Multi-class screening method for the analysis of doping agents in dried blood spot (DBS) samples

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Summary

In the last years, the use of Dried Blood Spots (DBS) as alternative sampling technique is reaching an increasing interest in the antidoping field. The collection of DBS is performed from small volumes of capillary blood lied on adsorbent support (e.g. cellulose) and let to dry. Compared to the conventional urine or blood collection, this technique presents several advantages as the sampling is less invasive, rapid and does not required specialized personnel to be performed. It also facilitates transport and storage, reducing the costs. Conversely, the reduced sample volume available could represent a limitation and requires the use of highly sensitive instrumentation.

This work presents a multi-class screening method for 100 compounds belonging to different groups of the World Anti Doping Agency prohibited list, including anabolic agents, beta-2 agonists, hormone and metabolic modulators, and diuretics, among others. DBS samples for method development were obtained depositing 20 µL of venous blood and letting them to dry on cellulose cards. The whole DBS spot was then punched out and extracted with organic solvents prior to the analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS).

The methodology was validated for qualitative purposes. Different parameters were evaluated. The vast majority of the compounds could be reliably detected at sub-ng/mL level. Satisfactory results were obtained in terms of recovery, precision and robustness. Also, matrix effects were negligible for most compounds, as expected considering the low volume of sample analyzed. As a final validation step, DBS samples collected after administration of boldenone, oxandrolone and tamoxifen to healthy volunteers were analyzed. The method showed good performance and robust results, making it fit-for- purpose for its application in sports drug testing.

Key words:
Doping. Dried blood spots. DBS. Doping agents.

Método de detección multiclase para el análisis de agentes dopantes en gotas de sangre seca (DBS)

Resumen

En los últimos años, el uso de gotas de sangre seca (DBS) como técnica de muestreo alternativa está alcanzando un interés creciente en el campo antidopaje. La recogida de DBS se realiza a partir de pequeños volúmenes de sangre capilar que se colocan en un soporte adsorbente, normalmente de celulosa, y se dejan secar. Esta técnica presenta varias ventajas respecto a las matrices convencionales (orina, sangre) ya que el muestreo es menos invasivo, rápido y no requiere personal especializado. También facilita el transporte y almacenamiento, reduciendo los costes. Por el contrario, el reducido volumen de muestra disponible podría representar una limitación y requiere el uso de instrumentación altamente sensible.

En este trabajo, se presenta un método de detección multiclase para 100 compuestos de la lista prohibida de la Agencia Mundial Antidopaje, incluyendo agentes anabólicos, agonistas beta-2, hormonas y moduladores metabólicos, y diuréticos, entre otros. Las muestras de DBS para el desarrollo del método se obtuvieron depositando 20 µL de sangre venosa y dejándola secar sobre tarjetas de celulosa. Después se cortó toda la mancha y se extrajo con disolventes orgánicos y se analizó mediante cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS).

La metodología fue validada con fines cualitativos. La gran mayoría de los compuestos se pudieron detectar de forma fiable a niveles inferiores al ng/ml. Se obtuvieron resultados satisfactorios en términos de recuperación, precisión y robustez. Además, el efecto matriz fue insignificante para la mayoría de compuestos, como se esperaba considerando el pequeño volumen de muestra analizada. Como paso final de validación, se analizaron muestras DBS obtenidas tras la administración de boldenona, oxandrolona y tamoxifeno a voluntarios sanos. El método mostró resultados sólidos, lo que lo hace adecuado para su aplicación en el control del dopaje en el deporte.

Palabras clave:
Dopaje. Gotas de sangre seca. DBS. Agentes dopantes.

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Introduction

Dried blood spots (DBS) are a microsample consisting of depositing a small volume of capillary blood generated by a finger, arm or heel puncture, on a support, generally made of pure cellulose[1]. DBS samples are used since the 1960s for the neonatal screening of phenylketonuria[2], and new applications have been described since then[3]. In recent years, there has been growing interest in the application of DBS sampling to other fields and, among them, the doping control field.

DBS samples have advantages over conventional liquid matrices currently used in doping control (urine, serum, plasma). Firstly, DBS sample collection is much less invasive and intrusive than conventional blood or urine samples, and it can be obtained without the need for specialized personnel, even it can be obtained by athletes themselves[4,5]. Furthermore, the stability of the analytes increases considerably even at room temperature and microbial growth is inhibited[1,3]. For these reasons, transportation and storage of DBS samples is simple and costs are reduced compared to conventional matrices. However, the small sample volume available (10-30 µL) can hinder the detectability of analytes, especially those that are present at very low concentrations. This is an important factor in antidoping analysis, as the ability to detect very low concentrations of certain analytes is required in order to ensure long detection windows. This drawback can be partially avoided by using very sensitive instrumentation and by the continuous improvement of the sensitivity and selectivity of analytical techniques[7], and by the increase in the frequency of controls allowed by the reduction of the cost of sample collection, transport and storage[8].

In 2021, the World Anti-Doping Agency (WADA) published a technical document harmonizing the use of DBS for doping control[9]. This document serves as the operational framework for the application of DBS samples for antidoping control purposes, regulating the collection, transportation, storage and analysis of samples. WADA has encouraged antidoping laboratories to develop analytical methodologies to detect doping agents in DBS samples. For that reason, there have been an increasing number of studies addressing the detection of doping agents in DBS[10-14].

In this work, a method to detect doping agents from different classes on the WADA prohibited list (anabolic agents, hypoxia inducible factor activating agents, growth hormone releasing factors, beta-2 agonists, hormone and metabolic modulators, and diuretics and masking agents) in DBS samples is presented. The DBS sample is extracted with organic solvents prior to the analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). The method has been validated following WADA guidelines, and has been applied to DBS samples obtained after administration of some doping substances to healthy volunteers.

Material and method

Materials and reagents

Tert-butyl methyl ether (TMBE), acetone, acetonitrile, methanol (LC gradient grade) and formic acid (LC-MS grade) were obtained from Merck (Darmstadt, Germany). Reference standards of the compounds under study were purchased from Sigma (St. Louis, MO, USA), Toronto Research Chemicals (Toronto, Canada) or NMI (Australia). A Milli-Q purification system was used (Millipore Ibérica, Barcelona, Spain).

Sample preparation

First, the whole spot of 20 µL was punched out and transferred into a polypropylene tube. Then, 10 µL of a methanolic solution of the ISTD (testosterode-d3, 3 ng/mL; furosemide- d5, 100 ng/mL) and 500 µL of methanol:acetonitrile (1:1 v/v) were added. The tubes were treated in an ultrasonication bath for 15 min. The supernatant was transferred into a fresh glass tube and the spot was extracted a second time with 500 µL of acetone (ultrasonication bath for 15 min). The combined solvents were evaporated to dryness under nitrogen stream in a water bath at 40ºC. The residue was reconstituted with 60 µL of a mixture of acetonitrile:water (50:50, v/v) and, then, diluted with 30 µL of water. The reconstituted extract was centrifuged for 5 min at 10.000 rpm, and the supernatant was transferred to the injection vial. A volume of 10 µL of the supernatant was analyzed by LC-MS/MS.

Analysis by liquid chromatography-mass spectrometry (LC-MS/MS)

LC-MS/MS analyses were carried out using a triple quadrupole (Xevo TQ MS) mass spectrometer (Waters Associates, Milford, MA, USA) coupled to an Acquity ultra-high performance liquid chromatographic system (Waters Associates). Nitrogen was used as desolvation gas and argon was used as collision gas. The desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. The nitrogen desolvation temperature was set to 450ºC and the source temperature to 120ºC. Capillary voltage of 3.5 kV was used in positive ionization mode.

Liquid chromatographic separations were performed using an Acquity BEH C18 column (100 x 2.1 mm i.d., 1.7 mm particle size) (Waters Associates). Water (solvent A) and acetonitrile (solvent B) both with formic acid (0.01%) were selected as mobile phase solvents. The percentage of organic solvent was linearly changed as follows: 0 min 3%, 1 min 3 %, 8 min 83%, 9 min 95%, 9.1 min 3%, 11 min 3% at a flow rate of 0.4 mL/min and at 45ºC.

Mass spectrometric analysis was performed in multiple reaction monitoring (MRM) mode in positive or negative electrospray ionization mode, by measuring two ion transitions per compound (Table 1).

Validation protocol

The following parameters were evaluated during method validation: selectivity and specificity, recovery, limit of detection, intra-day precision, robustness, and matrix effect. The validation was performed using DBS samples prepared by spotting 20 µL of human venous blood, containing EDTA as anticoagulant, onto Whatman® FTA DMPK-C cards, and allowing them to dry for at least 2 h at room temperature. DBS samples were protected from direct light sources, and were stored with suitable desiccant in zip-closure foil bags and kept at -20 ºC until analysis. The samples containing analytes were prepared by adding the appropriated stock solutions to the human venous blood and, then, the DBS sample was prepared and stored as already explained.

Table 1. Analytical conditions for the compounds included in the analytical method and the internal standards (ISTD): group of substances according to WADA Prohibited List[15], polarity in electrospray ionization (ESI), precursor ion and product ions used to monitor every target compound, and retention time (RT, min).

Group of substances	Compound	ESI polarity	Precursor ion (m/z)	Product ions (m/z)	RT (min)
Anabolic Androgenic Steroids (S1.1)	1-Androstendione	positive	287	185, 203	6.3
	1-Testosterone	positive	289	187, 205	6.0
	4-Chlorometandienone	positive	335	155, 149	6.3
	Bolasterone	positive	317	97, 123	6.2
	Boldenone	positive	287	135, 121	5.4
	Calusterone	positive	317	97, 123	6.4
	Clostebol	positive	323	143, 131	6.4
	Drostanolone	positive	305	269, 215	6.9
	Fluoxymesterone	positive	337	299, 281	5.1
	Gestrinone	positive	309	199, 241	5.9
	Mestanolone	positive	305	229, 269	6.6
	Mesterolone	positive	305	269, 173	6.5
	Metandienone	positive	301	121, 149	5.6
	Methasterone	positive	319	229, 283	7.2
	Methenolone	positive	303	83, 187	6.0
	Methylclostebol	positive	337	143, 131	6.6
	Methyldienolone	positive	287	269, 135	5.5
	Methylstenbolone	positive	317	201, 145	7.0
	Methyltestosterone	positive	303	97, 109	6.0
	Methyltrienolone	positive	285	277, 198	5.4
	Mibolerone	positive	303	107, 121	6.0
	Nandrolone	positive	275	109, 239	5.5
	Norclostebol	positive	309	143, 237	6.1
	Norethandrolone	positive	303	109, 121	6.4
	Oxabolone	positive	291	125, 149	5.6
	Oxandrolone	positive	307	271, 289	5.6
	Stanozolol	positive	329	81, 95	5.8
	Stenbolone	positive	303	187, 91	6.6
	Tetrahydrogestrinone	positive	313	241, 159	6.5
	Trenbolone	positive	271	253, 199	5.2
Other anabolic agents (S1.2)	AC-262,536	positive	279	195, 169	6.2
	ACP-105	positive	291	233, 167	6.7
	Andarine	negative	440	150, 205	5.7
	Clenbuterol	positive	277	203, 259	3.5
	GSK 2881078	positive	331	210, 311	5.7
	LGD_3303	positive	343	245, 293	6.2
	LGD_4033	positive	339	220, 240	6.7
	Osilodrostat	positive	228	81, 134	2.9
	Ostarine	negative	388	118, 185	6.2
	Ractopamine	positive	302	164, 121	3.1
	RAD140	positive	394	223, 170	6.0
	SARM S1	negative	401	261, 205	6.9
	SARM S9	negative	417	261, 127	7.2
	YK-11	positive	357	307, 197	7.9
	Zeranol	negative	321	277, 303	5.5
	Zilpaterol	positive	262	244, 185	2.2
HIF activating agents (S2.1)	Daprodustat	positive	394	230, 312	8.1
	Desidustat	positive	333	233, 204	5.9
	Enarodustat	positive	341	266, 295	6.0
	FG-2216	positive	281	206, 235	5.7
	IOX2	positive	353	278, 307	6.3
	JNJ-42041935	positive	347	276, 241	5.9
	Molidustat (BAY	positive	315	207, 233	3.3
	85-3934)	positive	353	278, 250	6.7
	Roxadustat (FG4592)	positive	307	232, 204	6.1
	Vadadustat				
Growth hormone releasing factors (S2.2)	Ibutamoren	positive	529	267, 263	4.7
Beta-2-Agonists (S3)	Bambuterol	positive	368	72, 294	3.8
	Fenoterol	positive	304	107, 135	2.6
	Formoterol	positive	345	149, 121	3.4
	Indacaterol	positive	393	375, 173	4.5
	Ritodrine	positive	288	121, 270	2.7
	Salbutamol	positive	240	148, 222	2.2
	Tulobuterol	positive	228	154, 118	3.5
	Vilanterol	positive	486	450, 159	4.9
Hormone and metabolic modulators (S4)	Aminoglutethimide	positive	233	188, 160	3.3
	Anastrazole	positive	294	225, 157	5.0
	Arimistane	positive	285	81, 107	6.6
	ATD	positive	283	171, 265	5.5
	Bazedoxifene	positive	471	126, 239	4.6
	Clomiphene	positive	406	100, 72	6.0
	Exemestane	positive	297	121, 135	6.1
	GW0742	positive	472	275, 206	8.0
	GW1516	positive	454	257, 188	7.8
	GW1516 sulfone	positive	486	257, 272	6.7
	Letrozole	positive	286	217, 190	5.0
	Meldonium	positive	147	58, 59	0.6
	Raloxifene	positive	474	112, 269	4.3
	SR9009	positive	438	125, 142	8.1
	Tamoxifen	positive	372	129, 72	6.0
	Testolactone	positive	301	121, 147	4.8
	Toremifene	positive	406	72, 58	6.0
	Trimetazidine	positive	267	166, 181	2.7
Diuretics and masking agents (S5)	Acetazolamide	positive	223	181, 164	2.4
	Althiazide	positive	384	342, 262	4.7
	Amiloride	positive	230	116, 143	2.2
	Bendroflumethiazide	positive	422	105, 271	5.4
	Brinzolamide	positive	384	217, 281	3.2
	Canrenone	positive	341	107, 105	6.1
	Chlorothiazide	negative	294	214, 179	2.5
	Chlortalidone	positive	339	322, 185	3.9
	Clopamide	positive	346	250, 169	4.3
	Dorzolamide	positive	325	135, 199	2.2
	Eplerenone	positive	415	163, 121	5.0
	Furosemide	negative	329	205, 285	4.7
	Hydrochlorothiazide	negative	296	269, 205	2.7
	Indapamide	positive	366	132, 117	5.0
	Piretanide	positive	363	238, 282	5.5
	Probenecid	positive	286	202, 185	5.9
	Torasemide	positive	349	264, 290	4.3
	Triamterene	positive	254	237, 104	3.0
Internal standards (ISTD)	Furosemide-d5	negative	334	290	4.7
	Testosterone-d3	positive	292	97	5.7

The selectivity and specificity were evaluated for all compounds by analyzing blank samples from different volunteers (n = 20) and verifying the absence of interfering substances at the retention times of the studied compounds.

The extraction recovery was calculated from the analysis of four replicates of DBS samples spiked with the compounds at one concentration level (5 or 20 ng/mL, depending on the compound), and four replicates of DBS blank samples to which the same concentrations of analytes were added after extraction of the blank matrix. The ratios of the peak areas between the analytes and the ISTD obtained from the extracted spiked samples were compared with the ratios obtained in the samples in which the analytes were added to extracted blank samples (representing 100% of extraction recovery).

To evaluated intra-day precision two quality control samples were prepared for each analyte (Low QC and High QC) at two different concentrations, which were set depending on the compound (High QC, 5 or 20 ng/mL; Low QC, 0.25 to 10 ng/mL). The intra-day precision was evaluated as the relative standard deviation (RSD) of the area ratios (analyte/ISTD) of the four replicates of the QC samples.

Robustness was evaluated by analysis in several days of a quality control (QC) DBS sample spiked with 5 ng/mL of the compounds.

Limit of detection (LOD) was established as the concentration a signal-to-noise ratio of 3 for the main ion transition.

Matrix effect was studied by the analysis (n = 2 replicates) of eight different blank blood samples spiked at 0.5 or 2 ng/mL, depending on the compound. The areas of the analytes and the ISTDs were compared with those obtained after the analysis of a water sample (n = 4 replicates) spiked with the compounds.

Administration study samples

DBS samples were collected after administration of boldenone, oxandrolone or tamoxifen to healthy volunteers. Samples were collected in Whatman FTA DMPK-C cards at the following times after administration: 72 h after intramuscular administration of 100 mg of boldenone, 24 h after oral administration of 20 mg of oxandrolone, and 24 after oral administration of 20 mg of tamoxifen. The samples were left to dry dried for 2 h and stored at -20ºC until analysis.

Results and discussion

Optimization of the analytical method

Several parameters were optimized in the sample preparation procedure such as extraction and reconstitution solvents, and extraction times. Regarding the extraction solvent, a mixture of methanol:tert-butylmethyleter (1:4,v/v) was initially used instead of methanol:acetonitrile (1:1 v/v) as first extraction solvent. However, better results were obtained in terms of extraction recovery with the latter specially for more polar compounds. For the reconstitution of the dry organic extracts, different mixtures of acetonitrile and water were tested, and the final selection was a mixture containing 50:50(v/v) to obtain adequate reconstitution of most of the analytes (specially the most lipophilic compounds) and, then, water needed to be added to obtain adequate retention and peak shape in the LC system. Different extraction times in ultrasonic bath were tested (15, 30 and 60 min) and no differences in the extraction recoveries were observed for most of the compounds and, for that reason, the shorter time was chosen (15 min).

LC conditions (mobile phase solvents and gradient elution) were optimized to obtain adequate separation of all the analytes in a reasonable analysis time. The run time in the final chromatographic conditions was 11 min, which considered adequate taking into account the high number of compounds included in the analytical method.

Regarding MS parameters, they were optimized for each compound (cone voltage, collision energy) to obtain the maximum signal for the specific precursor and productions. For each compound, different transitions were evaluated when available in terms of sensitivity and matrix interferences that could limit selectivity. The selected precursor and product ions are described in Table 1.

Validation results

A total of 100 compounds belonging to different classes of the Prohibited List (30 anabolic steroids, 16 anabolic agents, 10 HIF activating agents and growth hormone releasing factors, 8 β-2-agonists, 18 hormone and metabolic modulators and 18 diuretics and masking agents)[15] were validated for qualitative purposes using the optimized analytical method. In most of the cases, the parent compounds were included in the analytical procedure because they are the analytes present in blood.

The results of the validation study are presented in Table 2, and summarized in Figures 1 to 3. Results in Figure 1 are expressed as the percentage of compounds belonging to each defined category.

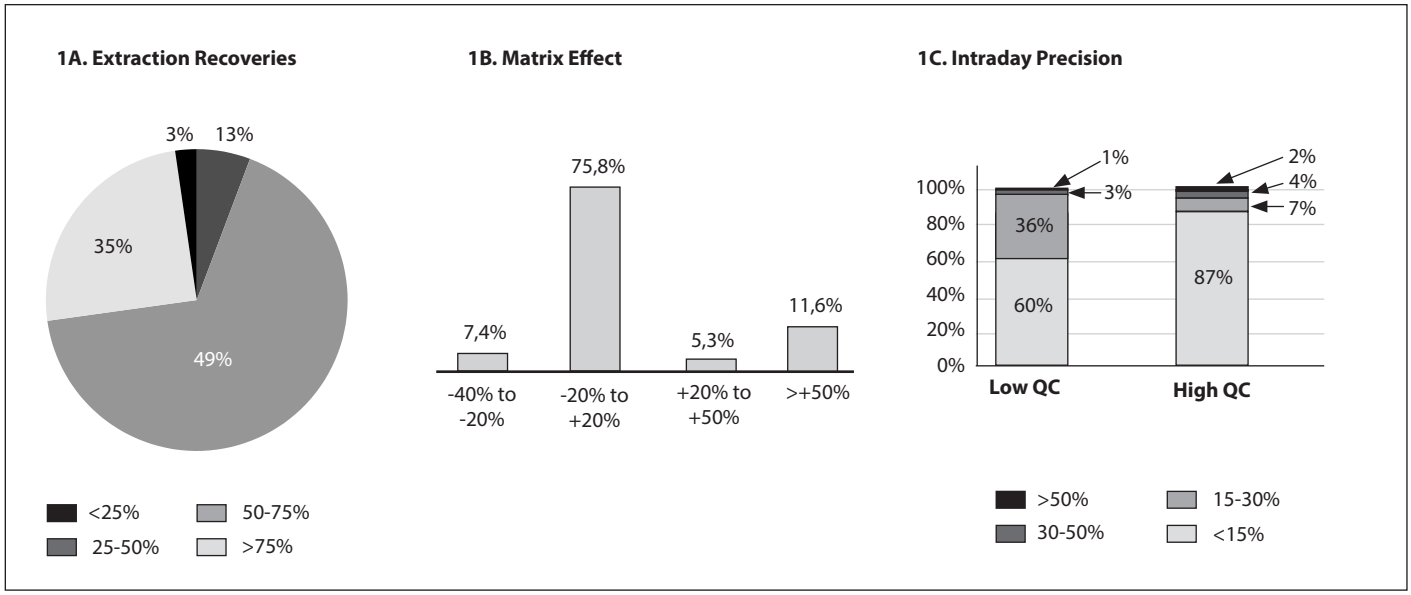
No significant interferences were observed at the retention times of the analytes and their ISTDs in the chromatograms of the corresponding ion transitions in drug-free DBS samples (Figure 2).

Extraction recoveries (Table 2, Figure 1A) were elevated for most of the compounds. Most of the compounds (84%) had extraction recoveries higher that 50%, and only a few analytes (3%) presented very low extraction recoveries (lower than 25%). For these latter analytes, the LODs were very good (lower than 1 ng/mL) and, therefore, the low recovery would not hamper a good detection of these compounds. The wide range of extraction recoveries was also expected and it is due to the different chemical structure and, hence, different physico-chemical properties of the target compounds included in the analytical method.

Regarding LODs, they were at the sub-ng/mL level for most of the compounds: 54% of the compounds showed LOD lower than 0.5 ng/mL, 34% of the compounds between 0.5 and 1 ng/mL, and only 12 % higher than 1 ng/mL (2.5 to 10 ng/mL). These LOD are low enough to detect the abuse of the compounds included in the analytical method in doping control tests according to data reported in the literature[16].

Negligible matrix effect was observed for the vast majority of compounds (Figure 1B), with 75.8% of the compounds in the range of 80 to 120% (±20%). For some compounds (7.4%) moderate ion suppression was observed and 16.9% of the compounds showed moderate to strong ion enhancement. For most of the compounds showing ion suppression, the limits of detection were below 1 ng/mL and, therefore, good detectability is guaranteed in spite of the moderate ion suppression. The negligible matrix effect for most of the compounds was expected due to the low volume of sample matrix (20 µL).

Figure 1. Summary of validation results. 1A. Extraction recoveries (%). 1B. Matrix effect (%). 1C. Intraday precision (%, relative standard deviation RSD).



All results are expressed as percentage of compounds included in the analytical method (%) included in each category.

Regarding the variability of the matrix effect among different blood samples, for most of the compounds (78.9%) the RSD of the signal among different matrices was below 15%, and only for 5% of the compounds was greater than 25%. These values are considered correct given the different chemical nature of the compounds analyzed.

Good intra-day precision results were also obtained (Table 2, Figure 1C). The intra-day precision was evaluated at two concentrations levels (Low QC and High QC, Table 2). As can be seen in Figure 1C, most of the compounds, specially for the High QC, showed RSD lower than 15%. For both samples, only a very small percentage of compounds had RSD greater than 30%.

Robustness of the analytical method was evaluated by the analysis of a QC sample spiked with some compounds in several days. Good results were obtained in terms of retention times stability and signal-to-noise ratio reproducibility. An example for some of the compounds is shown in Figure 2.

Analysis of samples collected after administration of doping agents

DBS samples collected after analysis of boldenone, oxandrolone and tamoxifen were analyzed as a final validation of the fitness-for-purpose analytical method. Results are shown in Figure 3. As it can be seen, in all samples collected after administration the parent compound was detected and in the case of tamoxifen three metabolites were also detected (two isomers of 4-hydroxy-tamoxifen, and 3-hydroxy-4-methoxy-tamoxifen). These metabolites were identified by comparison with reference standards. The concentrations of the compounds detected were estimated using a calibration sample containing a known concentration of the compounds. The concentrations detected were relatively low, specially for boldenone and oxandrolone, probably due to the extensive metabolism of these compounds.

To evaluate the windows of detection of these compounds in DBS samples and compare them with the current detection capabilities using urine samples, additional studies need to be performed with collection of DBS and urine samples at different time periods up to some days after administration.

Nevertheless, our results show the capability of the developed analytical method to detect doping agents after administration of the drugs, and its applicability to routine doping control.

Conclusions

In this study, a sensitive multi-class screening method has been developed to detect 100 doping agents in DBS samples. The method has been validated for qualitative purposes, obtaining limits of detection at sub-ng/mL level for the majority of compounds, and showing capability to ruggedly detect doping agents at very low concentrations. As a proof of concept, the analytical method was applied to DBS samples collected after administration of boldenone, oxandrolone and tamoxifen to healthy volunteers. In all three cases, the parent compound was detected and for tamoxifen, three metabolites were also detected. These results have demonstrated the capability of the analytical method to detect the administration of doping agents, and its applicability in routine doping tests.

Table 2. Validation results: group of substances according to WADA Prohibited List[5], limits of detection (LOD), extraction recoveries and intraday precisions obtained for the High QC and the Low QC.

Group of substances	Compound	LOD	Extraction Recovery		High QC		Low QC	
		(ng/mL)	Conc (ng/mL)	Recovery (%)	Conc (ng/mL)	RSD (%)	Conc (ng/mL)	RSD (%)
S1.1	1-Androstendione	2.5	20	49.2	20	7.2	5	11.9
	1-Testosterone	0.5	5	52.2	5	4.0	0.5	19.1
	4-Chlorometandienone	0.5	5	87.2	5	4.6	0.5	13.0
	Bolasterone	0.25	5	85.3	5	2.0	0.25	6.6
	Boldenone	0.25	5	86.3	5	2.6	0.25	10.6
	Calusterone	0.1	5	82.0	5	2.7	0.25	3.9
	Clostebol	0.5	5	85.1	5	5,3	0.5	9.7
	Drostanolone	1	5	80.7	5	6.6	1	9.8
	Fluoxymesterone	1	5	84.5	5	2.8	1	9.5
	Gestrinone	1	5	62.4	5	5.6	1	21.0
	Mestanolone	1	5	66.7	5	6.7	1	20.8
	Mesterolone	1	5	86.0	5	3.2	1	2.8
	Metandienone	0.5	5	85.7	5	4.3	0.5	13.7
	Methasterone	1	5	84.7	5	6.9	1	9.9
	Methenolone	0.25	5	86.2	5	7.5	0.25	8.1
	Methylclostebol	5	20	82.5	20	5.4	5	11.7
	Methyldienolone	1	5	72.8	5	11.7	1	27.0
	Methylstenbolone	1	5	86.9	5	4.0	1	6.9
	Methyltestosterone	1	5	72.0	5	7.6	1	21.7
	Methyltrienolone	0.25	5	67.7	5	11.8	1	9.0
	Mibolerone	1	5	81.5	5	6.0	1	9.7
	Nandrolone	0.5	5	77.7	5	2.7	0.5	8.5
	Norclostebol	2.5	20	73.2	20	11.8	5	7.3
	Norethandrolone	0.5	5	80.1	5	5.4	0.5	17.8
	Oxabolone	10	20	27.0	20	8.5	10	24.2
	Oxandrolone	2.5	5	87.8	5	4.6	2.5	15.2
	Stanozolol	0.25	5	73.4	5	11.5	0.25	20.8
	Stenbolone	1	5	83.3	5	6.7	1	3.3
	Tetrahydrogestrinone	1	5	73.6	5	6.0	1	13.2
	Trenbolone	1	5	52.8	5	8.7	1	14.9
S1.2	AC-262,536	1	5	61.6	5	11.6	1	20.6
	ACP-105	1	5	82.2	5	13.1	1	13.4
	Andarine	0.1	5	83.4	5	3.3	0.25	9.1
	Clenbuterol	0.1	5	69.6	5	4.0	0.25	4.0
	GSK 2881078	1	5	74.9	5	12.5	1	16.8
	LGD_3303	1	5	90.9	5	14.4	1	18.7
	LGD_4033	0.25	5	85.4	5	5.2	0.25	7.9
	Osilodrostat	0.1	5	86.4	5	8.0	0.25	5.3
	Ostarine	0.25	5	65.7	5	17.0	0.25	25.3
	Ractopamine	0.5	5	53.2	5	44.3	0,5	39.9
	RAD140	0.25	5	66.7	5	13.0	0.25	25.2
	SARM S1	0.1	5	69.8	5	13.4	0.25	14.5
	SARM S9	0.1	5	91.2	5	15.8	0.25	7.1
	YK-11	2.5	20	89.0	20	9.1	5	12.2
	Zeranol	1	5	73.8	5	2.8	1	11.6
	Zilpaterol	0.25	5	70.0	5	6.6	0.25	18.9
S2.1	Daprodustat	0.25	5	37.7	5	8.9	0.25	12.8
	Desidustat	5	20	58.8	20	8.8	5	16.1
	Enarodustat	0.25	5	54.3	5	13.4	0.25	27.4
	FG-2216	5	20	74.7	20	7.4	5	24.3
	IOX2	1	5	60.5	5	8.7	1	27.6
	JNJ-42041935	1	5	60.2	5	11.9	1	20.0
	Molidustat (BAY 85-3934)	5	20	41.8	20	22.4	5	12.8
	Roxadustat (FG4592)	0.1	5	68.2	5	6.8	0.25	12.5
	Vadadustat	0.1	5	74.2	5	2.6	0.25	16.5

(continued)

Table 2. Validation results: group of substances according to WADA Prohibited List[5], limits of detection (LOD), extraction recoveries and intraday precisions obtained for the High QC and the Low QC (continuation).

Group of substances	Compound	LOD	Extraction Recovery		High QC		Low QC	
		(ng/mL)	Conc (ng/mL)	Recovery (%)	Conc (ng/mL)	RSD (%)	Conc (ng/mL)	RSD (%)
S2.2	Ibutamoren	0.1	5	71.4	5	7.9	0.25	7.3
S3	Bambuterol	0.1	5	13.4	5	5.1	0.25	8.5
	Fenoterol	1	5	28.7	5	12.4	1	11.1
	Formoterol	0.1	5	33.6	5	5.9	0.25	11.0
	Indacaterol	5	20	28.3	20	26.9	5	24.9
	Ritodrine	1	5	44.1	5	8.2	1	15.9
	Salbutamol	0.5	5	52.7	5	5.1	0.5	7.0
	Tulobuterol	0.5	5	9.5	5	44.2	0.5	16.8
	Vilanterol	0.25	5	54.3	5	6.3	0.25	11.5
S4	Aminoglutethimide	1	5	54.0	5	49.6	1	26.6
	Anastrazole	0.05	5	88.6	5	2.9	0.25	3.9
	Arimistane	0.5	5	66.2	5	2.4	0.5	19.9
	ATD	1	5	49.9	5	6.9	1	14.4
	Bazedoxifene	1	5	9.3	5	3.9	1	26.3
	Clomiphene	0.5	5	54.1	5	48.4	0.5	39.0
	Exemestane	1	5	66.2	5	5.5	1	11.2
	GW0742	0.1	5	60.7	5	7.4	0.25	3.5
	GW1516	0.05	5	60.5	5	5.9	0.25	3.2
	GW1516 sulfone	0.1	5	69.4	5	4.4	0.25	2.9
	Letrozole	0.1	5	87.3	5	3.0	0.25	3.7
	Meldonium	5	20	72.9	20	6.0	5	9.9
	Raloxifene	0.25	5	38.9	5	3.3	0.25	7.9
	SR9009	0.25	5	92.2	5	19.9	0.25	15.8
	Tamoxifen	0.5	5	62.5	5	63.9	0.5	53.3
	Testolactone	0.5	5	80.1	5	9.9	1	11.8
	Toremifene	0.5	5	93.4	5	51.6	0.5	15.3
	Trimetazidine	1	5	65.1	5	28.8	1	30.6
S5	Acetazolamide	0.5	5	77.0	5	10.7	1	9.4
	Althiazide	1	5	64.8	5	11.6	1	13.8
	Amiloride	0.5	5	25.5	5	6.5	0.5	12.2
	Bendroflumethiazide	5	20	73.1	20	10.0	5	7.9
	Brinzolamide	1	5	75.0	5	9.8	1	15.8
	Canrenone	1	5	79.7	5	5.9	1	10.2
	Chlorothiazide	0.25	5	69.0	5	7.4	0.25	11.2
	Chlortalidone	1	5	86.6	5	2.4	1	8.5
	Clopamide	1	5	78.3	5	6.8	1	21.6
	Dorzolamide	1	5	73.9	5	9.6	1	19.0
	Eplerenone	1	5	74.1	5	8.6	1	18.0
	Furosemide	0.25	5	58.4	5	4.8	0.25	26.1
	Hydrochlorothiazide	0.25	5	80.8	5	9.0	0.25	28.7
	Indapamide	0.5	5	48.2	5	20.2	1	13.2
	Piretanide	0.25	5	56.4	5	5.1	0.25	15.7
	Probenecid	0.5	5	74.6	5	3.6	0.5	11.5
	Torasemide	0.05	5	69.5	5	3.7	0.25	4.1
	Triamterene	0.1	5	49.6	5	2.4	0.25	6.4

Conc: concentration; RSD: relative standard deviation.

Figure 2. Results obtained after analysis of a blank DBS sample (left) and a QC sample (DBS sample spiked at 5 ng/mL) in different days (days 1, 11 and 20). Chromatograms of the ion transitions of the different analytes.

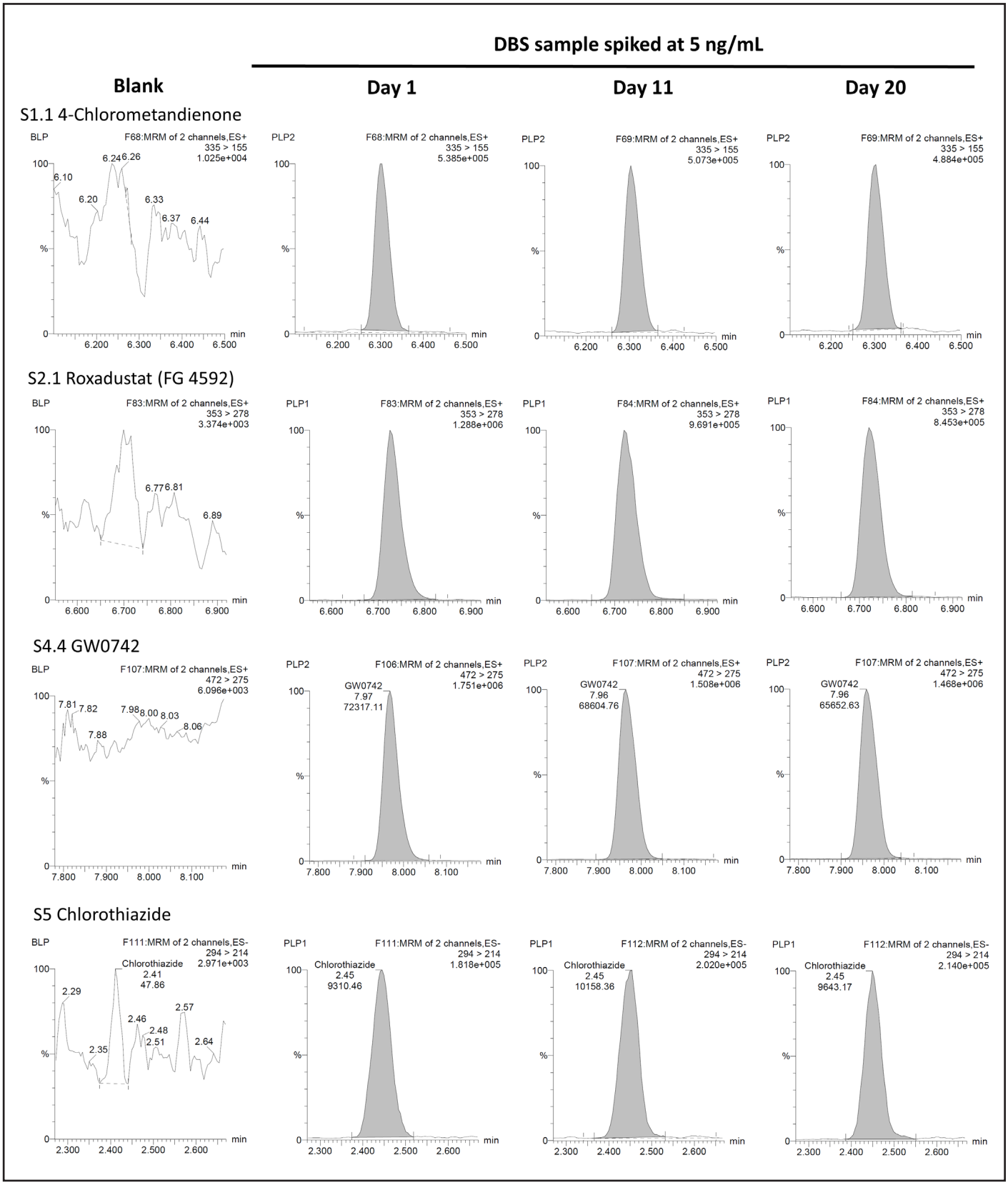
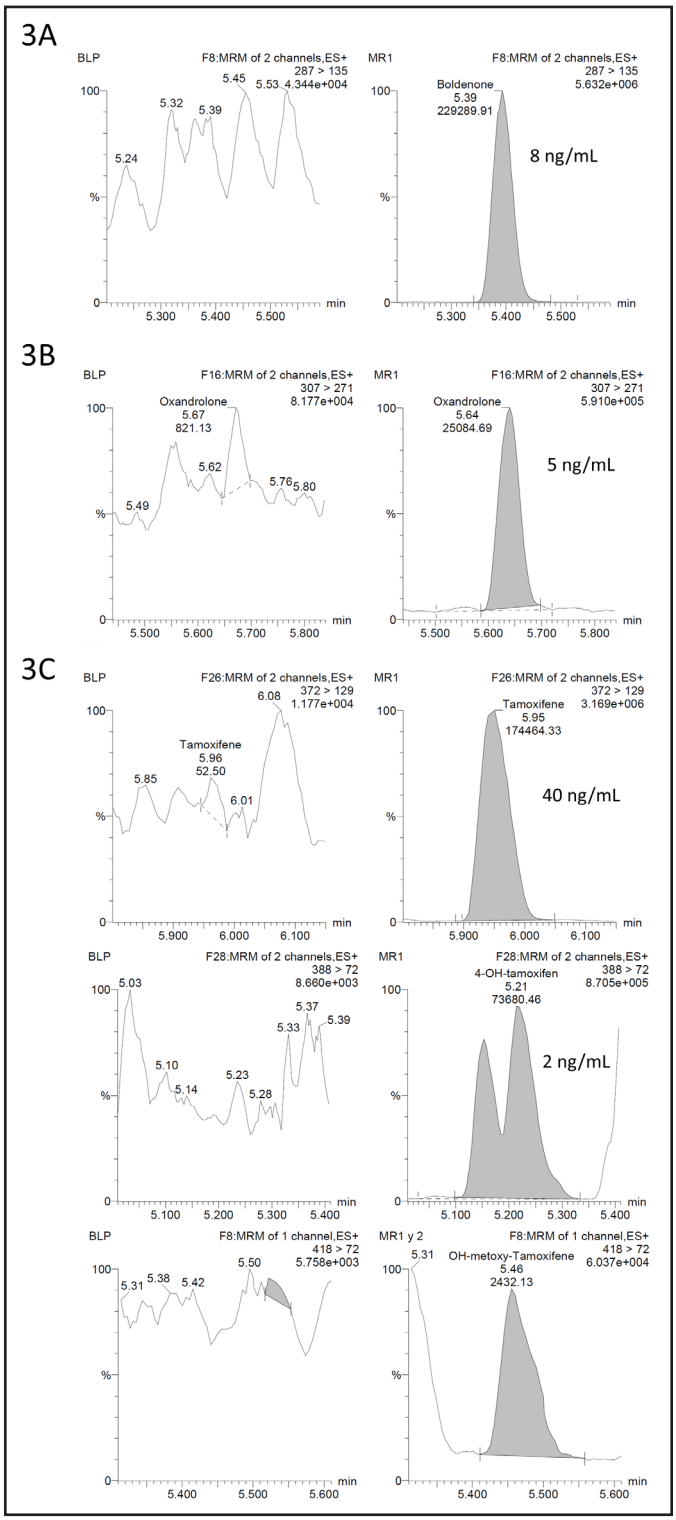


Figure 3. Results obtained after analysis of samples collected before (left) and after administration (right) of boldenone, oxandrolone or tamoxifen.



3A. Chromatograms of the characteristic ion transition of boldenone.
3B. Chromatograms of the characteristic ion transition of oxandrolone.
3C. Chromatograms of the characteristic ion transition of tamoxifen (top), 4-hydroxy- tamoxifen (middle) and 3-hydroxy-4-methoxy-tamoxifen (bottom).
Indicated concentrations were obtained using a single point calibration sample.

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Conflict of interest

The authors do not declare a conflict of interest.

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