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Simultaneous LC−MS/MS Determination of Reference Pharmaceuticals as a Method for the Characterization of the Caco-2 Cell Monolayer Absorption Properties

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Since its introduction a decade ago, the Caco-2 in vitro model for testing intestinal permeability has found wide application, in particular for screening new molecules in the pharmaceutical industry. An important issue for the validation of the model is to verify integrity and proper functionality of the Caco-2 cells monolayer, to ensure reproducibility and consistency of results. Several of these methods are based on measuring the apparent permeability coefficients (P_{app}) of well-characterized reference compounds, having known absorption characteristics, and comparing the observed values to those expected. Most of them use fluorescent or radioactive chemicals to test different parameters. The main limitation of such approaches is that each parameter to be tested is associated with a single specific method, so that multiple procedures are needed to adequately characterize the cell monolayer. This work describes the use of a unique LC−MS/MS method to simultaneously determine the P_{app} of a set of reference substances having well-characterized absorption behavior, i.e., phenylalanine, atenolol, and propranolol. The method is routinely used in our laboratory to check on model reproducibility and proved useful in verifying the consistency of the results derived from the experiments.

Since its introduction by Hidalgo et al. in 1989,[1] the Caco-2 cell monolayer model for testing drug absorption has been widely adopted. Different groups have demonstrated its usefulness in mimicking intestinal absorption in humans.[2−5] The model is commonly used by the pharmaceutical industry to evaluate the oral bioavailability potential of new synthetic molecule.[6−9]

One major concern of this model is its reproducibility,[3,4,10,11] requiring adequate control methods. This is necessary not only to check the quality of the culture but also to ensure full comparability of different sets of experiments.

Several methods are available to test the integrity of the monolayer,[1] and different approaches were followed to check on more characteristics such as the state of maturation of the culture.[4,11−13]

One particular method that has been used for testing the integrity of the cell monolayer is to measure the transepithelial electrical resistance (TEER): if the cells are confluent and tightly joined, they constitute a barrier to electrolyte movement and thus increase the electrical resistance across the monolayer. This technique is commonly used, but it is not very sensitive and is quite variable.[4,11,14] In particular, values appear different between laboratories; in our own experience, the trend followed by the TEER as a function of culture time was more significant than its absolute value: it was therefore necessary to monitor it continuously to draw reliable conclusions about the state of the culture.

One approach to determine the maturation stage of the culture is to measure the activity of some enzymatic systems, such as isomaltase,[13] present in mature enterocytes, or to determine the expression of the enzymes themselves (e.g., by western blot). These techniques are time and labor intensive, and although

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recommended for the original characterization of the cells in culture, they can hardly be applied on a routine basis.

The other general way of characterizing the cell transport properties is to use reference compounds[1,3,12] with a known behavior, which are applied to the Caco-2 monolayer, and the calculated apparent permeability coefficients (P_{app}) are compared to those expected. This results in quantitative values that can be used to make comparisons between experiments. Different groups have used fluorescent molecules for this purpose (e.g., lucifer yellow as an impermeable compound to test cell monolayer integrity),[1] only requiring a simple spectrofluorometer for the measurements. These methods can be applied to every transwell at the end of the experiment, allowing monitoring of the integrity of each cell monolayer. The main limitation of this approach is the availability of suitable fluorescent species. Another approach is the use of radiolabeled species:[1,5,15] this is more generally applicable as any desired species could be synthesized with labeled atoms. However, this powerful approach is limited by the need to synthesize the required radiolabeled species and, more importantly, by the risks associated with the manipulation of radioactive material and the regulatory restrictions to which they are subjected. A general limitation of the two above techniques is the difficulty of analyzing more than one compound with a single method, thus requiring multiple analytical procedures for an acceptable cell line characterization.

We considered that a more general methodology for the functional characterization of the Caco-2 cell culture absorption properties was desirable. Our aim was to develop a method that would allow the simultaneous analysis of different reference species indicative of different properties of the cell cultures. The approach used should allow us to extend the possible choice of reference species analyzed. For this work, we chose the technique of high-performance liquid chromatography−mass spectrometry (HPLC−MS), which is widely used for quantitative determination of compounds in Caco-2 media.[6,7,16] MS detection is suitable for the analysis of the vast majority of biologically relevant compounds; in addition, it is sensitive and specific, allowing measurement of even small amounts of analytes in complicated matrixes, while HPLC allows separation of the analytes from each other and from matrix components to eliminate risks of interferences and discrimination in ionization. Furthermore, HPLC−MS is the approach used for the quantitation of new molecules applied to the model in our laboratory, and the use of the same technique (and instrumentation) for both model characterization and screening purposes looked attractive in terms of speed and resource optimization. A disadvantage of this method is that it cannot be applied to each transwell used for the absorption tests but is used to characterize the batch of cells from a few wells only. In our laboratory, we make a coarse evaluation of the integrity of each monolayer by measuring TEER variation before and after the experiment and use the lucifer yellow and LC−MS methods on a small fraction of the transwells for a deeper monitoring of cell functionality. In our experience, the combined use of the above methods gives a good indication of the validity of an absorption experiment with reasonable effort.

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MATERIALS AND METHODS

Chemicals. Atenolol and propranolol were purchased from Sigma and phenylalanine was from Aldrich. All HPLC reagents and standards were of the highest grade available.

Cell Culture Supplements and Disposables. The Caco-2 cell line was obtained from the ECACC (European Collection of Cell Cultures, Salisbury, Wiltshire U.K.) at passage 38. Cells from passage 40−59 were used for the transport experiments. Penicillin, streptomycin, Dulbecco's phosphate buffer saline, and trypsin/EDTA were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). All other reagents for cell culture were from Sigma Chemical Co (St. Louis, MO). Transwell cell culture chambers (collagen-coated; diameter, 24 mm; pore size, 3 μm; growth area, 4.71 cm^2) and six-well plates were obtained from Corning Costar (Mainz, Germany). Plastic culture flasks were from Falcon (Becton Dickinson, Franklin Lakes, NJ).

Analytical Instrumentation. The HPLC instrument was a modular 1100 series chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a degasser, a high-pressure binary gradient pump, a 100-position autosampler, a thermostated column compartment with a column selection valve, and a photodiode array detector. The HPLC is controlled using the Chemstation software (version 08.01).

The detector was a LCQ mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with an atmospheric pressure chemical ionization (APCI) source. The LC eluent was introduced into the mass spectrometer going through the LCQ's divert valve. The mass spectrometer is controlled, and data analyzed, using the Xcalibur software suite (version 1.1). The two instruments communicate through a contact closure arrangement.

Analytical Methods. *HPLC Separation.* The separations were performed on Zorbax SB-Aq columns (5-cm length, 2.1-mm i.d., 5-μM particles) from Agilent Technologies. An on-line filter (pore size 0.2 μm) was inserted in the flow line between the autosampler and the analytical column. The gradient was obtained by high-pressure mixing of phase A (1% formic acid in water) and phase B (1% formic acid in acetonitrile). In initial conditions, the mobile-phase composition was 2% B; after injection, a linear gradient was applied to reach a composition of 95% B after 2 min; the column was then washed for 1.5 min maintaining the system at 95% B and then returned to initial conditions. The column was reequilibrated for 4 min before the following injection. The mobile-phase flow was maintained constant at 0.4 mL/min. The column compartment was maintained at 40 °C. Typically, 1 μL of untreated sample was injected on the column for analysis.

Standards were prepared from stock solutions of the singly prepared compounds at 0.1 M in DMSO. A mixed stock was prepared diluting 500 μL of singly prepared stocks in 50 mL of 50:50 acetonitrile/water: this solution contains a 1 mM concentration of each analyte. This mixed solution was aliquoted in plastic tubes; the aliquots were kept frozen (−20 °C) for up to 2 months. The working standards were prepared daily as follows: an aliquot of frozen mixed stock was thawed, and equal parts (generally 100 μL) were diluted in different volumes of culture buffer (Hank's balanced salt solution (HBSS) + HEPES) to obtain a series of standards in the range 1−100 μM. The quality control (QC)

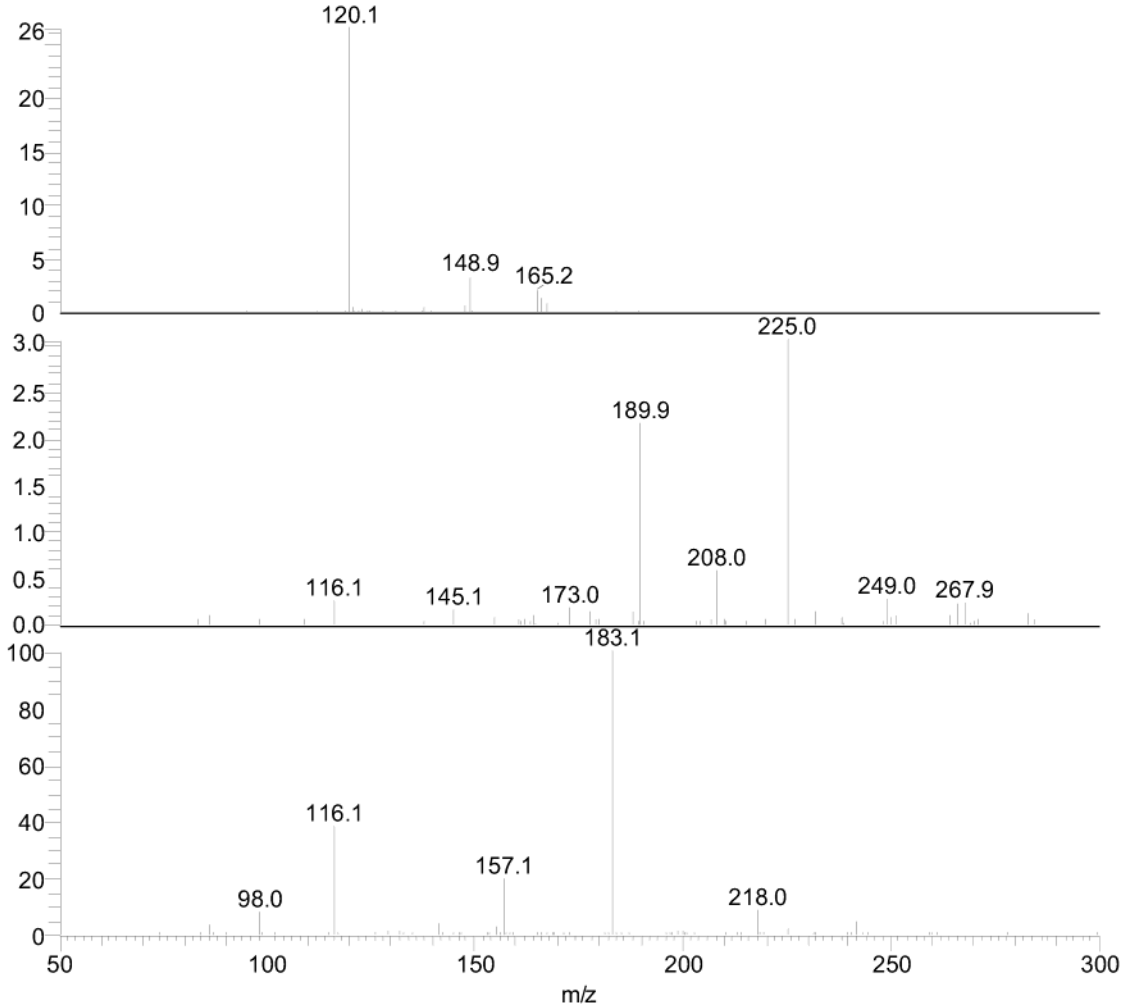

Figure 1. Product ion mass spectra of the three analytes. From top to bottom: phenylalanine, atenolol, and propranolol.

samples, at the 2, 20, and 100 μM concentration levels, were prepared using the same approach.

MS Detection. The HPLC flow was diverted to waste for the first 0.7 min; after this time, the HPLC eluent was introduced into the mass spectrometer, which then proceeded with detection until the end of the analysis (4.5 min after injection).

The ionization was positive APCI. The signal was monitored in tandem MS in scan mode: for each analyte, the quasi-molecular ion was isolated and fragmented and the spectrum of product ions recorded. Specific product ions were selected to produce mass-extracted chromatograms (one specific trace for each analyte), which were then integrated to measure analyte response. The different mass detector conditions, specific for the different analytes, were applied in consecutive time segments corresponding to the elution window of the different species. The product ion spectra of the analytes are displayed in Figure 1. The parameters used for analyte detection are described in Table 1.

In all experiments, the isolation width was 2.0 Da and the *Q* value was maintained at 0.25. The automatic gain control (AGC) was set on, with a MS/MS target of 2×10^7 counts and a maximum ion time of 400 ms; the number of microscans was 1.

The source current was set at 5.0 μA, and the vaporizer and capillary temperatures were 450 and 150 °C, respectively. Nitrogen was used as the sheath gas at a flow of 60 counts (arbitrary units).

Table 1. MS/MS Parameters Used for the Detection of Analytes

analyte	parent (m/z)	amplitude (%)	product ion range (Da)	extracted product ions (m/z)	time window (min)
atenolol	267	35	70−300	190, 225	0.7−1.5
phenylalanine	166	25	50−200	120	1.5−2.3
propranolol	260	35	70−300	116, 157, 183	2.3−3.3

Cell Culture and Transport Experiments. Cells were routinely cultured in 75-cm^2 culture flasks in the presence of minimum essential medium (MEM) supplemented with Earles salts, 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 1% MEM nonessential amino acids, and antibiotics (penicillin/streptomycin/amphotericin B cocktail) with medium change every 2 days in an atmosphere of 5% CO_2/95% air at 37 °C. When 80% confluency was reached, cells were subcultured using trypsin/EDTA (0.25% and 0.5 mM, respectively) and plated into new flasks at a density of 2×10^4 cells/cm^2. For the transport experiments, Caco-2 cells were seeded on presoaked collagen-coated membrane filters inside a transwell cell culture chamber at a density of 3×10^5 cells/filter (6.3×10^4 cells/cm^2). Each transwell insert was placed in a

35-mm well of a culture plate with 2.6 and 1.5 mL of culture medium in the basolateral and apical sides, respectively. The cell monolayers were fed with fresh culture medium every 2−3 days and were used 21−23 days after seeding and 18−24 h after feeding. At this point, cells have been fully differentiated as assessed by the development of TEER and the expression of sucrase/isomaltase activity.[13] TEER was monitored using an Evometer (World Precision Instruments) fitted with "chopstick" electrodes. Monolayer integrity during the transport experiment was determined by measuring the TEER before and after drug addition.

Transport Studies. Test and reference compounds were first solubilized in DMSO and then further diluted in assay buffer in order to achieve the final test concentration. The amount of DMSO was 0.1% in all the conditions tested in order to minimize adverse effects on cell permeability. Assay buffer was HBSS buffered at pH 7.4 with 12 mM HEPES and 0.35 g/L sodium bicarbonate.

Absorption is measured as the apparent permeability coefficient (P_{app}) of transport in the apical to basolateral direction; in contrast, secretion is measured as the apparent permeability (P_{app}) of transport in the basolateral to apical direction.

Cells were washed three times with HBSS and incubated for 1 h at 37 °C. To begin permeability experiments, the buffer in the donor compartment was aspirated and replaced with fresh buffer containing the compound to test at 100 μM concentration. Transwells were then placed in an orbital horizontal shaker placed in a CO_2 incubator. At time 20, 40, 60, and 120 min, an aliquot was removed from the receiver compartment (100 μL for absorption assay) and replaced with an equal volume of fresh buffer. Samples aliquots were transferred in autosampler vials and stored at −80 °C for LC−MS analysis.

Calculations. The apparent permeability coefficient, P_{app}, is expressed as cm/s $\times 10^6$. It is obtained by plotting the cumulative amount of compound transported (Q) versus time (T) and determining the linear appearance rate (slope, $\Delta Q/\Delta t$) of the compound on the receiver side. The appearance rate is determined by analyzing the linear part of the curve using a linear regression fitting: $Q = aT + b$, where a is the slope, which equals $\Delta Q/\Delta t$,and b is the intercept.The P_{app} value was obtained by the formula $P_{app} = (\Delta Q/\Delta t)/(AC_o)$, where $\Delta Q/\Delta t$ is expressed in millimoles per second, A is the surface area of the cell monolayers (in cm^2), and C_o is the initial concentration of the compound on the donor side (in mmol/cm^3).

The mass balance was calculated for all experiments. It is expressed as the total detected quantities of analytes at the end of the Caco-2 experiment (t = 120 min) to that originally introduced in the transwell ($t = 0$). A ratio value around 1 indicates good recovery. A value significantly lower than 1 could indicate loss of material in solution through adsorption to the transwell walls or membrane cellular uptake, metabolism, or degradation of the compounds. On the basis of the data available so far, typical mass balance values for atenolol, propranolol, and phenylalanine are 0.88 (relative standard deviation (RSD) = 7.9%), 0.77 (RSD = 16.9%), and 0.83 (RSD = 12.0%), respectively.

RESULTS AND DISCUSSION

Choice of Reference Compounds. The reference compounds used for the functional characterization of the Caco-2 cell culture absorption properties need to be indicative of the different parameters in play: in summary, they should allow us to determine when the cultures can be used to perform permeability experiments and give a quantitative reference value of the parameters tested. Another element in the choice of the reference compounds was the information already available in the literature. It was preferable to use well-studied chemicals as the experimental results could be compared to existing data. We sought compounds for which Caco-2 permeability values were available but had also been studied in vivo to have a reference as to their behavior in "real" conditions. Briefly, the cell growth on transwells proceeds in essentially three stages. In the first days after insemination on the transwell membrane, cells grow separated and spread over the surface. When the cells come to confluence, they start differentiating and acquiring characteristics typical of enterocytes: they form a brush border and tight junctions. In the following days, the cells continue their maturation process expressing enzymatic systems and transporter proteins as found in enterocytes in vivo. The cells become fully mature after ∼20 days in culture; from this point onward permeability tests can be performed that will take into account all the characteristics of a human intestinal epithelium. At more than 25 days, the cultures start to enter senescence and the cells lose their characteristics and usefulness again.

The parameters to test are mainly two: the monolayer integrity (presence of the tight junctions) and maturation of the cells. We also wanted to include a very permeable compound, to be used as a high absorption marker. After a preliminary selection of ∼20 biologically active compounds, we chose 2 β-blockers, atenolol and propranolol, which had been previously studied,[4] and a natural amino acid, phenylalanine.

To test the monolayer integrity, we used a poorly absorbed drug, atenolol. In fact, due to its hydrophilicity, this species can pass the intestinal epithelium only through the intercellular spaces (paracellular absorption).[11,17] As these are obstructed by the tight junctions between the cells, atenolol presents a low P_{app} once the culture has become confluent and the cells have gone through the differentiation process. In our laboratory, the maximum accepted P_{app} value for atenolol is 2.5×10^6 cm/s.

A value for high permeability is obtained using propranolol as the test compound. This drug is much more lipophilic than atenolol and easily permeates through the cell membranes (passive transcellular absorption).[11,17] Our minimum accepted P_{app} value for propranolol is 20×10^6 cm/s. Phenylalanine was used as a marker for cell maturation. This amino acid is very polar and does not easily cross the cell monolayer through passive absorption; however, it can be absorbed thanks to a specific amino acid active transporter protein[18] (many transporters allow hydrophilic nutrients to enter the organism in vivo). In the absence of the transporter, phenylalanine transepithelial permeability can be expected to be similar to that of atenolol, whereas if the cells are expressing the transporter, it is significantly higher. The absorption rate of phenylalanine is dependent on the availability of transporters (rather than the whole membrane surface area), requires energy, and depends on transporter affinity and activity. In absolute terms, this measure only gives an evaluation of the expression of the specific transporter(s) taking charge of phen-

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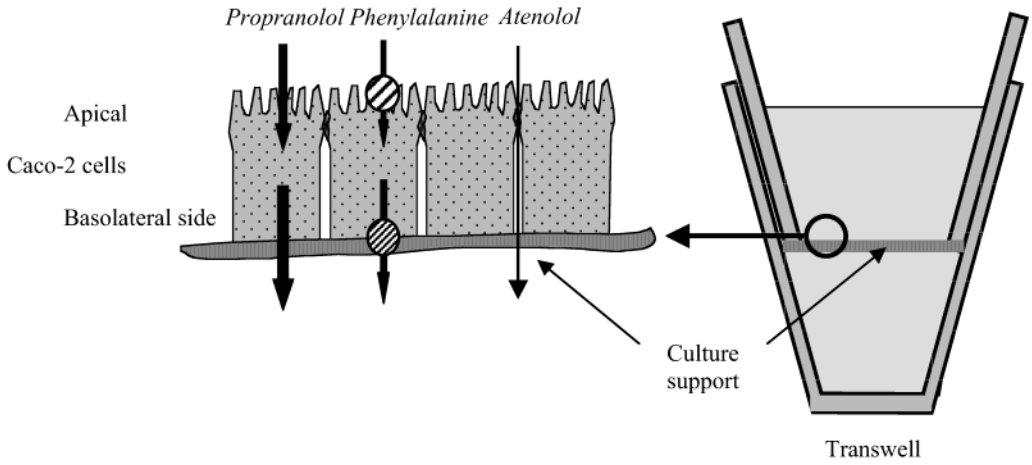

Figure 2. Schematic representation of the culture of Caco-2 cells on transwells and permeation behavior of the chosen reference compounds.

ylalanine, but we also used it as a general marker of cell maturation as transporters are synthesized only when the Caco-2 cells have reached maturity. The variation of phenylalanine permeability among different experiments gives a measure of the variation of transporter expression in the different culture batches. A P_{app} greater than 8.0×10^6 cm/s for phenylalanine is our currently accepted value to assess proper carrier expression.

In summary, atenolol is used to indicate the state of the cell monolayer (confluence, junctions); once the cells in culture have morphologically developed as enterocytes, it provides the lower extreme of the permeability scale while propranolol provides the high extreme. Phenylalanine, an actively transported species, is used as an indicator of the cells' biochemical maturation. A schematic representation of the culture of Caco-2 cells on transwells and the permeation behavior of the chosen reference compounds is given in Figure 2.

Method Development. One of the main functions of the HPLC step is to retain the analytes on the column while the buffer components are washed out. In our setup, this unretained material is sent to waste so that it does not contaminate the ion source. The analytes are then eluted, using a gradient, from the column into the mass spectrometer. Some separation of the analytes between one another is desirable as this diminishes the risk of ionization competition and allows us to set the mass spectrometer to specifically detect one single compound for a specified chromatographic segment, rather than monitor all analytes together, thus maximizing sensitivity and chromatographic peak sampling. Short gradients of 2 min were used throughout the study to allow us to operate analyses short enough for medium throughput.

The MS detection method following consisted of full-scan acquisition of the product ions; then, mass-extracted chromatograms for one or more specific fragment ions were built up postacquisition and used as analyte signal for chromatographic peak integration. This approach is slightly different from the one most typically used in LC−MS/MS quantitation, i.e., selected reaction monitoring (SRM) with a triple quadrupole instrument, in which only one or a few product ions are monitored instead of the entire spectrum. In our case, the high duty cycle of the ion trap analyzer in the full-scan mode allows acquisition of the product ion spectrum without significant loss in sensitivity compared to SRM, maintaining spectral information that would be otherwise lost. Other aspects of LC−MS/MS quantitation by ion trap versus triple quadrupole instruments have been discussed.[19−21] The LC−MS/MS chromatogram of a typical sample is shown in Figure 3.

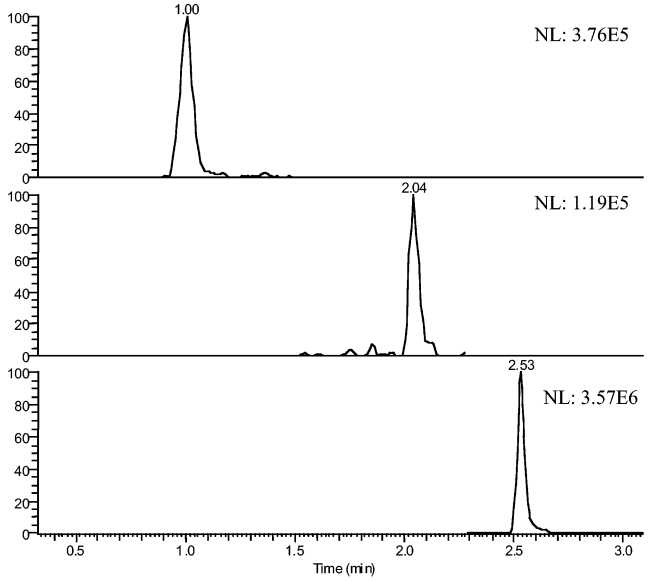

Figure 3. LC−MS/MS chromatogram of a typical sample. From top to bottom: phenylalanine (transition, *m*/*z* 166 to 120), atenolol (transition, *m*/*z* 267 to (190 + 225)), propranolol (transition, *m*/*z* 260 to (116 + 157 + 183)).

Validation. Linearity of response was tested over the range of interest, 1−100 μM, by injecting five standards of increasing concentrations. The calibration statistics obtained for a typical analytical sequence, injecting four sets of standards over a night time period (14 h), are presented in Table 2. All analytes showed linear response with regression coefficients higher than 0.995 ($r^2 > 0.995$) and back-calculated values differing by less than 15% from theory. The precision for those four sets, analyzed over an entire sequence, was good with RSDs under 15% at all concentra-

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Table 2. Calibration Curve Statistics for the Three Analytes (Four Sets of Standards Injected over 14 h)

theor concn (μM)	av back-calcd concn (μM)	inaccuracy (%)	RSD (%)
Phenylalanine: $Y = -23517.9 + 258847X$; $R^2 = 0.9979$			
1	1.0	3.5	8.6
4	4.1	2.1	11.0
10	9.8	−2.0	5.9
33.3	32.3	−2.9	4.7
100	101.3	1.3	3.6
Atenolol: $Y = 28298.9 + 1007820X$; $R^2 = 0.9989$			
1	1.0	1.1	6.4
4	4.0	1.2	5.7
10	9.7	−3.3	5.1
33.3	33.7	1.1	4.0
100	99.9	−0.1	2.7
Propranolol: $Y = -78355 + 502131X$; $R^2 = 0.9986$			
1	1.0	3.9	13.6
4	4.1	3.0	7.4
10	9.5	−5.0	0.4
33.3	33.2	−0.4	3.2
100	100.7	0.7	3.6

tions. Note that the lower limit of 1 μM does not represent the absolute lower limit of quantitation (LLOQ), which we did not investigate; the response was linear up to ~150 μM but goes out of the dynamic range beyond this value (data not shown).

The accuracy and precision were tested by the analysis of QC samples at low, medium, and high concentration (2, 20, and 100 μM, respectively). As for standards, the QC samples were analyzed repetitively between samples within each analytical sequence.

Table 3 presents the results obtained for intra-assay variability and precision.The average calculated values were accurate, with inaccuracies lower than 10%, and precision within 10% RSD. Retention time variation was under 3% RSD for phenylalanine and under 1% for the other two analytes.

Table 4 presents a summary for interassay reproducibility. It was evaluated by considering the first two of each QCs for four sequences ran over a 2-month period, for a total of eight values. The peak areas are given as well as the calculated values to show instrumental variability over this period. Although the peak areas were quite variable, with RSDs higher than 20% and up to 30%, the calculated concentrations were reproducible with RSDs within ~10%. This shows that although instrumentation performance can vary in time it is efficiently corrected for by preparing a calibration within each analytical sequence. The variation in peak area was "random" rather than a unidirectional tendency: this shows that it was probably due to time-specific changes in conditions (source and capillary cleanliness and mobile phases, essentially) rather than a continuous loss of instrumental performance (gradual loss of vacuum, soiling of ion path beyond sampling capillary, deterioration of the dynode). Use of an internal standard to increase method robustness was considered at a first stage of method development. We found that there was no significant improvement in RSDs using some common pharmaceuticals as internal references. On the other hand, we judged the intra- and interassay performance of the external standard method satisfactory and that the introduction of stably labeled analogues of the analytes as internal standards, which is the preferred approach for quantitative LC−MS, would have increased method complexity and analysis cost without any essential advantages for the purpose of the assay.

The response stability of the analytes kept at ambient temperature during the time of an analytical sequence was tested by injecting the less concentrated QC sample (2 μM) at regular intervals. The evolution of the analyte response over time is graphically presented in Figure 4. The signal was consistent for over 16 h (a typical sequence does not extend beyond 15 h run time) and varied randomly rather than decrease with time.

The influence of preparation mode of the standards and QCs was investigated to check whether assaying the analytes as single compound solutions or mixtures could have an effect, in case they influenced each other's response. Solutions were prepared as mixtures following the general scheme (see Materials and Methods) and as single compounds preparing all dilutions in culture buffer rather than using an intermediary preparation containing acetonitrile. No difference could be found in the quantities measured in solutions of the same concentration prepared with different procedures. The analytes do not influence each others' mass spectrometric response, and a small quantity of acetonitrile present in the sample solution does not modify the signal intensity.

Specificity was tested by injection of blank samples. No signal could be detected for any of the analytes in fresh culture buffer analyzed in a clean system. The carryover observed for blank injection immediately after the analysis of the top standard (100 μM) was under 1% in area and integrated to give values under 0.5 μM.

Blank injections were also done using cultured buffer, "blank" Caco-2 experiments were performed in typical conditions but not applying any test chemical, and the solutions in the transwell compartments on the apical and basolateral sides of the monolayer were collected after 2 h (time of the permeability experiments). Neither atenolol nor propranolol could be detected in either sample. On the other hand, small amounts of phenylalanine could be detected. The quantity detected in the apical side solution was lower than the lowest standard (<1 μM); the basolateral side solution contained a measurable quantity of 4 μM phenylalanine. This is not entirely surprising as phenylalanine is a common amino acid, endogenous to all cells, and is actively excreted by Caco-2 cells toward the basolateral compartment. The quantities thus naturally present in the basolateral receptacle are not high, accounting for less than 7% of total phenylalanine, and do not therefore significantly modify the analytical results for the phenylalanine artificially added at the start of the experiment. Moreover, the natural excretion of endogenous phenylalanine in itself proves the expression of the transporters (there is a significant difference between the apical and basolateral concentrations) and hence the maturity of the cells.

Application of Reference Compounds as a Mixture ("Cassette Dosing"). The simultaneous determination of mixtures of drug candidates after coadministration as an in vivo drug screening procedure (the so-called "cassette dosing") was described by Olah et al.[22] Applications of the same principle to the Caco-2 model were also described, using either LC−MS/MS[16] or LC with UV detection.[23]

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Table 3. Intra-Assay Repeatability[a]

	phenylalanine			atenolol			propranolol		
theor concn (μM)	2	20	100	2	20	100	2	20	100
found concn (μM)	1.8	19.3	99.0	2.1	21.7	106.1	2.1	20.7	108.3
	2.1	19.7	98.8	2.0	20.6	100.0	2.0	20.8	99.6
	2.2	17.0	98.2	1.9	20.2	100.9	2.1	19.4	101.9
	2.3	19.9	89.5	2.0	19.6	95.4	1.9	19.5	96.0
	2.3	20.3	97.3	2.1	19.7	95.4	2.2	20.2	100.7
	2.0	21.4	104.9	2.2	21.0	111.4	2.2	21.8	106.6
average (μM)	2.1	19.6	98.0	2.1	20.5	101.5	2.1	20.4	102.2
inaccuracy (%)	5.8	−2.0	−2.1	2.5	2.3	1.5	4.6	2.0	2.2
RSD (%)	9.2	7.5	5.0	5.1	3.9	6.2	5.4	4.4	4.5

[a] Calculated amount, $n = 6$.

Table 4. Interassay Reproducibility[a]

	2 μM		20 μM		100 μM	
	area	amt	area	amt	area	amt
	Analyte: Phenylalanine					
sequence 1	330 334	1.6	2 901 900	17.8	14 367 116	90.0
	365 256	1.8	3 014 414	18.5	17 934 889	112.5
sequence 2	448 551	1.8	4 966 667	19.3	25 603 505	99.0
	512 790	2.1	5 064 785	19.7	25 543 680	98.8
sequence 3	494 950	2.0	4 969 989	20.0	26 743 160	107.3
	547 105	2.3	5 109 647	20.6	27 428 886	110.1
sequence 4	299 823	1.9	3 139 889	22.2	14 771 539	104.8
	258 432	1.6	2 508 783	17.6	14 493 765	102.8
average	407 155	1.9	3 959 509	19.5	20 860 818	103.2
inaccuracy (%)		−5.0		−2.7		3.2
RSD (%)	26	11.6	29	7.8	29	7.0
	Analyte: Atenolol					
sequence 1	2 578 310	2.1	28 689 733	22.9	132 486 055	105.7
	2 652 659	2.2	24 077 719	19.3	119 009 755	95.0
sequence 2	2 163 709	2.1	21 876 808	21.7	106 977 200	106.1
	1 992 586	1.9	20 812 528	20.6	100 848 726	100.0
sequence 3	2 266 786	1.9	21 581 579	21.0	102 737 093	101.3
	2 510 212	2.2	22 198 232	21.6	100 966 690	99.5
sequence 4	1 224 822	1.8	14 997 248	22.6	67 105 085	101.4
	1 173 599	1.7	12 683 757	19.1	66 459 702	95.9
average	2 070 335	2.0	20 864 700	21.1	99 573 788	100.6
inaccuracy (%)		0.0		5.6		0.6
RSD (%)	28	8.5	24	6.7	23	4.0
	Analyte: Propranolol					
sequence 1	636 983	2.0	7 708 317	21.0	40 670 393	109.8
	693 569	2.1	7 092 398	19.4	37 091 868	100.1
sequence 2	964 707	2.1	10 312 711	20.7	54 300 152	108.3
	944 040	2.0	10 351 430	20.8	49 911 055	99.6
sequence 3	862 198	1.8	9 835 272	19.3	51 087 383	100.0
	1 017 591	2.1	9 476 459	18.6	54 772 100	101.4
sequence 4	668 672	2.1	7 417 789	21.0	36 051 778	101.3
	616 761	1.9	6 353 113	18.0	35 272 780	99.2
average	800 565	2.0	8568 436	19.9	44 894 689	102.5
inaccuracy (%)		0.5		−0.7		2.5
RSD (%)	20	5.9	19	5.9	19	4.0

[a] Areas and calculated amount, two first QCs of each sequence, four sequences spread over 2 months, and $n = 8$ in total.

The possibility of applying the reference compounds to the Caco-2 model as a mixture was investigated. We had already demonstrated that assaying the compounds as single-compound solutions or as mixtures did not influence the analytical results.

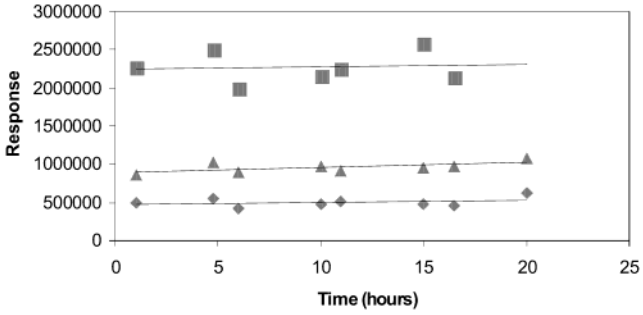

Figure 4. Stability of signals during the length of a sequence, measured as chromatographic peak areas. The three analytes are represented by squares (atenolol), triangles (phenylalanine), and diamonds (propranolol), respectively.

Table 5. Apparent Permeabilities (P_{app}, in cm/s × 10^6) for Reference Compounds Applied as Single Compounds or Mixture

	ref compds applied as	
	single species	mixture
atenolol	1.4	1.8
phenylalanine	11.9	12.3
propranolol	29.8	28.9

The aim of this particular experiment was to investigate possible effects at the biological level: drug−drug interactions on transport across the monolayer. The reference compounds were applied as single solutions and as a mixture, all with a concentration of 100 μM each in the apical side solution at the start of the experiment, in a single batch of cell culture (on the different transwells of a single plate). All experiments were conducted in duplicate for a total of eight transwells.

Table 5 presents the resulting calculated apparent permeabilities. There is no significant difference in the results obtained with one method and the other. All three reference compounds can be applied to the cell culture as a mixture without modifying each others' P_{app}. This constitutes an advantage as it limits the number of transwells necessary for checking the desired parameters in each culture batch—two transwells (duplicate experiment) rather than six (duplicate application of three single-compound solutions)—leaving more free space for experiments on new molecules.

Usefulness of the Method in Model Characterization. This method is now routinely used for characterizing the transport activity of each new batch of Caco-2 cultures. This allows us to

determine whether the model is reproducible in terms of its transport characteristics and whether variations are observed to identify their causes to possibly correct other experimental data. If atenolol presents an abnormally high P_{app}, this is indicative of poor monolayer integrity: the cells have not reached confluence, the tight junctions are not well formed, or they have been damaged. If phenylalanine presents a low P_{app}, it is indicative of poor expression of the transport protein and, in general, of immature cells. If propranolol presents a low P_{app}, it would be indicative of an unexpected barrier to passive diffusion (this is very unlikely to occur, the only possible cause being the impermeability of the porous membrane supporting the culture). Atenolol being poorly permeable and propranolol highly permeable, these two compounds give two extremes of a P_{app} window within which new chemicals tested in the model can be ranked. Overall the systematic use of the reference compounds helps in a better interpretation of the experimental results for other species tested in the system, which behavior is not known. During a period of ~5 months, the P_{app} values for our reference compounds were (average ± standard error, $n = 13$): atenolol, $(1.0 \pm 0.12) \times 10^6$ cm/s; propranolol, $(22.5 \pm 1.37) \times 10^6$ cm/s; phenylalanine, $(10.2 \pm 0.52) \times 10^6$ cm/s.

An advantage of the method presented here to characterize the transport activity of the Caco-2 model over more common approaches is that the assay is performed by LC−MS. As this analytical technique is also used for the measurements of new chemicals applied to the model, it allows us to run model checks and samples on the same system in an unattended manner. As the analytical instrumentation used is highly automatized and the method is routinely used, it requires very little human intervention at the analytical level. Another advantage of the approach is that it is more general in its application than fluorescence or radioactivity as it does not require the molecules used in the test to be fluorescent or radioactive. Since LC−MS can be used for the analysis of a varied range of compounds, this would make it easier to introduce other species as reference compounds within the method according to specific needs; for example, known substrates of specific transporters could be used to test the expression of different transport proteins of interest.

Beyond their routine use to check on model reproducibility, the reference compounds can be helpful to investigate some different uses of the model. We will give as an example their application to investigate the use of different temperatures to do experiments in the presence of active or inactive transporters. It may be necessary to identify whether a permeable compound crosses the monolayer by active or passive mechanisms, and in standard conditions this is difficult as both coexist. One possible approach to observe only passive phenomena could be to perform the experiments at a lower temperature (4 °C)[24] as the transport proteins are not expected to be active in these conditions. To test the applicability of this approach, we applied the reference compounds in the model at both 37 and 4 °C. The results obtained at both temperatures are compared in Table 6.

At 37 °C, as usual atenolol is poorly permeable, propranolol has a much higher P_{app}, and phenylalanine lies between for its apical → basolateral permeability. Propranolol has an equivalent P_{app} in both directions, and phenylalanine crosses the monolayer in an unidirectional manner, consistent with active absorption. At 4 °C, atenolol still presents poor permeability, indicating the low temperature does not affect the monolayer integrity. Phenylalanine presents poor permeability in both directions; as the transport protein is inactivated, it cannot cross the monolayer in either direction. Propranolol on the other hand retains significant permeability in both directions, and although its P_{app} is lower due to reduced diffusion at that lower temperature, it still efficiently crosses the membranes. The analytes mass balance values of the experiments at 4 and 37 °C were not significantly different, indicating that the P_{app} values were not influenced by decreased solubility at low temperature. Using the reference compounds in these conditions demonstrates that testing chemicals in the model at 4 °C allows us to observe their passive diffusion component alone.

Table 6. Apparent Permeabilities (P_{app}, in cm/s × 10^6) for Reference at 37 and 4 °C

	apical → basal (absorption)		basal → apical (secretion)	
	37 °C	4 °C	37 °C	4 °C
atenolol	1.1	<1		
phenylalanine	17.5	1.1	1.0	<1
propranolol	21.8	11	24.8	16.5

Possible Developments: Characterization of the Secretion Properties. Apart from the in vitro reproduction of absorption mechanisms, another interesting aspect of the Caco-2 cells in culture is their ability to express a secretion protein, P-glycoprotein (Pgp).[10,11,24] Pgp functions as a transporter of a wide range of chemicals, but rather than promote their absorption, it actively excretes them back out of the cells, preventing the entry of xenobiotics into the organism (at the intestinal level) or the other cells that express it. Indeed, Pgp is present not only at the surface of the intestinal epithelium but also in other cell types, particularly in tumor cells, and is the major cause of multidrug resistance. It is therefore useful to identify drug candidates that may be Pgp substrates early as this may impair their ability to enter the organism or specific target cells.

As for the absorption mechanisms, it would therefore be of value to monitor the expression of Pgp, to verify whether it is significantly active in the culture and follow its variation across experiments. Presently, we are using a fluorescent probe, Rhodamine 123, a known substrate of Pgp,[25−27] to evaluate the importance of efflux systems in cell preparations.

The easiest way of evaluating Pgp expression would be to use one of its substrates as a fourth reference compound. We are at present investigating some candidate compounds that could be analyzed concomitantly with the absorption reference chemicals.

CONCLUSION

A LC−MS method for the analysis of atenolol, phenylalanine, and propranolol as reference compounds for the functional

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characterization of the transport activity in the Caco-2 model was developed. The method was extensively validated and is now routinely used in our laboratory for checking each batch of cultures for permeability experiments. We also demonstrated that all three reference compounds can be applied simultaneously as a mixture to the model and subsequently analyzed simultaneously, saving time during both Caco-2 experiments and analyses.

As this assay uses the same technique as that for new chemicals, they can be performed along side using the same instrument. It is less time and work consuming than more common methods for transport characterization in the Caco-2 model based on fluorescence or radioactivity. As it uses a more general detection approach, not needing fluorescent chromophores or synthesis of molecules with radioactive isotopes, it can be easily adapted to other reference compounds of interest.

The method is not only useful in the routine characterization of the transport activity in each culture batch to check results consistency, it can also be instrumental in investigating the use of the cultures in different conditions, useful in further testing of new molecules, and monitor the exact effects on transport across the monolayer.

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