, Marcel Drekker, NY, 2000.
[10] M. Wessel, P. Jurs, J. Tolan, S. Muskal, J. Chem. Inf. Comput. Sci. 38 (1998) 726–735.
[11] M. Kansy, F. Senner, K. Gubernator, J. Med. Chem. 41 (1998) 1007–1010.
[12] P.V. Balimane, K. Patel, A. Marino, S. Chong, Eur, J. Pharm. Biopharm. 58 (2004) 99–105.
[13] S. Chong, S. Dando, R. Morrison, Pharm. Res. 14 (1997) 1835–1837.
[14] J. Alsenz, E. Haenel, Pharm. Res. 20 (2003) 1961–1969.
[15] E. Fung, I. Chu, C. Li, T. Liu, A. Soares, R. Morrison, A. Nomier, Rapid Commun. Mass. Spectrom. 17 (2003) 2147–2152.
[16] M. Markowska, R. Oberle, S. Juzwin, C. Hsu, M. Gryszkiewicz, A. Streeter, J. Pharmacol. Toxicol. Methods 46 (2001) 51–55.
[17] M. Wilm, M. Mann, Anal. Chem. 68 (1996) 1–8.
[18] M. Wilm, A. Shevchenko, T. Houthaeve, et al., Nature 379 (1996) 466–469.
[19] S. Zhang, C.K. Van Pelt, Expert Rev. Proteomics 1 (2004) 449–468.
[20] C.K. Van Pelt, S. Zhang, E. Fung, I. Chu, T. Liu, C. Li, W.A. Korfmacher, J. Henion, Rapid. Comm. Mass Spectrom. 17 (2003) 1573–1578.
[21] H. Liu, C. Sabus, G.T. Carter, C. Du, A. Avdeef, M. Tischler, Pharm. Res. 20 (2003) 1820–1826.
[22] J.A. Ruell, K.L. Tsinman, A. Avdeef, Eur. J. Pharm. Sci. 20 (2003) 393–402.
[23] K. Sugano, Y. Nabuchi, M. Machida, Y. Aso, Int. J. Pharm. 257 (2003) 245–251.
[24] S. Chong, S. Dando, K. Soucek, R. Morrison, Pharm. Res. 13 (1996) 120–123.
[25] G. Schultz, T. Corso, S. Prosser, S. Zhang, Anal. Chem. 72 (2000) 4058–4063.
[26] U. Fagerholm, A. Lindahl, H. Lennernas, J. Pharm. Pharmacol. 49 (1997) 687–690.
[27] A. Collett, N.B. Higgs, E. Sims, M. Rowland, G. Warhurst, J. Pharmacol. Exp. Ther. 288 (1999) 171–178.
[28] K. Lee, K. Brower, D. Thakker, J. Pharmacol. Toxicol. Methods 303 (2002) 574–580.
[29] H. Hasannejad, M. Takeda, K. Taki, H.J. Shin, E. Babu, P. Jutabha, S. Khamdang, M. Aleboyeh, M.L. Onozato, A. Tojo, A. Enomoto, N. Anzai, S. Narikawa, X.L. Huang, T. Niwa, H. Endou, J. Pharmacol. Exp. Ther. 308 (2004) 1021–1029.
[30] K.J. Ullrich, G. Rumrich, S. Kloss, Kidney Int. 36 (1989) 78–88.
[31] K. Obata, K. Sugano, M. Machida, Y. Aso, Drug Dev. Ind. Pharm. 30 (2004) 181–185.
[32] L. Laitinen, H. Kangas, A. Kaukonen, K. Hakala, T. Kotiaho, R. Kostianen, J. Hirvonen, Pharm. Res. 20 (2003) 187–197.
[33] J. Palmgren, J. Monkkonen, E. Jukkola, S. Niva, S. Auriola, Eur. J. Pharm. Biopharm. 57 (2004) 319–328.
[34] C. Tannergren, P. Langguth, K. Hoffmann, Pharmazie 56 (2001) 337–342.
[35] R. Juraschek, T. Dulcks, M. Karas, J. Am. Soc. Mass Spectrom. 10 (1999) 300–308.