CHROMSYSTEMS

THERAPEUTISCHES DRUG MONITORING
THERAPEUTIC DRUG MONITORING
SUIVI THÉRAPEUTIQUE DE MÉDICAMENTS
MONITORAGGIO DEI FARMACI
MONITORIZACIÓN DE FÁRMACOS

Instruction Manual for HPLC Determination

Antibiotics in serum/plasma

Order no. 61000

CE IVD

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1 Ordering information

Order no.	Product	
61000	**HPLC Reagent Kit** **Antibiotics in serum/plasma**	
	Contents for 100 determinations:	
	Mobile Phase A	2 x 750 ml
	Mobile Phase B	1 x 950 ml
	Internal Standard Mix	1 x 20 ml
	Dilution Buffer	1 x 10 ml
	Priming Solution	1 x 2 ml
	Reaction Vials, transparent	1 x 100 pcs.
	Components available separately	
61001	Mobile Phase A	750 ml
61002	Mobile Phase B	950 ml
61004	Internal Standard Mix	20 ml
61005	Dilution Buffer	10 ml
61012	Priming Solution	2 ml
	Accessories	
61100	HPLC Column (equilibrated, with test chromatogram)	1 pc.
18001	Precolumn Cartridge Holder 4/10	1 pc.
18061	Precolumn Cartridge 4/10	1 pc.
15070	Stainless Steel Prefilter Housing	1 pc.
15071	Stainless Steel Prefilter, 0.5 µm	5 pcs.
33006	Reaction Vials, transparent	100 pcs.
J0404	Autosampler Vials, amber glass 1.5 ml	100 pcs.
J0406	Crimp Caps, rubber/PTFE septa, 11 mm	100 pcs.
J0505	Micro-Inserts for Autosampler Vials, clear glass	100 pcs.
	Chromsystems calibrator and controls for antibiotics in serum/plasma	
61003	Plasma Calibration Standard	5 x 1.0 ml
61028	**3PLUS1**® Multilevel Plasma Calibrator Set	4 x 1.0 ml (lyoph.)
0183	Plasma Control Level I	5 x 1.0 ml (lyoph.)
0184	Plasma Control Level II	5 x 1.0 ml (lyoph.)

2 Introduction

2.1 Background information

Antibiotics as originally defined are metabolic products of fungi or bacteria that kill or inhibit the growth of other microorganisms. Current usage of the term includes synthetic, semisynthetic and genetically engineered agents developed for the treatment of bacterial infections.

Beta-lactam antibiotics are a large class of antibiotics consisting of agents whose molecular structure contains a beta-lactam ring as an active centre and chemical property. Antibiotics in the beta-lactam class include penicillins (ampicillin, piperacillin), cephalosporins (cefepime, ceftazidime), monobactams and carbapenems (meropenem). Beta-lactam antibiotics block certain bacterial enzymes (penicillin-binding proteins, PBPs), thereby inhibiting cell wall synthesis and ultimately leading to cell lysis and bacterial death.

There are various mechanisms of resistance to beta-lactam antibiotics. Many arise from the synthesis of beta-lactamases, others from reduced permeability of the outer membrane, reduced affinity with antibiotic-binding PBPs, or efflux pump activity.

Combination with **beta-lactamase inhibitors** can mitigate the inactivation of antibiotics by beta-lactamase-producing microorganisms. Beta-lactamase inhibitors are beta-lactam agents that do not possess antibiotic activity of their own (sulbactam, tazobactam). Combining ampicillin with sulbactam and piperacillin with tazobactam significantly expands the spectra of action of these antibiotics.

Linezolid, which belongs to a new class of antimicrobial substances called oxazolidinones, has a unique mechanism of action. It inhibits bacterial protein biosynthesis by binding to the bacterial ribosome and thus preventing the translation process. Resistances to linezolid are linked to point mutations in rRNA.

Special features of antibiotic treatment:
Antibiotic treatment has undergone a paradigm shift in the last number of years. It was formerly believed that antibiotic treatment needed to be continued even after symptoms disappeared, for a total treatment time of at least seven days, in order to minimise the development of resistant organisms. New insights, however, show that short treatment, preferably at a high dose, delivers the best results [8]. If the antibiotic dose is too low, long treatment is more likely to promote than prevent the development of resistant organisms.

The conclusion is that accurate dosing is one of the core elements of rational antibiotic treatment. The efficacy of antibiotics is essentially based on the duration of time during which the antibiotic concentration is higher than the minimum inhibitory concentration (MIC) of the disease-causing organism. However, the actual concentration of active substance in the blood is hard to predict. The outcome of antibiotic treatment can therefore be significantly improved by monitoring serum or plasma levels followed by dose adjustment. Critical care patients whose blood concentration should be four to five times higher than the MIC stand to benefit most from therapeutic drug monitoring.

2.2 Intended use

The reagent kit Antibiotics in serum/plasma is an *in vitro* diagnostic device to be used in clinical laboratories for the quantitative determination of ampicillin, cefepime, ceftazidime, linezolid, meropenem and piperacillin in human serum and plasma samples via high performance liquid chromatography (HPLC) with UV detection. It is intended as a test for patients to monitor the blood levels of the mentioned antibiotic drugs.

2.3 Principle of the reagent kit

This Chromsystems reagent kit allows you to determine serum or plasma concentrations of ampicillin, cefepime, ceftazidime, linezolid, meropenem and piperacillin effectively and reliably. First, patient samples are stabilized. Calibrators and controls already contain stabilizing additives. Subsequently, samples, controls and calibrators are prepared by simple precipitation. HPLC assay with subsequent UV detection takes place in two groups. Substance separation is by gradient elution for group 1. Substance separation for group 2 is isocratic with mobile phase B. Two internal standards are used for effective quantification. Stable matrix products facilitate reliable control of the method.

3 HPLC system

Caution:
When using the reagents comply with the hazard information in Appendix I.

3.1 Equipment and instrument parameters

The determination of ampicillin, cefepime, ceftazidime and meropenem requires a binary gradient system and the determination of linezolid and piperacillin an isocratic system with HPLC pump, coolable injector, coolable column oven and UV detector. The use of a column oven will avoid temperature variations and improve stability and reproducibility of the chromatographic separation. The use of a solvent degasser prevents air entrapment and ensures a stable baseline. To avoid evaporation, keep the mobile phases tightly closed even during operation, e.g. by using special safety caps.

Instrument settings:

Autosampler:	cooling of the samples necessary ≤ 10 °C
Injection volume:	group 1: 5 µl group 2: 10 µl
Run time:	group 1: 13 min group 2: 5 min
Flow rate:	1.0 ml/min
Column temperature:	+22 °C
UV detector:	group 1: cefepime, ceftazidime, meropenem: wavelength 290 nm, ISTD, ampicillin: wavelength 210 nm group 2: wavelength 252 nm
Needle rinsing solution for the injector:	10 % acetonitril

Gradient profile:
The gradient profile shown is intended as a basis for optimisation. Due to the different void volumes of individual HPLC systems the following gradient profile may have to be modified.

Group 1: Binary

Table 1: Gradient profile group 1

Time	Mobile Phase A	Mobile Phase B	Flow rate
0 min	100 %	0 %	1.0 ml/min
8.00 min	100 %	0 %	1.0 ml/min
8.01 min	0 %	100 %	1.0 ml/min
10.00 min	0 %	100 %	1.0 ml/min
10.01 min	100 %	0 %	1.0 ml/min
13 min	100 %	0 %	1.0 ml/min

Group 2: Isocratic, Mobile Phase B, flow rate 1.0 ml/min

3.2 HPLC column

The HPLC column for the determination of ampicillin, cefepime, ceftazidime, linezolid, meropenem and piperacillin is supplied equilibrated and tested, and is ready for use. It can be used directly. The backpressure of a new column at a flow rate of 1.0 ml/min is about 160 (±10) bar. It may increase with column age. As long as the separations are satisfactory, a raised backpressure is of no consequence.

Note:
To lengthen the column life, we recommend the use of a precolumn (order no. 18061 and 18001) or a prefilter (order no. 15070 and 15071).

Before starting a sequence of tests, prepare the HPLC system as follows:

1. Before installing the HPLC column rinse the system with ca. 50 ml ultrapure water (HPLC grade)
2. Rinse with ca. 30 ml of mobile phase A (for group 1 assay) or 30 ml mobile phase B (for group 2 assay) at a flow rate of 1.5 ml/min
3. Inject mobile phase A (for group 1 assay) or mobile phase B (for group 2 assay) repeatedly to clean the injector
4. Install the column
5. Equilibrate the system at a flow rate of 1.0 ml/min for 15 to 20 min until the baseline has stabilised
6. Inject the prepared calibrator repeatedly, until two successive chromatograms show identical retention times and peak areas/heights.

3.3 Shut-down

For interruptions in operation of up to 3 days, pump the mobile phase at a low flow rate (0.1 ml/min). The HPLC column remains connected in the system. To protect the lamp, turn off the detector. If the system will be out of use for more than three days, remove the HPLC column from the system. The column does not require any rinsing or preservation measures. Store the column in mobile phase at +20 to +25 °C. Insert a union to replace the column and rinse the HPLC system using about 50 ml of ultrapure water (HPLC grade)/methanol solution (90/10).

4 Chromatographic separation

The following table shows the approximate retention times of the analytes at a flow rate of 1.0 ml/min.

Table 2: Retention times group 1

Substance	Retention time (ca.)
Cefepime	4.0 min
Ceftazidime	5.0 min
Meropenem	5.6 min
Internal Standard	6.6 min
Ampicillin	8.7 min

Table 3: Retention times group 2

Substance	Retention time (ca.)
Internal Standard	3.0 min
Piperacillin	3.4 min
Linezolid	3.8 min

For a flow rate of 1.0 ml/min, the duration of chromatographic separation is about 13 minutes for group 1 and about 5 minutes for group 2. Retention times may vary slightly, for instance if there is a change in ambient temperature, if you use a new batch of mobile phase, or if you replace the HPLC column. Therefore, use a calibration chromatogram to determine current values.

5 Sample preparation

Caution:
When using the reagents comply with the hazard information in Appendix I.

5.1 Collection and storage of patient specimens

Use serum/plasma for analysis. The latter can be EDTA, lithium heparin, or sodium heparin plasma. Under the conditions indicated, the analytes in the sample are stable as follows:

Table 4: Storage and storage life of patient samples (heparin plasma and serum)*

Antibiotic	Storage temperature	Storage life in heparin plasma	Storage life in serum
Ampicillin	ambient temperature	6 hours	3 hours
	+2 to + 8 °C	48 hours	24 hours
	below –18 °C	7 days	7 days
	below –70 °C	2 months	2 months
Cefepime	ambient temperature	3 hours	1 hour
	+2 to + 8 °C	24 hours	24 hours
	below –18 °C	7 days	7 days
	below –70 °C	2 months	2 months
Ceftazidime	ambient temperature	3 hours	3 hours
	+2 to +8 °C	24 hours	24 hours
	below –18 °C	16 days	16 days
	below –70 °C	2 months	1 month
Linezolid	ambient temperature	48 hours	48 hours
	+2 to +8 °C	3 days	3 days
	below –18 °C	2 months	2 months
	below –70 °C	2 months	2 months

Antibiotic	Storage temperature	Storage life in heparin plasma	Storage life in serum
Meropenem	ambient temperature	1 hour	1 hour
	+2 to + 8 °C	6 hours	3 hours
	below –18 °C	2 days	4 days
	below –70 °C	1 month	1 month
Piperacillin	ambient temperature	3 hours	3 hours
	+2 to +8 °C	24 hours	6 hours
	below –18 °C	7 days	4 days
	below –70 °C	2 months	2 months

*Data based on internal investigations.

The addition of Priming Solution (order no. 61012) to the sample improves the storage life as follows:

Table 5: Storage life of patient samples after addition of Priming Solution (plasma and serum)

Antibiotic	Storage temperature +20 to +25 °C	Storage temperature +4 °C
Ampicillin	24 hours	3 days
Cefepime	24 hours	3 days
Ceftazidime	12 hours	3 days
Linezolid	48 hours	3 days
Meropenem	6 hours	24 hours
Piperacillin	24 hours	3 days

Note:
It is the responsibility of the individual laboratory to use all available references and/or its own studies to determine specific stability criteria for its laboratory.

5.2 Reconstitution of the calibrator

Prior to sample preparation, reconstitute the plasma calibrator (order no. 61028 or 61003) as follows:

1. Pipette 1.0 ml distilled water into the original vial
2. Reconstitute for 10 to 15 min at +20 to +25 °C, swirl repeatedly

Check that the vial contents are homogeneous. If undissolved substances are still visible, extend the reconstitution time.

Attention:
Do not add Priming Solution to calibrators. These already contain stabilizing additives.

The calibrator levels are traceable to initial weights of pure substances. The analyte concentrations in the calibrator are batch-dependent. Individual levels are given in the calibrator leaflet.

Caution:
This product is manufactured from pooled human plasma which has been tested by the manufacturer and found negative for infections by the human immunodeficiency virus (HIV), the hepatitis B virus (HBV), the hepatitis C virus (HCV) and the bacterium *Treponema pallidum*. Nevertheless, a potential risk of infection cannot be entirely excluded. Consider all products containing human source material as potentially infectious and exercise the same care in the handling of this product as in the handling of potentially infectious patient samples.

Storage life of the calibrators after reconstitution:
The calibrators dissolved in water have the following storage lives:

Table 6: Stability of the calibrators after reconstitution

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	8 hours	Light protection, tightly closed
+2 to +8 °C	3 weeks meropenem: 3 days	Light protection, tightly closed
below –18 °C	3 months meropenem: 7 days	Light protection, tightly closed
below –70 °C	3 months	Light protection, tightly closed
Freeze-thaw cycles	3 cycles	—

5.3 Reconstitution of the controls

Prior to sample preparation, reconstitute the plasma controls (order no. 0183, 0184) as follows:

1. Pipette 1.0 ml distilled water into the original vial
2. Reconstitute for 10 to 15 min at +20 to +25 °C, swirl repeatedly

Check that the vial contents are homogeneous. If undissolved substances are still visible, extend the reconstitution time.

Attention:
Do not add Priming Solution to controls. These already contain stabilizing additives.

The analyte concentrations in the controls are batch-dependent. Individual levels are given in the leaflet accompanying each control.

Caution:
This product is manufactured from pooled human plasma which has been tested by the manufacturer and found negative for infections by the human immunodeficiency virus (HIV), the hepatitis B virus (HBV), the hepatitis C virus (HCV) and the bacterium *Treponema pallidum*. Nevertheless, a potential risk of infection cannot be entirely excluded. Consider all products containing human source material as potentially infectious and exercise the same care in the handling of this product as in the handling of potentially infectious patient samples.

Storage life of the controls after reconstitution:
Controls dissolved in water have the following storage lives:

Table 7: Stability of the controls after reconstitution

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	8 hours	Light protection, tightly closed
+2 to +8 °C	3 weeks meropenem: 3 days	Light protection, tightly closed
below –18 °C	3 months meropenem: 7 days	Light protection, tightly closed
below –70 °C	3 months	Light protection, tightly closed
Freeze-thaw cycles	3 cycles	—

5.4 Sample preparation

To prepare patient samples, controls and calibrators for analysis, work through the following steps in the order given:

Note:
Adding priming solution prolongs the storage life of the analytes. More details can be found in chapter 5.1 (table 5).

Calibrator/control

1. Pipette 100 µl **calibrator/control** into a 1.5 ml reaction vial

Attention:
Do not add Priming Solution to calibrators and controls. These already contain stabilizing additives.

⇩

Sample

1. Pipette 100 µl **sample** into a 1.5 ml reaction vial
   Add 20 µl Priming Solution (order no. 61012) and vortex for 5 s

⇩

2. Add 200 µl Internal Standard Mix (order no. 61004) and vortex for 1 min
3. Centrifuge 5 min at 15000 x g
4. Transfer 100 µl of the supernatant into an autosampler vial with insert
5. Add 100 µl Dilution Buffer (order no. 61005) and vortex for 5 s
6. Inject 5 µl (group 1) or 10 µl (group 2) of the prepared sample into the HPLC system

5.5 Storage life of prepared samples

Samples prepared for analysis as indicated in section 5.4 have the following storage life:

Table 8: Storage life of the prepared samples

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	10 hours	Light protection, tightly closed, glass vials
+2 to +8 °C	2 days	Light protection, tightly closed, glass vials
below –18 °C	4 days	Light protection, tightly closed, glass vials

5.6 Handling of samples outside the calibration range

Patient samples with analyte concentrations above the calibration range are to be handled as follows:

Before sample preparation, dilute the original sample with isotonic saline solution (9.0 g/l), maximum ratio of 1:3, so that the analytical result is within the calibration range of the method regardless of the dilution factor. Subsequently prepare the sample as described in chapter 5.4.

When calculating the analyte concentrations of the diluted samples, consider the corresponding dilution factor.

6 Additionally required equipment

The HPLC determination of antibiotics in serum/plasma with automated sample preparation requires the following additional equipment not supplied with the reagent kit:

- HPLC gradient system
- Coolable autosampler
- UV detector
- Coolable column oven
- Vortex mixer
- Centrifuge for 1.5 ml reaction vials (minimum 15000 x g)

7 Data acquisition and evaluation

7.1 Calibration of the data analysis system

To calibrate your analysis system and verify the separation performance of the HPLC system, conduct a number of trial runs before analysing patient samples. Use the plasma calibrator (order no. 61028 or

61003) for this. The concentrations of the analytes in the calibrator are batch-dependent. The exact levels are given in the accompanying leaflet.

Repeatedly inject an aliquot of the prepared calibrator until two successive chromatograms are approximately identical in terms of retention times, peak resolution and peak areas and heights. Use the chromatogram of the last test injection for calibration of the analysis system (PC software, integrator).

Calibration curves are constructed by calculating the analyte to internal standard (ISTD) peak area/height ratio on the y axis against the concentration on the x axis. Then construct a calibration curve for all the analytes. If using 3+1 calibrator (order no. 61028), use linear regression and 1/x weighting.

Note
We recommend evaluation via peak heights having advantages with regard to precision and accuracy of the analysis results especially with low concentrations and in the presence of interferences.

To verify that HPLC conditions (retention times, calibration parameters) have remained constant throughout the analysis, inject the prepared calibrator again during and at the end of a sample series. Select the internal standard method for calibration in your analysis system.

7.2 Quantitative evaluation with internal standard

The use of an internal standard allows potential losses during sample preparation to be compensated for. A known amount of the internal standard is added to every specimen as well as to the calibrator and the controls. In the software, the peak of the internal standard from the calibration run (see chromatogram in chap. 13) is assigned accordingly. **Since the same amount of internal standard is added to the calibrator and to the samples, the concentration of the internal standard can be entered as "1".**

In chromatograms of group 1, an offset of the baseline may occur in isolated samples immediately after wavelength switch as shown in the following chromatogram. In this case, it might be beneficial to draw the baseline horizontally avoiding integration-related errors.

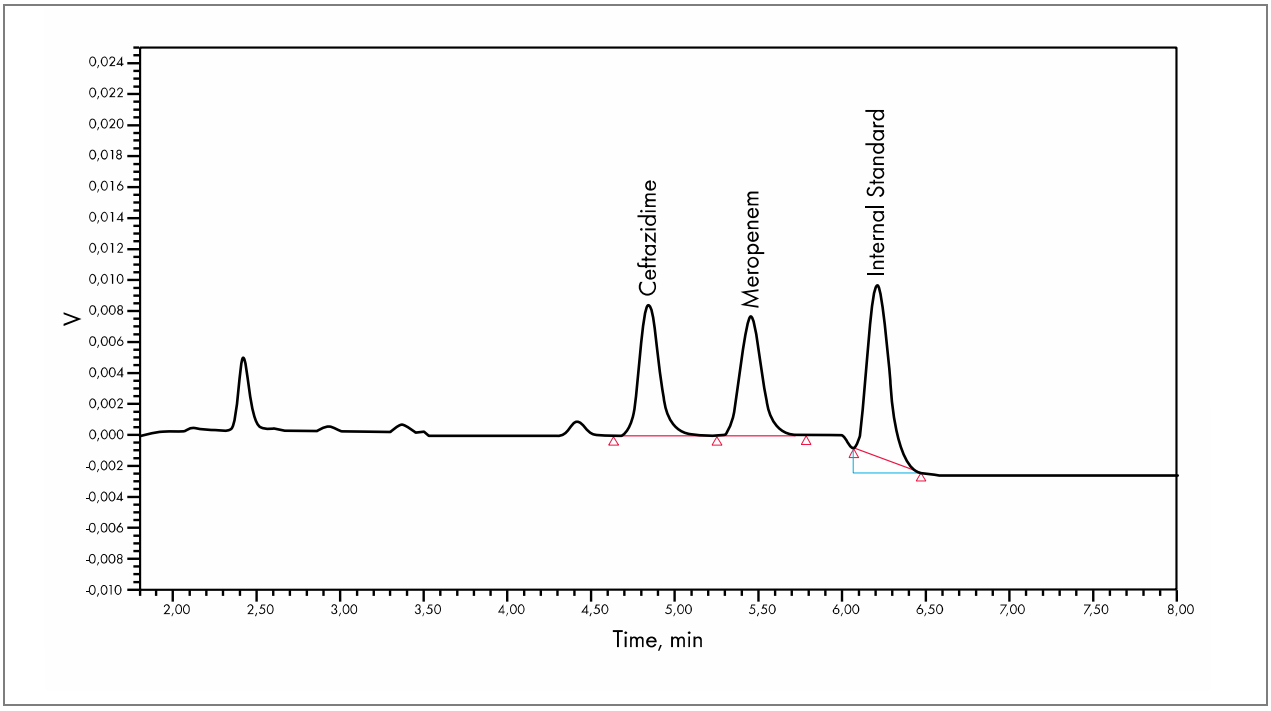

Figure 1: Chromatogram with two variants of peak integration of the internal standard of group 1

8 Quality control

Monitor precision and accuracy of the analyses by including additional controls (order no. 0183, 0184) in each analytical run. If the analysis of these controls yields values outside the range given on the accompanying information leaflet, check the system. If the discrepancy continues to exist, re-calibrate the system.

9 Therapeutic ranges

The S2k Guideline of the Paul-Ehrlich-Gesellschaft für Chemotherapie e.V. (Paul Ehrlich Chemotherapy Association) - "Kalkulierte parenterale Initialtherapie bakterieller Erkrankungen bei Erwachsenen – Update 2018" ("Calculated initial parenteral treatment of bacterial diseases in adults - update 2018") defines the objective of therapeutic drug monitoring in antibiotic treatment as follows:

„Viele Antibiotika sind durch erhebliche inter- und intraindividuelle Unterschiede der pharmakokinetischen Eigenschaften, vor allem im Eliminationsverhalten und Verteilungsvolumen, gekennzeichnet. Dies trifft im besonderen Maße auf Intensivpatienten mit schwerer Sepsis, septischem Schock und konsekutivem Multiorganversagen und starken Veränderungen in den Verteilungsräumen (z. B. kapilläres Leck und durch Infusionsbehandlungen) zu [...]. Dadurch können die resultierenden Plasmakonzentrationen nach Standarddosen in weiten Bereichen streuen [...], wodurch einerseits die Gefahr der Unterdosierung mit unzureichender therapeutischer Wirkung, andererseits überhöhte Plasmaspiegel mit dem Risiko unerwünschter toxischer Wirkungen drohen. Ziel des therapeutischen Drug-Monitorings (TDM) ist es, unter Berücksichtigung pharmakokinetischer Prinzipien und Messungen der Arzneimittelkonzentration im Patientenblut die individuell optimale Dosierung für den Patienten zu finden" [9: 66–67].

(Translation: "Many antibiotics are characterised by significant inter- and intraindividual differences in their pharmacokinetic properties, in particular in their elimination behaviour and volume of distribution. This applies in particular with regard to critical care patients with severe sepsis, septic shock and consecutive multiple organ failure and major changes in distribution spaces (e.g., capillary leakage and due to infusion treatments) [...]. Hence, the resulting plasma concentrations after standard doses are subject to broad fluctuation [...], with the associated risks on the one hand of underdosing and an inadequate treatment response, and on the other hand of excessive plasma levels with the risk of adverse toxicity. The objective of therapeutic drug monitoring (TDM) is to find the best dose for the individual patient, based on pharmacokinetic principles and monitoring of drug levels in the patient's blood" [9: 66–67].)

9.1 Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) is the lowest active drug concentration of an antimicrobial agent (e.g. an antibiotic) sufficient to prevent pathogen proliferation. The MIC levels in the table below are published by EUCAST ("The European Committee on Antimicrobial Susceptibility Testing") [3]. For continuous or prolonged infusions of beta-lactam antibiotics, bactericidal activity initially increases with rising concentrations of the antibiotic to levels of up to 4 to 5 times the MIC. Higher active substance levels cannot improve the response to treatment, however [9].

The stated minimum inhibitory concentrations are rough guides based on the literature [3]. They may differ from other published data. Please be sure to address national regulations in addition (e.g. CLSI, M100).

Table 9: Minimum inhibitory concentrations (MICs) (S - sensitive strains, R – resistant strains)

Pathogen	Substance	MIC [3]	
		S ≤	R >
Enterobacteriaceae	Ampicillin	8 mg/l	8 mg/l
	Ampicillin/sulbactam	8 mg/l	8 mg/l
	Piperacillin	8 mg/l	16 mg/l
	Piperacillin/tazobactam	8 mg/l	16 mg/l
	Cefepime	1 mg/l	4 mg/l
	Ceftazidime	1 mg/l	4 mg/l
	Ceftazidime/avibactam	8 mg/l	8 mg/l
	Meropenem	2 mg/l	8 mg/l
Pseudomonas	Piperacillin	16 mg/l	16 mg/l
	Piperacillin/Tazobactam	16 mg/l	16 mg/l
	Cefepime	8 mg/l	8 mg/l
	Ceftazidime	8 mg/l	8 mg/l
	Ceftazidime/avibactam	8 mg/l	8 mg/l
	Meropenem	2 mg/l	8 mg/l
Acinetobacter	Meropenem	2 mg/l	8 mg/l
Staphylococcus	Linezolid	4 mg/l	4 mg/l
Enterococcus	Ampicillin	4 mg/l	8 mg/l
	Ampicillin/sulbactam	4 mg/l	8 mg/l
	Linezolid	4 mg/l	4 mg/l
Streptococcus A,B,C,G	Linezolid	2 mg/l	4 mg/l
Streptococcus pneumoniae	Ampicillin	0.5 mg/l	2 mg/l
	Cefepime	1 mg/l	2 mg/l
	Meropenem (no meningitis)	2 mg/l	2 mg/l
	Meropenem (meningitis)	0.25 mg/l	1 mg/l
	Linezolid	2 mg/l	4 mg/l
Viridans group streptococci	Ampicillin	0.5 mg/l	2 mg/l
	Cefepime	0.5 mg/l	0.5 mg/l
	Meropenem	2 mg/l	2 mg/l
Haemophilus influenzae	Ampicillin	1 mg/l	1 mg/l
	Ampicillin/sulbactam	1 mg/l	1 mg/l
	Cefepime	0.25 mg/l	0.25 mg/l
	Meropenem (no meningitis)	2 mg/l	2 mg/l
	Meropenem (meningitis)	0.25 mg/l	1 mg/l
Moraxella catarrhalis	Ampicillin/sulbactam	1 mg/l	1 mg/l
	Cefepime	4 mg/l	4 mg/l
	Meropenem	2 mg/l	2 mg/l
Neisseria meningitidis	Ampicillin	0.125 mg/l	1 mg/l

Pathogen	Substance	MIC [3]	
	Meropenem (meningitis)	0.25 mg/l	0.25 mg/l
Anaerobes, Gram-positive	Ampicillin	4 mg/l	8 mg/l
	Ampicillin/sulbactam	4 mg/l	8 mg/l
	Piperacillin	8 mg/l	16 mg/l
	Piperacillin/tazobactam	8 mg/l	16 mg/l
	Meropenem	2 mg/l	8 mg/l
Anaerobes, Gram-negative	Ampicillin	0.5 mg/l	2 mg/l
	Ampicillin/sulbactam	4 mg/l	8 mg/l
	Piperacillin	16 mg/l	16 mg/l
	Piperacillin/tazobactam	8 mg/l	16 mg/l
	Meropenem	2 mg/l	8 mg/l
Listeria monocytogenes	Ampicillin	1 mg/l	1 mg/l
	Meropenem	0.25 mg/l	0.25 mg/l
Pasteurella multocida	Ampicillin	1 mg/l	1 mg/l
Corynebacterium	Linezolid	2 mg/l	2 mg/l
Aerococcus sanguinicola and urinae	Ampicillin	0.25 mg/l	0.25 mg/l
	Meropenem	0.25 mg/l	0.25 mg/l
Kingella kingae	Ampicillin	0.06 mg/l	0.06 mg/l
	Meropenem	0.03 mg/l	0.03 mg/l
Aeromonas	Cefepime	1 mg/l	4 mg/l
	Ceftazidime	1 mg/l	4 mg/l
PK-PD (non-species related) breakpoints	Ampicillin	2 mg/l	8 mg/l
	Ampicillin/sulbactam	2 mg/l	8 mg/l
	Piperacillin	4 mg/l	16 mg/l
	Piperacillin/tazobactam	4 mg/l	16 mg/l
	Cefepime	4 mg/l	8 mg/l
	Ceftazidime	4 mg/l	8 mg/l
	Ceftazidime/avibactam	8 mg/l	8 mg/l
	Meropenem	2 mg/l	8 mg/l
	Linezolid	2 mg/l	4 mg/l

9.2 Maximum plasma concentration (Cmax)

The term Cmax is a pharmacokinetics term designating the peak plasma concentration achieved after administration of a drug. MIC hence designates the lower limit and Cmax the upper limit of an antibiotic's plasma concentration. The table below lists the highest possible Cmax levels usually reached after intravenous administration of the highest dose. For further details, please consult the summaries of product characteristics of the drug manufacturers.

Table 10: Cmax

Substance	Cmax
Ampicillin (in combination with sulbactam)	95 mg/l
Cefepime	193 mg/l
Ceftazidime	170 mg/l
Linezolid	21.2 mg/l
Meropenem	115 mg/l
Piperacillin (in combination with tazobactam)	298 mg/l

10 Conversion factors

The following table lists conversion factors between mass and molar concentrations and conversely.

Table 11: Conversion factors

Substance	mg/l to µmol/l	µmol/l to mg/l
Ampicillin	x 2.86	x 0.349
Cefepime	x 2.08	x 0.481
Ceftazidime	x 1.83	x 0.547
Linezolid	x 2.96	x 0.337
Meropenem	x 2.61	x 0.383
Piperacillin	x 1.93	x 0.518

11 Storage and lifetime of the reagents

Unopened, and provided that transport and storage conditions are met, the reagents are stable until the expiry date stated on the label. Transport and store the reagents under the following conditions:

Table 12: Transport conditions for the reagent kit

Product	Transport temperature
Reagent kit (order no. 61000)	+18 to +30 °C

Immediately unpack reagents after transport and store individually as stated below:

Table 13: Storage conditions for the reagents

Product	Storage temperature
Mobile Phase A (order no. 61001)	+18 to +30 °C
Mobile Phase B (order no. 61002)	+18 to +30 °C
Internal Standard Mix (order no. 61004)	below –18 °C
Dilution Buffer (order no. 61005)	+18 to +30 °C
Priming Solution (order no. 61012)	+18 to +30 °C
Plasma calibrators (order no. 61003, 61028)	below –18 °C
Plasma controls (order no. 0183, 0184)	below –18 °C

Close the reagents immediately after use and store them at the specified temperature. The in-use shelf-life is one year but does not extend beyond the stated expiry date. Details of the stability of the reconstituted calibrators and controls are given in sections 5.2 and 5.3.

12 Waste disposal

Mobile Phase A, Mobile Phase B and Internal Standard Mix contain organic solvents. Dispose of product residues into a collection container for organic halogen-free solvents.

The Priming Solution contains a strong acid. Neutralise product residues and dispose into a collection container for salt solutions.

Residues of patient samples and prepared samples as well as controls and calibrators must be collected and disposed of as potentially infectious waste.

The mentioned solutions must not be disposed of together with domestic waste. Do not circulate into the main water supply. Dispose of in compliance with Directive 2008/98/EC on Waste and national and local requirements. The waste containers must be stored appropriately and only access permitted to authorised parties.

13 Examples of chromatograms

The following graphs provide several examples of chromatograms created using this method.

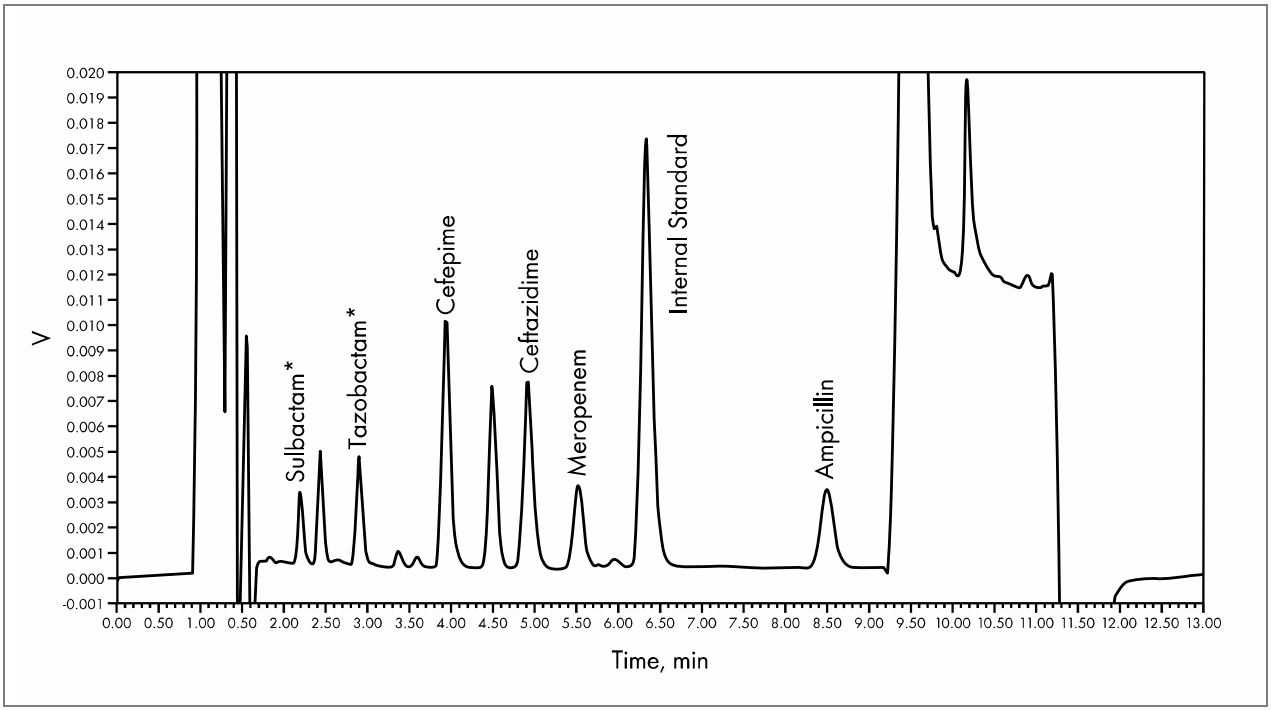

Figure 2: Chromatogram of a calibrator (order no. 61028/2), group 1
Concentration of the analytes: cefepime: 40 mg/l, ceftazidime: 33 mg/l, meropenem: 27 mg/l, ampicillin: 18 mg/l
*information on the beta-lactamase inhibitors tazobactam and sulbactam is qualitative only (measurement wavelength to be used: 210 nm). CE/IVD compliance does not apply.

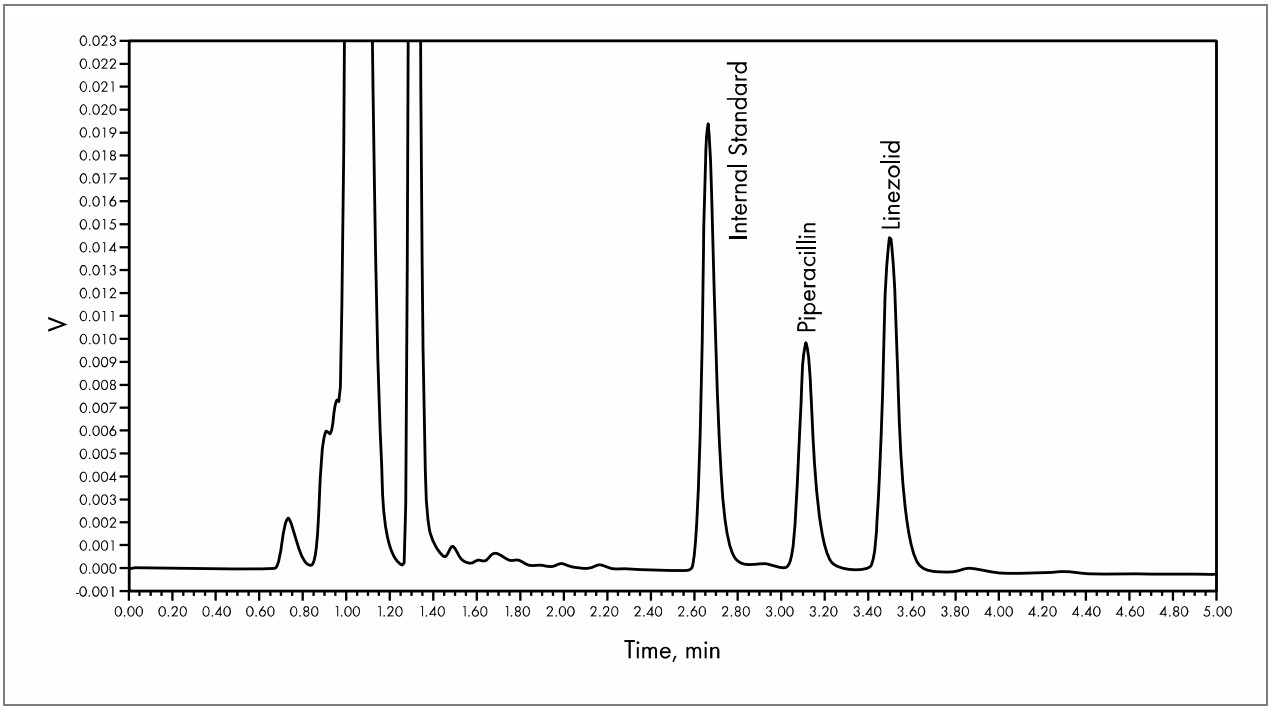

Figure 3: Chromatogram of a calibrator (order no. 61028/2), group 2
Concentration of the analytes: piperacillin: 46 mg/l, linezolid: 15 mg/l

14 Interference testing

The following drugs/metabolites were tested for any interferences:

Acetazolamide, acetylcysteine, acetylsalicylic acid, aciclovir, allopurinol, amikacin, 6-aminopenicillanic acid, amlodipine, amoxicillin, ampicilloic acid, ampicillin, azathioprine, azithromycin, bisoprolol, captopril, carbamazepine, carbamazepine-10,11-epoxide, 5-carboxy-4-[[5-[(dimethylamino)carbonyl]-3-pyrrolidinyl]thio]-3,4-dihydro-α-(1-hydroxyethyl)-3-methyl-2H-pyrrole-2-acetic acid),cephradine, chloramphenicol, chlordiazepoxide, cimetidine, ciprofloxacin, clarithromycin, dexamethasone, diazepam, diclofenac, digitoxin, digoxin, dihydrocodeine, disopyramide, dobutamine, enalaprilat, erythromycin, fluconazole, 5-flucytosine, furosemide, ganciclovir, gentamicin, hydrochlorothiazide, hydroxyethylglycin-metabolite (PNU 142586), hydroxyitraconazole, hydroxymidazolam, ibuprofen, isosorbide dinitrate, itraconazole, ketoconazole, levofloxacin, levothyroxine, lidocaine, lorazepam, metformin, methicillin, methylprednisolone, metoclopramide, metoprolol, midazolam, mycophenolic acid, mycophenolic acid glucuronide, N- acetylprocainamide, nadolol, sodium fluoride, N-desmethyldiazepam, neomycin, neostigmine, nifedipine, norverapamil, omeprazole, oxazepam, oxypurinol, pantoprazole, paracetamol, penicillin G, penicillin V, phenytoin, posaconazole, prazosin, prednisolone, prednisone, procainamide, propranolol, ranitidine, rifampicin, risperidone, salbutamol, simvastatin, streptomycin, sufentanil, sulfamethoxazole, torasemide, tramadol, triamterene, trimethoprim, valproic acid, vancomycin, verapamil, voriconazole

Salicylic acid, a metabolite of acetylsalicylic acid, elutes at the same time as the internal standard for Group 1. Approx. 10% of the ISTD area was recovered 9.5 hours after administration of 250 mg aspirin. Falsely low antibiotic results are possible.

All other substances examined have a negligible influence on the quantitative results (deviation ≤ 15 %).

If you have any questions concerning interferences, contact your local Chromsystems representative, call our hotline in Munich (phone: +49 89 18930-300) or send an e-mail to our HPLC support: HPLC-Support@chromsystems.com.

15 Clinical limitations

There are no universally valid therapeutic ranges for ampicillin, cefepime, ceftazidime, linezolid, meropenem and piperacillin in serum or plasma. Results obtained using different test methods cannot be compared. Laboratories should indicate the method used for assay in order to enable correct interpretation of the results.

Each treatment should be evaluated with care with regard to the results obtained. Before modifying treatment, the patient's clinical findings should be reviewed with care. Each user should determine their own therapeutic ranges based on clinical evaluation. Conversion factors between different assay methods should not be used to predict levels for individual patients.

The complexity of clinical status, individual differences in response to antibiotics and the side effects of ampicillin, cefepime, ceftazidime, linezolid, meropenem and piperacillin, and comedication with other drugs, dosage, method and duration of treatment, and a number of other factors may lead to different stipulations for optimum blood levels of antibiotics.

16 Troubleshooting

Table 14: Troubleshooting

Problem	Possible cause	Remedy
Baseline drifts	Detector lamp still cold	Wait
	Detector lamp too old	Replace lamp
	System not yet equilibrated	Inject calibrator repeatedly, until two successive chromatograms are identical
	Temperature drift	Use column oven
	Flow rate not constant	Check pump
Baseline unstable	HPLC pump	Check pump (air, leaks)
	Air in the system	Degas mobile phase
	Detector cell contaminated	Clean detector cell
Interference peaks	Air in the system	Degas mobile phase
	Injector contaminated	Clean injector
	Autosampler vials contaminated	Use new vials or clean vials
	HPLC column contaminated	Replace column
Broad peaks, tailing	HPLC column too old	Replace column
Double peaks	Dead volume in fittings	Renew fittings
	Dead volume in HPLC column	Replace column
No peaks	Injector leaks	Check injector
Reduced sensitivity	Detector lamp ageing	Replace lamp
	Detector cell contaminated	Clean cell
	Defective injection valve	Check injector
Retention times change	Temperature drift	Use column oven
	Irregular flow rate	Check HPLC pump, adjust flow rate
	System not yet equilibrated	Pump mobile phase for about 15 min through the system; inject calibrator repeatedly
No signal	Connection to integrator/recorder defective or interrupted	Check signal cable and connection
	Detector lamp	Check supply voltage, replace lamp if necessary

17 Literature

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Appendix I: Hazardous substance information

When using the reagents, note the following hazard information and take the relevant safety measures. More information can be gathered from our safety data sheets. These can be downloaded from our website www.chromsystems.com or are available upon request.

Table 15: Hazard and precautionary statements

Pictograms	Hazard and precautionary statements
Mobile Phase A (order no. 61001)	
	**Warning** H226 Flammable liquid and vapour. P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P241 Use explosion-proof electrical/ventilating/lighting equipment. P243 Take action to prevent static discharges. P280 Wear protective gloves/protective clothing/eye protection/face protection.
Mobile Phase B (order no. 61002)	
 	**Danger** H225 Highly flammable liquid and vapour. H319 Causes serious eye irritation. P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P241 Use explosion-proof electrical/ventilating/lighting equipment. P243 Take action to prevent static discharges. P280 Wear protective gloves/protective clothing/eye protection/face protection.
Internal Standard Mix (order no. 61004)	
  	**Danger** H225 Highly flammable liquid and vapour. H301+H311+H331 Toxic if swallowed, in contact with skin or if inhaled. H319 Causes serious eye irritation. H370 Causes damage to the central nervous system and the visual organs. P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P280 Wear protective gloves/protective clothing/eye protection/face protection. P301+P310 IF SWALLOWED: Immediately call a POISON CENTER/ doctor. P302+P352 IF ON SKIN: Wash with plenty of water. P403+P233 Store in a well-ventilated place. Keep container tightly closed.

Pictograms	Hazard and precautionary statements
Priming Solution (Best.-Nr. 61012)	
	**Danger** H290 May be corrosive to metals. H314 Causes severe skin burns and eye damage. P280 Wear protective gloves/protective clothing/eye protection/face protection. P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P310 Immediately call a POISON CENTER/doctor.
These components are not classified as dangerous according to European Union legislation: Dilution Buffer (order no. 61005) **3PLUS1**® Multilevel Plasma Calibrator Set (order no. 61028) Plasma Calibration Standard (order no. 61003) Plasma Controls (order no. 0183, 0184)	

Appendix II: Manual calculation

Calculation using the single point calibrator (order no. 61003)

For the calculation the following data are required:

- Peak area/height of the analyte in the chromatogram of the sample =A_{Sample}
- Peak area/height of the analyte in the chromatogram of the calibrator =$A_{Calibrator}$
- Peak area/height of the internal standard in the chromatogram of the sample =IS_{Sample}
- Peak area/height of the internal standard in the chromatogram of the calibrator =$IS_{Calibrator}$
- The concentration C of the analyte in the calibrator =$C_{Calibrator}$

Calculate the concentration of the analyte in the sample (C_{Sample}) as follows:

$$C_{Sample} = \frac{A_{Sample} \times IS_{Calibrator}}{A_{Calibrator} \times IS_{Sample}} \times C_{Calibrator}$$

Calculation using the 3PLUS1® Multilevel Plasma Calibrator (order no. 61028)

The quotients of the peak areas/heights of the analytes divided by the peak areas/heights of the internal standard plotted against the analyte concentrations produce a calibration curve by linear regression, 1/x weighted. The resulting equation is applied for each substance.

For manual calculation the following data are required.

- Peak area/height of the analyte A in the chromatogram of the sample =A_{Sample}
- Peak area/height of the internal standard in the chromatogram of the sample =IS_{Sample}
- Slope of the calibration curve =a
- Y-intercept of the calibration curve =b

Calculate the concentration of the analyte A in the sample (C_{Sample}) as follows:

$$C_{Sample} = \frac{(A_{Sample} / IS_{Sample}) - b}{a}$$

Note
We recommend evaluation via peak heights having advantages with regard to precision and accuracy of the analysis results especially with low concentrations and in the presence of interferences.

Appendix III: Performance data

The performance features were determined and verified on the following equipment:
– Agilent Technologies 1290 UHPLC system
– Shimadzu Nexera UHPLC system
– Waters Alliance 2690/2695 HPLC system

Recovery:
Analytical recovery was determined from calibration curve slopes after repeated spiking of plasma/serum and diluted standard solutions:

Table 16: Recovery rates

Substance	Recovery rate [serum]	Recovery rate [plasma]
Ampicillin	98 %	98 %
Cefepime	103 %	98 %
Ceftazidime	97 %	93 %
Linezolid	102 %	97 %
Meropenem	91 %	91 %
Piperacillin	102 %	104 %

Linearity and limit of quantitation (LLOQ):
Linearity was determined by spiking plasma and serum with defined quantities of standard substances. The lower limit of quantitation (LLOQ) was determined using defined dilutions of plasma and serum with analyte-free matrix.

The method is linear from the lower limit of quantitation (LLOQ) to the stated upper limit of quantitation (linear range).

Table 17: Limit of quantitation and linearity

Substance	LLOQ	Linear range up to at least
Ampicillin	1.0 mg/l	120 mg/l
Cefepime	2.2 mg/l	400 mg/l
Ceftazidime	1.8 mg/l	300 mg/l
Linezolid	0.5 mg/l	60 mg/l
Meropenem	1.1 mg/l	200 mg/l
Piperacillin	1.0 mg/l	400 mg/l

Intra-assay precision:
The coefficients of variation were determined on three different concentrations by repeated preparation (n = 10) of the same matrix sample in one sequence:

Table 18: Intra-assay precision

Substance	Coefficient of variation (concentration of analyte)		
Ampicillin	1.1 % (8.87mg/l)	0.8 % (13.8 mg/l)	0.6 % (26.4 mg/l)
Cefepime	1.3 % (15.8 mg/l)	0.5 % (37.1 mg/l)	0.6 % (79.1 mg/l)
Ceftazidime	1.0 % (16.7 mg/l)	0.5 % (29.7 mg/l)	0.8 % (61.1 mg/l)
Linezolid	1.1 % (7.87 mg/l)	0.7 % (10.5 mg/l)	0.7 % (22.0 mg/l)
Meropenem	1.1 % (13.1 mg/l)	0.5 % (19.4 mg/l)	0.8 % (43.7 mg/l)
Piperacillin	1.6 % (18.1 mg/l)	0.6 % (43.4 mg/l)	1.0 % (91.4 mg/l)

Inter-assay precision:
Determination of the inter-assay precision was done on three different concentrations by repeated preparation (n = 5) of the same matrix sample on 20 different days

Table 19: Inter-assay precision

Substance	Coefficient of variation (concentration of analyte)		
Ampicillin	3.1 % (8.87mg/l)	2.7 % (20.2 mg/l)	2.8 % (26.4 mg/l)
Cefepime	3.2 % (15.8 mg/l)	2.9 % (54.6 mg/l)	3.2 % (79.1 mg/l)
Ceftazidime	3.3 % (16.7 mg/l)	3.2 % (43.2 mg/l)	3.6 % (61.1 mg/l)
Linezolid	2.9 % (7.87 mg/l)	3.0 % (13.3 mg/l)	3.1 % (22.0 mg/l)
Meropenem	5.4 % (13.1 mg/l)	5.3 % (23.7 mg/l)	5.5 % (43.7 mg/l)
Piperacillin	2.8 % (18.1 mg/l)	2.5 % (65.1 mg/l)	2.7 % (91.4 mg/l)

These data have been established in our laboratory solely in order to verify the performance of the reagent kit and to fulfil regulatory requirements. We particularly emphasise that these data are not suitable to compare the measurement systems used, nor to make any statement concerning their general performance.

Drift
To identify any drift in analyte concentration over time, the concentration of all analytes in the three controls was compared over a period of 20 days. No drift was observed for any analyte.

Carry-over
The calibrator with the highest analyte concentration was measured followed immediately by measurement of the blank calibrator, and the peak areas in the blank were compared percentage-wise with the area of the calibrator. Review of the data thus generated showed no appreciable carry-over effects. The measured concentration of the blank calibrator was below the limit of quantitation in all of the assays.

Robustness:
The effect of certain modifications in sample preparation and HPLC system setup were reviewed during verification. The method is robust within the following tolerances provided the particular setup remains constant throughout a measurement series:

Table 20: Tolerance ranges HPLC system

HPLC system	Tolerance range
Injection volume	
Group 1	2.5–10 µl
Group 2	5–20 µl
Column temperature	22 ± 2 °C

Appendix IV: Symbols

We use EN ISO 15223-1 symbols on our labels, specifications and packaging. The meanings of each symbol are given in the table below:

Table 21: Symbols

Symbol	Meaning
	Manufacturer
	Use by
	Order number
	Batch/lot code
	See instructions for use
	Upper temperature limit: Store below a certain temperature
	Temperature limit: Store within a certain temperature range
	*In-vitro* diagnostic device