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In Vitro Permeability of Eight β-Blockers Through Caco-2 Monolayers Utilizing Liquid Chromatography/Electrospray Ionization Mass Spectrometry

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It is demonstrated that the apparent permeability (P_{app}) coefficients of β-adrenoceptor antagonist drugs can easily be determined for Caco-2 cell culture intestinal models utilizing liquid chromatography/mass spectrometry (LC/MS). The LC/MS method with electrospray ionization in the single ion monitoring mode showed an increased sensitivity of 1000-fold compared with LC/UV detection and enhanced selectivity with respect to both LC/UV and radioactivity assays. The P_{app} coefficients of β-adrenoceptor antagonists determined by LC/MS have the same ranking order as those determined by LC/UV and radioactivity assays. However, the P_{app} coefficients determined in this study showed significant discrepancies from those determined in other laboratories. There are several experimental factors that directly affect the absolute value of the P_{app} coefficients, including pH gradients, additional diffusion barriers (i.e. unstirred water layer and type of filter support), analyte concentration, detection method and possibly cell culture variations. These parameters should be controlled when generating Caco-2 P_{app} coefficients for different compounds. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: Caco-2; β-adrenoceptor antagonist drugs; apparent permeability; liquid chromatography/electrospray ionization mass spectrometry

INTRODUCTION

The deficiency of oral bioavailability of drug compounds may result from poor dissolution, chemical or enzymatic instability, low epithelial permeability or hepatic first-pass effects. The transport of drugs across the intestinal epithelial cell barrier is a major factor in determining *in vivo* oral bioavailabilities. That is, drug compounds that do not have adequate intestinal permeability will not be orally bioavailable. The human intestinal cell line Caco-2, which is derived from a human colorectal carcinoma, spontaneously differentiates on microporous filter membranes into polarized monolayers with tight cellular junctions.[1] This monolayer cell line has been used as an *in vitro* model to study drug transport and metabolism in the intestinal epithelium.[2,3] To determine the permeability of a drug through the Caco-2 cell barrier, drugs must be quantified from solutions containing significant quantities of salt. Typically, these quantitation studies are performed utilizing radiolabelled drug compounds with liquid scintillation counter detection[4,5] or drug compounds with liquid chromatography/ultraviolet (LC/UV) methods.[6–8] The objective of this paper is to describe a liquid chromatography/mass spectrometric method to quantitate drug compounds from a Caco-2 cell culture intestinal model. The apparent permeability (P_{app}) coefficients of eight β-adrenoceptor antagonist drugs (β-blockers) were measured utilizing liquid chromatography/electrospray ionization (ES) mass spectrometry (LC/MS) in the selected ion monitoring (SIM) mode. The P_{app} coefficients obtained by LC/MS are comparable to those obtained using radioactivity assays (RA) and LC/UV methods. We chose to study the β-blockers because their absorption characteristics have been well documented and the compounds are commercially available.[7,9] To our knowledge, no detailed report describing the use of LC/ESMS for the quantification of drug levels in Caco-2 transport studies has been published.

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EXPERIMENTAL

Chemicals

The following β-adrenoceptor antagonist drugs (β-blockers) and testosterone (T1500) were purchased from Sigma Chemical (St Louis, MO, USA): acebutolol (A3669), alprenolol (A8676), atenolol (A7655), metoprolol (M5391), oxprenolol (O0253), pindolol (P0778), propranolol (P0884) and timolol (T6394). The structures for these β-blockers are shown in Fig. 1. Radioactive [^{3}H]propranolol, [^{14}C]-D-mannitol and [^{14}C]PEG-4000 were purchased from New England Nuclear–

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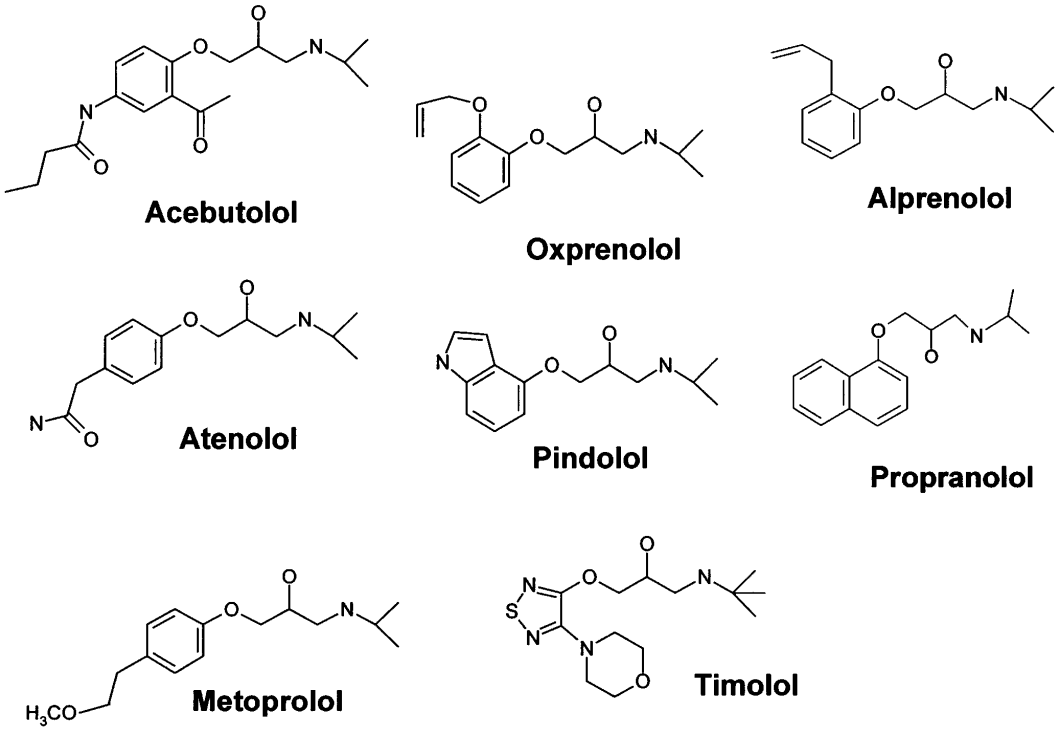

Figure 1. Structures of the β-adrenoceptor antagonist drugs (β-blockers).

DuPont (Wilmington, DE, USA) and [^{14}C]Testosterone from Amersham Life Sciences (Arlington Heights, IL, USA). All solvents and buffer components were of reagent grade.

Cell culture

Caco-2 cells (American Type Culture Collection, Rockville, MD, USA) were maintained in minimum essential medium (MEM) supplemented with 1% non-essential amino acids, 1% sodium pyruvate and 10% fetal calf serum at 37 °C in an atmosphere of 95% air and 5% CO_2. Cells were passaged by trysinization twice weekly. All medium components were obtained from GIBCO (Grand Island, NY, USA). For use in transport studies, Caco-2 cells were grown in Polycell filter insert units (Polyfiltronics, Rockland, MA, USA) in media containing penicillin, streptomycin and Fungizone. The inserts were seeded with ~60 000 cells (passage number 30–50). The Polycell insert unit was a single molded piece containing 24 wells with polycarbonate filter bottoms (surface area = 0.33 cm^2). The inserts rested in a standard 24-well plate also containing media. The media in the insert (apical cell side) and 24 well plate (basolateral cell side) were replaced three times per week. The integrity of the monolayer was determined by measuring the transepithelial electrical resistance using a custom epithelial voltmeter (World Electronics, Sarasota, FL, USA). The majority of the cell growth was between 3 and 8 days and our studies utilized cells cultured from 21 to 30 days. All cells grown for 21–30 days with a transepithelial electrical resistance greater than 300 Ω cm^2 were included. Several cell monolayers were chosen randomly and [^{14}C]PEG-4000, [^{14}C]-D-mannitol and [^{14}C]testosterone were used as standards to gauge the cell junction tightness between confluent cells. Transport studies were conducted as described in the next section. Monolayers were considered to be intact when less than 0.10 ± 0.05% [^{14}C]PEG-4000 per hour and 1.0 ± 0.2% [^{14}C]-D-mannitol per hour passed from the insert (apical) to the well (basolateral).[6,7] The apparent permeability coefficient [P_{app}; see Eqn (1) later] for [^{14}C]PEG-4000 was 0.67×10^{-6} cm s^{-1} and for [^{14}C]-D-mannitol it was 5.0×10^{-6} cm s^{-1}. The [^{14}C]PEG-4000 value was similar to that reported in the literature (0.78×10^{-6} cm s^{-1}).[5] The apparent permeability coefficient for [^{14}C]testosterone was 109.0×10^{-6} cm s^{-1} and again compared reasonably well with the 72.27×10^{-6} cm s^{-1} literature value.[5] Androstenedione was observed in the well (basolateral) compartment during the testosterone transport experiment and suggested that the Caco-2 cells contained an enzymatic system (i.e. 17-β-hydroxysteroid dehydrogenase) capable of metabolizing testosterone.[6] Radioactivity was counted using a liquid scintillation counter (Model 2500, Packard, Meriden, CT, USA).

Transport studies

Caco-2 cell monolayers grown on filter supports for 3–4 weeks were used in transport studies. On the day of the experiment, the complete culture medium was removed from both the insert (apical) and well (basolateral) compartments. The monolayers were washed (three times) and pre-incubated with 150 µl of apical buffer and 600 µl of basolateral buffer for 30 min at 37 °C on a shaker bath at 80 rpm. The apical buffer consisted of Hanks' Balanced Salt Solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.3 mM Na_2HPO_4 and 0.3 mM KH_2PO_4) and 25 mM D-glucose buffered at pH 6.5 with 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-

ethanesulfonic acid) (HEPES). The basolateral buffer was the same as the apical buffer except it was buffered at pH 7.4. To initiate an experiment, the apical buffer was removed and replaced with 150 μl of the desired drug at a concentration of 50 μM in apical buffer. The insert was placed in a well containing 600 μl of warmed basolateral buffer and incubated at 37 °C on a shaker at 80 rpm. At various time points (5, 7, 10, 20, 30, 45 and 60 min), the inserts were transferred to a fresh, pre-warmed 24-well plate and returned to the shaker. The well (basolateral) solutions were stored at −4 °C pending analyses by LC/MS. Experiments were run in triplicate and lasted ~1 h. Transepithelial electrical resistance readings were also taken pre- and post-analysis to ensure membrane integrity during transport studies.

LC/MS analysis

For detection and quantitation, a Finnigan TSQ 700 mass spectrometer (Finnigan MAT, San Jose, CA, USA) using electrospray positive ionization was utilized.[10] The instrument was operated in the SIM mode for two protonated molecular ions (i.e. analyte and internal standard) yielding a total scan time of 2 s with a ±0.25 u window. A HP-1050 liquid chromatograph (Hewlett-Packard, Wilmington, DE, USA) was used with a mobile phase consisting of acetonitrile (ACN)–water (0.1% trifluoroacetic acid) with gradient from 10% to 90% ACN in 7 min and a 5 min hold time at 90% ACN flowing at 250 μl min^{-1}. The column was a Phenomenex (Torrance, CA, USA) C_{18} (5 μm 300 , 50 × 2.0 mm i.d.). The method for atenolol was slightly modified using acetonitrile–water (pH 4, adjusted with acetic acid) with a gradient from 5% to 90% ACN in 7 min and a hold time of 5 min at 90% ACN flowing at 250 μl min^{1}. The column was a Phenomenex C_4 (5 μm, 300 , 50 × 2.0 mm i.d.). The injection volume for all samples was 10 μl. An automated divert valve was installed and all runs were diverted for 1 min to remove salts. The β-blockers typically had detection limits of 10 nM or better under these experimental conditions.

Calculations

The apparent permeability (P_{app}) expressed in cm s^{-1} was calculated with the following equation:[4]

$$P_{app} = V_R/(AC_0)\ dC/dt \quad (1)$$

where V_R is the volume of the well (basolateral) compartment (0.60 ml), A is the membrane surface area (0.33 cm^2), C_0 is the initial concentration of the β-blockers (50 μM) and dC/dt is the slope of the cumulative basolateral concentration (μM) with time (s). The apparent permeabilities were obtained under 'sink conditions,' i.e. less than 10% transported.

RESULTS AND DISCUSSION

The structures of the homologous series of β-adrenoceptor blocking drugs (β-blockers) chosen as model compounds are shown in Fig. 1. These compounds have similar molecular masses that range from 248 Da (pindolol) to 336 Da (acebutolol) and dissociation constants ($pK_a \approx 9$),[11] which minimize the influence of these parameters on their apparent permeability coefficients. However, their lipophilicity based on *n*-octanol buffer partition coefficients[11] and *in vivo* human absorption after oral administration displays a wide variability.[9] Since the inserts (apical) were adjusted to pH 6.5, all the β-blockers were in their protonated forms during the transport studies.

A typical LC/MS run is shown in Fig. 2 for a propranolol sample prepared in HBSS at a concentration of 0.5 μM at pH 7.4 and spiked with an internal standard (IS) of acebutolol at 0.1 μM (see Table 1). The internal standard acebutolol (m/z 337) and propranolol (m/z 260) are detected in a LC/MS single ion monitoring experiment with retention times of ~6.09 and 7.21 min, respectively. The ratios of the areas under the curves were measured and calibration graphs were obtained. The inset in Fig. 2 shows the calibration graph for propranolol over the concentration range 0.01–0.5 μM. Linear regression analysis for the propranolol example produced a correlation value (r^2) of 0.9998. In general, linear regression analysis for the other β-blockers produced $r^2 > 0.99$. The removal of salts prior to ESMS analysis was the key to obtaining good quantitative results.

For transport in the apical to basolateral direction, 50 μM of the β-blocker of interest was placed in the insert (apical side). The inserts was moved seven times over a 1 h period to wells containing fresh buffer. These well (basolateral) samples were spiked with an internal standard (see Table 1) and analyzed by LC/MS. The ratios of area of the drug to the area of the internal standard were computed and compared with calibration graphs to determine the concentration of drug in the well. Typical results are shown in Fig. 3 using propranolol as an example. These results suggested that the propranolol steady-state concentration of 0.6 μM is approached in ~1 h. The other β-blockers also approached their own steady-state concentrations within 1 h (data not shown). By using the data in Fig. 3, dividing it by the initial concentration and correcting for dilution effects, a cumulative plot of percentage of propranolol transported through the cell monolayer as a function of time was obtained (Fig. 4). For comparison purposes, we repeated the experiment under identical conditions and measured the percentage of [^{14}C]propranolol transported as a function of time utilizing a scintillation counter. The two linear regression lines have very similar slopes; however, there is a significant offset between the [^{14}C]propranolol and the LC/MS-measured propranolol regression lines, as evidenced by their intercept values. This offset may be due to metabolites of the [^{14}C]propranolol which cannot be discriminated from the parent drug. Since a liquid scintillation counter detects total radioactivity, any drug that is metabolized and retains the radioactive label will also be detected in addition to the drug. Hence the [^{14}C]propranolol regression line represents all compounds (propranolol plus metabolites) passing through the Caco-2 monolayer whereas the propranolol regression line represents only propranolol.

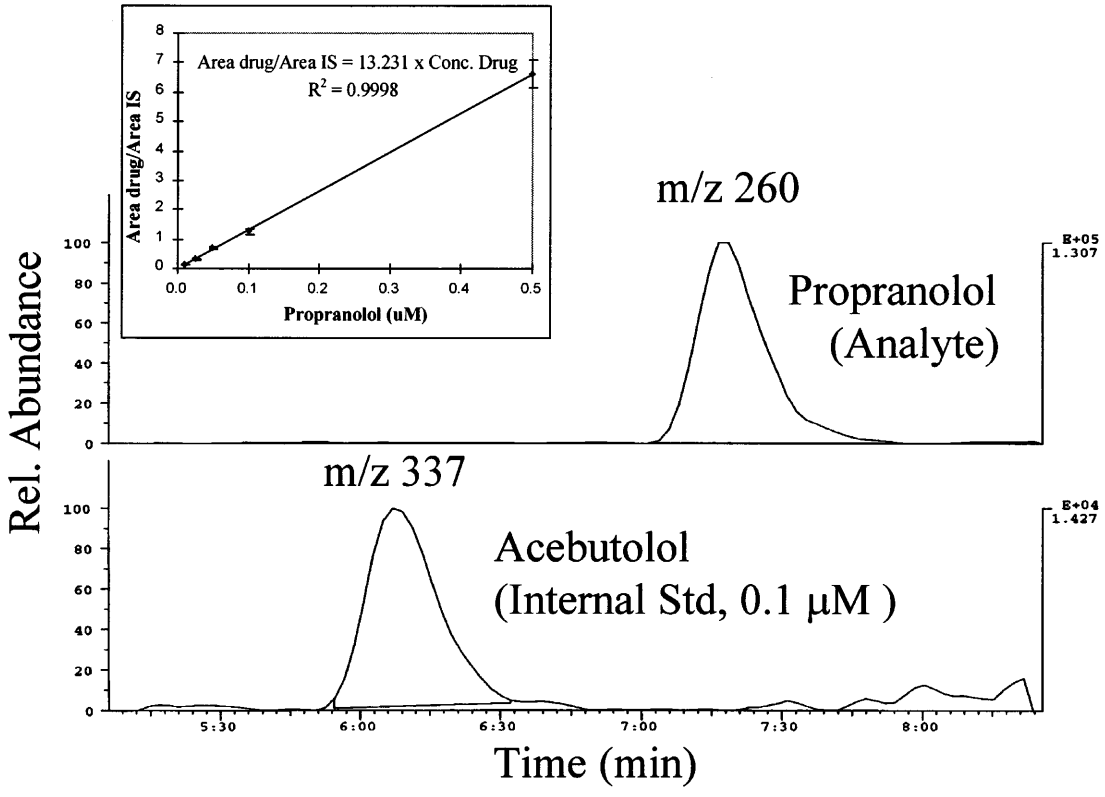

Figure 2. A typical LC/MS experiment with propranolol (*m/z* 260; 0.5 μM) and an internal standard (acebutolol; *m/z* 337; 0.1 μM). The mass spectrometer was set up in the SIM mode utilizing electrospray ionization and the LC parameters were as follows: a mobile phase consisting of acetonitrile (ACN)–water (0.1% trifluoroacetic acid) with a gradient from 10% to 90% ACN in 7 min and a 5 min hold time at 90% ACN; the column was a Phenomenex C_{18} (5 mm, 300 , 50 × 2.0 mm i.d.) flowing at 250 μl min^1; the injection volume was 10 μl. An automated divert valve was installed such that all runs were diverted for 1 min to remove salts. Under these conditions, the retention times for acebutolol and propranolol were ~6.09 and 7.21 min, respectively. The inset shows the calibration graph for propranolol over the concentration range 0.01–0.5 μM.

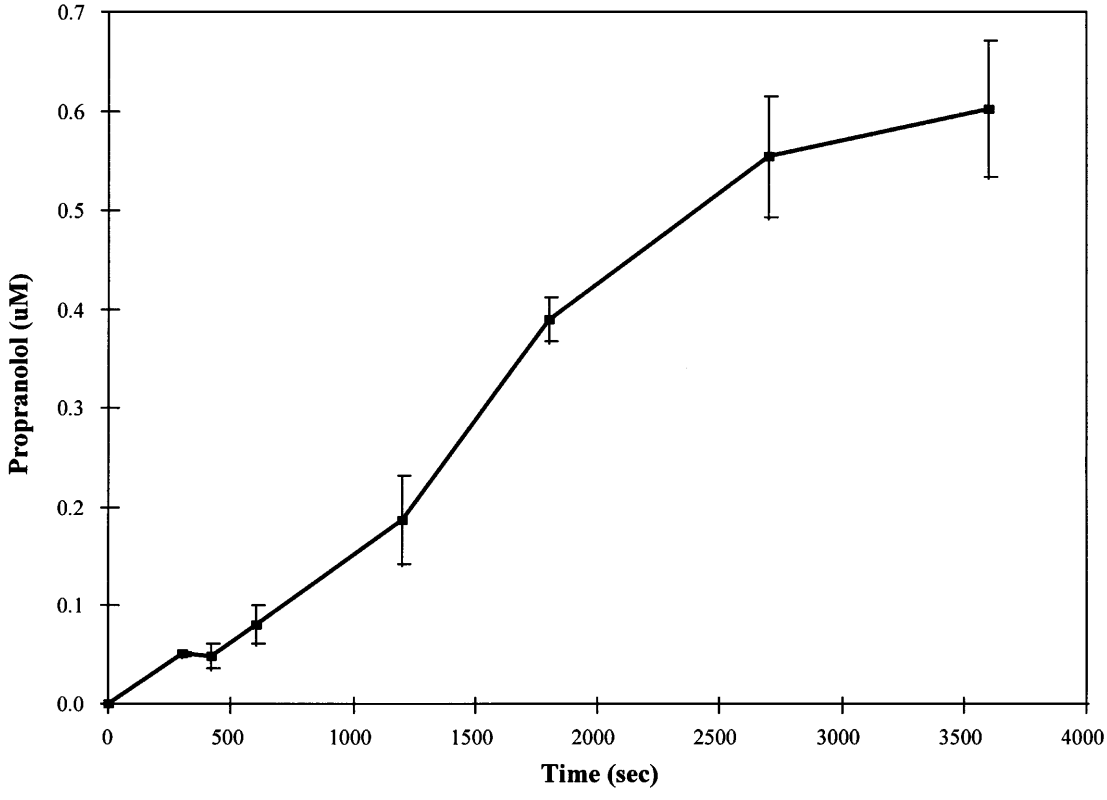

Figure 3. A 50 μM propranolol insert solution was moved seven times over a 1 h period to wells (basolateral) containing fresh solutions. These well samples were spiked with an internal standard (see Table 1) and analyzed by LC/MS to determine the concentration of propranolol. Each data point represents the mean ± one standard deviation for $n = 3$.

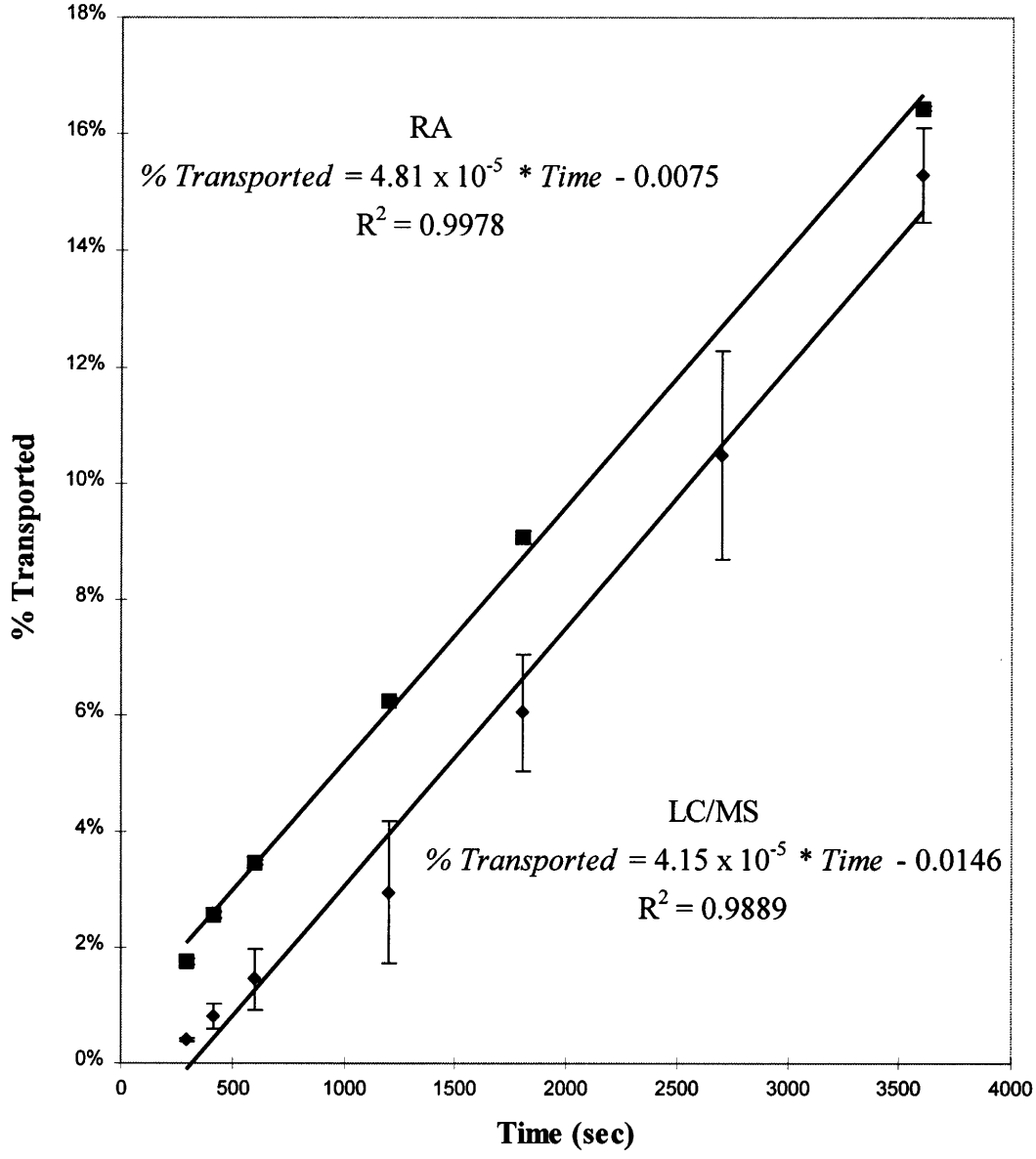

Figure 4. Cumulative plot of percentage transported of propranolol through the Caco-2 cell monolayer as a function of time. The data points represented by diamonds were collected utilizing the LC/MS techniques and those represented by squares utilizing a radioactive assay with [^{14}C]propranolol. The experiments were performed under identical conditions. Each data point represents the mean ± one standard deviation for $n = 3$.

It was of interest to examine the well solutions for the presence of propranolol metabolites. Several cations with m/z value consistent with metabolites of propranolol were observed in the mass spectrum. For example, the m/z 275 cation was consistent with naphtholic metabolites of propranolol and the m/z 392 cation was consistent with the glucuronide conjugates. The Caco-2 cells have been shown previously to contain enzymatic systems capable of metabolizing drugs.[6] Typical cumulative plots of the time course of the other β-blockers in the basolateral (well) compartment after the addition of 50 μM of the β-blockers to the apical (insert) compartment are shown in Fig. 5. The results in Fig. 5 suggest that acebutolol and atenolol are poorly absorbed, timolol and pindolol are moderately absorbed and oxprenolol, alprenolol, metoprolol and propranolol are highly absorbed. The β-blocker apparent permeability coefficients (P_{app}) were determined from the slopes of the percentage transported *vs.* time plots (Fig. 5) and Eqn (1). These values are summarized in Table 1 along with literature P_{app} coefficients determined utilizing either LC/UV methods or radiolabeled drugs.

The β-blocker P_{app} coefficients determined with LC/UV[7] and radiolabelled drug[4] assays correlate reasonably well with the present LC/MS results (see Table 1). With all three methods, the drugs show the same rank ordering of their P_{app} values. However, when the P_{app} (LC/UV) coefficients are compared with the P_{app} (LC/MS) coefficients, the propranolol and alprenolol values are similar, but the P_{app} (LC/UV) coefficients for the other three other β-blockers are noticeably higher. Several factors, such as initial β-blocker concentrations and pH values, may contribute to these discrepancies. The P_{app} (RA) coefficients significantly lower than the other data. The primary reason for discrepancies in the

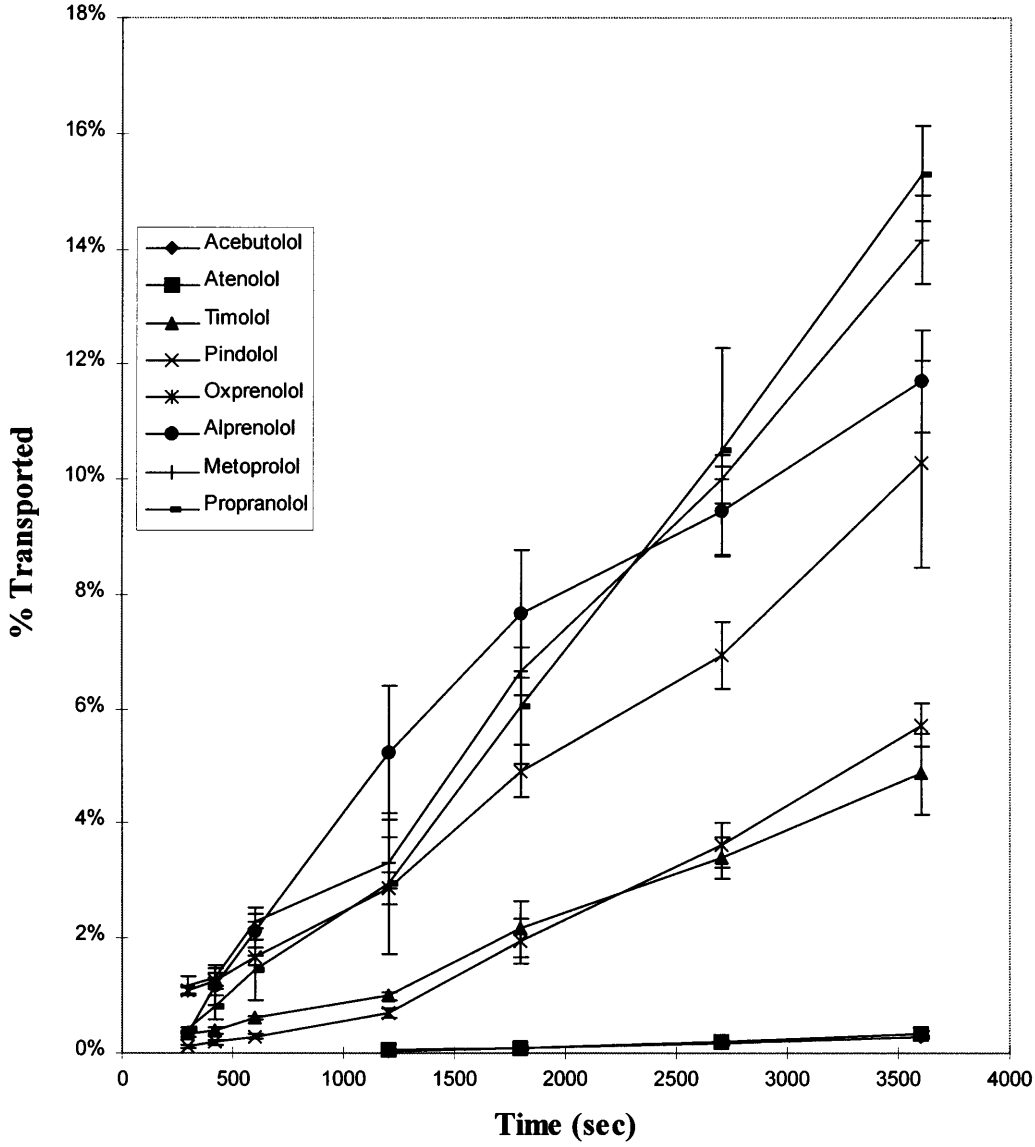

Figure 5. Cumulative plots of the time course of eight β-blockers in the basolateral (well) compartment after the deviation of 50 μM of the β-blockers to the apical (insert) compartment. Each data point represents the mean ± one standard deviation for $n = 3$.

P_{app} (RA) coefficients is presumably the lack of stirring during the experiment. There is an unstirred water layer at the interface between the bulk solution and the Caco-2 cell monolayer. For lipophilic analytes such as the β-blockers, the unstirred water layer exerts an additional diffusion barrier and hence P_{app} coefficients are underestimated.

It is evident based on this and other work[2–7] that factors such as temperature, pH gradient, additional diffusion barriers (i.e. unstirred water layers and type of filter support), source of Caco-2 cells, expression levels of bile acid transporters and phase I and phase II enzymes and analyte concentration need to be controlled in order to reduce discrepancies in the P_{app} coefficients between laboratories. As an example, the P_{app} coefficients for propranolol were measured repeatedly in several laboratories using [^{14}C]propranolol and the results differed by a factor of ~3 ((2.75–8.74) $\times 10^{-5}$ cm s^{-1}). Yee[5] measured $P_{app} = 2.75 \times 10^{-5}$ cm s^{-1} when the propranolol concentration was 1 μM and the cells were shaken at 80 rpm.[5] We measured $P_{app} = 4.55 \times 10^{-5}$ cm s^{-1} when the propranolol concentration was 1 μM and the cells were shaken at 80 rpm. Artursson and Karlsson[4] measured $P_{app} = 4.19 \times 10^{-5}$ cm s^{-1} when the propranolol concentration was 1 μM and the cells were not shaken. If we compare the propranolol P_{app} coefficients under nearly identical experimental conditions except for changes in the initial insert concentration from 50 μM ($P_{app} = 8.74 \times 10^{-5}$ cm s^{-1}) to 1 μM ($P_{app} = 4.55 \times 10^{-5}$ cm s^{-1}), we observe approximately a twofold decrease in the P_{app} coefficients. These results suggest that the absolute P_{app} coefficients can vary significantly (i.e. three-fold) under different experimental conditions.

Finally, if we compare the P_{app} coefficients with the *in vivo* human absorption data of the β-blockers (Table 2), we observe that the P_{app} coefficients can only distinguish the poorly absorbed β-blockers (acebutolol and atenolol) from the highly absorbed β-blockers. Since the P_{app} coefficients are somewhat variable and really can only distinguish the β-blocker *in vivo* data in a high/low manner, a new approach to ranking the β-blockers is

Table 1. Apparent permeability coefficients for β-adrenoceptor antagonist drugs (β-blockers)

β-Blocker	SIM ion (*m/z*)[a]	Internal standard (*m/z*)	$P_{app} \times 10^5$ (cm s^{-1}) LC/MS[c]	LC/UV[d]	RA[e]
Acebutolol	337	Alprenolol (250)	0.196 ± 0.019	0.449 ± 0.045	
Atenolol	267	Acebutolol (337)	0.219 ± 0.044		0.02
Timolol	317	Alprenolol (250)	2.52 ± 0.41	4.43 ± 0.28	
Pindolol	249	Acebutolol (337)	3.07 ± 0.21		
Oxprenolol	266	Acebutolol (337)	4.98 ± 0.79	6.55 ± 0.22	
Metoprolol	268	Acebutolol (337)	6.75 ± 0.33		2.70
Alprenolol	250	Acebutolol (337)	7.00 ± 0.91	7.50 ± 0.77	4.05
Propranolol	260	Acebutolol (337)	7.54 ± 1.21	8.20 ± 0.51	4.19
					8.74 ± 0.08[f]
					4.55 ± 0.06[g]
					2.75[h]

[a] LC/MS quantitation studies were performed in the SIM mode utilizing the MH^+ cations of the β-blockers.
[b] Internal standard and MH^+ cation utilized in the LC/MS quantitation study.
[c] See text and Eqn (1): all β-blockers were prepared in HBSS at 50 μM. The angle of the thermostated plate shaker was 0° and the rotational speed was set to 80 rpm. Each value represents the mean ± one standard deviation for $n = 3$.
[d] Results from Ref. 7 using an LC/UV assay. The β-blockers were prepared in HBSS at concentrations ranging from 600 to 830 μM except for propranolol, which was at 55 μM. The angle of the thermostated plate shaker was 2.5° and the rotational speed was set to 300 rpm.
[e] Results from Ref. 4 in which radiolabelled β-blockers were quantitated using a liquid scintillation counter. The β-blockers were prepared in HBSS at concentrations of 10–100 μM. The monolayers were not shaken. The standard deviation was stated to be generally less than 10% ($n = 3$).
[f] Present results in which [^{3}H]propranolol was quantitated with a liquid scintillation counter. Propranolol was prepared in HBSS at 50 μM and spiked with ~11 000 dpm of radiolabelled drug. The angle of the plate shaker was 0° and the rotational speed was set to 80 rpm. The value represents the mean ± one standard deviation for $n = 3$.
[g] Same as (f) except propranolol was prepared in HBSS at 1 μM.
[h] Results from Ref. 5 in which [^{3}H]propranolol was quantitated with a liquid scintillation counter. Propranolol was prepared in HBSS at 1 μM and the rotational speed was set to 80 rpm. The standard deviation was not stated.

Table 2. Absorption parameters for β-adrenoceptor antagonist drugs (β-blockers)

β-Blocker	P_{app} (Caco-2) (%)[a]	*In vivo* absorption (human) (%)[b]	Cumulative well (Basolateral) compartments (μM)[d]
Acebutolol	3	70	0.01(4%)
Atenolol	3	50	0.01(4%)
Timolol	33	>90	0.11(46%)
Pindolol	41	>90	0.10(42%)
Oxprenolol	66	≈90	0.18(75%)
Metoprolol	90	>90	0.22(92%)
Alprenolol	93	93[c]	0.20(83%)
Propranolol	100	>90	0.24(100%)

[a] LC/MS apparent permeability coefficients (see Table 1) normalized to propranolol.
[b] Ref. 9.
[c] Ref. 12.
[d] Each well solution of the various time points (5, 7, 10, 20 , 30, 45 and 60 min) was combined and the concentrations of the β-blocker were determined utilizing LC/MS. Values in parentheses are normalized to propranolol.

suggested. We repeated the β-blocker transport studies in an identical fashion as described in the Experimental section. Instead of analyzing every time point and generating cumulative basolateral concentration–time courses (see Figs 4 and 5), we combined all seven solutions and determined the overall β-blocker concentration. These results are given in Table 2 and clearly rank the β-blockers in the same order as the P_{app} coefficients. A large amount of LC/MS experimental time is saved when using this alternative approach. For example, the P_{app} coefficients required a measurement time ~1.5 days per drug whereas the ranking information from combining all time point solutions required ~4 h per drug. The ability to utilize this alternative method to distinguish drugs that have high/low absorption characteristics may be useful in a pharmaceutical drug discovery process.

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