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Human oral drugs absorption is correlated to their *in vitro* uptake by brush border membrane vesicles

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Abstract

Brush border membrane vesicles (BBMV) were prepared from the rabbit small intestine for testing drug absorption potency through the enterocyte's apical membrane, which is an important compartment for drug oral absorption. Some modifications have been made to the traditional vesicle assay for adapting it to the 96-well plate format. The accumulation of 23 reference drugs was measured, and the data showed a good correlation with human oral absorption with a correlation coefficient $R = 0.853$ ($P < 0.001$), with the exception of a few false positive results. As the measured drug absorption may contain a membrane/protein binding component as well as drug uptake into vesicles, these two fractions can be discriminated by changing extravesicular osmolarity using different mannitol concentrations. This model can be applied for evaluating drug absorption rate/mechanisms, and helping drug selection in early drug research and development.

Keywords: Absorption; Transport; BBMV; *In vivo–in vitro* correlation

1. Introduction

The oral absorption of an oral drug is an important feature for its successful treatment of diseases and has to be considered early in drug research and development. Intestinal drug absorption is a major barrier for its penetration into body following oral administration and is an important factor to estimate drug bioavailability, together with other pharmacokinetic parameters, such as distribution, metabolism and excretion.

The intestinal absorption depends on chemical structure of the drugs, permeability of the intestinal brush border membrane (BBM), and other biological factors such as intestinal motility, enzyme systems, food, and the physiological state of the human intestinal tissues. All these parameters emphasize the complexity of the absorption process. Despite the incomplete understanding of all aspects governing drug absorption in the intestine, more and more drugs were reported to be absorbed through the specialized system involving specific protein transporters localized in BBM (Katsura and Inui, 2003; Kunta and Sinko, 2004).

As the intestinal BBM is an important barrier responsible for drug absorption, an experimental model using BBM vesicles (BBMV) formed from isolated intestinal BBM and enriched in various transporters may provide important information regarding the drug absorption process. Such a model was first reported by Hopfer et al. (1973) demonstrating the possibility to measure glucose uptake into intestinal BBMV. Further simplifications (Maenz et al., 1991) made this model easier to use. Advantages and limitations of vesicles for transport kinetic studies were reviewed previously (Murer et al., 1984; Turner, 1983; Berteloot and Semenza, 1990), but essentially for the transport of natural substrates. At the same time, BBMV were also suggested as an attractive experimental model to evaluate drug absorption in different ways (Hori et al., 1988; Wood et al., 1990; Kohda-Shimizu et al., 2001).

One of the promising strategies for improving the oral absorption of poorly absorbed drugs is the utilization of membrane transporters to facilitate the absorption of appropriately modified drugs (Tamai and Tsuji, 1996; Tsuji and Tamai, 1996; Mizuma et al., 1994; Fujita et al., 1997; Nomoto et al., 1998; Han et

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al., 1998). The BBMV model is particularly interesting for such carrier-mediated oral drug delivery, since the BBMV preparation leads to a 10–20-fold enrichment of the apical membrane proteins as compared to the initial homogenate (Maenz et al., 1991). On the other hand, carrier-mediated transport influences hepatic drug distribution, elimination, renal drug reabsorption and clearance (Meijer et al., 1990; Zhang et al., 1998; Koepsell, 1998; Bendayan, 1996; Somogyi, 1996). The information obtained using the BBMV prepared from these tissues may become a very useful tool for predicting the PK/PD of drug candidates.

Many efforts have been made to predict the oral absorption by using different *in vitro* and *in situ* models for drug absorption (Hidalgo et al., 1989; Hori et al., 1988; Wood et al., 1990; Yu et al., 1996), and drug metabolism (Bai and Amidon, 1992). Traditional animal studies, aimed at predicting the extent of pharmacokinetic parameters in humans using allometric extrapolation were also reported (Mahmood, 2000). Roberts has reviewed the *in vitro* methods recently. He indicated that many cell culture systems had been used to study intestinal permeability, but with varying degrees of success. Caco-2 model presents a number of drawbacks, such as the relationship of Caco-2 predictions to *in vivo* human intestinal absorption is such that predictions for compounds with low-to-moderate Caco-2 permeability can be poor. In addition, paracellular passage is not modeled well with some water-soluble, low molecular weight compounds possessing high absorption predicted to have low permeability. The presence or absence of drug transporters in cell culture models can influence the interpretation of permeability data. P-glycolprotein (P-gp), for example, is present in Caco-2 and involved in the intestinal efflux of many compounds, but it is often overexpressed, resulting in the underestimation of the absorption potential of P-gp substrates. In addition, where compounds prove to be inhibitors of transporters, the potential for drug-drug interaction exists. Studies with Caco-2 cells can be time-consuming (Roberts, 2003). Comparing with Caco-2 model, BBMV are prepared directly from animal intestine, and may be used soon after thaw from frozen stock preparation (Schmitz et al., 1973).

In the present study, we have modified a generally used BBMV assay in order to adapt it for rapid compound selection in early drug discovery, research and development. Our BBMV model was validated by establishing a correlation between experimental data and drug's human oral absorption.

2. Materials and methods

2.1. Chemicals

All drugs and chemicals were purchased from Sigma (St. Louis, MI) and used without further purification. HPLC grade acetonitrile and water were used for all purposes. Other reagents were analytical grade and obtained through commercial resources.

The BCA (bicinchonique acid) protein assay kits were obtained from Pierce. Multiscreen Assay System consisting of a Vacuum Manifold and Multiscreen 96-well plates with Durapore membrane (PVDF, polyvinylidene fluoride) filters (0.45 μm pore size) were obtained from Millipore. Pump Gast (UL®746A) was used to provide the constant filtration pressure. Liquid handling and distribution were performed using multichannel Biohit Proline® Electronic Pipettors (Biohit 100 μl and Biohit 50–1200 μl).

2.2. Preparation of rabbit intestinal brush border membrane vesicles

New Zealand white rabbits were purchased from the *Ferme de Selection Cunipur* (St. Valerien, Quebec, Canada). Large batches of BBMV were prepared from the small intestine of male 2.0–2.5 kg New Zealand white rabbit as described previously (Maenz et al., 1991; Oulianova and Berteloot, 1996; Berteloot, 1984). Briefly, the small intestine was removed and flushed with ice-cold 0.9% NaCl. The mucosa was scraped on an ice-cold glass plate, introduced into the homogenate media (50 mM mannitol, 2 mM Tris–HCl buffer pH 7.0, EGTA 5 mM) at 1:20 ratio (w/v) and homogenized in a Warring blender for 1 min at full speed. Brush border membranes were precipitated by addition of 10 mM $MgCl_2$ (Maenz et al., 1991), purified in a P_2 fraction, resuspended in 50 mM HEPES–Tris buffer (pH 7.0, containing 0.1 mM $MgSO_4$) and 300 mM mannitol and subsequently frozen in liquid N_2 in 500 μl aliquots. On the day of assay or the previous day, P_2 were thawed and diluted 40-fold in the resuspension buffer (see below). BBMV were prepared as the final P_4 pellet and resuspended in the same buffer at the protein concentration of 30–35 mg/ml. Protein concentration was measured with the BCA kit (Pierce) using bovine serum albumine as a standard. The viability of the vesicles preparation was estimated using Na^+/glucose uptake at 37 °C, which exhibited a well characterized overshoot resulting from the activity of the SGLT1 protein.

2.3. BBMV assay

Drug uptake into BBMV was measured at controlled room temperature (RT, 22–23 °C) by the rapid filtration method with modifications. The RT was expected to slow down the BBMV uptake of rapidly absorbed compounds because their accumulation, being measured after 1 min of incubation, can be underestimated due to the overshoot's efflux phase as can be seen in Figs. 2 and 3. At the same time we expected, as a compromise for screening purposes, that 1 min of incubation at RT will be long enough to allow the uptake of low-to-moderate absorbed compounds. When experiment was carried out at 37 °C, the temperature is indicated in legends to the figures. Compounds were tested under physiological conditions: in the presence of inward H^+-gradient (Lucas, 1983) and inward Na^+-gradient. The tested drug concentration (1 mM) was chosen in anticipation of compounds with low permeability or for actively transported drugs with a high K_m value. In general, 20 μl of BBMV final P_4 suspension in 50 mM HEPES–Tris buffer (pH 7.5), containing 0.1 mM $MgSO_4$, 250 mM KCl and 100 mM mannitol were mixed with 130 μl of incubation media of 50 mM MES–Tris buffer (pH 6.0), containing 0.1 mM $MgSO_4$, 50 mM KCl, 200 mM NaCl and 100 mM mannitol, and 1 mM of investigated drug. Ice-cold

Stop solution contained 50 mM MES–Tris buffer (pH 6.0) with 0.1 mM $MgSO_4$, 250 mM NaCl and 100 mM mannitol. In the test discriminating substrate accumulation from its membrane binding, the osmolarity of the incubation media was increased by adding 50, 100, 300 or 481 mM mannitol. After incubation, sampled aliquots were immediately filtered into a 96-well plate (Millipore 96-well plate filter, HAWP, 0.45 μm of pore size), followed by washing with 100 μl ice-cold Stop solution. Acetaminophen and cefadroxil were selected as references for assay modifications and validation.

Non-specific binding to the filters was determined by repeating the above incubation/filtration procedure in the absence of BBMV. This amount was systematically subtracted from the total determination for each experimental point and the corrected value was considered as the drug uptake amount specific to BBMV.

At the end of the experiment, filters were punched out into 150 μl of distilled water, sonicated for 5 min, then sample were analyzed by valid HPLC-UV detector or HPLC/MS using a reverse-phase column (Luna C18, 2× 50 mm) with 5–95% acetonitrile (water contained 0.1% TFA) gradient. The concentration of drugs was determined from the peak area using standard curves.

3. Results and discussion

3.1. Modifications of a conventional RFT to accommodate it to a 96-well plate format

The conventional rapid filtration technique (RFT) (Artursson and Karlsson, 1991) consists of three steps: (a) incubation of the vesicles in the media with the investigated substrate; (b) ended by the addition of the Stop solution; (c) followed by the filtration and washing of the sample mixture in order to separate the vesicle-associated fraction from the incubation media. According to this method, BBMV were mixed with the incubation media at controlled RT. After 1 min incubation, the reaction was stopped by a 10-fold addition of an ice-cold Stop solution. Aliquots of 110 μl were sampled into a 96-well plate and immediately filtered. With this method, however, the observed signal/noise ratio was very low because values of uptake and non-specific binding for the test compounds were very close (Fig. 1), and it could not be improved by increasing the number of washing steps. That raised a doubt about the effectiveness of the Stop solution. Turner (1983) has demonstrated that quantitative interpretation of transport data using RFT depends critically on the ability of the Stop solution to prevent any further uptake or efflux of substrate from the vesicles. As for most of the drugs or drug candidates, the information regarding their specific mechanism and capacity to cross the BBM is unavailable, especially in early drug discovery, none of specific carrier inhibitors can be added to the Stop solution. In this case, the duration of the stopping process after incubation appears to be critical and has to be highly efficient.

After testing various modifications of the RFT in a 96-well plate, we have found that, after rapid filtration of 10 μl of the incubation mixture (consisting of 20 μl of the vesicles incubated in 130 μl media for 1 min at controlled RT), the following one washing step with 100 μl of the ice-cold Stop solution achieved almost complete removal of the non-specific binding (>97%) accompanied by a less pronounced decrease of BBMV associated fraction (>83%) for both cefadroxil and acetaminophen compared to the unwashed sample. This separated filtration/washing step significantly increased the signal/noise ratio comparing with the conventional RFT. Additional experiments as to the number of washing steps, filter temperature, their dry/wet condition as well as the filter pore size have been tested to optimize the signal/noise ratio (data not shown). As a result, all further experiments were performed in the plates maintained at controlled RT using only dry filters with 0.45 μm pore size. The difference of BBMV uptake between conventional and modified RFT method is illustrated in Fig. 1.

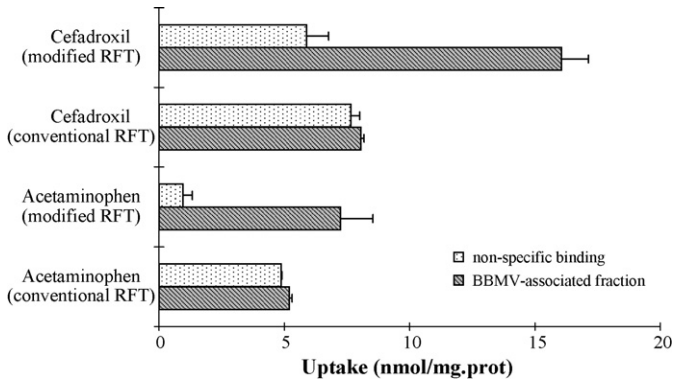

Fig. 1. Comparison of conventional RFT method and modified RFT method. In both methods, the initial mixture was prepared by the incubation of 20 μl of BBMV with 130 μl of incubation media for 1 min at controlled RT. In *conventional method*, 10 μl initial mixture was stopped by 100 μl ice-cold Stop solution, then sampled into 96-well plate and immediately filtered. In *modified RFT method*, 10 μl of the initial mixture was filtered and subsequently washed with 100 μl of ice-cold Stop solution. Uptake values are mean ± S.D. of 10 determinations.

Upon all above modifications, the RFT-96-well plate model allowed the dynamic approach to kinetic studies (Berteloot et al., 1991; Malo and Berteloot, 1991) through performing multiple sampling from the same uptake mixture for a time-uptake measurement. Owing to the fact that the filtration/stopping process needs to be carried out at different intervals with the washing step, we have tested the feasibility of the time-uptake in the 96-well plate by washing out all wells simultaneously upon the last time-point filtration. After 1 min incubation, the uptake mixture aliquots were sampled in all wells at 1 s interval and 100 μl of Stop solution was then added at the delay from 5 s to 4 min. Our result indicated that up to 4 min, there was no significant effect on uptake values for acetaminophen and cefadroxil (data not shown).

3.2. Identification of transporter systems using cefadroxil and zidovudine

The viability of the BBMV can be assessed by the expression of different transporters, particularly secondary active

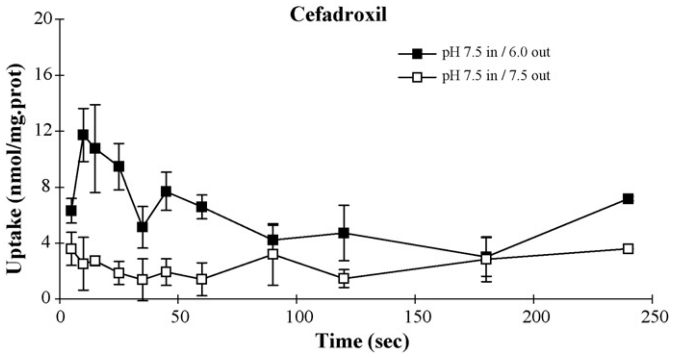

Fig. 2. Time-uptake of cefadroxil absorption. BBMV were mixed with incubation media (2:13, v/v) at 37 °C in the presence (pH $7.5_{in}/6.0_{out}$) or absence (pH $7.5_{in}/7.5_{out}$) of H^+-gradient. Sequential sampling of 11 aliquots from the same uptake mixture (10 μl each) was carried out at 5, 10, 15, 25, 35, 45, 60, 90, 120, 180 and 240 s. Filter was washed with 100 μl of the Stop solution at the end of the last filtration. Uptake values are mean ± S.D. of three distinct experiments each performed in quadruplicate. Data were corrected for non-specific binding.

co-transporters, which exhibit the highest rate of uptake. However, most human oral drugs, in general, exhibit lower transport rates than natural substrates. We thus tested the ability of the BBMV assay to produce the characteristic overshoot phenomenon for the known actively absorbed drugs. Cefadroxil is transported using the H^+/peptide carrier system as reported previously (Hori et al., 1988). In our assay, the time-uptake of cefadroxil in the presence (pH $7.5_{in}/6.0_{out}$) or absence (pH $7.5_{in}/7.5_{out}$) of a H^+-gradient was performed at 37 °C to amplify the overshoot phenomenon. Sequential sampling of the uptake mixture in 11 aliquots (10 μl each) was carried out at time from 5 s to 5 min. The time-uptake of cefadroxil transport demonstrated a specific H^+-dependent overshoot (Fig. 2). These results confirmed the presence of an active H^+/peptide transport system in BBMV preparation.

Zidovudine (AZT) is registered as a nucleoside drug for cancer and HIV treatment. It was previously reported to be transported by the Na^+/nucleoside carrier (Huang et al., 1994). The overshoot shown in Fig. 3A demonstrated that AZT uptake into BBMV occurred via a strictly Na^+-dependent transport system as described before. Lamivudine (3TC), another nucleoside drug, which is co-administred with AZT for HIV treatment, was also investigated. Its uptake exhibited the Na^+-dependent overshoot, which was very similar to AZT in BBMV, but with larger variation (Fig. 3B). Our BBMV assay showed the presence of an active Na^+-dependent carrier for 3TC. In our studies, the BBMV assay demonstrated a good measurable transport activity and sufficient sensitivity for evaluating the drug transport capability.

3.3. Establishment of in vivo–in vitro correlation

BBMV uptake measurement included two major fractions: protein/membrane binding and uptake into the BBMV by passive diffusion and transporters. This assay has to be able to mimic the absorption process of a drug in the intestine and therefore to predict the extent of *in vivo* absorption. Rabbit and human BBMV have been reported to have similar activities in the previ-

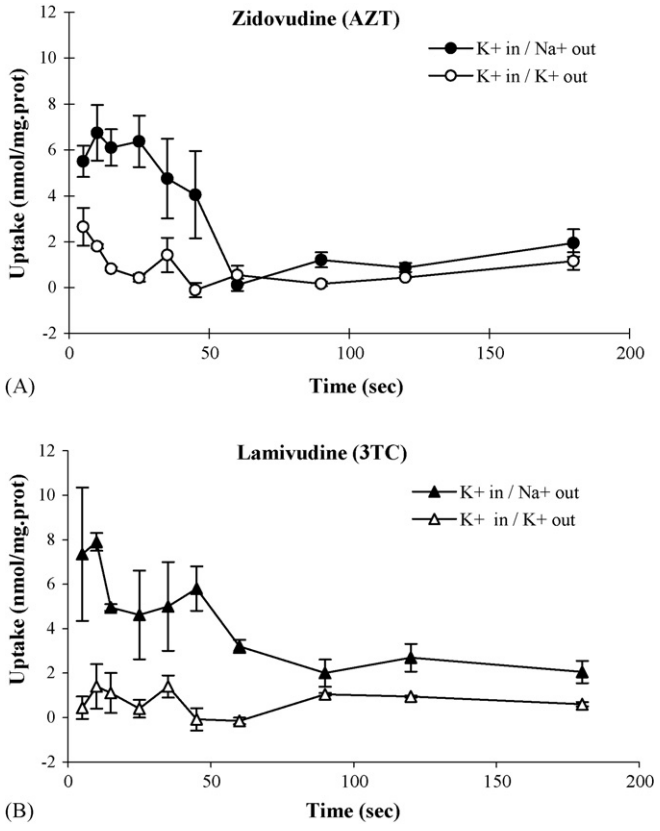

Fig. 3. Time-uptake of zidovudine (AZT) (A) and lamivudine (3TC) (B). BBMV were mixed with incubation media (2:13, v/v) at 37 °C in the presence or the absence of Na^+-gradient. Absence of Na^+-gradient were obtained by using 250 mM KCl instead of 250 mM NaCl in the incubation media. H^+-gradient (pH $7.5_{in}/6.0_{out}$) was kept in both conditions. Experiment was carried out as described in the legend to Fig. 2. Uptake values are mean ± S.D. of four determinations. Data were corrected for non-specific binding.

ous studies, such as penicillin G transport (Poschet et al., 1999), cholesterol transport activities (Schulthess et al., 1996), Pte-Glu transport (Said et al., 1987) and L-leucine transport system (Iannoli et al., 1999).

To establish a correlation between human oral absorption and drug uptake in the rabbit BBMV assay, 23 randomly selected reference drugs having different oral absorption in human were used (Table 1). For all test compounds, the drug uptake was measured after 1 min of incubation at controlled RT, followed by separated filtration/washing steps. A good correlation between *in vivo* human oral absorption and rabbit BBMV drug uptake was found with calculated correlation coefficient $R = 0.853$ ($P < 0.001$) or from data fitting by linear regression analysis, $Y = 9.01X + 2.7795$, $R^2 = 0.728$, except four false positive results observed for azlocillin, ouabaine, phenol red and doxorubicin (Fig. 4). The BBMV uptake of these four drugs may come from the high binding to the BBMV membrane/protein. To verify this assumption, the osmolarity test was used for some of those drugs for distinguishing drug intake from its binding, since only intravesicular drug accumulation is sensitive to the vesicle's volume change. The equilibrium drug uptake was measured under the increased osmolarity of the incubation media by adding mannitol (Fig. 5). Cefadroxil was selected as a positive control.

Table 1
BBMV uptake, human oral absorption and human plasma protein binding

Drug name	Drug uptake (nmol/g protein)	Human oral absorption (%)[a]	Human oral absorption (%)[b]	Plasma protein binding (%)
Acetaminoph	9.461	95	>95	<20
Cefadroxil	11.243	100	100[c]	28.1[d]
6-Mercaptopurine	10.891	90	High	20
Pirenzepine	4.9	25	20–30	10
Antipyrine	3.174	38	38[e]	58[f]
Atropine	6.787	95	95	50
Ampicillin	6.387	35	30–40	20
Brompheniramine	7.508	75	>50	72
Famotidine	3.436	40	40	20
Naloxone	11.331	95	95	50
Tubocurarine	2.274	0	0	40–50
Lisinopril	3.111	25	25	3–10
Methacycline[g]	6.508	58	58	78.5
Aspirin	8.429	90	>80	Variable
Cefalexin	7.536	90	80–100	6–15
Nadolol	4.221	30	30	30
Propranolol	12.452	98	>95	80–90
Cimetidine	6.714	80	>64[h]	13–25
AZT	6.4	90	90	34–38
Ouabaine	10.706	2.5	<5	Not bound
Phenol red[i]	16.802	0	Minimal	60–80
Azlocillin	8.504	0	Minimal	28
Doxorubicin	21.404	2	Minimal	74–76

[a] Data used for Fig. 4.
[b] Data taken from Dollery (1999), except indications.
[c] Data taken from Chiou and Barve (1998).
[d] Data taken from Nakanomyo et al. (1980).
[e] Data taken from Lennernas et al. (1992).
[f] Data taken from Wan et al. (2005).
[g] Data taken from Kunin (1962).
[h] Data taken from Zhao et al. (2000).
[i] Data taken from Smith and Smith (1938).

There was no slope observed for the plots of BBMV uptake versus 1/osmolarity for the above mentioned false positive drugs (Fig. 5) confirming that non vesicle accumulation occurred for azlocillin, phenol red and doxorubicin, in contrast to that of cefadroxil. Also, poor correlation ($R = 0.398$) between human plasma protein binding (Table 1) and BBMV uptake was found. The results indicate that the protein binding to BBMV may be different from that one occurring in human plasma.

In general, a high correlation coefficient between *in vitro* and *in vivo* absorption parameters is considered as the most important ruler for predicting human oral absorption. However, the extrapolation of the results from an *in vitro* model

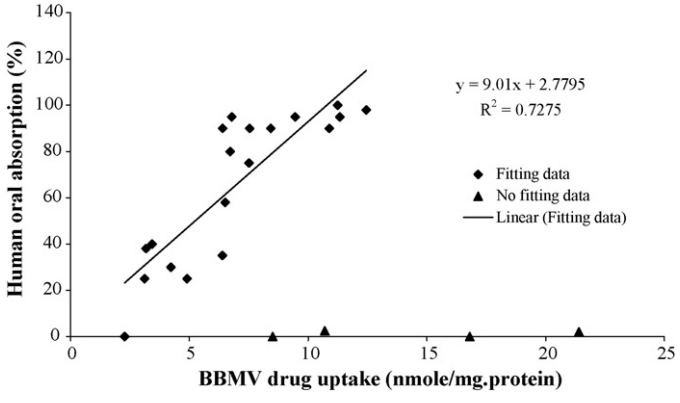

Fig. 4. Correlation between human oral drugs absorption and their BBMV uptake. Experiment was performed as described in the legend to Fig. 1 using modified RFT at controlled RT. Calculated correlation coefficient was $R = 0.853$ ($P < 0.001$) when azlocillin, ouabain, phenol red and doxorubicin were excluded. Data were fitted by linear regression analysis. Uptake values are mean for five determinations. Data were corrected for non-specific binding.

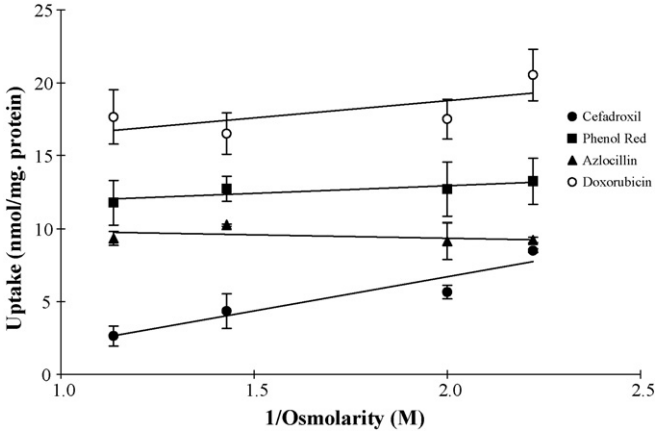

Fig. 5. Effect of osmolarity on drug uptake. Vesicles were loaded as described in Section 2.3. The incubation media contained 50, 100, 300 or 481 mM mannitol. Incubation was carried out for 30 min at controlled RT. Uptake values are mean ± S.D. for five determinations. Data were corrected for non-specific binding.

to the complex *in vivo* absorption should be done cautiously and with some limitations when interpreting data. A BBMV model will fairly predict the drug absorption in human only in cases when the main aspects of its absorption *in vivo* are limited to the events at the BBM level and therefore can be easily reproduced by the *in vitro* BBM assay. In other words, a good correlation between the BBMV assay and human absorption signify that the tested drug is mainly absorbed through BBMV translocation, and that, the additional factors subsequent to the BBM translocation (e.g. cellular metabolism, plasma transport, hepatic transport or metabolism) may play a minor role *in vivo*.

As the drug translocation through the BBM is a unique barrier for absorption in the BBMV assay (except the digestion by the BBM associated enzymes), and accounts for both protein/membrane binding, uptake and a passive diffusion into BBMV, this model is a very close simulation of intestinal absorption processes and is unlikely to give false negative results except, perhaps, for the drugs mainly absorbed through a paracellular route. For example, antipyrine (phenazone) absorption data obtained from human intestinal perfusion or from oral administration may vary from 38% (Lennernas et al., 1992) to 100% (Eichelbaum et al., 1982). It was also demonstrated that antipyrine absorption may be affected by factors such as net fluid absorption in a human jejunum (Fagerholm et al., 1995). The similar phenomenon was observed in the human rectum (Lennernas et al., 1995). It can be expected that trans-cellular and para-cellular pathways in both intestinal regions may contribute to the overall antipyrine absorption *in vivo*. In this case, the BBMV model will not predict the extent of *in vivo* absorption because it simulates exclusively jejunum BBM translocaton of the drug.

The false positive responses for some drugs are tolerable and acceptable as mentioned before by Chong et al. (1996) that, the ideal screening tool can tolerate false positive results for which additionally restricted selection may be applied. On the contrary, a high risk exists in the case of false negative results so that some promising drugs might be abandoned at early drug screening. The results from our 23 reference drugs correlated to human oral absorption indicates that uptake values from rabbit BBMV assay reliably reflects the ability of drugs to cross the human enterocyte's apical membrane in a similar way.

The BBMV transport model presents several advantages. First, the BBMV preparation can be frozen and stored in liquid N_2 for a long period of time. Only a small amount of vesicle suspension is used in this assay. The BBMV can be prepared from different species, such as from human intestine, including different intestinal segments for investigating regional intestinal drug absorption. The vesicles can also be prepared from different key tissues responsible for drug absorption and disposition, such as liver, brain, and kidney, which may provide important information for PK/PD studies.

The transport process in the BBMV is very rapid and now can be applied to a 96-well plate for a high-throughput screening. Protocol design can be adapted for performing different measurements, such as one-point accumulation, time-uptake, drug efflux, receptor–ligand binding and drug metabolism by the BBM associated enzymes.

As a good correlation was found between rabbit BBMV uptake and human oral absorption, the BBMV 96-well plate assay could be a suitable model for the fast absorption screening of drug candidates in early drug R&D. Also, it may allow the discrimination of different absorption mechanisms, such as carrier mediated, drug protein/membrane binding and passive diffusion into vesicles with additional *in vitro* assays.

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