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Comparison of drug analysis in whole blood and dried blood spots

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Abstract

Aim: Analysis of dried blood spots (DBS) becomes increasingly accepted in therapeutic drug monitoring whereas its application by analogy to forensic samples has not been further studied. Contrary to whole blood, DBS sampling is easier, allows storage without additional cooling and decreases the risk of infections with blood-borne viruses. The aim of our study was to investigate whether determination of alprazolam, risperidone, 9-hydroxyrisperidone, zopiclone, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA) and dexamphetamine from DBS is as reliable as from whole blood.

Methods: DBS and whole blood analysis was performed using 100 µL-specimens. Analysis was performed by LC/MS/MS following liquid-liquid extraction. Results from respective samples were compared using Bland-Altman difference plots.

Results and Discussion: The number of corresponding specimens, the mean concentration ratio (r), the mean difference between the 2 methods (d) and the limits of agreement (l, mean difference ± 1.96 SD) for each analyte were as follows:
Alprazolam: n=22, r=0.99, d= -0.09 ng/mL, l: -1.11 to 0.92 ng/mL; risperidone: n=10, r=1.07, d=0.83 ng/mL, l: -0.67 to 2,32 ng/mL; 9-hydroxyrisperidone: n=14, r=1.04, d=0.64 ng/mL, l: -1.13 to 2.40 ng/mL; zopiclone: n=45, r=1.19, d=3.99 ng/mL, l: -3.62 to 11.59 ng/mL; MDMA: n=35, r=0.99, d= -3.55 ng/mL, l: -14.34 to 7.25 ng/mL; MDA: n=30, r=0.99, d=0.02 ng/mL, l: -1.36 to 1.40 ng/mL; dexamphetamine: n=29, r=0.95, d= -1.03 ng/mL, l: -3.32 to 1.25 ng/mL. Variability of differences between methods was fairly constant across the range of measurement for all analytes. At least 95 % of all differences were within the limits of agreement.

Conclusion: For all analytes except zopiclone results from DBS exactly matched those from whole blood. The blood/DBS-ratio of zopiclone significantly differed from 1.00; the Bland-Altman difference plot showed 3 outliers, 2 of them were close to the limits of agreement. This may be due to zopiclone's lability, which is currently under investigation.

1. Introduction

Dried blood spots (DBS) have routinely been used in neonatal metabolic screening for over two decades, and have recently established themselves as a valuable tool in therapeutic drug monitoring [1-6]. Despite a limited sample size of 10-100 µL blood, analysis of DBS specimens has become feasible with the advent of increasingly sensitive MS technologies [7]. 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 from drug users [8, 9]. Being readily accessible also in subjects with limited venous access, such as e.g. injecting drug users, it represents a valuable and less invasive alternative to taking of a blood sample. DBS

sampling can also be performed by non-medical personnel. In addition, the use of DBS makes labile compounds such as ester type drugs less susceptible to degradation [9].
The main objectives of the present study were to check whether amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), risperidone and its active metabolite 9-hydroxyrisperidone, alprazolam and zopiclone can be determined in DBS as reliable as in whole blood samples. It is of interest whether DBS may be an alternative for the respective determination from whole blood in forensic cases.

2. Materials and methods

Whole blood and DBS samples from healthy volunteers were provided by European co-operating institutions who carried out driving experiments after ingestion of amphetamine-type drugs, risperidone, alprazolam and zopiclone. The study protocols were approved by the local Ethics Committees, and subjects provided informed consent prior to participation.

2.1. Materials

A custom made blood spot card was used for collection of DBS in the present investigation which was made from #903 specimen collection paper (GE Healthcare, Dassel, Germany) being a FDA listed class II medical device. Both, the manufacturing and post-printing quality of the paper were checked.

Zopiclone was purchased from Rhône-Poulenc Rorer GmbH (Köln); risperidone, 9-hydroxy-risperidone and didehydromethylrisperidone (internal standard) were obtained from Janssen-Cilag (Neuss, Germany). All other undeuterated and deuterated standards (1 or 0.1 mg/mL methanol) were provided by LGC (Wesel, Germany)
Methanol and acetonitrile (both HPLC grade) were obtained from Roth (Karlsruhe); all other solvents and chemicals were of the highest purity grade available.

2.2. Sample preparation

DBS samples were prepared by spotting 100 µL of blood onto filter paper which was dried at room temperature over night and stored at ambient temperature (20-24°C). Blood samples were kept frozen (-20°C) and thawed just prior to analysis using 100 µL aliquots. Before analysis, DBS were cut out completely with a punch (diameter 18 mm) and transferred into plastic tubes.

To each amphetamine and MDMA sample, calibrator or control 1 mL 0.01 M NaOH and the internal deuterated standards were added. Samples were extracted with 1.5 mL ethyl acetate and centrifuged (10 min, 4300*x*g). The organic layer was transferred to a silanized vial, acidified with 50 µL of methanolic hydrochloric acid (MeOH/HCl, 49:1, v/v) and evaporated to dryness under nitrogen at 40°C. The residue was reconstituted in 50 µL mobile phase (acetonitrile/methanol/4 mM ammonium acetate pH 3.2, 32:8:60, v/v/v).

Zopiclone and alprazolam samples were extracted by adding 1 mL borate buffer pH 8.5, the internal standards (lorazepam-d_4 and alprazolam-d_5, respectively) as well as 1 mL toluene/isoamylalcohol (95:5, v/v). Following centrifugation (10 min, 4300*x*g) and evaporation of the organic layer the residue was re-dissolved in mobile phase (zopiclone: 100 µL acetonitrile/methanol/4 mM ammonium acetate buffer pH 3.2, 48:12:40, v/v/v; alprazolam: 50 µL acetonitrile/methanol/4 mM ammonium acetate buffer pH 3.2, 44:11:45, v/v/v).
Extraction of risperidone and 9-hydroxyrisperidone was achieved by adding 1 mL borate buffer pH 8.5, the internal standard and 1.0 mL ethyl acetate. After extraction and

centrifugation (10 min, 4300*x*g) the organic phase was evaporated to dryness under nitrogen (40°C). The residue was re-dissolved in 50 µL of the mobile phase (acetonitrile/methanol/4 mM ammonium acetate buffer pH 3.2, 40:10:50, v/v/v).

2.3. Instrumentation

Analysis was performed on an API 4000 tandem mass spectrometer (AB Sciex, Darmstadt) with a TurboIon ionization source (positive mode) interfaced to a HPLC pump and an autosampler (1100 series, Agilent, Waldbronn). Separation of the amphetamine-type drugs as well as of risperidone and its active metabolite 9-hydroxyrisperidone was achieved on a Zorbax Eclipse XDB C8 column (2.1 x 150 mm, 5 µm particle size; Agilent, Waldbronn). Alprazolam and zopiclone were eluted from a Phenomenex Luna C8 column (2.0 x 150 mm, 5 µm particle size, Phenomenex, Aschaffenburg).

The mobile phase consisted of variable proportions of acetonitrile, methanol and 4 mM ammonium acetate pH 3.2.

Transitions monitored for quantitation were: risperidone m/z 411→191; 9-hydroxyrisperidone m/z 427→207; didehydromethylrisperidone m/z 421→201; amphetamine (-d_5) m/z 136→91 (141→124); MDMA (-d_5) m/z 194→163 (199→165); MDA (-d_5) m/z 180→163 (185→168); alprazolam (-d_5) m/z 309→205 (314→210), zopiclone m/z 389→245, lorazepam-d_4 m/z 325→307.

2.4. Evaluation

The methods were validated according to the current validation guideline of the GTFCh. The following parameters were checked: extraction efficiency, carry over, linearity, LLOD, LLOQ, ion suppression, linearity and imprecision, bench top stability as well as matrix effects [10, 11].

To check whether DBS analysis provides results which are equivalent to those using whole blood samples, results from blood and DBS were compared using blood/DBS ratios (b/DBS) and the corresponding relative standard deviations (RSD) for each analyte. Agreement of the two methods was further assessed by Bland-Altman difference plots [12].

3. Results and Discussion

3.1. Evaluation

Table 1 summarizes some of the evaluation data determined in DBS for all analytes. Additionally, matrix effects, extraction efficiency and bench top stability were checked; all values were in an acceptable range (data not shown). Carry over could not be observed for any analyte. There were significant differences between the validation results neither in whole blood nor in DBS; all parameters for whole blood were in the same range as given in table 1 or better. Matrix effects were slightly less distinctive in DBS than in whole blood.

Tab. 1. Validation results for the determination of drugs in DBS.

Analyte	LLOD [ng/mL]	LLOQ [ng/mL]	Between-run imprecision [%]	Within-run imprecision [%]	Linearity
Amphetamine	0.8	3.0	50 ng/mL: 5.1	50 ng/mL: 2.2	5-50 ng/mL r=0.9996
MDMA	1.6	5.7	50 ng/mL: 5.0 250 ng/mL: 4.1	50 ng/mL: 3.1 250 ng/mL: 2.8	50-400 ng/mL r=0.9987
MDA	0.1	0.4	50 ng/mL: 2.4	50 ng/mL: 1.9	2.5-30 ng/mL r=0.9990
Risperidone	0.3	1.2	6.7 ng/mL: 3.1 19.75 ng/mL: 3.9	6.7 ng/mL: 2.6 19.75 ng/mL: 3.9	5-25 ng/mL r=1.0000
9-Hydroxyrisperidone	0.3	1.3	10 ng/mL: 3.7 40 ng/mL: 5.8	10 ng/mL: 2.1 40 ng/mL: 4.9	15-75 ng/mL r=0.9983
Alprazolam	0.2	0.7	5 ng/ml: 7.2 30 ng/mL: 5.2	5 ng/ml: 5.3 30 ng/mL: 4.0	2.5-50 ng/mL r=0.9999
Zopiclone	0.3	1.2	10 ng/mL: 6.0 50 ng/mL: 4.2	10 ng/mL: 2.5 50 ng/mL: 2.0	2.5-50 ng/mL r=1.0000

3.2. Agreement of the determination from whole blood and DBS

Table 2 gives an overview of the b/DBS ratios and their respective RSD determined for the chosen analytes. Ideally, the mean b/DBS ratio should be equal to 1.00, which means that results from whole blood and DBS analysis do not differ.

Tab. 2. Blood/DBS ratio, RSD and blood concentration range.

Analyte	n	Concentration range in blood [ng/mL]	Blood/DBS range	Blood/DBS mean	RSD [%]
Amphetamine	29	10.8-40.7	0.87-1.07	0.95	5.40
MDMA	35	7.0-444.3	0.92-1.03	0.99	2.41
MDA	30	1.1-24.4	0.92-1.18	0.99	5.52
Risperidone	10	4.3-20.7	1.03-1.17	1.07	3.66
9-Hydroxyrisperidone	14	4.1-30.3	0.97-1.10	1.04	4.55
Alprazolam	22	3.7-20.7	0.80-1.09	0.99	6.13
Zopiclone	45	10.1-49.1	0.82-1.59	1.19	15.56

Ratios and RSD from paired samples containing amphetamine-type drugs such as MDMA, MDA and amphetamine account for equality of analysis between whole blood and DBS. The mean b/DBS ratio of risperidone was not as close to 1.00 as the respective ratios of the other analytes except zopiclone. The risperidone Bland-Altman difference plot (figure 1) may, however, be useful to evaluate whether its determination from DBS is equivalent to that from whole blood.

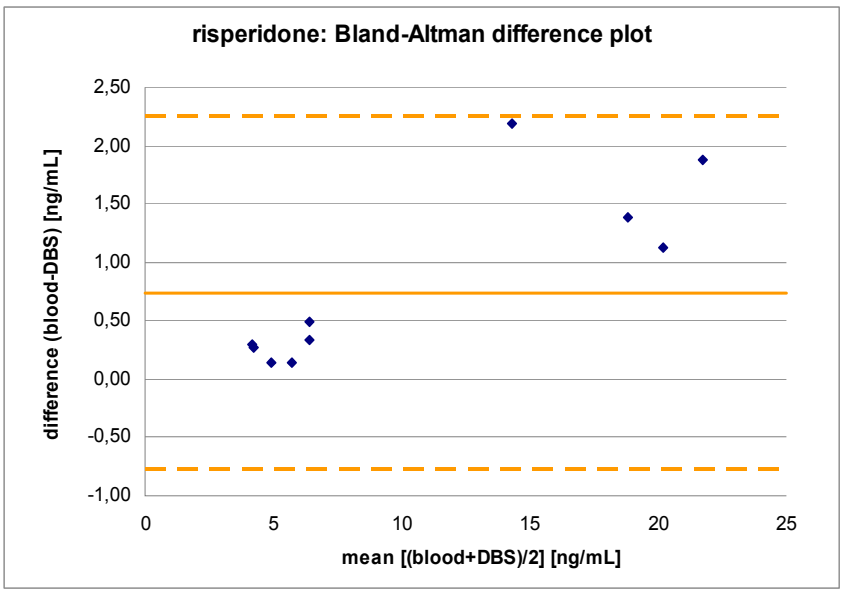

Fig. 1. Bland-Altman difference plot for risperidone. The solid line illustrates the mean difference of 0.83 ng/mL, the dotted lines indicate the limits of agreement set to 1.96*x*SD (-0.67 to 2.32 ng/mL).

The Bland-Altman difference plot of risperidone clearly indicated that determination of risperidone from either whole blood or DBS does not differ. None of the measured quantities was outside the limits of agreement. A mean difference of 0.83 ng/mL is quite low, and only accounts for 7.4 % with regard to a mean blood concentration of risperidone of 11.1 ng/mL. Overall, both methods can be regarded as equivalent.
A b/DBS ratio of 1.19 for zopiclone indicates an underestimation of the results from DBS compared to those from whole blood. This underestimation is supported by a mean difference of 3.99 ng/mL evaluated from the Bland-Altman difference plot of zopiclone (figure 2).

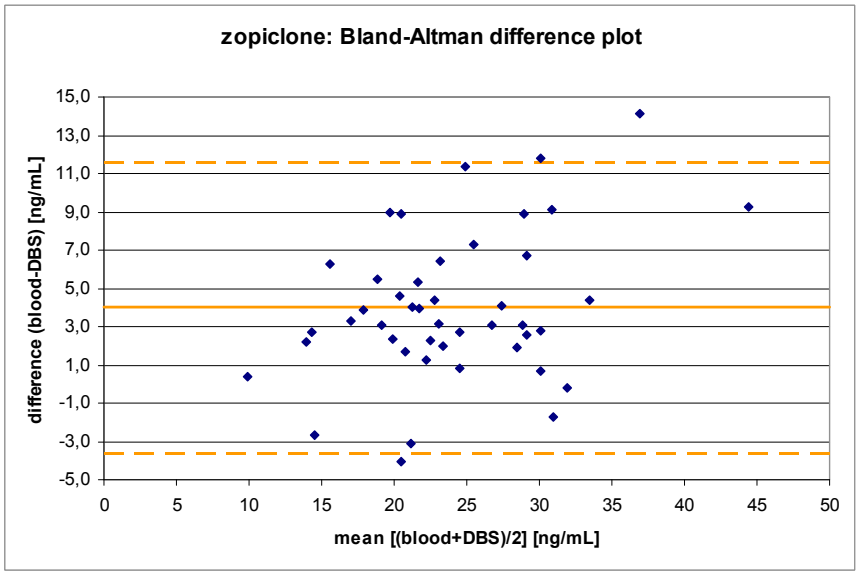

Fig. 2. Bland-Altman difference plot for zopiclone. The solid line illustrates the mean difference of 3.99 ng/mL, the dotted lines indicate the limits of agreement set to 1.96xSD (-3.62 to 11.59 ng/mL).

Whole blood samples were stored at -20°C prior to analysis, whereas DBS were kept at ambient temperature. With respect to the different temperatures of storage, degradation of zopiclone to 2-amino-5-chloropyridine may have occurred according to the scheme given in figure 3:

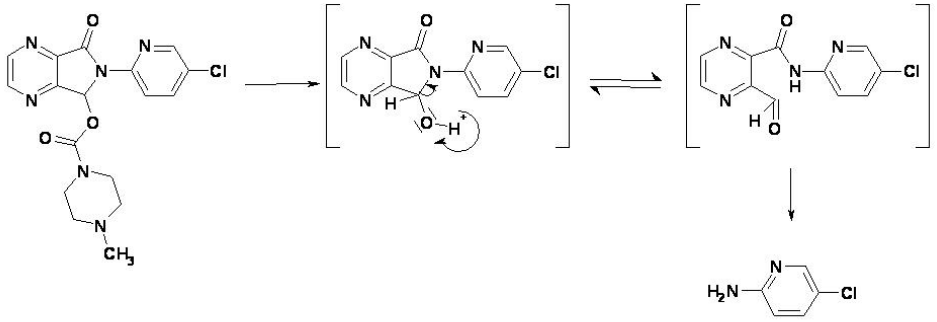

Fig. 3. Degradation scheme of zopiclone to 2-amino-5-chloropyridine modified according to [13].

Degradation of zopiclone has recently been investigated for whole blood samples by Nilsson et al. [14]. Currently, stability investigations are carried out to compare the degradation of the analyte in whole blood and DBS and to find out whether DBS will perform better and whether storage conditions can be improved.

MDMA and MDA could be quantified in DBS as reliable as in whole blood specimens [15]. Moreover, equivalence of the methods has been proven for amphetamine and alprazolam. Table 3 summarizes the results of the Bland-Altman analysis for all substances under investigation:

Tab. 3. Summary of the results from Bland-Altman analysis.

Analyte	**Mean difference blood-DBS [ng/mL]**	**Mean-1.96*x*SD [ng/mL]**	**Mean+1.96*x*SD [ng/mL]**	**Mean difference/mean blood concentration [%]**
Amphetamine	-1.03	-3.32	1.25	-5.01
MDMA	-3.55	-14.34	7.25	-1.94
MDA	0.02	-1.36	1.40	0.17
Risperidone	0.83	-0.67	2.32	7.44
9-Hydroxyrisperidone	0.64	-1.13	2.40	4.15
Alprazolam	-0.09	-1.11	0.92	-1.38
Zopiclone	3.99	-3.62	11.60	15.30

Obviously, for any analyte except zopiclone the mean difference did not exceed ±10 % of the mean blood concentration. Therefore, assays using DBS and whole blood methods for the investigated analytes can be regarded to be equivalent for all analytes under investigation.

4. Conclusions

DBS assay has potential as a precise and inexpensive option for the determination of amphetamine-type drugs, risperidone and 9-hydroxyrisperidone and alprazolam using a small blood volume. B/DBS ratios were very close to 1.00, except zopiclone which is prone to degradation. At present, a stability study in both media to compare the extent of degradation is running. Bland-Altman difference plots for all analytes under investigation showed, that their determination from either whole blood or DBS does not differ. Ninety five % of all differences were within the limits of agreement. Also, differences were uniformly distributed across the respective concentration range.

5. Acknowledgement

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6. References

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