Summary of the PhD thesis as a "thank you" for the GTFCh travel fund for presenting at the 2011 SOFT-TIAFT Meeting in San Francisco (CA)

Comparison of the determination of drugs with influence on driving performance in serum, whole blood and dried blood spots

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1. Introduction

A total of 29,700 cases of driving under the influence of drugs (DUID) has been registered in Germany in 2009 [1]. In these cases, a blood sample is regularly taken by a medicinal practitioner, which may cause a significant delay. Hence, the blood concentration may no longer reflect actual impairment. Roadside sampling is considered to be most advantageous over blood collection at the police office.

Furthermore, forensic investigations are often performed on whole blood; it is well known that drugs are unevenly distributed between the fluid and cellular phases of blood [2-5]. Thus, directly comparing results from blood to plasma or serum concentrations derived from pharmacokinetic studies is difficult. Especially in case of concentrations being close to legal limits or at the upper or lower therapeutic range may result in major implications.

The knowledge of blood/serum (B/S) ratios might help to interpret and evaluate results derived from whole blood. B/S ratios are influenced by the concentration of the analyte itself and the hematocrit value [4; 6]. In addition, B/S ratios determined from in vitro studies might differ from B/S ratios derived from authentic blood and corresponding serum samples.

Dried blood spots (DBS) are routinely used in neonatal metabolic screening for over two decades, and have recently established themselves as a valuable tool in therapeutic drug monitoring (TDM) [7-12]. Usually, a DBS sample is collected from finger tip or heel punctures. Despite of a limited sample size of 10-100 µL blood, analysis of DBS specimens has become feasible with the advent of increasingly sensitive mass spectrometry technologies [13]. DBS can be stored at room temperature and shipped by regular mail, in contrast to whole blood or plasma specimens. Use of DBS is an appropriate method to reduce virus infection risk to a minimum which is a major concern handling samples of drug users [14; 15]. In addition, the use of DBS makes labile compounds such as ester type drugs less susceptible to degradation [14]. Being readily accessible also in subjects with limited venous access, such as e.g. injecting drug users, it represents a less invasive alternative to taking of a blood sample. Since DBS sampling can be performed by non-medical personnel, DBS might be a valuable tool for roadside sampling. With respect to all these advantages, DBS might be a useful alternative to common blood samples within the field of forensic toxicology.

2. Aims

The first goal that has been pursued was to determine the B/S ratios of dexamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), morphine, hydromorphone, fentanyl, oxycodone, risperidone, alprazolam and zopiclone from corresponding authentic samples including major metabolites. Secondly, all analytes were determined from DBS to prove whether

analysis from this matrix is as reliable as that from whole blood. Both serum and DBS were directly prepared from the whole blood sample (Fig. 1). Zopiclone being unstable in biological samples, a follow-up stability study was performed also including stress conditions and monitoring formation of 2-amino-5-chloropyridine (ACP) in whole blood and DBS.

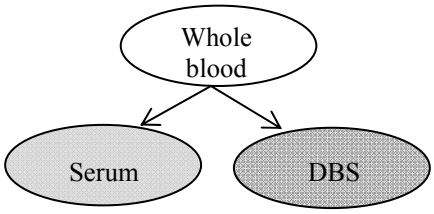

Fig. 1 Making up of a sample set for establishing blood/serum ratios and proving equivalence of DBS and blood analysis

3. Material and Methods

Sample sets (Fig. 1, whole blood and corresponding serum and DBS) from subjects were provided by several European institutions participating in the European DRIUD project. Blood samples were collected in the course of driving experiments either from healthy volunteers after ingestion of drugs (dexamphetamine, MDMA, alprazolam, zopiclone) or from patients on their regular medication (opioids, risperidone). Study protocols were approved by the particular local Ethics Committees, and subjects provided informed consent prior to participation. Participants were instructed to prepare DBS specimens by spotting 100 µL blood onto custom-made devices of Whatman #903 filter paper.

All analytes including major metabolites (3,4-methylenedioxyamphetamine (MDA), norfentanyl, noroxycodone and 9-hydroxyrisperidone (9-OH-risperidone) were determined by LC-MS/MS from either blood, serum or DBS by establishing or modifying analytical assays following validation. Validation was performed for each matrix and analyte according to the current guideline of the GTFCh [16] thus allowing a direct comparison of validation parameters between blood, serum and DBS. Analytes were isolated from the different matrices either by solid phase extraction (hydromorphone) or liquid/liquid extraction (all other analytes). For details regarding analytical procedures see references [16-18].

Mean B/S ratios, their standard deviation (SD) and range were estimated for each analyte. In addition, B/S ratios were graphically investigated to prove whether they are dependent on the analyte concentration within the respective concentration range. Lines representing the mean B/S ratio and ±1.96 SD (95% confidence interval) were added to the plot of the B/S ratios vs. the blood concentration. Ninety five % of all ratios are expected to lie within the respective confidence interval. In addition, linear regression analysis was performed (serum vs. blood concentrations).

Paired student's t-test was used to compare mean whole blood and DBS concentrations of each analyte. In addition, whole blood/DBS (WB/DBS) ratios as well as their relative standard deviations (RSD) were calculated. Statistical approaches to describe the relationship between DBS and whole blood measurement were linear regression, Bland-Altman difference, mountain and scatter plots as well as Passing-Bablok regression [19-21].

Stability of zopiclone was investigated in whole blood and corresponding DBS using freshly drawn, spiked blood (50 and 250 ng zopiclone/mL). In addition, degradation in authentic specimens (n = 10) was studied using whole blood samples from the DRUID study, which were thawed before allocating and preparation of corresponding DBS. Aliquots of 100 µL

spiked or authentic blood as well as respective DBS were stored at -20, 4, 20 and 40°C over a time period up to 30 days.

To study formation of ACP from zopiclone, a spiked blood sample (250 ng zopiclone/mL blood) and matching DBS were stored at 20°C over a time period of eight days. Further details are given in reference [18].

4. Results

4.1. Validation data

Validation parameters for all assays (serum, blood, DBS) met the criteria of the GTFCh guideline [22]. Validation data for the determination of zopiclone following liquid/liquid extraction with toluene/isoamyl alcohol (95:5 by vol.) are given in Table 1 as an example.

Tab. 1. Validation results for zopiclone measurement in serum, whole blood and DBS. RSD: relative standard deviation; LOD: limit of detection; LOQ: limit of quantitation; R: coefficient of correlation.

Validation parameter	Concentration [ng/mL]	Serum	Whole blood	DBS
Inaccuracy [RSD%]	10	-1.9	-3.4	-1.0
	50	-1.0	2.2	-0.8
Within-run imprecision [RSD%]	10	2.3	3.3	2.5
	50	3.3	3.8	2.0
Between-run imprecision [RSD%]	10	4.5	4.2	6.0
	50	4.8	3.8	4.2
Matrix effect	10	82.4	68.5	86.2
	50	80.7	80.8	91.8
Extraction efficiency	10	94.3	89.3	87.1
	50	92.7	79.2	78.9
Process efficiency	10	77.7	61.2	75.1
	50	74.8	64.0	72.4
LOD		0.3	0.3	0.1
LOQ		1.2	1.0	0.3
Linearity	2.5-50 ng/mL, y=0.02270x+0.00808, R=1.000			

Table 1 clearly shows a decrease of the matrix effect when using DBS. Extraction efficiencies were slightly lower in DBS than in whole blood or serum but did not result in higher limits of detection or quantitation. Nevertheless, an increase of process efficiency could be noticed for DBS compared to whole blood.

4.2. Blood/serum ratios

Table 2 gives an overview on the ranges of B/S ratios, their means, medians and RSDs. In addition, linear regression analysis (B/S ratios vs. blood concentrations) revealed correlation coefficients exceeding 0.92 for all analytes except for zopiclone (R = 0.855). Plotting B/S ratios against corresponding blood concentrations did not show any trend of the ratios over the particular concentration range for all analytes under investigation; there was no evidence of an inacceptable number of outliers for any analyte.

Tab. 2. Statistics of the blood/serum ratios of all analytes. n: number of the blood and serum samples.

Analyte	n	Range	Median	Mean	RSD [%]
Dexamphetamine	29	0.65-1.14	0.89	0.89	10.9
MDMA	36	0.74-1.46	1.16	1.17	10.4
MDA	29	0.95-3.02	1.63	1.69	32.1
Morphine	7	0.96-1.07	1.02	1.03	3.4
Hydromorphone	15	0.91-1.22	1.00	1.04	8.1
Fentanyl	13	0.62-1.02	0.91	0.87	14.0
Norfentanyl	13	1.03-1.29	1.22	1.19	6.8
Oxycodone	12	1.29-1.76	1.49	1.48	8.2
Noroxycodone	12	1.28-2.09	1.81	1.73	13.5
Risperidone	10	0.56-0.71	0.66	0.65	7.5
9-OH-Risperidone	14	0.61-0.91	0.73	0.73	12.3
Alprazolam	28	0.69-0.93	0.82	0.81	7.4
Zopiclone	45	0.66-1.29	0.86	0.89	16.1

4.3. Comparison of whole blood and DBS analysis

Results from whole blood and DBS were analyzed using WB/DBS ratios in addition to statistical and graphical method comparison. The number of samples as well as the particular mean and range of WB/DBS ratios are given in Table 3.

Tab. 3. Number of samples (n), blood/dried blood spot concentration ratio (WB/DBS) ranges, mean ratios and RSD of all analytes under investigation.

Analyte	n	WB/DBS range	WB/DBS mean	RSD [%]
Dexamphetamine	29	0.87-1.07	0.95	5.4
MDMA	36	0.92-1.09	0.99	2.9
MDA	32	0.80-1.18	0.98	6.4
Morphine	7	0.99-1.05	1.01	2.3
Hydromorphone	15	0.92-1.16	1.01	6.7
Fentanyl	13	0.84-1.11	1.01	7.9
Norfentanyl	13	0.96-1.19	1.04	6.2
Oxycodone	12	0.91-1.02	0.98	3.4
Noroxycodone	12	0.95-1.12	1.00	4.6
Risperidone	10	1.03-1.17	1.07	3.7
9-OH-Risperidone	14	0.97-1.10	1.04	4.6
Alprazolam	28	0.80-1.09	0.98	6.3
Zopiclone	45	0.82-1.59	1.19	15.6

The mean WB/DBS ratios of all compounds except zopiclone were within a range of 0.95-1.07, their RSDs spread from 2.3 to 7.9 %. For zopiclone, the mean WB/DBS ratio was 1.19 with a RSD of 15.6 %.

Additional statistical analyses such as the t-test, Bland-Altman difference-, mountain- and scatter plots as well as Passing-Bablok regression analysis were performed to evaluate equivalence of measurement between whole blood and DBS. Dexamphetamine and zopiclone were used as examples exhibiting the most and less accurate equivalence, respectively, between the two matrices (Table 4, Fig. 2A and B).

Tab. 4. Results of whole blood and DBS measurement analysis for dexamphetamine and zopiclone, P-B-regression: Passing-Bablok regression.

Test	Criterion	Dexamphetamine	Zopiclone
t-test	$mean_{blood} = mean_{DBS}$	no	no
Bland-Altman-difference plot	mean difference	-1.03	3.99
	expressed as % of $mean_{blood}$	-5.01	15.29
Mountain plot	median difference [ng/mL]	-1.05	3.11
	expressed as % of $mean_{blood}$	-5.10	11.92
Scatter plot	regression coefficient	0.9879	0.8558
P-B-regression	95 % CI of slope includes 1.0	yes	yes
	95 % CI of intercept incluces 0.0	yes	yes

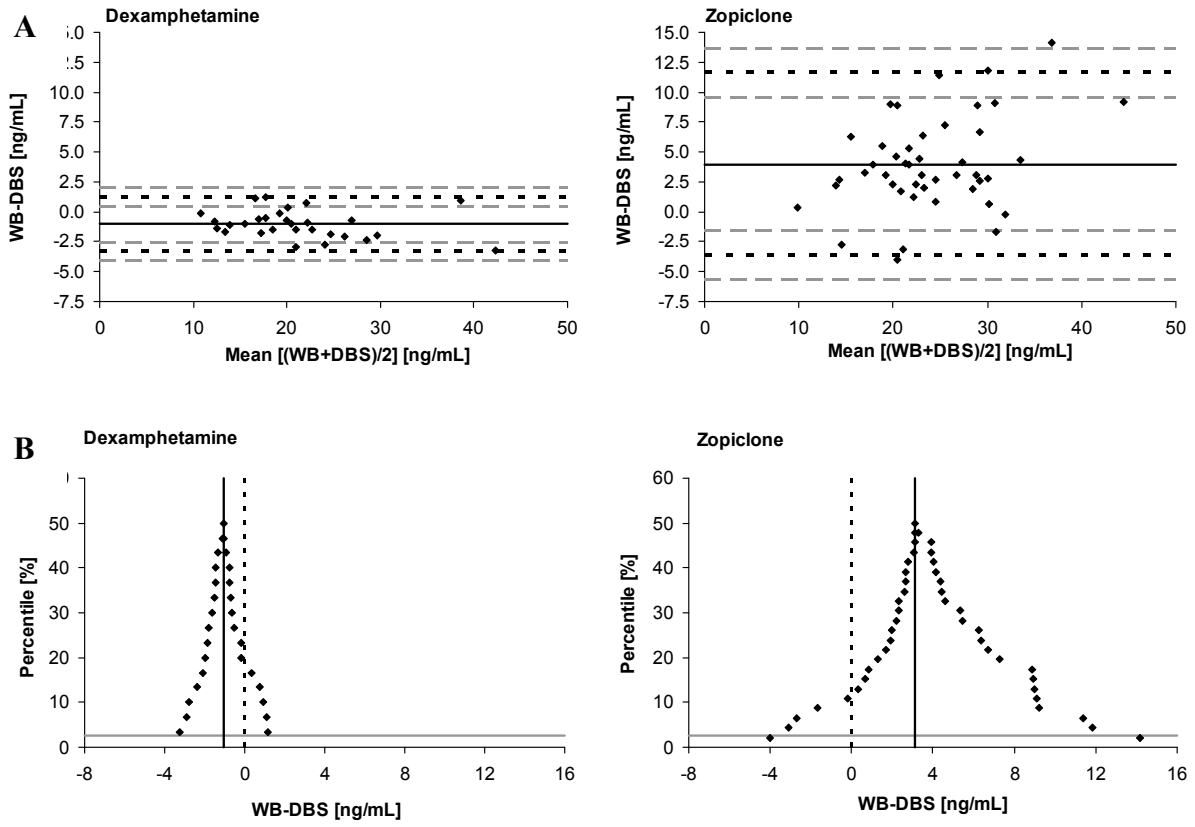

Fig. 2. A: Bland-Altman difference plot of dexamphetamine and zopiclone. Solid black lines: mean difference of whole blood and DBS concentrations; dotted black lines: the limits of agreement set to mean ± 1.96 x SD; dotted grey lines: 95 % confidence intervals of the limits of agreement.
B: Mountain plot of dexamphetamine and zopiclone, respectively. Grey lines: 2.5 % and 97.5 % percentile, respectively; dotted black lines: optimum median value of zero; solid black lines: median difference of whole blood and DBS concentrations.

It is obvious and supported by statistical and graphical analyses that measurement of dexamphetamine from DBS more closely agrees to that from whole blood compared to zopiclone. This may be caused by zopiclone's instability in biological samples. Therefore, a follow-up stability study was carried out in order to investigate degradation of zopiclone in spiked and authentic whole blood and DBS specimens.

4.4. Stability of zopiclone in corresponding whole blood and DBS samples

Generally, degradation of a compound increases with increasing temperature and time. In both spiked and authentic samples breakdown of zopiclone occurred faster in fluid blood than in corresponding DBS. In spiked samples, stability did not differ between low and high concentration levels.

The initial concentration of zopiclone in authentic blood samples averaged 20.1 ng/mL (11.5-26.9 ng/mL). Adapting the 15% degradation criterion of Nowatzke and Woolf [23], stability could be assured in frozen authentic whole blood samples for 15 days. After 30 days of storage at -20 and 4°C the mean initial concentration had decreased by 22 % and 52 %, respectively. At 20°C, more than 15 % of zopiclone had disappeared on day 3; after 8 days, only 15 % of the initial concentration could be detected. The analyte was not detectable in samples stored at 40°C for the same period of time, and had decreased by more than 15% already within one day. In corresponding DBS, 89, 84, 73 and 58 % of the initial mean concentration were still detectable after storage at -20, 4, 20 and 40°C for 30 days. It can be concluded that zopiclone is stable in authentic DBS for 30, 22, 8 and 3 days when stored at -20, 4, 20 and 40°C, respectively.

In the 3rd part of the study, 25 % and 87 % of the initial zopiclone concentration could be determined in whole blood and DBS, respectively, on day 8 at 20°C. ACP was formed from zopiclone in equimolar amounts in both media as shown in Figure 3.

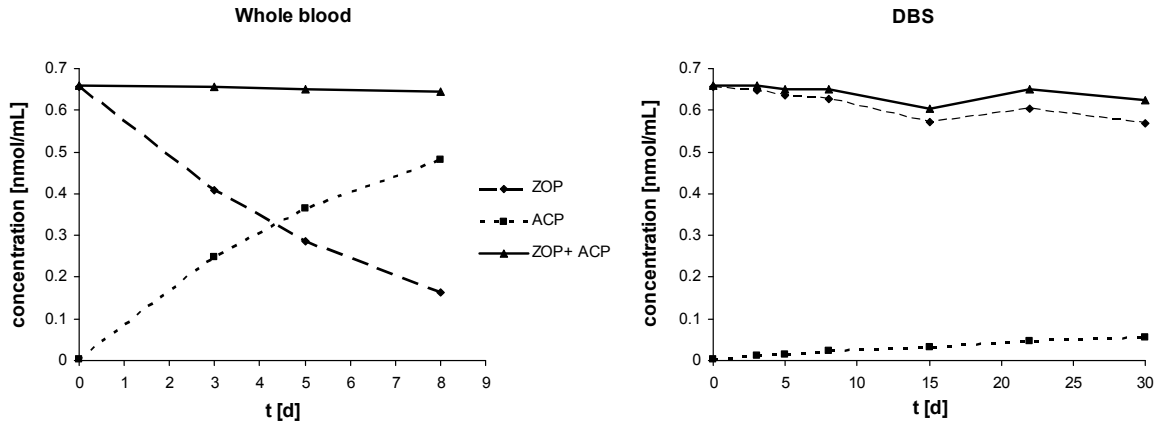

Fig. 3. Zopiclone degradation and ACP formation in spiked whole blood and DBS at 20°C.

5. Discussion

The present B/S ratios are widely in line with those reported in literature (Table 5) as far as available. Distribution ratios are generally derived from *in vitro* partition experiments where plasma water, plasma proteins and red blood cells are pooled. Some caution is advisable using these data. When spiked blood is diluted with autologous plasma water, erythrocytes may discharge the compound over proportionally compared to plasma proteins.

Tab. 5. B/S ratios of all analytes under investigation compared with literature data, as far as available. n.a.: not available; *determination of the B/S ratio from specimens containing racemic amphetamine.

Analyte	Concentration range [ng/mL serum]	B/S ratio; mean ±SD [%]	Published value	Reference
Dexamphetamine	12.2-41.1	0.89 ±0.10	0.91*	[5]
MDMA	0.6-388.6	1.17 ±0.12	1.16	[2]
MDA	0.9-13.5	1.69 ±0.54	1.27	[2]
Morphine	85.7-468.9	1.03 ±0.04	1.02	[24]
Hydromorphone	2.4-19.7	1.04 ±0.08	1.35	[25]
Fentanyl	0.2-8.7	0.87 ±0.12	1.01	[26]
Norfentanyl	0.07-1.82	1.19 ±0.08	n.a.	n.a.
Oxycodone	11.6-89.8	1.48 ±0.12	n.a.	n.a.
Noroxycodone	11.0-71.1	1.73 ±0.23	n.a.	n.a.
Risperidone	1.7-36.8	0.65 ±0.05	0.67	[27]
9-OH-risperidone	4.9-46.5	0.73 ±0.09	n.a.	n.a.
Alprazolam	3.1-27.5	0.81 ±0.06	0.80	[28]
Zopiclone	11.9-50.4	0.89 ±0.14	1.0	[29]

Generally, concentration ratios between blood and plasma may vary from 0.5 to 2.0 such as e.g. for phenytoin and maprotiline, respectively [15].

Published ratios of dexamphetamine, MDMA, morphine, risperidone and alprazolam could be confirmed. Differences between published and present B/S ratios could be observed for MDA, hydromorphone, fentanyl as well as for zopiclone. For norfentanyl, oxycodone, noroxycodone and 9-OH-risperidone, no such data exists.

In case of MDA, B/S concentration ratios scattered over a wider range compared to that of a study published by Garcia Boy et al. [2]. The lower limit of detection and serum values of the published data being significantly higher than those determined in the present study, this result is not unexpected. In relation to the small number of samples exceeding the lower limit of detection, the B/S ratio determined by Garcia Boy et al. [2] allows a rough estimation but should not be considered for a direct conversion.

For hydromorphone and zopiclone, published data significantly differ from B/S ratios determined in the present study. Parab et al. [25] published a considerably higher B/S ratio of 1.35 for hydromorphone. Also, the respective ratio of zopiclone given by Bramness et al. [29] differs from the present ratio. Unfortunately, neither Parab et al. [25] nor Bramness et al. [29] provide any information on the determination of B/S ratios. However, knowledge on the concentration range and if ratios have been determined from spiked, authentic or even postmortem samples is a major prerequisite for applying such data.

Bower and Hull [30] investigated the distribution of fentanyl between erythrocytes and plasma. The erythrocyte/plasma ratio has afterwards been cited as a blood/plasma ratio, irrespective of the significant difference between packed erythrocytes and whole blood. Packed erythrocytes were suspended in aqueous buffer thus not taking the protein binding of fentanyl in whole blood into account. Erythrocytes cannot be completely separated from whole blood sample by centrifugation whereby about 3.4-3.9 % of the total volume will remain as plasma between the erythrocytes [31]. Therefore, a comparison of the present value with that of Bower and Hull [30] will be meaningless.

The present B/S ratios being all derived following therapeutic dosages or administration of rather low amounts of drugs should cautiously be applied if highly dosed or toxic blood concentrations have to be evaluated.

DBS provide numerous advantages over conventional blood samples concerning collection, risk of infections, sample shipping, storage and analyte stability. Therefore, DBS might also be beneficial in the field of forensic toxicology. First of all, however, it has to be investigated if DBS analysis is in agreement with whole blood analysis for forensically relevant analytes. In the present study equivalence of whole blood and DBS methods could be proven for dexamphetamine, MDMA and MDA, morphine, hydromorphone, fentanyl and norfentanyl, oxycodone and noroxycodone, risperidone and 9-OH-risperidone as well as for alprazolam.

WB/DBS ratios of all drugs except zopiclone indicate equivalence between whole blood and DBS analyses. In addition, graphical analysis of the data which is given for dexamphetamine and zopiclone (Fig. 2A and B) shows that results from whole blood and DBS did not significantly differ.

The advantages of DBS analysis have already been shown for cocaine [14], benzoylecgonine [32], and morphine [33]. Furthermore, Schütz et al. [34] were able to reconstruct the chronology of drug intake and accident analyzing blood stains from the particular vehicle.

A zopiclone WB/DBS ratio of 1.19 indicates an underestimation of results obtained from DBS compared to those from whole blood. This is supported by a mean difference of 3.99 ng/mL, which can be deduced from Bland-Altman analysis (Tab. 4). A likely explanation is that whole blood samples had been stored at -20°C prior to analysis, whereas DBS had been kept at ambient temperature. Degradation of zopiclone to 2-amino-5-chloropyridine may have occurred during storage according to the scheme given in Fig. 4 [35].

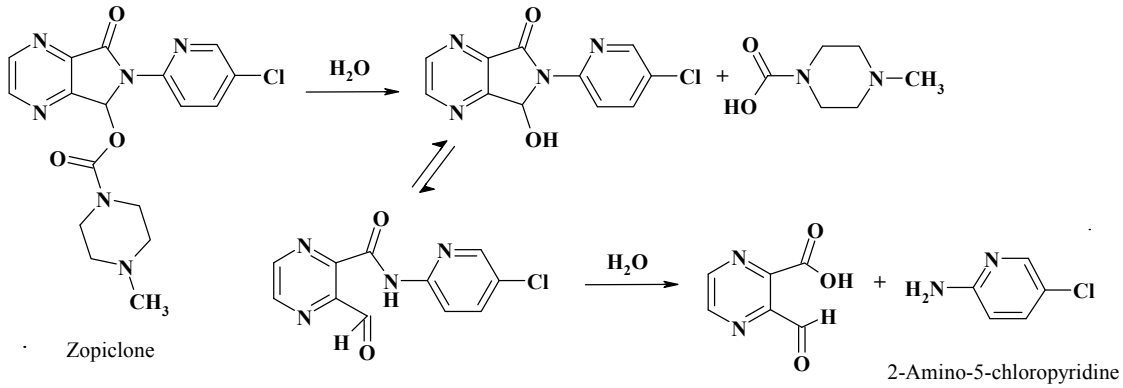

Fig. 4. Hydrolysis of zopiclone and formation of 2-amino-5-chloropyridine as a stable final product

To investigate the influence of storage time and temperature on the degradation of zopiclone in both whole blood and DBS a follow-up stability study has been performed. The results observed for spiked blood samples are in line with previously published data by Nilsson et al. [36] where degradation of zopiclone increases with increasing temperature and time.

Irrespective of the storage condition and whether spiked or authentic blood had been analyzed, it is evident that stability of zopiclone is enhanced using DBS instead of conventional blood (Fig. 3). A storage temperature of -20°C was the sole exception, where no significant difference between stability in whole blood and DBS could be observed. The water content in DBS is definitely lower and may therefore reduce degradation of zopiclone. The higher degradation rate in whole blood reflects that use of DBS is appropriate to stabilize zopiclone. In previously published reports, degradation of forensically relevant analytes prone to hydrolysis in whole blood such as cocaine or benzodiazepines could also be slowed using DBS [37].

In the last part of the stability study, it could be shown that ACP was formed from zopiclone in an equimolar amount not only in blood but also in DBS, suggesting that zopiclone undergoes hydrolysis only. Estimates of the mass balance for both whole blood and DBS at each assigned time largely coincide and can be ascribed to the initial concentration of zopiclone. Hence, it can be concluded that zopiclone is predominantly degraded to ACP, and hydrolysis is suggested as the exclusive degradation pathway of the drug in whole blood and DBS. These results are in line with those published for whole blood by Nilsson et al. [36], who also observed that ACP is formed from zopiclone in a stoichiometrically balanced reaction.

6. Conclusion

The B/S ratios determined in the present study are widely in line with literature data, as far as available. In addition, inter-subject variability could be estimated. Knowledge of these ratios and their range is mandatory to valuably compare drug concentrations measured either in whole blood or plasma and to reliably interpret results derived from whole blood analysis in forensic cases.

DBS assay has potential as a precise and inexpensive option for the determination of amphetamine-type drugs, opioids, risperidone and 9-hydroxyrisperidone and alprazolam using a small blood volume. WB/DBS ratios were very close to 1.00, except zopiclone which is prone to degradation.

A separately performed stability experiment could prove that zopiclone is more stable in DBS than in whole blood stored under the same condition. The degradation product ACP should be determined simultaneously to enable a rough estimation of the actual zopiclone concentration in either DBS or whole blood.

From the present results it can be concluded that DBS are not only beneficial in neonatal screening or TDM but may also valuably be employed in forensic toxicology.

7. Acknowledgements

It is with sincere gratitude that I acknowledge the support of my supervisor Professor Dr. Gisela Skopp for this very interesting dissertation topic, her expertise and feedback. I have to thank her for encouraging and challenging me. Special thanks go to Andrea Stadler (neé Faller) for her suggestions and discussions as well as for getting through this in friendship. In addition, I would like to thank all the staff for their help and support as well as for the friendly working atmosphere.

I would like to thank the EU for supporting this work by a grant from the EU-project DRUID (TREN-05-FP6TR-So7.61320-518404). I acknowledge the support from our partners Markus Schumacher from Bergisch-Gladbach, Wendy Bosker and Jan Ramaekers from Maastricht, Janet Veldstra and Karel Brookhuis from Groningen, Katerina Touliou from Thessaloniki and Gian Marco Sardi from Rome for their friendly collaboration and diligently providing of samples.

Finally, I greatly appreciate the support of the GTFCh - in particular Prof. Dr. H. Maurer - for providing the travel fund and giving me the opportunity to present my work at the 2011 SOFT-TIAFT Meeting in San Francisco (CA).

8. References

[1] Kraftfahrt-Bundesamt (KBA), Jahresbericht 2009, Flensburg, 2010.

[2] Garcia Boy R, Henseler J, Ramaekers JG, Mattern R, Skopp G. A comparison between experimental and authentic blood/serum ratios of 3,4-methylenedioxymethamphetamine and 3,4-methylenedioxyamphetamine. J Anal Toxicol 2009;33:283-286.

[3] Skopp G, Pötsch L, Mauden M, Richter B. Partition coefficient, blood to plasma ratio, protein binding and short-term stability of 11-nor-Δ^9-carboxy tetrahydrocannabinol glucuronide. Forensic Sci Int 2002;126:17-23.

[4] Skopp G, Pötsch L, Ganßmann B, Aderjan R, Mattern R. A preliminary study on the distribution of morphine and its glucuronides in the subcompartments of blood. J Anal Toxicol 1998;22:261-264.

[5] Skopp G, Jantos R, Blood to plasma ratios of amphetamine and MDMA and analysis of dried blood spots. DRUID project deliverable 1.2.1, 2010.

[6] Skopp G, Klinder K, Poetsch L, Zimmer G, Lutz R, Aderjan R, Mattern R. Postmortem distribution of dihydrocodeine and metabolites in a fatal case of dihydrocodeine intoxication. Forensic Sci Int 1998;95:99-107.

[7] Chace DH, Kalas TA, Naylor EW. Use of tandem mass spectrometry for multianalyte screening of dried blood spot specimens from newborns. Clin Chem 2003;49:1797-1817.

[8] Edelbroek PM, van der Heijden J, Stolk LML. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. Ther Drug Monit 2009;31:327-336.

[9] Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of human infants. Pediatrics 1963;32:338-343.

[10] Mei JV, Alexander JR, Adam BW, Hannon WH. Use of filter paper for the collection and analysis of human whole blood specimens. J Nutr 2001;131:1631S-1636S.

[11] Parker SP, Cubitt WD. The use of dried blood spot sample in epidemiological studies. J Clin Pathol 1999;52:633-639.

[12] Spooner N, Lad R, Barfield M. Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: considerations for the validation of a quantitative bioanalytical method. Anal Chem 2009;81:1557-1563.

[13] Li W, Tse FLS. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. Biomed Chromatogr 2010;24:49-65.

[14] Skopp G, Pötsch L. Nachweis von Cocain in Blutspots. Arch Kriminol 2001;207:81-88.

[15] Skopp G. Preanalytic aspects in postmortem toxicology. Forensic Sci Int 2004;142:75-100.

[16] Jantos R, Schuhmacher M, Veldstra JL, Bosker WM, Klöpping-Ketelaars I, Touliou K, Sardi GM, Brookhuis KA, Ramaekers JG, Mattern R, Skopp G. Bestimmung der Blut/Serum-Verhältnisse verschiedener forensisch-relevanter Analyten in authentischen Proben. Arch Kriminol 2011;227:188-203.

[17] Jantos R, Skopp G. Comparison of drug analysis in whole blood and dried blood spots. Toxichem Krimtech 2011;35:268-275.

[18] Jantos R, Vermeeren A, Sabljic D, Ramaekers JG, Skopp G. Degradation of zopiclone during storage of spiked and authentic whole blood and matching dried blood spots. Int J Legal Med 2012; Epub ahead of print:DOI: 10.1007/s00414-012-0696-4.

[19] Bland JM, Altman DG. Measuring agreement in method comparison studies. Stat Methods Med Res 1999;8:135-160.

[20] Krouwer JS, Monti KL. A simple, graphical method to evaluate laboratory assays. Eur J Clin Chem Clin Biochem 1995;33:525-527.

[21] Passing H, Balbok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. J Clin Chem Clin Biochem 1983;21:709-720.

[22] Peters FT, Hartung M, Herbold M, Schmitt G, Daldrup T, Mußhoff F. Anhang B zur Richtlinie der GTFCh zur Qualitätssicherung bei forensisch-toxikologischen Untersuchungen. Anforderungen an die Validierung von Analysenmethoden. Toxichem Krimtech 2009;76:185-208.

[23] Nowatzke W, Woolf E. Best practices during bioanalytical method validation for the characterization of assay reagents and the evaluation of analyte stability in assay standards, quality controls, and study samples. The AAPS Journal 2007; 9:E117-E122.

[24] Hand CW, Moore RA. Comparison of whole blood and plasma morphine. J Anal Toxicol 1988;12:234-235.

[25] Parab PV, Ritschel WA, Coyle DE, Gregg RV, Denson DD. Pharmacokinetics of hydromorphone after intravenous, peroral and rectal administration to human subjects. Biopharm Drug Dispos 1988;9:187-199.

[26] Bower S. The uptake of fentanyl by erythrocytes. J Pharm Pharmacol 1982;34:181-185.

[27] Mannens G, Meuldermans W, Snoeck E, Heykants J. Plasma protein binding of risperidone and its distribution in blood. Psychopharmacology (Berl) 1994;114:566-572.

[28] Crouch DJ, Rollins DE, Canfield DV, Andrenyak DM, Schultis JE. Quantitation of alprazolam and α-hydroxyalprazolam in human plasma using liquid chromatography electrospray ionization MS-MS. J Anal Toxicol 1999;23:479-485.

[29] Bramness JG, Arnestad M, Karinen R, Hilberg T. Fatal overdose of zopiclone in an elderly woman with bronchogenic carcinoma. J Forensic Sci 2001;46:1247-1249.

[30] Bower S, Hull CJ. Comparative pharmacokinetics of fentanyl and alfentanyl. Br J Anaesth 1982;54:871-877.

[31] Driessen O, Highley MS, Harper PG, Maes RA, De Bruijn EA. Description of an instrument for separation of red cells from plasma and measurement of red cell volume. Clin Biochem 1994;27:195-196.

[32] Henderson LO, Powell MK, Hannon WH, Miller BB, Martin ML, Hanzlick RL, Vroon D, Sexson WR. Radioimmunoassay screening of dried blood spot materials for benzoylecgonine. J Anal Toxicol 1993;17:42-47.

[33] Garcia Boy R, Henseler J, Mattern R, Skopp G. Determination of morphine and 6-acetylmorphine in blood with use of dried blood spots. Ther Drug Monit 2008;30:733-739.

[34] Schütz H, Gotta JC, Erdmann F, Rißе M, Weiler G. Simultaneous screening and detection of drugs in small blood samples and bloodstains. Forensic Sci Int 2002; 126:191-196.

[35] Mannaert E, Tytgat J, Daenens P. Detection of 2-amino-5-chloropyridine in urine as a parameter of zopiclone (Imovane) intake using HPLC with diode array detection. J Anal Toxicol 1997;21:208-212.

[36] Nilsson GH, Kugelberg FC, Kronstrand R, Ahlner J. Stability tests of zopiclone in whole blood. Forensic Sci Int 2010;200:130-135.

[37] Alfazil AA, Anderson RA. Stability of benzodiazepines and cocaine in blood spots stored on filter paper. J Anal Toxicol 2008;32:511-515.