

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.

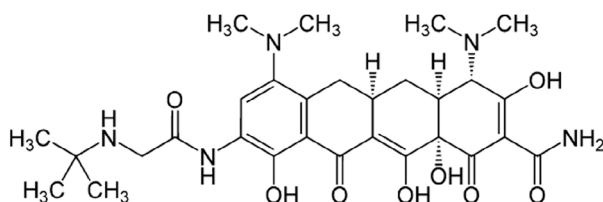


Figure 1. Structure of tigecycline.

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- $\mu\text{g/mL}$ method detection limits (MDLs). With the exception of Li et al.'s reported MDL of 50 $\mu\text{g/L}$, most other reports show MDLs around 1 $\mu\text{g/mL}$.³ In addition to HPLC-UV methods, HPLC-tandem mass spectrometry (MS/MS) methods with MDLs of 10 $\mu\text{g/L}$ 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.

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 $\mu\text{g/L}$ 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.⁶ 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 M $\cdot\text{cm}$ resistivity
- Acetonitrile (CH_3CN), HPLC Grade (Fisher Scientific P/N AC610010040)
- Methanol (CH_3OH), HPLC Grade (Fisher Scientific P/N AC610090040)
- Potassium Phosphate Monobasic (KH_2PO_4), HPLC Grade (Fisher Scientific P/N P286-1)
- Phosphoric Acid (H_3PO_4 , Fisher Scientific P/N AC20114-5000)
- Ammonium Sulfate ($(\text{NH}_4)_2\text{SO}_4$), Certified ACS, $\geq 99\%$ (Fisher Scientific P/N A702-500)
- Ethylenediaminetetraacetic Acid (EDTA) Disodium Salt Dihydrate (Fisher Scientific P/N S25687)
- Triethylamine ($\text{C}_6\text{H}_{15}\text{N}$ or TEA), HPLC Grade, $\geq 99\%$ (Fisher Scientific P/N O4884-100)

- Tigecycline Hydrate, HPLC Grade, $\geq 98\%$ (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 \times 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 \times 150 mm (P/N 063691)
Mobile Phase:	Phosphate buffer/ CH_3CN (85:15, v/v)
Flow Rate:	0.6 mL/min
Autosampler Temp:	4 $^\circ\text{C}$
Column Temp:	30 $^\circ\text{C}$
Detection:	UV absorbance at 247 nm

These conditions apply to Figures 2 and 3.

Preparation of Standard Solutions

Tigecycline Stock Standard 1 (1000 $\mu\text{g/mL}$)

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 $\mu\text{g/mL}$)

Make a 100-fold dilution of tigecycline stock solution 1 with DI water.

Tigecycline Stock Standard 3 (1 $\mu\text{g/mL}$)

Make a 10-fold dilution of tigecycline stock solution 2 with DI water.

Tigecycline Stock Standard 4 (0.1 $\mu\text{g/mL}$)

Make a 10-fold dilution of tigecycline stock solution 3 with DI water.

Oxytetracycline Stock Standard 1 (1000 $\mu\text{g/mL}$)

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 $\mu\text{g/mL}$)

Make a 100-fold dilution of oxytetracycline stock solution 1 with DI water.

Oxytetracycline Stock Standard 3 (0.25 $\mu\text{g/mL}$)

Make a 4-fold dilution of oxytetracycline stock solution 2 with DI water.

Tigecycline Stock Standard			Oxytetracycline Stock Standard (i.s.)			Vol of 75% Saturated (NH ₄) ₂ SO ₄ (mL)	Vol of DI Water (mL)	Final Vol of Calibration Standard (mL)	Final Concentration (µg/mL)		
Serial No.	Concn (µg/mL)	Volume (mL)	Serial No.	Concn (µg/mL)	Volume (mL)				Oxytetracycline (i.s.)	Tigecycline	
2	10	1.0	3	0.25	1.0	8	0	10	0.025	1.0	
		0.5								0.5	
3	1.0	1.0								0	0.1
		0.5									0.05
4	0.1	1.0								0	0.01
		0.5									0.005

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.² 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.

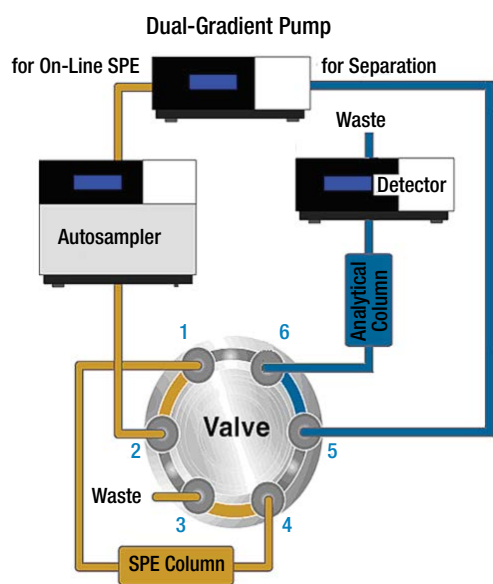


Figure 2. Flow schematic of on-line SPE.

Choice of Mobile Phase

Mobile phases based on phosphate buffer, acetonitrile, and an ion-pairing agent have been used to determine tigecycline.^{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.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 $\mu\text{g/mL}$ 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.

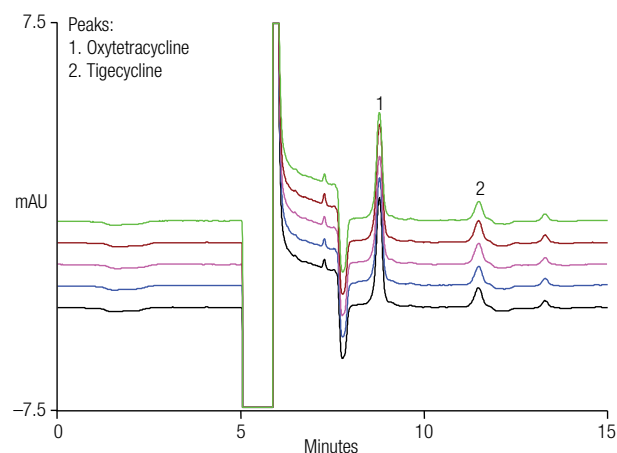


Figure 3. Overlay of chromatograms of five sequential injections of tigecycline.

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.

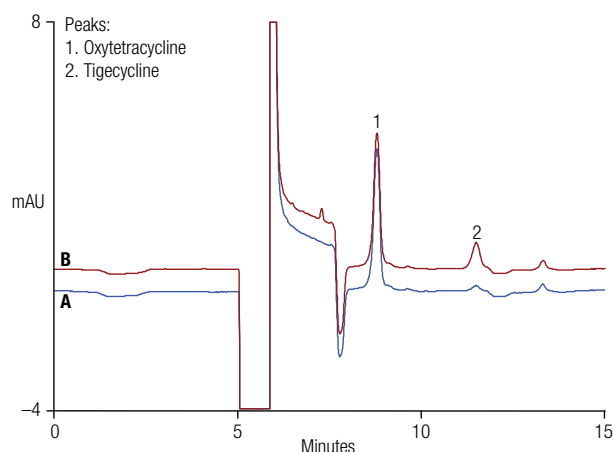


Figure 4. Cell lysate sample after tigecycline treatment (A) and the same sample spiked with 0.05 µg/mL tigecycline (B).

References

1. USP Reference Standard under Development, Item #1667643, Tigecycline, Projected Release Date Oct 1–Dec 30, 2014, U.S. Pharmacopeial Convention (USP); Rockville, MD.
2. Li, C.; Sutherland, C.; Nightingale, C.; Nicolau, D. Quantitation of Tigecycline, a Novel Glycylcycline, by Liquid Chromatography. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2004**, *811*, 225–229.
3. Da Silva, L.M.; Salgado, H.R. Validation of a Stability-Indicating RP-LC Method for the Determination of Tigecycline in Lyophilized Powder. *J. Chromatogr. Sci.* **2013**, *51* (2), 192–199.
4. Rodvold, K.; Gotfried, M.; Cwik, M.; Korth-Bradley, J.; Dukart, G.; Ellis-Grosse, E. Serum, Tissue and Body Fluid Concentrations of Tigecycline after a Single 100 mg Dose. *J. Antimicrob. Chemother.* **2006**, *58* (6), 1221–1229.
5. Muralidharan, G.; Micalizzi, M.; Speth, J.; Raible, D.; Troy, S. Pharmacokinetics of Tigecycline after Single and Multiple Doses in Healthy Subjects. *Antimicrob. Agents Chemother.* **2005**, *49* (1), 220–229.
6. Dionex (now part of Thermo Scientific) Application Note 256: Determination of Vitamin B12 in Beverages Using On-Line SPE Followed by HPLC with UV Detection. Sunnyvale, CA, 2010. [Online] www.dionex.com/en-us/webdocs/88015-AN256-LC-VitaminB12-Beverages-01Sep2010-LPN2574.pdf (accessed Dec. 6, 2013).

www.thermofisher.com/dionex

©2016 Thermo Fisher Scientific Inc. All rights reserved. Sigma-Aldrich is a registered trademark of Sigma-Aldrich Co. LLC. All other trademarks are the property of Thermo Fisher Scientific Inc. and its subsidiaries. This information is presented as an example of the capabilities of Thermo Fisher Scientific Inc. products. It is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details.

Africa +43 1 333 50 34 0
Australia +61 3 9757 4300
Austria +43 810 282 206
Belgium +32 53 73 42 41
Brazil +55 11 3731 5140
Canada +1 800 530 8447
China 800 810 5118 (free call domestic)
 400 650 5118

Denmark +45 70 23 62 60
Europe Other +43 1 333 50 34 0
Finland +358 9 3291 0200
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9494
Italy +39 02 950 591

Japan +81 6 6885 1213
Korea +82 2 3420 8600
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
Norway +46 8 556 468 00

Russia/CIS +43 1 333 50 34 0
Singapore +65 6289 1190
Sweden +46 8 556 468 00
Switzerland +41 61 716 77 00
Taiwan +886 2 8751 6655
UK/Ireland +44 1442 233555
USA +1 800 532 4752

AN70843_E 07/16S

Thermo
 SCIENTIFIC

Part of Thermo Fisher Scientific