



Simultaneous Quantitation of Polymyxin PMB1, PMB1-1 and PMB2 in Human Urine Using LC/MS/MS

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Introduction

Polymyxins are antibiotics with a general structure consisting of a cyclic peptide and a long hydrophobic fatty acid tail. Although the pharmacology of polymyxins is very limited and most data were obtained more than three decades ago, they have been increasingly used as the last resort for Gram-negative pathogens that are resistant to all other currently available antibiotics. Moreover, there is renewed interest in the exploration of other analog peptides that are active against Gram-positive bacteria ^[1,2]. Polymyxin B (PMB) is one of the two clinically used polymyxins. It is comprised mainly of PMB1, PMB1-1 and PMB2. Here we will present a sensitive, specific and high throughput LC-MS/MS method to simultaneously quantify PBM1, PMB1-1 and PMB2 in human urine treated with an equal volume of 0.5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) in 200 mM Phosphate Buffer pH=2.0.



Methodology

SAMPLE PREPARATION AND EXTRACTION:

1. Aliquot 200 μL of sample to corresponding wells of a 96-well deep well plate
2. Add 50.0 μL working internal standard [2000 ng/mL of CB-182,753 (an analog of PMB1) in water]
3. Add 200 μL of phosphate buffer (pH=2) and mix well.
4. Transfer samples from the 96-well plate to an Oasis WCX $\mu\text{Elution}$ plate which is pre-conditioned with 200 μL MeOH and 200 μL water.
5. Wash with 200 μL of 80:20 water:acetonitrile.
6. Elute samples with 2 x 25.0 μL of 1% TFA buffer.
7. Dilute samples with 200 μL of water.

CHROMATOGRAPHIC CONDITIONS

Column:	Xbridge™ C8, 2.1 x 50 mm
Mobile Phase:	A: 1% FA in water B: 1% FA in 50:50 MeOH:MeCN
Injection volume:	10 μL
Column temperature:	30 °C
Flow rate:	0.350 mL/min
Gradient:	18-30% B within 3.5 minutes
AS Temperature:	Ambient

MASS SPECTROMETER CONDITIONS

Instrument	Sciex API5000™
Ionization mode:	Turboionspray, Positive ion mode
Source Temperature:	500 °C
SRM transitions:	

PMB1		602.6 → 241.2
PMB1-1		602.6 → 241.2
PMB2		595.5 → 227.2
CB-182,753	Internal Standard	614.4 → 532.6

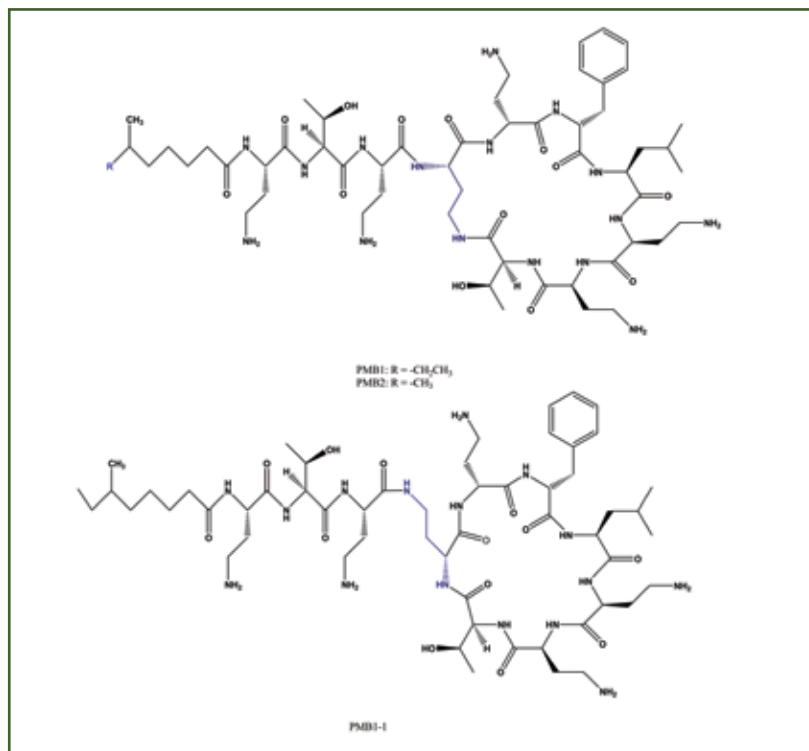


Results and Conditions

METHOD DEVELOPMENT

- PMB1 and PMB1-1 are isomers and therefore need to be chromatographically separated in order to be quantified separately.
- All three analytes are poly cationic molecules under physiological pH. They have strong non-specific binding properties to containers and matrix. The neat solutions need to be stored in polypropylene containers and at high concentrations.
- The adsorption loss of analyte in urine samples in storage containers is time dependent. Therefore, the urine samples need to be treated appropriately for long-term storage. No significant analyte loss was observed if the urine samples were treated with an equal volume of 0.5% CHAPS within 6 hours of collection.

FIGURE 1: Chemical Structures of PMB1, PMB2 (top) and PMB1-1 (bottom)





Results and Conditions (continued)

FIGURE 2: Chromatographic separation of PMB1, PMB1-1, PMB2 and the internal standard (IS) under the optimized conditions

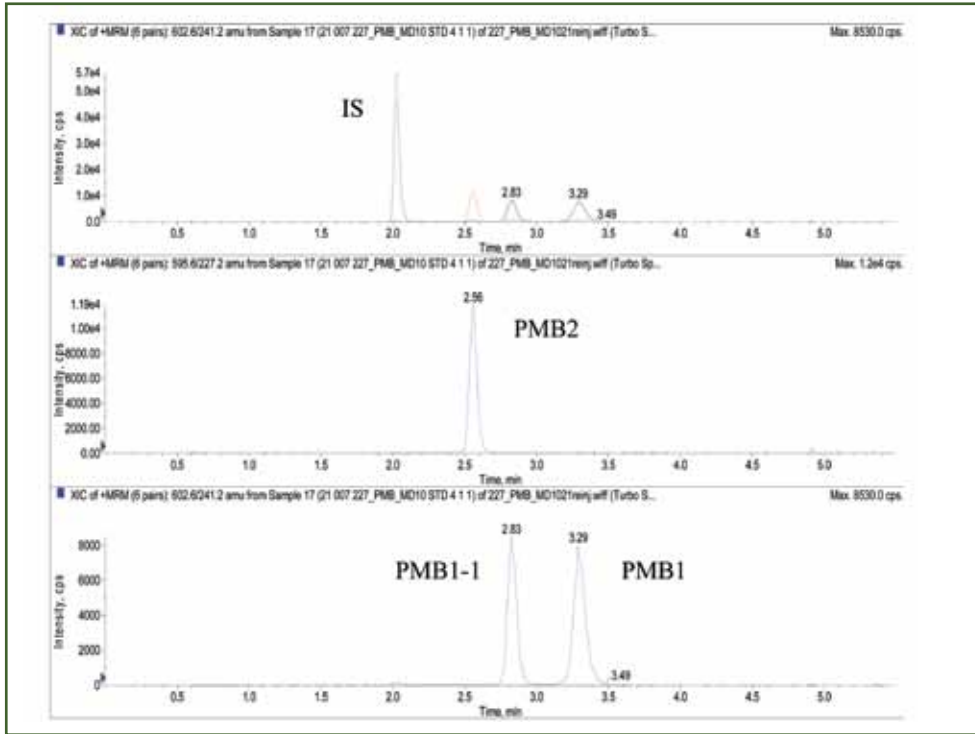
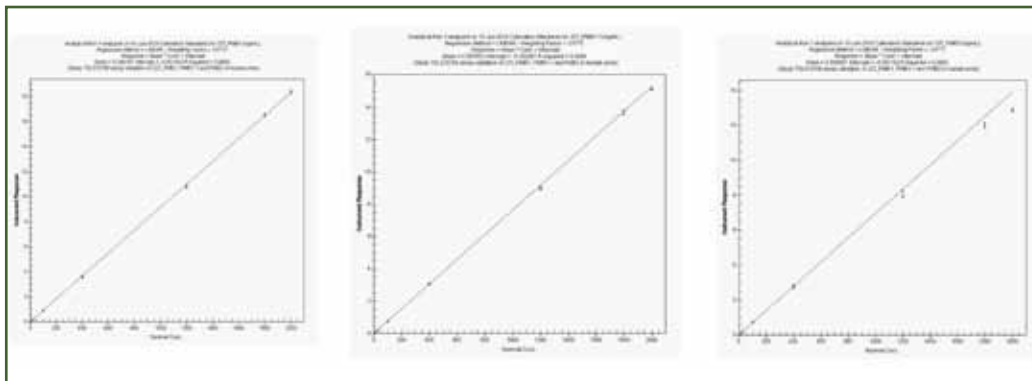


FIGURE 3: Representative calibration curves for PMB1 (left), PMB1-1 (middle) and PMB2 (right)





Results and Conditions (continued)

FIGURE 4: Individual "Analyte Only" High Standards(2,000 mg/mL) without Internal Standard for PMB1 (left), PMB1-1 (middle) and PMB2 (right) (The lower graph of each chart is the IS MRM transition)

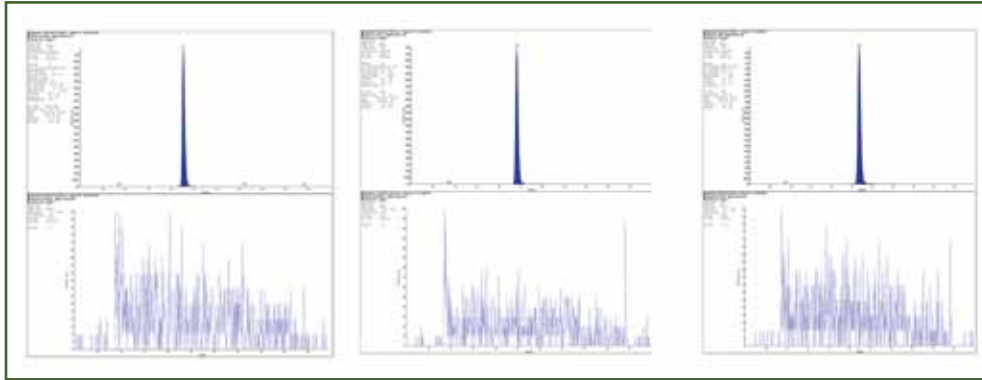
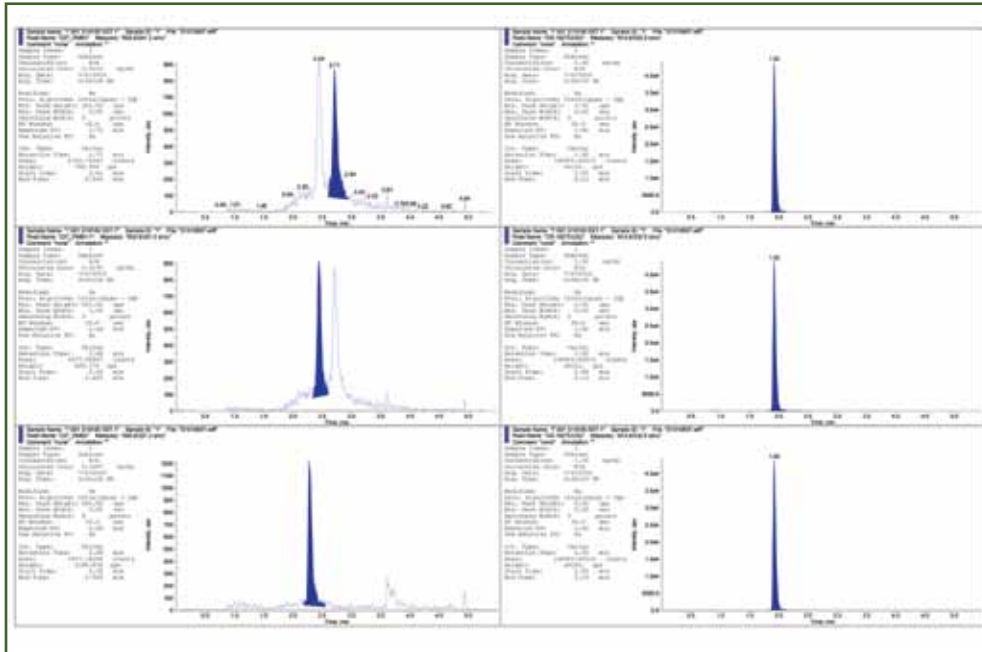


FIGURE 5: Representative Low Standard (5 ng/mL) for PMB1 (top), PMB1-1 (middle) and PMB2 (bottom) (The right side is the IS chromatogram)





Results and Conditions (continued)

FIGURE 6: Representative High Standard (2,000 ng/mL) for PMB1 (top), PMB1-1 (middle) and PMB2 (bottom) (The right side is the IS chromatogram)

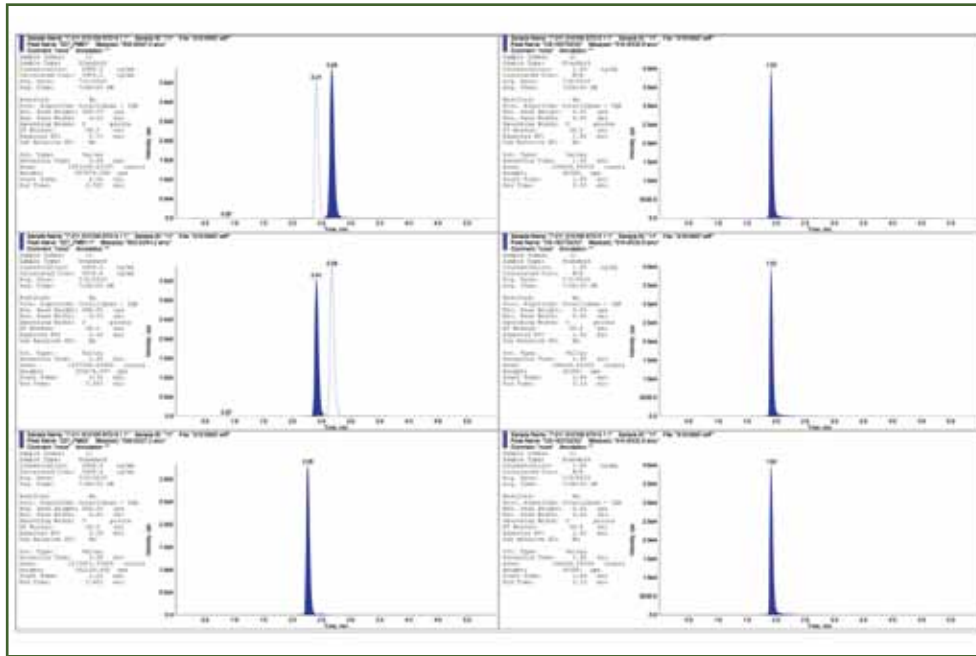
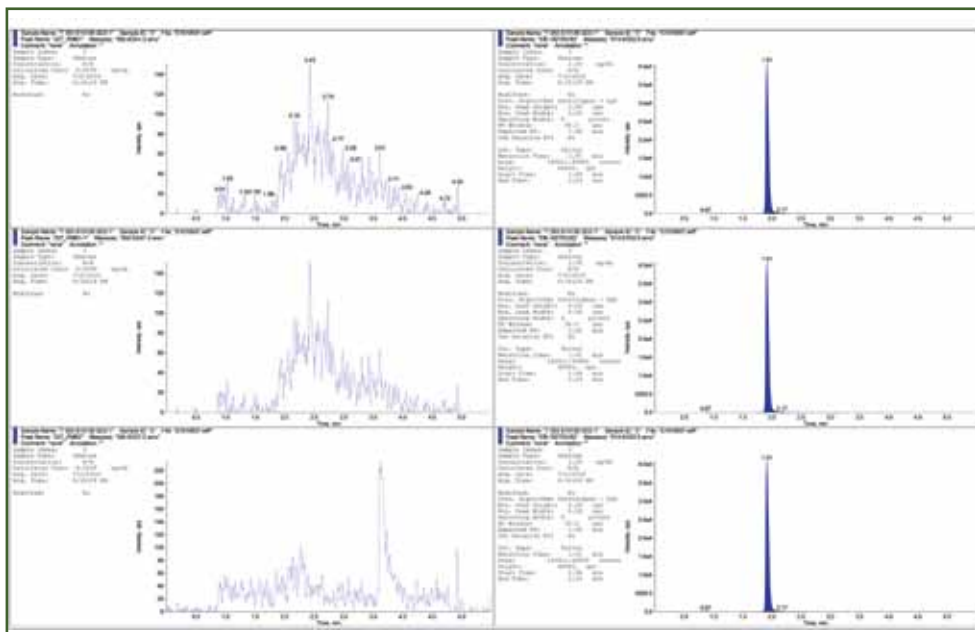


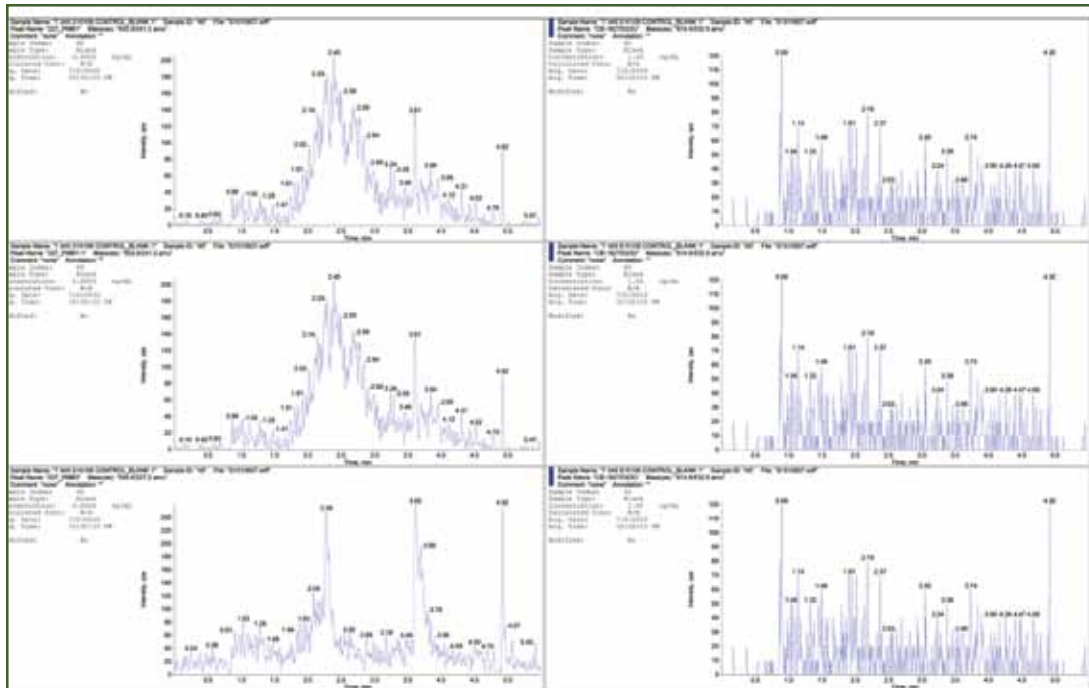
FIGURE 7: Internal Standard Only (500 ng/mL) (The right side is the IS chromatogram)





Results and Conditions (continued)

FIGURE 8: Representative Urine Control Blank



ASSAY VALIDATION

- Accuracy/precision:** Demonstrated (n=6 each run) at LLOQ, Low, Medium, High concentrations in 3 validation runs. (Table 2)
- Selectivity:** Demonstrated with blank and low QC concentrations in six sources of human urine. (Table 3)
- Ability to dilute:** Demonstrated (n=6) above ULOQ at DF=10. (Table 4)
- Extraction recovery:** Evaluated for analyte at Low, Medium, High concentrations and for Internal Standard (IS) at working concentration (n=6 each). (Table 5)
- Matrix factor:** Evaluated for analyte at medium QC concentration and for IS at working concentration (n=6 each). (Table 6)
- Carryover:** Evaluated in each run.



Results and Conditions (continued)

STABILITY

In solution: (Analyte and IS in 1% FA in water)

6 hrs at ambient temperature

90 days at 1-8 °C

In matrix: (Human urine with 0.25% CHAPS)

4 Freeze/Thaw cycles

6.7 hrs at ambient temperature

111 days at -70 °C and -20 °C

Reinjection

Reproducibility: 248 hours at ambient temperature

TABLE 1. Back-Calculated Concentrations of Calibration Standards for PMB1, PMB1-1 and PMB2

(All concentrations are expressed as ng/mL) (n=6 in 3 runs)

Run Date		5.00	10.0	25.0	100	400	1200	1800	2000
PMB1	Mean	5.07	9.78	24.4	102	402	1200	1790	2030
	S.D.	0.183	0.693	1.28	1.10	17.5	33.5	48.9	73.2
	%CV	3.6	7.1	5.2	1.1	4.4	2.8	2.7	3.6
	%Bias	1.4	-2.2	-2.4	2.0	0.5	0.0	-0.6	1.5
PMB1-1	Mean	5.05	9.87	24.5	103	409	1190	1770	2000
	S.D.	0.295	0.445	1.03	2.64	14.4	40.4	72.8	68.0
	%CV	5.8	4.5	4.2	2.6	3.5	3.4	4.1	3.4
	%Bias	1.0	-1.3	-2.0	3.0	2.3	-0.8	-1.7	0.0
PMB2	Mean	4.89	10.2	25.8	108	414	1170	1700	1880
	S.D.	0.200	0.681	1.78	5.13	23.1	41.2	53.5	76.6
	%CV	4.1	6.7	6.9	4.8	5.6	3.5	3.1	4.1
	%Bias	-2.2	2.0	3.2	8.0	3.5	-2.5	-5.6	-6.0



Results and Conditions (continued)

TABLE 2. Intra- and Inter-Assay Accuracy and Precision of Quality Control Samples for PMB1, PMB1-1 and PMB2 from ANOVA (n=18 in 3 runs)

Analyte		LLOQ QC 5.00 ng/mL	Low QC 15.0 ng/mL	Medium QC 200 ng/mL	High QC 1600 ng/mL
PMB1	Mean Observed Conc.	5.13	14.6	206	1650
	%Bias	2.6	-2.7	3.0	3.1
	Between Run Precision (%CV)	4.3	4.8	1.2	0.0
	Within Run Precision (%CV)	7.0	6.0	3.9	2.3
	Total Variation (%CV)	8.2	7.7	4.1	2.3
PMB1-1	Mean Observed Conc.	4.91	14.8	209	1630
	%Bias	-1.8	-1.3	4.5	1.9
	Between Run Precision (%CV)	0.0	0.0	0.0	0.9
	Within Run Precision (%CV)	10.1	5.8	3.1	2.4
	Total Variation (%CV)	9.8	5.8	3.0	2.6
PMB2	Mean Observed Conc.	4.60	15.9	208	1520
	%Bias	-8.0	6.0	4.0	-5.0
	Between Run Precision (%CV)	0.2	1.3	2.6	0.6
	Within Run Precision (%CV)	7.9	4.9	3.8	2.6
	Total Variation (%CV)	7.9	5.1	4.6	2.7

TABLE 3. Selectivity at the Low QC Concentration (15.0 ng/mL) for PMB1, PMB1-1 and PMB2 (n=6 in one run)

	PMB1 (ng/ml)	PMB1-1 (ng/ml)	PMB2 (ng/ml)
	15.7	15.8	15.1
	15.0	14.3	15.1
	15.6	14.7	14.9
	15.1	14.8	14.4
	14.7	14.3	14.4
	14.4	14.2	14.4
Mean	15.1	14.7	14.7
S.D.	0.504	0.598	0.354
%CV	3.3	4.1	2.4
%Theoretical	100.7	98.0	98.0
%Bias	0.7	-2.0	-2.0



Results and Conditions (continued)

TABLE 4. Dilution Quality Control Samples (8,000 ng/mL, DF=10) for PMB1, PMB1-1 and PMB2 (n=6 in one run)

	PMB1 (ng/ml)	PMB1-1 (ng/ml)	PMB2 (ng/ml)
	8080	7970	7860
	8060	8020	7960
	8110	8000	8090
	7970	7830	7640
	8180	8020	8130
	8110	7900	7760
Mean	8090	7960	7910
S.D.	69.5	76.6	190
%CV	0.9	1.0	2.4
%Theoretical	101.1	99.5	98.9
%Bias	1.1	-0.5	-1.1

TABLE 5. Relative Extraction Recovery for PMB1, PMB1-1, PMB2 and CB182735 (IS) (n=6 in one run)

	Low (15.0 ng/mL)	Medium (200 ng/mL)	High (1600 ng/mL)
Recovery for PMB1	58.2%	60.1%	58.3%
Recovery for PMB1-1	55.4%	59.3%	57.1%
Recovery for PMB2	50.6%	49.0%	46.3%
Recovery for CB182735 (IS, 500 ng/mL)	54.0%		

TABLE 6. Matrix Factor for PMB1, PMB1-1, PMB2 and CB182735 (IS)
(Results are expressed as area counts) (n=6 in one run)

	PMB1		PMB1-1		PMB2		CB182735	
	Extracted	Neat	Extracted	Neat	Extracted	Neat	Extracted	Neat
PMB1	175853	142193	149283	117194	181260	147840	181260	147840
	13044	4611	9139	5370	6584	4962	6584	4962
	7.4	3.2	6.1	4.6	3.6	3.4	3.6	3.4
Matrix Factor	1.2		1.3		1.2		1.2	



Conclusion

A simple, sensitive and robust LC-MS/MS method was developed and validated for simultaneous determination of PMB1, PMB-1-1 and PMB2 in treated human urine. CHAPS proved to be an effective modifier to prevent the analyte loss in urine for long-term sample storage.

Reference

- [1] Guoying C. et. al., Development and validation of a reversed-phase high-performance liquid chromatography assay for polymyxin B in human plasma, *Journal of Antimicrobial Chemotherapy* (2008) 62, 1009–1014
- [2] Cheng C, et. al., LC-MS/MS method development and validation for the determination of polymyxins and vancomycin in rat plasma, (2010), 878(28), 2831-8.