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A sensitive human bone assay for quantitation of tigecycline using LC/MS/MS

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ABSTRACT

Tigecycline (Tygacil®, Wyeth) is a first-in-class, broad spectrum antibiotic with activity against multiple-resistant organisms. In order to address the unexpectedly low tigecycline concentrations in human bone samples analyzed using a LC/MS/MS method developed elsewhere, we have developed and validated a new and sensitive human bone assay for the quantitation of tigecycline using LC/MS/MS. The new method utilizes the addition of a stabilizing agent to the human bone sample, homogenization of human bone in a strong acidic-methanol extraction solvent, centrifugation of the bone suspension, separation by liquid chromatography, and detection of tigecycline by mass spectrometry. Linearity was demonstrated over the concentration range from 50 to 20,000 ng/g using a 0.1 g human bone sample. The intra- and inter-day accuracy of the assay was within 100 ± 15%, and the corresponding precision (CV) was <15%. The stability of tigecycline was evaluated and shown to be acceptable under the assay conditions. The extraction recovery of tigecycline with the current method was 79% when using radio-labeled rat bone samples as a substitute for human bone samples. Twenty-four human bone samples collected previously from volunteers without infections who had elective orthopedic surgery after receiving a single dose of tigecycline were re-analyzed using the current validated method. Tigecycline concentrations in these samples ranged from 238 to 794 ng/g with a mean value 9 times higher than the mean concentration previously reported. The data demonstrated that the current method has significantly higher extraction efficiency than the previously reported method.

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1. Introduction

Tigecycline (Tygacil®, Wyeth, formerly GAR-936, chemical structure in Fig. 1) is a first-in-class glycylcycline antibiotic [1], on the United States market since June 2006. This broad-spectrum agent has activity against a wide range of Gram-positive, Gram-negative, atypical, anaerobic, and antibiotic-resistant bacteria. The penetration of many antibiotics into human bone for therapeutic use has been reported [2–11], but interpretation of the results is difficult as previous methods showed large variations in extraction recoveries. In humans, tigecycline is widely distributed in the body and has a long half-life [12]. A high degree of penetration of tigecycline into rat bone was reported in a radio-labeled tissue distribution study conducted in rats [13]. The ratio of rat bone exposure ($AUC_{0-\infty}$) to plasma exposure ($AUC_{0-\infty}$) was about 200 using a ^{14}C-tigecycline liquid scintillation counting (LSC) method. Therefore it was predicted that human bone would also have higher tigecycline concentrations compared with serum. However, the results from a 100 mg intravenous single dose clinical study [14] showed low concentrations of tigecycline in bone relative to serum and the exposure ($AUC_{0-\infty}$) ratio of human bone to serum was only 0.41. In the current study, we conducted a series of experiments to help determine if the previously observed low concentrations in bone were related to the bone assay [14]. Numerous methods have been used in an attempt to quantitate antibiotic levels in human bone. These reported methods can be divided into two types: one is high performance liquid chromatography (HPLC) [14–18] with different types of detection such as ultra-violet (UV), fluorescence spectrometry, or mass spectrometry (MS) and the other type is microbiological diffusion [5,19–24]. Both types of methods require sample extraction. The interpretation of antibiotic concentration is difficult as both types of methodology vary significantly due to low extraction recovery of the drug from bone. The majority of the HPLC extraction methods use acetonitrile for the extraction solvent. This acetonitrile-based extraction method is frequently used to analyze plasma or serum, however, acetonitrile cannot effectively dissolve the drug from the bone sample. Therefore, the extraction is inefficient and results in a low extraction recovery.

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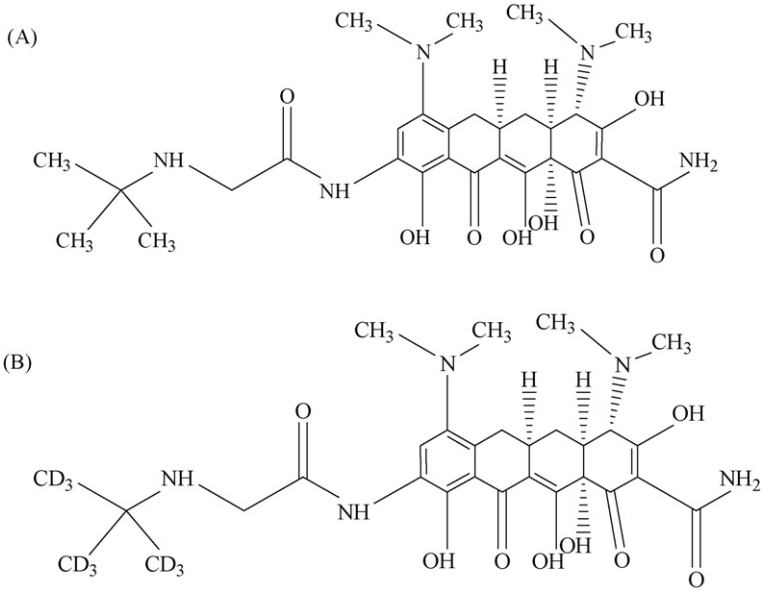

Fig. 1. The chemical structure of (A) tigecycline, molecular formula: $C_{29}H_{39}N_5O_8$, molecular weight 585.65 and (B) [*t*-butyl-d_9]-tigecycline (internal standard), molecular formula: $C_{29}H_{30}D_9N_5O_8$, molecular weight 594.70.

The previous method [14] for human bone tigecycline concentration was an acetonitrile-based extraction. Many microbiological diffusion methods reported the use of neutral phosphate buffer (pH 6.5–6.8) to soak the pulverized bone sample and then incubate the extracted tigecycline sample with bacteria-seeded gel for diffusion. The size of the diffusion diameter is then plotted against the tigecycline concentrations in a calibration curve. Again, the neutral buffer is only useful for bacteria–antibiotic binding in agar gel but is not effective for antibiotic extraction from bone. To develop a better method for human bone, we first developed and validated a more sensitive method for rat bone containing ^{14}C-tigecycline using an extraction solvent of perchloric acid, phosphoric acid, and methanol that resulted in a significantly higher recovery of tigecycline [25]. Using a similar methodology, modified slightly by the addition of a stabilizing agent, the human bone assay was subsequently developed and validated. The absolute extraction recovery of the human bone assay was estimated using the same extraction procedure to extract incurred rat bone samples from rats that received multiple ^{14}C-tigecycline doses. Absolute extraction recovery of the human bone assay could not be determined due to the difficulties of obtaining human bone samples with ^{14}C-tigecycline. This method has significantly higher extraction recovery due to the strong acidic extraction solvent used. The first objective of the current study was to develop an assay with an optimized extraction scheme for human bone samples. The second objective was to test the applicability of the newly developed antibiotic bone assay on previously collected bone samples obtained from uninfected volunteers administered single doses of tigecycline prior to elective orthopedic surgery. Future applications of this novel antibiotic bone assay will provide a new standard for human bone assays, not only for tigecycline, but also for other antibiotics and antimicrobials posing 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%) internal standard and ^{14}C-tigecycline (purity 96.0%) were synthesized by Wyeth Research, Radiosynthesis Group (Pearl River, NY). Methanol (HPLC grade), acetonitrile (HPLC grade), sodium bisulfite were purchased from EM Sciences (distributed by VWR Scientific Products, Newark, NJ). Oxalic acid dehydrate was from EMD Chemicals Inc. (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). L-Ascorbic acid and EDTA disodium salt dehydrate were purchased from J.T. Baker (Philipsburg, 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 Reverse Osmosis De-ionized Water System (GE Osmonics, Madison, WI). Control human bone was purchased from IIAM (Jessup, PA).

2.1.2. Solutions

Primary tigecycline stock solution (100,000 ng/ml) and primary internal standard (100,000 ng/ml) were prepared by adding 10 mg of tigecycline or [*t*-butyl-d_9]-tigecycline (weight corrected for purity) into a 100 ml low-actinic volumetric flask, filled to volume with methanol, mixed well and stored at −20 °C. The extraction solvent was prepared by adding an aqueous solution of perchloric acid (0.21 M) and phosphoric acid (0.14 M) at pH 0.9 to methanol at a ratio of 50:50 (v/v); mobile phase A consisted of deionized water, acetonitrile, methanol, and trifluoroacetic acid at ratios of 95.5:3.5:1:0.1 (v/v/v/v); mobile phase B was prepared in a volume ratio of methanol to acetonitrile of 22.2:77.8 (v/v).

2.1.3. Equipment

The tissue homogenizer (Kinematica Polytron® PT 10–35) and probe (Kinematica Polytron Aggregate® 7 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 mm × 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 PerkinElmer Life Sciences (Downers Grove, IL). ^{14}C-methyl methacrylate was purchased from Dupont Merck Pharmaceutical Corporation (Billerica, MA). The triple quadrupole mass spectrometer, model Sciex API 4000, was manufactured by Applied Biosystems (Toronto, Canada). The HPLC column (MetaChem Polaris C18-A 3 μm, 50 mm × 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. Clinical dosing and sample collection

The clinical trial has been described in Rodvold's paper [14]. Briefly, uninfected patients scheduled for elective surgical procedures received single 100-mg doses of tigecycline at varying times prior to their procedures. Tigecycline was measured in serum immediately after the infusion as well as in bone after collection of the surgical specimens. This current report focuses on re-analysis of the bone samples only.

Twenty-four human bone samples were collected from patients who underwent knee or hip replacement, rotator cuff repair, or surgery for shoulder endoprosthesis after administration of an intravenous infusion of a single dose of 100 mg tigecycline administered over 30 min. The collection time points were 4 (±2), 8 (±2), 12 (±2), or 24 (±2) h after the start of the infusion. Twenty to seventy grams of bone tissue were collected from each subject and stored in either 50 or 200-ml polypropylene containers at −70 °C until analysis.

These bone samples were collected during the period spanning from 11 November 2003 to 08 February 2005 and were originally analyzed and published using an LC/MS/MS method [14]. Due to possible underestimated concentrations found, these bone samples were subsequently shipped to our laboratory (Bioanalytical R & D, Wyeth Research, Pearl River, NY). The bone samples from the 24 subjects were analyzed in our laboratory on 23 March 2006 and 29 March 2006. The method and bone concentrations are reported in the current article.

2.2.2. Sample preparation

2.2.2.1. Incurred Human Bone Sample Preparation. Incurred human bone (IncHB) is defined as bone harvested and prepared from volunteers administered the study drug. Each incurred bone sample (20–70 g) was removed from a −70 °C freezer, quickly added liquid nitrogen to the bone sample in a mortar and waited for 1–2 min until liquid nitrogen became gas. The large piece bone sample was pulverized with a pestle. The smaller pieces of bone sample were ground for approximately 2–3 min in an industrial blender to produce bone particles of approximately 1 mm or less in diameter and stored at −70 °C for later analysis.

2.2.2.2. Control Human Bone Sample Preparation. Control human bone (CtrlHB) was purchased from a commercial source and prepared in the same manner as the IncHB.

2.2.3. Preparation of bone calibrators and control samples

Tigecycline working standard solutions: tigecycline working standard solutions (100, 200, 1000, 10,000, 35,000, and 40,000 ng/ml) were prepared daily from a stock solution (100,000 ng/ml in methanol) with appropriate dilution using 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.

CtrlHB calibrators (human bone standards), quality control/validation samples (CtrlHB QC), and incurred human bone quality control/validation samples (IncHB QC) were prepared as follows: Tigecycline CtrlHB calibrators: Approximately 0.1 g of CtrlHB was weighed, and then 120 μl of 0.10 M L-ascorbic acid solution was immediately added to the bone sample and incubated for approximately 5 min at room temperature. One milliliter of the extraction solvent was added to the above bone sample to create a mixture of bone and solvent. To prepare a range (50–20,000 ng/g) of CtrlHB calibrators, 50 μl of each respective tigecycline working standard solution was spiked into this mixture for each calibrator. Calibrators were prepared daily.

Tigecycline CtrlHB quality controls (or validation samples): Approximately 0.1 g of CtrlHB was weighed, and then 120 μl of 0.10 M L-ascorbic acid solution was immediately added to the bone sample and incubated at room temperature for approximately 5 min; 1.0 ml of the extraction solvent was added to the above bone sample to form a mixture of bone and solvent. To prepare a range of low (150 ng/g), mid (2500 ng/g), and high (15,000 ng/g) concentrations of tigecycline quality control (QC) samples, 50 μl of each 300, 5000, or 30,000 ng/ml of the tigecycline working solutions was spiked into this mixture for each respective QC sample. QC samples were prepared daily.

Tigecycline IncHB validation sample: Approximately 0.1 g of IncHB (ground incurred human sample from one volunteer with a relatively large amount of sample) that had been stored at −70 °C, was thawed, weighed, and then 120 μl of 0.10 M L-ascorbic acid solution was immediately added to the bone sample and incubated at room temperature for approximately 5 min; 1.0 ml of the extraction solvent was added to the bone sample 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 CtrlHB calibrators, CtrlHB QC, or validation samples.

2.2.4. Selection of stabilizing agent

One milliliter of extraction solvent, 50 μl of 10,000 ng/ml tigecycline standard solution, and 40 μl of working internal standard were added to a 2 ml HPLC vial, then the mix of 0.1 g of ground control human bone with 100 μl of a stabilizing agent after incubation for 5 min at room temperature was quickly added to the same vial. The above sample mixture was mixed and centrifuged. The supernatant of extracted sample was transferred to a 2-ml glass vial, and then the vial was placed on an HPLC autosampler at 4 °C. A 20 μl aliquot of the supernatant was injected onto the LC/MS/MS system to determine the peak ratio of tigecycline to the internal standard at different times (from 0 to 13 h after addition of the stabilizing agent). Several candidates for stabilizing agents, water (control for stabilizing agent), 0.5 M sodium bisulfite, 0.5 M oxalic acid, 0.5 M L-ascorbic acid, and 0.010 M disodium ETDA were tested in the same manner, respectively. Peak ratios versus time (hours after addition of stabilizing agent) were plotted. After identifying the best stabilizing agent, the optimum concentration of the stabilizing agent was evaluated in the same way.

2.2.5. Extraction procedure

Aliquots of approximately 100 mg of prepared IncHB or CtrlHB samples were accurately weighed and placed into 17 mm × 100 mm polypropylene tubes. Then 120 μl of 0.10 M L-ascorbic acid was added to the ground bone sample and incubated for 5 min at room temperature. Next 1 ml of extraction solvent, 50 μl of each tigecycline working standard solution (or 50 μl of methanol for study samples or IncHB 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 (with multiple 1 mm

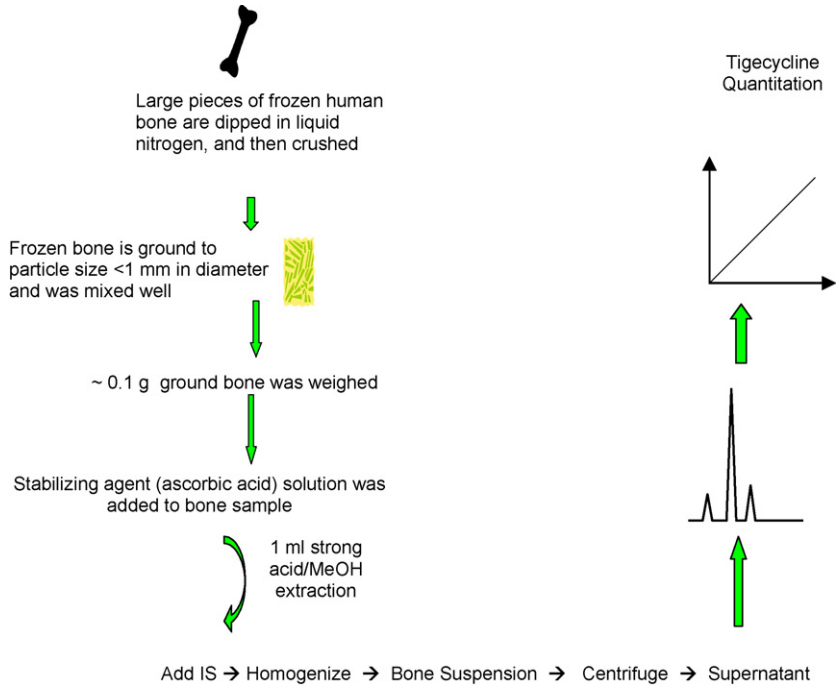

Fig. 2. Scheme of the extraction procedure for human bone samples.

sharp slits) 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 1–2 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 the probe for approximately 30 s, then immersion in 2 ml of methanol, operating the probe for approximately 30 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 determination of tigecycline concentration. A scheme of the extraction procedure is presented in Fig. 2.

2.3. Method validation

2.3.1. Precision and accuracy

Five replicates of each tigecycline validation sample (low, mid, and high) and the IncHB validation sample were analyzed with a human bone standard curve (6 standard 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 test

Stability tests for tigecycline in an incurred human bone sample were conducted in three sequential freeze/thaw bone steps (1–3 cycles, −70 °C/22 °C), over a 6-h period sitting on the bench-top at room temperature (22 °C), and after 313 days at −70 °C. Fifty-four hour bone extract stability of tigecycline was also evaluated with IncHB validation sample in a 4 °C autosampler. All stability tests were conducted using an IncHB sample with a relative large quantity.

2.3.3. Extraction recovery

In the previously published rat bone method [25], we reported the method used to obtain absolute extraction recovery for the rat bone assay. Samples from the same group of rats (Group B) were used for this experiment. Briefly, rats were dosed intravenously with multiple dose (bolus) of 3 mg/kg/day ^{14}C-tigecycline for 3 days and incurred bone samples were collected on day 3, 4 h post-dose. Five aliquots of pooled incurred rat bone sample (0.1 g each) were accurately weighed, placed into combustion cones, and allowed to air dry for approximately 3 days at room temperature. These 5 replicate samples were then oxidized in a Model 307/Oximate 80 sample oxidizer and counted in a Packard (PerkinElmer) liquid scintillation counter (LSC) using a toluene standard curve. The ng-equiv/ml concentrations were calculated using the specific activity of the dosing solution. In parallel, five aliquots of the incurred rat bone sample (0.1 g) were accurately weighed. The samples were extracted using the human bone extraction procedure described in Section 2.2.5. For the final supernatant, a portion (100 μl) was analyzed by liquid scintillation counting and another portion (20 μl) was injected onto LC/MS/MS for the determination of tigecycline parent drug concentration. The extraction recovery was calculated using the measured tigecycline concentration with LC/MS/MS divided by the concentration measured by the combustion and liquid-scintillation counting (LSC) method using the same pooled rat bone sample. Due to the feasibility of obtaining rat bone samples, the extraction recovery of human bone assay was estimated using a similar procedure with ^{14}C-tigecycline rat bone samples. The absolute extraction recovery (AER) was determined using the following equation:

$$\text{AER for parent drug (\%)} = \left[\frac{\text{total amount (ng) of tigecycline per gram of bone (determined by LC/MS/MS)}}{\text{total amount (ng equivalence) of tigecycline per gram of bone (determined by combustion-LSC)}}\right] \times 100$$

2.3.4. Cross-validation using rat bone calibration curve

To evaluate if human bone can be replaced with rat bone in the preparation of calibration curves and QCs, control rat bone was used as the biological matrix. The rat bone calibration curve was used to determine tigecycline concentration of the pooled incurred rat bone sample (5 aliquots, $n = 5$) from our previous rat bone study [25] and also in an incurred human bone sample ($n = 5$).

2.4. Determination of tigecycline concentrations in incurred human bone samples

Twenty-four human bone samples (0.1 g aliquots) were analyzed using the currently reported method. In each run, six CtrlHB calibrators ranging from 50 to 20,000 ng/g, six CtrlHB Quality control samples (150, 2500, and 15,000 ng/g, each in duplicate), four control human bone samples, and 12–20 study samples were extracted and analyzed with the currently reported method.

2.5. HPLC instrumentation

Separation procedures were carried out on a 50 mm × 2.0 mm (I.D., 3 μm particle size) analytical HPLC 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 refrigerated autosampler, an in-line degasser, and a quaternary solvent delivery system. The analytical column temperature was set at approximately 20 °C and 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 a gradient program as follows: 0–1 min: 100–100% mobile phase A (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.

2.6. 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 quadrupole Sciex API 4000 mass spectrometer was operated under positive electrospray ionization mode (ESI^+) in multiple reaction monitoring (MRM) mode. The optimal ionization conditions were tuned by infusing a 1 μg/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: duration 10.004 min, cycle time 0.41 s, number of cycles 1464, scan type positive MRM, Q1 resolution at low and Q3 at low, intensity threshold 0 cps, settling time 0 m/s, MR pause 5.007 m/s, curtain gas setting at 10.0, ion source temperature 400 °C, a nitrogen pneumatically assisted (software setting GS 1:35, GS 3:60) electrospray nebulizer set at 5000 V, collision energy cell setting at 8.0 (software setting CAD 8.0), and electronic multiplier 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 the following parameters: m/z 586.3 → 513.3 for tigecycline, m/z 595.4 → 514.3 for [t-butyl-d_9]-tigecycline, 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 ms for both the analyte and the internal standard.

2.7. Data analysis

Analyst software (Applied Biosystem, 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 (version 7.0.0.01). Six standards in duplicate that were injected at the beginning and re-injected at the end of the run 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 batch acceptance criteria for the rat bone standards were as follows: at least 75% of calibration standards (9 out of 12) must be within 100 ± 15% of their nominal values, except the lowest standard, which must be within 100 ± 20% of its nominal value. For the batch acceptance QC samples (CtrlHB QCs), CtrlHB QCs must have at least 4 out of 6 QCs be within 100 ± 15% of their nominal values. Two failed QCs samples cannot be at the same concentration. For stability data, if the difference between the initial and a stability time point is ±20%, the tigecycline is considered to be stable in that matrix.

3. Results

3.1. Analytical performance of the human bone assay

A linear relationship between the peak area ratios of tigecycline to internal standard versus human bone tigecycline concentrations was observed from 50 to 20,000 ng/g. Tigecycline concentrations (ng/g) were obtained using a $1/x$ weighted linear regression analysis of the 12 calibration standards (6 extracted standards injected in duplicate). Representative chromatograms of control human bone extracts, bone standards at the lower limit of quantitation (50 ng/g), and the incurred human bone validation sample (257 ng/g) are shown in Fig. 3A–C, respectively. The retention time of tigecycline was about 4.5 min. A typical human bone calibration curve (50–20,000 ng/g) is shown in Fig. 4. All standard curves from the three validation runs had coefficients of determination (r^2) ≥0.9952. The lower limit of quantitation (LLOQ) of this method was 50 ng/g (CV 4.3%, accuracy 111.7%, $n = 5$), which was equivalent to ~5 ng/ml of tigecycline in extraction solvent. The intra- and inter-day precision was expressed in terms of the coefficients of variation within a batch and among batches using spiked CtrlHB validation samples, at three different concentrations (150, 2500, and 15,000 ng/g) and an IncHB validation sample (257 ng/ml, Table 1). The nominal value for the IncHB validation sample was determined from the overall mean of the 3-day validation. The intra-day accuracy for all types of validation samples including LLOQ ranged from 96.1 to 111.7% with precision (CV) ranging from 1.6 to 8.4%. Inter-day accuracy ranged from 97.6 to 100% with a CV range from 3.7 to 7.9%.

3.2. Stability of tigecycline

The stability of tigecycline was evaluated using the incurred human bone sample. Results showed that tigecycline was stable in incurred human bone after 3 cycles of freeze/thaw, and after 6 h

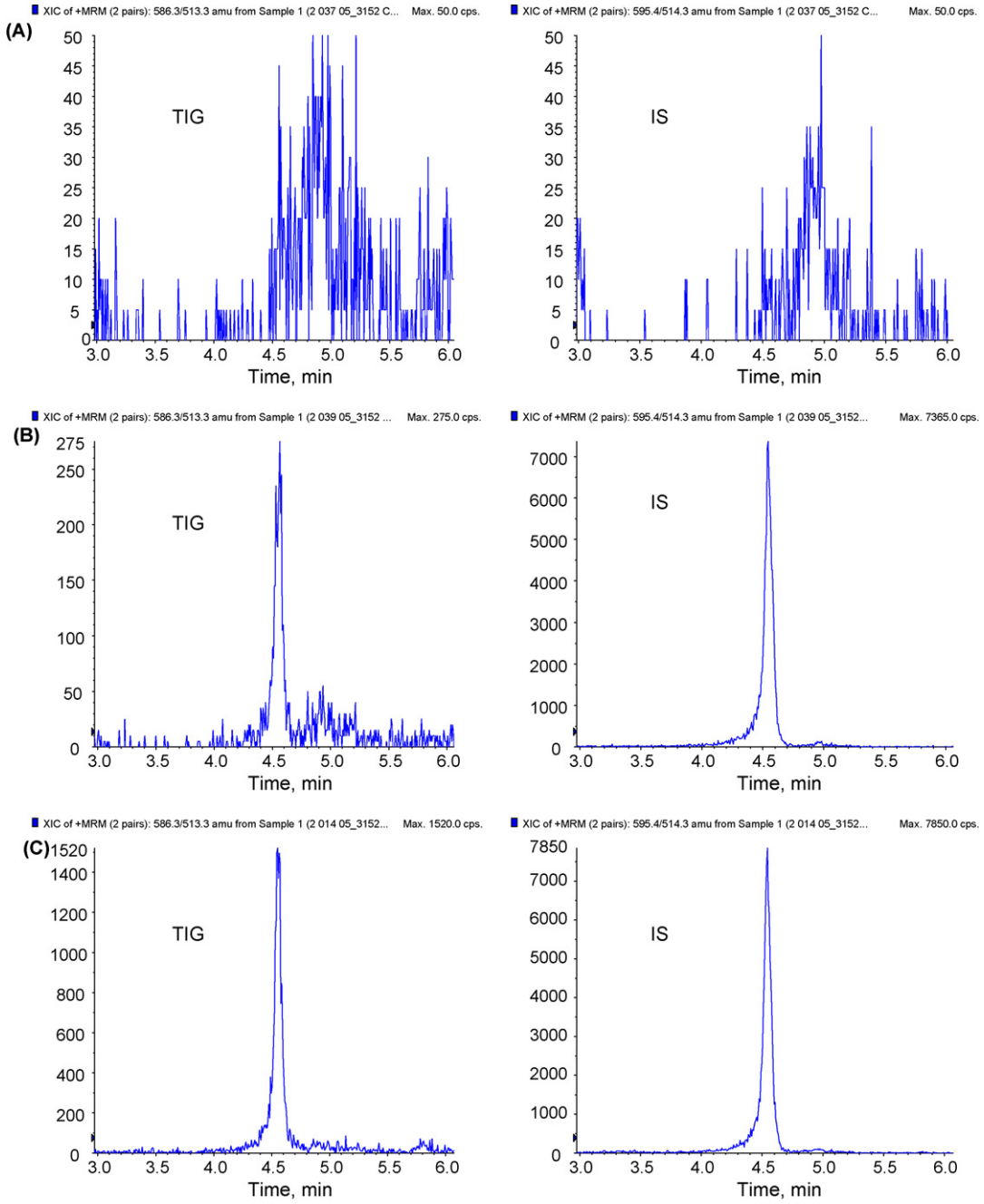

Fig. 3. Representative chromatograms of (A) blank human bone sample without IS; (B) lowest bone standard, 50 ng/g; (C) an incurred human bone sample, observed concentration was 257 ng/g.

at room temperature. Tigecycline was stable in human bone for at least 313 days after being stored at −70 °C. After addition of stabilizing agent, L-ascorbic acid, to the incurred human bone sample, extracted tigecycline was stable for 54 h at 4 °C (Table 2).

3.3. Cross-validation with rat bone standards

The mean concentration of tigecycline in the incurred human bone sample was 284 ng/g ($n = 5$) with a CV of 7.9% using the rat bone calibration curve. The difference from the inter-day mean (257 ng/g) for the incurred sample (% bias) using the human bone calibration curves was 10.5% (Table 3). The results demonstrate that the rat bone calibration curve is equivalent to the human bone calibration curve. This points out the future potential for using rat bone to prepare calibration standards and QC samples for analysis of human bone samples. The mean concentration of tigecycline in the pooled ^{14}C-tigecycline rat bone sample was 3400 ng/g ($n = 5$) with a CV of 3.8% using the rat bone standard curve (Table 3). The results for this incurred rat bone sample were very similar to the results obtained in the rat bone assay validation five months earlier.

3.4. Absolute extraction recovery

In the human bone assay, the addition of a stabilizing agent (L-ascorbic acid) to the human bone prior to adding the extraction solvent was the only difference in the extraction procedure

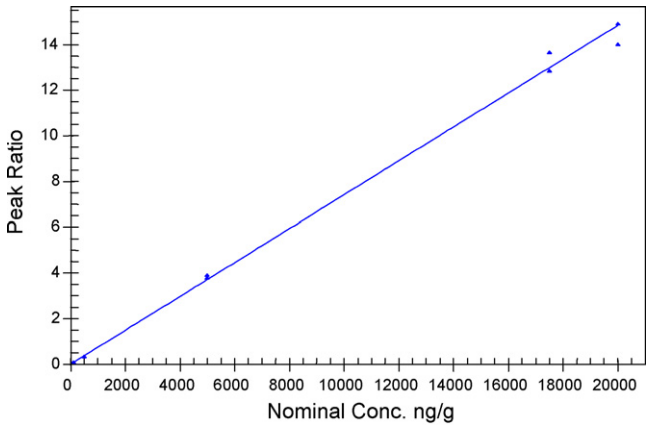

Fig. 4. Typical tigecycline calibration curve in CtrlHB. *Y*-axis is the peak ratio of tigecycline to internal standard and *X*-axis is the concentration of tigecycline in ng/g of human bone sample.

from the rat bone method [25]. The absolute extraction recovery of tigecycline from rat bone was 79.1% using the currently reported human bone method. This value serves as an estimate of the extraction recovery for the current human bone assay. The results are summarized in Table 4.

3.5. Optimum stabilizing agent

The peak area ratios of tigecycline to internal standard versus time are presented in Fig. 5. Sodium EDTA and oxalic acid did not have sufficient stabilizing ability since the tigecycline peak disappeared after 2–5 h. Sodium bisulfite and L-ascorbic acid were able to stabilize the tigecycline peak for at least 13 h. However, since the addition of sodium bisulfite in the solution resulted in an asymmetrical peak shape for tigecycline, L-ascorbic acid was chosen to be the best stabilizing agent for tigecycline bone extraction among the tested candidates.

3.6. Tigecycline concentrations from 24 human bone samples

Using this bone assay, the concentrations of tigecycline from 24 patient samples ranged from 238 to 794 ng/g. These bone samples were previously assayed with an acetonitrile-based extraction method [14]. Data from both laboratories are presented in Table 5. On average, the concentration of tigecycline from the present

Table 2
Stability data of tigecycline in incurred human bone

Stability tests	Mean concentration (ng/g)	Difference (%)
Baseline ($n=5$)	257	0
After 3 cycles freeze/thaw	247	−8.3
After 6 h at room temp. ($n=5$)	273	7.2
After 54 h in sample extract at 4 °C ($n=5$)	253	−0.7
After 313 days at −70 °C ($n=5$), baseline value was 270 ng/g[a]	219	−19.0

Note: Difference (%) = (mean of stability data − mean of baseline data)/mean of baseline data × 100%. If the %difference from the baseline results is <±20%, the drug is considered to be stable in the stability timeframe.

[a] This incurred sample was freshly collected at the time of analysis.

Table 1
Precision and accuracy of the LC/MS/MS human bone assay for determination of tigecycline concentration

Validation sample (concentration, ng/g)	LLOQ (50)	Low (150)	Mid (2500)	High (15,000)	IncHB (257)[a]
Intra-day precision (%CV, $n=5$/day for 3 days)	4.3	2.0–6.8	1.6–4.5	2.8–4.3	6.6–8.4
Intra-day accuracy (%Bias, $n=5$/day for 3 days)	111.7	97.7–99.0	98.3–99.1	96.9–101.4	96.1–105.1
Inter-day precision (%CV, global, $n=15$)	NA	4.8	3.7	3.8	7.9
Inter-day accuracy (%Bias, overall, $n=15$)	NA	97.6	100.0	99.2	100.0

[a] Since there was no theoretical value for the incurred human bone sample, the nominal value was obtained from the mean of the 3-day inter-day validation data ($n=15$).
NA: not applicable, only one run was conducted.

Table 3
Cross-validation of human bone assay using rat bone calibration curve

Validation sample (concentration, ng/g)	Low[a] (150)	Mid[a] (2500)	High[a] (15,000)	IncHB (257) [b]	IncRB[c] (3192)[d]
Mean	141	2386	14642	284	3400
CV (%)	5.7	4.7	5.5	7.9	3.8
Bias (%)	−5.8	−4.5	−2.4	10.5	6.5
n	5	5	5	5	5

[a] The appropriate tigecycline solution was spiked into human control bone (CtrlHB).
[b] The nominal value of the human incurred bone sample was obtained from the mean of 3 intra- and inter-day analytical runs ($n=15$) during the validation.
[c] Incurred rat bone (IncRB) sample was from the previous rat study [25] and the rats were dosed with ^{14}C-tigecycline.
[d] The nominal value of the incurred rat bone was from an intra-day validation run ($n=5$) when the rat bone sample was originally collected. The method used to obtain this nominal value was from the original rat bone assay [25] without addition of stabilizing agent during the extraction at that time.

Table 4
Absolute extraction recovery of tigecycline from incurred ^{14}C-tigecycline rat bone sample

Methodology	Measured ^{14}C counts (dpm/g)	Measured concentration (ng/g)	Extraction recovery (%) using concentration[b]
Combustion-LSC (0.1 g IncRB, $n=5$)	146872 ± 4561	4296[a]	NA
Current LC/MS/MS Bone Assay (0.1 g IncRB $n=5$)	131001 ± 8595[c]	3400	79.1

Note: the unit for liquid scintillation counting is disintegrations per minute per gram of bone (dpm/g). The unit for LC/MS/MS is ng/g.
[a] The unit is ng-equivalent/g of bone which was converted by a liquid scintillation counting standard curve.
[b] Extraction recovery% = (conc. obtained by LC/MS/MS)/(conc. measured by combustion and LSC) × 100.
[c] A portion of the supernatant (100 μl) from the acidic extraction was analyzed by liquid scintillation counting (LSC), dpm following extraction, then corrected for total volume of the supernatant.

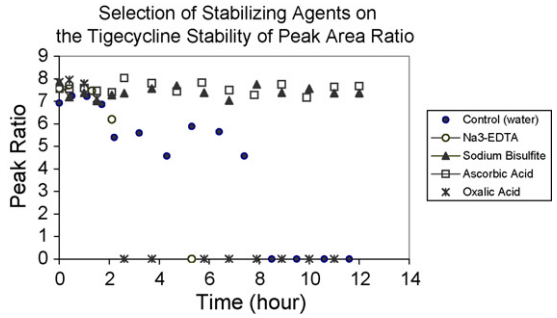

Fig. 5. Stability of tigecycline peak ratios in human bone supernatant in the presence of different stabilizing agents.

Table 5
Concentrations of tigecycline measured from incurred human bone samples: The previously reported method versus the improved Wyeth method

Subject ID	Contract lab results (ng/g)[b]	Wyeth bone assay results (ng/g)[c]	Ratio of Wyeth result to contract lab result[d]
00004	41.6	626	15.0
00006	46.5	281	6.05
00007	33.3	439	13.2
00008	79.3	794	10.0
00010	141	492	3.49
00012	<33.2[a]	550	16.6
00017	93.3	665	7.12
00020	269	431	1.60
00022	50.0	628	12.6
00024	<33.2[a]	269	8.10
00027	<33.2[a]	290	8.73
00030	<33.2[a]	335	10.1
00033	<33.2[a]	238	7.17
00038	<33.2[a]	240	7.23
00046	<33.2[a]	433	13.0
00048	<33.2[a]	560	16.9
00052	35.5	362	10.2
00053	<33.2[a]	492	14.8
00054	36.1	491	13.6
00057	139	595	4.28
00065	86.8	258	2.97
00075	33.3	323	9.69
00100	33.7	421	12.5
00108	148	411	2.78
Mean	65.2	443	9.49
S.D.	±57.5	±151	±4.52
n	24	24	24
Range	<33.2–269	238–794	

[a] The lower limit of quantitation (33.2 ng/g) value was used in the statistics calculation in the last column.
[b] The previous contract lab analysis period: 14 July 2004 to 9 March 2005, using the method in reference [14].
[c] Wyeth Analysis Dates: 23 March 2006 and 29 March 2006.
[d] Ratio = Wyeth bone assay result/contract lab bone assay result.

method are approximately 9 times higher than those reported earlier even though these bone samples had been stored at −70 °C for 13–28 months at the time of our analysis. The ratios of the new assay results to the acetonitrile-based bone method results for each individual sample ranged from 1.6 to 16.9.

4. Discussion

Tigecycline is currently indicated for use against susceptible pathogens isolated from complicated skin structure infections, and complicated intra-abdominal infections [12] and is highly effective against resistant organisms. Tigecycline is widely distributed in tissues and effectively penetrates bone in an animal model [13]. An expanded indication for infections localized in bone tissue could be explored if an accurate assay method for determining antibiotic concentrations in human bone were available. In the present study, we report a sensitive tigecycline assay for human bone with a high extraction recovery. The quantitation of tigecycline in human bone by LC/MS/MS required an improved extraction procedure due to the heterogeneity of bone tissue compared to biological fluids, the relatively tight binding of tigecycline to bone, the insolubility of bone matrix, and the instability of tigecycline in various extraction solvents. A previously developed assay method for tigecycline in bone used a similar extraction solvent (0.1% trifluoroacetic acid in acetonitrile) for the bone samples as that used for serum analysis. However, this solvent proved to be much less efficient in extracting tigecycline from the incurred bone samples. Since the degree of extraction recovery is dependent on the bone's particle size, the improved method also employed a more rigorous and labor-intensive grinding procedure. This process, coupled with a better extraction solvent and the addition of L-ascorbic acid as a stabilizing agent during the extraction procedure, contributed to the higher recovery of tigecycline using the improved method.

During the development of the human bone assay, we were unable to detect spiked tigecycline in ground control human bone when using the previously developed rat bone method. This may be because human bone contains more oxidative components than rat bone. As a general phenomenon, the extraction solvent destroys tigecycline in both rat bone and human bone during the extraction. However, the rat bone extraction process has a much slower decomposition rate for tigecycline than that in human bone. Tigecycline is easily oxidized or decomposed by various oxidative components in human bone during sample processing. Therefore, finding an optimal stabilizing agent was one of the key requirements to succeed in this human bone assay. The rationale for the selection of the stabilizing agents is to add a reducing agent such as ascorbic acid, oxalic acid, or sodium bisulfite to react with the oxidative components in human bone first so that it prevents tigecycline from being oxidized or decomposed. Therefore, tigecycline can be detected in the processed sample. Another thought is that many oxidation reactions need trace metal ion as a catalyst. EDTA can chelate many trace metal ions to form complexes so that oxidation reactions can be stopped or slowed down. Sodium ETDA was also chosen as a stabilizing agent candidate. The results showed that L-ascorbic acid was the best stabilizing agent among the several tested. Other reducing agents such as sodium bisulfite, oxalic acid or chelating agent (sodium ETDA) resulted in either the tigecycline peak shape to be asymmetrical or lower peak response. The sequence order of adding stabilizing agent and extraction solvent to the bone sample was also critical. The stabilizing agent must be added to the bone sample first, then incubated for 5 min, finally, the extraction solvent was added to the ground bone sample. The reason is that the oxidative components may react with ascorbic acid first, and then the strong acidic extraction solvent destroys the drug–bisphosphonate conjugation bonds [27] to release tigecycline from the bone.

The repeat analysis of the 24 human bone samples showed higher tigecycline concentrations than the results obtained with the previous LC/MS/MS method [14]. The significantly higher tigecycline concentrations in human bone verified that the previous acetonitrile-based extraction method had low extraction recovery. In our previously reported rat bone method [25], the acetonitrile-based extraction method showed 2.3% extraction recovery using a ^{14}C-tigecycline rat bone sample, while the acidic extraction method (without a stabilizing agent) showed 77% extraction recovery for tigecycline from the same rat bone sample. It should be clarified that there is not a direct linear relationship between the degree of extraction recovery improvement (from 2.3% to 77%, rat bone results) and the degree of extraction recovery improvement for

human bone results obtained from the two human bone methods (9-fold difference). The main reasons for the low extraction recovery in the previous method [14] could be due to: (a) the acidic acetonitrile solution used for the extraction solvent; and (b) the particle size of ground bone was not fine enough and was not well controlled. Both factors are directly related to extraction recovery. Acetonitrile is a protein precipitation agent, which does not dissolve bone and release drug from the bone. In our method, the particle size of the ground bone was approximately 1 mm or less in diameter. Smaller bone particle size should be related to larger surface area for exposure to the extraction solvent. The entire bone sample from each specimen (20–70 g) was ground once to approximately 1 mm or less particle size. This ground bone sample was a relatively homogeneous solid, so the results were reproducible when we re-assayed it multiple times (the incurred human sample in the validation is an example). It should be emphasized that if the grinding device/method is different from the current method, different final particle sizes will be produced, which can result in different tigecycline extraction recovery. This can be explained because solubility of bone in the extraction solvent is proportional to the surface area of exposure. Also, different locations within a piece of bone can have different amounts of drug penetration [26]. Therefore, when a bone sample was prepared, the entire piece of bone should be ground and mixed well for future analysis.

In the present human bone assay validation, a large incurred sample from one of the dosed volunteers was used to monitor the reproducibility of the drug's dissolution. Since the pooled incurred bone sample did not have a nominal value, the nominal value for this sample was generated from the mean value of fifteen replicate measurements obtained each day ($n = 5$) for three days. By evaluating the day-to-day observed concentration of the incurred sample, the reference value of the tigecycline concentration in the incurred sample was established. The validation results for this incurred human bone sample showed acceptable precision and accuracy.

All matrix-related tigecycline stability tests were conducted using an incurred human bone sample, since we discovered that a control bone sample spiked with tigecycline has a much longer stability time frame than that from an incurred human bone sample. Therefore, it is reasonable to use incurred bone samples to represent the real stability.

For a homogeneous biological fluid (plasma, urine, etc.), the absolute extraction recovery can be determined as the percentage of measured drug concentration from a known spiked drug concentration in plasma or a biological fluid. However, for heterogeneous tissues like bone, the amount of spiked drug in a bone suspension cannot represent the amount of drug extracted from incurred bone samples with the extraction solvent. In our previous rat bone method [25], ^{14}C-tigecycline was administered to rats and the pooled incurred rat bone sample was analyzed by both a LC/MS/MS and a combustion liquid scintillation counting method. The theoretical value of the incurred rat bone was the value obtained from the combustion/LSC method. Absolute extraction recovery is equal to the ratio of the result from LC/MS/MS to the result from the combustion/LSC method multiplied by 100%. In the current human bone method, we did not dose humans with radio-labeled ^{14}C-tigecycline, so the "true" value of tigecycline in the incurred human bone was unknown. In order to estimate the extraction recovery from human bone, we used the human bone assay to analyze the ^{14}C-tigecycline rat bone samples. The extraction recovery result was 79%, which was similar to the rat bone method that did not require the stabilizing agent (77.1%). The true concentration of tigecycline in human bone should be the measured value divided by 0.79, since 21% of tigecycline was not extracted from the bone sample. The cross-validation data showed that the incurred human bone sample had a similar tigecycline concentration using the rat bone calibration curve compared to the human bone. This indicates that in future studies, incurred human bone can be analyzed with a rat bone calibration curve using the present human bone assay procedure.

Although the 24 incurred human bone samples were stored about 13–28 months prior to analysis at our laboratory, the results still showed on average about 9 times higher tigecycline concentrations than the original concentration. Subsequent human bone stability results showed that tigecycline is stable for at least 313 days at −70 °C. The repeat analysis data verified that there was a significant improvement in the extraction recovery of tigecycline using the current human bone assay.

5. Conclusions

A sensitive, high extraction efficiency LC/MS/MS bone assay for the determination of tigecycline concentrations in human bone has been developed and validated. To the best of our knowledge, this is the first high extraction recovery human bone assay using a LC/MS/MS method. This assay employs the addition of a stabilizing agent and a strong acidic extraction solvent to the ground bone sample, homogenization of the bone tissue, with subsequent centrifugation of the bone mixture, and LC/MS/MS analysis. The estimated absolute extraction recovery was 79%. The present human bone assay has a linear range of 50–20,000 ng/g using 0.1 g human bone. Intra- and inter-day accuracy and precision were 100 ± 15% and <15%, respectively. Tigecycline was stable in human bone samples for 6 h at room temperature, and for 313 days at −70 °C. Tigecycline was also stable for 54 h in the acidic extraction solvent at 4 °C. The concentrations of tigecycline after re-analysis of the 24 incurred human bone samples were, on average, approximately 9 times higher than the initial concentrations of tigecycline obtained by the acetonitrile extraction LC/MS/MS method. The initial tigecycline concentrations in the incurred bone samples were significantly underestimated due to the lower efficiency of the original extraction solvent used, which potentially resulted in a marked underestimation of tigecycline levels in human bone.

This bone assay meets the analytical needs for the determination of tigecycline in human bone. It provides a new standard for antibiotic bone assays in humans, which may play an important role in providing evidence of tigecycline penetration in infectious bone diseases.

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