The influence of the sample matrix on LC-MS/MS method development and analytical performance

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Chapter 6

General discussion and future perspectives

This research focused on the various matrix dependent issues that can be encountered during the development of new analytical procedures for drugs and drugs of abuse using high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Matrix effects can severely deteriorate the analysis results and are not always detected and acknowledged. With this research, issues caused by the different effects of the used matrices were evaluated and overcome. Subsequently, analytical procedures were developed for the analysis of various substances in the human matrices whole blood, plasma, dried blood spots (DBS), hair and sweat patches. The parameters that were critical for a robust, selective, accurate and precise method were investigated and appropriate measures were taken. Finally, this research leads to a better understanding of the influence of various matrices on the performance of analytical methods.

WHOLE BLOOD

In chapter 2 we developed an analytical procedure for tacrolimus, sirolimus, everolimus and cyclosporin A in whole blood, which was required to perform better than the previously used immunoassay techniques regarding sensitivity, selectivity (metabolites), flexibility and linear range. In addition, the aim was to reduce the high costs per sample. The developed analytical procedure showed to be suitable for the analysis in whole blood with a fast and simple sample preparation, runtimes of 2.6 minutes, high sensitivity and chromatographic separation of interfering peaks. Overall, all patient samples could be analyzed and reported on the same day. The developed method for the analysis of immunosuppressants in whole blood has been used in daily routine for 8 years and more than 120,000 immunosuppressant samples from inpatients and outpatients have been analyzed in our laboratory. Calculations of the yearly costs showed that the replacement of the used immunoassay techniques with the LC-MS/MS (costs: 250,000 Euros) saved 160,000 Euros per year [1]. With the replacement of the LC-MS/MS in the year 2015 this added up to a total saving of 1,030,000 Euros (250,000 Euros depreciation included). This is a nice example of the impact of LC-MS/MS on clinical laboratories for both the technical advances and economic implications. Although the acquisition of a LC-MS/MS is costly, the benefits can be significant. It should be noted that the knowledge of the technical staff is crucial for a scientifically responsible and efficient use of the LC-MS/MS.

Our research has shown that the use of zinc sulphate and methanol in the sample preparation gives process efficiency results of about 100% for tacrolimus and cyclosporin A, while for

sirolimus and everolimus about 100% process efficiency is only achieved without the use of zinc sulphate. This has resulted in the use of two preparation methods, while still allowing the simultaneous analysis of cyclosporin A and tacrolimus or sirolimus and everolimus with a single LC-MS/MS system. In addition, the parameters of the LC-MS/MS method could be set optimal for tacrolimus and cyclosporin A, and for sirolimus and everolimus. Additional tests showed that the use of zinc sulphate mainly enhanced the peak height of cyclosporin A. Generally, the use of zinc sulphate provided a better and more homogeneous precipitation of the blood, while the use of methanol without zinc sulphate showed a less homogeneous precipitation and a higher chance of substance inclusion in the whole blood sample. This has adverse effects on the reproducibility of the method.

As a future perspective, a more sensitive LC-MS/MS system is supposed to increase sample throughput, with shorter runtimes and a larger autosampler capacity. With an increasing amount of patient samples, with this system it should still be possible to analyze and report results on the same day. In order to combine all four analytes in one method, the sample preparation with the use of zinc sulphate will also be re-investigated for sirolimus and everolimus. Despite the fact that our research has shown that the use of zinc sulphate decreased the recovery for sirolimus and everolimus by approximately 20%, the positive effect on the coefficient of variation may outweigh this loss of recovery. In addition, the increased sensitivity of the new LC-MS/MS will be able to compensate for the decreased signal due to the loss of recovery.

DRIED BLOOD SPOT

Although the use of dried blood spots (DBS) for therapeutic drug monitoring will certainly benefit the patient because of the ease of sampling and decreased patient travelling time, the development of suitable analytical procedures for this matrix is a challenge for the researcher. In chapter 3, different important aspects of optimal analytical procedures were investigated.

Although most methods describe the preparation of the desired hematocrit (HT), none of them refer to the DIN 58933-1 reference method [2]. The impact of two procedures to prepare the target HT value was therefore evaluated. In the first procedure, whole blood was centrifuged, red blood cells and plasma were separated and the proper volumes of red blood cells and plasma were mixed to obtain the target HT. This procedure resulted in a measured HT that was 11% lower than the target value. In the second procedure, blood was

centrifuged and a calculated volume of plasma was removed or added in order to adjust the HT. No difference was observed between the measured HT and the target value. The second procedure was therefore considered the preferred procedure. This research showed that there are discrepancies in the HT preparation procedures between DBS research projects and validations. This may lead to over or under estimation of the HT effects during DBS validations.

Moxifloxacin, used against multi drug resistant tuberculosis, was the first analyte for which a DBS analytical procedure was developed in our laboratory. The analytical procedure was fully validated and extra care was given to the effect of the HT, blood spot volume and DBS stability at various temperatures. The influence of paper type, the HT and the blood volume per spot on the estimated blood volume in a disc was investigated using camera images to measure DBS areas. The estimated blood volume in a disc showed to be highly influenced by HT while the effect of blood volume per spot appeared to be different among paper types. The large range of tested HT values produced significant analytical biases and could be partly corrected with the HT value. Although a HT correction for the measured DBS concentration was proposed, this is not feasible for patient DBS samples with unknown amount of blood volume and HT.

For rifampicin and clarithromycin the DBS method initially suffered from matrix effects, which were caused by the formation of metal ion complexes with rifampicin. The addition of both deferoxamine (DFX) and ethylenediaminetetraacetic acid (EDTA) to the extraction solutions recovered the responses of RIF in the DBS extraction to approximately 100%. The successful combination of EDTA and DFX for the extraction might be explained by their chelating properties. During the complex formation, DFX completely covers the surface of Fe3+, while EDTA is not able to completely shield the surface of the Fe3+ ion and forms an open (basket) complex. Although EDTA has a high stability constant for the formation of the EDTA and Fe^{3+} complex, other metal ions also form complexes with EDTA, making EDTA not a very specific chelating agent. DFX on the other hand, is known for its strong and specific binding affinity to Fe^{3+} and less affinity to other metals. This makes DFX better suitable as a complexing agent. However, the contribution of EDTA in the developed method is two-fold. First, it can form complexes with Fe^{3+}. Second, it facilitates in the precipitation of dissolved matrix after the DBS extraction, which is performed by the addition of acetonitrile.

The analytical procedure for the immunosuppressants was further optimized for DBS analysis on 31 ET CHR paper. The effect of the HT showed that the viscosity of the blood due to the HT is not the only parameter that affects the measured concentration. Additional recovery

tests proved that the combination of especially low HT and high drug concentration does not only affect the spot size but also affects the extraction recoveries of sirolimus and especially everolimus. At higher concentrations combined with lower HT values, recoveries deteriorated for everolimus and especially sirolimus. The relatively high number of hydrogen bond acceptors for sirolimus and everolimus was suspected to have more affinity to form hydrogen bonds with the cellulose of the paper, making the extraction less efficient. Although the parameters like HT and concentration were tested for a very large range and were not always likely to occur in routine analysis of outpatient samples, the fundamental effect of the combination of these parameters on extraction recoveries were proven with this research.

The analysis of creatinine in dried blood spots (DBS) could be a useful addition to DBS analysis in order to monitor creatinine levels or to adjust the dosage of renally excreted or nephrotoxic drugs. Therefore we developed a LC-MS/MS method for the analysis of creatinine in the same DBS extract that was used for the analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in transplant patients with the use of Whatman FTA DMPK-C cards. The method was validated using three different strategies; a 7-point calibration curve using the intercept of the calibration to correct for the natural presence of the creatinine in reference samples; a one point calibration curve at an extremely high concentration in order to diminish the contribution of the natural presence of creatinine and the use of creatinine-[2H3] with an eight-point calibration curve. It is known that in vitro creatinine concentrations significantly increase in serum and plasma within 24 hours to 3 days [3-5]. Ring closure of creatine in the matrix occurs while losing a molecule of water to form creatinine under influence of time, temperature and pH [6-9]. DBS is often promoted as a more stable matrix for a blood sample and this is also true for creatinine [10]. Our experiments showed that stability is now extended to 7 days at a maximum temperature of 32°C.

In order to investigate the influence of the number of hydrogen bond acceptors on the recovery of tacrolimus, ascomycin, sirolimus, everolimus and temsirolimus in dried blood spot analysis, we added the immunosuppressant analogues ascomycin and temsirolimus to the analytical method with the use of Whatman DMPK-C cards. Especially, the addition of temsirolimus was important because of the number of hydrogen bond acceptors of 16 which were even more than everolimus with 14 hydrogen bond acceptors. In this way, temsirolimus and ascomycin (12 hydrogen bond acceptors) were used to investigate the concentration and HT dependent recovery. This hypothesis was tested by evaluation of the extraction recoveries of tacrolimus, ascomycin, sirolimus, everolimus and temsirolimus, with 12, 12, 13, 14 and 16 hydrogen bond acceptors respectively. With an increasing number of

hydrogen bond acceptors of sirolimus, everolimus and temsirolimus, a decrease in recoveries was found, while ascomycin showed recoveries corresponding to those of tacrolimus. At increasing concentration from 3.0 to 50 ng/mL, the recovery of temsirolimus decreased with 32%, while the recoveries for everolimus and sirolimus decreased with 25% and 21% respectively. This showed that the number of hydrogen bond acceptors of the analyte of interest may influence the recoveries in DBS analysis and is a relevant factor to be investigated during future method development and validation.

A minimum drying time of 3 hours is frequently used in DBS protocols. This drying time showed to be insufficient for a robust DBS method. Our results showed that the recovery declined significantly within a period of 24 hours, followed by a stabilization of the recoveries. It was also noticed that the drying time effects were more distinct for sirolimus, everolimus and temsirolimus than for tacrolimus and ascomycin. The decreased recoveries due to increased drying times were in accordance with the increasing number of hydrogen bond acceptors of sirolimus, everolimus and temsirolimus respectively. The results showed that a drying time of at least 24 hours is necessary in order to minimize the risk on drying time related recovery effects for these analytes. This study showed that DBS drying time effects should be investigated for a period of at least 48 hours during future method development and validation. When the drying of patient DBS samples continues during transport in sealed bags with silica sachets, the impact may be minimal. However, if prepared calibration and control samples used for validation or routine analysis are insufficiently dried, the impact on the results may be significant.

The extraction recoveries and HT effects were also investigated for the following five types of DBS cards: Whatman 31 ET CHR, Whatman FTA DMPK-C, Whatman 903, Perkin Elmer 226 and Agilent Bond Elut DMS. We found that all DBS cards showed the same recovery pattern for sirolimus, everolimus and temsirolimus with decreasing HT and increasing concentration. The tested card types showed differences in performance, which were more emphasized at extreme concentrations and HT values. At high concentrations, the total HT effects were much more pronounced than at low concentrations for tacrolimus, sirolimus, everolimus, ascomycin and temsirolimus. Overall, the Whatman DMPK-C cards seemed to have the most constant and best performance. With regard to the correction of analysis results for only the HT value, a linear hematocrit correction method may be unsuitable and it may be more feasible to set up a point-to-point relation between recovery, HT and concentration. Recently, a DBS method for the determination of the HT by potassium measurement was published [11]. However, a second DBS is required for the potassium measurement, including

an immuno analyzer. This implies a significant amount of work for setting up the correction formula and the development of the analysis method and measurement of the HT value (by potassium measurement) in the (extra) DBS with an immuno analyzer. When no HT correction is applied, the method validation should include a framework for the HT and substance concentration in which the results are within acceptable limits.

Since future therapeutic drug monitoring will be performed with the use of Whatman FTA DMPK-C cards, the method was re-validated. The limitations of the DBS analysis method for immunosuppressants were influenced by parameters like concentration range, HT value and the patient population. During the validation, several parameters were adjusted to the specific patient population. The HT was set at 0.38 L/L, according to the patient population. The QC samples Low and Medium were set at therapeutic concentrations for trough levels and were used to assess the HT effect and recovery. The new and more restricted framework of parameters showed far less effects of the HT and the combination of HT and concentration dependent recovery. The HT effect showed biases within 15% within a HT range of 0.23 to 0.53 L/L, with an exception for cyclosporin A at 200 µg/L, which showed a bias of -17.8% at the HT level of 0.53 L/L. Initially, cyclosporin A showed a quadratic calibration curve from 20 to 2,000 µg/L. This was explained by the fact that at high cyclosporin A concentrations the binding capacity of leukocytes and erythrocytes becomes saturated [12]. The high affinity of cyclosporin A to distribute to plasma may explain the chromatographic effect seen at extremely low HT combined with a high concentration of the analyte. This causes a high degree of plasma bound cyclosporin A which has a lower viscosity than the erythrocytes, creating a chromatographic effect in the distribution of the DBS. The linear range for cyclosporin A was shortened to 1,000 µg/L and now showed a linear fit. This shortened range is too short for pharmacokinetic curves, but is suitable for trough levels, which are used for the monitoring of outpatients. For concentrations measured above the linear curve of 1,000 µg/L for cyclosporin A, a dilution of the DBS extract is not possible because of the unequal distribution of cyclosporin A in the DBS, which causes a quadratic curve at a concentration range up to 2,000 µg/L.

The more restricted framework of parameters made the adjusted validation much more suitable for therapeutic drug monitoring of outpatients. The observed HT effects were addressed by restricting the framework instead of HT based concentration correction. The approach of the restricted framework may make the analysis of an extra DBS for HT assessment unnecessary and creates a more efficient workflow in the laboratory.

Ongoing research and improved understanding of the parameters that influence DBS analysis results will ultimately result in well-founded guidelines for DBS analytical method validation.

In order to transfer whole blood analysis to DBS analysis, clinical validation studies need to be performed. First, DBS sampling instructions were documented in a flyer and a video. Second, health care personnel and patients were trained in correct DBS sampling. Third, the first part of the clinical validation of tacrolimus in DBS was performed with 42 patients. The first part of the clinical validation showed good correlation of DBS with whole blood analysis. The concentrations found in DBS were approximately 22% higher than in whole blood. This suggests that the reference values for tacrolimus could be adapted to the new DBS matrix. We will start the analysis of patient DBS samples for therapeutic drug monitoring of tacrolimus soon and the clinical validation of sirolimus, everolimus and cyclosporin A will follow subsequently. In addition, future investigation of automated DBS sample preparation may assure a cost-effective workflow for future DBS analysis.

PLASMA

Although plasma is considered to be an easier to matrix for LC-MS/MS analysis than the other matrices which have been investigated for this thesis, in some cases this matrix still poses challenges to the researcher.

Chapter 4 is dedicated to the plasma matrix and the synthetic opiate remifentanil, which is very unstable in whole blood and plasma because of digestion by endogenous esterases and chemical hydrolysis. The instability of remifentanil in these matrices makes the sample collection and processing a critical phase during bioanalysis. After withdrawal of the blood sample, the blood tube is placed in ice water and subsequently centrifuged at 4°C and 1 mL of plasma is then transferred into a tube containing 15 µL of 10% formic acid and mixed. In this matrix, remifentanil showed to be stable for 2 days at ambient temperature, 14 days at 4°C and 103 days at -20°C. When the developed sample collection and acidification procedure was not applied, remifentanil already showed a -14% decrease in concentration after 30 minutes at ambient temperature in whole blood. The observed drug instability was not observed in stock solution in water, which proved to be stable for 6 months at +4°C.

The understanding of the process of drug instability in the obtained sample should be part of the analytical method development and validation.

HAIR AND SWEAT PATCHES

Hair and sweat sampling is non-invasive and can be used to monitor a larger time window of drug use than blood or plasma. Chapter 5 is dedicated to the matrices hair and sweat, which are mainly interesting for monitoring compliance or non-adherence of drug use. There are however, some drawbacks like external contamination. In order to minimize false positive results for hair analysis, a wash procedure was developed with the use of dichloromethane which removed external contamination from the hair without already extracting the analyte of interest. The developed preparation method uses a ball mill to simultaneously pulverize and extract the hair sample with methanol containing isotopically labeled internal standards. The pulverized sample is then centrifuged and filtered prior to injection to the LC-MS/MS.

Since the sweat patch is covered by a non-occlusive membrane, external contamination seems unlikely. However, several investigations have shown that external contamination of the worn sweat patches can cause false positives [13]. The detection of metabolites may assist in overcoming this issue. Nevertheless, it may be concluded that sweat patches are not able to indisputably prove the use of drugs. However, the non-invasive character of hair and sweat patch testing can provide a useful complementary tool for physicians and health care professionals to evaluate possible drug use.

The use of qualifier mass transitions to enhance selectivity is advised when drugs of abuse analysis is performed and the European Commission has set maximum permitted tolerances for relative ion intensities for mass spectrometric techniques in the Official Journal of the European Communities [14].

In this document it is stated that the acceptable bias of a qualifier/quantifier ratio depends on the relative intensity of the qualifier mass transition compared to the quantifier mass transition. The qualifier/quantifier ratio is allowed to deviate 50% from its set value when a qualifier mass transition is more than 10 times less sensitive than its quantifier. This means that the less sensitive a qualifier, the less strict the rules for confirmation are. We found this unacceptable for the validation of the developed analytical methods.

In order to obtain enhanced selectivity during the MS/MS analysis of drugs of abuse, a qualifier mass transition was added to the analysis method and validated. Since no clear rules were described for the validation of qualifier mass transitions in literature, we developed and applied our own more strict rules of confirmation. During the method validation, the

ion ratios for the qualifier and quantifier were evaluated at the LLOQ, Low and Med levels of the accuracy and precision validation. All ratios of the qualifier and the quantifier found during the accuracy and precision validation were required to be within 20% of the ratio set in each validation run. Analytical cut-off concentrations were defined for each substance at the lowest concentration validated for accuracy and precision with a bias and CV within 15% and all qualifier/quantifier ratios within 20% of the set ratio during the whole validation. For some substances, the validation of qualifier/quantifier ratios has resulted in higher analytical cut-off concentrations than the validated LLOQs for accuracy and precision of these quantifiers. This shows that although the quantifier provides good accuracy and precision, below these analytical cut-off concentrations the accuracy of the ion ratio may be insufficient for confirmation of the detected substance. Although this increases the analytical cut-off concentrations for these substances, they are still within the screening cut-off concentrations set by the Society of Hair Testing for hair analysis and by the Substance Abuse and Mental Health Services Administration for the sweat patch analysis [15, 16]. This validation of the qualifier/quantifier ratios also underlines the selectivity and reliability of the described analytical procedures and should be incorporated in future validation protocols for the analysis of drugs of abuse.

CONCLUSIVE REMARKS

In order to provide personalized patient treatment, the requirements for a laboratory are high. A large number of analytical procedures is needed to measure a large variety of drugs in various human matrices, and short turnaround times are often desired for TDM patient samples. In addition, each patient setting may require a specific matrix. Sweat and hair samples may be used to monitor drug abuse or drug adherence. TDM performed with the use of blood and plasma samples may be suitable for in-patients, while DBS may be more suitable for outpatients. The use of DBS for TDM is very promising, but the investigated critical parameters showed not to be underestimated and need to be considered before performing patient analysis.

In the near future, personalized patient treatment will not only comprehend personalized dosing of patients, but also a personalized choice of matrix which best suits the patients' circumstances and optimal needs.

In this thesis, we have shown that the influence of the matrix can result in altered stability of the substance, formation of complexes with other substances or binding of the substance with the sampling material. More insight in these matrix effects will lead to improved analytical methods and performance, but will above all improve patient care and research.

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