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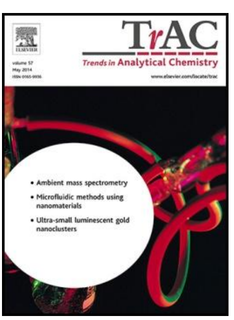

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1 **LC-MS/MS for Therapeutic Drug Monitoring of anti-infective drugs**

2

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29 Keywords: Liquid chromatography-tandem mass spectrometry (LC-MS/MS),

30 Therapeutic Drug Monitoring (TDM), pharmacokinetics/pharmacodynamics (PK/PD),

31 anti-infectives, matrix effects, antimicrobial stewardship (AMS), free drug

32 concentration, laboratory proficiency testing, dried blood spot analysis.

33

34 Abbreviations:

35 AMS: Antimicrobial stewardship

36 AUC: Area under the curve

37 DBS: Dried blood spot

38 C_{max}: Maximum concentration

39 C_{min}: Minimum concentration

40 CSF: Cerebrospinal fluid

41 ELF: Epithelial lining fluid

42 HIV: Human immunodeficiency virus

43 HPLC: High performance liquid chromatography

44 LC-MS/MS: high performance liquid chromatography tandem mass spectrometry

45 LLE: Liquid-liquid extraction

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46 LLOQ: Lower limit of quantification

47 MIC: Minimal inhibitory concentration

48 PK/PD: Pharmacokinetics/ Pharmacodynamics

49 PT: Proficiency testing

50 QC: Quality control

51 SPE: Solid-phase extraction

52 TDM: Therapeutic drug monitoring

53

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54 Highlights:

55 • TDM of anti-infective drugs plays an important role in treatment optimisation
56 of infectious diseases. TDM is mostly performed in blood, serum or plasma,
57 but other matrices are being explored.
58 • LC-MS/MS is a fast and accurate technique for quantification of anti-infective
59 drugs. Assays should be designed to enable a rapid turnaround time, enabling
60 the antimicrobial stewardship team to adopt and optimize treatment,
61 preferably within the first 24 hours.
62 • Under specific circumstances, free drug concentrations should be measured
63 instead of total drug concentrations.
64 • Assays and sampling should be fitted to the intended application, i.e. in- or
65 outpatients.
66 • Besides intralaboratory method validation, interlaboratory quality control is an
67 essential component of quality assurance.

68 **Abstract**

69 Therapeutic drug monitoring (TDM) is a tool used to integrate pharmacokinetic
70 and pharmacodynamic knowledge to optimise and personalize drug therapy. TDM is
71 of specific interest for anti-infectives: to assure adequate drug exposure and reduce
72 adverse events, to increase patient compliance and to prevent antimicrobial
73 resistance. For TDM, drug blood concentrations are determined to bring and keep
74 the concentration within the targeted therapeutic range. Currently, LC-MS/MS is the
75 primary analytical technique for fast and accurate quantification of anti-infective drug
76 concentrations. In addition to blood, several alternative matrices (cerebrospinal fluid,
77 inflammatory fluids, specific cells and tissue) and alternative sampling strategies
78 (dried blood spot and saliva) are currently being explored and introduced to support

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79 TDM. Here, we review the current challenges in the bioanalysis of anti-infective

80 drugs and give insight in the pre- and postanalytical issues surrounding TDM.

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83 ## 1. Introduction

84 Traditionally, therapeutic drug monitoring (TDM) was restricted to anti-
85 epileptic drugs and aminoglycosides, but also now covers - amongst others -
86 immunosuppressant drugs, drugs acting on the cardiovascular system, anti-HIV
87 drugs and antifungal drugs. For some classes of drugs, TDM has not only proven to
88 be beneficial for patient outcome, but also to be cost-effective [1,2].

89 With increasing pathogen resistance to anti-infective drugs, there is a clear
90 need for new agents. However, the development of new anti-infectives is time
91 consuming and expensive. Therefore, treatment optimization of the current anti-
92 infectives should be a focus of contemporary treatment. Due to its urgency,
93 development of antimicrobial resistance has a high priority for many organizations
94 and even entered the political agendas. Treatment optimization can be realized by
95 selecting the appropriate antimicrobial drug, assuring adequate drug exposure in
96 relation to the susceptibility of the micro-organism and reducing adverse events in
97 order to increase patient's compliance with treatment.

98 For many years, immunoassays and traditional high performance liquid
99 chromatography (HPLC) methods were the major techniques used to determine
100 concentrations of anti-infective drugs in human specimens. However, immunoassay
101 techniques are only available for a limited number of drugs and cross-reactivity, for
102 instance with drugs and their metabolites, is a problem. HPLC-UV often requires
103 extensive sample preparation and is therefore labour intensive. In addition, long
104 runtimes are often required in order to obtain a selective analysis method. In
105 addition, both immunoassays and HPLC-UV methods often lack sensitivity.
106 Nowadays, analytical challenges like these have been overcome with the
107 introduction of HPLC coupled with tandem mass spectrometry (LC-MS/MS). With the

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108 use of LC-MS/MS, sensitivity and selectivity has significantly improved, allowing
109 simple and fast sample preparations and short runtimes. This review will focus on
110 the bioanalytical hurdles related to the measurement of anti-infective drugs, but also
111 will give insight in pre- and post-analytical issues in order to help clinical chemists,
112 clinical pharmacologists and analytical technicians to raise their standards.
113

114 2. TDM

115 For over 30 years, TDM has been used as a tool to integrate pharmacokinetic
116 and pharmacodynamic knowledge to optimise drug therapy at the individual patient
117 level [3]. Pharmacokinetics describe the behaviour of a drug in the patient's body,
118 including absorption, distribution, metabolism and excretion, whereas
119 pharmacodynamics describe the biochemical or pharmacological effect of a drug on
120 the patient's body or micro-organism within the body. Together, both parameters
121 determine the pharmacological profile of the drug.

122 TDM uses drug blood concentrations to personalise drug therapy in order to
123 bring and keep the concentration within the targeted therapeutic range [4,5]. Below
124 this range the drug concentration is subtherapeutic or ineffective, whereas high
125 concentrations may result in adverse events or toxicity.

126 TDM is used when it is impossible to measure the pharmacodynamic effect of
127 the drug faster or in a more direct way, or it is used to optimise dosing in patients
128 with severely altered pharmacokinetic parameters (e.g. critically ill patients in ICU
129 [1,4]). For anti-infectives, it is both difficult and time-consuming to observe whether
130 the infection is being treated adequately. If the infection is not treated adequately, it
131 may be too late to turn the tide of illness, resulting in treatment failure including
132 patient morbidity or mortality or the emergence of antimicrobial resistance.

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133 Before TDM can be performed, several prerequisites have to be fulfilled. First,
134 a concentration effect relationship or therapeutic range should be established [4].
135 Secondly, large interindividual (e.g. sex, age or genetic variations) or intraindividual
136 variability (e.g. drug-drug interactions, decreased renal function or liver failure) in
137 pharmacokinetics should be observed, resulting in a large variation in blood
138 concentrations [4]. The final obvious prerequisite is that a sensitive and specific
139 assay must be available to determine the drug in blood or other biological matrices
140 [4,5].
141

142 2.1 Pharmacokinetic/pharmacodynamic relationships

143 For anti-infectives, the minimum inhibitory concentration (MIC), a measure of
144 potency of the drug for the micro-organism, is central to pharmacodynamics [6]. The
145 MIC is de lowest concentration at which an antibiotic inhibits visible growth of the
146 micro-organism after 18 to 24 hours incubation [7]. Unlike antibiotics, there is no
147 simple standard pharmacodynamic parameter, such as the MIC, that tests antiviral
148 susceptibility [7]. Although not applied in clinical practice, the half maximum inhibitory
149 concentration (IC_{50}) could be used to establish efficacy in an appropriate *in vitro* or
150 animal model [7].

151 The efficacy of anti-infective drugs not only is dependent on the pathogen's
152 MIC, but also on the exposure of the drug in the patient. This exposure is commonly
153 described by the area under the concentration-time curve (AUC) [6]. For many
154 drugs, the AUC/MIC ratio is the most relevant pharmacokinetic/pharmacodynamic
155 (PK/PD) index (Figure 1) [6].

156 In addition to the AUC/MIC ratio, other PK/PD indices also may be relevant.
157 An overview of the effective PK/PD indices of many antibiotics was previously

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158 provided by Roberts et al. [1]. For instance, beta-lactam antibiotics, such as
159 penicillins and carbapenems, display time-dependent pharmacodynamics, meaning
160 that the time of the unbound (or free) drug concentration exceeds the MIC ($fT_{>MIC}$) is
161 the most relevant PK/PD index [8]. For these drugs, both frequency of dosing and
162 duration of infusion are important [6]. Constant drug concentrations rather than high
163 peak concentrations result in more effective treatment [9]. Moreover, for these drugs
164 higher concentrations do not result in greater effectiveness. For these reasons,
165 continuous administration, preceded by a loading dose to quickly attain steady state,
166 has been suggested as an potentially improved strategy to conventional intermittent
167 dosing [9].

168 The peak level or maximum concentration of a drug (C_{max}) also may be
169 important. For instance aminoglycosides, exert their effectiveness and prevent from
170 drug resistance by the C_{max}/MIC [1].

171 Depending on the effective PK/PD index and the pharmacokinetics of the drug
172 one or more sampling times are usually chosen for TDM.

173

174 2.2 Multidisciplinary team

175 Although TDM is routinely performed for several anti-infective agents, optimal
176 treatment of the patient also depends on effective communication and cooperation
177 between many healthcare professionals (Figure 2). In general, drug treatment of
178 infectious diseases is selected based on clinically suspected pathogens. Adjustment
179 of the treatment is required after antimicrobial susceptibility testing results become
180 available. Since resistance to anti-infective drugs is a problem of increasing
181 magnitude, narrowing the anti-infective treatment is recommended based on the
182 susceptibility of the pathogen. Where antimicrobial resistance is observed, therapy

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183 should be changed to a more effective regimen. Subsequently TDM can be
184 performed, if a sensitive and accurate analytical method is available.

185 Antimicrobial stewardship (AMS) programmes have been developed to
186 optimise clinical outcomes and minimize unintended negative consequences of
187 antimicrobial use. An infectious disease physician and a clinical pharmacist with
188 infectious disease training are the core members of the AMS team [10,11]. Among
189 other factors, AMS is involved in appropriate treatment initiation and modification
190 where appropriate. Furthermore, dose optimization is a part of AMS, in which TDM
191 plays an important role for an increasing number of anti-infectives [10,11]. Therefore,
192 good collaboration between the infectious disease physician and clinical pharmacist
193 is necessary for the correct diagnosis and treatment of the infection, and the correct
194 interpretation and implementation of the TDM results. Additionally, a clinical
195 microbiologist can provide surveillance data on the susceptibility of the pathogen and
196 potential emergence of antimicrobial resistance. For implementation of
197 recommendations, computer support is necessary and an information system
198 specialist also may play an important role in AMS. Thus, to optimise clinical outcome
199 for the patient, good cooperation between these professionals plays a crucial role in
200 AMS and is cost-effective in many cases [10,12].

201

202 **3 LC-MS/MS in TDM**

203 LC-MS/MS has nowadays established itself as the primary analytical
204 technique to support TDM [13]. The commonly used matrices for TDM are blood,
205 plasma, and serum. More recently, dried blood spots (DBS) and saliva have been
206 introduced for TDM. Matrices like cerebrospinal fluid (CSF), inflammatory fluids,
207 specific cells and tissue are not routinely used for TDM, but may be relevant in

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208 specific cases [8]. However, each matrix has its analytical advantages and
209 disadvantages and the clinical interpretation of the results strongly depends on this
210 matrix. A number of guidelines on bioanalytical and clinical method validation have
211 been published in order to improve and ensure the quality of analytical method
212 validation and the generated analytical results. Among these are the Food and Drug
213 Administration (FDA) with the 'bioanalytical method validation', European Medicines
214 Agency Committee (EMEA) with the 'guideline on bioanalytical validation', and the
215 Clinical and Laboratory Standards Institute (CLSI) with the 'C62-A, Liquid
216 Chromatography-Mass Spectrometry Methods; Approved Guideline'[14–17].
217 LC-MS/MS has replaced HPLC-UV in many clinical laboratories in high
218 income countries. Unfortunately, the required broad repertoire of antimicrobial drug
219 assays necessary for an anti-infective TDM program will reduce the number of tests
220 per LC-MS/MS instrument annually, resulting in a relatively high price per test.
221 Although less attractive from a laboratory perspective, costs resulting from
222 inadequate antimicrobial treatment are much higher. If cheap, first-line anti-infectives
223 fail and have to be switched to salvage therapy with second-line anti-infective drugs,
224 costs will rise substantially. Before a hospital makes investments in an LC-MS/MS to
225 service a TDM program for antimicrobial drugs, one should make an business case.
226 In general 20.000-50.000 tests annually are considered to be an acceptable
227 justification of the investment [18]. For small hospitals, combining an LC-MS/MS for
228 other TDM programs as well (e.g. antidepressants, antipsychotics or
229 immunosuppressants), could result in cost-effective operation of an LC-MS/MS.
230 Another alternative could be sending a sample to a nearby reference center, if
231 turnaround time is acceptable. For low income countries, HPLC-UV still is an
232 alternative as long as sensitivity is not an issue. Hopefully, increased use of LC-

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233 MS/MS in clinical laboratories will result in lower investments costs enabling broader
234 implementation of LC-MS/MS.
235
236 3.1 Sample preparation
237 Because of the sensitivity and selectivity of the LC-MS/MS, extensive sample
238 extraction techniques like solid-phase extraction (SPE) and liquid-liquid extraction
239 (LLE) are often unnecessary. Therefore, fast and simple extraction techniques, like
240 protein precipitation or sample dilution, are feasible. However, due to the limited
241 sample preparation, endogenous compounds including lipids, phospholipids, and
242 fatty acids are not sufficiently removed from the sample with protein precipitation.
243 These compounds can interfere with the ionisation process resulting in ionisation
244 suppression. These so-called matrix effects are observed frequently and should be
245 solved for a reliable assay. Other types of matrix effects can originate from
246 substance interaction with the matrix. For example, the drug can form chelate
247 complexes with ferric ions, bind with heme groups, or can bind with the sampling
248 matrix [19–23]. Isotopically labelled internal standards may correct for matrix effects
249 better than structural analogues, but are unfortunately more expensive.
250 Ionisation suppression during the LC gradient can be visualized by continous
251 infusion of a high concentration of stock solution via a T-piece connection to the
252 mobile phase flow. Injection of a blank processed sample followed by the LC
253 gradient shows lowered substance response at periods of ionisation suppression in
254 a normally stable, but elevated baseline. By comparing the substance response of a
255 spiked neat sample with a spiked processed blank sample, the relative ionisation
256 suppression can be calculated.

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257 A structural analogue as internal standard is preferred to elute at the same
258 retention time and to have comparable ionisation characteristics. Since this is often
259 not possible, ionisation suppression should also be evaluated for the internal
260 standard. When ionisation suppression is present at the retention time of the
261 substance, the gradient should first be optimized in order to chromatographically
262 separate the ionisation suppression from the substance retention time. Dilution of the
263 processed sample or the use of another ionisation method, like Atmospheric
264 Pressure Chemical Ionisation (APCI), may also be used to avoid ionisation
265 suppression. Ultimately, an extensive sample preparation like SPE or LLE could be
266 performed, which will eliminate most of the ionisation suppression effects.

267 In some patient groups, especially new-borns, it is difficult to collect a
268 sufficiently large blood volume for HPLC-UV analysis. Due to its high selectivity and
269 sensitivity, sample volumes of 10 µL of plasma or serum are sufficient for LC-MS/MS
270 analysis [24]. Multiple analyses can be performed with LC-MS/MS using a single
271 blood sample or even a sample which was taken for other routine laboratory
272 measurements.

273 For analytical procedures used to analyse multiple compounds in a single
274 sample, it may be more efficient to apply protein precipitation instead of LLE or SPE.
275 The variation in physical and chemical properties of the different compounds to be
276 analysed complicates the development of a suitable LLE or SPE extraction method.
277 A LLE or SPE extraction method with acceptable recoveries for multiple compounds
278 will per definition be far less selective than an extraction method for a single
279 compound. If the use of protein precipitation allows the quantification of the
280 compound at the desired concentrations without ionisation suppression, it is the first
281 choice of sample preparation for LC-MS/MS.

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282 Although protein precipitation easily allows the simultaneous analysis of
283 multiple compounds in one LC-MS/MS method, differences in chemical and physical
284 properties might still complicate chromatographic separation. Alternatively, another
285 analytical column (with the use of a column switch) and/or mobile phase (with the
286 use of a quaternary pump) can be selected and reinjection of the samples can be
287 performed automatically [25].
288
289 3.2. LC-MS/MS turnaround time
290 Use of the LC-MS/MS analysis technique has significantly improved the
291 turnaround times for TDM samples. HPLC-UV and HPLC-DAD often require
292 extensive sample preparation to clean up and/or to concentrate the sample. In
293 addition, the chromatographic runtimes of these techniques often exceed ten
294 minutes. Runtimes of approximately five minutes are often feasible and use of an
295 ultra performance liquid chromatography (UPLC) method can even reduce runtimes
296 to less than two minutes.
297 In order to ensure short turnaround times, it also is useful to minimize
298 overhead injections. Bioanalytical method validation guidelines state that a sufficient
299 number of standards should be used to adequately define the relationship between
300 concentrations and response [14,15]. According to the guidelines for bioanalytical
301 studies a calibration curve of six to eight standards and quality control (QC) samples
302 should be incorporated in each analytical run. However for linear regression, multiple
303 concentration levels are unnecessary for reliable and accurate calibration. Instead,
304 two calibration concentrations (at the lower limit of quantification and at the higher
305 limit of quantification) are sufficient and proved to provide equal quality in analysis
306 results with QC samples at concentrations throughout the linear range [26]. A two-

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307 point calibration curve could be impaired when the curve becomes non-linear,
308 possibly due to changing ionization characteristics or overdue maintenance. An
309 isotopically labelled internal standard can compensate for changing ionization
310 characteristics. In addition, with the use of QC samples throughout the linear range,
311 linearity issues would result in unacceptable biases for the QC samples and run
312 rejection. Overhead samples put great pressure on the sample turnaround time,
313 especially when a run could consist of approximately 16 overhead samples and only
314 one patient sample. Minimizing overhead samples can be realized by validating a
315 two-point calibration curve in addition to an eight-point calibration curve, resulting in
316 a large reduction of injections. Subsequently, for the analysis of just one patient
317 sample, a QC sample before and after the patient sample may be sufficient.
318 Reduction in the turnaround time can make TDM more efficient.
319

320 **4. Free drug concentration**

321 Regularly, blood concentrations for TDM are determined as total drug
322 concentrations, i.e. the sum of the unbound and plasma protein bound fraction of the
323 drug. However, only the unbound, free drug can diffuse through biological
324 membranes to the site of action and exert its pharmacological and/or toxicological
325 effects [27,28]. Therefore for highly protein bound drugs, a small change in the
326 extent of protein binding may result in a major change in free fraction of highly
327 protein bound drugs [28,29].

328 In clinical practice, unbound drug concentrations of highly protein bound drugs
329 may be relevant for specific conditions, for instance in critically ill patients suffering
330 from hypoalbuminemia. This results in a higher free fraction of that particular drug
331 with subsequently several effects (Figure 3). Initially the unbound drug concentration

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332 increases. Since only the unbound drug can be removed from the blood, the amount
333 of drug cleared from the blood increases. Furthermore, the distribution of the
334 unbound drug from the blood to peripheral tissues is increased. As a result, the
335 unbound drug concentration decreases to the original value, while the total drug
336 concentration is decreased. Therefore, total drug concentrations may not be
337 representative for the effective PK/PD index and the unbound drug concentration
338 should be measured instead of total drug concentration, in particular for highly bound
339 drugs [8,28,30].
340
341 4.1 Methods of separation
342 There are several methods to separate the sample into unbound and bound
343 portions. The most commonly used methods are equilibrium dialysis,
344 ultracentrifugation, and ultrafiltration.
345 Due to its robustness, equilibrium dialysis is the reference method for
346 determining unbound drug concentrations. However, this method is less suitable in
347 clinical practice because of the long time to reach equilibrium. Another method to
348 separate bound and unbound drug concentration is ultracentrifugation. An important
349 advantage of ultracentrifugation, compared with equilibrium dialysis and
350 ultrafiltration, is the elimination of the possible interaction of the compound to the
351 filter membrane, since no filter membrane is used in ultracentrifugation. However,
352 the equipment used for ultracentrifugation is more expensive than the equipment
353 used for equilibrium dialysis and ultrafiltration [31]. Consequently, one of the most
354 commonly used methods in clinical practice is ultrafiltration, because of its simple
355 and rapid performance. Furthermore, with ultrafiltration all the proteins are filtered
356 out and further sample pre-treatment may not be necessary for LC-MS/MS analysis.

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357 With ultrafiltration, blood samples are centrifuged in systems that contain a
358 membrane with a certain molecular weight cut-off. The duration of centrifugation
359 differs for ultrafiltration, but is significantly shorter than equilibrium dialysis, which can
360 be more than 24 hours. Subsequently, the free drug concentration is measured in
361 the ultrafiltrate. For several anti-infective drugs, free drug concentrations are
362 determined using ultrafiltration. However, during method development, the possible
363 interaction of the compound to the filter membrane should be evaluated as well as
364 the influence of temperature, centrifugation time and centrifugal forces on protein
365 binding of the drug [27,29,31–33].

366

367 **5. Site of infection (alternative matrices)**

368 For TDM blood samples are predominantly used, while the site of infection is
369 located elsewhere. If there are no significant barriers, influx or efflux mechanisms at
370 the site of infection, it is expected that equilibrium is rapidly reached between the
371 drug concentration in tissue fluid and blood [34]. However, it is more accurately to
372 measure the drug concentration at the site of infection.

373

374 5.1 CSF

375 For central nervous system infections, the penetration of drugs from blood to
376 the site of infection may be variable. Due to inflammation associated with infection,
377 the blood brain barrier may initially be permeable for drugs, with the barrier then
378 being restored when the infection subsides. This results in reduced drug
379 concentrations in the central nervous system before the infection has been
380 completely resolved [34]. Therefore, it may be necessary to determine the
381 concentration of the drug in the CSF. The LC-MS/MS analysis of CSF is comparable

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382 to the analysis of ultrafiltrate. CSF contains very little proteins and is therefore
383 relatively clean. For the proteins that are present, a protein precipitation procedure is
384 sufficient as sample preparation. Obtaining blank CSF for method validation is
385 manageable, provided that institutional guidelines allow the use of left-over
386 materials. The use of an isotopically labelled internal standard is highly
387 recommended when different matrices are used between patient samples and
388 standards and QCs. Although CSF normally contains very low amounts of protein,
389 central nervous system infections and intracranial bleeding may significantly
390 increase the protein content in the patient sample. This may result in haemolytic CSF
391 and matrix effects, which affects the analysis results. This variation in protein
392 concentration between patient samples and standards and QCs should be
393 incorporated in the analytical method validation.
394

395 5.2 Pulmonary epithelial lining fluids and alveolar macrophages

396 Anti-infectives are frequently used in pulmonary infections. For extracellular
397 and intracellular respiratory pathogens, drug concentrations have been measured in
398 respectively pulmonary epithelial lining fluid (ELF) and alveolar macrophages or
399 bronchoalveolar lavage (BAL) fluid [8,35,36]. These studies are helpful as they show
400 whether a drug may be suitable for the treatment of pulmonary infections. In clinical
401 practice, ELF and alveolar macrophages concentrations, however, are rarely
402 measured due to the poor availability of assays and/or the invasive nature of sample
403 collection. Sometimes it is important to know whether the drug is present at sufficient
404 concentrations at the site of infection. In the absence of a validated assay, one may
405 use a standard addition method to obtain a semi-quantitative result.
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407 5.3 Intracellular

408 It may be of interest to measure intracellular concentrations for some drugs.
409 For example, for antiretroviral drugs since HIV replicates within the cells of the
410 immune system. Moreover, some of these drugs are administrated as prodrugs and
411 are converted intracellularly into an active form. Subsequently, several studies have
412 shown that the efficacy and toxicity of some antiretroviral drugs depend on
413 intracellular concentrations [37]. In clinical practice, intracellular concentrations are
414 not routinely measured for antiretroviral drugs, because for most antiretroviral drugs
415 like Non-Nucleoside Reverse Transcriptase Inhibitors and Protease Inhibitors a clear
416 relation exists between the plasma and intracellular concentration [37]. However, this
417 does not apply for Nucleoside Reverse Transcriptase Inhibitors and therefore
418 intracellular drug concentrations should be monitored for these. Together with the
419 isolation and counting of peripheral blood mononuclear cells, the analysis of
420 intracellular concentrations is still a major technical challenge. Intracellular drug
421 molecules are bound to membranes or proteins and therefore it will be difficult to
422 approximate the actual intracellular free drug concentration. Again, obtaining blank
423 matrix consisting of peripheral blood mononuclear cells is difficult and laborious.
424 Moreover, it could require additional sample preparation and concentration to
425 accurately quantify the very low intracellular concentrations with LC-MS/MS [37].
426

427 5.4 Tissue

428 In some situations, it may be helpful to quantify the drug concentration in
429 infected tissue material which has been obtained during operation. In addition to the
430 blood concentration, drug concentrations in tissue-homogenate may provide
431 information on the exposure of the tissue to the drug. The sample processing of the

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432 tissue material includes weighing and homogenization of the sample. After weighing,
433 the extraction solvent containing the internal standard can be added to the sample
434 and this will be centrifuged. The obtained supernatant can be analysed by LC-
435 MS/MS. This method is still in its infancy and exposure-response relations are not
436 described for the drug concentration in tissue-homogenate [38]. In addition, one
437 should realise, that drugs may be distributed unequally throughout the tissue, for
438 example during ischemia or when the drug is actively taken up by specific cells. In
439 summary, tissue homogenates are unlikely to be useful for drugs without equal
440 interstitial fluid and intracellular distribution and is likely to under represent
441 concentrations of drugs that do not penetrate intracellularly (e.g. beta-lactams).

442 A less invasive and more accurate sampling technique for measuring drug
443 tissue concentrations is microdialysis, which is increasingly being used in clinical
444 pharmacokinetic studies but is not commonly used in clinical practice. In contrast to
445 tissue biopsy, with microdialysis unbound drug tissue concentrations can be
446 measured directly and continuously in the interstitial space fluid in various tissues.
447 Therefore, microdialysis may provide extra information for patients with complicated
448 infections and where blood concentrations appear to be sufficient, but anti-infective
449 therapy is failing [39].

450

451 **6. Proficiency testing programme**

452 A variety of analytical methods has been published for the quantification of
453 anti-infective drugs in human serum or plasma. The reliability of these analytical
454 methods is essential to provide information on the drug concentration to the
455 antimicrobial stewardship that hopefully translates in the best outcome for our
456 patients.

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457 Intralaboratory (internal) method validation and intralaboratory QC
458 procedures, such as validation of equipment and qualification of technicians, should
459 ensure that these methods have sufficient accuracy, precision and specificity [14,15].
460 Participation in an interlaboratory (external) QC or proficiency testing (PT)
461 programme is an essential component of quality assurance and also provides
462 evidence of laboratory competence for clinicians, researchers, accrediting bodies
463 and regulatory agencies[40].
464 A PT programme is essential to verify whether the analytical method used for
465 TDM complies with the quality required for patient care. Many PT programmes exist
466 in the field of HIV, antifungal and antituberculosis drugs and have indeed led to
467 analytical improvement [40–42]. For instance, in a PT programme for the
468 measurement of antifungal drug concentrations, the results showed that one out of
469 five measurements was inaccurate. The performing laboratory was the main
470 determining factor for these inaccuracies, which probably means that intralaboratory
471 method validation was inaccurate [41]. In addition, the results of a PT programme for
472 antiretroviral drugs showed that the measurement of low antiretroviral concentrations
473 also was problematic and led to inappropriate dosing recommendations [42]. These
474 examples illustrate and emphasize the importance of PT programmes for analytical
475 methods used for TDM in clinical practice.
476

477 **7. Outpatient monitoring**

478 Routinely, blood samples are used for TDM which are often collected by vena
479 puncture [43,44]. However, this sampling strategy has several disadvantages. First,
480 venous sampling is difficult in some populations, such as neonates and patients
481 suffering from venous damage [43]. Second, there may be logistical setbacks. For

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482 venous sampling the patient needs to travel to the hospital or a designated
483 laboratory. This may not always be possible, for instance in resource-limited and
484 remote areas [43]. Another problem, especially in (sub)tropical areas, is sample
485 stability. Many drugs are not stable in serum or plasma at room temperature and
486 have to be stored and transported at -20 ˚C or lower [44]. To resolve these stability
487 problems, alternative sampling strategies have been developed, such as DBS, dried
488 plasma spots and microsampling [45–47].

489 DBS sampling is increasingly applied for optimizing drug dosages for many
490 drugs [43,44,48]. DBS is popular for its advantages like minimal invasive sampling,
491 sample stability and small blood volume required for analysis. In general, a DBS
492 sample consists of a peripheral blood sample obtained by a finger prick. With clear
493 instructions and after training, patients will be able to perform the procedure
494 themselves at home [44]. DBS methods have been published for several
495 antibacterial, antifungal and antiretroviral drugs [44,49]. Reference values for TDM
496 are traditionally based on serum or plasma drug concentrations and not on whole
497 blood concentrations. Therefore, clinical validation is required to translate capillary
498 blood-to-serum or -plasma concentration [44,48,50]. Another possible important
499 factor may be the interaction of the drug with the blood matrix or the DBS card
500 matrix. Rifampicin has demonstrated to interact with endogenous blood components,
501 like ferric ions from the red blood cells causing complex formation [22]. This causes
502 low recoveries from DBS extracts which can be improved by the addition of chelating
503 agents, such as EDTA and deferoxamine, to the extraction procedure. Also direct
504 binding of the drug by hydrogen bonding with the DBS card matrix may have an
505 effect on recovery [19,20]. Recovery also is influenced by haematocrit value,
506 substance concentration and drying time of the DBS card [20].This interaction is

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507 inherent to the current cellulose based card matrices [21]. An advantage of the dried
508 plasma spot technique over DBS is that it is not influenced by haematocrit value.
509 Quantification of anti-infective drugs using the dried plasma spot technique has been
510 described for fosfomycin, daptomycin, linezolid, triazole antifungal drugs and
511 antiretroviral drugs [45,47]. Although the use of DBS and dried plasma spot
512 techniques is not yet widely spread, both are a promising alternative for venous
513 blood sampling and in some cases (i.e. low resource and remote areas) the only
514 viable options.

515 Another patient friendly method of sampling is the use of saliva [43,51].
516 Compared to blood sampling, saliva is easy to collect and non-invasively with a
517 negligible chance of infections [52]. Furthermore, it is cheap and causes less stress
518 and discomfort to the patients [52]. As saliva is a very low protein matrix (~0.3%), the
519 measured concentration represents the unbound concentration of the drug. This may
520 require a very sensitive LC-MS/MS analysis method or an extensive sample
521 preparation procedure like SPE or LLE to concentrate the sample for drugs with high
522 protein binding. As there are many other determinants of the salivary drug
523 concentration, such as salivary flow rate, stability of the drug and its metabolites,
524 time of sample collection and ingestion of food or beverages [52], target
525 concentrations in saliva should be established on a drug-to-drug basis [43]. Saliva
526 methods using LC-MS/MS have been published for a few anti-infective drugs
527 (doxycycline, fluconazole, linezolid, lopinavir and oseltamivir) [52–54].

528

529 8. Conclusion

530 In conclusion, TDM plays an important role in the optimisation of treatment
531 with anti-infective drugs. To perform TDM adequately, it is essential to design assays

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532 with a rapid turnaround time, enabling the antimicrobial stewardship to quickly adjust
533 and optimise treatment if necessary. LC-MS/MS is a fast and accurate technique for
534 quantification of anti-infective drugs. If an analytical method is developed and
535 validated, interlaboratory quality control is an important component of quality
536 assurance.

537 In clinical practice blood is the most commonly used matrix for TDM since it
538 serves as a good surrogate for the site of infection. In general, it is easily obtained, in
539 contrast to other matrices. However, in complex infectious cases other matrices
540 could be used to optimise anti-infective treatment.
541

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715 **Figure legends**

716

717 Figure 1: The effective pharmacokinetic/ pharmacodynamic (PK/PD) indices of anti-
718 infective drugs. AUC, area under the concentration-time curve; C_{min}, minimum
719 concentration; C_{max}, maximum concentration; MIC, minimum inhibitory concentration;
720 $T_{>MIC}$, time that drug concentration is above the MIC.

721

722 Figure 2: The multidisciplinary team involved in the infectious disease treatment.

723

724 Figure 3: If the protein binding of a drug is decreased, the total drug concentration
725 (C_{tot}) is decreased due to increased distribution and an increased amount cleared,
726 while the unbound concentration of drug (C_u) remains the same. C_{tot}, total drug
727 concentration; C_u, unbound concentration of drug; F_u, fraction unbound; V_u, Volume
728 of distribution of unbound drug.

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