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

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Chapter 7

Summary

In this thesis, the influence of various matrices on the development and performance of analytical methods using high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was investigated. The parameters that were critical for substance stability in the matrix and a robust, selective, accurate and precise method were investigated and appropriate measures were taken. Finally, analytical procedures were developed for several substances in the matrices whole blood, plasma, dried blood spots (DBS), hair and sweat patches.

In **chapter 2** we focused on the matrix whole blood for the development of an analytical procedure for the analysis of the immunosuppressants cyclosporin A, tacrolimus, sirolimus and everolimus. The purpose of this method was to replace the immunoassay methods used in our laboratory. Several LC-MS/MS methods had been described so far, however most of them required complicated extraction procedures. The described analytical procedure uses a protein precipitation as sample preparation in combination with a chromatographic gradient of 2.6 minutes that is capable of separating otherwise interfering peaks. Our research showed that the use of zinc sulphate provides process efficiency results of about 100% for tacrolimus and cyclosporin A but only 81% and 87% for sirolimus and everolimus respectively. Therefore, two procedures were developed for the combined analysis of sirolimus and everolimus and for tacrolimus and cyclosporin A. With the developed sample preparation without zinc sulphate for sirolimus and everolimus, process efficiencies were 99% and 108% respectively. In conclusion, we found the developed methods to be cost saving, more flexible, more sensitive and to have larger linear ranges than the previously used immunoassay methods. Since implementation of the analytical methods in our laboratory in 2007, approximately 140,000 patient samples have been analysed in daily routine.

In **chapter 3.1** we aimed to determine the right blood hematocrit (HT) preparation procedure for standards and quality control samples for DBS analysis. Since HT effects on DBS analysis are widely acknowledged, a correct preparation of the target HT is vital. We compared two procedures for preparing specific HT values using a hematology analyzer. 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, which 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 for the right HT. No difference was observed between the measured HT and the target value. The second procedure was therefore considered the preferred procedure. In conclusion we recommended that as part of

quality assurance the target HT should be measured after preparation using a hematology analyzer.

In **chapter 3.2** we developed an analytical procedure for the determination of the antibiotic moxifloxacin in DBS and we investigated the impact of the HT and blood spot volume with Whatman N_0 3, 903 and 31 ET CHR paper. 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 effect of HT and blood volume per spot on the analytical results was also investigated and linear regression models were developed for each paper type. For the Whatman 31 ET CHR paper, which was used for the analytical validation, 89% of the variation of the estimated blood volume in a disc showed to be caused by the HT. While the effect of the blood volume was of no significant influence, suggesting that blood equally spreads on this paper regardless the size of DBS on Whatman 31 ET CHR paper. The regression models for Whatman N_0 3 and 903 showed that only 62% and 72% of the variation of the estimated blood volume in a disc could be explained by the HT and applied blood volume. A large range of HT values produced a significant analytical bias and could be partly corrected for the HT value. In conclusion, we found that the HT is main parameter that influences the analytical results but that the HT correction can not be performed without a known HT, which is normally unknown in DBS samples.

In **chapter 3.3** we investigated interactions and matrix effects of endogenous substances in blood with rifampicin, clarithromycin and their metabolites. Rifampicin can form chelate complexes with ferric ions or bind with heme groups, which are potentially present in the DBS extracts. The investigation focused on the interaction between rifampicin and endogenous substances in the DBS. The use of ethylenediaminetetraacetic acid (EDTA) and deferoxamine (DFX) as chelator agents improved the recovery of rifampicin with 51% and the eliminated the matrix effects. Finally, an analytical method was developed and validated to quantify rifampicin and clarithromycin and their metabolites desacetylrifampicin and 14-hydroxyclarithromycin in DBS samples. The role of EDTA and DFX as in the extraction may provide a solution for potential applications to other DBS analytical methods.

In **chapter 3.4** we developed an analytical procedure for the analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in DBS samples to facilitate therapeutic drug monitoring for transplant outpatients. Our research showed that the HT had a significant influence on the analytical results. Extensive recovery tests proved that the combination of especially low HT and high concentration does not only affect the spot size but can also affect the extraction

recoveries of sirolimus and especially everolimus. For example, the recovery of everolimus at HT 0.35 L/L and 3 µg/L was 87%, while the recovery at HT 0.25 L/L and 50 µg/L was lowered to 49%. 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 increasing number of hydrogen bond acceptors of the substances (tacrolimus 12, sirolimus 13, everolimus 14) and lowered level of protein binding in the blood was hypothesized to influence the formation of hydrogen bonds with the cellulose of the paper. This observed influence on the extraction efficiency gave new insights in the extraction methodology of DBS samples. The observed HT effects during the validation appeared to be negligible during the patient correlation between venous blood and venous DBS study as no concentration corrections for the HT values were needed. The patient correlation study showed good correlations for all four immunosuppressants with R^2 values higher than 0.87 between venous whole blood and venous DBS samples. Nevertheless, results from DBS samples with extremely high concentrations combined with extremely low HT values should be interpreted with caution.

In **chapter 3.5** we investigated if it was possible to measure creatinine in the same DBS extracts as used for the analysis of tacrolimus, sirolimus, everolimus and cyclosporin A. In order to monitor creatinine levels or to adjust the dosage of renally excreted or nephrotoxic drugs, the analysis of creatinine in DBS could be a useful addition to DBS analysis of immunosuppressants. Since creatinine free blood and thus DBS is not available and can not be prepared without changing the matrix, this issue had to be overcome. Therefore, our analytical 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-$[^2H_3]$ with an 8-point calibration curve. Creatinine concentrations significantly increase in serum and plasma within 24 hours to 3 days. Ring closure of creatine in the matrix occurs while losing a molecule of water to form creatinine under influence of time, temperature and pH. DBS is often promoted as a more stable medium for a blood sample and this is also true for creatinine. Our experiments showed that stability is now extended to 7 days at a maximum temperature of 32°C and at -20°C for 29 weeks.

In **chapter 3.6** we investigated the influence of the number of hydrogen bond acceptors on the recovery of tacrolimus, ascomycin, sirolimus, everolimus and temsirolimus in DBS

analysis. The hypothesis that the substance recovery was influenced by the number of hydrogen bond acceptors of the substance 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. For all tested substances, the recoveries were evaluated at varying concentrations and HT values. 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 the HT of 0.1 L/L and concentration of 50 ng/mL, the recoveries were 93% for tacrolimus, 93% for ascomycin, 63% for sirolimus, 59% for everolimus and 48% for temsirolimus. This study 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 method development and validation.

In **chapter 3.7** we investigated the influence of the DBS drying time for tacrolimus, sirolimus, everolimus, cyclosporin A, ascomycin and temsirolimus on the recovery. Investigation of the DBS drying time as part of the method validation is currently not included in DBS validations. The influence of the DBS drying time on the recovery was evaluated by measuring DBS with a fixed blood volume at a HT range between 0.1 and 0.6 L/L at 3, 24 and 48 hours of drying time. Results showed that the recovery of sirolimus, everolimus, temsirolimus and cyclosporin A was influenced by the DBS drying time, while the recovery of tacrolimus and ascomycin was not. At the low HT of 0.1 L/L, the recoveries of sirolimus, everolimus, temsirolimus and cyclosporin A declined with 24%, 26%, 27% and 14% respectively between 3 and 24 hours of drying time, followed by a stabilization of the recoveries. Therefore, we advised a drying time of at least 24 hours in order to stabilize drying time related recovery effects of sirolimus, everolimus, temsirolimus and cyclosporin A.

In **chapter 3.8**, the performance of five DBS card types was investigated for the DBS analysis of tacrolimus, sirolimus, everolimus, ascomycin, temsirolimus and cyclosporin A. For these substances, the extraction recoveries and HT effects were investigated for Whatman 31 ET CHR, Whatman FTA DMPK-C, Whatman 903, Perkin Elmer 226 and Agilent Bond Elut DMS DBS cards. We found that all DBS cards showed the same pattern in declining recoveries for sirolimus, everolimus and temsirolimus with decreasing HT at increasing concentration. The tested card types showed differences in recovery performance, which were more emphasized at concentrations and HT values outside the normal range. Overall, the Whatman DMPK-C cards seemed to have the most constant and best performance. The tested DBS cards showed little differences in performance regarding the formation of the DBS during partial

spot analysis. However, the total HT effects differed between Low and High concentrations. At high concentrations, the total HT effects were much more pronounced than at low concentrations for tacrolimus, sirolimus, everolimus, ascomycin and temsirolimus. Based on our research, a simple correction of analysis results for the HT value may be questioned, since the combination of the HT value and the substance concentration can affect the analysis results.

When no HT correction is applied, it is proposed that the method validation should include a range for the HT and substance concentration in which the results are within acceptable limits.

In **chapter 4** we investigated the matrix dependent stability of the synthetic opiate remifentanil in EDTA whole blood and acidified EDTA plasma. Remifentanil is rapidly metabolized in both blood and tissues and even after blood sampling remifentanil is unstable in whole blood and plasma through endogenous esterases and chemical hydrolysis. The instability of remifentanil in these matrices makes the sample collection and processing a critical phase for the bioanalysis of remifentanil. The stability of remifentanil was investigated in EDTA whole blood, EDTA plasma and acidified EDTA plasma at ambient temperature, 4°C, 0°C and at -20°C. The stability results of remifentanil in EDTA tubes, containing whole blood, placed in ice water showed a decrease of approximately 2% in 2 hours and 42% decrease at ambient temperature. The use of formic acid to acidify the EDTA plasma improved the stability of remifentanil, which now showed to be stable for 2 days at ambient temperature, 14 days at 4°C and 103 days at -20°C. The enhanced stability has made this analytical method and sample pre-treatment very suitable for remifentanil pharmacokinetic studies.

In **chapter 5.1** and **chapter 5.2** we developed two analytical procedures for abused drugs in hair and sweat patches. Both matrices provide a non-invasive alternative to urine or blood samples.

For hair analysis, an analytical procedure was developed and validated for amphetamine, methamphetamine, MDMA, MDA, MDEA, methylphenidate, cocaine, benzoylecgonine, morphine, codeine, heroin, 6-MAM, methadone, EDDP, THC, nicotine and cotinine in human hair. All the substances mentioned above were also incorporated for the sweat patch analysis method, except for THC.

The risk of false positive results caused by external contamination of the hair was minimized by a three-step wash procedure with dichloromethane, followed by simultaneous hair pulverization and extraction procedure with disposable metal balls.

The stability investigation of the abused drugs was performed in five sweat patches which were worn for one week by different non-drug using volunteers and were spiked with the drugs afterwards. The results showed that only heroin was unstable, with high individual and thus specific patient matrix variability.

We have set the requirements for the qualifier-quantifier ratios more clear and strict than previously described in literature. During the validation, the analytical cut-off concentrations were defined for each substance at the lowest validated accuracy and precision concentration with a bias and CV within 15% and qualifier/quantifier ratios within 20% of the set ratio. This resulted in lower analytical cut-off concentrations than those 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. The developed analytical methods were used for the analysis of 47 locks of hair (segmented into in 129 samples) and 96 sweat samples and proved to be useful for monitoring drug abuse.

In **chapter 6**, General discussion and future perspectives, we discussed the performed research in relation to the objectives of the thesis. Furthermore, we discussed the impact of the investigated matrices on the performed research and the final analytical methods that were developed. Finally, we discussed the future perspectives based on the performed research, where a personalized choice of matrix may be used in order to best suit the patients' circumstances.