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Research paper

Characterization of Caco-2 cell monolayer drug transport properties by cassette dosing using UV/fluorescence HPLC

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Abstract

Cassette dosing is commonly used in pharmacokinetic studies to decrease time, labor and expenditures. Cassette dosing (having several compounds in a mixture) has been used to screen in vitro permeabilities in Caco-2 cell monolayers. The cassette dosing method has accelerated both transport experiments and sample analyses, which are both part of Caco-2 permeability screening. In this study, a cassette dosing method was used for a mixture of heterogeneous test drugs, which are transported by various mechanisms across the Caco-2 cell monolayer. To characterize the Caco-2 cell monolayer absorption properties, we developed a new UV/fluorescence HPLC method for nine heterogeneous drugs. This new analytical method is fast and specific for high throughput analysis. Fluorescence detection was used to analyze the low concentration of drugs while UV detection was suitable for higher concentrations. The permeability results of single drugs and the mixture of drugs showed a high degree of similarity for each individual compound. All drugs can be applied to the Caco-2 cell culture as a mixture, and the cassette dosing method is suitable for permeability studies.
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Keywords: Cassette dosing; Caco-2 cell monolayers; Permeability screening; UV/fluorescence HPLC; High throughput analysis

1. Introduction

Cassette dosing is commonly used in pharmacokinetic studies to decrease time, labor and expenditure. Cassette dosing (having several compounds in a mixture, also called mixture dosing or *n*-in-one cocktail) has been used to screen in vitro permeabilities in Caco-2 cell monolayers in many laboratories. Many research groups have demonstrated its usability for permeability studies, and most results have shown that the permeabilities of drugs in a mixture also correlate strongly with permeabilities as single drugs [1–4]. The mixture dosing method has accelerated both transport experiments and sample analyses, which are both necessary for Caco-2 permeability screening. Analytical methods such as liquid chromatography coupled with mass spectrometry (LC/MS) have been effectively used to analyze the permeability of various compounds [1–3,5–7]. LC/MS has replaced traditional methods (e.g. UV, fluorescence and radiochemical detection), and increased sample throughput with high selectivity and sensitivity.

LC/MS method is a suitable method for the rapid analysis of numerous biological samples and even for the simultaneous analysis of several compounds in a mixture. In addition, the time needed for method development is greatly reduced by LC/MS. For example, Caldwell et al. [6] used LC/MS to analyze the absorption of eight β-adrenoceptor antagonists within a 12 min analytical time. Recently, Laitinen et al. [2] reported a 20 min total runtime for simultaneous permeability studies with 10 drugs. Although LC/MS is efficient, it has limitations, such as high price, ion suppression, contamination of the ion source after many samples and repeatability in some cases. UV spectrophotometric detection is currently the most popular and the most universally applicable method in pharmaceutical analysis. UV detection also has the advantage of low operating cost and straightforward use compared to MS. The sensitivity of UV detection is limited, however, but the combination of UV with fluorescence detection is an effective alternative approach to MS. Tannergren et al. [4] developed an UV-high-pressure liquid chromatography

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(HPLC) method for analyzing permeability samples of seven β-blockers and baclofen simultaneously. However, the total analysis time was 20 min and the drug concentrations were quite high. Ranta et al. [8] used a UV/fluorescence HPLC method to analyze β-blockers, which resulted in better sensitivity, but the runtime was 51 min. Clearly, such analytical runtimes are rather long for high throughput analysis, as permeability experiments screen hundreds of samples per day.

Caco-2 cells, which are derived from human colon adenocarcinoma, are widely used for screening the intestinal absorption of new drug candidates. Currently, there is great interest in establishing methods for the characterization of Caco-2 cell monolayer absorption properties, especially when the cells are modified by various techniques to better predict intestinal absorption [2–4]. For this purpose, we developed a cassette dosing mixture of heterogeneous test drugs, which are transported by various mechanisms across the Caco-2 cell monolayer (Table 1). The H^+-coupled oligopeptide transporter (PEPT1) has been shown to transport some peptidomimetic drugs, such as cephalexin [9]. Cephalexin was included in the test mixture, although Caco-2 cells express a low level of the PEPT1 [10]. Baclofen and ibuprofen are known to be carried by the amino acid transporter and by a pH-dependent monocarboxylic acid transporter, respectively [4,11]. Atenolol is used as a model compound for paracellular absorption, and both antipyrine and propranolol are used for transcellular transport studies [12]. Verapamil is a substrate for the P-glycoprotein efflux system [13]. The present analytical method was also developed for hydrochlorothiazide and metoprolol, which are alternatives to atenolol and propranolol, respectively. Many of the studied drugs are listed in the FDA Guidance for Industry as model drugs for permeability studies [14].

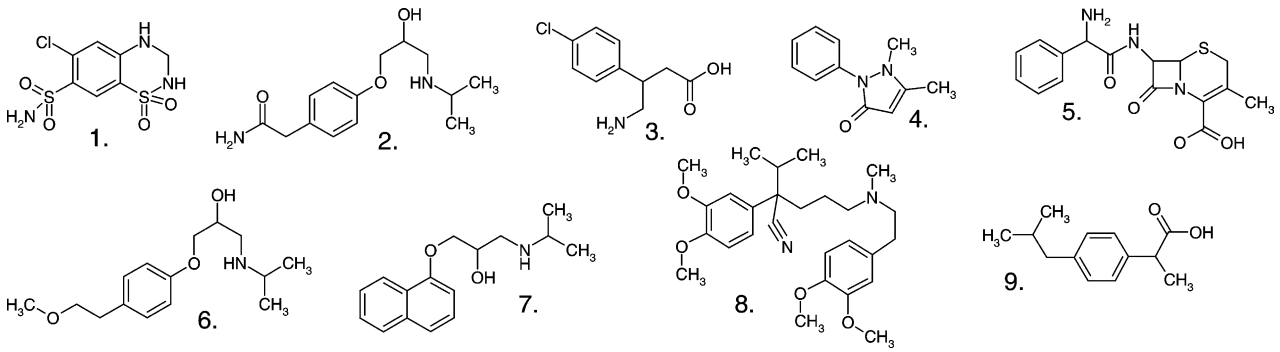
To characterize the Caco-2 cell monolayer absorption properties, we developed a new UV/fluorescence HPLC method for nine heterogeneous drugs. The new HPLC method has extremely good repeatability, it is fast, sensitive and specific at low concentrations, and it includes the quantitative analysis of these drugs with UV and fluorescence detection simultaneously. In this study, all studied drugs were analyzed within 5 min and the total runtime was only 10 min. To the best of our knowledge, no other direct UV/fluorescence HPLC method for investigating heterogeneous drug transport across Caco-2 cell cultures has been reported.

2. Materials and methods

2.1. Chemicals

Compounds used in the study were mannitol (NEN, Boston, MA), atenolol, propranolol, metoprolol, baclofen,

Table 1
Physicochemical properties and structures of the nine drugs used in this study



Compound	pK_a^a		log P^b	log D^c		Transport mechanism across Caco-2 cell culture [refs.]
	Acid	Base		pH 6.0	pH 7.4	
1. Hydrochlorothiazide	8.95, 9.49		−0.07	−0.089	−0.071	Paracellular [14,19]
2. Atenolol		9.17	0.10	−2.74	−1.66	Paracellular [12,14]
3. Baclofen	4.00	10.33	1.56	−0.94	−0.94	Amino acid transporter [4]
4. Antipyrine		1.21	0.27	0.27	0.27	Transcellular [12,14]
5. Cephalexin	3.12	6.80	0.65	−1.88	−2.48	H^+-peptide transporter [9,10]
6. Metoprolol		9.18	1.79	−1.05	0.026	Transcellular [12,14]
7. Propranolol		9.15	3.10	0.27	1.36	Transcellular [4,14]
8. Verapamil		9.04	4.91	2.14	3.28	P-glycoprotein substrate [13,14]
9. Ibuprofen	4.41		3.72	2.12	0.77	Monocarboxylic acid transporter [11]

[a,b,c]Represent the calculated values. Computational values were calculated by the ACDLABS ACD/pKa/logD program (Version 4.56/26 April 2000).

hydrochlorothiazide, antipyrine (Sigma, St Louis, MO), verapamil hydrochloride (ICN Biomedicals Inc., OH), ibuprofen (Orion, Espoo, Finland) and cephalexin (a gift from Viikki Drug Discovery Technology Center, Department of Pharmacy, University of Helsinki). All medium components and cell culture reagents were purchased from Bio Whittaker (Belgia) except FBS, which was purchased from Gibco (Germany). Acetonitrile (HPLC S grade) was obtained from Rathburn (Walkerburn, UK), anhydrous trifluoroacetic acid (TFA) from Sigma (St Louis, MO). Water was deionized and purified by a Millipore-Q UF Plus apparatus (Molsheim, France). All compounds and reagents were of the highest quality and used as received.

2.2. Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured at 37 °C and 7% CO_2 atmosphere, and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat inactivated fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 2 mM L-glutamine, 100 U/ml and 100 μg/ml penicillin and streptomycin, respectively. The cells were passaged at 80–90% confluence every 3–4 days using a trypsin–EDTA solution (0.25% trypsin and 450 μM EDTA) to detach cells from the culture flask. For transport studies, cells from passage number 41–50 were seeded on polycarbonate membranes (0.4 μm pore size, 12 mm diameter, Costar), at 9×10^4 cells to each insert. Cell monolayers were fed with fresh medium the day after seeding and then three times per week for at least up to 21 days, whereupon the cells were differentiated. The integrity of monolayers was controlled by [^{3}H]mannitol permeability studies for each experiment. Monolayers having a P_{app} of not more than 1×10^{-6} cm/s were considered to be tight enough for analysis.

2.3. Bidirectional transport procedure

The transport experiments were performed in a Hanks' balanced salt solution (HBSS) containing 10 mM of HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid], pH 7.4). Before the experiments, the Caco-2 cell monolayers were washed twice with HBSS-HEPES. Transwells were then placed in an orbital horizontal shaker (Heidolph Inkubator 1000, Titramax 1000, Germany) with constant stirring (100 rev./min) at 37 °C. Stock solutions of all test drugs were prepared in HBSS-HEPES and the final concentrations of each compound are presented in Table 5. Stock solutions (i.e. the mixture of seven drugs or solutions of each single drug) were added to the donor side of the cell monolayer and pure buffer was added to opposite, receiver side. Permeation of the sample (i.e. the mixture of seven drugs; atenolol, baclofen, antipyrine, cephalexin, propranolol, verapamil and ibuprofen) was studied in both directions, apical to basolateral and basolateral to apical, at an apical pH of 6.0 and basolateral pH of 7.4. The permeation of each single drug was studied in the same manner as the mixture under identical conditions. Further, mixture experiments were performed without a pH-gradient; i.e. pH 7.4 remained equivalent on both sides of the cell. The samples from the donor side were diluted before determination of the initial concentration for each drug. Sample aliquots of 100 μl were collected from the receiver side at 0, 10, 20, 30, 45, 60, 90, 120, 150, and 180 min and replaced with an equal volume of fresh buffer each time. The samples were stored at −20 °C until analyzed by HPLC without any purification procedure. All transport experiments were conducted in triplicate and under 'sink' conditions, where the concentration of drug in the receiver side was lower than 10% of the initial dosing concentration.

The apparent permeability coefficients, P_{app} (cm/s), for each single compound and the mixture of seven drugs were calculated according to the following Eq. (1)

$$P_{app} = J/AC_0 \quad (1)$$

where J (flux) is the amount of compound transported across the monolayer within a given time period, A is the membrane surface area (1.1 cm^2) and C_0 is the initial concentration of compound on the donor side.

Computational log P, log D and pK_a values were calculated by the ACDLABS ACD/pKa/logD software package (Version 4.56/26 April 2000). Log P was derived from the *n*-octanol–water partition coefficient for the neutral form of molecules, and the apparent partition coefficient (distribution coefficient), log D, was pH-dependent in the case of ionizable compounds [5].

2.4. Equipment

The HPLC system consisted of a Beckman System Gold Programmable Solvent Module 126, a Beckman System Gold Detector Module 168 with a diode array UV detector and a Beckman System Gold Autosampler 507e (Beckman Instruments Inc., CA) with a 20 μl sample loop. A MetaTherm column temperature controller (MetaChem Technologies, Torrance, CA) was used with the HPLC. A Hewlett-Packard 1046A fluorescence detector (Waldbronn, Germany) was used together with UV detection, and signals from the two detectors were collected and processed by the 32 Karat software package version 3.0.

2.5. Chromatographic conditions

Separations were performed on a Xterra C_{18} reversed-phase column (2.5 μm, 20 × 2.1 mm, Waters, Milford, MA). Solution A (pH 3) was water containing 0.05% (v/v) TFA. Solution B was acetonitrile–water (90% acetonitrile, ACN) containing 0.04% (v/v) TFA. The column temperature was set to +60 °C and flow rate of the mobile phase was 800 μl/min. The ACN/water (solution B) gradient was 2% from 0 to 1 min, 2–41%

from 1 to 2 min, 41–63% from 2 to 5 min and 63–2% from 5 to 7 min. Thereafter, the column was equilibrated at 2% ACN/water for 3 min before the next injection. Flushed loop injection was used with the autosampler, where the 20 μl sample loop was overfilled with 100 μl of sample to insure good reproducibility.

The UV detector operated at 250 nm for 0–0.35 min, then 225 nm for 0.35–2.30 min and finally at 205 nm for 2.30–10.0 min. The bandwidth was set at 4 nm and the data sampling rate at 8 Hz. The fluorescence detector was set at 230 nm (excitation) and 302 nm (emission), with a cut-off filter at 280 nm and a lamp flash frequency of 220 Hz. Response time of the fluorescence detector was 1000 ms, and the gain was 12.

2.6. Calibration and accuracy

Calibration mixtures (0.01, 0.05, 0.1, 0.25, 0.5, 1.0, 3.0 and 9.0 μM) and quality control mixtures (0.6, 2.0 and 8.0 μM) were prepared by diluting stock solutions with HBSS-HEPES. The calibration and control solutions contained nine different drugs; hydrochlorothiazide, metoprolol and the previously mentioned seven drugs. As for the calibration mixtures, quality control mixtures were analyzed repetitively between samples within each analytical sequence. Calibration curves were calculated by peak area versus concentration using linear regression. The precision was tested by control mixtures as within-day and between-day repeatabilities (on three different days). In determining the lowest limit of quantitation (LLOQ) for each compound, calculations were based on the FDA Guidance for Industry, Bioanalytical Method Validation [15]. Briefly, a precision and accuracy at the LLOQ should be 20 and 80–120%, respectively, with a signal-to-noise ratio of 5. The stability of each drug was studied by the analysis of quality control samples (2 μM), which were stored under light and at room temperature (22 °C) for 24 and 48 h.

3. Results and discussion

3.1. Choromatography and detection

The chromatographic separation of all nine drugs with UV detection and five drugs with fluorescence detection are shown in Fig. 1A and B, respectively. All compounds were eluted within 5 min with the fast gradient, column temperature controller and short reversed-phase column. An interfering compound from the mobile phase eluted just before 6 min (Fig. 1B), thus the separation was appropriate for analysis. The flow rate (0.8 ml/min) was relatively high for the small particle size column (2.5 μm). The high flow rate, however, led to considerable backpressure. In this case, the column operated at a lower backpressure level (2000 psi, 140 bar) by using the column oven ($T = 60$ °C). The flow rate that had been used for analysis allowed a fast re-equilibrating time for the column, thus the total chromatographic runtime was only 10 min. Hydrochlorothiazide eluted fast, and therefore the ACN concentration was increased after 1 min. Previously, problems with propranolol have been reported, such as contamination of the column or autosampler and long retention times [4,8]. In this study, propranolol and other drugs were analyzed efficiently by the short column without any contamination problems. However, potential contaminations were already minimized by analyzing low concentration samples first, and by using the flushed loop injection method.

Fluorescence detection was sensitive for atenolol, metoprolol, propranolol, verapamil and ibuprofen at an excitation wavelength of 230 nm and an emission of 302 nm (Fig. 1B). The use of fluorescence detection at similar wavelengths has been reported earlier for β-blockers, but not for verapamil and ibuprofen [8,16].

UV detection was performed in three parts; a detection wavelength of 250 nm was used for the time interval between 0 and 0.35 min in order to minimize a signal response from buffer salts or other impurities. A wavelength of 225 nm was chosen for 0.35–2.30 min, because it offered good sensitivity for hydrochlorothiazide, atenolol and baclofen. Antipyrine, cephalexin, metoprolol, propranolol, verapamil and ibuprofen were detected by using an UV wavelength of 205 nm from time points 2.30–10.0 min (Fig. 1A). Typically, the above-mentioned drugs had been analyzed by higher UV wavelengths in the presence of formic acid or phosphoric acid in the mobile phase [4,16–20]. In this study, the effect of mobile phase was tested by using different acids (formic, phosphoric and TFA) in the ACN–water solution. However, due to the low UV wavelength, the baseline of the formic acid solution was too noisy for sensitive analysis and chromatographic separation with phosphoric acid was ineffective. Fortunately, TFA is a common and effective mobile phase modifier and ion-pairing reagent [21]. Thus, the ACN–water–TFA solution offered the best sensitivity and enabled fast analysis for all drugs, even though such a low wavelength was used. The effect of TFA concentration on UV absorbance in mobile phase B is shown in Fig. 2. A concentration of 0.04% (TFA) was eventually chosen for analysis, as its re-equilibration time was shortest and it enabled the fastest analytical run.

3.2. Calibration and accuracy

Linearity was tested over the range of expected concentrations in the permeation studies. The calibration range of each compound for the fluorimetric and UV detectors is shown in Table 2. The equations for the curves were calculated by linear regression analysis, using five calibration points with three sets of replicate standards per curve. One curve covered a concentration range from 0.01 to 0.5 μM, and the second from 0.25 to 9.0 μM. For test samples, the concentration of the analytes was calculated from peak areas using equation derived from the linear

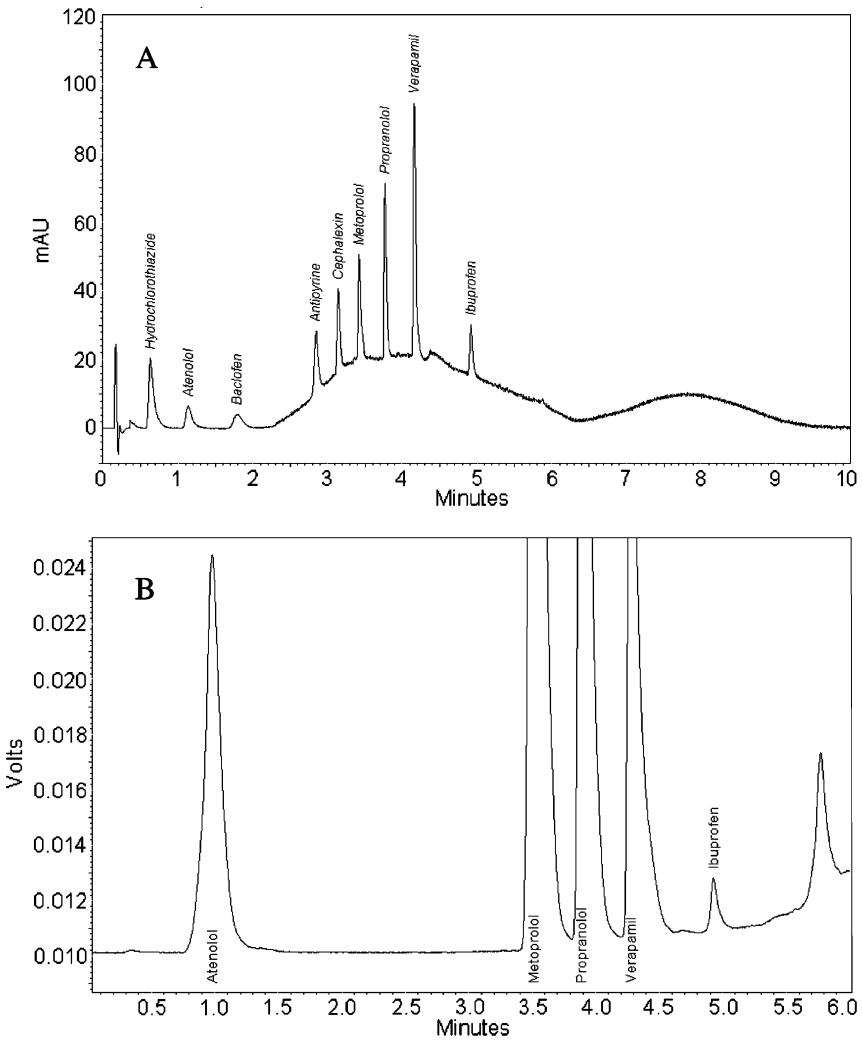


Fig. 1. The chromatographic separation of nine drugs (each 2 μM) with UV detection and five drugs (each 0.5 μM) with fluorescence detection in a single chromatographic run. (A) UV detection of hydrochlorothiazide, atenolol and baclofen was at 225 nm (0.35–2.30 min). Antipyrine, cephalexin, metoprolol, propranolol, verapamil and ibuprofen were detected by using wavelength 205 nm (2.30–10.0 min). (B) Fluorescence detection of atenolol, metoprolol, propranolol, verapamil and ibuprofen with excitation and emission wavelength at 230 and 302 nm, respectively.

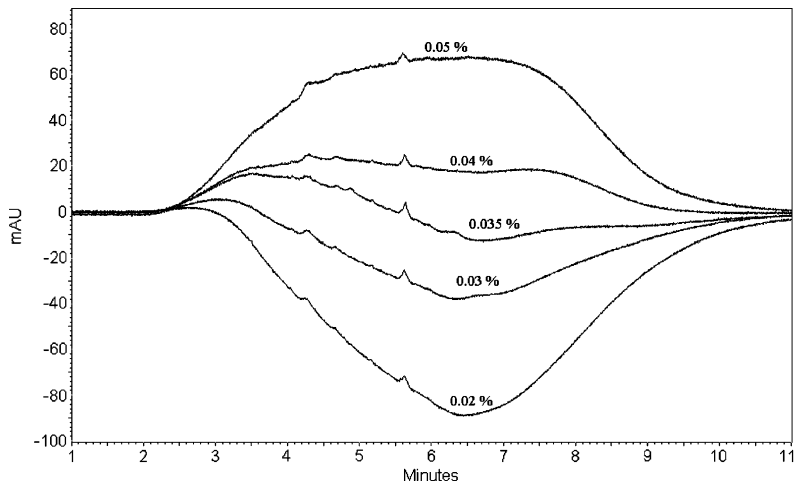


Fig. 2. Five different trifluoroacetic acid (TFA) concentrations in mobile phase B (acetonitrile–water solution) were used to compare for baseline absorbances. Analysis was performed by UV-HPLC (wavelength 205 nm).

Table 2
Calibration range, LLOQ and signal-to-noise ratio of the UV/fluorescence HPLC method

Compound	Calibration range (μM)		LLOQ (μM)		Signal-to-noise ratio	
	UV	FL[a]	UV	FL[a]	UV	FL[a]
Hydrochlorothiazide	0.10–9.0	–[b]	0.10	–[b]	3.7	–[b]
Atenolol	0.25–9.0	0.01–9.0	0.25	0.01	5.6	7.6
Baclofen	0.25–9.0	–[b]	0.25	–[b]	5.0	–[b]
Antipyrine	0.25–9.0	–[b]	0.25	–[b]	3.2	–[b]
Cephalexin	0.25–9.0	–[b]	0.25	–[b]	5.0	–[b]
Metoprolol	0.25–9.0	0.01–3.0	0.25	0.01	4.6	60
Propranolol	0.25–9.0	0.01–3.0	0.25	0.01	6.3	33
Verapamil	0.25–9.0	0.10–3.0	0.25	0.10	5.0	110
Ibuprofen	0.25–9.0	0.10–9.0	0.25	0.10	3.8	10

[a] Fluorescence detection.
[b] No fluorescence response.

regression. Linear regression analysis for verapamil produced a correlation coefficient (r^2) of 0.996 with fluorescence detection. The calibration curves for all other compounds showed good linearity with correlation values (r^2) better than 0.997 and 0.9999 for UV and fluorescence detections, respectively.

The within-day precision of the method was determined by analysis of control mixtures ($n = 6$) at 2.0, 8.0 μM and 0.6, 2.0 μM for UV and fluorescence detection, respectively (Table 3). Typically, relative standard deviations (RSD) were below 5%, however, ibuprofen was an exception at 6.45%. As seen in Table 3, fluorescence detection was more precise (within-day) than UV for the compounds of the mixture. The between-day precision was calculated within 3 days and RSD values of both detectors were lower than 5%. These results indicate the accuracy and repeatability of both detector systems and of overall analytical method.

The LLOQ for the nine studied compounds are given in Table 2. For fluorescence detection, the LLOQ for atenolol, metoprolol and propranolol was 0.01 μM, and the LLOQ for verapamil and ibuprofen was 0.10 μM. With UV detection, the LLOQ was 0.25 μM for all compounds, except hydrochlorothiazide, which was 0.10 μM. The RSD precision of LLOQ for each compound was lower than 13% ($n = 3$), which is better than the FDA requirement. The accuracy of the LLOQ was typically 80–120% of the stated concentration, except atenolol at 130%. The signal-to-noise ratios of the compounds were mostly near 5. These quantitation limits were suitable for the analysis of permeation samples, as true sample concentrations were well above the LLOQ.

The stability of each drug in solution during analysis was determined to evaluate the usefulness of the assay. The quality control samples were analyzed between samples

Table 3
Within-day repeatability and between-day precision of the analytical method

Compound[a]	Within-day repeatability						Between-day precision (3 days, $n = 9$)		
	UV detection ($n = 6$)			FL[b] ($n = 6$)			QC[c] (μM)	RSD (%)	
	QC[c] (μM)	Mean (μM)	RSD (%)	QC[c] (μM)	Mean (μM)	RSD (%)		UV	FL[b]
Hydrochlorothiazide	2.0	2.01	0.32	–[d]	–[d]	–[d]	2.0	0.92	–[d]
	8.0	8.33	0.86						
Atenolol	2.0	1.98	2.13	2.0	1.99	0.12	2.0	1.13	1.49
	8.0	7.80	2.16	0.6	0.59	1.49			
Baclofen	2.0	2.04	2.99	–[d]	–[d]	–[d]	2.0	1.91	–[d]
	8.0	8.06	0.57						
Antipyrine	2.0	1.92	3.61	–[d]	–[d]	–[d]	2.0	1.79	–[d]
	8.0	8.21	1.72						
Cephalexin	2.0	1.94	2.18	–[d]	–[d]	–[d]	2.0	3.13	–[d]
	8.0	7.80	2.69						
Metoprolol	2.0	2.07	2.34	2.0	2.00	0.34	2.0	2.55	1.08
	8.0	8.15	1.30	0.6	0.59	0.25			
Propranolol	2.0	1.96	4.22	2.0	2.03	0.60	2.0	1.80	0.77
	8.0	8.01	0.72	0.6	0.59	0.87			
Verapamil	2.0	2.19	1.48	2.0	2.18	0.73	2.0	1.29	4.89
	8.0	8.37	1.42	0.6	0.62	1.47			
Ibuprofen	2.0	2.19	6.45	2.0	2.03	0.86	2.0	4.70	1.19
	8.0	8.10	1.09	0.6	0.57	0.77			

[a] Compounds are in the order of their chromatographic elution.
[b] Fluorescence detection.
[c] Quality control sample.
[d] No fluorescence response.

Table 4
Stability of each drug in solution

Compound[a]	UV detection of quality control samples (2 μM, $n = 3$)		Fluorescence detection (QC = 2 μM, $n = 3$), Mean[b] ± RSD (%)
	Time (h)	Mean[b] ± RSD (%)	
Hydrochlorothiazide	0	100.5 ± 0.09	–[c]
	24	94.65 ± 0.73	
	48	91.65 ± 1.24	
Atenolol	0	99.58 ± 2.65	99.25 ± 0.08
	24	97.5 ± 0.85	99.2 ± 0.08
	48	99.2 ± 3.08	99.3 ± 0.23
Baclofen	0	101.93 ± 4.17	–[c]
	24	99.85 ± 1.78	
	48	103.6 ± 5.50	
Antipyrine	0	104.37 ± 4.35	–[c]
	24	100.75 ± 12.28	
	48	–[d]	
Cephalexin	0	98.18 ± 2.90	–[c]
	24	96.85 ± 8.57	
	48	94.85 ± 1.36	
Metoprolol	0	103.98 ± 0.84	100.2 ± 0.45
	24	101.5 ± 1.81	99.9 ± 0.10
	48	96.4 ± 3.56	100.5 ± 0.12
Propranolol	0	100.55 ± 2.94	101.8 ± 0.73
	24	100.7 ± 2.41	101.5 ± 0.22
	48	100.55 ± 2.84	102.85 ± 0.37
Verapamil	0	109.85 ± 1.70	109.0 ± 1.08
	24	114.95 ± 1.73	115.1 ± 1.81
	48	117.6 ± 2.55	128.9 ± 1.64
Ibuprofen	0	104.7 ± 2.94	101.0 ± 1.06
	24	108.65 ± 1.28	102.3 ± 0.18
	48	113.7 ± 2.75	102.1 ± 0.69

The quality control samples were stored at room temperature for 24 and 48 h until analyzed by HPLC with UV and fluorescence detection. Analytical sequence included standard quality control samples which represented time 0 min.

[a] Compounds are in the order of their chromatographic elution.

[b] Values are % of initial concentration.

[c] No fluorescence response.

[d] Not determined.

during the same analytical sequence; i.e. the analytical sequence also included a set of standard quality control samples which also served as the 0 min sample. The results (Table 4) indicate that all compounds were stable during analysis and degradation in solution was typically lower than 5% within 24 h of storage. The permeation samples were stored at − 20 °C before assay, and usually the samples were analyzed within 8 h.

3.3. Transport studies

Using the described HPLC method, the seven drugs that crossed the Caco-2 monolayer were detected as single compounds and as a mixture. Fig. 3 shows a typical chromatogram of the mixed sample (taken from the receiver side of the cell), observed at 45 and 120 min with fluorescence and UV detections, respectively. As seen in Fig. 3A, verapamil and an interfering compound eluted close to each other. The separation was, however, adequate for analysis. With UV detection (Fig. 3B), interfering peaks were not found close to the compounds. The intensity of baclofen was low, due to poor permeability and analytical response. Fluorescence detection was used to analyze the low concentration of drugs while UV detection was suitable for higher concentrations.

The permeability results of single drugs and the mixture were similar for each individual compound in the apical to basolateral (AP–BL) and basolateral to apical (BL–AP) directions at the apical pH of 6.0. Fig. 4 summarizes the P_{app} correlation of single drugs compared to mixture dosing. The correlation coefficients (r^2) of the linear regression for the AP–BL and BL–AP directions were 0.9747 and 0.9647, respectively. Recently [2,4], this statistical method has been used for the evaluation of mixture dosing.

In this study, the effect of a pH-gradient on P_{app} was also investigated. A recent study [2] reported that the carrier-mediated H^+ transporter for monocarboxylic acids was more effective at low pH. The P_{app} of ibuprofen in this study

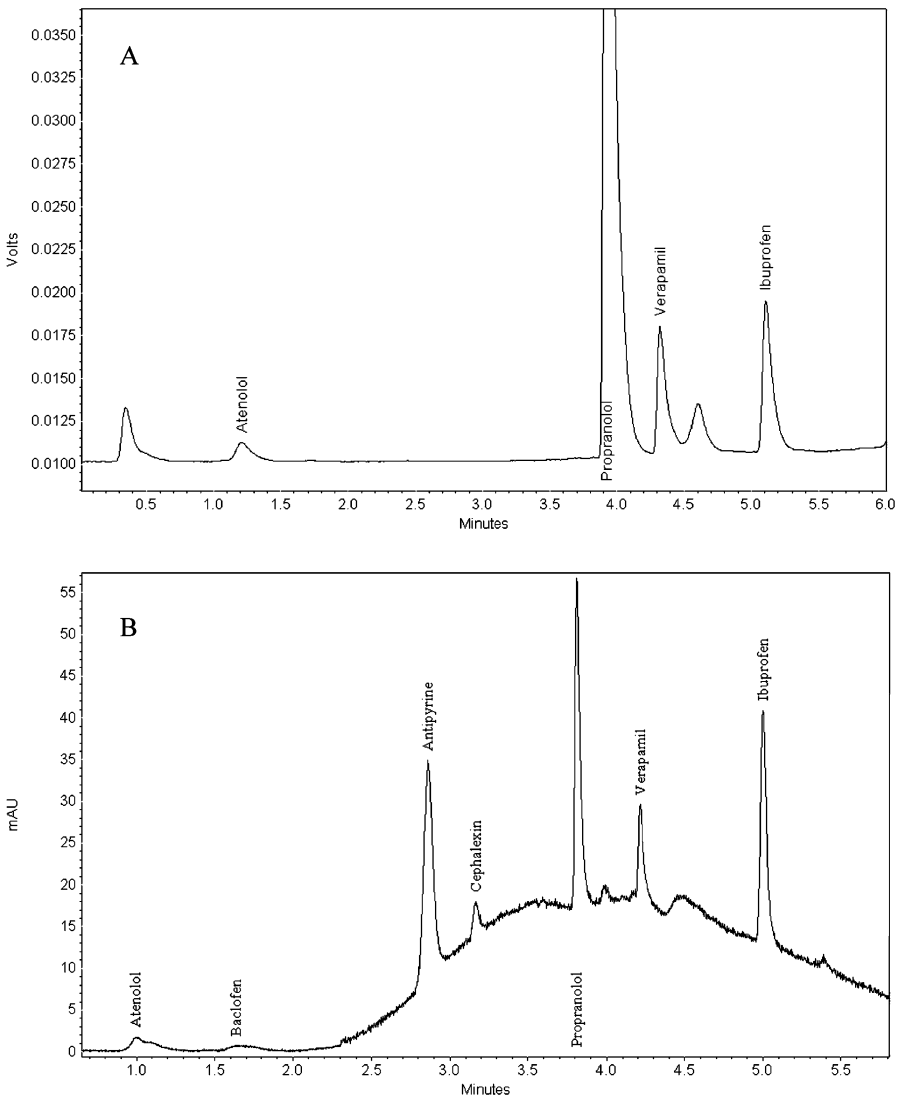


Fig. 3. Fluorescence and UV detections of seven drugs administered as a mixture. Permeability experiments were performed in the apical (pH 6.0) to basolateral (pH 7.4) direction and samples were taken at 45 min and at 120 min for fluorescence (A) and UV detections (B), respectively. Concentrations in (A): atenolol (0.065 μM), propranolol (0.516 μM), verapamil (0.399 μM) and ibuprofen (2.089 μM). In (B): atenolol (0.147 μM), baclofen (0.537 μM), antipyrine (3.386 μM), cephalexin (0.358 μM), propranolol (1.837 μM), verapamil (0.669 μM) and ibuprofen (4.487 μM). Conditions of detection as stated in Fig. 1.

is in line with that observation. As seen in Table 5, the AP–BL/BL–AP P_{app}-ratio of cephalexin was higher at pH 6.0 than at pH 7.4, which signifies that the same dependency on pH-gradient seems to occur with cephalexin and PEPT1 transporter in the Caco-2 cells. The permeability of baclofen was independent of the pH-gradient and, according to P_{app} results, Caco-2 cells have a weak amino acid transporter expression. Log D values for the passive transport of antipyrine at the both apical pH are equal. Therefore, AP–BL/BL–AP ratios (at both apical pH values) were similar and close to 1.0, which demonstrates independence of the transport direction. In the case of basic compounds, log D and pH may have combined effects on compound permeability. For example, atenolol, propranolol and verapamil have higher log D values at a pH of 7.4 (Table 1) than at pH 6.0, which indicates better lipophilicity and, thus, higher P_{app} values were observed at pH 7.4. This induced increment in the BL–AP permeability, and subsequently AP–BL/BL–AP ratios, were decreased when the apical pH was 6.0. Because passive permeability of verapamil is high at pH of 7.4, the P-glycoprotein efflux system may display a smaller transport action.

4. Conclusion

There was no significant difference in the P_{app} values obtained between the mixture and single dosing at an apical pH of 6.0, and the use of a pH-gradient was appropriate. All seven drugs can be applied to the Caco-2 cell culture as

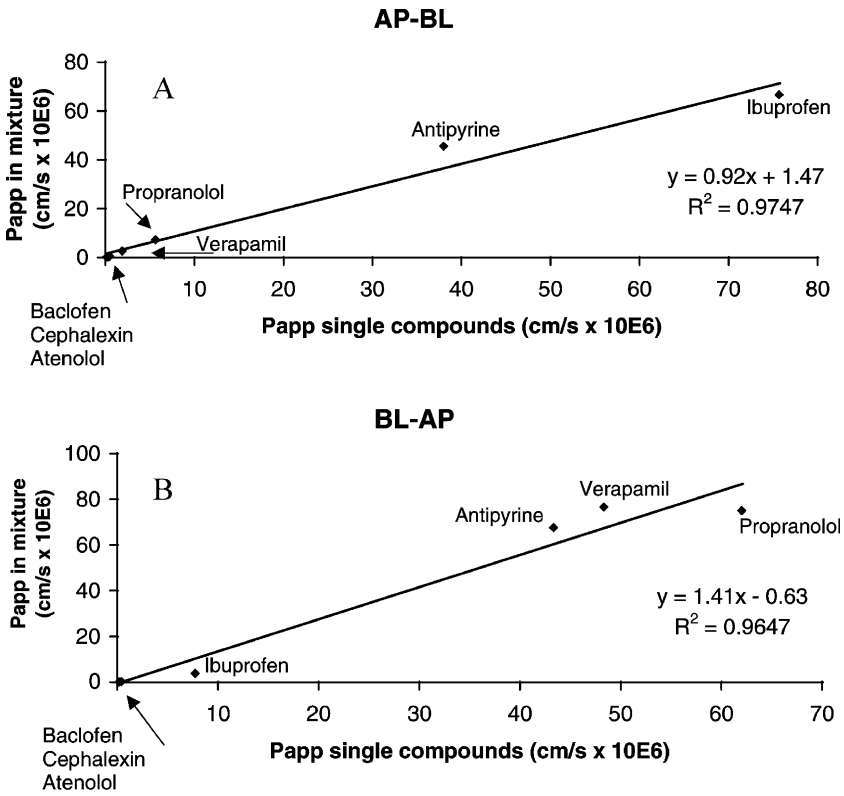


Fig. 4. Correlation between the single drugs and in the mixture. The linear regression for apical to basolateral direction is shown in (A), and basolateral to apical in (B). Baclofen, cephalexin and atenolol are close to the origin due to the scale of the axes. Experiments were conducted at the apical pH of 6.0 and basolateral at pH 7.4.

a mixture. The mixture of heterogenous drugs was suitable for permeability screening studies with Caco-2 cell monolayers. This study demonstrated that a typical UV/fluorescence HPLC method could be applied to high throughput cassette dosing analyses. The new analytical method, combined with the screening method, can be applied in the characterization of Caco-2 cell monolayer transport activity.

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Table 5
Bidirectional apparent permeability coefficients (P_{app}) of the seven drugs, analyzed as a mixture for Caco-2 cell monolayers

Compounds in mixture	P_{app} ($\times 10^6$ cm/s) + SD					
	AP to BL apical pH 6.0 ($n = 5$)	BL to AP apical pH 6.0 ($n = 5$)	Ratio[a] AP–BL/ BL–AP	AP to BL apical pH 7.4 ($n = 3$)	BL to AP apical pH 7.4 ($n = 3$)	Ratio[a] AP–BL/ BL–AP
Baclofen (500 μM)	0.20 ± 0.07	0.15 ± 0.01	1.35	0.12 ± 0.06	0.16 ± 0.03	0.75
Cephalexin (300 μM)	0.43 ± 0.17	0.10 ± 0.01	4.3	0 ± 0	0.054 ± 0.04	–
Ibuprofen (20 μM)	67 ± 11	3.9 ± 0.2	17.2	43 ± 10	54 ± 2.0	0.80
Antipyrine (20 μM)	46 ± 4.1	68 ± 30	0.68	41 ± 8.3	51 ± 6.0	0.80
Propranolol (50 μM)	7.2 ± 1.8	75 ± 14	0.1	32 ± 5.5	26 ± 3.6	1.23
Atenolol (200 μM)	0.14 ± 0.04	0.25 ± 0.02	0.56	0.23 ± 0.017	0.20 ± 0.035	1.15
Verapamil (20 μM)	2.7 ± 0.07 ($n = 2$)	77 ± 2.1 ($n = 3$)	0.04	29 ± 10	41 ± 5.5	0.71

Experiments were performed at an apical pH of 6.0 and 7.4, basolateral pH was 7.4. The P_{app} values across Caco-2 cell monolayers were calculated according to the equation $P_{app} = J/AC_0$ and results are the means ± SD.

[a] The ratio of apparent permeabilities in two directions.

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