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Determination of linezolid in plasma by reversed-phase high-performance liquid chromatography

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Abstract

An HPLC–UV method was developed for assay of linezolid in dog, rat, mouse, and rabbit plasma. Linezolid and the internal standard were extracted on a solid phase cartridge (SPE) and separated on a reversed-phase column (C8, 4.6 × 150 mm, 5 μm) with 20% acetonitrile in water as mobile phase. The SPE quantitatively recovered linezolid and the internal standard from plasma samples. The chromatographic peak height ratio or peak area ratio based on UV absorbency at 251 nm was used for quantitative analysis. The assay procedures were simple and the assay was specific and had adequate precision and accuracy. Calibration standards with concentrations over the range of 0.01–20 μg/ml were validated for routine sample analysis to support the pharmacokinetic and toxicology studies with linezolid in dog, rat, mouse, and rabbit. Analysis of quality control samples showed the coefficients of variation were usually $< 10\%$ and the measured and theoretical concentrations differed by $< 10\%$ in most assays. Linezolid in the plasma samples was stable when stored at below -20°C for at least 63 days, at room temperature (22–23°C) for up to 24 h, and after three freeze–thaw cycles. This HPLC method has been successfully used in multiple laboratories to assay plasma samples from pharmacokinetic and toxicology studies with linezolid in the animal species.

Keywords: Linezolid; Rat, dog, mouse, and rabbit plasma; Solid phase extraction; HPLC assay; UV detection; Method validation; Precision; Accuracy

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1. Introduction

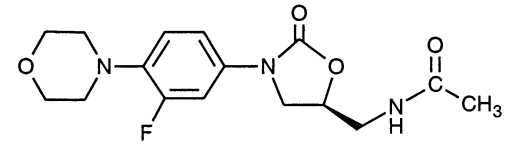
The recent increases in serious clinical infections resulting from the emergence of drug-resistant bacteria call for an urgent need for new antimicrobial agents for treatment of the infections [1]. Linezolid (PNU-100766) (**I**) is a novel oxazolidinone antimicrobial drug candidate currently under development for treatment of multidrug-resistant gram-positive bacterial infections [2]. In vitro microbiological studies showed that linezolid is effective against various antibiotic-resistant isolates of staphylococci, streptococci, enterococci, and pneumococci [3–10]. Recently, the antimicrobial activities of linezolid were validated in mice in vivo [11] and in early clinical studies [12]. Linezolid exhibits a unique mechanism of inhibition of bacterial protein synthesis at the initiation phase of translation [3,13].

A specific assay method with adequate specificity, sensitivity, precision, and accuracy has been developed to support the pharmacokinetic and toxicology studies in animal species during the pre-clinical phase of development of linezolid. This method is based on solid phase sample extraction (SPE), high-performance liquid chromatographic (HPLC) separation, and ultraviolet (UV) absorbency detection. It has been applied in the assay of linezolid in plasma samples from dog, rat, mouse, and rabbit after single and multiple dose administration of linezolid in pharmacokinetic and toxicology studies.

2. Experimental

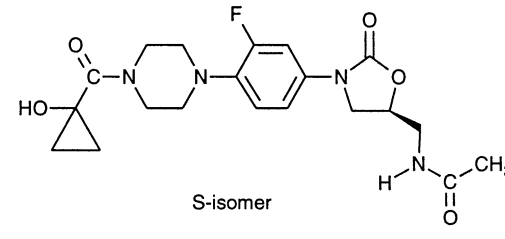
2.1. Chemicals and reagents

Linezolid, (*S*)-*N*-[[3-[3-fluoro-4-(4-morpholinyl) phenyl]-2-oxo-5-oxazolidinyl]methyl]-acetamide or PNU-100766 (**I**), and the internal standard (IS), (*S*)-*N*-[[3-[3-fluoro-4-[4-[(1-hydroxy c y c l o-propyl)carbonyl]-1-piperazinyl]phenyl]-2-oxo-5-oxazolidinyl]methyl]-acetamide or PNU-101145 (**II**), were provided by Medicinal Chemistry Research, Pharmacia & Upjohn, Kalamazoo, MI. HPLC grade acetonitrile and methanol were purchased from EM Science, Gibbstown, NJ. Water was purified through a Milli-Q UV Plus System (Millipore, Bedford, MA).



PNU-100766

I



S-isomer

PNU-101145

II

2.2. Instrumentation and chromatographic analysis

SPE cartridges (C2, 1 ml, 100 mg) were purchased from Varian (Harbor City, CA). Calibrated pipettes of various volume sizes (Rainin, Wobrun, MA) were used for quantitative transfer of biological samples and reagent solutions.

A typical HPLC system set up includes a Series 200 lc pump, Series 200 autosampler, and model 785 UV/VIS detector (Perkin Elmer, Norwalk, CT). A reversed-phase column (Zorbax RXC8, 4.6 × 150 mm, 5 μm, MAC-MOD, Chadds Ford, PA) with a precolumn (Zorbax C8, 4.6 × 12.5 mm, 5 μm) and a mobile phase of 20:80 (v/v) acetonitrile/water were used for the chromatographic separation. Other equivalent HPLC systems and components are suitable. The HPLC was interfaced with a computer data system to automate the sample injection and to capture and analyze the chromatographic data.

2.3. Standard solutions of linezolid and internal standard

A target amount of 1 mg of linezolid was

accurately weighed on an analytical balance (AE240, Mettler) and placed in a 10-ml volumetric flask to make a 10-ml stock standard solution in water with a target concentration of 100 μg/ml. The stock standard solution was diluted with water to yield the working standard solutions of 40, 12, 4, 1.2, 0.4, 0.12, 0.04, and 0.02 μg/ml. Similarly, a target amount of 1 mg of the internal standard was accurately weighed and dissolved in water to make 50 ml stock IS solution (target concentration: 20 μg/ml). This solution was diluted with water (0.5 ml–100 ml) to make a working IS solution of 0.1 μg/ml. The stock and working standard solutions and the stock and working IS solutions were stored in a refrigerator (5°C) when not in use. Under these conditions, the stock standard and IS solutions were stable for at least 2 months and the working standard and IS solutions were stable for at least 1 month from the day of preparation.

2.4. Calibration standard samples

For each chromatographic assay run, the calibration standard samples with nominal concentrations of 20, 6, 2, 0.6, 0.2, 0.06, 0.02, and 0.01 μg/ml in plasma were prepared by mixing 25-μl aliquots of the working standard solutions with 50 μl of blank plasma (dog, rat, mouse, or rabbit). In addition, a calibration standard with 0 μg/ml PNU-100766 was prepared by mixing the 50-μl blank plasma with 25 μl of water. Calibration standards over a different concentration range, for example 0.005–10 and 20–100 μg/ml, were prepared when necessary for assay of plasma samples from studies with very low or very high doses of linezolid.

2.5. Quality control (QC) samples

The QC samples were prepared at the target

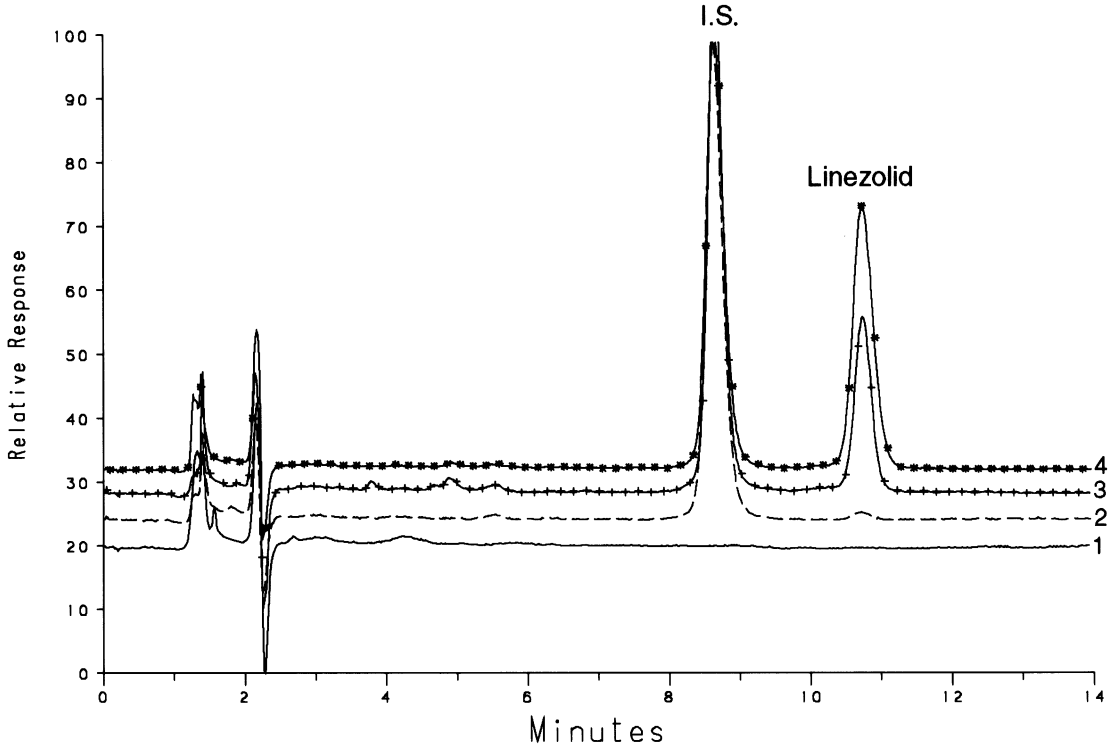


Fig. 1. Chromatograms from HPLC assay of linezolid in rabbit plasma. 1: a blank plasma sample without internal standard; 2: a quality control sample with 0.059 μg/ml linezolid; 3: a rabbit plasma sample with measured linezolid concentration of 1.20 μg/ml, and 4: a quality control sample with 2.38 μg/ml linezolid.

Table 1
Intra-assay and inter-assay precision and accuracy of HPLC analysis of linezolid in mouse plasma

Theoretical concentration (μg/ml)	Measured concentration (μg/ml)	Precision (%)	Accuracy (%)
Intra-assay 1 ($n=3$)			
0.0459	0.0441 ± 0.0009	2.1	96.1
1.90	1.77 ± 0.01	0.6	93.2
19.0	18.2 ± 0.1	0.5	95.6
Intra-assay 2 ($n=3$)			
0.0459	0.0442 ± 0.0003	0.8	96.3
1.90	1.78 ± 0.02	1.0	93.7
19.0	18.4 ± 0.2	1.1	96.8
Intra-assay 3 ($n=3$)			
0.0459	0.0455 ± 0.0021	4.7	99.1
1.90	1.79 ± 0.02	1.0	94.2
19.0	18.6 ± 0.1	0.6	97.9
Inter-assay ($n=9$)			
0.0459	0.0446 ± 0.0014	3.1	97.2
1.90	1.78 ± 0.02	0.9	93.7
19.0	18.4 ± 0.2	1.2	96.8

concentrations of approximately 20, 2, and 0.06 μg/ml in blank plasma (dog, rat, mouse, or rabbit). A target amount of linezolid was separately weighed to prepare the stock solutions for these QC samples. QC samples at other concentrations were prepared when necessary. The QC samples were divided into 100-μl aliquots in tightly closed microtubes and kept frozen at below -20°C until used for assay.

2.6. Sample preparation and chromatographic analysis

The calibration standards were prepared and analyzed concurrently with each assay run. A minimum of six QC samples, in duplicates of three concentrations, were prepared for each assay run. Generally, one QC sample was prepared for every 10 samples of the unknowns and calibration standards.

Aliquots of the unknown and QC samples (50 μl) were mixed with 25 μl water and 1 ml of the working IS solution. The calibration standard samples were also mixed with 1 ml of the working IS solution. These samples were loaded onto the SPE cartridges on an extraction manifold (Spelco, Bellefonte, PA). The SPE cartridges were preconditioned sequentially with 2×1 ml acetonitrile (or 1 ml methanol and then 1 ml acetonitrile) and 2×1 ml water. The loaded cartridges were washed with 1 ml water followed by 1 ml 5% (v/v) acetonitrile in water. Linezolid and IS were then eluted from the cartridges with 0.5 ml methanol. The methanol extracts were evaporated under a stream of nitrogen at below 40°C. The residues were dissolved in 200 μl of mobile phase for chromatographic analysis.

Aliquots (100 μl) of the prepared unknowns, calibration standards, and QC samples were injected onto the chromatographic system and analyzed The mobile phase flow rate was 1 ml/min. The column effluent was monitored for UV absorbency at 251 nm. The linezolid/IS ratios of peak heights or areas were measured for quantitative analysis.

3. Method validation

The HPLC method has been validated [14] in plasma samples from dog, rat, mouse, and rabbit for specificity, linearity range, precision, accuracy, and stability of linezolid.

3.1. Linearity range

During method validation, the lower limit of quantitation (LLOQ) and the range of linearity were established based on three separate runs of assay of freshly prepared calibration standards. The LLOQ was the concentration of the lowest concentration calibration standards with acceptable precision and accuracy. The range of linearity was from LLOQ to the highest concentration calibration standard that was either within the linear dynamic range of the detector response or necessary for the assay of the set of unknown samples.

The calibration curves were constructed from linear regression analysis of the peak height or peak area ratios versus the nominal concentrations of the calibration standards. The concentrations of linezolid in the plasma samples were calculated by inverse prediction from the calibration curve.

3.2. Precision and accuracy

In each assay validation run, a set of QC samples, in triplicates of three concentrations, were assayed concurrently with the calibration standards. The intra-assay precision was determined from the coefficient of variation (CV) of the QC samples in an assay run. The intra-assay accuracy was determined from the mean concentrations of the QC samples as the percentage of the theoretical concentrations (% recovery). Similarly, the inter-assay precision and accuracy were determined from the data of the QC samples assayed in the multiple assay runs.

3.3. Stability

The QC samples were assayed under three different conditions to assess the stability of linezolid in plasma samples. A set of QC samples were allowed to thaw and left at room temperature (approximately 22–23°C) for 4–24 h and then assayed. The results were compared to the data from assay of freshly thawed QC samples to evaluate sample stability at room temperature. A set of QC samples were subject to three freeze–thaw cycles and then assayed to evaluate freeze–thaw stability of linezolid in plasma. Long-term stability was studied by assaying samples that had been stored at below −20°C for a period of time.

Table 2
Inter-assay precision and accuracy of HPLC analysis of linezolid in rat, dog, and rabbit plasma

Theoretical concentration (μg/ml)	Measured concentration (μg/ml)	Precision (%)	Accuracy (%)
Rat plasma			
0.06 ($n = 10$)	0.0612 ± 0.0020	3.3	102.0
0.6 ($n = 12$)	0.570 ± 0.022	3.9	95.0
20.0 ($n = 10$)	19.6 ± 0.5	2.6	98.0
Rat plasma[a]			
0.0306 ($n = 9$)	0.0297 ± 0.0009	3.0	97.1
1.02 ($n = 9$)	0.999 ± 0.012	1.2	97.9
10.2 ($n = 8$)	10.5 ± 0.2	2.0	100.0
Dog plasma ($n = 9$)			
0.0647	0.0629 ± 0.0018	2.9	95.7
2.16	2.08 ± 0.06	2.9	96.3
21.6	21.8 ± 0.8	3.7	100.9
Rabbit plasma ($n = 9$)			
0.0594	0.0615 ± 0.0059	9.6	103.4
2.37	2.08 ± 0.057	2.7	87.8
47.54	48.12 ± 1.70	3.5	101.2

[a] Calibration curve concentration range: 0.005–10 μg/ml.

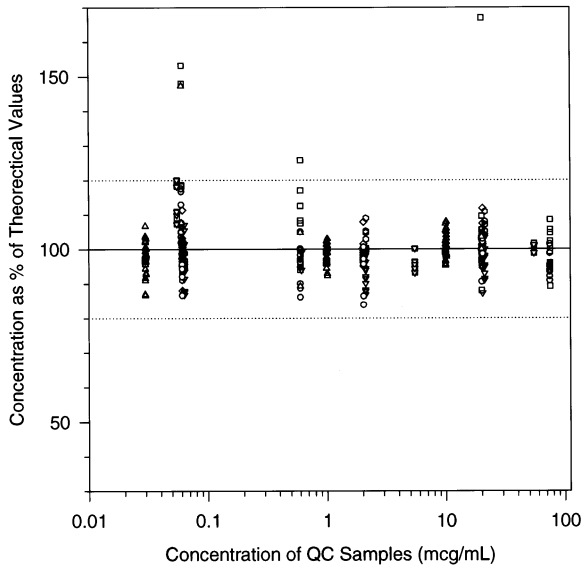


Fig. 2. Quality control data from assay of linezolid in rat plasma samples. Data were collected from assays of toxicokinetic samples in studies conducted in rats from 1994 through 1997. There were two samples with concentrations outside of the range of the *y*-axis. Each symbol represents an assay run; mcg/ml = μg/ml.

The SPE extracted samples in mobile phase solution were chromatographed when freshly prepared and re-chromatographed after storage in analytical vials in sample tray on autosampler for 48 h at room temperature. Comparison of these data evaluated the stability of linezolid in the prepared samples.

4. Results and discussion

4.1. SPE extraction and chromatography separation and specificity

Linezolid and the internal standard were quantitatively extracted from the plasma by SPE. Comparison of the peak responses from a direct solution fortification without extraction versus the plasma fortification and extraction showed the mean recoveries for linezolid and IS were 108.5 and 104.1%, respectively, across the entire range of the concentrations of the calibration curve.

Under the experimental conditions, linezolid and the IS were eluted within the retention windows of approximately 10–11 and 8–9 min, respectively. Blank plasma samples from dog, rat, mouse, and rabbit collected from multiple animals or purchased from commercial sources have shown no chromatographic peaks near the retention times of linezolid and IS that could have interfered with the assay. Pre-dose samples from pharmacokinetic and toxicokinetic studies in the animal species also did not show chromatographic interferences. Linezolid was catabolized to hydrophilic metabolites (data to be published) and the metabolites were eluted early under the chromatographic conditions and caused no interferes to the assay. These results established the specificity of the assay for linezolid. Representative chromatograms of a blank plasma sample, two QC samples, and a study sample from assay of linezolid in rabbit plasma are shown in Fig. 1.

Table 3
Stability of linezolid in mouse plasma samples stored at below −20°C

Theoretical concentration (μg/ml)	Concentration (μg/ml)[a]			
	Initial assay	14 days	35 days	63 days
0.0459	0.0441 ± 0.0009 (100)	0.0462 ± 0.0013 (104.9)	0.0446 ± 0.0002 (101.2)	0.0444 ± 0.0009 (100.8)
1.90	1.77 ± 0.01 (100)	1.79 ± 0.01 (101.1)	1.83 ± 0.05 (103.2)	2.10 ± 0.05 (118.5)
19.0	18.2 ± 0.1 (100)	18.8 ± 0.2 (103.3)	19.6 ± 0.1 (107.5)	20.0 ± 0.4 (110.1)

[a] Values are mean ± S.D. with percentage of initial concentration in parentheses.

Table 4
Stability of linezolid in dog and rat plasma samples at room temperature (22–23°C) and after three freeze–thaw cycles

Initial concentration (μg/ml)	Concentration (μg/ml) after 24 h at room temperature[b]	Concentration (μg/ml) after three freeze–thaw cycles[b]
Rat plasma[a]		
0.0608 ± 0.0009	0.0600 ± 0.0011 (98.7)	0.0607 ± 0.0003 (99.8)
2.01 ± 0.01	2.02 ± 0.02 (100.5)	2.01 ± 0.01 (100)
20.3 ± 0.1	22.4 ± 0.9 (110.3)	20.3 ± 0.1 (100)
Dog plasma[a]		
0.0585 ± 0.0017	0.059 ± 0.007 (100.9)	0.0583 ± 0.0017 (99.7)
1.83 ± 0.04	1.88 ± 0.02 (103.3)	1.91 ± 0.01 (104.3)
19.1 ± 0.1	19.0 ± 0.2 (99.4)	19.2 ± 0.2 (100.5.)

[a] Theoretical concentrations in rat plasma were 0.06, 2, and 20 μg/ml.
[b] Percentage of initial concentration in parentheses.

4.2. Linearity range

The LLOQ of 0.01 μg/ml and a linearity range of up to 20 μg/ml have been established based on the calibration curves in dog, rat, mouse, and rabbit plasma assayed in the method validation runs. For the majority of pharmacokinetic and toxicokinetic studies in the animal species, this concentration range of the calibration standards was adequate for assay of linezolid in the study samples. For other studies with linezolid doses at the low and high extremes, calibration curves with standards in concentrations ranging from 0.005 to 10 μg/ml and from 20 to 100 μg/ml were prepared to cover the low and high concentrations in the study samples, respectively. The calibration curves over these alternative concentration ranges have been validated. Aliquots of 100 μl of plasma samples were assayed when the calibration curves were over the range from 0.005 to 10 μg/ml.

For quantitative analysis, a linear equation of peak height ratios or peak area ratios versus linezolid concentrations using the reciprocals of the concentrations as weighting factors satisfactorily described the relationship between the detector response and concentration. Typically, the correlation coefficients ranged from 0.999 to unity. Alternatively, when the intercept of the linear regression line was not significantly different from zero ($P > 0.05$), a linear regression with forced zero intercept was used for quantitative analysis. Essentially identical results were obtained using either the peak height or peak area ratio for quantitative analysis.

4.3. Precision and accuracy

A representative set of data of intra- and inter-assay precision and accuracy of linezolid in mouse plasma is summarized in Table 1. The measured concentrations had CVs $\leq 4.7\%$ among the intra-assay samples ($n = 3$) and $\leq 3.1\%$ among the inter-assay samples ($n = 9$), indicating adequate intra- and inter-assay precision. The QC samples had measured concentrations in the range of 93.2–99.1% of the theoretical concentrations within an assay run and 93.7–97.2% between assay runs, indicating acceptable intra- and inter-assay accuracy. Table 2 summarizes the inter-assay precision and accuracy for assay of linezolid in rat, dog, and rabbit plasma. In these sample matrices, the inter-assay precision (CV, $n = 8$–12) was $\leq 9.6\%$ and the concentrations found were 95–103.4% of the theoretical concentrations, except for the mid-concentration QC samples in rabbit plasma which had a recovery of 87.8%. Thus, the assay of linezolid in rat, dog, and rabbit plasma also had acceptable intra-assay (data not shown) and inter-assay precision and accuracy.

Fig. 2 shows the QC data from assays in multiple laboratories supporting toxicology studies in rats conducted from 1994 through 1997. The theoretical concentrations of linezolid in the QC samples were in logarithmic scale on the x-axis

for reading convenience. Very few of QC samples had concentrations outside ±20% of the theoretical values. All assay runs completed thus far met the generally accepted criteria for acceptance of the analytical data [14]. These QC data showed reasonable central tendency around the theoretical 100% for concentrations ranging from approximately 0.03 to 75 μg/ml, indicating that the HPLC assays were well-controlled in multiple laboratories and over a period of 3 years.

4.4. Sample stability

Linezolid was stable in plasma samples. For example, Table 3 shows the long-term stability of linezolid in QC samples in mouse plasma. Compared to the theoretical linezolid concentrations at 0.0459, 1.9 and 19 μg/ml, the concentrations assayed initially and after storage of the samples at below −20°C for 14, 35, and 63 days showed no trend of decrease with time. These data indicated that linezolid in mouse plasma samples was stable for at least 63 days when stored frozen at below −20°C. Linezolid was also stable in plasma samples kept at room temperature (22–23°C) for 4 and 24 h and after three freeze–thaw cycles. For example, the linezolid concentrations in the QC samples in rat and dog plasma after 24 h room temperature storage showed no apparent degradation: the final concentrations were from 98.7 to 110.3% of the initial values in rat plasma and from 99.4 to 103.3% in dog plasma (Table 4). After three freeze–thaw cycles, linezolid in rat and dog plasma was also stable with its concentrations essentially unchanged (Table 4). Similar stability of linezolid in mouse and rabbit plasma at room temperature and three freeze–thaw cycles has been established. Linezolid extracted from mouse, rat, rabbit, and dog plasma samples in mobile phase was also stable for at least 48 h at room temperature.

4.5. Ruggedness

The ruggedness of the assay method has been demonstrated by the successful applications of the assay over time and among multiple laboratories in the quantitative analysis of linezolid in plasma samples from pharmacokinetic and toxicology studies in dog, rat, mouse, and rabbit. This method allowed variations in analytical equipment and in the processing of analytical data among the participating laboratories. For example, chromatographic separation at 35°C using a column oven was implemented in one laboratory. A procedure of switching the mobile phase to 100% acetonitrile and re-equilibrating the system with the mobile phase between samples was also successfully implemented. The acetonitrile flush of the column between samples was beneficial for long assay runs, but was not necessary for assay of 80 or fewer samples in a run. Measurement of UV absorbency at 251 nm (variable wavelength detector) and at 255 nm (diode array detector), using peak height and peak area for quantitative analysis, and using different linear regression models for calibration curves have generated acceptable assay results with adequate precision and accuracy to support pharmacokinetic and toxicology studies. More recently, human plasma and urine samples have been assayed using this HPLC method.

5. Conclusion

The HPLC–UV method was validated for assay of linezolid in dog, rat, mouse, and rabbit plasma. The assay procedures were simple and had adequate precision and accuracy with CVs $< 10\%$. The differences between the measured and theoretical concentrations of linezolid in the QC samples were generally $< 10\%$. The calibration standards with concentrations over the range of 0.01–20 μg/ml were validated for routine sample analysis to provide toxicokinetic support to toxicology studies in dog, rat, mouse, and rabbit. Alternative calibration curves with concentration ranges from 0.005 to 10 μg/ml and from 20 to 100 μg/ml have also been established for assay of samples from studies with very low and very high doses of linezolid. Linezolid was stable in plasma sample stored at below −20°C for at least 63 days. Linezolid concentrations in plasma did not decrease after leaving the sample at room temperature (22–23°C) for up to 24 h and after three

freeze–thaw cycles. Linezolid in the prepared sample in mobile phase solution was stable for at least 48 h. Multiple laboratories with variations in analytical facility and procedures have successfully adopted the assay method for analysis of plasma samples to support pharmacokinetic and toxicology studies.

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