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Research paper

Drug adsorption to plastic containers and retention of drugs in cultured cells under *in vitro* conditions

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Abstract

Loss of drug content during cell culture transport experiment can lead to misinterpretations in permeability analysis. This study analyses drug adsorption to various plastic containers and drug retention in cultured cells under *in vitro* conditions. The loss of various drugs to polystyrene tubes and well plates was compared to polypropylene and glass tubes both in deionised water and buffer solution. In cellular uptake experiments, administered drugs were obtained from cultured cells by liquid extraction. Samples were collected at various time points and drug concentrations were measured by a new HPLC–MS/MS method. Acidic drugs (hydrochlorothiazide, naproxen, probenicid, and indomethacin) showed little if any sorption to all tested materials in either water or buffer. In the case of basic drugs, substantial loss to polystyrene tubes and well plates was observed. After 4.5 h, the relative amount remaining in aqueous test solution stored in polystyrene tubes was $64.7 \pm 6.8\%$, $38.4 \pm 9.1\%$, $31.9 \pm 6.7\%$, and $23.5 \pm 6.1\%$ for metoprolol, medetomidine, propranolol, and midazolam, respectively. Interestingly, there was no significant loss of drugs dissolved in buffer to any of the tested materials indicating that buffer reduced surficial interaction. The effect of drug concentration to sorption was also tested. Results indicated that the higher the concentration in the test solution the lower the proportional drug loss, suggesting that the polystyrene contained a limited amount of binding sites. Cellular uptake studies showed considerable retention of drugs in cultured cells. The amounts of absorbed drugs in cellular structures were 0.45%, 4.88%, 13.15%, 43.80%, 23.57% and 11.22% for atenolol, metoprolol, medetomidine, propranolol, midazolam, and diazepam, respectively. Overall, these findings will benefit development and validation of further *in vitro* drug permeation experiments.

Keywords: Drug loss; Plastic instruments; Cultured cells; HPLC–MS/MS; Drug analysis

1. Introduction

Cell culture models like Caco-2 cells are commonly used to predict intestinal absorption properties of various drugs [1–3]. For transport experiments, cells are typically cultured in flasks and seeded on plastic membrane filters, where they form a monolayer. Each insert is placed in a well of a polystyrene plate in the presence of buffer solution. Test compounds are generally added to the apical side of the cell monolayer and after some incubation time samples from the basolateral side are collected for permeability analysis. The loss of drug content during experiments, however, can lead to a false assessment of permeability. Drug loss may arise from interactions with plastic surfaces or from absorption and retention within cultured cells [4,5]. Drug loss due to metabolism in Caco-2 cells is limited or insignificant, due to low expression levels of metabolizing enzymes of the cytochrome P450 class [6].

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In transport experiments, both initial sample and end-stage sample from the donor compartment are measured. Mass balance is evaluated based on the sum of the amount transported and amount remaining in the donor compartment against initial amount of donor. Reduced mass balance is generally observed in transport experiments. For example, 80–90% recoveries were reported for nine heterogeneous drugs that encompassed both acids and bases and 71% recovery was measured for procaine [4]. Recently, it has been reported that pH and concentration had an effect on the recovery of the acidic drug indomethacin [5]. Results showed that mass balance values decreased with decreasing pH and concentration. At the lowest pH and concentration recovery for indomethacin was only 50%. There was no significant adsorption of indomethacin to the plastic wells. Therefore, it was suggested that indomethacin had accumulated in the cell monolayer, but it was not directly shown.

Most cell culture instruments are made from polystyrene plastic, a long carbon chain polymer in which every other carbon is covalently bound to a phenyl group. Polystyrene is an amorphous, clear, and breakable all-plastic, which is used for many applications. The surface of untreated polystyrene is very hydrophobic and disallows the attachment of most cells. Thus, a variety of chemical (sulfuric acid) and physical (gas plasma, corona discharge, or irradiation) administration methods have been utilised to modify polystyrene plastic surfaces [7–11]. These methods modify the surface through addition of different chemical groups (hydroxyl, ketone, aldehyde, carboxyl, or amine) onto the polymer so that the surface becomes hydrophilic and/or charged [7–9]. Modified polystyrene (TC) allows for more efficient cell attachment and thus growth.

Sorption of drugs to plastic infusion bags composed of polyvinylchloride (PVC) and to plastic intravenous tubing is well documented, since drug loss in this manner might cause treatment failure. Generally, the sorption of samples has been analysed by UV spectrophotometry [12,13] or by UV-HPLC methods [14–18]. However, UV-based methods can have limitations with sensitivity and selectivity. During the last decade, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has shown its usefulness in diverse analytical fields. LC–MS/MS analysis is suitable for detecting small amounts of compounds in a heterogeneous mixture, and is fast, accurate, and reliable [2,3,19]. Due to the high selectivity and sensitivity of MS/MS detection, it is a very promising analytical method also for the study of drug sorption.

The aim of this study was to evaluate drug loss during *in vitro* cell permeability experiments either through adsorption to plastic cell culture material or retention in cultured cells. The LC–MS/MS-based assay system was developed for this purpose and a comprehensive set of test drugs with diverse physicochemical properties were selected. Many of the studied drugs are listed in the FDA Guidance for Industry as model drugs for permeability studies [20] and some drugs (e.g., diazepam, midazolam, and medetomidine) are known to interact with PVC and polystyrene plastic [15,18,21]. In the experiments, glass and polypropylene (PP) tubes were compared to TC well plates and TC tubes. To our knowledge, this is the first report which details both speculated elements of drug loss, that is (1) drug adsorption to the plastic instruments and (2) retention of drugs in cultured cells. Interaction between the heterogeneous drugs and negatively charged polystyrene is also illustrated. The results described here will be important in development and validation of *in vitro* drug permeation experiments.

2. Experimental

2.1. Chemicals and materials

The compounds atenolol, propranolol, metoprolol, antipyrine, diazepam, midazolam, naproxen, probenicid, ibuprofen, hydrochlorothiazide and indomethacin were obtained from Sigma (St. Louis, MO). Medetomidine was from Domitor® (Orion, Finland). Buffer solution components were purchased from Bio Whittaker (Belgium) and water was purified and deionised by a Milli-Q system (Millipore). Acetonitrile and methanol (HPLC S grade) were obtained from Rathburn (Walkerburn, UK). Ammonium acetate and formic acid were from Riedel-de Haen (Seelze, Germany). All compounds and reagents were of the highest quality. Borosilicate glass tubes (16 × 100 mm, Pyrex®), modified polystyrene culture tubes (16 × 125 mm), and well plates (12 well, Costar and Transwell®) were purchased from Corning Incorporated (NY) and polypropylene test tubes (10 mL) were from Sarstedt (Australia).

2.2. Drug recovery assay

2.2.1. Surficial binding of drugs to plastic and glass

All drugs were solubilised in both Hanks' balanced salt solution (HBSS) containing 25 mM of *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES, pH 7.4) and in Milli-Q water. Final concentrations of each compound are presented in Tables 3 and 4. Test solutions contained the mixture of all six basic (pH 7.05) or all four acidic (pH 6.65) drugs in water or correspondingly in buffer (pH 7.4). Recovery experiments were performed using methods and conditions from traditional *in vitro* permeability studies. Test solutions (1.5 mL) were added to TC well plates, TC culture tubes, glass, and polypropylene tubes. Tubes and well plates were placed in an orbital horizontal shaker (Heidolph Inkubator 1000, Titramax 1000, Germany) with constant stirring (300 rpm) at either 37 or 3 °C. Initial samples (200 μL) were collected from each test solution. Sample aliquots (200 μL) from well plates were collected at 15, 30, 60, 120, 180, and 270 min, and sample aliquots from test tubes were collected at 120 and 270 min. Equal amounts of internal standard (I.S.) (antipyrine and ibuprofen to basic and acidic mixtures,

respectively) were added to each sample to a final concentration of 90 nM. All recovery experiments were conducted in triplicate and samples were analysed during the experiment and at least within 2 h. The extent of binding to polycarbonate membranes was also tested. Membranes from well plates (insert membrane: 0.4 μm pore size, 12 mm diameter) were cut out and placed to glass tubes. Test solutions (1.5 mL) in buffer or in water were added to glass tubes, and samples were collected and prepared as stated above in the case of test tubes.

2.2.2. Extraction of drugs from TC culture tubes

After initial surface binding experiments, the remaining solution was discarded and TC-tubes were flushed twice with Milli-Q water with 2 min of shaking. Afterwards, methanol (1.5 mL) was added to TC-tubes, which were vigorously mixed for 5 min by vortex. Samples (200 μL) were then collected for the recovery determination. Extraction was also performed through addition of crystalline NaCl to the physiological concentration of 0.9%. After NaCl addition both TC-tubes and glass tubes were mixed for 15 min by vortex. Samples were collected for quantification both before and after addition of salt.

2.2.3. Cell culture and cellular uptake

Caco-2 cells, a human colon adenocarcinoma cell line, were purchased from ATCC (Manassas, VA). Cells were grown on filters as described previously [22]. Buffered solutions containing basic drugs at 23.75 μM, except for medetomidine at 3.125 μM, were administered to cells for 2 h at 37 °C in a temperature-controlled orbital shaker. Cells were washed twice with PBS buffer and lysed by addition of 0.1% Triton X-100 solution. Finally, cells were carefully scraped off the membranes, suspended by pipetting, and removed to microcentrifuge tubes. Donor samples (apical side) were collected before and after the experiments. All samples were stored at −20 °C until prepared, extracted, and analysed. Sample preparation and extraction were performed as described previously [22] with slight modification. Internal standard (antipyrine) was added to each sample at a concentration similar to that reported previously. Matrix effect for all the basic drugs (internal standard included) in cultured cells were determined as described earlier [22].

2.2.4. Liquid chromatography

The HPLC system included a Finnigan Surveyor MS pump and a Finnigan Surveyor autosampler (serial 1.4, San Jose, CA) with a 30 μL injection volume. The tray temperature and column oven control were set to +15 °C. The chromatographic separation was performed using a Xterra MS C_{18} reversed-phase column (2.1 × 20 mm, 2.5 μm, Waters, Milford, MA) with a flow rate of 200 μL/min. Solution A was water containing either 0.2 mM ammonium acetate or 0.1% formic acid for acidic or basic drugs, respectively. Solution B was composed of 80% acetonitrile and 20% of the corresponding solution A. The gradient profile for all the drugs was 0–80% acetonitrile in 6 min, and the column was re-equilibrated with solution A for 4 min before the next injection.

2.2.5. Mass spectrometry

Measurements were performed with a LTQ quadrupole ion trap mass spectrometer equipped with an electrospray ionisation (ESI) source (Finnigan Surveyor LTQ, serial 1.0 SPI, San Jose, CA). The mass spectrometer was operated in the positive and negative ion modes for basic and acidic compounds, respectively. The quantification was based on multiple reaction monitoring (MRM) of the most intense fragment ions (m/z). In the MS/MS experiments, precursor molecular ions ($[M + H]^+$ or $[M - H]^-$) were selected and fragmented in the ion trap. Mass spectrometric parameters were optimized by constant addition of a single analyte in water to the HPLC flow via a T-connector. The conditions and parameters employed for acidic drugs were: capillary temperature 250 °C, spray voltage 4.2 kV, sheath gas flow rate 35 (arbitrary units), capillary voltage −18 V, tube lens −65 V, and for basic drugs were: capillary temperature 275 °C, spray voltage 4.5 kV, sheath gas flow rate 35 (arbitrary units), capillary voltage 26 V, tube lens 75 V. In the ion trap, the relative collision energy ranged from 40% to 60% for all the monitored drugs. The flow from the HPLC was diverted to waste for the first 1.5 min and after 6 min to decrease ion source contamination. Data were processed using the Xcalibur software package version 1.4 SRI.

2.2.6. Standard solutions, calibration, and accuracy

Individual stock solutions (10 mM) of compounds were prepared separately in methanol, except medetomidine, which was commercially available in aqueous solution. Stock solutions were further diluted to 1 mM in Milli-Q water. Working solutions (40–400 μM) were prepared by diluting the stock solutions (1 mM) in water or in buffer solution. Furthermore, the working solutions were combined and further diluted with water or buffer solution. This mixture of basic or acidic compounds was used both for test solutions used in Section 2.2.1, and for calibration and quality control (QC) standards after serial diluting. The test solutions, calibration solutions, and QC standards contained either six basic or four acidic compounds. The calibration range and QC standard values of each compound are shown in Tables 1 and 2. Similarly, I.S. working solutions (1 μM, antipyrine or ibuprofen) were prepared by diluting stock solutions with water or buffer solution. Equal amounts of I.S. were added to each standard and sample solution to 90 nM. All stock and working solutions were stored in the dark at −20 °C until used. Test solutions, calibration solutions, and QC standards were prepared daily and analysed immediately after preparation.

Calibration curves were constructed by plotting chromatographic peak ratios of standard area/I.S. area versus concentration of the standard using linear regression. From these curves the coefficients of correlation (r^2) were calculated. The lowest limit of quantification (LLOQ) for each

Table 1
Calibration range, linearity (r^2), and LLOQ of the LC–ESI-MS/MS method for various compounds in buffer solution

Compounds in test solution	Calibration range (nM)	Linearity (r^2)[a]	LLOQ ($n = 3$)		
			nM	RSD%	Accuracy (%)
Bases					
Atenolol	2.5–50.0	0.9939	2.5	9.03	100.33
Metoprolol	2.5–50.0	0.9962	2.5	8.72	96.11
Medetomidine	1.0–20.0	0.9966	1.0	5.28	102.62
Propranolol	5.0–100.0	0.9919	5.0	8.78	97.07
Midazolam	1.0–20.0	0.9899	1.0	10.50	109.62
Diazepam	2.5–50.0	0.9970	2.5	6.84	102.08
Acids					
Hydrochlorothiazide	20.0–320.0	0.9946	20.0	4.22	111.08
Naproxen	20.0–320.0	0.9960	20.0	8.25	106.07
Probenicid	2.5–80.0	0.9957	2.5	5.80	99.44
Indomethacin	10.0–80.0	0.9944	10.0	2.19	106.68

[a] Correlation coefficient from six calibration points ($n = 3$).

compound was calculated based on the FDA Guidance for Industry, Bioanalytical Method Validation [23]. Briefly, the analyte response at LLOQ should be five times level of the baseline noise, and the analyte response at LLOQ should be determined with precision of <20% and accuracy of 80–120%.

Table 2
Within-day and between-day precision and accuracy of the LC–ESI-MS method for the various compounds used in this study

	In Milli-Q water					In buffer solution				
	Within-day variation ($n = 6$)			Between-day variation (3 days, $n = 9$)		Within-day variation ($n = 6$)			Between-day variation (5 days, $n = 15$)	
	QC[b] (nM)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	QC[b] (nM)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Bases[a]										
Atenolol	12.5	3.64	96.44	5.90	95.66	12.5	4.27	97.17	3.11	102.30
	20.0	4.55	104.39	3.39	97.24	20.0	3.35	96.60	5.21	98.76
Metoprolol	12.5	3.19	107.42	7.17	97.62	12.5	8.35	99.54	2.46	100.57
	20.0	4.03	97.60	2.20	98.73	20.0	3.49	97.05	2.48	100.78
Medetomidine	5.0	4.89	102.91	6.45	95.27	5.0	7.67	92.40	4.33	99.56
	8.0	6.88	92.85	4.29	97.04	8.0	5.23	94.20	3.24	99.49
Propranolol	25.0	5.66	101.82	4.90	92.62	25.0	5.03	99.31	4.05	99.22
	40.0	9.05	92.55	4.63	98.36	40.0	5.89	99.92	4.62	101.73
Midazolam	5.0	7.09	101.43	5.07	92.98	5.0	5.43	105.22	7.98	97.05
	8.0	9.94	103.13	4.91	105.69	8.0	4.67	106.60	6.83	106.27
Diazepam	12.5	5.38	96.90	6.17	93.85	12.5	6.58	92.94	4.70	95.25
	20.0	7.45	103.27	2.94	104.49	20.0	3.96	101.53	4.11	103.81
	In Milli-Q water					In buffer solution				
	Within-day variation ($n = 6$)			Between-day variation (3 days, $n = 9$)		Within-day variation ($n = 6$)			Between-day variation (3 days, $n = 9$)	
	QC[b] (nM)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	QC[b] (nM)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Acids[a]										
Hydrochlorothiazide	64.0	8.91	109.26	6.43	96.11	64.0	3.20	94.60	2.67	98.21
	100.0	8.71	103.51	4.51	102.83	100.0	4.28	90.65	3.17	93.24
Naproxen	64.0	4.88	101.73	2.61	100.07	64.0	2.49	99.13	1.65	101.24
	100.0	6.79	107.19	3.16	100.95	100.0	3.02	94.97	2.36	95.38
Probenicid	16.0	8.21	99.77	3.69	98.38	16.0	2.55	91.36	3.32	92.10
	25.0	8.28	102.01	3.58	102.55	25.0	2.13	92.34	2.87	91.77
Indomethacin	16.0	4.43	102.02	5.36	105.58	16.0	2.12	106.12	2.43	103.48
	25.0	6.37	97.86	3.74	104.99	25.0	2.52	109.49	4.21	107.28

[a] Bases and acids are in chromatographical order.
[b] QC, Quality control sample (nominal concentration).

Within-day accuracy and precision of the assay were determined by repetitive measurements ($n = 6$) of QC standards at two different concentrations. Precision was calculated as the relative standard deviation (RSD%) and accuracy was determined as the mean% [(mean measured concentration)/(expected concentration) × 100]. Between-day accuracy and precision were evaluated by performing repeated measurements of the same QC standards on three or five different days and calculated in the same manner as the within-day values. Both accuracy and precision were also tested according to FDA guidance through the following criteria: the accuracy and precision deviation values should be within 15% of the actual values.

3. Results and discussion

3.1. Liquid chromatography

All compounds were separated within 6 min (Fig. 1). The Xterra column properties allowed for fast analysis as the total chromatographic runtime was only 10 min. In surface binding experiments, the short analysis time was desirable because sample aliquots were collected within 15 min intervals (at the beginning of study) and the total experiment time was only 4.5 h. The runtime potentially could be shorter, however, the wide ranging lipophilicity of the set of drugs used here required a total run time to 10 min for the desired degree of separation. Because hydrophilic compounds such as atenolol and hydrochlorothiazide initially eluted quite early, the column oven was set to +15 °C, which resulted in uniform peak shapes, longer retention times, and better separation from salts and impurities. Due to the high specificity and selectivity of MS/MS detection, no interfering peaks from other compounds were found in ion channels specific for a given m/z value. Furthermore, the elution profile was sufficient to elute all of the drugs in a mixture, since pure water samples did not present any traces of carry-over.

3.2. Mass spectrometry

ESI source coupled with MS was chosen for this study because ESI-MS-based methods are commonly used and suitable for low molecular weight pharmaceutical compounds. Detection of acidic drugs using ion trap instruments has typically been performed using full MS mode [24,25]. In this study, both acidic and basic drugs were monitored by MS/MS detection. The operating parameters for ESI-MS were manually optimized to maximize the detection sensitivity, and general settings were used for each compound. The ionisation in the positive ion mode for all the basic drugs was sufficient, since abundant $[M + H]^+$ ions were observed for each compound. However, tuning of negative ion source for detection of acidic drugs was laborious and it was necessary to obtain a high level of ionisation with intense $[M - H]^-$ ions. The protonated and deprotonated molecules were both selected as precursor ions and product ions were detected by the MS/MS mode. The most intense product ion for each compound was used for quantification. Representative precursor and product ions are listed in Fig. 1. Furthermore, three different buffer compositions were used in the mobile phase to optimize peak intensity and retention times of acidic compounds. We observed that 10 mM ammonium acetate suppressed the signal of acidic compounds as reported previously [24]. Therefore, ammonia solution at pH 8.2 was tested, which resulted in better sensitivity, but peak shapes were uneven. Weak ammonium acetate (0.2 mM) buffer was ultimately selected for the analysis because it gave both uniform peak shapes and similar sensitivity as the ammonia solution.

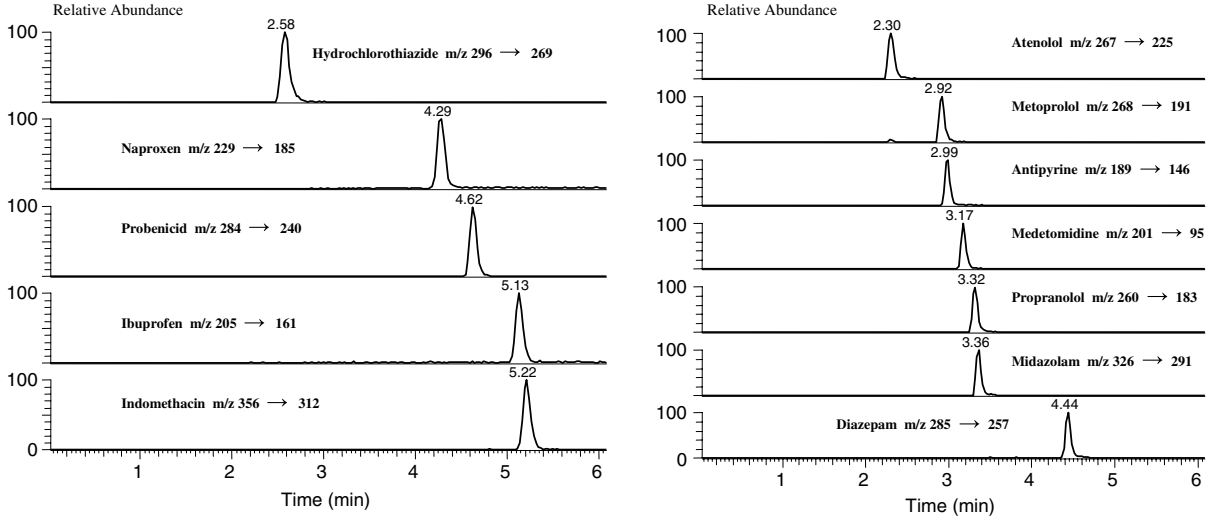

Fig. 1. Chromatography and tandem mass spectrometry of test compounds. Quality control mixtures of either acidic or basic drugs prepared in buffer solution were separated by reverse-phase LC over a 6-min gradient. Product ions of acidic compounds (left) or of basic compounds (right) were generated and measured by ESI/MS/MS.

3.3. Calibration and accuracy

Validation was performed for all four different conditions (acidic/basic drugs in water and buffer). The calibration curves of test drugs were linear over the used concentration ranges and correlation coefficients (r^2) were greater than 0.9899 (Table 1). Linearity was similar both in water and buffer solution, so only coefficients obtained from buffered samples are shown in Table 1. The equations for the curves were calculated using six calibration points with three replicate standards for each point ($n = 3$) per curve. To compensate for analyte losses during sample preparation and analysis, the sample assay was based on the internal standard method, which was calculated from the peak area ratios of unknown/I.S. versus the calibration curve. The LLOQ data in buffered solution are presented in Table 1. The accuracy range was 96–111% and the RSD precision was lower than 10.5% ($n = 3$) for all LLOQ values. Further, the signal-to-noise ratios obtained at the LLOQ were at least 20:1. The sensitivity of this assay is reflected by the LLOQ value of diazepam, which was 5000–50,000 times lower than concentrations measured by UV spectrophotometry [12,13].

Within-day precision was evaluated by performing six repetitive analyses of QC standards, which gave RSD values between 2.12% and 9.94%. The accuracy range was 92–110% (Table 2). The between-day accuracy of the methods ranged from 91% to 108% and the RSD precisions were lower than 8%. As seen in Table 2, RSD and accuracy values were similar both for samples in buffer and water. Overall, the tested parameters surpass the FDA criteria [23].

3.4. Adsorption experiment

In a typical *in vitro* permeability experiment, the donor drug concentration can range from 20 to 1000 μM [26,27]. Correspondingly, the final receiver sample can range from 1 to 50 μM. The first 60 min of the experiment is most often used for the calculation of coefficients and during that time the drug concentration can be observed at nM levels. In this study, both μM and nM concentration levels were studied. Each drug concentration was selected individually according to the detection intensity that was about 10–20 times the LLOQ value of each drug. Even though the concentrations in the test solutions were low, they were similar to those observed in traditional permeability studies. The accuracy and precision of the assay method was remarkable, which provided the ability for quantification of small losses of drugs. Therefore, the LC–MS/MS method development was validated extensively.

3.4.1. Loss of acidic drugs

For the most part, acidic drugs showed little if any sorption to all tested materials in either water or buffer. The lack of binding was seen both in the presence of glass and plastic materials. The only appreciable binding observed was with the lipophilic compound indomethacin in water to PP-tubes, where the concentration decreased to 69.5 ± 4.7% ($n = 3$) from that initially added (Table 3).

Table 3
Sorption of acidic drugs in water or buffer to different containers and materials after 270 min

Drug and material	% remaining ± SD ($n = 3$)	
Acids in test solution (nM)	Water +37 °C	Buffer +37 °C
Hydrochlorothiazide (100.0)		
Glass tube	103.7 ± 5.4	85.6 ± 3.7
Polypropylene tube	103.7 ± 10.2	87.4 ± 8.3
TC tube	93.7 ± 10.8	92.2 ± 3.2
TC well plate	87.6 ± 9.4	91.6 ± 5.0
Polycarbonate membrane	–	91.9 ± 5.8
Naproxen (100.0)		
Glass tube	95.3 ± 7.1	97.3 ± 2.7
Polypropylene tube	90.9 ± 10.8	100.7 ± 3.8
TC tube	94.6 ± 1.7	99.3 ± 2.0
TC well plate	92.2 ± 4.1	101.6 ± 3.7
Polycarbonate membrane	–	101.1 ± 7.0
Probenicid (25.0)		
Glass tube	90.8 ± 5.0	88.8 ± 1.5
Polypropylene tube	87.2 ± 8.0	92.9 ± 5.4
TC tube	88.6 ± 0.5	91.0 ± 2.2
TC well plate	91.0 ± 6.5	96.7 ± 3.6
Polycarbonate membrane	–	95.6 ± 5.7
Indomethacin (25.0)		
Glass tube	94.9 ± 8.3	92.1 ± 4.4
Polypropylene tube	69.5 ± 4.7	98.8 ± 1.3
TC tube	83.3 ± 2.6	102.4 ± 0.8
TC well plate	86.2 ± 5.1	101.4 ± 1.8
Polycarbonate membrane	–	101.6 ± 2.9

3.4.2. Loss of basic drugs in buffer (+37 °C)

Cell culture permeability screening is typically performed in buffered solutions (pH 7.4), so loss of drugs was studied in the presence of buffer. As summarized in Table 4 there was no significant loss of drugs dissolved in buffer to any of the tested materials indicating that buffer reduced surficial interaction. All drugs remained above 79.9% of their initial value, except propranolol, which had 72.7 ± 5.5% remaining in solution in TC-tubes.

3.4.3. Loss of basic drugs in water (+37 °C)

The loss of basic drugs to polystyrene well plates and TC-tubes in water was a rapid process. All the drug losses were achieved within the first 15 min (Fig. 2). After 4.5 h, the relative amount remaining in TC-tubes in aqueous solution was 64.7 ± 6.8%, 38.4 ± 9.1%, 31.9 ± 6.7%, and 23.5 ± 6.1% for metoprolol, medetomidine, propranolol, and midazolam, respectively (Table 4). Hydrophilic atenolol did not show loss in any of the different containers. As seen in Table 4, the loss of basic drugs to TC-plastic was much higher than to glass and PP-tubes. Midazolam had a strong affinity to TC-plastic in aqueous solution, but this affinity was greatly diminished in the presence of buffer. Fig. 3 summarizes the loss of midazolam in test solutions in different containers.

Table 4
Sorption of basic drugs in water or buffer to different containers and materials after 270 min

Drug and material	% remaining ± SD ($n = 3$)		
Bases in test solution (nM)	Water +37 °C	Buffer +37 °C	Water +3 °C
Atenolol (20.0)			
Glass tube	85.2 ± 1.3	99.0 ± 0.8	101.1 ± 3.1
Polypropylene tube	84.3 ± 3.0	97.0 ± 2.9	99.8 ± 3.8
TC tube	86.0 ± 3.6	94.6 ± 4.0	91.6 ± 2.8
TC well plate	98.0 ± 5.5	104.8 ± 7.7	96.1 ± 1.8*
Polycarbonate membrane	–	97.4 ± 3.2	96.1 ± 3.8
Metoprolol (20.0)			
Glass tube	88.1 ± 4.5	96.1 ± 7.4	99.7 ± 3.1
Polypropylene tube	88.9 ± 5.1	87.8 ± 8.2	104.1 ± 3.7
TC tube	64.7 ± 6.8	84.3 ± 2.8	79.2 ± 8.4
TC well plate	92.7 ± 8.9	98.1 ± 1.3*	91.0 ± 6.4
Polycarbonate membrane	–	95.1 ± 3.7	84.7 ± 4.9
Medetomidine (8.0)			
Glass tube	80.4 ± 1.3	92.7 ± 6.1	93.8 ± 4.2
Polypropylene tube	87.4 ± 8.5	84.8 ± 4.1	94.5 ± 0.7
TC tube	38.4 ± 9.1	79.9 ± 5.0	46.7 ± 4.9
TC well plate	54.2 ± 3.0	97.8 ± 2.8	38.3 ± 2.2
Polycarbonate membrane	–	87.6 ± 0.5	47.6 ± 0.8
Propranolol (40.0)			
Glass tube	78.9 ± 2.2	100.3 ± 6.8	107.4 ± 7.8
Polypropylene tube	82.9 ± 10.8	86.3 ± 1.8	103.5 ± 4.5
TC tube	31.9 ± 6.7	72.7 ± 5.5	43.1 ± 2.3
TC well plate	40.1 ± 4.9	91.0 ± 11.1*	28.9 ± 1.8
Polycarbonate membrane	–	88.8 ± 4.8	48.2 ± 1.7
Midazolam (8.0)			
Glass tube	92.1 ± 5.8	98.8 ± 3.2	91.4 ± 5.4
Polypropylene tube	80.5 ± 3.9	84.9 ± 4.8	87.6 ± 0.9
TC tube	23.5 ± 6.1	87.2 ± 6.8	35.8 ± 2.3
TC well plate	34.4 ± 5.9	91.6 ± 8.6*	18.5 ± 1.0
Polycarbonate membrane	–	83.4 ± 3.9	36.7 ± 5.0
Diazepam (20.0)			
Glass tube	94.4 ± 1.7	96.4 ± 4.4	99.2 ± 3.3
Polypropylene tube	77.1 ± 4.0	84.6 ± 1.8	90.0 ± 3.9
TC tube	82.7 ± 1.4	86.5 ± 5.2	92.4 ± 5.0
TC well plate	97.1 ± 5.8*	95.5 ± 6.6*	96.5 ± 1.9*
Polycarbonate membrane	–	89.4 ± 0.8	90.0 ± 1.1

* 180 min.

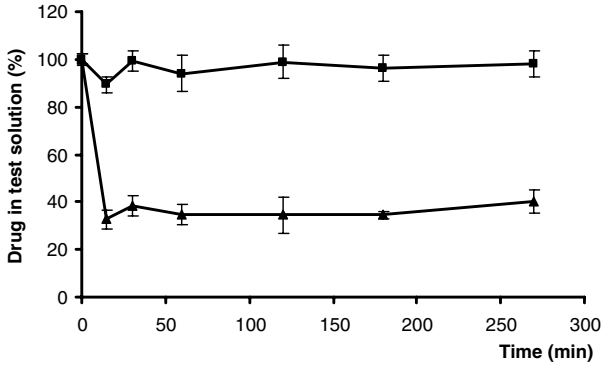

Fig. 2. Kinetics of basic drug sorption to polystyrene surfaces. Atenolol (■) and propranolol (▲) dissolved in aqueous test solution were added to wells from a TC cell culture plate to initiate the experiment. At various times, samples were collected and concentrations were determined with HPLC–ESI/MS/MS. Data points represent mean value ± SD ($n = 3$).

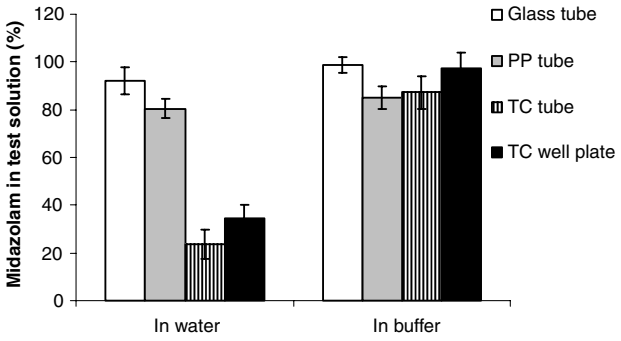

Fig. 3. Sorption of midazolam to various surfaces. Midazolam was prepared in a test solution of either water or buffer and was added to different containers for 270 min at +37 °C. The remaining concentration was determined by HPLC–ESI/MS/MS. Each value indicates mean ± SD ($n = 3$).

3.4.4. Loss of basic drugs in water (+3 °C)

Sorption experiments were repeated at +3 °C since this temperature is commonly used to study sorption kinetics to plastic [17] as well as transport mechanisms of drugs in cultured cells [5]. Generally, at +3 °C loss of basic drugs to TC-tubes was less than at +37 °C. On the contrary, loss of basic drugs increased in well plates at +3 °C compared to that at +37 °C (Table 4). This result may be due to a lower extent of evaporation at the colder temperature, since the concentration in wells increased over time at +37 °C. The effect of evaporation can be seen in Fig. 3, where the concentrations in well plates are higher than in TC-tubes. Sorption to polycarbonate membranes was also investigated. Membranes were cut out from inserts and placed to glass tubes for analysis. Results indicated that the loss of medetomidine, propranolol, and midazolam to polycarbonate membrane was similar to that observed in TC-tubes in water at +3 °C (Table 4).

3.4.5. The effect of drug concentration to sorption

The sorption of medetomidine, propranolol, and midazolam to TC-tubes was tested at a high concentration (1000 nM of each drug in water). At this concentration, these drugs remained above at 58% of the initial value and the losses were clearly lower than observed at 8–40 nM. When the drug concentration in the test solution was 2500 nM, no drug loss was detected at all. This suggests that the higher the concentration the lower the amount of drug loss, furthermore, the surface of tubes can interact with only a limited amount of drugs.

3.4.6. Extraction of drugs from culture tubes (TC-tubes)

To account for the drug loss observed in TC materials after initial sorption experiments, TC-tubes were flushed with water and treated with methanol to extract any bound drugs. Methanol addition recovered 24.0 ± 4.5%, 46.5 ± 9.2% and 72.5 ± 13.9% of initial amounts of medetomidine, propranolol, and midazolam, respectively. As seen in Table 5, methanol wash recovered almost all of the fraction lost for midazolam. Since buffer solution decreased the

Table 5
Methanol extraction of different compounds from TC tubes and the effect of salt on drug sorption to TC tubes

Drug	TC tubes ($n = 3$) (+37 °C water)		
	Remaining (%±SD) in test solution after 270 min	Drug found (%±SD) in test solution after NaCl addition	MeOH extraction recovery (%±SD)
Medetomidine, 8 nM	38.4 ± 9.1	98.8 ± 14.6	24.0 ± 4.5
Propranolol, 40 nM	31.9 ± 6.7	96.7 ± 4.6	46.5 ± 9.2
Midazolam, 8 nM	23.5 ± 6.1	58.8 ± 7.3	72.5 ± 13.9

losses of basic drugs to TC-plastic, we decided to test the effect of salt addition. After the 4.5 h experiment, NaCl was added to test solution for basic drugs in water. The amount of drugs recovered after salt addition was compared to the amount before salt addition. Addition of salt released both medetomidine and propranolol from TC surfaces as near 100% was recovered in each case. Salt addition removed some but not all of midazolam initially bound to TC surfaces as only 58.8 ± 7.3% was recovered after the salt addition (Table 5).

3.4.7. Mechanism of sorption

To ensure the stability of each drug in solution, initial test solutions were analysed before and after the experiments. Concentrations remained constant and no degradation products in either water or buffer were found indicating that observed drug loss was not due to degradation or poor solubility. It has been proposed that sorption of various drugs such as nitroglycerin, warfarin, and diazepam to PVC infusion bags could be described in terms of an absorption process, where adsorption played only a minor role in the overall loss [12]. Typically, the sorption profile of drugs acts as a function of time in the infusion bags. For example, loss of drugs is continuous throughout a 120 h storage time [12,13]. As stated in Section 3.4.2, the loss of basic drugs in the TC-plastic was rapid. All losses were seen within the first 15 min, which was about the time limit for the detection method used here. A similar profile has been measured for taxol in polystyrene tissue culture plates [14]. In this case, it was suggested that the loss was due to surface adsorption of containers.

Dahlström et al. [21] studied the adsorption of medetomidine to hydrophilic polystyrene plastic. This study characterized the mechanism of surface interaction and found that medetomidine had the ability to adsorb to hydrophilic polystyrene in deionised water. They concluded that the electrostatic binding was the most important mechanism for surface interaction although elements of hydrophobic interactions also contributed to the surface affinity of medetomidine. This is clear, because basic compounds are partially or fully positively charged at the acidic pH and, thus, ionic interaction between the compound and negatively charged polystyrene can occur. Our findings of basic drugs in water are in line with this idea. Lipophilic and positively charged drugs like medetomidine, propranolol, and midazolam in water solution at pH 7.05 had strong interaction with the negatively charged polystyrene. However, the positively charged atenolol did not interact with charged polystyrene. Atenolol and propranolol have similar pK_a values and molecular weights, so the different adsorption profiles may be explained by the lipophilicity of compounds. Since, the $\log D$ values are different (Table 6), the lipophilic propranolol should prefer to adsorb to plastic rather than remain in solution in water. The negative charge of acidic drugs should disallow interaction to negatively charged material surfaces. Indeed, acidic drugs in water solution at pH 6.65 did not show any significant adsorption to TC containers.

Table 6
Physicochemical properties of the drugs used in this study

Compound	pK_a[a]		$\log P$[b]	$\log D$[c] pH 7.4	M_w
	Acid	Base			
1. Atenolol		9.17	0.10	−1.66	266
2. Metoprolol		9.18	1.79	0.03	267
3. Medetomidine		6.75	3.17	3.02	200
4. Propranolol		9.15	3.10	1.36	259
5. Midazolam		6.20	3.70	3.69	326
6. Diazepam		3.40	2.80	3.86	285
7. Antipyrine (I.S.)		1.21	0.27	0.27	188
1. Hydrochlorothiazide	8.95, 9.49		−0.07	−0.07	297
2. Naproxen	4.10		3.18	0.03	230
3. Probenicid	3.40		3.21	−0.26	285
4. Indomethacin	4.50		4.27	−0.07	358
5. Ibuprofen (I.S.)	4.41		3.72	0.77	206

[a–c] Computational values calculated by ACDLABS ACD/pK_a/$\log D$ program (Version 4.56/26 April 2000).

When basic drugs were studied in buffer solution, there was no observed loss. It is probable that buffer ions compete for the plastic surface preventing interaction of the tested drugs. It should be noted that ion concentration of buffer was much higher than the drug concentration. The recovery of basic drugs in water after NaCl addition further supported an ion-pair reaction between adsorbed drugs and the TC surface. Furthermore, methanol extraction in most cases recovered the remainder of the bound fraction of drugs. All these results indicated that loss of basic drugs occurred by surface adsorption through ionic and lipophilic interactions.

3.5. Drug retention in cultured cells

The extraction procedure and analytical method was used to quantify basic drugs in Caco-2 cells. Liquid extrac-

tion using methanol/chloroform (1:1, v/v) was used for each basic drug. Extraction recovery and matrix effect were tested and performed by using spiked matrix samples, i.e., real spiked cell samples. The mean absolute recovery of drugs from the spiked cell samples ($n = 3$) ranged from 99.9% to 113.2%, showing that the extraction method was quite efficient. Matrix associated ion suppression of the response of the drugs was relatively minor with analytical method. Suppression values for the spiked cell samples ($n = 3$) for most cases ranged from −3.1% to +9.2%, except for atenolol which had a suppression value of 15%. No metabolites or degradation products of the drugs were detected in the extracted cell samples. Overall, the above-mentioned parameters demonstrate the usefulness of this extraction procedure.

In cellular uptake experiments, the solution of basic drugs was administered to a cell monolayer. After the appropriate incubation time, cells were washed with buffer and suspended prior to extraction. The drug amount in the sample was compared to the initial amount added. The amounts of absorbed drugs in cellular structures were 0.45%, 4.88%, 13.15%, 43.80%, 23.57% and 11.22% for atenolol, metoprolol, medetomidine, propranolol, midazolam, and diazepam, respectively. Laitinen et al. [4] reported a loss of 10–20% for midazolam and propranolol and procaine losses of 29% during the transport experiments. Additionally, indomethacin losses upwards of 50% have been previously reported [5]. Our results show that the absorption correlates well with lipophilicity of drugs, as lipophilic drugs may have an enhanced ability to absorb to cellular structures of cultured cells.

4. Conclusion

In conclusion, we have demonstrated that lipophilic and positively charged basic drugs adsorb to negatively charged surfaces of polystyrene plastic. When the buffer solution was used instead of aqueous solution, drug adsorption was minimal to polystyrene well plates or tubes. The results of this study show that under *in vitro* conditions in the presence of buffer and at high drug concentration the loss of drugs to cell culture plastic is insignificant. On the other hand, retention of lipophilic drugs in cultured cells should be taken into account in permeability experiments.

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