Characterisation of penicillin G uptake in human small intestinal brush border membrane vesicles

J F Poschet, S M Hammond, P D Fairclough

Abstract

***Background*—Many β lactams are well absorbed by the small intestine, although the reasons for this are poorly understood.**

***Aims*—To characterise the uptake of penicillin G into human small intestinal brush border membrane vesicles (BBMV) and to compare the uptake characteristics to those of rabbit BBMV.**

***Methods and results*—Uptake of penicillin G was studied in human BBMV. Penicillin G was actively transported into the lumen of BBMV via an H^+ dependent, Na^+ independent uptake system. The carrier mediated process was saturable and adhered to Michaelis-Menten kinetics (V_{max} 52 nmol penicillin G per mg protein per 30 seconds, K_m 13.9 mM). These results are similar to those previously reported in rabbit BBMV.**

***Conclusions*—It is suggested that penicillin G can be used as a model molecule for peptide and β lactam transport studies as it is cheap and readily available in isotopically labelled form. Furthermore, rabbit BBMV may be used as an acceptable substitute for human BBMV for the study of penicillin transport.**

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Keywords: brush border membrane vesicles; β lactam; antibiotic uptake; oral administration; pH gradient

β Lactam antibiotics are structural analogues of peptides containing a peptide bond between the β lactam ring structure and the adjacent subsidiary side chain. β Lactams are completely ionised at the pH of small intestinal contents, resulting in chemical and physicochemical properties which would predict poor absorption.[1] Nevertheless, many β lactams are well absorbed.[2 3] The reasons for this are poorly understood.

Early studies showed that small intestinal peptide transport was energy requiring, but distinct from amino acid uptake.[4–6] In the early 1980s it was discovered that peptide uptake was independent of a sodium gradient,[7 8] and soon after, Ganapathy and Leibach postulated that peptides were cotransported with protons.[9] The proton gradient across the brush border membrane of intact small intestinal enterocytes is thought to be maintained by the interaction of the sodium-potassium pump located at the basolateral membrane and the sodium-proton exchanger at the brush border membrane. As a result peptide uptake is best described as a "tertiary active" transport system. Furthermore, these interactions are thought to contribute to an acidic pH microclimate at the brush border membrane.[9–11]

Based on the structural analogies and many inhibitory studies it has been established that peptides and β lactams share a common transporter.[12–21] Although several H^+/peptide cotransporters (human peptide transporter 1 (HPT1),[22] peptide transporter 1 (PEPT1),[23 24] and peptide transporter 2 (PEPT2)[25]) have recently been cloned, little is known about the physiological mechanism of small intestinal peptide or β lactam transport. Nevertheless, as a result of cloning the human kidney PEPT2[25] and human small intestinal PEPT1[24] H^+/peptide cotransporters, it has been established that the two transporters are distinct, although they may share some common transport characteristics.[21 26]

The study of peptide transport in any small intestinal model is complicated by the fact that many dipeptides or tripeptides are susceptible to hydrolysis. Even some of the well studied and relatively hydrolysis resistant dipeptides may be hydrolysed during long incubations.[4 6 7 27 28] This problem can be overcome by the use of structural analogues such as β lactam antibiotics, which are not degraded by peptidases. However, the study of β lactam antibiotic uptake is associated with problems of species diversity. This was best illustrated by the finding that cephradine and ceftibuten uptake in rat small intestinal brush border membrane vesicles (rtBBMV) was more closely related to that in human BBMV (hBBMV), than results from rabbit BBMV (rbBBMV).[15] As all of the orally active β lactams are selected on the basis of animal studies, such different results will potentially lead to failure to consider potent orally active β lactams for human use. Due to the problems associated with obtaining sufficient human material for BBMV studies few such reports are available.[15 19 29] These problems underline the need for animal models which accurately reflect human absorption so that human β lactam uptake can be predicted.

Although penicillin G has been used as a marker or inhibitor of peptide or β lactam antibiotic uptake,[30–32] it was not until recently that it was shown to be actively transported into rbBBMV.[21] In the present paper we have characterised the uptake of penicillin G in hBBMV and compared the uptake characteristics to those of rbBBMV. On this basis we propose that rbBBMV are a suitable model for penicillin

Abbreviations used in this paper: HPT, human peptide transporter; PEPT, peptide transporter; BBMV, brush border membrane vessicle; rt, rat; rb, rabbit; h, human.

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uptake studies, although we cannot predict that this model will behave similarly for the study of cephalosporins or other β lactam antibiotics.

Methods

MATERIALS

Radiochemicals, benzyl[^{14}C]penicillin potassium (specific activity 59 mCi/mmol), and D-[U-^{14}C]glucose (specific activity 292 mCi/mmol) were from Amersham (Buckinghamshire, UK). Scintillation liquid, OptiPhase Safe, was from Wallac Ltd (Milton Keynes, UK). All other chemicals were of the highest purity available.

BBMV PREPARATION

After gaining informed consent from the relatives, 30 cm of proximal jejunum was removed from a transplant donor, thoroughly washed in ice cold 0.9% NaCl, and cut into pieces of approximately 12 cm^2. The tissues were wrapped in aluminium foil, frozen in liquid N_2, packed into airtight plastic bags, and stored at −80°C.

Human BBMV were prepared according to a modified salt precipitation method,[21] as described by Kessler and colleagues[33] and Shirazi-Beechey *et al.*[34] In short, on the day of preparation, tissue pieces were defrosted on ice and the mucosa scraped off using a plastic spatula. The tissue scrapings were suspended in 10 mM HEPES/Tris, 40 mM mannitol at 4°C and pH 7.1, and homogenised using an Ultra-Turrax homogeniser (Janke and Kunkel, IKA-Werk, Germany) at full output for five minutes on ice. The volume of the homogenate was adjusted to 350 ml and a 1 ml sample removed for purification factor assays. The remaining steps were as previously described for rabbit tissue.[21]

Magnesium precipitation was favoured over calcium precipitation, as it had been reported that calcium precipitation increased the proton conductancy in renal BBMV.[35] Such a potential increase in H^+ conductivity is clearly not desirable in an investigation of a pH dependent transport system.

BBMV CHARACTERISATION

Brush border membrane marker enzymes, K^+ activated phosphatase,[36] alkaline phosphatase,[37] aminopeptidase N,[38] and sucrase,[39] were measured. Glucose liberated by sucrase was assayed using a commercial kit (Glucose GOD-PAP method, Merck, Darmstadt, Germany). Protein concentration was determined using the bicinchoninic acid method (BCA-protein assay kit, Pierce Chemicals, Chester, UK).[40] The integrity of the BBMV was routinely tested by their ability to accumulate radiolabelled glucose in response to an Na^+ gradient, as described previously.[41]

PENICILLIN G UPTAKE STUDIES IN hBBMV

Penicillin G uptake studies were based on the method described by Okano *et al*,[42] with alterations as described previously.[21] The assay conditions for the individual experiments are described in the legends to the figures. At the end of the incubation period the uptake was stopped by adding 4°C stopping buffer (125 mmol/l NaCl, 50 mmol/l HEPES/Tris pH 7.1), and 1 ml was rapidly filtered through 0.22 mm GSWP filters (Millipore, Watford, UK) followed by 5 ml wash with the stopping buffer. The filters were dissolved in 4 ml of scintillation liquid OptiPhase Safe and counted in a LKB 1219 Rackbeta (LKB, UK). The counts were corrected for reagent blank (non-specific binding to filters) and vesicle blank (zero time uptake), allowing for non-specific binding to vesicles.

The kinetic experiments establishing V_{max} and K_m were carried out during the initial linear uptake phase (see fig 2) at 30 seconds and 37°C (fig 4). The effect of osmolarity on vesicle volume and penicillin G binding was established at 30 minutes and 37°C (fig 3).

All experiments were carried out at least three times in triplicate using different vesicle preparations for each experiments. Statistics, curves, and correlation coefficients (simple regression) were determined by Cricket-Graph for Windows, version 1.3.1 (Computer Associates, Malvern, USA).

Results

PURIFICATION

The enrichment factors studied were: K^+ activated phosphatase 0.53 (SD 0.65), alkaline phosphatase 9.46 (SD 0.95), aminopeptidase N 8.96 (SD 1.48), and sucrase 28.09 (SD 6.18). hBBMV were capable of transporting D-glucose against its concentration gradient. The maximal overshoot of 1295 pmol D-glucose/mg protein was reached after 60 seconds and was eightfold the equilibrium uptake, which was reached after 10 minutes (fig 1). These results compared well with those previously reported by us for rbBBMV[21] and those of other groups,[33 34] and showed that the hBBMV were functionally intact.

TIME COURSE OF PENICILLIN G UPTAKE

The time course of penicillin G uptake in hBBMV (fig 2) was a pH dependent process ($pH_{outside}$ (pH_o) = 5.0, pH_{inside} (pH_i) = 7.1) with a slightly more pronounced overshoot than that found in rbBBMV.[21] The maximal overshoot of 563 pmol/mg protein, was reached after 80 seconds and thereafter declined until 600

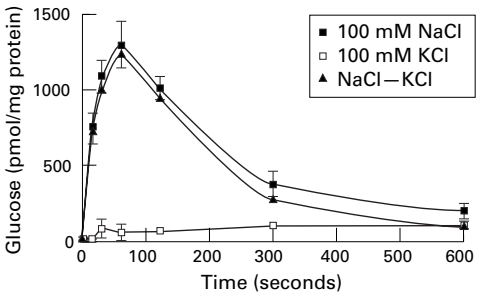

Figure 1 Time course of glucose uptake in hBBMV. Vesicles were loaded with 5 mM HEPES/Tris, 0.1 mM $MgSO_4$, 100 mM mannitol, 100 mM KCl at pH 7.1. The assay mixture contained 100 mM NaCl or 100 mM KCl, 100 mM mannitol, 0.1 mM glucose, and 5 mM HEPES/Tris pH 7.1. Samples were incubated at 37°C. Results represent the average of three experiments carried out in triplicate. Error bars represent standard deviation.

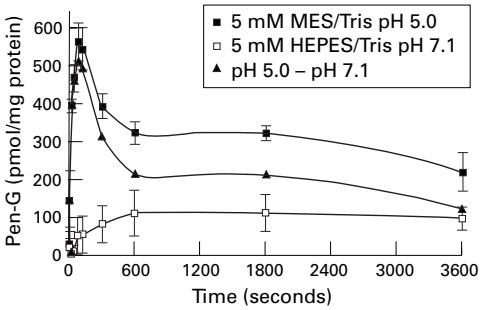

Figure 2 Time course of penicillin G uptake in hBBMV. Vesicles were loaded with 5 mM HEPES/Tris, 0.1 mM $MgSO_4$, 100 mM mannitol, 100 mM KCl at pH 7.1. The assay mixture contained 100 mM KCl, 100 mM mannitol, 0.1 mM penicillin G, and 5 mM MES/Tris pH 5.0, or 5 mM HEPES/Tris for pH 7.1, respectively. Samples were incubated at 37°C. Results represent the average of three distinct experiments carried out in triplicate. Error bars represent standard deviation.

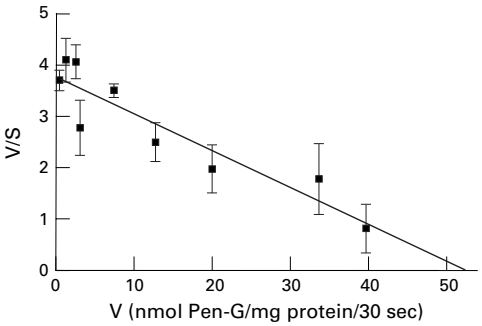

Figure 4 Eadie-Hofstee plot of penicillin G uptake in hBBMV. Vesicles were loaded with 50 mM HEPES/Tris, 0.1 mM $MgSO_4$, 50 mM mannitol, 100 mM KCl at pH 7.1. The external buffer contained 100 mM KCl, 0–50 mM mannitol (to correct for penicillin G addition), 50 mM MES/Tris pH 5.0, and 0.1–50 mM penicillin G. Samples were incubated for 30 seconds at 37°C. Results represent the average of three distinct experiments carried out in triplicate. Error bars represent standard deviation. V_{max} = 52 nmol/mg protein/30 sec; K_m = 13.9 mM; r=0.924.

seconds. During the initial phase the majority of penicillin G uptake was largely due to an active transport system (Δ pH 5.0 − pH 7.1), as indicated by the overshoot. This result is comparable with those described for other β lactams, peptides, and peptide analogues.[12–15 18 21 43–45] Over the remaining course of the experiment a slight fall in uptake was detected. Nevertheless, the equilibrium uptake compared with passive transport (pH_o = pH_i = 7.1) was not fully reached during the course of the experiment (60 minutes). This result suggests that not all of the penicillin G may be taken up into the intravesicular space. However, extrapolation to infinite osmolarity of the line relating equilibrium uptake to the reciprocal of the osmolarity of the external buffer suggested that almost 90% of penicillin G was transported into the vesicular space with the remaining 10% thought to be membrane associated (fig 3). This result was almost identical to that reported for penicillin G uptake in rbBBMV.[21] The uptake of penicillin G in the absence of a pH gradient (pH_o = pH_i) increased steadily to reach equilibrium after 10 minutes.

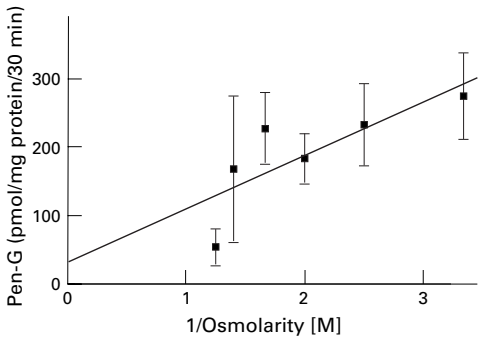

Figure 3 Effect of osmolarity on penicillin G uptake in hBBMV. Vesicles were loaded with 50 mM HEPES/Tris, 0.1 mM $MgSO_4$, 50 mM mannitol, 100 mM KCl at pH 7.1. The external buffer contained 100 mM KCl, 50–550 mM mannitol, 50 mM MES/Tris pH 5.0 and 0.1 mM penicillin G. Samples were incubated for 30 minutes at 37°C. The results represent the average of two distinct experiments carried out in triplicate. Error bars represent standard deviation. Intercept on y axis represents the amount of penicillin G associated with the membrane (12%); r=0.791.

KINETICS

The active component of the uptake process conformed to Michaelis-Menten kinetics during the initial linear phase of uptake at 30 seconds and 37°C: V_{max} 52 nmol penicillin G per mg protein per 30 seconds, K_m 13.9 mM (fig 4). V_{max} values in hBBMV and rbBBMV were of similar magnitude. K_m for the hBBMV was approximately 65% of that observed in rbBBMV.[21]

Discussion

The characteristics of hBBMV were similar to those previously reported by us for rbBBMV,[21] and compare well with the ones reported by others.[13 29 34 46] The results of the D-glucose uptake studies were only slightly different for hBBMV than for rbBBMV[21] and are of similar magnitude as previously reported for hBBMV.[13 34]

The characteristics of penicillin G transport in hBBMV appear to be similar to those in rbBBMV.[21] Penicillin G was transported against its concentration gradient, leading to a peak overshoot at 80 seconds (fig 2). Nevertheless, due to the different experimental conditions, such as buffer capacity, pH and temperature, it is difficult to compare these results with those of other investigators. However, two studies have shown an overshoot phenomenon for ceftibuten[13] and cephradine[15] in hBBMV. A pronounced overshoot for ceftibuten was only observed once the concentration was increased from 0.1 mM to 1 mM.[13] During the time course of the experiment penicillin G uptake did not reach full equilibrium, suggesting that not all of the penicillin G was actively taken up into the vesicles or that 60 minutes was insufficient time to reach equilibrium due to passive diffusion. The downward trend of the curve would suggest that an equilibrium could have been reached on extension of the experiment. Furthermore, the findings of the osmolarity experiments suggest that 88% of penicillin G was taken up into the vesicles (fig 3). This compares with similar studies with glycyl-glutamine,[29] glycyl-L-proline,[47] and glycyl-glycyl-L-proline,[46] which have reported a greater than 95% uptake into hBBMV.

Table 1 Kinetic parameters of β lactam antibiotic uptake

Reference	*β lactam (species)*	K_m *(mM)*	V_{max}
This study	Penicillin G (human)	13.9	52⋆
Poschet and colleagues[21]	Penicillin G (rabbit)	21.7	58⋆
Inui and colleagues[14]	Cefixime (rat)	0.8	6.2†
Yuasa and colleagues[18]	Cephradine (rat)	1.6	1.7†
	Cephradine (rabbit)	1.9	20.7†
Okano and colleagues[42]	Cephradine (rat)	9.4	5.7†
Tsuji and colleagues[12]	Cefixime (rat)	0.83	2.67⋆
	Ceftibuten (rat)	0.17	4.72†
Muranushi and colleagues[43]	Cefaclor (rat)	3.0	0.16†

⋆V_{max} = nmol/mg protein/30 seconds.
†V_{max} = nmol/mg protein/60 seconds.

The kinetic parameters of penicillin G uptake in hBBMV were determined by plotting the results of the initial concentration dependent uptake at 30 seconds as an Eadie-Hofstee plot. V_{max} was found to be 52 nmol/mg protein/30 sec and K_m was 13.9 mM (fig 4). However, in hBBMV K_m for penicillin G uptake was approximately half of that in rbBBMV.[21] This finding would imply that penicillin G has a slightly higher affinity for the human H^+/peptide cotransporter. Furthermore, as both preparations have a similar V_{max}, both transport systems would be expected to operate at the same rate under saturated substrate conditions. However, as the turnover rate of neither of the transporters is known, it is impossible to estimate the number of transporters present in either of the preparations.

As far as comparisons are possible, it became apparent that penicillin G and other β lactams lacking the α amino group[12 14 31 48 49] are more efficiently taken up at a pH below 6.0, whereas aminocephalosporins and aminopenicillins are better absorbed at a pH above 6.0. As Daniel and colleagues[49] have reported, transport of both is most likely to be mediated by the same transporter, PEPT1. Furthermore, it has recently been shown that anionic cephalosporins, such as ceftibuten and cefixime, are actively transported in oocytes expressing PEPT1 and PEPT2.[50]

Comparing the kinetic parameters of penicillin G uptake with those shown in table 1, it became apparent that penicillin G had a much lower affinity for the H^+/peptide cotransporter than any of the other β lactams reported.[12 14 18 42 43] In addition to its instability in a strongly acidic environment, such as the stomach, this partly accounts for the poor bioavailability of penicillin G in man. Penicillin G was used in the present studies as other penicillins were not readily available in an isotopically labelled form. The structural differences between penicillin G and penicillin V are, however, trivial and do not involve a position traditionally associated with intestinal transport of β lactam antibiotics. The fact that penicillin V inhibited penicillin G uptake in rbBBMV further suggests that they share the same carrier.[21] Additionally, to our knowledge there are no such studies available for penicillin transport in hBBMV, although some of the (amino-)cephalosporins and dipeptides have been studied.[13 29 34 46 47]

These results suggest that penicillin G can be successfully used as an easily available and relatively cheap marker for peptide and β lactam antibiotic uptake. As results found in hBBMV were similar to those found in rbBBMV,[21] it may also be suggested that penicillin G can be used as a model for penicillin uptake studies and that rbBBMV may constitute a suitable substitute should no human material be available.

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