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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 (metoprolol, propranolol, antipyrine, diazepam, midazolam, naproxen, probenicid, ibuprofen, hydrochlorothiazide, indomethacin, 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.)

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 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 calcu-

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.

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.

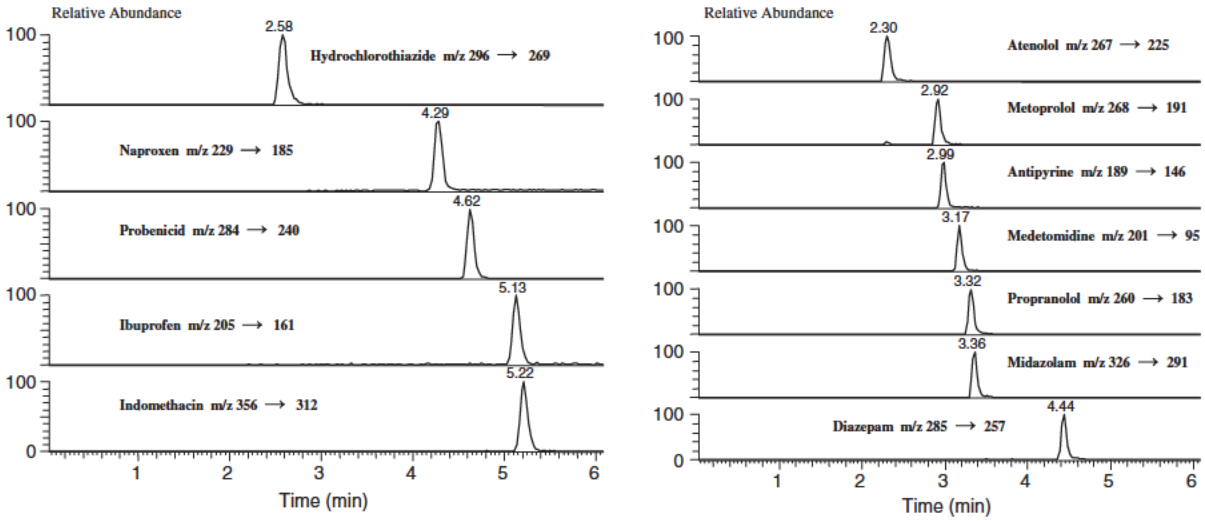

Fig. 1. Chromatography and tandem mass spectrometry of test compounds. Quality control mixtures of either acidic or basic drugs prepared in buffer solution were separated by reverse-phase LC over a 6-min gradient. Product ions of acidic compounds (left) or of basic compounds (right) were generated

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