**Bart Blanchaert**[1]
**Tine Wyseure**[2]
**Eefjan Breukink**[3]
**Erwin Adams**[1]
**Paul Declerck**[2]
**Ann Van Schepdael**[1]

[1]Department of Pharmaceutical and Pharmacological sciences, Pharmaceutical analysis, KU Leuven - University of Leuven, Leuven, Belgium
[2]Department of Pharmaceutical and Pharmacological sciences, Therapeutic and Diagnostic Antibodies, KU Leuven - University of Leuven, Leuven, Belgium
[3]Department of Biochemistry of Membranes, Bijvoet Center for Biomolecular Research, University of Utrecht, Utrecht, The Netherlands

Received April 22, 2015
Revised August 26, 2015
Accepted August 27, 2015

Research Article

# Development of a liquid chromatography/mass spectrometry assay for the bacterial transglycosylation reaction through measurement of Lipid II

Transglycosylation is the second to last step in the production of bacterial peptidoglycan. It is catalyzed by a transglycosylation site in class A penicillin-binding proteins (PBPs) or monofunctional glycosyl transferases. Several potential inhibitors have been suggested and need to be tested for activity. In this article, we describe the development and validation of an LC/MS assay for Lipid II, the substrate for transglycosylation. The developed assay can be used to monitor the transglycosylation activity of *Staphylococcus aureus* PBP2. There was no need for modification of Lipid II with a fluorescent tag that could alter affinity of inhibitors toward Lipid II. Recombinant PBP2 was produced in *Escherichia coli* and has been tested for activity. This LC/MS method is suitable for a transglycosylation assay for PBP2 and since it is relatively fast, it can be used to test inhibitors.

**Keywords:**
Bacterial transglycosylation / Lipid II / Liquid chromatography/mass spectrometry assay / Penicillin-binding protein 2 DOI 10.1002/elps.201500201

## 1 Introduction

The increasing antibiotic resistance of all bacteria, but in particular *Staphylococcus aureus* (*S. aureus*), has become a threat to human health. Today resistance to methicillin occurs in 50% of *S. aureus* isolates. Resistance to vancomycin has spread significantly as well [1]. This urges the need for development of new antibacterial drugs and drug targets. The bacterial cell wall still remains attractive because of the lethal effect of its disintegration, the easy accessibility of enzymes responsible for peptidoglycan synthesis and the lack of a eukaryotic counterpart. Several potential targets for drug action in bacterial cell walls remain to be exploited. Peptidoglycan is a 3D network of linear alternating, β 1,4-linked, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) glycan chains, cross-linked by short peptides [2]. The last two steps in peptidoglycan synthesis, transglycosylation and transpeptidation, are carried out as extracellular reactions by a class of membrane-bound enzymes called "penicillin-binding proteins" (PBPs) [2]. The PBPs can be divided into two classes: class A and class B. Class B enzymes only catalyze transpeptidation, whereas class A enzymes also have an N-terminal transglycosylation site making them capable of catalyzing both transglycosylation and transpeptidation [3–5]. In addition, monofunctional glycosyl transferases, enzymes with non-PBP-related transglycosylation activity, have been discovered [6]. Monofunctional glycosyl transferases take over transglycosylation in *S. aureus* in case of loss of activity of PBP2 [6,7]. Since all these enzymes have a high degree of similarity in their transglycosylase domain, it is likely that they will all be sensitive to the same inhibitors [8]. In our study, PBP2 of *S. aureus* is investigated because it is the most important transglycosylation enzyme in this microorganism [3, 6, 7, 9]. Since the acquired PBP2A in methicillin-resistant *S. aureus* does not catalyze transglycosylation, methicillin-resistant *S. aureus* is still susceptible to inhibitors of PBP2-mediated transglycosylation [10, 11].

The substrate for the transglycosylation reaction in Gram-positive organisms consists of a head group (*N*-acetylmuramoyl- (GlcNAc)-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala) coupled to a lipid undecaprenol carrier by a pyrophosphate [12] and is commonly referred to as Lipid II (Fig. 1). During transglycosylation, the C1 MurNAc of the growing glycan chain is transferred onto the C4 carbon of the glucosamine residue of Lipid II followed by the release of the undecaprenyl-pyrophosphate of the growing strand [3, 9]. Subsequently transpeptidation occurs. The peptide moieties in *S. aureus* peptidoglycan are cross-linked by a flexible pentaglycine chain allowing a cross-linking degree of 90% yielding a very rigid cell wall structure [13, 14].

**Correspondence:** Professor Ann Van Schepdael, Department of Pharmaceutical and Pharmacological sciences, Pharmaceutical analysis, KU Leuven - University of Leuven, O&N 2, PB 923, Herestraat 49, B-3000 Leuven, Belgium
**E-mail:** ann.vanschepdael@pharm.kuleuven.be
**Fax:**+3216323448

**Abbreviations:** ***E. coli***, *Escherichia coli*; **GlcNAc**, *N*-acetylglucosamine; **MurNAc**, *N*-acetylmuramic acid; **NCE**, normalized collision energy; **PBP**, penicillin-binding protein; ***S. aureus***, *Staphylococcus aureus*; **SRM**, selected reaction monitoring

 

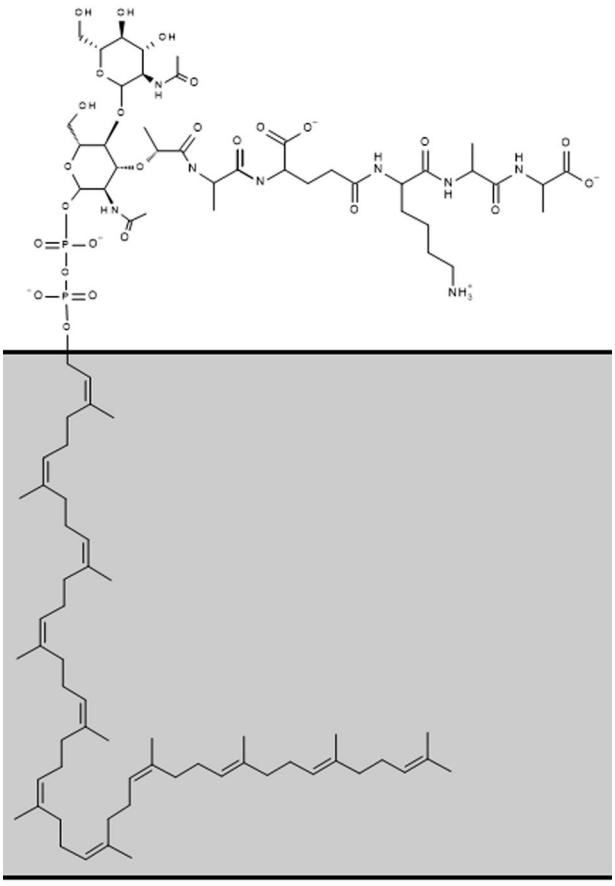

**Figure 1.** Lipid II, the substrate for the transglycosylation reaction, is anchored in the cell membrane by the undecaprenyl moiety.

The absence of a UV-chromophore in the structure of Lipid II, led to the development of assays using fluorescent or radiochemical labeling (see [15] for a review). However, label-free assays are interesting as well because there is no chance of altered affinity. A mass spectrometer is the most sensitive detector for the analysis of compounds lacking a chromophore or fluorophore. Despite the analytical power of MS, prior separation of the sample is recommended in order to avoid matrix effects and ionization suppression [16]. Separation of the sample can be done using several techniques. GC/MS is not useful for Lipid II analysis due to its low volatility. However, LC and CE could prove useful tools for this application. In comparison to LC, CE is faster, more efficient, and cheaper considering capillaries are used instead of expensive LC columns [17]. The high efficiency causes the peaks to be so narrow that only fast scanning mass analyzers can be used, most commonly the TOF analyzer [16]. Both platforms offer a high versatility toward various kinds of samples [16,17]. CE/MS interfaces with a sheath liquid are robust and offer the unique possibility of decoupling the separation and the ionization chemistry. Indeed, additives such as acids can be added to the sheath liquid to optimize ionization efficiency in the MS [18, 19]. Unfortunately, dilution in the sheath liquid decreases efficiency and sensitivity. Sheathless interfaces supply a nanoliter flow rate to the MS resulting in very good sensitivity. However, this interface has suffered from robustness issues [19]. Despite the advantages of CE/MS, LC/MS is still the platform of choice for most researchers because of the higher sensitivity achieved by a higher sample loading capacity [18, 20]. Also, LC results are generally more repeatable compared to those obtained by CE. Finally, despite the longer analysis times, LC is considered to be more time-efficient as it is more robust. While LC columns can run continuously for months, provided the mobile phase bottles are refilled, CE capillaries require more care and regeneration [21]. Therefore, we have chosen to develop an assay using LC/MS.

Lebar et al. have described an LC/MS assay for the study of PBP1b of *Escherichia coli* (*E. coli*) [22]. However, the work was focused on the effect of small alterations in Lipid II on the transglycosylation and transpeptidation activity of PBP1b. The analyte of the LC/MS method was not Lipid II, but the disaccharide units obtained upon hydrolysis of the glycan strands produced by PBP1b. In the present study, the goal is to develop an LC/MS assay for direct monitoring of Lipid II in order to eliminate time-consuming manipulations.

## 2 Materials and methods

### 2.1 Chemicals

A stock solution of 1.13 mM of undecaprenyl–diphospho-*N*-acetylmuramoyl-(GlcNAc)-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (Lipid II) in a mixture of water, chloroform, and methanol was produced according to the procedures of the Breukink lab [23]. The stock solution also contained 126 and 612 μM of the decaprenyl and dodecaprenyl variants of Lipid II, respectively. The total concentration of all Lipid II analogues has been determined by phosphate analysis. The ratio of Lipid II compared to related substances has been estimated using LC/MS.

LC/MS grade ACN was obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate and acetic acid, both LC/MS grade, were from Sigma-Aldrich (Steinheim, Germany). Also, HEPES, $CaCl_2$, $MgCl_2$, TCA, flavomycin, teicoplanin, octaethylene glycol monododecyl ether, MES and CHAPS were purchased from Sigma-Aldrich. DMSO was bought from Merck (Darmstadt, Germany). Sodium deoxycholate and sodium lauroyl sarcosinate (sarkosyl) were from Acros Organics (Geel, Belgium), Triton X-100 from VWR (Leuven, Belgium), and NaCl from ThermoFisher Scientific (Aalst, Belgium). Vancomycin was available in the lab. A Milli-Q purification system (Millipore, Bedford, MA, USA) was used to further purify demineralized water.

### 2.2 Expression of recombinant *S. aureus* PBP2 in *E. coli*

The genome of a wild-type species *S. aureus* NCTC 8325 was purified and the *pbp2* gene was multiplied by PCR. The primers for this PCR, 5′-GCGCTAGCATGACGGAAAACA

 

AAGGATCT-3' and 5′-GCGGATCCTTACTCGAGGTTG-AATATACCTGTTAATC-3′, were designed to introduce restriction sites for *Ava*I and *Nhe*I. Following digestion, the gene fragment was inserted into a digested and dephosphorylated pET21b(+) vector (Novagen). This vector contains a C-terminal His-tag code which is located right next to the *Ava*I restriction site. The ultimate isoleucine was replaced by leucine and glutamine as a result of the introduction of the *Ava*I restriction site. Changes in the sequence were kept to a minimum resulting in a total mass of the recombinant protein of 81.24 kDa. Subsequent to confirmation of the correct sequence by Sanger sequencing, competent *E. coli* BL21(DE3) cells were transformed by the vector. Expression of PBP2 was initiated by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside to the transformed cells grown to an optical density of 1 at 600 nm in Luria Broth medium. Cells were collected after 3 h through centrifugation followed by resuspension in a buffer containing 68 mM sarkosyl and subsequent lysis of the cells using a French press. PBP2 was purified using a Ni-column and purity was confirmed with SDS-PAGE. Yields were determined using the bicinchoninic acid assay [24]. Forty milliliters of a 6.7 μM solution of PBP2 in a buffer containing 0.10 M citric acid, 0.2 M NaOH, 0.5 M NaCl, and 10% glycerol was obtained.

### 2.3 LC/MS method development and validation

#### 2.3.1 LC/MS system

The LC/MS system consisted of a P680 HPLC pump from Dionex (Sunnyvale, CA, USA) and an AS100 autosampler from ThermoFinnigan (San Jose, CA, USA). Mass spectra were acquired on an LCQ IT mass spectrometer (ThermoFinnigan) with ESI interface operated in positive ion mode. The MS system was tuned by an automated procedure using 15 μM of Lipid II diluted in the mobile phase which was infused directly in the ESI source at a rate of 10 μL/min. The ESI needle voltage was set at 4.5 kV and the heated capillary was held at 250°C. The auto gain control regulated the number of ions stored in the trap. Following voltages were applied to obtain an optimal signal: capillary voltage 10 V, tube lens offset voltage −10 V, octopole 1 offset voltage −2.5 V, octopole 2 offset voltage −7 V, and interoctopole lens voltage −16 V. Nitrogen (Air Liquide, Liège, Belgium) served as sheath and auxiliary gas at a flow rate of 20 arb each. Helium was used as damping gas, but also to induce fragmentation by collision. Xcalibur 1.3 software (ThermoFinnigan) was used for instrument control, data acquisition, and processing. Since this LC/MS assay is only focused on the determination of the remaining Lipid II concentration after incubation with PBP2, the selected reaction monitoring (SRM) mode was used ensuring optimal sensitivity.

#### 2.3.2 Method development

Due to the lowered response obtained with flow rates above 0.2 mL/min by the LCQ IT mass spectrometer when equipped with an ESI source, only narrow bore columns were investigated as they are mostly operated at this flow rate. In this study, we compared two columns: the Symmetry Shield RP8 column (2.1 mm × 100 mm; 3.5 μm) and the XTerra RP18 column (2.1 mm × 100 mm; 3.5 μm) both from Waters (Milford, MA, USA). Methanol was compared to ACN to be used as organic modifier. Also, the influence of the pH and concentration of the buffer have been investigated.

#### 2.3.3 Final chromatographic conditions

LC/MS analyses of the incubation mixtures were performed on the Symmetry Shield RP8 column. The mobile phase was a gradient mixture of mobile phase A (0.05 M ammonium acetate (pH 4.5) in water–ACN, 50:50, v/v) and B (ACN) and was pumped at a flow rate of 0.2 mL/min. The gradient program (time [min]/%B) was set as follows 0/40, 5/40 to 13/88 to 13.5/40, 15/40. The injection volume was 5 μL. The mobile phases were degassed by sparging with helium. Figure 2 shows typical chromatograms obtained in SIM mode, to evaluate resolution between Lipid II and its analogues, and SRM mode, which was used for quantification.

#### 2.3.4 Method validation

The developed method was validated on several parameters necessary for ensuring a reliable assay for the enzymatic activity. Linearity, LOD, LOQ, precision and selectivity were investigated on samples dissolved in the incubation mixture.

### 2.4 Incubation and sample preparation

Incubations were performed at 30°C in 100 μL of a mixture containing 15 μM Lipid II, 35% DMSO, 0.04% Triton X-100, 50 mM MES buffer (pH 6.0), and 10 mM $CaCl_2$. Reactions were started by addition of 67 nM of PBP2 and stopped after 15 min unless indicated otherwise by addition of 100 μL ACN which causes PBP2 to precipitate. Incubation mixtures were centrifuged and the supernatant was injected onto the LC/MS system.

## 3 Results and discussion

### 3.1 LC/MS method development

#### 3.1.1 Column selection

Two columns have been compared: Symmetry Shield RP8 and XTerra RP18. Both were narrow bore columns with the main difference being their carbon chain length. When using gradient runs of ACN and water on the Symmetry Shield RP8 column, good peak shape and resolution between Lipid II and the related substances were observed. Due to the apolar nature of the analyte, interaction with the $C_{18}$ chains was very strong. Nearly 90% of ACN was necessary for elution of the analyte and severe carryover was observed between gradient

 

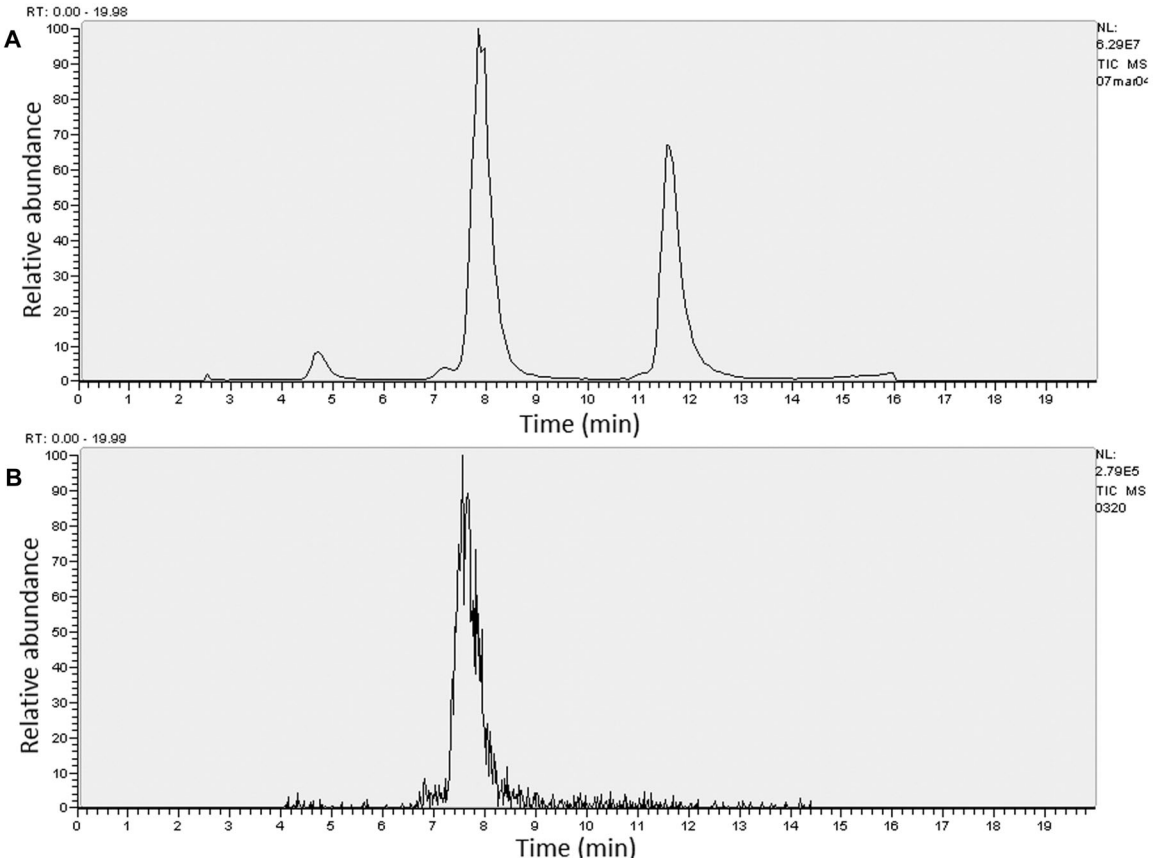


**Figure 2.** (a) Chromatogram obtained after injection of 150 μM of the Lipid II stock solution in methanol–water (70:30, v/v). SIM mode was set to scan for Lipid II ([M + $H^+$] *m/z* 1876) and the decaprenyl-([M + $H^+$] *m/z* 1810) and dodecaprenyl variants ([M + $H^+$] *m/z* 1943). Baseline separation of Lipid II from the impurities was obtained. (b) LC/MS chromatogram of 5 μM Lipid II sample in SRM mode.

runs on the XTerra RP18. Therefore, it was not suitable for analysis of this compound and the $C_8$-column was selected.

### 3.1.2 Selection of organic modifier

Methanol and ACN have been evaluated as organic modifier in the mobile phase. To the aqueous phase, 0.05 M ammonium acetate (pH 4.5) was added. In a slow gradient run, almost 100% of methanol was needed to elute the very apolar analyte from the $C_8$-column. This caused the baseline to be very noisy at the elution time which could impede reliable quantification. Furthermore, significant carryover was observed both for Lipid II and the dodecaprenyl variant. Therefore, ACN was preferred over methanol.

### 3.1.3 Influence of buffer pH and concentration

It was noticed in the early stage of method development that a nonbuffered mobile phase caused irreproducible retention times for the analyte as well as broad peaks. Therefore, 0.05 M ammonium acetate was added. Higher buffer concentrations, such as 0.15 M or 0.25 M, had a profound negative effect on ionization efficiency. The pH was set at 4.5. Lower pH (3.5) increased the retention with 3 min whereas higher pH (5.3) had no significant effect.

### 3.1.4 Optimization of SRM mode

To obtain maximal sensitivity, the SRM mode was used and set to scan for the fragmentation of Lipid II ([M + $H^+$], *m/z* 1876) into a fragment with [M + $H^+$] *m/z* 1127 or [M + $H^+$] *m/z* 949. These fragments are the sugar pentapeptide moieties with and without diphosphate, respectively. This has been confirmed by further fragmentation which yielded the fragment ions [M + $H^+$] *m/z* 746 (*N*-acetylmuramoyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala) and [M + $H^+$] *m/z* 561 (lactoyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala). An MS/MS spectrum is shown in Fig. 3. The fragmentation efficiency is influenced by the normalized collision energy (NCE) used for fragmentation inside the IT. A low NCE does not provide sufficient energy to fragment the parent ion. Higher NCE causes many smaller fragments. Various NCE levels between 10 and 40% were tested and 20% was found as optimal for this transition.

 

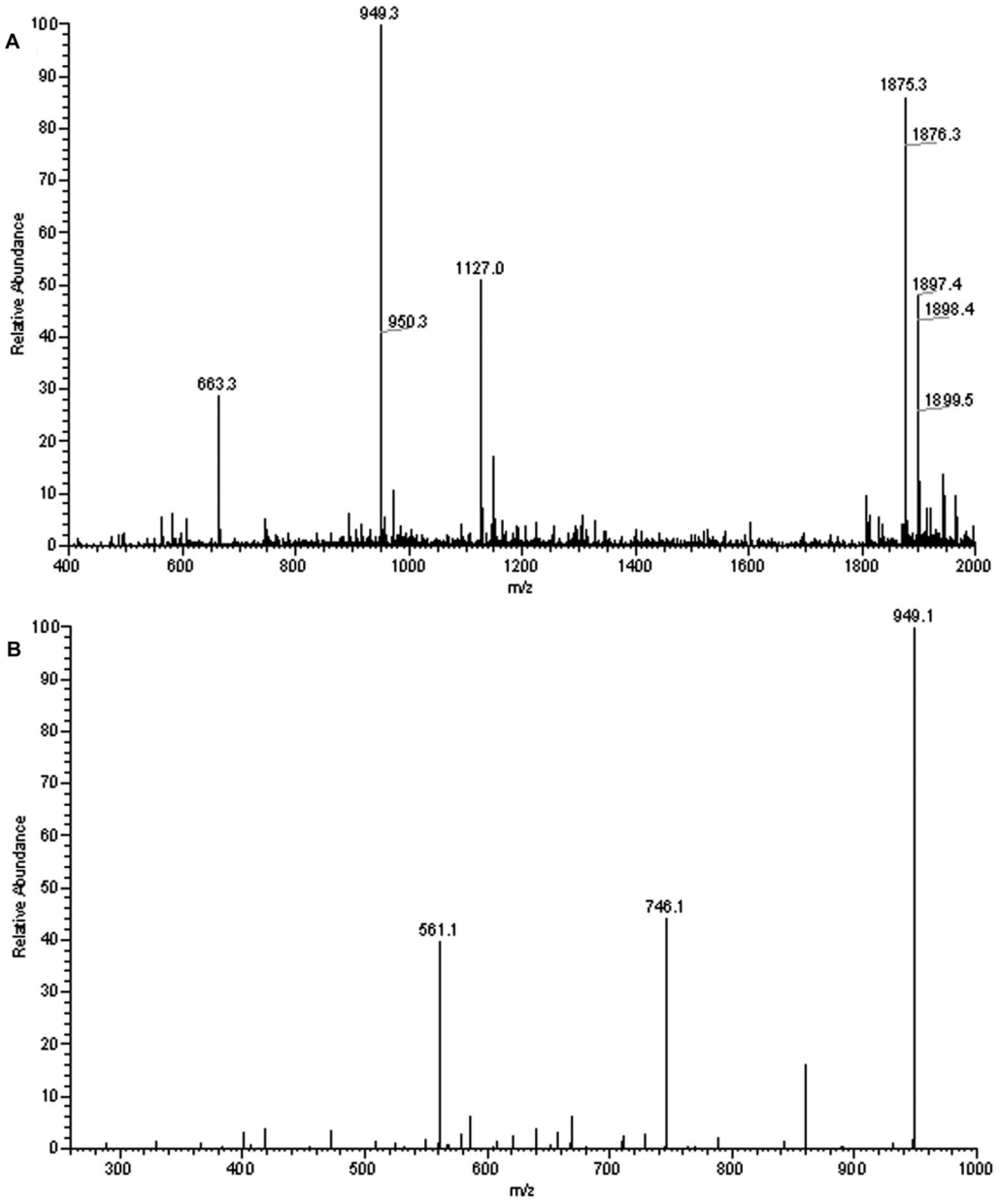


**Figure 3.** (a) MS spectrum obtained after direct infusion of Lipid II (15 μM in methanol–water (70:30, v/v)). Ions corresponding to Lipid II ($[M + H^+]$, *m/z* 1876), the sugar pentapeptide with and without diphosphate ($[M + H^+]$ *m/z* 1127 and $[M + H^+]$ *m/z* 949, respectively) could be observed. (b) The MS/MS spectrum (25% normalized collision energy) shows the fragment ions $[M + H^+]$ *m/z* 746 (*N*-acetylmuramoyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala) and $[M + H^+]$ *m/z* 561 (lactoyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala).

### 3.1.5 Matrix effects in LC/MS

Matrix effects occur when other molecules co-eluting with the analyte of interest, alter its ionization efficiency and thus the response obtained. When using LC/MS in bio-analysis, matrix effects should always be evaluated because severe ion suppression impedes proper, reliable, and repeatable quantification. The postextraction addition technique was used to estimate the altered ionization efficiency [25]. A blank incubation mixture was made to which PBP2 and ACN were added. Subsequent to centrifugation, Lipid II was added to the supernatant to obtain a final concentration of 15 μM. As a reference, Lipid II was diluted to the same concentration with mobile phase A. The difference in response between the postextraction addition sample and the reference divided by the response of the reference determines the degree of matrix

 

effect occurring on the analyte in question under chromatographic conditions. This test was performed in duplicate and the mean matrix effect was determined to be −9.6%.

### 3.2 Analytical validation of the LC/MS method

Validation was performed as prescribed by ICH and FDA guidelines [26, 27]. Linearity, sensitivity, and repeatability have been assessed as these characteristics are crucial for an assay.

#### 3.2.1 Linearity

Linearity was determined in the incubation mixture including inactivated PBP2. Dilutions of a 150 μM stock solution of Lipid II in $H_2O$–methanol (30:70, v/v) were made and transferred into Eppendorf tubes. After evaporation in vacuo, Lipid II was dissolved in 100 μL of the incubation mixture (see Section 2.4.) and 100 μL of ACN was added. Subsequently, PBP2 was added which immediately denatured and precipitated. All this was done to mimic the conditions after incubation as closely as possible. Final concentrations of 2, 4, 8, 12, 16, and 20 μM of Lipid II were prepared. This corresponded to a range of 10–100 picomoles injected onto the system as the injection volume was 5 μL. A linear calibration curve was constructed by LC/MS in the described range. The correlation coefficient was 0.9997, and the regression equation $y = 1\ 423\ 859x + 4\ 960\ 583$, where $x$ is the concentration of Lipid II (in μM) and $y$ is the area of the main peak. The determination coefficient was greater than 0.995 indicating a good linear relationship between detector response and the concentration of Lipid II [28].

#### 3.2.2 Repeatability

There are three subdivisions in precision: repeatability, intermediate precision, and reproducibility. Repeatability is the precision under the same conditions on the same day. Intermediate precision investigates the influence of different conditions within the same lab: different days, analysts, and equipment. Reproducibility is an interlaboratory comparison, for instance a collaborative study. In this research, only repeatability could be investigated because there was only one LC/MS system available in the laboratory. Repeatability was evaluated by analysis of six separately prepared samples of 7.5 μM Lipid II in incubation mixture including PBP2, as described for linearity. Averages of the area under the curve of three injections for each sample were compared. The relative standard deviation for these six averages was 1.4%.

#### 3.2.3 Sensitivity

It was important to have an estimate of the method's sensitivity since enzyme activity is being measured as a decrease in analyte concentration. LOD and LOQ are calculated based on the slope of the calibration curve [28] and were determined to be 0.28 and 0.94 μM, respectively. The sensitivity was concluded to be sufficient for this assay.

#### 3.2.4 Selectivity

Since a mass spectrometer allows mass-selective detection, specificity is not a big problem. Still, baseline separation for Lipid II and the deca- and dodecaprenyl variants was useful to avoid ionization suppression. This was evaluated during method development.

Despite the selective characteristics of the SRM mode, an additional control was performed. A blank incubation sample containing all components except Lipid II was prepared. After addition of ACN, the sample was centrifuged and injected onto the LC/MS system. No signal was observed at the expected elution time of Lipid II. This test was performed in duplicate (data not shown).

### 3.3 Optimization of incubation mixture

All necessary components for the enzyme activity were tested and evaluated. Besides the substrate there was also need for DMSO and a detergent to solubilize substrate and enzyme, ions to function as co-factors and a buffer to ensure pH stability. The influence of the temperature at which samples were incubated has been investigated as well.

#### 3.3.1 Detergent

Triton X-100 (0.04%), deoxycholate (0.01%), CHAPS (0.1%), and octaethylene glycol monododecyl ether (0.05%) have been tested; concentrations in 100 μL of incubation medium are mentioned between brackets. Samples containing Lipid II (7.5 μM), PBP2 (67 nM), DMSO (35%), MES buffer (50 mM, pH 6.0), NaCl (200 mM), $MgCl_2$ (25 mM), and $CaCl_2$ (10 mM) were supplemented with one of the detergents. Each sample was made in twofold of which one was immediately inactivated by addition of ACN and served as an inactivated control, whereas the other samples were shaken for 30 min at 30°C followed by inactivation with ACN. It was observed that the decrease in substrate concentration relative to the inactivated control was only significant in samples containing Triton X-100 and CHAPS (Fig. 4).

#### 3.3.2 Dimethylsulfoxide

DMSO concentrations were varied across a wide range (10–40%). Significant influences on enzyme activity were not found (data not shown).

#### 3.3.3 Co-factors

$CaCl_2$ (10 mM), NaCl (200 mM), and $MgCl_2$ (25 mM) were evaluated for their suitability to serve as co-factors

 

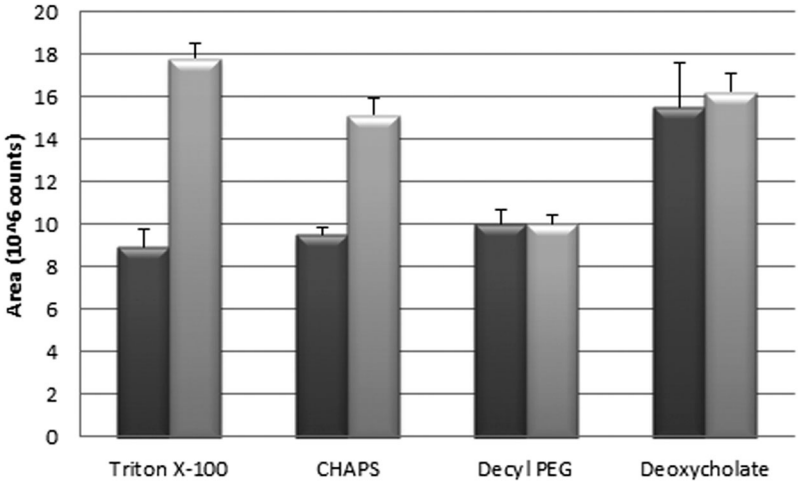


**Figure 4.** Comparison of the influence of several detergents on PBP2 activity. Normal samples (dark) and inactivated controls (light) have been prepared for each detergent. Only Triton X-100 and CHAPS seem to enable transglycosylation activity of PBP2. See Section 3.3.1.

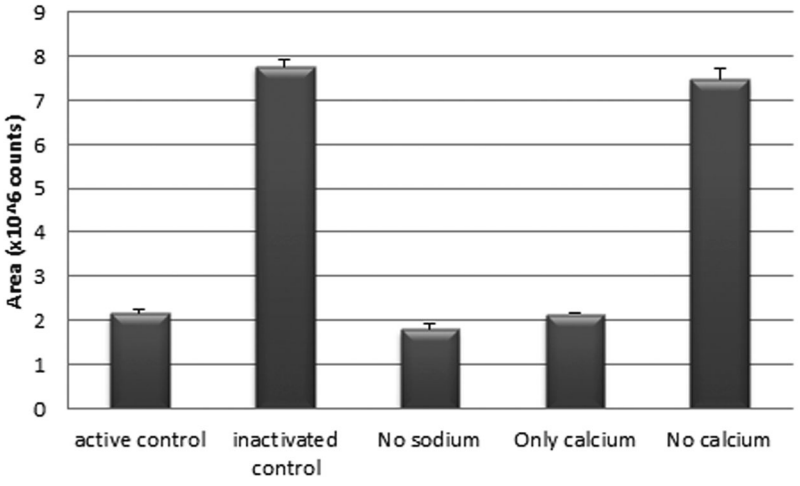


**Figure 5.** Influence of cations on PBP2 activity. The active and inactivated controls contain $Na^+$, $Ca^{2+}$, and $Mg^{2+}$. Transglycosylation can be observed in the case of omission of $Na^+$ (sample 3) and even of both $Na^+$ and $Mg^{2+}$ (sample 4). However, all activity is lost after omission of $Ca^{2+}$, even in the presence of $Na^+$ and $Mg^{2+}$. See Section 3.3.3.

in PBP2-mediated transglycosylation. Two control samples, containing all three salts, were made of which one was immediately inactivated, whereas the other one was incubated and treated as described above. Three samples did not contain all cations. In one sample, the effect of only sodium omission was investigated. A second sample contained only calcium and a third sample contained sodium and magnesium but no calcium. The latter two samples were prepared to determine whether calcium was sufficient and necessary for PBP2 activity. All samples contained 7.5 μM of Lipid II. All active samples were incubated for 30 min to evaluate whether other co-factors enabled transglycosylation at a lower rate. It was observed that omission of both $MgCl_2$ and NaCl did not influence enzyme activity, whereas presence of calcium ions seemed crucial (Fig. 5).

### 3.3.4 Incubation conditions

Small changes in pH might have an influence on enzyme stability and activity. Therefore, pH was varied between 5 and 7.5. No significant effect on enzyme activity was observed (data not shown). Incubation temperatures were varied from 25°C to 37°C. Enzyme activity was optimal at 30°C (data not shown).

## 3.4 Evaluation of precipitation techniques

A calibration curve was composed for samples of Lipid II in incubation mixtures containing immediately inactivated PBP2. It was observed that the response obtained was approximately ten times lower compared to that of samples containing Lipid II dissolved in methanol–water (70:30, v/v). Because significant matrix effects had already been ruled out, this could either be explained by a lower solubility in the incubation mixture or a co-precipitation of the analyte with the enzyme. The similar response obtained when dissolving Lipid II in incubation buffer without PBP2 and ACN compared to the analyte dissolved in methanol–water, confirmed the second hypothesis. ACN as precipitating agent was compared to two other strategies often used in literature, TCA (10%, w/v) and methanol [29]. Methanol was added in a 1:1 ratio and 50 μL of TCA was added to 100 μL of incubation mixture followed by

 

addition of 50 μL of ammonium formate 1 M (pH 3.6) after 1 h. The response relative to that obtained with ACN was 0.02 and 0.32 for methanol and TCA, respectively. Therefore, ACN was selected as precipitating agent. Since there is a linear relationship between the response and the concentration of Lipid II, the fraction of Lipid II that remained in the supernatant was constant over the range of concentrations used.

### 3.5 Biochemical validation of LC/MS method

It was necessary to ascertain that the decrease in substrate concentration was linked to PBP2-related transglycosylation. Therefore, the release of undecaprenyl-pyrophosphate was investigated as well as the inhibition of transglycosylation by a known inhibitor.

#### 3.5.1 Release of the undecaprenyl-pyrophosphate

Following the attachment of the C1 of MurNAc of the growing glycan chain to the C4 of GlcNAc in Lipid II, undecaprenyl-pyrophosphate is released from the growing chain [3]. Therefore, it is also a product of the transglycosylation reaction, just like the glycan strands are. A sample and an inactivated control were prepared and analyzed by ESI MS using direct infusion. The MS system was operated in negative ion mode. In the mass chromatograms obtained with samples containing active PBP2, a compound with a $[M - 2H]^{2-}$ $m/z$ 463 was observed, an $m/z$ value that corresponds to the undecaprenyl-pyrophosphate with a double negative charge. This ion could not be detected in the inactivated control.

#### 3.5.2 Inhibition of transglycosylation

Vancomycin was tested as positive control for inhibition. Although vancomycin was discovered as a transpeptidase inhibitor [30], its high affinity for the D-Ala-D-Ala C-terminus of the pentapeptide of Lipid II, causes vancomycin to inhibit both transglycosylation and transpeptidation [31]. Three samples were prepared as described in Section 2.4. One was inactivated and another one was supplemented with 10 μM vancomycin. Reactions were stopped by addition of ACN. Analysis of the active sample showed a decrease of substrate (mean area ± SD = 21 410 747 ± 280 246 counts) whereas substrate levels in the vancomycin supplemented sample (mean area ± SD = 38 436 954 ± 785 932 counts) did not diminish in comparison to the inactivated sample (mean area ± SD = 38 452 033 ± 77 071 counts). Therefore, we concluded that the decrease in Lipid II concentration in the sample without vancomycin can be attributed to a specific PBP2 activity, since this decrease was not observed in a sample where vancomycin inhibited enzyme activity.

## 4 Concluding remarks

In this article, we described for the first time an LC/MS method for the analysis of Lipid II which can be used to monitor the PBP2-mediated transglycosylation reaction. This has no need for fluorescent labeling that could interfere with the inhibitor's affinity for Lipid II. This method has been validated and can be used to evaluate potential inhibitors of the transglycosylation site of PBPs. This LC/MS method is much faster than other methods described in literature. As it is developed as an assay for Lipid II rather than the hydrolyzed glycan chains, a time-consuming hydrolysis step is avoided.

In contrast to the transpeptidation function of PBPs, the transglycosylation function has not led to any human therapeutic agents yet. Hopefully, that is about to change in the near future.

*The authors thank Professor Johan Van Eldere for the S. aureus NCTC 8325 strain from his library and Dr. Gaston Vondenhoff for the gift of E. coli BL21(DE3) cells. Support from the Faculty of Pharmaceutical Sciences is gratefully acknowledged.*

*The authors have declared no conflict of interest.*

## 5 References

[1] Bassetti, M., Righi, E., *Hematology Am. Soc. Hematol. Educ. Program* 2013, *2013*, 428–432.

[2] Vollmer, W., Bertsche, U., *Bioch. et Biophys. Acta* 2008, *1778*, 1714–1734.

[3] Scheffers, D. J., Pinho, M. G., *Microbiol. Mol. Biol. Rev.* 2005, *69*, 585–607.

[4] Graves-Woodward, K., Pratt, R. F., *Biochemistry* 1999, *38*, 10533–10542.

[5] Halliday, J., McKeveney, D., Muldoon, C., Rajaratnam, P., Meutermans, W., *Biochem. Pharmacol.* 2006, *71*, 957–967.

[6] Wang, Q. M., Peery, R. B., Johnson, R. B., Alborn, W. E., Yeh, W. K., Skatrud, P. L., *J. Bacteriol.* 2001, *183*, 4779–4785.

[7] Terrak, M., Nguyen-Distèche, M., *J. Bacteriol.* 2006, *188*, 2528–2532.

[8] Terrak, M., Sauvage, E., Derouaux, A., Dehareng, D., Bouhss, A., Breukink, E., Jeanjean, S., Nguyen-Distèche, M., *J. Biol. Chem.* 2008, *283*, 28464–28470.

[9] Lovering, A. L., de Castro, L. H., Lim, D., Strynadka, N. C., *Science* 2007, *315*, 1402–1405.

[10] Pinho, M. G., de Lencastre, H., Tomasz, A., *Proc. Natl. Acad. Sci. USA* 2001, *98*, 10886–10891.

[11] Pinho, M. G., Filipe, S. R., de Lencastre, H., Tomasz, A., *J. Bacteriol.* 2001, *183*, 6525–6531.

[12] de Kruijff, B., van Dam, V., Breukink, E., *Prostaglandins Leukot. Essent. Fatty Acids* 2008, *79*, 117–121.

[13] Tipper, D. J., Strominger, J. L., *J. Biol. Chem.* 1968, *243*, 3169–3179.

[14] Rohrer, S., Ehlert, K., Tschierske, M., Labischinski, H., Berger-Bächi, B., *Proc. Natl. Acad. Sci. USA* 1999, *96*, 9351–9356.

[15] Blanchaert, B., Adams, E., Van Schepdael, A., *Anal. Methods* 2014, *6*, 7590–7596.

 

[16] Lei, Z., Huhman, D. V., Sumner, L. W., *J. Biol. Chem.* 2011, *286*, 25435–2542.

[17] Robledo, V. R., Smyth, W. F., *Electrophoresis* 2014, *35*, 2292–2308.

[18] Dakna, M., He Z., Yu, W. C., Mischak, H., Kolch, W., *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2009, *877*, 1250–1258.

[19] Heemskerk, A. A., Deelder, A. M., Mayboroda, O. A., *Mass Spectrom. Rev.* 2014, *9999*, 1–13.

[20] Kok, M. G., Somsen, G. W., de Jong, G. J., *Talanta* 2015, *132*, 1–7.

[21] Büscher, J. M., Czernik, D., Ewald, J. C., Sauer, U., Zamboni, N., *Anal. Chem.* 2009, *81*, 2135–2143.

[22] Lebar, M. D., Lupoli, T. J., Tsukamoto, H., May, J. M., Walker, S., Kahne, D., *J. Am. Chem. Soc.* 2013, *135*, 4632–4635.

[23] Breukink, E., van Heusden, H. E., Vollmerhaus, P. J., Swiezewska, E., Brunner, L., Walker, S., Heck, A. J., de Kruijff, B., *J. Biol. Chem.* 2003, *278*, 19898–19903.

[24] Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C., *Anal. Biochem.* 1985, *150*, 76–85.

[25] Taylor, P. J., *Clin. Biochem.* 2005, *38*, 328–334.

[26] U.S. FDA, Guidance for Industry, Bioanalytical Method Validation, 2001.

[27] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Q2 (R2), *Validation of analytical procedures: text and methodology*, 2005.

[28] Harris, D. C., *Quantitative and Chemical Analysis*, Freeman, W. H. and company, New York 2007.

[29] Polson, C., Sarkar, P., Incledon, B., Raguvaran, V., Grant, R., *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2003, *785*, 263–275.

[30] Boger, D. L., *Med. Res. Rev.* 2001, *21*, 356–381.

[31] Barna, J. C., Williams, D. H., *Annu. Rev. Microbiol.* 1984, *38*, 339–357.

 