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Dried blood spots (DBS) for doping control analysis

Andreas Thomas,* Hans Geyer, Sven Guddat, Wilhelm Schänzer and Mario Thevis

Whole blood sample collection on cellulose paper has a more than 30-year-long tradition, especially in neonatal screening. The sampling is minimally invasive, fast, discreet, and robust against manipulation.

The present approach highlights the potential to determine doping agents in dried blood spots (DBS) after extraction and subsequent analysis by liquid chromatography-mass spectrometry (LC-MS). The assay is focused on selected model compounds of which the circulating target concentration is of particular interest. Here, pre- or post-competition testing with DBS allows probing for the conditions (i.e. presence or absence of relevant drugs) in the athlete's circulation during competition, which complements earlier approaches towards the identification of urinary indicators for the temporal application of substances prohibited in-competition only.

Precise (< 20%), linear, and robust conditions with limits of detection in low ng/ml range were accomplished by means of LC coupled to high resolution/high accuracy mass spectrometry for the selected model compounds benzoylecgonine, cocaine, pseudoephedrine, amphetamine, salbutamol, and JWH-018. Deuterium-labelled internal standards were used to yield reliable quantitative results. In addition, the non-targeted screening approach (positive/negative switching combined with tandem mass spectrometry (MS/MS) experiments) enables the retrospective qualitative data evaluation for a comprehensive selection of known and unknown substances as exemplarily shown by the extraction of 20 target compounds (corticosteroids, aromatase inhibitors, anabolic steroids, beta-blockers, etc.) at 20 ng/ml.

The simple and fast nature of the assay allows for an easy implementation into existing procedures and will potentially enhance the effectiveness of testing by reducing costs and effort of pre-analysis workload.

Keywords: sports drug testing; blood analysis; non-targeted screening; LC-MS

Introduction

The automation of laboratory processes has continuously been improved, and the simplification and acceleration of pre-analytical steps such as sample collection, transfer, and storage are desirable benefits in most medico-analytical arenas. The collection of whole blood samples, dried on a piece of paper, offers various advantages over conventional venopuncture-based blood sampling concerning time consumption, workload and costs without compromising the required quality.

The use of dried blood spots (DBS) was reported as early as the 1960s when this technique was applied to the sample collection for testing for phenylketonuria in newborns by a simple heel prick.[1] This pilot study initiated further applications, which finally led to the fact that nowadays more than 95% of newborns in the USA are screened for various diseases by means of DBS.[2,3]

In comparison to conventional venous cannula sampling, the collection of a drop of blood (usually 10–30 μl) from a heel, finger, or ear prick is considerably less invasive.[4–6] This minimizes the risk of infections especially for sensitive patients such as infants on the one hand, and enables the more frequent collection of samples in pharmacokinetic studies with small laboratory animals (mice, rats, etc.) on the other hand.[6] The stability of the cellulose-fixed target analytes is generally described to be superior to plasma, serum, or urine storage conditions due to inactivation of enzymatic degradation processes.[2,7,8] This is additionally supported by specifically modified collection card surfaces with denaturating agents.

Various applications for DBS are described for clinical (paediatrics), toxicological, pharmacological and (in one case) also doping control purposes. Besides the use of immunological assays, chromatographic (gas or liquid) separation approaches coupled to different detection systems (UV-Vis detector or mass spectrometry) were recently described.[7–26] All assays follow the common strategy to perform a more or less simple extraction, whereby the extraction conditions (organic/aqueous solvent ratio) strongly depend on the chemical properties of the target analytes. In the present study, an assay for selected prohibited threshold substances by means of liquid chromatographic separation coupled to high resolution/high mass accuracy mass spectrometry (LC-MS) is reported. Samples are manually punched from DBS cards, fortified with labelled internal standards, and extracted with an organic solvent prior to the LC-MS measurement. Target substances implemented in the method are cocaine, benzoylecgonine, salbutamol, ephedrine/pseudoephedrine, amphetamine and JWH-018, and validation for qualitative and quantitative purposes was performed.

* *Correspondence to: Andreas Thomas, Institute of Biochemistry/Center for Preventive Doping Research, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany.*
E-mail: a.thomas@biochem.dshs-koeln.de

Institute of Biochemistry/Center for Preventive Doping Research, German Sport University Cologne, Germany

Materials and methods

Chemicals

Acetonitrile, methanol, acetic acid, formic acid, ammonium hydrogen carbonate were of gradient resp. p.a. grade and purchased from Sigma (Deisendorf, Germany). Pseudoephedrine was from Cerilliant (Rock Round, TX, USA), salbutamol from Riedel-de-Haen (Seelze, Germany) and JWH-018 from LGC Standards (Wesel, Germany). The reference compounds for cocaine, benzoylecgonine and amphetamine were purchased from Sigma (Deisendorf, Germany). The labelled internal standards (ISTD) cocaine-d_3 and ephedrine-d_3 were obtained from Cambridge Isotopes (Andover, MA, USA) and salbutamol-d_3 was from Dr Ehrensdorfer GmbH (Augsburg, Germany).

Reference blood (MediDrug 3/09-C VB-plus, #AS 10927) was obtained from Medichem Diagnostica GmbH (Steinenbronn, Germany).

Materials

DBS cards (DMPK A, B, and C-cards) and puncher were obtained from Whatman GE (Uppsala, Sweden) and TFN filter paper (179 g/m^2) was purchased from Sartorius (Goettingen, Germany). The polypropylene tubes were from Eppendorf (Hamburg, Germany).

Sample preparation

The complete spots on the DBS card were punched out and transferred into an Eppendorf tube. The labelled ISTDs were added to the tubes with 0.6 ng each from a methanolic solution containing 0.1 ng/ml. Subsequently, the spots were suspended in 300 µl of a mixture of methanol/acetonitrile/aqueous acetic acid (2%) (1:1:1, *v/v/v*). After ultrasonication for 10 min, the tubes were centrifuged for 5 min at 17.000 *g*. The supernatant was transferred into a fresh tube prior to evaporation of the solvent in a vacuum centrifuge. The dry residue was reconstituted in 50 µl of aqueous acetic acid (2%)/acetonitrile (9:1, *v/v*), centrifuged for 5 min at 17.000 *g*, and 10 µl of the supernatant were used for injection.

Liquid chromatography

Liquid chromatography was performed on an Accela UPLC system (Thermo, Bremen, Germany) equipped with an analytical column Gemini C6, 2.1 x 10 mm, 3 µm particle size (Phenomenex, Aschaffenburg, Germany). Solvents consisted of A: 0.2% formic acid, and B: acetonitrile. The gradient started at 100 % A, held for 1 min, raised to 100% B in 9 min, followed by an reequilibration phase at starting conditions (5 min). The flow was set to 250 µl/min and the total run time was 15 min.

Mass spectrometry

The mass spectrometer used was an Exactive benchtop Orbitrap mass spectrometer (Thermo, Bremen, Germany) equipped with an electrospray ion source with scan-to-scan polarity switching. Full scan experiments without fragmentation were performed with a resolving power of 50 000 (full with at half maximum, FWHM) and additionally two higher energy collision-induced dissociation (HCD) experiments were acquired at 20 and 50 eV and a resolution of 25 000 (FWHM). The instrument was calibrated to ensure mass accuracies in low ppm range ($<$ 5 ppm) with the manufacturer's calibration kit containing caffeine, the tetrapeptide MRFA, and ultramark. Nitrogen generated by a CMC nitrogen generator (Eschborn Germany) was used as collision and damping gas in the curved linear ion trap and for the ESI source. Chromatograms were evaluated for the intact protonated molecules with an *m/z* window of 0.01 Da from the theoretically calculated molecular mass. Main parameters are summarized in Table 1.

Blood and urine samples

Blood spots for method development were collected from healthy male or female volunteers and aliquots of 20 µl were spotted on the targets of the collection cards by means of a calibrated pipette. The cards were allowed to dry for 2 h at room temperature. Post-administration study specimens were sampled after single application of 30 mg of pseudoephedrine-HCl (oral, combi-Preparation with 120 mg acetylsalicylic acid) to a 37-year-old male. Blood spots (20 µl) were collected after 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 24 h and spontaneous urine samples were aliquoted after 0, 2, 4, 7, 12, 23 h. Written consent from the volunteers and approval from the local ethics committee was obtained. The samples (urine and DBS cards) were stored refrigerated until analysis.

Reference blood (lyophilisate) was reconstituted in 2.5 ml of pure water following the manufacturer's instructions and 20 µl were spotted onto the cards.

Validation

Validation of the method's properties was performed with fortified whole blood samples. Therefore the blood was enriched

Table 1. Main mass spectrometric and chromatographic parameters of targeted analytes

Analyte	Molecular formula	Polarity	$[M+H]^+$	Retention time	Class of compound
			m/z	[min]	
Benzoylecgonine	$C_{16}H_{19}O_4N$	Positive	290.1387	5.3	Stimulant
Cocaine	$C_{17}H_{21}O_4N$	Positive	304.1543	5.2	Stimulant
Pseudoephedrine	$C_{10}H_{15}ON$	Positive	166.1226	4.0	Stimulant
Amphetamine	$C_9H_{13}N$	Positive	136.1121	4.1	Stimulant
Salbutamol	$C_{13}H_{21}O_3N$	Positive	240.1594	3.4	ß$_2$-agonist
JWH-018	$C_{24}H_{23}ON$	Positive	342.1852	11.5	Cannabinoid
Cocaine-d_3	$C_{17}[^2H_3]H_{18}O_4N$	Positive	307.1732	5.2	ISTD1
Pseudoephedrine-d_3	$C_{10}[^2H_3]H_{12}ON$	Positive	243.1783	3.8	ISTD2
Salbutamol-d_3	$C_{13}[^2H_3]H_{18}O_3N$	Positive	169.1415	3.4	ISTD3

and extensively mixed prior to spotting on the paper. The samples were spotted with a calibrated pipette (20 μl) into the predefined circles on the paper and dried at room temperature for at least 1 h.

Specificity

Ten DBS from healthy volunteers (6 male, 4 female) without medication were analyzed for presence of interfering signals. This experiment was performed with the Whatman as well as with the Sartorius cards.

Limit of detection (LOD)

Six samples fortified at the estimated LOD for each model compound were prepared and analyzed.

Linearity

Whole blank blood specimens were fortified with 0, 10, 25, 50, 75 and 100 ng/ml of each target analyte. Aliquots of 20 μL were spotted on a card, dried, and subsequently analyzed.

Recovery

Blood was fortified with 100 ng/ml of each target compound, and six spots of 20 μl were spotted on a paper. In addition to this, six blank spots of 20 μl volume were spotted also. All 12 spots were punched out completely and analyzed as already described. The six blank spots were then fortified with 100 ng/ml of each target substance into the readily prepared injection solution. These experiments were performed with Whatman and Sartorius cards.

Precision

Six spots with 50 ng/ml of each target were spotted and analyzed as described.

Robustness

The robustness regarding the different types of cards was evaluated by spotting aliquots of fortified blood samples on four different cards (Whatman type A, B, C) and Sartorius (TFN 179 g/m^2). Punches of these cards were analyzed and visually examined for interfering signals.

Stability

Storage of DBS is of utmost interest considering an enhanced stability of the target analytes under dry conditions. Therefore fortified (100 ng/ml) blood samples were spotted on a Whatman type C card and stored at 20 °C for 0, 1, and 2 days and at 4 °C for 0, 1, 2, 5, and 14 days. Subsequent analysis was performed according to the described procedure. The stability was tested for type C cards only, due to expected potential degradation for these untreated cards.

Accuracy

An independent proficiency test is a good option to compare quantitative results. The commercially available whole blood control sample MediDrug VB 09/3 was spotted twice with 20 μl on a Whatman type C card, dried and analyzed as described. Simultaneously, 5 aliquots of a blank specimen were fortified with respective amounts of cocaine (0, 10, 25, 50, 75 ng/ml) and benzoylecgonine (0, 50, 100, 150, and 200 ng/ml) and also spotted on a card to serve as calibration points. Concentrations of cocaine and benzoylecgonine in the reference blood were calculated by means of the resulting linear calibration curve (external) employing the analytes' ratio to the ISTD.

Ion suppression

The impact of ion suppression effects due to coeluting matrix components was evaluated by post-column infusion experiments. Therefore a 0.05 ng/μl solution of the target analytes were infused (with a syringe pump and 5 μl/min) to the regular gradient flow of a blank injection after the analytical column by means of a Tee-split-connector (post-column-split).[27]

Quantification of pseudoephedrine in administration study samples

DBS samples (20 μl) obtained after single oral application of pseudoephedrine-HCl were prepared as described and quantification was performed with an external calibration curve of fortified and spotted blank blood aliquots between 0 and 100 ng/ml. Concentrations are reported in ng/ml and the volume of the blood specimen (20 μl) was applied with a calibrated pipette to the paper. The spontaneous urine samples were analysed by direct injection (2 μl of urine) after addition of ephedrine-d_3 as ISTD.[28] Quantification was performed with an external calibration with fortified blank urine samples in the range between 1 and 100 μg/ml. The values were not corrected to density or creatinine.

Results and discussion

Considering the special requirements of sports drug testing, DBS offers the potential to directly determine the current concentration of circulating substances just prior to or after competition. This is of utmost importance because distinct classes of substances are prohibited in-competition only, whilst the use of such drugs during training periods are not sanctioned according to sports drug testing regulations.[29]

Another benefit of DBS in a doping control context is the fact that the sample collection occurs without invading the privacy of the athlete, independently of gender.[30] Simplified sample collection, storage, and transfer will have a considerable impact on the costs of anti-doping tests in the future. Moreover, manipulation of urinary doping control samples was frequently reported in the last years, and also here DBS represents an advantageous strategy.[31,32]

Beside these benefits, there are also drawbacks in DBS analysis. The most important one is the limited sample volume of 10–30 μl. In combination with very low concentrations of target analytes, for some classes of substances (e.g. anabolic agents, cannabinoids) the required LODs are not sufficient with actual performance and sensitivity of most available mass spectrometers.[33] Accordingly, for these substances, classical urine analysis is still the method of choice and DBS sampling can provide complementary information only. However, sensitive assays are recommended generally to reach the relevant LODs and, thus the ongoing process in developing more sensitive mass spectrometers will potentially enable adequate LODs for all targets in a single drop of blood in the future.

Whilst homogeneity in fluidic samples like plasma or urine is ensured *per se*, the potential heterogeneity of the DBS on the paper which is influenced mainly by the hematocrit of the sample is of major concern.[2] This has a reasonable impact especially on quantitative result interpretation.

Concentrations of prohibited substances in blood strongly depend on the class of compounds. While levels of stimulating agents are mainly in the range of 1–100 ng/ml, classical cannabinoids (e.g. marijuana) reach maximum plasma values of the active ingredients (e.g. tetrahydrocannabinol (THC) and OH-THC) of 0.5–5 ng/ml 2 h after consumption of a common dose.[33,34] Here, the described assay is unfortunately not sensitive enough. Recently a new class cannabinoids has reached the focus of doping controls and since 2010 JWH-018 and related substances were included in the list of prohibited substances.[29] Those are reported to reach C_{max} in serum of more than 10 ng/ml with a fast drop to sub-ng-levels within 3 h.[35] Accordingly, relevant concentrations during competition were sufficiently detected with the presented DBS methodology. This is also valid for the target substances cocaine, amphetamine, salbutamol and ephedrine with relevant plasma levels between 5 and 100 ng/ml or higher.[36–41]

Figure 1 shows an extracted ion chromatogram of a fortified blood sample (obtained from a finger prick) with abundant signals for benzoylecgonine (5 ng/ml), cocaine (5 ng/ml), pseudoephedrine (5 ng/ml), amphetamine (10 ng/ml), salbutamol (5 ng/ml) and JWH-018 (5 ng/ml). Additionally, the diagnostic ion traces for the ISTDs cocaine-d_3, ephedrine-d_3, and salbutamol-d_3 are illustrated. The typical retention-time difference between ephedrine/pseudoephedrine does not allow for baseline separation with the chosen chromatographic conditions but was found to be sufficient for differentiation between these stereoisomers.

The potential of the acquired data for detecting additional prohibited compounds is illustrated in Figure 2. Here the extracted ion traces (0.01 Da around the theoretical mass) of a fortified DBS sample is shown. The sample was spiked with 20 ng/ml of anastrozol, clenbuterol, clomiphene, exemestane, budesonide, stanozolol, metandienone, strychnine, cocaine, mesocarb, bisoprolol, furosemide, dexamethasone, selective androgen receptor modulators (SARMs, S9, S4, S1, S24), metoprolol, propranolol, and tamoxifen. The ISTD was cocaine-d_3. Scan-to-scan polarity switching enables the detection of different SARMs and furosemide as $[M\text{-}H]^-$ in negative ionisation mode, while all other substances were acquired in positive mode as $[M+H]^+$ within one chromatographic run.[42] A representative blank DBS sample provided no interferences in these selected ion traces (data not shown). Although the method was not validated for these compounds, the data underline the potential to enlarge the approach to other drugs and enable retrospective qualitative data evaluation.[42]

Validation

Blank blood samples from ten healthy volunteers analysed for specificity showed no interfering signals in the extracted ion traces at the respective retention times. This was valid for both types of card manufacturers. Samples fortified with every single target compound (at 50 ng/ml) provided unambiguous results for the respective compound; also the addition of ISTDs showed no interference in the diagnostic ion traces of the chromatograms. These observations were interpreted as crucial requirement for further experiments. The test for linearity should ideally enable the quantification of the target substances in the working range by external calibration using different ISTDs. All analytes yielded a coefficient of correlation between 0.97 and 0.99 with intercepts below 0.06. These results verify that linear approximation is possible and interferences from the blank matrix were not present. Imprecision, calculated as relative standard deviation from a sixfold determination at 50 ng/ml was below 20% for each

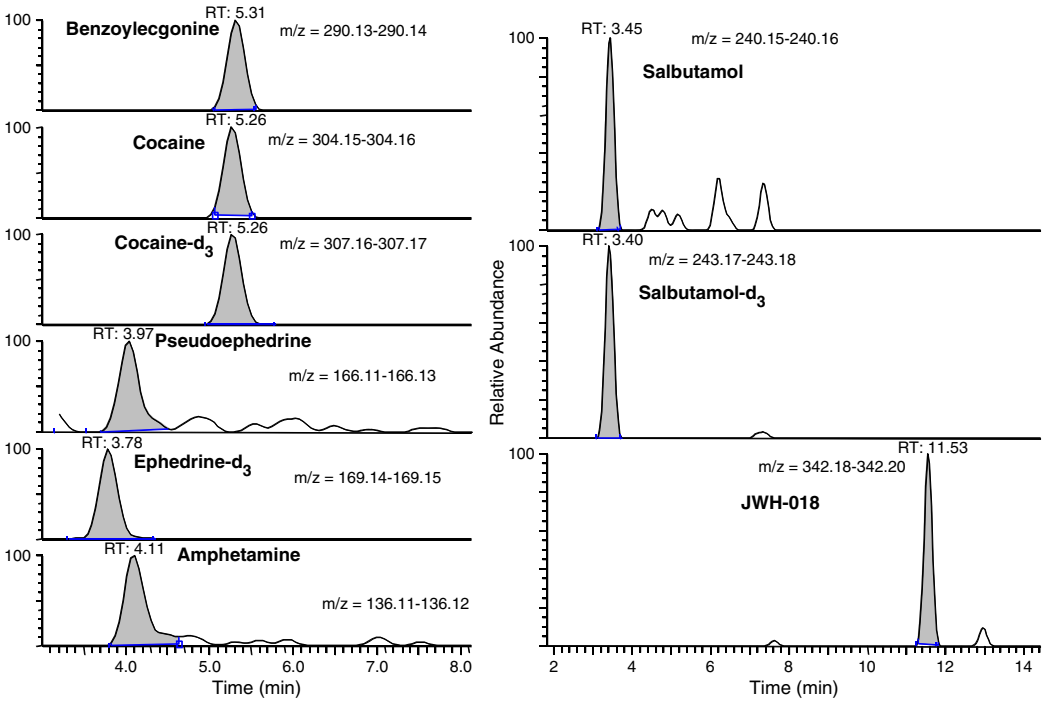

Figure 1. Extracted diagnostic ion traces of a fortified DBS sample with concentrations near the respective LOD: cocaine, benzoylecgonine, pseudoephedrine, salbutamol and JWH-018 at 5 ng/ml and amphetamine at 10 ng/ml.

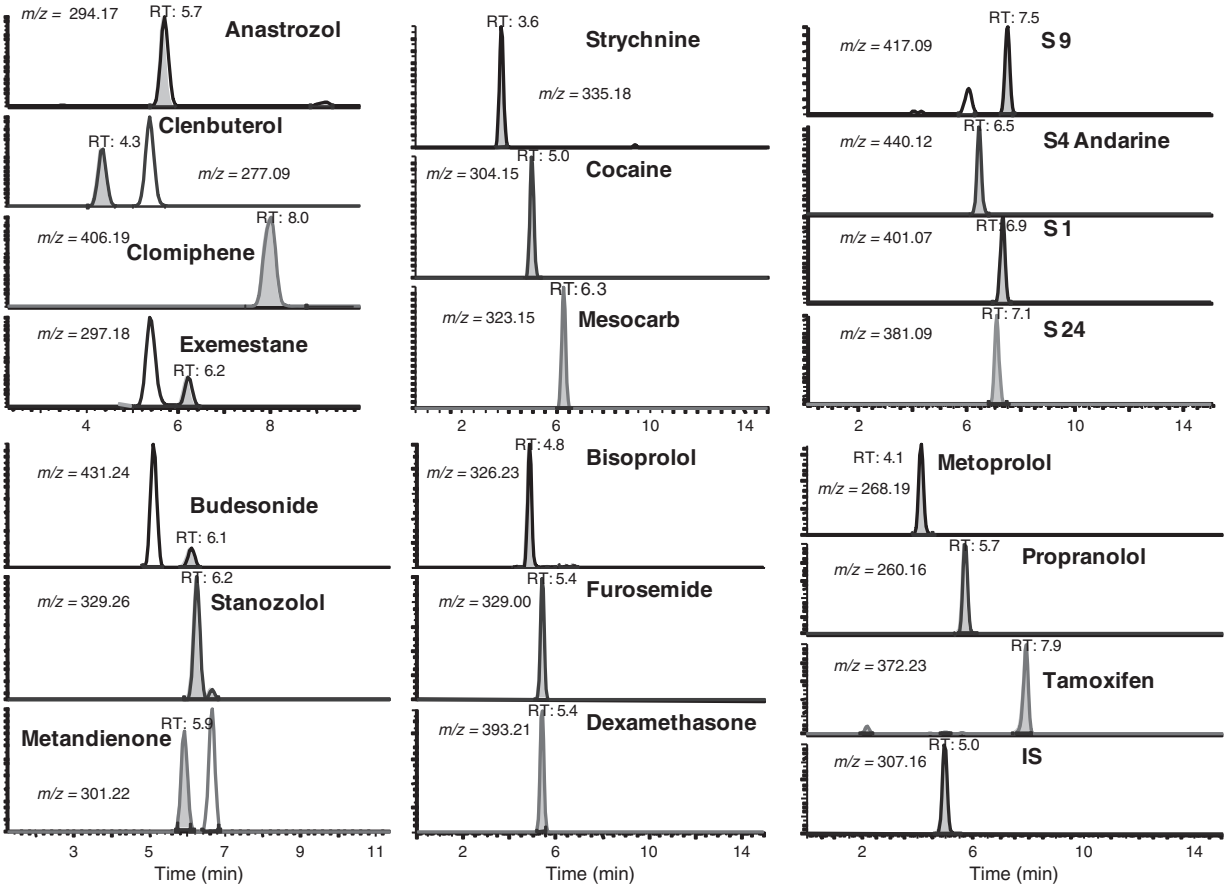

Figure 2. Principle of non-targeted approach illustrated by extracted ion traces (*m/z* window: 0.01 Da) of a fortified DBS sample (at 20 ng/ml) for selected prohibited substances from different classes of drugs.

analyte and the recovery typically ranged between 30 and 50% (for both types of cards). Especially the usage of labelled analogues should compensate for all effects during extraction, chromatography and subsequent mass spectrometric detection. Consequently, performance parameters for cocaine, ephedrine, and salbutamol (e.g. precision or coefficient of correlation) provided excellent results (Table 2) and corroborate the recommendation that labelled ISTDs are crucial for reliable quantitative analysis. Accuracy was determined by quantification of cocaine and benzoylecgonine in the spotted reference blood. Here the results of a twofold determination yielded values of 63 and 61 ng/ml for cocaine (acceptance range from the provider: 38.6–64.2 ng/ml) and 117 and 172 ng/ml (acceptance range: 114–177 ng/ml) for benzoylecgonine. Due to the fact that the complete spot is extracted and the sample was applied with a calibrated pipette, normalization to a volume of 20 µl was performed and, thus, comparison to classical volumetric approaches was enabled. The monitoring of potential ion suppression effects showed strong suppression in the retention time window near the dead volume of the system (0.8–1.5 min) and between 6.8 and 7.5 min, but the retention time windows of the selected model compounds were not affected (Figure 3). The LOD was evaluated by signal to noise (>3) with values between 2 and 10 ng/ml (Table 2). Significant differences between the cards were not observed for the selected model compounds and this was tested for potential interferences in blank specimens as well as for differences in recovery under the chosen conditions. Storage of spotted cards at room temperature (and at 4 °C) for two weeks showed no significant degradation of the selected target analytes; this is in concordance with former studies.[2,7] Here it is noteworthy, that these results were generally valid for the chosen model compounds (and likely their

Table 2. Main validation results

	Precision	Recovery	LOD		Linearity	(n = 7)	Stability	
	(n = 6)	(n = 6 + 6)	[ng/ml]	Intercept	slope	correlation (r^2)	20 °C	4 °C
Benzoylecgonine	15.7%	44.1%	2	0.004	0.002	0.964		
Cocaine	7.1%	43.3%	2	0.017	0.009	0.990		
Pseudoephedrine	10.1%	33.7%	5	0.017	0.007	0.994	> 2 days	> 2 weeks
Amphetamine	8.7%	48.0%	10	−0.022	0.005	0.944		
Salbutamol	14.3%	41.6%	2	0.059	0.010	0.976		
JWH-018	19.4%	28.5%	2	−0.004	0.001	0.983		

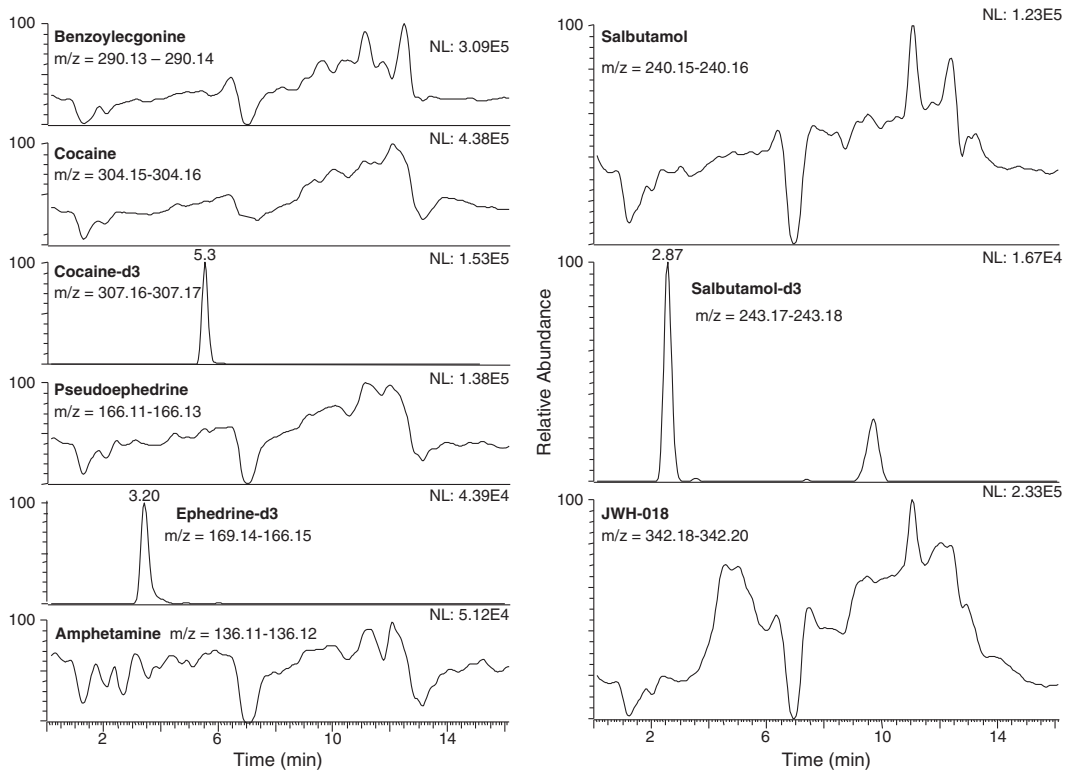

Figure 3. Extracted diagnostic ion traces of a typical blank sample with post-column reference-infusion for evaluation of potential ion suppression effects in the respective retention time windows of the target analytes.

related compounds) only and should be re-evaluated thoroughly for each additional analyte of interest.

Administration study

As one of the most important experiments the proof of principle was shown by application study specimens with pseudoephedrine and subsequent comparison of urinary and blood (DBS) concentrations over a 24-h period (Figure 4). C_{max}-values in blood were reached after 2–4 h (90 ng/ml), whereby maximal urine concentrations were determined after 7–9 h (23 μg/ml). Despite relevant levels for a doping violence after administration of 30 mg of pseudoephedrine-HCl were not reached in urine (limit: 150 μg/ml), it is obvious that pre- or post-competition urine testing only might miss the maximal values that were present in circulation during competition. Here the effective blood levels would be reliably determined with pre- or post-competition DBS sampling.

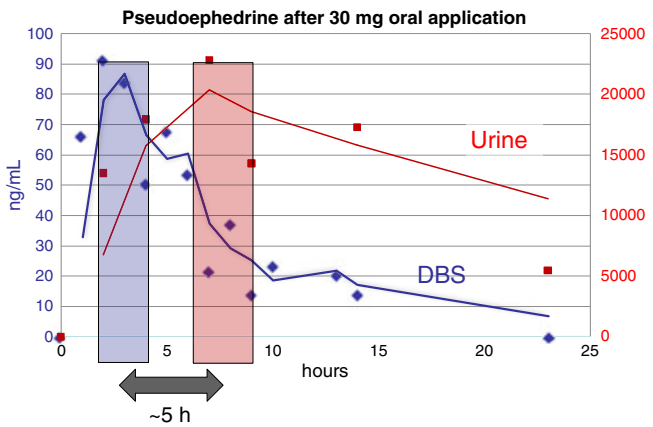

Figure 4. Comparison of DBS (blue) and urinary (red) concentrations after a single oral application of 30 mg of pseudoephedrine. C_{max} in blood (DBS) was reached after 2–4 h (~90 ng/ml) and after 7–9 h in urine (~23 μg/ml).

Conclusion

DBS in doping controls will certainly not replace classical urine (or blood) analysis, but the numerous benefits of this sample collection already led to an extensive use in preclinical metabolism/pharmacokinetic studies and would accordingly enhance the interpretability of doping control results.

On consideration of the ongoing engineering progress in building more sensitive mass spectrometers, the options provided by introducing DBS sampling in doping controls should not be missed, because with comparably low formal and instrumental workload for implementation of the technique a reasonable potential to enhance the effectiveness of testing is possible.

Hereby, the determination of the current concentration of circulating substances (banned in-competition) in competing athletes possesses benefits for both parties: the drug testing authorities are provided with the concentration of the active compound and thus can estimate its relevance more accurately. In the same context, the athlete has the opportunity to deliver a specimen to verify the absence of a prohibited substance, if in the respective urine sample an (inactive) metabolite is detected that is used to indirectly indicate a misuse. The additional information of the DBS analysis will surely have an impact on the consecutive sanction of the sportsmen in both scenarios.

In the presented study the analysis was focused on LC coupled to MS only, but after sufficient extraction also other analytical approaches based on gas chromatography or bioassay detection (ELISA, RIA, etc.) are conceivable. This will enlarge the number of potential target analytes to nearly all already established assays implemented in the World Anti-Doping Association (WADA) accredited laboratories worldwide using the existing instrumentation.

Another important benefit is given by the simple, long-time storage possibilities of a large number of cards (equivalent to doping specimens) for important sport events for re-analysis if new techniques or discoveries are available.

Altogether, DBS analysis in doping controls possesses very promising but not yet fully developed potential to yield more information by well-established analytical approaches.

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