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ABSTRACT

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

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

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

Keywords: tigecycline; UPLC; human plasma; quantification; therapeutic drug monitoring

INTRODUCTION

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

Tigecycline is active against clinically relevant susceptibility and multidrug resistant bacteria, such as methicillin resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae*, vancomycin resistant *Enterococci*, *Acinetobacter spp, Acinetobacter baumanni and Enterobacteriaceae*, including extended-spectrum β-lactamase-producing strains (ESBLs).

Importantly, for critically ill patients and those on multiple therapeutic agents, tigeycline does not interact with the cytochrome P450 enzyme family, including isoforms CYP1A2, CYP2C8, CYP2C19, CYP2D6 and CYP3A4, making pharmacokinetic drug interactions uncommon [3].

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

In order to increase knowledge about tigecycline pharmacokinetics, it is important to have a reliable and relatively inexpensive method to quantify tigecycline. Many methods have been published for the quantification of tigecycline in serum, plasma and other biological materials [4-13] using high-performance liquid chromatography (HPLC) coupled to different types of detector such as ultra-violet (UV-PDA), fluorescence spectrometry, or mass-spectrometry (MS) [4-13].

To date, to our knowledge, no ultra performance liquid chromatography (UPLC) method has been reported to analyze tigecycline. Our aim was to develop and validate a new UPLC assay to determine tigecycline concentrations in human plasma.

METHODS

Chemicals

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

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

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

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

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

Processing 0f clinical samples, calibrators and QCs

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

Chromatography

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

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

Specificity and selectivity

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

Accuracy, precision, and limit of quantification

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

Recovery

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

Stability

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

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

Patient Samples

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

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

RESULTS

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

Specificity and selectivity

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

Accuracy, precision, limit of quantification

Results of the validation are listed in Table 2. All observed data (accuracy, intra-day and inter-day precision [R.S.D.%]) were below 15.0%, according to the FDA guidelines [14]. LOQ and LOD were 0.024 µg/mL and 0.006 µg/mL, respectively.

Recovery

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

Analysis of plasma samples from patients

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

Stability

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

DISCUSSION AND CONCLUSION

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

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

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

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

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

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

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

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

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FIGURE LEGENDS

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

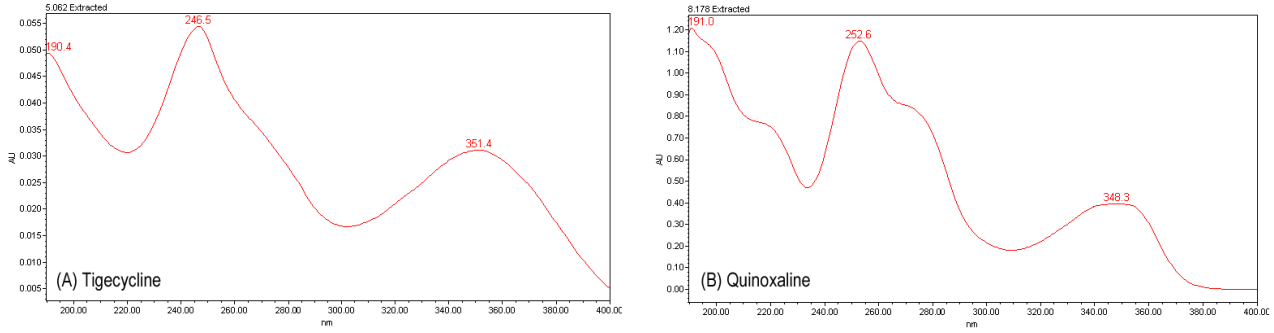

Figure 2. Overlay chromatograms of extracts of a blank human plasma sample and the LOQ/STD1 level [0.024 µg/mL of tigecycline].

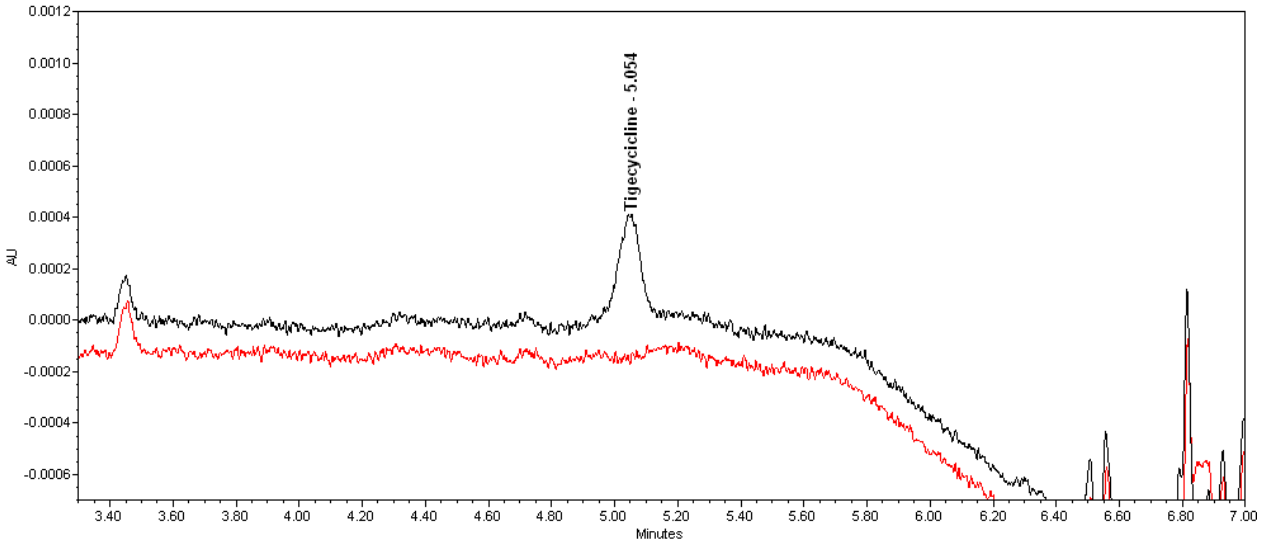

Figure 3. Overlay chromatograms of extracts of a blank human plasma sample and 5.0 µg/mL of IS quinoxaline.

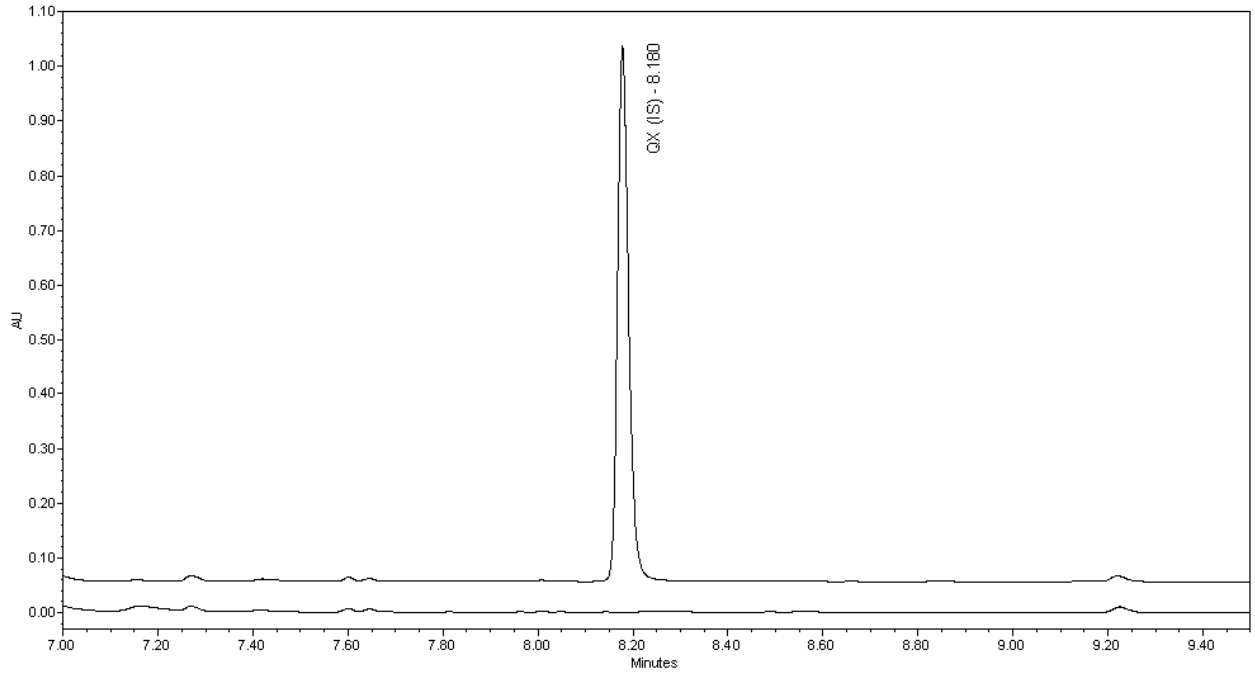

Figure 4. Chromatogram of QCH level [4.0 µg/mL of tigecycline]

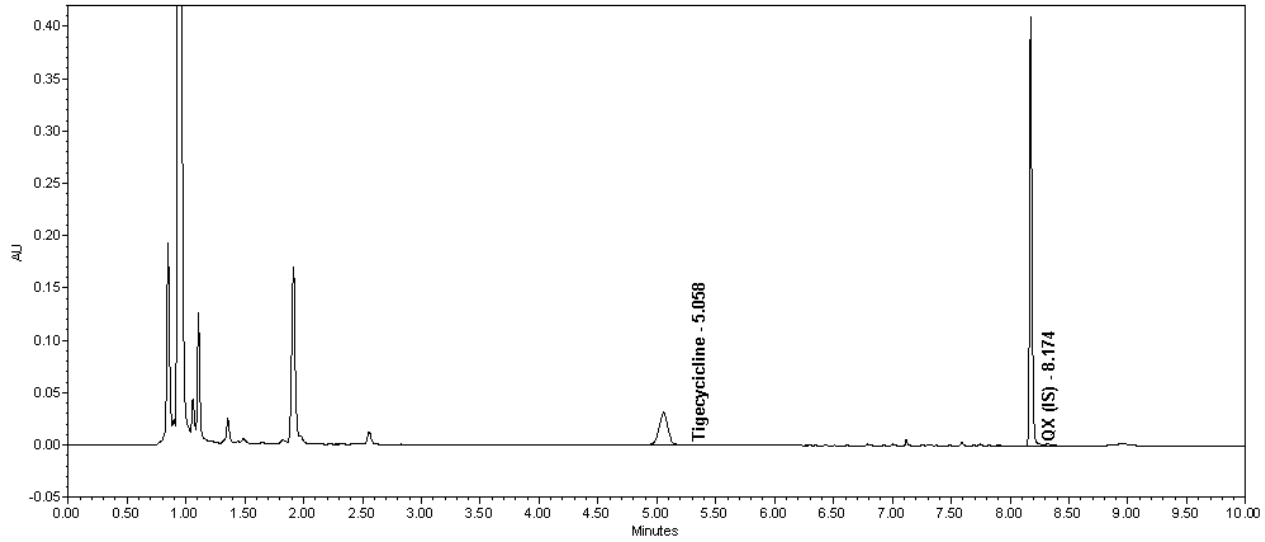

Table 1. Chromatographic conditions (gradient). Mobile phase A (KH_2PO_4 20 mM with ortho-phosphoric acid, final pH = 3.23) and mobile phase B (Acetonitrile).

TIME (minutes)	% Mobile Phase A	% Mobile Phase B	FLOW (mL/min)
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

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

QC, spiked Ticecycline concentration (µg/mL)	Accuracy %	Intra-day R.S.D. %	Inter-day R.S.D. %
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