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Compound mixtures in Caco-2 cell permeability screens as a means to increase screening capacity

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The purpose of this investigation was a study of simultaneous permeability measurement using compound mixtures (cassette dosing) as an alternative to single compound evaluation in order to increase the capacity of screens for intestinal drug permeability. Drug transport across Caco-2 monolayers was studied, both in the apical to basolateral and the basolateral to apical direction. The apparent permeability coefficients for ten compounds displaying different intestinal transport mechanisms were determined, first as single compounds and then as components of a mixture. Seven β-adrenoceptor antagonists and baclofen were analysed simultaneously using reversed phase HPLC with UV detection, D-glucose and mannitol were measured by scintillation counting. The results indicated that the P_{app} from the mixture as donor phase correlated well with that of the single compounds and merely small changes in the P_{app} of each compound were observed between the single compound and mixture experiments. This minor variation resulted in a change in rank-order of the poorly permeable compounds in the mixture, however, without affecting their association with the permeability class according to the biopharmaceutics classification system (BCS). It can be concluded that the use of compound mixtures is a suitable method for improving the capacity in permeability screens. Further improvement of the throughput may be expected upon automatisation of permeability measurements using robotics combined with increased selectivity using LC-MS analysis.

1. Introduction

Within the discovery process for new molecular entities in the pharmaceutical industry, more and more emphasis is put on the evaluation and optimisation of biopharmaceutical properties of compounds at an early stage of discovery [1, 2]. This development is due to several underlying causes one of which is the tendency to synthesise larger and more lipophilic compounds in the lead optimisation procedure to achieve high *in vitro* binding to the pharmacological targets of interest. In addition, the increased synthesis of biopharmaceutical peptides, proteins and oligonucleotides is apparent and these compounds may fail to result in any pharmacological response following peroral administration due to their poor intestinal permeability and extreme instability in biological systems.

Among the numerous preformulation tests to characterise drug candidates, the measurement of intestinal permeability is considered to be a key parameter to estimate peroral absorption in man [3–6]. Several techniques are available to experimentally determine intestinal permeability, but the use of intestinal cell cultures has turned out to be very widespread due to the reproducibility and the relative ease of the method.

In order to characterise the increasing number of substances emerging from combinatorial chemistry, there is a need to increase the capacity in permeability screening [5, 7]. One possible way to accomplish higher throughput of screening compounds is the automatisation of permeability measurements using robotics [8]. Another approach, which requires selective and sensitive analytical methods, is to perform several permeability measurements simultaneously using compound mixtures (cassette dosing). This new type of permeability screens has recently been proposed in the study of structure-transport relationships [7].

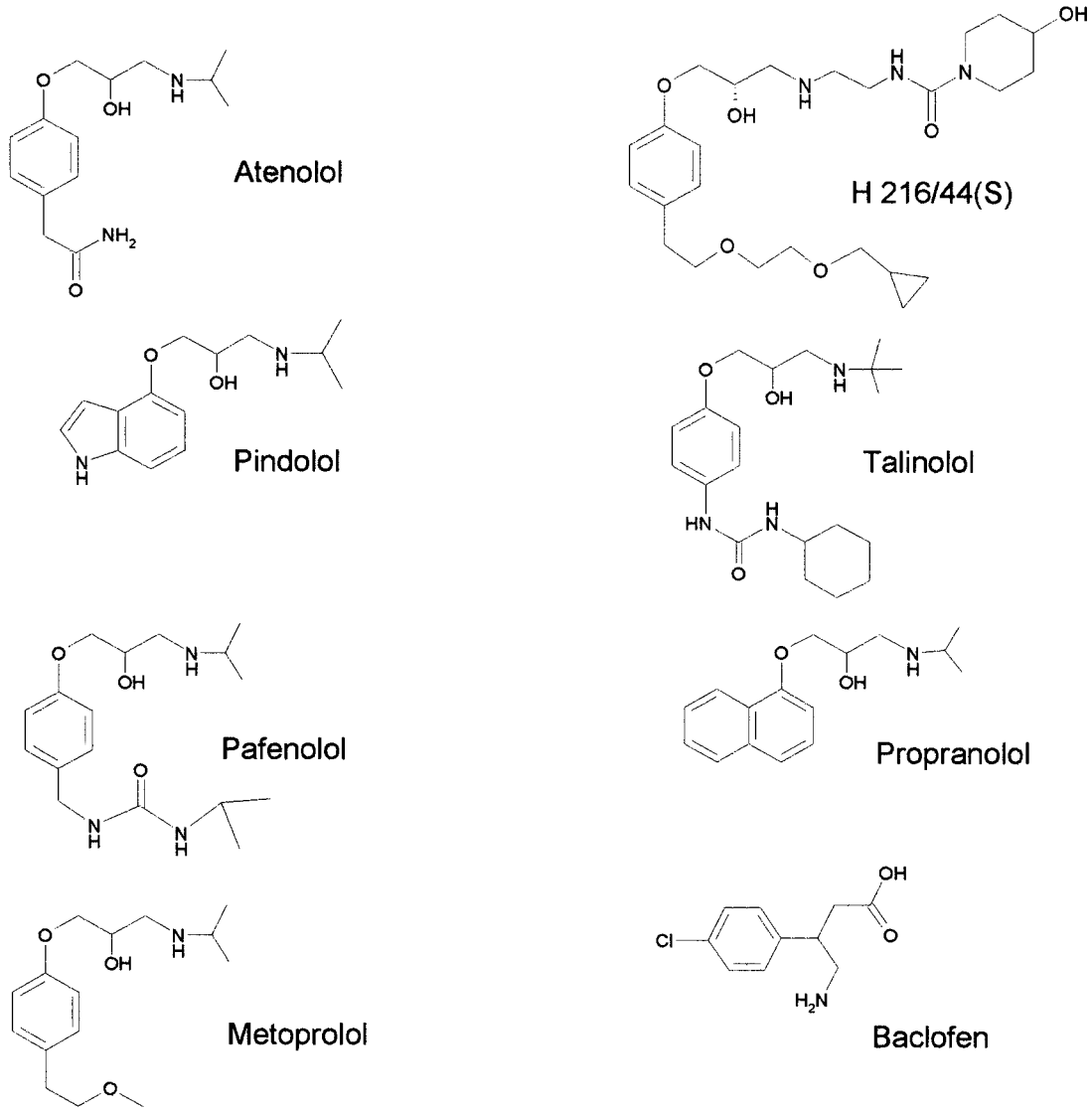
The objective of this study was to compare the apparent permeability coefficients (P_{app}) across Caco-2 cell monolayers for ten compounds that undergo different intestinal transport mechanisms. Initially, the compounds were studied as single entities and in the second phase as components of a mixture performing permeation experiments in both directions.

2. Investigations, results and discussion

2.1. LC-UV analysis

The physicochemical parameters of the β-adrenoceptor antagonists and baclofen are shown in Table 1, respectively. The compounds were chosen because they are transported by different transport mechanisms across the intestinal epithelium. Atenolol, mannitol, pindolol, metoprolol and propranolol are transported by passive diffusion, while baclofen and D-glucose are known to be transported by the neutral amino acid transporter and the glucose transporter, respectively. Talinolol, pafenolol and H 216/44(S) have been reported as substrates of the P-glycoprotein efflux system [1, 9].

Fig. 1 shows a representative chromatogram demonstrating separation of the seven β-adrenoceptor antagonists and baclofen in a donor mixture solution in a single run. The compounds were separated according to their lipophilicity, i.e. atenolol, the most hydrophilic β-adrenoceptor antagonist eluted first while the lipophilic propranolol and talinolol exhibited long retention times. The molar extinction coefficient of baclofen was lower as compared to the β-adrenoceptor-antagonists which resulted in decreased analytical response. The limits of quantification (LOQ) were set at 0.25 μM and 1 μM for all the β-adrenoceptor antagonists and baclofen, respectively. It should be noted that some of the compounds (e.g. pafenolol and talinolol) displayed signal to noise ratios significantly above 4 at 0.25 μM suggesting that their limit of quantification could be even lower. However, their true LOQ were not established because sample concentrations were in the μmolar range. Moreover the concentrations of some poorly permeable compounds (e.g. atenolol) were near their LOQ. The assay results for all compounds investigated are given in Table 1. The calibration curves were linear within the concentration range from the limit of quantification to 100 μM with r^2 values of 0.99–1 and no point of the calibration curve had a C.V. above 20%.



2.2. Drug permeabilities

The cassette dosing approach, i.e. the use of compound mixtures, has previously been used in drug discovery to screen for pharmacokinetic parameters (i.e. Cl, $t_{1/2}$, V) in animals *in vivo* [10–12].

In order to evaluate if permeability testing of compound mixtures could be a suitable approach to increase capacity in permeability screening, the P_{app} obtained from experiments with the single compound and the mixture were compared. In theory, compounds transported by passive diffusion across the Caco-2 monolayer should yield identical

Table 1: Physicochemical properties and HPLC validation data of the seven β-adrenoceptor antagonists and baclofen

Compd.	M[a]	ClogP[b]	K[c]	H bonds[d]		LOQ[e]	C.V.[e]	r^{2f}
	(g/mol)			Donors	Acceptors	(mM)		
Atenolol	266.3	−0.11	2.5	4	5	0.25	3.3	1
Baclofen	213.7	−1.33	5.7	3	3	1.0	7.9	0.9980
Pindolol	248.3	1.67	6.2	3	4	0.25	9.1	0.9999
Pafenolol	337.5	1.67	6.3	4	6	0.25	4.0	1
Metoprolol	267.4	1.2	7	2	4	0.25	5.1	0.9999
H 216/44 (S)	479.6	0.34	7.8	4	9	0.25	18.3	1
Talinolol	363.5	3.15	8.2	4	6	0.25	11.9	0.9999
Propranolol	259.4	2.75	8.7	2	3	0.25	10.4	0.9999

[a] Molecular weight
[b] Calculated octanol/water coefficient by CLOGP3 computer program from Daylight Chemical Information Systems, Inc. of Irvine, CA
[c] The capacity factor
[d] The number of hydrogen bond donors and acceptors
[e] Limits of quantification, set at this concentration, defined by a signal to noise ratio of 4 and a coefficient of variation (C.V.) < 20% (N = 5)
[f] Linear regression coefficients of the calibration curves in the concentration range LOQ-100 nM (N = 2)

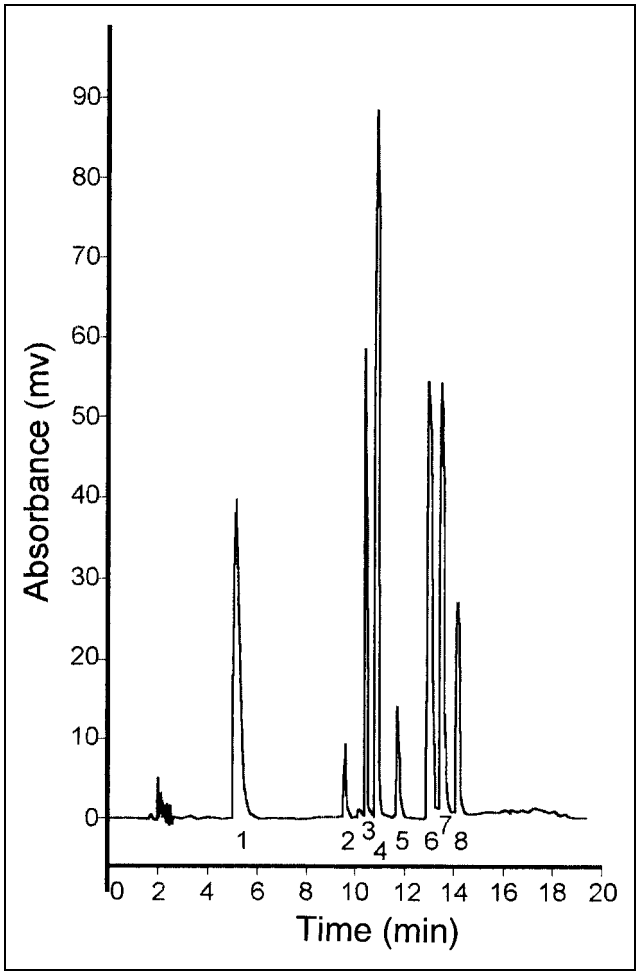


Fig. 1: HPLC separation and detection at 270 nm of seven β-adrenoceptor antagonists and baclofen in a donor solution (HBSS buffered with 25 mM MES to pH 6.5). Figures in the chromatogram refer to the identity of the compounds which were present at the following concentrations: 1 atenolol (1 mM); 2 baclofen (1 mM); 3 pindolol (0.1 mM); 4 pafenolol (1 mM); 5 metoprolol (0.1 mM); 6 H 216/44(S) (1 mM); 7 talinolol (1 mM); 8 propranolol (0.1 mM)

permeability values in a mixture as compared to a single compound study. The P_{app} value for compounds transported by a carrier-mediated process, however, may be altered when studied in a mixture due to saturation or inhibition of the transporter by another component in the mixture.

The apparent permeability coefficients, P_{app}, obtained from the apical to basolateral and basolateral to apical experiments are shown in Table 2. The TEER values obtained were 1500–1900 $\Omega \times cm^2$ demonstrating the intactness of the monolayers prior to the experiments. Furthermore the permeabilities of mannitol were below 0.2×10^{-6} cm/s, indicating intact monolayers during the experiments, except for two experiments with mixtures (see Table 2). In general the graphs of cumulative amount transported against time produced r^2 values above 0.9.

The P_{app} values obtained in the apical to basolateral experiments show that the P_{app} of each compound varied to some extent between single compounds and their mixtures (Table 2). The degree of variation, however, should be of minor concern in a screening situation with focus on rank order of various new chemical entities. Interestingly, P_{app}, of mannitol increased by a factor of 8 in the mixture (apical to basolateral direction) and this could not be explained by a general increase in paracellular permeability, since atenolol, another compound that is primarily transported via the paracellular pathway showed a slight decrease in permeability when studied in a mixture. The permeability behaviour of the P-glycoprotein substrates pafenolol and H 216/44(S) were not substantially changed when studied in a mixture as compared to the single-type compounds. For talinolol, however, there was a tendency towards higher absorptive permeability values for the compounds in a mixture and lower permeabilities in the secretory direction possibly due to a competition for the secretory carrier by the other components in the mixture. No obvious changes were observed for the permeabilities of baclofen and D-glucose, which are partly transported by a carrier mediated process. As shown in Table 2, the rank-order of the highly permeable pindolol, metoprolol, propranolol and D-glucose remained unaltered in the mixture as compared to the result with the single compound measurement. The rank-order of the poorly permeable compounds, however, was changed in the mixture but this was mainly due to very small changes in P_{app}, which were not high enough to alter their permeability class according to the Biopharmaceutical Classification System (BCS) [13]. As shown in Fig. 2, the P_{app} obtained in the mixture studies correlate strongly with the P_{app} of the single compounds both in the apical to basolateral and basolateral to apical direction ($r^2 = 0.9877$ and 0.9748, respectively). This statistical operation is the most commonly used validation procedure for cassette dosing [11, 12]. The results

Table 2: Apparent permeability coefficients (P_{app}) of the compounds measured individually or simultaneously as components of a mixture both in the apical to basolateral to apical direction

Compd.	Conc. (mM)	P_{app} ($cm/s \times 10^{-6}$) Apical to basolateral				P_{app} ($cm/s \times 10^{-6}$) Basolateral to apical			
		Single[a]	Rank[b]	Mixture[c]	Rank[b]	Single[a]	Rank[b]	Mixture[a]	Rank[b]
Atenolol	1.0	0.118 ± 0.026	**6**	0.033 ± 0.013	**10**	0.088 ± 0	**9**	0.207 ± 0.051	**10**
Baclofen	1.0	0.206 ± 0.026	**5**	0.277 ± 0.11	**6**	0.177 ± 0	**8**	0.384 ± 0.059	**7**
Pindolol	0.1	11.80 ± 1.28	**4**	8.85 ± 0	**4**	13.57 ± 0.51	**4**	13.57 ± 0.51	**3**
Pafenolol	1.0	0.074 ± 0.013	**8**	0.044 ± 0	**9**	0.678 ± 0.051	**6**	0.413 ± 0.051	**6**
Metoprolol	0.1	23.60 ± 2.55	**2**	20.46 ± 2.12	**2**	30.38 ± 3.58	**2**	35.10 ± 3.99	**2**
H 216/44 (S)	1.0	0.066 ± 0	**10**	0.166 ± 0.038	**8**	0.295 ± 0.051	**7**	0.324 ± 0.051	**8**
Talinolol	1.0	0.074 ± 0.013	**8**	0.188 ± 0.003	**7**	4.13 ± 0.51	**5**	0.885 ± 0	**5**
Propranolol	0.1	37.61 ± 2.21	**1**	27.66 ± 2.21	**1**	50.47 ± 2.70	**1**	61.95 ± 7.24	**1**
D-glucose	10.0	15.49 ± 0	**3**	9.96 ± 1.28	**3**	19.47 ± 1.77	**3**	13.57 ± 0.51	**3**
Mannitol	0.1	0.096 ± 0.013	**7**	0.791 ± 0.494	**5**	0.077 ± 0.005	**10**	0.295 ± 0.051	**9**

[a] Values indicate the mean apparent permeablity coefficient, P_{app}, ± standard devivation for N = 3
[b] Values show the rank-order from highest to lowest P_{app}
[c] Values indicate the mean apparent permeability coefficient, P_{app}, ± standard devivation for N = 4

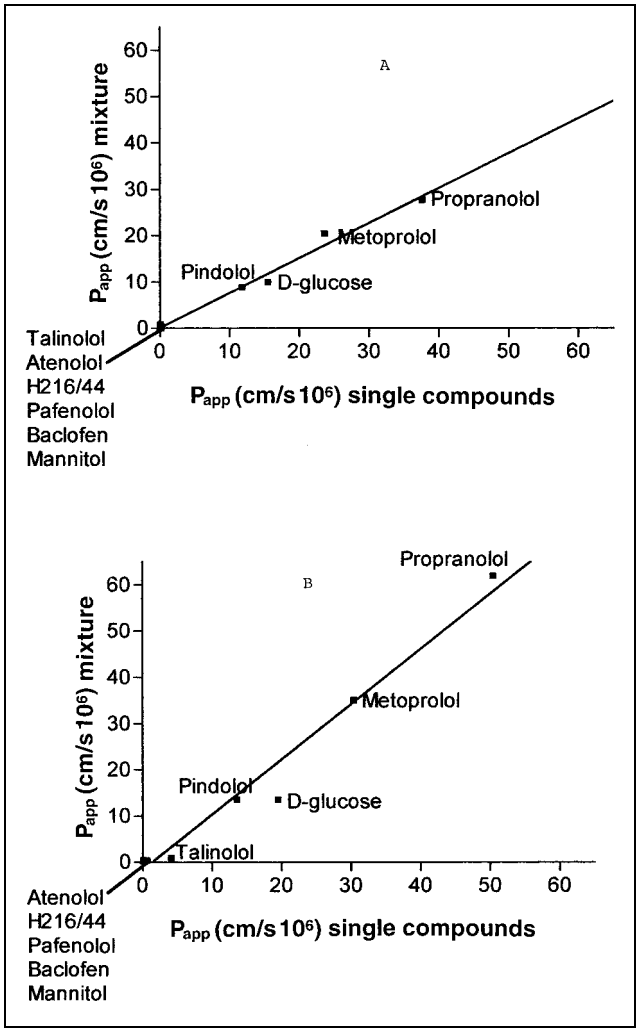


Fig. 2: Linear regression of the compounds P_{app} determined individually or in a mixture. A (upper graph) shows permeability data in the apical to basolateral direction, while B (lower graph) represents the results from the basolateral to apical experiments

suggest that mixtures can be used in the permeability screening process without severe compromise with respect to the precision of the permeability estimates as well as the permeability rank-order of a set of compounds.

For further characterisation of the transport mechanisms involved in the permeation of the compounds, the mixture and single compound permeabilities were also studied in the basolateral to apical permeation direction. As shown in Table 2, the P_{app} of the passively transported compounds was independent of the transport direction. The flux of the P-glycoprotein substrates pafenolol, H 216/44(S) and talinolol, however, were considerably higher in the basolateral to apical as compared to the reverse direction. The permeability of baclofen and D-glucose, substrates of an amino acid and D-glucose transporter, respectively, turned out to be independent of the transport direction. These results were unexpected. In the case of baclofen, they may be rationalised on the basis of a relatively high contribution of passive permeability to the total drug flux across the membrane. One of the reasons for this is the fact that the baclofen used in these studies was the racemic mixture of L- and D-baclofen and therefore it is obvious, that only 50% of the applied drug concentration can undergo carrier-mediated transport while the other half is transported via passive diffusion. A second explanation which in particular may serve to rationalize the permeability data of D-glucose, is the relatively high concentration applied in the donor solution (1 mM for baclofen, 10 mM for D-glucose) which may be saturating carrier-mediated processes. Nevertheless these concentrations represent typical concentrations applied in the cell culture permeability studies which partly are constrained by the sensitivity of the analytical method and the surface area to volume ratio of the Transwell equipment. In the case of D-glucose it is further known from studies with its optical antipode, L-glucose, that the absorptive permeability of the L-enantiomer in similarly conditioned Caco-2 cells is approximately 10-fold lower as compared to the D-enantiomer (Langguth, unpublished results). Hence it cannot be ruled out that carriers for D-glucose exist in Caco-2 cells, which can operate in both directions, i.e. the absorptive and the secretory direction.

The obtained P_{app} values were also evaluated for a correlation with clogP and K′, in order to test whether P_{app} could be predicted merely by these lipophilicity parameters. As shown in Fig. 3, this hold true only for those compounds that are transported mainly by passive diffusion processes, atenolol, pindolol, metoprolol and propranolol. In the case when intestinal secretion is involved (talinolol, pafenolol, H 216/44(S)), clog P and K′ will highly overpredict the resulting permeability in the absorptive direction. Consequently, the lipophilicity indicators like clogP and K′ may predict false positive P_{app} values.

Further optimisation in the screening of mixtures in permeability experiments can be expected from improved quantitative and qualitative analytical techniques such as LC-MS to replace LC-UV. Recently, Caldwell et al. [14] studied the *in vitro* permeability of a number of β-adrenoceptor antagonists, where the more selective LC-MS method gave the same rank-orders of compounds as earlier published LC-UV data, although the absolute values from the two methods differed somewhat. The introduction of LC with MS detection in permeability studies results in both increased sensitivity, particularly useful in quantitative analysis due to the low concentrations of the single compounds in a mixture as well as with respect to specificity which is valuable in mixtures due to the shorter development times for analytical methods. Moreover, the increased sensitivity may help to overcome the problems associated with the characterisation of low solubility substances in Caco-2 screening programs.

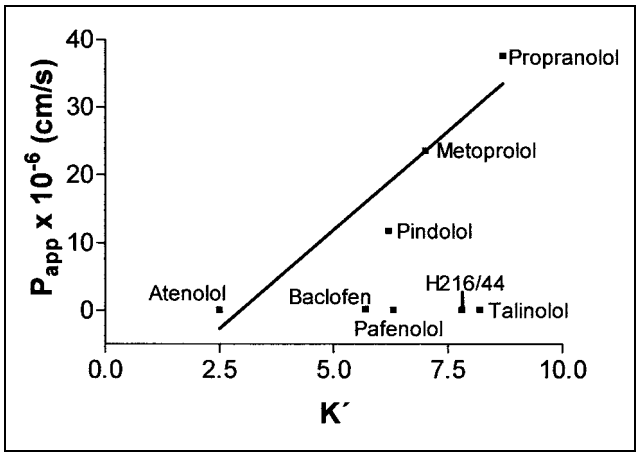


Fig. 3: Correlation between P_{app} and retention behaviour on reversed-phase HPLC (K′) of the eight compounds analysed by liquid chromatography. For the regression analysis, the compounds undergoing carrier-mediated intestinal transport (baclofen, talinolol, pafenolol, H 216/44(S)) were excluded

It can be concluded that the use of compound mixtures is a suitable method of improving the capacity of permeability screens for drug molecules, since there is a strong correlation between the P_{app} in a mixture and the corresponding P_{app} for the single compounds. Moreover the apparent permeability coefficients in the mixture did not differ to a relevant extent when compared with the single compound experiments. The method is especially applicable when a first permeability estimate is required. Furthermore the P_{app} across Caco-2 monolayer could not satisfactorily be predicted by the lipophilicity indicators like clogP and K′ which clearly emphasises the value of Caco-2 permeability testing in preformulation studies of new chemical entities.

Further improvement of the method towards higher screening capacity could be foreseen when (i) the mixture approach is combined with robotics to minimise the manual labour involved, (ii) LC-MS detection is used to facilitate quantitative analysis, speed up the analysis times and improve the detection selectivity and sensitivity.

An additional improvement with respect to the confidence in permeability estimates could be obtained, when "indicator" permeability standards are included in the mixture. Ideally such a cocktail with internal standards should include marker compounds for the paracellular pathway and for the various intestinal carriers present in the absorptive and secretory direction.

3. Experimental

3.1. Chemicals and solutions

Compounds used in the transport experiments were mannitol (KEBO Lab, Spånga, Sweden), atenolol, baclofen, propranolol, pindolol (Sigma, Stockholm, Sweden), H 216/44(S), metoprolol, pafenolol (AstraZeneca R&D, Mölndal, Sweden) and talinolol (H. Spahn-Langguth, University of Halle, Germany). [^{3}H]D-glucose and [^{14}C]mannitol with specific radioactivity of 15.5 and 51.5 mCi/mmol, respectively, were purchased from NEN-DuPont Life Science Products (Stockholm, Sweden). An aqueous stock solution of 6 M formic acid (Fluka, Buchs, Switzerland) was diluted with water to give a 2% solution at pH 3.5 which was used together with gradient grade acetonitrile (KEBO Lab, Spånga, Sweden) for preparation of the mobile phases used in the HPLC analysis.

3.2. Sample analysis

A reversed phase-HPLC method with gradient elution and UV detection was developed in order to quantify the seven β-adrenoceptor antagonists and baclofen (Fig. 1) individually or simultaneously in a mixture. The HPLC system consisted of a Pharmacia LKB HPLC Pump 2248 (Bromma, Sweden), a Spectra Focus UV detector (Spectra Physics, San José, CA, USA) operated at 270 nm and an automatic sample injection system (ASPEC XL, Gilson, Villiers-le-Bel, France). The analytical column was a 5 μm Kromasil C_{18} column (15 cm × 4.6 mm i.d. Eka Nobel, Surte, Sweden) which was protected by a cyano Brownlee guard column (7 μm, 15 mm × 3.2 mm) from Applied Biosystems (San José, CA, USA). The chromatograms were stored and reprocessed by means of peak area calculation using a computerised data integration program (Spectra Physics, Spectra system software-PC 1000).

The two mobile phases used were composed of 10% (A) and 40% (B) acetonitrile in an aqueous formic acid solution (2%, pH 3.5). The solvent gradient program was formed by a LKB LC Controller 2252 equipped with a low-pressure mixer. At a flow rate of 1 ml/min the gradient elution profile was 100% (A) for 0–2 min, 0–100% (B) for 2–14 min and 100–0% (B) for 14–15 min. Thereafter the system was equilibrated for 5 min. before injection of the next sample. The samples were injected directly without pre-treatment and the injection volume was 100 μl.

Initial calibration curves were obtained by preparing a mixture of atenolol, metoprolol and propranolol at 1 mM in mobile phase A. This stock solution was then further diluted in mobile phase A to a final concentration of 100; 50; 10; 5; 1; 0.5 and 0.25 μM, respectively. Each standard solution was analysed in duplicate by HPLC and the calibration curves were obtained by plotting the mean peak area against the known concentration of the analytes. The corresponding calibration curves for pindolol, pafenolol, H 216/44(S), talinolol and baclofen were constructed by adding one compound at a time at a concentration of 1 mM to the above stock solution followed by dilution and HPLC analysis as given above.

Validation of the analytical method was based on linearity, precision (repeatability) and determination of the limit of quantification (LOQ) for each compound. The linearity was assessed by calculating the linear regression coefficient, r^2, for each calibration curve. Precision was evaluated by determining the coefficient of variation, C.V., for each point in the calibration curves. The LOQ of each compound was defined by a signal to noise ratio of about 4. The solution at that concentration was analysed repeatedly (N = 5) and was defined as LOQ if the C.V. was less than 20%.

[^{14}C]-Mannitol and ^{3}H-D-glucose were analysed using a Wallac WinSpectral 1414 liquid scintillation counter (Turku, Finland).

3.3. Culturing of Caco-2 cells

The Caco-2 cells were obtained from ATCC (Rockville, USA) and used in the transport experiments between passage 76–87. They were cultured at 37 °C, 90% relative humidity and 5% CO_2 atmosphere. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine containing 10% fetal bovine serum (FBS), 1% non-essential amino acids (MEM), 100 U/ml and 100 μg/ml penicillin and streptomycin, respectively. All medium components used were purchased from Life Technologies AB (Täby, Sweden) and the culturing medium was changed every other day.

After reaching 70–90% confluency, the cells were detached from the culture flask by addition of a trypsin-EDTA solution (0.25% trypsin and 0.2% EDTA) and seeded at a density of 88500 cells/cm^2 on 12 mm (internal diameter) Transwell polycarbonate membrane (0.4 μm pore size) inserts and placed in Transwell 12 well clusters purchased from Costar (Life Technologies AB, Täby, Sweden). The cells grew 14–18 days prior to any transport experiment in order to obtain differentiated monolayers.

3.4. Apical to basolateral transport studies

The experiments were conducted in Hank's Balanced Salt Solution (HBSS) buffered with 25 mM 2-morpholinoethanesulfonic acid monohydrate to pH 6.5 as transport buffer (MES, Sigma-Aldrich AB, Västra Frölunda, Sweden). Culturing medium was removed and the monolayers were washed twice with HBSS. The inserts were then placed in basolateral chambers containing 1.5 ml transport buffer prewarmed to 37 °C. Experiments were initiated by adding 0.7 ml of a prewarmed drug solution which was prepared from the radiolabelled and unlabelled compounds dissolved in transport buffer. The concentrations used were 1 mM for atenolol, baclofen, pafenolol talinolol and H 216/44(S) and 0.1 mM for pindolol, metoprolol and propranolol. The [^{14}C]mannitol solution had a concentration of 0.1 mM at 1 μCi/ml. The [^{3}H]-D-glucose solution was 10 mM at 1.5 μCi/ml, respectively. The initial concentration on the donor side was determined by analysing two 50 μl samples in duplicate. The monolayers were agitated by an orbital shaker (SO 1, Stuart scientific, Surrey, UK) at constant stirring rate (107 rpm) at 37 °C and 90% relative humidity. At regular time intervals the inserts were placed into another basolateral chamber containing fresh transport buffer. In the single compound experiments the sampling intervals were 120 min for atenolol, baclofen, pafenolol and H 216/44(S), 60 min for [^{3}H]D-glucose, [^{14}C]mannitol and pindolol while 15 min intervals were used for metoprolol and propranolol. The concentration of the labelled and unlabelled compounds in the basolateral chambers and for the apical samples was determined by liquid scintillation counting and HPLC, respectively. The apical samples were diluted ten times in transport buffer in order to obtain concentrations within the range of the calibration curves. Each experiment was performed in triplicate.

In the experiment with mixtures, a solution containing the same compounds and concentrations as mentioned above was studied under identical conditions as the single compounds, except that the inserts were placed in new basolateral chambers at 120 min intervals for 240 min. The experiment was performed in quadruplicate.

3.5. Basolateral to apical transport studies

The basolateral-to-apical transport experiments of the single compounds and the mixtures were performed under the same conditions as above except for the following changes. The inserts were placed in basolateral chambers containing 1.5 ml drug solution. Samples of the donor solution were taken prior to the experiment. The transport buffer (0.6 ml) was then added to the apical chamber to initiate the experiment. At regular time intervals the inserts were placed in another chamber and apical samples were taken and replaced with transport buffer. Sixty minute's intervals for a duration of 120 min were used for all single compounds except for metoprolol and propranolol where 30 min intervals for a duration of 60 min was deemed appropriate. The sampling interval in the mixture experiment was 60 min for a total duration of 120 min. Each experiment was performed in triplicate.

3.6. Epithelial integrity tests

In order to assess the integrity of a monolayer prior to an experiment the transepithelial electrical resistance (TEER) was measured. Moreover the

paracellular marker [^{14}C]mannitol at a concentration of 0.1 mM and 1 μCi/ml was coincubated with the other compounds. Monolayers were considered to be intact when the permeability of mannitol was less than 0.2×10^{-6} cm/s.

3.7. Calculation of the apparent permeability coefficients

The apparent permeability coefficients (P_{app}) for each single compound and component of the mixture were calculated using the equation [1]:
$P_{app} = k^* V_R/A^* 60$ (cm/s) where k (min^{-1}) is the slope obtained from a plot of cumulative amount transported as a function of time (minutes), V_R is the volume in the receiver chamber (1.5 ml or 0.6 ml in the basolateral and apical chamber, respectively) and A is the surface area of the membrane (1.13 cm^2).

Acknowledgements: This work was performed at the Department of Pharmacokinetics and Drug Metabolism, AstraZeneca R&D, Mölndal. Financial support by the Dr. Robert Pfleger-Stiftung, Bamberg and the Fonds der Chemischen Industrie, Frankfurt to P.L. is acknowledged. The authors thank Ms. Marie Östgren, AstraZeneca R&D Mölndal for skilful technical assistance during the permeability studies and Prof. H. Spahn-Langguth for helpful discussions.

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Received April 18, 2000
Accepted September 18, 2000

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