Measurement of antibiotics using LC-MS/MS

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LC-MS(/MS) in quantitative determination of antibacterial drugs – A Review

Introduction

The aim of this review is to show how far the measurement of antibiotics using LC-MS(/MS) has evolved, what technics are used and what validation standards are applied. This section shows where recent research on antibiotics has been focused and gives an overview of available methods. Since LC-MS methods are often not easily converted between laboratories or even different equipment, a broad overview over successful methods gives the reader a good idea on how to approach their analytical problem. In this review I included papers that present LC-MS(/MS) methods for measuring antibiotics used in human treatment.

For the further interest of the reader in TDM and LC-MS, three reviews are recommended: In 2010 Rentsch and Mueller published a review on LC-MS methods for TDM including anti-infective drugs [1]. They could only include a handful of methods for antibiotics back then, which shows the rapid increase of methods published and available during the last years. Decosterd et al. published a very interesting review in 2016 about the role of LC-MS in TDM [2]. They discuss the rationale behind TDM, the influence of LC-MS technologies on TDM and how to take TDM measurements and interpret them correctly. Veringa et al. [3] give a broader view on TDM and include all kinds of assays for anti-infective drugs. They review the current challenges in the bioanalysis of anti-infective drugs and give insight into the pre- and postanalytical issues surrounding TDM.

Papers for this review were identified using a literature search of PubMed from 1990 to September 2020 as well as references from within relevant papers. The search terms included: (LC-MS AND antibiotic) and (TDM AND antibiotic), as well as the search term LC-MS and specific antibiotic classes.

Methods were grouped together by the class of antibiotics they are referring to, because Antibiotics of one class are often structurally related and show similar physicochemical properties, so drugs of the same class can be expected to behave similar during HPLC and MS analysis. Grouping by class also makes sense from a clinical view, since in clinical settings often specific classes of antibiotics will come into the focus of interest. If any recent reviews on some or several of the reviewed antibiotics have been found, they are pointed out and methods included in these reviews were not duplicated here.

Interpretation of Validation Data

All provided validation data was surveyed and listed according to the rules of the FDA or EMA, as stated in their most recent "Guidance for Industry" concerning bioanalytical method validation [4] and "Guideline on bioanalytical method validation" [5]. If authors presented data according to the following list, this validation parameter was included.

- **Accuracy**: a minimum of five determinations per concentration at a minimum of three concentrations. Mean value should be within 15%, at LLOQ 20% is acceptable. Within-run and between-run accuracy should be accessed
- **Precision**: a minimum of five determinations per concentrations at a minimum of three concentrations. precision should be within 15% of the coefficient of variation, at LLOQ 20% is acceptable. Within-run and between-run precision should be accessed
- **Selectivity:** blank plasma samples from at least six sources should be analyzed to exclude interferences from the matrix
- **Sensitivity:** determination of the lowest limit of quantification (LLOQ)
- **Reproducibility**: replicate measurements and reinjection reproducibility by injecting the same sample several times
- **Stability:** At least three samples at two concentrations should be tested. Results should be within 15% of nominal value
    - Freeze-thaw: minimum of three cycles
    - Short-term: should cover conditions during laboratory handling, 12-24h at room temperature are recommended.
    - Long-term: should equal or exceed the time the samples are stored, e.g. frozen at the intended storage temperature
    - Stock solution stability: when samples are stored in a different state (solid vs. solved) or buffer condition than the reference standard, stock solution stability should be performed
    - Autosampler stability: stability of the processed samples in the autosampler
- **Dilution integrity**: measurement of samples over the upper limit of quantification by dilution
- **Matrix effect:** ion suppression or enhancement should be tested using an appropriate method
- **Recovery:** extracted, spiked samples should be tested against analyte in solvent to access the rate of recovery
- **Linearity**: blank sample (no internal standards), zero sample (only internal standard), and at least six concentrations, including the LLOQ. For the validation, runs with 4

concentrations suffice. At least three runs on different days should be analyzed. The simplest model possible should be used. At least 75% of non-zero standards should meet the criteria

  - Coefficient of variation and inaccuracy should be below 15%
  - LLOQ: Signal to noise should be bigger than 5, the coefficient of variation and inaccuracy should be below 15%
  - At least 5 samples determined
  - QCs: at least three QCs in duplicate should be incorporated. At least 67% of the QCs should be within 15% of their expected values for the run to be accepted and at least 50% of QCs on each level.

- **Dried blood spots:** storage and handling temperature, homogeneity of sample spotting, hematocrit, stability, carryover, and reproducibility should be recorded. Correlative studies with traditional sampling should be conducted during drug development

Macrolide antibiotics

The most recent review on the analysis of macrolides via LC-MS in food, biological and environmental matrices was presented by Wang et al. in 2008 [6]. Therefore, no papers on macrolides dating before 2008 were included in this review. A review on HPLC, LC-MS and LC-MS/MS methods for azithromycin was published in 2013 by Sharma et al. [7], therefore, also no azithromycin papers before 2013 were included. Both papers are strongly recommended to the reader. Macrolide methods can be found in table 1.

Most methods for the analysis of macrolides were done for bioequivalence studies in humans e.g. [8–11] or pharmakokinetik and clinical studies in humans and animals. Most papers determined macrolides in human plasma, one paper additionally determined saliva concentrations of clindamycin in humans [12] and one detected azithromycin in vaginal tissue sampled using vaginal swabs [13].

Vu et al. [14] presented a method for dried blood spots, a further development of their paper from 2009. One paper measuring non-human biological matrices was included for measuring interesting non-blood compartements. The group around Oswald et al. measured not only horse plasma, but epithelial lining fluid and bronchoalveolar cells to research pulmonal penetration of arithromycin, and rifampicin [15].

The quality of the papers on macrolides varies strongly. Some papers did not present any validation data, though they claimed to present a fully validated method, e.g. Li et al. [11]. Other methods were quite well validated, mostly with current FDA or EMA standards.

Only some methods used deuterated internal standards, including rifampicin-d8 and azithromycin-13c1,d2 [14, 16, 17], while others used structurally related macrolides, which also gave good results in repeatability and precision.

In the paper by Cai et al. a method validated for erythromycylamine was used for the determination of the prodrug dirithromycin. Their presented LOQ was not determined empirical, but rather mathematical [8]. Liu et al. also measured dirithromycin as erythromycilamine, but used only a single massspectrometer [9]. The group around Li [11] also only used a single mass spectrometer to measure Clindamycin. They also didn't present their validation data but claimed to have validated their method as advised by the current FDA guidelines. All papers except those from Li et al. and Liu et al. used tandem mass spectrometer to produce results. ESI in positive ion mode was the favored ionization method used in all groups, only two groups did not specify their polarization mode [11, 12], while in one paper describing tigecycline measurements the polarization mode was described (positive), but not the ionization method [18].

A wide range of different sample preparations was used to prepare the macrolide samples, but the lowest LOQs were reached by either liquid-liquid extraction or SPE e.g. [8, 9, 15]. For analytical separation most commonly C18 columns with different endcappings were used, but also in one case a phenyl-hexyl phase column [8] and one normal phase column was used [19].

For other papers describing the analysis of macrolides as part of a larger group of antibiotics, see "methods for several compound classes": [20] [21] [22].

Table 1 Macrolide antibiotics

Compound	Sample Material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Erythromycilamine [8]	Human plasma	Azithromycin	Solid phase extraction using Strata-X polymeric reversed phase cartridges, dilution	Xtimate™ Phenyl–Hexyl	Water with 20mM ammonium acetate adjusted to pH 3.9 with formic acid/ acetonitrile (75/25) Isocratic	LC-MS-MS ESI+ MRM	n.s.	Selectivity, accuracy, precision, recovery, matrix effect, stability (short-term, Long term, freeze-thaw), LOQ	0.0005-0.44
Erythromycylamine [9]	Human plasma	Midecamycin	Liquid-liquid extraction,	Hypersil HyPURITY C18	Water with 10mM ammonium acetate adjusted pH to 6.40 with acetic acid/ acetonitrile/ methanol (50/10/40) Isocratic	LC-MS ESI+ SIM	n.s.	Selectivity, accuracy, precision, recovery, matrix effect, stability (short-term, long term, freeze-thaw), LOQ	0.0045-0.72
Clarithromycin, rifampicin and their main metabolites [15]	Horse plasma, epithelial lining fluid, broncho-alveolar cells	Roxi-thromycin	Liquid-Liquid extraction,	XTerra® MS C18	Water with 25mM ammonium acetate buffer adjusted to pH 4/ acetonitrile (45/55) Isocratic	LC-MS-MS ESI+ MRM	3 min	Selectivity, accuracy, precision, recovery (only in plasma), matrix effects, stability (short-term, autosamplers, freeze-thaw, stock solution)	Plasma: 0.0025-0.25
Clarithromycin, rifampicin, 14-hydroxyclarithromycin, 25-O-desacetylrifampicin [19]	Human plasma	Cyano-imipramine	Protein precipitation	HyPurity Aquastar	Acetonitrile/ water/ water with 130mM ammonium acetate, acetic acid and trifluoroacetic anhydride (99.8/0.000033/0.2) Gradient	LC-MS-MS ESI+ MRM	3.6 min	Selectivity, accuracy, precision, recovery, stability (freeze-thaw, autosampler, short-term), LOQ, matrix effects	Clarithromycin and 14-hydroxyclarithromycin:0.10–10.0 rifampicin and 25-desacetylrifampicin: 0.20–5.0
Clarithromycin, rifampicin, 14-hydroxyclarithromycin, 25-O-desacetylrifampicin [23]	Dried blood spot	Cyanoimipramine Rifampicine-d8	Liquid extraction, protein precipitation	HyPurity C18	Acetonitrile/ water/ water with 130mM ammonium acetate, acetic acid and trifluoroacetic anhydride (99.8/0.000033/0.2) Gradient	LC-MS-MS ESI+ MRM	3.5 min	Selectivity, accuracy, precision, recovery, stability (short-term, long-term), LOQ, matrix effect, hematocrit, dilution integrity, carry-over	Clarithromycin: 0.05-10 Rifampicin: 0.15-30 Hydroxyclari-thromycin: 0.05-10 Desacetylrifampicin: 0.15-10

Compound	Sample Material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Roxithromycin [10]	Human plasma	Clarithromycin	Liquid-liquid extraction.	YMC ODS-A C_{18}	Acetonitrile/ water with 50 mM ammonium acetate (80/20) Isocratic	LC-MS-MS, ESI+ MRM	1.6 min	Accuracy, precision, selectivity, recovery, stability (freeze-thaw, processed-sample, long-term, short-term)	0.05-20
Clindamycin [12]	Human plasma and saliva	Lincomycin	Protein precipitation	Zorbax SB-C18 Pre-column: security guard C18	Methanol/ water + trifluoroacetic acid 0.01% (40/60) Isocratic	LC-MS-MS ESI (polarization mode n.s.) SRM	1.5 min	Selectivity, precision, accuracy, recovery, matrix effect, stability (long-term, short-term), LOQ	0.05-15
Clindamycin [11]	Human plasma	Roxithromycin	Liquid extraction	Shim-pack VP-ODS C18	Methanol/ water + 10 mM ammonium acetate (70/30) Isocratic	LC-MS ESI (polarization mode n.s.) SIM	n.s.	Selectivity, calibration curve, precision, accuracy, stability (long-term, freeze-thaw, short-term), LOQ	0.02-10
Azithromycin [16]	Human plasma	Azithromycin-13c,d2	Liquid-liquid extraction	Symmetry C18	Water +0.05mM ammonium acetate/ acetonitrile (9/1) and water + 0.05mM ammonium acetate/ methanol (1/9) Gradient	LC-MS-MS, ESI+ MRM	8 min	Selectivity, precision, accuracy, linearity, carry-over, dilution integrity, stability (autosampler, freeze-thaw, short-term, long-term), recovery, matrix effect	0.0005-0.25
Azithromycin [13]	Vaginal swabs	Leucine enkephalin	Liquid-liquid extraction	Agilent Porshell 120 SB-C18	Water/ acetonitrile / MeOH + 20mM ammonium Gradient	LC-MS-MS, ESI+ MRM	13 min	Accuracy, precision, selectivity, stability (freeze-thaw, short-term, long-term), linearity, recovery	0.01-1

n.s. = not specified, ESI+ = electron spray ionization in positive ion mode, ESI- = electron spray ionization in negative ion mode. $APCI^+$ = atmospheric pressure chemical ionization in positive ion mode, LOQ = lower limit of quantification, MRM = multiple reaction monitoring, SRM = single reaction monitoring, SIM= singe ion monitoring

Beta-lactam antibiotics

A review on beta-lactams was published in 2015 by Carlier et al. [24] A very good comparisons of the different analytical approaches with focus on chromatographic methods, but also including immunoassays and biosensors. Since not all the listed methods here are included in Carliers review, no beta-lactam methods were excluded from this review. In tables 2 to 5 all methods regarding cephalosporins, carbapenems, penicillins and general beta-lactam methods can be found.

Cephalosporins

In 2014, Jin et al [25] published a review on third generation cephalosporins and their quantification using LC-MS/MS, therefore no papers concerning cephalosporins of the third generation published before 2014 were included in this review.

Twelve papers described the use of tandem mass spectroscopy, only two [26, 27] used single ion measurement (SIM) methods. All of the here described cephalosporin methods used ESI as an ionization method, and all except the two methods measuring cefuroxime [28, 29] did so in positive ionization mode.

Most methods here were validated in human plasma, one method was additionally validated in urine [26] while cefazoline was additionally validated in plasma ultrafiltrate and cord blood by Crutchfield at al. [30] and Kan et al. [31] measured only free cefoperazone from plasma ultrafiltrate. Sutherland et al. [32] presented a HPLC–MS/MS method for the determination of ceftolozane/tazobactam in bronchoalveolar lavage fluid. This method was not as well validated as others, especially since they substituted bronchoalveolar lavage fluid with NaCl solution in the validation without checking its applicability.

Another paper described the extraction of ceftriaxone not only from human plasma but also from dried blood spots [33]. This paper from Page-Sharp et al. shows that Ceftriaxone can be reliably measured from dried blood spots and showed a good correlation between plasma and DBS concentrations. They validated their method thoroughly, including comparison of the DBS results with results from plasma of the same patients. They used the same method and instrumentation to receive these results, though the FDA recommends using different methods and instrumentation to avoid systematic errors. They also validated some important DBS specific issues, like the influence of the site of chad sampling and the effect of the hematocrit value on results. One drawback of their method is the use of cefazoline as internal standard, which allows for possible interferences. This paper is strongly recommended for readers with an interest in DBS validation.

The method presented by Sillén et al. [34] is actually two separate methods for ceftazidime and avibactam. This means two separate measurements need to be performed on every sample and does not represent modern LC-MS standards anymore.

The papers by Mendes et al. [29] and Gong et al. [26] do not show the validation data for their methods, nor do they show how the method was validated. All other methods were appropriately validated according to either FDA or EMA rules.

As internal standards, various substances were used, such as phenacetin, flucloxacillin or b-oxyphylline. Only three papers used deuterated internal standards [32, 34, 35]. Interesting to note is that for other antibiotic classes preferred internal standards have arisen, this seems not to have been the case so strongly with the cephalosporin antibiotics. Nowadays, for nearly all cephalosporin antibiotics deuterated standards are available and should be used whenever possible in the context of TDM.

Protein precipitation, in two cases followed by liquid extraction [29, 33] is the favored method of sample preparation for plasma samples. One method reached a LOQ of cefaclor as low as 0.002 mg/L by using only protein precipitation [36]. However, most of the other methods described did not reach LOQs as low as this. Most papers used tandem mass spectroscopy, but [26] and [37] (used single mass spectrometer in their methods.

Noteworthy and of special interest is the method by Wu et al. [37] for the quantification of cephalexin using a self-made molecularly imprinted polymer micro-column.

Rigo-Bonnin et al. present a method for ceftolozane and tazobactam using ceftazidime-d5 and sulbactam as internal standards [38]. Since they use the same LC-MS method as in their other multi-analyte method from 2017 [39] this method is not described here further. See “multi-analyte methods” for additional methods including cephalosporins.

Carbapanems

Only one paper used a deuterated standard [40] even though only faropenem is not available deuterated at the time this paper was written. All other papers used undeuterated drugs as internal standard.

The most common sample preparation for carbapanems is protein precipitation with organic solvents, as this was used in every method. Only Pickering et al. [41] used liquid-liquid extraction as a second sample preparation step. Only one paper did not validate their method in human matter, but in rat and monkey plasma and mouse whole blood. Since no other paper for the single measurement of imipenem could be found, this paper was still included. They measured three different drugs simultaneously, imipenem as well as cilastatin and an investigational b-lactamase inhibitor [42].

ESI was used by all authors, the preferred polarization mode being positive. Ertapenem was measured in positive ionization mode [43] as well as in negative ionization mode [40, 44]. While all authors used protein precipitation to clean up their samples, Pickering at al. additionally followed this step by liquid-liquid extraction.

The quantification limits of all described methods are similarly in the therapeutical range of the described drugs. All Ertapenem papers were validated following the current FDA standards.

No method for the single determination of other carbapanems like doripenem or meropenem was found.

This is not due to a low interest in carbapenems in general, but rather to the abundant inclusion of carbapenems in many multi-analyte methods.

Further methods can therefore be found in "Methods testing for several compound classes". For meropenem and imipenem see [20, 45], several methods including meropenem are [46][47][48–51] and for ertapenem see [52].

Penicillins

Eleven methods were published dealing with the quantification of penicillins using LC-MS(/MS). Four papers alone dealt with the measurement of amoxicillin with or without clavulanic acid and three papers measured piperacillin/ tazobactam, while flucloxacillin was featured in two papers. Temocillin, cloxacillin, ampillin and penicillin G were determined by one group each.

The most common sample preparation technique was protein precipitation and only one method used SPE for the clean-up of amoxicillin in plasma samples [53]. Piperacillin and tazobactam in plasma total and plasma ultrafiltrate, urine, and renal replacement therapy effluent was evaluated by [54]. Page-Sharp et al. [55] presented a method for penicillin G in dried blood spots and [56] for piperacillin/ tazobactam. Other compartments were measured by Popoxicz et al. (Plasm, plasma ultrafiltrate, urine, renal replacement therapy effluent) [57].

Many structurally differs drugs have been used as internal standards for penicillin drugs: Prazosin, an alpha-blocker, was used by Barco et al. [56]and bisoprolol, a beta-blocker was used by Dong et al. [53]. Terbutaline, which was used by Yoon et al. [58] and clenbuterol, used by Zhang et al. [59] are two sympathomimetic. Neither of them is structurally related to penicillin.

By far the most popular column choice for penicillins were C18 column, with only two groups choosing either a C8 or a phenyl endcapping for different selectivity.

Some of the methods provide rather low and narrow analytical ranges, which might be applicable in pharmacokinetic studies, but would not provide enough coverage for a TDM situation. For these methods, validation of dilution integrity should be performed before adopting these for TDM.

General methods for beta-lactam antibiotics

Seven method were found describing a method for the determination of beta-lactames belonging to different groups (cephalosporines, penicillines or carbapanemes) [45, 60–65]. In 2015 Carlier et al. published a faster method with less analytes with very similar conditions to their paper from 2012.

Most methods were intensively validated using current FDA and EMEA standards, including matrix effects, which are, even in newer papers, often neglected. Many authors used several deuterated standards for the seven beta-lactam analyzed. This is clearly to be preferred over the use of fluconazole, ethylparaben and cefazolin, which was used by Sime et al., Ohmori et al. and Abdulla et al. respectively [62, 64, 65] as internal standards, especially in a TDM environment, were the intake of other antibiotics is not only possible, but very likely. Two methods included the quantification of beta-lactam inhibitors [60] [61] and two groups used HILIC methods [45, 62].

Most methods used similar mobile phases, water with 0.1% formic acid and methanol or acetonitrile with 0.1% formic acid. This is remarkable since mobile phases containing acetonitrile clearly seem to be favored by most other researchers measuring beta-lactams. The method by Abdulla et al. stands out, because of their use of a BEH Amide column in a HILIC method [62].

The use of protein precipitation, in some cases followed by dilution was used in six methods, only Ohmori et al. presented a solid phase extraction sample preparation.

Most of the multi-analyte methods including drugs from several classes feature at least one penicillin, therefore a look at the section “Methods testing for several compound classes” is advised. Some recommended papers include: [20, 22, 47, 49, 66].

Table 2 Cephalosporins

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Cefazedone [27]	Human plasma	Metronidazole	Protein precipitation, dilution	Ultimate XB-CN Pre-column: Inertsil ODS guard column	Acetonitrile/ water with 20mM ammonium acetate and 0.1% formic acid (15/85) Isocratic	LC-MS-MS ESI+ MRM	4.5 min	Selectivity, accuracy, precision, matrix effect, stability (freeze-thaw, bench-top, long-term, processed samples), LOQ	0.2-401.12
Cefuroxime axetil [29]	Human plasma	Cefoxitin	Protein precipitation, Liquid-liquid extraction	Jones Chromatography C18	Acetonitrile/ water/ (30/70) + 11.5 mM formic acid and acetonitrile/ water (90/10) + 11.5 mM formic acid.	LC-MS-MS ESI- MRM	4 min	Recovery, stability (freeze-thaw, processed sample), LOQ	0.1-20
Cefuroxime [28]	Human plasma	Cefoxitin	SPE Oasis HLB cartridges	LiChrospher 60 RP Select B	Acetonitrile:5mM ammonium acetate solution:glacial acetic acid (70:30:0.020)	UPLC-MS-MS ESI- MRM	n.s.	Selectivity, sensitivity, linearity of response, LOQ, accuracy, precision, recovery, matrix effect, stability (short-term, processed sample, long-term)	0.08-16.0
Ceftriaxone [33]	Human plasma and dried blood spots	Cefazolin	Plasma: Protein precipitation, dilution DBS: Liquid extraction, dilution	Acquity T3 C18 pre-column: VanGuard C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ MRM	5 min	Accuracy, precision, recovery, matrix effect, LOQ, LOD, stability (DBS and plasma: freeze-thaw, bench-top, long-term, processed sample,) site of chad sampling, effect of hematocrit value	Plasma and DBS: 1 - 200
Cefaclor [36]	Human plasma	Flucloxacillin	Protein precipitation	UPLC BEH C18	Water/ formic acid (99.9/0.1) and acetonitrile Gradient	LC-MS-MS ESI+ MRM	7.5 min	Accuracy, precision, recovery, matrix effect, stability (freeze-thaw, bench-top, stock solution), LOQ	0.002-10
Cefaclor [26]	Human plasma and urine	B-oxyphylline	Plasma: Protein precipitation Urine: dilution	BDS Hypersil C18	Methanol/ water/ acetonitrile (2/10/88) Isocratic	LC-MS ESI+ SIM	n.s.	n.s.	n.s.

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Cephalexin, cefradine, cefadroxil [37]	Human serum	Sulindac	Molecular imprinting SPE	Molecular imprinting microcolumn (self made)	Methanol/ trifluoracetic acid (99.9/0.1) Isocratic	LC-MS ESI+ SIM	4 min	Recovery, matrix effect, calibration curve, LOD, LOQ	Cephalexin: 0.3-25
Cefepime, enmetazobactam [35]	Human plasma	Cefepime-13c2d3 Enmetazobactam-d3	Automated protein precipitation	Acquity BEH HILIC	Water+ 20mM ammonium formate and acetonitrile Gradient	UPLC-MS-MS ESI+ MRM	4 min	Selectivity, linearity, precision, accuracy, matrix effects, recovery, stability	Enmetazobactam: 0.05-50 cefepime: 0.5-500
Cefoperazone [31]	Human plasma ultrafiltrate	Ceftiofur	Ultrafiltration and protein precipitation	Acquity UPLC BEH C18	Water/ formic acid (99.9/0.1) and acetonitrile Gradient	UPLC-MS-MS ESI- MRM	5 min	Selectivity, linearity, lower limit of quantification, matrix effect, accuracy, precision, recovery, stability	0.05-5
Cefazolin [30]	Human plasma, ultrafiltrate and cord blood	Cloxacillin	Plasma and cord blood: protein precipitation Ultafiltrate: ultrafiltration and dilution	Phenomenex Kinetex C8	Acetonitrile/ water/ formic acid (2/97.9/0.1) and acetonitrile/ formic acid (99.9/0.1) and acetonitrile/ isopropanol/ acetone (45/45/10) Gradient	LC-MS-MS ESI+ MRM	5 min	Accuracy, precision, carryover, matrix effects, recovery, stability	Total cefazolin: 0.48-480 free cefazolin: 0.048-48
Cefprozil diastereomers [67]	Human plasma	Cephalexin	Protein precipitation	Phenomenex C18 with Phenomenex C18 guard column	Methanol/ 2% formic acid (25:75) Isocratic	LC-MS-MS ESI- MRM	n.s.	Selectivity, carry-over, calibration curves, accuracy and precision, matrix effects, extraction recovery, stability (short-term, freeze-thaw, processed samples)	Cis-cefprozil: 0.125 and 0.0403 trans-cefprozil :0.0403-1.72
Ceftolozane, tazobactam [32]	Broncho-alveolar lavage fluid	Tazobactam-15N3	Dilution	Zorbax Eclipse C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ SRM	8 min	Accuracy, precision, linearity, LOQ, stability (short-term, long-term, freeze-thaw), recovery	0.02-0.5

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Ceftazidime [34]	Human plasma	Ceftazidime-d5	Protein precipitation, drying, redissolving	Acquity HSS T3	Water +10mM Ammonium formate (pH 3) and acetonitrile: Gradient	LC-MS-MS ESI+ SRM	2.5 min	Accuracy, precision, selectivity, LOQ, reproducibility, stability (short-term, long-term, freeze-thaw, processed samples, bench-top)	0.0438-87
Avibactam [34]	Human plasma	Avibactam-13C-5,15N-1	Solid phase extraction using Oasis WAX 10	Acquity BEH amide	Water + 100 mM Ammonium formate (pH 9)/ acetonitrile (5/95) Isocratic	LC-MS-MS ESI+ SRM	0.9 min	Accuracy, precision, selectivity, LOQ, reproducibility, stability (short-term, long-term, freeze-thaw, processed samples)	0.01-10

n.s. = not specified, ESI+ = electron spray ionization in positive ion mode, ESI- = electron spray ionization in negative ion mode. APCI$^+$ = atmospheric pressure chemical ionization in positive ion mode, LOQ = lower limit of quantification, MRM = multiple reaction monitoring, SRM = single reaction monitoring, SIM= singe ion monitoring

Table 3 Carbapanems

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Imipenem [42]	Rat plasma, monkey plasma and mouse whole blood	Cilastatin analogue	Protein precipitation	Atlantis HILIC	Water + 15mM ammonium formate (pH 3)/ acetonitrile (20/80) Isocratic	LC-MS-MS ESI+ MRM	4 min	Accuracy, precision, selectivity, stability (freeze-thaw, bench-top), matrix effect	0.1–100
Faropenem [68]	Human plasma and urine	Cefalexin	Plasma: protein precipitation, dilution Urine: dilution	Zorbax SB-C18	Water + formic acid 0.1%/ methanol (45/55) Isocratic	LC-MS-MS ESI+ MRM	n.s.	Selectivity, stability (freeze-thaw, long-term, bench-top), matrix effect, precision, accuracy	0.005-4
Ertapenem [41]	Human plasma	Meropenem	Protein precipitation. Liquid-liquid-extraction, filtration	Acquity BEH	Acetonitrile and water/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ and ESI- MRM	n.s.	Matrix effect, selectivity, stability (freeze-thaw, bench-top), precision, accuracy, LOQ	0.5–50
Ertapenem [44]	Human plasma	Ceftazidime	Protein precipitation	Synergi 4µ Polar-RP	Water + 2 mM ammonium hydroxide/ acetic acid (99.9/0.1) and methanol Gradient	LC-MS ESI- SIM	8 min	Selectivity, accuracy, precision, stability (long-term, processed samples), LOQ, matrix effect	0.1-50

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Ertapenem [40]	Human plasma	Ertapenem-D4	Protein precipitation, drying, reconstitution	HyPURITY C18	Water/ acetonitrile/ water + 10g/l ammonium acetate, 35 mg/l acetic acid, 2mg/l trifluoroacetic anhydride Gradient	LC-MS-MS ESI+ MRM	4 min	Selectivity, linearity, accuracy and precision, recovery and dilution integrity, stability (bench-top, processed samples, freeze-thaw)	0.1-125

n.s. = not specified, ESI+ = electron spray ionization in positive ion mode, ESI- = electron spray ionization in negative ion mode. APCI$^+$ = atmospheric pressure chemical ionization in positive ion mode, LOQ = lower limit of quantification, MRM = multiple reaction monitoring, SRM = single reaction monitoring, SIM= singe ion monitoring

Table 4 Penicillins

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Amoxicillin [53]	Human plasma	Bisoprolol	Protein precipitation, dilution	Sepax Sapphire C18 Pre-column: security guard-C18	Acetonitrile and water/ formic acid (99.9/0.1) Gradient	LC-MS-MS, ESI+ MRM	11 min	Accuracy, precision, recovery, matrix effect, stability (freeze-thaw, processed samples, long-term, bench-top), LOQ	Amoxicillin:0.005-20
Amoxicillin [69]	Human plasma	Ampicillin	SPE on HLB Oasis Cartridges	Hypersil Gold	Water + 10mM ammonium formate (pH 5.0)/ acetonitrile (10/90) Isocratic	LC-MS-MS ESI+ MRM	2 min	Accuracy, precision, recovery, stability (freeze-thaw, processed sample, bench-top, long-term, stock solution), matrix effect	0.17 - 17
Amoxicillin, clavulanic acid [59]	Human plasma	Amoxicillin: clenbuterol Clavulanic acid: none used	Protein precipitation, dilution	Shim-pack XR-ODS	Water/ formic acid (99.8/0.2) and acetonitrile/ formic acid (99.8/0.2) Gradient	LC-MS-MS, ESI- and ESI+ MRM	n.s.	Accuracy, precision, recovery, stability (freeze-thaw, long-term, bench-top), LOQ	Amoxicillin: 0.005–16 clavulanic acid: 0.05–2
Amoxicillin, clavulanic acid [58]	Human plasma	Terbutaline	Protein precipitation	Zorbax RX C8	Formic acid/ water/ acetonitrile (2/1000/100) Isocratic	LC-MS ESI- SIM	3 min	LOD; LOQ, recovery, accuracy, precision, stability (long-term, stock solution)	Amoxicillin: 0.125-8 clavulanic acid: 0.0625-4
Flucloxacillin, ampicillin [70]	Human plasma	Cloxacillin	Protein precipitation, dilution	Acquity BEH C18	Water + 10 mM ammonium formate/ acetonitrile (68/32) Isocratic	LC-MS-MS ESI+ MRM	3.5 min	Accuracy, precision, selectivity, recovery, matrix effect, stability (freeze-thaw, processed samples, bench-top)	Both: 0.2–500

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Flucloxacillin, cloxacillin [71]	Human plasma and microdialysis samples	Oxacillin	Protein precipitation	Acquity BEH C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	UPLC-MS-MS ESI+ SRM	2 min	Selectivity, LOQ, accuracy, precision, recovery, stability (freeze-thaw, bench-top, long-term, processed samples), matrix effect	Plasma: 6–30 ringer solution: 0.25-5
Temocillin total and unbound [72]	Human serum	Ticarcillin	Unbound: Ultrafiltration Unbound and total: Protein precipitation	XBridge® phenyl	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ MRM	6min	LOQ, LOD, precision, accuracy, matrix effect, selectivity	Total: 1 - 500 unbound: 0.5 - 300
Penicillin G [55]	Human dried blood spots	Penicillin G-d7	Plasma: protein precipitation DBS: liquid extraction	Kinetex XB-C18	Water/ formic acid (99.9/0.1)/methanol/ formic acid (99.9/0.1) (50/50) Isocratic	LC-MS-MS ESI+ MRM	n.s.	LOQ, calibration, accuracy, precision, matrix effect, hematocrit	0.1-100
Piperacillin, tazobactam [54]	Plasma total and unbound, urine, renal replacement therapy effluent	Piperacillin-d5 sulbactam	Dilution and protein precipitation	C18 Shimadzu Shim-pack XR-ODS III	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	UPLC-MS-MS ESI+ and ESI- MRM	4.5 min	Matrix effects, precision and accuracy, specificity, stability	Piperacillin: 0.5-500 tazobactam: 0.625-62.5
Piperacillin, tazobactam [57]	Human plasma and pleural fluid	Piperacillin-d5, sulbactam	Plasma: protein precipitation and dilution	Acquity BEH C18	Water/ ammonium hydroxide (99.6/0.4) + 10 mM ammonium bicarbonate and methanol/ ammonium hydroxide (99.6/0.4) Gradient	UPLC-MS-MS ESI+ MRM	3.2 min	Selectivity, specificity, precision, accuracy, LOQ, matrix effects, recovery, stability	Piperacillin: 0.25-352 tazobactam: 0.25-50.5
Piperacillin, tazobactam [56]	Human dried blood spots	Prazosin	Liquid extraction	Kinetex C18	Water/ formic acid (99.9/0.1) and acetonitrile Gradient	LC-MS-MS ESI+ and ESI- MRM	5.5min	Selectivity, accuracy, precision, recovery, stability (freeze-thaw, processed sample, bench-top, long-term, stock solution), matrix effect, carry-over, LOQ	Piperacillin: 0.6-100 tazobactam: 0.1-40

n.s. = not specified, ESI+ = electron spray ionization in positive ion mode, ESI- = electron spray ionization in negative ion mode. APCI$^+$ = atmospheric pressure chemical ionization in positive ion mode, LOQ = lower limit of quantification, MRM = multiple reaction monitoring, SRM = single reaction monitoring, SIM= singe ion monitoring

Table 5 General methods for beta-lactam antibiotics

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Amoxicillin, cefuroxime, ceftazidime, meropenem, piperacillin [63]	Human plasma	Piperacillin-d5 ceftazidime-d6 amoxicillin-4 meropenem-d6 cefuroxime-d3	Protein precipitation, dilution	Acquity UPLC BEH C18	Water/ formic acid (99.9/0.1) with 2 mM ammonium acetate and methanol/ formic acid (99.9/0.1) with 2 mM ammonium Gradient	LC-MS-MS ESI+ MRM	2.5min	Precision, accuracy, matrix effect, selectivity, carry-over, stability (processed samples, freeze-thaw, stock solution), LOQ	Amoxicillin and cefuroxime: 1-100 meropenem and ceftazidime: 0.5 - 80 piperacillin :1 -150
Amoxicillin, ampicillin, cefuroxime, cefazolin, ceftazidime, meropenem, piperacillin, clavulanic acid, tazobactam [60]	Human plasma	Piperacillin-d5 ceftazidime-d6 amoxicillin-4 ampicillin-d5 meropenem-d6 cefuroxime-d3	Protein precipitation and liquid extraction	Acquity UPLC BEH C18 + guard column	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	UPLC-MS-MS ESI+ and ESI- MRM	5.5 min	Precision, accuracy, linearity, matrix effect and recovery	Piperacillin: 1.5-100 all others: 0.5-100
Cefepime, meropenem, piperacillin, tazobactam [61]	Human plasma	Meropenem-d6 piperacillin-d5	Protein precipitation, dilution	Phenomenex Synergy C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ SRM	7 min	Precision, accuracy, sensitivity, selectivity, dilution integrity, matrix effect, extraction recovery, hemolysis effect, carry-over	Cefepime: 0.5-150 meropenem and piperacillin: 0.1-150 tazobactam: 0.25-150
Amoxicillin, benzylpenicillin, cefotaxime, cefuroxime, ceftazidime, flucloxacillin, imipenem, meropenem, piperacillin [62]	Human plasma	Cefazolin	Protein precipitation	Waters Acquity UPLC BEH Amide	Acetonitrile/ 100 mM ammonium formate (80:20) pH 6.5 Isocratic	UPLC-MS-MS ESI+ MRM	5.2 min	Linearity, LOQ, recovery, matrix effects, precision, accuracy, carry-over, stability (processed samples)	Amoxicillin: 0.1-20 benzylpenicillin: 0.05-10 cefotaxime: 0.25-13 cefuroxime: 0.3-65 ceftazidime: 0.1-25 flucloxacillin: 0.75-150 imipenem: 0.2-40 meropenem: 0.2-40 piperacillin: 0.4-80
Piperacillin, benzylpenicillin, flucloxacillin, meropenem, ertapenem, cephazolin and ceftazidime [65]	Human plasma	Fluconazole	Protein precipitation, dilution	Kinetex C18	Water/ formic acid (99.9/0.1) and methanol/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ MRM	7 min	Precision, accuracy, stability (bench-top, processed samples, freeze-thaw), recovery, matrix effects, LOQ, selectivity	flucloxacillin: 0.25-50 all other: 0.1-50

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Cefepime, imipenem, meropenem [45]	Human plasma, cerebrospinal fluid	Cefepime-d3 meropenem-d6	Protein precipitation	Hypersil GOLD HILIC, precolumn: Turboflow Cyclone-MCX	Water/ formic acid (99.9/0.1) + 10 mM ammonium acetate and acetonitrile/ methanol/ formic acid (49.95/49.95/0.1) + 10 mM ammonium acetate and acetonitrile/ acetone/ 2-propanol (1/1/1) Gradient	LC-MS-MS ESI+ MRM	12.75 min	Precision, accuracy, stability (short-term, long-term, stock solutions, processed samples), recovery, matrix effects, LOQ, selectivity, dilution integrity	Cefepime: 1-100 imipenem: 0.6-60 meropenem: 0.4-40
Ampicillin, cefazolin, cefepime, cefmetazole, cefotaxime, doripenem, meropenem, piperacillin [64]	Human serum	Ethylparaben	Solid phase extraction using Oasis HLB cartridges	Unison UK-C18 RP porous ODS	Water/ formic acid (99.9/0.1) +1 0 mM ammonium formate and methanol Gradient	LC-MS-MS ESI+ and ESI- SRM	13 min	Precision, accuracy, linearity, recovery, matrix effects	Doripenem: -0.5-50 all others: 0.1–50

n.s. = not specified, ESI+ = electron spray ionization in positive ion mode, ESI- = electron spray ionization in negative ion mode. $APCI^+$ = atmospheric pressure chemical ionization in positive ion mode, LOQ = lower limit of quantification, MRM = multiple reaction monitoring, SRM = single reaction monitoring, SIM= singe ion monitoring

Quinolones

The quality of published quinolone methods is quite varied. Some methods cannot be backed up by well described method parameters or substantial validation data [73, 74]. Other methods were well described and validated, even in slightly older papers, e.g. Bian et al. [75].

Quinolones were measured in very different human matrices, including besifloxacin in tears [76], nemonoxacin in feces and urine [77] or levofloxacin in human plasma, bronchoalveolar lavage and alveolar cells [73]. Moxifloxacin was also analyzed as total drug and as free fraction from plasma ultrafiltrate [78].

Most popular column to separate quinolones were C18 columns, but also diphenyl [76] and a not closer described propyl column [73] were used. Internal standards were used in all found methods and ranged from related quinolones to dextrorphan. No deuterated standards were used in any of the methods.

Other methods including quinolones include: [20, 66, 79–81].

Table 6 Quinolones

Compound	Sample Material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Norfloxacin, ciprofloxacin, ofloxacin [74]	Human urine	Enrofloxacin	Solid phase extraction using 3M-Empore MPC cartridges	Kromasil C8	Acetonitrile/ water + ammonium acetate (pH 2.5) (20/80)	LC-MS ESI+ SIM	15 min	Recovery, specificity, LOQ, precision	0.01-10
Moxifloxacin, free and total [78]	Human plasma, plasma ultrafiltrate, and cerebrospinal fluid	Cyano-imipramine	Protein precipitation	Thermo Electron HyPurity C18	Water and acetonitrile and water/ trifluoracetic anhydride (99.8/0.2) + 10 g/L ammonium acetate + 35 mg/L acetic acid Gradient	LC-MS-MS ESI+ MRM	3.5 min	Selectivity, accuracy, precision, recovery, matrix effect, stability (freeze-thaw, short-term, autosampler), carry over, LOQ	0.05 - 5.0
Besifloxacin [76]	Human tears	Sparfloxacin	Protein precipitation	Pursuit® Diphenyl, guard column diphenyl	Water + 5mM ammonium formate (pH 3.25) and acetonitrile/ methanol (20/80) Gradient	LC-MS-MS ESI+ MRM	4 min	Recovery, accuracy, precision, recovery, and stability (freeze-thaw, short-term, autosampler, long-term), selectivity, carry over	0.002-2
Balofloxacin [75]	Human plasma	Naphazoline	Liquid-liquid extraction	ZORBAX 300SB C18	Methanol/ water + 10mM ammonium acetate (pH 3.0) (40/60) Gradient	LC-MS ESI+ SIM	5 min	Matrix effect, precision, accuracy, selectivity, recovery, stability (freeze-thaw, short-term, autosampler, long-term), LOQ	0.03-3
Levofloxacin [73]	Human plasma, bronchoalveolar lavage and alveolar cells	Ciprofloxacin	Protein precipitation	Propyl column	Acetonitrile/ water/ trifluoroacetic acid/ ammonium phosphate dibasic (90/10/0.06/ 0.0006) Isocratic	LC-MS-MS APCI+ MRM	n.s.	Precision	Plasma: 0.025-4 bronchoalveolar lavage and alveolar cells: 0.005-0.8
Nemonoxacin and metabolite [77]	Human urine and feces	Gatifloxacin	Liquid-liquid extraction	Symmetry Shield RP18	Formic acid/ water (0.1/ 99.9) and acetonitrile (84/16) Isocratic	LC-MS-MS ESI+ SRM	2 runs: 4 min and 3.5 min	Matrix effect, precision, accuracy, selectivity, recovery, stability (freeze-thaw, short-term, autosampler, long-term), LOQ, carry-over	Metabolite in urine: 0.001 -0.2 in feces: 0.03-3 nemonoxacin: in feces: 0.12-48

Sitafloxacin [82]	Human plasma	Dextrorphan	Liquid-liquid-extraction, drying and reconstitution	ZORBAX SB-C18	Methanol/ water/ formic acid (46/53.946/0.054) Isocratic	LC-MS-MS ESI+ MRM	4 min	Matrix effect, precision, accuracy, selectivity, recovery, stability (freeze-thaw, short-term, autosampler, long-term), LOQ	0.005-2.5
Ciprofloxacin [83]	Human plasma	Ofloxacin	Liquid-liquid extraction	Agilent C18	Water/ formic acid (99.9/0.2)/ methanol (10:90) Isocratic	LC-MS-MS ESI+ SRM	3.0 min	Specificity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, stability (short-term, auto-sampler, freeze-thaw, long-term)	0.01-5
Moxifloxacin, levofloxacin [84]	Human serum	Enrofloxacin	Protein precipitation	Atlantis dC18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) (60/40) Isocratic	LC-MS-MS ESI+ MRM	4 min	Selectivity, accuracy, precision, linearity, recovery, matrix effects, stability (long-term, short-term, autosampler)	Moxifloxacin:0.023-1 levofloxacin: 0.00013-1

n.s. = not specified, ESI+ = electron spray ionization in positive ion mode, ESI- = electron spray ionization in negative ion mode. $APCI^+$ = atmospheric pressure chemical ionization in positive ion mode, LOQ = lower limit of quantification, MRM = multiple reaction monitoring, SRM = single reaction monitoring, SIM= singe ion monitoring

Tuberculostatic drugs

While most groups analyzed human serum or plasma for tuberculostatic drugs, two groups analyzed dried blood spots for rifampicin, clarithromycin, desacetyl-rifampicin, and 14-hydroxyclarythromcin or rifapentine and desacetyl-rifapentine, respectively [14, 85]. The measurement of ethambutol from plasma, bronchoalveolar lavage fluid and alveolar cells by Conte et al. [86] and of rifampicin in cerebrospinal fluid by Srivastava et al. [87] is also noteworthy. Since anti-tuberculostatic treatment can be carried out over several months, the interesting opportunity arises to use hair samples to assess treatment adherence. This was shown to be possible by one group for several tuberculostatic drugs [88–90]. They claim not only treatment adherence assessment, but even TDM can be achieved using hair samples.

Some methods were developed for use in pharmacokinetic or large clinical studies while several, especially more recent methods, were developed for TDM and one was developed for use in a bioequivalence study. All methods tested calibration ranges that make them applicable in TDM. One paper in particular shows an extremely wide calibration range of 0.005-40 mg/l for rifampicin [91]. One group used LC-MS [92] even though they produced the results on a triple quadrupole mass spectrometer, which is capable of tandem LC-MS. All other papers showed the use of LC-MS-MS. ESI in positive ionization mode was used in all cases except in the determination of ethambutol and pyrazinamide by Gong et al., who used APCI in positive ionization mode [93].

In most papers deuterated standards were used. Other methods used structurally related antibiotics (e.g. [87], [92], [94], [86]); one used nicotinic acid [95] and one used nialamide [96]. None of the three methods determining cycloserine used a deuterated standard, but used cytosin, nicotinic acid and mildronate instead. Most papers used protein precipitation as their only cleaning step, only in four methods a solid phase extraction was employed [91, 95, 97, 98]. Dried blood spots were extracted using either water or a 1:1 mix of methanol and 50 mM ammonium formate [14, 85].

Interesting is the wide range of analytical columns used in the separation of tuberculostatic drugs. While various C18 columns were by far the most common, also more polar phases like the Synergi Polar RP or C8 column, as well as silica and HILIC columns were used. For ethambutol alone, very different phases were employed [86, 88, 94]. Quite often trifluoracetic acid or heptafluorobutyric acid were used in mobile phases, which is a rather uncommon additive in LC-MS, e.g. [85, 86, 95]. Several groups used an additional pre-column to protect the analytical column [92, 95, 96].

Other papers that describe the analysis of tuberculostatic drugs, see “methods for several compound classes”: [15, 66, 99, 100].

Table 7 Tuberculostatic drugs

Compound	Sample Material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Runt time	Validation Data	Analytical Range [mg/l]
Isoniazid, ethambutol, ethionamide, prothionamide, linezolid, pyrazinamide, pretomanid, clofazimine, levofloxacin, moxifloxacin, bedaquiline [88]	Human hair	Deuterated standards, n.s.	Pulverization of hair, liquid extraction	Phenomenex Synergi Polar RP	Water/ formic acid (99/1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ MRM	23 min	LOQ, linearity, matrix effect, recovery, accuracy, precision,	0.005-100 ng/mg
Isoniazid, rifampicin, pyrazinamide, and ethambutol [94]	Human serum	Rifabutin, 6-aminonicotinic acid	Protein precipitation	Hydrosphere C18	Formic acid/ water (0.3/99.7) and formic acid/ methanol (0.3/99.7) Gradient	LC-MS-MS ESI+ MRM	4 min	Accuracy, LOQ, matrix effect, recovery	Isoniazid:0.5-8 rifampicin: 0.5-80 pyrazinamide: 5-80 ethambutol: 5-8
Rifampicin, isoniazid and their major metabolites [97]	Human plasma	Rifampicin-d3, 25-desacetyl-rifampicin-d3. isoniazid-d4, acetylisoni-azid-d4, isonicotinic acid-d4	Solid phase extraction using Captiva ND Lipids cartridge	Zorbax SB-Aq	Water + 5 mm ammonium acetate and acetonitrile/ water with formic acid 0.01% (90/10) Gradient	LC-MS-MS ESI+ MRM	7 min	Accuracy, precision, selectivity, matrix effect, recovery, stability (freeze-thaw, processed samples), LOQ	Rifampicin: 0.025–50 25-desacetylrifampicin: 0.00.2.5–5 isoniazid: 0.005–10 acetylisoniazid: 0.0125–5 isonicotinic acid: 0.0125–5
Rifampicin, rifabutin, rifapentine and their active desacetyl metabolites [101]	Human plasma	Rifabutin-D6 rifampin-D8	Protein precipitation and dilution	ACE® C18	Formic acid/ water/ acetonitrile (0.5/55/45) Isocratic	LC-MS-MS ESI+ MRM	2 runs; 3.85 min and 2.1 min	Precision, accuracy, matrix effect, selectivity, recovery, stability (freeze-thaw, short-term, processed samples), LOQ	Rifampicin, rifabutin, rifapentin: 0.075–30

Pyrazinamide, isoniazid, ethambutol, streptomycin, rifampicin [102]	Human plasma	N.s.	Protein precipitation	ZORBAX SB-C18	Heptafluorobutyric acid/ formic acid/ methanol (0.02/0.2/99.8) and heptafluorobutyric acid/ formic acid/ water (0.02/0.2/99.8) Gradient	LC-MS-MS ESI+ SRM	8.5 min	Selectivity, matrix effect, linearity, precision, accuracy, stability (freeze–thaw, short-term, long-term), LOQ	Pyrazinamide: 0.2-4 isoniazid: 0.08-2 ethambutol: 0.0002-1 streptomycin:2-200 rifampicin: 0.2-4
Isoniazid, rifampicin, ethambutol, pyrazinamide [103]	Human plasma	Isoniazid-D4, Rifampicin-D3, Ethambutol-D4, Pyrazinamide-15N,D3	Two times protein precipitation, dilution	Chromolith Flash RP-18	Methanol/ water/ formic acid (5/94.54/0.45) + 5 mm ammonium acetate and methanol/ water/ formic acid (95/4.54/0.45) + 5 mm ammonium acetate and water + 5 mm ammonium acetate Gradient	LC-MS-MS ESI+ SRM	10.2 min	Selectivity, linearity, precision, accuracy, LOQ, recovery, matrix effect, stability (autosampler), carry-over.	Isoniazid: 0.5-10 rifampicin: 0.75-30 ethambutol: 0.25-10 pyrazinamide: 4-80
Pyrazinamide, isoniazid, rifampicin, ethambutol [104]	Human serum	Pyrazinamide-D3, isoniazid-D4, rifampicin-D3, rifampicin-D8, ethambutol-D4	Protein precipitation	Xselect HSS T3	Trifluoroacetic acid/ water (0.1/99.9) + 5mM ammonium formate and trifluoroacetic acid/ methanol (0.1/99.9) + 5mM ammonium formate Gradient	LC-MS-MS ESI+ SRM	5 min	Selectivity, precision, accuracy, linearity, linearity, LOQ, carryover, stability, matrix effect, recovery	Pyrazinamide: 1.02-60 isoniazid: 0.152-10 rifampicin: 0.500-30 ethambutol: 0.0998-5.99
Pyrazinamide, pyrazinoic acid, 5-hydroxy pyrazinoic acid [105]	Human plasma	Pyrazinamide-d3, pyrazinoic acid-d3, 5-hydroxy pyrazinoic acid-13C3	Liquid-liquid extraction	Zorbax Eclipse XDB C18	Methanol/ 0.1% acetic acid (65/35) Isocratic	LC-MS-MS ESI+ MRM	4 min	Carry-over, selectivity, LOQ, accuracy, precision, recovery, matrix effect, dilution integrity, stability (short-term, long-term), incurred sample analysis	Pyrazinamide: 0.1-30 pyrazinoic acid: 0.03-9 5-hydroxy pyrazinoic acid: 0.002-0.6

Rifampicin, clarithromycin, desacetyl rifampicin, 14-hydroxyclarythromcin [14]	Dried blood spots	Rifampicin-d8, cyano-imipramine	Liquid extraction	Hypurity C18	Acetonitril and water and water + ammonium acetate 10 g/L, acetic acid 35 mg/L, trifluoroacetic anhydride 2 mg/L, ph 3.5 Gradient	LC-MS-MS ESI+ SRM	3.5 min	Selectivity, specificity, carry-over, linearity, incurred sample analysis, dilution integrity, accuracy, precision	Rifampicin: 0.15-60 desacetylrifampicin: 0.15-20 clarithromycin: 0.05-20 14-Hydroxy clarithromycin:0.05-20
Ethambutol and pyrazinamide [93]	Human plasma	Ethambutol-d4, pyrazinamide-d3	Protein precipitation, supernatant was dried and reconstituted	Chromolith speedrod RP-C18 endcapped	Trifluoroacetic acid/ water (0.1/99.9) and trifluoroacetic acid/ methanol (0.1/99.9) Gradient	LC-MS-MS $APCI^+$ MRM	3.8 min	Precision, accuracy, selectivity, recovery, matrix effect, stability (short-term, freeze-thaw, long-term, processed samples, stock solution), LOQ	Ethambutol: 0.01-5 pyrazinamide: 0.05-25
Ethambutol [86]	Human plasma, bronchoalveolar lavage fluid and alveolar cells	Neostigmine and propranolol	Protein precipitation	Hypersil^{tm} Silica	Acetonitrile/ water/ trifluoroacetic acid (80/19.9/0.1) with 4mm ammonium acetate Isocratic	LC-MS-MS ESI+ MRM	2.8 min	Precision, accuracy, recovery, stability (long-term), LOQ	Plasma: 0.05-2.4
Rifampicin [87]	human plasma and cerebrospinal fluid	Rifapentine	Protein precipitation	Hypurity C18	Acetonitril/ formic acid (99.95/0.05) and water + 15 mm ammonium formate Gradient	LC-MS-MS ESI^+ MRM	6 min	Selectivity, accuracy, precision, LOQ, recovery, matrix effect, stability (freeze–thaw, processed samples and heat inactivation)	0.025-6.4
Rifampicin [92]	Human plasma	Rifamycin	Protein precipitation and dilution	Betasil Phenyl-Hexyl Pre-column: betasil hexyl	Water with 10mm Ammonium acetat/ Acetonitril (60/40) Isocratic	LC-MS ESI+ SIM	6 min	Recovery, stability (heat inactivation, freeze-thaw), matrix effect, LOQ	0.1-12.8
Rifampicin [91]	Human plasma	Rifampicin-D8	Protein precipitation, solid phase extraction using Captiva ND Lipids filtration plates	Kinetex C18	Water/ formic acid (99.9/0.1) and acetonitrile Gradient	LC-MS-MS ESI+ MRM	2.4 min	Selectivity, accuracy, precision, linearity, recovery, matrix effects, stability (freeze-thaw, short-term, autosampler)	0.005-40

D-Cycloserine [95]	Human plasma	Nicotinic acid	Protein precipitation and solid phase extraction using Oasis MCX extraction cartridge	YMC-Pack SIL-06 HILIC Pre-column: YMC-Pack Silica Column guard	Methanol/ propanol-2/ water with trifluoroacetic acid 0.075 % (66.5/28.5/5) Isocratic	LC-MS-MS ESI+ MRM	N.s.	Recovery, precision, accuracy, stability (freeze– thaw, short-term, long-term, processed sample), matrix effect, LOQ	0.3-30
Cycloserine [98]	Human plasma	Cytosine	Solid phase extraction using copure Poly MCX Cartridges	BDS Hypersil C18	0.2% formic acid in water/ methanol/ acetonitrile (70/15/15) Isocratic	LC-MS-MS ESI+ SRM	3.1 min	Selectivity, LOQ, matrix effect, linearity, precision, accuracy, recovery, dilution integrity, stability (autosampler, freeze–thaw, short-term, long-term), incurred sample analysis	0.2-20
Cycloserine [106]	Human plasma	Mildronate	Protein precipitation, dilution	Shim-pack XR-ODS	Methanol/ water + 0.01% formic acid (70/30) Isocratic	LC-MS-MS ESI+ SRM	2 min	Selectivity, matrix effect, linearity, precision, accuracy, carry-over, stability (autosampler, freeze–thaw, short-term, long-term, stock solutions), LOQ	0.3-90
Rifabutin [107]	Human plasma	Raloxifene phenacetin	Two liquid-liquid extractions	Discovery HS C18	Water + 10 mM ammonium acetate, pH 4.5/ acetonitrile (15/85) Isocratic	LC-MS-MS ESI+ SRM	3.5 min	Selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, stability (autosampler, freeze–thaw, short-term, long-term)	Rifabutin: 0.001-1
Isoniazid [89]	Human hair	Isoniazid-d4	Pulverization and liquid extraction	Phenomenex Synergi Polar-RP	Water/ formic acid (99.8/0.2) Isocratic	LC-MS-MS ESI+ MRM	5 min	Specificity, accuracy, precision, recovery, linearity, stability (short-term, long-term)	0.05-50 µg/kg
Isoniazid [96]	Human plasma	Nialamide	Protein precipitation	Hypersil^{tm} Silica Pre-column: guard column and pre-column filter, unspecified	Acetic acid/ water (0.1/99.9) + 2.5 mM ammoniumacetate and acetic acid/ acetonitrile (0.1/99.9) Gradient	LC-MS-MS ESI^{+} MRM	10 min	Accuracy, precision, LOQ, recovery, matrix effects, stability (freeze-thaw, long-term, short-term)	0.05-10
Bedaquiline [90]	Human hair	Bedaquiline-d6	Pulverization of hair, liquid extraction	Phenomenex Synergi Polar RP	Water/ Formic acid (99/1) and acetonitrile/ 0.1% formic acid (99.6/0.4) Gradient	LC-MS-MS ESI+ MRM	14 min	LOQ, linearity, accuracy, precision,	0.005–20 ng/mg

Bedaquiline, N-monodesmethyl bedaquiline [108]	Human serum	Bedaquiline-d6	Protein precipitation	Hypurity C18	Water and acetonitrile and water+ 10 g/l ammonium acetate + 35 mg/l acetic acid + 2 ml/l trifluoroacetic anhydride Gradient	LC-MS-MS ESI+ SRM	2.6 min	Selectivity, LOQ, linearity, accuracy, precision, recovery, matrix effect, recovery, dilution integrity.	0.05-6
Rifapentine, desacetyl-rifapentine [85]	Dried blood spots	Rifampin-d3	Liquid extraction	Waters BEH C8	Water + 5 mM ammonium formate and DMSO/ acetonitrile (3/97) Gradient	LC-MS-MS ESI+ SRM	4 min	Accuracy, precision, linearity, recovery, LOQ, matrix effects, recovery, selectivity, stability (long-term, short-term, freeze-thaw)	0.050-80

n.s. = not specified, ESI+ = electron spray ionization in positive ion mode, ESI- = electron spray ionization in negative ion mode. $APCI^+$ = atmospheric pressure chemical ionization in positive ion mode, LOQ = lower limit of quantification, MRM = multiple reaction monitoring, SRM = single reaction monitoring, SIM= singe ion monitoring

Peptide antibiotics

Several methods for the quantification of peptide antibiotics have been published in the last years. There has been an abundance of colistin methods using LC-MS(/MS) published, but only one since the recent review by Dagla et al. [109]. In this review about different analytical methods to measure colistin in biological material, detailed descriptions of LC-MS and LC-MS/MS methods are summarized. Also, analytical challenges in colistin measurement are discussed and critical aspects of the method of detection, the sample pretreatment methodology etc. are compared. No single analyte method for colistin is therefore included in this review.

Even though immunoassays for the detection of vancomycin are available, there have still been several methods using LC-MS(/MS) published. Those ten, which measure vancomycin as a single analyte method can be found in table 8. They are characterized by a wide range of different columns used, from HILIC columns to C8 and BEH C18. Still, their use of mobile phases is quite similar over all ten methods: mostly water with 0.1% formic acid in a combination with acetonitrile or methanol with 0.1% formic acid is used. The investigated matrizes range from plasma or serum to cerebrospinal fluid, dried blood spots and volumetric absorptive microsampling. Several methods have also been published for daptomycin, for example by Dei Cas et al., who looked daptomycin in plasma and breast milk to determine the safety of breast feeding while the mother was undergoing antibiotic treatment [110]. Both Bazoti et al. and Verdier et al [111, 112] measured daptomycin using reserpine as internal standard. Interestingly, the use of deuterated standards for peptide antibiotics seems to be quite rare, as only three out of 24 methods found used those, even though Andriguetti et al. Used deuterated creatinine to quantify vancomycin and creatinin [113–115]. Most authors rely on related drugs or use even sulfamethoxazole as standard for teicoplanin [116]. Several groups have also shown reliable results using custom made drug analogs [117, 118] or glycine or leucine adducts [119, 120]. These analogs are closely related to the drug analyzed and therefore well suited as an IS, also it cannot occur naturally in any sample. On the other hand, synthesizing of an internal standard is time consuming and not every laboratory may have the equipment and skill to do so.

Of special interest is the quantification of polymyxin B and teicoplanin, since both are a mixture of several closely related chemicals. Since these differ in their molecular masses, determination using mass spectrometers represents a challenge for these drugs. Therefore, methods differ in the specific components of the drugs measured. Hee et al. measured the four components polymyxin B1, B2, B3 and isoleucine-polymyxin B1 [121], while Covelli et al. and Thomas et al. [122, 123] only determined polymyxin B1 and B2. They also might quantify each component separately, like in the presented polymyxin methods or as the sum of all components together, as is done for teicoplanin.

The most common sample preparation method for peptide antibiotics is protein precipitation, while only three methods used solid phase extraction, but these reached comparatively lower limits of detection.

Peptide antibiotics are common in multi-class methods, further methods are: [20, 48, 50, 66, 99, 124–128]

Table 8 Peptide antibiotics

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Vancomycin, teicoplanin, daptomycin, colistin [129]	Human plasma	Polymyxin B	Protein precipitation, dilution	Kinetex C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ MRM	10min	Precision, accuracy, LOD, LOQ selectivity, recovery, matrix effect, stability (long-term, freeze-thaw, processed samples)	Vancomycin: 0.50–100.0 colistin B: 0.27–54.0 colistin A: 0.13–26.0 teico A3-1: 0.27–10.8 teico A2-1: 0.14–5.6 teico A2-2 & A2-3: 0.32–63.8 teicoA2-4 & A2-5: 0.14–28.0 daptomycin: 0.50–100.0
Vancomycin [119]	Human serum	Vancomycin-glycine	Protein precipitation, ultrafiltration	Fortis C8	Water/ formic acid (99.9/0.1) and methanol/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ MRM	21 min	Selectivity, matrix effect, accuracy, precision, stability (autosampler)	1.06 - 84.4
Vancomycin [130]	Human serum	Atenolol	Solid phase extraction using Strata-X-C cartridges, drying and reconstitution	ACE-3-C8 Pre-column: SecurityGuard C18	Water/ acetonitrile/ formic acid (89.9/10/0.1) Isocratic	LC-MS ESI+ full scan mode	5 min	Precision, recovery, accuracy, matrix effect, stability (freeze-thaw, processed sample), LOD, LOQ	0.05–10
Vancomycin [120]	Human plasma	Vancomycin-des-leucine formiate	Protein precipitation	Acquity UPLC BEH HILIC Pre-column: C18 guard	Acetonitrile/ formic acid (99.9/0.1) and water/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ MRM	5 min	Precision, recovery, accuracy, matrix effect, stability (Freeze-thaw, short-term, long-term, autosampler), LOQ, carry-over, selectivity	0.3-100
Vancomycin [131]	Human dried blood spots and plasma	Teicoplanin	Liquid extraction	Accucore C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS, ESI+ MRM	8.5 min	Linearity, accuracy, precision; LOQ, stability (long-term, short-term, autosampler), hematocrit, matrix effect, recovery	1-100

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Vancomycin [132]	Human plasma	Linezolid	Protein precipitation	Hypersil GOLD aQ C18	Water/ formic acid (99.9/0.1) and acetonitrile Gradient	UPLC-MS-MS, ESI+ MRM	3 min	Linearity, accuracy, precision; LOQ, stability (long-term, short-term, autosampler, freeze-thaw), matrix effect, recovery, carry-over, incurred sample analysis	0.1-128
Vancomycin [133]	Human cerebrospinal fluid	Methotrexate	Protein precipitation	Waters BEH C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	UPLC-MS-MS, ESI+ MRM	3.5 min	Selectivity, (LOQ), carry-over, linearity, accuracy, precision, recovery, matrix effect, dilution integrity, stability (long-term, short-term, autosampler, freeze-thaw),	1-400
Vancomycin [113]	Human plasma and dog plasma	Vancomycin-d12	Protein precipitation	Waters Acquity BEH C18	Water/ acetonitrile/ formic acid (94.9/5/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	UPLC-MS-MS, ESI+ MRM	4 min	Linearity, accuracy, precision, selectivity, matrix effect, recovery, stability (long-term, autosampler, freeze-thaw), incurred sample analysis	0.05-100
Vancomycin [115]	Volumetric absorptive microsampling	Creatinine-d3	Liquid extraction	Accucore C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS, ESI+ MRM	8.5 min	Linearity, accuracy, precision, selectivity, LOQ, matrix effect, recovery, stability (long-term, autosampler, short-term)	1-100
Vancomycin [114]	Human plasma (total and unbound), urine, renal replacement therapy effluent	Vancomycin-d12	Unbound fraction: ultrafiltration Urine: dilution, filtration All: protein precipitation	SeQuant zic-HILIC	Acetonitrile/ acetone/ 0.1% formic acid in water (30/10/60) and acetonitrile/ acetone/0.1% formic acid in water (70/10/20) Gradient	UPLC-MS-MS, ESI+ MRM	4.5 min	LOQ, linearity, precision, accuracy, matrix effects, incurred sample reanalysis, stability (freeze–thaw, short-term, stock solutions)	1-100
Vancomycin [134]	Human serum	Tobramycin	Protein precipitation	Acquity UPLC RP BEH C18	Formic acid/ acetonitrile/ water (0.1/5/94.9) + 2mM ammonium acetate and formic acid/ water/ methanol (0.1/5/94.9) + 2mM ammonium acetate Gradient	UPLC-MS-MS, ESI+ MRM	5 min	Linearity, precision, accuracy, recovery, LOQ, matrix effect, stability (autosampler)	0.1-100

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Daptomycin [111]	Human plasma	Reserpine	Protein precipitation, supernatant was dried and reconstituted	Acquity BEH C18 Pre-column: VanGuard BEH C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ full scan mode	3.5 min	Selectivity, precision, accuracy, recovery, matrix effect, stability (short-term, long-term, processed sample, freeze-thaw), LOQ	0.01–10
Daptomycin [112]	Human plasma	Reserpine	Protein precipitation, dilution	C18 Hypersil Gold	Water with formic acid 0.1%/ water/ acetonitrile (10/70/20) and acetonitrile Gradient	LC-MS-MS ESI+ MRM	11 min	Selectivity, precision, accuracy, recovery, matrix effect, stability (long-term, autosampler, freeze-thaw), LOQ, LOD	1 -120
Daptomycin [110]	Human plasma and breast milk	Erythromycin	Plasma: dilution, protein precipitation Breast milk: protein precipitation, filtration	Agilent Poroshell 120 SB-C8	Water/ formic acid (99.9/0.1) + 10mM ammonium formate and acetonitrile Gradient	UPLC-MS-MS, ESI+ MRM	10 min	Specificity, precision, accuracy, linearity, LOQ, LOD	Plasma: 19–199 breast milk: 0.12–0.32
Daptomycin, free and total [135]	Human plasma (total and unbound)	Diazepam	Free fraction: ultrafiltration Both: protein precipitation, filtration	Aeris Peptide ODS XB-C18 Guard column C18-Peptide	Methanol/ water/ acetic acid (60/49.9/0.1) Isocratic	UPLC-MS-MS, ESI+ SRM	10 min	Precision, accuracy, matrix effect, recovery, specificity, linearity, carry-over, stability short-term, long-term, freeze-thaw, autosampler, stock solution)	Plasma: 1-100 filtrate: 0.1-10
Teicoplanin A2-1, A2-2, A2-3, A2–4A, 2-5 [136]	Human plasma	Polymyxin B	Protein precipitation	Phenomonex Kinetex C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS, ESI+ SRM	5 min	Linearity, LOQ, accuracy, precision, carry-over, matrix effect, recovery	12.0–89.0
Teicoplanin [137]	Human serum	Ristocetin	Protein precipitation	Acquity UPLC BEH C18	Water + 1 g/l ammonium acetate and water/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ MRM	n.s.	Stability (autosampler), matrix effects, recovery, precision, LOQ, LOD	0–200
Teicoplanin A3-1, A2-1, A2-2, A2-3, A2-4, A2-5 [116]	Human plasma	Sulfa-methoxazole	Dilution	Cadenza HS-C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS, ESI+ SRM	6.5 min	Specificity, linearity, LOQ, precision, accuracy, matrix effect, stability (short-term, long-term, freeze-thaw)	1-50

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Teicoplanin A2-1, A2-2, A2-3, A2-4, A2-5 [138]	Human plasma	Vancomycin	Protein precipitation	Hypersil Gold C8, precolumn: TurboFlow Cyclone	Water/ formic acid (99.9/0.1) + 10mM ammonium acetate and methanol/ acetonitrile/ formic acid (49.95/49.95/0.1) + 10mM ammonium acetate and acetone/acetonitrile/2-propanol (1/1/1) Gradient	LC-MS Orbitrap, ESI+ Exact masses	10.7 min	Selectivity, linearity, precision, accuracy, LOQ, matrix effect, stability (autosampler)	0-100
Polymyxin B1, polymyxin B2 and polymyxin B1-1 [117]	Human plasma and urine	CB-182,753, a proprietary semi synthetic cyclic peptide	Automated solid phase extraction using Oasis HLB RP well plates	Waters XbridgeTM C8 UNIGUARD guard cartridge	Water/ formic acid (99.9/0.1) and methanol/ acetonitrile/ formic acid (49.95/49.95/0.1) Gradient	LC-MS-MS, ESI+ SRM	5.5 min	Linearity, accuracy, precision, specificity, recovery, matrix effects, stability (long-term), LOQ	Both: 0.005-2
Polymyxin B1 and B2 [123]	Human plasma	None used	Dilution and solid phase extraction using Oasis HLB cartridges	Atlantis C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS, ESI+ SRM	12 min	Accuracy, precision, specificity, recovery, matrix effects, LOQ, stability (short-term, long-term, freeze-thaw)	Both: 0.1-2.5
Polymyxin B1 and B2 [122]	Human and rat plasma and cation-adjusted Mueller-Hinton broth	Colistin B	Broth: protein precipitation and dilution plasma: protein precipitation, drying and reconstitution	Human plasma: XTerra MS C18 Others: XSelect CSH C18	Formic acid/ trifluoracetic acid/ water (0.5/0.01/99.5) and formic acid/ trifluoracetic acid/ acetonitrile/ methanol (0.5/0.01/49.75/ 49.75) Gradient	LC-MS-MS, ESI+ SRM	20 min	Linearity, accuracy, precision, recovery, specificity, matrix effects, stability (stock solutions, freeze-thaw), incurred sample analysis	CAMHB: 0.1-8.0µg/mL, rat and human plasma: 0.05-4.0µg/mL
Polymyxin B1, B2, B3 and isoleucine-polymyxin B1 [121]	Human plasma	None used	Protein precipitation and dilution	Zorbax Bonus-RP	Water/ formic acid / trichloroacetic acid (99.8/0.1/0.1) and water/ acetonitrile/ formic acid (10/98.9/0.1) Gradient	UPLC-MS-MS, ESI+ SRM	6.5 min	Linearity, recovery, matrix effects, accuracy, precision, stability (short-term, long-term, autosampler)	PB1 (50–5000 ng/ml); PB2 (10–1000 ng/ml); PB3 (5–500 ng/ml); Ile-PB1 (10–1000 ng/ml).
Ramoplanin [139]	Human dried blood spots	Teicoplanin	Liquid extraction	BEH phenyl	Water / formic acid (99.8/0.2) + 10 mM ammonium formate and acetonitrile/ formic acid (99.8/0.2) Gradient	LC-MS-MS ESI+	n.s.	Selectivity, matrix effects; recovery; LOQ, stability (in whole blood; in dried blood spots, autosampler)	0.01-5

Compound	Sample material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Dalbavancin [118]	Human plasma and urine	Homolog of dalbavancin	Protein precipitation, dilution	Fortis, Phenyl C18	Water/ formic acid (99.9/0.1), acetonitrile/ methanol (50/50) and acetone Gradient	LC-MS-MS, ESI+ MRM	3.5 min	Precision, accuracy, carryover, selectivity, stability (long-term, freeze–thaw)	Plasma: 0.5 – 500 urine: 0.05-50 in urine

n.s. = not specified, ESI+ = electron spray ionization in positive ion mode, ESI- = electron spray ionization in negative ion mode. $APCI^+$ = atmospheric pressure chemical ionization in positive ion mode, LOQ = lower limit of quantification, MRM = multiple reaction monitoring, SRM = single reaction monitoring, SIM= singe ion monitoring

Other antibiotics

Other often used antibiotics which could not be classified into any other group are described here and in table 9.

Fosfomycin

Fosfomycin is a phosphoenolpyruvate analogue antibiotic produced by *Streptomyces fradiae* [140]. Several methods for its detection have been recently published. The group around Parker at al. published two methods, one for the determination of fosfomycin in plasma and urine in a pharmacokinetic study in humans [141] and one regarding its detection in dried blood spots [142]. They used an isocratic HILIC method and ethylphosphonic acid as internal standard. The same standard was used by Papakondyli et al. [143], while Martens et al. used propylphosphonic acid [144] and two methods used deuterated fosfomycin [145, 146]. The paper by Papakondyli et al. stands out, because of the rare use of APCI and because they used a simultaneous extraction and derivatization step to convert fosfomycin into pentafluorobenzyl ester before analyzation.

Linezolid

Linezolid is an oxazolidinone antibiotic that works through inhibiting bacterial protein synthesis through rRNA-binding [147]. Linezolid is a frequently used drug in intensive care and several methods for its quantification have been published. Zander et al. presented a very well validated method using linezolid-d3 as internal standard and a fast isocratic separation on a ZB-C18 column [148]. La Marca et al. published a dried blood spot method without the use of any internal standard and insufficient validation of the influence of hematocrit or how to back calculate to the amount of blood used in each dried blood spot [149]. Souza et al. measured linezolid and its primary two metabolites to determine the influence of these metabolites on the toxicity observed in patients with renal impairment [150].

Metronidazole

Metronidazole is the most important drug of nitroimidazole antibiotics. All three found methods used liquid-liquid extraction for sample preparation [151–153]. While not only metronidazole itself, but also one of its main metabolites, hydroxymetronidazole shows antibacterial activity, only one group included hydroxymetronidazole in their method. Jeffery et al. [153] extracted metronidazole and its metabolite from human feces to research impact of metronidazole concentrations in *Clostridium difficile* treatments. The high lipid concentration in feces samples made an additional filtering and dilution step before analyzation necessary.

Tetracyclines

Four papers were found dealing solely with the determination of tetracyclines, mostly tigecycline, but also for the relatively new compound minocycline. Shao et al. included an overview over the published LC-MS/MS methods for tigecycline in biosamples in their method report but since their list was not comprehensive, we still included methods for tigecycline before 2018 here [17].

Mei et al. [154] detected tigecycline in cerebrospinal fluid as well as plasma, while Ozcimen et al. present a method for rabbit vitrous humour, aqeous humor and plasma to assess the spread of tigecycline into different eye compartements following i.v. administration [18]. For the determination of tigecycline by Ozcimen et al. no internal standard was used, while the other two papers used tigecycline-d9. In one case the sample preparation was also described insufficiently [18].

Minocycline in plasma for a pharmakokinetic study was measured by Araujo et al. They presented a very quick (3 min) isocratic separation method with a wide analytical range [155].

Lerbech and her group [81] analyzed several antibiotics, including tetracycline and doxycycline in human urine. They were looking for antibiotic residues and therefore their analytical range is very small as well and does not cover the therapeutic range. A further method including tigecycline is by Cazorla et al. [20].

Sulfonamides and trimethoprim

Bedor et al. compared a newly developed LC-MS/MS method to a HPLC-UV for determination of sulfamethoxazole and trimethoprim in plasma [156]. They found the LC-MS/MS method to be approximately six times more sensitive than the method with ultraviolet detection. Also, a much shorter run time and greater ruggedness could be achieved with the new method.

Simultaneous determination of trimethoprim and sulfamethoxazole in dried plasma and urine spots was presented by Gonzales et al. [157]. As dried plasma spot specific parameters they validated the influence of punch carryover and sample volume variation. One advantage of dried plasma spots over dried whole blood spots is the independence of hematocrit for the quantification. They compared their dried plasma and urine spot results with from fresh plasma and urine and found quite a great variance from -30 to +20%.

Besides sulfamethoxazole and trimethoprim, Dijkstra et al. measured the sulfamethoxazole metabolite sulfamethoxazole-N-acetyl. While their validation results were good, their calibration range of 0.2-10 mg/l is quite narrow and may not be sufficient for TDM [158].

Aminoglycosides

A paper published in 2015 [159] dealt with aminoglycosides in biological matrices. This detailed review describes published methods of all kinds to detect aminoglycosides in food stuff, the environment and human plasma or serum. Farouk et al. describe detection challenges, sample clean-up approaches and chromatographic options for LC-MS(/MS) but also includes non-chromatographic methods.

Since 2015, only two publications about aminoglycosides were published, even though they are routinely measured during treatment, probably due to the availability of enzyme assays that are widely established. Still, in multi-analyte methods amikacin, tobramycin and gentamicin can also be found: See also [20, 99, 127, 160] in "Methods testing for several compound classes".

Interesting is the paper by Lucha et al. [161] especially for their quantification of different gentamicin congeners. They characterized bought gentamicin standards by NMR-spectroscopy to determine the gentamicin congener concentrations. They also included specially synthesized gentamicin-glycerins for all analytes and included several kinds of serum and plasma, (K2-EDTA, K3-EDTA and lithium heparin plasma) in their validation. Dijkstra et al. described a method for amikacin and kanamycin using apramycin as internal standard. They also compared their results from 17 clinical samples with a validated immunoassay method and found very good comparability between both [162].

Table 9 Other antibiotics

Compound	Sample Material	Internal Standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Data	Analytical Range [mg/l]
Fosfomycin [141]	Human plasma and urine	Ethyl-phosphonic acid	Urine: filtration, dilution plasma: dilution, protein precipitation	SeQuant zic-HILIC, pre-column: SeQuant zic-HILIC guard	Acetonitrile/ water + 2mM ammonium acetate (pH 4.8) (85/15) Isocratic	LC-MS-MS ESI- MRM	n.s.	Matrix effects, LOQ. selectivity, precision, accuracy, stability (freeze-thaw, stock solution), recovery	Plasma: 1-2000 urine: 100-10000
Fosfomycin [142]	Dried human plasma spots	Ethyl-phosphonic acid	Liquid-extraction	SeQuant zic-HILIC, pre-column: SeQuant zic-HILIC guard	Acetonitrile/ water + 2mM ammonium acetate (pH 4.8) (85/15) Isocratic	LC-MS-MS ESI- MRM	n.s.	LOQ, selectivity, precision, accuracy, matrix effects, recovery, stability (storage and transport), sample spot volume	5–2000
Fosfomycin [143]	Human plasma	Ethyl-phosphonic acid	Dilution, derivatization, drying and reconstitution	ACE C18-PFP	Acetonitrile/ water (30/70) Isocratic	LC-MS APCI- SIM	n.s.	Stability (long-term, freeze-thaw, short-term. Autosamplers), selectivity, precision, accuracy, recovery, LOQ	0.05-12
Fosfomycin [146]	Human plasma, urine and aqueous fluid	Fosfomycin -13c3	Protein precipitation, dilution	Luna Omega PS C18	Water/ formic acid (99.95/0.05) + 20mM ammonium formate and methanol/ formic acid (99.95/0.05) +20mM ammonium formate Gradient	UPLC-MS-MS ESI- MRM	2 min	Precision, accuracy, LOQ, linearity, matrix effect, recovery, stability (freeze-thaw, autosamplers, long-term, short-term, autosampler), dilution integrity	Plasma: 12.5-800 urine: 62.5-4000 NaCl: 1-160
Fosfomycin [144]	Human plasma	Propyl-phosphonic acid	Protein precipitation	Atlantis HILIC silica	Ammonium formate/ formic acid/ water (0.1/0.05/99.9) and ammonium formate/ formic acid/ acetonitrile (0.1/0.05/99.9) Gradient	LC-MS-MS ESI- MRM	12.5 min	Precision, accuracy, LOQ, linearity, stability (long-term, short-term, autosampler), matrix effect	Two calibration ranges: 5-150 and 100-750
Fosfomycin [145]	Human plasma and urine	Fosfomycin -13c3 benzylamin e	Dilution and ultrafiltration	Acquity UPLC BEH Amide	Water +4mM ammonium formate (pH 7)/ acetonitrile (20/80) Isocratic	UPLC-MS-MS ESI- SRM	4 min	Precision, accuracy, LOQ, linearity, stability (long-term, short-term, autosampler, autosamplers), matrix effect, carry-over, recovery	0.75–375
Linezolid [149]	Human dried blood spots	None used	Liquid extraction	Zorbax Eclipse Plus C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ MRM	22 min	Precision, accuracy, recovery, stability (short-term, long-term), LOQ, sensitivity	1–100

Linezolid [148]	Human serum	Linezolid-D3	Protein precipitation	Kinetex XB-C18, Pre column: Waters Oasis HLB	Water/ acetonitrile/ formic acid (59.9/40/0.1) Isocratic	LC-MS-MS ESI+ MRM	4 min	Accuracy, precision, recovery, LOQ, selectivity, carry-over, stability (short-term, autosamplers, long-term, freeze-thaw)	0.13-32
Linezolid and two primary metabolites [150]	Human serum	Linezolid-d3	Protein precipitation	Waters X-bridge C18	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ MRM	15 min	Selectivity, precision, accuracy, LOQ, linearity, matrix effect, carry-over, recovery	Linezolid: 0.1-50 PNU-142300: 0.1-50 PNU-142586: 0.1-25
Metronidazole [152]	Human plasma	Zidovudine	Liquid–liquid extraction	Varian C18 Microssorb, pre-column: Phenomenex AJO-4287 C18	Acetonitrile/ water + 10mM ammonium acetate/ formic acid (79.9/20/0.1) Isocratic	LC-MS-MS ESI+ SRM	5 min	Selectivity, recovery, calibration curve, precision, accuracy, stability (freeze-thaw, short-term, autosamplers)	0.05–8.00
Metronidazole [151]	Human plasma	Metro-nidazole-d4	Liquid–liquid extraction	ACE C18	Acetonitrile/ water + 10mm ammonium + formic acid, (pH 4) (80/20) Isocratic	LC-MS-MS Ionization method not specified + SRM	n.s.	Incurred sample analysis, calibration curve, precision, accuracy, carry-over, stability (short-term, short-term), dilution integrity	0.01-10
Metronidazole, hydroxy-metronidazole [153]	Human faecal samples	Zidovudine	Liquid extraction, filtration, and dilution	Waters Acquity UPLC BEH C18	Water/ formic acid (99.9/0.1) +2 mM ammonium acetate and methanol/ formic acid (99.9/0.1) + 2 mM ammonium acetate Gradient	UPLC-MS-MS ESI+ MRM	3 min	Calibration curve, precision, accuracy, recovery, specificity, carry-over, stability (long-term, short-term)	0.066-30
Sulfamethoxazole and trimethoprim [156]	Human plasma	Benz-nidazole	SPE on Waters Oasis HLB cartridges	Purospher® star C18 Pre-column: C18 security guard	Acetonitrile/ water (50/50) Isocratic	LC-MS-MS ESI+ SRM	2.5 min	Selectivity, recovery, accuracy, precision, stability (freeze-thaw, short-term, autosampler, long-term)	Sulfamethoxazole: 0.5–60.0 trimethoprim: 0.05–5.0
Trimethoprim, sulfa-methoxazole [157]	Dried human plasma und urine spots	Trimetho-prim-D3, Sulfa-methoxazole-D4	DPS and DUS: liquid extraction, dilution	ACE PFP	Water/ formic acid (99.9/0.1) and acetonitrile/ formic acid (99.9/0.1)	LC-MS-MS ESI+ MRM	n.s.	Selectivity, accuracy, precision, recovery, matrix effect, carryover, stability (long-term, autosamplers) punch carryover, sample volume variation	Trimethoprim: DPS: 0.1-50 and DUS: 0.5–250 sulfamethoxazole: DPS and DUS: 1–500
Sulfa-methoxazole, Sulfa-methoxazole-N-acetyl, trimethoprim [158]	Human serum	Sulfametho xazole-d4, tri-methoprim -d9, Sulfa-methoxazol e-N-acetyl-d4	Protein precipitation	Hypurity Aquastar C18	Ammonium acetate (5.0 ag/l), acetic acid (100%, 35 ml/l) and trifluoroacetic acid (100%, 2 ml/l)	LC-MS-MS ESI+ SRM	3 min	Selectivity, accuracy, precision, stability (freeze-thaw, short-term, autosampler), recovery, matrix effects	All: 0.2-10

Tigecyclin [154]	Human plasma and cerebrospinal fluid	Tigecyclin-d9	Plasma: Protein precipitation CSF: Protein precipitation and dilution	Kromasil C18	Water/ trifluoroacetic acid (99.9/0.1) and acetonitrile/ methanol/ trifluoroacetic (75/25/0.1) Gradient	LC-MS-MS, ESI+ SRM	6 min	Selectivity, LOQ, carry-over, linearity, recovery, matrix effect, accuracy, precision, stability (freeze-thaw, short-term, long-term, autosampler)	Plasma: 0.025-2 cerebrospinal fluid: 0.25-100
Tigecyclin [17]	Human plasma	Tigecyclin-d9	Protein precipitation, drying and redissolving	Waters Acquity UPLC® BEH-C18	Water/ formic acid (99.8/0.2) + 10 mm ammonium formate and acetonitrile Gradient	UPLC-MS-MS, ESI+ SRM	4.5 min	Selectivity, carry-over, linearity, sensitivity, accuracy, precision, matrix effect, recovery, stability (freeze-thaw, short-term, long-term, autosampler)	0.01-5
Tigecyclin [18]	Rabbit vitrous humour, aqeous humor and plasma	None used	SPE, drying and reconstitution	Inertsil ODS-4	Water with formic acid (99.9/0.1)/ acetonitrile with formic acid (99.9/0.1) Gradient/ Isocratic n.s.	LC-MS-MS (ionization mode n.s.)+ MRM	n.s.	n.s.	0.01-15
Minocycline [155]	Human plasma	Clarithromycin	Liquid–liquid extraction	Zorbax RX-C8	Acetonitrile/ water/ trifluoracetic acid (80/19.9/0.1) Isocratic	LC-MS-MS ESI+ SRM	3 min	Linearity, precision and accuracy, recovery, LOQ	0.005-2
Gentamicin C1, C1a, C2, C2a and C2b [161]	Human serum and plasma, (K2-EDTA, K3-EDTA and lithium heparin plasma)	Self-made gentamicin-glycines	Dilution, protein precipitation	XSelect HSS PFP	Water/ trifluoroacetic acid (99.93/0.07) and acetonitrile Gradient	LC-MS-MS ESI+ SRM	16 min	Linearity, matrix effects, precision, accuracy, specificity, LOQ, stability (autosampler)	Total gentamicin: 0.13-15.2
Amikacin and kanamycin [162]	Human serum	Apramycin	Protein precipitation	HyPURITY C18	Water/ heptafluoro-butyric acid anhydride (99/1) and water and methanol Gradient	LC-MS-MS ESI+ SRM	6 min	Selectivity, accuracy, precision, linearity, recovery, matrix effects, stability (short-term, autosampler), dilution integrity	Amikacin: 0.25-25 kanamycin: 0.1-25

n.s. = not specified, ESI+ = electron spray ionization in positive ion mode, ESI- = electron spray ionization in negative ion mode. APCI$^+$ = atmospheric pressure chemical ionization in positive ion mode, LOQ = lower limit of quantification, MRM = multiple reaction monitoring, SRM = single reaction monitoring, SIM= singe ion monitoring

Methods testing for several compound classes

Multi-analyte methods have the advantage of providing fast results for a wider variety of analytes. This is especially relevant in clinical situations or in environmental exposure control as opposed to in clinical or pharmacokinetic studies, were only a certain set of analytes must be evaluated. Therefore, it is not surprising that most of the published methods including several classes of antibiotics were evaluated for a clinical use. The methods presented here contain between two and twenty-one analytes. The most common antibiotics featured are the beta-lactams piperacillin, amoxicillin, meropenem, cefepime and ceftazidime as well as vancomycin, linezolid, and ciprofloxacin.

The most extensive methods is the one by Cazorla-Reyes et al. from 2014 [20]. They reported an ultra-high-performance liquid chromatography-(UHPLC)-MS/MS method for 21 antibiotics in human serum, urine, cerebrospinal fluid and bronchial aspiration. Since their MS could not be run in alternating ionization mode, two runs had to be performed on each sample with slightly different gradients; one in ESI positive mode and one in ESI negative mode to detect clavulanic acid and sulbactam. They used only a short protein precipitation for sample preparation and since the two runs needed for each sample could be done in approximately 12 minutes, their presented method is still quite fast. Their method does have some drawbacks; they had to accept up to 20% deviation from the nominal value for all their samples and measured strong matrix effects for several analytes. They also did not use any internal standards, which may explain their quite large deviation results. The analytical range in this method varies from 0.1 to 5 mg/l for analytes in all matrices and is therefore quite narrow.

The group around Barco published a method for 13 quite diverse antibiotics and tazobactam using a Accucore Polar Premium column and deuterated standards for nearly all their analytes [127]. They used two different protein precipitation methods, depending on the analyte, and an additional dilution step was needed for three analytes. Therefore, not all 14 analytes have been measured in one run. They did not explain why two different approaches were needed but stated, that the extra dilution step was needed to reach acceptable linearity. The method was very well validated including extensive stability data.

Lefeuvre et al. published a method for 15 antibiotic drugs using a full scan approach on a quadrupole-orbitrap mass spectrometer [163]. Triple-quadrupole devices were used in nearly all other of the published methods. Lefeuvre presented a real multi-analyte method for all analytes, since the same sample preparation (protein precipitation and dilution) and only one run was needed. They claim the method to be easily expandable and could present very good data in terms of precision and accuracy. In comparison to the other two methods with over 10 analytes per

method, they did not include any peptide antibiotics and their analytical range of 0.5 to 32 mg/l does not sufficiently cover the expected concentrations of all antibiotics.

Generally, C18 columns seem to be the most versatile separation option and were used in most of the multi-analyte methods, especially those including many analytes and including both small and peptide-based antibiotics. Still, a wide range from C8 [80] to phenyl endcapped [164] columns were employed. Nearly half of all methods here used deuterated analytes for internal calibration, but their use seems to have risen in recent years, probably due to the broad availability of deuterated standards for most antibiotics nowadays. Many of the non-deuterated standards used were drugs with clinical use, like dicloxacillin [47, 49], oxacillin [48] or hydroxycarbazepine [50], which are not good choices in any setting outside of controlled studies.

The majority of methods measured plasma or serum, while three included in urine [20, 79, 81]. One method assessed the penetration of metronidazole and spiramycin I into human plasma, saliva and gingival crevicular fluid [21]. Two methods were used to detect antibiotics in human tissue samples [22, 125], which is quite common for analysis of food stuff, but seldomly done in humans. Kraft et al. [125] detected antibiotic residues in stored allograft cells and did therefore not bring their method to a clinical use. Barco et al. adopted their method published in 2015 and presented a volumetric absorptive microsampling method for the simultaneous quantification of four antibiotics in 10µL blood [165]. In contrast to their dried blood spot method, it allowed an accurate quantification without hematocrit influence.

Protein precipitation clearly seems to be the method of choice when detecting diverse analytes from biological matrices. Solid phase extraction was only used by Tuerk et al. and Lerbech et al., both groups to detect antibiotics from urine [79, 81]. Szultka et al. tested three different sample preparation techniques: protein precipitation, solid phase extraction and microextraction in a packed syringe. Between the sample preparation techniques, microextraction in a packed syringe provided the best results [166]. They also compared an HPLC-UV to their LC-MS/ method and found lower LOD and LOQ values for the UV method.

The method by Zander et al from 2015 is worth mentioning since they used a semi-automated sample preparation approach [164], while Jourdil et al. did not only measure five antibiotics, but three antifungals, an antineoplastic agent (imatinib), and an antiretroviral (raltegravir) in plasma, making it one of the most diverse methods presented here [66].

Chen et al. gave an overall interesting look into method development in their paper for cefepime, meropenem, piperacillin, tazobactam, daptomycin and vancomycin by examining the influence of formic acid concentration on the peak intensity of their analytes [124]. They found that even a small concentration of as low as 0.1 mM formic acid increased signal strength greatly.

Table 10 Methods testing for several compound classes

Compound	Sample Material	Internal standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Parameters	Analytical Range [mg/l]
Cefepime, meropenem, piperacillin, tazobactam, daptomycin, vancomycin [124]	Human plasma	Cefepime-d3 Meropenem-d6 Piperacillin-d5 (no IS used for tazobactam and vancomycin)	Protein precipitation	ZORBAX Eclipse Plus C18	Formic acid/ water (0.1/99.9) and formic acid/ acetonitrile (0.1/99.9) Gradient	LC-MS-MS ESI+ MRM	6 min	Precision, accuracy, selectivity, stability (short-term, long-term, freeze-thaw, autosampler), incurred sample analysis	All: 0.2-50
Piperacillin-tazobactam, meropenem, linezolid, ceftazidime [167]	Human plasma	Prazosin	Protein precipitation and filtration	Kinetex C18 column	Formic acid/ water (0.1/99.9) and acetonitrile Gradient	LC-MS-MS ESI+ and ESI- MRM	5.5 min	Precision, accuracy, selectivity, LOQ, matrix effect, recovery, stability (short-term, long-term, freeze-thaw, short-term)	Piperacillin, ceftazidime: 3.125-200 tazobactam, meropenem, linezolid: 1.25-40
Piperacillin-tazobactam, meropenem, linezolid, ceftazidime [165]	Volumetric absorptive micro-sampling and DBS	Piperacillin-d5	Re-hydration and protein precipitation	Kinetex C18 column	Formic acid/ water (0.1/99.9) and acetonitrile Gradient	LC-MS-MS ESI+ and ESI- MRM	5.5 min	Precision, accuracy, selectivity, LOQ, matrix effect, recovery, carry-over, stability (short-term, long-term, freeze-thaw), hematocrit,	Piperacillin, ceftazidime: 3.125-200 tazobactam, meropenem, linezolid: 0.625-40
Vancomycin, teicoplanin A2-1, A2-2, A2-3, A2-4, A2-5, A3-1, meropenem, voriconazole [50]	Human plasma	10-hydroxy-carbazepine	Protein precipitation, dilution	Agilent Zorbax SB-C18	Formic acid/ water (0.1/99.9) and acetonitrile Gradient	LC-MS-MS ESI+ MRM	5 min	Precision, accuracy, selectivity, LOQ, matrix effect, recovery, stability (long-term, freeze-thaw, autosamplers),	Meropenem: 0.3-30.0 teicoplanin, vancomycin: 1.0-100 voriconazole: 0.3-10.0
Amoxicillin, metronidazole [168]	Human plasma	Metronidazole-d4, ampicillin	Protein precipitation	ZIC-HILI and ZIC-HILI pre-column	Formic acid/ water (0.1/99.9) and formic acid/ acetonitrile (0.1/99.9) Gradient	LC-Orbitrap ESI+ SIM of exact masses	20 min	Selectivity, accuracy, precision, calibration, lower limit of quantification (LOQ), extraction recovery and matrix effect	Both: 0.1-6.4
Cefoxitin, vancomycin, lincomycin, polymyxin B [125]	Decellular-ized tissue-engineered heart valves	None reported	Liquid extraction and filtration	Synergi 4µ Max RP C12	Formic acid/ water (0.1/99.9) 5mm ammonium acetate and formic acid/ methanol (0.1/99.9) 5mM ammonium acetate Gradient	LC-MS-MS ESI+ MRM	15 min	n.s.	All: LOD 0.2 mg/kg

Compound	Sample Material	Internal standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Parameters	Analytical Range [mg/l]
Ampicillin, cefuroxime, ciprofloxacin, meropenem, metronidazole, piperacillin, rifampicin, tazobactam [49]	Human plasma	Dicloxacillin piperacillin-d5	Protein precipitation	Kinetex F5 core–shell RP and F5 pre-column	Formic acid/ water (0.1/99.9) and formic acid/ acetonitrile (0.1/99.9) Gradient	LC-MS-MS ESI+ and ESI- MRM	2 runs: each 10 min	Sensitivity, specificity, linearity, accuracy, precision, dilution integrity, carry-over, recovery, matrix effects, stability (stock solution and working solution, freeze-thaw, autosamplers)	All: 1-100
Linezolid, meropenem, piperacillin, teicoplanin [126]	Human plasma	Meropenem-d6, piperacillin-d5, linezolid-d8, daptomycin	Liquid-liquid extraction and dilution	MassTox TDM C18	Formic acid/ water (0.1/99.9) and formic acid/ methanol (0.1/99.9) Gradient	LC-MS-MS ESI+ MRM	7 min	Precision, accuracy, selectivity, LOQ, sensitivity, matrix effect, recovery, stability (short-term, long-term, freeze-thaw), dilution integrity	Linezolid, meropenem, piperacillin: 1.0-45.0 teicoplanin: 1.75-63.0
Amikacin, amoxicillin, ceftazidime, ciprofloxacin, colistin, daptomycin, gentamicin, linezolid, meropenem, piperacillin, teicoplanin, tigecycline, tobramycin, vancomycin, tazobactam [127]	Human plasma	Amoxicillin-d4, ceftazidime-d5, ciprofloxacin-d8, daptomycin-d5, linezolid-d3, meropenem-d6, piperacillin-d5, tigecycline-d9, tobramycin-d11/d12, vancomycin-d12	Protein precipitation	Accucore Polar Premium	Formic acid/ water (0.1/99.9) and formic acid/ acetonitrile (0.1/99.9) Gradient	LC-MS-MS HESI+ and HESI- MRM	5 min	Selectivity, linearity, carry over, calibration curve, accuracy, precision, LOQ, stability, dilution integrity, incurred sample reanalysis, matrix effect, recovery	Amikacin, amoxicillin, tazobactam, tobramycin, gentamicin, linezolid: 0.4-40 ceftazidime, daptomycin, piperacillin: 2-200 meropenem, teicoplanin, tigecycline, vancomycin: 1-100 ciprofloxacin: 0.1-10 colistin A: 0.3-26 colistin B: 0.5-54
Amoxicillin, levofloxacin, amikacin, ceftriaxone, vancomycin, ampicillin, tobramycin, tazobactam, piperacillin, ceftazidime, linezolid, imipenem, sulbactam, cefepime, moxifloxacin, teicoplanin, clavulanic acid, meropenem, tigecycline, daptomycin, clarithromycin [20]	Human urine, serum, cerebro-spinal fluid and bronchial aspiration lavage	None	Urine: protein precipitation Serum: protein precipitation Cerebrospinal fluid: dilution Bronchial aspiration lavage: dilution with dl-dithiothreitol	Acquity UPLC BEH C18	methanol and formic acid/ water (0.1/99.9) Gradient	LC-MS-MS ESI+ and ESI- MRM	2 runs: 6 min and 5.8 min	Precision, accuracy, recovery, matrix effect, selectivity, sensitivity, LOQ, stability (long-term, autosampler)	serum and urine, all: 0.1-5

Compound	Sample Material	Internal standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Parameters	Analytical Range [mg/l]
Piperacillin, tazobactam, cefepime, meropenem, ciprofloxacin, linezolid [164]	Human serum	Cefepime-13c,d3, ciprofloxacine-d8, meropenem-d6, linezolid-d3	Protein precipitation	Acquity UPLC BEH Phenyl Pre-column: Oasis HLB	Formic acid/ water (0.1/99.9), methanol/ acetonitrile (75/25) and methanol/ acetonitrile (80/20) Gradient	LC-MS-MS ESI+ MRM	5 min	Accuracy, precision, selectivity, matrix effect, recovery, stability (short-term, long-term), carry over, LOQ	Piperacillin: 0.5-60 tazobactam: 0.25-20 cefepime: 0.13-50 meropenem: 0.25-50 ciprofloxacin: 0.05-8 linezolid: 0.1-32
Daptomycin, ciprofloxacin, oxacillin, levofloxacin, rifampicin [66]	Human plasma	Levofloxacine-13c-d3, voriconazole-d3, imatinib-d8	Protein precipitation	Kinetex PFP Pre-column: POROS R1	Formic acid/ water (0.1/99.9) + 10 mM ammonium formate (ph 3.0), formic acid/ acetonitrile (0.1/99.9) and formic acid/ methanol (0.1/99.9) Gradient	LC-MS-MS ESI+ MRM	5 min	Recovery, matrix effect, accuracy, precision, selectivity, stability (short-term, long-term), LOQ	daptomycin: 0.6-120 ciprofloxacin: 0.04-8 levofloxacin: 0.04-8 oxacillin: 0.2-40 rifampicin: 0.2-40
Daptomycin, amikacin, gentamicin, rifampicin [99]	Human plasma	Quinoxaline	Protein precipitation	Synergy 4µ Hydro-RP pre-column: C18	Formic acid/ water (0.05/99.95) and formic acid/ acetonitrile (0.05/99.95)	LC-MS-MS ESI+ SRM	20 min	Recovery, matrix effect, accuracy, precision, selectivity, stability (long-term, short-term, autosamplers, freeze-thaw), LOQ, sensitivity	Daptomycin: 1.6-130 amikacin: 2.3-150 gentamicin: 0.6-40 rifampicin:0.6-40
Efazoline, cefotiame, cefuroxime, chloramphenicol, ciprofloxacin, ofloxacin, sulfamethoxazole, trimethoprim [79]	Human urine	None	SPE on a Bakerbond C18 cartridge, filtration	Nucleodur C18 EC	Formic acid/ water (0.1/99.9) and formic acid/ acetonitrile (0.1/99.9)	LC-MS-MS ESI+ and ESI- MRM	5.8 min	Accuracy, precision, recovery, matrix effect, calibration, sensitivity	All: 0.02-5
Amoxicillin, ampicillin, metronidazole, cefuroxime, ciprofloxacin, trimethoprim, sulfamethoxazole, tetracycline, doxycycline [81]	Human urine	Trimethoprim-d3, ciprofloxacin-8, penicillin-d5, sulfamethoxazole-d4	SPE on an Oasis HLB cartridge, drying, reconstitution	XTerra MS C18 Pre-column: XTerra MS RP18 guard	Water /acetonitrile/ triethylamine/ formic acid (989.8/10/0.1/0.1) + 0.35 g ammonium formate and Water/acetonitrile/ triethylamine/ formic acid (50/949.8/0.1/0.1) + 0.35 g ammonium formate	LC-MS-MS ESI+ and ESI- MRM	20 min	LOQ, sensitivity	All: 0.1-5

Compound	Sample Material	Internal standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Parameters	Analytical Range [mg/l]
					Gradient				
Ampicillin, piperacillin, tazobactam, meropenem, metronidazole [47]	Human plasma	Dicloxacillin	Protein precipitation	Aquasil C18 and Ultra Aqueous C18	Formic acid/ water (0.1/99.9), and formic acid/ methanol (0.1/99.9) Gradient	LC-MS-MS ESI+ and ESI- MRM	2 runs, 6min and 5min	LOQ, LOD, matrix effect, Recovery, accuracy, precision, selectivity, stability (long-term, short-term, autosamplers, freeze-thaw)	Ampicillin: 0.3-150 piperacillin: 0.3-150 tazobactam: 0.15-75 meropenem 0.1-50 metronidazole: 0.05-25
Neomycin, bacitracin [169]	Human and rabbit serum	Kanamycin	Protein precipitation	Luna C18	Water + 20mM formic acid and 10 mM nonafluoro-pentanoic acid and methanol + 20 mM formic acid and 10 mM nonafluoro-pentanoic acid Gradient	LC-MS ESI+ SIM	n.s.	Stability (long-term, short-term, autosamplers, freeze-thaw), accuracy, precision, selectivity, recovery, carry over, LOQ, LOD	0.2-50
Spiramycin I, metronidazole [21]	human plasma, saliva and gingival crevicular fluid	Ornidazole	Plasma: liquid–liquid extraction, drying, reconstitution	Kromasil C18	Acetonitrile/ water/ formic acid (15/85/0.1) and acetonitrile/ water/ formic acid (50/50/0.1) Gradient	LC-MS-MS ESI+ SRM	10min	Selectivity, recovery, matrix effect, stability (freeze-thaw, short-term, autosamplers), accuracy, precision	Plasma: metronidazole: 0.05-5 spiramycin: 0.015-2
Linezolid, amoxicillin [166]	Human plasma	None for linezolid, Gemifloxacin for amoxicillin	Protein precipitation or SPE on C2 Chromabond cartridges or microextraction in packed syringe	Linezolid: ACE C8 Amoxicillin and gemifloxacin: ACE C18	Linezolid: methanol/ water + 5 mM ammonium acetate (50/50) amoxicillin: methanol/ water/ formic acid (10/89.9/0.1) Gradient	LC-MS-MS ESI+ SRM	n.s.	Precision, accuracy, recovery, matrix effects, LOQ, LOD, stability (autosamplers)	Linezolid: 1–30 amoxicillin: 1–50
Amoxicillin, meropenem, cefazolin, cefotaxime, deacetyl-cefotaxime, ceftriaxone, vancomycin [48]	Human plasma	Oxacillin	Protein precipitation, drying, reconstitution	Acquity UPLC BEH C18	Water/ formic acid (99.9/0.1) and methanol/ formic acid (99.9/0.1) Gradient	LC-MS-MS ESI+ MRM	3.5min	Accuracy, precision, matrix effects, selectivity, LOQ, stability (autosamplers, freeze-thaw, short-term, long-term, short-term)	Vancomycin: 0.7-70 amoxicillin: 0.2-80 meropenem: 0.80 cefazolin: 0.5-250 cefotaxime: 0.2-100 deacetylcefotaxime: 0.2-100 ceftriaxone: 2-360

Compound	Sample Material	Internal standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Parameters	Analytical Range [mg/l]
Amoxicillin, flucloxacillin, piperacillin, benzylpenicillin, clindamycin, clavulanic acid, tazobactam [22]	Human plasma, ultrafiltrate and tissue	Amoxicillin-d4, flucloxacillin-13C4,15N piperacillin-d5	Free fraction: ultrafiltration All: Protein precipitation	Accucore XL C18 Precolumn: TurboFlow MAX	Water +10 mM ammonium carbonate (pH 8) and methanol/ acetonitrile/ formic acid (49.95/49.95/ 0.1) + 10 mM ammonium acetate and acetonitrile/ isopropanol/ acetone (33/33/33) Gradient	LC-MS-MS ESI+ and ESI- MRM	10.25 min	Accuracy, precision, recovery, matrix effects, selectivity, carry-over, stability (autosampler, short-term, long-term, stock solutions)	Tissue: 0.2–25 mg/kg free fraction of flucloxacillin and clindamycin: 0.05–20 amoxicillin, piperacillin: 3.125–125 clavulanic acid, tazobactam: 1–40 benzylpenicillin: 0.25–40 flucloxacillin: 1.5–60 clindamycin: 0.05– 8
Trimethoprim-sulfamethoxazole, ciprofloxacin, ertapenem, cefaclor, cefazolin, cefpodoxime, ceftazidime, cefuroxime [52]	Human plasma, ultrafiltrate	Trimethoprim-d9, sulfamethoxazole-d4, ciprofloxacin-d8	Protein precipitation free fraction: ultrafiltrate	Phenomenex Synergi 12C pre-column TurboFlow Cyclone	Water/ formic acid (99.9/0.1) + 10 mM ammonium acetate and methanol/ acetonitrile/ formic acid (49.95/49.95/ 0.1) + 10 mM ammonium acetate and acetonitrile/ isopropanol/ acetone (33/33/33) Gradient	LC-MS-MS ESI+ MRM	9.2 min	Accuracy, precision, recovery, matrix effects, selectivity, carry-over, stability (autosampler, short-term, long-term, stock solutions)	Trimethoprim: 0.125-25 sulfamethoxazole: 1.5-300 ciprofloxacin: 0.5-10 ertapenem: 0.5-100 cefaclor: 0.25-25 cefazolin: 1-100 cefpodoxime: 0.1-10 ceftazidime: 1-100 cefuroxime: 1-100
Cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid, piperacillin [80]	Human serum	Meropenem-d6, ciprofloxacin-d8, moxifloxacin hydrochloride-13c1,d3, linezolid-d3, and piperacillin-d5, cefepime-13c1,d3	Protein precipitation, dilution	Fortis 3µm C8	Water/-formic acid (99.9/0.1) +10mM ammonium formate and acetonitrile. Gradient	LC-MS-MS ESI+ MRM	4 min	Linearity, specificity, LOQ, accuracy, precision, carry-over, selectivity, dilution integrity, recovery, matrix effect, stability (autosampler, short-term, long-term, stock solutions, freeze-thaw)	Cefepime: 0.25–200 meropenem: 0.25–120 ciprofloxacin: 0.05–10 moxifloxacin: 0.125–10 linezolid: 0.125–50 piperacillin: 0.5–400
Amikacin, gentamicin, vancomycin [160]	Human plasma	Kanamycin B	Protein precipitation	Hypurity Aquastar C18 polar endcapped	Water, acetonitril and water with 200mM perfluoro-pentanoic acid and 130mM ammonium acetate Gradient	LC-MS-MS, ESI+ MRM	7.5 min	Selectivity, accuracy, precision, recovery, matrix effect, stability (long-term, autosamplers, freeze–thaw, short-term, stock solution), LOQ	Amikacin and gentamicin: 0.3–50 vancomycin:1.0–100

Compound	Sample Material	Internal standard	Sample preparation	Stationary Phase	Mobile Phase	Detection	Run time	Validation Parameters	Analytical Range [mg/l]
Amoxicillin, azithromycin, cefotaxime, ciprofloxacin, meropenem, metronidazole, piperacillin [170]	Human serum	Amoxicilline-d4, azithromycin-d5, cefotaxime-d3, ciprofloxacin-d8, meropenem-d6, metronidazole-d4, piperacilline-d5	Protein precipitation, drying and reconstitution	Acquity UPLC HSS T3	Water +5 mM ammoniumacetate (pH 2.4)and acetonitrile/ formic acid (99.9/0.1) Gradient	UPLC-MS-MS, ESI+ SRM	2.75 min	Selectivity, accuracy, precision, linearity, recovery, matrix effect, dilution integrity, stability (long-term, freeze–thaw, short-term, autosampler)	All: 0.1-50
Amoxicillin, oxacillin, piperacillin, ticarcillin, cefepime, cefotaxime, ceftazidime, ceftriaxone, ertapenem, imipenem, meropenem, clindamycin, ofloxacin ciprofloxacin, tazobactam [163]	Human serum	Cefepime-d3, cefotaxime-d3, ciprofloxacin-d8, clindamycin-d3, levofloxacin-d8, meropenem-d6, piperacillin-d5	Protein precipitation, dilution	Accucore C18	Formic acid/ acetonitrile (0.1/99.9) + 2mm ammonium formate and formic acid/ acetonitrile (0.1/99.9) Gradient	UPLC-MS (Orbitrap), ESI+ Accurate masses were measured	9 min	Selectivity, specificity, accuracy, precision, linearity, recovery, matrix effect, dilution integrity, stability (long-term, freeze–thaw, short-term, autosampler, stock solutions), carry-over, incurred sample analysis	All: 0.5-32

n.s. = not specified, ESI+ = electron spray ionization in positive ion mode, ESI- = electron spray ionization in negative ion mode. $APCI^+$ = atmospheric pressure chemical ionization in positive ion mode, LOQ = lower limit of quantification, MRM = multiple reaction monitoring, SRM = single reaction monitoring, SIM= singe ion monitoring

Discussion

In most TDM applications, LC-MS(/MS) is considered the gold standard for quantitative measurements, not only for antibacterial drugs, but also for a wide range of other clinically relevant parameters. The number on published methods on analytical methods for quantitative measurement of antibacterial drugs is steadily increasing. While there are guidelines available [4, 5] and most authors claim their methods were validated according to one or both of the guidelines, it is rarely comprehensively done so. In most cases not the complete set of validation parameters is tested, or not in the recommended repetitions. Often also the recommended testing of lipemic, hemolyzed and icteric samples is omitted (or not mentioned) as well as the use of different batches of matrices to evaluate matrix effects (EMA guidelines only). In addition, stability data should conclusively show the stability of samples in clinical situations and not only focus on laboratory conditions. More recent papers show a much greater adherence to the guidelines.

The most prominent detection method is the use of LC-MS/MS coupled to ESI in positive mode, while APCI applications and negative mode are the exception. These alternatives should nonetheless be kept in mind, as some substances are hard to detect in ESI positive mode, as is the case with clavulanic acid or fosfomycin. Even though TDM of some antibiotics, especially aminoglycosides, is done routinely, many of the published methods were not used for TDM but were reported as part of bioequivalence studies or residue determinations. Many were developed for use in study situations, other methods were used in the clinical setting, which might explain the use of common drugs as analytical standards in some. The newest publications clearly show an increased use of deuterated standards, probably owing to the commercial availability of more deuterated antibiotics since then. Regarding sample preparation, protein precipitation is the most widely used form, and sometimes even combined with liquid-liquid extraction or solid phase extraction. New forms of sample preparation like molecular imprinting solid phase extraction or supported liquid membranes are still very rare, but offer exciting alternatives, as they might be able to lower detection levels and increase sensitivity.

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RESEARCH PAPER

A 2D HPLC-MS/MS method for several antibiotics in blood plasma, plasma water, and diverse tissue samples

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Abstract

An analytical method using 2D high-performance liquid chromatography followed by tandem mass spectrometry for the quantification of the beta-lactam antibiotics amoxicillin, flucloxacillin, piperacillin, benzylpenicillin, the beta-lactamase inhibitors clavulanic acid, and tazobactam, as well as the macrolide antibiotic clindamycin, is presented. All analytes were measured in human plasma, while amoxicillin, clavulanic acid, flucloxacillin, and clindamycin were also analyzed in human tissue samples. Because of its high-protein binding, additionally, the free fraction of flucloxacillin was measured after ultrafiltration. As internal standards, deuterated forms of the beta-lactams were used. Sample preparation for all matrices was protein precipitation followed by online extraction on a TurboFlow MAX column, while sample separation was performed on an Accucore XL C18 column. Calibration curves were linear over 0.2–25 mg/kg for the tissue samples and 0.05–20 mg/l for the free fraction of flucloxacillin. In plasma, the calibration curves for amoxicillin and piperacillin were linear over 3.125–125 mg/l, for clavulanic acid and tazobactam over 1–40 mg/l, for benzylpenicillin 0.25–40 mg/l, and for flucloxacillin and clindamycin over 1.5–60 mg/l and 0.05–8 mg/l respectively. In plasma and plasma ultrafiltrate, inaccuracy and imprecision for any analyte were always less than 15%. In tissue, the accuracy and precision varied up to 16%, respectively, 20%, when various tissues were analyzed using a calibration in water.

Keywords Therapeutic drug monitoring · Tandem mass spectrometry · Chromatography · High-pressure liquid · Clindamycin · Beta-lactams · Beta-lactamase inhibitors

Introduction

Evidence is growing that therapeutic drug monitoring (TDM) of antibiotics should not be restricted to those drugs traditionally monitored due to their high toxicity (aminoglycosides and glycopeptides) but should also be expanded to a wider range of antibiotic classes, especially the beta-lactams. Not only the toxicity of the drug is a valid reason to monitor antibiotic plasma levels, also the attainment of sufficiently high levels should be assisted by TDM [1]. Especially since antimicrobial resistances are spreading rapidly, and minimal inhibitory concentrations of most bacteria are rising, TDM has become a tool to ensure the efficiency of antibiotic treatment.

Many critically ill patients receive antibiotic courses over weeks or even months. During that time, changes in their renal clearance, hepatic function or the use of supportive extracorporeal therapies like continuous renal replacement therapy, can alter drug levels unpredictably [2]. The prediction of plasma levels, especially of the free, unbound fraction, therefore remains difficult. Since only the free fraction of a drug is pharmacologically active, target levels are always based on the free concentration [3]. For most, only moderately bound drugs, free concentrations can be estimated from the total plasma concentration with ease. For drugs with higher protein binding, already a small change in the concentration of the binding protein leads to a significant rise or decrease of the unbound fraction. Therefore, the quantification of the unbound fraction for drugs with high-protein binding increases the efficacy of TDM. Flucloxacillin is bound to mostly albumin at about 93–96% in healthy and moderately ill people [4, 5]. Clindamycin is bound at about 94% [6]. Since these two drugs are highly bound to proteins and their target population is often severely ill, with a high risk for changes in plasma

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protein levels and drug binding, measurement of the free fraction is more informative than measuring total plasma levels [7].

In a bacterial infection, apart from sepsis, the site of colonization is not the blood, even though this is the compartment in which drug levels are measured. Drug levels in blood do therefore not necessarily represent drug levels at the target site [8]. In many cases, the site of infection is actively separated from blood, like the brain and cerebrospinal fluid [9]. In addition, joint capsules and even encapsulations caused by the bacteria themselves can lead to reduced penetration of drugs into the site of infection. Reversely, inflammation and bacterial invasion mechanisms often lead to increased permeability of barriers [10–12]. Measurement of tissue material can therefore bring insight into drug levels at target site.

Various methods measuring beta-lactams in plasma, plasma water, or tissue have been published. Several methods describe the quantification of some of the here presented analytes in plasma, but not tissue [13–16]. Many tissue related publications deal with the quantification of veterinary antibiotic residues in animals [17–19]. They usually use a great amount of tissue material and have lengthy extraction procedures unsuitable for unstable compounds such as clavulanic acid. Other methods were only validated for bone material [20]. Several papers have been published describing the determination of one or two antibiotics in human tissue [21–26]. Martin et al. presented a HPLC-UV method for measuring amoxicillin and clavulanic acid in fatty tissue and colonal wall tissue [21]. A method for measuring benzylpenicillin with amoxicillin and ampicillin was published by Cross et al. using HPLC-UV and a fast and easy sample preparation [22]. An HPLC-UV method for flucloxacillin and cephadin in vertebral discs was presented by Gibson et al., even though they could not detect any drug level in patient samples [23]. Frank et al. measured flucloxacillin concentration in heart valves [24] and Averono et al. measured amoxicillin in tonsils after tonsillectomy [25], both using LC-UV.

Cazorla-Reyes et al. presented a very interesting and well-validated method, measuring 21 diverse antibiotics in plasma and several human tissues [26]. However, they did not include flucloxacillin, benzylpenicillin, or clindamycin and used at least 800 μg of tissue sample.

No method has been published until now measuring the set of antibiotics presented here, which represent the penicillins most often used in critically ill patients in Switzerland, combined with clindamycin and the beta-lactamase inhibitors tazobactam and clavulanic acid. No method has been published for application with plasma, plasma water, and a wide range of tissue materials. In addition, the very short and easy tissue extraction procedure combined with a minimal amount of material needed sets aside this method from those published before.

Material and methods

Chemicals and reagents

Blank lithium-heparinized plasma was obtained from volunteers and tested for antibiotic residues before use. For blank tissue, leftover biopsy material from patients undergoing surgical restoration was used. The reasons for surgical restoration were divers, the most common indications being artificial joint infections and cellulitis. Samples were included if sufficient material was sent in for microbiological examination and no prior antibiotic treatment with any of the measured antibiotics had been performed. Amoxicillin trihydrate, amoxicillin-D4, piperacillin, piperacillin-D5, flucloxacillin sodium, flucloxacillin-13C4 15N sodium and clavulanic acid potassium salt were purchased from Toronto Research Chemicals (Toronto, Canada). Clindamycin hydrochloride and tazobactam were purchased from Sigma Aldrich (Buchs, Switzerland). All analytes used were of analytical grade. Centrisart 1 with 20,000 u mass weight cutoff (MWCO) and VIVACON 500 30,000 MWCO ultracentrifugation devices were purchased from Sartorius (Goettingen, Germany), while Amicon Ultra 0.5 ml 30,000 MWCO were purchased from Merck (Darmstadt, Germany). Acetone, acetonitrile, methanol, 2-isopropanol (all of HPLC grade), ammonium acetate, ammonium carbonate, acetic acid, and formic acid were purchased from Merck (Darmstadt, Germany). Heparinized plasma of healthy volunteers was collected in Sarstedt tubes (Nuembrecht, Germany) without gel.

Instrumentation

Samples were analyzed using a Thermo Scientific HPLC system (UltiMate 3000) coupled to a triple quadrupole mass spectrometer (TSQ ENDURA) (Thermo Scientific, Reinach, Switzerland) equipped with an electrospray ionization source. Online sample preparation used a Thermo TurboFlow MAX 50 × 0.5 mm column, while chromatographic separation was performed on an Accucore™ XL C18 4 μm 150 × 4.6 mm column, both by Thermo Fisher Scientific (Reinach, Switzerland). A binary gradient was used to elute the analytes. Mobile phase A was composed of 10 mM ammonium carbonate in water, adjusted to pH 8 with acetic acid, while mobile phase B was composed of 10 mM ammonium acetate and 0.1% formic acid in methanol/acetonitrile (1/1, v/v). For column cleaning, mobile phase C, consisting of acetonitrile/isopropanol/acetone (1/1/1/, v/v/v), was used. The HPLC method and the column set up can be found in Table 1 and Fig. 1. The total run time of the method was 10.25 min and from minute 2.5 to 8.5 flow was directed to the MS. The auto sampler kept samples at 10 °C before analysis and the whole equipment including the column was kept in an air condition–controlled room at approximately 24 °C.

Table 1 HPLC method

Time min	Connection EC–AC	Loop	Loading pump				Eluting pump			
			Flow ml/min	MP A %	MP B %	MP C %	Flow ml/min	MP A %	MP B %	MP C %
0.00	Out	Out	1.5	100	0	0	0.6	90	10	0
0.50	In	In	0.2	100	0	0	0.4	90	10	0
1.25	Out	In	1.5	100–0	0–100	0	0.6	90–20	10–80	0
3.25	Out	In	1.5	0	100	0	0.6	10–0	80–100	0
5.75	Out	In	1.5	0	0	100	0.6	0	100	0
6.75	Out	In	1.5	0	100	0	0.6	0	0	100
7.75	Out	In	1.5	60	40	0	0.6	90	10	0
8.25	Out	Out	1.5	100	0	0	0.6	90	10	0
10.25	End									

MP mobile phase, *EC* extraction column, *AC* analytical column

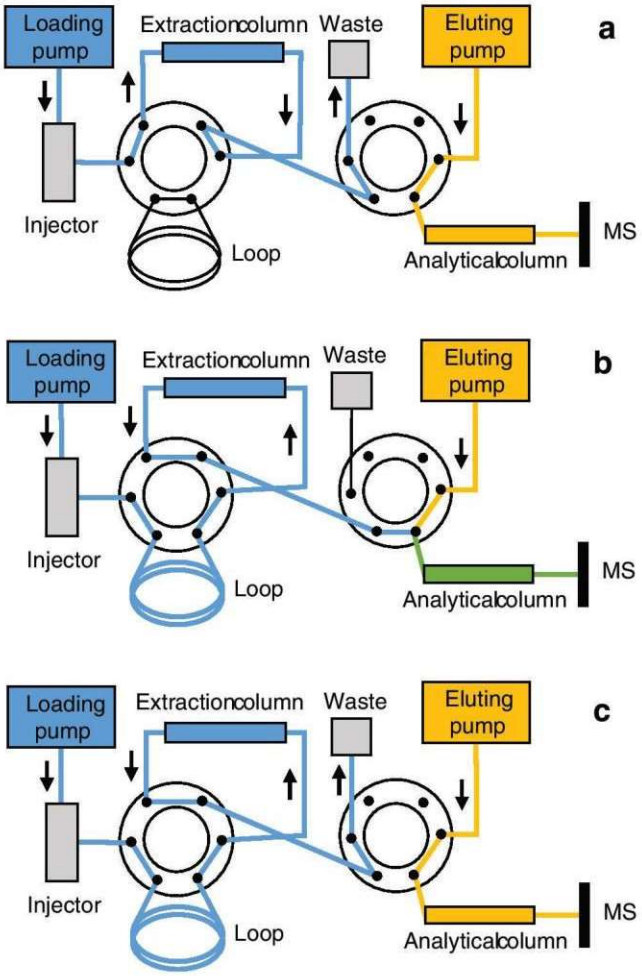


Fig. 1 Column settings. The sample is loaded unto the online extraction column using setting **a** and then the analytes are flushed unto the analytical column using the organic solvent stored in the loop according to setting **b**. The analytes are separated on the analytical column using setting **c** while the loop is refilled with organic solvent. For equilibration, setting **a** is used again

The mass spectrometer was operated in alternating mode, acquiring in both positive and negative ion mode using the following settings: spray voltage 3500 V, ion transfer tube temperature 200 °C; vaporizer 350 °C; sheath gas flow 6.5 l/min; auxiliary gas flow 8 l/min. The analytes were quantified by multiple reaction monitoring (MRM, see Table 2).

Preparation of solutions, standards, and quality controls

Stock solutions for quality controls (QC) and calibrators of each analyte were separately made by weighting in and dissolving the analyte in water (tazobactam at 2.5 g/l, flucloxacillin at 2.5 g/l, clavulanic acid at 8 g/l), methanol (piperacillin at 12.5 g/l, clindamycin at 2 g/l, benzylpenicillin at 5 g/l), or water/methanol 20/80 (amoxicillin at 3.125 g/l). Subsequently, 8 calibrators, including one blank, and additionally three QC were made by spiking blank plasma, plasma water or water for plasma, plasma water, and tissue analysis respectively. Calibrators and QC were prepared from separate stock solutions. For plasma and ultrafiltrate, calibrators and QC were stored as 100 µl aliquots until use. Tissue calibrators were stored as 5 µl aliquots. For tissue QC, tissue was finely cut, mashed using a plastic skewer and weighed in at 100 mg. Each QC level was made from a different tissue sample to ensure correctness between different kinds of tissue. One hundred microliters of stock solution containing all analytes at the correct concentration was added and mixed with 2800 µl of methanol. The QC were then divided into aliquots of 155 µl. Later, during sample preparation, these QC only had the internal standard added, as they already contained 150 µl methanol each.

Internal standards were dissolved in methanol and kept as stock solutions at 0.1 mg/ml. All Stock solutions of the internal standards were mixed and diluted with methanol to give

Table 2 Instrument settings. Calibration ranges and MRM settings, including one quantifier and two qualifiers for all analytes

Analyte	Corresponding internal standard	Calibration range (mg/l)	Precursor ion (*m/z*)	LV (V)	SF (V)	Quantifier		Qualifier 1		Qualifier 2	
						Product ion (*m/z*)	CE (V)	Product ion (*m/z*)	CE (V)	Product ion (*m/z*)	CE (V)
Amoxicillin	Amoxicillin-D4	3.125–125	365.9	118.3	0.0	349.0	10.3	207.9	13.9	134.0	32.1
Amoxicillin-D4			371.2	120.7	0.0	354.0	10.3	212.9	13.4	115.1	20.7
Benzylpenicillin	Piperacillin-D5	0.25–8.00	335.0	125.9	0.0	176.1	11.5	160.1	10.3	114.1	31.5
Clavulanic acid	Amoxicillin-D4	1.00–40.0	198.3	79.1	0.0	108.1	10.3	112.1	10.3	136.1	10.3
Clindamycin	Flucloxacillin-13C4 15N	0.05–8.00	425.1	209.0	0.0	126.2	29.6	389.2	17.5	377.2	19.2
Flucloxacillin	Flucloxacillin-13C4 15N	1.50–60.0	454.0	143.8	0.0	160.1	10.6	294.9	14.4	114.1	37.4
Flucloxacillin-13C4 15N			460.0	150.1	0.0	300.9	14.1	199.9	32.3	162.0	11.9
Piperacillin	Piperacillin-D5	3.125–125	518.2	154.4	50.0	143.1	19.2	358.9	10.3	302.0	11.4
Piperacillin-D5			523.2	171.1	50.0	148.1	17.6	364.1	10.3	307.1	13.6
Tazobactam	Piperacillin-D5	1.00–40.0	300.9	157.1	0.0	207.0	15.9	168.0	14.2	122.1	21.6

CE collision energy, *LV* lens voltage, *SF* source fragmentation voltage

the required concentrations for each preparation. All stock solutions, QC, calibrators, and IS were stored at − 80 °C.

Sample preparation

Plasma

One hundred microliters lithium-heparinized plasma (calibrators, QC, and patient samples) was precipitated with 300 μl of internal standard solution containing flucloxacillin-13C4 15N, piperacillin-D5, and amoxicillin-D4 at 10 mg/l in methanol. The samples were vortexed and then shaken for 10 min at room temperature. The samples were centrifuged at 4 °C and 16,000*g* for 10 min. One hundred microliters of the supernatant was transferred to a glass vial and diluted with 500 μl of mobile phase A. Fifty microliters of this mixture was injected into the HPLC.

Ultrafiltrate

To achieve equilibration of the protein binding at body temperature, a 500 μl aliquot of plasma was placed in an ultracentrifugation device (Vivacon 500, 30,000 MWCO) and kept in a water bath at 37 °C for 1 h. Following that, the samples were quickly transferred to a centrifuge and centrifuged at 37 °C and 16,000*g* for 15 min. A 100 μl aliquot of the resulting ultrafiltrate was then mixed with 300 μl of methanol containing 2.5 mg/l flucloxacillin-13C4 15N as internal standard and vigorously vortexed. One hundred microliters of this mixture was injected into the HPLC.

Tissue

Tissue samples from surgical restoration procedures were finely cut and weighed in to achieve approximately 5 mg. The exact weight was noted down for calculation of the exact concentration. Fifty microliters internal standard containing flucloxacillin-13C4 15N and amoxicillin-d4 at 2.5 mg/l in methanol and 150 μl methanol was added. The samples were mashed using a plastic skewer and vigorously vortexed before being shaken for 30 min at room temperature. The samples were then transferred to a centrifuge and centrifuged for 10 min at 16,000*g* and 4 °C. One hundred fifty microliters of this mixture was transferred into an HPLC vial and 100 μl were injected into the HPLC.

Method validation

Linearity, accuracy, and precision

Linearity was evaluated by measuring seven calibration levels on six separate days. The linearity was evaluated using R^2 and the deviation of individual points from the calibration curve. An R^2 of at least 0.99 and a deviation of the single calibration points of no more than 15% was considered satisfactory. Accuracy and imprecision were determined using three QC levels: six within-run replicates and six replicates on separate days were measured. Within-run and between-run accuracy and imprecision should lower than 15%, except for the lowest control level, for which 20% deviation was acceptable.

Matrix effect and recovery

As a qualitative evaluation of matrix effects, analytes and internal standards were directly infused into the MS at 5 μl/min with 10 ng/μl, while blank samples from six different sources were run. Drops and peaks in the baseline indicate ion suppression or ion enhancement.

To quantitatively measure matrix effect and recovery, three sets of spiked samples were measured. Each set consisted of five samples. Set A consisted of plasma samples spiked at three different concentrations (QC low, QC middle, and QC high). Set B consisted of water spiked at the same concentrations as Set A. Set C consisted of blank plasma samples. While sets A and B were worked up like normal samples, the samples in set C were first precipitated with pure methanol and then diluted with Eluent A containing analytes and internal standards. The final concentrations of all substances in the worked up samples were the same in each set. Mean peak areas of the analytes in each set were used to calculate matrix effect and recovery according to the following equations:

$$Recovery\ (\%) = \frac{Area_A}{Area_C} \times 100$$

$$Matrix\ effect\ (\%) = \frac{Area_A}{Area_B} \times 100$$

Compensated matrix effect was calculated according to the following equation:

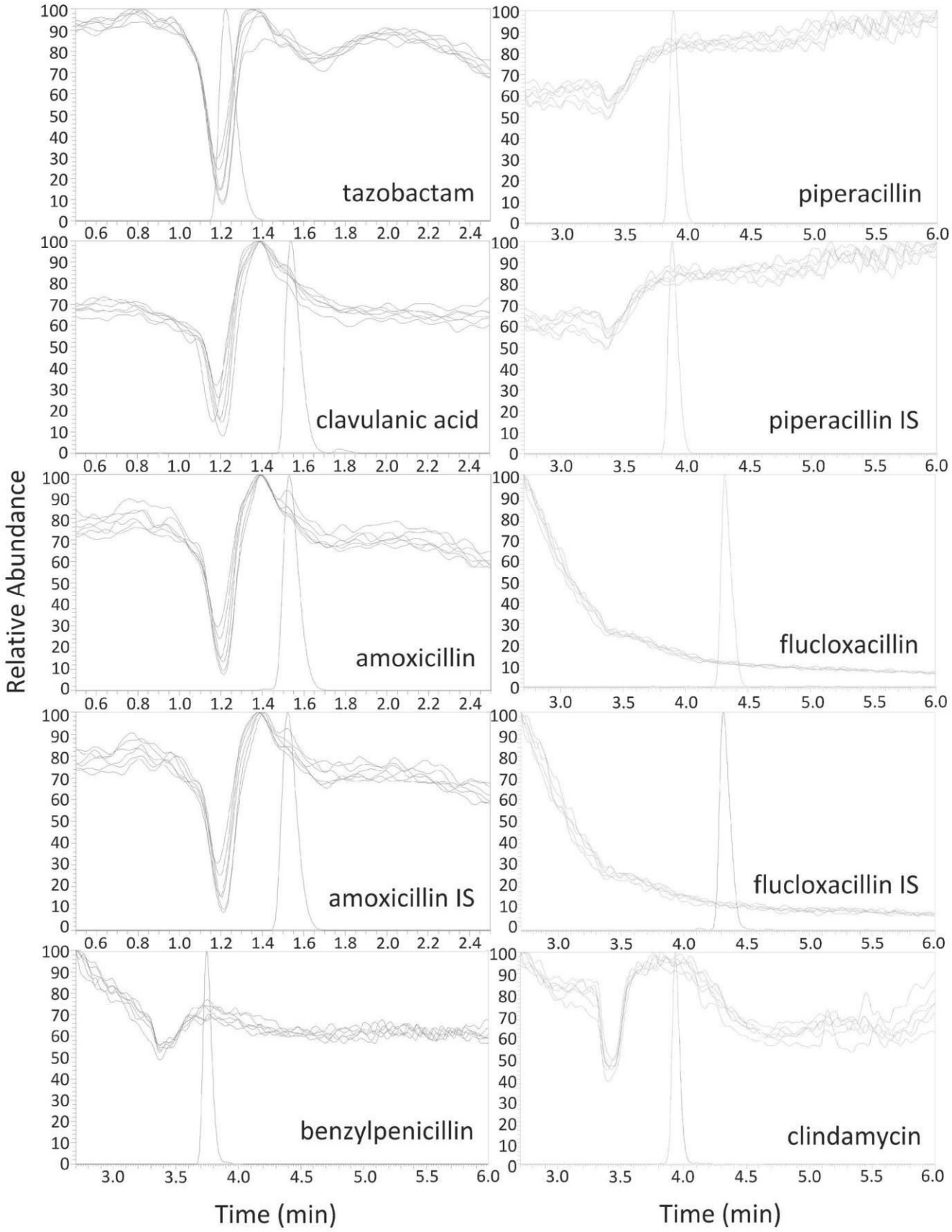
$$Compensated\ matrix\ effect\ (\%) = \frac{Matrix\ effect_{analyte}}{Matrix\ effect_{internal\ standard}} \times 100$$

Due to the use of a TLX system for extraction, only the manual steps of the sample extraction could be evaluated.

Table 3 Accuracy and precision. Mean of six measurements at three concentrations. Mean inaccuracy and mean CV are below 15% for all analytes. Only in tissue at the lowest quality control level inaccuracy and mean CV varied up to 20% for clavulanic acid

Analyte	Plasma			Tissue		
	Concentration (mg/l)	Accuracy ± CV (%)		Concentration (mg/l)	Accuracy ± CV (%)	
		Intra-day	Inter-day		Intra-day	Inter-day
Amoxicillin	4.4	95.2 ± 1.8	98.2 ± 3.1	0.4	113 ± 16	106 ± 6.2
	44	93.3 ± 4.3	93.0 ± 2.2	11	107 ± 12	97.5 ± 7.3
	110	101 ± 3.6	99.1 ± 2.6	22	98.2 ± 7.7	91.9 ± 8.2
Benzylpenicillin	0.8	93.1 ± 1.8	105 ± 5.9			
	12	100 ± 1.4	98.0 ± 3.9			
	32	102 ± 3.0	98.1 ± 5.8			
Clavulanic acid	1.4	109 ± 10	110 ± 8.7	0.4	105 ± 20	115 ± 17
	14	98.1 ± 4.6	96.9 ± 4.6	11	90.1 ± 9.8	88.2 ± 6.6
	35	102 ± 3.1	101 ± 6.6	22	93.8 ± 9.5	89.3 ± 8.1
Clindamycin	0.16	85.4 ± 3.4	89.4 ± 9.0	0.4	111 ± 11	102 ± 5.1
	2.8	98.5 ± 1.7	93.4 ± 14	11	113 ± 8.1	99.5 ± 4.5
	7	96.5 ± 2.5	101.1 ± 12	22	115 ± 9.6	108 ± 6.6
Flucloxacillin	2.2	105 ± 4.2	102 ± 4.2	0.4	90.9 ± 14	88.7 ± 13
	22	93.7 ± 2.6	95.0 ± 3.5	11	98.5 ± 6.6	99.7 ± 2.8
	55	100 ± 4.0	105 ± 3.8	22	95.5 ± 7.5	97.1 ± 6.7
Flucloxacillin, free fraction	0.18	100 ± 5.6	95.0 ± 5.3			
	9.0	103 ± 2.6	98.9 ± 2.8			
	18	105 ± 4.1	99.8 ± 3.2			
Piperacillin	4.4	94.5 ± 2.1	93.1 ± 3.2			
	44	90.4 ± 1.7	89.7 ± 1.1			
	110	95.8 ± 1.8	93.8 ± 1.6			
Tazobactam	1.4	85.3 ± 4.2	91.3 ± 13			
	14	93.5 ± 2.2	98.9 ± 5.5			
	35	96.1 ± 3.9	98.0 ± 7.4			

CV coefficient of variation


Relative Abundance
tazobactam
piperacillin
clavulanic acid
piperacillin IS
amoxicillin
flucloxacillin
amoxicillin IS
flucloxacillin IS
benzylpenicillin
clindamycin
Time (min)
Time (min)

◀ **Fig. 2** Qualitative matrix effect. Six blank plasma chromatograms from different patients are overlaid with a chromatogram of the lowest calibrator. No interferences between matrix and analyte can be seen. Only tazobactam shows some ion suppression

Stability

Stability of the analytes in whole blood at room temperature for 6 h was evaluated, which corresponds to the maximal duration for samples to be transferred to the laboratory. Stability in plasma at room temperature and at 8 °C was tested over the course of 3 days. The influence of gel containing plasma heparin tubes was also evaluated over the same duration. Long-term stability in stock solution, working solutions, and in plasma samples was tested over 12 months at - 20 °C as well as − 80 °C. Autosampler stability over 24 h was tested at 10 °C. A loss of 15% of analyte was determined to be acceptable for stability.

Ultrafiltration

Most ultracentrifugation devices used for the filtration of plasma are not validated for the preparation of the free fraction of plasma but are sold as DNA purification filters. Therefore, adsorption of drugs onto the filter must be validated to ensure the accuracy of the method. Centrisart 1 20,000 molecular weight cutoff (MWCO) and VIVACON 500 30,000 MWCO ultracentrifugation devices from Sartorius, as well as Amicon Ultra 0.5 ml 30,000 MWCO and Centrifree 30,000 MWCO filters, both from Merck, were tested and compared for adsorption. Water was spiked with stock solution containing all seven analytes. The solution was analyzed directly and after undergoing the normal plasma ultrafiltration procedure.

Results

Linearity

R^2 of all measured calibration curves was 0.995 or better for all plasma and ultrafiltrate calibrations and at least 0.99 for calibration curves in water.

Accuracy and precision

Accuracy and precision for all analytes in different matrices are shown in Table 3. All results are within the expected limits.

Matrix effect and recovery

The result of the qualitative matrix effect experiment is displayed in Fig. 2. A chromatogram of the lowest calibrator is shown overlaid with six chromatograms of a direct infusion into the MS. For the internal standards, a typical chromatogram can be seen. Quantitative matrix effect and compensated matrix effect values are shown in Fig. 3. Recovery was between 70% (amoxicillin) and 83% (flucloxacillin). Clindamycin showed a recovery of 110%. Standard variation of up to 13% was mainly due to the recovery difference at different concentrations (flucloxacillin: 92% at 2.2 mg/l vs 74% at 55.0 mg/l). Recovery was slightly higher for all analytes at lower concentrations.

Stability

At − 20 °C, none of the analytes was stable in neither stock solution, working solution, nor plasma over the course of 12 month. Clavulanic acid and amoxicillin even showed degradation up to 100% when stored at − 20 °C. Surprisingly, at − 80 °C in plasma, all analytes showed very good stability. Clavulanic acid turned out to be problematic also during short-term stability, being only stable for up to 2 h at RT as well as at 8 °C. Therefore, samples containing clavulanic acid must be stored on ice as soon as possible before transportation to the laboratory and frozen before analysis. Full stability data can be found in Table 4.

Comparing the use of blood collecting tubes with and without separating gel, we could see no effect on the recovery or stability of the tested antibiotics, neither directly after centrifugation nor after storage on gel for 72 h.

Ultrafiltration

Ultrafiltration is a common technique to separate protein bound analyte from the free, unbound fraction of antibiotic drugs. However, loss of analyte during the filtration

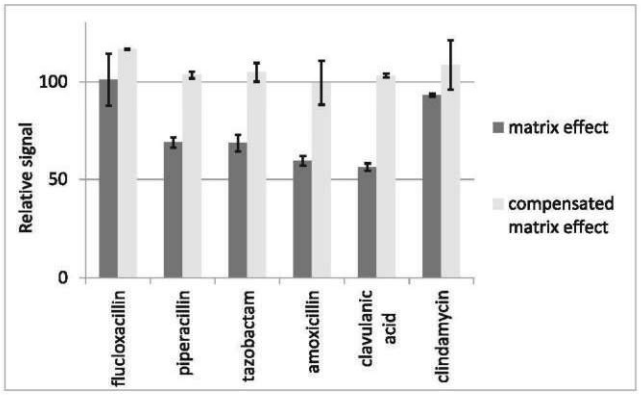


Fig. 3 Quantitative matrix effect and compensated matrix effect. Mean values of six replicates at three concentrations. Bars show standard deviation. Matrix effect was between 56% and 101%. By using the internal standard, the matrix effect could be nearly completely compensated. Compensated matrix effect was calculated as the ratio between matrix effect of analyte and matrix effect of internal standard

Table 4 Stability of the analytes under different conditions. The condition was deemed stable, when the mean concentration at all three control levels was within ± 15% of the nominal concentration

	Amoxicillin	Benzylpenicillin	Clavulanic acid	Clindamycin	Flucloxacillin	Piperacillin	Tazobactam
Whole blood, RT	6 h	3 h	3 h	6 h	6 h	6 h	6 h
Plasma, 4 °C	72 h	48 h	2 h	24 h	72 h	72 h	72 h
Plasma, RT	24 h	8 h	2 h	8 h	8 h	8 h	8 h
Plasma, − 20 °C	< 6 months	< 6 months	< 6 months	< 6 months	6 months	< 6 months	< 6 months
Plasma, − 80 °C	12 months	12 months	12 months	12 months	12 months	12 months	12 months
Working solution, − 20 °C	6 months	< 6 months	< 6 months	6 months	< 6 months	6 months	< 6 months
Working solution, − 80 °C	12 months	12 months	6 months	12 months	12 months	12 months	12 months
Stock solution, − 20 °C	6 months	< 6 months	< 6 months	6 months	6 months	6 months	6 months
Stock solution, − 80 °C	12 months	12 months	6 months	12 months	6 months	12 months	12 months
Autosampler	24 h	24 h	12 h	24 h	24 h	24 h	24 h

process can occur either due to adsorption of the analyte onto the filter or due to degradation of the analyte during processing of the samples. Table 5 shows the recovery of analytes in water after centrifugation compared to samples analyzed before centrifugation. The extreme loss of analyte by adsorption unto the filter for flucloxacillin and clindamycin of more than 45% and 97%, respectively, could also be seen in spiked plasma samples, leading to extremely low apparent free concentrations of 1.7% and 0.9%, respectively, when using the Amicon Ultra filter device. Since the Vivacon 500 filters showed no adsorption for flucloxacillin or clindamycin and very good results for all other analytes, we choose the Vivacon 500 filters for plasma ultrafiltration. This resulted in free plasma concentrations of 5.6% and 5.0% respectively for flucloxacillin and clindamycin in spiked plasma samples of healthy volunteers, which corresponds to published data [4–6].

Table 5 Ultracentrifugation recovery. Recovery of analytes from water after ultracentrifugation using different devices, average of three measurements. Strong adsorption could be seen in the Amikon Ultra and the Centrisart 1 device. Only the Vivacon device showed no adsorption of clindamycin and flucloxacillin

Analyte	Amikon Ultra	Vivacon	Centrisart 1	Centrifree
	Recovery in water (%)			
Amoxicillin	96.3	101	98.9	98.5
Benzylpenicillin	66.6	100	95.2	91.7
Clavulanic acid	83.4	93.7	95.9	96.0
Clindamycin	2.4	104	73.9	90.4
Flucloxacillin	55.2	101	78.2	89.4
Piperacillin	49.8	103	98.4	89.3
Tazobactam	84.4	89.1	94.1	90.0

Practical implementation

In Fig. 4 chromatograms of a plasma sample from a patient receiving piperacillin-tazobactam (a) and a tissue sample of a patient receiving amoxicillin-clavulanic acid and flucloxacillin (b) are shown. Figure 5 shows results from a line of measurements undertaken within a patient receiving 2 g flucloxacillin 4 times daily. Plasma samples were measured for total and free plasma concentration, also a sample of biopsy material was analyzed.

Discussion

Different calibrations were tested for quantitation of the antibiotics in tissue. Calibration using spiked tissue did not result in better accuracy and precision than calibration using spiked water, especially when comparing different kinds of tissue against each other. Since this method should be applicable to a wide range of tissues, from fatty tissue to muscle, to liquor or puss, it was not applicable to create calibration standards for each kind of sample. Differences in the recovery of the analytes when analyzing different types of tissue material could be seen, but they were all within the allowable values. By using spiked tissue as quality controls, using a different tissue material for each control level, we could ensure the correctness of the calibration and extraction procedure.

The use of as little sample material as 5 mg made it possible to prepare diverse tissues using the same simple sample preparation. The already very small pieces could be cut even smaller using surgical scissors and the manual mashing of the samples always allowed good blending of the samples. Of course, hard materials, like bone, could not be analyzed using this method.

The use of ultracentrifugation devices as a tool to measure free concentrations of drugs is widely spread and accepted

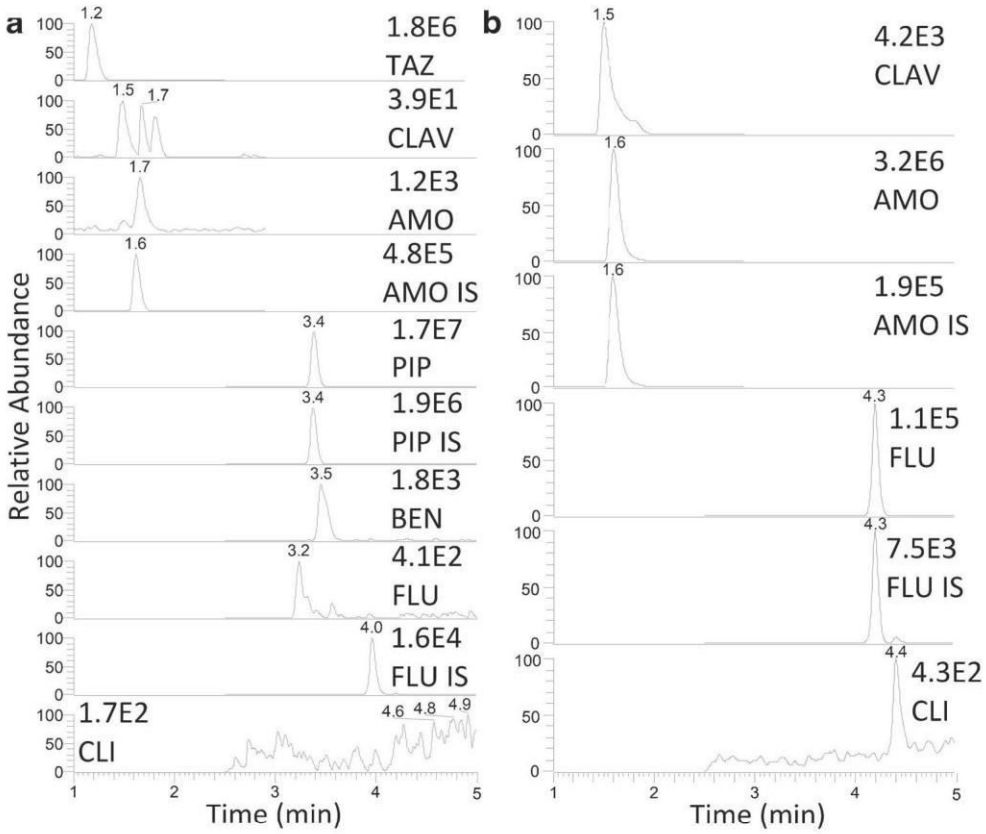


Fig. 4 Chromatograms of two patients. **a** Plasma of a patient with 264 mg/l piperacillin and 37 mg/l tazobactam. **b** Tissue of a patient with 46 mg/kg amoxicillin, 1.7 mg/kg clavulanic acid, and 18 mg/kg flucloxacillin

[27–31]. Albumin has a molecular weight of about 66,000 Da and α-glycoprotein weights about 43,000 Da. These two plasma proteins are responsible for most of the drug-protein binding [32, 33]. Therefore, a cutoff weight of maximal 30,000 Da should be used in ultracentrifugation devices, to ensure both proteins are not able to pass the filter membrane.

During method development, we found extremely low free fractions of clindamycin and flucloxacillin when using the

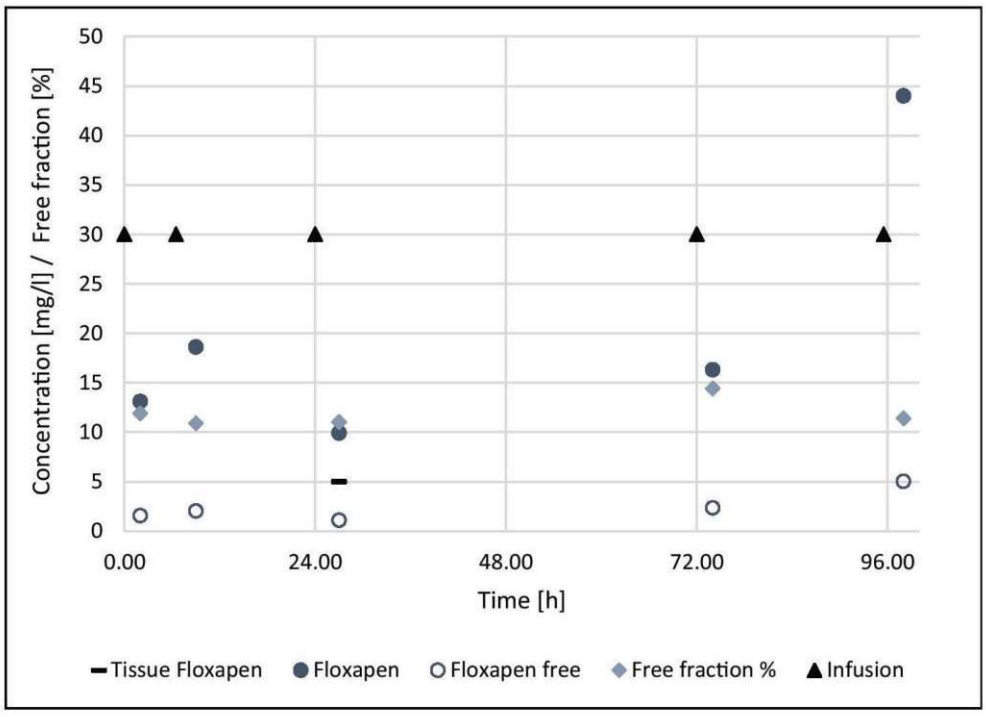


Fig. 5 Patient under flucloxacillin treatment. Plasma levels, free plasma levels, and calculated free fraction, as well as one tissue concentration (biopsy of a knee fistula) of a patient receiving 2 g flucloxacillin 4 times a day. The time points of last infusions before each sample collection are depicted as triangles and are always between 2 and 3 h before sampling. Plasma concentrations were between 10 to 45 mg/l. Free fraction of unbound flucloxacillin was quite stable at about 10–15%. Concentrations of knee tissue was 5.0 mg/kg, which was between the plasma concentration (9.9 mg/l) and the unbound plasma concentration (1.1 mg/l) at that time point

Amikon Ultra device, which could not be explained, save for adsorption onto the filter. Consequently, we tested several filters from different manufacturers, finding that only one out of four gave acceptable results. We tested adsorption from water, which can give misleading results, as the proteins from plasma should deactivate the filter membrane, preventing adsorption. Therefore, the absolute adsorbed percentage might be different in plasma, but the adsorption itself was still clearly observable. This strong adsorption is in stark contrast to what other groups described [29, 30, 34]. Our findings were confirmed in repeat measurements using the same production batch of filters. Repeat measurement using a different batch showed much higher recovery of both clindamycin and flucloxacillin, though they were still only about 45% of the recovery observed using devices of other manufacturers. These findings highlight the high influence of the devices on successful separation of bound and unbound drugs. It is not implausible, that differences between production batches could also be found in other manufactures filter devices.

The method has been routinely used for the measurement of both plasma and free plasma levels of patients from the university hospital and has proven to be stable and reliable. We employed this method for the quantification of tissue material from the microbiology ward and will use it in several upcoming studies dedicated to tissue penetration of antibiotics.

Conclusion

We presented a fast and easy method for measuring the penicillins amoxicillin, benzylpenicillin, flucloxacillin, and piperacillin and the beta-lactamase inhibitors clavulanic acid and tazobactam, as well as the macrolide clindamycin. Quantitative measurement in plasma and tissue as well as in plasma water is possible. Its usability in the clinical setting and its application for the different matrices has been shown.

Compliance with ethical standards

The analyzed authentic samples belonged to individuals who provided an informed consent for their use and all analyses were carried out according to the ethical standards of the University Hospital Basel.

Conflict of interest The authors declare that they have no competing interests.

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HILIC LC-MS/MS method for the quantification of cefepime, imipenem and meropenem

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ABSTRACT

A high performance hydrophilic interaction chromatography method combined with tandem-mass spectrometry for the quantification of cefepime, meropenem and imipenem in plasma and cerebrospinal fluid is presented. A solution of 0.5 M 3-Morpholinopropanesulfonic acid and ethylene glycol (1:1) was added to the samples before analysis to ensure stability of analytes during work up and storage. Deuterated forms of cefepime and meropenem were used as internal standards. Protein precipitation prior to injection into the LC–MS/MS system provided a fast and easy sample preparation. For online extraction, a Turboflow Cyclone-MCX column was used and the chromatographic separation was carried out on a Hypersil GOLD HILIC column. Linear calibration curves were obtained in the concentration range of 0.4-40 mg/l, 0.6-60 mg/l and 1-100 mg/l for meropenem, imipenem and cefepime, respectively. The intra- and interday imprecision and inaccuracy values were below 10 % for plasma and 13 % for cerebrospinal fluid using a calibration in plasma. The method was employed for therapeutic drug measurements in a university hospital.

1. Introduction

The two carbapenems imipenem and meropenem as well as the cephalosporin cefepime are polar compounds with logP values below zero. As all three components are amphoteric, they all bear a negative and a positive charge in the same molecule at physiological pH, which strongly increases their polarity. They are not orally bioavailable and therefore must be administered intravenously, or in the case of cefepime, also as intramuscular injection. Structural formula of the analytes can be found in Fig. 1. Due to their high polarity, all three drugs can cross the blood-brain barrier easily and penetrate into the cerebrospinal fluid (CSF) [1]. Their high CSF penetration also leads to high incident numbers of neurotoxicity [2,3].

Cefepime, imipenem and meropenem have been in use for several decades, but new administration regimens such as prolonged or continuous infusion, as well as the rising threat of antimicrobial resistances, make therapeutic drug monitoring (TDM) of these analytes desirable. CSF level measurements may help distinguish drug-induced neurotoxicity from other causes. In addition, several expert boards recommend therapeutic drug monitoring of beta-lactam antibiotics in the critically ill [4–6].

Because of their polar nature, retention of these analytes on standard reversed-phase columns is insufficient and often results in peak tailing. Hydrophilic interaction chromatography (HILIC) is a variant of normal-phase separation. It is a rather uncommon separation method for drug quantification, as well as in other fields of analytical chemistry. The HILIC design consists of a polar stationary phase and relatively high concentrations of organic solvents as mobile phase and enables good separation of polar and ionic compounds [7–9]. Besides their high polarity, the low stability of beta-lactams in aqueous solutions make reliable quantification difficult. Beta-lactams can be hydrolyzed in acidic, as well as in alkaline conditions. Organic solvents like ethylene glycol disrupt the ordered water bridges needed for the hydrolyzation of the beta-lactam ring. Since both acidic and basic pH increase hydrolyzation, buffering at neutral pH increases the stability of beta-lactam antibiotics greatly [10].

To date, there have been several methods published dealing with the quantification of imipenem, meropenem or cefepime. A HILIC UPLC-MS/MS method of imipenem and meropenem and other beta-lactam antibiotics was published by Abdulla et al. [11]. They did not use any kind of stabilization. They also found quite large variations in matrix effect and recovery between samples, which indicates a suboptimal sample preparation. Two UPLC-MS/MS methods for cefepime, meropenem and other antibiotics were presented by Zander et al. and Rigo-Bonnin et al. [12,13]. Both groups present well validated methods but did not use any stabilizer to ensure stability of meropenem during workup and

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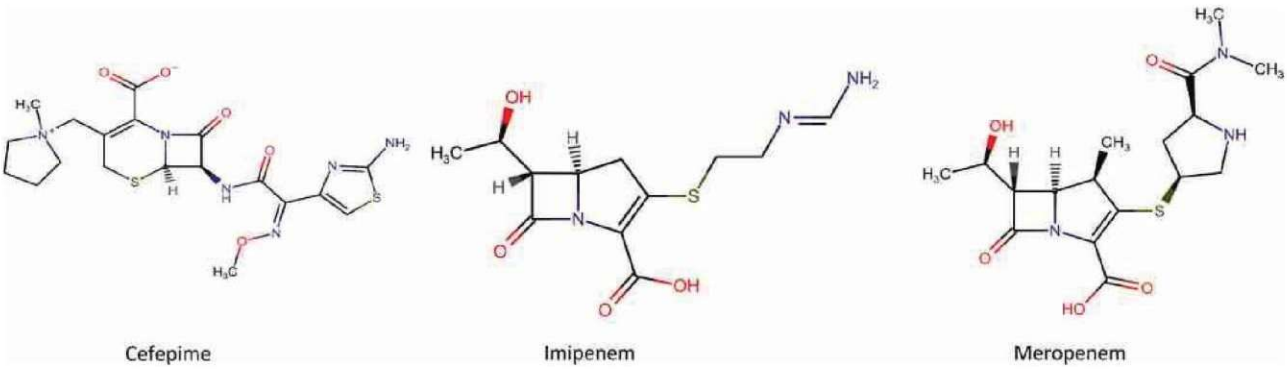

Fig. 1. Structural formulas of cefepime, imipenem and meropenem. All three analytes are amphoteric at physiological pH, which makes them strongly water soluble, results in low oral bioavailability and makes them easily pass the blood brain barrier.

storage. Several methods quantifying only meropenem in plasma using reversed phase LC-MS have been published [14–16]. Xu et al. presented a HILIC-LC-MS method for imipenem, cilastatin and an investigational beta-lactamase inhibitor using ethylene glycol and 2-(N-morpholino)-ethanesulfonic (MES) [17]. None of these methods could be used to measure imipenem, cefepime and meropenem simultaneously. Only one group measured all three analytes along with other beta-lactams in one UHPLC-MS/MS method [18]. Cazorla-Rey et al. did not use any stabilization, though. They concluded that the maximum stability of one week at -80 °C for meropenem was good enough for their study, but not being able to store quality controls and calibrators for a longer time is not suitable for routine analysis.

There are a some HPLC-UV methods described which measure one or two of the desired analytes, but none dedicated to all three of the here presented compounds. Interestingly, all of these methods measuring imipenem use MES or 3-Morpholinopropanesulfonic acid (MOPS) stabilizing solutions [19–24].

Until now, there has been no method described for the determination of imipenem, meropenem and cefepime using HILIC MS/MS in combination with an effective stabilizer. The possibility to measure simultaneously plasma and CSF samples is another advantage of the analytical approach described here.

TDM of beta-lactam antibiotics for patients with specific indications was introduced as standard of care in our hospital and is part of our antibiotic stewardship program. Indications for beta-lactam TDM include admittance to the ICU ward, renal failure, sepsis, severe over- or underweight as well as infections with multi-resistant bacteria. The method presented here, along with other methods developed by our group [25] were put into effect to ensure quick and efficient TDM.

2. Material and methods

2.1. Chemicals and reagents

Blank lithium-heparinized plasma was obtained from the blood donation center Basel and tested for antibiotic residues before use. CSF was obtained from leftover material in the clinical laboratory and tested for antibiotic residues before use. Imipenem monohydrate, cefepime-d3 sulfate and meropenem-d6 were purchased from Toronto Research Chemicals (Toronto, Canada). Cefepime hydrochloride monohydrate, meropenem trihydrate sodium carbonate and MOPS were obtained from Sigma-Aldrich (Buchs, Switzerland). Ethylene glycol (EG) was obtained from Honeywell (Seelze, Germany). Acetone, acetonitrile, methanol and 2-isopropanol were purchased from Merck (Darmstadt, Germany), as well as ammonium acetate, ammonium carbonate, acetic acid, sodium hydroxide and formic acid. All analytes used were of analytical grade, all other chemicals of HPLC grade.

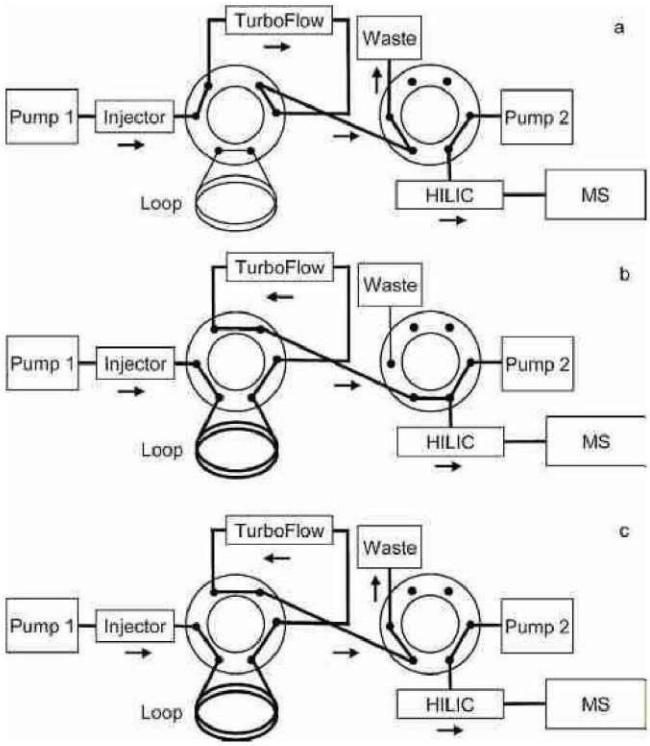

Fig. 2. Column configurations. The sample is loaded onto the online extraction column using setting a). The analytes are flushed onto the analytical column using the organic solvent stored in the loop according to setting b). The analytes are separated on the analytical column while the loop is refilled with organic solvent using setting c). For equilibration, setting a) is used again. Adapted from Rehm et al. [24].

2.2. Instrumentation

The chromatography was performed on an UltiMate 3000 HPLC system (Thermo Fisher Scientific). For online extraction a Turboflow Cyclone-MCX column (50 × 0.5 mm, Thermo Fisher Scientific) was used, while for chromatographic separation a Hypersil GOLD HILIC column (150 × 4.6 mm, 5 µm, Thermo Fisher Scientific) was employed. The mobile phases consisted of A: ammonium acetate (10 mM) and formic acid (0.1 %), B: acetonitrile-methanol (1:1, v/v) with ammonium acetate (10 mM) and formic acid (0.1 %). Mobile phase C was used for cleaning the TurboFlow column after separation and was prepared from acetonitrile-acetone-2-propanol (1:1:1, v/v/v). The HPLC program and column configurations are given in Table 1 and Fig. 2, respectively. Detection was performed on a TSQ ENDURA triple quadrupole mass spectrometer (Thermo Fisher Scientific) equipped with a turbo ion spray interface operated in positive-ion mode. No column oven was

Table 1
HPLC method. A, B and C stand for mobile phase A, mobile phase B and mobile phase C, respectively. Tee stands for the connection of the TurboFlow column with the separation column.

Duration (s)	Tee	Loop	Flow (ml/min)	Pump 1 (%)					Flow (ml/min)	Pump 2 (%)		
				A		B		C		A		B
30	Out	Out	1.5	80	:	20	:	0	0.6	20	:	80
15	Out	In	0.3	20	:	80	:	0	0.6	20	:	80
30	In	In	0.3	20	:	80	:	0	0.3	20	:	80
180	Out	Out	0.3	0	:	100	:	0	0.6	20→50	:	80→50
180	Out	Out	0.3	0	:	0	:	100	0.6	50	:	50
60	Out	Out	1.5	100	:	0	:	0	0.6	50	:	50
60	Out	Out	1.5	0	:	100	:	0	0.6	20	:	80
60	Out	Out	1.5	0	:	0	:	100	0.6	20	:	80
30	Out	In	1.5	20	:	80	:	0	0.6	20	:	80
120	Out	Out	0.3	80	:	20	:	0	0.6	20	:	80

used, but the whole equipment was in a temperature-controlled room, which kept room temperature adjusted to approximately 24 °C. The sample compartment of the autosampler was set at 4 °C.

Interface settings were as following: spray voltage 3500 V, ion transfer tube temperature 300 °C; vaporizer temperature 400 °C; sheath gas flow 6.5 L/min; auxiliary gas flow 8.0 L/min. Quantification was performed using multiple reaction monitoring (MRM). In Table 2, the calibration ranges, MRM transitions, collision energies and lens voltages are described.

2.3. Solutions, calibrators and quality control samples

A 0.5 M MOPS buffer (adjusted to pH 6 with NaOH) : ethylene glycol (1:1) solution was used as a stabilizing agent to prevent degradation of meropenem in the stock solution and the plasma samples.

Stock solution for calibrators and quality controls were weighted in separately. Stock solutions were prepared for cefepime at 5 mg/ml in methanol, for imipenem at 1 mg/ml in methanol and for meropenem at 2 mg/ml in stabilizing solution. Working solutions were prepared by adding the appropriate amount of stock solution to stabilizing solution. Both stock solutions of deuterated standards were prepared in methanol at a concentration of 100 mg/l. The final IS contained 10 mg/l cefepime-d3 and 4 mg/l meropenem-d6 in methanol.

Seven calibrators, three quality controls (QC), and an additional blank were prepared by spiking blank plasma with the appropriate amount of stock solution and adding stabilizing solution to the plasma at a ratio of 1:1. All solutions, QC and calibrators were stored at -70 °C until use.

2.4. Sample preparation

A 50 µL aliquot of lithium-heparinized plasma or CSF was mixed with 50 µL stabilizing solution and precipitated with 200 µL IS. The samples were vortexed and shaken for 10 min at room temperature, followed by centrifugation at 4 °C and 14′000 *g* for 10 min. The supernatant was transferred to a glass vial and 50 µL was injected for analysis.

3. Method validation

3.1. Analytical performance

By measuring seven calibration levels on six separate days, linearity was assessed by evaluating R^2, which should be at least 0.99. According to FDA guidelines [25], the deviation of individual points from the calibration curve should be less than 15 %. Accuracy and precision were assessed at three different QC levels: six within-run replicates and one replicate on each of six separate days were measured. Within-run and between-run inaccuracy and imprecision, expressed as coefficient of variation (CV), should be less than 15 % [25]. CSF quality controls were quantified using a calibration in plasma. Samples above the highest calibrator were measured as is, without dilution, to test the extent of the linear range. Imipenem was tested up to 200 mg/l, cefepime up to 500 mg/l and meropenem was tested up to 400 mg/l. If samples above the linear range could not be analyzed correctly, they were diluted 1:5 using mobile phase A and retested. Carry over was investigated by running blank samples after the highest calibrator in six runs. Carry over should not exceed 20 % of the lowest calibrator.

3.2. Matrix effect and recovery

Matrix effects were assessed qualitatively and quantitatively. We used post-column infusion to qualitatively evaluate matrix effects. Six different blank plasma heparin samples were prepared and run, while the three analytes and the two IS were directly infused into the MS via a T-piece at 0.05 µg/min. Deviations from the baseline signal imply the occurrence of matrix effects.

For the quantitative evaluation of matrix effect and recovery, three sets of samples were compared for plasma and CSF, respectively. Each set consisted of six blank batches of plasma or CSF from different sources, spiked at three concentration levels. Each sample was measured in duplicate. Set A consisted of spiked plasma or CSF. Recovery was evaluated using set B, a blank plasma or CSF set, which was spiked with analytes and IS after sample workup was completed. For calculation the formula: $Recovery\ (\%) = \frac{Area_A}{Area_B} * 100$ was used. Matrix effect was evaluated using set C, spiked water, using the formula: $Matrix\ effect\ (\%) = \frac{Area_A}{Area_C} * 100$.

Compensated matrix effect was calculated as the area ratio of analyte and IS and variation between the six batches of the same matrix should be less than 15 %.

3.3. Stability

Short term stability in plasma was determined at room temperature (RT) in both untreated plasma and in plasma mixed with stabilizing solution for a maximum of 24 h. Stability at 4 °C was determined in untreated plasma for a maximum of 72 h. Processed samples stored at 4 °C in the autosampler were reanalyzed after 24 h for auto sampler stability. Long-term stability of plasma was compared with stabilizer at -20 °C and without stabilizer at -20 °C and -70 °C. Tested were six replicates of each control level, high and low. Stock solutions and working solutions were compared at -20 °C and -70 °C after three weeks, 6 months and 12 months. All solutions were separately diluted six times with water and measured according to the directions for sample preparation. A maximum loss of 15 % of analyte was determined to be acceptable for stability in all conditions.

Table 2
Calibration range and MRMs with their respective collision energies and lens voltages. Underlined product ions were used for quantification.

Analyte (corresponding IS)	Calibration range (mg/l)	Lens voltage (V)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)
Cefepime (Cefepime-d3)	0.4–40	165.0	481.2	323.9	15.8
				166.9	24.4
				86.2	16.1
Cefepime-d3		114.6	485.0	396.9	10.3
				325.0	15.1
				167.0	22.6
Imipenem (Meropenem-d6)	0.6–60	113.7	300.2	141.9	29.0
				169.9	16.6
				194.9	13.5
Meropenem (Meropenem-d6)	1.0–100	148.3	384.2	141.1	16.7
				254.1	16.6
				340.1	10.3
Meropenem-d6		112.5	390.2	147.2	16.0
				260.1	15.7
				346.1	10.3

Table 3
Intra-day and inter-day precision and accuracy for all three analytes at three concentrations. Mean of six measurements at three concentrations. Mean inaccuracy and mean CV are below 15 % for all analytes. CSF quality controls were calibrated using plasma calibrators. CV, Coefficient of variation.

Analyte	Concentration [mg/l]	Plasma		CSF	
		Intra-day Accuracy ±CV [%]	Inter-day	Intra-day	Inter-day
Cefepime	2.0	107 ± 4.8	103 ± 1.4	92.2 ± 2.7	106 ± 6.5
	50	108 ± 2.2	101 ± 4.2	88.5 ± 1.5	93.4 ± 6.3
	90	106 ± 1.9	98.4 ± 7.0	91.0 ± 2.3	94.5 ± 5.8
Imipenem	1.2	95.6 ± 7.2	98.0 ± 6.8	98.4 ± 2.7	105 ± 5.3
	30	102 ± 4.3	97.0 ± 4.5	109 ± 4.3	110 ± 5.1
	54	101 ± 3.5	90.6 ± 9.7	110 ± 4.9	113 ± 4.4
Meropenem	0.80	101 ± 6.3	105 ± 6.3	103 ± 5.1	111 ± 7.7
	20	98.0 ± 1.0	102 ± 4.5	101 ± 1.1	101 ± 2.6
	36	98.7 ± 1.4	102 ± 3.4	104 ± 2.1	104 ± 2.4

4. Results

4.1. Analytical performance

R^2 for meropenem was always better than 0.999, for cefepime 0.998 and for imipenem 0.997. Intra- and inter-day accuracy and precision can be found in Table 3.

Samples containing imipenem could be quantified up to 200 mg/l, samples containing cefepime up to 500 mg/l and samples containing meropenem could be quantified up to 100 mg/l without dilution. When diluted with mobile phase A at a ratio of 1:5, samples containing meropenem could be correctly quantified up to 300 mg/l. Carry over in blank samples after the highest calibrator was 11 % for cefepime, 16 % for imipenem and 10 % for meropenem, which was within the defined limitations.

4.2. Matrix effect and recovery

The results of the qualitative matrix effect tests can be found in Fig. 3. The six tested batches of plasma showed similar suppression chromatograms and no major matrix suppression peak or valley was found to elute at the same time as the analytes. Quantitative matrix effect, compensated quantitative matrix effect, as well as recovery values for plasma and CSF can be found in Table 4. Compensated matrix effect variation between the six tested batches of plasma and CSF was no higher than 6.4 % and 5.6 % respectively. Recovery in plasma and CSF differ up to 30 % in low concentration meropenem samples. Since recovery of the internal standards closely fits those of the analytes, this effect is compensated. Because sample preparation included a TLX system, only the manual part of the sample extraction could be evaluated using this method.

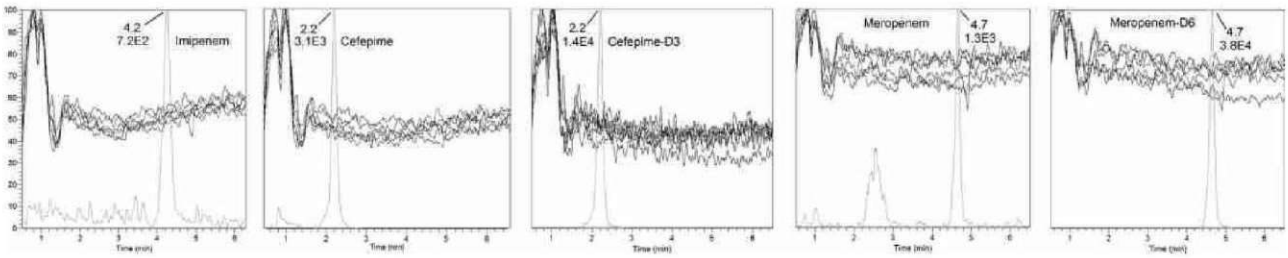

Fig. 3. Qualitative matrix effect. Chromatograms of six blank samples were detected while a methanol solution containing all analytes was directly infused into the MS at 0.05 µg/min. The chromatograms are shown overlaid with one chromatogram of the lowest calibrator.

Table 4
Matrix effect and recovery. IS concentration was stable over all three QC levels. Matrix effect and recovery were tested in six different batches of plasma and CSF each; all data points were measured in duplicate.

Analyte	Concentration [mg/l]	Plasma		CSF	
		Matrix effect Accuracy ±CV [%]	Recovery	Matrix effect	Recovery
Cefepime	2.0	72.0 ± 8.1	83.2 ± 11	64.9 ± 8.5	96.1 ± 14
	50	72.1 ± 9.7	86.9 ± 11	70.6 ± 6.5	95.6 ± 13
	90	69.5 ± 8.0	90.2 ± 6.6	71.0 ± 10	102 ± 13
Cefepime: compensated matrix effect	2.0	98.6 ± 2.8		101 ± 2.3	
	50	107 ± 2.6		109 ± 1.6	
	90	104 ± 2.7		109 ± 3.3	
Cefepime-d3		91.8 ± 10	87.6 ± 10	64.6 ± 9.4	95.8 ± 13
		86.7 ± 10	86.9 ± 11	64.9 ± 6.6	96.8 ± 14
		96.7 ± 7.0	96.6 ± 7.0	65.1 ± 13	106 ± 14
Imipenem	1.2	62.3 ± 8.7	77.5 ± 8.8	73.2 ± 7.8	96.3 ± 11
	30	61.9 ± 1.7	86.2 ± 5.0	72.4 ± 3.4	100 ± 4.0
	54	57.9 ± 5.0	87.5 ± 15	67.0 ± 11	96.8 ± 12
Imipenem: compensated matrix effect	1.2	104 ± 6.4		106 ± 5.6	
	30	105 ± 1.7		106 ± 2.4	
	54	101 ± 6.3		106 ± 5.0	
Meropenem	0.80	55.9 ± 7.8	76.1 ± 11	67.2 ± 5.9	103 ± 7.3
	20	56.6 ± 2.4	86.6 ± 6.5	66.5 ± 2.5	98.8 ± 4.0
	36	54.4 ± 2.9	89.9 ± 9.8	61.9 ± 5.3	99.6 ± 5.8
Meropenem: compensated matrix effect	0.80	94.2 ± 1.8		97.2 ± 4.0	
	20	95.8 ± 1.3		97.6 ± 2.6	
	36	95.0 ± 4.8		97.8 ± 1.4	
Meropenem-d6		59.3 ± 7.3	83.1 ± 9.8	69.2 ± 3.9	104 ± 6.9
		59.1 ± 2.3	89.2 ± 6.6	68.2 ± 3.7	99.5 ± 3.9
		57.3 ± 3.3	94.3 ± 8.6	63.3 ± 6.5	100 ± 7.1

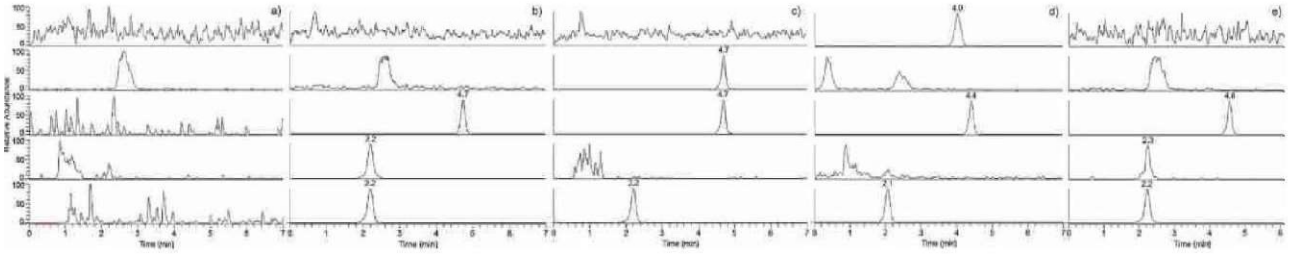

Fig. 4. Chromatogram of patient samples. Shown are a blank plasma without IS a), plasma of a patient with 69.1 mg/l cefepime b), 18.2 mg/l meropenem c), 5.2 mg/l imipenem d) and a CSF sample of a patient with 0.5 mg/l cefepime e). The corresponding plasma level at the time of the CSF sampling was 1.5 mg/l cefepime.

4.3. Stability

Results of stability tests can be found in Table 5. Cefepime showed acceptable stability under most tested conditions. The carbapenems imipenem and meropenem were found to be less stable. Both analytes could not be kept at -20 °C, neither in plasma nor in stock solution, for any prolonged amount of time. In untreated whole blood and plasma at RT, both carbapenems were very unstable as well. The addition of EG:MOPS increased the stability of these compounds considerably.

4.4. Application to clinical samples

Representative chromatograms of patient samples of both, plasma and CSF, can be found in Fig. 4. Fig. 5 depicts results of routine measurements from patients of the University Hospital Basel. According to the standard procedure protocol, imipenem and cefepime concentrations are measured at steady state at through level right before the next infusion. Meropenem is delivered as a continuous infusion in the ICU ward, the great majority of the meropenem measurements are therefore from continuous infusions. TDM samples of meropenem are collected at least 6 h after onset of infusion.

Table 5
Stability of cefepime, imipenem and meropenem under different conditions. The condition was deemed stable, if the mean concentration at both high and low control level was within ± 15 % of the nominal concentration.

	Cefepime	Imipenem	Meropenem
Whole blood, RT, no stabilization	7 h	2 h	2 h
Plasma, 4 °C, no stabilization	72 h	4 h	24 h
Plasma, RT, no stabilization	7 h	< 2 h	4 h
Plasma, RT, with stabilization	24 h	7 h	24 h
Plasma, -20 °C, with stabilization	12 months	< 3 weeks	<6 months
Plasma, -70 °C, no stabilization	12 months	< 3 weeks	12 months
Plasma, -70 °C, with stabilization	12 months	6 months	12 months
Stock solution, -20 °C	12 months	< 3 months	12 months
Stock solution, -70 °C	12 months	12 months	12 months
Auto sampler	24 h	24 h	24 h

5. Discussion

To our knowledge, we present the first method using HILIC MS/MS for the determination of imipenem, meropenem and cefepime in combination with EG : MOPS as stabilizer. The approach of using plasma calibration to quantify CSF samples, permitted by the use of a TurboFlow pre-column, is novel as well.

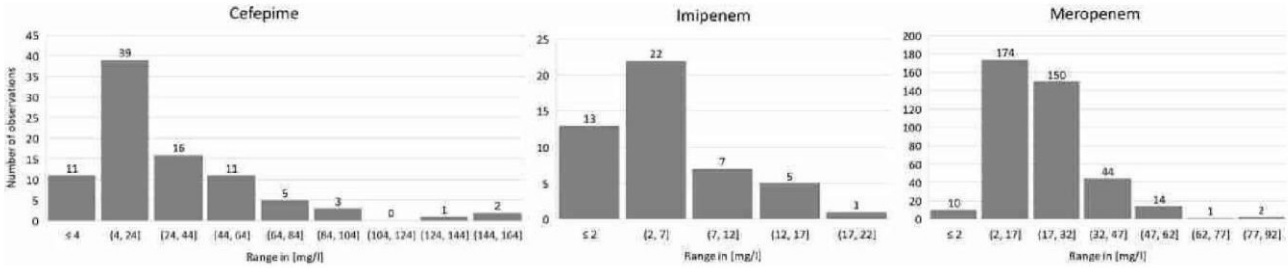

Fig. 5. Results of routine measurements. Histograms of over 500 routine measurements. The results show that most patients show beta-lactam levels above EUCAST breakpoints (≥2/4 mg/l). Some patients had very high concentrations associated with clinically observed toxicity.

HILIC is an interesting alternative to the usual reversed phase chromatography used for the analysis of pharmaceutical compounds. It is especially interesting when used together with a TurboFlow pre-column in a 2-D approach. After loading of the sample onto the pre-column and washing out salts and debris using an aqueous mobile phase, organic mobile phase is used to elute the analytes from the pre-column onto the separation column. In reversed phase approaches, this large volume of organic solvent often causes problems on the separation column, leading to e.g. broadened peaks. Since HILIC needs high percentages of organic solvent at the start of the method, the 2-D approach fits HILIC well. One disadvantage of HILIC is the long equilibration time these columns require, which make methods quite time consuming. In our method, we needed 4.5 min equilibration time to bring the HILIC column back to its starting condition.

During development of the method, it became clear that without any kind of stabilization of imipenem and meropenem, routine measurements would not be possible. Even a normal work up led to intolerable loss of both analytes. In addition, storage of QC and calibrators for more than a few weeks was not possible. The same was experienced by other groups working with those compounds [18,26].

Stability of carbapenems and cefepime are clearly dependent on solvent. In watery solutions like plasma, stability was much lower than in stock solutions. Adjusting the pH to 6 and disrupting water bridges using EG drastically improves stability [21]. The stabilizing solution used here was already successfully used by other groups [21,27] to increase stability of imipenem or meropenem and proofed to be convenient and effective in our method too.

Due to the bad short-term stability of the carbapenems and since it is not feasible to add stabilization already on the ward, samples containing imipenem and meropenem have to be send to the lab cooled by ice water or cold packs. Samples are kept frozen at -20 °C before analysis on the same day and the stabilizer is added to the samples as soon as they are thawed. To ensure stability of calibrators, QC and stock solutions, they are stored at -70 °C.

During HPLC method development, strong negative matrix effects could be observed for cefepime. Therefore, the gradient time was prolonged and a higher percentage of polar solvents was used in the loading phase for loading of the sample unto the precolumn. Thanks to these changes, matrix effects were reduced for all analytes and cefepime was moved out of the affected time range completely. There is still about 30–50 % of reduced signal for all analytes due to matrix effects, but the effect is very stable and not affected anymore by slight variations in retention time of the analytes. Now CSF and plasma can be analyzed in the same run, since there is no significant difference in recovery and matrix effect in both matrices. It is possible to analyze both, plasma and CSF, using a calibration in plasma.

Up to now, more than 500 TDM samples of cefepime, imipenem and meropenem have been analyzed, including some CSF samples for cefepime and imipenem. We also have successfully participated in several external quality assessments for the analytes cefepime and meropenem. As of now, there is no external quality assessment available for imipenem.

Over-curve linearity was tested successfully, but patient samples with levels above the calibration range have only been detected for cefepime so far. For cefepime, the first 10 patient samples above the linear range were additionally diluted and retested, resulting in variation from the original measurement of no more than 12 %.

The majority of the measured concentrations were above the EUCAST breakpoints [28] for most relevant bacteria. These breakpoints are relevant in the determination of empirical therapies and lie between 0.25 mg/l and 8 mg/l for cefepime, imipenem and meropenem for the most relevant bacteria (streptococci, enterococci, pseudomonas). Concentrations of beta-lactam antibiotics should stay above the minimal inhibitory concentration, or even above 4 times the minimal inhibitory concentration, at all times. Therefore, the measuring ranges chosen in this method do not extend below 0.4 mg/l, since any levels lower than this would be considered too low, independent of the exact value. The data gained from routine measurements show, most patients results lie well above the recommended concentration levels. For imipenem, six of the observed concentrations below 2 mg/l were between 1–2 mg/l. Some patients showed very low levels, but of these samples, many were measured as proof of complete clearance of the drug after administration had been already stopped a while ago. An example of such a case is patient X, whose CSF chromatogram can be found in Fig. 4. Patient X developed epileptic seizures under cefepime treatment. Drug administration was subsequently stopped and later, plasma and CSF samples were measured to exclude persistent cefepime as the reason for the continued seizures. The measurements showed a cefepime CSF level of 0.5 mg/l and a corresponding plasma level of 1.5 mg/l.

Concerning are the very high levels which some patients experience. Impaired renal function, encephalopathy and subsequent death was often associated with very high through levels. TDM clearly is useful in these cases to avoid adverse effects.

The method presented here has been successfully implemented as part of the antibiotic stewardship program of our hospital. TDM of beta-lactam antibiotics helps dosage adjusting in patients with renal impairment or complicated infections and provides help in detecting beta-lactam associated neurotoxicity. The method proofed to be robust over a period of over one year and accurate when compared to external quality assessments.

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CRediT authorship contribution statement

Sophia Rehm: Conceptualization, Methodology, Writing - original draft, Validation, Visualization. **Katharina M. Rentsch:**

Conceptualization, Supervision, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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LC-MS/MS method for nine different antibiotics

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ABSTRACT

Background and aims: TDM of antibiotics can bring benefits to patients and healthcare systems by providing better treatment and saving healthcare resources. We aimed to develop a multi-analyte method for several diverse antibiotics using LC-MS/MS.

Materials and methods: Sample preparation consisted of protein precipitation with methanol, dilution and online extraction using a Turboflow Cyclone column. Separation was performed on a Synergi 4 µm Max RP column and deuterated forms of three antibiotics were used as internal standards.

Results: We present a LC-MS/MS method for the quantitative determination of nine antibiotics, including five cephalosporins, the carbapenem ertapenem, the fluoroquinolone ciprofloxacin as well as the combination drug trimethoprim-sulfamethoxazole from plasma. Additionally, unbound ertapenem and cefazolin were analyzed in plasma water after ultrafiltration using plasma calibrators. Results from routine TDM show the applicability of the method.

Conclusion: The presented method is precise and accurate and was introduced in a university hospital, permitting fast TDM of all nine analytes. It was also used in a clinical study for measuring cefazolin free and total concentrations.

1. Introduction

During the last years, Therapeutic Drug Monitoring (TDM) of diverse antibacterials has become more common, however, only for aminoglycosides and glycopeptides is it already widely established [1,2]. Evidence is now growing, that plasma measurements may also be beneficial for beta-lactams and other drug classes like the fluoroquinolones. TDM of antibiotics may not only help in treating infections in the critically ill and high-risk patients, but also helps lower health costs by shortening the length of hospital stays and lowering the risk of antibiotic induced adverse events like nephrotoxicity. Several expert boards already recommend TDM of beta-lactam antibiotics in the critically ill [2–4]. These recommendations are part of the fight against growing antibacterial resistances and originate from the growing understanding that the one-size-fits-all approach of antibiotics in use during the last decades is not sufficient to treat the highly variable situation of the seriously ill [5].

Not only total plasma concentrations vary greatly in some high-risk patient populations, also the protein binding of drugs is altered by changes in the patients' renal clearance, hepatic function or through the use of supportive extracorporeal therapies like continuous renal replacement therapy [6]. Drugs with higher protein binding are affected more strongly, as already a small decrease in protein binding leads to a significant rise of the unbound fraction. Quantification of the unbound plasma concentration increases the information gained for those highly bound drugs. Cefazolin is usually stated to be bound between 80 and 90%, but its protein binding has been shown to be both concentration dependent and highly variable between patients [7]. The protein binding of ertapenem is concentration-dependent as well and varies between 85 and 95% in healthy patients depending on total plasma concentrations [8].

Aminoglycosides and glycopeptides can be quantified by commercially available immunoassays, which provide fast and relatively cheap testing. This is the foundation of the widely established TDM routine for these analytes. Currently though, there are no commercial tests available for most of the other antibiotics of interest. Therefore, laboratories still need to develop their own methods to provide hospitals with quick and reliable drug measurements. Since a broad range of antibiotics needs to be measured, multi-analyte methods including different antibiotic classes have become more common, since they save time and are cost-efficient.

Several papers are already published regarding single analyte

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methods or combination methods measuring two or three of the analytes presented here. Several multi-analyte methods including ceftazidime [9] and ciprofloxacin [9–14] have been published. In a review from 2014 about HPLC methods coupled to UV and MS Pickering et al. discussed published methods for the analysis of ertapenem [15]. The use of ertapenem-D_4 as internal standard for ertapenem sets aside the more recent method by van Rijn et al. [16].

Jin et al. presented a thorough method review about third generation cephalosporins including cefpodoxime and ceftazidime [17]. Cefazolin was featured in several multi-analyte methods using LC-MS, together with three other antibiotics [18] or together with ceftazidime, ertapenem and other antibiotics [19]. Cefazolin in serum and adipose tissues was measured by Lillico et al. [20]. Parker et al. measured cefazolin total and free plasma concentrations [21]. Both bound and unbound cefazolin and ertapenem were measured by Kratzer et al. [22], who used HPLC-UV.

Fewer methods using LC-MS have been published for cefaclor [23] or cefpodoxime in plasma [24].

There are several methods published for the quantification of cefuroxime and ceftazidime all using UPLC but very different columns. Abdulla presented an HILIC based method [25], others used reversed phase C18 columns [26,27] or a BEH C18 column [28]. Ceftazidime in rat plasma was measured by Beaudoin et al. [29] and in human plasma by a British group [30]. Two multi-analyte methods including ceftazidime were also presented [31,32].

The combination drug trimethoprim-sulfamethoxazole was featured in several papers: A well validated method for plasma, urine and dried plasma as well as urine spots was presented by Gonzalez et al. [33]. Plasma, as well as tissue were analyzed by Stein et al. [34] while Bedor et al. compared a LC-UV to a LC-MS method for the quantification of trimethoprim-sulfamethoxazole from plasma [35]. Trimethoprim-sulfamethoxazole combined with ciprofloxacin and 8 other antibiotics was quantified by El-Najjar [36] using UPLC-MS/MS.

While there are several methods for one or some of the here described analytes, to our knowledge, no method offers quantification of the nine antibiotics presented here. Another advantage of our method is the possibility to measure free ertapenem and cefazolin from plasma water after ultrafiltration.

2. Material and methods

2.1. Chemicals and reagents

Blank lithium-heparinized plasma was purchased from the blood donation center Blood Transfusion Centre, Swiss Red Cross (Basel, Switzerland) and tested for antibiotic residues before use.

Cefaclor monohydrate, cefazolin, cefpodoxime, ceftazidime pentahydrate, ciprofloxacin-D_8 hydrochloride hydrate, sulfamethoxazole, sulfamethoxazole-D_4, trimethoprim and trimethoprim-D_9 were bought from Sigma-Aldrich (Buchs, Switzerland). Ciprofloxacin hydrochloride, cefuroxime sodium salt and ertapenem monosodium was purchased from TRC (Toronto, Canada). Acetone, acetonitrile, methanol, and 2-isopropanol were purchased from Merck (Darmstadt, Germany), as well as ammonium acetate and formic acid. All analytes used were of analytical grade, all other chemicals of HPLC grade. The ultrafiltration devices Centrisart 1 20′000 molecular weight cut-off (MWCO) and Vivacon 500 30′000 MWCO, were bought from Sartorius (Göttingen, Germany), while Amicon Ultra 0.5 ml 30′000 MWCO and Centrifree 30′000 MWCO filters, were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation

The LC-MS/MS instrument was an UltiMate 3000 HPLC coupled to a TSQ ENDURA triple quadrupole mass spectrometer (both Thermo Fisher Scientific, Reinach, Switzerland). A TurboFlow Cyclone 50 × 0.5 mm column (Thermo Fisher Scientific) was used for online extraction while separation was performed on a Synergi 4 µm Max column 75 × 2 mm, RP 80 Å (Phenomenex, Basel, Switzerland). The mobile phases consisted of A: ammonium acetate (10 mM) and formic acid (0.1%), B: acetonitrile-methanol (1:1, v/v) with ammonium acetate (10 mM) and formic acid (0.1%). Mobile phase C was used for cleaning the columns after separation and was prepared from acetonitrile-acetone-2-propanol (1:1:1, v/v/v). In Table 1 and Fig. 1, the HPLC program and column configurations are described. The ionization chamber was equipped with an electrospray ionization interface operated in positive-ion mode and the interface settings were as follows: sheath gas flow 5.5 l/min; auxiliary gas flow 5 l/min, ion transfer tube temperature 300 °C; vaporizer temperature 400 °C; spray voltage 3500 V. Quantification was performed using multiple reaction monitoring (MRM). Calibration ranges, MRM transitions, collision energies and lens voltages are presented in Table 2. The whole LC-MS equipment was kept in a temperature-controlled environment at 24 °C. Samples stored in the autosampler were kept at 4 °C before analysis and 25 µl were injected for analysis.

2.3. Stock solutions, calibrators and quality control samples

Calibration and quality control (QC) stock solutions were prepared by separately weighting in and solving all analytes. Ciprofloxacin was prepared at 0.5 mg/ml, sulfamethoxazole at 15 mg/ml, trimethoprim at 1.25 mg/ml, cefpodoxime at 1 mg/ml and cefuroxime at 10 mg/ml in methanol. Cefaclor 2.5 mg/ml, cefazolin 5 mg/ml, ceftazidime 5 mg/ml, and ertapenem 5 mg/ml were prepared in water-methanol (1:1, v/v). The analytes were dissolved in water and diluted with the same amount of methanol.

Working solutions were prepared by adding the appropriate amount of stock solution to methanol. Stock solutions of deuterated standards were prepared in methanol at a concentration of 100 mg/l. The final internal standard (IS) contained 10 mg/l ciprofloxacin-D_8, 10 mg/l trimethoprim-D_9 and 75 mg/l sulfamethoxazole-D_4 in methanol.

We prepared seven calibrators, three quality controls, and an additional blank by spiking blank plasma with the appropriate amount of stock solution. Because of the high amount of analyte that had to be added to the plasma, two separate sets of calibrators and QC were prepared, one set containing ciprofloxacin, sulfamethoxazole, trimethoprim and ertapenem and the other containing the five cephalosporins. All solutions, QC and calibrators were stored at −70 °C until use.

2.4. Sample preparation

For the determination of unbound ertapenem or cefazolin, a 500 µl aliquot of plasma was placed in a Vivacon 500, 30′000 MWCO ultrafiltration device by Sartorius (Goettingen, Germany) and kept in a water bath at 37 °C for 1 h. The samples were quickly transferred to a centrifuge and centrifuged at 37 °C and 16′000*g* for 15 min.

A 100 µl aliquot of lithium-heparinized plasma or ultrafiltrated plasma water was precipitated with 200 µl IS. The samples were vortexed and shaken for 10 min at room temperature, followed by centrifugation at 4 °C and 14′000*g* for 10 min. The supernatant was transferred to an autosampler vial and diluted 1:5 with mobile phase A.

To assess the influence of experimental conditions on observed free fractions, we tested ertapenem ultrafiltration using different conditions. Influence of temperature was tested at 4 °C, 20 °C and 37 °C. Centrifugal forces tested were 1′000*g*, 2′000*g*, 8′000*g*, and 14′000*g*, while centrifugation time was varied between 5, 15 and 30 min. Influence of pH was tested by buffering plasma to pH 7.4, 7.8 and 8.4. The four ultrafiltration devices Centrisart 1, Vivacon 500, Amicon Ultra 0.5 and Centrifree were compared for unspecific binding.

Table 1
HPLC Method. MP: Mobile Phase, EC: Extraction Column, AC: Analytical Column.

			Loading Pump				Eluting Pump			
Time [min]	Connection EC - AC	Loop	Flow [ml/min]	MP A %	MP B %	MP C %	Flow [ml/min]	MP A %	MP B %	MP C %
0.00	out	out	1.5	95	5	0	0.4	97	3	0
0.50	in	in	0.2	95	5	0	0.2	97	3	0
1.17	out	in	0.2	95–0	5–100	0	0.4	97–20	3–80	0
4.17	out	in	1.5	0	100	0	0.4	0	100	0
4.67	out	in	1.5	0	0	100	0.4	0	100	0
5.17	out	in	1.5	60	40	0	0.4	97	0	3
5.33	out	in	1.5	60	40	0	0.4	0	0	100
5.88	out	out	1.5	60	40	0	0.4	0	0	100
6.33	out	out	0.2	95	5	0	0.4	97	3	0
9.33	End									

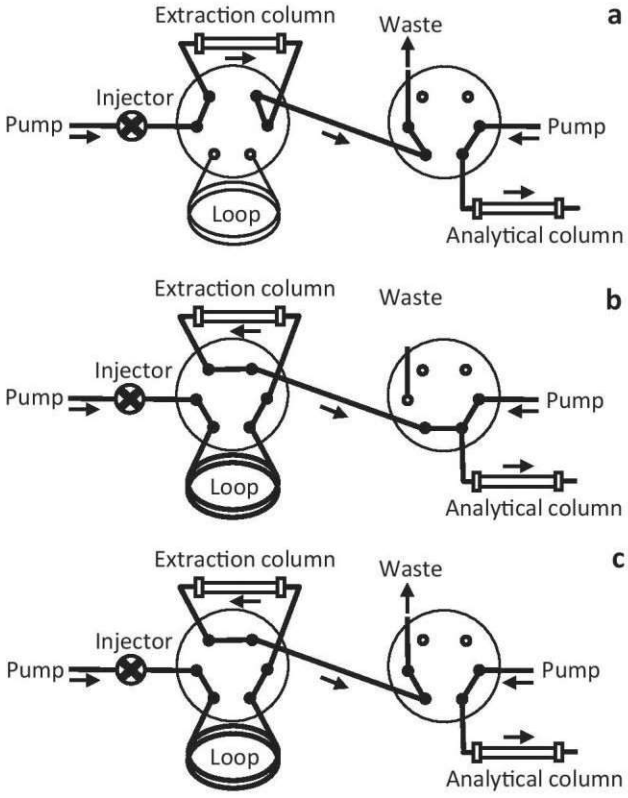

Fig. 1. Column configurations. The sample is pumped from the injector to the online extraction column using setting (a). The sample is then flushed onto the analytical column using the organic solvent stored in the loop according to setting (b). The sample is separated on the analytical column while the loop is refilled with organic solvent using setting (c). The columns are equilibrated using setting (a). Adapted from Rehm et al. [37].

3. Method validation

3.1. Analytical performance

We followed the FDA recommendations for bioanalytical method validation, except for some exceptions and we extended some acceptation criteria. We did not test freeze–thaw stability, since QC and calibrators should not be thawed more than once before analysis, we did not do incurred sample analysis and we only tested three QC levels instead of the recommended four.

Not more than one back calculated concentration of the calibration standards after regression should exceed ± 15% of its nominal value, while the mean concentration of QCs should be within 15%. All calibrations, after exclusion of maximum one calibrator, should show an R^2 of at least 0.99 for acceptable linearity. Accuracy and precision were assessed using three QC levels and measured on six separate days for inter-day replication and six consecutive times for intra-day replication. Plasma water QC were quantified using a calibration in plasma. Inter-day and intra-day inaccuracy and imprecision, expressed as coefficient of variation (CV), should be better than 15%. Carry over should be no more than 20% of the lowest calibrator.

3.2. Matrix effect and recovery

To explore recovery and matrix effect, six batches of plasma were prepared, including one from hemolytic and one from lipemic samples according to [38]. Matrix effect was quantitatively assessed by comparing spiked plasma and spiked water samples. Recovery was quantitatively assessed by comparing spiked extracted plasma and plasma spiked with the corresponding amount of analyte and IS only after extraction. Both times, three concentrations (low, middle, high) were analyzed. Measured analyte ratios of the corresponding plasma or watery samples were compared and the mean and CV of the six measured batches was calculated. Compensated matrix effect was calculated as the area ratio of analyte and corresponding IS. Compensated matrix effect should be no bigger than 15%.

Matrix effects were also assessed qualitatively, using a post-column infusion technique as proposed by Bonfiglio et al [39]. Six different blank plasma heparin samples, including one hemolytic and one lipemic plasma, were analyzed via injector while all analytes and IS were directly infused into the MS via a T-piece at 0.03 µg/min. Deviations from the baseline signal imply the occurrence of matrix effects.

3.3. Stability

Short-term stability was determined in plasma at 4 °C over 72 h, at RT over 24 h and in non-centrifuged whole blood at RT over 6 h. Blood collection tubes containing separation gel were tested against tubes without gel at 4 °C. One medium concentration level was tested for short-term stability. Processed samples stored in the autosampler at 4 °C were reanalyzed up to 24 h to establish auto-sampler stability. Long-term stability of plasma at −70 °C was determined after 6 months, stability of stock solutions at −70 °C after 12 months. Long-term and auto-sampler stability in plasma was determined at two levels (low and high). A maximum deviation of 15% from the original concentration was considered acceptable in all conditions.

4. Results and discussion

4.1. Analytical performance

A chromatogram of the method is shown in Fig. 2, depicting analytes at the lower limit of quantification and absolute signal heights. No more than one calibrator per calibration showed more than ± 15% deviation from its nominal value. R^2 was better than 0.993 for all analytes during

Table 2
Instrument settings. Calibration ranges and MRM settings, including one quantifier and two qualifiers for all analytes. CE, collision energy; LV, lens voltage; SF, source fragmentation voltage.

Analyte	Corresponding IS	Calibration range [mg/l]	Precursor ion [m/z]	LV [V]	Quantifier		Qualifier 1		Qualifier 2	
					Product ion [m/z]	CE [V]	Product ion [m/z]	CE [V]	Product ion [m/z]	CE [V]
Cefaclor	Trimethoprim-D9	0.25–25	368.24	137.09	191.06	10.25	174.06	13.54	118.11	33.36
Cefazolin	Trimethoprim-D9	1.0–100	455.08	117.67	322.99	10.25	295.00	15.92	156.06	15.97
Cefpodoxime	Trimethoprim-D9	0.1–10	428.11	154.99	324.00	12.38	240.99	14.05	210.00	18.65
Ceftazidime	Trimethoprim-D9	1.0–100	547.21	164.39	468.04	12.23	440.04	16.88	396.04	16.52
Cefuroxime	Trimethoprim-D9	1.0–100	442.11	137.09	364.00	10.25	336.00	15.16	318.00	20.62
Ciprofloxacin	Ciprofloxacin-D8	0.05–10	332.18	165.90	314.18	19.66	245.00	22.79	231.00	37.81
Ciprofloxacin-D8			340.15	131.63	296.11	18.19	249.04	25.02	234.99	39.02
Ertapenem	Trimethoprim-D9	0.5–100	476.21	168.30	432.04	10.25	390.06	10.25	345.99	14.80
Sulfamethoxazole	Sulfamethoxazole-D4	1.5–300	254.06	131.60	156.07	16.37	108.04	23.05	92.10	26.08
Sulfamethoxazole-D4			258.00	97.65	160.00	16.22	112.11	24.46	96.15	27.14
Trimethoprim	Trimethoprim-D9	0.125–25	291.15	148.00	275.04	25.37	257.04	31.29	230.11	24.71
Trimethoprim-D9			300.18	171.37	280.04	26.69	264.06	26.43	234.04	25.37

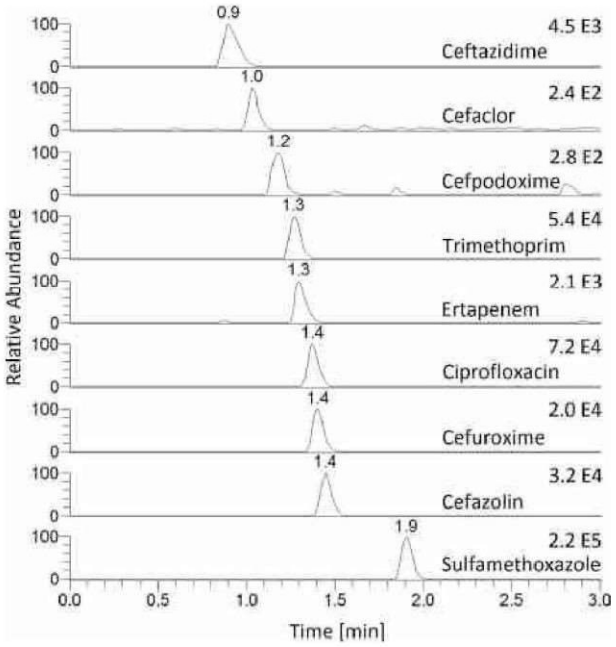

Fig. 2. Chromatogram of the lowest calibrator. Shown are retention times and absolute signal heights of the analytes and internal standards.

all measurements of the validation, except for ertapenem, which presented one calibration with an R^2 of 0.967, but was otherwise better than 0.997. Values of accuracy and precision for plasma and plasma water can be found in Table 3. Carry over in blank samples after the highest calibrator was below 20% for all analytes.

Imprecision and inaccuracy of all analytes were below 15% and therefore acceptable. Measuring ertapenem and cefazolin from plasma water was possible using a calibration in plasma. Even so, during routine measurements, QC made from spiked plasma water as well as unfiltered plasma are included in every run to ensure ongoing accuracy of the method. Plasma water samples could be analyzed using unfiltered plasma calibrators which indicates recovery and matrix effects of the analytes cefazolin and ertapenem to be similar in both matrices.

The 2 D column set-up used in this method allows the use of a TurboFlow pre-column, which acts as an online extraction. Analytes are flushed on to the pre-column using a watery mobile phase. The analytes are retained on the column by the TurboFlow system, while salts and other hydrophilic compounds are flushed to waste. The analytes are then eluted to the analytical column via the front end of the TurboFlow column. Since debris is eluted to waste and not retained on the TurboFlow column, as it happens in most other pre-columns, the 2 D system allows for much longer life-times (at least 1000 injections), while keeping the analytical column free from dirt. The good performance of the system in reducing matrix effects is demonstrated by the fact that matrix differences between plasma and plasma water are minimized and both can be analyzed using the same calibration.

No difference in free fraction of ertapenem could be found when using any of the four tested ultrafiltration devices. Because of their ease of use and good results with other analytes, we decided on the Vivacon filters. Generation of free fraction was tested at 4 °C, 20 °C and 37 °C degrees. While at 4 °C, measured free fraction was 4.8%, at 20 °C it was 10.1%, and at 37 °C 13.8%. Centrifugal forces also had a great influence on observed free fractions. The Vivacon filters used in this method should be used at 14′000 g, according to the manufacturer. Using lower centrifugal forces led to markedly higher free fractions. We observed a difference of 7.8% and 40% between 2′000 g and 8′000 g, and between 2′000 g and 14′000 g. On the contrary, at 1′000 g observed free fraction was vastly reduced. Varying centrifugal time from 5 to 15 or 30 min did not show any significant differences in free fraction, therefore 15 min were chosen, since sufficient filtrate was generated during that time. We also tested the influence of pH changes, which regularly occur in blood and plasma through centrifugation, contact with air and freezing. Between pH 7.4 and 7.8, no differences were noted, but at pH 8.2, the observed free fraction rose by 15%. These inconsistences were already described by several studies and are mostly in line with findings from Kratzer et al. or Zeitlinger et al. [22,40]. We decided to perform ultrafiltration using Vivacon 500 filters at 37 °C at 14′000*g* for 15 min, but decided against buffering plasma before filtration, since filtration usually happens before freezing, which could lead to a pH as high as 8.2 [41], and the effect was small at lower pH.

We successfully partook in the bi-annual external quality assessment "Antibiotic drugs II" provided by the SKML for the antibiotics ceftazidime, sulfamethoxazole-trimethoprim, and ciprofloxacin. We are therefore confident in the accuracy and precision of this method. As of now, there are no external quality assessments available for the other analytes.

4.2. Matrix effect and recovery

The quantitative matrix effect, compensated quantitative matrix effect, and recovery test results can be found in Fig. 3. Compensated quantitative matrix effect ranged from 84% for cefpodoxime to 95% for trimethoprim. Recovery ranged from 72% for ciprofloxacin to 99% for ertapenem. The use of deuterated analytes as IS must be regarded as gold

Table 3
Accuracy and precision. CV, Coefficient of variation. Mean of six measurements at three concentrations. Mean inaccuracy and mean CV were below 15% for all analytes. Plasma water values were quantified using a calibration in plasma.

Analyte	Plasma			Plasma water		
	Concentration [mg/l]	Accuracy ± CV [%]		Concentration [mg/l]	Accuracy ± CV [%]	
		Intra-day	Inter-day		Intra-day	Inter-day
Cefaclor	1.0	111 ± 5.7	114 ± 13			
	10	108 ± 3.6	97.9 ± 5.6			
	20	111 ± 2.9	105 ± 4.1			
Cefazolin	4.0	107 ± 4.3	94.3 ± 10	1.2	101 ± 3.2	98.7 ± 7.1
	40	102 ± 1.3	108 ± 9.0	12	94.1 ± 3.1	93.6 ± 6.0
	80	105 ± 3.7	106 ± 8.1	60	113 ± 1.4	109 ± 8.1
Cefpodoxime	0.4	102 ± 7.2	94.5 ± 13			
	4.0	102 ± 4.6	110 ± 14			
	8.0	110 ± 7.6	112 ± 13			
Ceftazidime	4.0	103 ± 7.9	89.0 ± 8.3			
	40	101 ± 3.7	101 ± 5.6			
	80	103 ± 3.2	104 ± 1.9			
Cefuroxime	4.0	114 ± 3.0	90.4 ± 8.9			
	40	110 ± 6.9	105 ± 7.9			
	80	114 ± 4.9	100 ± 5.7			
Ciprofloxacin	0.16	102 ± 4.9	107 ± 5.0			
	4.0	97.8 ± 1.3	96.2 ± 5.4			
	8.0	101 ± 3.9	98.8 ± 7.5			
Ertapenem	1.6	102 ± 10	102 ± 6.1	0.6	97.5 ± 7.0	95.3 ± 10
	40	103 ± 3.3	103 ± 6.3	6.0	86.3 ± 0.3.0	89.3 ± 4.2
	80	103 ± 3.6	104 ± 7.9	30	91.1 ± 2.0	99.8 ± 8.4
Sulfamethoxazole	4.8	91.7 ± 3.0	93.1 ± 4.6			
	120	98.0 ± 1.7	92.7 ± 5.3			
	240	96.0 ± 1.7	91.9 ± 3.9			
Trimethoprim	0.4	97.6 ± 3.1	93.9 ± 4.9			
	10	97.1 ± 3.3	100 ± 3.6			
	20	97.7 ± 2.9	100 ± 4.2			

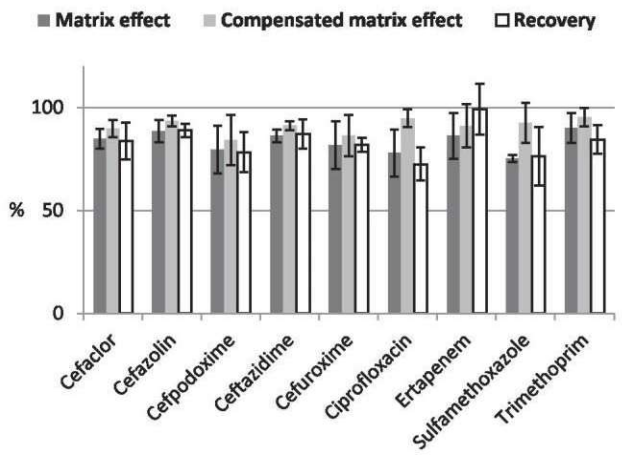

Fig. 3. Quantitative matrix effect, compensated quantitative matrix effect and recovery.

standard in quantification using LC-MS. Deuterated molecules show physicochemical properties extremely similar to their non-deuterated partners, which is crucial in compensating for recovery or matrix variations. They do have several downsides though: deuterated analytes may cause undesired matrix effects, they are generally quite expensive, and they may not be commercially available. While matrix effects can be validated, cost and availability limit the number of deuterated IS used in a method. Since we developed a multi-analyte method with nine different antibiotics, it was not feasible to include deuterated standards for every analyte. The IS used in our method were comparatively cheap, available from several manufacturers, which is important to assure supply safety and showed very good long-time stability. While it would have been preferable to use at least one deuterated cephalosporin, these analytes were either non-available at the time of method development, extremely expensive or unusable because of their low stability, like the cefaclor deuterated standards. The use of ceftriaxone-D_9 as IS was also not successful since ceftriaxone showed strong carry over which influenced the quantification. Trimethoprim proved to be an acceptable IS for all cephalosporins. If acceptance criteria for any of the cephalosporins cannot be met, the method could easily be expanded to include newly available deuterated cephalosporin analogues.

The results of the qualitative matrix effect testing can be found in Fig. 4.

4.3. Stability

Results of the stability testing can be found in Table 4. No influence could be seen when comparing the gel containing plasma tubes to non-gel tubes on stability, neither directly after centrifugation nor after storage in the tubes for up to 72 h.

Most analytes showed acceptable stability in all tested conditions. Only cefaclor exhibits very low stability in plasma and whole blood at room temperature. Therefore, cefaclor samples had to be stored on ice water immediately after blood withdrawal and sent to the laboratory

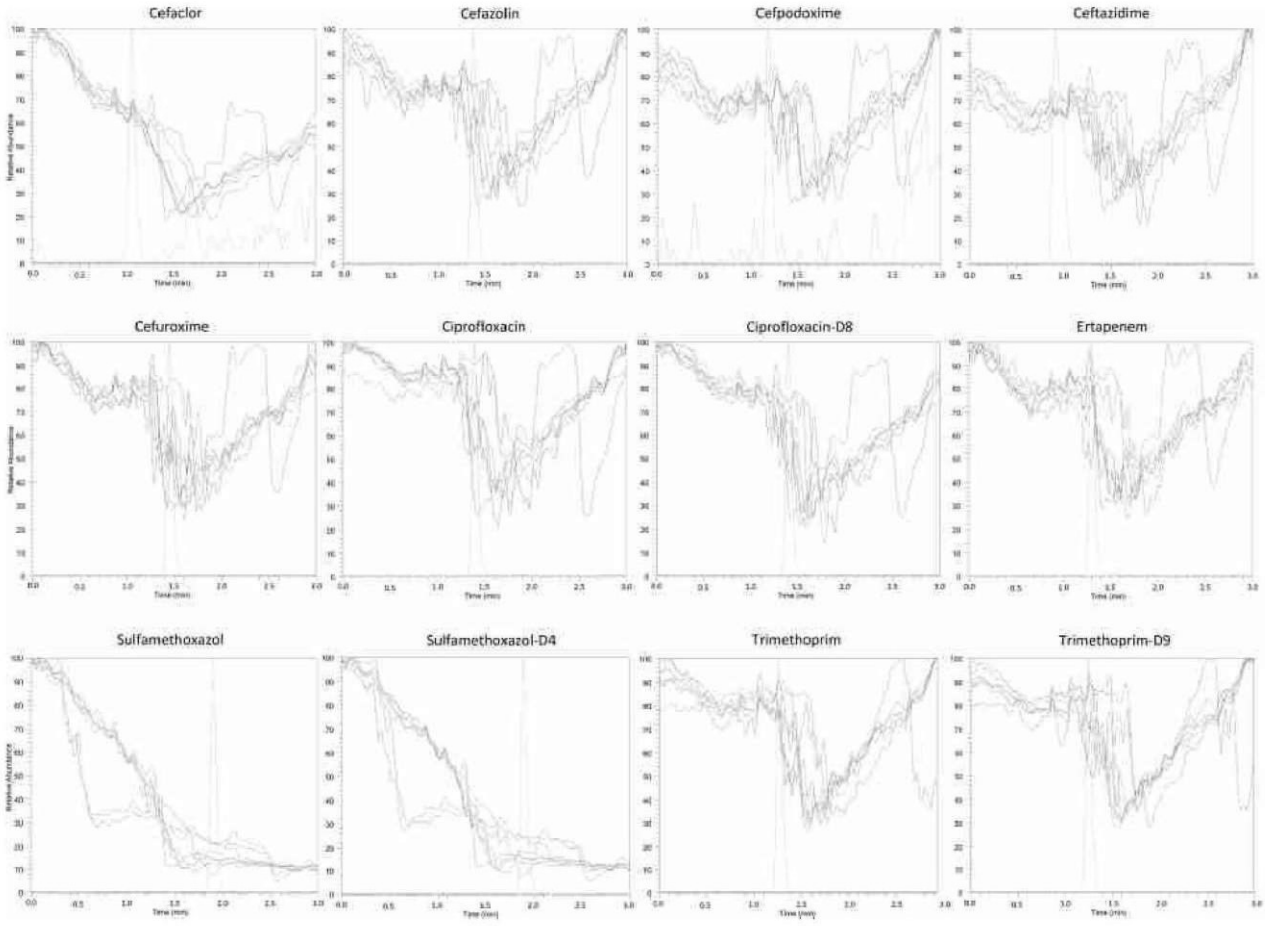

Fig. 4. Qualitative matrix effect of all analytes and IS. Shown are six blank samples, including one lipemic and one hemolytic sample, overlain with a chromatogram of the lowest calibrator.

Table 4
Stability of the analytes in different conditions. The condition was deemed stable, when the resulting concentration was within ± 15% of the nominal concentration.

	Whole blood, RT	Plasma, 4 °C	Plasma, RT	Plasma, −70 °C	Stock solution, −70 °C	Auto-sampler, 4 °C
Cefaclor	1 h	8 h	<2h	6 months	12 months	12 h
Cefazolin	6 h	72 h	72 h	6 months	12 months	24 h
Cefpodoxime	6 h	72 h	72 h	6 months	12 months	12 h
Ceftazidime	6 h	48 h	24 h	6 months	12 months	24 h
Cefuroxime	6 h	72 h	72 h	6 months	12 months	24 h
Ciprofloxacin	6 h	72 h	24 h	6 months	12 months	24 h
Ertapenem	6 h	48 h	24 h	6 months	12 months	24 h
Sulfamethoxazole	6 h	72 h	24 h	6 months	12 months	24 h
Trimethoprim	6 h	72 h	24 h	6 months	12 months	24 h

either cooled using cool-packs (in-house samples) or frozen and shipped on dry ice (external samples). All other in-house samples could be transported at room temperature. Samples from external senders were shipped on dry ice nevertheless, to insure stability during the longer transport. An autosampler stability of at least 12 h makes repeat measurements of samples possible.

4.4. Application to clinical samples

The presented method has been used in clinical routine for about two years and in a clinical study measuring cefazolin total and unbound plasma concentrations. About 100 routine patient samples have been analyzed. Most samples were analyzed for ciprofloxacin and trimethoprim-sulfamethoxazole (both 37 samples). Plasma concentrations can be found in Figs. 5 and 6 respectively.

Optimal therapeutic ranges and TDM testing schemes for antibiotics depend largely on the antibiotic class as well as the target bacteria. For beta-lactams, free concentrations should stay above the minimal inhibitory concentration (MIC) of the pathogen during the full dosing interval, or even above 4 to 8 times the MIC, especially in critical care patients, while higher concentrations give rise to a higher risk of adverse events [3]. Therefore, beta-lactam TDM should be performed at though level and the therapeutic target should be the free concentration >1-8x MIC during the full dosing interval.

The ciprofloxacin therapeutic target is a ratio of Cmax over MIC of about 10. Borderline susceptible breakpoints according to EUCAST are at 0.5–1 mg/l [42]. Even though it can't be ascertained what target range would have been optimal for each individual patient in the group presented here, most samples showed acceptable ciprofloxacin levels between 0.5 and 8 mg/l [43,44].

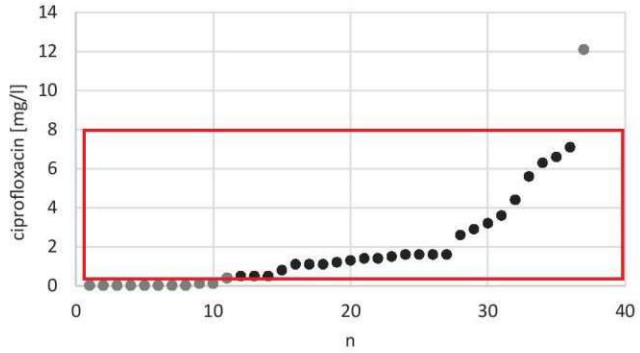

Fig. 5. Ciprofloxacin concentrations of patient samples. The red box contains all samples inside the target range of 0.5–8 mg/l. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

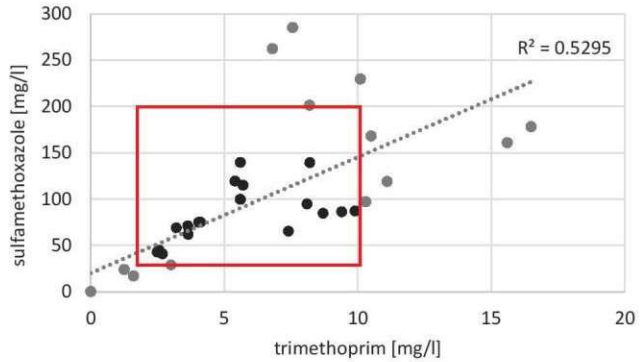

Fig. 6. Sulfamethoxazole vs. trimethoprim concentrations of patient samples. The red box contains all samples inside the target range of 30–200 mg/l for sulfamethoxazole and 1.5–10 mg/l for trimethoprim. The correlation between both concentrations in one sample was $R^2 = 0.5295$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

There is no consent yet, on what the pharmacokinetic–pharmacodynamic relationship is for sulfamethoxazole-trimethoprim; Cmax over MIC or AUC [45]. Therefore, Cmax and Cmin measurements might be used in TDM. Sulfamethoxazole-trimethoprim showed great individual plasma level distribution. Only 60% (18/30) of the positive samples presented plasma concentrations inside the target range of 30–200 mg/l for sulfamethoxazole and 1.5–10 mg/l for trimethoprim [46,47]. The correlation between sulfamethoxazole and trimethoprim concentrations in the same patient was rather low, with an R^2 about 0.5. This further underlines the fact that in severely ill patients, as were most of the tested patients here, unpredictable changes in volume distribution or renal clearances can occur. Predicting plasma concentrations in severely ill patients is clearly difficult and TDM is therefore advised.

Eight samples showed levels below the limit of quantification for ciprofloxacin and seven showed undetectable levels of sulfamethoxazole-trimethoprim. In most cases, these levels do not present normal TDM measurements, but rather checks to confirm if residual antibiotics are the cause of some observed complication. Those negative results do therefore not necessarily signify inadequate dosing.

5. Conclusion

The method presented here has been used for TDM in a university hospital and for a clinical study researching cefazolin and cefazolin free concentrations. The method is accurate and precise, uses a short and simple sample preparation and enables measurements of ultra-centrifuged plasma water concentrations of ertapenem and cefazolin using a calibration in unfiltered plasma.

CRediT authorship contribution statement

Sophia Rehm: Conceptualization, Methodology, Investigation, Writing - original draft, Validation, Visualization. **Katharina M. Rentsch:** Conceptualization, Supervision, Resources, Writing - review & editing.

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Validation of ultrafiltration techniques for TDM

Introduction

Measuring free fraction means measuring the pharmacological active part of a drug. However, the true unbound fraction of a drug in a sample is not known and cannot be known. We can only define a "gold standard" – an analytical method against which to compare all other measurements. Equilibrium dialysis is generally regarded as this gold standard, but it has its own flaws. During equilibrium dialysis, two chambers are separated by a semipermeable membrane. One chamber is filled with plasma and the other chamber contains a buffer solution, usually isotonic phosphate-buffered saline. When equilibrium is reached after 4 to 24 hours, the free drug will have diffused through the membrane and free drug concentrations on both sides will be equal [1]. During the process, not only the drug, but also other small molecules (e.g. salts) can pass the membrane, changing their concentrations in the sample. The process is very time consuming; therefore, analytes instable at higher temperatures will degrade during the process.

Ultrafiltration is a widely used alternative to equilibrium dialysis. In ultrafiltration, a plasma sample is filtrated though a membrane at high pressure produced by g forces. These membranes are available with well-defined pore sizes, allowing the exclusion of molecules above a specific weight, mostly 10 to 30'000 Dalton. Only small molecules can pass, keeping proteins and all protein-bound drugs in the reservoir. The ultra-filtrate will have the same concentration of the drug as the unbound fraction. This procedure takes only about 15 minutes of centrifugation, is comparatively cheap and needs only small sample volumes compared to equilibrium dialysis. Ultrafiltration presents its own set of problems though: The ultrafiltration process may change the equilibrium in the sample, since not only the drug but also water, salts and small peptides are excreted during the filtration process, changing the concentrations of these in the reservoir. Analyte binding to the membrane or parts of the filter may disturb analyte concentrations [2].

Unfortunately, protein binding of drugs is variable depending on patient- individual conditions. Chin et al. recently showed that predicting free flucloxacillin levels from total flucloxacillin in hospitalized patients is unreliable [3].

The conditions applied during filtration can have a huge impact on the measured free fraction, resulting in clinically relevant variations of the results. Several factors influence protein binding of drugs, and therefore perceived free fractions, can be altered by laboratory conditions. These factors are the filtration devices used, the centrifugal force and time as well as the temperature and pH during the filtration [4].

Two proteins dominate drug binding to plasma proteins: albumin and alpha-1-acid glycoprotein. Albumin is the main component in drug-protein binding and at 40 g/l makes up nearly 60% of total protein in plasma. It has a molecular weight of 66'000 Da and possesses two main binding

sites for drugs with different binding specificity [2, 5]. One of these is known to have a pH dependent binding capacity. Albumin binds mostly acidic compounds, but also some neutral and basic compounds. Albumin concentration is kept constant in healthy individuals, but can decrease significantly in patients with liver failure, injuries, after surgeries, through malnutrition or inflammation [2, 6].

Alpha-1-acid glycoprotein is the second most important protein concerning drug binding in plasma. It has a molecular weight of ca 44'000 Da and has one major binding site relevant for xenobiotics [7]. Several glycosylated states with different binding properties and kinetics exist. Physiological concentrations vary from 0.5 to 1 g/l, but are increased in infection, inflammation, or cardiovascular diseases, as it acts as an acute-phase one protein. Alpha-1-acid glycoprotein binds mostly basic compounds [2, 7].

Material and Methods

The four ultracentrifugation devices Centrisart 1 20'000 molecular weight cut-off (MWCO) and VIVACON 500 30'000 MWCO, both from Sartorius (Göttingen, Germany), as well as Amicon Ultra 0.5 ml 30'000 MWCO and Centrifree 30'000 MWCO filters, both from Merck (Darmstadt, Germany) were compared. The conditions temperature, pH, centrifugal force, and centrifugal time were adjusted. The fixed angle rotor centrifuge microcentrifuge 5424 by Eppendorf® (Schönenbuch, Switzerland) and the free-swinging centrifuge Heraeus™ Megafuge™ 40 by Thermo Scientific™ (Reinach, Switzerland) were used. Several antibiotics as well as the antiepileptic drugs valproic acid and phenytoin were used as model analytes. Antiepileptic drugs were measured using GC-MS, while all antibiotics were measured using LC-MS/MS.

Ciprofloxacin hydrochloride, ertapenem monosodium, amoxicillin trihydrate, piperacillin, flucloxacillin sodium and clavulanic acid potassium salt were purchased from Toronto Research Chemicals (Toronto, Canada). Cefazolin, sulfamethoxazole, trimethoprim, clindamycin hydrochloride, valproic acid, phenytoin, penicillin g potassium salt and tazobactam were purchased from Sigma Aldrich (Buchs, Switzerland).

Procedure

Spiked plasma was incubated at 4°C, room temperature or 37°C for 30 min, then all ultrafiltration devices were filled with 450 µl plasma and the plasma left on the filters for another 20 minutes. After preliminary tests, I concluded that the longer samples stayed in contact with the filters, the greater the adsorption. Since waiting times cannot be totally avoided, I decided to set a fixed time to make results more comparable.

I evaluated the following factors influencing the concentration of the free drug fraction in the plasma water:

- ultrafiltration device
- centrifugal force
- length of centrifugation
- temperature
- pH

When not testing for influence of centrifugal forces, the following settings were used as recommended by the manufacturer: Centrisart I and Centrifree 1'200 g, Vivacon and Amicon Ultra 14'000 g. Regular spinning time was 15 min. When testing for the temperature influence at 4°C, because of very low yields, spinning time had to be prolonged to 30 minutes. When not testing for influence of pH, frozen, non pH stabilized serum was used. When not testing for temperature, plasma was warmed to 37°C and centrifuges were set to 37° C. Only the comparison between water, plasma water and plasma were done at 20°C.

All filtrations and measurements were performed in duplicate and the mean of both results was used for calculation. Table 1 displays the analyte concentrations used during the experiments.

Table 1 Drug concentrations used in the ultrafiltration tests.

	[mg/l]
Amoxicillin	24
Benzylpenicillin	8
Ciprofloxacin	1.0
Clavulanic acid	8
Clindamycin	1.2
Ertapenem	10
Flucloxacillin	12
Phenytoin	1
Piperacillin	24
Sulfamethoxazole	30
Tazobactam	8
Trimethoprim	2.5
Valproic acid	5

All filtered concentrations were compared to measured unfiltered concentrations from the same batch of plasma, measured in the same run. For temperature dependent values, unfiltered plasma was stored for the same amount of time at the relevant temperature to include all loss of analyte by thermal breakdown.

Results and Discussion

The observed free drug fractions varied strongly between serum and plasma. Plasma gave smaller free fractions for nearly all tested instants, for seven tested analytes and four tested devices. Plasma/serum ratio mean was 1.07 for Centrifree, 1.09 for Centrisart I, 1.17 in Vivacon filters and 1.10 using Amikon Ultra devices. This might be due to differences in pH, as pH can greatly influence protein binding, as shown in Figure 1. Regretfully, I did not test pH of the used serum and plasma pools.

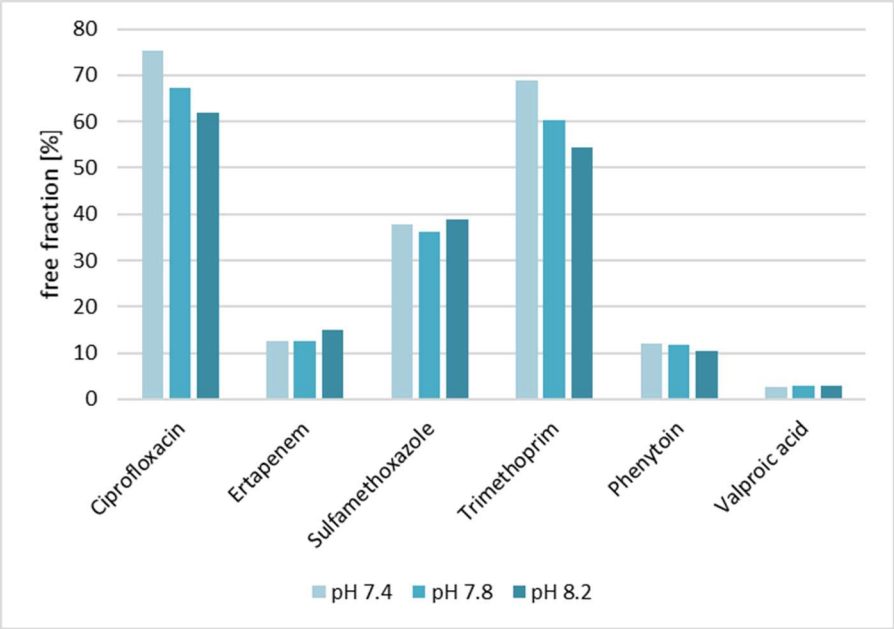

Figure 1: Free fractions at pH 7.4, 7.8 and 8.2. Analytes filtered from plasma, at 37°C using Vivacon filters. The physiological blood pH of 7.4 rises rapidly after centrifugation. A pH of 7.8 was measured in thawed plasma. A pH as high as 8.2 was measured after repeated freezing and prolonged standing [1].

I observed mostly lower free drug fractions at higher pH, especially for ciprofloxacin and trimethoprim, while ertapenem showed higher free fraction at higher pH (12.7% at pH 7.4 to 14.9% at pH 8.2). The other tested analytes were not greatly influenced by the pH changes.

Recovery of the analytes from water and plasma water after ultracentrifugation was quite low for some analytes and not a good representation of recovery from plasma. Plasma water proofed to give better predictions. The reason for this observation could be that plasma proteins can deactivate the membranes of the ultrafiltration devices. Free fractions varied up to 100% between

filtration devices. This effect was especially distinct in analytes with strong protein binding. Exact data can be found in table 2.

Table 2: Recovery from water compared to recovery from plasma water and plasma of some selected analytes. Recovery of analytes from water after ultracentrifugation using different devices, average of three measurements. Only the Vivacon device showed no adsorption of clindamycin and flucloxacillin. Measurements at 20°C, 15 min spinning time.

Analyte	**Amikon Ultra**			**Vivacon**			**Centrisart 1**			**Centrifree**		
Recovery [%]	Water	Plasma -water	Plasma	Water	Plasma -water	Plasma	Water	Plasma -water	Plasma	Water	Plasma -water	Plasma
Amoxicillin	96.3	101	82.7	101	104	72.6	98.9	103	83.4	98.5	105	85.1
Benzylpenicillin	66.6	95.3	31.0	100	103	36.5	95.2	101	36.5	91.7	101	39.7
Clavulanic acid	83.4	87.3	86.9	93.7	87.6	72.6	95.9	93.2	88.6	96.0	95.9	94.4
Clindamycin	2.4	40.8	3.3	104	103	9.4	73.9	84.0	6.0	90.4	90.9	6.7
Flucloxacillin	55.2	81.9	4.6	101	101	10.6	78.2	95.4	6.4	89.4	102	6.3
Piperacillin	49.8	95.4	65.6	103	100	60.0	98.4	96.5	78.2	89.3	95.0	82.3
Tazobactam	84.4	106	72.7	89.1	105	91.2	94.1	96.1	95.6	90.0	95.1	97.9
Ciprofloxacin	14.7	84.1	64.1	140	98.5	64.7	81.9	96.4	67.7	72.7	93.7	70.3
Ertapenem	72.9	94.4	10.1	95.6	89.0	6.03	83.1	91.3	9.7	91.0	90.1	12.1
Sulfamethox-azole	95.3	96.8	31.2	100	96.4	29.6	32.9	93.5	30.4	95.0	98.1	34.2
Trimethoprim	69.6	90.5	40.0	95.2	90.5	47.4	73.3	40.6	24.5	81.7	93.3	52.3
Phenytoin	72.0	94.8	1.9	97.8	98.3	2.4	17.6	45.3	1.1	96.4	100.3	2.5
Valproic acid	77.5	98.5	12.2	96.4	97.6	13.4	92.5	98.7	12.1	92.5	98.7	13.7

Influence of g force during ultrafiltration was tested at 1’000, 2’000, 8’000 and 14’000 g. 1’000 to 2’000 g are the recommended values for Centrisart I and Centrifree filters. 14’000 g is the recommended g force for Vivacon and Amicon Ultra devices. Since according to the manufacturer, Centrisart I and Centrifree devices should not be used at such high g forces, I decided to use only the Vivacon filter in this test. Results for all analytes can be found in figure 2.

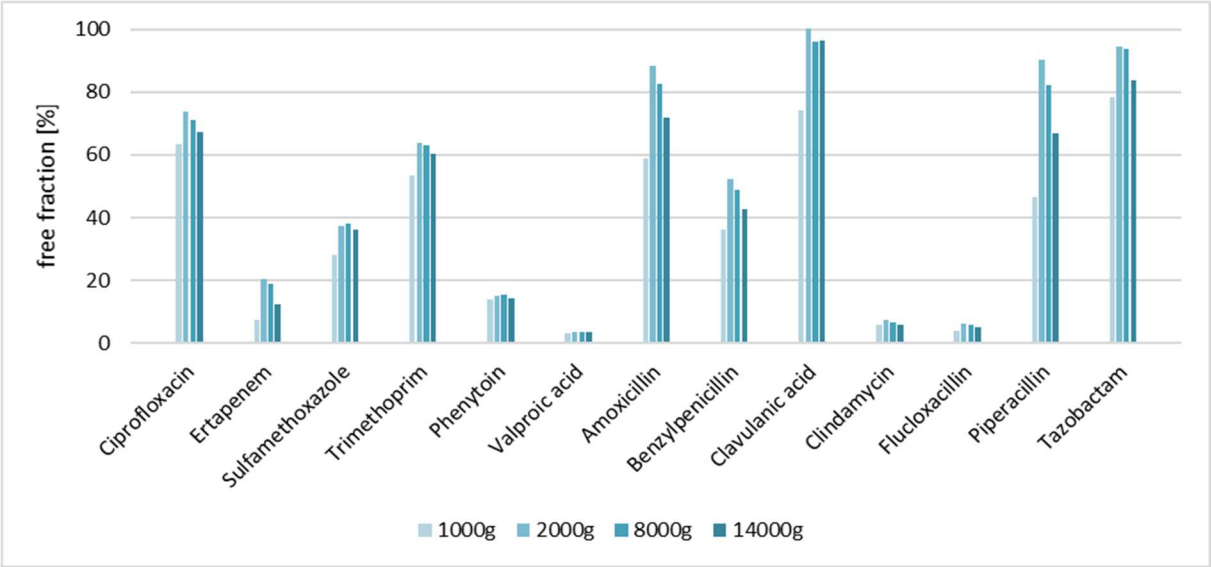

Figure 2: Influence of centrifugation force on free fraction. Centrifugation time 15 min at 37°C. Vivacon filters. 14'000 g is the recommended centrifugation force for the Vivacon filters.

It has been proposed that higher centrifugal forces will lead to a "bleeding" effect, resulting in lower observed free drug concentration fractions [4]. Kratzer et al. observed about 30% lower free fractions for ertapenem when comparing filtrates from 1'000 g to 10'000 g. In line with their data, I observed a difference of -7.8% and -40% between 1'000 g and 8'000 g, and between 1'000g and 14'000g, respectively, in the free fraction of ertapenem. It is remarkable though, that the strongly bound phenytoin and valproic acid, were not strongly influenced by centrifugal force (4-5% lower binding at higher g), but clindamycin, flucloxacillin and ertapenem, which are also strongly bound were influenced to a greater degree (15-40% lower binding at higher g). A reason for this might be the higher molecular mass of these analytes (see table 3).

Table 3 Molecular masses of some highly protein bound analytes.

	Molecular mass [g/mol]
Ertapenem	475
Flucloxacillin	453
Clindamycin	423
Phenytoin	252
Valproic acid	144

Greater centrifugation forces generally yielded smaller free fractions, but to a widely varying degree between analytes (Figure 2). Piperacillin and tazobactam for example, are both about 30% protein bound. The apparent piperacillin binding rose from 10 to 33% between 2'000g and 14'000g. Apparent tazobactam binding rose from 6.4 to 16%.

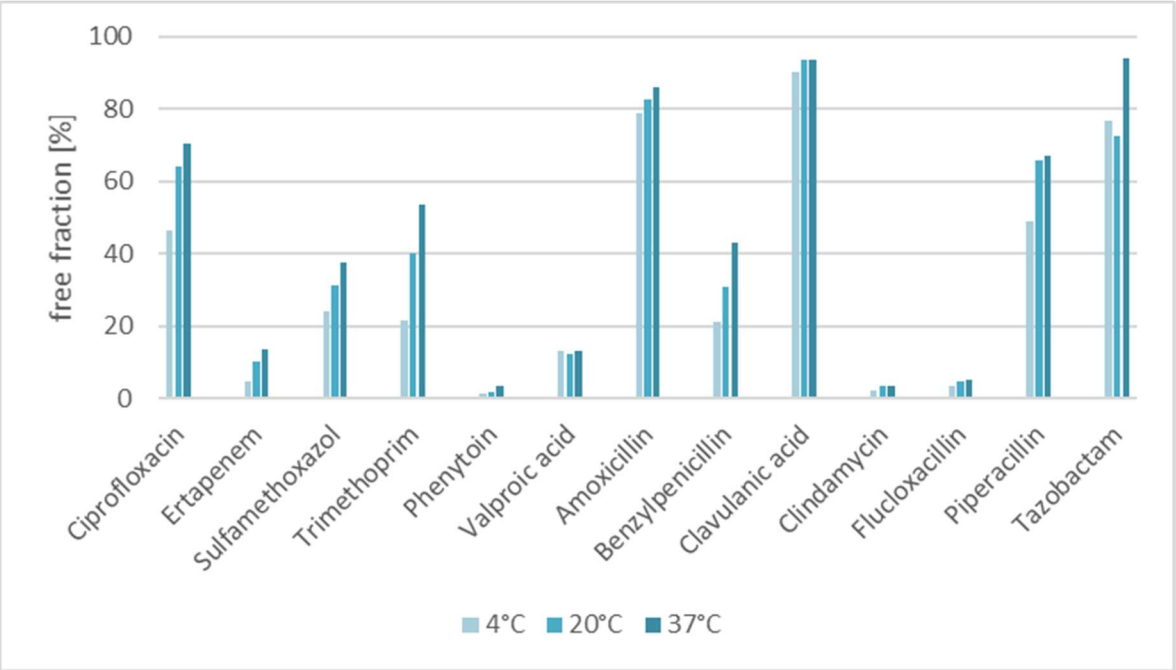

Figure 3: Influence of temperature on free fractions. All temperature results measured in plasma, mean of all four devices, pH not adjusted.

Temperature can clearly have a great effect on measured free fractions, but the effect size is analyte dependent (see figure 3). While for valproic acid centrifugation even at 4°C is possible and the only slightly bound amoxicillin and clavulanic acid are also not greatly influenced, all of the other tested compounds should be filtered at 37°C. Ertapenem at 4°C showed a free fraction of 4.8%, which is compliant with literature data from healthy individuals, but at 37°C the free fraction was 13.8%. This inconsistency was already described by several studies [8, 9].

Influence of length of centrifugation was compared after 5 minutes, 15 minutes and 30 minutes. For results, see figure 4.

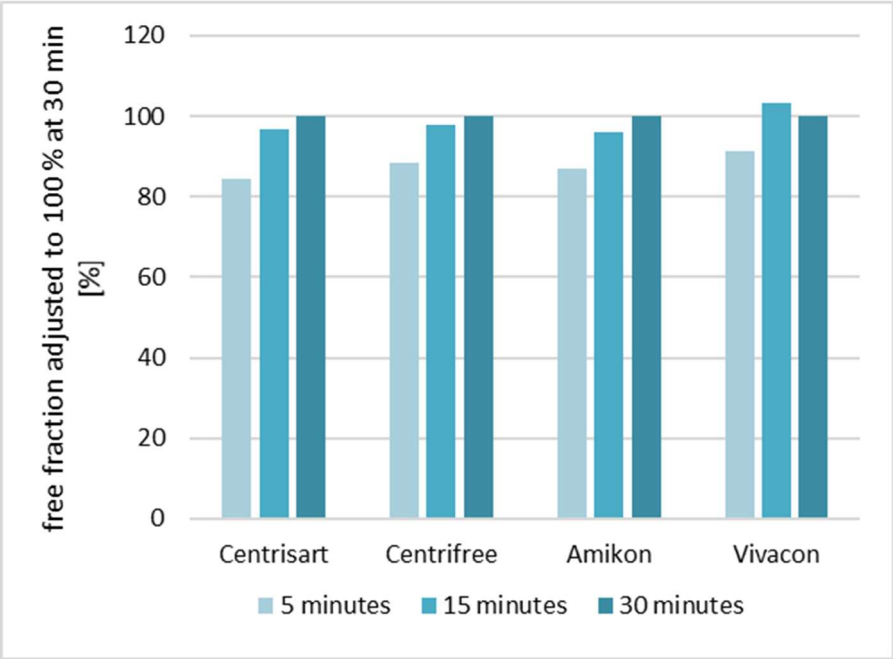

Figure 4: Influence of centrifugation length. Shown are the mean difference of six measured analytes (sulfamethoxazole, trimethoprim, ciprofloxacin, ertapenem, valproic acid, phenytoin), adjusted to the mean free fraction after 30 minutes.

Length of centrifugation has been claimed to have no effect on free fraction [2]. The effect of lower free fractions I experienced after 5 minutes compared to longer centrifugation times might be the same effect observed in very low centrifugal forces: the amount of filtrate produced after very short centrifugation times may be diluted by the glycerin used in the filter membranes. All tested devices contain glycerine; Centrisart I, Vivacon 500 and Centrifree contain 2 µl, the Amikon Ultra-0.5 contains "trace amounts of glycerine". When comparing recoveries of the filters, it becomes clear that dilution by glycerine cannot be the answer to this problem. After 5 minutes, the Centrifree devices yieled the lowest ultrafiltrate volume of approximately 45 µl. This volume could be affected by an extra 2 µl glycerine, but the results show that Centrifree devices were not stronger affected by spinning time than the Centrisart or Amikon devices, though their approximate yield was 130 µl and 280 µl, respectively, after 5 minutes.

The differences may be due to the fast cooling down of the small samples while being transferred from the water bath to the centrifuge. The centrifuge as well, is unable to maintain 37°C for long, as soon as the centrifuge is heated up, the samples need to be transferred and the filtration has to be started. Since samples and centrifuge may both not have exactly 37°C in the beginning of the filtration, this could explain the lower free fractions seen after 5 minutes. Probably, during this time, the samples heat up and longer centrifugation times do not influence the result anymore.

This could explain why differences found between 15 minutes and 30 minutes spin time are neglectable and within method deviation.

Table 4 Literature review of expected free fractions. Data from this study are measurements in different devices in plasma at 37°C, pH not adjusted.

	Free Fraction according to literature [%]	Free Fraction found in this study	Reference
Amoxicillin	80	73-85	[10]
Benzylpenicillin	35-65	31-40	[11]
Ciprofloxacin	60-80	64-70	[12]
Clavulanic acid	70-75	73-84	[10]
Clindamycin	6-40 5-19 (in ICU)	3.3-9.4	[13, 14]
Ertapenem	5-8 (up to 55 in ICU)	6.3-12	[8, 15, 16]
Flucloxacillin	4-7	4.6-11	[17, 18]
Phenytoin	6-42	10-12	[19]
Piperacillin	70	60-82	[20]
Sulfamethoxazole	40	30-34	[21]
Tazobactam	70	73-98	[20]
Trimethoprim	50	25-52	[21, 22]
Valproic acid	5-10 at 20mg/l, up to 52 at 430mg/l	12-14	[23]

In a recent study on flucloxacillin protein binding Dorn et al. compared Vivacon/Vivafree from Sartorius (Göttingen, Germany) to Nanosep filters from VWR (Darmstadt, Germany) [14]. With both ultrafiltration devices the same values for free clindamycin were acquired. In contrast, the free fractions of flucloxacillin and tedizolid were significantly lower with Nanosep compared to Vivafree. They did not find any influence of centrifugal forces used during centrifugation. Temperature changes from 37°C to 20°C to 4°C resulted in about 15% lower free fraction for flucloxacillin and a 50% lower free fraction of tedizolid, but a 40% higher free fraction of clindamycin. In contrast to their findings, I found consistently lower free fractions of all analytes at lower temperatures. Regarding pH changes, Dorn et al. found tedizolid to be not affected, clindamycin free fraction nearly halved between pH 7.4 and 8.2 and flucloxacillin free fraction was about 20% higher at higher pH.

Kratzer et al. compared several filtration devices and influence of pH, temperature, centrifugation time and relative centrifugal force using among others, cefazolin and ertapenem [4]. They used Vivacon and Centrifree and Amicon-Ultra 0.5 devices as I did and also included the Nanosep Omega PES (VWR, Ismaning, Germany) and Vivaspin 500 PES (Sartorius, Göttingen, Germany). They also observed an increase in free fraction at higher temperatures for most analytes except ceftriaxone, which is in line with my findings. At 37°C Kratzer et al. found ertapenem free fractions of about 20%. (I found about 14% at 37° C). They did not find any differences between plasma and serum, but they were controlling for pH, which I did not. They found increased free fractions of cefazolin at higher pH (8.5 vs 7.5) while ertapenem showed lower free fractions at higher pH.

Divergences from the literature in this study might be due to the small amount of methanol in the samples, which of course might interact with drug-protein binding. Since this project was mainly done to evaluate the influence of filtering devices and filtering conditions and the amount of methanol was the same during all tests, this influence is negligible.

Problematic is also the equilibration time at 37°C since the plasma, after transference to the filters, will quickly cool down again. In addition, the small centrifuge used could not maintain 37°C without spinning for a certain time to equilibrate samples beforehand inside the centrifuge.

All unfiltered values were measured in the same run with filtered values and those were used for calculation, not the calculated spikes values. This was done to incorporate daily deviations in measurements. Also, for the temperature dependent filtered values, unfiltered plasma was stored at the relevant temperature for the same amount of time as the filtered samples. This way, thermal breakdown was included in the calculation, as we were not interested in the stability of the compounds during work up, but only in ultrafiltration efficacy.

During this study I found slightly different values for the same experiment while comparing different conditions against each other. I take this as proof of how sensitive the ultrafiltration system is to underlying conditions. A different batch of plasma for example, or a slightly different room temperature can produce divergent results.

While it has been proposed that adsorption onto membranes should not be a problem in the presence of plasma proteins [24], this effect was clearly seen in several devices (compare table 2).

Some limitations of the study were the use of a free swinging centrifuge for the Centrisart I and Centrisart filter instead of a fixed angle centrifuged as specified by the manufacturer. I did not use the same serum or plasma pool for all experiments. Albumin and total protein content of my serum is not known. Since I mixed several drugs together, interactions between drugs influencing protein binding cannot be excluded. I tried to minimize this problem by using small but

therapeutic analyte concentrations. I only tested at one concentration. Residual methanol of 2% may have influenced protein binding. As I used frozen plasma and serum, I cannot exclude that freezing might have had an influence on protein configuration and surely had an impact on serum pH. I did not measure pH during the complete set of experiments, but only in one pool of serum.

Conclusion

The results show a big impact of filtration conditions on the observed free fractions of the drugs. Temperature, pH and filtration forces affect all analytes, but to widely varying degrees. Some filtration devices strongly absorb drugs, leading to erroneous low free fractions. Therefore, validation of the ultrafiltration process is the key in obtaining reliable free fractions. Whenever reporting free fractions, detailed information on the method, material and conditions used to obtain these numbers should be made available.

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Antibiotic tissue penetration in clinical practice

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Abstract

Objectives: The aim of this study was to investigate the distribution of antibiotics into different compartments, especially the cerebrospinal fluid, in samples screened for microbiological colonization. Special emphasis was put on the influence of inflammation and microbial colonization on the penetration of the drugs.

Patients and methods: Samples sent into the microbiology laboratory of the hospital were screened for antibiotic i.v. exposure of the following antibiotics: amoxicillin, piperacillin/tazobactam, penicillin G, ceftriaxone, and vancomycin. Measured tissue concentrations were compared to the minimal inhibitory concentrations according to EUCAST, the found colonizing bacteria and to individual patient variables e.g. estimated glomerular filtration rate

Results: Across all examined antibiotic drugs 265 samples were included. Penetration into most tissues was sufficient for lower, but often not for higher MICs of potential pathogens.

Conclusions: At normal eGFR some antibiotics were at elevated risk of underexposure at the site of infection. Plasma levels were often not a good indicator of tissue levels. Colonization by bacteria seems to lead to lower concentrations in tissue for amoxicillin, but not for any of the other investigated antibiotics.

Introduction

Beta-lactams antibiotics are one of the oldest anti-infective agents in use. Still, data about penetration of these anti-infectiva into compartments other than blood, namely tissues, cerebrospinal fluid (CSF) or joints, are still scarce [1]. Most information on tissue penetration ratios stems from in vitro tests, or animal models. Studies in humans have been mostly conducted in healthy patients receiving antibiotic as prophylactic treatment [2–6].

This fact is especially worrying since it is well known that transport of drugs across natural barriers like the blood brain barrier or joint capsules can by strongly influenced by infectious processes [7, 8]. Pathogens may also protect themselves by abscess formation or formation of biofilms which keep the immune systems and antibiotics at bay. Nonetheless, research in severely ill patients and infected tissues are scarce and the studies contain small participant numbers [9, 10]. Vancomycin has been approved in Switzerland since the 60s, but only limited penetration data into infected tissue is available [11, 12].

One of the problems in investigating tissue penetration in clinical situations is that the site of interest is not easily accessible, as it is with blood samples. Inadequate penetration of antibiotics into the site of infection is probably one of the reasons why soft tissue infections can often be treated only insufficiently [13].

This study helps shedding light into this issue in two fashions. First, it examined the relationship between infection and tissue or CSF penetration of some of the most commonly used antibiotics in Switzerland. It gives a wide range of data on tissue concentrations that have been determined in clinically relevant situations, by studying patients undergoing surgical restorations and tested for infection of the CSF. Additionally, this study demonstrates a new, non-invasive way of studying tissue penetration, which can be done without the need for additional invasive procedures.

The fight against microbial resistances is one of the biggest challenges of modern medicine. In a 2019 WHO report, the worldwide growth of antibiotic resistances was called a “global crisis” [14]. One result of growing resistances it the fact that ever higher doses of antibiotics have to be prescribed to achieve eradication of bacterial infection, leading to increased risk of toxicity. At the same time, inadequate antibiotic concentrations enable bacterial strains to develop resistances. That is why therapeutic drug monitoring (TDM) in plasma is conducted in more and more hospitals. TDM for beta-lactams is still discussed controversially [15–17], even though several international guidelines recommend beta-lactam TDM in high risk patients [18–20]. Still, as long as only insufficient data can be provided about the tissue penetration of antibiotics into the site of infection, regulations based on plasma concentrations remain inconclusive, especially for the severely ill.

Reviewing the literature, the following data are available for the selected drugs.

Amoxicillin

Penetration into healthy and infected gingiva under 500 mg/3d of amoxicillin were measured by Amid et al. [21]. The concentrations after 24 h and 7 days of administration were 25.9 ± 4.1 and 124.8 ± 18 μg/mL, respectively in infected gingiva, but undetectable in healthy tissue. No plasma samples were taken.

Averono et al. measured amoxicillin levels in plasma and tissue after tonsillectomy [9]. Amoxicillin/ clavulanic acid dosing was based upon patients' weight. Median plasma and tissue amoxicillin concentrations were 4.7 mg/ml and 1.1 mg/g, respectively. Poor correlation between plasma and tissue concentration was found, and often no amoxicillin could be detected in either one or both tonsils, leading them to suggest that fibrosis may have hampered antibiotic penetration.

Martin et al. [3] found concentrations of amoxicillin in fatty tissues were 12 to 23% of the levels in serum. In the colonic wall, the concentrations of amoxicillin were 27 to 49% of the levels in serum after administration of 2000 mg of amoxicillin. The concentrations of clavulanic acid in fatty tissues were 12 to 23% of the levels in sera. In the colonic wall, the concentrations of clavulanic acid were 52 to 63% of the levels in sera

Vancomycin

Blassman et al. examined vancomycin penetration into CSF in patients with CNS infections [11]. The median (range) Cmax and Cmin concentrations in CSF were 0.65 (0.24–3.83) mg/L and 0.58 (0.24–3.95) mg/L, respectively with a median daily dose of 2500 (500–4000) mg. Vancomycin demonstrated poor penetration into CSF, with a median CSF/serum ratio of 0.03 and high inter-subject pharmacokinetic variability of its penetration, which they could not explain with any of the studied covariates.

Young et al. [12] measured subcutaneous fat tissue after a systemic regime of 1g vancomycin. Mean subcutaneous fat tissue concentration increased from 2.7 to 4.4 mg/kg during the 30 minutes of the surgery and decreased to 2.4 mg/l after 80 minutes into the surgery (corresponding plasma levels during surgery 11.4 mg/l). Tissue: plasma ratio therefore was at about 0.2 - 0.4.

Piperacillin

Chandorkar at al.[22] enrolled healthy subjects to receive three doses of piperacillin/tazobactam 4.5 g administered every 6 h via a 30 min infusion. Mean maximum concentration and AUC in epithelial lining fluid (ELF) from time 0 to the end of the dosing interval (AUC) were 58.8 mg/L and 94.5 (mg*h)/L. The ELF/plasma AUC ratio was 0.26. The mean plasma Cmax values ±SD for piperacillin and tazobactam were 314.6±62.4 and 35.0±7.5 mg/L, respectively, and AUC values were 357.3±65.9 and 46.1±8.7 (mg*h)/L, respectively.

A recent study by Bue et al.[23] investigated[24] plasma and subcutaneous tissue concentrations at the upper arm via microdialysis after 4g piperacillin 3 times daily. Their ten patients underwent renal replacement therapy during their antibiotic course. This treatment resulted in 65.08 ±43.27 mg/l piperacillin concentrations in the microdialysates at through level.

Murao et al.[25] measured plasma, peritoneal fluid samples as well as peritoneum samples during surgery after administration of 0.4 and 0.5g of piperacillin-tazobactam. Piperacillin concentrations ranged from 12.6–534.0 mg/L in plasma, 14.6–297.8 mg/L in peritoneal fluid, and 14.4–220.2 mg/kg in the peritoneum; TAZ concentrations ranged from 1.5–59.2 mg/L, 2.0–33.4 mg/L, and 1.8–27.2 mg/kg, respectively. The ratio of peritoneal fluid to plasma was 0.75–0.79 and the ratio of peritoneum to plasma was 0.49–0.53; the mean PIP:TAZ ratio was 8.1 in both the peritoneal fluid and the peritoneum.

Hayashi[26] et al. in 2010 published a review of the pharmacokinetics of piperacillin-tazobactam including the most important published tissue measurements at the time. They concluded, that both analytes penetrated well into skin and lung tissues (>90%), to a lesser extent into gastrointestinal tissue (~50%) and to less than 30% into fatty tissue, muscle, cancellous bone and cortical bone. The healthy, non-inflamed blood brain barrier into the CSF is only crossed by about 5% (piperacillin) or 17% (tazobactam).

Sörgel and Kinzig [24] concluded that within 30 min of infusion, piperacillin/tazobactam achieves 16–85% of plasma concentrations in skin, muscle, lung, gallbladder, and intestinal mucosa, providing best penetration into skin and lung tissue and lower penetration into fatty tissue.

The group around Daschner[27] measured piperacillin in heart valves and heart muscles during open-heart surgery of 28 patients. Subcutaneous tissue and muscle concentrations varied between 1.7 and 23.8 mg/kg , after a preoperative bolus injection of 4 g piperacillin. Concentrations in cardiac valves were found to be higher than those in muscle and fat and all tissue concentrations.

During transurethral resection of the prostate, prostate tissue samples of 47 patients were collected at 0.5 h (completion of infusion), 1 and 1.5 h after initiation of infusion of piperacillin-tazobactam. Prostate tissue/plasma ratio of piperacillin was about 36% both for the maximum drug concentration (C_{max}) and the area under the drug concentration–time curve (AUC)[28].

Penicillin G

Karlsson et al.[29] measured median concentrations of penicillin G in serum of 37, 5.6 and 0.5, µg/ml 1 and 3-6h after drug administration of 3 g every 6 h i.v. CSF concentrations were 0.5 (range 0.3 to 1.6) µg/ml after 2 to 3 h.

Rahaave studied the concentration of benzylpenicillin in serum and subcutaneous tissue homogenates in 13 patients who underwent surgery of the colon and/or rectum. After infusion of 5 Mio IU concentrations of 76.8 mg/l in serum and 170.8 mg/l in tissue homogenate have been determined, respectively[30].

Ceftriaxone

Ceftriaxone is especially used in the treatment of skin and soft tissue infections, as it shows good penetration into tissue and bone and has a broad antibacterial activity. It has a long half life of 6 to 10 hours, and long proven good penetration into major tissues, making it an ideal candidate for deep tissue infections[31, 32].

Leone et al. administered 1g ceftriaxone to 11 patients undergoing nephrectomy and collected plasma and abdominal as well as renal fat tissue. Tissue to serum ratio ranged from 0.28 in fat to 1.6 in the renal cortex with tissue concentrations between 18 ±7 mg/kg and 110 ±78 mg/kg, respectively[5].

Martin et al. measured ceftriaxone during cardiac bypass operation in 20 patients after 1 g or 2 g iv. administration. Concentrations reached 5-12 mg/kg in thoracic wall fat and 2-8 mg/kg in the sternal bone and all cardiac tissues[33].

The aim of this study was to investigate the distribution of antibiotics into diverse compartments, especially the CSF. Special emphasis is put on the influence of inflammation and microbial colonization on the penetration. We aimed to investigate the significance of classical laboratory parameters and microbiologic results on the tissue concentrations to be expected in patients. The measured tissue levels have also to been compared to EUCAST minimal inhibitory concentrations

(MIC), as well as regional MIC encountered at the University Hospital Basel and if available, to MIC of the corresponding colonizing bacteria.

Based on their usage in soft-tissue infections and infections of the CNS we included the following antibiotics: amoxicillin/clavulanic acid, piperacillin/tazobactam, penicillin G, ceftriaxone and vancomycin.

Materials and Methods

An observational study has been performed using samples obtained from the microbiological laboratory sent in for microbiological screening. We enrolled samples from patients receiving an i.v. antibiotic therapy with amoxicillin, piperacillin, penicillin G, ceftriaxone or vancomycin prior to sample taking.

This study has received approval by the Ethics Committee of Northwest and Central Switzerland EKNZ Project-ID: 2019-02377.

Included were only biopsy or liquor samples from deep, sterile conditions from patients of the University Hospital Basel, who had signed the general research consent of the hospital and who had an antibiotic i.v. regime with amoxicillin, piperacillin, vancomycin, penicillin G or ceftriaxone since at least four hours before sampling. Heparinated plasma of the same patients was included if it was drawn ± 24 h from the time of biopsy sampling.

Samples were excluded when no signed consent was available. Additionally, dilution of samples with e.g. saline solution or if more than 72 hours passed between sampling or blood collection and the time of study inclusion led to exclusion of the sample. If less than 300 mg or 500 µl of biopsy/CSF material or plasma sample, respectively was available, samples were excluded to ensure follow up tests could be performed unimpeded. Samples taken from non-sterile sites were also excluded, as well as samples with no information on dosing time schedule or dosing available. If the last antibiotic dosing has been more than 2 hours longer ago, than the normal dosing schedule would prescribe, the samples were excluded.

Data collected for each sample included health data like age and sex, antibiotic and dosing regimen of the study antibiotics and any other antibiotic drug given at the same time. Dosing time points before sampling of biopsy or CSF and plasma, microbial colonization including the bacterial strain, sampling site and material, albumin, creatinine, leucocyte count and neutrophilic count as well as C-reactive protein (CRP).

Samples were stored for a maximum of 72 hours at 4°C before inclusion into the study. CRP, albumin, leucocyte count and neutrophilic count were measured immediately after blood sampling. Plasma and tissues were stored at -70°C and antibiotic concentrations subsequently measured in batches.

The bioMérieux BacT/ALERT® FA/FN Plus (bioMérieux Suisse S.A., Geneva, Switzerland) system was used for the identification of positive blood cultures. Antibiotic sensitivity was screened using the VITEK system and to obtain MIC, the Etest® was used, both by bioMérieux.

Vancomycin plasma and tissue/CFS levels were measured using the Cobas ONLINE TDM Vancomycin Gen.3 by Roche Diagnostics (Rotkreuz, Switzerland), a KIMS based test using photometric detection. The test has been validated for tissue using spiked CSF and tissue homogenate. As hemolytic samples above a hemolytic index of 1000 could potentially disturb measurements, they were diluted and re-measured. Validation data for vancomycin tissue measurements can be found in the supplementary material.
Plasma concentrations, free plasma concentrations, tissue and CSF concentrations of amoxicillin + clavulanic acid, piperacillin + tazobactam and benzylpenicillin were analyzed using a previously described method using high pressure liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [34]. Ceftriaxone was measured using a validated LC-MS/MS method. The method and validation data used to measure ceftriaxone in tissue, plasma and plasma water can be found in the supplementary material.

All continuous variables were compared using either the Mann-Whitney-U test, reporting median and interquartile ranges (IQR) or the Student's t-test, respectively ANOVA in case of more than two sample groups, then reporting mean and standard deviation (SD). Multivariable logistic regression models (GLM) were performed to analyze associations between patient variables. Using Akaike-Information-Criterion (AIC) and Bayesian-Information-Criterion (BIC) we tried to find the most exact and at the same time most simplistic model to predict tissue and CSF concentrations. We considered statistical significance if the two-sided p-value was less than 0.05. All analyses were performed with the use of Stata lC software (StataCorp LLC, Texas, USA, Version 03 Feb 2020).

Results and Discussion

We included 247 samples, including 18 samples with more than one study antibiotic, resulting in 265 measured concentrations. The most common combination was vancomycin with ceftriaxone

(12 samples). Number of samples per antibiotic and distribution over sampling sites can be found in table 1. Population parameters of the study cohort can be found in table 2.

Table 1 Number of samples per antibiotic and sampling site.

	Biopsy	CSF	Joint puncture	Ascites	Pleura puncture	Abscess	All
Amox (+Clav)	59	4	3	5	7	5	83
Ceft	12	15	3	6	5	1	42
Piper (+Tazo)	44	2	-	4	23	3	76
Pen G	13	9	-	-	1	-	23
Vanco	11	27	-	1	2	-	41
All	139	57	6	16	38	9	265

Table 2 Population parameters for all included samples as well as separated by antibiotic. Number of samples taken after the patient received a certain daily dose: n x daily dose.

	Age median (IQR)/ mean (SD) [years]	Sex f/m (f%)	eGFR median (IQR)/ mean (SD) [ml/min]	Crp median (IQR)/ mean (SD) [mg/l]	Daily dose n x daily dose	Concentration median (IQR)/ mean (SD) [mg/kg]
All	66 (54-75)/63.4 (14.4)	91/263 (35%)	96 (70-127)/ 96.4 (43.2)	84 (45-160)/ 117.4 (79.2)	-	-
Piper (+Tazo)	67 (61-76)/ 67.0 (11.4)	17/59 (29%)	88 (58-110)/ 85.0 (41.5)	140 (64-219)/ 153.4 (105.2)	10x 2x4.5 g/d 60x 3x4.5 g/d 6x 4x4.5 g/d	Piper: 46 (19-73)/ 54.1 (44.8) Tazo: 6 (3-11)/ 7.3 (5.6)
Amox (+Clav)	68 (61-78)/ 66.7 (13.7)	20/48 (31%)	88 (70-111)/ 90.4 (40.8)	87 (35-211)/ 134 (116)	1x 2x0.5 g/d 3x 2x1.2 g/d 7x 3x1.2 g/d 60x 3x2.2 g/d (7x only Amox) 12x 4x2.2 g/d (1x only Amox)	Amox: 10 (4-24)/ 14.9 (13.8) Clav: 0 (0-1)/ 0.5 (0.7)
Ceft	63 (45-75)/ 59.3 (15.7)	21/42 (50%)	101 (69-132)/ 100.2 (45.5)	94 (40-131)/ 94.9 (62.7)	11x 2x2 g/d 31x 2x1 g/d	14 (2-50)/ 36.9 (53.7)
Vanco	55 (44-61)/ 54.1 (14.4)	18/41 (44%)	123 (105-146)/ 125.7 (33.5)	87 (31-146)/ 98.7 (72.2)	1x <1g/d 24x 1-2g/d 16x 2-3g/d	2 (1-11)/ 6.1 (7.4)
Pen G	64 (45-75)/ 62.2 (16.0)	16/24 (67%)	78 (53-142)/ 93.6 (44.6)	69 (48-93)/ 79.0 (60.3)	21x 4x5 Mio IU/d 3x 6x5 Mio IU/d	15 (2-30)/ 24.6 (28.9)

Piperacillin + tazobactam

Piperacillin and tazobactam concentration values were not normally distributed, therefore square root transformation was used on tissue and plasma concentrations before statistical evaluation, resulting in acceptable skewness and kurtosis and making the use of parametric tests possible.

Penetration of piperacillin and tazobactam into the tissue was good and correlated well with plasma levels. Mean ratio of piperacillin to tazobactam was slightly higher in tissue than in plasma. Mean tissue piperacillin:tazobactam ratio was 7.6 (SD ±2.6) compared to 6.0 (SD ±1.5) in plasma (figure 1).

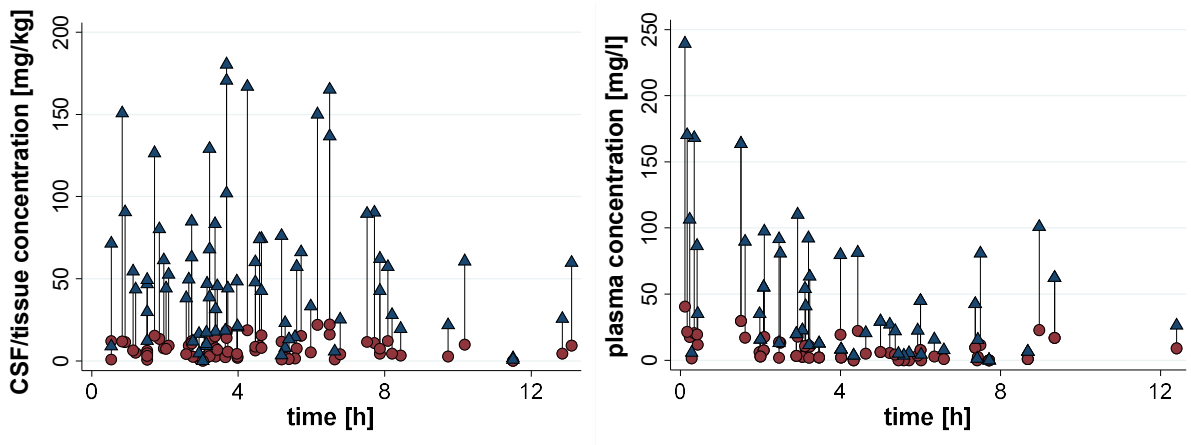

Figure 1a) Piperacillin (triangle) and tazobactam (circle) concentration in all tissue and CSF samples. **1b)** Piperacillin (triangle) and tazobactam (circle) concentration in plasma samples.

Calculated GFR correlated strongly and statistically significantly with measured tissue and CSF concentrations. When controlled for doses, CRP, time of sampling, leucocyte count, microbial colonization and material using a generalized linear model, GFR remained the strongest predictor of tissue and CSF concentration with $p<0.01$ (figure 2a).

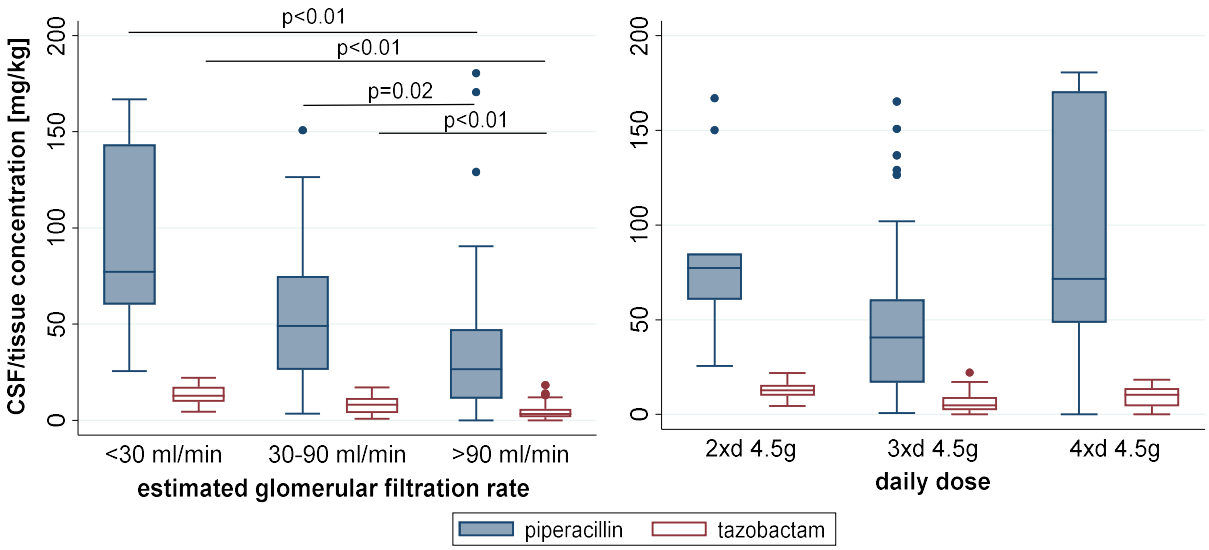

Figure 2 a) Piperacillin and tazobactam concentrations in tissues and CSF according to eGFR. **2 b)** Piperacillin and tazobactam concentrations in tissue and CSF according to daily dose. No significant difference between the groups was found.

The adjustment of the daily doses seems mostly appropriate in our patient group. The means between the dosing groups differ, but all reach appropriate, comparable mean concentrations of both piperacillin and tazobactam (figure 2b). (Target range piperacillin: 16-100 mg/l) There were some cases, in which tissue concentrations were extremely low. For example, no concentrations could be detected in a sample of necrotic skin from the lower leg of a skin transplant patient.

EUCAST Breakpoints of piperacillin range from 0.25 to 16 mg/l. Since there are no breakpoints determined for tazobactam, but drugs usually contain the drugs at a ratio of 8:1 ratio, we decided on breakpoints for tazobactam from 0.2 (lower limit of quantification) to 2 mg/l.

34 samples were positive for bacterial colonization. They showed 59 separate positive identifications and 26 different bacteria. 46 of the 59 positive findings were backed up by information on susceptibility by either Vitek or E-Test or could be unambiguously determined as resistant or sensitive based on the species. Of those 46, 40 were found to be susceptible to the bacteria found, 6 were resistant. Of these, one additionally received daptomycin, covering the susceptibility range of the problematic species. Measured MICs were between 0.008 and 3 mg/l (for pseudomonas aeruginosa).

Of the 34 samples, only one showed no detectable piperacillin and tazobactam concentration, but it was the only sample not reaching a 0.5 mg/kg piperacillin concentration (2.9%). At a breakpoint of 4 mg/kg three samples (8.2%) did not reach this concentration and a breakpoint of 16 mg/kg

was not reached by 10 samples (29.3 %). The same number of samples did not reach a 2 mg/kg concentration of tazobactam, but only three samples were below 0.2 mg/kg.

When the threshold is 8 /1 mg/l nine samples showed insufficient levels of either one or both drugs. Notably, all except one of these patients presented a GFR above 90 ml/min.

16 out of 76 samples had levels below 16 and 2 mg/l for either one or both drugs. 3 out of 15 (one: no information available) had eGFR above 90 ml/min. Higher eGFR is associated with higher risk of not reaching desired target levels in tissue.

Only 20.0% (3/15) of samples that did not reach 16 mg/kg piperacillin had a GFR of below 90 ml/min and therefore renal insufficiency, while 60.7% (37/61) of all samples that did reach 16 mg/kg had a GFR of below 90 ml/min. Pearson Chi square test gives an $p=0.005$ for samples with a GFR of below 90 for having a higher chance of reaching sufficient tissue levels (piperacillin above 16 mg/kg).

Using GLM the time of sampling (negative correlation), eGFR (negative correlation) and site of sampling: abscess and CSF (both indicating lower concentrations), could be identified as factors with a significant influence ($p<0.05$) on tissue concentration. Non-significant but relevant correlations could be found with plasma concentrations (positive correlation) and daily doses of 16 g compared to lower doses.

Neither leukocyte count, CRP nor bacterial colonization were found to be predictive for tissue or CSF concentration.

Amoxicillin

Amoxicillin concentrations were not normally distributed, square root transformation was used on tissue and plasma concentrations before statistical evaluation, resulting in acceptable skewness and kurtosis and making the use of parametric tests possible.

Due to the limited stability of clavulanic acid in aqueous solutions, not only at room temperature but also cooled or frozen (details see previous publications [34, 35]) the very low levels of clavulanic acid detected in this study are probably misleading. Measured tissue clavulanic acid was never higher than 1.9 mg/l and in 31 of 74 samples (42%) clavulanic acid concentrations were below the detection limit. Both sera and tissue and CSF samples are routinely processed at room temperature before storage, all samples probably spending around 2 to 4 hours at RT before being stored at 8°C for 0-72 hours. This would lead to an approximate loss of clavulanic acid of between 5-70% before samples can be stored at -70°C to stop degradation. In comparison, amoxicillin should undergo no more than 10% degradation during this time[34]. Therefore,

unfortunately we could not include clavulanic acid into the evaluation. Because of the low incident numbers of very low daily doses 1 g/day and 2 g/day, these were combined with 3 g/d samples into one category "1-3 g/d."

Penetration of amoxicillin into the tissue was sufficient in most samples. Mean ratio of amoxicillin to clavulanic acid was 15.3 (SD ±10.3) in plasma and 41.5 (SD ±41.2) in tissue. This high variability is probably due to degradation of clavulanic acid in the matrix.

At through point amoxicillin should be above 8 mg/l. In figure 3a and b amoxicillin concentrations in tissue and plasma are depicted.

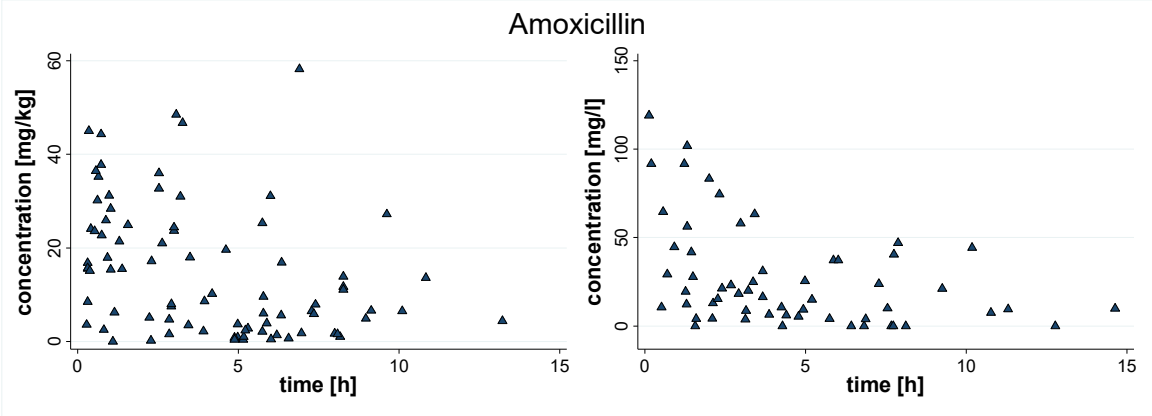

Figure 3 a): Amoxicillin and clavulanic acid in tissue and CFS. **3 b):** Amoxicillin and clavulanic acid in plasma

Amoxicillin tissue levels are much less dependent on eGFR than piperacillin. Mean eGFR of amoxicillin patients was 89 ml/min (SD ±45%) while piperacillin patients had a mean GFR of 85 ml/min (SD ±49%), therefore a difference between cohorts can not explain this finding.

Using generalized linear models, CRP ($p<0.01$) and the time of sample taking ($p<0.01$) had an significant influence on tissue concentrations, while eGFR ($p=0.17$) and colonization ($p=0.10$) had a strong, but not signifikant influence, even after controlling for daily dose, leucocyte count, plasma concentration and site of sample taking (figure 4).

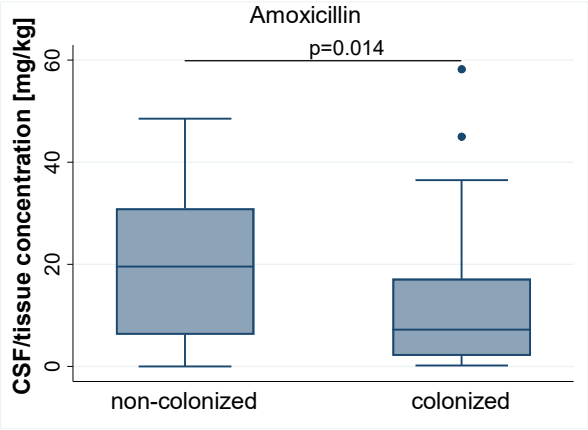

Figure 4: Colonization vs. Concentration of amoxicillin p=0.014

Ceftriaxone

Since ceftriaxone concentrations were not normally distributed, log transformation was used on tissue and plasma concentrations before statistical evaluation.

42 samples were collected 28 of which showed no infection with bacteria. 14 had some colonization with one (13) or two (1) type of bacteria. Three bacterial strains were tested resistant to ceftriaxone, and only one of these patients received additional antibiotics appropriate to this strain.

Ceftriaxone breakpoints according to EUCAST are at 1 mg/l, meaning only 3 out of 42 (7.1%) samples (2 CFS, 1 ascites) were below that threshold. In figure 5 a and b ceftriaxone concentrations in tissue and plasma are depicted.

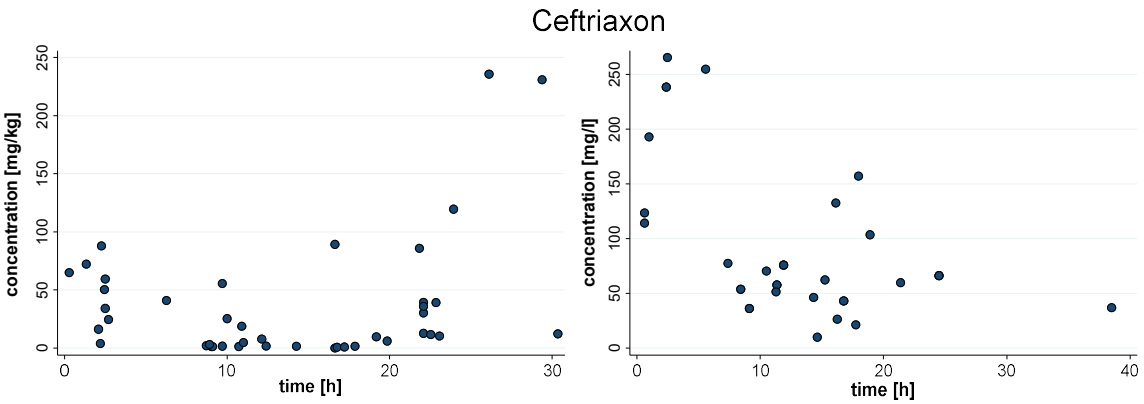

Figure 5 a): Ceftriaxone in tissue and CFS**. Figure 5 b):** Ceftriaxone total plasma concentration. Ceftriaxone was given as continuous infusion.

Even though dosing is adjusted to eGFR, lower eGFR is still associated with higher ceftriaxone tissue concentrations (figure 6). None of the patients in the cohort presented with an eGFR of less than 10 ml/min, so no dose adjustment was performed for this reason, according to our in-house policy. Eleven patients (26%) received a high dose regime of 2x 2g daily, in accordance with suspected meningitis.

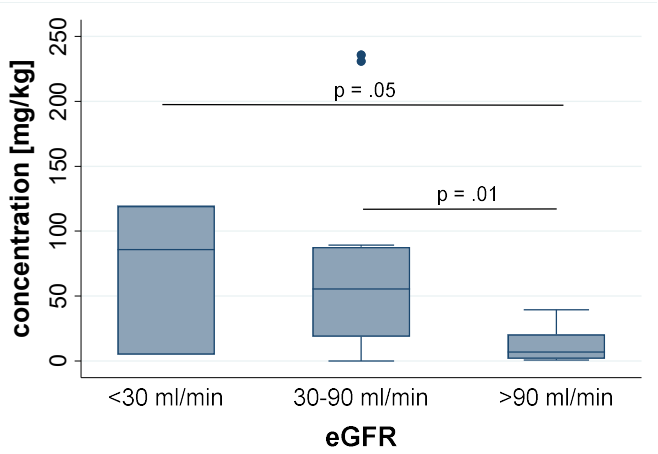

Figure 6: Ceftriaxone concentration in tissue and CSF according to eGFR.

For ceftriaxone, no influence of microbial colonization on tissue concentration could be found (p=1.0). Plasma concentration free or total was also not found to correlate with tissue concentrations very well (p=0.4) when considering the full GLM model but using only the free or total concentration as predictor showed significant, but low correlation. Using the free fraction did not show better correlation than using the total plasma concentration for modeling.

The influence of leucocytes on ceftriaxone concentration give reason to expect a connection between inflammation and ceftriaxone concentration, but neither CRP nor microbial colonization could support this theory.

Vancomycin

Vancomycin penetrated only insufficiently into the CFS. Since penetration was vastly different into CSF from into tissues, both materials were analyzed separately. Plasma and tissue or CSF concentrations can be found in figure 7 a and b.

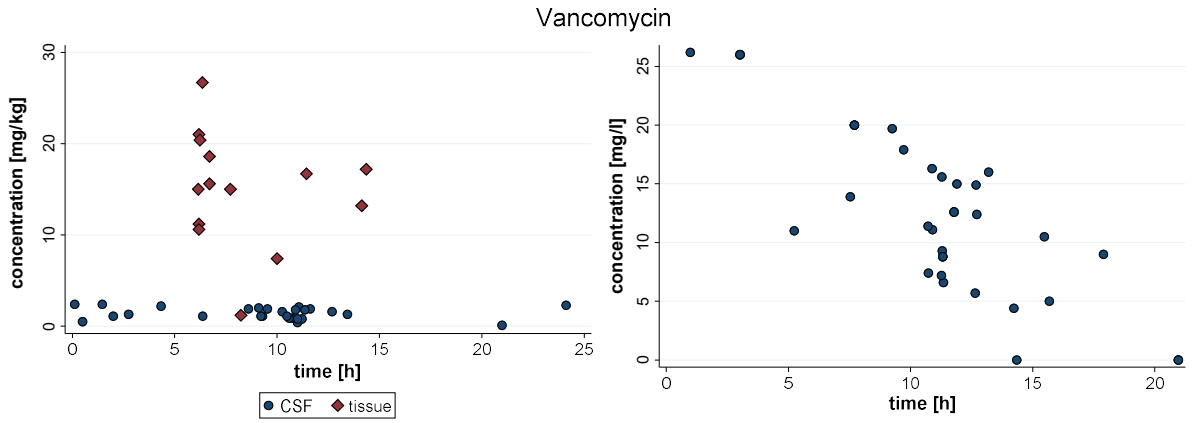

Figure 7 a): CSF (circles) and tissue (diamonds) vancomycin concentration in all tissue and CSF samples. **b):** Vancomycin plasma concentrations.

With a median eGFR of 123 (IQR 105-146) the vancomycin population had the highest renal clearance of the studied populations. Since only three samples had eGFR values below 90 ml/min, this factor could not be studies accordingly. Also, the vancomycin population was one of the youngest studied (median age 55 years (IQR 44-61).

Both materials showed no correlation to time of sampling in their concentration, which is still unexpected, even taking into consideration vancomycin's comparatively long elimination half-life time of 6h. No correlation with eGFR could be found, probably due to the population, which showed much higher median and mean eGFR than the average study population, and included no eGFR values below 90 ml/min. Higher CRP levels were a strong indicator for higher tissue concentrations $p(<0.05)$

None of the CSF samples reached relevant levels of vancomycin (maximum 2.4 mg/l). Desired through levels in plasma are 10-15 mg/l or 15-20 mg/l for MRSA infections.

Looking at microbial colonization, 22 of the 41 samples were positive. Most samples showed either cutibacterium acnes (11/22 50%) and staphylococcus epidermidis (8/22 36%). Vancomycin was an appropriate choice of antibiotic for most samples, excluding three samples positive for pseudomonas aeruginosa. Therefore 89.7% of samples were appropriately treated. Only 8/29 (27.6%) strains were backed up by susceptibility testing, though, most of them staph. epidermidis. 24 out of 29 samples (83%) reached 1 mg/l while only 13 out of 29 (45%) reached a concentration of 4 mg/l (EUCAST Cut-Off for several coagulase negative staphylococci).

Penicillin G

Using log-transformation, penicillin G tissue and plasma samples were transformed, making the use of parametric tests possible. Penicillin G penetration into the CSF is quite low, and concentrations were very different between CSF and tissues, both materials were analyzed separately. The differences in concentration between biopsy samples (mean 39.0 mg/kg ±7.7) and CSF (mean 2.3 mg/kg ±0.5) were strongly significant (P<0.001).

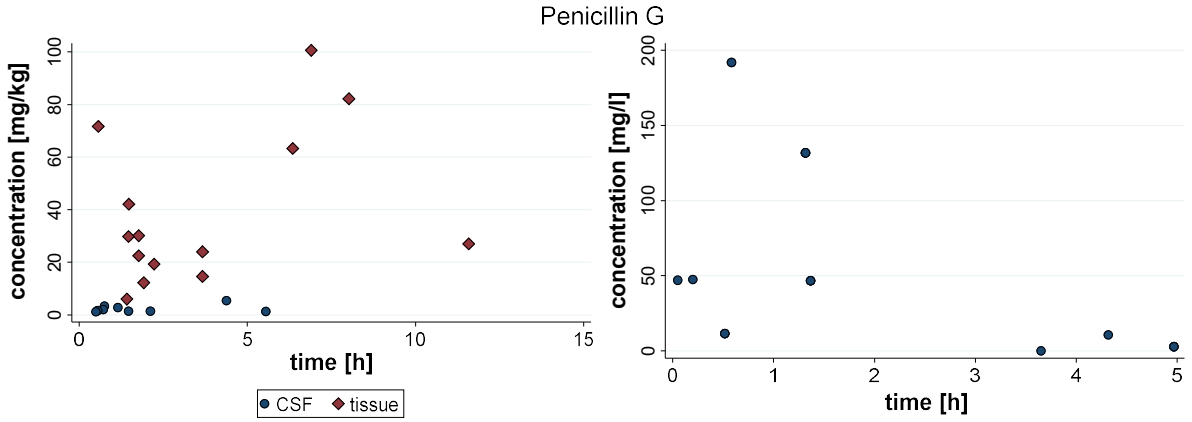

Figure 8 a): CSF (circle) and tissue (diamond) penicillin G concentration in all tissue and CSF samples. **Figure 8 b):** Penicillin G plasma concentrations.

In samples stemming from patients with no renal impairment (GFR>90 ml/min) mean tissue concentration was 36.4 (SD 7.9), while the mean concentration was 9.2 (SD 7.0) in patients with renal impairment.

This finding is confounded by the fact that all nine included CSF samples had eGFR above 90 ml/min while only 1 of 14 biopsy samples had a eGFR above 90 ml/min (figure 9a). The sampling site was strongly significant (p<0.001) when correlated against eGFR, CRP, leucocyte count, time of sampling and dosing. Mean biopsy concentration was 39 mg/kg (SD 29), while mean CSF concentration was only 2.3 (SD 1.4). Because of the strong influence of the blood brain barrier on concentration, the impact of eGFR is hard to predict. Using generalized linear models to look at biopsies and CSF separately, eGFR showed a non-significant negative correlation with tissue concentrations.

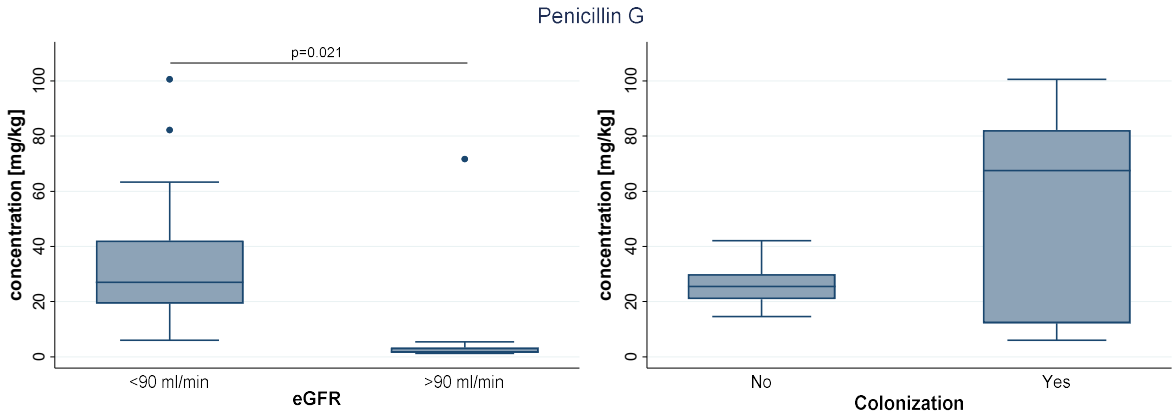

Figure 9 a): Penicillin G concentration in tissue and CSF according to eGFR. No eGFRs lower than 30 ml/min were recorded for penicillin G. **b):** Penicillin G concentration in tissue and CSF according to microbial colonization.

CRP showed the strongest correlation to measured tissue concentration R^2=0.30, p<0.01, and stayed significant when including covariates. Of the 23 penicillin G samples collected, in 17 no bacterial colonization could be found, including all of the CSF samples. Influence of colonization on tissue means was compared only in biopsies, since the generally lower CSF penetration would confound the findings. Non infected tissue mean was 26.2 mg/kg (SD 3) and colonized tissue mean was 56 mg/kg (SD 15.7) (Figure 9b). At p=0.37 the difference is not significant, indicating no influence of bacterial colonization on tissue concentration.

We developed models to predict piperacillin and amoxicillin tissue concentrations using generalized linear models, since these were the two antibiotics for which the most samples could be acquired. Covariates included were sampling time, plasma concentration, CRP, leukocyte count, site of sampling, GFR, daily dose and bacterial colonization. In figure 10 the correlation between model values and measured piperacillin tissue concentrations can be found.

The resulting model for piperacillin included the time of sampling, eGFR, site of sampling and daily dosage. The resulting equation is depicted as Equation 1.

Equation 1:

$$\sqrt{tissue\ conc.\left[\frac{mg}{kg}\right]} = 9.5664 - eGFR\left[\frac{ml}{min}\right] * 0.0254 - sampling\ time[h] * 0.3620 + \sqrt{plasma\ conc.[\frac{mg}{l}]} * 0.2037$$

Adjustments for daily dose and sampling material were made.

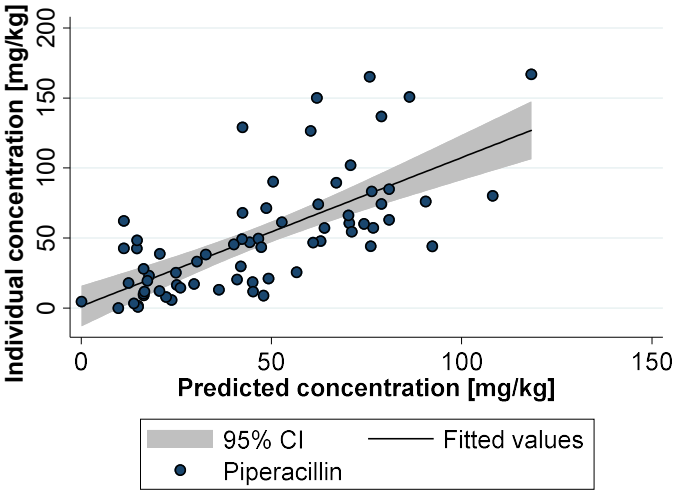

Figure 10: Predicted vs. individual piperacillin values. R^2 0.48 predicted and measured values.

Amoxicillin tissue concentrations were predicted using a general linearized model including the variables CRP, eGFR, sampling time, site of sampling, daily dose and microbial colonization. The resulting equation is depicted as Equation 2. Figure 11 shows the correlation between model values and measured amoxicillin tissue concentrations.

Equation 2:

$$\sqrt{tissue\ conc.\left[\frac{mg}{kg}\right]} = 4.5945 - eGFR\left[\frac{ml}{min}\right] * 0.0062 - sampling\ time[h] * 0.1735 + CRP * 0.0043$$

Adjustments for microbial colonization, daily dose and sampling material were made.

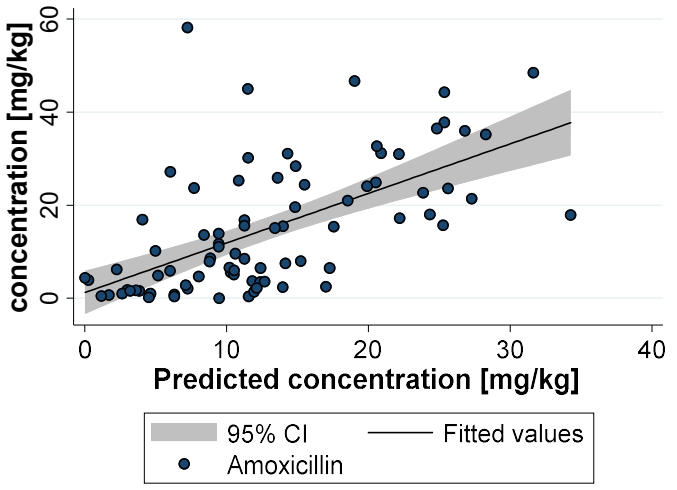

Figure 11: Predicted vs. individual amoxicillin values. R^2 0.37 predicted to measured values.

One of the strong points of this study is the non-invasive approach using sample materials from patients undergoing surgical restorations or punctures with the aim of microbiological screening. This approach gives a great opportunity to gather high numbers of samples with the lowest inconvenience and risk to patients possible.

One disadvantage of this study is that it relies on the information routinely gathered during patient treatment. Time points of infusion or the time of surgical extraction may not be as exact as when collected especially for the purpose of a study. Antibiotic treatment prior to admittance to the hospital could not be evaluated, neither was the influence of oral intake of antibiotics prior to the i.v. regime considered. Oral prescriptions were not included in this study because it was impossible to gather exact dosing times and because of the more complicated pharmacokinetics of oral administration.

The reliance on routinely gathered information also means that a great number of samples had to be excluded because of missing data. Another disadvantage is the wide range of treatment regimes, underlying conditions, reasons for surgical interventions and time points of sample taking after administration of the antibiotic. These factors make for a wide range of results and make it harder to extrapolate the reason for variations in such a varied sample pool. Greater sample numbers or more constricted inclusion criteria might help alleviate these problems in further studies of a similar kind.

Supplementary Material

Vancomycin Quantification

Table 1 Accuracy and precision of the vancomycin method for tissue.

Vancomycin	Concentration [mg/l]	Intra-day	Inter-day
		Accuracy ±CV [%]	Accuracy ±CV [%]
Tissue / CFS	5.76	96.1 ±8.8	104 ±8.2
	7.68	100 ±4.1	110 ±7.5
	27.18	112 ±2.0	113 ±5.1

Ceftriaxone Quantification

Sample preparation for tissues and CSF was the same as reported in Rehm et al. [34] using ceftriaxone-d3 (2 mg/L in MeOH) as internal standard.

Stock solutions for calibrators and QC were weighted in separately at 3 mg/ml in MeOH. Seven calibrators and three QCs were prepared in plasma or filtered plasma water by adding appropriate amounts of stock solution. Calibration ranged from 1 to 300 mg/l in plasma and 0.2 to 30 mg/l in plasma water. For tissue calibrators, seven calibrators and one blank, ranging from 0.2 to 25 mg/kg, were prepared in water. Three quality controls were prepared by adding appropriate amounts of stock solution to three different batches of CSF and tissue. After adding 150 µl MeOH per sample, the tissue was homogenated by thorough mixing using a plastic skewer and subsequent aliquoted.

Sample preparation for plasma and filtered plasma water consisted of diluting 50 µl plasma with 300 µL IS, vortexing, shaking for 10 minutes and centrifugation at 13'200 rpm for 10 minutes. Afterwards, 50 µl of the cleaned sample was diluted with 500 µl Mobile Phase A. For tissue and CSF sample preparation, to 5 µg tissue or CSF 50 µL IS and 150 µL MeOH was added. The samples were thoroughly mixed using a plastic skewer. Samples were vortexed, shaken for 30 minutes and subsequently centrifuged for 10 minutes at 13'200 rpm.

All matrices were analysed using the same LC-MSMS method.

For online sample preparation, a precolumn for AED ClinMass by Chromsystems (Gräfelfing, Germany) was used while chromatographic separation was carried out on an Accucore™ XL C18 4 µm 150 x 4.6 mm column, both by Thermo Fisher Scientific (Reinach, Switzerland). Mobile phase A was composed of 10 mM ammonium carbonate in water, adjusted to pH 8 with acetic acid, while mobile phase B was composed of 10 mM ammonium acetate and 0.1% formic acid in

methanol/acetonitrile (1/1, v/v). For column cleaning, mobile phase C, consisting of acetonitrile/isopropanol/acetone (1/1/1/, v/v/v), was used. The HPLC method is shown in detail in table 1. 20 µL sample were injected into the HPLC. The total run time of the method was 8.0 minutes and from minute 3.5 to 6.5 the flow was directed to the MS. The mass spectrometer acquired in positive ion mode using the following settings: spray voltage 3500 V, ion transfer tube temperature 300 °C; vaporizer 400 °C; sheath gas flow 6.5 l/min; auxiliary gas flow 8 l/min. The analytes were quantified by multiple reaction monitoring (MRM, see Table 2). Accuracy and precision of the method can be found in table 3.

Table 2 Column configurations. A, B and C stand for mobile phase A, mobile phase B and mobile phase C, respectively.

Duration (s)	Tee	Flow (ml/min)	A		B (%)		C	Flow (ml/min)	A	B (%)	C
0	Out	0.6	100	:	0	:	0	0.5	95	5	0
120	In	0.6	50	:	50	:	0	0.0	50	50	0
60	Out	0.6	100	:	0	:	0	0.5	100		0
30	Out	0.6	100	:	0	:	0	0.5	100		0
15	Out	0.6	0	:	0	:	100	0.5	5	95	0
31	Out	0.6	0	:	100	:	0	0.5	0		100
15	Out	0.6	0	:	100	:	0	0.5	0		100
30	Out	0.6	0	:	100	:	0	0.5	5	95	0
150	Out	0.6	100	:	0	:	0	0.5	5	95	0

Table 3 MRM settings, including one quantifier and two qualifiers for ceftriaxone and its internal standard ceftriaxone-d3.

Analyte	Precursor ion (m/z)	Lens voltage (V)	Product ion (m/z)	Collision energy (V)
Ceftriaxone	555.2	187	395.9	13.5
			323.9	17.3
			167.0	26.1
Ceftriaxone-d3	558.2	188	398.9	13.2
			327.0	17.4
			243.9	20.8

Table 4 Accuracy and precision of the ceftriaxone method

Ceftriaxone	Concentration [mg/l]	Intra-day	Inter-day
		Accuracy ±CV [%]	Accuracy ±CV [%]
Plasma	15	113 ± 2.1	108 ± 7.2
	135	111 ± 3.2	107 ± 7.9
	255	95.3 ± 4.5	92.8 ± 8.6
Plasma water	1.25	99.5 ± 2.2	96.7 ± 4.8
	12.5	105 ± 2.1	102 ± 3.7
	25	98.9 ± 1.9	95.7 ± 3.7
Tissue / CFS	0.4	99.8 ± 2.9	90.1 ±13.3
	11	96.6 ± 1.8	97.3 ± 4.0
	22	94.2 ± 1.7	93.2 ± 4.6

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Drug stability testing of benzylpenicillin in elastomeric pumps

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Abstract

Outpatient Parenteral Antimicrobial Therapy (OPAT) refers to the administration of a parenteral antimicrobial in an outpatient setting with the explicit aim of facilitating early discharge or avoiding admission. The use of benzylpenicillin in OPAT needs special consideration because of its limited stability at elevated temperatures. We tested benzylpenicillin stability in elastomeric pumps at different concentrations in saline and in buffered solution using sodium citrate. Storage conditions were seven days at 4°C and up to 48 hours at 37°C to imitate storage at home and elevated temperature encountered during infusion. Sodium citrate was an effective buffer to raise stability of benzylpenicillin to no more than 6% break down during 7 days at 4°C and 24 hours at 37°C. Stability was concentration dependent. Several breakdown products were detected and adduct formation of breakdown products was observed. Patients receiving benzylpenicillin during OPAT presented with adequate levels of benzylpenicillin.

Introduction

The economic pressure on the healthcare system in Switzerland is increasing. To reduce the financial burden, over the last years more effort has been put on early discharge from the hospitals and on outpatient treatments. Intravenous antimicrobial therapy often prolongs hospital stay unnecessarily [1]. An alternative to inpatient care is Outpatient Parenteral Antimicrobial Therapy (OPAT). OPAT has already shown cost reductions in several countries (e.g. United Kingdom [2]).

OPAT refers to the administration of a parenteral antimicrobial in an outpatient setting with the explicit aim of facilitating early discharge or avoiding admission. Infectious diseases most commonly treated in the OPAT program include urogenital infections, cellulitis, bone and joint infections, and infective endocarditis. Patient welfare, reduction of risk of health care associated infections and cost-effective use of hospital resources are the main drivers for OPAT. The safe practice of OPAT depends on a team approach with careful patient selection and antimicrobial management with programmed and adaptable clinical monitoring and assessment of outcome [3].

Beta-lactam antibiotics (BA) administration using elastomeric pumps has been associated with good clinical outcome [4]. However, stability and safety of one BA drug is not transferable to the whole drug group, the main limitation for its use in elastomeric pumps is the chemical stability of the active pharmaceutical ingredient. BAs are unstable in watery solutions - this can be as pronounced as near 100% degradation in solution after 24 hours at slightly elevated room temperatures. This was shown for benzylpenicillin [5]. Studies investigating the temperature in elastomeric pumps when carried near to the body are available. Volunteers were used to carry elastomeric infusion devices fitted with temperature sensors during 24 h in order to mimic the continuous administration of the drugs in OPAT. These studies show that typical administration during OPAT can lead to exposure of analytes to temperatures of above 30°C [6, 7]

Elastomeric pumps release intravenous drug solutions at an almost steady rate and it has been shown that drug levels are comparable to levels achieved using continuous infusion by standard infusion devices for stationary units for several antibiotics already [4]. Such evidence is still missing for benzylpenicillin.

A review on BA drug stability including benzylpenicillin showed that several groups studied stability of benzylpenicillin at elevated room temperatures, but only one researched the full time span (8 days) and temperature range (up to 37°C) that might be encountered in an OPAT setting [8]. According to their findings, benzylpenicillin is not sufficiently stable for OPAT programs when applied in water for injection and shows a quick chemical decomposition [6]. According to McDougall et al. [5], stability is much better when buffered with a citrate buffer. McDougalls group

could show that stability is sufficient in citrate buffer after 7 days at 4°C, followed by 24 hours at 37°C.

Because of the limited evidence for benzylpenicillin use in OPAT and the strong buffer dependency of the stability, we conducted a replication study of the aforementioned study concerning benzylpenicillin stability in a simulated OPAT scenario. The acceptable stability limit was 95% in the elastomeric infusion devices when stored at 4°C for 7 days and after subsequent storage in a simulated infusion at 37 °C during 24 h. Furthermore, no toxic products should be formed when benzylpenicillin is chemically decaying. The release rate of the elastomeric pump system used by our hospital was tested to ensure a steady infusion over the full 24 hours. We will furthermore gather therapeutic drug monitoring (TDM) data for five OPAT patients to explore if plasma levels achieved during administration via elastomeric pumps are sufficient.

Methods and Materials

Benzylpenicillin potassium salt (Sigma Aldrich, Buchs, Switzerland) and benzylpenicillin-d5 potassium salt (Toronto research Chemicals, Toronto, Canada), were both of analytical grade. Penicillin „Grünenthal“ 10 Mio IU for infusion (Grünenthal Pharma AG, Mitlödi, Switzerland) was used to fill the elastomaric pumps Easypump® II ST/LT (B. Braun Medical AG, Sempach, Switzerland). For preparation of the elastomeric pumps, sodium chloride (NaCl) 0.9 % for injection (B. Braun Medical AG, Sempach, Switzerland) and in-house pharmacy-produced 40 mg/ml sodium citrate solution with sodium citrate Ph. Eur. (Hänseler AG, Herisau, Switzerland) where used.

For the quantitative measurement of benzylpenicillin we adapted a previously described LC-MS/MS method[9]. To increase accuracy and precision we used deuterated benzylpenicillin as an internal standard for all measurements. Measured parent mass for benzylpenicillin-d5 was 340.2, lens voltage was kept at 139 V and product ions with their respective collision energies were 114.1 (32.4 V), 160.1 (10.3 V) and 181.1 (13.2 V). Patient samples were measured according to the described protocol, while drug infusions were diluted 500 times with water before work up.

To identify degradation products of benzylpenicillin, qualitative measurements of the drug infusions were performed by adapting the HPLC method to exclude the online extraction, as a clean-up of the samples was not necessary. The MS was first used in scan mode, measuring masses from 145 to 1000 m/z at a scanning speed of 1000 Da/sec. In further measurements product scanning mode was employed. Parent masses identified in scan mode were further fragmented

using 1.5 mTorr collision gas and collision energy ramped from 15 to 35 V, while scanning speed was kept at 1000 Da/sec. Samples were diluted 1 to 50 before qualitative measurement.

Elastomeric pump flow of a single pump over 24 h was measured at room temperature, the flow restrictor was lead through warm water of about 31°C and the output was dripped into a beaker on a balance. The balance readout was taken automatically every 60 seconds via computer script. The pumps were filled with a standard dose of Penicillin "Grünenthal" in water.

Two vials of Penicillin „Grünenthal" 10 Mio IU were dissolved in 17 ml sodium citrate 40 mg/ml. An appropriate amount was filled into an elastomeric pump and the pump was filled up to 240 ml using NaCl 0.9%. Pumps were filled with three different concentrations: 10 Mio IU, 20 Mio IU, and 40 Mio IU, corresponding to 23, 46 and 93 mg/ml benzylpenicillin. For each concentration, three pumps were prepared. Unbuffered pumps were prepared by solving the drug product in 0.9% NaCl only.

Elastomeric pumps were kept in a cold room at 4-8°C and an incubator at 37°C. Both were temperature controlled and a ventilator circulated air inside to ensure uniform air distribution. At allocated time points, samples were drawn and kept frozen at -80°C until measurement. Samples were drawn immediately after constitution, after 7-day refrigeration (168 hours) including a 30 min. warm up phase at room temperature, as well as after 6, 12, 18, 21, 24, 27 and 48 hours at 37°C.

On each sampling point, a separate sample was taken and frozen at -80°C for pH measurement. pH of samples was measured using a Metrohm 780 pH meter equipped with a microelectrode "Biotrode" (Methrom Schweiz AG, Zofingen, Switzerland).

Patients who received benzylpenicillin and were scheduled to receive it further during OPAT were included for TDM measurements. We measured one trough level while the patients received short-term infusions while still an in-patient. Second measurement was taken at least 24 hours after continuous infusion was started and the third measurement was taken at least five days after the second. Benzylpenicillin measurements were taken as part of routine control. Both blood samples during OPAT were taken during the patient's weekly visits at the OPAT unit. Renal capacity was calculated using creatinine measurements from patient data and eGFR using the Chronic Kidney Disease Epidemiology Collaboration study equation. Mean values were calculated from blood samples taken on all study days, and standard deviations (SD) were calculated.

Results

Designated pump flow of Easypumps 270 ml should be 10 ml/h at 31°C. Measured pump flow was slightly time-dependent and slowed down over time, but overall was very constant. Flow varied no more than 11% from 10 ml/h.

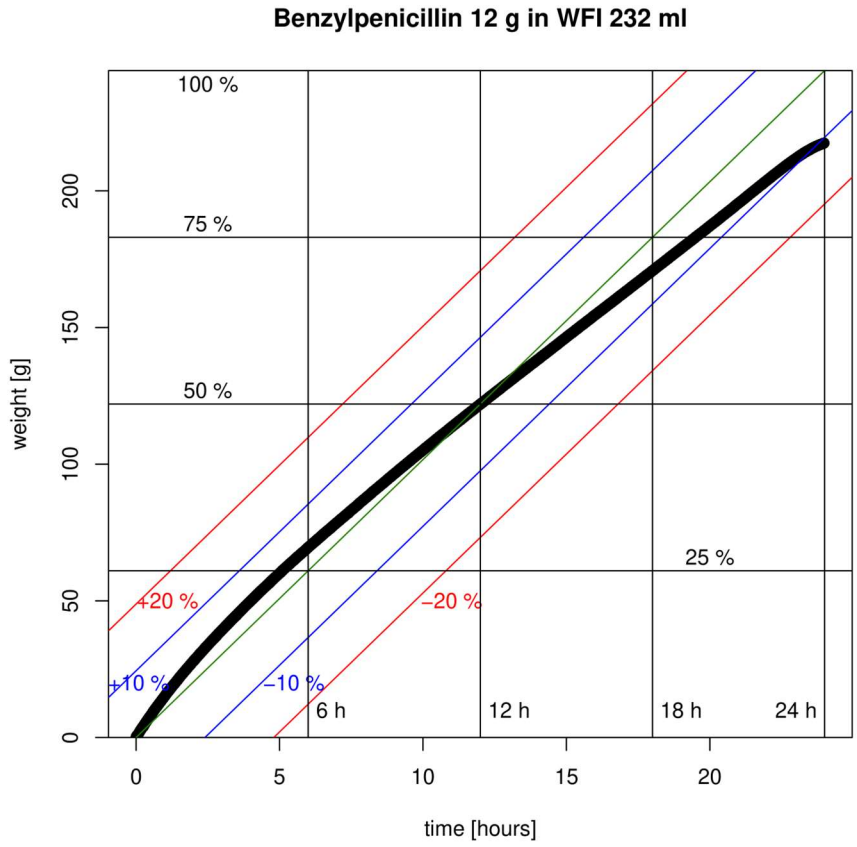

Figure 1 Flow rate of 20 Mio IU benzylpenicillin pump (standard conc.) prepared with water for injection (WFI). The pump was dripping the solution on a beaker on the balance. Due to this open system, parts of the solution evaporated during measurement, this effect was not compensated.

The validated cold room was set to 2 – 8°C, temperature over the storage period was at a mean of 4.5 ±0.9°C during the 7 days, never exceeding 6.9 °C. The incubator was set to about 38°C but was only able to keep temperature at a mean of 36.3 ±2.0°C due to door handling during sample retrieval. Thus, during the measured 48 hours, by measuring with 4 sensors, minimum and maximum temperature of 25.8°C and 38.8°C where recorded.

In figures 2 – 4 the means of three pumps are depicted, while error bars describe the standard deviation.

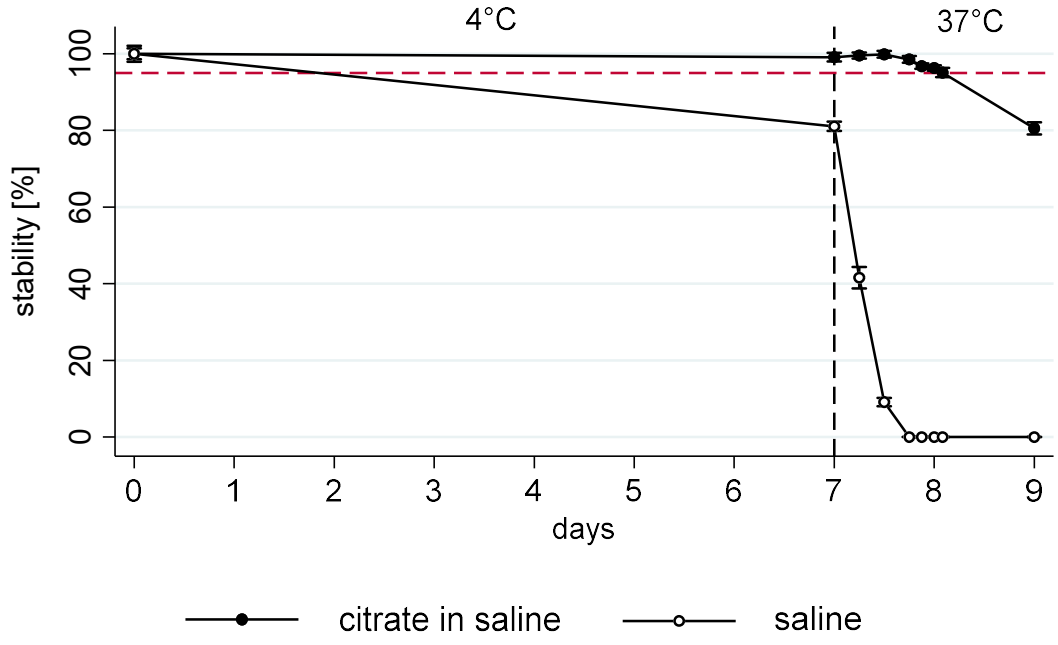

Figure 2 Comparison of buffered and unbuffered 20 Mio IU solutions. Error bars at day 0 depict the standard deviation of the difference in starting concentration between the three pumps of each condition.

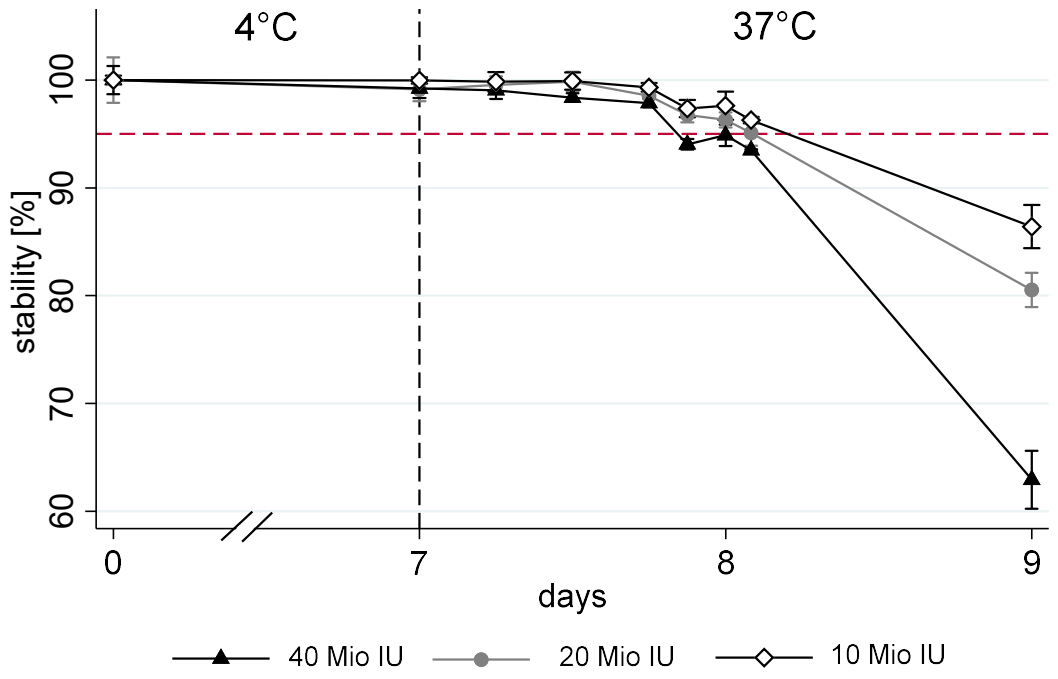

Figure 3 Comparison of three concentrations in citrate buffer. Error bars at day 0 depict the standard deviation of the difference in starting concentration between the three pumps of each condition. For easier readability, the 7 day refrigerator period is shown stinted.

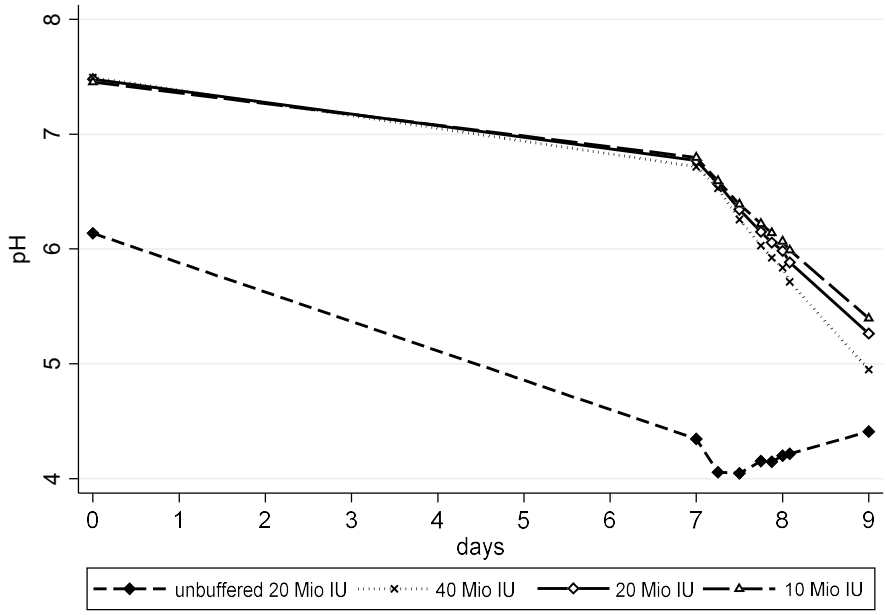

Figure 4 Starting pH in both buffered and unbuffered pumps over time.

Unbuffered benzylpenicillin solutions had no measurable analyte concentration left after 8 days. Buffered solutions showed acceptable stability at all three tested concentrations of 10 Mio IU, 20 Mio IU, and 40 Mio IU. At 10 Mio IU, 97.6% of initial concentration was left after 8 days, while at 40 Mio IU only 94.9% was left. Only the highest concentrated infusion did not achieve 95% stability during all measurements with the single lowest measured concentration presenting after 22 hours at 37°C, with a degradation of 6.5%.

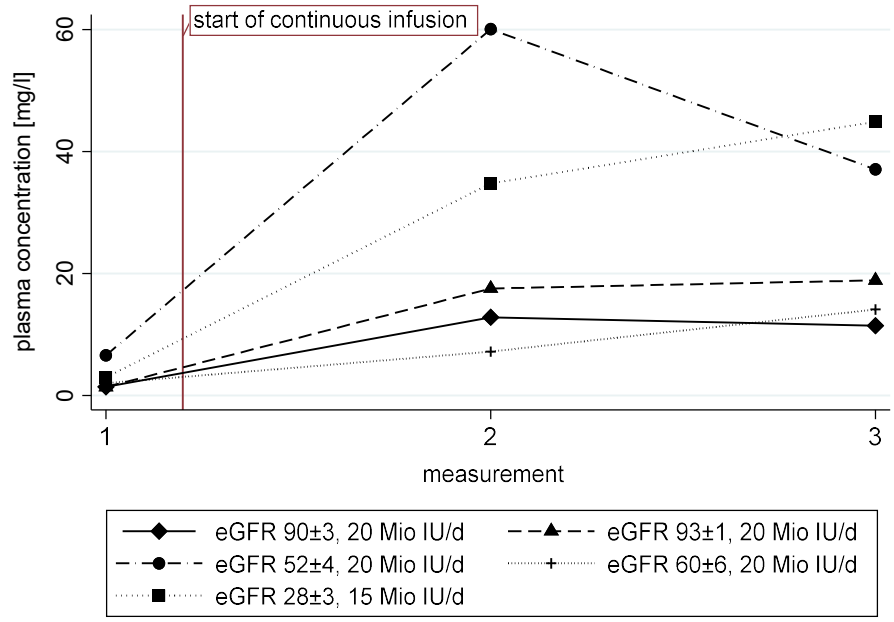

Figure 5 Five patients receiving benzylpenicillin as short infusion during the first measurement and subsequently as continuous infusion. eGFR: estimated glomerular filtration rate in ml/min/1.73m².

Patients of our tertiary-care hospital, who were scheduled to receive benzylpenicillin in OPAT, were included. Standard dosing of benzylpenicillin is 20 Mio IU per day. Only one patient presented with a mean eGFR of below 30 ml/min and received a lower dosage of 15 Mio IU per day. Patients received the same daily dose before and during continuous infusion. 20 Mio IU per day was given as 5 Mio IU 4x/d and 15 Mio IU/d as 5 Mio IU 3x/d via short infusions.

Through levels measured after intermittent bolus dosing ranged from 0.5 to 6.6 mg/l. Median time between first and second blood withdrawal was 5 days (range 2 to 10 days). Second OPAT measurement was taken 14 days (median) (range 11 to 17 days) after the first measurement. Levels measured during OPAT ranged from 7.2 to 60 mg/l and levels did not differ significantly between both samples from the same patient (two-sided student's t-test for dependent samples; p=0.85).

Using a qualitative LC-MS/MS method, we identified several breakdown products in stored benzylpenicillin solutions. Scans show the complete disappearance of the benzylpenicillin peak in unbuffered solution after storage, while several new peaks have emerged (figure 6).

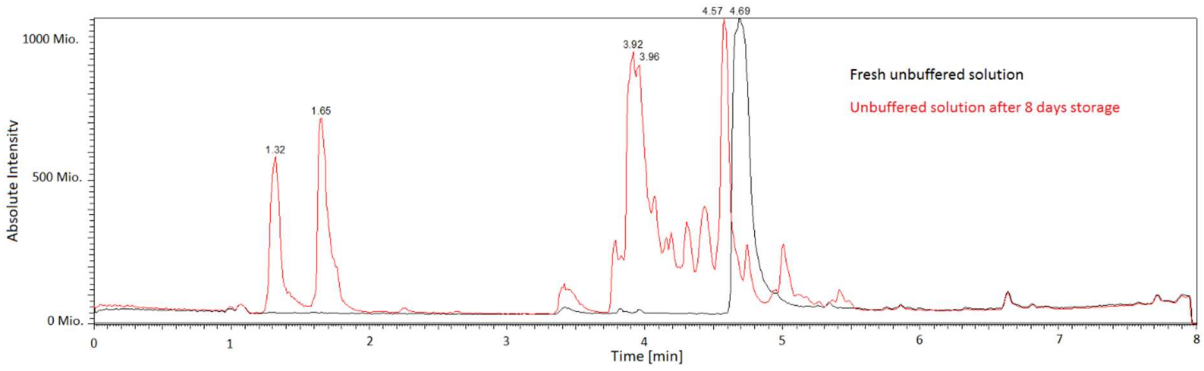

Figure 6 Scans of unbuffered solution in elastomeric pumps directly after constitution and after eight days storage: seven days refrigerated at 4°C and one day at 37°C.

Subtracting the spectra gained from fresh solutions from a scan of the stored solution, yielded higher quality chromatograms, with lower background noise (figure 7).

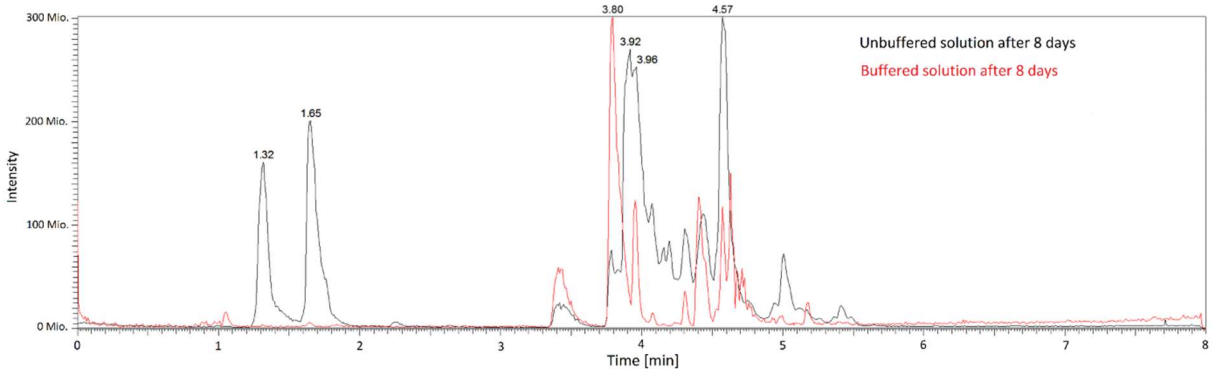

Figure 7 Chromatograms of breakdown products of buffered and unbuffered solutions in elastomeric pumps after eight days storage: seven days refrigerated at 4°C and one day at 37°C. (Background subtracted chromatograms)

Peaks identified in those spectra were further fragmented using LC-MS/MS. Results from these experiments can be found in figure 8 and table 1. Where available, breakdown products were identified with the help of previously published analyte spectra [10–12].

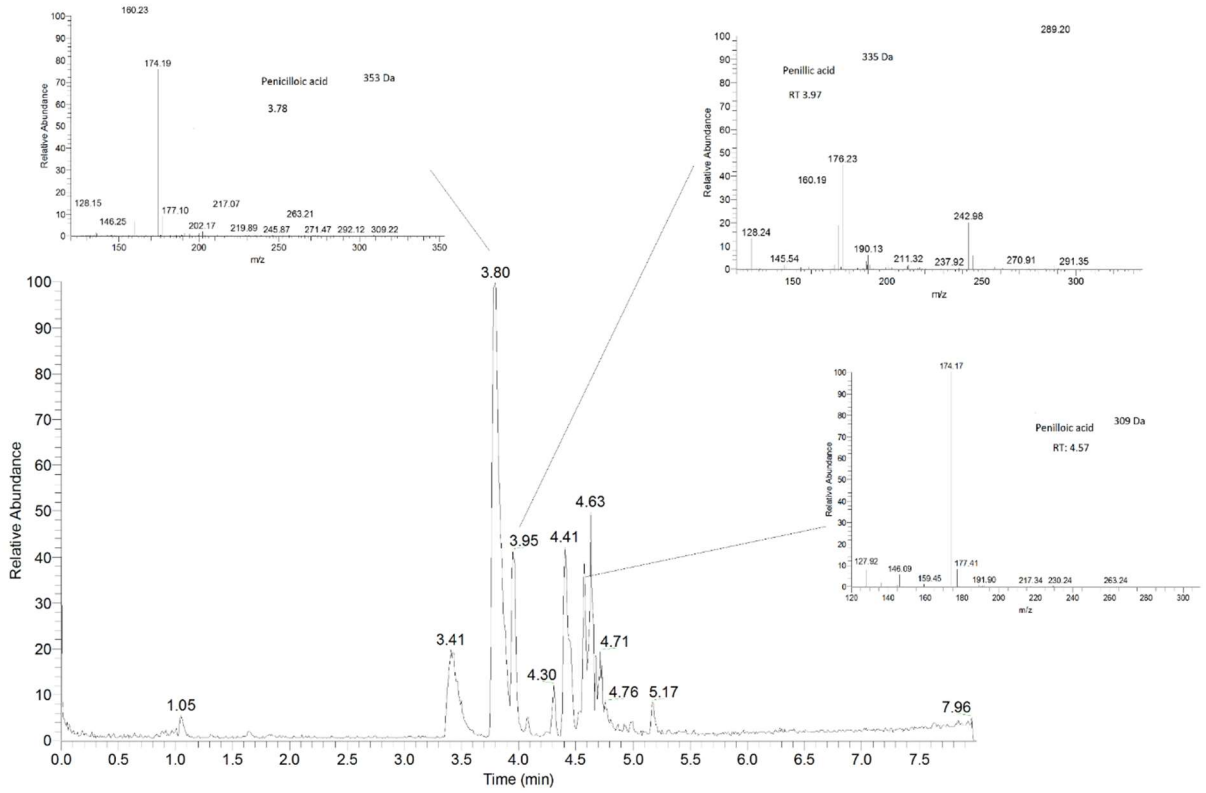

Figure 8 Chromatogram of break down products in buffered solution. Spectra of some important products are added.

Table 1 Identified degradation products and adducts, their retentions times, parent and product masses, including their relative intensity.

Analyte	Retention time [min]	Parent mass [m/z]	Product masses [m/z] (relative intensity)
Benzylpenicillin (M)	4.75	335	176.2 (100), 160.2 (90)
Penilloic acid	4.57	309	174.4 (100), 177.4 (10), 127.9 (10)
Penillic acid	3.95	335	289.1 (100), 176.1 (50), 160.1 (35), 243.1 (30), 174.1 (15), 128.1 (15)
Penicilloic acid	3.41+3.78	353	160.2 (100), 174.2 (80), 217.2 (15), 128.2 (15), 177.1 (10)
Isopenillic acid/ Penicillenic acid	1.32 /1.65	335	159.1 (100), 185.1 (65), 203.1 (30), 283.2 (25), 289.2 (20)
Predicted Adducts			
2 Penillic acid+Na	4.15+4.41	691	357.2 (100)
M+CH3OH	4.63	367	335.1 (100), 160.1 (10), 176.1 (10)
2 Penillic acid+H	4.20	669	335.2 (100), 289.2 (10), 160.1 (10), 176.1 (10)
2 M+H	5.20	669	335.2 (100), 217.0 (35), 317.3 (30), 176.1 (10), 160 (10)
2 Penilloic acid+H	4.30	616.4	309.2 (100), 160.1 (15), 263.2 (15)

Discussion

Infusions of benzylpenicillin solved in saline (NaCl 0.9%) resulted in insufficient stability for the OPAT program. After 7 days at 4°C, only 81% stability could be noted. At 37°C, degradation was strongly increased, resulting in only 10% of analyte left after 12 hours. After 18 hours at 37°C, benzylpenicillin concentrations fell below the quantification range of 1 mg/l. Neither at 4°C, nor at 37°C was stability high enough for prolonged storage.

Buffering with citrate sodium was confirmed to be a proficient way to greatly increase stability of benzylpenicillin. Stability of benzylpenicillin at 4°C was very good when buffered with citrate sodium. Solutions showed no more than 2.2% degradation in any of the nine tested pumps after the first 7 days. At 37°C, stability was lower but still sufficient over the course of 24 hours, resulting in a maximal degradation of 6.5% in one of the 40 Mio IU pumps. On average, degradation after 8 days was 2.4%, 3.7% and 5.1% in 10 Mio IU, 20 Mio IU, and 40 Mio IU pumps, respectively. The conditions tested depict a worst-case scenario, in which infusion pumps are stored for a whole week and are afterwards, during infusion, exposed to high temperatures, as could occur in summer or in areas with elevated average temperatures.

Even though the effect was not very notable after 24 hours, after a prolonged storage of 48 hours at 37°C, stability differences between 10 Mio IU, 20 Mio IU, and 40 Mio IU were significant, resulting in 86%, 81%, and 63% stability, respectively. The amount of citrate buffer was proportional to the benzylpenicillin dose. Thus, with lower or higher benzylpenicillin concentrations also buffer concentrations where adapted. The lowest concentrated analyte / buffer mixture resulted in the lowest degradation rate. We hypothesize that building up of degradation products leads to lowering of pH at first and by that to an acceleration of further degradation. According to this theory, the pH should decrease less in less concentrated solutions. Our pH measurements are consistent with this theory.

We tested a concentration range of 10 Mio IU to 40 Mio IU, to give clinicians and pharmacists security in choosing different daily dosages. A 240 ml pump containing 47 mg/ml benzylpenicillin corresponds to a standard daily dose of 20 Mio IU, while 10 Mio IU pumps are of interest in pediatric cases or in patients with decreased renal capacity. Higher daily dosages of 40 Mio IU may be needed in the treatment of endocarditis or meningitis.

Starting pH decreased over time in both buffered and unbuffered pumps. This is probably due to analyte degradation, since two major degradation products, penicilloic acid and penillic acid have two carboxyl groups, while benzylpenicillin has only one. The additional carboxyl group provide an additional H^+, decreasing pH. When these two products are further degraded, they again lose the second carboxyl-group, resulting again in a rise in pH. pH dropped about 0.7 units during the 7 days refrigerated storage period in buffered solutions, even though only a minimal benzylpenicillin breakdown of 1-2% has occurred. While stronger degradation of benzylpenicillin seems to lead to a stronger drop in pH, correlation between pH and degradation is not very good. The complex build-up and breakdown of different metabolites and their varying pKa_S lead to a non-linear change in pH, which is hard to link to degradation. Since degradation of benzylpenicillin and its metabolites is pH dependent [12, 13], the breakdown itself accelerates degradation by lowering the pH to a more aggressive environment.

Benzylpenicillin and benzylpenicillenic acid have been identified in forming allergy-inducing protein-conjugates, with benzylpenicillenic acid being the more reactive species [14]. Qualitative measurements detected several breakdown products, including penilloic acid, penicilloic acid and penillic acid. Penicillenic acid and isopenillic acid could be tentatively identified, but only in unbuffered solutions, which agrees with the breakdown processes postulated by several authors, which agree, that both degradation products are formed later on (in the break down process) from other breakdown products[12, 15]. While liquid chromatography revealed several peaks in the degraded infusion solution, not all these could be identified. Most of the peaks were identified as adducts of benzylpenicillin or one of its breakdown products.

None of the other regularly found human metabolites, like penamaldic acid, penilloaldehyde, aminopenicillanic acid, penaldic acid or penicillamine was found, even in solutions where all benzylpenicillin had been degraded. Other papers researching benzylpenicillin degradation outside of humans/ in the environment have also no reports of any of these metabolites [10, 12]. It is still possible, that we missed those and other breakdown products in our method, e.g. because analytes did not retain well on the column used here.

We included five patients for TDM measurement. All patients presented sufficient levels during short-term infusion, measured at through level. During continuous infusion, all five patients presented sufficient to high levels of benzylpenicillin. Concentrations of β-lactams should be maintained above MIC, or even 4-times above MIC for the entire dosing interval [16]. The European Committee on Antimicrobial Susceptibility EUCAST defines non-species related benzylpenicillin breakpoints at 2 mg/l, while for most bacteria, breakpoints are lower [17]. The levels measured in our study cohort were therefore slightly too low to be appropriate during short time infusion and appropriate to elevated during OPAT. Since constantly elevated levels probably increase toxicity, during continuous infusion, TDM should be performed, to decrease the risk of toxicity.

In this study we could replicate the results by McDougall et al. [5], while expanding the available information. We showed a concentration dependency of benzylpenicillin degradation as well as well as a correlation between pH and degradation. A pH change of benzylpenicillin infusion solutions did not correlate well with the degradation of benzylpenicillin, owing to the complex build-up and breakdown of products with different pKa_s. TDM data showed sufficient to high plasma levels when patients received benzylpenicillin as continuous infusions during OPAT.

Benzylpenicillin buffered with sodium citrate is a safe and convenient option for use in continuous infusions and OPAT.

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17. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters Version 10.0, 2020

Probability of target attainment with flucloxacillin in *Staphylococcus aureus* bloodstream infection: a prospective cohort study of unbound plasma and individual minimal inhibitory concentrations

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Abstract

Background: Methicillin-susceptible *Staphylococcus aureus* (MSSA) bloodstream infection (BSI) is associated with considerable mortality. Data regarding the pharmacology of the anti-staphylococcal penicillin flucloxacillin are scarce.

Objectives: Determination of the probability of optimal pharmacological target attainment (100% $fT_{>MIC}$) in blood plasma at different time points in patients with MSSA-BSI. We measured unbound fractions of flucloxacillin and determined the MIC of each MSSA strain.

Methods: In total, 50 patients with MSSA-BSI were included. 231 plasma flucloxacillin concentrations were measured at five defined time points during antibiotic treatment. MIC of MSSA strains were determined by broth microdilution (flucloxacillin) and Etest® (oxacillin). The time of unbound drug concentration above MIC ($fT_{>individual\ MIC}$) and associated factors were analysed.

Results: Most of the patients included suffered from bone and joint infections (n=16; 32%). Median MIC of MSSA strains was 0,06 mg/L for flucloxacillin and 0.38 mg/L for oxacillin. On study day 1, median mid-dose concentration of unbound flucloxacillin was 4.8 mg/L (IQR 1.9-15) and median trough concentration 1.7 mg/L (IQR 0.4-9.3). The pharmacological target was attained in 45/50 patients (90%) according to flucloxacillin MICs and in 13/50 patients (26%) according to EUCAST epidemiological cut-off values. Target attainment was associated with more severe disease and a higher daily flucloxacillin dose but not with better clinical outcome. 9/50 patients (18%) had excessive unbound flucloxacillin concentrations (>20mg/L).

Conclusions: Variability of unbound flucloxacillin concentration in patients with MSSA-BSI is substantial. While pharmacological target attainment is improved in critically ill patients, risk of excessive concentrations is high. Therefore, in MSSA-BSI, therapeutic drug monitoring of unbound flucloxacillin concentrations is desirable.

Abbreviations

AKIN	acute kidney injury index
BSI	blood stream infection
CI	confidence interval
DILI	drug induced liver injury
ECOFF	epidemiological cut-off value
EEG	electroencephalogram
eGFR	estimated glomerular filtration rate
Etest	Epsilometertest
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GCS	Glasgow coma scale
ICU	intensive care unit
IQR	interquartile range
IV	intravenous
MIC	minimal inhibitory concentration
MSSA	methicillin susceptible *Staphylococcus aureus*
SD	standard deviation

Introduction

Blood stream infections (BSI) with methicillin-susceptible *Staphylococcus aureus* (MSSA) remain a devastating disease with a high mortality rate between 18-30% [1]. Appropriate antibiotic therapy, aggressive management including identification of infectious foci, early surgery, and consultation of an infectious diseases specialist are the cornerstones in the management of MSSA-BSI [2]. Flucloxacillin is a β-lactam antibiotic frequently used in the treatment of MSSA infections. It has a half-life of about 1 h, is metabolized to a limited extent [3] and is eliminated by renal (glomerular filtration and tubular secretion) [4] and non-renal mechanisms [5]. Approximately 95% of the drug is bound to serum proteins. The extent of binding to plasma proteins is highly relevant, as it is the unbound fraction of a drug that is responsible for the pharmacological effect [6].

β-lactams are time-dependent antibiotics. Consequently, the time that unbound drug concentrations remain above the minimal inhibitory concentration (MIC) of the pathogen ($fT_{>MIC}$) is recognized as the pharmacological parameter that best correlates with antimicrobial activity and outcome. Clinical outcome may be improved if concentrations of β-lactams are maintained above the MIC for extended periods (100% $fT_{>MIC}$ or even 100% $fT_{>4xMIC}$) [7]. Flucloxacillin is usually administered as intermittent bolus four to six times a day. Despite its common use in MSSA infections, data on target attainment in patients with standard dosing are scarce. Simulation studies based on a small number of MSSA patients have either demonstrated flucloxacillin underdosing of patients [8, 9] or a high variability of the free flucloxacillin fraction especially in critically ill patients [10, 11]. Importantly, potentially toxic free flucloxacillin concentrations were observed in almost 1/4 of patients when more than 6g/24h were administered intravenously (IV) as continuous infusion [12]. Hence, measurement of the free drug concentration has been recommended especially in critically ill patients presenting with hypalbuminaemia [9], which is rarely performed in practice. Furthermore, little is known about the relationship between the unbound drug concentration or target attainment and the clinical outcome of patients. Determination of the exact patient-individual MSSA-MIC by Epsilometertest (Etest®) or broth microdilution is rarely performed in the published studies, but rather target attainment was calculated using the standard epidemiological cut-off values (ECOFF) of MSSA (2 mg/L).

Given the scarcity of pharmacological data in critically and non-critically ill patients with MSSA-BSI, the aim of this study was to determine the probability of optimal pharmacological target attainment (100% $fT_{>MIC}$) in patients with MSSA-BSI by measuring the unbound fraction of flucloxacillin when administered as standard intermittent bolus dosing and by determining the flucloxacillin MIC of each MSSA strain to calculate patient-individual target attainment.

Materials and methods

We performed a longitudinal, observational, and prospective cohort study in a 750-bed tertiary care hospital between January 2018 and December 2019. The study was approved by the Ethics Committee of Northwest and Central Switzerland (EKNZ Project-ID: 2017-02072), was in accordance with the Declaration of Helsinki and all patients provided written informed consent for participation in the study.

Patient characteristics and management

All adult patients ≥18 years who were admitted to our hospital, who had at least one blood culture with growth of MSSA and who received flucloxacillin treatment were screened for eligibility. Patients from whom MSSA positive blood cultures were recently drawn in another hospital and who were transferred to our hospital for further management could also be included. Exclusion criteria included age <18 years, participation in the study within the last 30 days, hemodialysis, pregnancy, outpatient treatment, polymicrobial BSI (with the exception of bacteria regarded as contamination), planned stop of flucloxacillin within the next 24 hours or planned discharge within the next 48 hours.

Collection of clinical data

Demographic and clinical data were prospectively collected and included age, gender, weight, comorbidities (diabetes mellitus, chronic renal failure, cardiovascular disease, and IV drug use), focus of BSI and calculation of the Charlson comorbidity score. Immunosuppression was defined as daily dosage of more than 5 mg prednisone or equivalent, treatment with classical immunosuppressive drugs or monoclonal antibodies, a neutrophil count less than 500 per microliter, HIV CDC category C, and liver cirrhosis. Severity of illness was classified by calculating the PITT Bacteremia score and the SOFA score on admission and on every study day. Plasma albumin and creatinine (including estimated glomerular filtration rate (eGFR) as calculated by the Chronic Kidney Disease Epidemiology Collaboration study equation) were determined from the blood samples collected for the measurement of plasma flucloxacillin concentrations.

Blood cultures and MIC determination

The BacT/ALERT® FA/FN Plus system (bioMérieux) was used for the identification of positive blood cultures. MALDI-TOF-based identification of colonies grown on subcultures was used for the identification of MSSA. Resistance testing was performed with the VITEK2 system (bioMérieux). Oxacillin MICs of MSSA were determined by Etest® (Liofilchem Diagnostici). Flucloxacillin MICs were measured by using microdilution with cation-adjusted Mueller-Hinton

broth. According to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), *S. aureus* was considered susceptible to oxacillin and flucloxacillin, if the MIC was ≤2 mg/L. For patients with missing flucloxacillin MIC, a MIC of 0.25 mg/L was defined, as this was the highest of all measured flucloxacillin MICs in our study.

Plasma sampling and drug assay

Plasma samples were drawn at study day 1 (mid-dose and trough), 3 (mid-dose and trough) and 7 (trough only) (Figure 1). Plasma samples were immediately sent to the central laboratory of the University Hospital Basel, centrifuged, aliquoted and stored at -80°C to ensure stability of the analyte. Samples were subsequently analysed in batches.

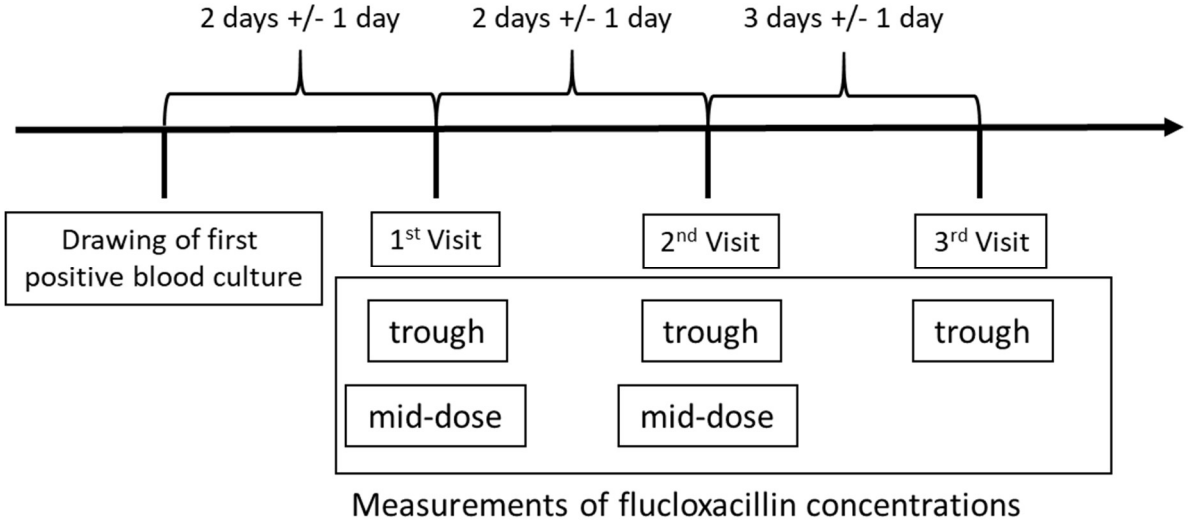

Figure 1: Overview of study visits and concentration measurements of flucloxacillin.

Total plasma concentrations, as well as unbound plasma concentrations of flucloxacillin were measured using a validated high-performance liquid chromatography–mass spectrometry method using isotope dilution [13]. Free concentrations were determined using ultrafiltration prior to analysis. Sample preparation consisted of manual protein precipitation and online extraction. Analyte and deuterated internal standard were separated, fragmented using positive electrospray ionization and three major fragments were detected.

Endpoints

The primary endpoint was defined as the probability of optimal target attainment (100% $fT_{>MIC}$) in blood plasma on all occasions. Target attainment was defined as a measured trough plasma concentration of flucloxacillin above the MIC as determined by microdilution. In addition, target attainment was calculated using the EUCAST ECOFF for flucloxacillin of 2 mg/L.

Secondary endpoints included the probability of pharmacological target attainment for the minimum target (≥50% $fT_{>MIC}$) and the maximum target (100% $fT_{>4xMIC}$) in blood plasma. Additional secondary endpoints were the association of patient parameters such as age, comorbidities, renal function, amount of daily infusions, requirement of dialysis/vasopressors/ventilation, albumin, inflammatory proteins, focus of infection, surgical intervention etc. with target attainment (100% $fT_{>MIC}$). Finally, intra-individual variability of flucloxacillin plasma concentrations and unbound fraction as well as the association of pharmacological target attainment (100% $fT_{>MIC}$) and unbound flucloxacillin trough concentrations with clinical outcome (30-day mortality) were investigated. In a post-hoc analysis, toxicity of flucloxacillin was evaluated by analyzing the association of unbound trough flucloxacillin concentration (100% $fT_{>10xECOFF}$) with organ toxicity [14].

Definition of flucloxacillin toxicity

Potential renal and hepatic toxicity of flucloxacillin was evaluated by using the acute kidney injury index (AKIN) [15] and the drug induced liver injury (DILI) criteria [16]. For the determination of neurotoxicity, we assessed need for intubation, alterations of the Glasgow coma scale (GCS), electroencephalograms (EEG) and documented neurological symptoms [17].

Statistical analysis

In our 750-bed tertiary care hospital, approximatively 60-80 cases of MSSA bacteremia are diagnosed every year. We estimated that about 50 cases are necessary to obtain robust observational data and to be able to define up to five covariates predicting the achievement of the primary outcome.

All continuous variables were compared using either the Mann-Whitney-U test, reporting median and interquartile ranges (IQR) or the Student's t-test, then reporting mean and standard deviation (SD), where appropriate. We used the chi-square and Fisher's exact test for comparisons of categorical variables where appropriate. A mixed-effect model using the restricted maximum likelihood method was employed to analyse intra-individual variability of unbound flucloxacillin trough concentrations and unbound fractions. In addition, the coefficient of variation of the intra-individual variability was calculated by dividing the individual standard deviation by the mean of all free fractions of an individual patient. Correlations between the unbound fraction and laboratory and clinical parameters were analysed using the Spearmen correlation coefficient.

Multivariable logistic regression models including potentially confounding variables were performed to analyse associations between patient variables with target attainment (using the EUCAST ECOFF), toxicity or outcome.

We considered statistical significance if the two-sided p-value was less than 0.05. All analyses were performed with the use of SPSS Version 26 (IBM SPSS Statistics for Windows. Armonk, U.S.A.) and Prism Version 8 (GraphPad Software, San Diego, U.S.A.).

Results

Patients and clinical characteristics

Between January 2018 and December 2019 148 patients were screened for eligibility, of whom 50 patients were included in our study (Figure 2). The median time from the first blood culture with growth of MSSA to administration of flucloxacillin and to inclusion of the patient (study visit 1) was 2 days (IQR 1-3) and 4 days (IQR 3-6), respectively.

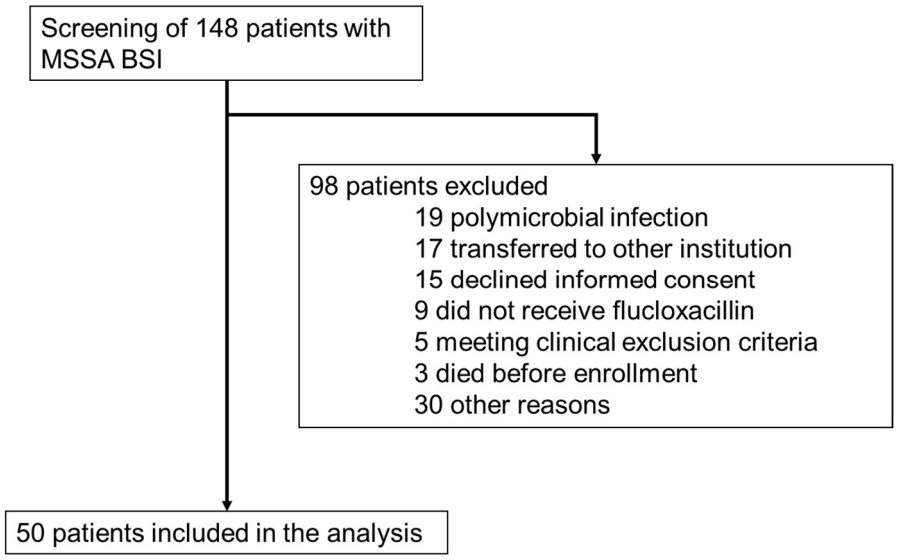

Figure 2: Flow chart of screened and included patients.

Table 1 summarizes the baseline patient characteristics. Bone and joint infections were the most common infections (n=16; 32%) followed by endocarditis (n=11, 22%) and skin and soft tissue infections (n=10; 20%). Median length of hospital stay was 26 days (IQR 16.0-50.5). Eight patients died within 30 days after collection of the first positive blood culture (16%).

Most (n=46, 92%) patients received empirical treatment in the form of a β-lactam antibiotic, most frequently amoxicillin-clavulanic acid. Only three patients remained untreated before blood cultures turned positive. A switch to flucloxacillin was performed 2 days (IQR 1-3) after initiation of antibiotic therapy. On the first study day, 68% of patients (n=34) received 12 grams

flucloxacillin IV per day and 32% (n=16) lower dosages depending on the suspected focus of infection.

Table 1: Characteristics and outcome of 50 patients with a bloodstream infection caused by methicillin-susceptible *Staphylococcus aureus*

Variable	n (%) or median (IQR)
Female sex	13 (26)
Age (years)	64.7 (49.9-76.8)
BMI (kg/m^2)	25.7 (22-29.5)
Length of stay (days)	26 (16-50.5)
Comorbidities	
Diabetes mellitus	12 (24)
Liver cirrhosis	4 (8)
Chronic renal disease	13 (26)
Cardiovascular disease	20 (40)
Chronic lung disease	5 (10)
Hematooncological malignancies	6 (12)
IVDU	9 (18)
Charlson comorbidity score	3 (1.5-6)
Disease severity at BSI onset and study inclusion, respectively	
PITT bacteremia score	1 (0-2), 1 (0-1.5)
SOFA score	3 (2-5.5), 3 (1.5-6)
Laboratory results at BSI onset and study inclusion, respectively	
CRP (mg/L)	179 (45-281), 112 (69-171)
Leukocytes (10^6/L)	11 (8-15), 10 (7-14)
Creatinine (µmol/L)	89 (63-153), 82 (58-122)
eGFR (ml/min/1.7m^2)	79 (37-104), 81 (47-111)
Albumin (g/L)	28 (23-34), 21 (18-26)
Focus of BSI	
Osteomyelitis or arthritis	16 (32)
Endocarditis	11 (22)
Skin and soft tissue	10 (20)
Catheter or foreign material	4 (8)
Respiratory tract	1 (2)
Primary origin	8 (16)
Complications and outcome	
Vasoactive treatment	6 (12)
ICU admission	24 (48)
30-day mortality	8 (16)

Abbreviations, BMI, Body mass index; IVDU, intravenous drug use; SOFA score, Sequential organ failure score; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate, BSI, blood stream infection; ICU, intensive care unit

Pharmacological data

Initial flucloxacillin concentrations were determined during steady state after a median of 2 (IQR 1-3) days of treatment. Median mid-dose unbound concentration was 4.8 mg/L (IQR 1.9-14.9) and 5.2 mg/L (IQR 1.7-16.4) on study day 1 and 3, respectively. Median unbound trough concentration was 1.7 mg/L (IQR 0.4-9.3), 1.9 mg/L (IQR 0.4-6.2) and 1.0 mg/L (IQR 0.6-3.4) on study days 1, 3 and 7, respectively (Figure 3). Higher daily doses were associated with increased unbound concentrations (e.g. day 1 median unbound trough concentration 2.2 mg/L (IQR 0.9-13.8) vs. 0.5 mg/L (0.2-1.4), p=0.004, for a daily dose of 12 g vs. 8 g, respectively). Trough unbound concentrations remained stable during the 7-day period despite a change in doses in 11/50 (22%) of patients (p>0.05). Median unbound fraction ranged from 7.3% (study day 7) to 13.5% (mid-dose on study day 1) with a minimum of 1.1% and a maximum of 64.7%. A significant correlation (all p-values <0.005) was observed between the unbound fraction and serum albumin (r=-0.67), eGFR (r=-0.4) (Figure 4), total flucloxacillin concentration (r=0.49), C-reactive protein (r=0.41), platelet count (r=-0.45), vital signs (systolic blood pressure r=-0.40, respiratory rate r=0.52) and disease severity (SOFA score r=0.49, PITT score r=0.49).

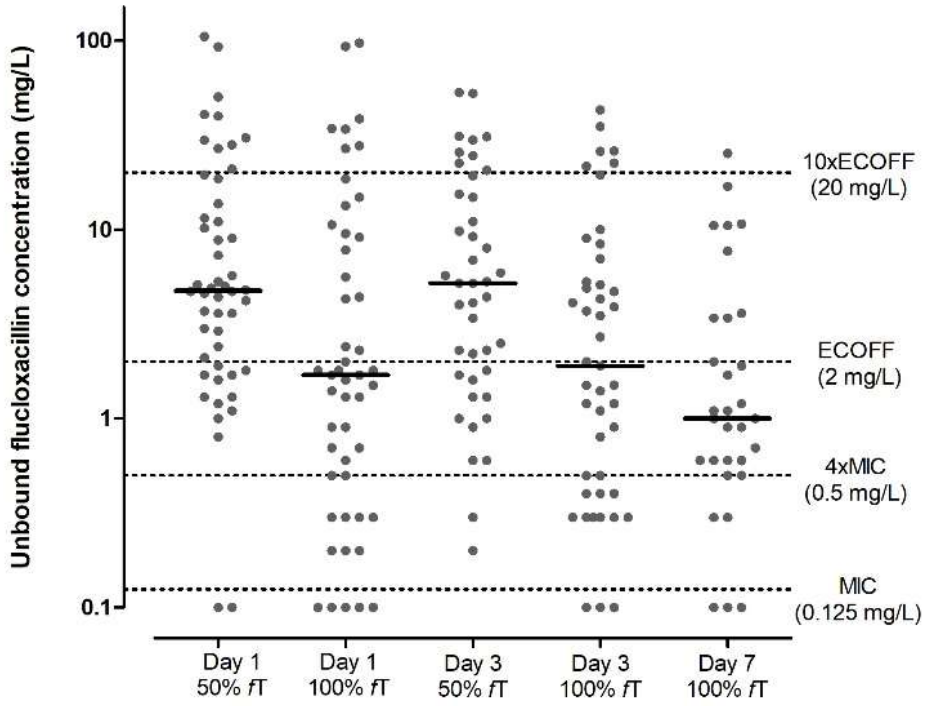

Figure 3: Unbound flucloxacillin concentration during the 7-days study period.

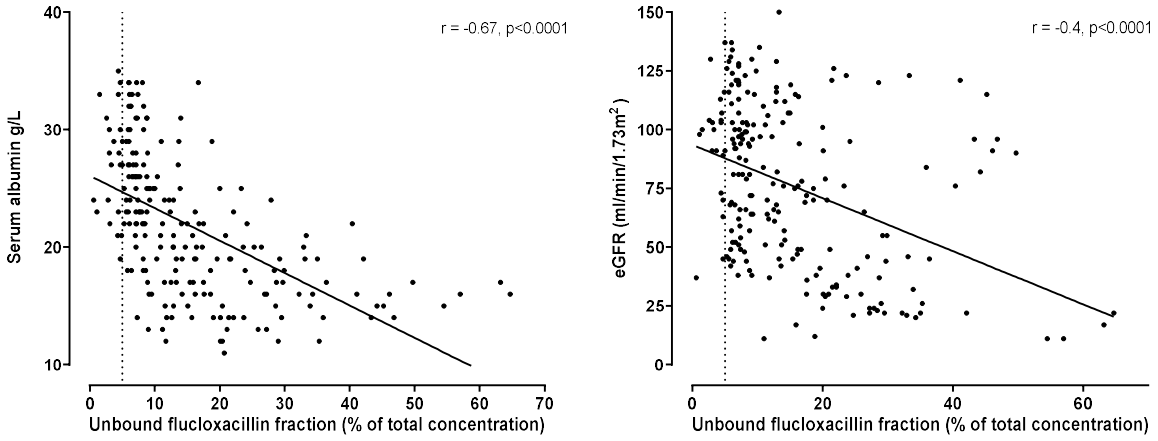

Figure 4: Correlation of serum albumin and eGFR with unbound flucloxacillin fraction.

Although significant (p=0.02), intra-individual variation in the unbound flucloxacillin fraction over time was modest. The median intra-individual coefficient of variation was 27%, ranging from 0 to 69% (Figure 5). There was no significant correlation between the intra-individual coefficient of variation and age, disease severity, mean eGFR or mean serum albumin (data not shown).

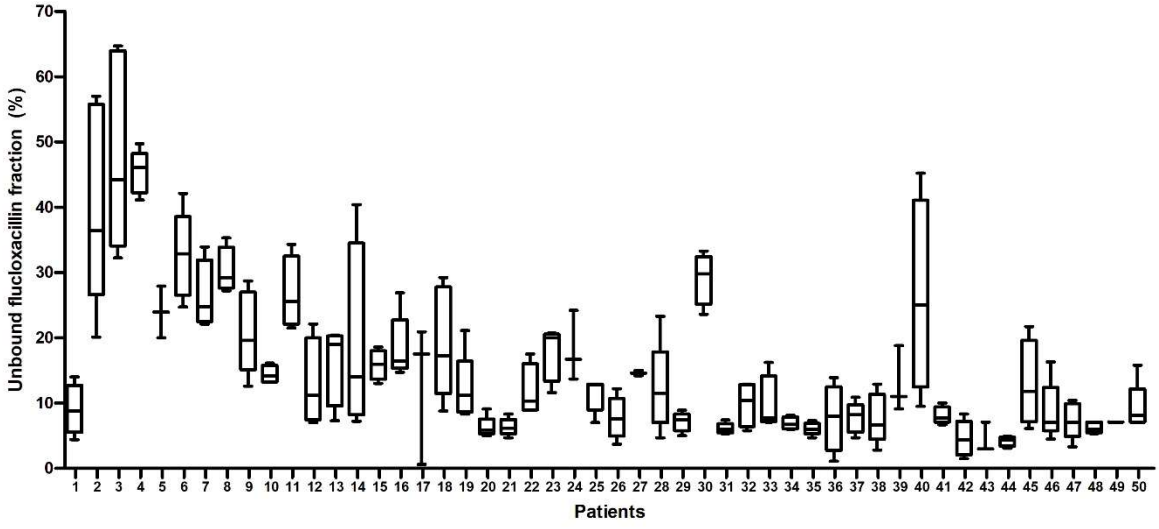

Figure 5: Individual patient unbound flucloxacillin fraction (%) including all mid-dose and trough measurements during the 7-day study period.

In critically ill patients we observed higher unbound flucloxacillin concentrations and a higher unbound fraction (median unbound trough concentration 4.2 mg/L (IQR 0.9-23.6) vs. 1.1 mg/L

(IQR 0.3-10.9), p=0.008, median unbound fraction (mid-dose and trough) 16.0 mg/L (IQR 9.7-30.4) vs. 8.0 mg/L (6.7-12.8), p=0.001), whereas intra-individual variability was not different.

Microbiological data

MICs of flucloxacillin and oxacillin were measured in bacterial isolates of 37 (74%) and 44 (88%) patients, respectively. Median MIC of flucloxacillin determined by broth microdilution was 0.06 mg/L (IQR 0.06-0.10). Median flucloxacillin MIC increased to 0.125 mg/L (IQR 0.06-0.25) when missing MICs (defined as 0.25 mg/L, the highest measured flucloxacillin MIC) were included. Median MIC of oxacillin determined by Etest® was 0.38 mg/L (IQR 0.3-0.5).

Pharmacological target attainment

The primary target (100% $fT_{>MIC}$) was attained by 45 patients (90%) on all occasions (Table 2), and the majority (34 patients, 68%) also achieved the maximum target (100% $fT_{>4xMIC}$). In contrast, flucloxacillin trough concentrations were above the EUCAST ECOFF (100% $fT_{>ECOFF}$) in only 13 (26%) patients on all occasions, and even the most conservative target (≥50% $fT_{>ECOFF}$) was only achieved in 32 (64%) patients. (Table 2 and Figure 6). Of note, critically ill patients were more likely to achieve pharmacological targets compared to non-critically ill patients (100% vs. 81% for 100% $fT_{>MIC}$, p=0.05, 83% vs. 54% for 100% $fT_{>4xMIC}$, p=0.04, and 38% vs. 15% for 100% $fT_{>ECOFF}$, p=0.1)

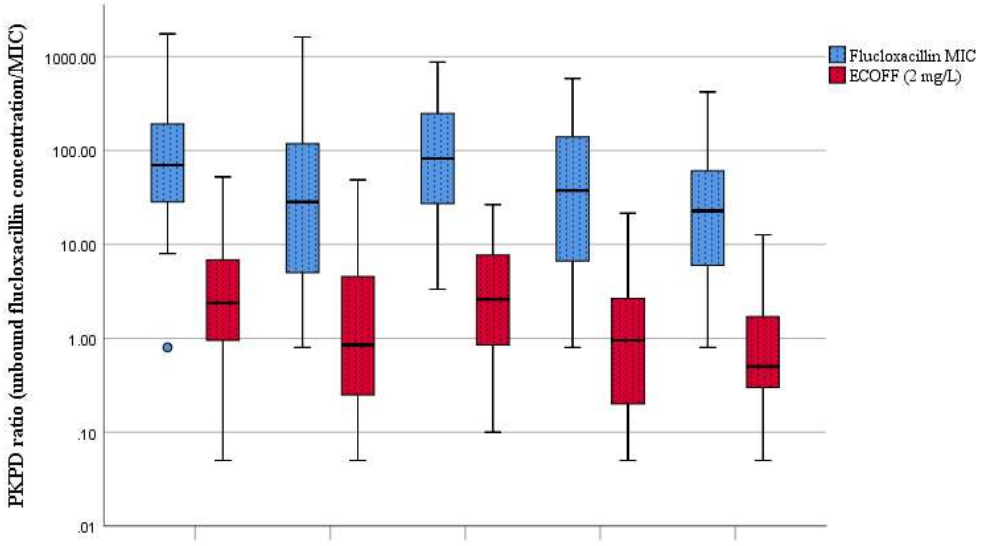

Figure 6: PK/PD ratios (unbound flucloxacillin concentrations divided by individual flucloxacillin MIC or ECOFF (2 mg/L)) at 50% and 100% of the dosing interval during the 7-day study period.

Table 2: Target attainment at different time points (percentage)

	Study day 1		Study day 3		Study day 7		Cumulative	
	flucloxacillin MIC	ECOFF	flucloxacillin MIC	ECOFF	flucloxacillin MIC	ECOFF	flucloxacillin MIC	ECOFF
≥50% $fT_{>MIC}$	96	74	100	71.4			96	64
≥50% $fT_{>4xMIC}$	94	36	90.5	38.1			90	30
100% $fT_{>MIC}$	91.8	40.8	97.8	48.9	93.5	32.3	90	26
100% $fT_{>4xMIC}$	71.4	26.5	82.2	22.2	83.9	16.1	68	14

Abbreviations: MIC, minimal inhibitory concentration; ECOFF, EUCAST epidemiological cut-off value; % $fT_{>NxMIC}$, percentage of dosing period where unbound concentration of flucloxacillin is above n-times the MIC.

Table 3: Predictors of cumulative flucloxacillin target attainment 100% $fT_{>ECOFF}$ (worst case scenario), univariate and multivariate analysis. All variables measured at BSI onset.

Predictor	Univariate OR (95% CI)	p-value	Multivariate OR (95% CI)	p-value
Age (years)	1.03 (0.99.-1.07)	0.1		
Female gender	1.38 (0.34 -5.59)	0.7		
BMI (kg/m^2)	0.99 (0.86-1.15)	0.9		
eGFR (ml/min/1.72m^2)	0.96 (0.94-0.98)	0.002	0.95 (0.92-0.99)	0.006
SOFA Score	1.63 (1.20-2.22)	0.002		
PITT Score	2.38 (1.22-4.65)	0.01	3.6 (1.2-11.0)	0.03
Charlson Score	1.03 (0.82-1.30)	0.8		
Daily dose 12g iv vs. <12g iv	8.2 (0.96-69.75)	0.06		
ICU admission	3.3 (0.86-12.71)	0.08		

Logistic regression analyses using the worst-case scenario (100% $fT_{>ECOFF}$) identified disease severity (as measured by the PITT bacteraemia score on the day of first study visit) and renal function as independent predictors of optimal target achievement (table 3).

Clinical endpoints

30-day mortality was 16% (8/50 patients) and median length of stay was 26 days (IQR 16-50). Target attainment (100% $fT_{>MIC}$) was not associated with length of stay or 30-day mortality (data not shown). However, 30-day mortality was higher in patients achieving vs. not achieving 100% $fT_{>MIC}$ (8/45 (18%) vs. 0/5 (0%), p=0.6) and 100% $fT_{>ECOFF}$ (5/13 (39%) vs. 3/37 (8%), p=0.02), respectively. In line with those results, the average unbound flucloxacillin trough concentration over the study period was significantly higher in deceased patients (median 14.8 mg/L (IQR 1.2-31.8) vs. 1.7 mg/L (IQR 0.6-5.1), p=0.01). After adjusting for age and PITT bacteremia score on admission, these associations were not significant anymore (100% $fT_{>ECOFF}$ OR 1.6 (95% CI 0.2-12.8), p=0.6; average unbound flucloxacillin trough concentration OR 1.4 (95% CI 0.96-1.13), p=0.3).

Flucloxacillin toxicity

An unbound flucloxacillin concentration of 10 times the ECOFF (i.e. >20 mg/L) is accepted as threshold for potentially toxic drug concentration [14]. This threshold was exceeded in 9 of 50 patients (18%; the majority (89%) critically ill patients) at least once during their hospital stay (median 34.2 mg/L, IQR 27-68). Excessive unbound concentration (100% $fT_{>10xECOFF}$) was more frequently observed in critically ill patients (8/24 (33%) vs. 1 (4%), p=0.01) and associated with higher 30-day-mortality (4/9 (44%) vs. 4/41 (10%), p=0.03).

Acute kidney injury (AKI) and potential neurotoxicity, but not DILI, occurred more frequently in patients with 100%$fT_{>10xECOFF}$ (AKI 8/9 (89%) vs. 14/41 (34%), p=0.007; neurotoxicity 7/9 (78%) vs. 8/41 (20%), p=0.002). In line with these findings, median creatinine peak concentration was 304 µmol/L (IQR 210-514) versus 89 µmol/L (IQR 70-139) in patients without trough concentrations above this threshold (p<0.001). In addition, median peak of total bilirubin concentration was also higher (76 µmol/L (IQR 49-244) vs. 11 µmol/L (IQR 8-19), p=0.04).

Elevated average unbound flucloxacillin trough concentration was associated with potential neurotoxicity (p<0.0001) but not acute liver injury and AKI occurring after the start of flucloxacillin treatment (Figure 7) in univariate analysis. It remained an independent predictor of neurotoxicity (OR 1.12 per 1 mg/L increase, 95% CI 1.02-1.23, p=0.02) after adjusting for age, baseline renal function, the Charlson comorbidity score and the PITT bacteremia score.

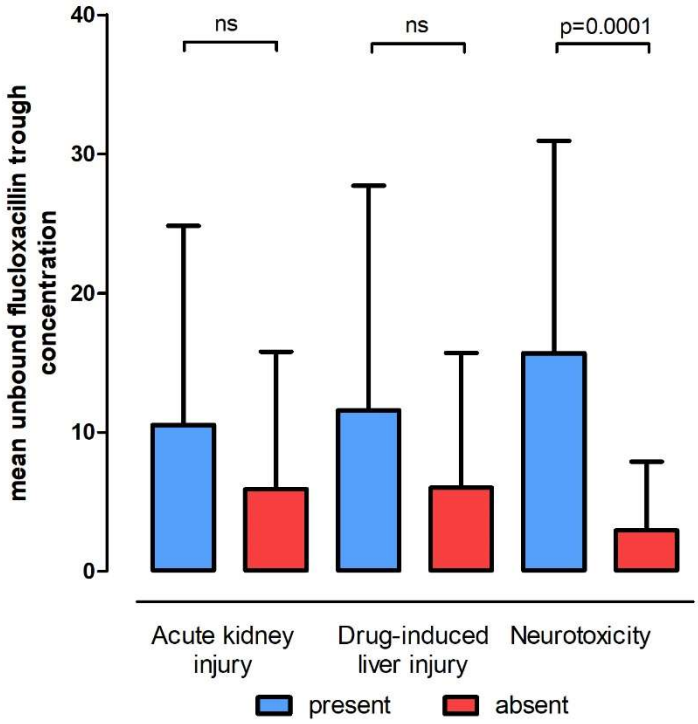

Figure 7: Mean unbound flucloxacillin trough concentration (three study visits) in patients with acute kidney injury, acute liver injury and potential neurotoxicity occurring after the start of flucloxacillin treatment.

Discussion

This is, to the best of our knowledge, the first longitudinal observational study that systematically analysed the probability of optimal pharmacological target attainment (100% $fT_{>MIC}$) in patients with MSSA-BSI by measuring the unbound fraction of flucloxacillin when administered as standard intermittent bolus dosing at several time points. Furthermore, MICs of MSSA strains were determined by the current reference method to accurately calculate patient-individual $fT_{>MIC}$.

We observed high variability of unbound trough flucloxacillin concentrations between patients but only small intra-individual differences over the study period of 7 days. Concerning the inter-individual unbound plasma fraction, mid-dose values ranged widely from 1.1% to 64.7% showing substantially higher values than reported for healthy individuals (2-8%) [15]. A modest intra-individual variability was observed in particular in patients with impaired renal function, hypoalbuminaemia and more severe disease. All of these findings are in line with previous studies that observed similar correlations and also a great variability in the unbound fraction of

flucloxacillin in patients with MSSA-BSI (range 3-65%) [20]. As it is the unbound fraction of the drug that is mostly responsible for the pharmacological effect [6], knowledge about the unbound drug concentration is crucial. Dosage adjustments should be in accordance with the unbound and not with the total flucloxacillin concentration [18]. Prediction of the unbound fraction by using pharmacological models is not reliable and hence not recommended especially in critical ill patients with hypoalbuminaemia and in highly protein-bound antibiotics like flucloxacillin [11, 19]. Measurement of the unbound concentration might be the best option to obtain reliable information about target attainment. Because there is only a modest variability in intra-individual unbound concentrations, in particular in non-critically ill patients, our data emphasize that measurement of only one value in steady state might be sufficient to evaluate target attainment, when albumin and creatinine are stable.

In our study, 90% of all patients and 100% of ICU patients attained the optimal target (100% $fT_{>MIC}$) with patient-individual MIC, whereas only 38% of critically ill patients attained the target concentration when it was defined as 100% $fT_{>ECOFF}$. We identified disease severity and renal function as independent predictors of optimal target attainment in the worst-case scenario 100% $fT_{>ECOFF}$). In PKPD studies, ECOFF [18] or EUCAST clinical species-specific breakpoints [20] of the targeted bacterium are often used to define target plasma concentrations. In MSSA ECOFF and clinical breakpoint are identical (2 mg/L). In our study, all measured flucloxacillin MICs ranged between <0.06 mg/L and 0.25 mg/L and were therefore 16x and four dilutions lower than the ECOFF and the breakpoint, respectively. This highlights that we must critically evaluate if ECOFF is the appropriate reference value on which to base optimal drug concentrations or if a patient-individual MIC should be preferred. Mouton et al. critically discussed measurement of patient-individual MIC to guide antibacterial drug dosing. They state that individual MICs may not be appropriate due to high variability (1-2 dilutions) between repeat measurements and variations according to the determination method [21]. They state that TDM-guided dosing adjustments must consider these variations in MIC determination. However, even a variability of 1-2 dilutions is less than the difference between measured MIC and ECOFF we observed. Considering that not only in our study, MICs are far below the ECOFF but the MIC of a vast majority of wildtype MSSA strains is below 1 mg/L [22], the use of ECOFF to define optimal target concentration of flucloxacillin risks exposing patients to unnecessary toxic drug concentrations.

Our data show that especially in patients admitted to ICU the proportion of patients with excessive unbound flucloxacillin concentrations (100% $fT_{>10xECOFF}$) is significant (33% vs. 4%, p=0.01). If the measured MIC had been taken as reference, 17 patients (34%) would have exceeded the threshold. In patients with MSSA-BSI it is difficult to differentiate between drug induced and sepsis related organ damage and patients in septic shock receive a wide variety of drugs that may

harm the organ system. β-lactams are known for their potential for neurological, renal and hepatic toxicity, especially when administered in high dosages [23]. The observed organ damages in our study cohort are therefore hard to link with one sole therapeutic agent. Nevertheless, our data are similar to already published studies that analysed β-lactam toxicity [23, 24].

In critically ill patients, both unbound flucloxacillin concentrations and unbound fractions were substantially higher than in non-critically ill patients, but both patient groups did not show any differences concerning intra-individual variability. These higher concentrations are consistent with the increased toxic side effects in patients admitted to the ICU. This highlights that more is not necessarily better for the patient and warns of underestimating the toxic side effects of β-lactams. Clinicians must carefully assess if a patient might be at risk for developing excessively high drug concentrations. Patients admitted to the ICU have more severe disease and suffer more likely from intravascular MSSA infections like endocarditis than non-ICU patients. Consequently, higher flucloxacillin dosages (12 g/d) are prescribed according to the recommendations of most of the international guidelines [25, 26]. In our cohort, 68% of the patient initially received 12 g/d, although bone and joint infection were the most frequent underlying diseases. Our in-house policy advices the administration of 12 g/d until endocarditis can be excluded by transoesophageal echocardiography. Facing the risk of overdosing and potentially harming the patient, this approach might be questioned. Maybe a more conservative approach, as it is pursued in the UK in patients with endocarditis (8 g/d if patients weight is below 85 kg), should be discussed. Similarly, in patients with impaired renal function we have to carefully evaluate if dosage reduction has to be performed not only when eGFR is below 10 ml/min/1.73 m^2 [27] but even earlier.

Despite an increasing number of studies, there is still limited evidence for association of target attainment or general plasma drug concentrations with clinical outcome [20]. Our study showed that target attainment was not associated with 30-day mortality. However, our sample size was too small to obtain robust data.

Cefazolin is a first-generation cephalosporin that is increasingly used in the treatment of MSSA-BSI, especially in patients with impaired renal function [28]. Despite several study limitations, a review of the literature by Loubet et al. showed that there is no significant difference in relapse or mortality rate in patients receiving anti-staphyloccoccal penicillins (ASPs), including flucloxacillin, or cefazolin for the treatment of MSSA-BSI. A meta-analysis of Weis et al. [29] postulated that cefazolin seems to be at least equally as effective as ASPs. The administration of ASPs was associated with higher nephrotoxicity and more frequent discontinuation of treatment due to adverse events, mostly involving kidneys and skin [28]. It remains unknown if adverse events were linked to toxic ASP concentrations, because the analysis did not include TDM. Studies on cefazolin concentrations and target attainment in MSSA-BSI are scarce. It would be desirable

to evaluate if excessive drug concentrations (100% $fT_{>10xECOFF}$) and clinically manifesting side effects may also be observed during cefazolin treatment, particularly in critically ill patients.

A strength of our study is that, to the best of our knowledge, this is the first prospective cohort study of nearly equally distributed ICU and non-ICU patients with several measurements of bound and unbound flucloxacillin concentrations, the determination of unbound fractions and of patient-individual flucloxacillin MICs by microdilution. Further, our study population is quite heterogeneous and represents a typical MSSA cohort concerning age, sex, site of infection and 30-day mortality (16%). All patients were evaluated by infectious diseases specialists. Determination of flucloxacillin concentration was performed in a laboratory experienced in performing TDM measurements. Hence, high quality data could be guaranteed. Only in patients that died could the study protocol not be completed.

Limitations of our study include its single centre character and the small sample size of only 50 patients. Furthermore, we were not able to obtain initial positive blood cultures of all patients, because they partially were drawn in other institutions and their transfer to our hospital was not possible. Our optimal target (100% $fT_{>MIC}$) may be considered too conservative in the light of recent guidelines advocating an unbound concentration of even 100% $fT_{>2-5xMIC}$ [30]. However, these recommendations lack validation in large prospective trials. Another limitation of our study concerns the underrepresentation of immunosuppressed patients and the exclusion of patients undergoing hemodialysis. Furthermore, we could not assess mortality rates appropriately due to our small sample size. The study period was only 7 days, while for patients with complicated MSSA-BSI treatment recommendations are 4-6 weeks. Especially in critically ill patients with potentially improving renal function over time, re-assessment of target attainment must be considered.

In conclusion, we were able to demonstrate a large inter-variability of unbound flucloxacillin concentration and fractions in patients with MSSA-BSI. Critically ill patients attain the pharmacological target (100% $fT_{>MIC}$) more often but are at risk of excessive flucloxacillin concentrations resulting in toxic damage of kidneys and CNS. Therefore, TDM of not only total, but unbound flucloxacillin concentrations in MSSA-BSI is desirable to assess optimal, patient-individual dosage, as they strongly depend on severity of illness. Measurement of patient-individual MIC might be discussed in critically ill patient, to avoid overdosing and toxic drug concentrations. Future large, prospective studies assessing the association of optimal target attainment with mortality are mandatory.

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Statement of authorship

The review as well as the work presented in the chapters «Validation of ultrafiltration techniques for TDM» were done completely by me, including all lab and analytical work as well as the writing of the manuscripts.

For the publications «A 2D HPLC-MS/MS method for several antibiotics in blood plasma, plasma water, and diverse tissue samples», «HILIC LC-MS/MS method for the quantification of cefepime, imipenem and meropenem» and «LC-MS/MS method for nine different antibiotics» all research, method development and validation was done by me. Routine clinical analysis of patient samples was done largely by the members of the clinical chemistry team of the University Hospital Basel. I wrote all manuscripts, with suggestions and support by Prof. K.M. Rentsch.

The chapter «Antibiotic tissue penetration in clinical practice» is an original study established by Prof. Rentsch and me. I planed the project aims and procedures with help from Prof. Rentsch. I collected all patient samples, including plasma and biopsies. I collected all patient data. I did most of the analytical measurements, with some help from members of the clinical chemistry team, especially for the measurement of vancomycin. The MIC presented in this work were mostly collected from routine patient data, additional measurements were realized by Dr Vladimira Hinic from the University Hospital Basel, Clinical Bacteriology and Mycology. All data analyzation, illustration and interpretation were done by me. The manuscript is planned to be handed in to the «International Journal of Antimicrobial Agents».

The work presented in the chapter «Drug stability testing of benzylpenicillin in elastomeric pumps» was a collaboration with Julian Meier from the Hospital Pharmacy of the University Hospital Basel. We were both involved equally in writing the research proposal handed in to GSASA, which financed this project. We both equally contributed to the planning of the project. I was responsible for the development and validation of the quantitative and qualitative methods and measurement and interpretation and illustration of all stability and patient data. Mr. Meier measured the flow rates of the elastomeric pump, manufactured all pumps and was responsible for storage and sample drawing in all conditions. Dr. Osthoff and Dr. Khanna acquired patient samples and set the initiative for this project. I wrote the main part of the manuscript sections “Methods and Materials” and “Results and Discussion”, while Mr. Meier was mainly involved in writing the section “Introduction “. As soon as completed, we plan to hand this paper in to the «Journal of Antimicrobial Chemotherapy».

The manuscript «Probability of target attainment with flucloxacillin in Staphylococcus aureus bloodstream infection: a prospective cohort study of unbound plasma and individual minimal

inhibitory concentrations» was a collaboration work. I was involved in the study design and responsible for the organization of sample transport, storage, measurement and processing whenever related to measurements of flucloxacillin, creatinine and albumin. I developed the method for the measurement of free and total flucloxacillin and wrote parts of the original version of the Introduction and Discussion section of the manuscript. For this thesis, I edited the whole manuscript.

Further Publications, Posters and Talks

Publications

1. Weibel J, Lin Y-S, Landolt H-P, Garbazza C, Kolodyazhniy V, Kistler J, Rehm S, Rentsch K, Borgwardt S, Cajochen C, Reichert CF (2020) Caffeine-dependent changes of sleep-wake regulation: Evidence for adaptation after repeated intake. Progress in neuro-psychopharmacology & biological psychiatry 99:109851
2. Reichert CF, Veitz S, Bühler M, Gruber G, Deuring G, Rehm SS, Rentsch K, Garbazza C, Meyer M, Slawik H, Lin Y-S, Weibel J (2020) Wide awake at bedtime? Effects of caffeine on sleep and circadian timing in male adolescents - A randomized crossover trial. Biochem. Pharmacol.:114283
3. Weibel J, Lin Y-S, Landolt H-P, Berthomier C, Brandewinder M, Kistler J, Rehm S, Rentsch KM, Meyer M, Borgwardt S, Cajochen C, Reichert CF (2020) Regular caffeine intake attenuates REM sleep promotion and sleep quality in healthy men. bioRxivorg.
4. Weibel J, Lin Y-S, Landolt H-P, Kistler J, Rehm S, Rentsch KM, Slawik H, Borgwardt S, Cajochen C, Reichert CF (2020) The impact of daily caffeine intake on nighttime sleep: signs of overnight withdrawal? bioRxivorg.

For these four publications, I developed a quantitative LC-MS/MS method for caffeine, theobromine, paraxanthine and theophylline together with a master student. I validated this method and used it to measure two sets of samples (each approximately 120 samples) belonging to different studies. I analyzed the results together with the first authors and helped classify them in the context of the study.

5. Ruperti-Repilado FJ, Haefliger S, Rehm S, Zweier M, Rentsch KM, Blum J, Jetter A, Heim M, Leuppi-Taegtmeyer A, Terracciano L, Bernsmeier C (2019) Danger of Herbal Tea: A Case of Acute Cholestatic Hepatitis Due to Artemisia annua Tea. Frontiers in medicine 6:221

In colaboration with Dr. Bernsmeier and Dr. Leuppi-Taegtmeyer I measured samples of a Artemisia annua tea using a qualitative approach to exclude a contamination of the herbal medicine with drugs and confirm its source as artemisia annua.

6. Mack I, Sharland M, Brussee JM, Rehm S, Rentsch KM, Bielicki J Insufficient stability of clavulanic acid in widely used child-appropriate formulations

To quantify the stability of amoxicillin and clavulanic acid in different formulations (syrup and dispersible tablets), I measured the stability of several formulations during a duration of up to seven days using the published method for «A 2D HPLC-MS/MS method for several antibiotics in

blood plasma, plasma water, and diverse tissue samples». I took part in designing the experiment and discussion of results for the publication.

Posters

1. «Validation of ultrafiltration techniques for TDM»
2. «A 2D HPLC-MS/MS method for several antibiotics» (Winner of the Best Poster Award)

Presented at the annual assembly of the Schweizerische Gesellschaft für Klinische Chemie SGKC 28.08.2019 - 30.08.2019 in Zurich

Talks

1. «Beta-lactam Antibiotika - TDM im Praxistest – Vorteile des Monitorings freier Antibiotika-Konzentrationen am Beispiel Flucloxacillin»

Presented at the 16. Annual assembly of the Deutsche Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V., 25.09.2019-28. 09.2019 in Magdeburg

2. «Drug stability testing of continuous infusion of β-lactam antibiotics in elastomeric pumps and determination of drug plasma levels in patients for improved outpatient parenteral antimicrobial therapy (OPAT)»

Presented at the annual assembly of the Schweizerischer Verein der Amts- und Spitalapotheker GSASA 22.08.2019 in Bern.

3. «Antibiotikaspiegelmessungen von A bis Z»

Presented 22.08.2019 at University Hospital Basel as part of a monthly internal staff training.

4. «TDM der Antibiotika am USB»

Presented 18.06.2020 at University Hospital Basel as part of an internal staff training for infectious disease specialists.