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Development and validation of a LC–MS quantification method for the lantibiotic MU1140 in rat plasma

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ABSTRACT

This study reports the first ever development and validation of a quantification method for a lantibiotic in plasma. This method was developed for the quantification of total MU1140 in Sprague Dawley rat plasma. The procedure involved acidification of plasma samples with formic acid followed by precipitation of plasma proteins using isopropanol, filtration, and analysis by RPLC–MS. The lantibiotic gallidermin was used as an internal standard (ISTD). The analyte and ISTD were eluted using a gradient of isopropanol and water, both acidified with 0.3% formic acid (v/v), at a flow rate of 250 μl/min. Positive electrospray ionization was utilized at the ion source and the analyte and ISTD were both detected by selected-ion monitoring (SIM). Total run time was 15 min. This method was validated for selectivity, sensitivity, linearity, recovery, accuracy, and precision. The method was shown to be selective, with a quantitative linear range of 0.39–100 μg/ml using 25 μl samples. The bias, intra- and inter-day percent relative standard deviation at all concentrations tested was lower than 15%. MU1140 mean extraction recovery was 96.1%. The analyte was shown to be stable to freeze/thaw and for short- and long-term storage. Extracted MU1140 was stable at 4 °C for over 5 days.

This method was successfully applied to a preliminary pharmacokinetic study of intravenously administered MU1140 in Sprague Dawley rats. Overall, this method was shown to be applicable for quantification of MU1140 in plasma samples for the purpose of further MU1140 ADME or bioequivalence studies.

1. Introduction

The excessive and improper use of antibiotics has lead to the selection and spread of bacterial strains resistant to many of the currently used antibiotics. The United States FDA noted that antibiotic resistance problems must be detected as they emerge, and actions taken to contain them, or else the world could be faced with previously treatable diseases that have again become untreatable, as in the days before antibiotics were developed [1]. This sharp increase in bacterial antibiotic resistance can be contained by the development and commercialization of new classes of antimicrobials [2].

The lantibiotics is a class of antimicrobials that is gaining much attention due to their activity against drug resistant pathogens. Lantibiotics are so named for their content of lanthionine residues [4], which are amino acids that are composed of two alanine residues linked by a thioether bridge through their β-carbons. Lantibiotics are produced by bacteria to protect themselves and their habitat from encroaching bacterial strains. Although the first lantibiotic was discovered in 1928 [6], and approximately 50 more have been identified subsequently, their development as pharmaceutical agents for treatment of infectious diseases has been hindered by the lack of cost effective production and/or purification [7].

MU1140 (Fig. 1A) is a lantibiotic bacteriocin produced by the microorganism *Streptococcus mutans* strain JH1140 [3]. It belongs to the family of antimicrobial peptides known as lantibiotics. MU1140 has been shown to exert its antimicrobial effect on Gram-positive bacteria by a novel mechanism involving lipid II abduction, in which aggregates of MU1140 bind to molecules of lipid II and translocate them from sites of active cell wall biosynthesis [5]. The result is inhibition of cell wall synthesis. In the case of MU1140, production of sufficient amounts of essentially pure product has been achieved in order to perform a number of pre-clinical tests [8]. These indicate the potential usefulness of MU1140 in the treatment of certain Gram-positive infections, including those caused by wild-type and drug resistant variants of *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis*.

A reliable bioanalytical method for the quantification of drugs is crucial for their development [9]. Determination of the drug's concentration is needed for many studies, among which is the assessment of the pharmacokinetic properties of the drug and the subsequent dose design, evaluation of stability, assessment of

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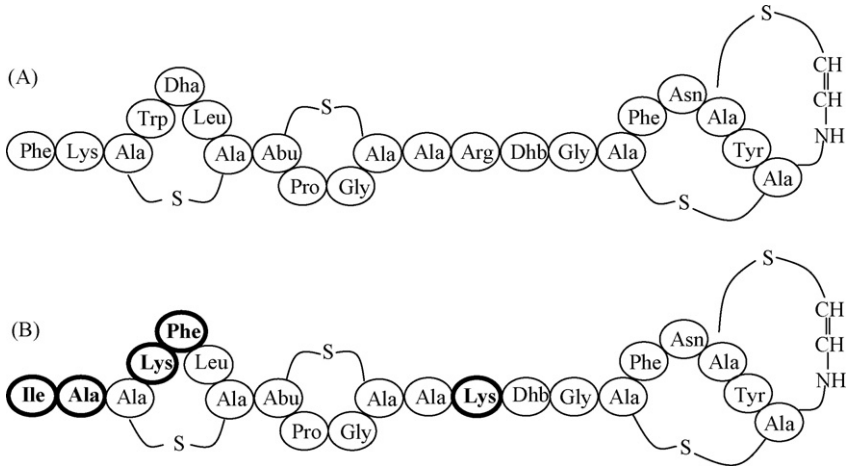

Fig. 1. (a) MU1140 [3]; (b) gallidermin [10]. Amino acids different from MU1140 are highlighted.

patient compliance, therapeutic drug monitoring, and determination of the bioequivalence of generics and follow on biologics. To date, no analytical method has been reported for the quantification of free or total lantibiotics in a biological matrix. This study describes the development and validation of an LC–MS method for the quantification of total MU1140 in rat plasma. The method was validated with regard to its accuracy, precision, selectivity, sensitivity, reproducibility, and stability. It was used successfully in a preliminary pharmacokinetic study of intravenously administered MU1140 in Sprague Dawley rats.

2. Experimental

2.1. Materials and stock solutions

MU1140 was produced by Oragenics, Inc. (Alachua, FL) and gallidermin (Fig. 1B), which was used as an internal standard (ISTD), was purchased from Alexis Biochemicals (San Diego, CA). MU1140 and the gallidermin ISTD stock solutions were prepared in 1:1 (v/v) mixture of isopropyl alcohol (IPA):water at a concentration of 25 μg/ml and stored at −80 °C until used. Mass spectrometry grade IPA, water, and formic acid were purchased from Sigma (St. Louis, MO). Microcon® Centrifugal Devices (10 kDa cutoff) were purchased from Millipore (Bedford, MA). Drug-free, male Sprague Dawley rat plasma with EDTA was purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA), aliquoted, and stored at −20 °C until used.

2.2. Equipment and analysis conditions

The LC–MS analysis system used consisted of a Surveyor plus autosampler and pump (ThermoFisher Scientific, San Jose, CA) coupled to an API SCIEX 150EX single quadrupole mass spectrometer (Concord, ON, Canada) equipped with electrospray ionization. A Clipeus C-18 analytical column (100 mm × 2.1 mm; 5 μm particle size; Higgins, MA, USA) with a pre-column in-line filter (0.5 μm, MacMod, PA) was used for separation at room temperature. Samples (25 μl) of standards and unknowns were injected onto the column. Proteins were eluted with an acidified (0.3% (v/v) formic acid) IPA:water gradient at a flow rate of 250 μl/min. The gradient went from 5% to 95% IPA:water (v/v). Electrospray ionization was used for ions generation, with positive ion detection. Optimal sensitivity was achieved when ion source temperature was maintained at 475 °C and a voltage of 5.5 kV was applied to the sprayer needle. Nitrogen was used as the nebulizer and curtain gas. Single ion monitoring (SIM) was used for detection of analyte and ISTD. SCIEX Analyst software 1.4 was used for data collection and integration of the chromatographic peaks. The peak area ratios of MU1140 to ISTD were plotted as a function of MU1140 concentration in standard solutions. A linear curve fit with no weighing was used to generate the regression line. The regression equation of the calibration curve was used to calculate the concentrations of the quality control samples and all unknowns.

2.3. Standards and quality control samples

Working solutions of MU1140 (1 μg/μl) and gallidermin (0.1 μg/μl) were prepared in 10% IPA. These solutions were used to prepare calibration curve standards and QC samples. Calibration standards of MU1140 in rat plasma were prepared by addition of MU1140 working solution to an initial concentration of 100 μg/ml and nine serial two-fold dilutions were prepared. Quality control (QC) samples were prepared at three concentrations, including low (1 μg/ml, LQC) medium (10 μg/ml, MQC), and high (50 μg/ml, HQC). Both the calibration standards and quality control samples were spiked with the working solution of the ISTD to a final concentration of 6 μg/ml.

2.4. Sample preparation

Plasma samples were spiked with the ISTD working solution to give a final concentration of 6 μg/ml. Samples from the pharmacokinetic study were allowed to thaw unassisted and fortified with the ISTD for a final concentration of 6 μg/ml. The samples were mixed for 30 s at medium speed using a vortex (VWR, Chicago, IL, USA). MU1140 and ISTD were detached from plasma proteins by acidifying the samples with 100% formic acid to a final concentration of 2% (v/v) and vortexing for 30 s. Plasma proteins were precipitated by the addition of an equal volume of 100% IPA and vortexed for 10 s to ensure complete mixing, after which the samples were centrifuged at 16,000 × *g* for 30 min in a table top centrifuge (Eppendorf, Hamburg, Germany) at room temperature. The supernatant was transferred to Microcon ultrafiltration device and centrifuged to dryness at 10,000 × *g* at room temperature. The ultrafiltrate was analyzed by LC/MS as described above.

2.5. Method validation

The method was validated for selectivity (specificity), sensitivity, linearity, accuracy, precision, recovery, and stability.

The selectivity of this method was verified by treating blank rat plasma samples from six different lots and analyzing the samples

for interfering peaks with the same m/z ratio at the analyte and ISTD retention times.

Sensitivity was assessed by determining the lowest quantifiable concentration (LLOQ) of MU1140. The LLOQ was established as the lowest concentration of MU1140 used in the calibration curve with accuracy and precision of 100% ± 20%. Bias and relative standard deviation were used as measures of accuracy and precision respectively, and were computed using

$$\text{Bias} = \frac{\text{theoretical concentration} - \text{mean observed concentration}}{\text{theoretical concentration}} \times 100$$

and

$$\%\text{Relative standard deviation} = \frac{\text{standard deviation}}{\text{mean}} \times 100.$$

Linearity was assessed by plotting MU1140:ISTD peak area ratios *versus* concentrations of calibration curve standards.

Accuracy and precision of the method were assessed by injecting QC samples in pentuplicate and quantifying the MU1140 concentration using the regression line equation of the calibration curve. Bias and relative standard deviation were used as measures of accuracy and precision, respectively, and calculated as mentioned above. A run was rejected if more than a third of the QC sample concentrations showed a deviation from the theoretical concentration equal to or greater than 20%.

The developed method's ability to recover MU1140 was estimated by quantifying the MU1140 content of QC samples extracted by our method using a calibration curve constructed from unextracted standards. Unextracted standards were prepared by fortifying extracted, drug-free plasma filtrate with MU1140 and ISTD. These samples represent 100% recovery and normalize for matrix effect, if any.

Stability of MU1140 under different conditions was assessed as part of the method's validation procedure. MU1140 stock solution stability was assessed at −80 °C for up to 30 days. Every 10 days, three aliquots were thawed, spiked with ISTD, extracted, and analyzed. Bench top (short-term) stability was determined at three concentrations (LQC, MQC, and HQC). Plasma aliquots were fortified with MU1140 and incubated at room temperature for 1.5, 3, and 6 h. After incubation, the ISTD was added to the samples and the samples were extracted and analyzed for their MU1140 content. Freeze and thaw stability was evaluated by subjecting rat plasma samples, spiked with MU1140 at three different concentrations (2.5, 10, and 40 μg/ml), to three freeze–thaw cycles. Samples were frozen for 24 h at −80 °C then allowed to thaw unassisted at room temperature. This process was repeated two more times, and after the third cycle, samples were spiked with ISTD, extracted, and analyzed. To determine the post-preparative stability of MU1140, plasma samples were spiked with the MU1140, samples were extracted as per the developed method and incubated in autosampler vials at 4 °C for up to 4 days. ISTD was added to the samples prior to analysis.

2.6. Preliminary pharmacokinetic study

Jugular vein cannulated Sprague Dawley rats (200–220 g) were purchased from Charles River Laboratories (Raleigh, NC), caged separately, and provided diet and water *ad libitum*. After 48 h acclimation, MU1140 doses equivalent to 12.5 mg/kg or 25 mg/kg rat body weight were administered via the indwelling jugular cannula to one rat per dose level as a rapid iv infusion (<1 min) and plasma samples were drawn via the cannula at 5, 10, 20, 30 min, and 1, 2, 4, and 6 h post-dosing in EDTA containing tubes. Blood samples were centrifuged at 500 × *g* for 10 min to separate the plasma. Plasma samples were immediately collected and stored at −80 °C until analyzed. The validated method was used to quantify the MU1140 content in the rat plasma samples.

3. Results and discussion

3.1. LC–MS detection and method selectivity

Analysis conditions for LC–MS were optimized using MU1140 and gallidermin (ISTD) in 50% IPA. The run time of the chromatographic method was 15 min with retention times of the analyte

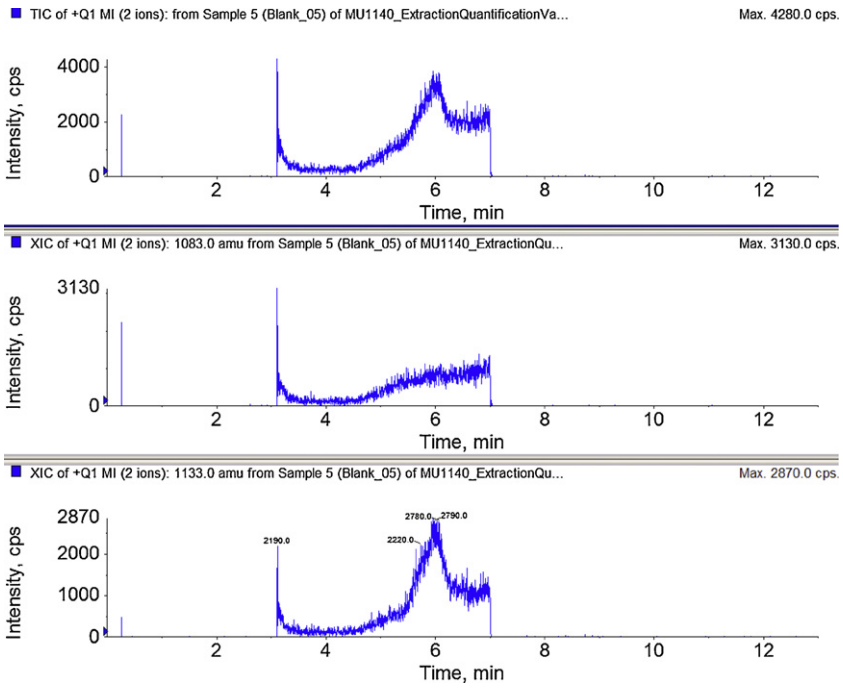

Fig. 2. Representative LC–MS chromatogram of extracted drug-free rat plasma. (A) Total ion current (TIC) of the two ions. (B) Extracted ion chromatogram (XIC) for m/z 1082–1084. (C) XIC for m/z of 1132–1134.

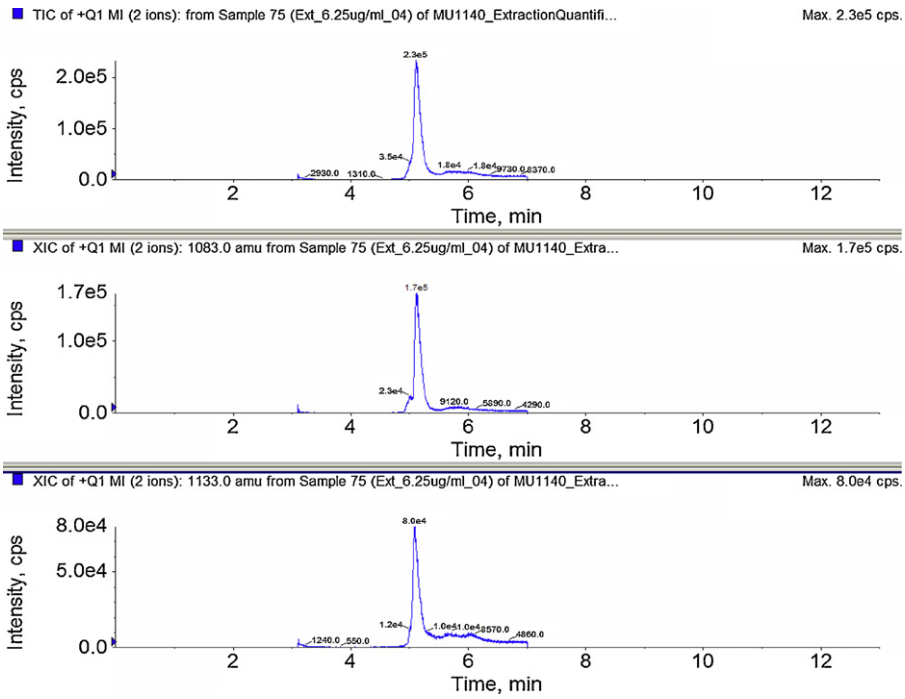

Fig. 3. Representative LC–MS chromatogram of plasma sample fortified with MU1140, *m*/*z* 1133 and gallidermin, *m*/*z* 1083. (A) TIC of the two ions. (B) XIC for *m*/*z* 1082–1084. (C) XIC for *m*/*z* of 1132–1134.

and ISTD being approximately 5.2 and 5.3 min, respectively. These methods were used in the following studies. Chromatograms of rat plasma spiked with MU1140 and ISTD and then extracted revealed that each molecular species was dominant in its doubly protonated molecular ion form $[M+2H]^{2+}$, detected at *m*/*z* of 1133 and 1083, respectively. These values accord with their known molecular formulas. Six different lots of drug-free rat plasma were treated as per the developed method and analyzed by LC–MS. No endogenous matrix ions were observed at *m*/*z* 1133 or 1083 at the retention times of MU1140 and ISTD. Data are presented in Figs. 2 and 3. This ensured the selectivity of the method and its applicability to quantify these lantibiotics in rat plasma.

The relatively short run time allowed increased sample throughput, thus making this method specifically suitable for quantitation needs of studies of large sample size such as pharmacokinetic or bioequivalence studies. To increase sensitivity, selective ion monitoring (SIM) was used for quantification. Gallidermin was found to be a suitable internal standard due to its structural similarity to MU1140.

3.2. Linearity and sensitivity

The calibration curve was linear over the range of 0.39–100 μg/ml when 25 μl of sample was injected onto the column. The relatively small sample volume allowed multiple injections from the same sample, thereby improving precision of quantitation. The correlation coefficient (r^2) was >0.995 for all validation batches.

The limit of quantification for MU1140 was far below the established MIC of MU1140 for susceptible organisms [8]. This finding suggests that accurate quantification of MU1140 in the concentration range of interest should be readily achieved, and lead to accurate determination of the pharmacokinetic parameters.

3.3. Accuracy, precision, and recovery

The inter-day accuracy and precision of the method were determined at the LLOQ as well as at three different QC concentrations in two different days. The accuracy of the method was described by

Table 1
Intra-run and inter-day accuracy and precision of the bioanalytical method at the LLOQ and three concentrations of MU1140.

	Theoretical concentrations			
	LLOQ (0.39 μg/ml)	1 μg/ml	10 μg/ml	50 μg/ml
Measured concentrations (μg/ml)				
Day 1				
Within run mean (n = 5)	0.36	1.07	9.64	49.39
% Bias	−6.67	6.86	−3.58	−1.23
%R.S.D.	10.7	11.87	8.47	5.1
Day 2				
Within run mean (n = 5)	0.35	1.10	9.85	48.9
% Bias	−10.26	10.0	−1.50	−2.20
%R.S.D.	15.7	7.92	6.69	4.68
Inter-day comparisons				
Inter-day mean (n = 2) concentration	0.36	1.09	9.70	49.2
% Bias	−8.97	8.50	−2.55	−2.14
%R.S.D.	1.99	1.95	1.52	0.70

Table 2
Recovery of MU1140 from plasma samples.

	Theoretical concentration (μg/ml)		
	2.5	10	40
Mean ($n = 6$) observed concentration (μg/ml)	2.39	9.64	38.46
%R.S.D.	10.7	9.1	6.4
Recovery (%)	95.6	96.4	96.15

the bias of theoretical *versus* measured concentrations, while the percentage of the relative standard deviation (%R.S.D.) served as a measure of precision. Table 1 summarizes the intra-run, as well as the inter-day accuracy and precision of this bioanalytical method, measured on two different days. The intra-run bias was <11% for all concentrations with intra-run %R.S.D. of <16% at the LLOQ level, and <11% for all other concentrations. The mean inter-day deviation from the nominal concentration was <8% and the inter-day R.S.D. was <2% for all tested concentrations. These data are in compliance with FDA guidance on bioanalytical method validation [10].

The developed method's percentage recovery of MU1140 from the plasma samples was estimated by comparing the ratio of the analyte peak areas from extracted samples to that from the unextracted samples. The mean recovery of MU1140 for all samples was 96.1% with R.S.D. of <11%. Data are summarized in Table 2.

The data presented above confirms that the developed method is capable of accurately and precisely quantifying MU1140 in Sprague Dawley plasma. This high recovery ratio improves the ability to detect and quantify MU1140.

3.4. Stability

Stability of the MU1140 stock solution constituted at 25 μg/ml in 50% IPA/water (v/v) was assessed after freezing for 10, 20, and 30 days at −80 °C. The original solution was aliquoted into 12 tubes. Three tubes were analyzed immediately and the others were frozen at −80 °C. Three tubes were thawed and analyzed at the indicated times. Each vial was sampled in triplicate. In all cases, MU1140 was detectable at levels equal to or greater than 96% (R.S.D. < 8%) compared to MU1140 freshly prepared at the same concentration. Data are presented in Table 3. This suggests that the standard solution of MU1140 was stable for at least 30 days when stored at −80 °C.

Bench top stability at room temperature of MU1140 in plasma was investigated at the three QC concentration levels, LQC, MQC, and HQC. Just before termination of the incubation period the ISTD was added. Incubation at room temperature was stopped at 0, 1.5, 3, and 6 h by addition of formic acid and isopropanol as per the extraction procedure and samples were analyzed for MU1140 content. There was no measurable loss of MU1140 in plasma at room temperature for more than 6 h. Data are summarized in Table 4.

Plasma samples spiked with MU1140 were subjected to three freeze and thaw cycles, after which ISTD was added, and the samples were processed by extraction and quantification. A mean percentage change of <0.5% with a %R.S.D. of <10% was observed. Data are summarized in Table 5. This result confirms that multiple freezing and thawing of MU1140-containing plasma did not affect the stability of MU1140.

Table 3
MU1140 stock solution (25 μg/ml) stability at −80 °C for up to 30 days.

Day	Mean ($n = 3$) concentration (μg/ml)	% Bias	%R.S.D.
0	24.94	0.24	6.38
10	24.09	3.64	7.65
20	25.73	−2.92	6.4
30	25.13	−0.52	7.89

Table 4
Bench top stability of MU1140 in Sprague Dawley plasma at room temperature. %R.S.D. is shown in parenthesis.

Time (h)	Theoretical concentration (μg/ml)		
	1	10	50
Calculated concentration (μg/ml)			
0	0.98 (9.5)	10.2 (7.9)	51.1 (4.7)
1.5	1.10 (10.6)	9.95 (8.2)	49.9 (4.5)
3	1.20 (11.3)	103 (8 6)	51.6 (3.3)
6	0.95 (8.8)	9.50 (10.5)	50.3 (5.5)

Table 5
Freeze/thaw stability assessment of MU1140 in plasma.

Theoretical concentration (μg/ml)	Mean ($n = 6$) concentration (μg/ml)	% Bias	%R.S.D.
2.5	2.29	0.06	9.1
10	10.0	0.36	8.4
40	42.5	0.49	6.3

Post-preparative stability of MU1140 was also determined at three concentrations at 4 °C for up to 4 days. Less than 5% (<12% R.S.D.) change in the intensity of the MU1140 signal was evident. Data are summarized in Table 6. This result indicates that MU1140 extracted from plasma was stable for at least 4 days at 4 °C.

Optimal storage and handling condition of MU1140-containing plasma samples were tested by the long-term and short-term stability studies, multiple freeze/thaw studies, and post-preparative stability studies which all showed no appreciable degradation and loss of the lantibiotic. These findings indicate that plasma samples from dosed animals can be conveniently stored at −80 °C and thawed, processed on the bench top at room temperature, and placed in a refrigerated autosampler for extended periods without significant loss of MU1140.

3.5. Preliminary pharmacokinetic (PK) study of MU1140 in Sprague Dawley rats

The purpose of this PK study was to evaluate the validated method for the quantification of MU1140 content in *in vivo* samples. The method was successfully applied in a preliminary PK study of MU1140 in two rats which received either 12.5 or 25 mg/kg dose. All QC samples were found to be within acceptable limits for precision and accuracy. Plasma concentration–time data were subjected to noncompartmental analysis (NCA) and dose linearity of the calculated PK parameters was established. C_{max} (8.86, 15.9 μg/ml) and $AUC_{0-\infty}$ (12.39, 24.69 h μg/ml) were dose-dependent for the 12.5, 25 mg/kg doses, respectively. The half-life and clearance were dose-independent, indicating the linearity of the PK of MU1140 in that dose range. The PK profiles are presented in Fig. 4 and the PK data are presented in Table 7. Overall, it appears that the developed method is reliable for the *in vivo* quantification of MU1140.

Table 6
Post-preparative stability assessment of MU1140 after 4 days at 4 °C in autosampler.

Day	Theoretical concentration (μg/ml)		
	2.5	10	40
Mean ($n = 3$), concentration (μg/ml)			
0	2.30 (11.3)	8.64 (8.1)	38.9 (5.3)
1	2.40 (9.6)	9.30 (10.2)	37.2 (6.2)
2	2.30 (9.2)	10.2 (9.7)	37.7 (5.8)
3	2.55 (9.8)	10.0 (8.7)	40.5 (7.4)
4	2.45 (10.3)	10.1 (9.1)	36.5 (6.1)
% Bias	−3.00	−3.37	−4.60

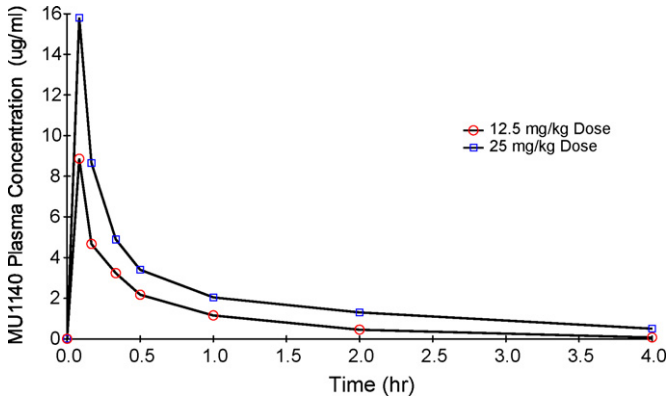

Fig. 4. MU1140 plasma concentration–time profiles after IV bolus administration of a single dose of 12.5 mg/kg or 25 mg/kg to two different rats.

Table 7
MU1140 pharmacokinetic parameters determined using NCA analysis of plasma concentration–time data.

Dose	C_{max} (μg/ml)	$AUC_{0-\infty}$ (h μg/ml)	$t_{1/2}$ (h)	Clearance (l/h/kg)
Pharmacokinetic parameters				
12.5 mg/kg	8.86	12.39	1.33	1.00
25 mg/kg	15.9	24.69	1.56	1.01

4. Conclusions

This paper describes the first bioanalytical method for the quantification of a lantibiotic in a biological matrix. The method was developed specifically for the quantification of MU1140 in rat plasma samples, and uses a simple and inexpensive liquid–liquid extraction followed by a rapid, sensitive LC/MS separation and detection procedure. This method was validated to be selective, accurate, precise, and sensitive, and the stability of MU1140 was not compromised during sample handling and processing. The validated method was successfully tested in a pilot *in vivo* MU1140 PK study in Sprague Dawley rats using two MU1140 dose levels. Clear dose-dependent response was observed, indicating the validity and reliability of this quantification method. Collectively, the results indicate that this method should be applicable to quantitative studies of MU1140 and should serve as a starting point for optimization of bioanalytical methods for other lantibiotics.

Acknowledgement

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