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Development and validation of an ultra-high performance liquid chromatography – high resolution mass spectrometry method for the quantification of total and free teicoplanin in human plasma

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ABSTRACT

Objectives: The antibiotic teicoplanin, used for the treatment of infections caused by Gram-positive bacteria, is highly bound to plasma proteins (percentage protein binding, %PB, around 90%) and therapeutic plasma levels of total teicoplanin are 10–100 mg/L. Because of the low free concentrations (i.e. < 1–10 mg/L), current immunoassays are not able to quantify free teicoplanin concentrations, although they might be more relevant in therapeutic drug monitoring than total concentrations.

Design and methods: In this study, an ultra-high performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS) method for the quantification of total and free teicoplanin in K_2EDTA plasma samples was developed and validated. Furthermore, %PB obtained by ultrafiltration was compared with that obtained by equilibrium dialysis using spiked samples from healthy subjects. Analytes were separated using a phenylhexyl column, gradient mobile phase analysis was used, total run time was 4.5 min and teicoplanin was detected by orbitrap MS.

Results: The precision and accuracy were below 15% and within ± 15%, respectively and teicoplanin was found to be stable for at least 14 days in plasma at 4 °C. The %PB of teicoplanin in spiked plasma from healthy subjects as obtained by ultrafiltration (94.1 ± 1.3%) was in good agreement with that obtained by equilibrium dialysis (93.6 ± 0.4%), whereas mean %PB of teicoplanin in samples from infected patients who received the antibiotic was 87.7 ± 4.2% (range: 79.6–95.4%).

Conclusion: A novel highly sensitive UHPLC-HRMS method was developed and validated for the quantification of total and free teicoplanin in human K_2EDTA plasma samples. Amongst others, this method is suitable for therapeutic drug monitoring.

1. Introduction

It is considered that only the free drug fraction is biologically active because only this fraction can travel through cell membranes, by e.g. passive diffusion, towards the site of action [1]. Alterations in free fractions can be of clinical relevance, especially for drug compounds with a high level of protein binding having a narrow therapeutic range or when drug resistance is possible. A change in percentage protein binding (%PB) from e.g. 95% to 90% implicates a doubling in free fraction and may have an impact on the pharmacokinetics (PK) and pharmacodynamics (PD) of that drug. Therefore, knowledge of the free drug concentrations may be more relevant than total concentrations to fully understand PK/PD as for instance with therapeutic drug monitoring (TDM) [2–4].

The most widely used methods to separate free fractions are equilibrium dialysis (ED) and ultrafiltration (UF) [5–7]. Although ED is considered to be the reference, its major drawback is the long equilibration time (i.e. equilibration of the free fraction between the serum/plasma and buffer chamber), which is 5–6 h for small compounds or even 24 h for large compounds. In addition, equilibration time also depends on the molecular weight cut-off of the membrane. Conversely, UF is a more rapid method that separates the free fraction in only 20–30 min by centrifugation, which forces water and free drugs through the filter membrane. It should however be mentioned that both ED and UF sometimes suffer from non-specific adsorption (NSA) to the used membrane. In ED, this can be solved by measuring the

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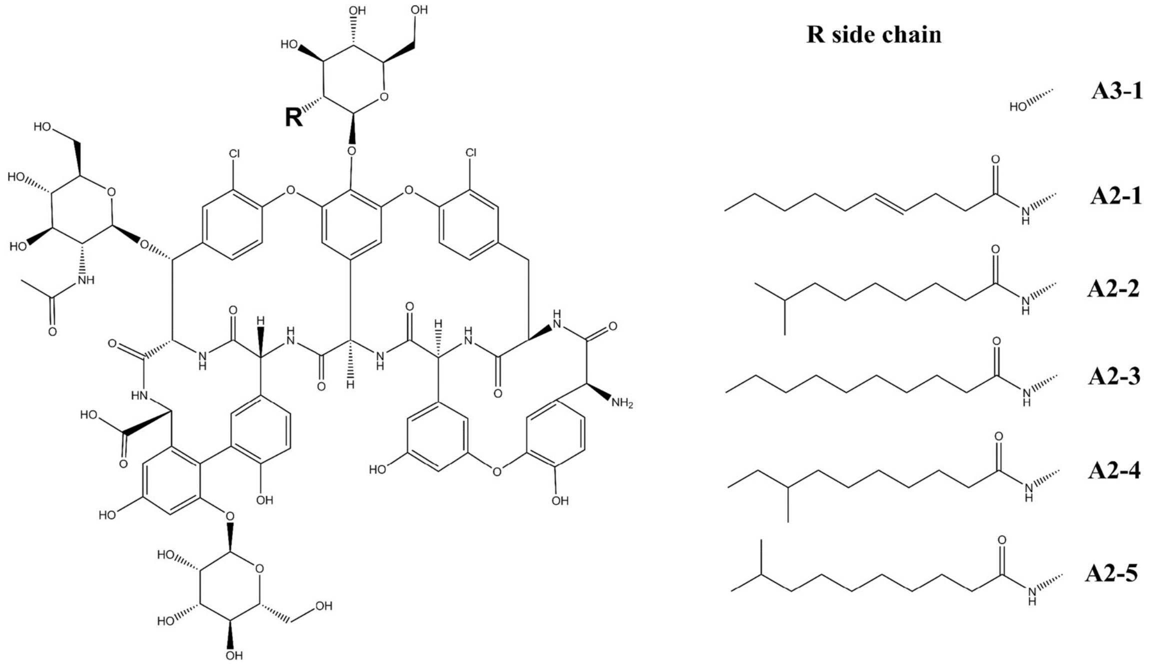

Fig. 1. Chemical structures of the 5 different major teicoplanin compounds, A2–1 to A2–5, and the more polar compound A3–1, which only differ in the alkyl side chain on the core group.

concentration in both the buffer and serum/plasma chamber, whereas in UF, correction for NSA can be readily performed by filtering the calibration standards, together with the unknown samples.

Besides vancomycin, the glycopeptide antibiotic teicoplanin can also be used for the treatment of infections caused by Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) [8–11]. Teicoplanin is composed of a mixture of compounds that only differ in the alkyl side chain (see Fig. 1). These compounds include 5 major (A2–1 to 5) compounds accounting for 90–95% of the total product, a more polar compound (A3–1) and 4 minor (RS-1 to 4) compounds [8,12]. Teicoplanin binds to plasma proteins, with a %PB reported to range from around 70% to 99% [2,13,14] and is less toxic than vancomycin, particularly in terms of nephrotoxicity [15,16]. Therapeutic levels of total teicoplanin in plasma range from 10 to 100 mg/L [17–19] and hence, theoretical free teicoplanin concentrations range from below 1 up to 10 mg/L [2,13]. Due to the small number of studies, data correlating total teicoplanin concentrations to clinical outcome are limited [20,21] and even absent for free teicoplanin. Nevertheless, TDM is important to ensure its therapeutic effectiveness [14,19]. However, since the free drug fraction is responsible for therapeutic effects and the inter-patient variability is large [2,3], free concentration monitoring might be more relevant than total concentration. Despite this theoretical importance of monitoring free teicoplanin concentrations, no clinical studies have confirmed this so far.

Throughout the years, different analytical methods have been developed for the quantification of total teicoplanin in serum or plasma samples, including fluorescence polarization (FPIA) [13,22–24] and homogeneous particle-enhanced turbidimetric [25] immunoassays as well as high performance liquid chromatography techniques in which teicoplanin was quantified by ultraviolet detection [2] or mass spectrometry [26–30]. In the small number of papers discussing the quantification of free teicoplanin, there was no evidence for the accuracy of the obtained free fractions (e.g. by comparison with ED) or these free fractions were obtained at physiologically irrelevant temperatures (4 °C) [2,13,23].

Therefore, we developed and validated a sensitive method for the quantification of total and free teicoplanin in plasma samples by ultra-high performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS). HRMS devices offer the advantage of high sensitivity, very high identification power together with easy development of quantitative methods [31]. In our method, free fractions were obtained by UF at 37 °C and the used UF settings were compared with the reference method ED.

2. Materials and methods

2.1. Chemicals

Acetone, acetonitrile, methanol and formic acid (all LC-MS grade) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Teicoplanin European pharmacopoeia reference standard (CRS 50), ammonium formate and trimipramine-D_3 100 mg/L in methanol were obtained from Sigma Aldrich (Saint-Louis, MO, USA). The teicoplanin reference standard consisted of 5 major compounds (with different relative abundances), including teicoplanin A2–1 (6.2%), isomers A2–2 & A2–3 (67.5%) and isomers A2–4 & A2–5 (21.1%) as well as the more polar compound A3–1 (5.2%) (Fig. 1). Ultrapure water (conductivity < 1 μS and filtered through a 45 μm filter) was used for all dilutions and prepared using an Elga Medica R7 instrument (Veolia water solutions & Technologies, Ede, The Netherlands).

2.2. Teicoplanin solutions

2.2.1. Standard solutions

A stock solution of teicoplanin European pharmacopoeia reference standard with a theoretical concentration of 1400 mg/L was prepared and used for both total and free teicoplanin analyses. A pool of K_2EDTA plasma from healthy volunteers (i.e. blank plasma) was used to prepare calibration curves. For the determination of total teicoplanin, 8 calibrator stock solutions (10 × the final concentration) were prepared and for each calibrator level, 100 μL was added to 900 μL blank plasma. For free teicoplanin, 7 calibrator stock solutions (10 × solution of the final concentration) were prepared and 100 μL was added to 900 μL ultrafiltrate, which was obtained by ultrafiltration of blank plasma using Centrifree Ultrafiltration filters (Merck, Darmstadt, Germany; 30 min, 1885 *g*, RT – Beckman Coulter X-15R centrifuge). Subsequently, these

Table 1
Retention times (t_R) and instrument settings. For each teicoplanin compound, z = 2.

	t_R (min)	m/z 1	m/z 2	m/z 3	m/z 4	m/z 5
Scan segment 1: 0.0–1.4 min (*m/z* 780–788)						
A3–1	0.83	*782.183*	782.685	783.183	783.683	784.184
Scan segment 2: 1.4–2.1 min (m/z 936–951)						
A2–1	1.72	*938.778*	939.280	939.779	940.280	940.780
A2–2 & A2–3	1.72	*939.786*	940.287	940.786	941.286	941.786
A2–4 & A2–5	1.87	946.793	947.295	947.794	*948.295*	948.794
Scan segment 3: 2.1–4.5 min (m/z 290–300)						
Internal standard	2.35	298.236	n.a.	n.a.	n.a.	n.a.

m/z: mass-to-charge ratio; total teicoplanin: only m/z in italic; free teicoplanin: scan segments and m/z 1 to 5; n.a.: not applicable.

calibrator levels in ultrafiltrate were filtered under the same conditions in order to account for NSA to the UF membrane.

For each calibrator level, aliquots of 50 µL were transferred into 1.5 mL Eppendorf tubes (Hamburg, Germany) and stored at −80 °C until analysis.

2.2.2. Quality control samples

In house quality control (QC) samples (low, mid and high) were prepared in a pool of blank plasma (total teicoplanin) or in blank plasma ultrafiltrate (free teicoplanin). For total teicoplanin, theoretical concentrations were 7 mg/L (QCL_T), 70 mg/L (QCM_T) and 140 mg/L (QCH_T) whereas for free teicoplanin, theoretical concentrations were 0.93 mg/L (QCL_F), 5.6 mg/L (QCM_F) and 12.6 mg/L (QCH_F).

2.3. Sample collection

Blood samples from healthy subjects (*n* = 6) and infected patients (*n* = 41) to whom teicoplanin was administered were collected in K_2EDTA and lithium heparin plasma tubes as well as in serum separating tubes (SST) (all Becton Dickinson Company, New Jersey, USA). Subsequently, blood was centrifuged for 10 min at 2095 *g* at room temperature (RT) (Beckman Coulter X-15R centrifuge - VWR, Leuven, Belgium) and the obtained plasma and serum were stored at −80 °C. The method described here was developed and validated using K_2EDTA plasma only. Lithium heparin plasma and serum were only used to study the effect of blood tube type on the quantification of teicoplanin, for method comparison and to determine the %PB of teicoplanin in patient samples.

This study was conducted according to the Declarations of Helsinki, was approved by the Ethics Committee of Ghent University Hospital (2017/0162 for healthy volunteers and 2015/004 for patients) and all participants gave their written informed consent.

2.4. Sample preparation

The internal standard trimipramine-D_3 (50 µL, 0.5 mg/L in acetone) was added to 50 µL calibrator, quality control, patient plasma or ultrafiltrate (obtained by Centrifree filters, 30 min, 1885 *g*, 37 °C – Beckman Coulter X-15R centrifuge) followed by thorough mixing. Subsequently, 200 µL of a cold (4 °C) solution containing methanol and acetonitrile (50/50 *v*/v %) was added to precipitate the proteins and the samples were mixed again. After cooling down (30 min, −20 °C), each sample was mixed, placed in a Thermomixer for 5 min at 1400 rpm and 10 °C (Eppendorf, Hamburg, Germany). Finally, the samples were centrifuged (5 min, 16,162 *g*, RT – Beckman Coulter Microfuge 16) and 100 µL supernatant was transferred into an autosampler vial and placed in the autosampler at 10 °C.

2.5. UHPLC – HRMS analysis

The chromatographic system consisted of a Dionex Thermo Scientific UltiMate 3000 system (Waltham, Massachusetts, USA) equipped with a binary pump and autosampler with thermostat, which was set at 10 °C. The injection volume was 1 µL and 2 µL for total and free teicoplanin, respectively. Chromatographic separation was performed at 40 °C using an Accucore phenylhexyl column (100 × 2.1 mm, 2.6 µm particle size) (Thermo Scientific, Waltham, Massachusetts, USA). A linear gradient elution was used at 0.4 mL/min and started at 75% of mobile phase A (2 mM ammonium formate and 0.1% formic acid in water) and 25% of mobile phase B (2 mM ammonium formate, 0.1% formic acid, and 1% water in 50/50 *v*/v% methanol/acetonitrile). Subsequently, the composition of the mobile phase was changed to 80% B during the first 2.5 min and was maintained for one minute. Finally, a composition change to 90% B was achieved in 0.25 min and maintained for 0.75 min, followed by re-equilibration of the column at 75% A during 0.5 min. The total run time was 4.5 min.

Analytes were detected using a Q-Exactive Hybrid quadrupole orbitrap mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). Heated electrospray ionization was used in positive mode at a sheath gas (N_2) flow of 45 (arbitrary units, a.u.), a temperature of 300 °C and a spray voltage of 3.5 kV. Auxiliary gas (N_2) flow and temperature were 15 a.u. and 350 °C, respectively. Full scan analysis (*m/z* 200–2000) was applied for total teicoplanin whereas three scan segments were introduced (Table 1) for free teicoplanin to increase sensitivity. Automatic gain control (AGC) targets were $1\ 10^6$ and $2\ 10^5$ for total and free teicoplanin, respectively. For both teicoplanin analyses, the resolution was set at 70,000. Possible shifts in the mass spectrum were corrected by lock mass at m/z 391.284. Teicoplanin compounds A2–2 & A2–3 and teicoplanin compounds A2–4 & A2–5 are both pairs of isomers and were therefore detected simultaneously, whereas compounds A2–1 and A3–1 were detected separately, as summarized in Table 1. Teicoplanin compounds A2–1 to 5 and A3–1 were summed up for calibration as well as for unknowns. Minor compounds (RS1–4) were not included. Data acquisition and processing were performed using TraceFinder (Thermo Scientific, Waltham, Massachusetts, USA) software, version 3.3.

2.6. Assay validation

Both total and free teicoplanin assays were validated according to the European Medicines Agency (EMA) guidelines for selectivity, carry-over, limit of quantification (LOQ), linearity, accuracy (within-run and between-run), precision (within-run and between-run), matrix effect, storage and freeze/thaw stability.

Selectivity for both teicoplanin and internal standard was demonstrated by using 6 individual blank K_2EDTA plasma samples and QC samples, respectively. For both selectivity validations, 50 µL internal standard was replaced by 50 µL ultrapure water. In addition, selectivity was tested in patient samples collected in different types of blood tubes (i.e. K_2EDTA, lithium heparin and SST serum). Absence of interfering compounds was accepted when the response was < 20% of LOQ for teicoplanin and < 5% for the internal standard.

For carry-over effects, six individual blank plasma samples were injected after the highest calibrator. Carry-over was assumed to be negligible when the response was < 20% of LOQ for teicoplanin and < 5% for the internal standard.

The LOQ was considered to be the lowest calibration standard and was run 3 times. The LOQ was accepted if the response was at least 5 times the signal of a blank sample.

Calibration curves were evaluated when prepared in blank plasma and blank plasma ultrafiltrate for total (eight levels) and free (seven levels) teicoplanin, respectively (*n* = 5). A separate calibration curve was constructed for each teicoplanin compound (i.e. teicoplanin A3–1, A2–1, A2–2 & A2–3 and A2–4 & A2–5) and data shown here are the sum of these compounds. Results were expressed as coefficients of variation (CV%) and the bias (%) to the nominal value was calculated. The calibration curves were accepted when the back calculated concentrations of the calibrator levels were within ± 15% of the nominal value, except for the LOQ for which ± 20% was accepted. The reproducibility was accepted when ≤15% for all levels and ≤20% for the LOQ.

Precision and accuracy were assessed using *in house* made QC samples for both total and free teicoplanin. For within-run precision and accuracy, $LOQ_{T\&F}$, $QCL_{T\&F}$, $QCM_{T\&F}$ and $QCH_{T\&F}$ were prepared and analyzed in one batch. Between-run precision and accuracy were determined by preparing $LOQ_{T\&F}$, $QCL_{T\&F}$, $QCM_{T\&F}$ and $QCH_{T\&F}$ after 1, 4, 7 and 14 days storage at −80 °C. Also here, results were expressed as CV% and the bias (%) to the nominal value was calculated. The assays were assumed to be precise and accurate when the CV% and bias were ≤15% for $QCL_{T\&F}$, $QCM_{T\&F}$ and $QCH_{T\&F}$ and ≤20% for $LOQ_{T\&F}$ and within ± 15% for $QCL_{T\&F}$, $QCM_{T\&F}$ and $QCH_{T\&F}$ and ± 20% for $LOQ_{T\&F}$, respectively.

Matrix effects were investigated using six blank plasma samples from healthy volunteers. For both total and free teicoplanin, plasma samples and ultrapure water were prepared for analysis but after extraction, 180 µL supernatant was spiked with 20 µL of low and high concentration calibrator stock solution. Matrix effects were evaluated by calculating the matrix factor (MF), which is the ratio of the peak area of teicoplanin or internal standard measured in plasma to the peak area of teicoplanin (MF_{teico}) or internal standard (MF_{IS}) measured in water. Matrix effects were assumed to be negligible when MF_{teico}/MF_{IS} was within ± 15% for both low and high concentration sets. For calculations, the mean of the obtained peak areas was used.

Storage and freeze/thaw stability was studied using QCL_T, QCM_T and QCH_T samples. For storage stability, these QC samples were stored at 4 °C for 1, 4, 7 and 14 days and the obtained total and free concentrations were compared to the same samples which were stored at −80 °C. Freeze/thaw stability was evaluated by comparing obtained total and free concentrations of QCL_T, QCM_T and QCH_T after three freeze/thaw cycles (at −80 °C) with those which were stored at −80 °C. Stability was assumed when total and free concentrations were within ± 15% of the concentrations found for QC samples which were stored at −80 °C.

2.7. Equilibrium dialysis versus ultrafiltration

Free fractions of spiked healthy plasma were obtained by both ED and UF. For ED, an HTDialysis 96b system (HTDialysis, Connecticut, USA) was used with membranes of regenerated cellulose (molecular weight cut-off of 12–14 kDa). These membranes were hydrated and used according to the manufacturer's guidelines. Dialysis was performed against blank plasma ultrafiltrate during 24 h at 37 °C (equilibration time was determined in a pilot experiment, data not shown). An adhesive film was used to seal the ED wells to avoid water losses through evaporation and shifts in pH.

Two different UF devices were checked for NSA, i.e. Amicon Ultra – 0.5 mL Centrifugal filters and Centrifree Ultrafiltration filters (both Merck, Darmstadt, Germany). Amicon filters were spun at 4520 *g* during 20 min at RT (based on [32]) whereas Centrifree filters were spun at 1885 *g* during 30 min at RT or 37 °C (Beckman Coulter X-15R), as previously described to obtain free vancomycin [33].

Non-specific adsorption of teicoplanin to both the ED device and membrane and to both UF membranes was checked by determining the recovery after ED or UF at RT, using calibrator level 6 for free teicoplanin quantification (i.e. 14 mg/L) which was prepared in blank plasma ultrafiltrate (all $n = 3$).

Free (C_F) teicoplanin concentrations in spiked plasma samples obtained by ED (37 °C) and UF (RT and 37 °C, Centrifree filters only) were compared. Additionally, the %PB was calculated from the measured total (C_T) and C_F concentrations: $\%PB = [1 - (C_F/C_T)] \times 100\%$. For this experiment, blank plasma samples from 6 healthy volunteers were individually spiked with teicoplanin to reach final concentrations of either 7 (low), 70 (mid) or 140 (high) mg/L, with each level in duplicate.

2.8. %PB in patient samples

%PB of teicoplanin was also determined in anonymized left-over plasma (K_2EDTA and lithium heparin) and serum samples ($n = 41$) from patients who received teicoplanin, which were collected in the core laboratory of Ghent University Hospital.

2.9. Effect of blood collection tubes: spiked plasma samples

Plasma (K_2EDTA and lithium heparin) and serum (SST) from healthy volunteers (all $n = 6$) were collected to study the possible effect of different blood tube types on total and free teicoplanin concentrations as well as on the %PB. These plasma and serum samples were spiked after thawing with either a low (final concentration: 11.2 mg/L) or high (final concentration: 56 mg/L) concentration of teicoplanin. Subsequently, these samples were incubated for 30 min at RT prior to sample preparation. Concentrations were determined using a calibration curve in a pool of blank K_2EDTA plasma (total teicoplanin) or blank K_2EDTA plasma ultrafiltrate (free teicoplanin) only.

2.10. Method comparison: patient and spiked samples

For method comparison, the same left-over plasma (K_2EDTA and lithium heparin) and serum samples ($n = 41$) from patients who received teicoplanin were used. K_2EDTA plasma samples ($n = 7$) from healthy subjects which were spiked with teicoplanin were included as well. Each sample was stored at −80 °C immediately after collection until batch analysis was performed. Total teicoplanin concentrations were assayed by a validated homogeneous particle-enhanced turbidimetric (i.e. quantitative microsphere system, QMS® teicoplanin) immunoassay [25,28] on an Architect c4000 instrument (Abbott, IL, USA) as well as by the UHPLC-HRMS method described here. Plasma and serum samples were quantified using a calibration curve prepared in the corresponding matrix. Subsequently, the obtained concentrations were compared by Passing-Bablok regression and a Bland-Altman plot, using Medcalc software (Medcalc BVBA, Ostend, Belgium).

3. Results

3.1. Chromatography

The described UHPLC-HRMS method was used to separate teicoplanin compounds A3–1, A2–1, A2–2 & A2–3 (pair of isomers) and A2–4 & A2–5 (pair of isomers) for the quantification of total and free teicoplanin. For both assays (i.e. total and free), the same separation procedure was used and the total run times were 4.5 min. The retention time of each teicoplanin compound is provided in Table 1. For total teicoplanin assay, compounds were detected (*m/z* values in italic; Table 1) in full scan whereas for free teicoplanin, three scan segments were introduced and 5 mass isotopes (*m/z* 1 to 5 in Table 1) were monitored to enhance sensitivity. A representative chromatogram for each compound is shown in Fig. 2.

3.2. Assay validation

3.2.1. Selectivity

No detectable interferences were found in the individual blank EDTA plasma samples for teicoplanin. The QC samples showed no interference with the internal standard and in both types of analyses, a small peak was detected at the m/z ratio of the internal standard but its response was < 5%. However, in a number of patient samples which were collected in different blood tubes, an interfering peak for A2–4 &

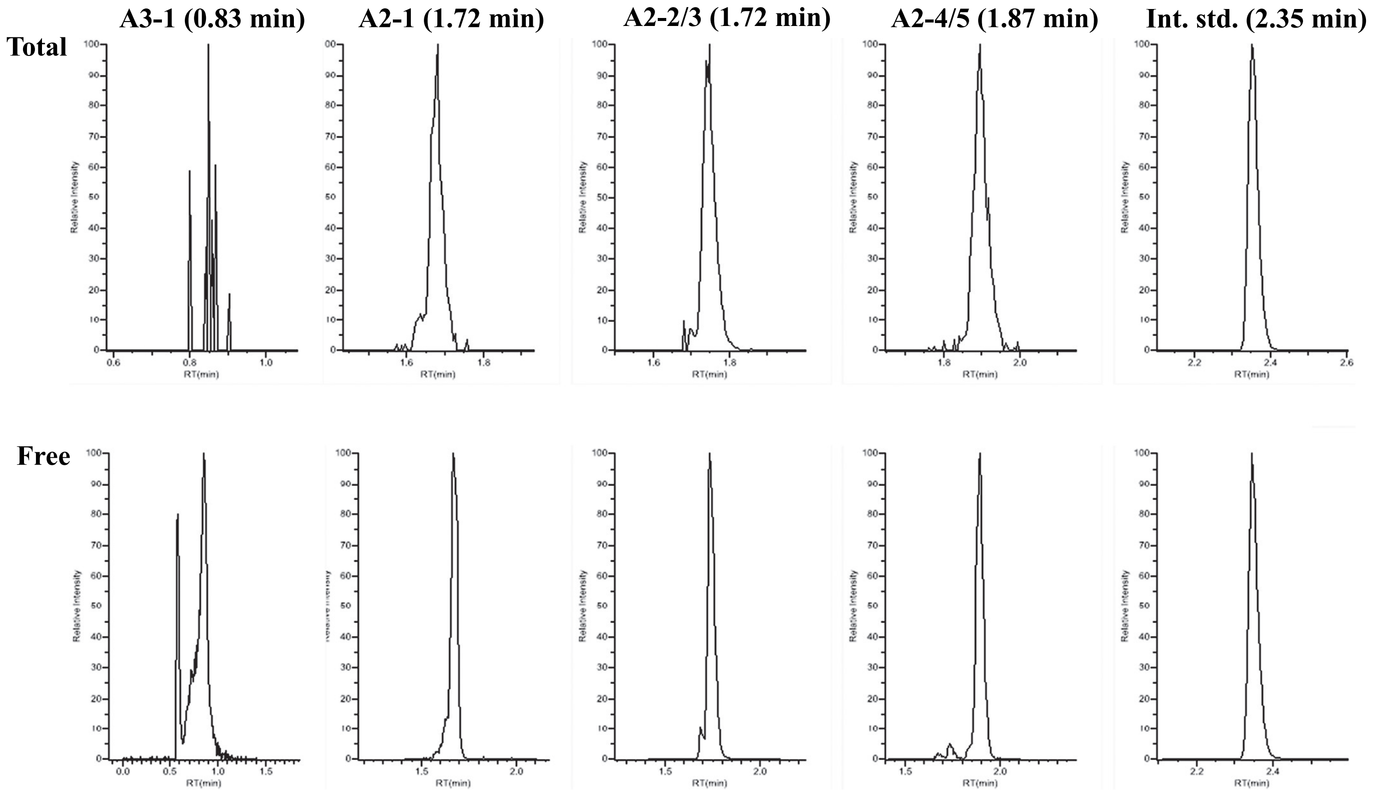

Fig. 2. Representative chromatograms obtained from the highest calibrator standard for total and free teicoplanin compounds (A3–1, A2–1, A2–2 & A2–3 and A2–4 & A2–5), as well as for the internal standard (int. std.).

A2–5 detection was observed at m/z = 946.793. Therefore, we selected m/z = 948.295 for interference-free A2–4 & A2–5 detection (Table 1). Thus, the method was selective for all teicoplanin compounds and the internal standard at these settings.

3.2.2. *Carry-over*

No detectable peaks were found in individual blank plasma samples which were analyzed after the highest calibrator standard.

3.2.3. *LOQ*

No peaks were detected in blank samples and the LOQ was 1.4 and 0.3 mg/L for total and free teicoplanin, respectively.

3.2.4. *Calibration curves*

Appropriate calibration regressions and weightings were selected based on a recently described mathematical model [34,35]. For total teicoplanin, a quadratic curve was used with weighting factor x^{-2} ($R^2 = 0.99 \pm 0.004$; $n = 5$), whereas for free teicoplanin, a linear curve was used with weighting factor x^{-2} ($R^2 = 0.99 \pm 0.008$; $n = 5$). Table 2 summarizes the precision of the back calculated concentrations and the accuracy to their corresponding nominal value. Except for a high bias for calibrator standard 3 of free teicoplanin (15.4%), all other calibrator standards had a precision < 15% and a bias within ± 15% of the nominal value. Therefore, the calibration curves were accepted.

3.2.5. *Precision and accuracy*

Data on both within-run and between-run precision and accuracy are tabulated in Table 3. The within-run precision was 5.2% and 4.4% at LOQ level and ranged from 6.3 to 12.9% and from 3.1 to 6.6% at QC levels, for total and free teicoplanin, respectively. The between-run precision was 1.7 and 15.4% at LOQ level and ranged from 1.8 to 7.6% and from 4.8 to 11.5% at QC levels, for total and free teicoplanin, respectively. Within-run accuracy values ranged from −10.4 to 9.6%. Between-run accuracy ranged from −10.5 to 11.7%.

Table 2
Calibration curve information.

	Theoretical value (mg/L)	Calculated value (mg/L)	Precision (CV %)	Accuracy (% Bias)
Total teicoplanin				
Cal 1	1.4	1.4 ± 0.1	5.2	2.1
Cal 2	2.8	2.7 ± 0.2	7.2	−4.4
Cal 3	7.0	6.9 ± 0.9	13.5	−1.1
Cal 4	28.0	30.6 ± 2.5	8.2	9.3
Cal 5	70.0	63.2 ± 6.2	9.9	−9.7
Cal 6	105.0	108.1 ± 10.3	9.6	3.0
Cal 7	140.0	140.4 ± 14.8	10.5	0.3
Free teicoplanin				
Cal 1	0.3	0.3 ± 0.01	4.4	−4.9
Cal 2	1.4	1.6 ± 0.2	12.5	13.0
Cal 3	2.8	2.4 ± 0.2	7.2	−15.4
Cal 4	7.0	7.4 ± 0.3	4.0	6.3
Cal 5	10.5	10.1 ± 0.8	7.6	3.7
Cal 6	14.0	14.1 ± 0.8	5.5	1.0

Calculated values are expressed as mean ± standard deviation; CV%: coefficient of variation; $n = 5$ for both total and free teicoplanin.

3.2.6. *Matrix effects*

Peak areas of total and free teicoplanin as obtained in spiked extracts of blank plasma, blank plasma ultrafiltrate and ultrapure water with a low and a high concentration are provided in Table 4, together with peak areas of the internal standard. Except for low total teicoplanin, MF_{Teico}/MF_{IS} was within ± 15% for each concentration set.

3.2.7. *Storage and freeze/thaw stability*

QC samples were stable over a period of 14 consecutive days when stored at −80 °C (all below 10%, data not shown). QC samples proved

Table 3
Assay precision and accuracy.

	Theoretical value (mg/L)	Within-run			Between-run		
		Calculated value (mg/L)	Precision (CV%)	Accuracy (%Bias)	Calculated value (mg/L)	Precision (CV%)	Accuracy (%Bias)
Total teicoplanin							
$LLOQ_T$	1.4	1.4 ± 0.1	5.2	2.1	1.4 ± 0.02	1.7	2.0
QCL_T	7.0	7.3 ± 0.9	12.9	3.9	7.7 ± 0.1	1.8	10.5
QCM_T	70.0	76.7 ± 4.8	6.3	9.6	78.2 ± 2.3	2.9	11.7
QCH_T	140.0	132.5 ± 9.0	6.8	−5.4	139.7 ± 10.6	7.6	−0.2
Free teicoplanin							
$LLOQ_F$	0.3	0.3 ± 0.01	4.4	−4.9	0.3 ± 0.04	15.4	−10.5
QCL_F	0.9	0.9 ± 0.04	4.1	−3.6	0.9 ± 0.04	4.8	−3.7
QCM_F	5.6	5.0 ± 0.2	3.1	−10.4	5.0 ± 0.3	5.3	−9.9
QCH_F	12.6	11.9 ± 0.8	6.6	−5.7	12.3 ± 1.4	11.5	−2.4

Calculated values are expressed as mean ± standard deviation; CV%: coefficient of variation; $n = 5$ (within-run) or $n = 4$ (between-run: after 1, 4, 7 and 14 days) for both total and free teicoplanin.

to be stable when stored at 4 °C as well, when compared to those which were stored at −80 °C, for both total and free teicoplanin (all within ± 15%). In addition, three freeze/thaw cycles of each QC sample had no effect on the stability of teicoplanin (all within ± 15%).

3.3. Equilibrium dialysis versus ultrafiltration

Teicoplanin recovery was 112.3 ± 12.1% in ED analyses, and 23.1 ± 2.7% and 79.5 ± 8.0% in UF analyses using Amicon and Centrifree filters, respectively. Total (C_T) and free (C_F) teicoplanin concentrations as obtained by ED (37 °C) and UF (RT and 37 °C, Centrifree filters only) in individually spiked plasma samples were used to calculate the %PB of teicoplanin in each sample (Table 5). The overall mean value of C_T, C_F and %PB is also given in Table 5. The overall mean %PB was 93.6 ± 0.4%, 94.1 ± 1.3% and 95.8 ± 1.1% as obtained by ED (37 °C), UF (37 °C) and UF (RT), respectively.

3.4. %PB in patient samples

The mean total and free teicoplanin concentrations as determined in 41 patient samples were 23.9 mg/L (range 6.5 to 53.3 mg/L) and 2.7 mg/L (range 0.2 to 6.7 mg/L), respectively. The mean %PB of teicoplanin as determined in 41 patients was 87.7% and ranged from 79.6 to 95.4%.

3.5. Effect of blood collection tubes: spiked plasma samples

Total and free teicoplanin concentrations as determined in spiked plasma (K_2EDTA and lithium heparin) and serum (SST) samples from healthy donors are summarized in Table 6. For both low and high concentration sets in K_2EDTA plasma, total teicoplanin concentrations corresponded to their nominal values. Because of the limited number of repetitions ($n = 3$), values were not statistically compared, but a trend of higher total concentrations when determined in lithium heparin plasma and in serum samples was observed as compared to those in K_2EDTA plasma. This is due to a higher peak area of teicoplanin and not to a lower peak area of the internal standard (data not shown). However, no clear difference was found in free teicoplanin concentration amongst the different matrices. Consequently, a small increase in %PB was calculated in lithium heparin and serum samples as compared to in K_2EDTA samples.

3.6. Method comparison: patient and spiked samples

Total teicoplanin concentrations determined by QMS® teicoplanin analysis and the UHPLC-HRMS method described here were compared by Passing-Bablok regression and a Bland-Altman plot, provided in Fig. 3 and Fig. 4 respectively. For Passing-Bablok regression, the correlation coefficient between both methods was 0.82 (95% confidence interval: 0.70–0.90). Total teicoplanin concentrations were slightly higher when measured with the newly developed UHPLC-HRMS method as compared to the QMS® method. Bland-Altman analysis revealed a mean bias of 3.2% between both methods and therefore, the here described UHPLC-HRMS method was accepted.

4. Discussion

A novel UHPLC-HRMS method for the quantification of total and free teicoplanin in K_2EDTA plasma samples was developed and validated according to EMA guidelines. The here described method requires only 200 μL of plasma to quantify both the total and free teicoplanin concentration, which is much lower as compared to methods described

Table 4
Peak areas of teicoplanin (Teico) and internal standard (Int. std.) as measured in plasma and water, to study the matrix effect.

	Plasma	Water	MF	MF_{Teico}/MF_{IS}
Total teicoplanin				
Teico (L)	3,926,253 ± 497,008	3,651,793 ± 317,795	1.08	1.20
Int. std. (L)	64,898,049 ± 3,951,204	72,712,426 ± 6,139,386	0.89	
Teico (H)	70,762,594 ± 12,554,319	8,336,444 ± 8,181,394	1.03	1.13
Int. std. (H)	66,193,315 ± 3,720,803	72,488,916 ± 2,533,207	0.91	
Free teicoplanin				
Teico (L)	7,453,429 ± 936,331	7,424,363 ± 1,197,619	1.00	1.02
Int. std. (L)	123,306,400 ± 73,059,824	125,210,396 ± 10,686,508	0.98	
Teico (H)	89,200,239 ± 5,338,651	104,190,165 ± 24,525,044	0.86	0.92
Int. std. (H)	124,503,213 ± 12,100,533	133,167,832 ± 43,036,386	0.93	

Peak areas are expressed as mean ± standard deviation; L: low concentration; H: high concentration; all $n = 6$.
MF: matrix factor.

Table 5

Total (C_T) and free (C_F) teicoplanin concentrations and the corresponding percentage protein binding (%PB) as obtained by equilibrium dialysis at 37 °C and ultrafiltration at 37 °C and room temperature (RT) in blank plasma samples, which were spiked with a low, mid or high concentration of teicoplanin.

	C_T (mg/L)	C_F (mg/L)			%PB (%)		
		ED (37 °C)	UF (37 °C)	UF (RT)	ED (37 °C)	UF (37 °C)	UF (RT)
Low	**7**[a]						
Sample 1	7.5	0.5	0.5	0.4	93.1	93.0	94.4
Sample 2	6.7	0.4	0.5	0.4	93.9	92.2	94.4
Mid	**70**[a]						
Sample 3	76.5	4.7	4.3	3.0	93.9	94.3	96.1
Sample 4	73.7	5.1	3.3	2.6	94.1	95.6	96.5
High	**140**[a]						
Sample 5	124.8	8.6	6.8	4.3	93.1	94.6	96.6
Sample 6	149.7	9.8	7.3	4.5	93.5	95.1	97.0
Overall	**73.2 ± 58.8**	**4.9 ± 3.9**	**3.8 ± 2.9**	**2.5 ± 1.8**	**93.6 ± 0.4**	**94.1 ± 1.3**	**95.8 ± 1.1**

[a] Theoretical values.

Table 6

Effect of blood tube type on the total (C_T) and free (C_F) teicoplanin concentration and the corresponding percentage protein binding (%PB).

	C_T (mg/L)	C_F (mg/L)	%PB (%)
Low	**11.2**[a]		
K_2EDTA plasma	11.1 ± 1.9	1.0 ± 0.1	91.3 ± 0.6
Lithium heparin plasma	15.6 ± 1.4	1.2 ± 0.1	92.3 ± 0.5
SST serum	19.1 ± 2.7	1.1 ± 0.1	94.2 ± 1.1
High	**56.0**[a]		
K_2EDTA plasma	54.1 ± 5.4	4.9 ± 1.4	90.7 ± 3.6
Lithium heparin plasma	66.8 ± 9.7	4.7 ± 0.3	92.9 ± 1.3
SST serum	77.8 ± 10.5	4.3 ± 0.9	94.4 ± 0.7

[a] Theoretical values; calculated values are expressed as mean ± standard deviation; n = 3 for both low and high concentrations of teicoplanin.

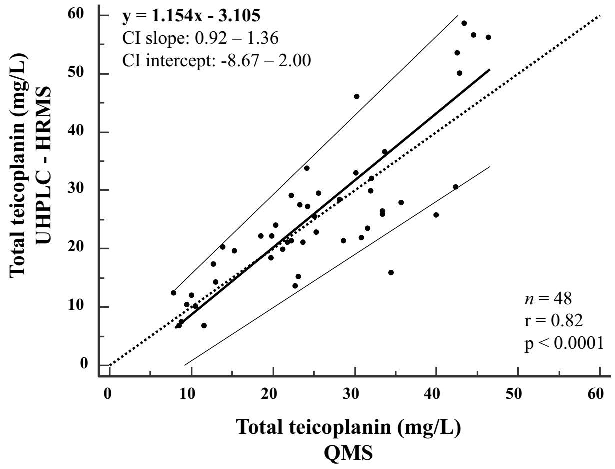

Fig. 3. Passing-Bablok regression for method comparison of total teicoplanin between the QMS® teicoplanin assay and the newly developed UHPLC-HRMS method.

elsewhere (700 µL in [2] and > 1 mL in [13]), and covers the complete therapeutic window of teicoplanin: ranging from 1.4–140 and 0.3–14 mg/L for total and free teicoplanin, respectively. In literature, only a limited number of papers discussed the analysis of both total and free teicoplanin [2,13,23]. To the best of our knowledge, only one paper described the analysis of total teicoplanin (i.e. A2–1, A2–2 & A2–3 and A2–4 & A2–5) using the same HRMS instrument [28]. However, in our method, four teicoplanin compounds (i.e. A3–1, A2–1, A2–2 & A2–3 and A2–4 & A2–5) were separated by UHPLC resulting in a total run time of only 4.5 min, which is half the time as presented by Mueller et al. where HPLC was used [28].

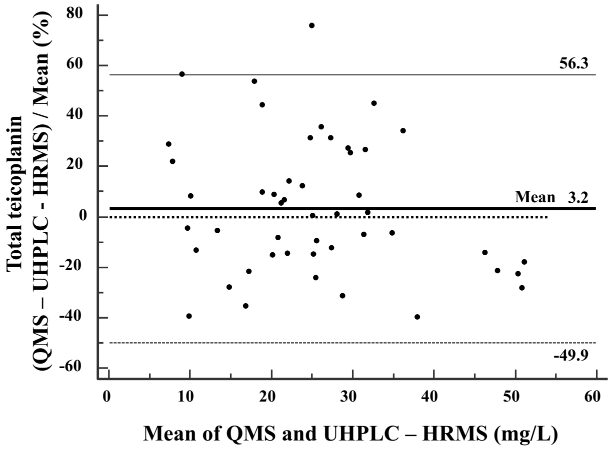

Fig. 4. Bland-Altman plot for method comparison of total teicoplanin between the QMS® teicoplanin assay and the newly developed UHPLC-HRMS method.

This method was selective for both teicoplanin and the internal standard in K_2EDTA samples. The precision and accuracy of the total and free assay were within the requirements of the EMA guidelines, for both within-run and between-run analyses. *In house* prepared QC samples were all stable over a period of 14 consecutive days and three freeze/thaw cycles.

A signal increase of teicoplanin was observed in spiked lithium heparin samples and in spiked serum samples as compared to spiked K_2EDTA plasma samples. This effect could possibly be explained by a difference in ionization efficiency of the teicoplanin compounds between the different tested sample types, as previously found for other drug substances [36,37]. In a number of patient samples, an unknown interfering compound was present for A2–4 & A2–5 detection at *m/z* = 946.793. Therefore, we selected a less abundant isotope at m/z = 948.295 for interference-free A2–4 & A2–5 detection. In this study, we were not able to elucidate the origin of this interfering compound and to the best of our knowledge, this is the first time that the presence of an interfering compound for teicoplanin A2–4 & A2–5 is reported. Therefore, we can only formulate a number of hypotheses which should be studied in future research, including the presence of concomitantly administered drugs, heparins as anticoagulant in lithium heparin blood tubes or compounds migrating from the separator gel in serum blood tubes (probably others than those used here in spiking experiments), as previously reported for other compounds [38,39].

It should also be mentioned that no deuterated teicoplanin was used as internal standard. Consequently, there was no compound-specific correction for sample loss during sample preparation, sample injection or sample ionization. However, the method described here showed an acceptable precision and the obtained results were accurate. Therefore, the usage of expensive deuterated teicoplanin seemed to be unnecessary in our method, as well as in other mass spectrometry based teicoplanin assays [26–30].

Free teicoplanin concentrations were obtained by ultrafiltration at 37 °C and were close to the values obtained by equilibrium dialysis, which was also performed at 37 °C to mimic the human body's temperature. Ultrafiltration at room temperature should be avoided since this leads to a small decrease (not statistically tested because $n = 3$) in free teicoplanin concentrations, resulting in a slightly higher %PB. Furthermore, ultrafiltration should not be carried out at 4 °C because of the apparent higher %PB of teicoplanin [13]. The use of Amicon filters is not recommended either because of their low recovery for teicoplanin. Calibrator levels for free teicoplanin calibration were also ultrafiltered after dilutions in ultrafiltrate to correct for the NSA to the Centrifree filters, which was around 20%. The %PB (90–95%) reported here for teicoplanin in spiked plasma samples was in good agreement with in vivo values reported in the literature where ultrafiltration was performed at 37 °C, but where no evidence for the accuracy of the obtained free fractions (e.g. by comparison with ED) was presented [2,3]. In addition, the %PB of teicoplanin was found constant in the studied physiological concentration range, i.e. from 7 to 140 mg/L. The in vivo %PB values as obtained in patient samples was 87.7% (range: 79.6–95.4%), pointing out the large inter-patient variability, which was also reported in [2,3].

In routine analyses, the QMS® teicoplanin assay is often used for quantification of total teicoplanin because of its short turn-around time as no sample preparation is required. This QMS® teicoplanin assay may, however, suffer from non-specific interferences since it is hypothesized that the used antibodies can also interact with compounds other than teicoplanin [28]. Therefore, the major advantages of the present method, as compared to the QMS® teicoplanin assay, are its higher selectivity and sensitivity [40]. The correlation ($r = 0.82$) between the newly developed UHPLC-HRMS method and the QMS® teicoplanin assay was moderate, but in line with the correlation reported by Mueller et al. who used the same MS instrument, but only for total teicoplanin analysis purposes [28]. Not only K_2EDTA plasma samples, but also serum en lithium heparin samples were used for method comparison. Hence, our UHPLC-HRMS method was developed and validated using K_2EDTA plasma samples, but is also applicable for analysis of serum samples and even lithium heparin samples, but then, calibration curves should be constructed in the corresponding matrix.

This new UHPLC-HRMS method can be useful in TDM. Although there is no consensus on the therapeutic value for free teicoplanin so far, it would be of additional value since the free fraction is biologically active and thus of therapeutic relevance. Nowadays, this free fraction is often estimated by a general percentage of around 10%, despite the large inter-patient variability as demonstrated in this paper and in [2,3]. With our method, the target attainment of free teicoplanin can be determined in a more reliable way as compared to the estimation of 10%.

5. Conclusion

In conclusion, a novel highly sensitive and fast UHPLC-HRMS method was developed and validated according to EMA guidelines for the quantification of total and free teicoplanin in human K_2EDTA plasma samples. Furthermore, our method is applicable for interference-free total and free teicoplanin analysis in serum and lithium heparin samples. Amongst others, this method can be useful in therapeutic drug monitoring, especially when knowledge of the free teicoplanin concentration is important.

Author contributions

OD and TM researched data. OD, TM and AGV discussed the results. OD wrote the manuscript, SE and AGV reviewed the manuscript.

Author disclosure

The authors have approved the final version and declare no conflict of interest.

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