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APPENDIX



APPENDIX A

The particle size and percent remainings of antibacterial suspension
formulations

Table A-1 Particle size of all formulations after heating cooling cycle (n=3).

Formulations	Particle size (μm) (Mean \pm SD)			
	Cycle 0	Cycle 2	Cycle 4	Cycle 6
CT20	35.5 \pm 36	32.4 \pm 30	30.8 \pm 50	31.6 \pm 28
CT80	34.9 \pm 31	32.5 \pm 28	31.6 \pm 28	35.4 \pm 24
COT20	34.7 \pm 47	34.9 \pm 36	36.1 \pm 42	32.3 \pm 36
COT80	34.2 \pm 34	30.9 \pm 27	32.7 \pm 26	34.2 \pm 23

Table A-2 Particle size of antibacterial suspension formulations after 3 months storage (n=3).

Code	Conditions	Particle size (μm) (Mean \pm SD)				
		Day 0	2 weeks	1 month	2 months	3 months
COT201	30 $^{\circ}\text{C}$	31.9 \pm 34	31.8 \pm 31	44.1 \pm 34	40.3 \pm 42	44.87 \pm 43
COT201	40 $^{\circ}\text{C}$	31.9 \pm 34	30.9 \pm 30	41.4 \pm 37	49.7 \pm 50	51.82 \pm 47
COT202	30 $^{\circ}\text{C}$	30.2 \pm 26	31.3 \pm 30	44.2 \pm 34	46.6 \pm 31	49.44 \pm 42
COT202	40 $^{\circ}\text{C}$	30.2 \pm 26	37.6 \pm 35	38.6 \pm 27	48.4 \pm 45	51.62 \pm 43

Table A-3 The percent remainings of amoxicillin in suspensions after 3 months storage (n=3).

Formulations	Conditions	percent remainings of amoxicillin (Mean \pm SD)			
		2 weeks	1 month	2 months	3 months
COT201	30 $^{\circ}\text{C}$	99.52 \pm 0.24	100.38 \pm 0.07	99.41 \pm 0.57	101.57 \pm 0.71
COT201	40 $^{\circ}\text{C}$	98.12 \pm 0.30	95.50 \pm 0.67	95.13 \pm 0.53	95.65 \pm 0.30
COT202	30 $^{\circ}\text{C}$	97.35 \pm 0.06	97.65 \pm 0.66	99.62 \pm 1.39	96.42 \pm 0.63
COT202	40 $^{\circ}\text{C}$	98.24 \pm 0.66	95.82 \pm 0.52	94.97 \pm 1.02	84.17 \pm 1.46



Table A-4 The percent remainings of enrofloxacin in suspensions after 3 months storage (n=3).

Formulations	Conditions	percent remainings of enrofloxacin (Mean±SD)			
		2 weeks	1 month	2 months	3 months
COT201	30 °C	99.98 ± 0.20	99.97 ± 0.86	99.55 ± 0.20	98.81 ± 2.22
COT201	40 °C	98.80 ± 0.12	95.66 ± 0.06	94.83 ± 0.79	92.59 ± 0.58
COT202	30 °C	99.29 ± 0.10	97.13 ± 0.68	97.04 ± 0.05	95.75 ± 2.49
COT202	40 °C	100.46 ± 0.06	97.19 ± 1.74	96.02 ± 0.64	94.21 ± 0.28





APPENDIX B

Validation of HPLC method

Partial validation for the quantitative analysis of amoxicillin and enrofloxacin by HPLC method

The HPLC method was modified from Numan et al. (2009) and Manceau et al. (1999). The UV-Vis detector was set at 229 nm and 277 nm detect amoxicillin and enrofloxacin, respectively. The analytical parameters used in the partial validation of HPLC method were specificity, linearity, accuracy and precision.

1. Specificity

The specificity of HPLC method was evaluated by comparing the assay results of amoxicillin and enrofloxacin in the suspension and in the receptor medium with those in the standard solution. The peak of amoxicillin and enrofloxacin must be completely separated from interference and other components in the sample.

2. Linearity

Six standard solutions of amoxicillin and enrofloxacin mixture (concentration 5 - 60 $\mu\text{g/mL}$) were prepared and analyzed (three sets). The relationship between concentrations and peak area was plotted by using linear regression. The linearity was determined from the coefficient of determination (r^2).

Acceptance criteria: The coefficient of determination (r^2) must be more than 0.9990.

3. Accuracy

The accuracy of the analytical method was determined from the percentage of analytical recovery. Three concentrations (low, medium, high) of five sets of amoxicillin and enrofloxacin mixture at 5, 20, and 60 $\mu\text{g/mL}$ were prepared and analyzed. The percentage of recovery of each concentration was calculated.

Acceptance criteria: The percentage of analytical recovery must be within 98.0-102.0% of each nominal concentration.



4. Precision

4.1 Intraday precision

The intraday precision was determined by analyzing three concentrations of five sets of amoxicillin and enrofloxacin mixture at 5, 20, and 60 $\mu\text{g}/\text{mL}$ in the same day. The relative standard deviations (%RSD) of the peak area responses vs concentration were determined.

4.2 Interday precision

The interday precision was determined by analyzing three concentrations of of amoxicillin and enrofloxacin mixture at 5, 20, and 60 $\mu\text{g}/\text{mL}$ on five different days. The relative standard deviations (%RSD) of the peak area responses at each concentration were determined.

Acceptance criteria: The percentage of relative standard deviation (%RSD) for both intraday and interday precision must be less than 2%.



Partial validation for the quantitative analysis of amoxicillin and enrofloxacin by HPLC method

The developed HPLC system was applied to analyze amoxicillin and enrofloxacin content in suspension. The analytical method was partially verified under following topic.

1. Specificity

Potassium phosphate buffer pH 3.0/acetonitrile was used as the mobile phase. The typical chromatograms of potassium phosphate buffer pH 3.0/acetonitrile and amoxicillin and enrofloxacin combination standard solution are presented in Figures 33 – 35. All chromatograms are presented under the same attenuation and scale.

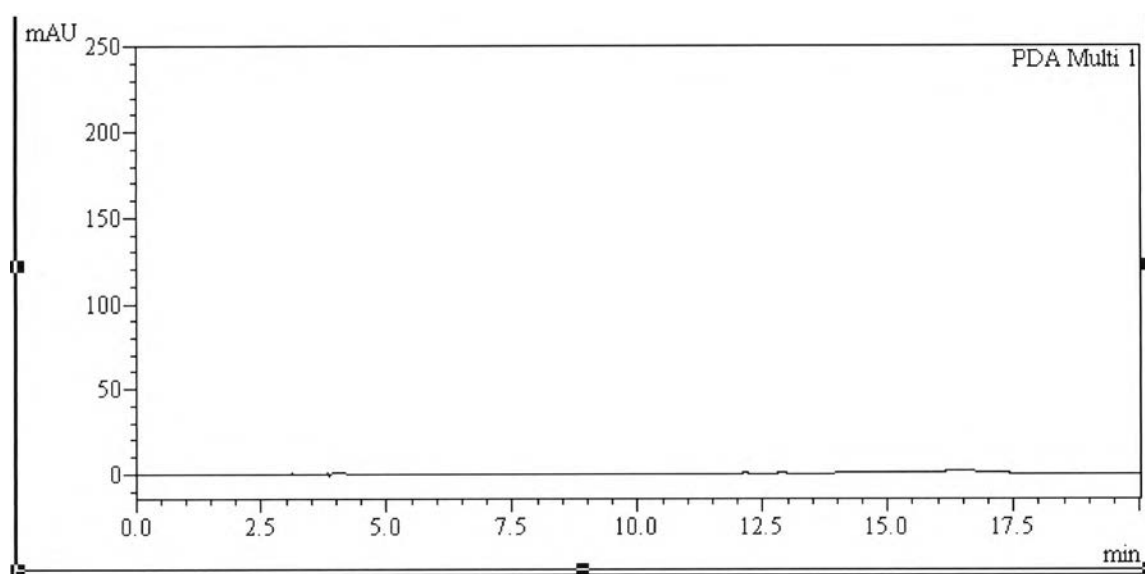


Figure 34 HPLC chromatogram of potassium phosphate buffer pH 3.0/acetonitrile medium (90:10 v/v).



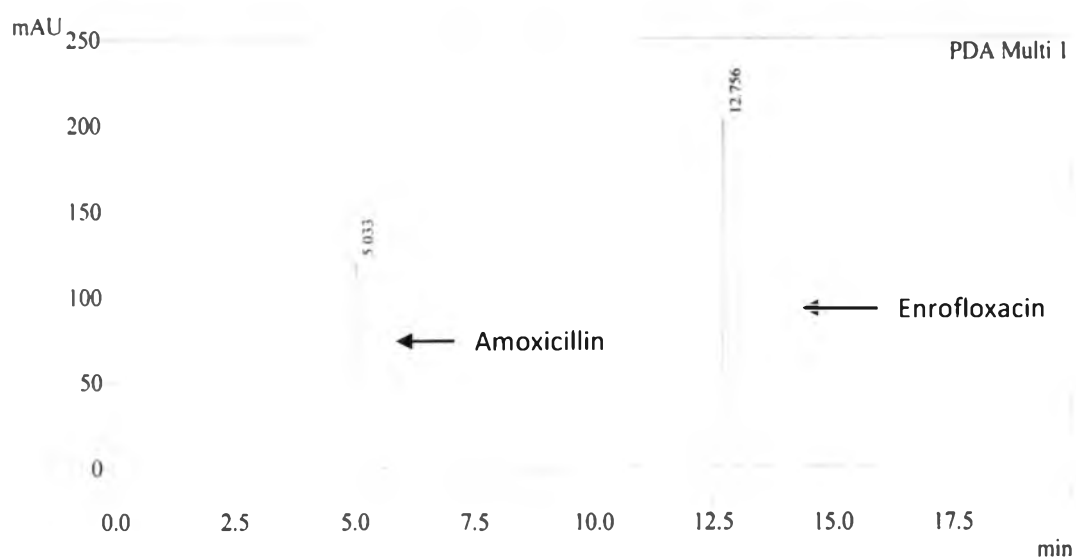


Figure 35 HPLC chromatogram of amoxicillin and enrofloxacin at wavelength 229 nm.

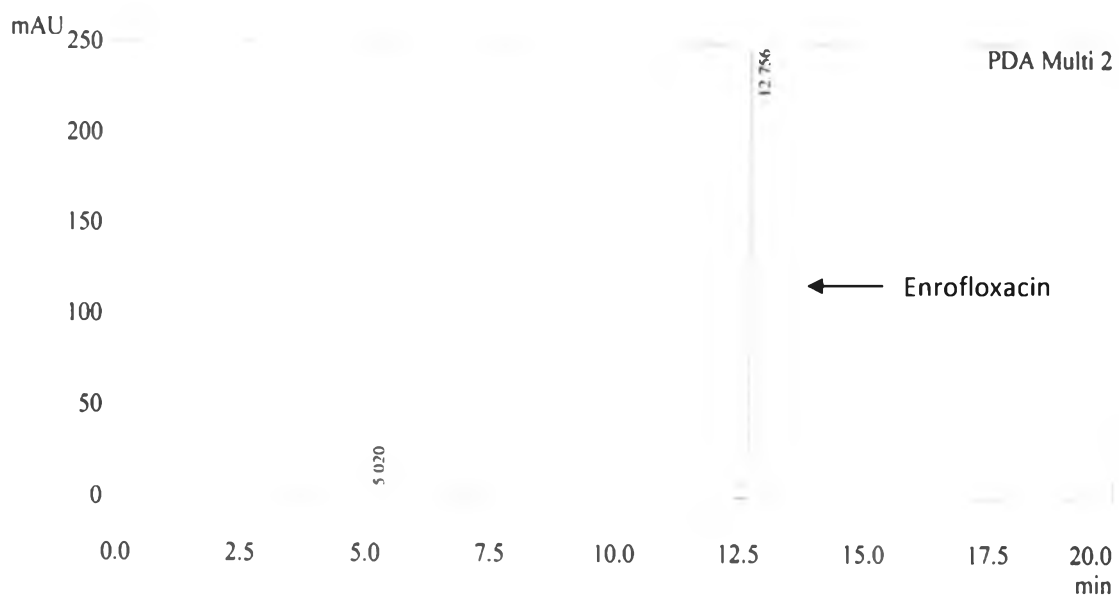


Figure 36 HPLC chromatogram of enrofloxacin at wavelength 277 nm.

From Figures 34 - 36, the retention time of amoxicillin and enrofloxacin peak were obtained at about 5.03 and 12.76 minutes, respectively. There was no interference from other components in the chromatograms including the solvent peak of solvent system, mobile phase, oils and surfactants. Thus, the HPLC system was acceptable for specificity.

2. Linearity

The calibration curve data of amoxicillin and enrofloxacin are presented in Table B-1 and B-2, respectively. The calibration curve was plotted between the peak area and amoxicillin and enrofloxacin concentrations (Figure 37). Linear regression analysis was performed with the coefficient of determination (r^2) of 1.0000. These results indicated that the HPLC system was acceptable for quantitative analysis of amoxicillin and enrofloxacin in the studied range.

Table B-1 Data for calibration curve of amoxicillin trihydrate by HPLC method.

Concentration of amoxicillin trihydrate ($\mu\text{g/mL}$)	Peak area			Mean	SD
	Set 1	Set 2	Set 3		
5.0	137315	128990	128550	131618	4939
10.0	275506	266390	266705	269533	5175
20.2	542334	535335	528513	535394	6911
40.3	1079228	1052262	1047967	1059819	16945
50.4	1349314	1312634	1312797	1324915	21130
60.5	1620228	1568327	1569724	1586093	29570
r^2	1.0000	0.9999	1.0000	1.0000	-

Table B-2 Data for calibration curve of enrofloxacin base by HPLC method.

Concentration of enrofloxacin base ($\mu\text{g/ml}$)	Peak area			Mean	SD
	Set 1	Set 2	Set 3		
5.1	814328	917539	727636	819835	95071
10.1	1531612	1549414	1422477	1501168	68727
20.2	2888062	2984678	2768506	2880415	108288
40.4	5706481	5735719	5530973	5657724	110739
50.5	7134899	7073461	6919863	7042741	110760
60.6	8554125	8390342	8255809	8400092	149397
r^2	1.0000	0.9999	1.0000	1.0000	-



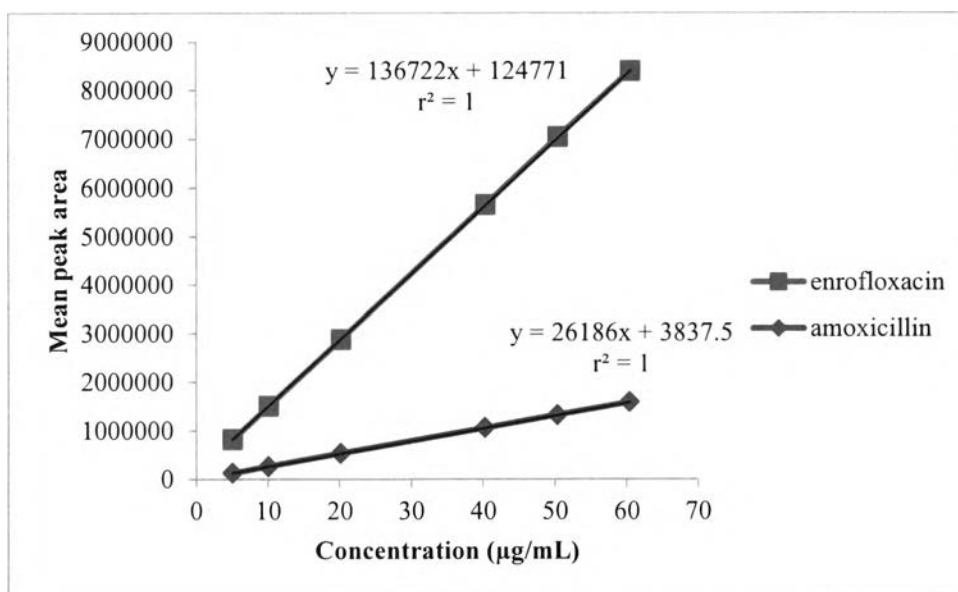


Figure 37 Calibration curve of amoxicillin trihydrate and enrofloxacin base by HPLC method.

3. Accuracy

The accuracy as percent recovery was obtained from the mean of observed concentrations calculated from the calibration curve then divided by the mean of actual concentrations and multiplied by 100. The percentages of analytical recovery of amoxicillin and enrofloxacin were in the range of 98.73 – 99.88% and 99.96 – 100.10%, respectively, which were in the range of 98.00 – 102.00%. The data are shown in table B-3 and B-4. The results indicated that HPLC method could be used to accurately determine amoxicillin and enrofloxacin within the concentration range (5 – 60 µg/mL).



Table B-3 The percentage of analytical recovery of amoxicillin trihydrate by HPLC method.

Concentration of amoxicillin trihydrate ($\mu\text{g/mL}$)	%Analytical recovery					Mean \pm SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
5.0	99.21	99.86	99.87	100.50	99.96	99.88 \pm 0.46
20.0	100.84	99.35	100.01	99.42	99.69	99.86 \pm 0.60
60.0	99.27	99.31	98.39	97.98	98.70	98.73 \pm 0.57

Table B-4 The percentage of analytical recovery of enrofloxacin base by HPLC method.

Concentration of enrofloxacin base ($\mu\text{g/ml}$)	%Analytical recovery					Mean \pm SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
5.0	98.39	100.05	100.52	100.36	100.48	99.96 \pm 0.90
20.0	99.64	99.75	99.22	101.26	100.54	100.08 \pm 0.81
60.0	99.46	100.69	99.26	99.61	101.46	100.10 \pm 0.94

4. Precision

The precision was determined in terms of percent relative standard deviation (%RSD) or percent coefficient of variation (%CV) of series of measurements. Table B-5 to B-8 showed the data of intraday precision and interday precision, respectively, of amoxicillin and enrofloxacin. The ranges of relative standard deviation values of intraday precision of amoxicillin and enrofloxacin were 0.73 – 0.98% and 0.59 – 0.65%, respectively. Interday precision, relative standard deviation values were in range of 0.90 – 1.67% and 1.33 – 1.45% for amoxicillin and enrofloxacin, respectively. %RSD of an analytical method should be less than 2%. So, the HPLC method was precise for the quantitative analysis of amoxicillin and enrofloxacin in the studied range.



Table B-5 Data of intraday precision of amoxicillin trihydrate by HPLC method.

Concentration of amoxicillin trihydrate ($\mu\text{g/mL}$)	Estimated Concentration ($\mu\text{g/mL}$)					Mean \pm SD	%RSD
	Set 1	Set 2	Set 3	Set 4	Set 5		
5.0	5.0	5.0	4.9	4.9	4.9	4.9 \pm 0.04	0.73
20.0	19.2	19.6	19.4	19.2	19.1	19.3 \pm 0.19	0.98
60.0	56.6	56.9	56.4	55.8	57.3	56.6 \pm 0.53	0.94

Table B-6 Data of intraday precision of enrofloxacin base by HPLC method.

Concentration of enrofloxacin base ($\mu\text{g/mL}$)	Estimated Concentration ($\mu\text{g/mL}$)					Mean \pm SD	%RSD
	Set 1	Set 2	Set 3	Set 4	Set 5		
5.000	5.0	5.0	5.0	5.0	4.9	5.0 \pm 0.03	0.60
20.000	20.1	20.1	20.0	20.0	20.0	20.0 \pm 0.12	0.61
60.000	60.3	60.1	60.0	59.3	60.2	60.0 \pm 0.38	0.64

Table B-7 Data of interday precision of amoxicillin trihydrate by HPLC method.

Concentration of amoxicillin trihydrate ($\mu\text{g/mL}$)	Estimated Concentration ($\mu\text{g/mL}$)					Mean \pm SD	%RSD
	Set 1	Set 2	Set 3	Set 4	Set 5		
5.0	5.0	5.0	5.0	5.1	5.0	5.0 \pm 0.05	0.90
20.0	19.6	20.5	20.2	20.1	20.1	20.1 \pm 0.33	1.66
60.0	57.3	59.5	59.0	59.3	59.7	59.0 \pm 0.98	1.67

Table B-8 Data of interday precision of enrofloxacin base by HPLC method.

Concentration of enrofloxacin base ($\mu\text{g/mL}$)	Estimated Concentration ($\mu\text{g/mL}$)					Mean \pm SD	%RSD
	Set 1	Set 2	Set 3	Set 4	Set 5		
5.0	5.0	5.0	5.2	5.1	5.1	5.1 \pm 0.07	1.36
20.0	20.1	20.2	20.9	20.5	20.3	20.4 \pm 0.30	1.45
60.0	60.1	61.0	59.4	60.4	61.5	60.5 \pm 0.81	1.33



In conclusion, the analysis of amoxicillin and enrofloxacin by HPLC method developed in this research exhibited good specificity, linearity, accuracy and precision. Therefore, this HPLC method was used for determination of amoxicillin and enrofloxacin in suspension.



VITA

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