Journal of Chromatography B, 879 (2011) 2632–2641

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Dalbavancin: Quantification in human plasma and urine by a new improved high performance liquid chromatography–tandem mass spectrometry method

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ARTICLE INFO

Article history:
Received 27 April 2011
Accepted 20 July 2011
Available online 26 July 2011

Keywords:
Dalbavancin HPLC–MS/MS in plasma
Non-specific binding
Pediatric pharmacokinetic sampling
Urine sample collection
Validation
ISR

ABSTRACT

Dalbavancin is a novel second-generation lipoglycopeptide antibiotic with activity against broad range of Gram-positive pathogens. In order to determine the pharmacokinetics (PK) of dalbavancin in pediatric patients, a new High Performance Liquid Chromatography–Tandem Mass Spectrometry (HPLC–MS/MS) bioanalytical method has been developed for quantification of dalbavancin in plasma and in urine. The plasma method was validated for dalbavancin in the linear range from 0.5 μg/mL to 500 μg/mL using 50 μL of K_2 EDTA plasma. For dalbavancin spiked in urine, non-specific binding (NSB) of the drug to polypropylene (PP) urine collection containers was observed. The loss amounted to about 10% per transfer. After successfully establishing the collection/sampling procedure for urine by addition of Triton X-100 to the collection vessels (with a purpose of preventing NSB), the method was validated for dalbavancin in the range from 0.05 μg/mL to 50 μg/mL, using 100 μL of urine. These methods were used to quantify dalbavancin in plasma and urine of hospitalized children in a pediatric dalbavancin PK study. Eighteen percent of the total number of plasma study samples was reassayed for incurred samples reproducibility (ISR) and all the reassayed dalbavancin concentrations were within the ±20% limits. For urine, all the collected samples were reassayed for ISR and the original dalbavancin concentration was confirmed within the ±20% limits for 17 (94%) samples; the one remaining urine sample had its reassayed concentration confirmed within ±25% of the original result.

1. Introduction

Dalbavancin is a novel, semi synthetic lipoglycopeptide antibiotic developed for parenteral use [1]. It has been studied for the treatment of complicated skin and skin structure infections (cSSSI) in adults and shown to be effective for the treatment of cSSSI caused by Gram-positive organisms such as *Staphylococcus aureus* (including MRSA) [2,3]. Dalbavancin has both renal and non-renal routes of elimination, with about one third of the administered dose excreted through kidneys, most of it as the intact drug. A minor metabolite, hydroxyl-dalbavancin (OH-D) has been found in human urine [4].

Following a request for a bioanalytical method that minimizes the volume of blood to be collected in a typical pediatric PK study with sick infants and toddlers, a sensitive bioanalytical method for determination of dalbavancin in plasma was developed. For the youngest pediatric population 0.5 mL of blood for each collection time point would suffice and an intravenous or capillary blood collection system (like Microvette 500 from Sarstedt or similar) could be used. For older children and adults or when infrequent sampling is required, 1–2 mL of blood per time point is drawn into standard commercial collection tubes.

Although the preferred collection of urine is by direct voiding, it was necessary to develop a sample collection procedure that would include catheterized children and/or children requiring baby urinal bags. Adsorption of dalbavancin to the plastic urine collection containers was impacting the integrity of the PK urine samples; therefore this issue had to be resolved prior to the study start.

Two previously published methods used to quantify dalbavancin [5,6] did not meet our requirements regarding the reduced blood sample volume and lower limit of quantitation (LLOQ).

2. Experimental

2.1. Chemicals and materials

Dalbavancin, OH-D and internal standard (IS, a diethyl amide homologue of dalbavancin) were received from Pfizer Inc. (Groton, CT). HPLC grade acetonitrile, methanol, acetone, and formic acid (min 98%) were purchased from EMD Chemicals (Gibbstown, NJ). Triton X-100 was purchased from Sigma–Aldrich (St. Louis, MO)

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doi:10.1016/j.jchromb.2011.07.027

and water was supplied in house from a Barnstead Diamond RO system, Thermo Fisher Scientific (Waltham, MA).

2.2. Instrumentation – LC–MS/MS

The HPLC system used for the analysis of dalbavancin was a Shimadzu LC10Avp (Columbia, MD). Chromatographic separations were performed on Fortis, Phenyl C18, 2.1 mm × 50 mm, 5 μm analytical columns (Fortis Technologies, Neston, Cheshire, UK) at ambient temperature. Three mobile phases were used in the chromatography. Mobile phase [A] was 0.1% formic acid (aqueous), mobile phase [B] was acetonitrile:methanol (50:50, v:v), and mobile phase [C] was 100% acetone. The total run time was 3.50 min. The elution program for plasma was: initial flow of 400 μL/min with 55% [A]: 45% [B]: 0% [C], isocratic for 1.60 min, followed by a wash out step of 5% [A]: 95% [C] at flow of 0.600 μL/min to 2.50 min, and finally a re-equilibration step to initial conditions up to 3.50 min.

The elution program for urine was the same with the exception of the flow rate being 300 μL/min and the wash out step consisting of 5% [A]: 95% [B].

Analyses were performed on a Sciex API 4000 (Concord, ON, Canada) triple quadrupole mass spectrometer, operating in positive ion mode, using TurboIonspray interface and multiple reactions monitoring (MRM). Settings were: ion spray voltage 5000 V, gas was ultra-pure nitrogen with gas 1 at 65 psi, gas 2 at 65 psi, curtain gas at 15 psi, CAD setting at 8, temperature at 450 °C, declustering potential of 60 V, exit potential of 10 V, collision energy of 40 V for dalbavancin and 35 V for hydroxyl-dalbavancin and CXP at 13 V. MRM transitions monitored were for dalbavancin: m/z 909.2 → 340.1, for OH-D: m/z 917.2 → 338.2, and for IS: m/z 923.2 → 340. Analyst software version 1.2.4 was used to acquire data and Watson LIMS, Ver. 7.2 was used as the data processing and reporting software (Thermo Electron Corp., Bellefonte, PA).

2.3. Preparation of standards

2.3.1. Stock solutions

2.3.1.1. Dalbavancin stock solution. Duplicate dalbavancin stock solutions of 50.0 mg/mL were prepared by accurately weighing 50–100 mg of dalbavancin and dissolving in the required volume of acetonitrile:water (25:75, v:v). A comparison of the two stocks by LC–MS/MS resulted in a less than 3% difference between solutions. After agreement between the two stocks, one (Stock A) was used as a stock for calibrators spiking solutions and the other (Stock B) as a stock for quality control spiking solutions. The solutions were kept at −20 °C in a polypropylene (PP) container. Due to dalbavancin tendency to absorb water from the atmosphere, every time dalbavancin was weighed, a sample was taken to be tested for water content. Once the water content was determined, the purity of the weighings performed on that day had to be adjusted for water content.

2.3.1.2. Dalbavancin spiking solutions. A dalbavancin diluted solution at 10.0 mg/mL was prepared from Stock A for use in spiking plasma calibration standard samples, and at 1.00 mg/mL for use in spiking urine calibration standard samples. A dalbavancin diluted solution at 200 μg/mL and 20.0 μg/mL were prepared from Stock B for use in spiking quality control (QC) plasma samples. A dalbavancin diluted solution at 1.00 mg/mL and 10.0 μg/mL were prepared from Stock B for use in spiking quality control (QC) urine samples. Diluted solutions were prepared with acetonitrile:water (25:75, v:v) and were stored in PP containers at −20 °C.

2.3.1.3. Internal standard stock solution. Approximately 10 mg of Internal Standard (IS) was accurately weighed and dissolved in the required volume of acetonitrile:water (20:80, v:v) to give a 2.50 mg/mL stock solution. The solution was stored in a PP container at −20 °C.

2.3.1.4. Internal standard working solution (ISWS). ISWS was prepared at 25 μg/mL (for use with plasma samples) and at 1.00 μg/mL (for use with urine samples) by diluting the 2.50 mg/mL stock with acetonitrile:water (20:80, v:v). This solution was stored refrigerated at 1–8 °C in a PP container.

2.3.2. Plasma calibration standards and quality control (QC) samples

One mL of each of the calibration standards at 500 μg/mL, 450 μg/mL, 250 μg/mL, 50 μg/mL, 10 μg/mL, 5 μg/mL, 1 μg/mL and 0.5 μg/mL were prepared fresh daily in plasma. The 500 μg/mL and 450 μg/mL standards were prepared by directly spiking K_2EDTA control human plasma with 10 mg/mL dalbavancin spiking solution (prepared from Stock A). All remaining calibration standards were prepared by subsequent dilution of the higher standard with plasma.

Quality control samples (QCs) were prepared at six levels of dalbavancin in plasma: 0.50 μg/mL, 1.50 μg/mL, 20.0 μg/mL, 200 μg/mL and 400 μg/mL. The sixth level, Dilution QC, was prepared at 2500 μg/mL. QC samples were prepared in human plasma and stored in cryogenic vials (∼0.300 mL into each vial) in a freezer set at −20 °C.

The total matrix (plasma) content in calibration standards and QC samples was kept above 95%.

2.3.3. Urine calibration standards and QC samples

Calibration standards at 50 μg/mL, 45 μg/mL, 10 μg/mL, and 5 μg/mL were prepared fresh daily in PP test tubes using dalbavancin spiking solution at 1 mg/mL (prepared from Stock A) into blank human urine containing 1% Triton X-100. The remaining four calibration standards (1 μg/mL, 0.500 μg/mL, 0.1 μg/mL and 0.05 μg/mL) were prepared by subsequent dilution of the higher standard with blank human urine containing 1% Triton X-100.

Six levels of dalbavancin QC samples in urine (at 0.050 μg/mL, 0.150 μg/mL, 2.00 μg/mL, 20.0 μg/mL and 40.0 μg/mL) were used. The Dilution QC for urine was prepared at 250 μg/mL. In order to prevent NSB of dalbavancin to the PP containers, blank urine was pretreated with Triton X-100 solution at 1% (volume of Triton X-100 to volume of blank urine being treated). Quality controls (QCs) are prepared in human urine with 1% Triton X-100 and stored in cryogenic vials (∼0.300 mL into each vial) in a freezer set at −20 °C.

2.4. Analytical procedure

2.4.1. Human plasma sample preparation

Calibration standards were prepared fresh daily in K_2EDTA blank plasma. Stored QC samples were thawed at room temperature. A 50 μL volume of a calibration standard, QC or incurred plasma sample was aliquoted to a 2-mL-well 96 well block (Nalg Nunc, Rochester, NY) to which 50 μL of ISWS (25 μg/mL) was added. Samples were then diluted with 1000 μL of water. A TomTec Quadra 3 liquid handling system (TomTec, Hamden, CT) was used to mix the samples, after which a 50 μL aliquot was aspirated to a new block. Next, 400 μL of acetonitrile:water (20:80, v:v) was added to all samples. The block was vortexed briefly for mixing and 10 μL was injected onto the HPLC system for analysis.

2.4.2. Human urine sample preparation

Calibrators were made fresh daily in human urine containing 1% Triton X-100 (v:v). Stored QC samples containing 1% Triton X-100 were thawed at room temperature. A 100 μL volume of a calibration standard, QC or incurred urine sample was aliquoted

to a 2-mL-well 96 well block to which 50 μL of ISWS (1.00 μg/mL) was added. Samples were then diluted with 1000 μL of acetonitrile:water (25:75, v:v). A TomTec Quadra 3 liquid handling system (TomTec, Hamden, CT) was used to mix the samples after which a 100 μL aliquot was aspirated to a new block. Further, 500 μL of acetonitrile:water (25:75, v:v) was added to all samples. The block was vortexed briefly for mixing and 10 μL was injected onto the HPLC system for analysis.

2.5. Validation

The above described procedures for determination of dalbavancin in human plasma and urine were validated according to the CDER Bioanalytical Guidance [7] with respect to linearity, limits of quantification, intra- and inter-assay precision and accuracy, carryover, selectivity and stability.

All stocks, solutions, spiking solutions and ISWS, as well as the frozen matrix QC samples, were used within their confirmed stability.

Accuracy and precision was determined over three analytical runs at $n = 6$ for all QC levels. The run design was to assay QC samples in repeating ($n = 1$) profile order bracketed by two calibration curves. Blanks were evaluated for carryover after the highest calibrators.

The method selectivity was evaluated in six different lots of human plasma and urine respectively, at the LLOQ level of dalbavancin. The precision and accuracy of the assay, stability in the matrix at room temperature, stability after 4 freeze and thaw cycles from −20 °C to room temperature, and long term stability in the matrix were evaluated. The potential interference of vancomycin and teicoplanin with the dalbavancin human plasma assay was also tested.

2.6. Collection procedure for study samples

2.6.1. Collection of plasma samples

Blood, 2 mL, to allow for at least 0.7 mL of plasma, was collected via a heparin/saline lock or indwelling venous cannula from the arm contra lateral to the infusion site.

All samples were collected by syringe and immediately transferred into tubes containing K_2EDTA as an anticoagulant. To harvest plasma, blood specimens were centrifuged at 1000–1200 × *g* (~3000 rpm) at 4 °C for 10 min. After centrifugation, the upper plasma layer was transferred with a disposable pipette to a PP vial. The vials with plasma were frozen (−20 °C) within 1 h of blood collection and kept in a frozen state until assayed.

Plasma samples for PK assessment were taken at the specified time points starting with 0 h (prior to the start of the infusion) and followed by 12 others including one during the infusion and following up to 8 weeks post-start-of-infusion.

2.6.2. Collection of urine samples

Samples from 24-h urine collection were taken to further assess dalbavancin. Prior to the dalbavancin infusion, one aliquot (approximately 10 mL) of urine was collected and retained as a pre-dose sample.

24-h urine collections were performed on study days 1 and 2. The urine was collected by direct voiding into 500 mL PP beakers. Immediately after voiding, the urine was transferred into the 3 L PP container and kept refrigerated (2–8 °C). One beaker was used per patient per collection day. At the end of each urine collection period, the total volume was measured and recorded. To counteract any NSB and recover dalbavancin from the PP container, 1% (v:v) of Triton X-100 was added to the urine, formulated as a Triton X-100:acetonitrile (50:50, v:v) solution. First, the beaker used to collect urine was rinsed by vigorous swirling of the measured Triton X-100:acetonitrile solution (divided in two approximately equal volumes) and added to the collected urine. The urine was then mixed thoroughly and a 10 mL aliquot was withdrawn for the measurement of drug concentrations. The sample was frozen (−20 °C) and kept in a frozen state until assayed.

3. Results and discussion

3.1. Method development

Quantitative determination of dalbavancin in human plasma and urine poses many challenges. They are associated with the tendency of dalbavancin to adhere to the proteins in plasma and adsorption/NSB of dalbavancin in urine to the collection containers. OH-D was also monitored in urine to assure that specificity of dalbavancin determination was not compromised by this minor metabolite.

3.1.1. Human plasma

The initial method development approach was to use a protein precipitation extraction procedure. This approach was abandoned because of relatively low and variable recovery due to dalbavancin absorption to precipitated proteins. Since the required LLOQ was 0.5 μg/mL, a simple sample dilution was used. A two step process was employed: the first step was addition of 1000 μL of water to (combined) 50 μL of plasma and 50 μL of ISWS (in acetonitrile:water, 20:80, v:v), resulting in a 22-fold dilution. In second step, a 50 μL aliquot of the preceding dilution was diluted further with 400 μL of acetonitrile:water (20:80, v:v). The organic solvent content of the second dilution was necessary to prevent adsorption of dalbavancin to the PP containers, yet it was still low enough to prevent precipitation.

3.1.2. Human urine

Diluting the samples into analytical range was the initial approach to develop an extraction procedure for dalbavancin in human urine. However, this did not address the adsorption of dalbavancin to the PP containers and had to be resolved.

The study was conducted in hospitalized children. Expecting that some children may be catheterized and that toddlers in potential future studies may need to have urinal bags attached in order to collect the 24-h urine, we set out to develop an urine collection protocol that would not impair the analyte's concentration due to NSB of dalbavancin to the wall of the collection container. Experiments were designed to evaluate the loss of dalbavancin due to NSB urine collection bags and sample transfer containers.

Based on those experiments, urine and urine container treatments were developed and sample integrity assured. Such an experiment, designed to evaluate adsorption of dalbavancin to the PP containers used for sample collection, storage and sample processing, can be observed from Table 1. Two pools of human urine spiked with dalbavancin at 0.200 μg/mL and at 20.0 μg/mL were prepared. Each pool was divided into 3 new pools; one was placed into a urine catheter bag, the other was placed into a pediatric urinal bag, and the third was left with no exposure to plastic of the urine collection device. After 7 h at room temperature, mimicking the average time between the transfers of collected urine from the catheter bag into the refrigerated 3 L PP container, triplicate urine samples were taken from each of the pools and analyzed for dalbavancin. The quantitative results for dalbavancin subjected to the urine collection devices when compared to a control pool treated the same way minus the exposure to the collection bag, showed a loss of approximately 25–35% at the 0.200 μg/mL concentration level, and a loss of approximately 5–10% at the 20.0 μg/mL concentration level. The observed loss is concentration dependant, being larger at lower concentrations (Table 1). No significant difference

Table 1
Adsorption of dalbavancin to urine collection devices. Calculation of loss from QC pool = [(PAR of sample mean − PAR of QC pool mean)/PAR of QC pool mean] × 100. PAR is peak area ratio.

QC1 0.200 μg/mL	QC1 pool (no bag exposure)	QC1 – in urine bag at room temp. for 7 h	QC1 – in pediatric urinal bag at room temp. for 7 h
Replicate #		Peak area ratio	
Mean (n = 3)	0.101	0.0709	0.0670
Loss from QC1 pool [%]	0.0	−29.9	−33.8
QC4 20.0 μg/mL	QC4 pool (no bag exposure)	QC4 – in urine bag at room temp. for 7 h	QC4 – in pediatric urinal bag at room temp. for 7 h
Replicate #		Peak area ratio	
Mean (n = 3)	12.2	11.0	11.5
Loss from QC4 pool [%]	0.0	−10.4	−6.0

was observed between the two collection devices, pediatric versus larger catheter bag.

Triton X-100 was tested and chosen as an additive at 1% (v:v) in urine to counteract NSB of dalbavancin to PP containers. Recovery of dalbavancin from the walls of PP urine containers was independent of the order in which the additive and the urine were placed in the container. Table 2 summarizes the results for QC1 and QC 4 adsorption to PP containers. Without 1% Triton added to the sample adsorption losses were greater than 80% and 60% respectively after 5 transfers of the urine sample to new containers. Losses were ∼15% and 10% per transfer for QC1 and QC4, respectively.

To prevent NSB, the blank urine used for preparation of calibration standards and QC samples was pretreated with Triton X-100 solution at 1% (volume of Triton X-100 to volume of blank urine being treated). Positive displacement pipettes were used to transfer this viscous liquid. The recovery of dalbavancin following addition of 1% and 2% Triton X-100:acetonitrile solution was shown to be equivalent. The final Triton X-100 concentration in urine was 1%, same for calibrators and incurred samples.

A batch of QC samples spiked into urine treated with 2% of Triton X-100:acetonitrile (50:50, v:v) was compared with a similar batch spiked into urine pretreated with 1% Triton X-100 only, and the equivalence was confirmed.

In the clinic, the human urine samples from the two 24-h collection periods were pretreated with 2% (v:v of urine) of Triton X-100:acetonitrile solution (50:50, v:v). Diluting Triton X-100 with acetonitrile reduced its viscosity and made it easier to apply in the clinical setting.

3.1.3. Chromatography

An isocratic elution was applied to limit column carry over. A wash out step was used in both plasma and urine methods. In the plasma assay, acetone was used to remove phospholipids, while the highly retained endogenous compounds in the urine method were washed out with acetonitrile:methanol (50:50, v:v).

In the plasma assay, dalbavancin and IS were eluting at approximately 0.87 min and 0.96 min, respectively. The chromatography for urine was developed to accommodate monitoring OH-D in addition to dalbavancin. The retention times for dalbavancin, OH-D and IS in the urine assay were 1.2 min, 0.6 min, and 1.3 min, respectively.

3.1.4. MS transitions

For all three compounds, the transitions monitored were doubly charged species; e.g. the m/z 909 represents the doubly charged protonated dalbavancin species, MW 1816/2 = 908 + 1 = 909. The following MRM transitions were monitored: m/z 909.2 → 340.1 for dalbavancin, m/z 917.2 → 338.2 for OH-D, and m/z 923.2 → 340 for IS.

3.2. Validation in human plasma and human urine

Under the above described conditions, a linear, 1/concentration squared weighted, least squares regression algorithm, was used to construct the calibration curve. All concentrations were then calculated from their PARs (peak area ratio of dalbavancin to the internal standard) against the calibration line. The validated range was 0.5–500 μg/mL in human plasma and 0.05–50 μg/mL in human urine.

Average back-calculated values (n = 6) from each of the 8 levels of the calibration curve in the 3 plasma validation runs had a bias (% difference from theoretical) from −2.2% to 2.0% and precision (%CV) ≤5.0%.

Five levels of validation QC samples including LLOQ QC were analyzed at n = 6 in each run. The overall assay accuracy and precision in human plasma were −6.7% to −2.0%, and ≤8.9%, respectively (Table 3). The intra-run assay accuracy and precision (Table 3) for the 3 validation runs were, respectively: −3.6% to 3.9%, and ≤12.4%

Table 2
Adsorption of dalbavancin to polypropylene containers with and without Triton X-100. Calculation of loss from QC pool = [(PAR of sample mean − PAR of QC pool mean)/PAR of QC pool mean] × 100. PAR is peak area ratio.

Sample ID	Number of transfers	Peak area ratio	
		Mean (n = 2)	% Loss from 0 X
QC 1 with 1% Triton	0×	0.340	0.0
	5×	0.356	4.7
QC 1 no Triton added	0×	0.251	0.0
	5×	0.0466	−81.4
QC 4 with 1% Triton	0×	14.2	0.0
	5×	14.1	−0.7
QC 4 no Triton added	0×	12.0	0.0
	5×	4.64	−61.2

Table 3
Summary of the dalbavancin validation samples in human plasma.

Dalbavancin concentration in human plasma	Run	LLOQ QC 0.505 μg/mL	QC1 1.52 μg/mL	QC2 20.2 μg/mL	QC3 202 μg/mL	QC4 405 μg/mL	QC5 2530 μg/mL DF = 10[a]
	1						
Intrarun mean		0.487	1.58	20.3	198	394	2470
Intrarun SD		0.0209	0.196	1.10	2.73	3.13	38.3
Intrarun %CV		4.3	12.4	5.4	1.4	0.8	1.6
Intrarun %Bias		−3.6	3.9	0.5	−2.0	−2.7	−2.4
n		6	6	6	6	6	6
	2						
Intrarun mean		0.457	1.45	19.4	192	385	
Intrarun SD		0.0315	0.0441	0.462	3.35	7.82	
Intrarun %CV		6.9	3.0	2.4	1.7	2.0	
Intrarun %Bias		−9.5	−4.6	−4.0	−5.0	−4.9	
n		6	6	6	6	6	
	3						
Intrarun mean		0.470	1.44	19.3	193	393	
Intrarun SD		0.0321	0.0677	0.405	3.58	6.74	
Intrarun %CV		6.8	4.7	2.1	1.9	1.7	
Intrarun %Bias		−6.9	−5.3	−4.5	−4.5	−3.0	
n		6	6	6	6	6	
Mean concentration found (μg/mL)		0.471	1.49	19.7	194	391	2470
Inter-run SD		0.0296	0.132	0.816	3.96	7.16	38.3
Inter-run %CV		6.3	8.9	4.1	2.0	1.8	1.6
Inter-run %Bias		−6.7	−2.0	−2.5	−4.0	−3.5	−2.4
n		18	18	18	18	18	6

[a] Diluted 10-fold with control human plasma.

(Run 1), −9.5% to −4.0%, and ≤6.9% (Run 2), and −6.9% to −3.0%, and ≤6.8% (Run 3).

The analyte proved to be stable in K_2 EDTA human plasma after 4 freeze and thaw cycles from −20 °C to room temperature and for 24 h bench top storage at room temperature. Long term stability for dalbavancin in human plasma at −20 °C was established for 256 days. A representative chromatogram of a dalbavancin LLOQ standard in human plasma is given in Fig. 1.

Average back-calculated values ($n = 6$) from each of the 8 levels of the calibration curve in the 3 urine validation runs had a bias

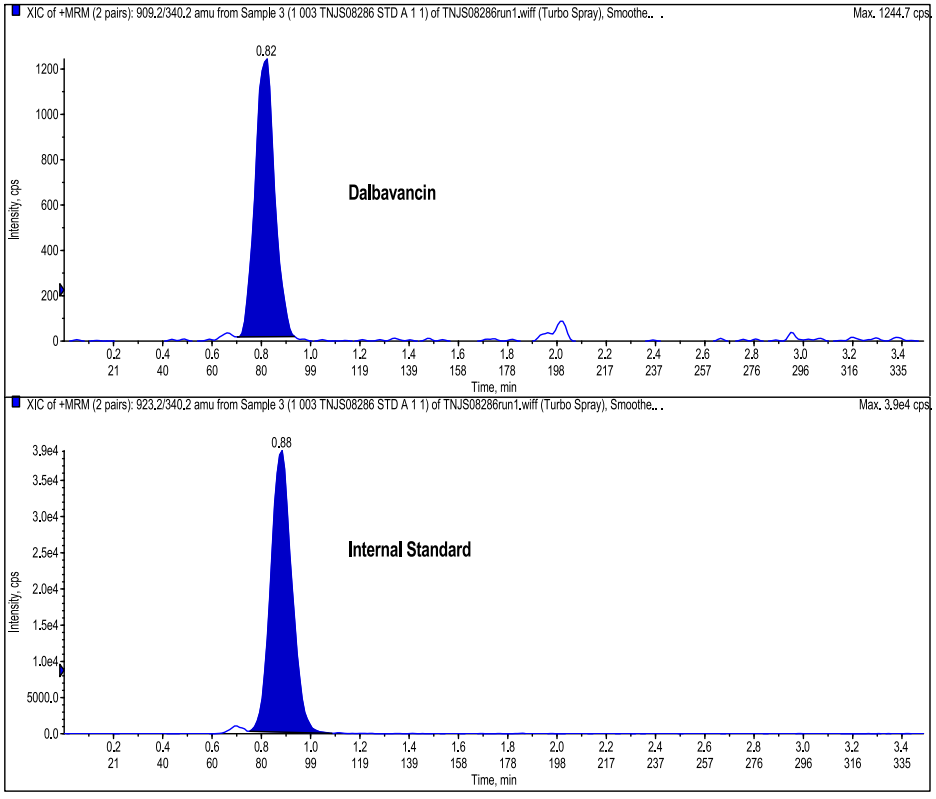

Fig. 1. Dalbavancin LLOQ calibration standard (0.5 μg/mL) in human plasma; *x*-axis = time [min], *y*-axis = signal intensity [cps].

Table 4
Summary of the dalbavancin validation samples in human urine.

Dalbavancin concentration in human urine	Run	LLOQ QC 0.0500 μg/mL	QC1 0.150 μg/mL	QC2 2.00 μg/mL	QC3 20.0 μg/mL	QC4 40.0 μg/mL	QC5 250 μg/mL DF = 10[a]
	1						
Intrarun mean		0.0466	0.150	2.02	20.0	36.4	227
Intrarun SD		0.00192	0.00387	0.0280	0.308	0.934	7.66
Intrarun %CV		4.1	2.6	1.4	1.5	2.6	3.4
Intrarun %Bias		−6.8	0.0	1.0	0.0	−9.0	−9.2
n		6	6	6	6	6	6
	2						
Intrarun mean		0.0440	0.147	2.00	19.3	35.5	
Intrarun SD		0.00270	0.00740	0.0488	0.564	0.422	
Intrarun %CV		6.1	5.0	2.4	2.9	1.2	
Intrarun %Bias		−12.0	−2.0	0.0	−3.5	−11.3	
n		6	6	6	6	6	
	3						
Intrarun mean		0.0427	0.140	1.97	19.6	36.1	
Intrarun SD		0.00160	0.00631	0.0513	0.489	1.17	
Intrarun %CV		3.7	4.5	2.6	2.5	3.2	
Intrarun %Bias		−14.6	−6.7	−1.5	−2.0	−9.8	
n		6	6	6	6	6	
Mean concentration found (μg/mL)		0.0444	0.145	1.99	19.6	36.0	227
Inter-run SD		0.00262	0.00715	0.0464	0.542	0.930	7.66
Inter-run %CV		5.9	4.9	2.3	2.8	2.6	3.4
Inter-run %Bias		−11.2	−3.3	−0.5	−2.0	−10.0	−9.2
n		18	18	18	18	18	6

[a] Diluted 10-fold with control human urine.

(% difference from theoretical) from −7.6% to 5.6%, and precision (%CV) ≤3.8%.

Five levels of validation QC samples including LLOQ QC were analyzed at $n = 6$ in each run. The overall assay accuracy and precision in human urine containing Triton X-100 were −11.2% to −0.5%, and ≤5.9%, respectively (Table 4). The intra-run assay accuracy and precision (Table 4) for the 3 validation runs were, respectively: −9.2% to 1.0%, and ≤4.1 (Run 1), −12.0% to 0.0%, and ≤6.1 (Run 2), and −14.6% to −1.5%, and ≤4.5 (Run 3).

Dalbavancin proved to be stable in treated human urine after 4 freeze and thaw cycles from −20 °C to room temperature and for 24 h bench top storage at room temperature. Long term stability for dalbavancin in human urine at −20 °C was established for 204 days. An example of a chromatogram of an LLOQ standard in human urine is given in Fig. 2.

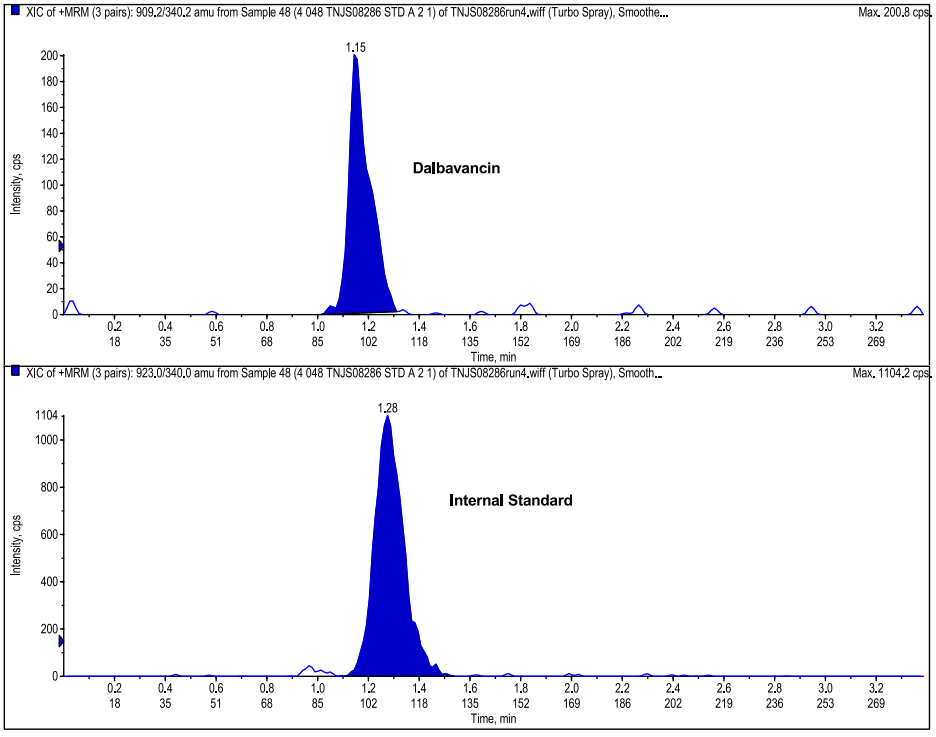

Fig. 2. Dalbavancin LLOQ calibration standard (0.05 μg/mL) in human urine; *x*-axis = time [min], *y*-axis = signal intensity [cps].

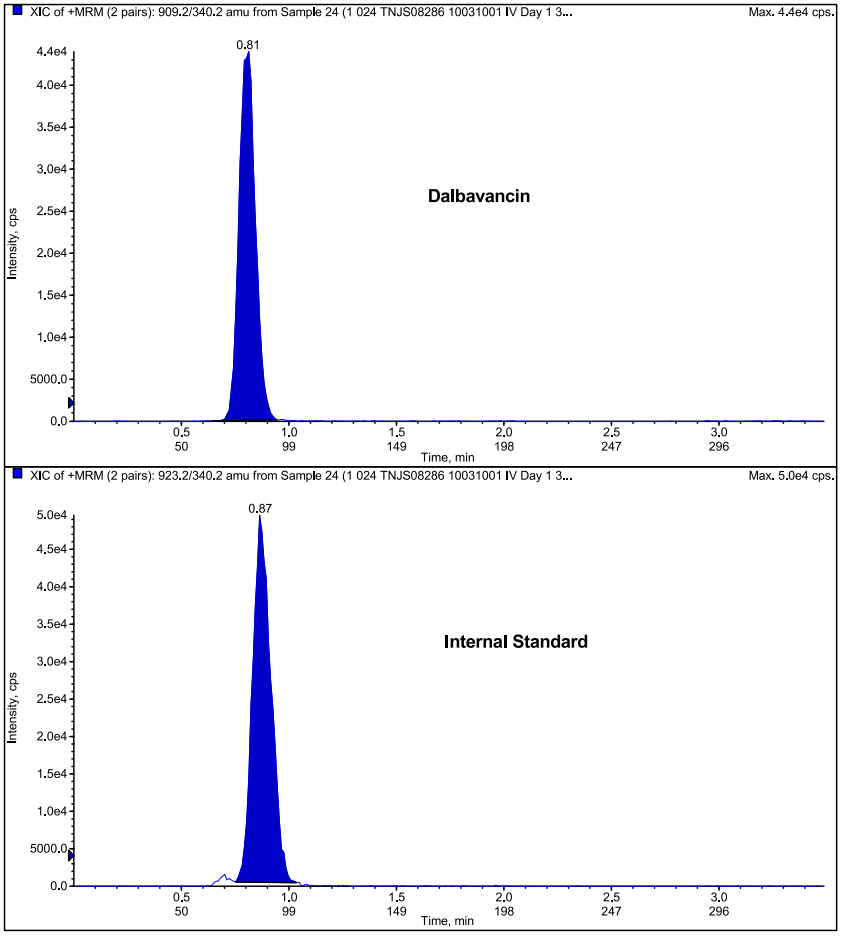

Fig. 3. Chromatogram of an incurred plasma sample, containing 14.0 μg/mL dalbavancin; *x*-axis = time [min], *y*-axis = signal intensity [cps].

The calibration curve range and placement of QCs spanned and adequately reflected the dalbavancin concentrations of the study samples. Examples of typical chromatograms pertaining to an incurred dalbavancin plasma sample and an incurred dalbavancin urine sample are shown in Figs. 3 and 4, respectively. Representative blank plasma and urine samples can be viewed in Figs. 5 and 6, respectively.

The dalbavancin carryover in plasma validation runs ranged from 17.4% to 48.4% of the lowest LLOQ sample. All double blank samples used to assess carryover were followed by double blank samples with peaks <20% of the lowest dalbavancin response. To mitigate carryover during sample analysis, additional double blanks are to be included in each run, and samples analyzed in PK order.

In urine validation runs dalbavancin carryover ranged from 0.0% to 26.1% of the lowest LLOQ response. All double blank samples used to assess carryover were followed by double blank samples with no peaks at the retention time of dalbavancin.

The internal standard used for this assay was an analog of dalbavancin, therefore it was important to assess the potential matrix effect on the ionization caused by plasma and urine from different individuals. The matrix effects were evaluated by analysis of six different lots of human plasma and urine, respectively, spiked at the LLOQ level for dalbavancin. The accuracy for each

Table 5
Lot to lot matrix accuracy of dalbavancin in human plasma and urine. Plasma and urine were fortified with dalbavancin at the LLOQ level.

Dalbavancin theor. conc. in plasma (μg/mL)	0.505		Dalbavancin theor. conc. in urine (μg/mL)	0.0500	
Plasma lot #	Found conc.	%Deviation	Urine lot #	Found conc.	%Deviation
#1	0.499	−1.2	#1	0.0432	−13.6
#2	0.477	−5.5	#2	0.0479	−4.2
#3	0.493	−2.4	#3	0.0482	−3.6
#4	0.529	4.8	#4	0.0478	−4.4
#5	0.496	−1.8	#5	0.0456	−8.8
#6	0.516	2.2	#6	0.0471	−5.8
Mean (μg/mL)	0.502			0.0466	
SD	0.0183			0.00192	
%CV	3.6			4.1	
%Theoretical	99.4			93.2	
n	6			6	

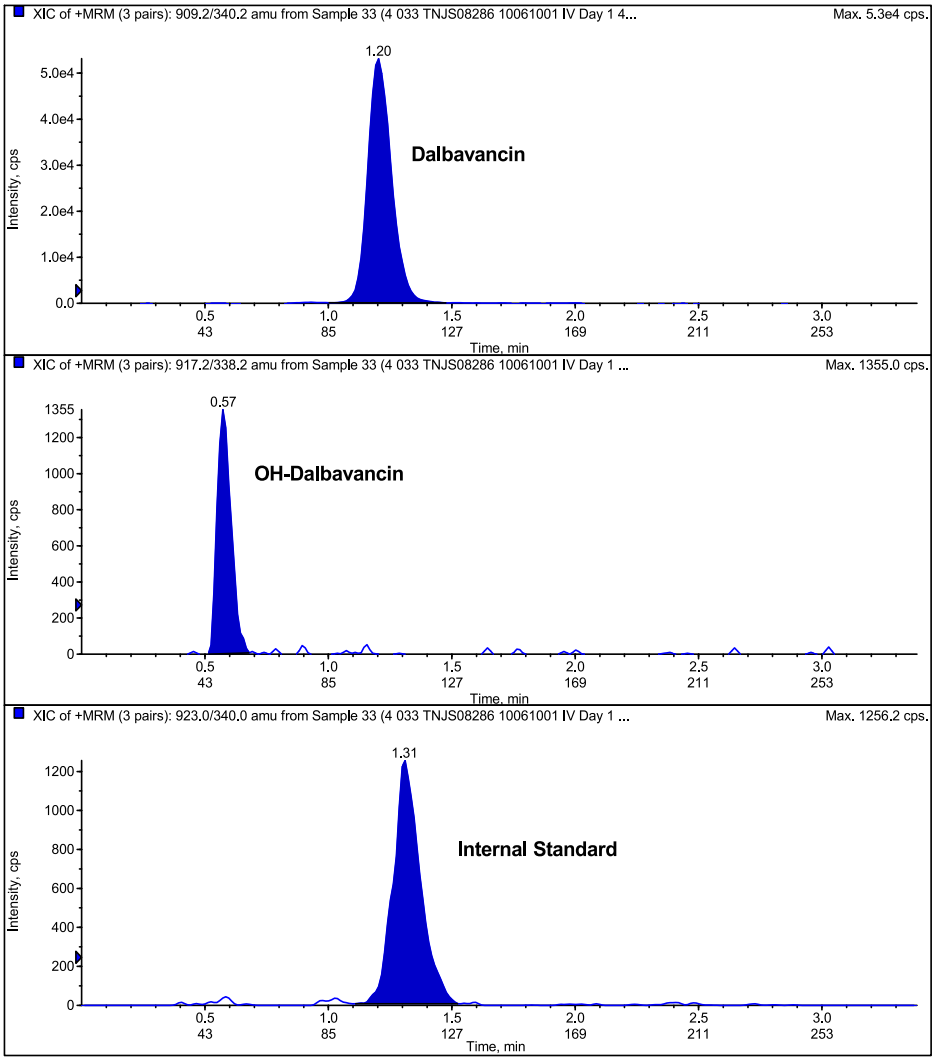

Fig. 4. Chromatogram of an incurred urine sample, containing 12.9 μg/mL dalbavancin; *x*-axis = time [min], *y*-axis = signal intensity [cps].

lot was determined to be less than 6% deviation from theoretical for plasma and less than 14% deviation from theoretical for urine (Table 5).

In addition, the dalbavancin plasma method allows for the determination of dalbavancin in the presence of co-administered drugs teicoplanin and vancomycin with no effect on the precision and accuracy of the assay (Table 6). Teicoplanin and vancomycin are usually the first choice drugs given to patients with the disease dalbavancin is indicated for. It is very likely that the patients undergoing dalbavancin therapy were previously exposed to either of the two, therefore accuracy and precision was determined with these compounds present.

3.3. Incurred sample reproducibility

Incurred sample reproducibility (ISR) analysis was conducted for dalbavancin for both plasma and urine samples. Of the 130 analyzed plasma samples, 24 (18%) were reassayed for ISR. Samples were chosen over the whole range of concentrations, from Cmax to the terminal elimination phase in the PK profile. All 24 samples confirmed their original concentration within the ±20% limits. For urine, all 18 samples collected on day 1 and day 2 were reassayed for ISR. The original dalbavancin concentration was confirmed within the ±20% limits for 17 (94%) samples; the one remaining urine sample had its reassayed concentration confirmed within ±25% of the

Table 6
Concomitant medication interference assessment (vancomycin and teicoplanin) in dalbavancin human plasma assay. Human plasma was fortified with dalbavancin at the low (QC1) and high (QC4) levels. These dalbavancin plasma samples were also fortified with teicoplanin and vancomycin at the approximate concentrations of two times the average maximum concentration found in humans.

Dalbavancin concentration (μg/mL)	QC1 1.52	QC4 400	QC1 1.50	QC4 400
Interference concentration	Vancomycin 80 μg/mL	Vancomycin 100 μg/mL	Teicoplanin 100 μg/mL	Teicoplanin 100 μg/mL
Mean dalbavancin concentration found (μg/mL)	1.42	403	1.50	402
SD	0.0403	5.91	0.0325	15.9
%CV	2.8	1.5	2.2	4.0
%Bias	−6.6	0.8	0.0	0.5
n	6	6	6	6

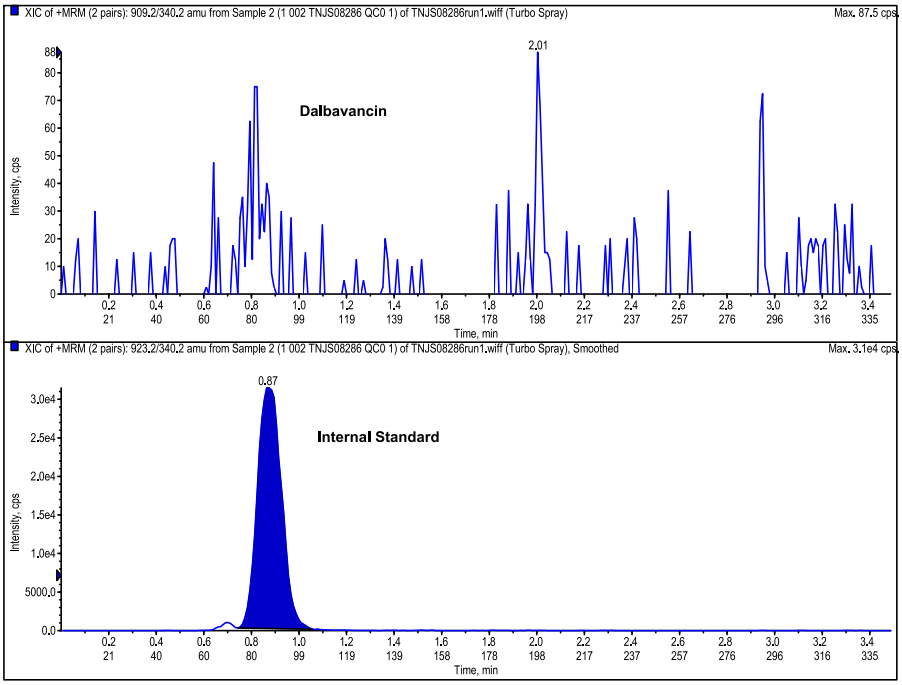

Fig. 5. Chromatogram of a blank plasma sample containing Internal Standard; *x*-axis = time [min], *y*-axis = signal intensity [cps].

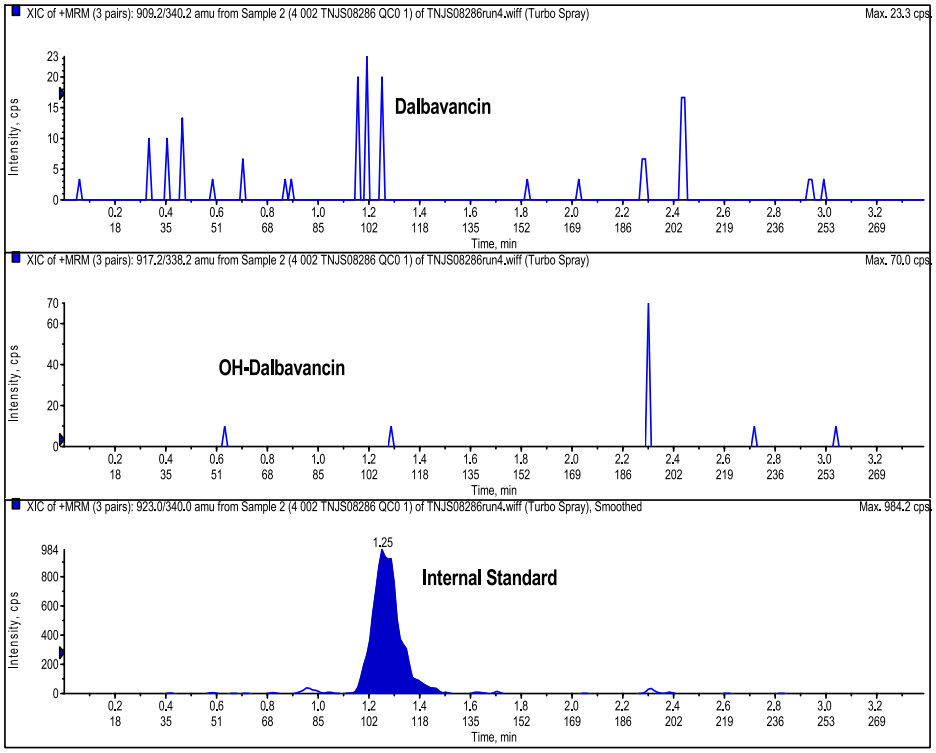

Fig. 6. Chromatogram of a blank urine sample containing Internal Standard; *x*-axis = time [min], *y*-axis = signal intensity [cps].

original result. The ISR met acceptance recommendation on ISR for incurred samples from Crystal City III Workshop [8].

4. Conclusions

A new, very sensitive, reliable and rugged LC–MS/MS method was developed for quantification of dalbavancin in human plasma and urine. The low sample volume (50 μL of plasma and 100 μL of urine) is suitable for pediatric studies. The method was successfully validated and used to analyze dalbavancin pharmacokinetic plasma and urine samples from hospitalized children.

Acknowledgements

The bioanalytical assay described in this manuscript was developed in collaboration with Tandem Labs and validated by Tandem Labs. This work was funded by Pfizer Inc. Roger Demers and Laura Cojocaru are full-time employees of Tandem Labs. Tanja Alebic-Kolbah is a full-time employee of Pfizer. The authors would like to thank Vivek Purohit from Clinical Pharmacology, Pfizer, for helpful discussions during procedure development for PK sample collection and study conduct. Barbara Duncan is acknowledged for help in reviewing and editing of the manuscript.

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