Determination of Tigecycline in a Cell Lysate

Chen Jing,1 Dai Zhenyu,1 Xu Qun,1 Lina Liang,1 and Jeffrey Rohrer2
1Thermo Fisher Scientific, Shanghai, People’s Republic of China; 2Thermo Fisher Scientific, Sunnyvale, CA, USA

Key Words
Antibiotics, On-Line SPE, HPLC, Bioanalysis, Large-Volume Injection

Goal
To develop an on-line solid-phase extraction–high-performance liquid chromatography (SPE-HPLC) method for the simple and sensitive determination of tigecycline in a cell lysate sample

Introduction
Due to the overuse and misuse of antibiotic therapy, multidrug-resistant bacteria have appeared and their prevalence—especially in hospitals—has increased. Therefore, new antibiotics against resistant pathogens are needed for clinical practice. Tigecycline, a novel broad-spectrum antibiotic, exhibits activity against bacteria resistant to other antibiotic classes. Although it is a structural analogue of the tetracycline family, tigecycline resists deactivation by most known tetracycline resistance mechanisms.

Tigecycline (structure shown in Figure 1) is a newly commercially available antibiotic, so few HPLC–ultraviolet (UV) methods have been described for its determination.

A new U.S. Pharmacopeia (USP) monograph using an HPLC-UV method for the determination of tigecycline in drugs was recently proposed.1 Li et al. reported quantification of tigecycline in Hank’s balanced salts solution, human serum, and neutrophils.2 Due to UV detector sensitivity limits and sample volume capacity, these methods usually reported low-μg/mL method detection limits (MDLs). With the exception of Li et al.’s reported MDL of 50 μg/L, most other reports show MDLs around 1 μg/mL.3 In addition to HPLC-UV methods, HPLC-tandem mass spectrometry (MS/MS) methods with MDLs of 10 μg/L also have been described for determination of tigecycline in various biological fluids and tissues.4,5 The reported HPLC-UV methods are not readily transferred to HPLC-MS/MS methods because the mobile phases of the HPLC-UV methods for tigecycline include phosphate buffer, an ion-pairing agent, and acids such as ethylenediaminetetraacetic acid (EDTA), which are essential for good peak shape but detrimental to mass spectrometry.

Figure 1. Structure of tigecycline.
Although routine HPLC-UV methods provide sufficient sensitivity to quantify tigecycline in various biological tissues and fluids, in applications such as measuring the intracellular pharmacokinetics of tigecycline, which require quantification of tigecycline at μg/L concentrations in the cell, a more sensitive method is needed. Large sample-volume injections combined with on-line SPE using a Thermo Scientific™ Dionex™ UltiMate™ 3000 x2 Dual Rapid Separation RSLC system with a DGP-3600RS Dual Ternary Rapid Separation Pump have proven to significantly increase method sensitivity without MS. Therefore, the authors developed an on-line SPE-HPLC-UV method to quantify trace level tigecycline in a tigecycline-treated cultured cell lysate.

**Equipment**
- UltiMate 3000 x2 Dual RSLC system, including:
  - DGP-3600RS Dual Ternary Rapid Separation Pump (P/N 5040.0066)
  - SRD-3600 Integrated Solvent and Degasser Rack (P/N 5035.9230)
  - WPS-3000TRS Rapid Separation Wellplate Sampler, Thermostatted (P/N 5840.0020) with a 2500 μL sample loop and a 2500 μL syringe
  - TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000) or TCC-3000SD Thermostatted Column Compartment (P/N 5730.0010) equipped with two 2p–6p valves
  - DAD-3000RS Rapid Separation Diode Array Detector (P/N 5082.0020) with 13 μL flow cell
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software, version 7.1
- Thermo Scientific™ Orion™ 2-Star Benchtop pH meter

**Consumables**
- Thermo Scientific™ Target2™ Nylon Syringe Filters, 0.45 μm, 30 mm (P/N F2500-1)
- Autosampler Vial Screw Thread Caps, 8 mm (P/N 60180-514)

**Reagents and Standards**
- Deionized (DI) water, 18.2 MΩ·cm resistivity
- Acetonitrile (CH3CN), HPLC Grade (Fisher Scientific P/N AC610010040)
- Methanol (CH3OH), HPLC Grade (Fisher Scientific P/N AC610090040)
- Potassium Phosphate Monobasic (KH2PO4), HPLC Grade (Fisher Scientific P/N P286-1)
- Phosphoric Acid (H3PO4, Fisher Scientific P/N AC20114-5000)
- Ammonium Sulfate ((NH4)2SO4), Certified ACS, ≥99% (Fisher Scientific P/N A702-500)
- Ethylenediaminetetraacetic Acid (EDTA) Disodium Salt Dihydrate (Fisher Scientific P/N S25687)
- Triethylamine (C6H15N or TEA), HPLC Grade, ≥99% (Fisher Scientific P/N O4884-100)
- Tigecycline Hydrate, HPLC Grade, ≥98% (Sigma-Aldrich® P/N PZ0021)
- Oxytetracycline Hydrochloride (Fisher Scientific P/N AC30127-5000)

**Conditions**

**On-Line SPE**
- Column: Thermo Scientific™ Acclaim™ PolarAdvantage II (PA2) Guard Cartridge, 5 μm, 4.6 × 10 mm (P/N 069699)
- Mobile Phase: DI water
- Flow Rate: 1.0 mL/min
- Injection Volume: 1500 μL on the on-line SPE cartridge

**Separation**
- Column: Acclaim 120, C18, 3 μm Analytical, 3.0 × 150 mm (P/N 063691)
- Mobile Phase: Phosphate buffer/CH3CN (85:15, v/v)
- Flow Rate: 0.6 mL/min
- Autosampler Temp: 4 °C
- Column Temp: 30 °C
- Detection: UV absorbance at 247 nm

These conditions apply to Figures 2 and 3.

**Preparation of Standard Solutions**

**Tigecycline Stock Standard 1 (1000 μg/mL)**
Dissolve accurately weighed 10 mg of tigecycline in methanol in a 10 mL volumetric flask and bring to volume with methanol.

**Tigecycline Stock Standard 2 (10 μg/mL)**
Make a 100-fold dilution of tigecycline stock solution 1 with DI water.

**Tigecycline Stock Standard 3 (1 μg/mL)**
Make a 10-fold dilution of tigecycline stock solution 2 with DI water.

**Tigecycline Stock Standard 4 (0.1 μg/mL)**
Make a 10-fold dilution of tigecycline stock solution 3 with DI water.

**Oxytetracycline Stock Standard 1 (1000 μg/mL)**
Oxytetracycline is used as the internal standard (i.s.) in the determination of tigecycline. Dissolve accurately weighed 10 mg of oxytetracycline in methanol in a 10 mL volumetric flask and bring to volume with methanol.

**Oxytetracycline Stock Standard 2 (10 μg/mL)**
Make a 100-fold dilution of oxytetracycline stock solution 1 with DI water.

**Oxytetracycline Stock Standard 3 (0.25 μg/mL)**
Make a 4-fold dilution of oxytetracycline stock solution 2 with DI water.
Mixed Working Standards of Tigecycline and Oxytetracycline for Calibration

Dissolve 51.6 g of ammonium sulfate in 100 mL of DI water to make a 75% saturated salt solution. For the preparation of mixed working standards for calibration, add appropriate volumes of tigecycline and oxytetracycline stock standards into 10 mL glass vials and bring to 10 mL with 75% saturated ammonium sulfate. Refer to Table 1 for details.

Sample Preparation

A customer provided cultured cell lysate with tigecycline treatment.

Dissolve 7.74 g of ammonium sulfate in 15 mL of cell lysate, add 150 μL of 2.5 μg/mL oxytetracycline to the cell lysate, and centrifuge at 10000 rpm for 10 min. Collect the supernatant for use as a sample. This sample will contain oxytetracycline (i.s.) with a concentration of 0.025 μg/mL.

Dissolve 7.74 g of ammonium sulfate in 15 mL of cell lysate, add 75 μL of 10 μg/mL tigecycline and 150 μL of 2.5 μg/mL oxytetracycline to the cell lysate, and centrifuge at 10000 rpm for 10 min. Collect the supernatant for use as a spiked sample. This sample will contain oxytetracycline (i.s.) with a concentration of 0.025 μg/mL and spiked tigecycline with a concentration of 0.05 μg/mL.

Store the samples in brown bottles at 4 °C before analysis.

Preparation of Phosphate Buffer for Mobile Phase Preparation

Dissolve 0.50 g of EDTA, 6.805 g of KH₂PO₄, and 10 mL of TEA in 0.98 L of water; adjust the pH to 6.2 with H₃PO₄ and bring volume to 1 L with DI water.

Results and Discussion

Choice of Agents to Precipitate Proteins in Samples

For biological samples such as serum and cell culture samples, protein depletion by precipitation is a necessary step for quantification of small molecules. Adding an organic solvent such as acetonitrile or salt such as ammonium sulfate are routinely accepted procedures to precipitate protein.

Because a large volume of acetonitrile in the sample will make on-line SPE trapping difficult, the authors chose ammonium sulfate to treat samples. Tigecycline in 75% saturated ammonium sulfate was trapped on the SPE (Acclaim PA2) column and the salt was rinsed off the SPE column before the tigecycline was transferred for HPLC analysis.

Trichloroacetic acid (TCA) also precipitates protein in biological samples. TCA does not interfere with on-line SPE trapping as long as the sample pH after adding TCA does not affect the stability of tigecycline or the i.s. Though not tested here, TCA is another possible choice for protein precipitation.
Evaluation of On-Line SPE

Figure 2 shows a typical flow schematic of on-line SPE directly coupled to the HPLC column using one 6-port (2p-to-6p) valve. The method works as follows: Directly inject a prepared sample solution into the system and deliver it to the SPE column (Acclaim PA2 column) for enrichment (1-2 position) using a DI water mobile phase delivered by the first pump. Simultaneously equilibrate the analytical Acclaim C18 column using the second pump of the dual-pump module. After the analyte—tigecycline—is bound to the SPE column and impurities are washed out, switch the SPE column into the analytical flow path (6-1 position) to elute the bound tigecycline that is then separated on the analytical column using a phosphate/acetonitrile mobile phase and detected by the UV detector. This method is easily accomplished using the UltiMate 3000 x2 Dual RSLC system.

Choice of Mobile Phase

Mobile phases based on phosphate buffer, acetonitrile, and an ion-pairing agent have been used to determine tigecycline.\textsuperscript{2,3} Peak tailing was observed when tigecycline was analyzed at pH 7.1, whereas a mildly acidic pH of 5.5 will prevent peak tailing.\textsuperscript{2}

Tigecycline elutes too fast (1.5 min) at mobile phase pH 3. Therefore, the authors chose a mobile pH of 6.2 to determine tigecycline. The ion-pairing agent TEA was added to minimize peak tailing and improve peak symmetry. Tigecycline is known to easily form complexes with metal cations, which may affect its separation by HPLC. Therefore, EDTA was added to the mobile phase to prevent tigecycline/metal complex formation. The percentage of acetonitrile in the mobile phase was adjusted to 15% to achieve adequate retention of tigecycline and good separation between tigecycline and oxytetracycline.

Reproducibility, Linearity, and Detection Limits

Method reproducibility was estimated by making five replicate injections of 0.05 μg/mL tigecycline (injection volume 1500 μL). Retention time reproducibility was 0.04 (RSD) and peak area reproducibility was 5.17 (RSD). An overlay of the five injections is shown in Figure 3.

![Flow schematic of on-line SPE](image)
Calibration linearity for tigecycline was investigated by making three replicate injections of a mixed standard prepared at six different concentrations (0.005, 0.01, 0.05, 0.1, 0.5, and 1 μg/mL). The i.s. method was used to calculate the calibration curve and to quantify tigecycline in samples. Linearity was observed from 0.01 to 1 μg/mL when plotting the concentration versus the peak area, and the coefficient of determination was 0.9987. Five replicate injections of a cell lysate sample spiked with 0.01 μg/mL of tigecycline standard were used for estimating MDLs using the single-sided Student’s t-test method. The estimated MDL was 3.0 μg/L.

Sample Analysis

A sample of cell lysate after tigecycline treatment and the same sample spiked with 0.05 μg/mL tigecycline were analyzed. Figure 4 shows chromatograms of both samples. The detected amount of tigecycline is 0.012 μg/mL. The calculated spike recovery is 108%. The data show good spike recovery for tigecycline, which indicates method accuracy and demonstrates that this on-line SPE-HPLC method is suitable for the determination of tigecycline in cell lysate.

Precautions

- The authors observed interinjection decline of tigecycline peak area when tigecycline samples were not stored in a thermostatted autosampler chamber. Therefore, a thermostatted autosampler is recommended for this application.
- Tigecycline peak area declined when glass sample vials were used, especially in low-concentration samples. Sample loss was attributed to adsorption from the glass surface/silanol interaction. Therefore, plastic sample vials are recommended to prevent sample loss.

Conclusion

This work describes an on-line SPE-HPLC with UV absorbance detection method for determining tigecycline in a cell lysate. The determination was performed on the UltiMate 3000 x2 Dual RSLC system controlled by Chromeleon CDS software. The low-μg/L MDL achieved using UV detection supported by on-line SPE provides a convenient method to determine trace amounts of tigecycline in tigecycline-treated cells. These low concentrations cannot be determined using a routine HPLC-UV detection method.

References