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Determination of Meropenem in Bacterial Media by LC-MS/MS

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Abstract

To support the development of a dynamic *in vitro* human Pharmacokinetic/Pharmacodynamic simulation model for biofilm-mediated infections and study stability of meropenem, an LC-MS/MS method for the determination of meropenem in Luria Bertani (LB) media was developed and validated in an API2000 LC-MS/MS system. A partial validation was also performed in M_9 media. Sample aliquots of 100 μL (or 25 μL for M_9 media) were mixed with the internal standard (IS) ceftazidime and filtered. The filtrate was directly injected onto a C_8 column eluted with ammonium formate (10mM, pH4) and acetonitrile (0.1% formic acid) in a gradient mode. ESI^+ and MRM with ion pair *m/z* 384 → 68 for meropenem and *m/z* 547 → 468 for the IS were used for quantification. The calibration curve concentration range was 50 to 25,000 ng/mL. The recovery was over 98%. In LB media, significant signal suppression was observed throughout the time period of detection when compared with mobile phase solvents, but the matrix effect was compensated well with the IS. In M_9 media, much less signal suppression was observed. The method is simple, fast, and reliable. Using the method, stability of meropenem in LB and M_9 media were tested. No significant degradation was observed for at least 8 hours in both LB media (37 °C) and M_9 media (30 °C), but more than 15% degradation was observed overnight (~20hr). The method was transferred to an API5000 LC-MS/MS system using meropenem-d_6 as the IS.

Keywords

LC-MS/MS; meropenem; M_9; LB Media; stability

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1. Introduction

Meropenem is a carbapenem antibiotic with broad-spectrum *in vitro* activity to a wide range of gram positive, gram negative, and anaerobic bacteria including methicillin-susceptible *Staphyloccocus aureus* and *Pseudomonas aeruginosa*. Carbapenems, like all β-lactam antibiotics, inhibit bacterial cell wall synthesis by binding to and inactivating penicillin-binding proteins [1]. Meropenem is known for its activity against gram-negative organisms that are resistant to other β-lactam antibiotics (such as cephalosporins and β-lactam inhibitor combinations) because of its stability against hydrolysis by most β-lactamases, including AmpC and extended spectrum β-lactamases (ESBL). Meropenem is generally administered to humans intravenously (*IV*), exhibits a half-life of approximately 1 hour, and is predominantly renally eliminated. Meropenem has excellent penetration into most bodily fluids and tissues, including the central nervous system and lung tissue. Meropenem exhibits time-dependent killing. Thus, the percentage of time the free meropenem concentrations exceed the minimum inhibitory concentration (*f*T>MIC) during a dosing interval is most predictive of clinical and microbiological success and is known to be 40% [2]. Meropenem is commercially available as single use clear glass bottles contacting 500 mg or 1 g sterile meropenem powder (Merrem® Astra-Zeneca; package insert). Once reconstituted, meropenem *IV* dosing (or infusion) solution can only be stored for 2 hours at concentrations up to 50 mg/L at temperatures of 15-25° C per the manufacturer (Merrem package insert). Accordingly, *IV* dosing or analysis of meropenem samples should be complete in a few hours at room temperature.

While the pharmacokinetics/pharmacodynamics (PK/PD) of antibiotics on planktonic bacteria (e.g., grown in liquid culture) have been well characterized, the PK/PD of antibiotics on bacterial biofilms are virtually unknown [3]. To support studies of meropenem in a newly developed *in vitro* PK/PD biofilm simulator, an analytical method to quantitate meropenem in bacterial media is needed.

In respect to instability of meropenem samples at room temperature, it is beneficial to develop an analytical method with a short run time. Numerous analytical methods for quantitation of meropenem have been reported [4-8]. Most of them are based on high performance liquid chromatography coupled with ultra-violet/visible spectrophotometer (HPLC-UV), requiring long run times. Two methods using liquid chromatography-tandem mass spectrometry (LC-MS/MS) were reported for determination of meropenem in plasma [9] and serum [10]. Here we report LC-MS/MS methods to quantify meropenem in Luria Bertani (LB) and M_9 bacterial media. To expedite sample analysis to meet the need of the project, the methods were developed on two LC-MS/MS systems: AB Sciex API2000 and API5000. The run times were 5-7min. The methods were validated based on AIDS Clinical Trial Group (ACTG) guidelines [11], and applied to studies of *Pseudomonas aeruginosa* biofilm infection in a newly developed *in vitro* human PK/PD simulation model.

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2. Experimental

2.1. Chemicals and materials

Meropenem (Figure 1) was obtained from AstraZeneca Pharmaceuticals. Ceftazidime (the internal standard, IS) was purchased from A.K. Scientific Inc. (Mountain View, CA, U.S.A.). Meropenem-d_6 (IS for the modified method on API5000) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Acetonitrile (MeCN), water, ammonium formate (NH_4FA), and formic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). LB Media was purchased from Sigma-Aldrich (St. Louis, MO, USA). Difco™ M_9 minimal salts and dextrose were purchased from VWR (Visalia, CA, USA). All chemicals were of HPLC grade. LB Media (1/2 strength) was prepared by adding 0.5g LB broth into 50mL water. M_9 Media was prepared by adding 11.3g M_9 minimal salts, 1mL 0.1M $CaCl_2$, 1mL 1M $MgSO_4$ and 54mg glucose to 1L water.

2.2. Instrumental

2.2.1. LC-MS/MS method with API2000—The AB Sciex API 2000 triple quadrupole mass spectrometer was coupled with the PE Biosystems 200 series autosampler and two series 200 micro HPLC pumps. The gases were supplied from a liquid nitrogen tank, and the system was managed with the software Analyst® 1.5.1. Chromatographic separation was achieved on a C_8 analytical column (50 × 2.1 mm, 5 μm; Agilent Inc., Santa Clara, CA, USA) equipped with a C_8 guard column (10 × 2.1 mm, 5 μm) from the same source. Solvent A was 10 mM aqueous NH_4FA at pH 4.0 and solvent B was MeCN with 0.1% formic acid. The column was eluted at a flow rate of 0.3 mL/min in a gradient program consisting of 7% solvent B (0 -1min), from 7 to 90% B (1- 2.5 min), 90% B (2.5-3.5 min), 90%-7% B (3.5-3.6 min), and 7% B (3.6-6.6min). The IS was ceftazidime. The retention times for meropenem and IS were 1.7 and 1.9 min, respectively, and injection volume was 10 μL. The Mass Spectrometer (MS) conditions for meropenem and the IS were optimized by separate infusion of meropenem or IS (10 μg/mL) in 50% MeCN containing 0.1% FA into the MS at a flow rate of 5 μL/min constantly while adjusting MS parameters to achieve maximal signal. Electro-spray ionization in positive ion mode (ESI^+) was used for ionization and multiple reactions monitoring (MRM) mode was chosen for quantitation. The precursor-product ion pair was m/z 384 → 68 for meropenem and m/z 547 → 468 for the I.S on the API2000. The optimized acquisition parameters were listed in Table 1

2.2.2. LC-MS/MS method with API5000—API5000 triple quadrupole mass spectrometer was coupled with Shimadzu Prominence $20AD^{XR}$ UFLC pumps and SIL-$20AC^{XR}$ autosampler, and the system was managed with the software Analyst® 1.5.1. The gases were supplied by an LC-MS Gas Generator (Source 5000™, Parker Balston Inc., Haverhill, MA, USA). The column and mobile phases were the same as API2000-based method but deuterated meropenem (MP-d_6) was used as the IS and the run time shortened by 1.4 min. The column was eluted at a flow rate of 0.4 mL/min in a gradient program consisting of 3% solvent B (0 -0.2min), from 3 to 30% B (0.2- 3.2 min), from 30 to 90% B (3.2- 3.3 min), 90% B (3.3-4 min), 90% to 3% B (4-4.01 min), and 3% B (4.01-5.2min). The retention times for meropenem and IS were 2.09 and 2.08 min, respectively. Injection volume was 5 μL. The MS conditions for meropenem were optimized following the same

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procedure as described above on API2000 with a 200-fold diluted meropenem solution (50 ng/mL). The precursor-product ion pair was *m/z* 384 → 141 for meropenem and *m/z* 390 → 147 for the IS. The optimized acquisition parameters are listed in Table 1.

2.3. Standards and quality controls preparation

Primary stock solutions of meropenem and IS were prepared at 1 mg/mL for each in water. These solutions were diluted with water to prepare for working solutions. The working solutions of meropenem were spiked to blank bacterial media (LB or M_9) to obtain calibration standards of 50, 100, 250, 500, 1000, 2500, 5000, 10000, and 25000 ng/mL. QC samples were spiked at 150, 3000, and 20000 ng/mL by adding the working solutions into blank bacterial media at 1:50 ratio. For API5000, the calibrators were 50, 100, 250, 500, 1000, 2500, 5000, and 10000 ng/mL and the QC levels were 150, 1500, and 8000ng/mL, prepared in M_9 media. Calibration standards and QC samples were prepared from separately weighted stock solutions. The stock solutions, standards, QC samples, and the IS working solution (1 μg/mL) were stored frozen at -70 °C between uses.

2.4. Sample preparation

If LB media was used: a 100 μL aliquot of each sample was added into a centrifugal filter device (Millipore Ultrafree Durapore® PVDF 0.1μm, 0.5 mL capacity), mixed with 100 μL IS (1 μg/mL ceftazidime), and centrifuged at 10,000 rpm (8900 g) for 4 min. The filtrate was transferred to autosampler vials for LC-MS/MS analysis. Standard, QC, and blank media without bacteria were mixed with IS in eppendorf tubes without filtration. If M_9 media was used: a mixture of 25 μL sample and 25 μL IS was diluted with 150 μL water, transferred to an autosampler vial and injected (10 μL) onto LC-MS/MS API2000 system, or diluted with 950 μL water, transferred to an autosampler vial and injected (5 μL) onto LC-MS/MS API5000 system.

2.5. Validation

The method was validated according to ACTG guidelines [11], which were based on United States Food and Drug Administration (FDA) guidelines [12]. Precision was reported as relative standard deviation (RSD) and accuracy as percent deviation of the nominal concentration (% dev). Recovery was determined by comparing the peak area of un-filtered samples (direct injection) to those of filtered samples at the low (150 ng/mL) and high (20000 ng/mL) concentration levels. Matrix effect was evaluated using LB media with 2-, 4-, and 8-fold dilution with water. Medium QCs were prepared in triplicates with each of LB media, processed and injected onto LC-MS/MS system. Their concentrations were compared with nominal concentration (3000 ng/mL). Matrix effect was also evaluated by infusion experiment: A mixture of meropenem and the IS (each at 4 μg/mL in water) was infused into the eluent from LC column via a T-connector by a syringe pump at a constant flow rate of 10 μL/min. A solution of pure meropenem and IS in water was injected into the LC-MS/MS systems yielded retention times of 1.7 and 1.9 minutes, respectively. The retention times from this injection were used as references. The blank LB media (10 μL) was injected onto the column and the signal (in MRM mode) was monitored for 15 min. Stability

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of meropenem in LB media was evaluated at 37 °C, and stability in M_9 was tested at 30 °C and -70 °C by comparing to freshly prepared samples

2.6. Application

This method was used to validate a novel dynamic PK/PD model designed to study the effects of human simulated meropenem concentrations on *Pseudomonas aeruginosa* biofilms grown *in vitro*. The concentration-time curve of a single 2 gram intravenous bolus dose was simulated based on human population PK parameters [13]. Samples were taken at *t*=0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7. 7.5, 8 and 24 hours. All samples were immediately stored at -80°C after collection, shipped to our analytical lab on the same day with dry ice overnight delivery, and stored at -70 °C freezer until analysis. Samples were typically analyzed within a week.

3. Results and discussion

Meropenem could be detected by ultra-violet/visible spectrophotometer detector, and historical methods mainly utilized HPLC-UV system [4-8]. LC-MS/MS method has the advantage of high selectivity and can achieve a short run time, this is especially beneficial for unstable drugs like meropenem, which is only stable for a few hours at room temperature. Initial efforts were made to develop an HPLC-UV method to determine meropenem in LB Media. Interference from LB media presented a big challenge to keep the LLOQ at an acceptable level while maintaining a short run time. Therefore, we switched to an LC-MS/MS system.

3.1. LC-MS/MS optimization

Three different columns were tested: Synergi polar RP column (2.0×50 mm, 4μm, Phemonenex Inc. Torrance, CA, USA), Zorbax XDB-C_{18} and XDB-C_8(2.1×50 mm, 5μm, Agilent Tech. Inc., Santa Clara, CA, USA). Zorbax XDB C_8 was used as the retention times for meropenem and ceftazidime (the IS) were similar (1.7 and 1.9 min, respectively) under the final LC gradient elution conditions. The run time per sample was 6.6min. While on the API5000 system, a deuterated IS (MP-d_6) was used and the run time was shortened by 1.4 min. The optimized MS parameters were listed in table 1. Noticeably, the quantification ion pair of meropenem on API5000 was 384/141 that was less abundant than 384/68. Because the API5000 is so sensitive that the samples need to be diluted further by 5-fold and less abundant ion pair 384/141 was chosen for quantification to reduce signal intensity. In addition, the upper limit of quantification decreased by 2.5–fold to 10000ng/mL to avoid signal saturataion.

3.2. Sample preparation

The method was initially developed in LB media to support the *in vitro* PK/PD model. Aliquots of 100 μL samples were mixed with equal volume of IS solution (1000ng/mL ceftazidime in water) and a 10 μL of the mixture injected directly onto the LC column. Later M_9 media was adopted for the *in vitro* PK/PD model establishment. The method was adjusted accordingly. It was found the MS signal of meropenem increased by ~4-fold compared to that in LB media. Signal saturation was observed at the high end of calibration

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curve. Thus sample volume reduced to 25 μL, mixed with 25 μL IS and 150 μL water in a microcentrifugal tube. A 10 μL aliquot of the mixture was injected onto the LC-MS/MS API2000 system. If API5000 system was used, 25 μL meropenem in M_9 was mixed with 25 μL IS (100ng/mL MP-d_6 in water) and 950 μL water in a 1mL sample vial and vortex-mixed 15 sec. A 5 μL aliquot of the mixture was injected onto the LC-MS/MS API5000 system. The Millipore centrifugal filtration device was only used when bacteria presented in the media. The recovery after filtration was close to 100% for both meropenem and the IS.

3.3. Validation

Based on the need of the *in vitro* PK/PD model, we determined an LLOQ of 50ng/mL is sufficient. The method was initially validated on API2000 system in LB media. Samples were prepared in 1/2strength LB media (10g/L) and diluted with equal volume of IS solution before injected onto API2000 system. At 50ng/mL, s/n ratio was 23 (Figure 2). The calibration range was 50-25000ng/mL with least square linear regression weighted by $1/x^2$. The back calculated values are all within 15% deviation from the nominal values (Table 2). The intra- and inter-day precision (RSD) and accuracy (%deviation) were all within 15% (Table 3). Infusion experiment demonstrated significant ion suppression for both meropenem and the IS when a blank LB media was injected (data not shown). As the *in vitro* model experiments were planned to run with ½ strength LB media and sample preparation resulted in further 2-fold dilution, matrix effect of 1/2, 1/4, and 1/8 strength LB media were tested by spiking meropenem at 3000ng/mL in the LB media diluted by 2-, 4-,and 8-fold with water, significant matrix effect was observed (Table 4). However, the difference of peak area ratio was less than 15%, suggesting IS compensated the matrix effect. Noticeably, injection of 7-8 blank LB media in the beginning of run helped to reach a stable MS signal intensity.

Based on the validated method in LB media, a partial validation was performed in M_9 media. Noticeably, M_9 media was much cleaner and no significant ion suppression was observed. The sample needs to be further diluted by 4-fold to obtain the same calibration range in API2000 (1:1,v/v for LB versus 1:7,v/v for M_9 media). In API5000 system more dilution (1:39,v/v) was required, and the range of the calibration curve (fitted with 1/x weighted least square linear regression) was narrowed to 50-10000ng/mL.

3.4. Comparison of sensitivity of meropenem on API2000 and API5000

In M_9 media, the S/N ratio of meropenem at 50ng/mL was 206 for the ion pair 384/68 on API5000, while no S/N ratio was available on API2000 due to absence of baseline signal. The peak height of meropenem was about 10-fold higher in API5000 compared to API2000 (Figure 2). The injected sample was (50ng/mL/40)×5μL=6.25pg for the API5000 system versus (50ng/mL/8)×10μL=62.5pg for API2000. Thus, the sensitivity of meropenem on API5000 was ~100-fold higher than API2000.

3.5. Stability

Meropenem in M_9 media was stable at -70 °C storage for at least 6 months(Table 5). Noticeably, no significant degradation (<15%) was observed within 8hr at room temperature and 30 °C in M_9 media and 37 °C in LB media. Therefore, the *in vitro* human PK/PD

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simulation model experiments could be performed in a period of 8 hr without concern for degradation.

Interestingly, other research groups reported mixed stability data [14-16]. Similar stability has been reported by two groups [14,15]. Keel et al reported that meropenem in saline was stable for 12hr at 30 °C, 8hr at 35 °C and 6hr at 40 °C [14], while Berthoin et al reported that meropenem degradation was time, temperature and concentration dependent [15]. If meropenem>40mg/mL, over 10% degradation was found when stored at 25 °C for 12hr. We found 50mg/mL meropenem in M_9 media can be stable for up to 8 hr at room temperature (20-25 °C). Another study demonstrated that meropenem in saline is degraded by 5.80% at 20 °C and 11.85% at 32-37 °C after 8hr [16], slightly lower than our results. It is possible that degradation might occur during analysis if the method required a long run time. We noticed that meropenem signal was dropped significantly when the same sample was reinjected on the following day, however, if the sample rack was cooled at 15 °C, the samples could be stable for 3 days. Pure drug should be white in powder or clear in solution while the degraded solution is yellow.

3.6. Application

The method was applied to determine meropenem concentration from an *in vitro* human PK/PD biofilm silumation model. The PK/PD analysis will be reported elsewhere. A representative concentration-time curve from the model is showed in Figure 3.

4. Conclusion

Methods for determination of meropenem in bacteria media were developed in two LC-MS/MS systems. The method on API 5000 system is ~100-fold more sensitive than that on API2000 system. Matrix effect from LB media was significant, but the IS (both ceftazidime and MP-d_6) compensate the matrix effect. There is no significant matrix effect from M9 media. The method was successfully used in the study of an *in vitro* human PK/PD simulation model. Meropenem in bacterial media was stable for up to 8 hr at ambient temperature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- Quantification of meropenem in bacteria media with a short run time
- Applied to study an *in vitro* pharmacokinetic/pharmacodynamic biofilm model.
- Stability of meropenem in bacteria media.
- Comparison of sensitivity between API2000 and API5000.

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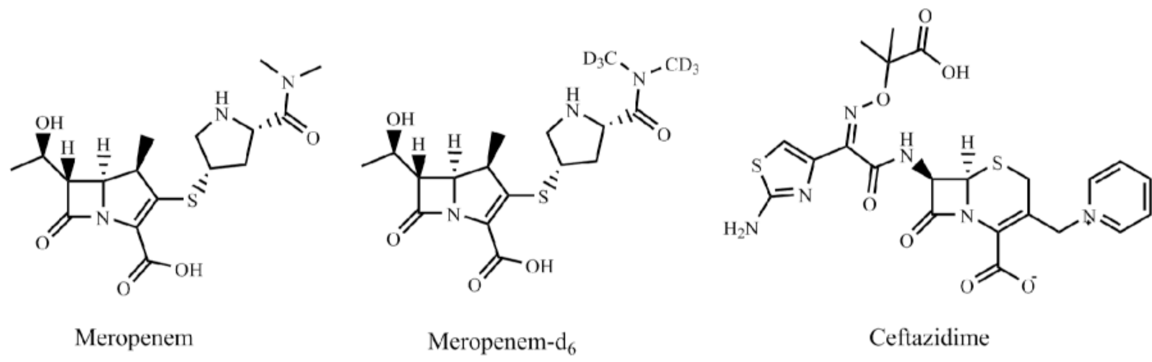

Figure 1.
Chemical structures of meropenem, meropenem-d_6 and ceftazidime.

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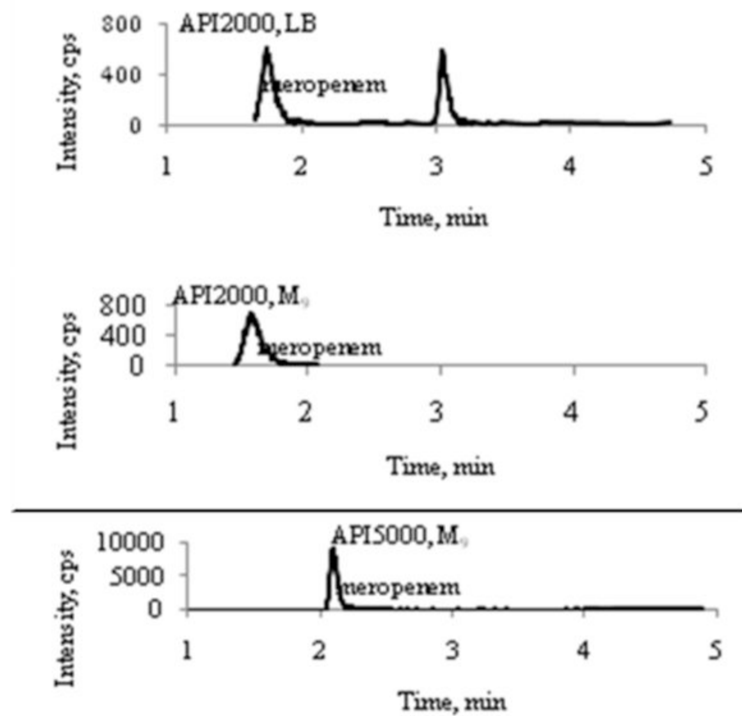

Figure 2.

Chromatograms of meropenem at 50ng/mL (LLOQ) in LB and M_9 media. The same ion pair m/z 384/68 was monitored. In M_9 media, API5000 (lower panel) is about 100-fold more sensitive compared to API2000 (middle panel), counting on a half of injection volume and 5-fold additional sample dilution.

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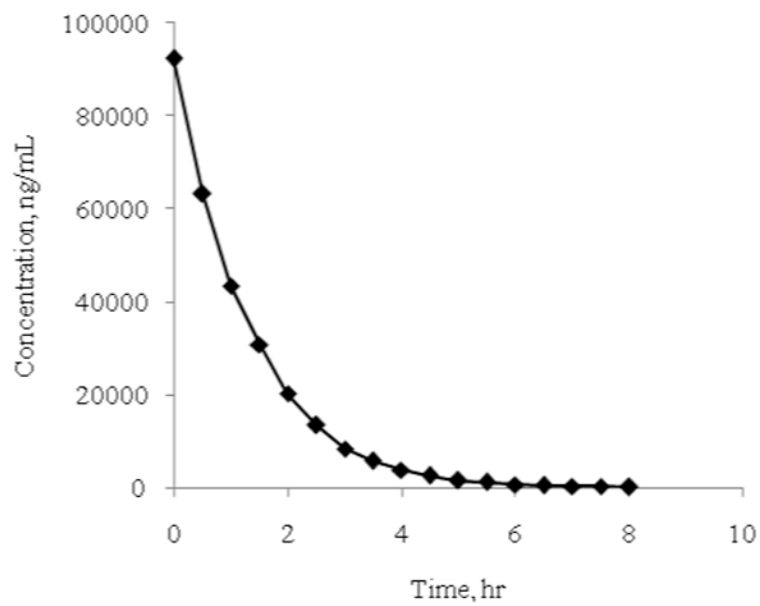

Figure 3.
A representative concentration-time profile of meropenem in an *in vitro* PK/PD biofilm model.

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Table 1

Optimized MS parameters

Source parameters*	**TEM, °C**	**IS, v**	**CUR, psi**	**Gas1, psi**	**Gas2, psi**	**CAD, psi**	
API2000	**400**	**5000**	**20**	**40**	**70**	**4**	
API5000	**500**	**5500**	**40**	**50**	**60**	**12**	
Compound parameters*	DP, v	FP, v	EP, v	CE, v	CEP, v	CXP, v	Dwell time, ms
API2000							
m/z 384/68 (meropenem)	11	350	7.5	61	16	8	250
m/z 547/468 (ceftazidime, IS)	11	350	7.5	19	16	20	250
API5000							
Quantitation ion pair m/z 384/141 (meropenem)	81		10	25		20	80
Confirmation ion pair m/z 384/68(meropenem)	81		10	75		16	80
m/z 390/147(meropenem-d_6, IS)	81		10	25		20	80

Note: parameters were defined as follows: TEM, source temperature; IS, ion spray voltage; CUR, curtain gas; Gas1, Nebulizer gas; Gas2, Auxiliary(turbo) gas; CAD, collision-activated dissociation gas; DP, declustering potential; FP, focusing potential; EP, entrance potential; CE, collision energy; CEP, collision cell entrance potential; CXP, collision cell exit potential.

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Table 2

Inter-day average back-calculated standard concentrations (n = 5)

Nominal conc, ng/mL	50	100	250	500	1000	2500	5000	10000	25000	slope	Y-intercept	R
Mean, ng/mL	49.1	101	254	525	1028	2501	4996	9636	23296	0.0030	0.0089	0.9980
Precision (RSD,%)	2.46	2.43	2.67	4.07	2.99	3.08	3.85	6.91	1.13			0.142
Accuracy (% dev)	1.80	0.82	1.76	5.00	2.78	0.03	-0.08	-3.64	-6.81			
n	5	5	5	5	5	5	5	5	5			

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Table 3

Intra- and inter-day precision and accuracy of meropenem in LB media.

	Intra-day				Inter-day			
Nominal, ng/mL	**50**	**150**	**3000**	**20000**	**50**	**150**	**3000**	**20000**
mean, ng/mL	44.2	141	2880	17740	46.5	149.4	3040.8	18252
SD	1.1	5.1	79.7	288	3.7	7.8	172.1	961.8
RSD,%	2.4	3.6	2.8	1.6				
%dev	-11.5	-6.0	-4.0	-11.3	-6.9	-0.4	1.4	-8.7
n	5	5	5	5	25	25	25	25

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Table 4

Matrix effect of LB media (n=3): LB media of different strengths diluted with water was spiked with meropenem at 3000ng/mL. RSD, relative standard deviation. %diff, percent difference compared to the control (1/4 LB media).

	LB conc., g/L	meropenem peak area	RSD, %	%diff	peak area ratio	RSD,%	%diff
1/2 LB	10	1.80×10^5	2.2	-25.7	10.24	4.3	-2.4
1/4 LB (control)	5	2.48×10^5	4		10.49	0.9	
1/8 LB	2.5	2.73×10^5	1.3	5.8	10.92	1.3	4.1

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Table 5

Stability of meropenem (n=3).

conditions	Conc., mg/L (mean±SD)	% remaining
M_9, 7 months, -70 °C		
Low(0.150mg/L)	0.136±0.006	91.5%
High (20mg/L)	18.6±0.3	90.6%
LB media (10g/L), 37 °C		
0hr	0.144±0.002	100%
	19.8±0.6	100%
8hr	0.137±0.016	94.6%
	18.8±0.5	95.2%
16hr	0.134±0.001	92.8%
	16.8±0.8	84.8%
M_9 media, 30 °C		
0hr	0.179±0.001	100%
	19.2±0.4	100%
8hr	0.167±0.002	93.7%
	17.0±0.3	93.8%
22hr	0.146±0.006	81.5%
	16.8±0.8	83.5%
Stock in M_9, ~25 °C		
0hr	$(50.0±1.3)×10^3$*	100%
8hr	$(45.8±1.5)×10^3$*	91.6%
24hr	$(37.3±2.5)×10^3$*	74.6%

*Note: Concentration of stock in M9 was measured along with *in vitro* samples once each day in 5 different days (n=5). SD, standard deviation.