Chromatography
Separation Techniques

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Development of a High Performance Liquid Chromatography Method for the Determination of Tedizolid in Human Plasma, Human Serum, Saline and Mouse Plasma

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Abstract

A simple and reliable high performance liquid chromatography method was developed and validated to analyze tedizolid in human plasma, human serum, saline and CD-1 mouse plasma. An ultra violet detector set at 251nm was used with a reverse phase column. The mobile phase consisted of sodium acetate, deionized water and acetonitrile at a flow rate of 1.0 ml/min. 4-nitroaniline was used as the internal standard. The standard curves were linear over a range of 0.2 to 5 µg/ml. Accuracy and precision for the validations were within the acceptable 10% limit. This method was successfully used to assay samples previously analyzed by liquid chromatography with tandem mass spectrometric detection.

Keywords: Tedizolid; HPLC; Validation

Abbreviations

UV: Ultra Violet; FDA: Food and Drug Administration; ABSSSI: Acute Bacterial Skin and Skin Structure Infection; HPLC: High Performance Liquid Chromatography; DMSO: Dimethyl Sulfoxide; K3EDTA: Tri-potassium Ethylene Di-amine Tetra Acetic Acid; QC: Quality control; LLQ: Lower Limit of Quantification; LC-MS/MS: Liquid Chromatography – Tandem Mass Spectrometry; SD: Standard Deviation

Introduction

Tedizolid phosphate (SIVEXTRO®, Cubist Pharmaceuticals) is a novel oxazolidinone antibiotic recently approved by the Food and Drug Administration (FDA) (Figure 1). Tedizolid phosphate is rapidly converted in vivo by phosphatases to the microbiologically active antibiotic tedizolid. Tedizolid phosphate is indicated for treatment of adults with acute bacterial skin and skin-structure infections caused by the following Gram-positive microorganisms, *Staphylococcus aureus* (including methicillin-resistant and methicillin-susceptible pathogens), *Streptococcus pyogenes*, *Streptococcus agalactiae, Streptococcus anginosus* group (including *Streptococcus anginosus*, *Streptococcus intermedius* and *Streptococcus constellatus*) and *Enterococcus faecalis* [1]. Tedizolid phosphate is available as both oral (i.e., tablet) and intravenous (administered over 1 hour) formulations both administered at the same dose of 200 mg once daily for the treatment of acute bacterial skin and skin structure infections (ABSSSI) [1]. The purpose of this present study was to develop a simple, reproducible, and selective high performance liquid chromatography (HPLC) method for the detection of tedizolid in human plasma, human serum, saline and mouse plasma that is consistent with FDA guidance [2].

Materials

Chemicals

Tedizolid standard powder was provided by Cubist Pharmaceuticals (Lexington, MA.). Commercially available 4-nitroaniline was purchased from Sigma (St. Louis, MO) and used as the internal standard. Sodium acetate, trichloroacetic acid, ethyl alcohol and dimethyl sulfoxide (DMSO) were also purchased from Sigma. Acetonitrile from Avantor Performance Materials, Inc. (Center Valley, PA) was used without further purification. Deionized water was obtained from Millipore's Milli-Q analytical deionization system (Bedford, MA). 0.9% sodium chloride injection USP was purchased from Hospira, Inc., (Lake Forest, IL).

Instrumentation

An HPLC system consisting of a Waters 515 gradient pump (Waters Associates, Milford, MA) and WISP 717 plus autosampler (Waters) was equipped with a 5 µm SphereClone-80 ODS2 column (4.6 x 150 mm, Phemonenex, Torrance, CA) coupled to a Bondapak C_{18} Guard-pak precolumn (Waters). The autosampler was cooled to 10°C. The column was maintained at room temperature. A programmable Hitachi L 2400 ultra violet (UV) detector (Model 526; ESA Inc., Chelmsford, MA) set at 251nm was used to detect the analytes. The run time for each human plasma and serum sample was 28 minutes. The run time for each saline and mouse plasma sample was 20 minutes. The EZChrom Elite chromatography data system (Scientific Software Inc., Pleasanton, CA) was used to quantify the peak heights.

Mobile phase preparation

The mobile phase consisted of a mixture of 0.0192 M sodium acetate buffer and 23% acetonitrile. The flow rate was 1.0 ml/min. All chromatographic procedures were performed at room temperature.

Standard solutions and control

Tedizolid standard solutions were made in a volumetric flask using DMSO to dissolve and dilute the stock standard. The internal

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standard, 4-nitroaniline 6 µg/ml, was prepared in ethanol according to the manufacturer's recommendations. Drug free adult human plasma with tri-potassium ethylene di-amine tetra acetic acid (K3EDTA) and serum from 6 different individuals as well as the CD-I non-immunized mouse plasma (K3EDTA) were purchased from Bioreclamation Inc. (Hicksville, NY). Separate standard curves were made for tedizolid in pooled human plasma, mouse plasma and saline. Tedizolid was spiked into human plasma and saline to make 8 standard solutions (0.2, 0.4, 0.6, 0.8, 1.5, 2, 3, and 5 µg/ml) and three quality controls (QC) (0.3, 1, and 4 µg/ml) for each validation. Tedizolid was spiked into mouse plasma to make 6 standard solutions (0.2, 0.4, 0.8, 2, 3, and 5 µg/ml) and two quality controls (0.3 and 4 µg/ml). Additional quality controls (0.3, 1, and 4 µg/ml) were made in human serum for a cross matrix validation against the human plasma standard curve. Aliquots of the standards and internal standard were stored at -80°C until analysis.

Method Validation

200 µl of sample and 50 µl of internal standard was pipetted into a polyethylene tube. 150 µl of a 7.5% tri chloro actetic acid was then carefully added down the side of the tube to deproteinize the sample. The sample was vortexed for 30 seconds and then centrifuged for 10 minutes at 3600rpm. 200µl of the sample was then placed into a WISP vial (Waters) for injection.

Assay validation

The peak height ratio of tedizolid and the internal standard was plotted to generate the calibration curve. The linear regression equation was generated by applying the weighted (1/concentration) least square regression analyses. Linear regression was used to calculate the concentrations of the quality controls and unknown samples. Linearity of the standard curve was assessed with the correlation coefficient by plotting the peak height ratio of tedizolid versus the theoretical concentrations.

A human plasma and saline calibration curve consisted of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and eight non-zero samples covering the expected concentration range of the unknown samples, including the lower limit of quantification (LLQ) for the human plasma and saline validations. The precision and accuracy were evaluated with the three quality controls with low, middle, and high concentrations. Precision was determined by taking the standard deviation divided by the average. Accuracy was determined by the relative error from the theoretical concentrations taking the calculated concentration divided by the theoretical concentration and subtracting from 100%. The LLQ for the assay was evaluated on five samples with 0.2 µg/ml of tedizolid for each validation.

Human plasma and saline quality control samples were run in triplicate to determine tedizolid stability at room temperature, freeze and thaw, in stock solution, and stability in the autosampler. Room temperature studies were done at 23°C. Each quality control sample was thawed and kept at room temperature for 6 hours which is the maximum length of time it takes to process the samples in a run. Freeze and thaw stability was assessed by completely thawing the quality controls at room temperature and then refreezing them at -80°C for 24 hours. This freeze and thaw cycle was repeated two more times and analyzed after the third freeze and thaw cycle. The stability of the drug stock solution and the internal standard were evaluated at room temperature for 6 hours and compared to that of freshly prepared solutions. The autosampler stability of a sample after extraction was verified to ensure that all samples stored in the cooled auto sampler during a run do not experience degradation in the time required to complete the assay. This stability assessment was performed on three aliquots of each quality control concentration. To determine long term stability, aliquots of each of the quality control concentrations were stored at -80°C. The concentrations of the long term stability samples were compared to the mean of back-calculated values for the appropriate standards from the first day.

Recovery experiments were performed in triplicate for each of the human plasma and saline quality control concentrations. Values of percent recoveries of tedizolid were calculated by comparing the peak height ratio of tedizolid and the internal standard in plasma to that of non-extracted pH adjusted saline solutions for human plasma. For the saline assay values of percent recoveries of tedizolid were calculated by comparing the peak height ratio of tedizolid and the internal standard in saline to that of water.

Since tedizolid is likely to be determined in other matrices, a cross matrix validation was performed in human serum against the human plasma standard curve. A full validation of tedizolid was conducted in human plasma and saline. A partial validation was conducted in mouse plasma.

To assess the absolute recovery, precision, and accuracy of the current analytical methodology, we re-assayed human plasma samples from a previously conducted pharmacokinetic study. These samples were originally assayed using a validated liquid chromatography – mass spectrometry and liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) method and stored at -80°C [3].

Results

Chromatography

Human plasma from six individuals was tested for interference using this assay. Figure 2 represents a typical chromatogram with blank human plasma. The chromatogram shows no interfering peaks with tedizolid or the internal standard. The retention time of the internal standard and tedizolid were 8.8 and 15.1 minutes for the plasma assay. Figure 3 represents a typical chromatogram with blank saline and shows no interfering peaks with tedizolid or the internal standard. A slight shift in retention time was seen on the saline standard curve. The internal standard and tedizolid were 9.4 and 16.3 minutes respectively. The retention time of the internal standard and tedizolid were 8.1 and 14.2 minutes for the mouse plasma standard curve. Figure 4 represents a chromatogram with blank mouse plasma.

Linearity, precision and accuracy

Linearity was demonstrated with the correlation coefficient (r) for each calibration curve of ≥0.999 for plasma (n=11) and saline (n=6).

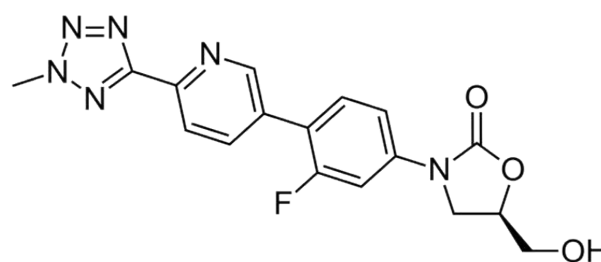

Figure 1: Chemical structure of Tedizolid.

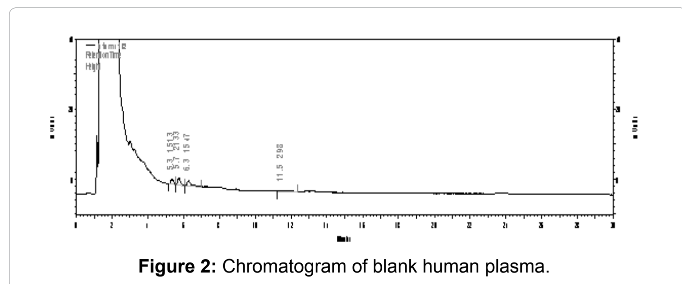

Figure 2: Chromatogram of blank human plasma.

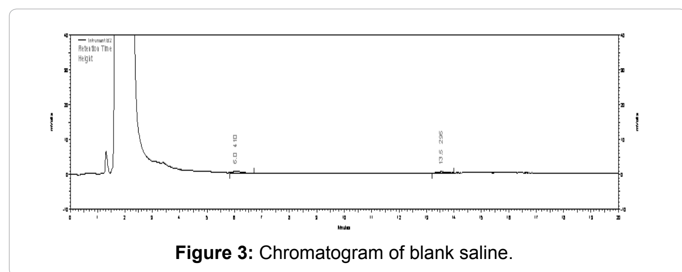

Figure 3: Chromatogram of blank saline.

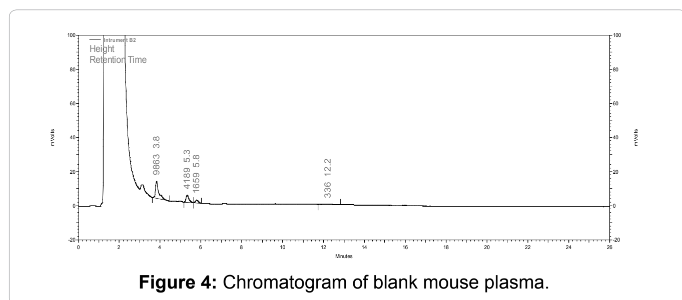

Figure 4: Chromatogram of blank mouse plasma.

The plasma slope was 0. 4350 ± 0.013 (mean ± standard deviation (SD)) and the intercept was 0.0041 ± 0.005 (mean ± SD). The saline slope was 0.4904 + 0.066 (mean ± SD) and the intercept was 0.0017 ± 0.004 (mean ± SD). Linearity for the mouse plasma (n=3) was demonstrated with the correlation coefficient (r) for each calibration curve of ≥0.999. The mouse plasma slope was 0.4796 ± 0.033 (mean ± SD) and the intercept was 0.0078 ± 0.006 (mean ± SD).

The summary data for the inter- and intra-day precision and accuracy in tedizolid-spiked human plasma are shown in Table 1. Summary data for the saline inter- and intra-day precision and accuracy are shown in Table 2. Summary data for the mouse plasma standard curve inter- and intra-day precision and accuracy are shown in Table 3.

Lower limit of quantification and recovery

The LLQ of 0.2 µg/ml for tedizolid was chosen as the concentration for the lowest standard sample (Figure 5). The precision and accuracy of LLQ (n=5) for tedizolid in human plasma was 2.25% and 3.75%, respectively while the precision and accuracy of the LLQ (n=5) for tedizolid in saline (Figure 6) was 0.50% and -8.44%.

Recovery experiments were performed in triplicate by comparing the analytical results for extracted plasma samples at the quality control concentrations with unextracted controls in a 0.1M phosphate buffer of pH 8 that represents 100% recovery. The pH of 8 was chosen to closely resemble that of the plasma and serum used. The recovery of the 0.3, 1 and 4 µg/ml samples was 81.09% 0.045%, 77.22% ± 0.039%, and 82.66% ± 0.004%, respectively. Similarly recovery experiments were performed in triplicate for extracted saline samples at the quality control concentrations with unextracted controls in water that represents 100% recovery. The recovery of the 0.3, 1 and 4 µg/ml samples was 104% + 0.0283, 102.5% + 0.042, and 101.8% + 0.017.

Stability

Room temperature stability was determined in triplicate for each of the three quality control concentrations. Each sample was thawed at room temperature (23°C) and kept at this temperature for 6 hours. Tedizolid plasma quality controls were stable at room temperature with <9% degradation. Tedizolid saline quality controls were stable at room temperature with < 10 % degradation. The 4-nitroaniline standard solution of 6 µg/ml was stable at room temperature for 6 hours with <3% degradation. Tedizolid plasma and saline quality controls were stable for three freeze thaw cycles with <10% degradation. Stock solution stability of the drug and the internal standard were evaluated on each of the three aliquots of quality control concentration at room temperature for 6 hours with <9% degradation. The autosampler stability of a sample after extraction is verified to ensure that all samples stored in the cooled autosampler during a run do not experience degradation in the time required to complete the assay. The stability was performed on three aliquots of each quality control concentration. The plasma and saline quality control samples after extraction in the 10°C autosamplerr were stable for 24 hours with <8% degradation. Tedizolid plasma quality controls were stable at -80°C for 28 days with <9% degradation while the tedizolid in saline quality controls were stable at -80°C for 11 days with <9% degradation

Cross matrix validation

The summary data for the human serum inter- and intra-day precision and accuracy for tedizolid are shown in Table 4.

Comparison of HPLC and mass spectrometry

A total of 9 samples were assayed with concentrations ranging from 0.36 to 2.97 µg/ml. Average recovery of these samples was 91.01% ± 0.052 as shown in Table 5. Precision and accuracy were 5.71% and -9.16% respectively [3].

Conclusion

An efficient and reliable HPLC method has been developed to assess tedizolid concentrations in human plasma, human serum, saline, and mouse plasma. Precision and accuracy of the QC samples were within the acceptable limits of 10% as defined by FDA guidelines [2]. Moreover, this methodology was successfully used to replicate the results obtained previously using a LC-MS/MS detection method. While LC-MS/MS detection is more sensitive, this current HPLC method is more cost effective and has the advantage of less variability.

	Tedizolid concentration (µg/ml)		
	Low (0.3)	Medium (1)	High (4)
Inter-run (n=11)			
Mean	0.31	1.01	4.20
SD	0.007	0.028	0.133
Precision	2.16%	2.72%	3.16%
Accuracy	4.88%	1.02%	4.92%
Intra-run (n=10)			
Mean	0.32	1.06	4.31
SD	0.009	0.026	0.062
Precision	2.75%	2.48%	1.43%
Accuracy	6.40%	5.65%	7.86%

Table 1: Precision and accuracy of Tedizolid in human plasma.

	Tedizolid concentration (µg/ml)		
	Low (0.3)	Medium (1)	High (4)
Inter-run (n=6)			
Mean	0.31	1.04	4.18
SD	0.010	0.010	0.072
Precision	3.30%	1.01%	1.72%
Accuracy	4.44%	3.50%	4.45%
Intra-run (n=10)			
Mean	0.30	1.04	4.24
SD	0.013	0.019	0.104
Precision	4.35%	1.86%	2.5%
Accuracy	0.46%	4.45%	6.03%

Table 2: Precision and accuracy Tedizolid in saline.

	Tedizolid concentration (µg/ml)	
	Low (0.3)	High (4)
Inter-run (n=3)		
Mean	0.30	4.12
SD	0.006	0.080
Precision	1.95%	1.95%
Accuracy	-1.11%	2.92%
Intra-run (n=10)		
Mean	0.29	4.11
SD	0.012	0.058
Precision	4.30%	1.4%
Accuracy	-3.86%	2.73%

Table 3: Precision and accuracy of Tedizolid in mouse plasma.

	Tedizolid concentration (µg/ml)		
	Low (0.3)	Medium (1)	High (4)
Inter-run (n=4)			
Mean	0.32	1.01	4.11
SD	0.008	0.039	0.066
Precision	2.55%	3.92%	1.61%
Accuracy	6.67%	0.75%	2.62%
Intra-run (n=10)			
Mean	0.31	0.96	4.06
SD	0.010	0.027	0.120
Precision	3.09%	2.79%	2.95%
Accuracy	4.05%	-3.54%	1.51%

Table 4: Precision and accuracy of Tedizolid in human serum.

Subject	Sample Time	Re-assayed Concentration	Original Concentration	%Accuracy	%Recovery
104	0h	0.36	0.37	-3.25%	96.23%
104	1h	2.97	3.25	-8.50%	91.50%
104	8h	1.25	1.46	-14.32%	85.68%
101	0h	0.48	0.48	-0.32%	100.52%
101	1h	1.88	2.10	-10.29%	89.71%
101	8h	1.23	1.39	-11.41%	88.59%
118	0h	0.45	0.48	-5.49%	94.12%
118	1h	2.42	2.88	-15.81%	84.19%
118	8h	1.37	1.55	-11.45%	88.55%

Table 5: Comparison of tedizolid concentrations (µg/ml) in HPLC and mass spectrometry.

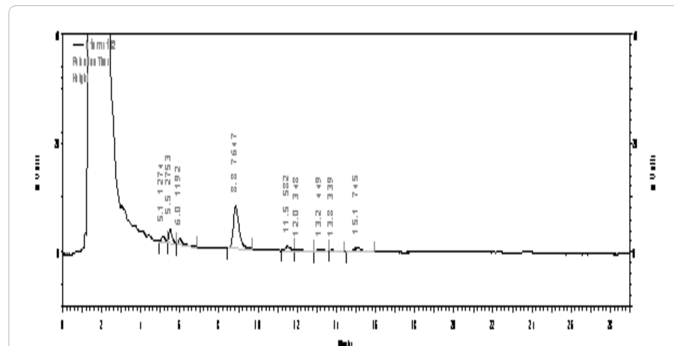

Figure 5: Chromatogram of LLQ in human plasma.

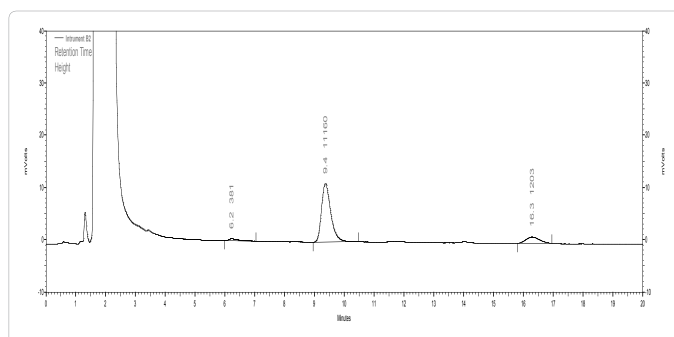

Figure 6: Chromatogram of LLQ in saline.

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