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Optimized LC–MS/MS quantification method for the detection of piperacillin and application to the development of charged liposaccharides as oral penetration enhancers

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Abstract

Piperacillin, a potent β-lactam antibiotic, is effective in a large variety of Gram+ and Gram− bacterial infections but its administration is limited to the parenteral route as it is not absorbed when given orally. In an attempt to overcome this problem, we have synthesized a novel series of charged liposaccharide complexes of piperacillin comprising a sugar moiety derived from D-glucose conjugated to a lipoamino acid residue with varying side-chain length (cationic entity) and the piperacillin anion. A complete multiple reaction monitoring LC–MS/MS method was developed to detect and characterize the synthesized complexes. The same method was then successfully applied to assess the *in vitro* apparent permeability values of the charged liposaccharide complexes in Caco-2 monolayers.
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Keywords: Piperacillin; Charged liposaccharide; Penetration enhancer; LC–MS/MS; Caco-2; Permeability

1. Introduction

Piperacillin is a broad spectrum β-lactam antibiotic, commonly used in combination with tazobactam for the treatment of patients with polymicrobial infections (Perry and Markham, 1999). It is however only available intravenously and intramuscularly, thus requiring medical supervision when administered. Attempts to develop new orally active formulations of this drug have been made, yet no satisfactory compound has emerged. Recent approaches include the use of penetration enhancers, such as lipids (Chancellor et al., 2003) and charged liposaccharides (Ross et al., 2004). Penetration enhancers have the great advantage of improving the absorption properties of a substance without altering its chemical structure, and therefore its biological activity. Ion-pairing is one of several techniques developed for penetration enhancement (Neubert, 1989; Quintanarguerrero et al., 1997), and has recently been successfully applied to the oral administration of β-lactam antibiotics (Mrestani et al., 2006). Since piperacillin is usually supplied as a sodium salt, it is a potential candidate for complex formation with a suitable cationic ion-pairing agent. Under appropriate conditions, the cationic counter ions, which includes a hydrophobic moiety, can establish electrostatic interactions with the anionic molecule, resulting in the formation of a neutral complex with enhanced hydrophobic properties.

In this study, six complexes (**P1–P6**) were synthesized using charged liposaccharides (1-amino-D-glucose derivatives coupled to α-amino acids with alkyl side chains) as oral penetration enhancers (Scheme 1). The membrane permeability of the compounds was evaluated *in vitro* using Caco-2 cell monolayers. Originally derived from a human adenocarcinoma, Caco-2 cells have been extensively studied as a model for predicting the transport of pharmaceuticals across the intestinal epithelium (Gan and Thakker, 1997; Artursson et al., 2001).

Ion-pair complexes were identified and characterized by electrospray mass spectrometry (ESI-MS). A complete liquid chromatography – mass spectrometry tandem (LC–MS/MS) method was designed and optimized to allow the quantification

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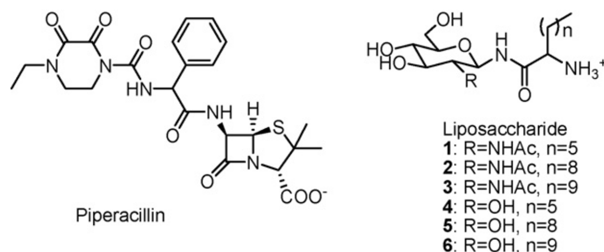

Scheme 1. Structure of piperacillin and charged liposaccharide (**1**–**6**). The resulting complexes are labelled **P1**–**P6**.

of piperacillin in the synthesized complexes and establish the apparent permeability values of the complexes through Caco-2 monolayers. The developed method includes two ion-transition monitorings (MRM) of the main residue, as recommended by the European Commission (Regulations of Analytical Methods and Analyses of Results),[1] and low limit of detection (LOD) and quantification (LOQ) were determined.

2. Materials and methods

2.1. Chemistry

All solvents and reagents were obtained at the highest available purity from Sigma–Aldrich (Castle Hill, NSW, Australia). ESI-MS and LC–MS/MS analyses were carried out on a PE Sciex API3000 triple quadrupole mass spectrometer, using a mixture of solvent A (1% formic acid in water) and B (1% formic acid in 90% acetonitrile/water) at 0.1 mL/min. For LC–MS/MS experiments, a Phenomenex luna C18 column (5 μm, 50 mm × 2.0 mm) was attached to the mass spectrometer and gradient methods of A and B were used for elution at a flow rate of 0.5 mL/min with a 1:10 splitter upstream from the ionisation source (Shimadzu LC-10AT system). Data were acquired with Analyst 1.4 software (applied Biosystems/MDS Sciex, Toronto, Canada). Proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra of the liposaccharides were recorded on a Bruker 300 MHz instrument in $CDCl_3$ (data not shown).

Liposaccharides **1–6** were prepared following a procedure previously described by Drouillat et al. (1998) and their structure and purity confirmed by ESI-MS and NMR. The complexation of these liposaccharides with piperacillin was successfully achieved by simply lyophilising an equimolar solution of liposaccharide and piperacillin solution in acetic acid at −80 °C and <150 Psi. Each complex was then characterised by ESI-MS.

2-Acetamido-2-deoxy-*N*-(1-amino-(*R*/*S*)-octoyl)-β-D-glucopyranosylamine (**P1**): yield, 98%; ESI-MS [M (complex **P1**) = 878.4; M^1 (liposaccharide) = 361.2] *m*/*z* (%) 879.9 $[M+H]^+$, 1396.9 $[(M\text{-piperacillin})+H]^+$, 1241.3 $[(M\text{-}M^1)+H]^+$, 362.6 $[M^1+H]^+$.

2-Acetamido-2-deoxy-*N*-(1-amino-(*R*/*S*)-undecoyl)-β-D-glucopyranosylamine (**P2**): yield, 99%; ESI-MS [M (complex **P2**) = 920.4; M^1 (liposaccharide) = 403.3] *m*/*z* (%) 921.9 $[M+H]^+$, 1439.8 $[(M\text{-piperacillin})+H]^+$, 1325.1 $[(M\text{-}M^1)+H]^+$, 404.5 $[M^1+H]^+$.

2-Acetamido-2-deoxy-*N*-(1-amino-(*R*/*S*)-dodecoyl)-β-D-glucopyranosylamine (**P3**): yield, 98%; ESI-MS [M (complex **P3**) = 934.4; M^1 (liposaccharide) = 417.3] *m*/*z* (%) 935.8 $[M+H]^+$, 1452.9 $[(M\text{-piperacillin})+H]^+$, 1353.1 $[(M\text{-}M^1)+H]^+$, 418.7 $[M^1+H]^+$.

N-(1-amino-(*R*/*S*)-octoyl)-β-D-glucopyranosylamine (**P4**): yield, 97%; ESI-MS [M (complex **P4**) = 837.4; M^1 (liposaccharide) = 320.2] *m*/*z* (%) 838.6 $[M+H]^+$, 1357.0 $[(M\text{-piperacillin})+H]^+$, 1158.4 $[(M\text{-}M^1)+H]^+$, 321.0 $[M^1+H]^+$.

N-(1-amino-(*R*/*S*)-undecoyl)-β-D-glucopyranosylamine (**P5**): yield, 99%; ESI-MS [M (complex **P5**) = 879.4; M^1 (liposaccharide) = 362.2] *m*/*z* (%) 880.9 $[M+H]^+$, 1398.0 $[(M\text{-piperacillin})+H]^+$, 1243.4 $[(M\text{-}M^1)+H]^+$, 363.8 $[M^1+H]^+$.

N-(1-amino-(*R*/*S*)-dodecoyl)-β-D-glucopyranosylamine (**P6**): yield, 99%; ESI-MS [M (complex **P6**) = 893.4; M^1 (liposaccharide) = 376.3] *m*/*z* (%) 894.3 $[M+H]^+$, 1411.7 $[(M\text{-piperacillin})+H]^+$, 1270.4 $[(M\text{-}M^1)+H]^+$, 377.8 $[M^1+H]^+$.

2.2. Cell culture

Cell culture reagents were purchased from Gibco-BRL (Grand Island, NY) except of Hank's balanced saline solution (HBSS) and ^{14}C-labelled mannitol which came from Sigma–Aldrich (Castle Hill, NSW, Australia) and Amersham Biosciences (Piscataway, NJ), respectively. Tissue culture flasks (75 cm^2) were ordered from Becton Dickinson (North Ryde, NSW, Australia).

Caco-2 cells were obtained from the American type culture collection (Rockville, MD) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and 1% non-essential amino acids at 95% humidity and 37 °C in an atmosphere of 5% CO_2. The medium was changed every second day. After reaching 80% confluence, the cells were subcultured using 0.2% ethylenediaminetetraacetic acid (EDTA) and 0.25% trypsin. 6000–8000 cells (passage number 47–50) were seeded onto polycarbonate Transwell® inserts supplied by Coastar (Cambridge, MA; mean pore size = 0.45 μm, 6.5 mm diameter) and cultivated in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin and 100 mg/mL streptomycin. The cells were allowed to grow for 21–28 days and the medium was changed every other day.

2.3. Drug transport experiment

The tested compounds were dissolved in HBSS–HEPES (HBSS buffered with 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) at pH 7.4) to a final concentration of 0.5 mM. Prior to the transport studies, the Caco-2 monolay-

[1] Cf. Commission Decision (2002/657/EC) of 12 August 2002 concerning the performance of analytical methods and the interpretation of results, Official Journal of the European Communities L221, Belgium, 2002, pp. 8–36.

ers were washed three times with pre-warmed HBSS–HEPES and the integrity of the monolayers was assessed by measuring the transepithelial electrical resistance (TEER) values using a Millicell-ERS system (Millipore Corp., Bedford, MA). Observed values were typically above 1 kΩ cm^{-2} and no significant decrease was observed during or at the end of the study. The drug solutions (100 μL) were then added to the donor side of the monolayers and the Transwell® plates placed on an incubator sharker (Heidolph Inkubator & Titramax 1000, Schwabach, Germany) set at 400 rpm and 37 °C for the duration of the experiment. At determined time points (30, 90, 120 and 150 min), samples (400 μL) were taken out from the receiver chambers and replaced with the same amount of HBSS–HEPES. All compounds were tested in two to five independent assays, using at least three inserts each time. Results were then analysed by LC–MS/MS. The transport of ^{14}C-mannitol (200 μM), a marker of paracellular transport, was also measured in parallel inserts. After dilution of samples (400 μL) with 4 mL of Wallac OptiPhase HiSafe 3 liquid scintillation cocktail, radioactivity was measured using a Beckman Coulter LS650 Multipurpose liquid scintillation spectrometer and permeability values assessed.

2.4. LC–MS/MS analyses

Concentrations of piperacillin in samples were determined by LC–MS/MS in MRM mode with positive electrospray ionisation. The tested compounds were eluted with a 30–100% B gradient over 4 min at a flow rate of 0.5 mL min^{-1}. The ion spray voltage of the source was set at 5200 V; compressed air was nebulised at 10 L min^{-1} while nitrogen was used as the curtain gas (12 L min^{-1}) and collision gas (4 L min^{-1}).

2.5. Determination of permeability coefficients

All experiments were carried out under sink conditions. Apparent permeability coefficients (P_{app}; cm s^{-1}) were determined according to the following equation:

$$P_{app} = \frac{dC}{dt} \times \frac{V_r}{A \times C_0}$$

where dC/dt is the steady-state rate of change in the chemical in the receiver chamber (mM, or dpm mL^{-1}), V_r is the volume of the receiver chamber (mL), A is the surface area of the cell monolayers (cm^2) and C_0 is the initial concentration in the donor chamber (mM, or dpm mL^{-1}) (Artursson and Karlsson, 1991).

2.6. Statistical analysis

All experimental data were expressed as a mean ± standard deviation (S.D.) and subject to statistical analysis using a one-way analysis of variance (ANOVA) at a significance level of 0.05 ($p < 0.05$), followed by Tukey's post hoc test (multiple pairwise comparison of means). Values were computed with GraphPad Prism v4.03 for Windows (GraphPad Software, San Diego, CA) and Excel add-in Analyse-it™ v1.73 (Analyse-it Software Ltd., Leeds, UK).

3. Results and discussion

3.1. Development and optimization of an LC–MS/MS method for the detection and quantification of piperacillin in the charged liposaccharide complexes ***P1–P6***

Common elution conditions for the detection of β-lactam antibiotics include the use of acetonitrile/water gradient-based mobile phases, with variable amounts of additives such as acids (formic acid (Rizzo et al., 2005) or acetic acid (Sulton et al., 2005)), accelerating solvents ($CHBr_3$, $CHCl_3$) (Horimoto et al., 2002) or buffers (ammonium acetate (Flarakos et al., 2005; Pozo et al., 2006) formate (Jemal, 2000)).

Our first concern was to investigate the influence of solvent composition on the detection and quantification of piperacillin. Although methods using methanol have been reported (Pozo et al., 2006; Ghibellini et al., 2006), acetonitrile was retained as the first choice for the organic eluant, as β-lactams are known to be susceptible to degradation in methanol by hydrolysis and ring opening (Tyczkowska et al., 1992; Deshpande et al., 2004). The effect of acidification was also determined by infusing a 20 μM solution in acetonitrile/water (1:1) containing formic acid (0.1, 0.5 and 1%) or ammonium acetate (1, 5 and 10 mM), and MS/MS parameters were assessed in both negative and positive mode. The best signal/noise ratio and sensitivity were obtained in positive mode with 1% formic acid, with the main ions observed being m/z 518.1 $[M+H]^+$ and fragments 142.7 and 160.2. Optimized parameters are presented in Table 1.

To be consistent with the Caco-2 permeability assays which were carried out in HBSS–HEPES buffer, additional measurements were performed with 0.001–5 μM piperacillin solutions in HBSS–HEPES. Reproducibility and linearity of the results were established within the range 0.005–5 μM (typical correlation coefficients of 0.999). Under the previous optimized conditions, the strongest response was observed with the 518 > 142.7 MRM transition, which was therefore used for quantification purposes. The linear response of piperacillin ranges from 0.05 to 5 μM

Table 1
MS/MS optimized parameters for the detection of piperacillin in acetonitrile/water (1:1) with 1% formic acid (positive mode; dwell time: 150 ms)

	Q1 mass (amu)		Q3 mass (amu)	Acquisition potentials (V)			CE (V)	CXP (V)
				DP	FP	EP		
Observed	518.1	>	142.7	31	140	10	23	14
transition (m/z)	518.1	>	160.2	31	140	10	15	16

DP: declustering potential; FP: focusing potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential.

with high correlation coefficients (typically $r = 0.999$, $n = 8$). The limit of detection, defined as three times the baseline noise, was found to be LOD = 0.002 μM (0.04 pmol, 21 pg) and the limit of quantification was set at LOQ = 0.01 μM (0.2 pmol, 103 pg), the concentration of the second lowest calibration standard.

3.2. *In vitro evaluation of the cationic liposaccharide complexes of piperacillin–Caco-2 permeability assays*

In recent years, our group has been particularly interested in the development of new charged, liposaccharide-based penetration enhancers (Toth et al., 2002; Ross et al., 2004). The conjugation of lipoamino acids (LAAs), α-amino acids with an alkyl side chain, to poorly absorbed drugs has been shown to enhance their ability to cross biological membranes (passive transport) by increasing the overall lipophilicity of the molecule (Toth et al., 1999, 2002; Wong and Toth, 2001). The incorporation of a sugar moiety usually improves water solubility – a requirement for oral drug delivery – and may promote drug uptake through active transport mechanisms, including sugar transporters (Wong and Toth, 2001; Blanchfield and Toth, 2004; Wu et al., 2006). In this study, six complexes of piperacillin incorporating two different sugars (*N*-acetyl-D-glucosamine and D-glucose) and three lipoamino acids (containing side-chain alkyl groups of 6, 9 and 10 carbon atoms, respectively) were synthesized and evaluated *in vitro* on Caco-2 monolayers in comparison to piperacillin alone.

All six complexes displayed increasingly higher concentrations over time in the basolateral compartment of the Transwell® inserts compared to piperacillin alone, and their absorption profiles were found to be very similar to that of piperacillin (Fig. 1). Significant improvements were noticed for D-glucose complexes **P4–P6**, which were nearly eight times more concentrated than piperacillin alone (**P0**), after 30 min. Despite a rapid permeation of the Caco-2 monolayer in the first 90 min of the assays, all complexes showed a steady, flatter trend after 2 h.

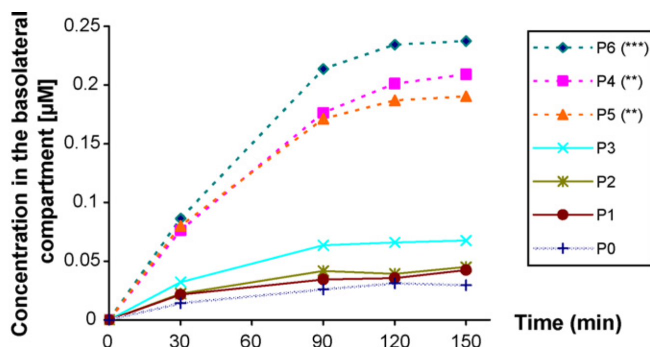

Fig. 1. Concentration over time through Caco-2 monolayers of piperacillin alone (**P0**) and complexed with charged liposaccharides **1–6** (**P1–P6**). Samples ($n = 4$) were taken in the basolateral compartment of the Transwell® inserts at 30, 90, 120 and 150 min and their concentrations determined by LC–MS/MS based upon 8 point standard curves ($r > 0.99$) established for each compound (results not shown). Statistical significance was noted by $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ with reference to piperacillin alone (**P0**). Comparison within groups (i.e. (**P1–P3**) and (**P4–P6**)) showed no significant difference.

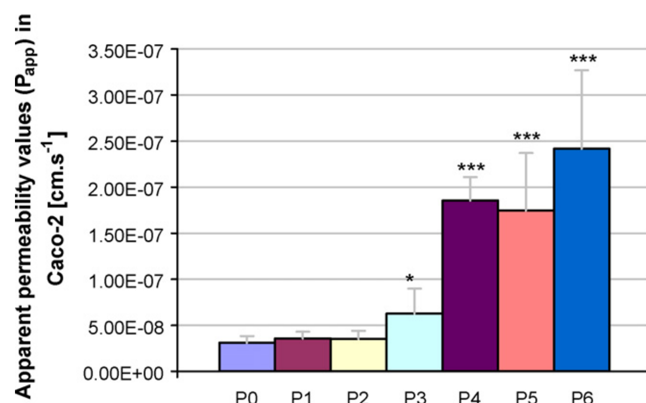

Fig. 2. Apparent permeability coefficients in Caco-2 monolayers (P_{app}, cm s^{-1}) of piperacillin alone (**P0**) and complexed with charged liposaccharides **1–6** (**P1–P6**). Statistical significance was noted by $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ with reference to piperacillin alone (**P0**). Tukey's post hoc multiple comparison test between D-glucose (**P1–P3**) and *N*-acetyl-D-glucosamine derivatives (**P4–P6**) indicated a p-value < 0.01.

The apparent permeability value of piperacillin was found to be very low ($P_{app} = 3.25 \pm 0.14 \times 10^{-8}$ cm s^{-1}). However, when tested in association with charged liposaccharides, notable improvements were noticed, particularly with the D-glucose complexes **P4–P6** (Fig. 2).

N-acetyl-D-glucosamine-containing liposaccharides (**1**) and (**2**) did not significantly modify the apparent permeability value of the piperacillin in Caco-2; when increasing the length of the lipoamino acid to 12 carbon atoms (**3**), some improvement was noticed, with a nearly two-fold increase in the P_{app} values. This is consistent with previous studies reporting the effect of C12 lipoaminoacid derivatives on tight junctions opening and their efficiency as penetration enhancers compared to other lipoamino acids such as C8 or C10 (Lindmark et al., 1995, 1998; Yata et al., 2001). Even more promising results were obtained with D-glucose conjugates **P4–P6**, which displayed apparent permeability values up to eight times greater than the piperacillin alone. As with complexes **P1–P3**, the largest increase was observed with the C12 liposaccharide **6** (Fig. 2).

Such differences between *N*-acetyl-D-glucosamine and D-glucose derivatives can be explained by the type of sugar transporters expressed in Caco-2 cells, mainly GLUT1, GLUT3 and GLUT5 (Harris et al., 1992). GLUT1 and GLUT3 are D-glucose (and D-galactose) transporters while GLUT5 is a D-fructose transporter (Gould and Holman, 1993). However, none of these transporters has been shown to be involved in the transport of *N*-acetyl-D-glucosamine. The large and significant increase ($p < 0.05$, Tukey's post hoc test) in permeability values observed with D-glucose complexes **P4–P6** in comparison to *N*-acetyl-D-glucosamine complexes **P1–P3** therefore suggests that the complexes formed between piperacillin and liposaccharides **4–6** might be transported *via* an active mechanism involving one or more D-glucose transporters, while the *N*-acetyl-D-glucosamine derivatives **P1–P3** were not.

Drugs with good oral bioavailability (80–100% in humans) usually exhibit apparent permeability values of 10^{-6} cm s^{-1} or greater in Caco-2 assays (Artursson et al., 2001). The perme-

ability values observed with the piperacillin complexes were lower (maximum of $P_{app} = 2.42 \pm 0.85 \times 10^{-7}$ cm s^{-1} for complex **P4**) and it is therefore likely that these complexes would not be completely absorbed through the intestinal epithelium. Yet, the significant increase noticed in the case of the D-glucose derivatives and the probable involvement of an active transport mechanism *via* glucose transporters as an alternative to passive diffusion are very encouraging results and will form the basis for the future design and synthesis of more effective, glucose-based charged liposaccharides.

4. Conclusion

D-Glucose- and *N*-acetyl-D-glucosamine-based charged liposaccharides were investigated as potential penetration enhancers for the oral delivery of piperacillin. A sensitive, LC–MS/MS method (MRM, ESI positive mode) was developed and optimized for the detection and quantification of piperacillin and its ion-pair complexes in HBSS–HEPES solution, achieving low LOD and LOQ (0.002 and 0.01 μm, respectively). The apparent permeability values of the synthesized complexes have been assessed through Caco-2 monolayers and significant improvements were observed with the D-glucose complexes **P4–P6** which displayed a six- to eight-fold increase in permeability values compared to piperacillin alone. The absence of noticeable variations in the TEER values during the assay indicates that the compounds do not seem to exhibit particular toxicity towards Caco-2 cells. Although passive diffusion was the targeted route in this study, the results obtained with the D-glucose derivatives on the permeability values suggest the possible involvement of active transport mechanisms, and reflect the importance of the sugar moiety in the design and choice of liposaccharide-based penetration enhancers. Further analyses, including *in vivo* experiments, will be carried out to examine the pharmacological profile of the synthesized complexes.

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