N-in-One Permeability Studies of Heterogeneous Sets of Compounds across Caco-2 Cell Monolayers

Leena Laitinen,[1,2,5] Heli Kangas,[1,3] Ann Marie Kaukonen,[1,4] Kati Hakala,[1] Tapio Kotiaho,[1] Risto Kostiainen,[1,3] and Jouni Hirvonen[1,4]

Received August 13, 2002; accepted November 1, 2002

Purpose. The purpose of the study was to evaluate several n-in-one cocktails of heterogeneous compounds to increase the throughput of permeability studies across Caco-2 monolayers, to investigate the reliability and applicability of the method, and to develop fast and sensitive analysis for the compounds. Compounds with potential interactions in efflux and/or active transport were chosen.

Methods Permeability experiments with verapamil, propranolol, midazolam, hydroxyzine, timolol, buspirone, procaine, naproxen, ketoprofen, and antipyrine as single compounds and in cocktails of 5–10 compounds were performed at 50 μM concentration both in the apical-to-basolateral and basolateral-to-apical direction. The compounds were quantified by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI/MS/MS). Toxicity tests were performed to determine cellular damage.

Results The analytical method was sensitive, accurate, and rapid. The individual permeabilities of compounds in cocktails correlated well with permeabilities as single compounds. No significant interactions between the compounds within the mixtures were observed, except for acidic compounds. The studied mixtures did not show any toxicity.

Conclusions The use of n-in-one cocktails is a suitable method to improve the capacity in routine permeability experiments and higher throughput screening of drug candidates, although potential interactions should always be borne in mind. The use of LC-ESI/MS/MS technology provides an excellent tool in fast and accurate analysis of small amounts of heterogeneous compounds.

KEY WORDS: Caco-2 monolayers; permeability; n-in-one cocktails; LC-ESI/MS/MS analysis; increased throughput.

INTRODUCTION

Successful and economical drug development requires optimization of the experimental procedures in the testing of early absorption, distribution, metabolism, and excretion (ADME) drug properties. Because of the expensive 3-week period of Caco-2 cell growth and differentiation, new approaches for increased efficiency are needed. One way to accelerate the productivity of cell culture permeability studies is to perform these studies with more than one compound at the same time (n-in-one cocktails). Experiments with several compounds in mixtures have been published recently (1,2). Tannergren *et al.* (2) attempted to increase the efficiency of permeability screenings for new drug compounds. The drug concentrations in these experiments were high (0.1–1.0 mM) and the sampling interval long, allowing sample analysis to be performed by high-performance liquid chromatography with limited throughput and sensitivity. Liquid chromatography/tandem mass spectrometry (LC-MS/MS) has shown its usefulness in the analysis of samples from Caco-2 cell monolayer studies (1,3,4). The LC-MS/MS offers sensitivity, specificity, and sample capacity that is ideal especially for low sample concentrations and n-in-one cocktails.

Many absorption experiments in Caco-2 cell monolayers are performed under conditions that do not mimic the intestinal conditions *in vivo*. An acidic microclimate maintained by an active secretion of H^+ is found above the enterocytes in the upper parts of small intestine. Because of the absence of a prominent mucus layer on the surface of Caco-2 cell monolayers produced *in vivo* by goblet cells, the apical pH will mainly be determined by the transport medium (5,6). Therefore, a better prediction of the per oral absorption should be gained, if the apical pH is 5.5–6.5 rather than 7.4 (7). This can be performed without compromising the cells, as Palm *et al.* (6) have shown. The integrity of the Caco-2 cell monolayers was unaffected when the apical pH value varies between 5.5 and 7.5. Not only is the pH gradient across the enterocyte membrane more predictive for the *in vivo* permeability of passively permeated compounds, it will also provide the driving force for active transport (7,8). The intestinal permeability of weak organic acids (like salicylic acid) is strongly enhanced by the decrease in apical pH. This phenomenon cannot entirely be explained by the pH-partition theory. Several experiments suggest the involvement of carrier-mediated transport across intestinal mucosa for monocarboxylic acids, mainly due to a proton-cotransport mechanism, also present in Caco-2 cells (7,9).

In addition to absorptive transporters, the pH gradient ameliorates the function of efflux transporters present in the Caco-2 cell lines. The efflux transporters protect the membrane and the cells against potential toxic agents like drugs. P-glycoprotein (P-gp) is perhaps the most important multidrug resistance enzyme in the small intestine. P-gp is a 170-kDa phosphorylated glycoprotein encoded by the multidrug resistance (MDR1) gene, which functions as an ATP-dependent efflux pump (10). Substrates of the P-gp are believed to enter from the lipid bilayer and bind to one or more nonidentical binding sites at the cytoplasmic leaflet (10–12). Experiments on P-gp-mediated transport of different substrates and inhibitors suggest noncompetitive interactions and different substrate specificity between the binding sites (13). It has been demonstrated that inhibition of P-gp by high concentrations of verapamil increases the absorption and decreases the efflux of vinblastine and vincristine (14,15), thus decreasing the ratio between basolateral-to-apical (BL-to-AP) and apical-to-basolateral (AP-to-BL) transport. However, depending on the origin and culturing conditions of the Caco-2 cells and the studied compounds, interactions between different substrates and inhibitors of P-gp may or may not have tangible effects on their individual permeabilities across Caco-2 cell monolayers (16).

[1] Viikki Drug Discovery Technology Center (DDTC), Department of Pharmacy, University of Helsinki, P.O. Box 56, Fin-00014 University of Helsinki, Finland.
[2] Division of Biopharmaceutics and Pharmacokinetics.
[3] Division of Pharmaceutical Chemistry.
[4] Division of Pharmaceutical Technology.
[5] To whom correspondence should be addressed. (Tel: +358-9-191 59 157; Fax: +358-9-191 59 144)

0724-8741/03/0200-0187/0 © 2003 Plenum Publishing Corporation

The main objective of the present study was, in addition to the increased throughput of the permeability studies across Caco-2 cell monolayers, to investigate the reliability and applicability of the method with several sets of structurally heterogeneous compounds. On the apical side of the cell monolayers the pH was adjusted to 5.5 to better mimic intestinal drug absorption. Representatives of various absorption mechanisms were chosen in order to probe potential interactions regarding both efflux (verapamil, propranolol, hydroxyzine, timolol, buspirone, and procaine) and active absorption (naproxen and ketoprofen). Midazolam and antipyrine, as passively permeating compounds, were expected to remain practically unaffected by others. At the same time, rapid and sensitive LC-MS/MS analytical methodology was developed for the quantitation of individual compounds and the cocktails studied.

MATERIALS AND METHODS

Chemicals

Of the compounds studied, verapamil hydrochloride, propranolol hydrochloride, ketoprofen, and naproxen were purchased from ICN Biomedicals Inc. (Aurora, OH, USA); antipyrine (= phenazon) from Aldrich Chemical Company Inc. (Milwaukee, WI, USA); midazolam from Hoffman-La Roche (Basle, Switzerland); procaine hydrochloride was a gift from the Helsinki University Pharmacy, Finland and timolol maleate from Orion Pharma (Espoo, Finland); hydroxyzine and buspirone were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle medium, nonessential amino acids, heat inactivated (+56°C for 30 min) fetal bovine serum, L-glutamine (200 mM), antibiotic mixture (1000 IU/mL penicillin G, 1000 μg/mL streptomycin), Dulbecco's phosphate-buffered saline, Hank's balanced salt solution (HBSS), and HEPES solution (10 mM) were purchased from Gibco Invitrogen Corp. (Life Technologies Ltd. Paisley, Scotland). 2-(*N*-morpholino)-ethanesulfonic acid was purchased from Sigma Chemical Co. [^{14}C]-mannitol (specific activity = 51.50 μCi/mmol) as a marker for membrane integrity was obtained from DuPont NEN (Boston, MA, USA). 3-(4,5 dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. and CytoTox 96® kit from Promega (Madison, WI, USA).

All the organic solvents and other chemicals for the analyses were of analytical or chromatographic grade. Acetonitrile was purchased from Rathburn (Walkerburn, Scotland) and water was purified in a Milli-Q water purification system (Millipore, Molsheim, France). Ammonium acetate and formic acid were purchased from Merck (Darmstadt, Germany).

Cell Culture

The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Cells were seeded at 6.8×10^4 cells/cm^2 on polycarbonate filter membranes with a pore size of 0.4 μm and growth area of 1.1 cm^2 in clusters with 12 wells (Corning Costar Corp., Cambridge, MA, USA). The cells were grown in a medium comprised of Dulbecco's modified Eagle medium containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, penicillin (100U/mL), and streptomycin (100 μg/mL). Cultures were maintained at 37°C in an incubator (BB 16 gas incubator, Heraeus Instruments GmbH, Germany) in an atmosphere of 5% CO_2, 95% air, and 95% relative humidity. The growth medium was changed three times a week until the time of use. Cells from passage numbers 34 to 37 were used for the transport experiments. The cell monolayers were used in experiments at the age of 23–27 days.

Permeability Studies

All the single-compound and n-in-one transport experiments across Caco-2 cell monolayers were conducted under so-called "sink conditions," that is, the quantity of transported compound in the acceptor chamber did not exceed 10% of the quantity in the donor compartment. The experiments were performed by the same person during three days so that the cocktails numbers 1 and 4; 2 and 3; and 5 and 6 were performed on the same day with Caco-2 cells of the same passage number and growing time. The permeability of single compounds (verapamil, propranolol, midazolam, hydroxyzine, buspirone, timolol, procaine, naproxen, ketoprofen, and antipyrine) and six different mixtures of five to 10 drugs (Table I) was studied in both AP-to-BL and BL-to-AP direction at apical pH of 5.5 and basolateral pH of 7.4.

Before the permeability experiments, the cell monolayers were washed twice with HBSS containing 10 mM HEPES, pH 7.4. After the washing procedure, the apical transport buffer (HBSS, containing 10 mM 2-(*N*-morpholino)-ethanesulfonic acid and pH adjusted to 5.5), and the basolateral transport buffer (HBSS, containing 10 mM HEPES) and pH adjusted to 7.4) were added. After 30 min of equilibration at 37°C in humidified atmosphere, the integrity of the monolayers was checked by measuring the transepithelial electrical resistance (TEER) by Millicell® ERS (Bedford, MA, USA). The TEER values varied between 470 and 540 Ω·cm^2 before the transport experiments. HBSS containing 0.05 mM of the studied compounds as single or in different sets of cocktails (Table I) was added to the apical (0.5 mL, pH 5.5) or the basolateral (1.5 mL, pH 7.4) compartment. Samples were obtained every 15 or 30 min over 90 or 120 min by moving the cell inserts into a new well with fresh medium (AP-to-BL direction) or by emptying the apical compartment by pipetting 0.5 mL (the whole volume of the apical compartment) and adding new medium (BL-to-AP direction). All the transport experiments were conducted in triplicate. The samples were kept at –22°C until they were analyzed.

The apparent permeability coefficients, P_{app} (cm/s), were calculated according to the equation:

$$P_{app} = \frac{(dQ/dt)}{A * 60 * C_0}$$

where dQ/dt is the amount of compound transported within a given time period; A is the surface area of the insert; 60 is the conversion from minutes to seconds; C_0 is the initial concentration of compound on the donor side. Data are presented as the average P_{app} (cm/s) ± SD from three monolayers. A ratio of the BL-to-AP/AP-to-BL of the P_{app} values was calculated in each case to reveal the possible involvement of active transport/efflux. At ratios other than unity, the possibility for ac-

Table I. Summary of Apparent Permeability Coefficients (P_{app}) for Compounds as Single or in Different Sets (1–6) of N-in-One Cocktails across Caco-2 Cell Monolayers: Apical-to-Basolateral (AP-to-BL); Basolateral-to-Apical (BL-to-AP) Direction; and Calculated Ratio BL-to-AP/AP-to-BL

Sample	Verapamil	Propranolol	Midazolam	Hydroxyzine	Timolol	Buspirone	Procaine	Naproxen	Ketoprofen	Antipyrine	Total Conc. mM
$P_{app} \cdot 10^6$ cm/s AP-to-BL[a]											
Single	1.82 ± 0.18	2.02 ± 0.08	14.8 ± 1.5	2.36 ± 0.16	1.02 ± 0.08	2.84 ± 0.46	1.94 ± 0.15	101 ± 7	81.6 ± 4.3	52.2 ± 2.5	0.05
1	1.00 ± 0.08	2.00 ± 0.25	20.5 ± 1.9	2.17 ± 0.28	1.09 ± 0.06	2.81 ± 0.33	3.90 ± 0.22	–	–	–	0.35
2	–	2.12 ± 0.09	16.8 ± 0.8	1.93 ± 0.12	1.16 ± 0.11	2.54 ± 0.08	2.54 ± 0.13	–	–	–	0.30
3	–	–	19.8 ± 1.8	2.41 ± 0.21	1.29 ± 0.03	3.10 ± 0.21	2.90 ± 0.17	–	–	–	0.25
4	–	–	18.4 ± 0.5	2.05 ± 0.07	1.01 ± 0.02	2.64 ± 0.08	3.55 ± 0.36	–	66.0 ± 6.2	–	0.30
5	–	–	17.3 ± 0.1	2.59 ± 0.18	1.63 ± 0.26	3.10 ± 0.31	1.28 ± 0.13	27.9 ± 2.4	35.8 ± 2.4	35.0 ± 2.3	0.40
6	1.98 ± 0.15	2.56 ± 0.13	17.5 ± 0.2	2.59 ± 0.11	1.54 ± 0.02	3.03 ± 0.19	2.97 ± 0.28	34.8 ± 1.5	44.5 ± 2.0	34.8 ± 1.6	0.50
$P_{app} \cdot 10^6$ cm/s BL-to-AP[a]											
Single	72.4 ± 3.1	76.1 ± 2.5	56.0 ± 1.0	68.7 ± 0.7	37.7 ± 4.1	76.1 ± 4.7	99.6 ± 1.6	4.26 ± 0.06	4.56 ± 0.02	59.4 ± 2.8	0.05
1	57.1 ± 3.4	57.5 ± 2.5	59.2 ± 4.1	60.1 ± 4.0	32.1 ± 1.3	68.6 ± 5.1	58.3 ± 2.8	–	–	–	0.35
2	–	69.4 ± 2.7	55.7 ± 1.9	52.4 ± 1.3	34.2 ± 1.4	72.7 ± 3.0	36.8 ± 4.3	–	–	–	0.30
3	–	–	57.6 ± 1.9	53.4 ± 1.6	37.3 ± 1.4	75.1 ± 1.9	26.6 ± 3.4	–	–	–	0.25
4	–	–	53.9 ± 1.4	47.2 ± 2.0	36.6 ± 0.7	60.6 ± 2.5	57.5 ± 7.4	–	5.35 ± 0.55	–	0.30
5	–	–	62.4 ± 1.1	53.4 ± 3.0	40.6 ± 2.3	67.8 ± 0.7	82.1 ± 5.1	3.89 ± 0.44	3.71 ± 0.28	53.4 ± 5.1	0.40
6	55.1 ± 3.2	68.3 ± 7.7	59.8 ± 1.9	48.8 ± 5.1	30.4 ± 4.8	64.1 ± 8.7	69.9 ± 3.0	4.30 ± 0.16	3.71 ± 0.08	50.8 ± 1.8	0.50
RATIO P_{app} BL-to-AP/AP-to-BL											
Single	39.8	37.7	3.78	29.1	37.0	26.8	51.3	0.04	0.06	1.14	
1	57.4	28.8	2.89	27.7	29.5	24.4	15.0	–	–	–	
2	–	32.7	3.32	27.2	29.5	28.6	14.5	–	–	–	
3	–	–	2.91	22.2	28.9	24.2	9.2	–	–	–	
4	–	–	2.93	23.0	36.2	23.0	16.2	–	0.08	–	
5	–	–	3.61	20.6	24.9	21.9	64.1	0.14	0.10	1.53	
6	27.8	26.7	3.42	18.8	19.7	21.2	23.5	0.12	0.08	1.46	

[a] The apparent permeability P_{app} was conducted at 50 μM donor drug concentration. The apical pH was 5.5 and basolateral pH 7.4. Results are expressed as means ± SD (n = 3).

tive transport in either AP-to-BL or Bl-to-AP direction exists (17).

After adding the donor solution, a sample was collected from the donor compartment to determine the actual initial concentration of each drug. Depletion of donor drugs during the course of the study was analyzed at the end of the transport study. Mass balance was evaluated based on the sum of the cumulative amount transported and amount remaining in the donor compartment against initial amount of donor. Typically, 80–90% of the initial drug content was accounted for, except for procaine (71%), where lower mass balance may be caused by compound retained in cellular structures or adsorbed to the plastic device.

After each experiment, TEER values between 470 and 660 $\Omega \cdot cm^2$ were measured. The monolayer status was further probed with ^{14}C-mannitol (a paracellular marker molecule). The monolayers were washed twice with fresh prewarmed HBSS, and mannitol in HBSS (pH 5.5 or 7.4) was added to the apical or basolateral compartment, respectively. After 60 min, 100-μL aliquots were removed from the receiver compartments, 4 mL of scintillation cocktail HiSafe 2 (Wallac Scintillation Products, Fisher Chemicals, Loughborough, UK) added, and the concentration of ^{14}[C]-mannitol determined by liquid scintillation counting (Wallac, Turku, Finland). The permeation rate of mannitol did not exceed 0.5% per 60 min.

Cellular Damage

MTT and lactate dehydrogenase (LDH) tests were performed to investigate the possible cellular damage caused by the drugs. MTT (thiazolyl blue) is a tetrazolium salt that is cleaved by mitochondrial dehydrogenases of living cells to give a dark-blue formazan product (18). Damaged or dead cells show a reduced or no dehydrogenase activity. The details for the MTT test have been described previously (19). The mitochondrial toxicity of the studied drugs as single compounds and different mixtures was calculated as the percent of vital activity compared with the blank.

LDH is a stable cytosolic enzyme that is released upon cell lysis into the extracellular fluid and can be detected with an enzymatic assay. The reactions with LDH will end up to the conversion of a tetrazolium salt into a red formazan product (20). The absorption of the formazan can be measured at λ 490 nm in a multiwell scanning spectrophotometer (Model 550 Microplate Reader, Bio-Rad, Japan). The cellular damage of the single compounds and mixtures was calculated as a percentage of the maximal cytotoxicity as compared to the blank.

None of the studied compounds showed any toxicity as single compounds or as different sets of cocktails (data not shown). The viability of Caco-2 cells was calculated to be >90% in all the cases, as compared with the corresponding controls.

Analytical Procedures

A standard stock mixture containing 0.1 mg/mL of each compound studied (Table II) was diluted to appropriate concentration with HBSS at pH 5.5 or 7.4. The samples were filtered by Millex-HV (0.45 μm) polyvinylidene diflouride membrane (Millipore Corp., Bedford, MA).

N-in-one analyses were performed with an LC-MS/MS system using atmospheric pressure ionization (API). The liquid chromatographic set-up consisted of an Agilent 1100 binary pump, a column switching system, and an autosampler (Hewlett-Packard GmbH, Waldbronn, Germany). The column was a Purospher STAR® C_{18} 5.5 × 2 mm with a particle size of 3 μm (Merck, Darmstadt, Germany) and the column temperature was set to +30°C. Flow rate of the mobile phase was 350 μL/min. The injection volume was 50 μL. Acetonitrile was chosen for organic mobile phase and 15 mM ammonium acetate at pH 3.5 (adjusted by formic acid) for aqueous phase after experimental optimization and Drylab® simulations. The gradient program was 0.1–1.5 min, 10% acetonitrile; 1.5–6.5 min, 10–100% acetonitrile; 6.5–10 min, 100% acetonitrile; 10.0–11.0 min, 100–10% acetonitrile; 11–20 min, 10% acetonitrile. The eluent was introduced to waste for 1.5 min, after which the flow was directed by column switching to MS. Before introduction to a Sciex API 3000 triple quadruple mass spectrometer equipped with an ion spray (Applied Biosystems, Sciex, Toronto, Canada) the flow was split with the ratio of 1:6.

Compounds were detected by positive ion multiple reaction monitoring. Ion spray voltage was 5500 V. Declustering potential, collisional energy, and collision cell exit potential

Table II. Precursor Ion and Product Ion (m/z) Used for Quantitation, within-Day and between-Day (4 Days) Precision (250 ng/mL), Limits of Detection, and the Linearity of the LC-ESI/MS/MS Method

Compound	Precursor/ product (m/z)	Within day precision % (n = 7)	Between-day precision % (n = 8)	Limit of detection (ng/mL)	Linearity r^2
Antipyrine	189/104	7.5	8.5	5	0.994
Naproxen	231/170	5.0	14.0	5	0.990
Ketoprofen	255/105	4.9	4.7	5	0.994
Propranolol	260/116	3.1	3.1	1.5	0.996
Procaine	237/100	16.4	23.9	1.5	0.990
Timolol	317/244	4.0	6.0	1	0.992
Midazolam	326/291	6.8	9.1	0.5	0.994
Hydroxyzine	375/201	7.0	8.8	0.5	0.990
Buspirone	386/122	5.8	11.9	1	0.992
Verapamil	455/165	3.0	3.5	1	0.998

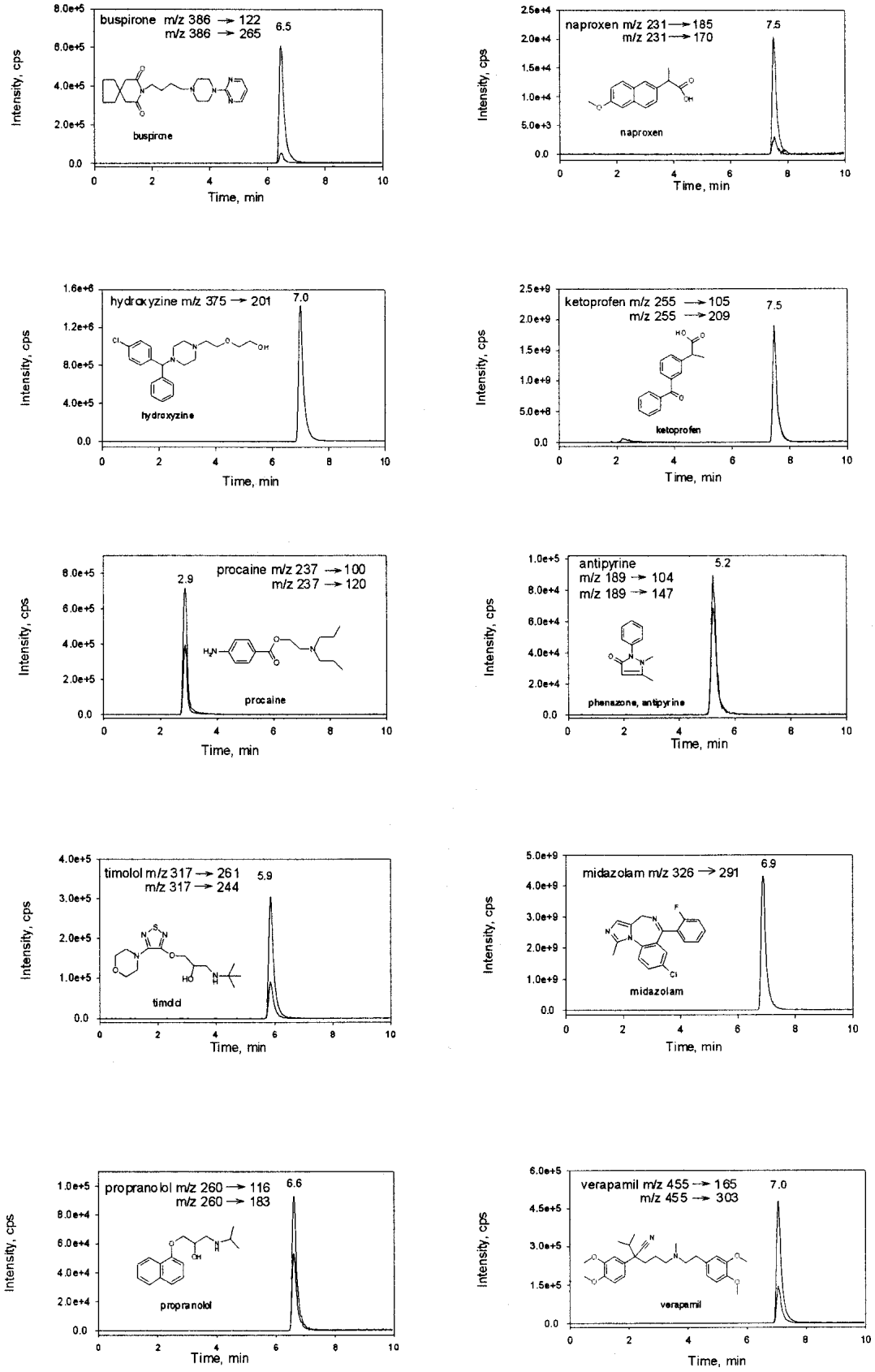

Fig. 1. The compounds used in Caco-2 experiments and the representative LC-ESI/MS/MS chromatograms

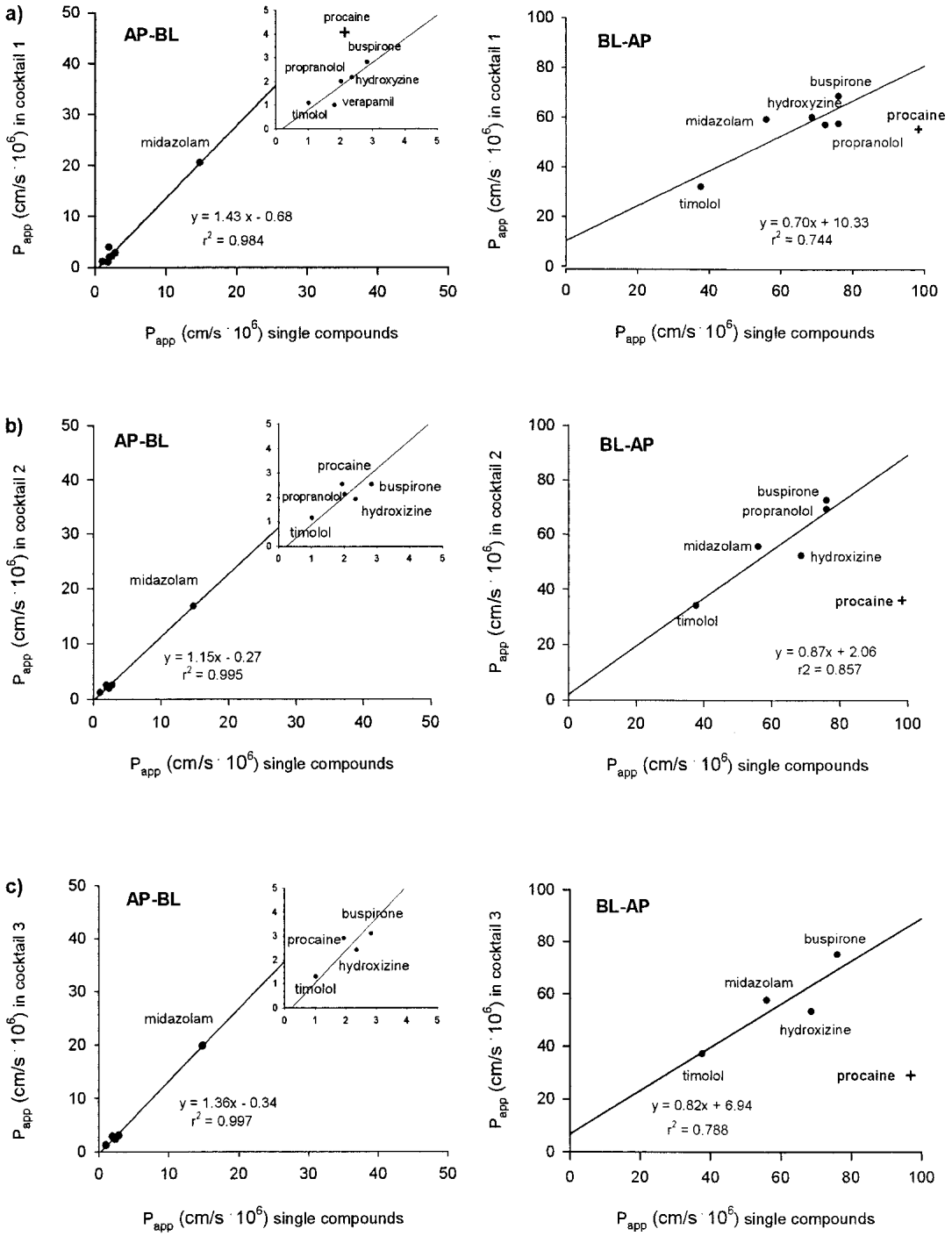

Fig. 2. Linear regression of the P_{app} values between the compounds as single and in cocktails: (a) cocktail 1; (b) cocktail 2; (c) cocktail 3; (d) cocktail 4; (e) cocktail 5; and (f) cocktail 6. The graph on left side represents the regression in the AP-to-BL and on the right side BL-to-AP direction. Linear regression of insets in (d), (e), and (f) have been recalculated without acidic compounds.

were individually optimized. Nitrogen, generated by a nitrogen generator (Generator system 75–720, Whatman Inc., Haverhill, MA) was used as a nebulizing, curtain and collision gas. The dwell time was 70 ms of each monitored ion pair. The precursor ions and monitored product ions are presented in Fig. 1. The data was collected and processed with Analyst 1.1 software.

RESULTS AND DISCUSSION

Analytical Section

The high specificity of tandem mass spectrometry allowed the use of fast gradient in LC separation. All the compounds were eluted within 8 min with good resolution (Fig.

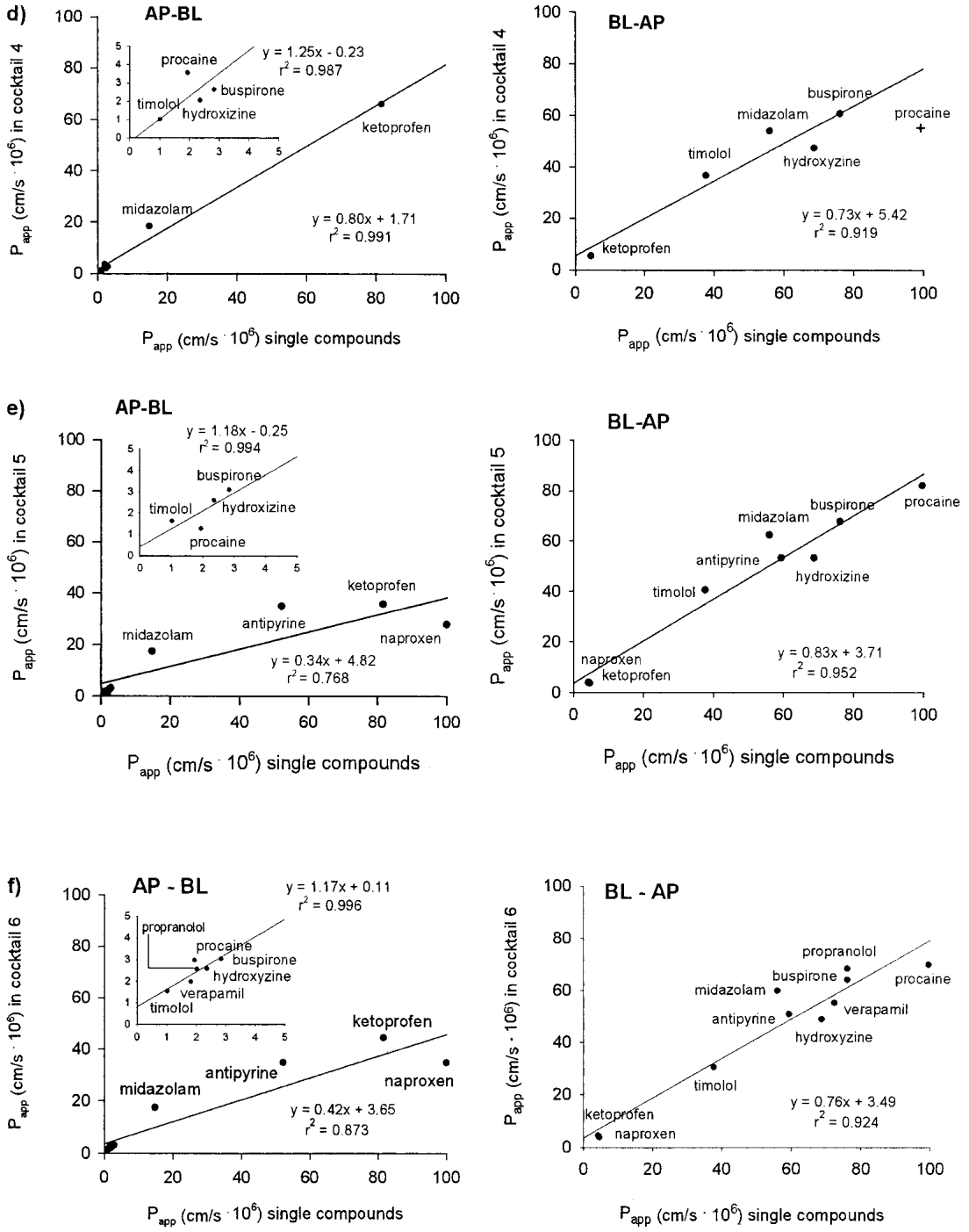

Fig. 2. Continued.

1). To avoid instrument contamination and, therefore, poor reproducibility and decreased sensitivity, it was necessary to use column switching. Salts and other possible nonvolatile materials in Caco-2 samples were flushed to waste over 1.5 min, after which the eluent was directed to MS. Without column switching the sensitivity decreased significantly because of the contamination of the sampling orifice of the API source after 10–20 runs. The use of column switching ensured also that no suppression of the ionization process was observed and good analytical reproducibility was achieved, also for the co-eluting compounds.

The ionization efficiency in the positive ion mode for all the compounds studied was good and the spectra showed very abundant $[M+H]^+$ ion (protonated molecule) with minimal fragmentation. However, for the acidic drugs, the addition of formic acid was necessary to achieve good ionization efficiency. The protonated molecule was chosen for the precursor ion and two specific and intense product ions were chosen for each compound for multiple reaction monitoring. The other product ion was used for quantitation (Table II) and the other was a qualifier. External standard method was used in the quantitative analysis instead of internal standard method

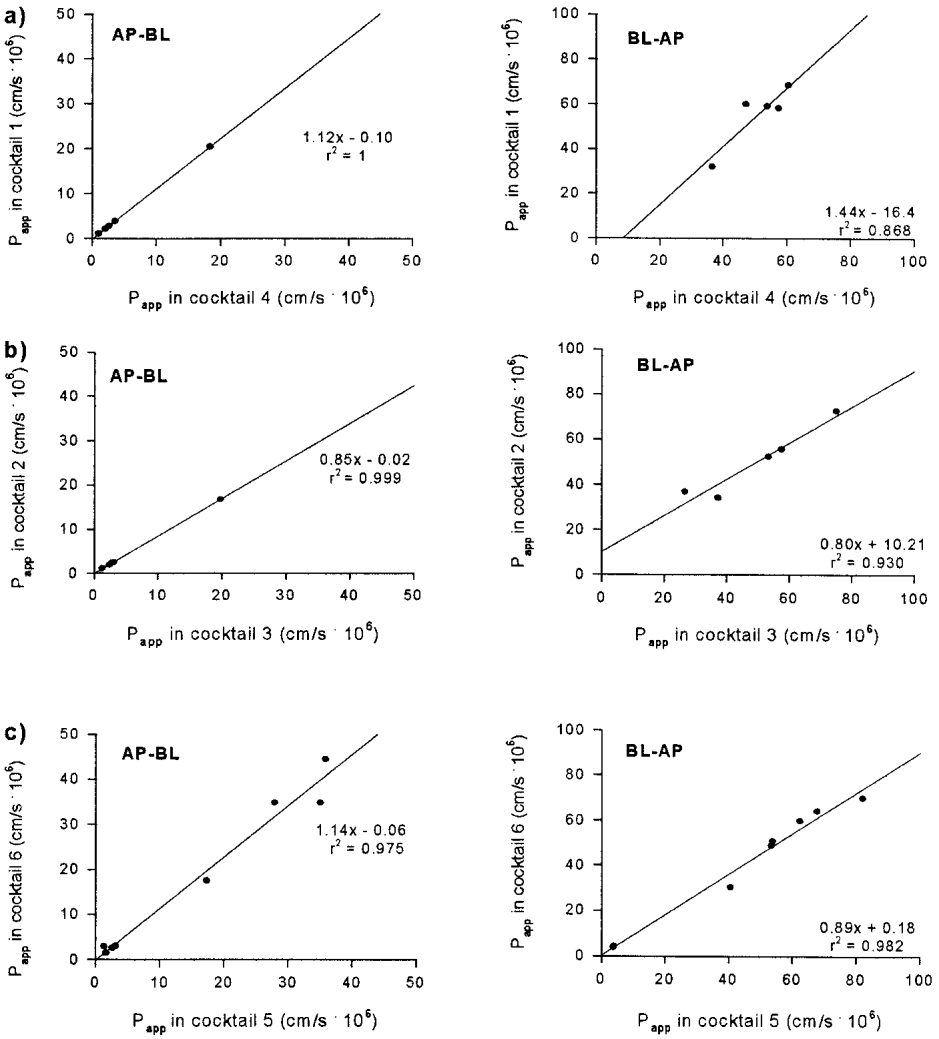

Fig. 3. Linear regression of the P_{app} values determined across two sets of cocktails: (a) cocktails 1 and 4; (b) cocktails 2 and 3; and (c) cocktails 5 and 6. The graphs on the left represent the regression in the AP-to-BL and on the right in the BL-to-AP direction.

because of the heterogeneous characters of the compounds studied.

The linearity of the method was studied between 5 and 2000 ng/mL (Table II). Calibration graphs were constructed using spiked samples of at least six different concentrations in each series to cover the quantitative range. The calibration curves for all the compounds showed good linearity with correlation coefficients (r^2) better than 0.990. The within-day precision of the method was evaluated with seven samples (250 ng/mL) and between-day precision with eight samples

Table III. Directional Permeabilities across Caco-2 Cell Monolayers at Apical pH of 5.5 and 7.4 and Basolateral pH of 7.4

	$P_{app} \cdot 10^6$ cm/s[a]					
Compound (0.25 mM)	AP-to-BL apical pH 5.5	BL-to-AP apical pH 5.5	Ratio[b] BL-to-AP/ AP-to-BL	AP-to-BL apical pH 7.4	BL-to-AP apical pH 7.4	Ratio[b] BL-to-AP/ AP-to-BL
Verapamil	1.74 ± 0.10	47.8 ± 1.4	27.5	23.3 ± 0.5	25.5 ± 0.6	1.09
Propranolol	4.46 ± 0.20	152 ± 9	34.1	32.5 ± 0.4	42.0 ± 1.8	1.29
Ketoprofen	92.2 ± 5.6	4.59 ± 0.61	0.05	24.2 ± 0.4	41.4 ± 0.1	1.71
Naproxen	123 ± 5	4.59 ± 0.61	0.04	29.1 ± 2.3	40.6 ± 1.7	1.40
Antipyrine	51.0 ± 2.8	62.2 ± 1.2	1.22	49.5 ± 1.7	58.3 ± 5.1	1.18

[a] The permeability was determined during a 120 min exposure to studied drugs. The drugs were added on either the apical or the basal side of the monolayers. Transport rates are means ± S.D., n = 4.
[b] The ratio was calculated according to P_{app} (BL to AP)/P_{app}(AP to BL).

within 4 days. Relative standard deviations in the within-day and between-day experiments remained typically below 10%, indicating acceptable precision of the analytical method. Procaine was an exception for which the within-day precision was 16.4%**.** The use of column switching allowed injection volumes of 50 μL that improved the limit of detection (LOD). Without column switching the injection volumes of 50 μl contaminated the instrument rapidly. The LODs (S/n = 5) of the drugs were between 0.5 and 5 ng/mL being higher for the acids than for the bases, which is expected in positive ion spray. All the LODs were very satisfactory for the Caco-2 experiments.

Transport of Compounds across Caco-2 Cell Monolayers

Correlation of Single Compounds vs. N-in-One Cocktails

The individual permeability data of the single compounds and the cocktails containing 5–10 compounds have been presented in Table I (AP-to-BL and BL-to-AP). All six cocktails included hydroxyzine, timolol, buspirone, and procaine, which are potential substrates of efflux transport systems (21,22). Each cocktail also contained midazolam, a substrate for CYP3A isoenzymes but a nonsubstrate for efflux transport (23,24), expected to exhibit passive permeability. Another passively permeating compound, antipyrine, was added to cocktails 5 and 6. Verapamil, a known inhibitor and substrate for P-gp (13), was included in cocktails 1 and 6 to probe whether inhibition of the efflux system enhanced AP-to-BL permeation of other P-gp substrates. Propranolol, another potential substrate/modulator for P-gp (25,26), was added to cocktails with (cocktails 1 and 6) and without (cocktail 2) verapamil to further investigate interactions within the efflux system. The acidic compounds ketoprofen (cocktails 4, 5, and 6) and naproxen (cocktails 5 and 6) were added to elucidate the functionality of the active H^+ co-transporter, especially in view of apparent interactions.

The P_{app} values from the experiments using single compounds were compared with those obtained from the different sets of cocktails (Fig. 2a–f). The individual permeabilities in the cocktails correlated strongly with those obtained from single experiments. The AP-to-BL permeabilities of the basic compounds hydroxyzine, timolol, buspirone, and midazolam in all the cocktails were extremely consistent with the results obtained using single compounds. This was evidenced by the linear slope values close to unity and high r^2 values. In cocktail 1, which contained seven compounds, including verapamil and propranolol, a somewhat higher slope was obtained (slope = 1.4), indicating slightly higher permeabilities of the individual compounds present in the cocktail (Fig. 2a). The P_{app} values of all the compounds (including ketoprofen) in cocktail 4 correlated well with the P_{app} values of the single compounds (Fig. 2d). In cocktails 5 and 6, the correlation was poorer, as evidenced by slope values of 0.34 and 0.42 and r^2 values of 0.77 and 0.87, respectively. However, this may be explained by the reduced permeability of the two acidic compounds (see insets of Fig. 2e and f), indicating competitive utilization of a shared active transport system. In cocktails 1–4, the BL-to-AP permeabilities of all the compounds, with the exception of procaine, correlated well with the permeabilities of individual single compounds. In cocktails 5 and 6, the permeability of all the eight and ten compounds in the cocktails was adequately consistent with the permeability of the same compounds as single (slopes of 0.83 and 0.76, r^2 values of 0.95 and 0.92).

Overall, verapamil, propranolol, midazolam, hydroxyzine, timolol, buspirone, and antipyrine showed extremely similar permeabilities as single compounds and in the different cocktails (Table I). Midazolam, present in each cocktail as a model passive permeant, demonstrated the excellent reproducibility of the experiments. The P_{app} values of midazolam were 18.4 ± 1.7 and 58.1 ± 3.4 ($\times 10^6$ cm/s; mean ± SD, n = 18) in the AP-to-BL and BL-to-AP directions, respectively. The apparent polarity of midazolam permeability may be ascribed to increased extent of ionization in the apical compartment, which will be further discussed below.

Within-Day Comparisons

When the P_{app} values from experiments performed on the same day are compared, a further decrease in the degree of variation was observed as compared to the variation of single compounds / different cocktails across different days. When cocktails 1 and 4, 2 and 3, and 5 and 6 are compared as pairs by linear regression (Fig. 3a–c), they exhibit a very strong correlation in both directions (AP-to-BL and BL-to-AP), as suggesting the absence of interactions. In addition, the correlation between cocktails 1 and 4 shows the negligible effects of verapamil and propranolol in cocktail 1 on the permeability of the other basic compounds in cocktail 4, where no verapamil or propranolol was present. The situation was similar for cocktails 2 and 3, and 5 and 6. The only difference between the cocktails 2 and 3 was the presence of propranolol in cocktail 2. The slope of 0.85 and $r^2 = 0.99$ indicates that the permeability values of the compounds lie very near to each other in both the cocktails, and that propranolol did not affect the permeability of other drugs (Fig. 3b). Again, the permeability values in cocktail 6 (verapamil and propranolol being present with all the other basic and acidic compounds) correlated strongly with those present in cocktail 5, where neither verapamil nor propranolol was present (Fig. 3c). Note also that this comparison does not reveal the interaction between naproxen and ketoprofen because both are contained in the cocktails.

The apparent lack of effects of verapamil and propranolol on the permeability of other compounds in the cocktails may be the result of the relatively high donor concentrations. Inability to detect polarized permeation based on the efflux has been ascribed to compounds exhibiting high passive permeabilities and/or saturation of the efflux system (17,27). However**,** too low concentrations of verapamil may also explain the results, as previously reported experiments with verapamil as an inhibitor of the P-gp efflux system have used concentrations in the range of 0.2–0.5 mM (27,28). In these studies the P-gp inhibition was used to ascertain the passive permeability of individual compounds.

The BL-to-AP/AP-to-BL Ratio

The BL-to-AP/AP-to-BL ratios of the P_{app} values are shown in Table I. For the basic compounds, verapamil, propranolol, hydroxyzine, timolol, and buspirone, the BL-to-AP/AP-to-BL ratios varied between 18 and 57. For the weak bases antipyrine and midazolam (pK_a values of 1.2 and 6.2,

respectively), the BL-to-AP permeability was 1.1- and 3.8-fold higher, respectively, than the AP-to-BL permeability. The acidic compounds, ketoprofen and naproxen, showed a higher permeability in the AP-to-BL than BL-to-AP direction (low ratios of BL-to-AP/AP-to-BL, 0.06 and 0.04, respectively) as individual compounds.

Verapamil, propranolol, hydroxyzine, and timolol are considered potential substrates/modulators for the P-gp efflux system (13,21,22,25,26). The permeability of these substrates is characterized by a directional difference in favor of a higher BL-to-AP permeation. When the pH of the AP and BL transport medium is the same, the permeability of passively diffused compounds should be equal in both directions (17), whereas substrates of P-gp efflux system may display only a small increment in the BL-to-AP permeability. Hence, without the pH gradient, substrates have been classified as likely substrates of the P-gp efflux system at P_{app} ratios (BL-to-AP vs. AP-to-BL) higher than 2.

When the pH of the apical transport medium was lowered to 5.5 and the basolateral medium was maintained at 7.4, the permeability profiles of the studied compounds changed dramatically. The basic compounds, verapamil, propranolol, midazolam, hydroxyzine, timolol, buspirone, and procaine, exhibited higher BL-to-AP than AP-to-BL permeability, nicely according to the pH partition theory (Table I). The relative contribution of the unionized form of a base with a pK_a of about 8 is below 0.1% at AP (pH 5.5) and about 5% at BL (pH 7.4). This causes a strong increment in the BL-to-AP permeability, and thus increases the ratio BL-to-AP/AP-to-BL for both substrates and nonsubstrates of the P-gp efflux system. For example, the ratio between BL-to-AP and AP-to-BL permeabilities for verapamil and propranolol was reduced from 28 to 1.1 and from 34 to 1.3, respectively, when the apical pH was changed from 5.5 to 7.4 (Table III). These experiments were performed at 0.25 mM concentration, which may explain the low ratios without pH gradient. For many substrates of P-gp, passive permeability will dominate at high drug concentrations, if both the affinity to P-gp and the passive permeability is high as in the case of verapamil (27). The apparent passive permeation of propranolol corroborates recent studies, where it has been described as a nonsubstrate for transport, but a potential modulator of ATPase activity (12,17).

The unionized form of antipyrine at the both apical pH values used is practically 100%, therefore no directional difference was expected according to the pH partition theory (Tables I and III). In fact, the BL-to-AP/AP-to-BL ratio near 1.0 for antipyrine indicates that the transport across Caco-2 monolayers consists entirely of passive diffusion (17,29). Midazolam, although assumed to be a passive permeant, displayed a BL-to-AP permeability that was 3- to 4-fold higher than the AP-to-BL permeability (Table I). However, corrected permeability values based on the unionized fraction of the compound at respective pH more than compensates for the difference (6).

At apical pH of 5.5, weak acids with a pK_a value higher than the apical pH (ketoprofen, pK_a 5.9) are to a high degree unionized, which favors the AP-to-BL permeability. For stronger acids like naproxen (pK_a 4.1), the relative contribution of the unionized form is only 4% in the apical chamber even at pH 5.5. This should lead to a relatively low AP-to-BL permeability, if no active transport mechanism were involved. A directional difference of this magnitude (0.04, Table I) suggests a clear presence of an active transport mechanism for naproxen, as well as for ketoprofen (0.06). The pH dependence of the transcellular AP-to-BL transport is ascribed mainly to the carrier-mediated H^+ cotransporter (7,30). The permeability of ketoprofen and naproxen at AP pH 7.4 was much lower in AP-to-BL direction and higher in BL-to-AP direction than it was at AP pH 5.5 (Tables III and I). According to these results, the carrier-mediated H^+ co-transporter seems to function more effectively at apical pH 5.5 than 7.4. In cocktails 5 and 6, where both the acidic drugs were present, possible competition of the co-transport mechanism caused an increment in the ratio of P_{app} values, when the AP-BL permeability was strongly reduced (Table I) as compared with the results of naproxen or ketoprofen alone.

CONCLUSIONS

In conclusion, we have demonstrated that the use of LC-ESI/MS/MS technology provides a fast and reliable tool in detecting small amounts of heterogeneous compounds in Caco-2 samples. Caco-2 cell culture is an excellent absorption model for n-in-one permeability studies for passively permeated drugs, actively effluxed basic compounds, and actively absorbed monocarboxylic acid-like drugs. Adjustment of the pH in the AP chamber to 5.5 enhances the use of the experimental model. This way, the Caco-2 cell monolayers mimic the conditions *in vivo* and give more reliable information about the absorption of the drugs across the enterocyte membrane. The use of n-in-one cocktails is an extremely suitable method for improving the throughput of Caco-2 cell permeability tests even of highly heterogeneous drugs. The good correlation between the P_{app} values of compounds as single and in mixtures as well as in different sets of mixtures most probably enables permeability evaluation also for new drug candidates, although potential interactions should always be borne in mind. The use of n-in-one cocktails in cell culture experiments also reduces the normal variation caused by different passage numbers and the age of the cell culture. If the experiments are performed during the same day and by the same person, the variation is further minimized.

ACKNOWLEDGMENTS

We thank the National Technology Agency, Orion Pharma Ltd, Juvantia Pharma, United Laboratories Ltd and Hormos Medical (Finland) for financial support and encouragement.

REFERENCES

1. M. Markowska, R. Oberle, S. Juzwin, C.-P. Hsu, M. Gryszkiewicz, and A. J. Streeter. Optimizing Caco-2 cell monolayers to increase throughput in drug intestinal absorption analysis. *J. Pharmacol. Toxicol. Methods* **46**:51–55 (2001).
2. C. Tannergren, P. Langguth, and K.-J. Hoffmann. Compound mixtures in Caco-2 cell permeability screens as a means to increase screening capacity. *Pharmazie* **56**:337–342 (2001).
3. Z. Wang, C. Hop, K. Leung, and J. Pang. Determination of in vitro permeability of drug candidates through a Caco-2 cell monolayer by liquid chromatography/ tandem mass spectrometry. *J. Mass Spectrom.* **35**:71–76 (2000).
4. H.-Z. Bu, M. Poglod, R. Micetich, and J. Khan. High-throughput Caco-2 cell permeability screening by cassette dosing and sample pooling approaches using direct injection/on-line guard cartridge

extraction/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **14**:523–528 (2000).
5. C. Bailey, P. Bryla, and W. Malick. The use of intestinal epithelial cell culture model, Caco-2, in pharmaceutical development. *Adv. Drug Deliv. Rev.* **22**:85–103 (1996).
6. K. Palm, K. Luthman, J. Ros, J. Gråsjö, and P. Artursson. Effect of molecular charge on intestinal epithelial drug transport: pH-dependent transport of cationic drugs. *J. Pharmacol. Exp. Ther.* **291**:435–443 (1999).
7. T. Ogihara, I. Tamai, H. Takanaga, Y. Sai, and A. Tsuji. Stereoselective and carrier-mediated transport of monocarboxylic acids across Caco-2 cells. *Pharm. Res.* **13**:1828–1832 (1996).
8. S. Yamashita, T. Furubayashi, M. Kataoka, T. Sakane, H. Sezaki, and H. Tokuda. Optimized conditions for prediction of intestinal drug permeability using Caco-2 cells. *Eur. J. Pharm. Sci.* **10**:195–204 (2000).
9. H. Takanaga, I. Tamai, and A. Tsuji. pH-dependent and carrier-mediated transport of salicylic-acid across Caco-2 cells. *J. Pharm. Pharmacol.* **46**:567–570 (1994).
10. A. Shapiro and V. Ling. Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. *Eur. J. Biochem.* **250**:130–137 (1997).
11. C. Pascaud, M. Garrigos, and S. Orlowski. Multidrug resistance transporter P-glycoprotein has distinct but interacting binding sites for cytotoxic and reversing agents. *Biochem. J.* **333**:351–358 (1998).
12. E. Wang, C. Casciano, R. Clement, and W. Johnson. Two transport binding sites of P-glycoprotein are unequal yet contingent: initial rate kinetic analysis by ATP hydrolysis demonstrates intersite dependence. *Biochim. Biophys. Acta* **1481**:63–74 (2000).
13. S. Orlowski, L. Mir, J. Belehradek, and M. Garrigos. Effects of steroids and verapamil on P-glycoprotein ATPase activity: progesterone, desoxycorticosterone, corticosterone and verapamil are mutually non-exclusive modulators. *Biochem. J.* **317**:515–522 (1996).
14. T. Tsuruo, H. Iida, S. Tsukagoshi, and Y. Sakurai. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res.* **41**:1967–1972 (1981).
15. J. Hunter, B. H. Hirst, and N. L. Simmons. Drug absorption limited by P-glycoprotein- mediated secretory drug transport in human intestinal epithelial Caco-2 cell layers. *Pharm. Res.* **10**: 743–749 (1993).
16. P. Anderle, E. Niederer, W. Rubas, C. Hilgendorf, H. Spahn-Langguth, H. Wunderli-Allenspach, H. Merkle, and P. Langguth. P-glycoprotein (P-gp) mediated efflux in Caco-2 cell monolayers: the influence of culturing conditions and exposure on P-gp expression levels. *J. Pharm. Sci.* **87**:757–762 (1998).
17. J. Polli, S. Wring, J. Humphreys, L. Huang, J. Morgan, L. Webster, and C. Serabjit-Singh. Rational use of in vitro P-glycoprotein assays in drug discovery. *J. Pharmacol. Exp. Ther.* **299**:620–628 (2001).
18. T. Mosmann. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**:55–63 (1983).
19. L. Gyürösiová, L. Laitinen, J. Raiman, J. Cižmárik, and E. Sedlárová. and J. Hirvonen. Permeability profiles of malkoxysubstituted pyrrolidinoethylesters of phenylcarbamic acid across Caco-2 monolayers and human skin. *Pharm. Res.* **19**:162–168 (2002).
20. T. Decker and M. L. Lohmann-Matthes. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J. Immunol. Meth.* **115**:61–69 (1988).
21. W. M. Kan, Y. T. Liu, C. L. Hsiao, S. Y. Shieh, J. H. Kuo, J. D. Huang, and S. F. Su. Effect of hydroxyzine on the transport of etoposide in rat small intestine. *Anticancer Drugs* **12**:267–273 (2001).
22. T. Terao, E. Hisanaga, Y. Sai, I. Tamai, and A. Tsuji. Active secretion of drugs from the small intestinal epithelium in rats by P-glycoprotein functioning as an absorption barrier. *J. Pharm. Pharmacol.* **48**:1083–1089 (1996).
23. M. F. Paine, D. D. Shen, K. L. Kunze, J. D. Perkins, C. L. Marsh, J. P. McVicar, D. M. Barr, B. S. Gillies, and K. E. Thummel. First-pass metabolism of midazolam by the human intestine. *Clin. Pharmacol. Ther.* **60**:14–24 (1996).
24. R. Kirn, C. Wandel, B. Leake, M. Cvetkovic, M. Fromm, P. Dempsey, M. Roden, F. Belas, A. Chaudhary, D. Roden, A. Wood, and G. Wilkinson. Interrelationship between substrates and inhibitors of human CYP3A and P-glycoprotein. *Pharm. Res.* **16**:408–413 (1999).
25. J. Hunter and B. H. Hirst. Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Adv. Drug Deliv. Rev.* **25**:129–157 (1997).
26. J. Yang, K.-J. Kim, and V. H. L. Lee. Role of P-glycoprotein in restricting propanolol transport in cultured rabbit conjunctival epithelial cell layers. *Pharm. Res.* **17**:533–538 (2000).
27. S. Doppenschmitt, H. Spahn-Langguth, C. G. Regårdh, and P. Langguth. Role of P-glycoprotein-mediated secretion in absorptive drug permeability: an approach using passive membrane permeability and affinity to P-glycoprotein. *J. Pharm. Sci.* **88**:1067–1072 (1999).
28. J. Gao, O. Murase, R. Schowen, J. Aube, and R. T. Borchardt. A functional assay for quantitation of the apparent affinities of ligands of P-glycoprotein in Caco-2 cells. *Pharm. Res.* **18**:171–176 (2001).
29. J. Karlsson, S.-M. Kuo, J. Ziemniak, and P. Artursson. Transport of celiprolol across human intestinal epithelial (Caco-2) cells: mediation of secretion by multiple transporters including P-glycoprotein. *Br. J. Pharmacol.* **110**:1009–1016 (1993).
30. A. Tsuji, H. Takanaga, I. Tamai, and T. Terasaki. Transcellular transport of benzoic acid across Caco-2 cells by a pH-dependent and carrier-mediated transport mechanism. *Pharm. Res.* **11**:30–37 (1994).