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1 **Ultra Performance Liquid Chromatography PDA method for determination of**  
2 **tigecycline in human plasma**

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16

1     **ABSTRACT**

2     A simple UPLC-PDA method for the quantification of human plasma concentrations of  
3     tigecycline was developed and validated.

4     Quinaxoline, used as internal standard, was added to 500  $\mu$ L of plasma before adding 1 mL of  
5     protein precipitation solution. The extracts were dried in a vacuum centrifuge system at 60°C and  
6     reconstituted with 60  $\mu$ L of water and acetonitrile (95:5; v/v), 5  $\mu$ L was injected onto an  
7     ACQUITY UPLC™ H-Class system. Chromatographic separation was performed on a C18  
8     ACQUITY UPLC™ HSS T3 column using a gradient of potassium phosphate buffer (pH 3.2)  
9     and acetonitrile. Detection was performed using a PDA detector at 350 nm.

10    Relative error at three quality control concentrations ranged from -2.49 to -8.74%. Intra-day  
11    (CV%) and inter-day (CV%) precision ranged from 3.93 to 12.27% and from 9.53 to 13.32%,  
12    respectively. LOQ and LOD were 0.024  $\mu$ g/mL and 0.006  $\mu$ g/mL, respectively. Mean recovery  
13    was 95%. The calibration curve was linear up to 6  $\mu$ g/mL. This concentration range proved to be  
14    adequate to measure tigecycline concentrations in patients treated with the drug, therefore this  
15    method would be suitable for therapeutic drug monitoring.

16  
17    **Keywords:** tigecycline; UPLC; human plasma; quantification; therapeutic drug monitoring

## 1 INTRODUCTION

2 Tigecycline, a derivative of minocycline, is the first of a new glycycline class of extended-  
3 spectrum antibiotics that have an expanded broad spectrum of in vitro activity. It was developed  
4 to overcome the two key resistance mechanisms, efflux pumps and ribosomal protection, that  
5 limit the use of tetracyclines. Due to its steric hindrance afforded by a large D-ring substituent,  
6 tigecycline is not affected by these common mechanisms of resistance and also by target site  
7 modifications, enzymatic degradation of the drug molecule and DNA gyrase mutations of  
8 antibiotic resistance<sup>1</sup>. Tigecycline is formed by the addition of a side-chain to minocycline, in the  
9 form of a 9-tert-butyl-glycylamido moiety that occurs at the 9<sup>th</sup> position of the D ring. This  
10 derivative was ultimately shown to possess expanded microbiological and therapeutic benefits<sup>1,2</sup>,  
11 by binding to the 30S ribosomal subunit and so inhibiting protein synthesis.

12 Tigecycline is active against clinically relevant susceptibility and multidrug resistant bacteria,  
13 such as methicillin resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae*,  
14 vancomycin resistant *Enterococci*, *Acinetobacter spp*, *Acinetobacter baumannii* and  
15 *Enterobacteriaceae*, including extended-spectrum  $\beta$ -lactamase-producing strains (ESBLs).

16 Importantly, for critically ill patients and those on multiple therapeutic agents, tigecycline does not  
17 interact with the cytochrome P450 enzyme family, including isoforms CYP1A2, CYP2C8,  
18 CYP2C19, CYP2D6 and CYP3A4, making pharmacokinetic drug interactions uncommon<sup>3</sup>.

19 The primary route of tigecycline elimination is biliary excretion (59%) as unaltered drug.  
20 Secondary routes of elimination include renal excretion (22% unchanged through urine), and  
21 glucuronidation<sup>4</sup>. Tigecycline is indicated for adult patients aged 18 years old or older, and the  
22 pharmacokinetics of tigecycline appear to be unaffected by age, food, renal disease or mild-to-  
23 moderate hepatic dysfunction. Dosage adjustment, by administering half of the usual daily dose,  
24 is recommended in severe hepatic dysfunction (Child-Pugh class C)<sup>3</sup>.

1 In order to increase knowledge about tigecycline pharmacokinetics, it is important to have a  
2 reliable and relatively inexpensive method to quantify tigecycline. Many methods have been  
3 published for the quantification of tigecycline in serum, plasma and other biological materials<sup>4-13</sup>  
4 using high-performance liquid chromatography (HPLC) coupled to different types of detector  
5 such as ultra-violet (UV-PDA), fluorescence spectrometry, or mass-spectrometry (MS)<sup>4-13</sup>.  
6 To date, to our knowledge, no ultra performance liquid chromatography (UPLC) method has been  
7 reported to analyze tigecycline. Our aim was to develop and validate a new UPLC assay to  
8 determine tigecycline concentrations in human plasma.

9

## 1 **METHODS**

### 2 ***Chemicals***

3 Tigecycline and the internal standard (IS), quinoxaline (QX), were obtained from Sigma–Aldrich  
4 (St. Louis, MO). Acetonitrile HPLC grade was purchased from J.T. Baker (Deventer, Holland).  
5 Methanol HPLC grade was from VWR (Milan, Italy). Potassium dihydrogen phosphate and ortho-  
6 phosphoric acid were from Sigma–Aldrich (St. Louis, MO). HPLC grade water was produced with  
7 a Milli-DI system coupled to a Synergy 185 system by Millipore (Billerica, MA, USA). Blank  
8 plasma from healthy donors was kindly supplied by the Blood Bank of Maria Vittoria Hospital  
9 (Turin, Italy).

10

### 11 ***Stock solutions, calibrators (STD) and quality control samples (QC)***

12 Tigecycline and IS stock solutions were made in a solution of water and water:methanol (90:10;  
13 v/v), respectively, to obtain a final concentration of 1 mg/mL; IS stock solution was refrigerated at  
14 4°C while tigecycline stock solution was stored a -80°C until use, within 1 month.

15 IS working solution was made with QX in water:methanol (50:50; v/v) at a concentration of 50  
16 µg/mL. The highest calibrator (STD 9) and the quality controls (QCs) were prepared by adding a  
17 precise volumes of stock solutions to blank plasma. The calibration curve was prepared by serial  
18 dilutions from STD 9 to STD 1. A blank sample (STD 0), without tigecycline, was used to confirm  
19 the absence of interfering peaks. The calibration range was from 0.024 µg/mL to 6 µg/mL, and QC  
20 concentrations were 4 µg/mL (QC H), 1 µg/mL (QC M), 0.1 µg/mL (QC L).

21 STDs and QCs were stored at –80°C until use for no longer than 3 months.

22

### 23 ***Processing Of clinical samples, calibrators and QCs***

1 Blood samples, collected in lithium heparin tubes (7 mL), were obtained from patients who had  
2 received tigecycline. To avoid thawing cycles each patient plasma sample was aliquoted into two  
3 criovials of 600  $\mu$ L. Plasma was collected after centrifugation at  $1400\times g$  (3000 rpm) for 10 min at  
4  $+4^{\circ}\text{C}$  (Jouan Centrifuge, Model BR4i, Saint-Herblain, France). 1000  $\mu$ L of protein precipitation  
5 solution (acetonitrile 100%) and 50  $\mu$ L of IS working solution were added to 500  $\mu$ L of plasma  
6 samples, in a PTFE microfuge tube. After vortexing for 30 seconds, the mixture was centrifuged at  
7 12,000 rpm for 10 minutes at  $4^{\circ}\text{C}$ . The supernatant was transferred into a glass tube and dried in a  
8 vacuum centrifuge system at  $60^{\circ}\text{C}$  for 1 hour. Samples were reconstituted with 60  $\mu$ L of water and  
9 acetonitrile (95:5; v/v) and 5  $\mu$ L was injected in UPLC system. All analyses were performed in  
10 duplicate, and all procedure steps were carried out at room temperature.

11

## 12 ***Chromatography***

13 The instrument used was a ACQUITY UPLC™ H-Class system (Waters, Milford, CT, USA)  
14 composed of a quaternary solvent manager, a sample manager FTN (Flow-Through Needle), a  
15 column heater and a photo-diode array (PDA). Empower 2 Pro software (version year 2005:2008,  
16 Waters; Milford, CT, USA) was used. Chromatographic separation was performed at  $45^{\circ}\text{C}$ . The  
17 chromatographic column was an ACQUITY UPLC™ HSS T3, 1,8  $\mu\text{m}$ , 2.1 x 150 mm protected by  
18 a Waters ACQUITY UPLC™ Column In-Line Filter (Waters, Milford, CT, USA).

19 Mobile phases were: Mobile Phase A (20 mM of potassium dihydrogen phosphate buffer at pH 3.2  
20 with ortho-phosphoric acid) and Mobile Phase B (acetonitrile, 100%). Tigecycline and QX were  
21 monitored at 350 nm, on the basis of their absorbance spectrums (Figures 1 A and B).  
22 Chromatographic separation was performed at 0.4 mL/min with a 10 minute gradient run as  
23 reported in Table 1.

24

## 25 ***Specificity and selectivity***

1 Interference from endogenous compounds was investigated by the analysis of six different blank  
2 plasma samples.

3

#### 4 ***Accuracy, precision, and limit of quantification***

5 Accuracy and intra-day and inter-day precision of the method were determined by assaying 10  
6 spiked plasma samples at three different concentrations (QCs). Accuracy was calculated as the  
7 percent deviation from the nominal concentration. Inter-day and intra-day precision were expressed  
8 as the standard deviation at each QC concentrations. Each calibration curve was obtained using nine  
9 calibration points, ranging from 0.024 to 6 µg/mL, and it was analyzed in duplicate as requested by  
10 FDA guidelines <sup>14</sup>. The calibration curve was created by plotting the peak area ratio of drug to the  
11 IS against the various drug concentrations in the calibrators. A linear regression forced through zero  
12 (STD 0) curve was used and no weighting was applied. The limit of detection (LOD) in plasma was  
13 defined as the concentration that yielded a signal-to-noise ratio of 3/1. Percent deviation from the  
14 nominal concentration (measure of accuracy) and relative standard deviation (measure of precision)  
15 of the concentration considered as the limit of quantification (LOQ) had to be <20%, and it was  
16 considered the lowest calibration standard, as requested by the FDA <sup>14</sup>.

17

#### 18 ***Recovery***

19 Average recovery of tigecycline and IS were determined by comparing the peak area of the analytes  
20 extracted from spiked plasma samples (4.0, 1.0 and 0.1 µg/mL for tigecycline and 50 µg/mL for IS)  
21 with those obtained by direct injection of the same amounts of drug.

22

#### 23 ***Stability***

24 Long term stability of tigecycline in plasma samples was assessed by storing QC samples at 4°C, at  
25 -20°C and at -80°C for three months. Freeze/thaw stability was tested by thawing plasma samples



1 at room temperature (24°C) and freezing at -80°C for three consecutive cycles. Bench-top stability  
2 was assessed over a 6-h period at room temperature (24°C), and autosampler stability was tested by  
3 keeping samples for 6-h in a refrigerated autosampler at 4°C.

4

#### 5 *Patient Samples*

6 The method was tested using samples from 10 patients receiving treatment with tigecycline.  
7 Samples were collected after different days of treatment. Blood sampling at the end of the dosing  
8 interval (C<sub>trough</sub>), was performed after written informed consent had been given. Some samples  
9 (n=5) were collected to evaluate the C<sub>max</sub>. All samples were stored at -80°C until analyses and for  
10 no more than 1 month.

11 The co-administered drugs received by these patients were the following: meropenem, teicoplanin,  
12 seleparina, vancomycin, caspofungin, esomeprazole, methylprednisolone, dopamine,  
13 methyltetrahydrofolate calcium penta hydrate, levothyroxine sodium, nora, cyclosporine,  
14 propafenone, ursodeoxycholic acid, furosemide, ondansetron, pantoprazole, pentaglobin,  
15 citalopram, ranitidine, sucralfate, ademetonine, carvedilol, emtricitabine, tenofovir, efavirenz,  
16 seretide, cefepime, diltiazem hydrochloride, digoxin, flurazepam monohydrochloride, canrenone,  
17 lansoprazole, voriconazole, clarithromycin, amikacin, phenobarbital, omeprazole, morphine,  
18 oxybutynin, linezolid.

19

## 1 **RESULTS**

2 Representative chromatograms of a blank plasma extracted, as STD 0, and STD 1 are shown in  
3 Figure 2. In Figure 3 overlay chromatograms are shown of extracted blank plasma and STD 1. In  
4 addition, a chromatogram of QC H extracted is shown in Figure 4. The retention times of the  
5 analytes were  $5.1 \pm 0.30$  and  $8.2 \pm 0.30$  for tigecycline and IS, respectively. The mean regression  
6 coefficient ( $r^2$ ) of all calibration curves was more than 0.995. As clearly indicated in the FDA  
7 guidelines, the simplest model that adequately describes the concentration-response relationship  
8 was used, and the forcing the line through zero gave a higher curve reliability.

9

### 10 ***Specificity and selectivity***

11 The six blank plasma samples did not show any endogenous interferences, considering the retention  
12 time windows of the analytes (Figure 2 and Figure 3). The assay did not show any significant  
13 interferences, probably since tigecycline and QX were monitored at 350 nm. Moreover, no  
14 interferences from concomitant drugs in patients were observed.

15

### 16 ***Accuracy, precision, limit of quantification***

17 Results of the validation are listed in Table 2. All observed data (accuracy, intra-day and inter-day  
18 precision [R.S.D.%]) were below 15.0%, according to the FDA guidelines<sup>14</sup>. LOQ and LOD were  
19  $0.024 \mu\text{g/mL}$  and  $0.006 \mu\text{g/mL}$ , respectively.

20

### 21 ***Recovery***

22 Multiple aliquots ( $n = 6$ ) at each of the three QC concentrations were assayed and mean recovery  
23 for tigecycline was above 95% and 90% for IS.

24

1 ***Analysis of plasma samples from patients***

2 Plasma samples for Ctrough were obtained at a median of 12.15 h (IQR 11.75–12.90) after the last  
3 intake of tigecycline. The average Ctrough and Cmax tigecycline plasma concentrations were 0.063  
4 µg/mL and 1.020 µg/mL, respectively.

5

6 ***Stability***

7 Stability results showed that tigecycline was stable for at least 3 months when stored at -80°C, but a  
8 significant degradation (>20%) of tigecycline was observed after 3 days at 4°C and around 3  
9 months at -20°C. Freeze/thaw stability tests showed that tigecycline was stable after 3 freeze/thaw  
10 cycles, as well as after 6 h at room temperature and in a refrigerated autosampler at 4°C.

11

## 1 **DISCUSSION AND CONCLUSION**

2 Recently, PK-PD analyses for safety have shown that higher plasma exposure of tigecycline was  
3 predictive of nausea and/or vomiting<sup>15</sup>. Therefore, having a method which allows quantification of  
4 tigecycline in plasma in a quick and reliable manner may be useful in clinical practice.

5 In this paper, we described the validation of the first ultra performance liquid chromatographic  
6 method for the determination of tigecycline in human plasma. The results showed the method to be  
7 accurate and precise, simple to perform and that it does not require the use of very complex and  
8 expensive instrumentation, such as mass detectors. In fact, we have chosen to use a UPLC-PDA  
9 system and to monitor the tigecycline and the internal standard at a wavelength of 350 nm. This is a  
10 highly selective wavelength that allows very few endogenous and exogenous (due to concomitant  
11 medications) interferences. Moreover, it is known that relatively fewer endogenous and exogenous  
12 constituents of human plasma absorb intensively at that wavelength and so interference is less  
13 probable, as observed in our patient samples. In addition, we have developed a 10 minute long  
14 chromatographic run, by exploiting the high selectivity and the high number of theoretical plates of  
15 the column to 150 mm (ACQUITY UPLC™ HSS T3, 1,8 µm, 2.1 x 150 mm). The selection of QX  
16 as the internal standard was based on our previous experience. It was used and fully validated in our  
17 published methods for other drugs<sup>16-26</sup>. It is not a prescribed drug, it is cheap, easy to purchase, and  
18 it has intermediate chemical characteristics useful for our method.

19 Recovery seems to be adequate (95%), and the data on accuracy, precision (Table 1) and LOQ/LOD  
20 support this statement.

21 To date, only one other published work describes the determination of tigecycline in human plasma  
22 using UV<sup>8</sup> detector, while other assays used mass detectors<sup>4-7, 9-13, 27</sup>. The advantage of using  
23 instruments coupled to UV detector is that they are less expensive and easier to use than those  
24 coupled to mass detectors. Our method, compared with Li et al.<sup>8</sup>, is characterized by higher  
25 chromatographic selectivity, and it does not use a chemotherapeutic drug as IS. Moreover, we have

1 included in the same run time a column wash step that ensures the removal of possible "ghost  
2 peaks" in sequential runs. For these reasons our UPLC-PDA method appears more reliable from the  
3 chromatographic point of view.

4 We have also verified the stability of tigecycline in human plasma. Long-term stability tests showed  
5 that tigecycline was very stable in plasma kept at -80°C for at least 3 months. Tigecycline was  
6 found to be stable at room temperature, during the extraction process and in an autosampler, for at  
7 least 6 hours.

8 In addition, the analysis of tigecycline in plasma from patients treated with tigecycline confirmed  
9 the adequate sensitivity of the method and the suitability of the calibration range used. Moreover,  
10 patients treated with more than one drug did not show any plasma interferences with the peaks of  
11 the analytes of interest, due to the high selectivity of the method.

12 Thus, we have developed and validated a relatively simple and cheap UPLC-PDA method which  
13 can be used to promote the practice of tigecycline TDM.

14

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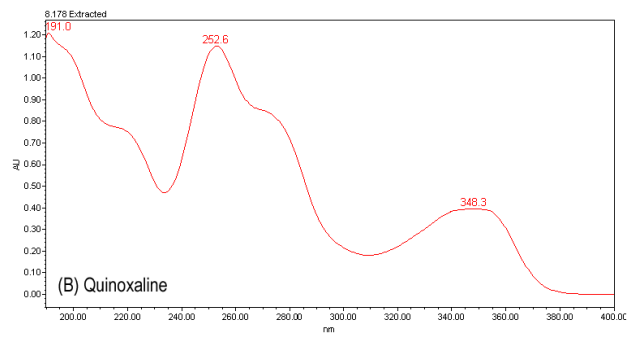
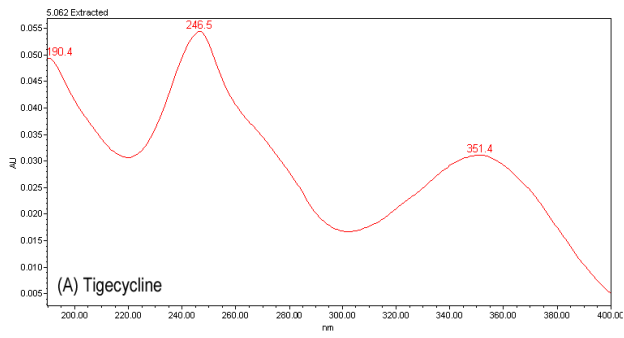
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21  
22

23

1 **FIGURE LEGENDS**

2 **Figure 1.** UV absorbance spectrum for tigecycline (A) and quinoxaline (B).



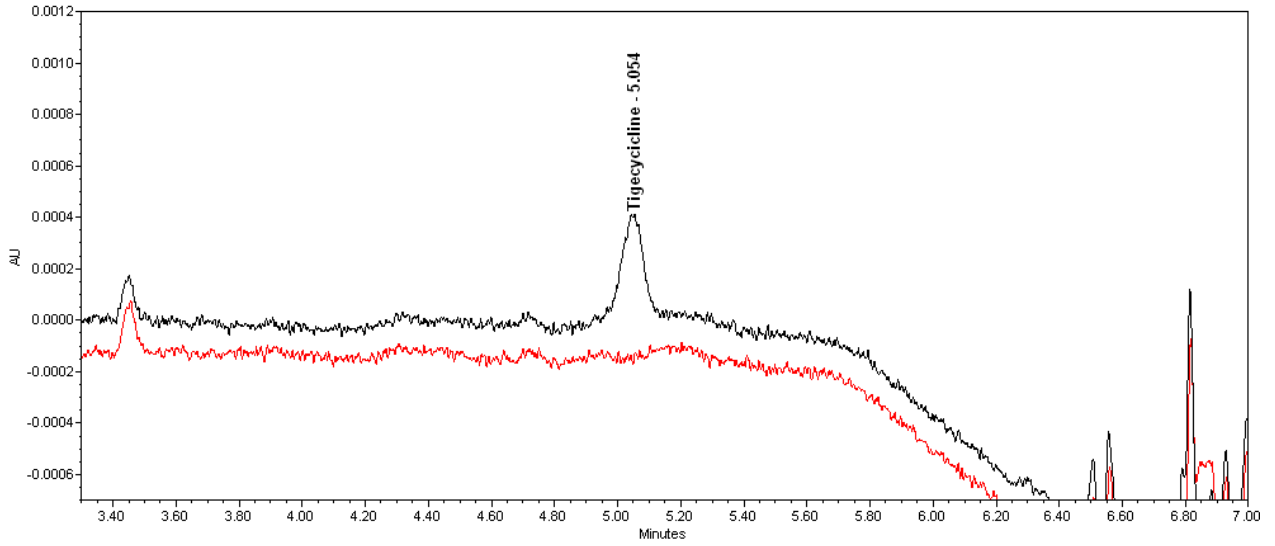
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1

2 **Figure 2.** Overlay chromatograms of extracts of a blank human plasma sample and the LOQ/STD1  
3 level [0.024 µg/mL of tigecycline].

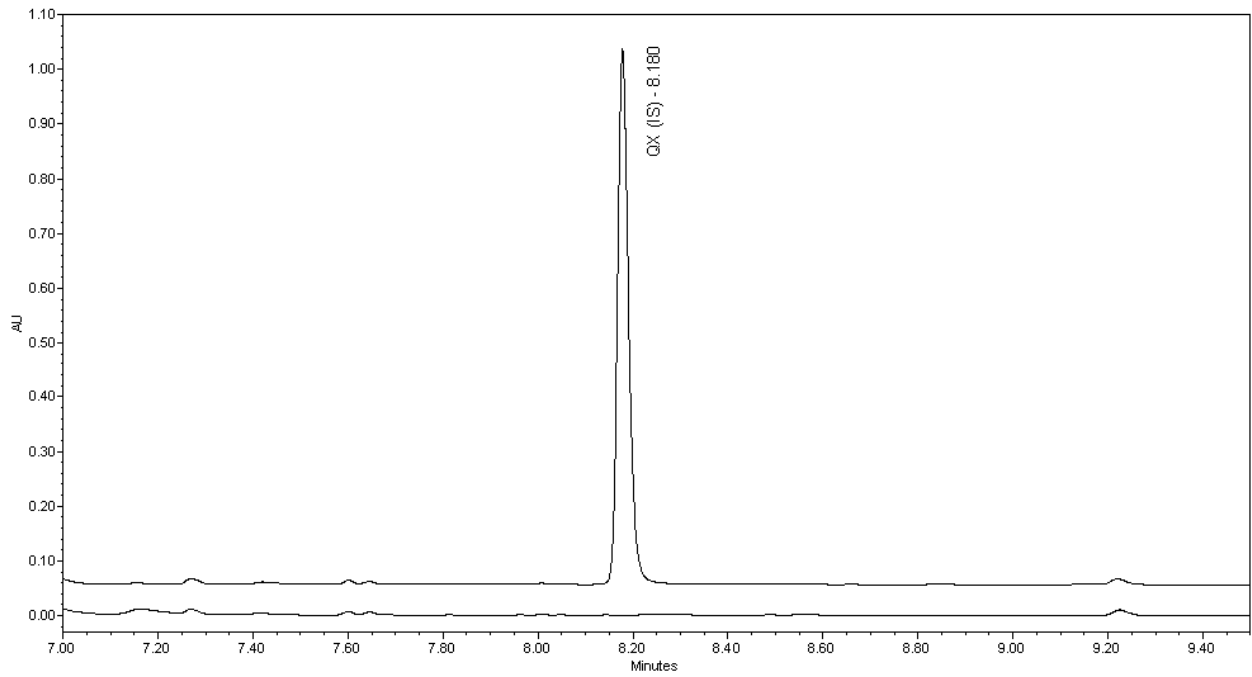


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2 **Figure 3.** Overlay chromatograms of extracts of a blank human plasma sample and 5.0  $\mu\text{g/mL}$  of IS  
3 quinoxaline.

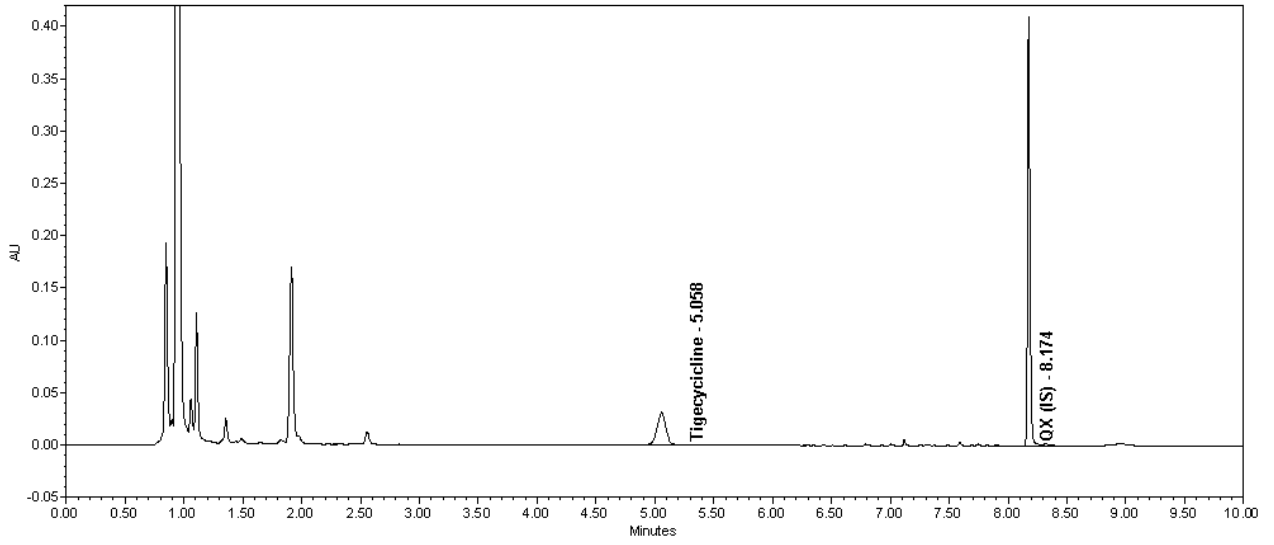


4

5

1

2 **Figure 4.** Chromatogram of QCH level [4.0 µg/mL of tigecycline]



3

4

1 **Table 1.** Chromatographic conditions (gradient). Mobile phase A (KH<sub>2</sub>PO<sub>4</sub> 20 mM with ortho-  
2 phosphoric acid, final pH = 3.23) and mobile phase B (Acetonitrile).

3

<b>TIME (minutes)</b>	<b>% Mobile Phase A</b>	<b>% Mobile Phase B</b>	<b>FLOW (mL/min)</b>
0.0	93	7	0.4
4.0	90	10	0.4
6.5	30	70	0.4
8.2	30	70	0.4
8.3	93	7	0.4
10.0	93	7	0.4

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1 **Table 2.** Intra-day and inter-day precision for the analysis of tigecycline in plasma QCs ( $n = 10$ )  
2 [mean R.S.D.%].

<b>QC, spiked Ticecycline concentration (<math>\mu\text{g/mL}</math>)</b>	<b>Accuracy %</b>	<b>Intra-day R.S.D. %</b>	<b>Inter-day R.S.D. %</b>
QC-H (4.0)	-2.49	7.55	9.53
QC-M (1.0)	-7.80	12.27	10.71
QC-L (0.1)	-8.74	3.93	13.32

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