Adhesive blood microsampling systems for steroid measurement via LC–MS/MS in the rat

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1. 1. Introduction

Recently, Liquid Chromatography Tandem Mass-Spectrometry (LC–MS/MS) has evolved as a valuable tool for endocrine steroid research and routine steroid plasma analysis [1]. It allows for the direct, (semi-)automated analysis of multiple hormones in a single probe with minimal usage of total sample volume. Due to these benefits and the accuracy of the method, the establishment of LC-MS/MS as endocrine gold standard for plasma analysis is under current debate [1,2], possibly replacing classic (radio-)immunoassays in many fields in the future [3]. Moreover, we have shown recently that LC–MS/MS can also be sufficiently used to perform steroid analysis in endocrine organs themselves, such as the human and rat placenta [4,5]. In animal studies, especially in rodent-based fetal research, adequate handling of small sample

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volumes is pivotal. Blood sampling of the rodent fetus mainly relies on microsampling, which is classically based on the conventional dried blood spot (DBS) technique.

While DBS has a long history (>50 years) in neonatal care as a routine screening technique for endocrine and metabolic disorders [6,7], it has since gained increasing popularity for various other applications requiring quantitative analysis of smaller blood samples (reviewed by [8]). The success of DBS is partly rooted in the simplicity of sampling and sample handling (e.g. non-hazardous shipping at room temperature). Furthermore, DBS can be used with small blood volumes (<20 μL), which are common in both human [8] and rodent perinatal research [9]. Importantly, the technique allows for the spotting of an approximate volume of blood by researchers and clinicians, as accurate analytical measurement is achieved by taking a fixed diameter subpunch of the spot in the laboratory later [10]. However, subpunching is associated with challenges that center around the volume of blood spotted onto the card, the hematocrit of the blood and the homogeneity of the spot [8,11]. As these issues might negatively affect analytic quality, volumetric absorptive microsampling (VAMS) was introduced as an alternative to the DBS approach for quantitative bioanalysis. This method aims to overcome issues of subpunch analysis and hematocrit-based sample inhomogeneity [8,11]. As EDTA plasma is commonly used to perform steroid assays [12], we compared two VAMS-devices and the standard DBS method to EDTA plasma. We set out to compare these tools for steroid analysis in the rat, aiming to improve inter-system comparability. VAMS might be of special interest for settings that require small sample volumes (i.e. rodent, fetal and neonatal research). It further enables long-term follow-up rodent studies requiring repetitive blood sampling thereby potentially minimizing cohort sizes.

2. Experimental

2.1. Human blood samples

Human surplus EDTA-Plasma was used anonymously. The procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional or regional) and were carried out in accordance with *The Code of Ethics of the World Medical Association* (Declaration of Helsinki) for experiments involving humans.

2.2. Animals

All procedures performed on animals were in line with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The *EU Directive 2010/63/EU* for animal experiments was followed. The experimental protocol was approved by the appropriate Institutional and Governmental Review Boards (Regierung von Mittelfranken, AZ #54-2531.31-31/09). Wistar rats were ordered form Charles River (Sulzfeld, Germany). Animals were housed under standard conditions with free access to standard chow (No. 1320; Altromin, Lage, Germany) and tap water. Mixed arterio-venous EDTA blood was drawn under inhalative isoflurane anesthesia via syringe and collected in K3 EDTA blood collection tube (Sarstedt, Nümbrecht, Germany) at day of sacrifice via heart puncture.

2.3. Sample processing and LC-MS/MS analysis

We compared Whatman® 903 Protein Saver Cards, the volumetric absorptive microsampler (VAMS) devices Noviplex™ Plasma Prep Cards and the Mitra™ Microsampling device referring to EDTA plasma. Table 1 gives an overview of the adhesive blood collection systems that were tested and the respective suppliers. We used deuterium-labeled steroids (cortisol-d4, corticosterone-d8, progesterone-d9 and 17-OH-progesterone-d8) as internal standards (IS). LC-MS/MS reagents were ordered from Sigma-Aldrich (Nümbrecht, Germany) unless stated otherwise at the highest purity level (>98%). Deuterated steroids came from Cambridge Isotope Laboratories, Inc. (via Euriso-top GmbH, Saarbrücken, Germany) at the highest purity level (>98%). We determined the plasma volume factor using different approaches: Firstly, we spiked pooled rat blood samples with different cortisol concentrations. Additionally, we compared the volume factors of each system using pooled adult human (n = 5) and Wistar rat (n = 8, 18 weeks, mixed gender) blood, respectively. Finally, we validated these factors in individual samples of juvenile (n = 11, 21 days) and adult (n = 6, 3 female, 3 male, 18 weeks) Wistar rats. Hematocrit was determined for each animal using the Sysmex XN-1000™ Hematology Analyzer (Sysmex Deutschland GmbH, Norderstedt, Germany). Steroid levels were determined via LC–MS/MS, as described before [4,5]. In short, cortisol concentrations in the different calibrators ranged from 0.967 to 250 μg/L. The calibration concentrations of the steroids corticosterone and 11-dehydrocorticosterone were 0.976–500 μg/L and 0.1–25.0 μg/L for testosterone and progesterone. As steroid IS, a solution consisting of cortisol-d4 (250 μg/L), corticosterone-d8 (500 μg/L), progesterone-d9 (5 μg/L) and 17-OH-progesterone-d8 (30 μg/L) was used [13].

2.4. Cortisol spiking experiment

EDTA blood supplemented with different cortisol concentrations was used to obtain plasma volume correction factors for each system. In order to avoid possible interactions with endogenous steroids, human cortisol was used, since we found it absent in rodents [5]. Solutions of cortisol (final concentrations 1.00–250 μg/L) were added to aliquots of pooled arterio-venous EDTA blood (1/99, v/v) of 3 female and 3 male adult Wistar rats. Non-coagulated blood samples were transferred to Whatman® 903 Protein Saver Cards and the absorptive microsampler devices Noviplex™ Plasma Prep Cards and Mitra™ Microsampling Devices. In parallel, EDTA plasma was obtained by centrifugation of the corresponding blood samples at 1400*g* for 10 min. Subsequently, LC–MS/MS was used to determine cortisol levels in these absorptive microsampling systems in comparison to the original EDTA sample. Furthermore, corticosterone and 11-dehydrocorticosterone plasma concentrations were measured.

For the Whatman® 903 Protein Saver Cards, 60 μL of EDTA blood sample supplemented with cortisol were spaced per spot and dried for 3 hours at ambient laboratory conditions. Our ambient laboratory conditions for sample drying consisted of free circulating laboratory air at 21 °C, relative air humidity of 50–55%.

For the Noviplex™ Plasma Prep Cards, 40 μL of EDTA blood sample were spread per spot and dried for 3 min before removing the top layer containing red blood cells. The lower layer containing only EDTA plasma was dried for another 15 min at ambient labora-

Table 1
Overview of the adhesive blood collection systems tested and the respective suppliers.

Blood collection systems	Supplier
K3 EDTA blood collection tubes	Sarstedt, Nümbrecht, Germany
Mitra™ Microsampling Device	Phenomenex, Aschaffenburg, Germany
Whatman® 903 Protein Saver Cards	Sigma-Aldrich Chemie GmbH, München, Germany
Noviplex™ Plasma Prep Cards	Shimadzu Scientific Instruments, Columbia, USA

tory conditions. All preparation steps were performed in accordance with the manufacturer's instruction given (Table 1).

Mitra™ Microsampling Devices were filled, as recommended by the supplier (Table 1) and as previously described in the literature [8]. Tips were dipped into EDTA blood at an angle of up to 45° from the vertical just braking the surface shoulder for an additional 2 s after it had become completely colored (total sampling time of 4–6 s). After sufficient blood absorption the tips were dried for 3 h at ambient laboratory conditions. In order to achieve the best sampling precision we did not handle the tip before use and the tips did not touch each other or their surroundings at any time. Furthermore tips were not submerged in blood, as recommended [8,11].

As an initial step for further preparation, all dried blood samples needed to be removed from the respective blood collection system for further preparation. Per sample on a dried Whatman® 903 Protein Saver Card, a defined circular segment with a diameter of 3 mm was punched out. The dried plasma disc on the Noviplex Plasma Prep Cards was taken out. Only the soaked, dried tip of the Mitra™ Microsampling Device was further used in its manufactured state and placed in an extraction tube after removal.

Whatman® 903 Protein Saver Card segment, Noviplex Plasma Prep Card disc and Mitra™ Microsampling Device tip were further prepared separately. Each of these samples was mixed with 100 μL of water/methanol (1:1; LC–MS grade) and 100 μL of the steroid IS solution before ultrasonication for 5 min at room temperature. Similarly, 100 μL of the EDTA plasma was mixed with 100 μL of the steroid IS solution and equilibrated at 4 °C for 15 min. All samples were precipitated by the addition of 200 μL of a $ZnSO_4$ solution and centrifuged for 10 min at 23,000*g*. The resulting supernatant was transferred into a microtiter plate, which was kept in the autosampler at 15 °C for steroid analysis via LC–MS/MS.

2.5. Plasma volume factors of individual samples

In a second step the glucocorticoid concentrations of pooled EDTA blood samples of two species (human blood (n = 5) and rat blood (n = 8)) were spotted separately on adhesive blood microsampling devices and compared to the EDTA plasma concentration of the pooled blood sample, respectively (Table 2, calibration set). To validate the obtained plasma volume factors, the glucocorticoid concentrations were measured in an additional validation set of samples from juvenile (21 days, n = 11) and adult (18 weeks, n = 6) rats. In this experiment the plasma volume factors were calculated for each individual sample (Table 2). Moreover, testosterone and progesterone levels were measured (Table 3). The hematocrit was determined for each sample (Sysmex XN-1000™ Hematology Analyzer) individually.

2.6. Statistical analysis

Data processing and graphic presentation were performed with Microsoft Excel 2010 (Microsoft, Bellevue, USA) and GraphPad Prism Version 4.0c (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard deviation (SD), unless otherwise stated.

3. Results

3.1. Cortisol spiking of rat blood samples

The use of human cortisol as supplement in rat blood samples was chosen based on the absence of endogenous cortisol in the rat, which was tested beforehand (data not shown). Since all steroids share a similar chemical structure, transferability of the cortisol results to the other steroid hormones was expected. The detected cortisol levels showed a linear correlation in the spiked whole blood samples of adult rats (coefficients of correlations: 0.993–0.999) containing a hematocrit of 38.0%. All sampling devices reflected the linear accumulation of cortisol found in EDTA-Plasma (Fig. 1).

3.2. Plasma volume factor correction, recovery and bias

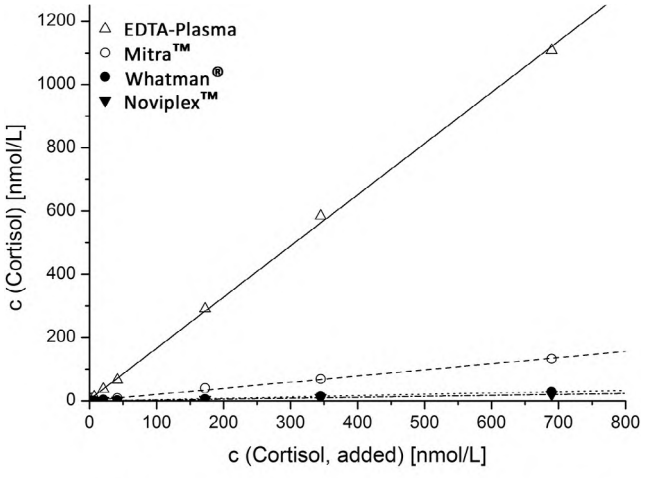
Plasma volume correction factors for adhesive blood microsampling devices were determined in a calibration (pooled human blood and pooled rat blood of mixed gender) and a validation set (juvenile and adult rats, not pooled, gender-specific). Although cortisol-spiking revealed linear correlations of different adhesive blood microsampling devices towards EDTA-Plasma, we were unable to establish one single volume factor for every steroid in each system (Table 2). Instead, volume factors had to be adjusted for the recovery rate of each steroid individually for every device. Interestingly, these factors deviated from the theoretical blood volume supplied by the manufacturers of all devices (Table 2). In our validation set, we determined the recovery rate of the internal standards (IS) corticosterone-d8 and cortisol-d4 (Fig. 2). We were able to recover >96% of cortisol-d4 and >80% of corticosterone-d8 in juvenile and adult rats in EDTA-Plasma. The recovery rates of IS from blood microsampling devices was comparable to EDTA-Plasma, except for corticosterone-d8, which showed a hormone-specific adsorption effect to serum-coated Noviplex™ Plasma Prep Cards, with a significant pre-analytic loss of the steroid and recovery rates <30% (Fig. 2).

Table 2
Calculated blood volumes for different adhesive blood microsampling systems. Calculation was based on detected glucocorticoid levels. Calibration was performed on pooled human and pooled rat blood samples of mixed gender with and without cortisol addition. Juvenile and adult rat blood samples were used for validation purpose. Coefficients of variation (CV) are represented in brackets. Statistical significance is presented with * for P < 0.05 and *** for P < 0.001, Std. = standard.

	Samples	n	Hematocrit %	Whatman® μl (CV)	Mitra™ μl (CV)	Noviplex™ μl (CV)	Glucocorticoids
Theoretical blood volume				7.5	10	2.5 (Serum)	
Calibration set	Human	5	28.1%	6.4 (12%)	7.9 (22%)	2.5 (17%)	Cortisol
				8.8 (27%)	9.5 (32%)	4.4 (56%)	Cortisone
	Rat adult	8	38%	6.2 (6.0%)	8.0 (6.0%)	2.3 (26%)	Corticosterone
				5.6 (21%)	7.1 (24%)	2.2 (12%)	11-Dehydrocorticosterone
	Std. addition	8		5.0	12	1.7	Cortisol
Validation set	Rat total	17	37.9%	5.7 (10%)	7.0 (18%)	3.9 (10%)	Corticosterone
				7.8 (18%)	9.7 (29%)	2.5 (22%)	11-Dehydrocorticosterone
	Rat juvenile	11	28.6%	5.9 (10%)	7.8 (9.0%)	4.1 (7.0%)	Corticosterone
				8.5 (13%)	11 (25%)	2.6 (22%)	11-Dehydrocorticosterone
	Rat adult	6	44.1%***	5.3 (6.0%)*	5.6 (11%)***	3.6 (13%)*	Corticosterone
				6.5 (8.0%)	7.4 (13%)	2.2 (18%)	11-Dehydrocorticosterone

Table 3
Steroid analysis of rat blood samples using adhesive blood microsampling systems. Steroid levels determined by the different adhesive blood microsampling devices are given for juvenile (j = 11) and adult (a = 6), as well as female (f = 8) and male (m = 9) rats, with juvenile male (jm, n = 6), juvenile female (jf, n = 5), adult male (am, n = 3) and adult female (af, n = 3). Testosterone and progesterone levels were calculated with the factors found in all rat samples (n = 17), for corticosterone (5.7 for Whatman®, 7.0 for Mitra™) and 11-dehydrocorticosterone (2.5 for Noviplex™). Abbreviations: a = adult, j = juvenile, f = female, m = male, LLOQ = lower limit of quantification; # concentration of two samples < LLOQ, ## factor 5.7 for Whatman®, 7.0 for Mitra™, 2.5 for Noviplex™.

	Group	Plasma	Whatman®	Mitra™	Noviplex™
Corticosterone (B)	jm	219.2 ± 42	225.0 ± 44.7	235.8 ± 41.7	237.0 ± 49.5
[nmol/L, mean ± SD]	jf	361.5 ± 122	383.7 ± 144	410.9 ± 125	369.4 ± 128
	am	794.7 ± 253	714.7 ± 245	631.9 ± 172	810.0 ± 293
	af	1775 ± 68.1	1706 ± 39.6	1376 ± 52.1	1538 ± 233
11-Dehydrocorticosterone (A)	jm	17.9 ± 3.22	19.1 ± 4.74	18.1 ± 1.62	20.1 ± 5.2
[nmol/L, mean ± SD]	jf	19.3 ± 3.61	21.8 ± 4.79	23.7 ± 6.8	17.9 ± 2.71
	am	65.7 ± 11.0	57.6 ± 13.3	53.4 ± 8.6	66.2 ± 15.9
	af	111.1 ± 13.3	88.0 ± 8.8	78.1 ± 13.3	87.5 ± 13.4
Ratio B/A	jm	12.3 ± 1.6	12.0 ± 1.0	12.9 ± 1.5	12. 0 ± 1.6
[mean ± SD]	jf	18.8 ± 5.8	17.3 ± 4.4	18.1 ± 6.1	20.8 ± 7.2
	am	11.8 ± 2.3	12.1 ± 1.9	11.6 ± 1.4	11.8 ± 1.8
	af	16.2 ± 1.7	19.5 ± 1.5	18.1 ± 3.4	17.7 ± 1.7
Testosterone ##	jm	1.2 ± 1.8	1.3 ± 1.3	1.5 ± 2.0 #	< LLOQ
[nmol/L, mean ± SD]	jf	0.09 ± 0.05	< LLOQ	< LLOQ	< LLOQ
	am	14.9 ± 6.1	15.5 ± 6.1	19.0 ± 8.5	13.4 ± 4.6
	af	0.27 ± 0.22	< LLOQ	< LLOQ	< LLOQ
Progesterone ##	jm	2.5 ± 1.3	3.9 ± 1.1	2.2 ± 0.6	3.4 ± 0.6
[nmol/L, mean ± SD]	jf	3.3 ± 2.0	4.9 ± 2.6	2.9 ± 1.2	3.8 ± 1.1
	am	6.7 ± 3.5	7.5 ± 3.7	2.3 ± 0.7	5.9 ± 2.4
	af	83.3 ± 40.5	95.1 ± 47.8	51.7 ± 51.2	39.4 ± 13.5



Applied cortisol [nmol/L]	EDTA-Plasma [nmol/L]	Mitra™ [nmol/L]	Whatman® [nmol/L]	Noviplex™ [nmol/L]
0	<LLOQ	<LLOQ	<LLOQ	<LLOQ
2.76	5.24	0.408	<LLOQ	<LLOQ
6.90	12.6	0.621	0.074	<LLOQ
20.7	36.4	3.14	0.654	0.513
41.4	65.6	8.06	1.52	1.42
172	290	39.5	5.30	3.59
345	584	68.7	14.9	9.60
690	1108	132	28.1	19.8

Fig. 1. LC–MS/MS measurement of linear accumulation of human cortisol in adult Wistar rat EDTA-Plasma. X-axis: Concentration (c) of human cortisol (ng/ml) in rat EDTA-Plasma and the tested microsampling blood collection systems determined by LC–MS/MS. Y-axis: Concentration (c) of human cortisol (ng/ml) added to rat EDTA-Plasma. The table below indicates applied and detected cortisol levels of spiked rat blood samples in the different blood collection systems. Cortisol concentrations below the lowest limit of quantification are indicated as <LLOQ. Abbreviations: △ = K3 EDTA blood collection tubes, ○ = Mitra™ Microsampling Device, ● = Whatman® 903 Protein Saver Cards and ▼ = Noviplex™ Plasma Prep Cards.

3.3. Variation of reproducibility

In humans, the coefficient of variation (CV) for cortisol ranged from 12 to 22% and for cortisone 27–56%, depending on the microsampling system (Table 2, Human). In juvenile and adult rats (Table 2, Rat total) the CV for corticosterone ranged from 10 to 18% and for 11-dehydrocorticosterone the CV range was 18–29%. Across species, Whatman® 903 Protein Saver Cards had the lowest CV among the microsampling devices tested (Table 2).

3.4. Influence of hematocrit

The introduction of novel volumetric microsampling techniques aims to facilitate sampling of fixed accurate volumes, thereby overcoming the drawback of hematocrit-dependent DBS sampling (non-volumetric) [8]. Using different sample sets (i.e. human, juvenile and adult Wistar rats) enabled us to examine the performance of various microsampling devices at a range of hematocrit levels (28.1–44.1%, Table 2). Our results obtained with the novel VAMS devices in the rat show that the hematocrit must not be neglected. In fact, a clear bias (Fig. 2) of the individual samples occurred when a uniform plasma volume correction factor from our calibration set (Table 2) was applied. Hence, haematocrit-adapted volume factors are mandatory.

3.5. LC–MS/MS glucocorticoid measurement of Wistar rat blood specimen

Using the determined individual volume factors from the validation set, the different blood withdrawal systems revealed comparable values (Table 3, Supplementary Fig. 1). Using the obtained volume factors for the different microsampling devices we established reference values for our Wistar rats at different stages of development. The results of our steroid analysis revealed that juvenile Wistar rats exhibited ~3–5-fold lower levels of both active and inactive glucocorticoid, as well as progesterone when compared to adult rats (Table 3, Supplementary Fig. 1). This is analogous to humans, where steroid levels show age-specific differ-

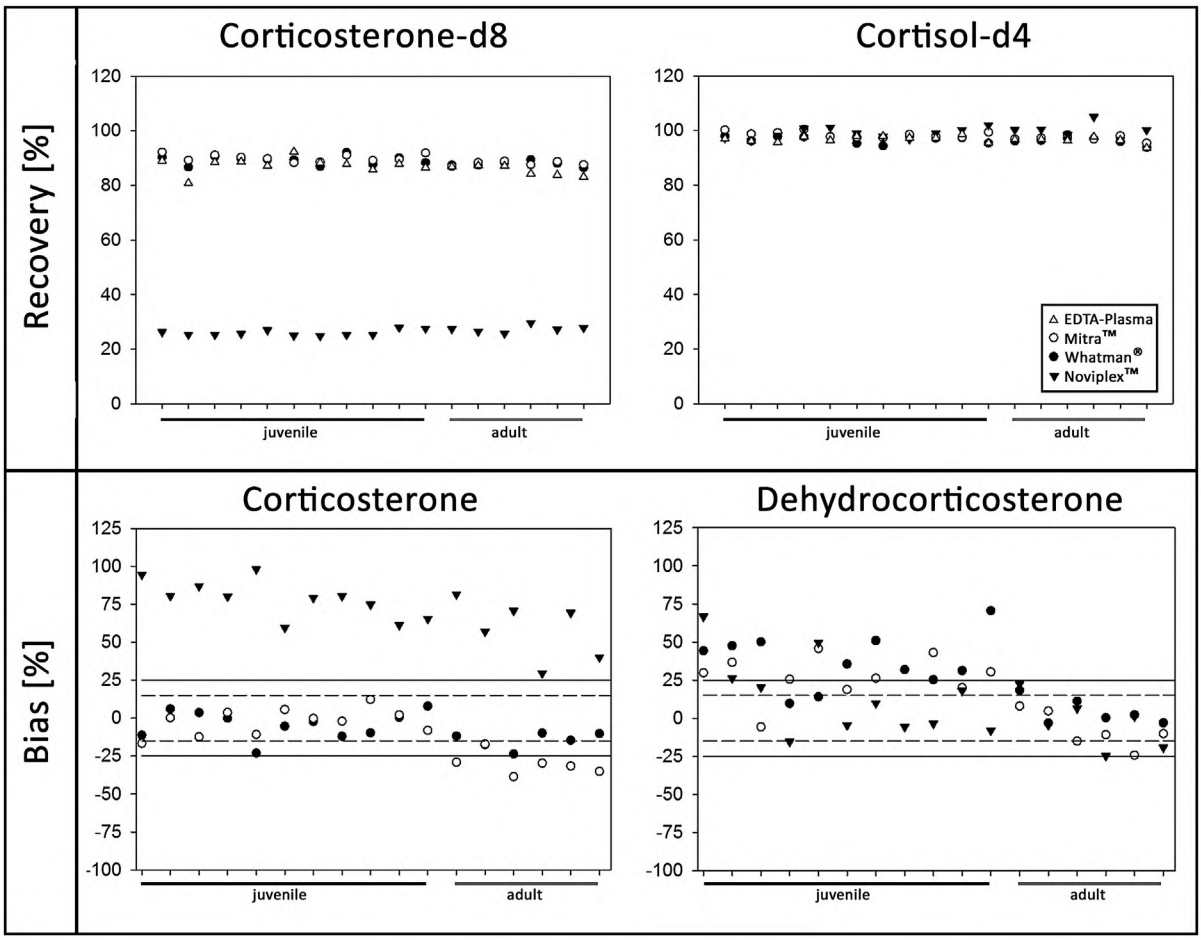


Fig. 2. Glucocorticoid recovery and bias in the different blood withdrawal devices A uniform plasma volume correction factor from our calibration set (see Table 2) was applied. Values were found for △ = K3 EDTA blood collection tubes, ○ = Mitra™ Microsampling Device, ● = Whatman® 903 Protein Saver Cards and ▼ = Noviplex™ Plasma Prep Cards in the validation experiment using juvenile and adult rat blood samples (see labeling of x-axis).

ences [14–17]. Sex-specific differences were determined with higher EDTA-Plasma levels of corticosterone, 11-dehydrocorticosterone and progesterone in female juvenile and especially in female adult rats, when compared to their male counterparts. Interestingly, basal glucocorticoid levels of both rats and humans were described as generally sex-independent [18], while the adaptive response to stress is sex-dependent [18–21], which also includes the corticosterone response to decapitation, anesthesia and euthanasia [22]. As expected [23], higher plasma levels of testosterone were found in male rats, with increasing levels towards masculinization in adulthood.

4. Discussion

We set out to compare two novel volumetric absorptive microsampling (VAMS) devices and classic non-volumetric dried blood sampling (DBS) for LC–MS/MS glucocorticoid analysis in human and rat EDTA-blood.

The comparison of adult and juvenile rat samples showed an unexpectedly strong hematocrit-dependency. Interestingly, volume factors that were determined for the different blood collection systems concerning cortisol were not transferable to other steroids. A possible explanation for this phenomenon could be varying adsorption behavior of the different steroids on the different collection materials, which was already described in the literature [24]. The observed effect was more pronounced than in the classic non-volumetric DBS-device.

As a consequence, volume factors had to be adjusted for the recovery rate of each steroid individually before usage, to allow an accurate calculation of final concentrations for individual steroids.

Adsorption affinity could be further influenced by the use of different extraction solvents (polar, non-polar) as they potentially promote steroid solubilization (from adhesion on the Microsampling Device surface) to varying extents [25]. These effects need to be further investigated in future studies. Our finding that Noviplex™ Cards showed pre-analytical loss of corticosterone-d8 (component of the steroid IS) due to adsorption, may have been caused by polarity-specific adsorption to the Noviplex™ material. Corticosterone adsorption may have occurred either in the plasma-containing lower layer or in the red blood cell containing upper layer, which might have impaired corticosterone permeability towards the plasma-containing lower layer.

It was shown by others, that Mitra™ VAMS offers a reliable alternative for drug detection of acetaminophen, midazolam [8] and caffeine [11] in plasma. To our knowledge, its use for steroid analysis in the rat has not been demonstrated previously. There is no evidence of selective absorption by the tip of the plasma component over whole blood [8,11]. In our study EDTA-blood was used as matrix of comparison allowing for distribution of uncoagulated blood to the respective sampling devices. Usually, VAMS blood samples *in vivo* does not contain EDTA. In contrast, quantitative bioanalysis relies on the preparation of calibration standards and quality control (QC) samples from anti-coagulated control blood.

Interestingly, Spooner et al. [8,11] were able to show that preloading of Mitra™ tips with EDTA resulted in acceptable precision and accuracy values when measuring plasma acetaminophen levels via LC–MS/MS. Introducing EDTA at the extraction stage, we were able to match the matrix between samples and calibrants/QCs at the point of analysis. However, it remains to be determined in further experiments, whether mismatch of matrices by anti-coagulation of calibrants/QCs might negatively affect the quality of steroid analysis using microsampling techniques in regular *in vivo* settings.

Although the Mitra™ VAMS approach showed no advantage over the classic DBS method with regard to hematocrit-dependence, it preserved the recognized advantage of the DBS method, especially the technical ease. Use of the Mitra™ VAMS device simplifies the sample collection process and work flow (e.g., no need of sub-punching) in research settings and in the analytical laboratory, which might help to boost comparability between results. Its design further enables the future integration into automated sample processing procedures. However, we believe that suitable staff training is required before routine deployment to avoid analytical pitfalls linked to mal-handling of the device, as seen by others [8]. A conceivable setting that might benefit from the use of Mitra™ for steroid analysis could include situations where EDTA-Plasma analysis is not feasible, e.g. in long-term follow-up rodent studies, thereby helping to optimize sample quality and minimize cohort sizes.

Our study revealed a variation of reproducibility (coefficient of variation (CV), Table 2) for each sampling system: We found that Whatman® 903 Protein Saver Cards offered the lowest CV when compared to the VAMS Noviplex™ and Mitra™.

As certain steroids are present at physiologically low levels only, higher sample volumes might be beneficial, taking into account the lower limit of quantification (Fig. 1). In this respect, the Mitra™ device offered the lowest limit of quantification in our experiments, along with the highest sample volume (10 μl vs. 7.5 μl Whatman®, 2.5 μl Noviplex™).

Taken together, our findings provide plasma volume correction factors for LC–MS/MS steroid analysis of volumetric and non-volumetric microsampling systems in comparison to EDTA-Plasma. Our study argues for a thorough analysis of chromatographic effects before the use of novel volumetric systems for steroid analysis. A detailed validation for each steroid and sample set remains a strong prerequisite.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

The present work was performed by Kirsten Heussner in partial fulfillment of the requirements for obtaining the degree “Dr. rer. nat.” at the Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Germany.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2017.01.006.

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