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A novel antibiotic bone assay by liquid chromatography/tandem mass spectrometry for quantitation of tigecycline in rat bone

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Abstract

Tigecycline (Tygacil®) is a first-in-class, broad spectrum antibiotic with activity against antibiotic-resistant organisms. In rats and humans, tigecycline readily distributes to bone tissue but its accuracy of quantitation via liquid chromatography/mass spectrometry (LC/MS/MS) is hindered by a low extraction recovery when using a conventional plasma extraction method. To overcome this issue, we have identified an effective extraction solvent for quantitation of tigecycline in rat bone. The current LC/MS/MS bone assay is novel, simple, and sensitive, and has a wide linear range of 50–10,000 ng/g. The assay requires homogenization of the rat bone in a strong acidic-methanol extraction solvent, centrifugation of the bone suspension, separation of the supernatant with liquid chromatography, and detection of tigecycline with tandem mass spectrometry. The incurred pooled rat bone samples obtained from rats given 3 mg/kg/day of [^{14}C]-tigecycline and non-radio-labeled tigecycline were analyzed with the current method. The absolute extraction recovery of the bone assay for tigecycline was 77.1%. The intra-day accuracy ranged from 91.7 to 106% with precision (CV) of 1.9–10.7%, and inter-day accuracy ranged from 96.1 to 100% with a precision of 6.3–8.7%. In addition, biological activity was demonstrated for the tigecycline extracted from incurred rat bone. This bone assay provides an important analytical tool for the determination of drug concentrations (especially, antimicrobials) in rodent bone tissues and has served as the foundation of development and validation of a similar bone assay for tigecycline in human bone tissues.
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1. Introduction

Tigecycline (Tygacil®, Wyeth, formerly GAR-936, chemical structure in Fig. 1) is a recently approved, first-in-class, glycylcycline antibiotic [1,2]. This broad-spectrum agent has activity against a wide range of Gram-positive, Gram-negative, atypical, anaerobic, and antibiotic-resistant bacteria [3,4]. The use of tigecycline and other antibiotics or antimicrobials for bone diseases has not been extensively studied due to insufficient information on their disposition and relationship between bone concentration and pharmacological effects [5]. In rats, tigecycline showed a high volume of distribution and high concentrations of tigecycline in several tissues, including bone, bone marrow, salivary gland, thyroid, spleen, and kidney. The exposure (area under curve) in bone was 13-times higher than the next highest tissue, bone marrow [6]. In humans, tigecycline is widely distributed in the body and has a long half-life [7,8]. However, results from two human studies ([8] and a Wyeth contract lab results) showed relatively low concentrations of tigecycline in bone (0.35-fold) relative to serum. It was unclear if this was due to poor distribution of tigecycline to human bone or due to poor extraction of tigecycline from the human bone for analysis by the LC/MS/MS method. To investigate low extraction recovery in human bone tissue, tigecycline quantitation methods for human biological fluids and tissues were reviewed. In these LC/MS/MS or LC/UV methods [8-11], tigecycline was

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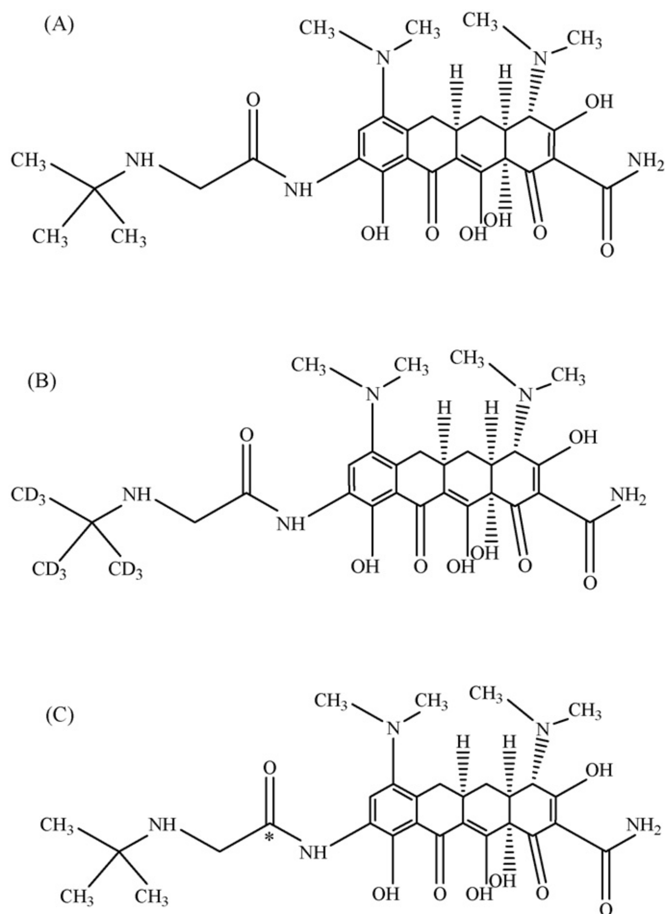

Fig. 1. The chemical structure of (A) tigecycline (molecular weight 585.65); (B) [t-butyl-d_9]-tigecycline (internal standard, molecular weight 594.70); (C) ^{14}C-tigecycline (molecular weight 587.65), * is the site of ^{14}C label at carbonyl group.

extracted with acetonitrile, a protein precipitation agent. A similar method was used for extraction of tigecycline from human bone [8]. The protein precipitation method has a major weakness in extracting tigecycline out of the bone tissue because acetonitrile cannot dissolve bone tissue. One study [12] reported the measurement of antimicrobial agents in human bone (obtained from hip or knee replacement surgery) using a microbiological disk diffusion method. In the microbiology method, the antibiotics were extracted from pulverized bone using a neutral buffer solution (pH 6.8). The buffer is suitable only for microbiology testing, but not suitable for bone extraction due to the insolubility of this tissue. Also, the microbiology assay itself has a higher detection limit (1–5 μg/ml in serum, and 0.5–3.6 μg/g in bone), making this method unsuitable to detect tigecycline in the ng/g range.

In this study, we conducted a series of experiments to develop an effective extraction solvent and also determined absolute extraction recovery using an incurred pooled radio-labeled ^{14}C-tigecycline rat bone sample. The first objective of the current study was to develop a LC/MS/MS method with an optimized extraction scheme in an animal model (rat) and demonstrate a high extraction recovery. The second objective was to validate the tigecycline bone assay and also to provide additional verification that the bone extract (from the extraction solvent) was biologically active using standard antimicrobial assays. The application of this novel antibiotic bone assay, particularly with respect to incurred bone samples, has built a foundation for the development of a tigecycline human bone assay [13], which can also be used for other antibiotics and antimicrobials possessing similar bone disposition properties.

2. Experimental

2.1. Materials

2.1.1. Chemicals

Tigecycline (purity 99.0%) was synthesized by Wyeth Research, Chemical and Pharmaceutical Development (Pearl River, NY). [t-butyl-d_9]-tigecycline (purity 94.7%) and ^{14}C-tigecycline (purity 96.0%) were synthesized by Wyeth Research, Radiosynthesis Group (Pearl River, NY). Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from EM Sciences (distributed by VWR Scientific Products, Newark, NJ). Formic acid, acetic acid, perchloric acid (70–72%) and phosphoric acid (85–88%) were purchased from J.T. Baker (Phillipsburg, NJ). Trifluoroacetic acid was obtained from Burdick & Jackson (Muskegon, MI). Liquid nitrogen was purchased from Airgas Inc. (Radnor, PA). Deionized water was obtained from an in-house deionization system at Wyeth Research (Pearl River, NY). Control rat bone was purchased from Bioreclamation Inc. (Hicksville, NY). Microbiology materials included: nutrient agar (Remel, Lenexa, KS); Agarose (Sigma–Aldrich Inc., St. Louis, MO); saline solution (0.85% sodium chloride) (Wyeth Research, Pearl River, NY); Trypticase Soy Agar Blood Plates (Becton Dickinson, Sparks, MD).

2.1.2. Solutions

Primary tigecycline stock solution (100,000 ng/ml) was prepared by adding 10 mg of the drug (weight corrected for purity) into a 100 ml low-actinic volumetric flask filled to volume with methanol and stored at −20 °C. Stock internal standard solution (100,000 ng/ml) was prepared by adding 10 mg of [t-butyl-d_9]-tigecycline (weight corrected for purity) into a 100 ml low-actinic volumetric flask and diluting to volume with methanol and stored at −20 °C in 50-ml conical polypropylene tubes. An extraction solvent was prepared in final concentrations of perchloric acid 0.21 M and phosphoric acid 0.14 M in 50:50 (v:v) MeOH:H_2O solution. Mobile Phase A consisted of deionized water, acetonitrile, methanol, trifluoroacetic acid at volume ratios of 95.5:3.5:1:0.1(v/v/v/v); Mobile Phase B was prepared in volume ratio of methanol to acetonitrile, 22.2:77.8 (v/v).

2.1.3. Equipment

The tissue homogenizer (Kinematica Polytron® PT 10–35) and probe (Kinematica Polytron Aggregate® 12 mm-PTA7) were purchased from Brinkmann Instruments (Westbury, NY). The blender (Waring Model 51BL32) was from Waring Commercial (Tarrington, CT). The centrifuge (Sorvall RT 6000D) was from Dupont (Newtown, CT). The polypropylene tubes

(17 × 100 mm) and polypropylene low volume autosampler vials (300 μl) were purchased from VWR Scientific Products (Bridgeport, NJ). The sample oxidizer (Model 307/Oximate 80) and liquid scintillation counter (Model Tri-carb 3100 TR) were manufactured by Perkin-Elmer Life Sciences (Downers Groves, IL). ^{14}C-methyl methacrylate was purchased from Dupont Merck Phar. Co. (Billerica, MA) and the Nunc Bio-Assay dish (243 × 243 × 18 mm) was from Nalge/Nunc International Inc. (Rochester, NY). The bacterial culture, *Bacillus cereus* ATCC 11778 (GC 4561), was from American Type Culture Collection (Rockville, MD). The triple quadrapole mass spectrometer, model Sciex API 4000, was made by MDS Sciex Applied Biosystems (Concord, Canada). The HPLC column (MetaChem Polaris C18-A 3 μm, 50 × 2.0 mm) was from Varian Inc. (Torrance, CA). The syringe pump was from Harvard Apparatus (Holliston, MA) and the HPLC controller (Alliance 2795) was purchased from Waters Corporation (Milford, MA).

2.2. Procedures

2.2.1. Animal dosing

Bone samples from two groups of male CD VAF Sprague–Dawley rats (body weight range of 0.455–0.572 kg) dosed intravenously (bolus) with either tigecycline or ^{14}C-tigecycline were used for developing the optimum extraction procedure (incurred rat bone samples) and for evaluating the extraction recovery. For Group A, 12 male rats were dosed intravenously (bolus) with a single 3 mg/kg tigecycline solution (concentration of 1 mg/ml in sterile saline solution). At 4 h post-dose, these rats were euthanized and the femoral bones were harvested. For Group B, 20 male rats were dosed intravenously (bolus) with a ^{14}C-tigecycline solution (3 mg/kg/day, 1 mg/ml in sterile saline solution) for 3 days. On the last day of dosing, the rats were euthanized at 4 h post-dose and the femoral bones were harvested.

2.2.2. Sample preparation

2.2.2.1. Incurred rat bone sample preparation. Incurred rat bone (IncRB) is defined as bone harvested and prepared from rats administered the study drug. Two different groups of incurred rat bone were prepared: Group A represented bone collected from rats administered a single dose of tigecycline and Group B represented bone collected from rats administered multiple doses of ^{14}C-tigecycline. To prepare the bone for extraction procedures, two pieces of femoral bone from each rat in each group were collected. The femoral bones were cleaned with saline to remove excess blood and bone marrow. The femoral bones were then air-dried and combined to form two pooled samples of incurred rat bone: Group A and Group B. The pooled bone samples were each ground for approximately 2 min in an industrial blender to produce bone particles <1 mm in diameter. The ground bone samples for each group were stored at −70 °C for later analysis.

2.2.2.2. Control rat bone sample preparation. Control rat bone (CtrlRB) was purchased from a commercial source and prepared in the same manner as the incurred rat bone.

2.2.3. Preparation of bone calibrators and quality control samples

Tigecycline working standard solutions (100, 200, 1000, 10,000, 16,000, and 20,000 ng/ml) were prepared daily from stock solution (100,000 ng/ml in methanol) with appropriate dilutions of methanol. A working internal standard solution of 5000 ng/ml [*t*-butyl-d_9]-tigecycline was prepared by a 1:20 dilution of the stock solution with methanol.

Calibrators (CtrlRB calibrator or bone standards), quality control/validation samples (CtrlRB QC), and incurred rat bone quality control/validation samples (IncRB QC) were prepared as follows:

Tigecycline CtrlRB calibrators: Approximately 0.1 g of CtrlRB was weighed and dissolved in 1.0 ml of the extraction solvent to form a mixture of bone and solvent. To prepare a range (50–10,000 ng/g) of CtrlRB calibrators, 50 μl of each tigecycline working standard solution was spiked into this mixture. Calibrators were prepared daily.

Tigecycline CtrlRB quality control samples (or validation samples): Approximately 0.1 g of CtrlRB was weighed and dissolved in 1.0 ml of the extraction solvent to create a mixture of bone and solvent. To prepare the low level (150 ng/g), mid level (1000 ng/g), and high level (7500 ng/g) of quality control (QC) samples, tigecycline QC spiking solutions of 300, 2000, and 15,000 ng/ml were spiked into this mixture. QC samples were prepared daily.

Tigecycline IncRB quality controls (or validation samples): Approximately 0.1 g of IncRB (Group A), which had been stored at −70°, was thawed, weighed and dissolved in 1.0 ml of the extraction solvent to create a mixture of bone and solvent. Then, 50 μl of methanol were added to match the volume of working standard solutions added in CtrlRB calibrators or CtrlRB QC or validation samples.

2.2.4. Extraction procedure

Aliquots of approximately 100 mg of prepared IncRB or CtrlRB sample were accurately weighed into 17 × 100 mm polypropylene tubes. One ml of extraction solvent, 50 μl of each tigecycline working standard solution (or 50 μl of methanol for study samples or IncRB sample) and 40 μl of working internal standard solution (5000 ng/ml [*t*-butyl-d_9]-tigecycline in methanol) were added to each tube. All sample tubes were vortexed for about 60 s. A tissue-homogenizing probe was introduced into the mixture (small particles of prepared bone samples in extraction solvent) to further break up the bone particles. The homogenizing probe was operated at a setting of 4 (∼17,000 rpm) for about 2 min until the bone particle mixture became a cloudy, white suspension. The probe was removed from the suspension and cleaned between each sample preparation by immersion in 2 ml of water, operating from 30 to 60 s, then immersion in 2 ml of methanol, operating from 30 to 60 s, and then wiping dry. Each sample tube containing bone suspension was centrifuged at approximately 3000 rpm at room temperature for about 5 min. The supernatant (200 μl) was transferred to a 250-μl conical low volume polypropylene autosampler vial and re-centrifuged for another 5 min before loading into the HPLC autosampler (4 °C). A 20 μl aliquot of

the supernatant was injected onto the LC/MS/MS system for tigecycline determination.

2.3. Method validation

2.3.1. Precision and accuracy

Five replicates of each tigecycline validation sample (low, mid, and high levels of QCs) and the IncRB validation sample (or IncRB QC) were analyzed with a bone standard curve (six calibrator points with initial injection at the beginning of the run and re-injection at the end of the run) for intra- and inter-day precision and accuracy.

2.3.2. Stability tests

Rat bone samples from IncRB Group A were conducted in three sequential freeze/thaw bone processes (3 cycles, −70 °C/22 °C), and over a 4-h period while sitting on the bench-top at room temperature (∼22 °C). Extract stability of tigecycline in the IncRB Group B sample (^{14}C) was evaluated in an autosampler at 4 °C at different times. Results were plotted as the peak-area ratio against time.

2.3.3. Absolute extraction recovery

2.3.3.1. Combustion method for theoretical value of IncRB QC samples. Four aliquots of IncRB Group B pooled sample (0.1 g each) were accurately weighed, placed into combustion cones, and allowed to air dry for approximately 3 days. These four samples were then oxidized in a Model 307/Oximate 80 sample oxidizer, using Carbosorb® E (7 ml) as a trapping agent and PermalFluor® ET (10 ml) as a scintillant. Oxidation efficiency was determined by oxidation of ^{14}C-methyl methacrylate and was found to be 99%. The oxidized samples were counted in a Packard (Perkin-Elmer) liquid scintillation counter using a toluene standard curve. The ng-equiv/ml concentrations were calculated using the specific activity of the dosing solution.

2.3.3.2. The current extraction method. In parallel to the above (a), five aliquots of the IncRB Group B pooled sample (0.1 g) were accurately weighed. The samples were extracted using the extraction procedure described in Section 2.2.4. The final supernatant (100 μl of the 1.09 ml supernatant from extraction) was sent for liquid scintillation counting (LSC) and 20 μl of the 1.09 ml supernatant was injected onto the LC/MS/MS for tigecycline parent drug concentration determination.

The absolute extraction recovery (AER) was determined using the following equation:

$$\text{AER for Parent Drug, tigecycline (\%)} = \frac{[\text{total amount (nanogram) of tigecycline per gram of bone (determined by LC/MS/MS)}]}{[\text{total amount (nanogram) of radioactivity per gram of bone (determined by combustion} - \text{LSC)}]} \times 100$$

$$\text{AER for total radioactivity (tigecycline and its metabolites, \%)} = \frac{[\text{total dpm per gram of bone in LC/MS/MS extract (determined by LSC)}]}{[\text{total dpm per gram of bone (determined by combustion} - \text{LSC)}]} \times 100$$

2.3.3.3. Comparison of methods. A Wyeth contract laboratory method was used to extract the same-pooled rat bone sample ($n = 5$, IncRB QCs from Group B). The bone extracts were quantified with the liquid scintillation counting method and mass spectrometry, respectively. The extraction recoveries from the current method and the Wyeth contract lab method were compared. The Wyeth contract lab method for tigecycline quantitation is briefly summarized here: 0.20 g of crushed bone was added to 3.0 ml of 0.1% trifluoroacetic acid acetonitrile solution. Sample was homogenized and centrifuged at approximately 3000 rpm, the supernatant was dried down and reconstituted with 0.2 ml of the mobile phase A. Twenty μl of the sample were injected onto the LC/MS/MS.

2.4. HPLC instrumentation

Separation procedures were carried out on a 50 × 2.0 mm (i.d., 3 μm) HPLC analytical column with a pre-column in-line solvent filter (2.0 μm PEEK filter) and a LC/MS switching valve. PEEK tubing (1/16 in. × 0.005 in.) connected the separation module, the analytical column, the LC/MS switching valve, and the mass spectrometer. The separation module included a 4 °C autosampler, an in-line degasser, and a quaternary solvent delivery system. The analytical column temperature was at approximately 20 °C; the autosampler temperature was maintained at 4 °C. The eluting components were separated from the bone extracts using a mobile phase flow rate of 0.300 ml/min with the following gradient program: 0–1 min: 100–100% mobile phase A; 1–2 min: 100–90% A; 2–4 min: 90–20% A; 4–7 min: 20–20% A; 7–7.1 min: 20–100% A, 7.1–11 min: 100–100% A. To minimize contamination of the mass spectrometer, the unwanted eluted components were diverted to waste without passing through the mass spectrometer.

2.5. Mass spectrometric detection

The LC/MS switch valve program used was as follows: 0–3 min: switch 2 on (to waste); 3–6 min: switch 1 on (to mass spectrometer); 6–11 min: switch 2 on (to waste). The triple quadruple Sciex API 4000 mass spectrometer was operated under the positive electrospray ionization mode (ESI^+) in multiple reaction-monitoring (MRM) mode. The optimal ionization conditions were tuned by infusing a 1000 ng/ml tigecycline solution in mobile phase A: mobile phase B (50:50, v/v) at a flow rate of 10.0 μl/min with a syringe pump. The mass spectrometry conditions were as follows: Run duration 10.004 min, cycle time 0.41 s, number of cycles 1464, scan type positive MRM, Q1 and Q3 resolution set to low, intensity threshold 0 cps, settling time

0 msec, MR pause 5.007 msec, curtain gas setting at 10.0, ion source temperature 400 °C, a nitrogen pneumatically assisted (software setting GS 1:35, GS3:60) electrospray nebulizer set at 5000 V, collision energy cell setting 8.0 (software setting CAD 8.0), electronic multiplier setting at 1800 V. Full scan spectra of Q 1 were acquired over the *m/z* range of 100–800. Multiple reaction monitoring (MRM) mode was used for analyte quantitation with a transition of precursor ion to product ion: *m/z* 586.3 > 513.3 for tigecycline, *m/z* 595.4 > 514.3 for [*t*-butyl-d_9]-tigecycline; and API-4000 mass spectrometer parameters were declustering potential at 37 V for both the analyte and the internal standard, entrance potential at 10 V for both compounds, collision cell exit potential was 24 V for tigecycline and 23 V for the internal standard, collision energy at 43 V for tigecycline and 45 V for the internal standard, and dwell time was 200 milliseconds for both the analyte and the internal standard.

2.6. Data analysis

"Analyst" software (MDS Sciex Applied Biosystems, Version 1.3.1) was used for mass spectrometer data acquisition and processing. The peak area ratios of tigecycline to internal standard [*t*-butyl-d_9]-tigecycline were plotted versus the known tigecycline concentrations for the calibration curve using Watson software (Thermo Scientific, Version 7.0.0.01). Six standards in duplicate were plotted as one calibration curve. $1/x$ weighted linear regression was used to calculate the concentrations. The relationship between peak-area ratios (y) and analyte concentrations (x, ng/g) was calculated. The tigecycline concentration (ng/g) in each sample was calculated by interpolation from the regression line using the following formula: $y = a + bx$, where y is the peak-area ratio (analyte/internal standard); a is the intercept; b is the slope; and x is the analyte concentration. The run acceptance criteria for the rat bone standards were as follows: at least 75% of calibration standards (9 out of 12) must be within $100 \pm 15\%$ of their nominal values, except the lowest standard, which must be within $100 \pm 20\%$ of its nominal value. For the run acceptance QC samples (CtrlRB QCs and IncRB QCs), CtrlRB QCs must have at least four out of six QCs be within $100 \pm 15\%$ of their nominal values. Two failed QCs samples cannot be at the same concentration. Additionally 3 IncRB QC samples were included in the study sample runs to monitor drug extraction recovery from the incurred samples. At least two out of the three IncRB QC samples must be within $100 \pm 15\%$ of their established concentration value (the mean value from the previous inter-day runs).

2.7. Verification of tigecycline microbiological activity

2.7.1. Standard curve preparation

A stock solution of tigecycline standard powder, at a concentration of 1000 μg/ml, was prepared in normal saline. Dilutions were prepared in normal saline at concentrations of 125, 250, 500, 1000, 2000, and 4000 ng/ml for the preparation of the standard curve. To validate the standard curve, a check standard (1000 ng/ml) was also made from stock solution and run with the standard curve.

2.7.2. Preparation of Inoculum

An overnight trypticase soy agar blood plate culture (incubated at 30 °C) of *Bacillus cereus* ATCC 11778 was adjusted to a McFarland 0.5 standard in saline. This suspension yielded a bacterial density of approximately 10^8 colony-forming units (CFU)/ml.

2.7.3. Preparation of bioassay agar plates

The agar medium was prepared by adding nutrient broth (8 g) and agarose (11 g) per 1000 ml of distilled water (8:11:1000, w/w/v). After autoclaving at 121 °C for 15 min, the medium was allowed to equilibrate to a temperature of 48–50 °C for approximately 1 h in a water bath. The adjusted *B. cereus* culture was used to inoculate the cooled agar to a final concentration of 1% (1:100, v/v). A volume of 100 ml was added to a Nunc bioassay dish and the agar was allowed to solidify at room temperature on a level surface. After cooling, wells were cut into the surface of the agar assay plate using a vacuum well cutting device. The standard curve and unknown samples were placed into the wells (50 μl) in a pre-determined array with three wells each per concentration. A check standard (1000 ng/ml of tigecycline) was also tested in triplicate. For comparison of tigecycline activity in the ground rat bone with its bone extracts, 0.735 ml of saline solution was added to 0.725 g of ground bone sample to form a slurry. Then 0.1 g of the slurry was weighed and added to a pre-seeded bacteria agar plate in triplicate. The slurry was overlaid with 1.1% agarose in order to maintain contact with the seeded agar. Bone extracts were obtained as follows: One gram of ground rat bone was added to 10 ml of the extraction solvent. Then the sample mixture was homogenized with a homogenizing probe and centrifuged (described in Section 2.2.4). Approximately 10 ml of the supernatant as bone extract was transferred to a clean beaker. Three bone extracts were treated as: (a) neutralized with 50% concentrated ammonium hydroxide in water (50:50, v/v) to pH 7.0 and then evaporated (methanol) before microbiology test; (b) evaporated (methanol) and then neutralized with 50%ammonium hydroxide in water to pH 7.0 before microbiology test; (c) evaporated (methanol) only. The above three bone extracts (50 μl) were applied to the pre-bacteria seeded agar plate in triplicate. The plates were pre-diffused at 4 °C for 2 h then incubated at 30 °C for 18–24 h.

2.7.4. Determination of tigecycline concentration using a microbiology method

The diameters of the zones of inhibition for the standards and the samples were measured using electronic calipers. The concentrations of the standard curve were then plotted on a semilogarithmic scale versus their corresponding zone diameters to give a standard regression curve. The concentrations of the samples and the check standard were determined by comparing the mean zone size of the samples to the zone sizes of the standard curve and their corresponding concentrations. A bioassay data analysis program was used to perform the calculations and plots.

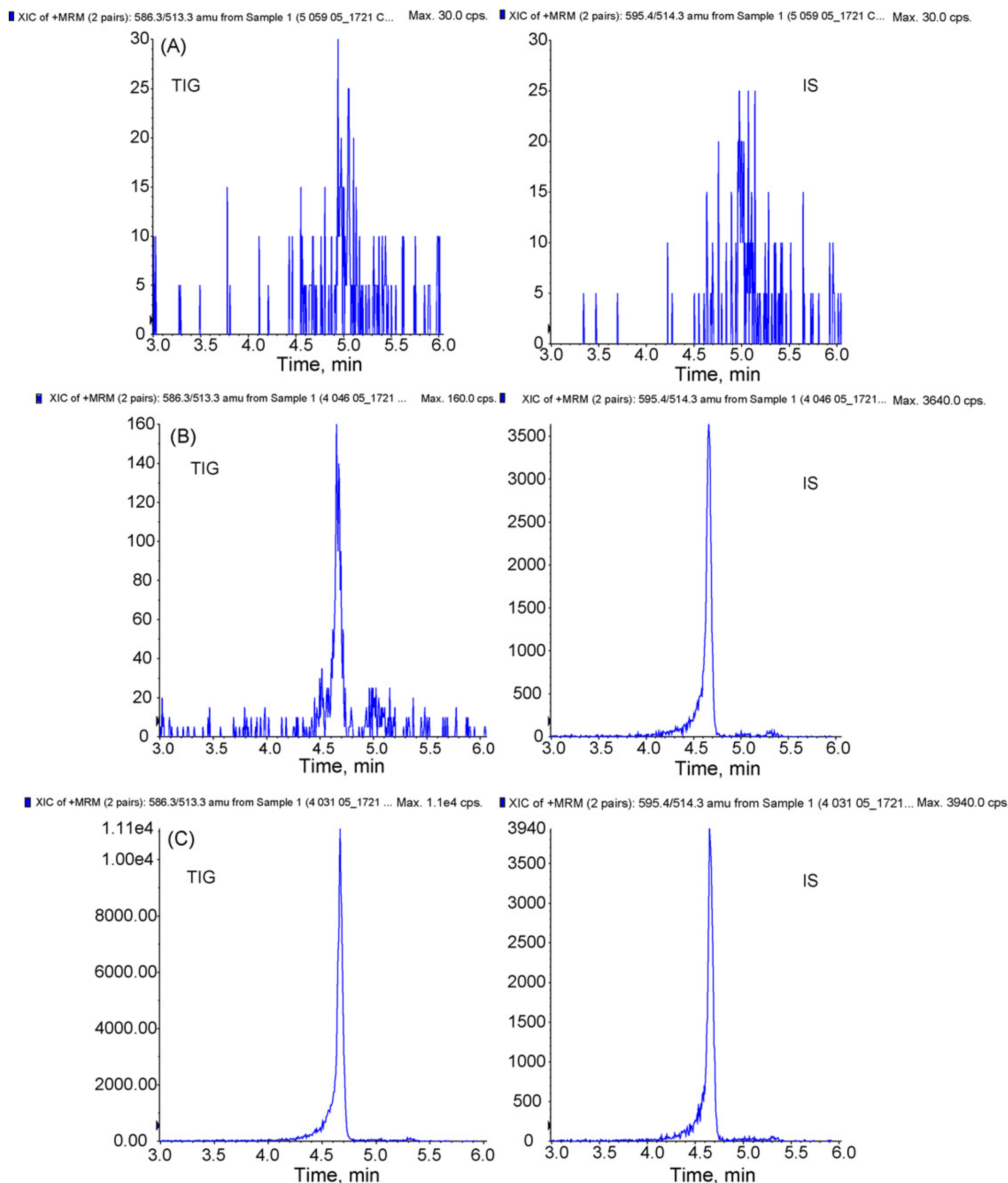

Fig. 2. Representative chromatograms of (A) control rat bone (CtrlRB) without internal standard; (B) lower limit of quantitation 50 ng/g (CtrlRB) in rat bone; (C) incurred rat bone (IncRB) QC sample from Group B (mean observed concentration was 3192 ng/g). The *Y* axis represents intensity of the mass, counts per second (cps) and the *X* axis represents retention time in minutes. TIG is tigecycline and IS is internal standard.

3. Results

3.1. Analytical performance of tigecycline bone assay

Representative ion chromatograms of control rat bone extracts, bone standards at the lower limit of quantitation (50 ng/g), and an incurred rat bone QC sample (987 ng/g) are shown in Figs. 2A through 2C, respectively. The retention time of tigecycline was about 4.7 min. A typical rat bone calibration curve (50–10,000 ng/g) is shown in Fig. 3. All standard curves from four validation runs had linear correlation coefficients ≥0.9980. The lower limit of quantitation (LLOQ) of this method was 50 ng/g (CV 8.4%, accuracy 111%, $n = 5$), which was equal to 4.59 ng/ml of tigecycline in the final solution before injection. The assay is linear from 50 to 10,000 ng/g. The intra- and inter-day precision at three different concentrations (150, 1000, 7500 ng/g) of CtrlRB samples and an IncRB validation sample (Group A) is presented in Table 1. The nominal value for the IncRB validation sample was determined from the overall mean of the 3-day validation. The intra-day accuracy for all validation samples ranged from 91.7 to 106% with precision (CV) ranging from 1.9 to 10.7%. Inter-day accuracy ranged from 96.1 to 100% with CV ranging from 6.3 to 8.7%.

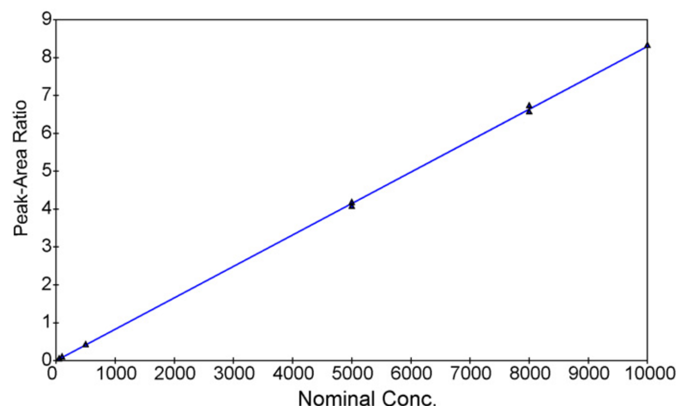

Fig. 3. Rat bone calibration standard curve of tigecycline. The Y axis represents the peak area ratio of tigecycline to internal standard and the X axis represents the tigecycline concentration in rat bone (ng/g).

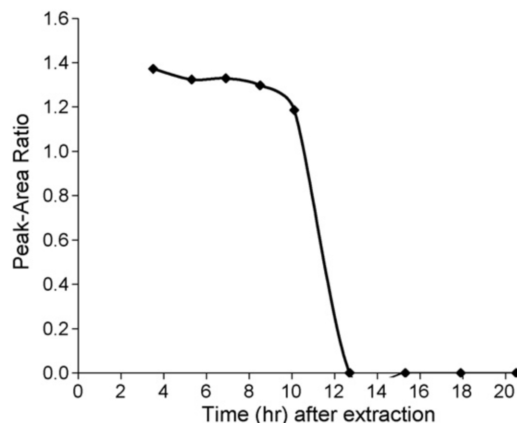

Fig. 4. Stability of tigecycline in extracted sample at 4 °C. The Y axis represents the peak area ratio of tigecycline to internal standard and the X axis represents hours after extraction at 4 °C.

3.2. Absolute extraction recovery

The current LC/MS/MS method uses a strong acidic extraction solvent to extract tigecycline from bone, the absolute extraction recovery of total radioactivity (tigecycline and possible metabolites) from incurred rat bone samples was 87.2% ($n = 5$). The absolute extraction recovery for the parent drug was 77.1% using tigecycline concentrations obtained from the mass spectrometry methodology. The Wyeth contract laboratory method showed an absolute extraction recovery 2.3% for total ^{14}C labeled-tigecycline and its metabolites using LSC readings in the bone extracts. The mass spectrometer could not detect a quantifiable analyte peak from the bone extract. These results are summarized in Table 2.

3.3. Stability of tigecycline in rat bone samples and in the acidic extracts

The stability of tigecycline was evaluated using the incurred rat bone sample. Results showed that tigecycline was stable in incurred rat bone after 4 freeze/thaw cycles, and after 4 h at room temperature. Tigecycline was stable in an incurred rat bone sample for at least 5 months when stored at −70 °C. The extracted tigecycline from the incurred rat bone sample (Group B) was stable for only 10 h (Fig. 4). Therefore, the number of injections per run was limited to not more than 40 (∼12 min per injection).

Table 1
Precision and accuracy of the LC/MS/MS rat bone assay for determination of tigecycline concentrations

Validation sample (conc., ng/g)	LLOQ (50)	Low (150)	Mid (1000)	High (7500)	IncRB (987[a])
Intra-day precision (%CV, $n = 5$/day for 3 days)	8.4	4.7–10.7	4.1–7.6	3.6–8.5	1.9–9.5
Intra-day accuracy ($n = 5$/day for 3 days)	110.8	91.7–100.0	94.7–104.0	96.3–102.5	97.1–106.2
Inter-day precision (%CV, $n = 15$, global)	NA[b]	8.7	6.8	6.3	7.9
Inter-day accuracy ($n = 15$, global)	NA[b]	96.1	98.2	98.6	100.0

[a] There is not a nominal value for the incurred rat bone sample; the listed value was from the mean of a 3-day inter-day validation ($n = 15$).
[b] NA: not applicable.

Table 2
Comparison of absolute extraction recovery of tigecycline from various methods using pooled ground rat bone sample from Group B

Methodology	Measured ^{14}C counts (dpm/g)	Extraction recovery (%) using dpm	Measured conc. (ng/g)	Extraction recovery (%) using conc.
Combustion (0.1 g IncRB, $n = 4$)	141451 ± 12318	NA	4137[a]	NA
Current bone assay (0.1 g IncRB n = 5)	123398 ± 3855[b]	87.2	3192[c]	77.1
Wyeth contract lab method (0.1 g IncRB $n = 5$)	3311 ± 233[b]	2.3	BQL[c,d]	ND[e]

dpm: Disintegrations per minute, NA: not applicable, the value serves as a theoretical recovery 100%.
[a] The unit is ng-equivalent/g of bone.
[b] A portion of the supernatant from the bone-extraction solvent suspension was used for ^{14}C counting to determine tigecycline concentration.
[c] Another portion of the same supernatant from the bone-extraction solvent suspension was used for LC/MS/MS analysis.
[d] Below quantifiable limit (BQL, 10 ng/g) and no analyte peak was observed.
[e] ND: Not determined. Extraction solvent in this method did not provide high enough levels of tigecycline from the bone extraction supernatant to calculate extraction recovery.

Table 3
Tigecycline concentrations in pooled incurred rat bone samples

Dose group	Tigecycline dose (number of rats)	Time point	Number of replicates in the bone assay	Measured conc. mean ± S.D. (ng/g)	Corrected mean conc.[a] (ng/g)
A, single dose	3 mg/kg ($n = 12$)	Day 1, 4 h	5	1048 ± 88[b]	1359
B, multiple dose	3 mg/kg[c] ($n = 19$)	Day 3, 4 h	5	3192 ± 93[b]	4140

[a] The corrected true concentration in rat bone was calculated using measured concentration dividing by 0.771 (absolute extraction recovery was 77.1%).
[b] The pooled bone sample results were from the first day analysis, $n = 5$.
[c] ^{14}C-tigecycline was administered.

Table 4
Tigecycline activity of samples and diluent determined by microbiological assay

Matrix	Zone size (mm)		
	Sample ($n = 3$)	Diluent control ($n = 3$)	Sample minus diluent
Ground bone (IncRB Group B)[a]	0	0	0
Neutralized bone extract A (pH 7.0)[b]	0	0	0
Neutralized bone extract B (pH 7.0)[c]	0	0	0
Bone extract (non-neutralized) (pH 1.7)[d]	33.3	21.5	11.8

[a] 0.1 g slurry of ground rat bone in saline solution.
[b] The bone extract was from IncRB Group B. Neutralization was done prior to methanol evaporation.
[c] The bone extract was from IncRB Group B. Neutralization was done after methanol evaporation.
[d] The bone extract was from IncRB Group B, methanol was evaporated without neutralization.

3.4. Tigecycline concentrations determined from incurred rat bone

The concentrations of tigecycline in pooled rat bone samples following either a single (Group A) or multiple (Group B, once daily for 3 days) 3 mg/kg dose of tigecycline were determined by the LC/MS/MS bone assay using five aliquots from each pool. These results, as presented in Table 3, showed a mean concentration of 1048 ng/g for Group A (CV 8.4%) and a mean concentration of 3192 ng/g for Group B (CV 2.9%). If the absolute extraction recovery of the bone assay (77.1%) is applied to the measured tigecycline concentrations in rat bone, the estimated concentration of tigecycline in the rat bone is 1359 ng/g for Group A and 4140 ng/g for Group B rat bone pooled sample (Table 3).

3.5. Microbiological activity of tigecycline from extracted rat bone

The results of the tigecycline biological activity test are presented in Table 4. Ground bone pool (Group B IncRB) and neutralized bone extracts did not exhibit biological activity. However, the supernatant of an extracted incurred rat bone sample (Group B, IncRB) and control rat bone (CtrlRB) without neutralization (pH 1.7) showed an average zone of inhibition of 21.5 and 33.3 mm, respectively. The significant difference (11.8 mm) between these sizes of zones of inhibition indicates that biologically active tigecycline is in the sample. The inhibitory activity of the diluents and their effect on the standard curve did not allow for quantitation of the amount of tigecycline.

4. Discussion

The pharmacologic management of bone infections is difficult and systemic antimicrobial therapy alone does not usually eradicate bacteria because of poor drug penetration into bone. Adverse effects are increased when high doses of antibiotics are administered over long durations of treatment. Confounding this issue is the increasing prevalence of highly resistant pathogens [14]. Tigecycline is currently indicated for the treatment of susceptible pathogens isolated from complicated skin, skin structure infections, and complicated intra-abdominal infections. Tigecycline is widely distributed and effectively penetrates bone. It is highly effective against resistant organisms. An expanded indication for infections localized in bone tissue could be explored if accurate assay methods for determining antibiotic concentrations in bone were available.

4.1. Tigecycline extraction and LC/MS/MS detection

The measurement of antibiotic or antimicrobials in bone tissue by an LC/MS/MS methodology requires extracting drug from the bone and detecting the intact molecule in solution using mass spectrometry. Several studies [15–17] have reported the use of various acids, such as hydrochloric acid, the mixture of nitric acid and hydrochloric acid [18], and perchloric acid [19] to dissolve animal bone, human bone, or teeth. A limitation of these methods is their application to the detection of stable inorganic ions only. In these methods, fluoride, phosphate, calcium and other trace metal ions were measured with their respective ion-selective electrodes or atomic absorption methods without an instability issue for the analyte. Using these strong acids to dissolve the rat bone would cause instability of the drug (e.g., tigecycline) and result in difficulties in drug quantification by LC/MS/MS.

In the current method, an extraction solvent containing perchloric acid and phosphoric acid in a methanol/water solution was employed. The unique combination of perchloric acid and phosphoric acid at the specified concentrations in the extraction

solvent was very important in order to achieve dissolution of the bone and detection of the tigecycline. Extracted tigecycline was separated from the bone constituents by liquid chromatography and detected by mass spectrometry. No endogenous interfering peak at the retention time of the analyte and its internal standard was observed. The signal to noise at the LLOQ level (50 ng/g) was approximately 1:5. These results suggest that the LC/MS/MS bone assay is selective for tigecycline quantitation in rat bone. The current bone assay had a LLOQ of 50 ng/g that was equivalent to ~5 ng/ml in the final extracted sample with an injection volume of 20 μl. It was noticed that tigecycline and its internal standard peaks showed fronting, so that attempts to sharp the peak shape using mobile phase modifiers were tried but we did not obtain satisfactory results. Since the slightly asymmetrical peaks did not affect the accuracy of tigecycline quantitation, therefore, the chromatograms were accepted. The wide linear range of 50–10,000 ng/g of the current LC/MS/MS method is suitable for studies where high concentrations of tigecycline are anticipated. One might ask if strong acids in the final injection solution affected the mass spectrometer's response. The effect of different acids in the final reconstitution solution on the response of tigecycline and its internal standard was evaluated. Different acids such as formic acid alone, perchloric acid alone and a combination of phosphoric and perchloric acids (the extraction solvent of the current method) were tested. The results showed that there was no significant difference among these acids in the final reconstitution/injection solutions. This was due to the presence of trifluoroacetic acid in the existing mobile phase and small sample injection volume, which had little or no impact on changing pH or the ion concentration of tigecycline and internal standard in the mass spectrometer's source since perchlorate and phosphate ions went to waste for the first 3 min of the gradient program. The matrix effect of the current method showed an absolute ion enhancement of 39% for tigecycline and 45% for the internal standard. Since quantitation was based on peak area ratio (analyte/internal standard), the variability of the matrix effect was cancelled out by the stable isotope internal standard (matrix factor was 0.997) [20].

4.2. The role of incurred bone sample in the method validation

For insoluble tissue (like bone) assays, an incurred sample from a dosed animal is helpful to monitor the reproducibility of the drug dissolution from incurred bone samples. Since the incurred bone sample pool does not have a nominal value for tigecycline concentration, the so-called nominal concentration (established nominal value) for this pooled sample was based on the mean of fifteen replicate measurements from the incurred sample on each day ($n = 5$) for three days. By evaluating the day-to-day observed concentration of the incurred sample, the reference value of tigecycline in the incurred sample was established (without considering 87.2% absolute extraction recovery) and used as its nominal value. The inter-day precision of the incurred rat bone sample was 7.9% and the overall mean from 3 days ($n = 15$) was 987 ± 78 ng/g (mean ± S.D.).

4.3. The role of ^{14}C-tigecycline in the evaluation of absolute extraction recovery

Bone is a heterogeneous solid tissue. Unlike a plasma or urine sample, the absolute extraction recovery for bone cannot be determined with a drug-spiked rat bone sample. Therefore, it is necessary to measure the actual amount of drug (or a surrogate such as radioactivity) in an incurred rat bone sample to determine the absolute extraction recovery. Using combusted incurred rat bone samples from Group B (obtained from rats administered ^{14}C-tigecycline), the amount of labeled material in bone was measured using liquid scintillation counting (LSC). The recovery of spiked drug in bone suspension (usually 100%) may not equal the amount of drug extracted from incurred bone samples with the extraction solvent (usually < 100% of tigecycline in bone can be extracted out). In the current method, the theoretical amount of the drug in incurred bone (represented by the total radioactivity) was determined by combustion-LSC using bone samples taken from rats dosed with radio-labeled tigecycline (Group B). Based on the measured concentration from the LC/MS/MS bone assay, 77.1% of the parent drug, tigecycline, in the bone was recovered. All concentrations obtained from this bone assay should be corrected by dividing by 0.77 in order to obtain the approximate true concentration of tigecycline in the rat bone.

Since tigecycline is not extensively metabolized by rats or humans in vivo, large quantities of metabolites were not expected in bone. Furthermore, examination of the extraction recoveries for total radioactivity (87.2%) and tigecycline itself (77.1%) indicates that at least 77% of the extracted radioactivity represents tigecycline. In fact, the modest 10% difference between the two values in extraction recovery could be considered as an acceptable level of inter- or intra-methods variation. Collectively, this information suggests that the use of total radioactivity as a surrogate for parent drug in un-extracted bone probably had little impact on the results. In comparison, the extraction recovery from the method used by a contract laboratory, which employed a modified protein precipitation method normally reserved for human serum, was only 2.3% for total radioactivity (parent and metabolites). The LC/MS/MS analysis of the extract obtained using the contract laboratory method did not detect measurable tigecycline in the incurred rat bone sample, which was likely due to the very poor recovery. The low extraction recovery is the likely reason for low concentrations detected in human bone in the previously reported study also [8].

4.4. Stability of tigecycline in extracted samples

Tigecycline was stable for a minimum of 24 h in a neat solution of extraction solvent. The stability of tigecycline in spiked rat bone samples (CtrlRB QCs or validation samples) was found to be much longer than that of incurred rat bone samples. Therefore, all stability tests (freeze/thaw, bench-top, and extracted tigecycline in the extraction solvent) were conducted using incurred bone sample (either from Group A or Group B). The peak area of extracted tigecycline from an incurred

sample in the extraction solvent decreased approximately 50% over a period of 8 h. Data indicate that one or more oxidants from the incurred rat bone samples cause degradation of tigecycline. Fortunately, the stable labeled internal standard had the same degradation profile as the analyte peak during the course of extraction. Therefore, the peak area ratios of tigecycline to internal standard remained the same and the accuracy was not affected as long as the peak areas could be quantified. To ensure peak area sufficiency during the 8-h run time in the autosampler (4 °C), the calibration curve was injected at the beginning of a batch and re-injected at the end of the batch.

5. Conclusions

A novel, simple, and sensitive antibiotic/antimicrobial bone assay for the determination of tigecycline concentrations in rat bone has been developed and validated. To our knowledge, this is the first bone assay to demonstrate high recovery using a LC/MS/MS method. This assay employs homogenized bone tissue added to a strong acid solvent, with subsequent centrifugation of the bone mixture and HPLC separation of tigecycline. Tigecycline was detected and quantified by tandem mass spectrometry. This bone assay results in a very high ($\geq$77%) absolute extraction recovery for tigecycline. It has a wide linear range (50–10,000 ng/g) with high precision (CV $\leq$ 8.7%, n = 15) and accuracy (100 $\pm$ 3.9%). This bone assay meets the analytical needs for the determination of tigecycline in rat bone. In addition, this novel bone assay provides a foundation for the determination of other acid stable drugs in the bones of other species. It has been used as a good foundation for the development of a tigecycline bone assay in humans and will play an important role in the future development of therapeutic indications for tigecycline in infectious diseases of bone. We also demonstrated that tigecycline extracted from rat bone maintained biological activity. Success with this bone assay has set the stage for the design of future clinical trials with tigecycline.

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References

[1] D.M. Livermore, J. Antimicrob. Chemother. 56 (2005) 611–614.
[2] A.K. Meagher, P.G. Ambrose, T.H. Grasela, E.J. Ellis-Grosse, Clin. Infect. Dis. 41 (2005) S333–S340.
[3] C.H. Jones, P.J. Petersen, Drugs Today 41 (2005) 637–659.
[4] I. Chopra, M. Roberts, Microbiol. Mol. Biol. Rev. (2001) 232–260.
[5] D. Stepensky, L. Kleinberg, A. Hoffman, Clin. Pharmacokinet. 42 (2003) 863–881.
[6] N.L. Tombs, I. Chaudary, R. Conant, J. Kantrowitz, Abstract Book of 39th Interscience Conference on Antimicrobial Agents and Chemotherapy (San Francisco), Washinton D.C.: American Society for Microbiology, 1999, p. 302.
[7] A.K. Meagher, P.G. Ambrose, T.H. Grasela, E.J. Ellis-Grosse, Diagn. Microbiol. Infect. Dis. 52 (2005) 165–171.
[8] K.A. Rodvold, M.H. Gotfried, M. Cwik, J.M. Korth-Bradley, G. Dukart, E.J. Ellis-Grosse, J. Antimicrob. Chemother. 58 (2006) 1221–1229.
[9] C.H. Li, C.A. Sutherland, C.H. Nightingale, D.P. Nicolau, J. Chromatogr. B 811 (2004) 225–229.
[10] G. Muralidharan, M. Micalizzi, J. Speth, D. Raible, S. Troy, Antimicrob. Agents Chemother. 49 (2005) 220–229.
[11] J.E. Conte Jr., J.A. Golden, M.G. Kelly, E. Zurlinden, Int. J. Antimicrob. Agents 25 (2005) 523–529.
[12] J.D. Smilack, W.H. Flittie, T.W. Williams, J.R. Antimicrob, Agents Chemother. 9 (1976) 169–171.
[13] A.J. Ji, J.P. Saunders, P. Amorusi, N.D. Wadgaonkar, M. Leal, E.N. Fluhler, Abstract Book of 46th Interscience Conference on Antimicrobial Agents and Chemotherapy (San Francisco), Washinton D.C.: American Society for Microbiology, 2006, p. 32.
[14] H. Winkler, O. Janata, C. Berger, W. Wein, A. Georgopoulos, J. Antimicrob. Chemother. 46 (2000) 423–428.
[15] J.T. Elliston, S.E. Glover, R.H. Filby, J. Radioanal. Nucl. Chem. 263 (2005) 301–306.
[16] A. Demirbas, Y. Abali, E. Mert, Resour. Conserv. Recycl. 26 (1999) 251–258.
[17] M. Christgau, K.A. Hiller, G. Schmalz, C. Kolbeck, A. Wenzel, J. Periodontal Res. 33 (1998) 138–149.
[18] N.B. Roberts, H.P.J. Walsh, L. Klenerman, S.A. Kelly, T.R. Helliwell, J. Anal. At. Spectrom. 11 (1996) 133–138.
[19] H. Zakrzewska, A. Machoy-Mokrzynska, M. Materny, I. Gutowska, Z. Machoy, Arch. Oral Biol. 50 (2005) 309–316.
[20] C.T. Viswanathan, S. Bansal, B. Booth, A.J. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, R. Weiner, AAPS J. 9 (2007) E30–E42.