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Perspective

Do dried blood spots (DBS) have the potential to support result management processes in routine sports drug testing?

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Abstract

Dried blood spots (DBS) have been considered as complementary matrix in sports drug testing for many years. Especially concerning substances prohibited in-competition only, the added value of DBS collected concomitantly with routine doping control urine samples has been debated, and an increasing potential of DBS has been discussed in the scientific literature. To which extent and under which prerequisites DBS can contribute to enhanced anti-doping efforts is currently evaluated. As a proof-of-principle, two analytical applications, one targeting cocaine/benzoyl ecgonine and the other prednisone/prednisolone, are presented in this perspective to indicate potential added value but also presently existing limitations of the DBS approach.

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Current Status

The currently enforced World Anti-Doping Agency (WADA) Prohibited List is composed of ten classes of prohibited substances and three classes of prohibited methods,[1] with four classes (including stimulants, narcotics, cannabinoids, and glucocorticoids) being considered as prohibited during the in-competition period only. This period typically commences 12 h before competition and ends with the sample collection process related to the competition at the end of the contest. All other substances and methods of doping itemized in WADA's Prohibited List are banned at-all-times.

In principle, adverse analytical findings (AAFs) concerning routine doping control urine samples are to be issued if drugs (or corresponding metabolites or markers) prohibited in-competition are detected and so-called therapeutic use exemptions are not granted. However, for a subset of substances, urinary decision limits have been established and enforced, particularly concerning the stimulants cathine (norpseudoephedrine), ephedrine, methylephedrine, and pseudoephedrine, the analgesic morphine, and the cannabinoid tetrahydrocannabinol (THC, targeted via its metabolite 11-nor-9-carboxy-Δ^9-THC).[2] In addition, reporting levels apply for other compounds summarized in the aforementioned classes of substances banned in-competition. For most stimulants for instance, the reporting level corresponds to 50% of the minimum required performance level (MRPL)[3], *i.e*. 50 ng/mL, while the reporting level for glucocorticoids has been set at 30 ng/mL. Only if these urinary decision limits or reporting levels are exceeded and the other predefined reporting criteria are met, AAFs are reported.

Potential alternative/complementary strategy

On the one hand, the approach of employing urinary reporting levels and decision limits has offered substantial practicality and harmonization at both laboratory and result managing authority (RMA) level; on the other hand, individualized case management cannot be guaranteed,[4] and differing drug potencies and, consequently, pharmacologically relevant blood concentrations and corresponding urinary levels are not fully accounted for. Information complementing the urine sample analytical data are desirable that support and particularize the decision-making process by the RMA, ideally by either verifying or falsifying the presence of pharmacologically relevant blood levels of a prohibited substance in an athlete's sample collected in-/post-competition.[5] Especially for drugs of abuse including stimulants such as *e.g*. cocaine and narcotics such as *e.g*. morphine, blood concentrations indicating an individual's impairment were reported[6] and cut-off levels have been recommended for different areas of application (*e.g*. workplace or roadside drug testing) concerning drugs of abuse.[7, 8] Also for glucocorticoids such as dexamethasone, prednisolone, and methylprednisolone, values representing effective drug plasma concentrations are available, defined (amongst others) by suppressed blood cortisol levels,[9, 10] and the concept of considering relevant/irrelevant drug plasma and corresponding urine concentrations has further been established in the context of equine sports drug testing.[11, 12]

Translated into practice of human sports drug testing programs, a conceivable scenario would consist of paired urine and blood sampling for in-competition doping controls.[13] In case of an AAF regarding a substance prohibited in-competition only, triggered by exceeding established urinary cut-off / reporting levels (collectively referred to in the following as "urinary trigger concentration" - UTC), the corresponding blood sample will undergo quantification of the active principle. If an effective blood concentration of the drug is determined (Table 1), the doping offence is analytically corroborated and anti-doping rule violation (ADRV) proceedings will follow as stipulated by prevailing anti-doping

regulations. If the further analysis however returns blood concentrations of the prohibited substance below recommended/accepted levels that indicate an influence on the athlete at the time of competition, the RMA obtains vital information for a more individual case-management taking into consideration the additional evidence in favor of the athlete's defense. A major limitation in that scenario has been the invasive and costly collection/transport of blood samples. A conceivable alternative in this context have become dried blood spot (DBS) matrices, which are readily produced, comparably cheap, and which offer stabilizing features for the target analyte.[14] In combination with recent advances in sampling techniques, (semi-)automated DBS sample preparation options,[15-18] and the considerably enhanced instrumental sensitivity in bioanalysis, the collection of matched pairs of urine and DBS samples in routine doping controls would offer a substantial added value for the wider overview on the testing.

An excellent example of complementary information provided by DBS in a sports drug testing context was recently presented by Kojima *et al*., reporting on pilot studies concerning ephedrine and methylephedrine.[19] Following the oral administration of 25 mg of methylephedrine hydrochloride, urinary methylephedrine concentrations exceeded the relevant threshold of 10 µg/mL 20 h post-administration, while concomitantly collected DBS samples returned blood concentrations below 25 ng/mL, *i.e*. below the minimum effective blood concentration of the drug.[20, 21] Of note, urine samples collected within the first 7 h after drug ingestion, *i.e*. plausibly within an athlete's active in-competition period, returned analyte concentrations substantially below WADA's decision limit. The corresponding blood concentrations ranged between 100 and 125 ng/mL, suggesting pharmacological effects on the athlete. Cocaine ranked 3rd in WADA's 2017 annual statistics among the class of stimulants detected in in-competition doping controls.[22] Also here, the question whether the drug administration occurred out-of-competition is frequently raised. While defining the time of drug use from a single sample is particularly difficult, contextualizing a given blood concentration present in-competition in an athlete's organism with a potential influence on the individual appears feasible, especially in consideration of existing cut-offs suggested and/or established in guidelines applied to *e.g*. investigations concerning driving under the influence.[6-8] Here, cocaine blood concentration cut-offs of 10-24 ng/mL were presented. This information combined with the reported viability of quantifying cocaine and its main metabolites (*e.g*. benzoyl ecgonine) in DBS[23-27] could allow adapting a strategy by routine doping controls that utilizes both routine urine sports drug testing samples and DBS. If a urine sample is found to contain cocaine and/or its major metabolite(s) such as e.g. benzoyl ecgonine, above the currently enforced reporting level of 50 ng/mL, the concomitantly collected DBS sample will be analyzed for cocaine (and benzoyl ecgonine), and recommended cut-off levels could be applied by the RMA to facilitate their anti-doping result management.

Proof-of-principle pilot study data – benzoyl ecgonine/cocaine

In the course of a proof-of-concept pilot study, urine and DBS samples (collected prior to and after the ingestion of 250 mL of freshly prepared coca tea) and a 10 mL aliquot of a coca tea brew were obtained from Paraguay. First, in accordance with the manufacturer's instructions, one tea bag (containing 0.8 g of dry ground coca leaves) of commercially available tea was submerged in hot water for 5 min before the tea bag was discarded. A 10 mL aliquot of the tea was immediately frozen (-20°C) and prepared for shipment to the testing laboratory. Following written consent, one volunteer consumed tea prepared in the same way but using two tea bags, and the entire tea volume of 250 mL was ingested within 10 min after brewing. Urine and DBS samples were collected before and 1, 2, and 3.5 h post administration. The DBS samples were allowed to dry for 2 h at room temperature before being deposited in plastic bags containing desiccant gel. Urine and DBS samples were then frozen (-20°C)

and, together with the tea aliquot, all specimens were shipped on dry ice to the Center for Preventive Doping Research in Cologne, Germany.

Using established liquid chromatographic-mass spectrometric test methods (LC-MS(/MS)),[24, 28] the tea aliquot as well as the urine and DBS samples were analyzed. Similar to literature data,[29] the approximate amount of cocaine and benzoyl ecgonine contained in the consumed 250 mL of tea prepared from two tea bags was 3.8 mg and 1.0 mg, respectively. The volunteer's cocaine and benzoyl ecgonine urine and DBS concentrations are presented in Figure 1. The WADA reporting level for urinary benzoyl ecgonine (50 ng/mL, blue line) relevant for athletes undergoing doping controls was exceeded at all post-administration sample collection time points (1 h, 2 h, and 3.5 h) as illustrated by the blue bars. DBS cocaine levels (orange bars) never exceeded 5 ng/mL and hence remained substantially below commonly recommended cut-off levels indicative for impairment (10-20 ng/mL, orange line).[6] DBS benzoyl ecgonine concentrations were found between 40 ng/mL and 70 ng/mL (grey bars), thus exceeding at one point (2 h) the cut-off level of 50 ng/mL suggested by Walsh[7] but never the cut-off referred to in the German road traffic act (§24a (2))[30] set at 75 ng/mL (grey line).

Representing merely three post-administration time points, the pilot study data at hand can only serve for indicating situations where urinary reporting levels of target analytes are exceeded and concomitantly existing blood concentrations are (presumably) pharmacologically ineffective. Further investigations into elimination profiles appear warranted and necessary when pharmacokinetic interpretations are required. Nevertheless, the principle applicability and relevant contribution of DBS to doping controls is conceivable, and the set of herein analyzed DBS and urine samples demonstrated that urinary benzoyl ecgonine levels beyond the reporting level of 50 ng/mL currently enforced in doping controls can correspond to (potentially) irrelevant blood cocaine and benzoyl ecgonine concentrations. Blood concentrations however could be considered as a critical information for the anti-doping result management proceedings, contributing to obtaining a more comprehensive picture of the AAF. Of note, a variety of aspects (e.g. the administration route, development of tolerance, individual drug response, comparability of whole (venous) blood drug concentrations *vs*. DBS (capillary) drug concentrations, etc.) are not entirely accounted for.

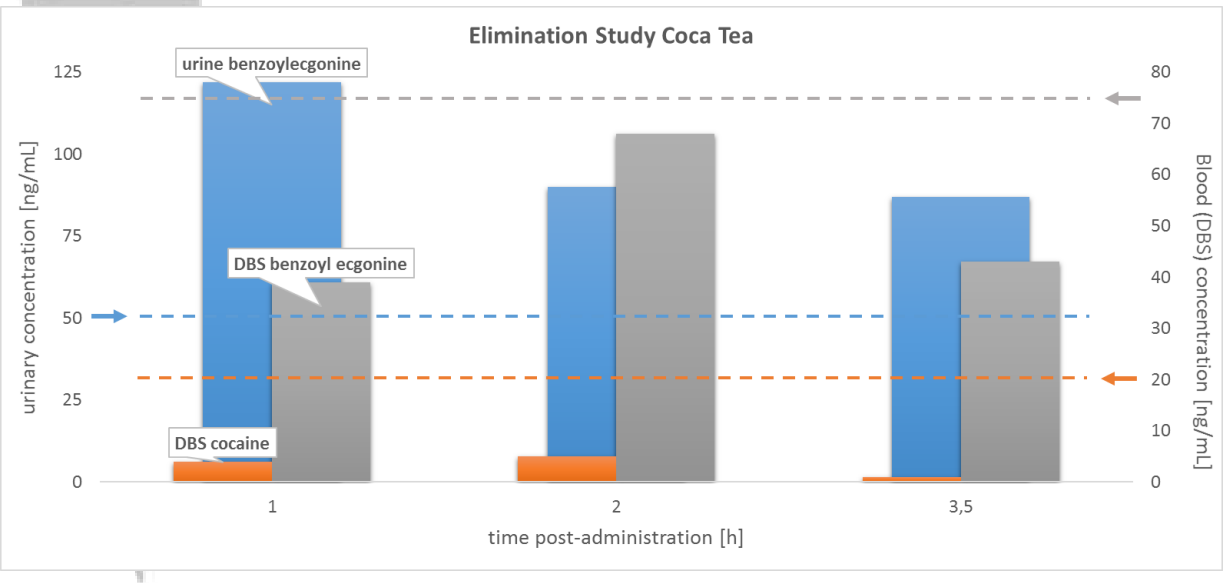


Figure 1: Proof-of-principle elimination study results. A volume of 250 mL of coca tea (accounting for approximately 3.8 mg of cocaine and 1 mg of benzoyl ecgonine), was administered, resulting in exceeding the reporting level for urinary benzoyl ecgonine (50 ng/mL, blue bars and blue line) at all post-administration sample collection time points (1 h, 2 h, and 3.5 h). Concomitantly collected DBS samples returned cocaine levels (orange bars) substantially below commonly recommended cut-off levels indicative for impairment (20 ng/mL, orange line).[6] DBS benzoyl ecgonine concentrations were found between 40 ng/mL and 70 ng/mL (grey bars), *i.e*. the cut-off referred to in the German road traffic law (§24a (2))[30] set at 75 ng/mL (grey line) was not reached.

Table 1: Examples of potential urinary trigger concentrations (UTC) and cut-off levels for anti-doping case management based on and derived from currently utilized urinary reporting levels[3], recommended impairment limits[6] and consensus cut-offs[7], relative drug potencies and effective plasma concentrations[9].

Drug	Hypothetical UTC [ng/mL]	Hypothetical DBS cut-off [ng/mL]
Stimulants		
Cocaine	50	20
Benzoyl ecgonine	50	75
Amphetamine	50	20
Narcotics		
Morphine	1000	10
Corticoids		
Betamethasone	10	0.5
Dexamethasone	10	0.5
Methylprednisolone	30	5
Prednisolone	30	5

Proof-of-principle pilot study data – prednisone/prednisolone

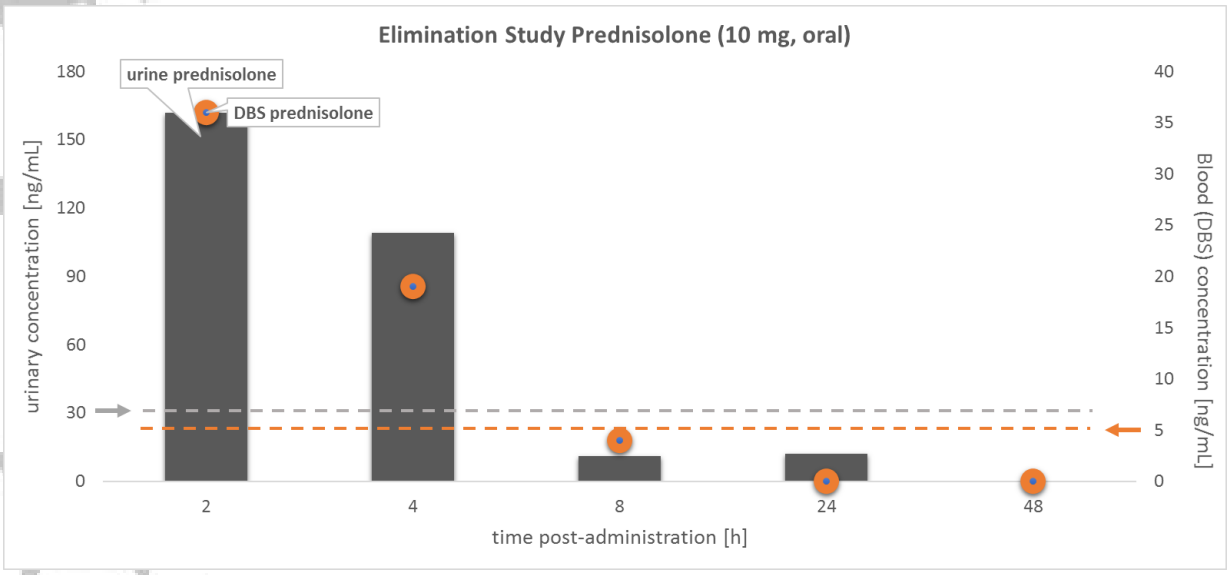
A considerable number of studies into urinary glucocorticoid eliminations following the administration of different drug formulations and varying routes of administration has been conducted, supporting

today's result interpretation in case of AAFs related to glucocorticoids as necessitated by the permitted use of glucocorticoids during out-of-competition periods as well as via routes that are not considered to result in systemic effects and effective blood concentrations.[31-36] To date, AAFs concerning glucocorticoids are issued when a glucocorticoid is detected at concentrations exceeding the reporting level of 30 ng/mL,[3] thus largely disregarding the potencies and correspondingly required therapeutic dosages of glucocorticoids. In the light of the considerably different relative potencies of glucocorticoids, drug-specific minimum effective blood concentrations (MECs) could provide a more objective basis for deciding whether an athlete competed under the (systemic) influence of glucocorticoids or not. A substantial amount of information on systemic corticoid activity has been published, utilizing for instance endogenous cortisol levels and T helper lymphocyte cell counts as parameters indicating the synthetic glucocorticoid's action.[9, 10] In case of intravenous dexamethasone administrations (4-7 mg), cortisol suppression was observed up to 32 h post-injection and the corresponding IC_{50} plasma concentration was estimated with 0.1-0.2 ng/mL. Suppression effects caused by prednisolone and methylprednisolone, whose differing potency was accounted for by increased dosing (ca. 6-fold and 5-fold compared to dexamethasone, respectively), lasted for ca. 16 h and IC_{50} plasma concentrations were reported with ca. 0.5-2 ng/mL.[9] A compendium composed of literature data on relevant / effective blood concentrations of glucocorticoids and cut-off levels for other drugs, regardless of the route of administration, could contribute to a more comprehensive assessment of a reported AAF in support of the RMAs' decision-making process. Of note, this would considerably affect the paradigm underlying the permitted use of drugs through selected routes of administration. In contrast to earlier approaches, any administration resulting in a systemic and pharmacologically relevant blood (plasma) drug concentration would then result in an AAF, regardless of how the substance (*e.g*. intramuscular *vs*. intraarticular) was introduced.

Similar to the above-mentioned proof-of-principle elimination study regarding cocaine, urine and DBS drug concentrations were determined in the context of a pilot study concerning prednisolone. Following ethical approval of the local ethics committee of the German Sport University Cologne (#107/2018) and written consent, a single therapeutic dose of 10 mg of prednisolone was orally administered by one healthy male volunteer, and urine as well as DBS samples were collected prior to and 2, 4, 8, 24, and 48 h post-dosing. Also here, existing test methods based on LC-MS(/MS) were employed to semi-quantitatively determine prednisolone and prednisone in both test matrices[24, 37] with LODs of 1 ng/mL. As illustrated in Figure 2, urinary prednisolone concentrations (specific gravity-adjusted to 1.020, dark grey bars) were found above the reporting level of 30 ng/mL (grey line) at 2 h and 4 h post-administration. Subsequent samples collected at 8 and 24 returned concentrations below 30 ng/mL and below the assay's LOD (48 h). Urinary prednisone concentration, however, remained above the reporting level of 30 ng/mL even at 24 h, which would have resulted in an AAF if the sample had been an athlete's doping control specimen and the exogenous nature of prednisone had been confirmed by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS).[38] With the next spot urine sampled at 48 h, the prednisone concentration fell below the assay's LOD. The corresponding DBS tested for prednisolone (orange circles) and prednisone (orange squares) yielded concentrations above a hypothetical DBS cut-off level of 5 ng/mL (deduced from published half maximal inhibitory plasma concentrations, IC_{50}, concerning cortisol[9, 10]) for 4 h post-administration, suggesting a systemic effect on the individual within a time period corresponding to an in-competition time window. The DBS sample collected at 24 h post-administration tested negative for both prednisolone and prednisone, indicating no remaining systemic effect of the drug administration on the test person. Also here, the current pilot study data are only indicators for the potential situations where urinary reporting levels of target analytes are exceeded and concomitantly existing blood concentrations are (presumably) pharmacologically ineffective. If transferred to a doping control

scenario, such additional analytical information would be particularly helpful for the anti-doping case management: the urinary trigger concentration (UTC, 30 ng/mL) is exceeded and the analysis of the corresponding DBS sample (collected together with the doping control urine sample) can be requested for additional analyses. If a (yet) hypothetical DBS cut-off is also exceeded, the ADRV is corroborated; if the glucocorticoid concentration in the DBS falls below the cut-off as exemplified in the 24 h sample collection of this pilot study, an effect of the drug on the tested individual is unlikely.

(A)



(B)

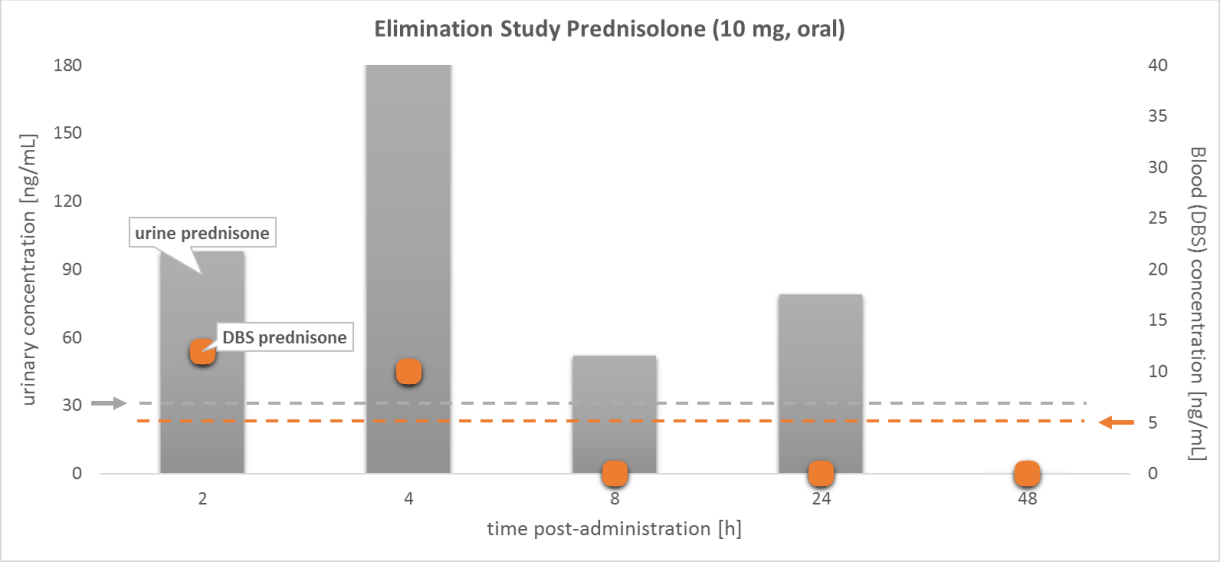


Figure 2: Proof-of-principle elimination study results with prednisolone. Following an oral administration of 10 mg, urine and DBS samples were collected up to 48 h post-dosing and results for prednisolone (A) and prednisone (B) are presented separately. The WADA urinary reporting level of 30 ng/mL (grey line) was exceeded up to 24 h in the case of prednisone (B, grey bars) with and without specific gravity-adjustment), while urinary prednisolone concentrations (A, black bars) fell below the reporting level at 8 h post-administration. Corresponding DBS samples returned blood concentrations for prednisolone (A, orange circle) and prednisone (B, orange squares) above 5 ng/mL (hypothetical cut-off, orange line) only during the first 4 h. This suggests the absence of systemic glucocorticoid effects thereafter.

Conclusions

The knowledge of blood concentration levels concerning drugs banned in-competition only would be a benefit in routine doping controls.[5, 13] The procedure and costs associated with whole blood sampling for sports drug testing purposes has been a limiting factor in the past; the more recently accomplished analytical sensitivity, however, has enabled the consideration of alternative matrices such as dried blood spots as a particularly convenient add-on to routine doping controls by representing a complementary source of information. This source can facilitate the provision of data on the blood concentration of target analytes and, thus, provide considerable support to the result management processes.[13] Ideally, requests concerning this information will only be issued when urinary reporting levels are exceeded. Thus, the concomitant collection of DBS together with conventional doping control urine samples is necessary in in-competition settings, and the development of platforms allowing for quantitative DBS analyses, where both pre-analytical (i.e. sample collection strategy), scientific (availability of pharmacokinetic data, etc.) and analytical aspects (sensitivity, specificity, hematocrit effects, etc.) have to be taken into consideration, is required. If these conditions are met, DBS will have great potential to complement and improve routine doping controls; nevertheless, the use of substances banned in-competition is and remains prohibited during in-competition periods; consequently, compliance with anti-doping rules via utmost care and vigilance will have to remain a priority to elite sports athletes.

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