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Determination of free ertapenem in plasma and bronchoalveolar lavage by high-performance liquid chromatography with ultraviolet detection

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Abstract

A sensitive assay for the determination of unbound ertapenem in human plasma and bronchoalveolar lavage (BAL) was developed using ultrafiltration of plasma and BAL samples. A rapid HPLC method was used with ultraviolet detection set at a wavelength of 305 nm and a separation on a Prontosil AQ C18 column, with imipenem used as internal standard. This assay was linear over the concentration range of 0.5–100 µg/mL and 0.25–50 µg/mL in plasma and BAL, respectively. Limits of detection and quantitation were respectively 0.05 and 0.25 µg/mL. Validation data for accuracy and precision were CV < 2.48 and 8.25%, accuracy in the range 98.1–104.2% and 102.2–108.4%, respectively, for intra and inter-day.
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1. Introduction

Ertapenem (Ivanz®, MK-0826, Merck & Co) is a new once-a-day parenteral β-lactam antibiotic effective for the treatment of community-acquired and mixed infections caused by Gram-positive and Gram-negative aerobic and anaerobic bacteria [1].

Ertapenem differs from imipenem by a unique anionic side-chain and a 1-β methyl group (Fig. 1). It is resistant to human renal dihydropeptidase-I (DHP-I) probably due to the presence of the 1-β methyl and the anionic side-chain presumably increases the binding to plasmatic proteins. Those characteristics give to ertapenem a longer plasma half-life than imipenem, about 4.9 h versus 1 h for imipenem, which allows for a once-daily administration [2–4]. Ertapenem inhibits bacterial cell wall synthesis by binding to penicillin binding proteins (PBPs).

Ertapenem is especially effective against *Bacteroides fragilis* and other anaerobic bacteria, also against Gram-negative aero-bic bacilli. It has little effect on *Pseudomonas aeruginosa* and *Acinetobacter baumanii*. The major indication is community-acquired infections, particularly intra-abdominal or pelvic infection caused by aerobic or anaerobic Gram-negative bacteria, and community-acquired pneumonias; with other indications such as skin and skin structure infections and complicated urinary infections. Ertapenem can be also used in deep anaerobic infections of bone tissue in diabetic patients [1,2]. In plasma, some of the drug binds to plasma proteins or blood cells, or diffuses into the blood cells. Some drug also remains unbound in plasma and can move freely in the body. A similar scenario also occurs in the tissue. Most infections occur in the tissues of the body rather than in the blood so that it is accepted today that appropriate antibiotic therapy requires achievement of significant concentrations of antibiotics at the sites of infection. Several studies determined ertapenem concentrations in plasma [4–6], urine [5,7], cerebrospinal fluid [8], but no data on BAL concentrations is available. Determination of ertapenem plasma and BAL concentrations in critically ill patients on mechanical ventilation with community-acquired pneumonia is necessary to assess its pharmacokinetic parameters and to ensure its effi-

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cacy in this particular subset of patients who often present some pathophysiological conditions that may alter the pharmacokinetic behavior of this agent. The difference between the total plasma concentrations and the free plasma or tissue concentrations can be quite significant when the protein binding of the antibiotics is high. Therefore, the free plasma concentration is an interesting parameter for the rational dosing of antibiotics and the unbound drug concentration at the infection site should be preferred. Moreover, it is important to realize that in vitro MIC values are determined in the presence of the free antibiotic concentrations and the protein binding of the antibiotic is frequently not taken account.

The present paper describes the development and validation of a high-performance liquid chromatographic method (HPLC) coupled with ultraviolet (UV) detection for determination of free ertapenem in human plasma and BAL. This method will be used and adapted for patients in intensive care unit (ICU), to determine free ertapenem plasma and BAL concentrations in order to know the pharmacokinetic profiles of ertapenem in ICU infected patients. The new plasma pharmacokinetic parameters calculated could be used to optimize the design of administration of ertapenem by comparing them with pharmacodynamic parameters such as minimum inhibitory concentration (MIC). Finally, this specific assay might contribute to a better understanding of the distribution of ertapenem in different tissues such as BAL to quantify the actual concentrations met in respiratory tract.

2. Experimental

2.1. Chemicals

Ertapenem and imipenem, the internal standard, were obtained from Merck & Co (USA) (Fig. 1).

Acetonitrile HPLC quality was purchased from Scharlau (Barcelona, Spain). Di-natrium hydrogen phosphate Na_2HPO_4
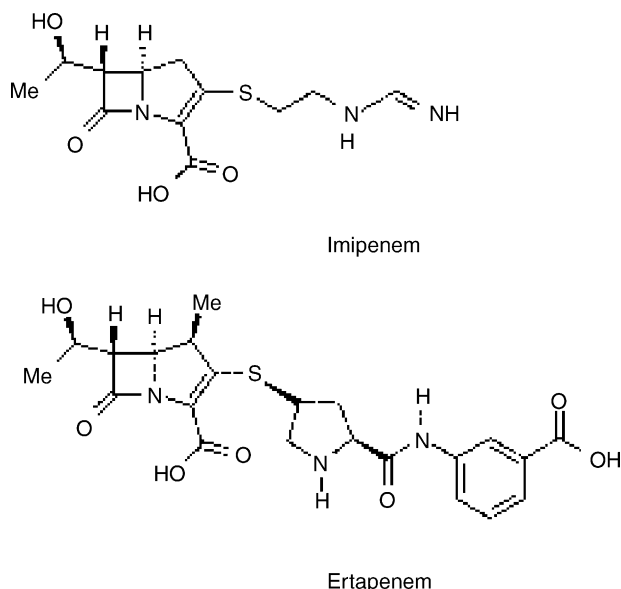

Fig. 1. Chemical structures of imipenem and ertapenem.

and concentrated orthophosphoric acid were from Prolabo (Nogent sur Marne, France).

2.2. Equipment

The HPLC system consisted of Agilent 1100 series (Agilent technologies, Weldbroon, Germany): a model G1311A quaternary pump, a model G1315B DAD UV detector and an Agilent Chemstation for LC systems.

Ultracentrifugation was performed in a Sigma 2 MK centrifuge (Bioblock Scientific, Strasbourg, France).

2.3. Ultrafiltration

All the samples (calibration standards, quality controls or clinical) were thawed at room temperature. In a haemolysis tube, 200 μL of sample were mixed with 350 μL of 40 mM phosphate buffer containing 25 μg/mL imipenem pH 4.0. The solution of imipenem was prepared just before analysis; it was stable for 12 h at room temperature.

A 400 μL aliquot was transferred to a Microcon^{TM} YM10 centrifugal device with a molecular cut-off of 10,000 Da (Millipore, Bedford, USA), which was centrifuged at $15,000 \times g$ at room temperature for 20 min in the sigma model 2 MK centrifuge. Then, 125 μL of the filtrate were transferred in an auto-sampler vial and 40 μL were injected into the chromatographic system.

All those operations were performed at room temperature.

2.4. Chromatography

The mobile phase consisted of 10 mM phosphate buffer adjusted to pH 6.5 with concentrated orthophosphoric acid (phase A), and mixed with acetonitrile (phase B); a gradient elution was performed with the two pumps: the gradient began with 94 and 6%, phase A and B, respectively, from 0 to 2 min, became 82 and 18%, phase A and B, respectively, from 2 to 7 min and came back to original conditions between 7 and 14 min. The mobile phase was filtered through a 0.45 μm filter from Millipore (Saint Quentin en Yvelines, France); the flow rate was set at 1 mL/min. The analytical column was a Prontosil 120 AQ+ C18 (4.6 mm × 150 mm, 5 μm) from Bischoff chromatography (Leonberg, Germany). The sample injection volume was 40 μL. The UV absorbance detection was set at 305 nm with a bandwidth of 4. The chromatographic run time was 14 min.

2.5. Preparation of calibration standards and quality controls

2.5.1. Plasma calibration and quality controls

A working stock solution of 1 mg/mL of ertapenem was prepared by appropriate dilution into pH 6.5, 10 mM phosphate buffer. It was diluted into free human plasma to obtain a concentration range from a 0.5 to 100 μg/mL of ertapenem for calibration. For quality controls, concentrations were different

from those used for calibration and represented 0.75, 15 and 75 μg/mL of ertapenem in plasma.

2.5.2. Bronchoalveolar lavage calibration and quality control

The working stock solution, the same as plasma, was diluted in free BAL to obtain a concentration range from 0.25 to 50 μg/mL of ertapenem for calibration. Quality control concentrations representing 0.5, 7.5 and 30 μg/mL of ertapenem were prepared in drug-free BAL.

2.6. Sample treatments

The blood samples, received in a BD Vacutainer system (Becton-Dickinson, Le Pont-de-Claix, France), were centrifuged at $1200 \times g$ for 10 min and the plasma was stored at $-80\,^\circ$C in cryovials until analysis. After being thawed, they were immediately treated by ultrafiltration and analyzed by HPLC. All BAL samples were stored as soon as possible at $-80\,^\circ$C.

2.7. Calibration and calculation procedure

Spiked matrix samples were prepared in a concentration range from 0.5 to 100 μg/mL for plasma, 0.25 to 50 μg/mL for BAL; each calibration curve consisted of a blank sample and six calibrator concentrations. Calculations were made using the ration of the observed peak areas of ertapenem and internal standard (imipenem). Linear regression analysis of the calibration data was performed using the equation $y = mx + b$ where y was the peak area ratio, x the concentration of ertapenem, m and b two constants; unknown concentrations were computed from the linear regression equation of the peak area ratio against concentration for the calibration curve.

2.8. Limit of detection and limit of quantitation

The limit of detection (LOD) was defined by the concentration with a signal-to-noise ratio of 3. The limit of quantitation (LOQ) was the minimum injected amount that gave precise measurements (relative error and relative standard deviation (R.S.D.) both less than 15%) and was determined by a number of samples with decreasing amounts of the analytes injected five times.

2.9. Accuracy, precision and recovery

Accuracy was determined by the mean of the measured QC concentration relative to the theoretical value and was reported in percentage. Precision was defined as the ratio of the standard deviation of the observed QC concentration to the mean observed QC concentration. The intra-day precision and accuracy for plasma and BAL filtrate QCs were calculated from three concentrations (0.75, 15 and 75 μg/mL for plasma; 0.5, 7.5 and 30 μg/mL for BAL) analyzed six times on the same day. The inter-day variability was estimated from sixplicate analysis of three plasma and BAL filtrate QCs (0.75, 15 and 75 μg/mL for plasma; 0.5, 7.5 and 30 μg/mL for BAL) on four separate days.

The overall mean precision was defined by the coefficients of variation (CVs) as defined by the FDA guidelines [9]. Recovery of ertapenem was evaluated by comparing the mean peak areas of the different QC samples ultrafiltrated with those prepared by adding compounds to ultrafiltrate plasma and BAL blanks at corresponding concentrations. The variability of recovery results was determined.

2.10. Specificity and selectivity

Five blank plasma samples were investigated for interference of endogenous matrix components. Specificity was assessed in the presence of epinephrine (2 μg/mL), norepinephrine (2 μg/mL), isoprenaline (2 μg/mL), ritonavir (5 μg/mL), lopinavir (8 μg/mL), indinavir (5 μg/mL), atazanavir (3 μg/mL), nelfinavir (5 μg/mL), amprenavir (4 μg/mL), nevirapin (5 μg/mL), efavirenz (10 μg/mL), tenofovir (4 μg/mL), acyclovir (2 μg/mL), itraconazole (0.5 μg/mL), amphotericin (1 μg/mL), ribavirin (3 μg/mL), ofloxacin (1.5 μg/mL), gentamicin (2 μg/mL), rifampicin (5 μg/mL), ceftazidime (15 μg/mL), ciprofloxacin (2 μg/mL), amikacine (1.5 μg/mL), cloxacillin (5 μg/mL) and ceftriaxone (6 μg/mL).

2.11. Stability

Stability of ertapenem in plasma and BAL filtrates was evaluated after short-term storage (12 h) at room temperature and at 5 °C. Low, middle and high QC concentrations (0.75, 15 and 75 μg/mL for plasma, 0.5, 7.5 and 30 μg/mL for BAL) were prepared in six samples in plasma and BAL from a freshly made stock solution. A set of samples were tested after 12 h stored in the HPLC auto-sampler at room temperature; another similar set was tested at 5 °C for 12 h. Data were compared with results obtained from freshly prepared QC samples.

The stability of stock solutions of ertapenem and imipenem were assessed at room temperature for 12 h. The long-term storage stability of ertapenem in plasma and BAL were evaluated at 0.75, 15 and 75 μg/mL for plasma; 0.5, 7.5 and 30 μg/mL for BAL at $-80\,^\circ$C. These QCs were analyzed at 3 and 6 months.

3. Results

3.1. Chromatography characteristics

Fig. 2 shows analytical chromatograms for blank samples in plasma and BAL and spiked samples. Fig. 3 shows patient's chromatograms. The retention times of imipenem and ertapenem were, respectively, 2.4 and 6.2 min.

3.2. Calibration curve

The plasma assay was linear from 0.5 to 100 μg/mL in plasma, from 0.25 to 50 μg/mL in BAL and showed good coefficient of correlation r^2 (0.9982 and 0.9991, respectively, for plasma and BAL). Regression intercepts were not significantly different from zero.
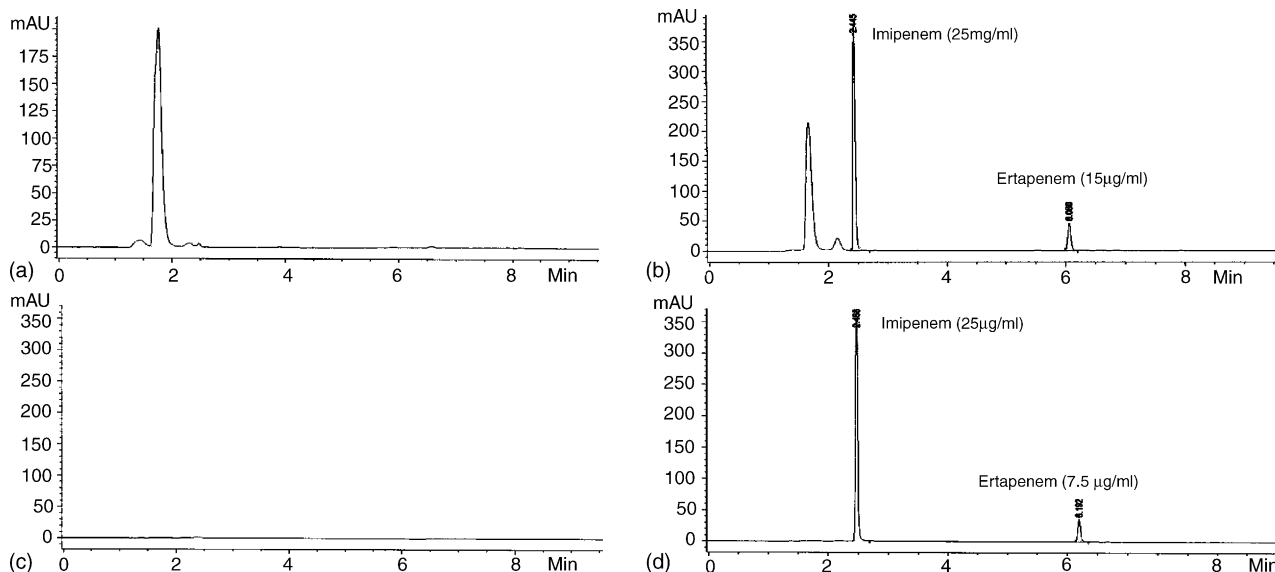
Fig. 2. Chromatograms used for validation analytical assay: chromatograms of blank human plasma sample (a), human plasma sample spiked with 15 μg/mL of ertapenem and 25 μg/mL of imipenem (b), blank human BAL sample (c), human BAL sample spiked with 7.5 μg/mL of ertapenem and 25 μg/mL of imipenem (d).

3.3. Limit of detection, limit of quantitation

The LOD and LOQ were determined, respectively, as 0.05 and 0.25 μg/mL in plasma and BAL.

3.4. Specificity and selectivity

No endogenous substance did interfere with imipenem and ertapenem in blank plasma and BAL. Potentially co-administered drugs tested had retention times that were different from ertapenem or were not detected.

3.5. Accuracy, precision and recovery

Intra-day evaluation of plasma filtrate QCs ($n = 6$) gave good results with precision from 1.41 to 2.48% and 1.51 to 4.48% for CV and accuracy from 98.1 to 104.2% and 101.6 to 103.2%, respectively, for plasma and BAL (Table 1).

The inter-day variability for plasma filtrates, calculated from three samples six-fold injected on 4 days was low, with CV ranging from 3.82 to 8.25% and 3.10 to 6.54% and accuracy from 102.2 to 108.4% and 98 to 104.3% for plasma and BAL, respectively (Table 2). Accuracy and CV data respected the criteria for
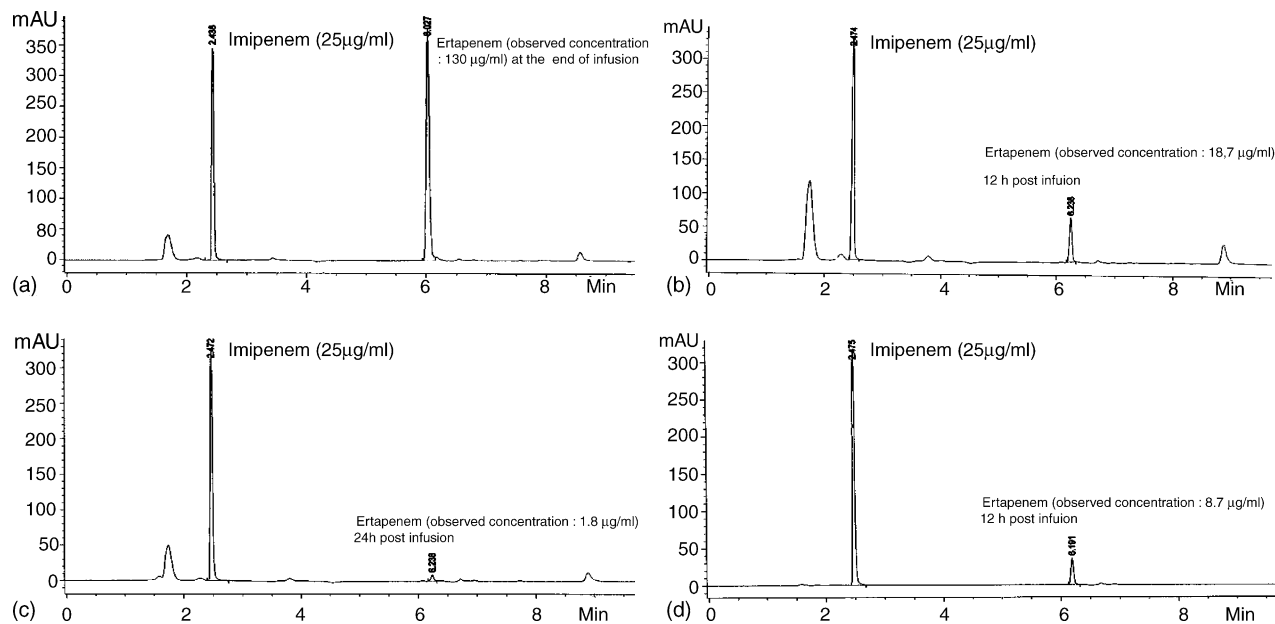

Fig. 3. Chromatograms used for determination of ertapenem in patient's plasma and BAL: chromatograms of patient plasma sample at the end of infusion with an observed concentration of 130 μg/mL (a), 12 h after infusion with an observed concentration of 18.7 μg/mL (b), 24 h after infusion with an observed concentration of 1.8 μg/mL (c), patient BAL sample 12 h after infusion with an observed concentration of 8.7 μg/mL (d).

Table 1
Intra-day accuracy and precision from the determination of ertapenem

Theorical concentration	Intra-day concentration found (mean ± S.D.)	Accuracy (%)	R.S.D. (%)	n
Plasma (μg/mL)				
0.75	0.78 ± 0.019	104.2	2.48	6
15	14.72 ± 0.207	98.1	1.41	6
75	79.82 ± 1.273	106.4	1.60	6
BAL (μg/mL)				
0.50	0.51 ± 0.023	102.6	4.48	6
7.5	7.61 ± 0.115	101.6	1.51	6
30	30.97 ± 0.571	103.2	1.84	6

validation specified within the FDA guidelines [9]. The ultrafil-trate mean recoveries of ertapenem from QC samples on four separate days ranged from 97.81 to 99.78% with an average of 99% for plasma and from 96.54 to 100.38% with an average of 98.5% for BAL.

3.6. Stability

Stock solutions of ertapenem and imipenem in buffer showed no perceptible degradation between solutions kept at room temperature for 12 h and freshly prepared solutions.

Long-term stability studies showed no significant degradation of QC samples stored at −80 °C and analyzed at 3 and 6 months. Mean ertapenem concentrations ranged from 96.9 to 102.8% for plasma, and from 98.6 to 103.5% for BAL, compared to freshly prepared QCs. Freeze-thawed stability of these plasma and BAL QC samples was assessed over three cycles: samples were frozen, thawed and analyzed three times; average stability results ranged from 98.4 to 101.7% and 96.4 to 103.9%, respectively, for plasma and BAL. Ultrafiltrates were stable (>95%) for 12 h at room temperature and at 5 °C.

4. Discussion and conclusion

A simple and sensitive HPLC assay for ertapenem in human plasma and human bronchoalveolar lavage was developed using ultrafiltration and UV detection ($\lambda = 305$ nm).

Previously reported HPLC-UV methods used ultracentrifugation and on-line extraction. Concerning our study, we use of a previous assay about imipenem to obtain the ultrafiltration, elution and detection of ertapenem and took imipenem as the internal standard. Sensitivity of UV detection was thought sufficient to quantitate the lowest through concentrations found in both human plasma and BAL. Our objective was to study the penetration of ertapenem in infectious sites by comparing its amounts in plasma with those in BAL so that we thought necessary to use the same method for treating several matrixes. Therefore, ultrafiltration and HPLC coupled with UV detection was chosen for the determination of ertapenem in human plasma and BAL.

Chromatography for ertapenem in urine by reversed-phase HPLC with UV absorbance detection had been reported previously using column-switching for on-line extraction by Musson et al. [5]. The method was not always selective for the urine collections of some clinical subjects. The use of column-switching allowed several parameters to be modified for enhanced selectivity of chromatography of ertapenem. A modified method was adapted from the previous one by changing the on-line extraction column to enhance the selectivity of the column for ertapenem over endogenous material by decreasing the secondary interactions [7].

Because ertapenem and other β-lactams were unstable in an acidic environment and tended to convert to the open-ring form [5,8], we used a quasi neutral (pH 6.5) mobile phase in our assay which provided a very good stability of ertapenem in the chromatographic system. Kitchen et al. used an acidic mobile phase but no conversion of ertapenem to the open-ring form was detected during the assay, possibly because of the short time the drug was exposed to the mobile phase. Our assay provided a simple and fast preparation of the mobile phase with a very good selectivity and sensitivity because of the step of ultrafiltration. Our LOD and LOQ in plasma were the same comparing to these assays and were, respectively, 0.05 and 0.25 μg/mL. Moreover,

Table 2
Inter-day accuracy and precision from the determination of ertapenem

Theorical concentration	Inter-day concentration found (mean ± S.D.)	Accuracy (%)	R.S.D. (%)	n
Plasma (μg/mL)				
0.75	0.77 ± 0.063	102.2	8.25	24
15	15.46 ± 0.690	103.1	4.46	24
75	81.30 ± 3.103	108.4	3.82	24
BAL (μg/mL)				
0.5	0.53 ± 0.049	104	6.54	24
7.5	7.35 ± 0.347	98	4.72	24
30	31.28 ± 0.971	104.3	3.10	24

large injection volumes (200 μL) were necessary to attain the required sensitivity using column-switching. Our assay needed 200 μL for ultrafiltration and only 40 μL were injected. Moreover, we observed in our assay that when the small volume of extraction was flushed to the analytical column, the peak remained sharp with repeated injections despite the observations described by Kitchen et al. without using column-switching. The author described progressive deterioration in the peak shape of chromatography of ertapenem [8]. This last point, small volume of ultrafiltration (200 μL) and the absence of deterioration of the peak shape of chromatography of ertapenem represented the major advantages of our assay. In conclusion, our assay was rapid, simple, reproducible and sensitive (LOQ = 0.25 μg/mL in plasma and BAL). Calibration range was based on human plasma ertapenem concentrations in pharmacokinetic steady-state when ertapenem was administered at 1000 mg once a day intravenously. Lastly, our assay is adapted and will be applied to pharmacokinetic analysis of free ertapenem penetration in human infectious sites such as BAL especially in ICU patients.

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