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Determination of polymyxin E_1 in rat plasma by high-performance liquid chromatography

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Abstract

A precise and accurate HPLC assay for polymyxin E_1 in rat and dog plasma has been validated. Samples and standards are extracted from plasma with a 96-well C_8 extraction disk plate. Sample extracts are derivatized with dansyl chloride, and polymyxin E_1 derivative is quantitated on a C_8 column by HPLC with fluorescence detection. The assay is linear in the range of 0.050–5.00 μg/ml for polymyxin E_1. The precision and accuracy of polymyxin E_1 plasma assay was well within the recommended limits set in the FDA Guidance for Bioanalytical Method Validation. Polymyxin E_1 stability in rat and dog plasma for 24 h at room temperature and through three freeze–thaw cycles was demonstrated.

Keywords: Polymyxin E_1

1. Introduction

Polymyxin E_1 is the major component of colistin [1]. Colistin (also known as polymyxin E) is a mixture of polymyxins, a generic name for a group of chemically related fatty acyl decapeptide antibiotics, produced by strains of *Bacillus polymyxa* var. *colistinus* [1]. Polymyxins are all small cyclic decapeptides with unusual amino acids and fatty acid tails.

The current regulatory climate favors the use of single component over multiple component drugs. The benefit of developing a colistin component, which allows administering and monitoring a well characterized, single entity (such as polymyxin E_1), is clear when compared to the current colistin products, which comprise 15–20 pharmacologically equivalent components. To monitor polymyxin E_1 concentrations in rat and dog plasma after administration of the purified component, a selective assay needed to be developed and validated.

EIA and microbiological assays for colistin in tissues exist [2,3], but they are not specific for polymyxin E_1. Two published HPLC assays for colistin [4,5] require complex automated pre or post column derivatization procedures after sample preparation of 2–10 g of sample. A rapid assay with a smaller sample size, with simpler chromatography, and with selectivity for polymyxin E_1 was needed to assay hundreds of samples generated during toxicology studies. One such assay in plasma [6] utilizes TCA precipitation and automated OPA derivatization to quantitate colistin, but the assay has a very narrow concentration range of 28–250 ng/ml. Another assay

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employing OPA derivatization [7] is intended for feeds. A recently published method [8,9] has similar features to the method reported here but has slightly longer run times.

Assaying polymyxin E_1 in plasma by HPLC presents a challenge because it is a single peptide in a complex mix of plasma peptides. Polymyxin E_1 contains no amino acids with chromophores, so its absorbance maximum at the low end of the ultraviolet spectrum is too weak to assay with adequate sensitivity and selectivity by HPLC with UV detection. The presence of five primary amine functional groups in the molecule presents an opportunity for selective extraction based on pH and facile derivatization. Tagging polymyxin E_1 with a fluorescent molecule allows for a very sensitive and specific assay [10], and the primary amines of the γ-diaminobutyric acids are likely candidates for derivatization. Thus, we tried reactions with 2,4-dinitrofluorobenzene, *ortho*-phthalaldehyde, Waters AccQ-Tag, 1-naphthylisocyanate, 9-fluorenylmethyl chloroformate, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole, and dansyl chloride were tried with varying degrees of success. Dansyl chloride was subsequently chosen because of the successful derivatization of colistin reported by Tamura et al. [11], which we were able to reproduce.

The final assay consists of solid-phase extraction of plasma using 96-well, C_8 extraction disk plates, derivatization with dansyl chloride, and HPLC on a C_8 column with fluorescence detection.

2. Experimental

2.1. Materials

Control EDTA rat plasma and control EDTA dog plasma used in polymyxin E_1 assays were obtained from commercial sources (Pel Freez, Rogers, AR). Polymyxin E_1 was the current standard lot (Chiron, Seattle, WA) potency 748 μg/mg (≥96% HPLC purity). Acetonitrile, methanol, and acetic acid were HPLC grade (J.T. Baker, Phillipsburg, NJ, USA). Water was deionized via a Milli-Q UV plus ultrapure water system (Millipore, Bedford, MA, USA). Dansyl chloride, >97%, was from Sigma (St. Louis, MO, USA). Sodium carbonate and sodium bicarbonate were analyzed reagent grade (J.T. Baker). Trifluoracetic acid purity was >99% (Burdick and Jackson, Muskegon, MI, USA).

2.2. Polymyxin E_1 assay

All polymyxin E_1 stock solutions were prepared and stored in water in disposable polypropylene test tubes. Stock solutions were stored refrigerated at approximately 4 °C and were stable for at least 3 months. The standard spiking solutions (SSS) and plasma standards were prepared fresh on each assay day. Plasma QC samples were stored frozen at −80 °C.

2.2.1. Polymyxin E_1 standards and controls

Separately prepared stock solutions of polymyxin E_1 free base, 1.0 mg/ml were used to make SSS or quality control spiking solutions (QCSS). SSS were prepared at 1, 2, 4, 10, 20, 40, and 100 μg/ml by diluting the stock solution. Plasma standards were prepared by adding 25 μl SSS to 475 μl rat or dog plasma in 1.5-ml polypropylene microcentrifuge tubes which resulted in concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 μg/ml. QCSS were prepared at 1.0, 3.5, 30, 80, and 200 μg/ml. An aliquot of 0.5 ml of each spiking solution was added to 9.5 ml plasma, which resulted in QCs of 0.05 (LLOQ), 0.175 (Low), 1.5 (Mid), 4.0 (High), and 10.0 (Dilution) μg/ml.

2.2.2. Polymyxin E_1 assay procedures

Fresh SSS were prepared and spiked into plasma to make standards as described above. The appropriate QCs and unknown samples were thawed and mixed thoroughly. Sufficient wells on an Empore 96-well C_8 (standard density) disk extraction plate were conditioned to accommodate all standards, all controls, and single samples. The wells were conditioned with 2×100 μl methanol and 1×200 μl water. After each addition vacuum was applied until the liquid was drawn through all the disks. Into separate wells were pipetted 200 μl of each standard in duplicate, 200 μl of each QC in duplicate and each plasma sample singly. Samples with <200 μl (but not less than 20 μl) volume were pipetted into a well containing enough blank plasma (up to 180 μl) to make the total volume 200 μl. Vacuum was

applied until all plasma was drawn through the disks. The wells were washed with 1×1000 μl water and 3×50 μl methanol. After each addition vacuum was applied until the liquid was drawn through all the disks. Polymyxin E_1 was eluted with 4×100 μl eluting solution, acetonitrile–water–trifluoroacetic acid 600:400:2 (v/v/v), into 1200-μl polypropylene microtubes. After the last addition the vacuum was applied long enough to ensure complete recovery of the eluting solution. Extracts were evaporated to dryness under nitrogen at 50 °C. A 100-μl aliquot of methanol was added to each dried extract and evaporated to dryness at 50 °C to concentrate the samples in the bottom of the microtubes and remove any excess water. The samples were reconstituted with 100 μl of 0.1 *M* bicarbonate buffer, sonicated for 5 min, and vortexed for 10 s. Dansyl chloride reagent was prepared (dansyl chloride 0.5 mg/ml in acetonitrile), 100 μl were added to each tube, and the tubes were vortexed thoroughly. Samples were allowed to react for 1 h in the dark at room temperature after which the solutions were transferred to 0.7-ml polypropylene autosampler vials. Each sample was split into duplicate vials for re-injection if necessary.

2.3. HPLC conditions

The HPLC system consisted of Waters equipment including a 600E solvent delivery system with a column heater, a Waters 717 plus autosampler, and a Waters 474 Scanning Fluorescence Detector, which were all controlled by Waters Millennium Client/Server LC Software. The column used was a Zorbax Eclipse XDB C_8, 5 mm, 3.0×150 mm heated to 45 °C. The mobile phase was methanol–0.2% acetic acid (glacial acetic acid in water, v/v) 87:13 (v/v) delivered at a flow-rate of 0.5 ml/min. The injection volume was 25 μl, the run time was 11 min, and the detector settings were: excitation wavelength 344 nm, excitation bandwidth 40 nm, emission wavelength 518 nm, noise filter (Response) 3 s, and gain 100.

2.4. Rat plasma validation

The following scheme was used to validate the polymyxin E_1 method in rat plasma. In three separate runs spanning at least 7 days a calibration curve of duplicate polymyxin E_1 standards including a plasma and a reagent blank was assayed along with six replicates of the lower limit of quantitation (LLOQ), low, mid, high, and dilution pooled QC. In one run a set of non-extracted (recovery) standards was analyzed along with a typical set of plasma standards to determine the extraction efficiency of the assay. The extraction recovery of polymyxin E_1 from plasma was determined by comparing the peak heights of extracted plasma standards to the peak heights of absolute, non-extracted standards at each concentration. Six blank plasma samples from six different normal rats (three males and three females) were assayed to determine the assay selectivity. Four replicates of the low and high QCs were subjected to three freeze–thaw cycles and analyzed. Four replicates of the low and high QCs were kept at room temperature for 24 h and analyzed to determine bench stability. A duplicate set of derivatized samples from the one split run was injected after remaining refrigerated overnight to determine derivatized sample stability for 24 h.

2.5. Dog plasma partial validation

A partial validation was performed for polymyxin E_1 in dog plasma. A calibration curve of duplicate polymyxin E_1 standards including a plasma and a reagent blank was assayed along with six replicates of each LLOQ, low, mid, high, and dilution pooled QC. A set of non-extracted (recovery) standards was analyzed to determine the extraction efficiency of the assay. Six blank plasma samples from six different normal dogs were assayed to determine selectivity.

2.6. Penta-dansyl polymyxin E_1

Penta-dansyl polymyxin E_1 was synthesized, and characterized by LC–MS.

2.6.1. Synthesis

Polymyxin E_1, 252 mg (0.152 mmol), was dissolved in 24 ml borate buffer (pH~8.9). To the polymyxin E_1 solution was added 410 mg of dansyl chloride in 24 ml of acetonitrile solution. The reaction mixture was stirred at room temperature. Sodium hydroxide (solid) was added to maintain the

pH at 8.9. After overnight stirring acetonitrile was removed by roto-vap. The remaining water phase was extracted with ethyl acetate several times until no fluorescent material could be detected. The combined ethyl acetate extracts were dried over sodium sulfate. Filtration and evaporation gave a yellow powder, which was purified with a Sephadex LH-20 column (methanol) to yield 147 mg of the desired product (41.9%).

2.6.2. Characterization

The synthesized sample of dansyl derivatized polymyxin E_1 was analyzed by LC–MS in order to confirm the structure by determining the molecular mass. The molecular formula was determined to be $C_{113}H_{155}N_{21}O_{23}S_5$ and the exact mass range was 2334.021–2341.028 u. Because this mass was outside the mass range of the spectrometer, the doubly charged molecular ion $(M+2H)^{2+}$ mass range 1168–1171.5 u was used to identify the compound. The MS analysis was performed on a Finnigan SSQ7000 (ThermoFinnigan, San Jose, CA) in the positive ion electrospray mode. The spray voltage was set to maintain a spray current of 1.00 μA. The capillary temperature was 250 °C. The LC system consisted of a Thermo Separations Products Constametric 4100 solvent delivery system, a Rheodyne injector and a Microsorb C_8 column (150×4.6 mm). The mobile phase was composed of methanol–10 m*M* ammonium acetate (89:11, v/v) pumped at a flow-rate of 0.8 ml/min. The sample was dissolved in the mobile phase and flow injections of 5 μl were made. The scan range was 250–2000 u at a scan rate of 1.5 scans/s. The retention time of the major peak (scan 446) was 5.0 min. One other minor peak (scan 246) was observed at 2.7 min. The major peak consisted of material with an *m*/*z* 1168.3–1170.8 u consistent with the doubly charged ion of penta-dansyl polymyxin E_1.

3. Results and discussion

3.1. Chromatographic separation and selectivity

The dansyl derivative of polymyxin E_1 is well separated from the HPLC solvent front and endogenous peaks in both rat and dog plasma as shown in the representative chromatograms in Figs. 1–4. The mean retention time of polymyxin E_1 determined from the validation standard curves and QCs was approximately 7 min. No interfering peaks were found at the retention time of polymyxin E_1 in the blank plasmas from six control rats and six control dogs.

3.2. Dansyl polymyxin E_1

Polymyxin E_1 derivatized with dansyl chloride has excitation maxima at 218, 260, and 344 nm and has

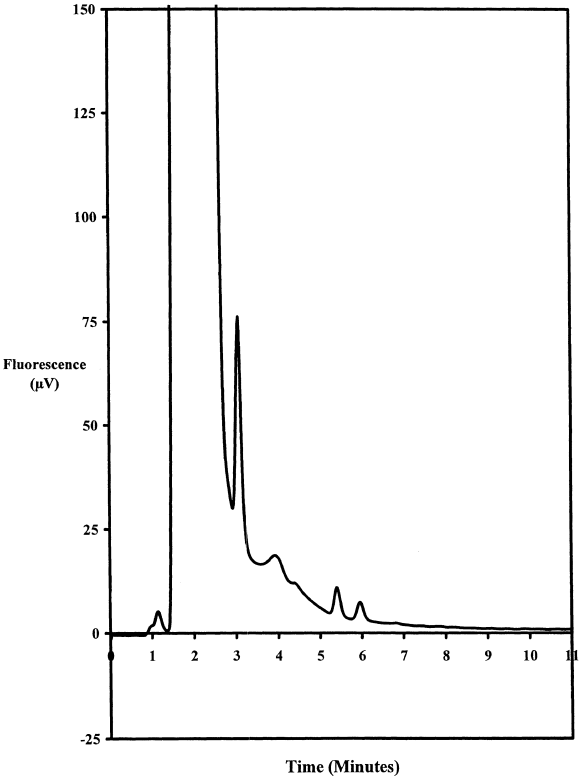

Fig. 1. A typical chromatogram of blank rat plasma. The column used was a Zorbax Eclipse XDB C_8, 5 mm, 3.0×150 mm, which was heated to 45 °C. The mobile phase was methanol–0.2% acetic acid (87:13, v/v) with a flow-rate of 0.5 ml/min. The injection volume was 25 μl, the run time was 11 min, and the detector settings were: excitation wavelength 344 nm, excitation bandwidth 40 nm, emission wavelength 518 nm, noise filter (Response) 3 s, and gain 100. The polymyxin E_1 retention time was 7.7 to 7.9 min.

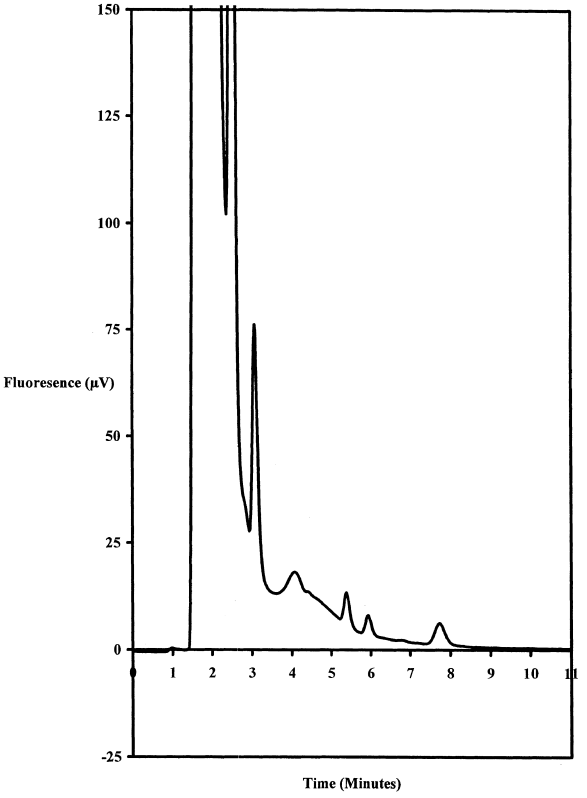

Fig. 2. A typical chromatogram of polymyxin E_1 rat plasma, spiked at 0.05 μg/ml. Conditions as in Fig. 1. The polymyxin E_1 retention time was 7.7 min.

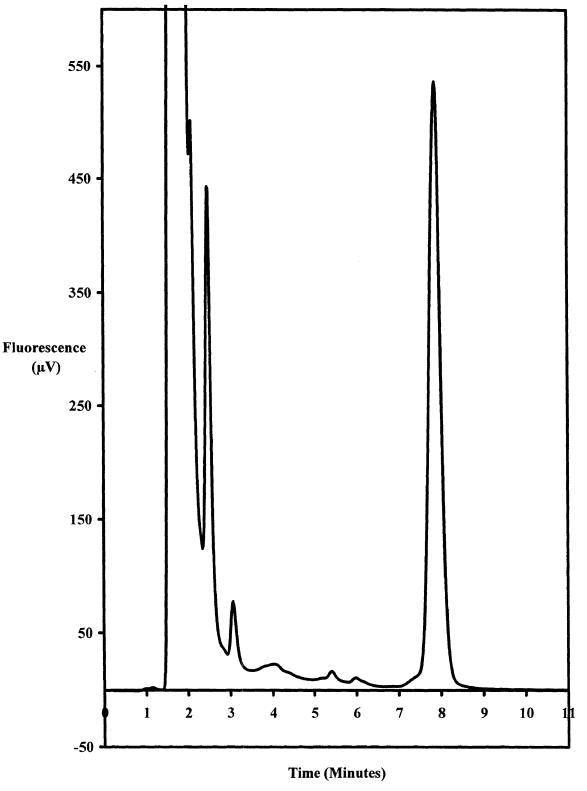

Fig. 3. A typical chromatogram of polymyxin E_1 rat plasma, spiked at 5.0 μg/ml. Conditions as in Fig. 1. The polymyxin E_1 retention time was 7.9 min.

an emission maximum at 518 nm. The difference between the excitation and emission wavelengths allows the use of the maximum bandwidth setting for the excitation wavelength. Dansyl derivatized stock polymyxin E_1 was compared chromatographically to synthesized penta-dansyl polymyxin E_1. Retention times were identical and detector response factors for each compound based on theoretical concentration were within 3%. The response factor agreement demonstrates that the polymyxin E_1 derivatization using the conditions described for this assay is nearly 100%.

The dansyl derivative of polymyxin E_1 was not stable in autosampler vials after the first injection was made, but the derivative was stable if injected from a duplicate set of vials, which were refrigerated.

3.3. Linearity

The regression parameters and back-calculated standard curve (weighted $1/x$) results are given in Table 1. The polymyxin E_1 calibration curves were linear in the range of 0.050–5.00 μg/ml, with correlation coefficients (R)≥0.997. There was no bias observed at any concentration regardless of matrix.

3.4. Precision

The intra-assay precision of the polymyxin E_1 rat plasma assay from a single assay was ≤5.1% RSD. The inter-assay precision of the assay determined for the three QCs from three consecutive runs was ≤8.2%. The precision of the polymyxin E_1 dog

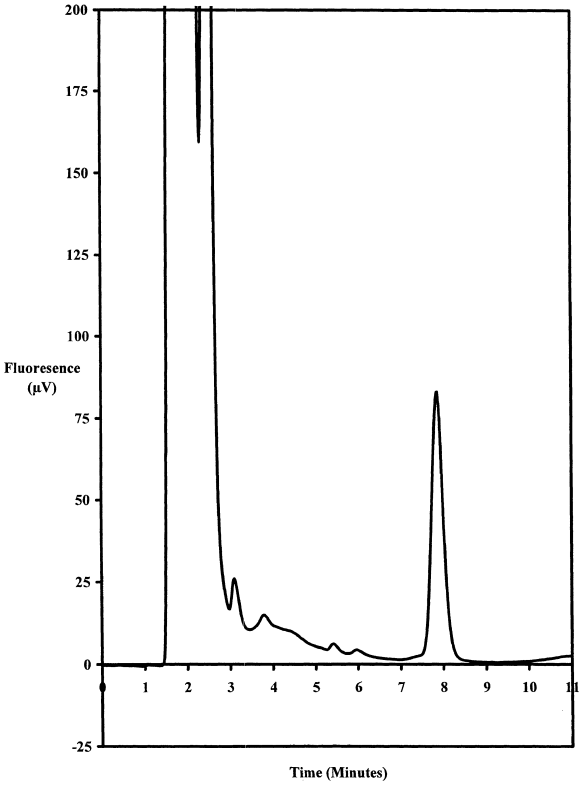

Fig. 4. Plasma sample from a rat exposed to polymyxin E1 by inhalation. Concentration found was 0.922 μg/ml. The polymyxin E_1 retention time was 7.9 min.

plasma assay from a single run was ≤8.7% RSD. The precision results are summarized in Table 2.

3.5. Accuracy

For the polymyxin E_1 rat plasma method, the inter-assay accuracy (n=18) ranged from 94 to 98%. For the polymyxin E_1 dog plasma method, the accuracy ranged from 94 to 99% (Table 2).

3.6. Sample dilution

A QC pool (QCD), prepared at a concentration above that of the highest standard in the standard curve, was made to determine the accuracy of diluting sample plasma with blank plasma. This dilution was performed as a partial volume dilution directly in the wells of the extraction plate. For the polymyxin E_1 rat plasma method, the overall accuracy of sample dilution was 99% and the overall precision was 4.0%. For the polymyxin E_1 dog plasma method, the accuracy of sample dilution was 92% and the precision was 6.0% (Table 2).

3.7. Lower limit of quantitation

The LLOQ for polymyxin E_1 was 0.050 μg/ml in rat and dog plasma. Quantitation of lower concentrations was attempted during method development, but concentrations less than 50 ng/ml were not reproducibly detected or quantitated. The overall accuracy of the rat plasma assay at the LLOQ, determined in three successive runs, was 98% and the precision was 3.6% RSD. The accuracy of the dog plasma assay at the LLOQ was 90% and the precision was 5.5% RSD (Table 2).

3.8. Recovery

The mean extraction recovery of polymyxin E_1 from rat plasma was 92% (3.2 %RSD) and was 107% (2.9 %RSD) from dog plasma.

Table 1
Calibration curve parameters

Matrix	Validation day	Slope (B)[a]	Standard error	Intercept (A)[a]	Standard error	R^2[b]
Rat plasma	1	105 572	843	−606	403	0.9996
Rat plasma	2	98 511	795	−920	380	0.9996
Rat plasma	3	107 657	682	−802	326	0.9998
Dog plasma	1	130 548	2709	−336	1296	0.9974

[a] Linear regression equation: $Y = A + BX$ (weighted $1/x$).
[b] R, linear correlation coefficient.

Table 2
Accuracy and precision of assays for polymyxin E_1 plasma

Species	Concentration (μg/ml)	Accuracy (%)	Intra-assay precision (%RSD)	Inter-assay precision (%RSD)
Rat plasma	0.05	98	2.9	3.6
	0.176	94	3.3	8.2
	1.501	95	5.1	5.7
	4.004	98	2.9	4.6
	10.01	99	0.9	4.0
Dog plasma	0.05	90	5.5	
	0.175	99	4.4	
	1.503	94	8.7	
	4.008	94	2.5	
	10.02	92	6	

3.9. Stability

Polymyxin E_1 rat plasma QCs at approximately 0.175 (low) and 4.0 (high) μg/ml were thawed and allowed to remain at room temperature for at least 24 h before assay to test for bench top stability. Overall there was no significant decrease observed (−0.2, and 1.1% difference, respectively) in the low and high QC mean concentrations (%RSD ≤5.3) compared to the overall QC means, and polymyxin E_1 was considered to be stable in rat plasma at room temperature for 24 h. Polymyxin E_1 QCs at 0.175 (low) and 4.0 (high) μg/ml were subjected to three freeze–thaw cycles. Overall, there was no significant decrease observed (2.4 and −0.2% difference, respectively) in the low and high QC mean concentrations (%RSD ≤5.8) compared to the overall QC means and polymyxin E_1 was considered to be stable for three freeze–thaw cycles.

Polymyxin E_1 was found to be stable in rat plasma frozen at −80 °C for 6 weeks. The percent differences between the mean concentrations and the nominal were −1.0, −4.4, and 0.8 for the low, medium, and high concentrations, respectively, with acceptable precision of 2.4, 2.6, and 1.6 %RSD for the respective means. Polymyxin E_1 was also found to be stable at −80 °C for 6 weeks in the mid and high dog plasma QC samples used for stability assessment. The percent differences between the mean concentrations and the time zero were −18, +11, and +6 for the low, medium, and high concentrations, respectively, with acceptable precision of 1.1, 1.3, and 3.9 %RSD for the respective means. Since the mid and high QCs were acceptable and the low only slightly below the acceptable range (−15%), polymyxin E_1 was considered to be stable under these conditions.

The duplicate set of day 2 derivatized polymyxin E_1 standards and quality controls were injected the following day after refrigeration at approximately 4 °C overnight. The standards, standard curve parameters, and QC results obtained for this batch were comparable to the original results with an overall percent difference of ≤0.9% for the QCs. These results demonstrated that the derivatized sample extract solutions were stable for at least 24 h under these storage conditions.

4. Conclusions

The assay method reported has been shown to have acceptable linearity, precision, accuracy, sensitivity, and recovery for the determination of polymyxin E_1 in rat and dog plasma samples (Fig. 5). In addition, bench top stability and mid-term stability of polymyxin E_1 in rat plasma at approximately −80 °C and after three freeze–thaw cycles was confirmed. The use of 96-well extraction plate technology gives this assay the potential for automation. The assay of Li et al. [8] has the potential for automation and should also be applicable to any colistin monocomponent.

Polymyxin E_1 is a purified component of the

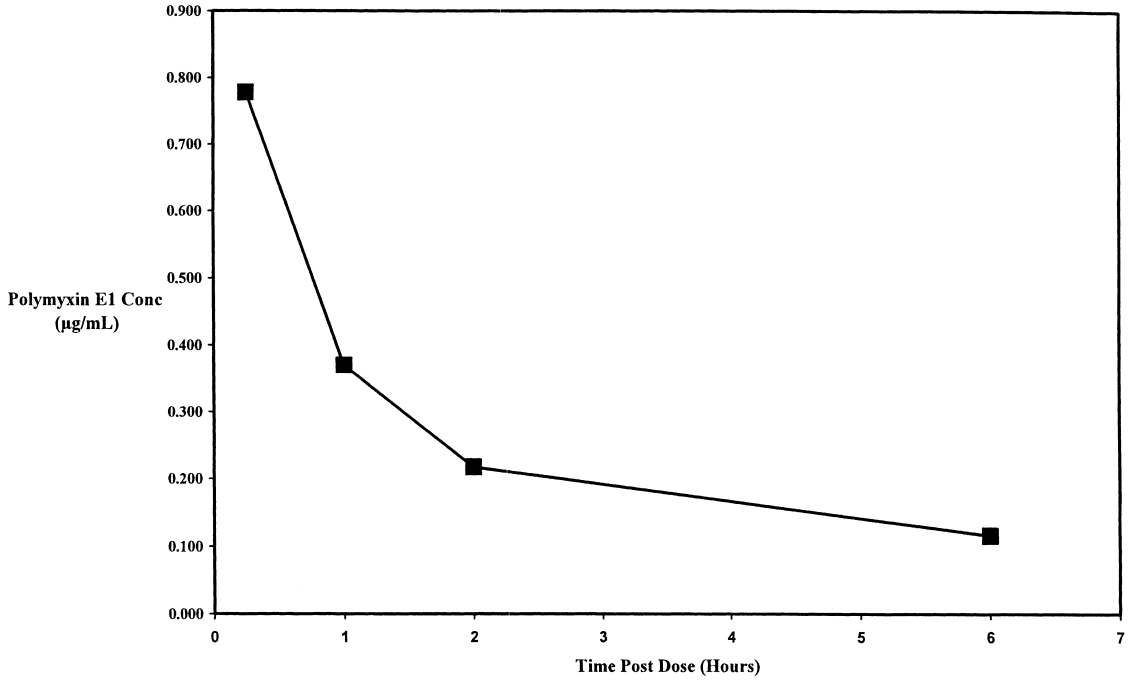

Fig. 5. Polymyxin E_1 concentration (μg/ml) in rat plasma versus time (h) past exposure (by inhalation). Each point is an average of two rats.

fermentation product colistin, which suggests that this assay might be applied to the complex mix. We have used this assay for colistin at similar concentrations. The individual components, once derivatized, are not well resolved, but co-elute as a group of two or three (depending on the concentration) partially resolved peaks. While this partial separation of colistin components does not satisfy the expected criteria of assay selectivity, the assay, when applied to colistin, is still useful. The application of this assay to colistin, with the potential for automation, would speed the assay of the large numbers of samples involved in a toxicological or clinical study.

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