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High-performance liquid chromatographic methods for the determination of a new carbapenem antibiotic, L-749,345, in human plasma and urine

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Abstract

A column-switching, reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of a new carbapenem antibiotic assay using ultraviolet detection has been developed for a new carbapenem antibiotic L-749,345 in human plasma and urine. A plasma sample is centrifuged and then injected onto an extraction column using 25 m*M* phosphate buffer, pH 6.5. After 3 min, using a column-switching valve, the analyte is back-flushed with 10.5% methanol–phosphate buffer for 3 min onto a Hypersil 5 μm C_{18} BDS 100×4.6 mm analytical column and then detected by absorbance at 300 nm. The sample preparation and HPLC conditions for the urine assay are similar, except for a longer analytical column 150×4.6 mm. The plasma assay is specific and linear from 0.125 to 50 μg/ml; the urine assay is linear from 1.25 to 100 μg/ml.

Keywords: Carbapenem antibiotic; L-749,345

1. Introduction

L-749,345 (Fig. 1A) is a broad spectrum antibiotic. It is structurally similar to imipenem with a different sulfhydryl side-chain (2-[[3-carboxylphenyl) amino]carbonyl]-pyrrolidine-4-yl) and a methyl substituent in place of a proton on C-4. Unlike imipenem [1, 2], L-749,345 is more stable to chemical hydrolysis of the β-lactam moiety and is resistant to both β-lactamase and human renal dehydropeptidase-I. A column-switching HPLC assay method using ultraviolet absorbance detection was developed for L-749,345 in human plasma and urine to support human pharmacokinetic studies. Similar methodology [3] has been reported for the carbapenem L-739,428 in rat and monkey plasma. The method does not require sample preparation involving plasma deproteination or analyte extraction prior to HPLC analysis. The approach minimizes drug loss that might occur during an isolation procedure prior to HPLC, i.e., drug degradation due to hydrolysis of L-749,345 to its open-lactam form (Fig. 1B); the approach also eliminates the search for an appropriate internal standard with similar stability. The lower levels of quantitation for the plasma and urine

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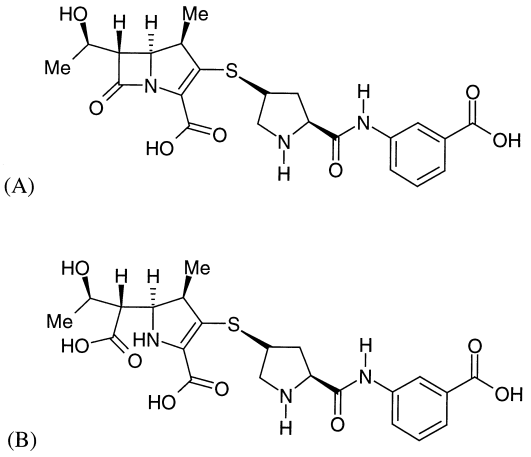

Fig. 1. Structures for L-749,345 acid form (A) and its open-lactam (B).

assays are 0.125 μg/ml and 1.25 μg/ml, respectively, using an injection volume of 50 μl.

2. Experimental

2.1. Chemicals and reagents

L-749,345, (4*R*,5*S*,6*S*,8*R*,2′*S*,4′*S*)-3-[[2-[[3-carboxyphenyl) amino] carbonyl] -pyrrolidin-4-yl] thio]-4-methyl-6-(1-hydroxy)-7-oxo-1-azabicyclo[3.2.0]-hept-2-en-2-carboxylic acid mono-sodium salt, (MK-0826) [4] was obtained from Merck (Rahway, NJ, USA). 2-[*N*-Morpholino]ethane-sulfonic acid (MES acid) and its respective sodium salt (MES sodium salt) and ethylene glycol were purchased from Sigma (St. Louis, MO, USA). Sodium phosphate dibasic anhydrous, ACS grade, *o*-phosphoric acid, 85% and methanol, optima were obtained from Fisher (Fair Lawn, NJ, USA). All chemicals were used as received.

2.2. Equipment

The HPLC system consisted of equipment from Waters (Milford, MA, USA) and Applied Biosystems (Foster City, CA, USA): Model 717plus autosampler, a 600E system controller and pump, a 6000A solvent delivery system and a Spectroflow 783A absorbance detector. The detector signal was acquired and processed by a Perkin Elmer Nelson Access*Chrom data acquisition system (Cupertino, CA, USA). The column switching was performed by an Autochrom M10 column switching valve (10-port) which was purchased from Valco Instruments (Houston, TX, USA).

2.3. Chromatographic conditions

2.3.1. Plasma

An extraction column Maxsil 10 μm ODS (50 mm×4.6 mm) from Phenomenex (Torrance, CA, USA) is in-line after Pump 1 (Fig. 2), injector and prep filter, using a 10-port valve. The analytical column BDS Hypersil C_{18}, 5 μm (100 mm×4.6 mm) from Keyston Scientific (Bellefonte, PA, USA) is in-line after Pump 2 and prefilter and before the detector. Pump 1 and 2 flow-rates were set at 1.5 ml/min and 2.0 ml/min, respectively. The autosampler was programmed with an injection volume of 50 μl, a carousel temperature of 5°C and a run time of 13 min. The absorbance wavelength on the detector was set at 300 nm. The pneumatic switching

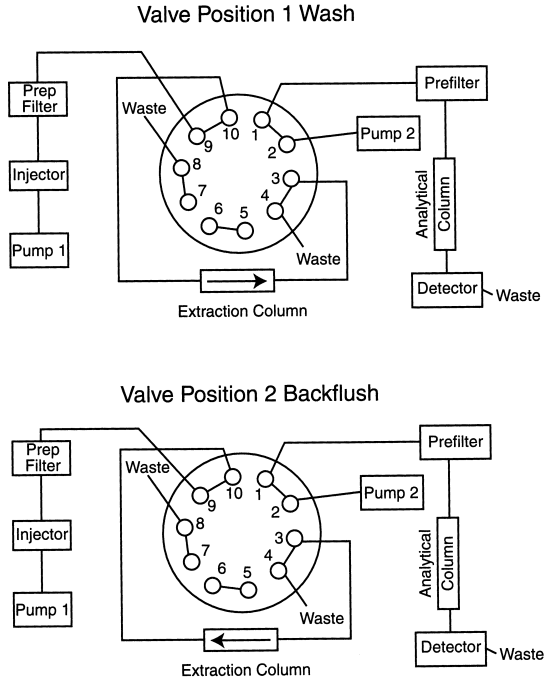

Fig. 2. Diagram of the different column-switching positions of the HPLC system.

valve was activated by contact closures on the Waters 600E System Controller or contact closures on the PE Access Chrom A/D box. The valve was in position 1 (Fig. 2) for 3 min after sample injection of the analyte onto the extraction column and wash of the column with the phosphate buffer, pH 6.5; the valve was switched to position 2 for 3 min during backflush of the analyte on to the analytical column. After 6 min from injection, the valve moved back to position 1, waiting for the next injection.

Mobile phase for pump 1 was 25 m*M* sodium phosphate buffer, pH 6.5 (7.1 g sodium phosphate dibasic anhydrous in 2000 ml of water, pH adjusted to pH 6.5 with *o*-phosphoric acid, 85%); for pump 2, 10.5% methanol in 25 m*M* phosphate buffer, apparent pH 6.5. The mobile phases were degassed under vacuum by ultrafiltration using 0.45 μm Nylon-66 filters (Rainin, Woburn, MA, USA).

2.3.2. Urine

Chromatographic conditions for the urine assay were identical to the plasma assay except for the analytical column and the mobile phase. A longer column (BDS Hypersil C_{18} 5 μm, 150 mm×4.6 mm) was necessary to separate the analyte from endogenous urine background. For further separation (as necessary for a particular subject), mobile phase for pump 2 was adjusted between 7 and 11% methanol in phosphate buffer.

2.4. Short-term stability of L-749,345 in plasma and urine at 5°C and 24°C

2.4.1. Plasma

Stability samples were prepared at 1.5, 3.0, 6.0, 15.0, 30.0, and 60.0 μg/ml in plasma and in plasma mixed with an equal volume of stabilizing solution. The stabilizing solution was prepared by mixing ethylene glycol with 0.1 *M* MES acid, 1:1 v/v (19.5 g of MES acid in 1000 ml water).

2.4.2. Urine

Stability samples at 2.5, 5, 12.5, 25, 50 and 100 μg/ml were prepared in urine and in urine mixed with an equal volume of the 0.1 *M* MES buffer, pH 6.5 (15.5 MES sodium salt and 5.6 g MES acid in 1000 ml water).

The plasma and urine samples were analyzed over time in an autosampler set at 5°C or 24°C. The time intervals for the plasma and urine stability studies were about 12 and 24 h, respectively. A stability sample was placed in an autosampler and periodically analyzed directly using the column-switching HPLC procedure. The changes in analyte concentrations with time were measured by comparing peak areas (in percentages), starting with 0-time.

2.5. Preparation of plasma and urine quality controls (QCs) for long-term stability and freeze–thaw cycles

For QC at 0.25 μg/ml, an aliquot 50 μl from a 500 μg/ml L-749,345 stock solution was transferred to a 100-ml volumetric flask and was diluted to the mark with human control plasma (SeraTec, New Brunswick, NJ, USA) and mixed by vortex. The 100-ml plasma solution was stabilized by adding an equal volume of 0.1 *M* MES acid–ethylene glycol 1:1 v/v solution. The stabilized plasma QC was divided into 750 μl aliquots in conical polypropylene tubes with caps and stored at −70°C. The procedure was repeated for the high QC (40 μg/ml) by transferring 400 μl from a 10.0 mg/ml stock solution of L-749,345 to a 100-ml flask and diluting with human plasma.

Similarly, urine QCs were prepared at concentrations 10.3 and 41.2 μg/ml. Urine solutions of L-749,345 were prepared and then mixed with an equal volume of 0.1 *M* MES buffer, pH 6.5 (stabilizer). The stabilized urine solutions were divided into 1.0-ml aliquots in previously described tubes and stored at −70°C.

The QCs were removed from storage at specific times for analysis to determine assay interday precision and drug stability. A set of QCs was also thawed at room temperature, analyzed and frozen to determine freeze–thaw stability. This freeze thaw cycle was repeated three to four times.

2.6. Solid-phase extraction (SPE) procedure for plasma and urine

A SPE cartridge Varian C_{18} 3cc 200 mg (Harbor City, CA, USA) was prepared for analyte isolation by wetting the packing with a one-column volume of methanol followed by one-column volume of water

on a vacuum manifold, a SPE™-21 (J.T. Baker, Phillipsburg, NJ, USA) . A 1-ml aliquot of a plasma sample containing L-749,345 and stabilizer was added to the SPE followed by vacuum. The SPE column was then washed with 1 ml water, and the analyte was eluted with 1 ml of 30% methanol in sodium phosphate buffer, pH 6.5. The sample was then injected directly onto the HPLC without the column-switching valve and the extraction column.

A 1-ml aliquot of urine sample containing stabilizer is added to a preconditioned SPE cartridge. The cartridge was washed with 3 ml water, and the analyte was eluted with 1 ml methanol–water (30:70, v/v).

2.7. Preparation of calibration standards

2.7.1. Plasma

A 0.5-mg/ml stock solution was prepared as the carboxylic acid in 0.1 *M* MES buffer, pH 6.5. Appropriate volumes of the acid solution were transferred to volumetric flasks and diluted with MES buffer to attain concentrations at 2.5, 5.0, 10.0, 20.0, 50.0, 100.0 and 250.0 μg/ml. Appropriate volumes of the above working standards (50–100 μl) and control human plasma were mixed to obtain plasma standards from 0.125 to 50 μg/ml. Stabilizer (0.1 *M* MES acid–ethylene glycol) was added to each plasma standard in a 1:1 (v/v) ratio.

2.7.2. Urine

Urine standards were similarly prepared as plasma standards. Stock solutions at 0.50 and 0.05 mg/ml in 0.1 *M* MES buffer, pH 6.5 were prepared and appropriate volumes were mixed with human control urine to obtain concentrations 1.25 to 100 μg/ml. MES buffer, pH 6.5 was added to each urine standard in a 1:1 (v/v) ratio.

Aliquots of the stock solutions were found to be stable for at least 5 days at −70°C.

2.8. Sample preparation for direct-injection HPLC

Frozen plasma samples and QCs were thawed at room temperature, mixed by vortex and aliquots were transferred to 12×75-mm polypropylene conical test tubes. The tubes are centrifuged at 750 *g* at 5°C for 5 min. The supernatants were transferred to autosampler tubes and refrigerated at 5°C until analysis. Samples with measured concentrations greater than the upper calibration standard were diluted with a 1:1 (v/v) mixture of plasma and the 0.1 *M* MES buffer, pH 3.5–ethylene glycol stabilizer and then reanalyzed. Frozen urine samples and QCs were prepared as described for plasma samples, but the dilutions were prepared with the 0.1 *M* MES buffer, pH 6.5.

3. Results and discussion

3.1. Chromatography

The HPLC of the carbapenem in plasma involves the initial retention of the analyte on a reversed-phase C_{18} MAXSIL guard column (extraction column) for 3 min using a phosphate buffer, pH 6.5 mobile phase. The analyte is then back-flushed off the column with 10.5% methanol–phosphate buffer to a BDS Hypersil C_{18} analytical column (100×4.6 mm). The column-switching valve returns the extraction column to the phosphate buffer mobile phase after 3 min, waiting for the next injection. The chromatography is specific for the analyte in human plasma (Fig. 3), which includes different commercial control plasmas and many predose subject plasmas from different clinical studies. The open-lactam form of L-749,345 (Fig. 1) is the major metabolite and it elutes before the analyte as a broad peak ($\alpha=2$). The lower limit of detection is 0.064 μg/ml ($S/N=3:1$).

The timing for the column-switching valve and the type of extraction and analytical columns used were investigated for peak shape, sensitivity and specificity. For example, the 3-min time interval for back-extraction of the analyte was shortened to 1 min. The time difference appeared to sharpen the analyte peak, but the reproducibility of the peak area for standards varied with use. The chromatography of the extraction column most likely was changing with use and not all of the analyte was eluted from the extraction column, and thus, the peak shape of the analyte was changing.

Chromatography of the analyte could be improved (e.g. sharper peak) by using a different extraction column, BDS Hypersil 5 μm (50 mm×4.6 mm). However, compared to the MAXSIL 10-μm column,

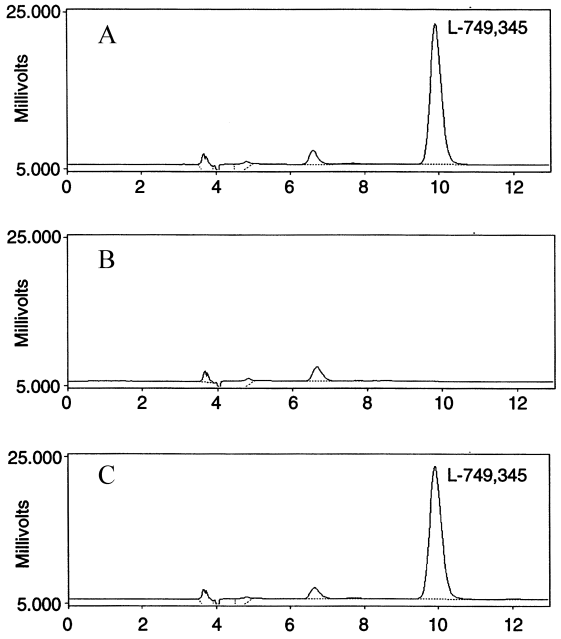

Fig. 3. Chromatogram of (A) plasma standard containing 25 μg/ml of L-749,345 (T_R=9.9 min), (B) a predose subject sample and (C) a postdose subject sample containing drug at 134.2 μg/ml (sample diluted with plasma/stabilizer, 1:1, v/v).

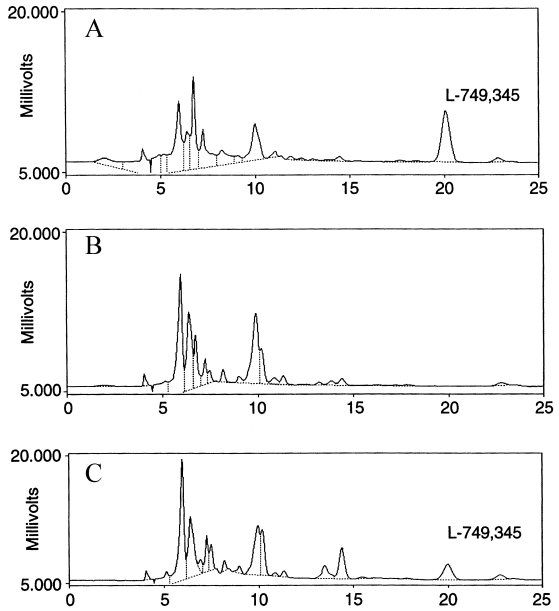

Fig. 4. Chromatograms of (A) urine standard containing 25 μg/ml of L-749,345 (T_R=20.0 min), (B) a predose subject sample and (C) a postdose subject sample containing drug at 9.97 μg/ml.

the Hypersil extraction column did not survive much repeated use, resulting in increasingly greater back-pressure. For the Maxsil column, routine use eventually resulted in peak broadening that was observed after the analysis of about 150–200 samples and was remedied by replacement. For similar reasons, the analytical column was changed about every 350 to 400 samples.

Chromatography of the carbapenem in urine involves conditions similar to the plasma assay. Specificity of the assay is demonstrated in Fig. 4. Chromatography of the analyte in urine requires a longer analytical column (150 mm×4.6 mm) to achieve specificity. Occasionally, a subject's urine did contain an endogenous interfering peak. Specificity was obtained by adjusting the methanol content of the mobile phase for pump 2 between 7 and 11%. The calibration standards, QCs and the subject's samples were then analyzed together with the modified mobile phase. The lower limit of detection was 0.3 μg/ml (S/N=6.7:1).

3.2. Solid-phase extraction

Sample preparation using SPE was evaluated for L-749,345 in plasma over the concentration range 0.175 to 35.0 μg/ml using Varian C_{18} 3 cm^3 200-mg cartridges. Intraday precision, linearity and accuracy were determined using SPE over the calibration range with five replicates at each standard concentration. The eluates were analyzed by HPLC without column switching and compared with the peak area response of neat standards in MES buffer, pH 6.5. Linear regression of the peak areas demonstrated a linear calibration curve (r^2=0.9995) and reproducibility at <10% coefficient of variation (CV) over the concentration range, but absolute recovery changed steadily from 101.5% at 35 μg/ml to 72.8% at 0.875 μg/ml to 47.5% at 0.175 μg/ml. Modifications to the procedure did not improve the recoveries. An internal standard was not used in the assay while testing SPE. A structurally similar analog was not readily available with similar stability and chromatography.

SPE of urine samples containing L-749,345 was

investigated for improved assay specificity and sensitivity. The procedure was evaluated for intraday precision, accuracy, and specificity over the calibration range 0.25 to 25 μg/ml. Precision and recovery varied beyond acceptable limits: Precision varied from 5.6 to 16% using peak areas; recovery, from 65 to 83% based on neat standards.

3.3. Linearity, reproducibility and accuracy

Standard plasma curves ($n=30$) run daily over the calibration range 0.125 to 50 μg/ml gave mean± SD r^2 and slope at 0.9997±0.0009 and 14 537±426, respectively. Intraday reproducibility and accuracy at each standard concentration are listed in Table 1 and show precision at <10% CV ($n=5$) and accuracy at 102.3 ± 2.1% based on nominal concentrations (each concentration is calculated from an average regression curve). Interday precision and accuracy of QCs at the low concentration 0.25 μg/ml are 6.1% CV and 100.0 ± 8.0%, and for the high concentration 40 μg/ml, 2.6% CV and 95.3 ± 2.5%, respectively. Plasma QCs were measured for about 11 months ($n=42$).

Similarly for the urine assay, standard curves ($n=38$) run daily over the concentration range 1.25 μg/ml to 100 μg/ml gave a mean±S.D. r^2 and slope at 0.9993±0.0006 and 6784±346, respectively. Intraday precision and accuracy are listed in Table 1: 0.94 to 5.0% CV; 100.5±6.0 % accuracy. Interday QCs were prepared at 10 μg/ml and 75 μg/ml. Day-to-day measurements ($n=38$) for the low QC gave

Table 1
Linearity, reproducibility and accuracy of the direct-injection HPLC assays for L-749,345 in human plasma and urine

Intraday validation			
Matrix	Concentration (μg/ml)	Precision[a] (% CV)	Accuracy[b] (%)
Plasma	0.125	7.88	103.5
	0.25	7.02	101.2
	0.5	6.74	98.6
	1.0	2.81	99.0
	2.5	2.92	98.8
	5.0	5.95	102.8
	12.5	3.93	97.7
	25.0	4.82	98.8
	50.0	9.17	102.0
Urine	1.25	5.04	93.3
	2.5	4.2	97.4
	5.0	2.4	112.6
	12.5	2.45	101.7
	25	1.12	97.9
	50	2.81	101.5
	100	0.94	99.3

Interday validation			
Matrix	Linearity	Accuracy and precision	
		Low QCs	High QCs
Plasma	r^2=0.9997±0.0009	0.25±0.02[c]	38.1±1.0[c]
	slope=14 537±0.426	(6.1%)	(2.6%)
Urine	r^2=0.9993±0.0006	9.97+0.36[d]	74.5+2.1[d]
	slope=6784±346	(3.7%)	(2.9%)

[a] $n=5$, coefficient of variation of peak areas.
[b] Percent of nominal.
[c] Nominal concentrations are 0.25 and 40.0 μg/ml ($n=30$).
[d] Nominal concentrations are 10.0 and 75.0 μg/ml ($n=38$).

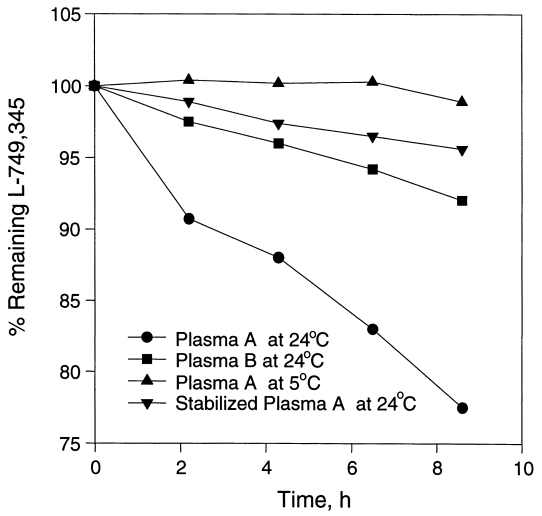

Fig. 5. Stability of L-749,345 in plasma and stabilized plasma (30.0 ug/ml) at 24°C and plasma at 5°C.

3.66% CV and 99.7±3.6% accuracy and for the high QC, 2.9% CV and 99.3±2.8 %, respectively. Urine QCs were measured for about 12 months (n=16).

3.4. Stability

The β-lactam moiety in a carbapenem is chemically and enzymatically vulnerable to hydrolysis to the open-lactam form (1,2) and thus, the stability of L-749,345 was studied under different chemical conditions. After 2 h at room temperature at pH 2 (0.1 *M* HCl), only 50% of L-749,345 (by peak area) remained; at pH 12 (0.1 *M* NaOH) the drug completely degraded with formation of a new chromatographic peak eluting before L-749,345; at pH 6.0 (0.1 *M* MES buffer) the drug was stable. The major degradation product in acidic or alkaline conditions was the open lactam based on reference material.

Table 3
Freeze–thaw stability[a] of L-749,345 in stabilized human plasma at 0.25 μg/ml and 40 μg/ml (n=3–6)

Freeze–thaw cycle	Concentration (μg/ml)	
Nominal	0.25	40.0
Initial	0.24±0.01	40.6±0.1
1 Cycle	0.24±0.01	40.7±0.2
2 Cycle	0.25[b]	38.9±0.1
3 Cycle	0.26±0.01	39.1±0.1
4 Cycle	0.25±0.01	38.9±0.4

[a] The frozen samples were thawed at room temperature and refrozen at −70°C.
[b] n=2.

At room temperature, the drug candidate was stable (>95% remaining) in 0.1 *M* MES buffer, pH 6.5 for at least 23 h. In urine, it was stable for about 8 h (2.5 to 100 μg/ml). In plasma, stability varied from 2 to 4 h depending on the lot of plasma (Fig. 5). In stabilized plasma, it was stable for about 9 h (% remaining >95%). At 5°C, L-749,345 was stable in plasma without stabilizer for about 11 h; with stabilizer, the amount remaining at 11 h was about 98% at every concentration studied (1.5 to 60.0 μg/ml). Table 2 summarizes the results of similar stability studies with L-749,345 in urine.

Long-term storage stability of stabilized samples at −70°C was evaluated with L-749,345 in plasma containing MES buffer–ethylene glycol (1:1 v/v) and in urine containing MES buffer, pH 6.5 (1:1 v/v). Stability was acceptable for about 12 months in stabilized plasma and 11 months for stabilized urine (Table 2). The buffer was added to urine(s) in order to bring final pH to about 6.5.

Freeze–thaw data for plasma and urine QCs are given in Tables 3 and 4, and the data overall demonstrate drug stability in the different matrices after each freeze–thaw cycle from initial measure-

Table 2
Stability (≥95%) of L-749,345 in human control plasma and urine and in stabilized plasma and urine

Matrix	Short-term stability at 24°C (h)	Short-term stability at 5°C (h)	Long-term stability at 70°C (months)
Plasma	>2	11	>6
Stabilized plasma	9	>11	>12
Urine	8	>24	
Stabilized urine	12	>24	>11

Table 4
Freeze–thaw stability of L-749,345 in stabilized human urine at 10.3 μg/ml and 41.2 μg/ml (n=3–6)

Freeze–thaw cycle	Concentrations (μg/ml)	
Nominal	10.3	41.2
Initial	10.2±0.7	39.4±0.4
1 Cycle	9.3±0.2	39.2±0.7
2 Cycle	9.1±0.1	36.4±0.8
3 Cycle	10.4±0.6	41.6±0.8

ments before freezing. The differences in mean concentration between the freeze–thaw cycles appear to be due to interday assay variability and not degradation of the antibiotic during freeze–thaw.

3.5. Application

Clinical samples generated from a Phase I, a single rising intravenous dose study in healthy subjects, were analyzed by the described assay methods. Fig. 6 shows plasma levels with time of a representative subject (#5) infused (30 min) intravenously at 250 mg and at 1000 mg. The plasma concentrations for Subject #5 at 250-mg and 1000-mg doses varied from 44.9 μg/ml and 158.2 μg/ml, respectively, at 0.5 h post dose to below the lower limit of quantification at 48 h. Urinary concentrations of the analyte for this subject at 250-mg dose varied from 148.3 μg/ml at 0–2 h collection to 3.7 μg/ml at the 36–48 h collection.

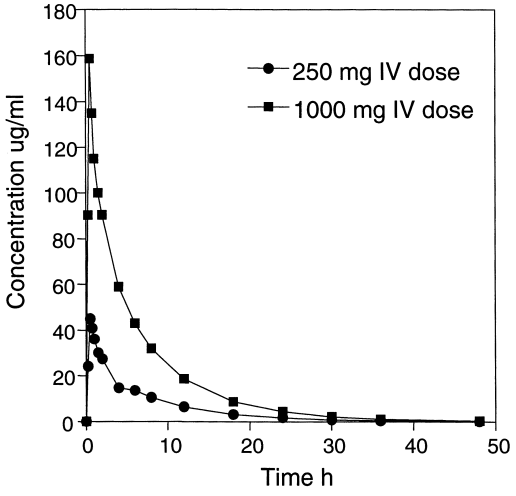

Fig. 6. Representative plasma concentrations of L-749,345 from a healthy subject administered a single i.v. infusion of 250 mg or 1000 mg.

4. Conclusion

Precise and accurate HPLC assays have been developed for the quantitation of L-749,345 in plasma and urine using column switching for direct injection of a sample aliquot. The technique precludes the need for sample preparation procedures that might enhance drug degradation and allows for direct evaluation of the carbapenem stability in biological fluids. The column-switching method does require the replacement of the in-line extraction column every 150 to 200 samples. Evaluation of other off-line isolation procedures such SPE and protein precipitation using an organic solvent were unsuccessful. Application of the assays on samples from subjects administered 250 mg intravenously showed adequate assay sensitivity for clinical studies.

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