Recent advances in therapeutic drug monitoring of immunosuppressive drugs

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A R T I C L E   I N F O

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A B S T R A C T

The transplantation of a solid organ is always followed by a lifelong immunosuppressive therapy to guarantee the survival of the organ in the recipient. Immunosuppressive drugs have to be applied in order to preserve the graft and at last the patient’s life. These drugs are strongly recommended for therapeutic drug monitoring (TDM) in order to adjust the adequate dose for each patient to avoid rejection or adverse effects of the therapy. This finely tuned therapy would not be possible without the proper analytical tools and techniques. Either chromatography methods or immunoassays based on e.g. fluorescence or colorimetric detection principles are used in practice. All of them have to cope with the challenging, individually differing matrix whole blood. It is difficult to reach the relevant levels of quantification.

The focus of this review is on the explanation, comparison and future outlook of the current analytical techniques for TDM of immunosuppressants.

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1. Introduction

For a long time, organ failure was the immediate death sentence of the patient. Nowadays, organ transplantations of livers, kidneys and hearts save many lives each year. Unfortunately, there are not enough organs to save each patient’s life. The available organs need to be used as efficient as possible and the function has to be ensured continuously. It is essential to perform an immunosuppressive therapy to avoid rejection of the transplanted organ [1]. The therapeutic index of the used immunosuppressive drugs is extremely narrow. Consequently, a lifelong monitoring of blood levels needs to be done.

Abbreviations: CsA, cyclosporine A; DBS, dried blood spots; FKBP12, FK506 (tacrolimus) binding protein 12; ISD, immunosuppressive drug; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MPA, mycophenolic acid; mTOR, mammalian/mechanistic target of rapamycin; POCT, point-of-care testing; PSI, proliferation signal inhibitors; SPE, solid phase extraction; TBS, Tris buffered saline; TDM, therapeutic drug monitoring; UHPLC (UPLC), ultra high performance liquid chromatography.

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Generally, there are different approaches of monitoring laboratory parameters. The classic way is to draw a sample once or twice a day and analyze it at the central laboratory of the hospital. Several hours pass until the physician has the result in his or her hands. A faster decision needs to be taken, if the patient is in critical conditions, e.g. multimeribid or early after transplantation. As the most recent tool, Point-of-care testing (POCT) devices, can conduct semi-continuous measurements at the bedside of the patient, favored with regenerative biosensors [2]. One measurement is conducted at the bedside within minutes, making a faster on-site decision of physicians possible. A real-time measurement, as performed in online blood gas analysis [3], is not practicable for Therapeutic drug monitoring and not essentially needed.

Currently, immunosuppressive drugs are monitored at central laboratories by chromatography methods like UPLC-MS/MS or LC-MS/MS and immunoassays based on e.g. fluorescence or colorimetric detection principles. The measurements are mainly performed once or twice a day, right before the administration of a new dosage. In critical conditions of the patient, measurements in shorter time intervals are needed to be done to get clear information about pharmacokinetics of the applied drug. The sample matrix is whole blood in the majority of cases. The high and individually fluctuating content of proteins and products of metabolism poses a challenge for all analytical methods. It often has to be facilitated by a preparative precipitation step to perform reliable measurements.

Nevertheless, it is difficult to reach the relevant levels of quantification, especially in some cases when the assay needs to differentiate between the drug, active and inactive metabolites of it. Additionally, the required drug levels are being lowered more and more to avoid long term toxicity of the applied immunosuppressants. The focus of this review is on the multifaceted realization of therapeutic drug monitoring of immunosuppressive drugs. It points out how current analytical techniques, being used in practice, work and how they perform. Furthermore a glance on possible future developments is given, as they might overcome the obstacles of the currently used techniques.

2. Therapeutic drug monitoring (TDM)

Therapeutic drug monitoring (TDM) describes the measurement of drug concentrations in blood and is done if there is no clinical marker or parameter, which can easily be monitored. Therefore, a relationship between dose and blood concentration and between blood concentration and therapeutic effect of the drug has to exist [4,5]. Criteria for a drug to be suitable for TDM [4] are:

- Relationship between concentration and effect
- Narrow therapeutic index
- Inter-individual pharmacokinetic variability: poor relationship between dose and drug response
- Pharmacological response should be difficult to assess or distinguish from adverse effects

The goal is to individualize therapy of difficult-to-manage medication, assuming knowledge about pharmacokinetics and pharmacodynamics and at last to optimize clinical outcome. The list of drugs, which are recommended for therapeutic drug monitoring, is further growing. Important therapeutic groups are anticonvulsants, cardio active drugs, some antibiotics, psychotropics, immunosuppressants, cytotoxics and hormones [6].

The monitoring of immunosuppressive drugs (ISD) after organ transplantation is strongly recommended [7] for several reasons. The narrow therapeutic index of the drugs leads to severe adverse effects, e.g. nephotoxicity, if the drug is overdosed whilst underdosage can result in graft rejection. Another characteristic of immunosuppressive drugs is the high degree of inter-individual differences in bioavailability. Pharmacokinetics like differences in absorption, distribution, metabolism and elimination have to be considered, because they can be influenced by gender, age, genetical polymorphisms and renal or biliary insufficiency of the patient. Drug interactions and inflammation can influence pharmacokinetics additionally [8]. Immunosuppressant drug interactions require prompt action: Identification and intensified TDM with adequate dosing responses [9].

3. Immunosuppressants and their mechanism of action

In 1949 Hench et al. discovered the immunosuppressive effect of Cortisol [10], followed by cyclosporine A (CsA) in 1976 [11], sirolimus (rapamycin) in 1977 [12], tacrolimus in 1987 [13] and mycophenolic acid (MPA) in 1991 [14], to name the most important immunosuppressive drugs [15]. The currently most commonly used immunosuppressive drugs are listed in Table 1.

Tacrolimus and CsA are calcineurin inhibitors (CsIs). They bind to immunophilins and block the effect of calcineurin. This results in a reduced production of IL-2 and reduced proliferation of T lymphocytes, which are crucial for immune response. CsA binds to the immunophilin cyclophilin, whereas tacrolimus binds to the immunophilin FKBP12. The complex of tacrolimus and FKBP12 inhibits the calcium dependent protein phosphatase activity of calcineurin. Calcineurin, calmodulin complex, inhibiting both T lymphocyte signal transduction and IL-2 transcription by blocking TNFα gene transcription [16]. Calcineurin inhibitors induce vasocstriction in the kidney, which leads in combination with the overexpression of transforming growth factor to interstitial fibrosis [17]. To avoid these side effects, the calcineurin inhibitor concentration is lowered by combining them with MPA or sirolimus.

The group of antiproliferative drugs or proliferation signal inhibitors (PSIs) consists of sirolimus (rapamycin) and everolimus. They

<table>
<thead>
<tr>
<th>Immunosuppressive Drug</th>
<th>Mechanism of action</th>
<th>TDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine A</td>
<td>Kinase and phosphatase inhibitors (Calcineurin, CNK/38 kinase)</td>
<td>yes</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>Kinase and phosphatase inhibitors (Calcineurin, CNK/38 kinase)</td>
<td>yes</td>
</tr>
<tr>
<td>Sirolimus (Rapamycin)</td>
<td>Kinase and phosphatase inhibitors (Cycin kinase cascade)</td>
<td>yes</td>
</tr>
<tr>
<td>Everolimus</td>
<td>Kinase and phosphatase inhibitors (Cycin kinase cascade)</td>
<td>yes</td>
</tr>
<tr>
<td>Mycophenolic acid</td>
<td>Inhibition of purine synthesis (inosine-5'-monophosphate dehydrogenase)</td>
<td>(yes)</td>
</tr>
<tr>
<td>Mycophenolate mofetil</td>
<td>Inhibition of purine and pyrimidine synthesis (Thymidylylate synthase, several enzymes)</td>
<td>yes</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Regulation of gene expression (Glucocorticoid receptors)</td>
<td>no</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>Antibodies against human T lymphocytes</td>
<td>no</td>
</tr>
<tr>
<td>Basiliximab (antibody)</td>
<td>CD 25, IL2-R-α-chain</td>
<td>no</td>
</tr>
<tr>
<td>Anti-thymocyte globulin</td>
<td>6-mercaptopurine is metabolized to thioguanine nucleotids; cytocytotoxicity resulting in immunosuppression and inhibition of DNA synthesis (polymorphisms or activity of thiopurine methyltransferase are checked before administration)</td>
<td>yes</td>
</tr>
<tr>
<td>6-mercaptopurine/ Azathioprine (prodrug)</td>
<td>Inhibition of CD28 mediated costimulation of T cells (CD 80 and CD 86 on antigen presenting cells)</td>
<td>no</td>
</tr>
</tbody>
</table>
are both inhibitors of mammalian/mechanistic target of Rapamycin (mTOR), a cell cycle kinase. By forming a complex with the immunophilin FKBP12, which binds and inhibits mTOR, it blocks the cell cycle and cytokine driven proliferation and activation of T lymphocytes. Although sirolimus and everolimus have a very similar chemical structure, there is a significant difference in the plasma half-life: 60 h for sirolimus and 30 h for everolimus. The side effects of the PSIs are comparable to tacrolimus and CsA, but they show different frequencies of occurrence.

Mycophenolic acid is available in two formulations: As sodium salt and as the prodrug mycophenolate mofetil (MMF). CsA has a different target in the cell, than the above mentioned immunosuppressants. It reversibly inhibits inosine monophosphate dehydrogenase, which is involved in the guanine monophosphate synthesis in the de novo purine synthesis. Proliferation of B and T lymphocytes in particular is inhibited. Other cell types are able to recover purines via a separate salvage pathway.

Current immunosuppressive therapy consists of the combination of at least two immunosuppressants with different targets to obtain a reduced dose for each drug [18]. Thereby, toxic side effects can be reduced and rejection rates minimized. The most common regimes are a combination of CsA and MPA or tacrolimus and MPA [19–21], often combined with glucocorticoids. These reduced drug concentrations require ambitious analytics for the determination in whole blood (Table 2), which have been partially solved in recent years.

4. Sample drawing for TDM

Sampling matrices for therapeutic drug monitoring of immunosuppressive drugs can be blood (whole blood, plasma, serum), tissue (e.g. hepatic tissue), cells (e.g. lymphocytes) or saliva. Müller et al. describe for example the analysis of CsA in hair samples [27], whereas Mendonza et al. investigate salvia as matrix [28]. The blood is usually drawn by venous blood sampling. If only a small volume is needed, capillary blood can be used (fingerprick sampling), as it is done in blood glucose monitoring. In clinical practice, most of the through levels of immunosuppressive drugs in whole blood are determined once or twice a day [23,29]. The samples are usually analyzed in the central laboratory by LC-MS/MS [30–32] or immunoassays. Based on the results, the next dosage of the drug is adapted. Recent studies discovered that a calculation of the dosage based on the determination of the area under the curve (AUC) is more precise, as the important pharmacokinetic information is included (Fig. 1). For this reason, reliable and fast determination of whole blood levels is eligible.

Different pharmacokinetics of the drugs dictate the point in time, when the samples for TDM of the immunosuppressants should be obtained. The therapeutic drug monitoring of CsA correlates best with the AUC 0–4 h after drug administration. Within this period most variability of pharmacokinetics occurs. The Cₐ₀ (before the dose) and C₂ (2 h after the dose) are significant predictors of the AUC 0–4 h. C₂ has been clinically validated for kidney, liver and heart transplanted patients [33]. Obtaining CsA samples at C₀ showed no significant difference in patients’ outcome [34]. The tacrolimus assays lack of standardization, however sampling at C₀ is correlated with the AUC during the first months after transplantation [35,36]. Sirolimus and everolimus can be as well monitored with C₀ sampling intervals, but only few studies have proven this sampling approach. MPA efficacy is correlated with the AUC. Sampling points between C₀ and C₄ can easily predict the AUC [37]. The need of TDM for MPA is still under discussion.

Fig. 1. C₀ blood level (through level before the next dose) in comparison with the area under the curve: C₄ levels can be determined by classical LC-MS methods, whereas the monitoring of the area under the curve can be done with a POCT device, measuring the blood level in short time intervals (blue dots). Red arrows show the sampling for the LC-MS; immediately the next dose is applied.

5. Analytical methods

The beginning of therapeutic monitoring of immunosuppressive drugs in the late 1970s and early 1980s significantly improved graft survival and has been further improved until today. Nevertheless, there are advantages and disadvantages for each analytical approach, which results in a variety of different techniques for the TDM of immunosuppressive drugs. The most relevant and recent research in this field will be illuminated in the following.

5.1. Chromatography methods

As described before most clinical laboratories use LC-MS/MS methods as gold standard for therapeutic drug monitoring. The
Fig. 2. Distribution of immunosuppressive drugs in blood: Tacrolimus and CsA are mainly bound to blood cells, in plasma the protein binding is higher than 90%, resulting in a very low free fraction of the drugs.

The advantage of this technique is that it is well established. Using a MS/MS as detector allows the achievement of very low limits of detection and quantification, which is essential, as the dosages in modern immunosuppressive therapy are very low.

Additionally to the sensitivity, the specificity is very good. By the detection of the mass-to-charge ratio (m/z), the discrimination between the drug and its similar metabolites is feasible with minor problems compared to immunoassays. Multiplex testing is possible, which is an important advantage because in most of the regimes a combination of two or more different drugs is applied. The high time consumption and the costly instrumentation are the main disadvantages of this technique. Patient’s samples have to be brought to a central laboratory where several samples are collected before an extensive sample extraction of the whole blood is started. Direct injection of whole blood is not possible due to high protein content, leading to an increased column backpressure and poor separation. Depending on the matrix, ionization efficiency of the target compound and the internal standard can be enhanced or suppressed [39]. Many methods request a protein precipitation with ZnSO₄, followed by an extraction procedure. The most common methods are solid-phase-extraction (SPE) either offline or online [40,41] directly in front of the chromatography column and liquid-liquid extraction. Liquid-liquid extraction of the lipophilic drugs is mostly done by addition of different organic and non-organic solvents, including methanol [42], acetonitrile [43],acetone or mixtures of organic solvents. Advantages of liquid-liquid extraction are the simple treatment and the reduction of costs for SPE columns. By the use of SPE, ion suppression can be eliminated. It also allows extraction of larger volumes than liquid-liquid extraction. In the chromatography system, reverse phase columns are useful for separation as all of the drugs are lipophilic. C18 cartridges are common columns for chromatography. Appropriate internal standards are necessary for the quantification of the drugs. By the determination of the ratio of analyte to internal standard, the loss of analyte during sample preparation is corrected. Deuterated internal standards are available for all immunosuppressive drugs. Ascomycin is commonly used as internal standard in tacrolimus assays (Waters Mass Trac kit). Cyclosporine D is an alternative to deuterated drug in CsA assays. LC is mostly interfaced to a mass spectrometer by an electrospray ion source (ESI). Ions are generated in positive mode and mass transitions of the analyte and the internal standard are monitored [44].

There are also HPLC methods for the determination of immunosuppressive drugs using UV as detectors [45,46]. They are useful for CsA (220 nm), sirolimus or everolimus (277 nm) and MPA (254 nm). Tacrolimus is not detectable with an UV detector, as there is no chromophore in the molecule. Furthermore, absorbance of the amide bonds in CsA is not as intense or distinctive as of many other drugs characterized by aromatic rings.

Especially the extraction process causes a lack of automation of this technique and a high time consumption. In addition, qualified stuff is needed to proceed the different steps and evaluate the results later on. The instrumentation and technical support is very expensive and is only profitable for high sample throughput in large clinical laboratories [47].

Karapirli et al. describe an UPLC-MS/MS for simultaneous determination of CsA, tacrolimus, sirolimus, and everolimus in whole blood [40]. After the addition of precipitation reagent, including ZnSO₄ in water/methanol and internal standards (Ascomycin and CyD) to the EDTA-treated blood samples, online SPE was used for extraction of the drugs. Chromatography took place in a short phenyl hexyl RP column to reach short analysis times of 2.5 minutes. An atmospheric pressure ionization tandem mass spectrometer (API-MS/MS) with triple quadrupole was used for detection in the multiple reaction monitoring (MRM) mode. In this method, sample preparation is done by online SPE. The authors state that their procedure is very simple and requires only short time. Simultaneous measurements of four ISDs are feasible in a robust setting. Sensitivity and selectivity are comparable to other LC-MS/MS methods.

Tszyrsznic et al. developed an UPLC-MS/MS method for CsA, tacrolimus, sirolimus and everolimus in whole blood and for MPA in plasma [48]. Because of the high distribution in erythrocytes (40–60% for CsA and 95% for tacrolimus), these drugs were quantified in whole blood, whereas MPA is almost exclusively found in plasma. In contrast to the UPLC-MS/MS, developed by Karapirli et al., deuterated internal standards were used (d₃-MPA, etc.). For the treatment of plasma, the addition of ZnSO₄ was omitted and methanol was used as solvent instead of acetonitrile for the internal standard. Liquid-liquid extraction with acetonitrile was used in both described procedures. Both methods were validated and patient samples were analyzed with the new method. The results were compared to those obtained from immunoassays. In this study, immunoassays significantly overestimated the concentration of ISDs.

A high throughput method for LC-MS/MS measurements in whole blood has been developed by Koster et al. [47]. The described method uses a chromatographic gradient in combination with one single protein precipitation step as sample preparation. The sample preparation was conducted with the analysis time of 2.6 min, on a triple quadrupole. LC was done with a C₁₈ column at 60°C. Sirolimus and everolimus were measured separately from CsA and tacrolimus due to different sample preparations, different capillary temperatures (280°C and respectively 360°C) and a low number of scan masses for a better signal to noise ratio. The sample preparation was optimized: CsA and tacrolimus were precipitated with ZnSO₄ by adding
500 μL of methanol containing 100 μg/L ascomycin. Sirolimus and everolimus were precipitated without ZnSO₄ by adding 500 μL of methanol: acetonitrile (50:50 (V/V)) as protein precipitation agent containing 100 μg/L ascomycin. Afterwards all the samples were vortexed, frozen at −20°C for 10 minutes and centrifuged before the injection into the column. A comparative study showed that correlations of more than 0.85 R² values were found when comparing the measurements with immunoassays. Controls showed a coefficient of variation of less than 8.0%. Methods were in use for more than 20,000 patients’ samples, 70 patients’ samples were tested on one day. Stabilities of the immunosuppressants were tested at room temperature in whole blood (stable at least for 3 days), processed samples at 10°C in the autosampler (stable at least for 2 days) and during 3 freeze-thaw circles (no observed instability trend). Different spiked solutions and samples were tested: whole blood from individuals, pooled blood and blank water (no carryover or interfering peaks). The assay has passed the tests of the international Proficiency Testing Scheme (round robin testing scheme) several times. When the method was compared to immunoassays, CsA showed 17% lower signals and everolimus 30% lower signals. Koster et al. blame the lower selectivity of the immunoassays for these findings. Immunoassays detect as well some metabolites of the drugs, compared to the LC-MS/MS method. Interestingly, tacrolimus and sirolimus showed no difference between LC-MS/MS and immunoassays.

The developed LC-MS/MS assays seems to be very promising for clinical laboratories with high sample throughput. Koster et al. mention, that their method is cheaper than immunoassays, if more than 8000 samples a year are measured.

Klepaki et al. developed an UHPLC-MS/MS assay for the quantification of MPA and its major metabolites MPA-glucuronide and MPA-acetyl-glucuronide in human plasma and urine [49]. D3-MPA was used as internal standard for MPA and d3-MPAG for both metabolites. Commercial protein precipitation solution containing internal standards was used before sample injection. A C18 column was used for chromatography and ESI for ionization. The assay was validated for use in clinical TDM and showed robust and reliable results during the measurement of samples from several pharmacokinetic trials. The method is furthermore highly automatized using a liquid-handling robotic extraction.

Koster et al. describe a LC-MS/MS method for simultaneous measurement of immunosuppressive drugs by the use of dried blood spots (DBS) as matrix [50]. In their method, 50 μL blood is pipetted on nitrocellulose paper, dried overnight and an 8 mm disc is punched from the central part of the spot and put into an Eppendorf cup. The sample is extracted by a methanol-water mixture, containing deuterated internal standards for tacrolimus, sirolimus, everolimus and CsA. After vortex and sonication steps, protein precipitation in the extract takes place in a glass insert at −20°C. The supernatant is injected in the LC-MS system. The method was validated and effects of the matrix, especially the influence of hematocrit were investigated. Recovery was determined by punching blank paper, which was afterwards spiked with high and low concentrations of immunosuppressive drug. Hematocrit was adjusted to 0.35 L/L by the addition of red blood cells to serum. Recovery could be calculated. Hematocrit influences the viscosity of the blood and in this way the permeability through the paper. When hematocrit is high, the blood spots get smaller and therefore a fixed diameter punch would contain a higher blood volume. For the investigation of effects of the hematocrit, samples with different hematocrit values and different drug concentrations were prepared and 10 μL were spotted on blank, punched paper. It was shown that the effect of the DBS spot size, caused by the hematocrit is not the only parameter that can cause the deviating biases. Measuring real patient samples showed, that the hematocrit depends on the chosen cohort: Patients in hospital differ from outpatients.

Normally, venous whole blood samples are taken from the patient in hospital or at the doctor’s office. The best way is to measure the blood levels immediately or to store it at the latest for some hours in the fridge (4°C) until a defined volume is taken for analysis. For long term storage, samples have to be kept in the freezer (−20°C) [51]. An advantages of the DBS method, compared to whole blood, is the high stability of the sample for at least 7 days at 22°C. It facilitates the TDM for the patient, who can take the sample at home and send it to hospital by mail. Only a low blood volume is needed and there is a lower risk of biohazard. A major disadvantage is the effect of hematocrit on the measured value, which has to be corrected. Koster et al. show that required LOQs are reached with this method and there is a good correlation between venous blood and venous DBS. Protein binding in the blood and hydrogen binding to the cellulose of the paper seem to play an important role. These findings give new insights in the extraction methodology of DBS samples and have to be further investigated.

Szerkus et al. report the development and validation of an UHPLC method for determination of CsA in ocular rabbit tissue [46]. This technique makes it possible to determine the ocular pharmacokinetics of CsA after topical ophthalamic administration. HPLC methods are mentioned as the gold standard for determination of CsA [52]. The authors state that changes of stationary and mobile phase could not improve accuracy and precision of the method. In their work, they combined UHPLC with an UV detector, decreasing analysis time, costs for instrumentation and consumption of solvents, whereas keeping high method resolutions. Cyclosporine D (CsD) was utilized as internal standard. Chromatography was carried out on a C18 reverse phase column with the use of gradient elution.

The major advantage of the use of the UV detector instead of a MS is the cost issue. There is a benefit in particular for smaller laboratories, which cannot afford a mass spectrometer. Nevertheless, expensive UHPLC equipment is needed and the use of the UV detector is not possible for all analytes, because of the lack of a chromophore. Furthermore, if an UV-detector is used, extensive and time consuming sample preparation needs to be done to avoid interferences of blood compounds at low wavelengths.

5.2. Mass spectrometry with paper-spray ionization

In a recent publication Shi et al. describe the determination of tacrolimus using paper-spray ionization [53]. Advantages of this method are the saving of time and the small sample volume. No LC system and extraction procedure is needed. For sample preparation, internal standard is added to the blood sample and a drop of blood (10 μL) is spotted onto paper. Solvent is added after the blood has dried and a high voltage of 3–5 kV is applied on the moist paper. An electrospray is induced at the sharp tip of the triangular shaped paper in the cartridge, which is held stationary in front of the inlet of the MS. Samples were analyzed in a quadrupole mass spectrometer in the selected reaction monitoring (SRM). The method was evaluated in a clinical laboratory and tested with real patient samples. Accurate results were obtained. Advantages of this method are the simple sample preparation, the complete elimination of chromatography, which saves costs and the lower consumption of solvent.

5.3. Immunoassays

Immunoassays are an alternative method to LC-MS/MS. Their advantages are the fast assay procedure, low sample volumes, the opportunity to incorporate it into an automated system and the possibility to conduct the assay into a point-of-care-testing (POCT) device. Costs for instrumentation and technical support are drastically lower than for LC-MS/MS, whereas the costs for reagents are generally higher. Disadvantages are the limited analytical performance (LOQs are slightly higher compared to LC-MS/MS) and cross
Table 3
A selection of the latest chromatography methods, common commercial immunoassays and three recently published non-commercial immunoassays are listed. Many assays can be conducted with several immunosuppressive drugs. Examples and properties of the commercially available immunoassays are listed in Table 4.

<table>
<thead>
<tr>
<th>Detection of:</th>
<th>Cyclosporine A</th>
<th>Tacrolimus</th>
<th>Sirolimus</th>
<th>Everolimus</th>
<th>Mycophenolic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatography (non-commercial):</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>UPLC-MS/MS by Karapirli et al. (simultaneously)</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>UPLC by Tzyszczuk et al.</td>
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<td>LC-MS/MS (dried blood spot) by Koster et al.</td>
<td>X</td>
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<td>LC-MS/MS (high throughput) by Koster et al.</td>
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<td>UHPLC-MS/MS (incl. metabolites) by Klepacki et al.</td>
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<td>Paper spray-tandem mass spectrometry (PS-MS/MS) by Shiet al.</td>
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<td></td>
</tr>
<tr>
<td>Immunoassays (non-commercial):</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>AFM by Menotta et al.</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
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<tr>
<td>Semisynthetic biosensor by Griss et al.</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td></td>
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<tr>
<td>LSAW by Chang et al.</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
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<tr>
<td>Sandwich assay by Wei et al.</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

Reactivity. Especially active and inactive metabolites [23] and also autoantibodies in the blood sample like rheumatoid factor interfere depending on the antibody and kind of metabolite [54]. Matrix effects like variable hematocrit or protein values can also influence the analytical performance [55].

There are several immunoassays available on the market (Table 3). The current guidelines of therapeutic ranges in whole blood of the immunosuppressants are based on measurements with standard chromatography methods. Immunoassays need to be standardized with those methods, as they tend to add the metabolites to the measured drug levels [56].

In the following, some examples of different analytical techniques are explained (see Table 4 and Fig. 3).

5.3.1. Immunoassays (commercially available)

5.3.1.1. ACMIA (antibody conjugated magnetic immunoassay, affinity column-mediated immunoassay). Anti-ISD antibody/β-galactosidase conjugate is added to whole blood samples. Analyte (ISD) in the sample binds to the antibody-enzyme conjugate. Magnetic ISD coated particles remove the excess of free antibody-enzyme conjugates. The supernatant is mixed with the substrate chlorophenol red-β-galactopyranoside, which is converted into the dye chlorophenol red for colorimetric measurements at 577 nm [57]. The amount of dye is proportional to the amount of analyte in the sample.

5.3.1.2. CEDIA (cloned enzyme donor immunoassay). The CEDIA assay is a homogeneous enzyme immunoassay, based on β-galactosidase, which has been genetically engineered into two inactive fragments (enzyme donor and acceptor). If the enzyme re-associates, active enzyme cleaves a substrate. The colored product can be measured spectrophotometrically. In this assay drug in the sample competes with drug derivative, coupled to enzyme donor of β-galactosidase for limited antibody binding sites. The amount of free enzyme donor and resulting re-associated and therefore active enzyme is direct proportional to the amount of analyte in the sample [58].

5.3.1.3. CMIA (chemiluminescent microparticle immunoassay). Whole blood samples are collected in EDTA tubes. Protein is precipitated with ZnSO4 in methanol. The assay uses anti-ISD antibody-coated paramagnetic microparticles and an acridinium tracer, which is coupled to the drug derivative [59]. After incubation of antibody with the sample, free binding sites are occupied by the acridinium drug derivative. Chemiluminescence is detected. The chemiluminescence signal is inversely proportional to the amount of analyte in the sample.

5.3.1.4. ECLIA (electrochemiluminescence immunoassay). Whole blood samples are pretreated to release the analyte from the proteins. The pretreated sample is incubated with anti-ISD biotinylated antibody and a ruthenium complex labeled ISD-derivative. The antibody binding sites are partially occupied with analyte and with ruthenylated hapten, depending on analyte concentration in the sample. After addition of streptavidin-coated microparticles, the antigen-antibody complex binds to streptavidin via biotin. The mixture is aspirated into the measuring cell and microparticles are magnetically captured onto the surface of the electrode. Application of a voltage to the electrode induces chemiluminescence emission which is measured by a photomultiplier [60]. The chemiluminescence signal is inversely proportional to the amount of analyte in the sample.

5.3.1.5. ELISA (enzyme-linked immunosorbent assay), e.g. PRO-Trac™ II FK 506 ELISA assay (DiaSorin). The blood samples are extracted and added to the wells of the microtiter plate. The analyte (ISD) in the sample is bound to the specific antibody, which is captured on the plate by a secondary goat anti-mouse antibody. After incubation at room temperature, ISD horseradish peroxidase conjugate is added and again incubated. Afterwards, the wells are washed and chromogen is added. This reaction is stopped by sulfuric acid and the absorbance in each well is read out at dual wavelength of 450/630 nm. The amount of dye is inversely proportional to the amount of analyte in the sample. This manual-technical method has about eight steps.

5.3.1.6. EMIT (enzyme-multiplied-immunoassay technique). A derivative of the analyte is labeled with the enzyme glucose-6-phosphate dehydrogenase, which catalyzes the conversion of glucose
6-phosphate and NAD$^+$ to 6-phosphogluconate and NADH + H$^+$.

5.3.1.7. **FPIA (fluorescence polarization immunoassay).** In this assay, a fluorescent-labeled drug competes with unlabeled drug for antibody binding sites. The sample is excited with plane polarized light and emits plane polarized light at higher wavelengths. Small, free fluorescent-drug rotates fast, leading to less polarized emission than larger antibody-fluorescent-drug complex [53–55]. The fluorescence polarization signal is inversely proportional to the amount of analyte in the sample.

5.3.1.8. **MEIA (microparticle enzyme immunoassay).** After an initial precipitation step, the blood is centrifuged. The supernatant is conducted into the sample well and both anti-ISD antibody coated microparticles and ISD-phosphatase conjugate are added. The ISD and its conjugate competitively bind to the anti-ISD microparticles forming antibody–antigen and antibody–antigen–alkaline phosphatase complexes. An aliquot of the reaction mixture is transferred to a glass fiber matrix, where the microparticles bind irreversibly. In this manner, unbound compounds are removed by washing. The substrate 4-methylumbelliferyl phosphate is added and the fluorescent product (methyl-umbelliferone) is measured spectrophotometrically by determining the NADH concentration at 340 nm [61,62].

### Table 4
Commercially available immunoassays sorted by the immunosuppressive drugs they can detect. The detection limits and detection ranges are indicated according to the datasheets of the manufacturers. To be able to compare the immunoassays to HPLC methods, a comparison row is added. When there is no data in the cell, the value wasn’t explicitly mentioned.

<table>
<thead>
<tr>
<th>Immunosuppressant</th>
<th>Immuoassay:</th>
<th>Reportable Range:</th>
<th>LOQ:</th>
<th>Sample material:</th>
<th>Manufacturer:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine</td>
<td>ADVIA Centaur® Cyclosporine immunoassay Architect® system</td>
<td>30–1500 ng/mL</td>
<td>30 ng/mL analytical</td>
<td>EDTA whole blood</td>
<td>Siemens</td>
</tr>
<tr>
<td></td>
<td>CEDIA Cyclosporine PLUS Assay</td>
<td>30–1500.0 ng/mL</td>
<td>30 ng/mL (minimum reportable value)</td>
<td>EDTA whole blood</td>
<td>Abbott Diagnostics (CMIA assay principle)</td>
</tr>
<tr>
<td></td>
<td>Dimension Cyclosporine (CsA) and Cyclosporine Extended Range (CsAE) Assays</td>
<td>25–450 ng/mL Low Range 450–2000 ng/mL High Range</td>
<td>CsA: 25–500 ng/mL CsAE: 350–2000 ng/mL</td>
<td>EDTA whole blood</td>
<td>Thermo Scientific (CEIA assay principle)</td>
</tr>
<tr>
<td></td>
<td>Elicys® Cyclosporine</td>
<td>30–2000 ng/mL 50 ng/mL (LOD: 30 ng/mL)</td>
<td>EDTA whole blood</td>
<td>Siemens (ACMIA assay principle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emit® 2000 Cyclosporine A (CsA) Assay</td>
<td>CSA: 40–500 ng/mL CsA: 350–2000 ng/mL</td>
<td>CSA: 40 ng/mL analytical CsA: 350 ng/mL functional</td>
<td>EDTA whole blood</td>
<td>Roche (ECLI assay principle)</td>
</tr>
<tr>
<td></td>
<td>Comparison to Immunoassays: LC-MS/MS (Koster et al.)</td>
<td>10–2000 ng/mL 10 ng/mL (LLOQ)</td>
<td>EDTA whole blood</td>
<td>Siemens (EMIT assay principle)</td>
<td></td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>Architect® system</td>
<td>2–30 ng/mL 2 ng/mL (minimum reportable value)</td>
<td>EDTA whole blood</td>
<td>Siemens (CMIA assay principle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CEDIA® Tacrolimus assay</td>
<td>2–30 ng/mL 2 ng/mL</td>
<td>EDTA whole blood</td>
<td>Thermo Scientific (CEIA assay principle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dimension integrated chemistry systems Tacrolimus (Tac) assay</td>
<td>1.0–30.0 ng/mL 1.0 ng/mL (LOD: 0.7 ng/mL)</td>
<td>EDTA whole blood</td>
<td>Siemens (ACMIA assay principle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Elicys® Tacrolimus</td>
<td>0.5–40 ng/mL 1.0 ng/mL (LOD: 0.5 ng/mL)</td>
<td>EDTA whole blood</td>
<td>Roche (ECLI assay principle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emit® 2000 Tacrolimus (TCAR) Assay</td>
<td>2–30 ng/mL 2.0 ng/mL analytical 2.8 ng/mL functional 1.0 ng/mL</td>
<td>EDTA whole blood</td>
<td>Siemens (EMIT assay principle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thermo Scientific QMS® Tacrolimus Immunoassay</td>
<td>1.0–30 ng/mL</td>
<td>EDTA whole blood</td>
<td>Thermo Scientific (Quantitative Microsphere System)</td>
<td></td>
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<tr>
<td>Sirolimus</td>
<td>Comparison to Immunoassays: LC-MS/MS (Koster et al.)</td>
<td>2.50–50.0 ng/mL 2.50 ng/mL (LLOQ)</td>
<td>EDTA whole blood</td>
<td>Siemens (CMIA assay principle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Architect® system</td>
<td>2–30 ng/mL 2 ng/mL (minimum reportable value)</td>
<td>EDTA whole blood</td>
<td>Siemens (CMIA assay principle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Comparison to Immunoassays: LC-MS/MS (Koster et al.)</td>
<td>2.50–50.0 ng/mL 2.50 ng/mL (LLOQ)</td>
<td>EDTA whole blood</td>
<td>Siemens (CMIA assay principle)</td>
<td></td>
</tr>
<tr>
<td>Everolimus</td>
<td>Thermo Scientific QMS® Everolimus Immunoassay</td>
<td>2.0–20 ng/mL 2.0 ng/mL</td>
<td>EDTA whole blood</td>
<td>Siemens (CMIA assay principle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Comparison to Immunoassays: LC-MS/MS (Koster et al.)</td>
<td>1.00–50.0 ng/mL 1.00 ng/mL (LLOQ)</td>
<td>EDTA whole blood</td>
<td>Siemens (CMIA assay principle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CEDIA® Mycophenolic acid application</td>
<td>0.3–10 μg/mL</td>
<td>EDTA plasma</td>
<td>Siemens (CMIA assay principle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emit® 2000 Mycophenolic acid (MPA) Assay</td>
<td>0.1–15 μg/mL</td>
<td>EDTA or Heparinized plasma</td>
<td>Siemens (CMIA assay principle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Comparison to Immunoassays: UHPLC-MS/MS</td>
<td>0.098 μg/mL–200 μg/mL</td>
<td>EDTA plasma</td>
<td>Siemens (CMIA assay principle)</td>
<td></td>
</tr>
</tbody>
</table>

5.3.1.9. **RIA (radioimmunoassay), e.g. CYCLO-Trac-NS radioimmunoassay (Diasorin).** Blood sample are extracted with methanol and centrifuged. The sample is mixed with 125I-Cyclosporine A and
Fig. 3. Schemes of commercial available immunoassays as described above. All of them are using specific antibodies as recognition element for the drug and a label (fluorophore or enzyme). Either fluorescence, chemiluminescence or absorption of a colored product is detected.
Anti-CYCLO Trac SP ImmunoSep (a mixture of a specific monoclonal mouse anti-CsA antibody and secondary donkey anti-mouse IgG antibody). The solution is incubated for 1 hour. \(^{125}\)I-CsA and CsA compete for the binding to the specific antibody. After centrifugation, the supernatant is discarded. The immune complexes remain in a pellet at the bottom of the tube. The bound radioactivity of samples are measured using a gamma scintillation counter [67]. The amount of radioactivity in the pellet is inversely proportional to the CsA concentration in the sample.

5.3.1.10. QMS (quantitative microsphere system). Free drug molecules in the blood sample compete with a drug-coated microparticle for a limited number of antibody binding sites. Free drug, which binds to the antibody, inhibits the antibody to agglutinate the drug-coated microparticles. The rate of absorbance is measured photometrically. It is proportional to the rate of agglutination of the microparticles [68]. The rate of absorbance is inversely proportional to the analyte concentration in the sample.

Radioimmunoassays were the first highly sensitive and specific immunoassay methods for the determination of biomarkers in blood. Concerns about working place safety and the disposal costs of the radioactive waste promoted immunoassay techniques without radioactive labels, foremost enzyme-linked immunosorbent assays. At the beginning of the development, these heterogeneous assays were not able to reach the same sensitivity as RIAs. Continuous improvements were done to perform precise and highly sensitive immunoassays. One disadvantage today is that a lot of manual steps are needed to perform the assay and an automatization is difficult. To overcome this disadvantage, different companies in the field of clinical diagnostics developed various assay kits, containing all the needed reagents. They can be conducted with step by step instructions or automatized systems, containing as little manual steps as possible.

The analysis platforms have to be bought by the laboratory and can be used for various analytes. In literature, different systems are compared to each other in multicenter clinical trials with real patients’ samples. Analytical results of different systems and assay types show deviations, especially compared to HPLC-MS/MS. Due to the cross reactivity to metabolites of the drug, most of the immunoassays indicate higher blood levels, than measured by HPLC-MS/MS [69]. Nevertheless, the results which are obtained by immunoassays are in most of the cases reliable and can be used for TDM of immunosuppressive drugs [70].

5.3.2. Immunoassays (non-commercial)

Furthermore, there are some immunoassays described in literature, using label-free detection methods. The detection of a tacrolimus immunoassay with AFM is unique in literature. Menotta et al. describe the quantification of tacrolimus in biological samples by atomic force microscopy [71]. Anti-tacrolimus-antibody has been immobilized on a mica surface and incubated with a sample solution containing the drug. The surface was washed with Tris buffered saline (TBS), containing 0.05% Tween 20 and afterwards with TBS (without Tween 20). FKBP12 was immunocomplexed before with anti-FKBP12 antibodies in HEPES buffer and incubated with the micas. The surface was washed with TBS, dried by nitrogen flow and imaged.

The results show that the determination by AFM is feasible in principle and a validation can be done. Nevertheless, small changes of the surface can’t be detected by AFM properly. Therefore, a sandwich assay was used, causing a significant change at the surface. Sandwich assays are practicable for many analytes, but not for small molecules showing only one binding site. In this method, the measurement of the surface was done after it has been dried by nitrogen. There are some receptor-ligand interactions, which are destroyed by drying and measurements in liquid medium might be more suitable. Further investigations concerning this detection technique will be necessary to find out more about compatibility with biological samples and the questionable usage in clinical practice.

Wei et al. showed that a sandwich assay for tacrolimus using two anti-tacrolimus antibodies is feasible [72]. This approach is completely new, because steric hindrances between the two binding sites of the small molecule couldn’t be avoided till now. They use an intact tacrolimus molecule, which is covalently bound to a carrier protein via two different position as hapten for the production of antibodies. These positions are separated by 10 carbon atoms. By the use of a sandwich assay, the sensitivity and specificity will increase.

Griss et al. report from a novel semisynthetic bioluminescent sensor for point-of-care therapeutic drug monitoring of immunosuppressants, antiepileptics, anticancer agents and antiarrhythmics [73]. Minimal volumes of blood sample are spotted on a paper and the detection is done by the use of a simple digital camera. This technique uses luciferase-based indicators of drugs (LUCID), which is composed of a receptor protein for the drug of interest, a luciferase and a synthetic molecule containing a fluorophore and a ligand for the receptor protein. The attached ligand binds to the receptor protein, bringing the fluorophore close to the luciferase. Bioluminescent resonance energy transfer (BRET) occurs. The intramolecular fluorophore emits light. Analyte, displacing the ligand from receptor protein, causes decreased BRET efficiency. The ratio of light emitted from luciferase (blue) and the synthetic fluorophore (red) permits quantification of the analyte. The sensor protein is a fusion protein, consisting of the receptor for the drug of interest, the luciferase NanoLuc, a 30-prolin linker, a SNAP-tag (self-labeling protein tag for the introduction of a fluorescence marker in a protein during expression), the fluorophore Cy3 and an intramolecular ligand. The 30-prolin linker ensures low BRET efficiency in open state by pushing luciferase and fluorophore away. Permutated receptors can improve efficiency by the change of affinity to intramolecular ligand. For the detection of CsA, a mutant of human Cyclophilin A is used. FKBP12 was used as receptor for tacrolimus and sirolimus. Both termini of the FKBP12 protein lie far away from the active site, leading to low BRET efficiency in closed state. An intramolecular ligand was chosen spanning both binding sites of FKBP12. Low LODs were reached (Fig. 4).

Bilirubin in the blood absorbs blue light. Samples were spread as a thin layer on chromatography paper to reduce the distance the light has to travel through serum. In this way, the problem of absorption has been solved. For detection of bilirubinolucence during the development of the method, a microplate reader was used. As peaks of NanoLuc and Cy3 are separated and overlap with blue and red color channel of standard CMOS and CCD, digital cameras were used. Thirty patients’ samples were analyzed, showing good correlation to a standard FPIA.

Advantages are the low cost, low time and low sample volume consumption. Disadvantages are the need of intensive development of the fusion protein, including mutation of the receptor or the choice of clever intramolecular ligands. The affinity of the ligand to the receptor plays an important role regarding the BRET efficiency and the working range of the obtained calibration. Considering the relatively simple structure of the detection module, this assay approach could be integrated in a POCT device. Personalized dosage at bedside at home without the presence of qualified stuff could be made possible.

Chang et al. developed a leaky surface acoustic wave (LSAW) immunosensor array for CsA [74]. Anti-CsA antibody was immobilized on the gold surface of 100 MHz LiTaO\(_3\) piezoelectric crystals via Protein A. If CsA binds to the antibody, mass loading of the immunosensor increases, which leads to phase shifts of LSAW. A LOD of 0.89 μg/L and a working range of 1–1000 μg/L has been reached.
6. Whole blood concentration and immunosuppressive effect

All of the before mentioned assay types are optimized for determination of immunosuppressive drugs in whole blood. Several studies indicated that whole blood concentrations are suitable for therapeutic drug monitoring of these drugs. Having in mind that most of the drugs are bound on red blood cells and plasma proteins, only the unbound drug can interact with the targets. Therefore, the determination of the free drug concentration could contain more information on the immunosuppressive effect than whole blood levels. The major problem of this approach is first of all the adaption of extraction protocols and furthermore the achievement of very low limits of quantification (free drug fraction of tacrolimus is for example lower than 1% of whole blood concentration). Another approach is the determination of intracellular concentrations (e.g. in lymphocytes) or the effect on the target protein (e.g. calcineurin) lower than 1% of whole blood concentration). Another approach is the determination of intracellular concentrations (e.g. in lymphocytes) or the effect on the target protein (e.g. calcineurin) [38,75,76]. In this way it could be shown that whole blood concentration doesn’t always correlate with immunosuppressive effect and inter-patient variabilities play an important role.

7. Conclusion and future perspectives

Since the application of first immunosuppressive drugs after organ transplantation in the 1970s, the therapy as well as analytical methods have continuously improved. LC-MS methods are the gold standard for TDM of ISDs until now. Definitely, the standardization of this widely used method is essential and has been already tackled in several papers. Immunoassays have been developed since the late 1980s as a cheaper alternative and for Point-of-care application without the need of a central laboratory. The main disadvantage until now is the cross-reactivity to active and inactive metabolites in the sample, which cannot be completely circumvented. For this reason, it is essential to know about the strengths and weaknesses of each technique. Another important point, which is just being investigated, is that specimen can be obtained out of different matrices. Due to their different binding to blood cells and plasma proteins, the optimal sample matrix has to be found for an exact determination of drug. Additionally the amount of drug in the obtained matrix has to correlate with the immunosuppressive activity. Otherwise the obtained value has no clinical value. In the future, one promising approach might be to determine only the drug fraction, which can be effective at the corresponding receptor in cell.

The funding of several projects by the European Union show that there is a great demand of continuous measurement of drug concentrations and of personalized medication in immunosuppressive therapy. Today’s analytical techniques have to be further adapted to clinicians needs: Whilst the overarching goal in the past was to reach extremely low limits of detection, the aim is now to investigate alternative matrices and improve sampling strategies. One concept for the future will be the continuous measurements of immunosuppressive drugs in a POCT device. The determination of the free drug fraction is for example picked out in the NANODEM project (NANophotonic DEvice for Multiple therapeutic drug monitoring) [77]. In this interdisciplinary project a continuous monitoring of immunosuppressive drugs and related metabolites in the early phase of medication is targeted. In this approach, the patient will be connected to a POCT device by an intravenous microdialysis catheter to allow hourly measurements during 48–72 hours. Each dose will be calculated individually. Pharmacokinetics of the drug are considered by applying the AUC concept. The device will consist of different elements: A microdialysis unit, microfluidics, a multiparameter biochip, where the immunoassays are situated, and an optoelectronic device. Specific antibodies are used as recognition elements, which are immobilized on magnetic beads, and a fluorescence marker for detection. The research project BIO-DrIM (BIOmarker-Driven personalized IMMunosuppression) focuses on biomarker-based early patient stratification to personalize immunosuppressive therapy. Five clinical studies are designed and carried out by the consortium.

All mentioned analytical methods, chromatography techniques as well as immunoassays possess a right to exist. They combine advantages and disadvantages. You have to keep in mind that the result depends on the respective assay type you are using. From clinical point of view, avoiding acute rejection (mediated by T lymphocytes) in the first year after transplantation is an essential contribution to the survival of the graft. The patient’s profile continuously changes during therapy as well as the graft function and histology, requiring an adaption of the medication. This results in an increasing number of immunosuppressive drugs combinations during the life course of a transplanted organ [9]. An accurate therapeutic drug monitoring and the knowledge about pharmacokinetics and –dynamics of the respective drug is essential. Finally, clinical outcome, including quality of life of the patient, is the key parameter for the quality of therapeutic drug monitoring.

Acknowledgements

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References


