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Practical application of blood microsampling: an effective VAMS-based workflow for anabolic androgenic steroid analysis

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Abstract

Volumetric absorptive microsampling (VAMS) has emerged as a promising alternative to traditional biological sampling methods, offering a high-reliability, field-deployable solution for steroid analysis with practical applications in anti-doping, forensic science and clinical testing. This study presents the optimisation of a VAMS-based workflow for the determination of anabolic androgenic steroids (AAS) in whole blood using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The research primarily focuses on refining microsampling and pretreatment strategies and addressing critical parameters such as volumetric accuracy, extraction efficiency, haematocrit effect mitigation and matrix interference. The workflow assesses both endogenous and exogenous steroids, overcoming limitations associated with traditional venous blood sampling. Experimental evaluations included assessments of sample homogeneity, recovery rates (87-95%), stability over time (up to 30 days) and the impact of haematocrit variability (-7% - +9%) on sampled volume. Strategies for internal standard (IS) addition to VAMS were also optimised to further enhance analytical accuracy. By integrating microsampling with the high reliability of mass spectrometric analysis, this study bridges the gap between laboratory research and practical applications in anti-doping testing, forensic science and clinical bioanalysis. The study provides a validated, cost-effective alternative to traditional sampling methods, confirming that VAMS is a promising minimally invasive tool for steroid detection in whole blood and highlighting its potential for broader applications in translational chemistry and personalised medicine.

Keywords: Microsampling; volumetric absorptive microsampling; anabolic androgenic steroids; HPLC-MS/MS; anti-doping testing

Introduction

The detection of anabolic androgenic steroids (AASs) in sports drug testing remains a crucial challenge due to their widespread misuse for performance enhancement. AASs significantly modify muscle strength, increase the lean-to-fat mass ratio and improve athletic endurance, leading to their strict regulation by the World Anti-Doping Agency (WADA) [1,2]. However, AASs misuse is not limited to professional athletes and recreational users also frequently engage in steroid consumption, raising concerns regarding public health risks and severe physical and psychological side effects [3-5]. Prolonged steroid use has been associated with cardiovascular diseases, hepatic dysfunction, neuropsychiatric disorders and hormonal imbalances [6-9].

Traditional anti-doping tests primarily rely on urine and venous blood analysis using liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) for steroid determination [10]. While urine sampling remains the preferred matrix due to higher steroid metabolite concentrations and non-invasiveness, in some cases it can present drawbacks such as microbial degradation, storage instability and risk of sample adulteration [11-13]. Blood-based methods offer high selectivity and short detection windows, yet venous blood collection is invasive, logistically complex and requires trained personnel [14-16]. To address these limitations, microsampling techniques such as dried blood spoting (DBS) and volumetric absorptive microsampling (VAMS) have emerged as promising alternatives [17]. DBS has been widely studied for AASs analysis, providing low sample volume requirements, easy transport and storage stability [18-20]. However, DBS can suffer from inherent drawbacks such as the lack of volume control, uneven blood diffusion on the cellulose support and haematocrit (HCT)-dependent volume variability [21].

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VAMS, on the other hand, offers fixed-volume sampling regardless of HCT levels, improved accuracy and enhanced reproducibility [22-24]. These advantages make VAMS a superior choice for AAS analysis in anti-doping applications.

Translational chemistry plays a pivotal role in bridging the gap between fundamental analytical research and its practical applications in real-world scenarios, particularly in the development of innovative bioanalytical tools for anti-doping, forensic sciences and clinical workflows. The ability to translate advances in microsampling technology into robust, field-deployable solutions highlights the interdisciplinary nature of this research, integrating analytical chemistry, clinical applications and regulatory compliance [19,20,25-31]. By refining microsampling techniques such as VAMS, this study contributes to the advancement of sustainable, cost-effective and minimally invasive approaches for steroid detection, directly impacting both elite and amateur sports communities. Recent studies have validated VAMS-HPLC-MS/MS workflows for steroid and glucocorticoid assessment in urine microsamples, demonstrating their applicability in forensic toxicology, endocrinology and doping control [32,33]. While the chromatographic and mass spectrometric parameters for steroid analysis using HPLC-MS/MS are well-established [10,34], additional research is needed to optimise sampling accuracy and precision, analyte extraction, (HCT) independence of sampling and matrix effect evaluation in blood-derived micromatrices.

This study aims to develop and validate an optimised VAMS-based workflow for the analysis of AASs in whole blood, aligning with the principles of translational chemistry by providing a highly applicable and scientifically rigorous solution to real-world doping control challenges, as well as forensic and clinical analysis needs. Five representative AASs have been included, namely: testosterone, nandrolone, stanozolol, methandienone and boldenone (chemical structures in **Figure 1**). Study focus is placed on sampling and pretreatment optimisation, including volumetric accuracy assessment, solvent extraction efficiency, internal standard (IS) addition strategies and HCT-related interferences, with the final aim of developing and validating a robust, high-throughput microsampling workflow using VAMS for AAS detection in whole blood, overcoming limitations of conventional sampling methods in doping control and clinical analysis. By improving the robustness and reliability of VAMS for AASs testing, its applicability in sports drug testing programs is enhanced, making it a significant contribution to the broader field of translational chemistry.

Materials and Methods

1. Chemicals and solutions

All reagents and solvents used for sample preparation and HPLC-MS/MS analysis were analytical grade. Methanol (MeOH), acetonitrile (ACN), formic acid (FA), ammonium hydroxide and ultrapure water (18.2 MΩ·cm) were obtained from Merck Life Science (Milan, Italy). VAMS devices (30 µL) were purchased from Trajan Scientific and Medical (Ringwood, Victoria, Australia). AAS certified reference standards, namely testosterone (17β-hydroxyandrost-4-en-3-one), nandrolone (19-nor-17β-hydroxyestr-4-en-3-one), stanozolol (17α-methyl-2'H-androst-2-eno(3,2-c)-pyrazol-17β-ol), methandienone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-one) and boldenone (17β-hydroxyandrost-1,4-dien-3-one) and their respective isotopically labelled ISs were obtained from LGC Standards (Teddington, UK). Stock solutions (1 mg/mL) were prepared in MeOH and stored at −20°C when not in use. Working solutions of AASs and ISs were freshly diluted in water/ACN (50:50, v/v) with 0.1% FA before analysis. All solutions were stored in amber glass vials certified for mass spectrometry (Waters, Milford, MA, USA) and kept protected from light.

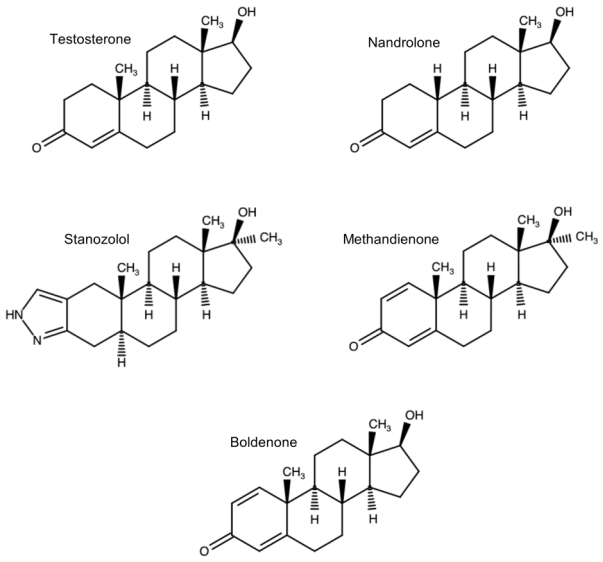

Figure 1 | Chemical structures of the five anabolic androgenic steroids analysed in this study.

2. HPLC-MS/MS method

The HPLC-MS/MS conditions followed those previously established for VAMS-based steroid analysis in urine microsamples, ensuring consistency in chromatographic and mass spectrometric parameters [25]. Chromatographic separation was performed using a Raptor C18 column (50 × 2.1 mm, 2.7 µm; Restek, Bellefonte, PA, USA), equipped with a C18 guard column (5 × 2.1 mm, 2.7 µm). The mobile phase consisted of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in ACN (Solvent B). The gradient elution program was as follows: 0.0–1.5 min: 30% B (hold); 1.5–3.0 min: 30% to 70% B; 3.0–10.0 min: 70% B (hold); 10.0–12.0 min: 70% to 30% B (re-equilibration); 12.0–15.0 min: 30% B (hold). The flow rate was set at 300 µL/min and the injection volume was 10 µL. Mass spectrometric detection was performed using electrospray ionisation (ESI) in positive mode. The ion spray voltage was set at 4.00 kV and the source temperature was maintained at 120°C. Other settings included: desolvation temperature: 150°C; desolvation gas flow: 750 L/h (nitrogen); collision gas: argon. The mass spectrometer operated in multiple reaction monitoring (MRM) mode, monitoring specific precursor-to-product ion transitions for each analyte and IS. A full list of MRM transitions, cone voltage and collision energy values is reported in **Table 1**.
This analytical method had been validated for the determination of endogenous and exogenous AASs [25], with the following key performance indicators: limit of detection (LOD) ≤ 0.5 ng/mL, limit of quantitation (LOQ) ≤ 1.5 ng/mL, precision as relative standard deviation (%RSD) ≤ 7.6% and absolute recovery (>77.1%).

3. Whole blood collection and handling

Fluid whole capillary blood was collected by means of a Winnoz (New Taipei City, Taiwan) Haiim device. After pricking a fingertip with a disposable lancet, the pricked fingertip was placed on the designated device inlet. Once powered up, the device draws up to 500 µL of blood through the inlet and into a anticoagulant-coated blood collection microtube by vacuum activity.

4. VAMS collection and pretreatment

The miniaturised sampling strategy and pretreatment protocol developed during this experimental work was tested by using whole blood drawn from six healthy volunteers. Aliquots of 30 µL of blood were collected by touching the blood surface in the microtube with a VAMS tip, held at a 45° angle and taking care not to completely immerse the tip. The filled VAMS device was left to dry for 45 min at room temperature. When the sample was dry, the tip was detached from the handle and placed in an amber vial, into which 500 µL of MeOH were added. The vial was then subjected to ultrasonic assisted extraction (UEA) for 5 min and centrifuged at 4000 RPM for 5 min at 4°C. The supernatant was brought to dryness exploiting a Thermo Fisher Savant SpeedVac SPD 1030 vacuum concentrator and redissolved with 100 µL of MeOH.
VAMS were fortified with 30 µL of a standard solution containing the analytes at known concentrations. To obtain fortified samples, three different procedures potentially suitable for blood microsampling were studied and namely:

1. Touching the surface of an analyte standard solution with an unused 30 µL VAMS tip, leaving it to dry for 45 min and then sampling blood with the same device as described above;

2. Pipetting 30 µL of standard solution on an unused VAMS tip, leaving it to dry for 45 min and then sampling blood with the same device as described above;

3. Sampling 30 µL of blood with a VAMS as described above, leaving it to dry for 45 min and then touching the surface of a standard solution with the VAMS tip and leaving the tip to dry again.

The obtained fortified blood samples were then subjected to pretreatment and HPLC-MS/MS analysis.

Table 1 | MRM transitions, cone voltage and collision energy for each analyte and IS.

Compound	Parent ion (*m/z*)	Daughter ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Testosterone	289.5	109.2	25	25
Nandrolone	275.0	109.0	25	25
Stanozolol	329.5	81.1	55	31
Methandienone	301.5	121.2	25	25
Boldenone	287.4	121.4	23	30
Testosterone-d3 (IS1)	292.4	112.1	25	25
Nandrolone-d3 (IS2)	278.2	112.0	25	23
Stanozolol-d3 (IS3)	332.5	84.1	55	30
Methandienone-d3 (IS4)	304.4	124.3	23	25
Boldenone-d3 (IS5)	290.4	124.4	25	30

5. VAMS performance assessment

5.1. Volumetric accuracy, repeatability and HCT independence

Blood aliquots at different HCT values in the 20-70% range (namely: 20, 30, 40, 50, 60 and 70%) were sampled by VAMS six times at each HCT value. The mean sampled volume was plotted as a function of HCT value to highlight any volume/HCT variability and possible relationships. Acceptability criterion: $r^2 < 0.5$. Moreover, volume accuracy, expressed as mean percentage volume error and volume repeatability, expressed as percentage relative standard deviation of volumes over six samplings, were obtained at each HCT value. Acceptability criteria: mean RE ≤ ±15%; RSD ≤ 10%.

5.2. Extraction yield

Different extraction procedures of the analytes from VAMS were tested using different solvents, namely: MeOH, ACN, water/MeOH (10/90, 20/80, 30/70) mixtures, water/ACN (10/90, 20/80, 30/70) mixtures, MEOH/ACN (70/30, 50/50, 30/70) mixtures. The samples were subjected to UAE, microwave-assisted extraction (MAE) and vortex-assisted extraction (VAE). Extraction times were tested within the 1-10 min range for UAE, within the 10-180 s range for MAE and within the 30-300 s range for VAE. Acceptability criterion: mean extraction yield ≥ 80%.

5.3. Matrix effect

In order to evaluate the extraction procedure effectiveness in analyte purification, IS-corrected matrix effect was evaluated by analysing six blank VAMS replicates, fortified post-extraction by adding known analyte concentrations at three different levels to blank blood VAMS extracts. The mean analyte/IS peak area ratios for each added concentration were compared with analyte/IS peak area ratios from standard solutions at the same theoretical concentration and the resulting percentage was calculated. Acceptability criterion: response = 100 ± 15%.

5.4. Stability

Short- and medium-term stability of the analytes in the matrix at room temperature (RT) was tested. VAMS samples fortified with two analyte concentrations (a low and a high concentration of the respective calibration curve) were analysed by HPLC-MS/MS at time zero and at set time intervals, corresponding to 1, 7, 14, 20 and 30 days. Subsequently, the analyte concentrations found at each time interval were compared to those found at time zero. For the whole duration of the study, fortified samples were stored at RT, protected from light, heat sources and humidity. Acceptability criterion: mean analyte recovery ≥ 80%.

Results and discussion

1. VAMS accuracy and reproducibility

The results of VAMS sampling performance assessment assays (in terms of volumetric accuracy and repeatability) were very satisfactory and are detailed in **Table 2**. As one can see, mean accuracy was well within the ±15% threshold at all HCT values, as was repeatability. For the latter, some HCT dependence is noted, since RSD% becomes higher at extreme HCT values. VAMS demonstrated negligible volume dependency from HCT levels ($R^2 = 0.0051$), confirming its suitability for standardised microsampling across diverse patient populations (**Figure 2**).

2. Optimised extraction and matrix effect

A fast and simple solvent extraction procedure was devised for this analytical workflow. Indeed, more complicated procedures such as solid phase extraction and its variants were deemed unnecessary due to the high selectivity and sensitivity of the HPLC-MS/MS

Table 2 | Evaluation of haematocrit independence of VAMS sampled volume (30 µL).

Haematocrit value (%)	Mean accuracy (RE%*)	Repeatability (RSD%**)
20	-7	6.3
30	+4	4.7
40	+9	4.1
50	-8	2.2
60	-7	5.5
70	+5	6.4

* RE% = Percent relative error. Calculated as $\frac{V_{actual}-V_{expected}}{V_{expected}}$ %.

** RSD% = Percent relative standard deviation.

Calculated as $\sqrt{\frac{\sum_{i=1}^{n}(V_i-\bar{V})^2}{(n-1)\bar{V}^2}}$ %.

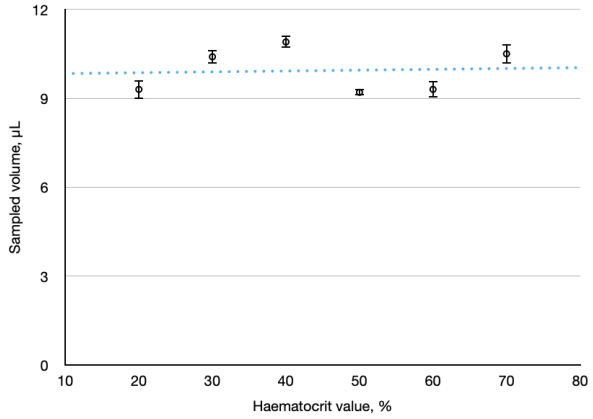

Figure 2 | Haematocrit dependence of sampled volume in VAMS (R^2 = 0.0051), demonstrating volumetric stability. The blue dotted line is the least-squares linear regression line.

method and the fact that the very absorption of blood on the polymeric tips constitutes a first sample preparation step, capable of selectively retaining specific matrix compounds and analytes; in a similar way, the extraction procedure from the tip can be likened to a selective elution step of the analytes from a polymeric sorbent.

The detailed study of extraction conditions showed that the best procedure involved the use of 500 µL of MeOH and UAE for 5 min. All other solvents and solvent mixtures tested provided either lower extraction yields, or interference, or unacceptably high matrix effect, as detailed in **Table 3**. In a similar way, MAE and VAE generally produced lower analyte yields than UAE (**Table 3**) and it was ascertained that their combinations did not provide any improvement over simple UAE. Finally, extraction time proved to be critical for method performance, with 5 min (in the case of UAE) being sufficient to reach satisfactory yields and longer times not significantly improving results (**Table 3**).

The HPLC-MS/MS chromatogram of a blood VAMS sample fortified with the analytes is shown in **Figure 3**. Peak shape and efficiency are satisfactory, no significant interference is detected and baseline noise is acceptable.

Table 3 | Performance of the main tested extraction conditions.

Solvent	Extraction procedure	Extraction time (s)	Extraction yield range (%)*	Interference	Matrix effect range (% response)*
MeOH	UAE	60	84-90	-	90-95
		300	88-95	-	93-101
		600	87-96	+	78-84
	MAE	10	66-75	-	88-90
		90	67-80	+	80-92
		180	55-89	++	85-95
	VAE	30	52-89	+	77-89
		150	52-82	+	74-95
		300	54-71	+	76-87
ACN	UAE	60	76-80	+	105-113
		300	70-86	++	104-110
		600	68-79	+++	106-113
	MAE	10	54-66	+++	110-120
		90	61-78	+++	112-119
		180	44-70	++	106-113
	VAE	30	42-65	-	90-99
		150	44-78	-	89-95
		300	54-81	+	90-105
MeOH/ACN (50/50)	UAE	60	80-88	+	98-104
		300	85-89	+	99-106
		600	82-87	+	92-99
	MAE	10	70-80	-	99-105
		90	65-79	+	96-106
		180	65-80	++	96-104
	VAE	30	54-67	-	84-94
		150	56-77	+	82-95
		300	49-69	++	83-96

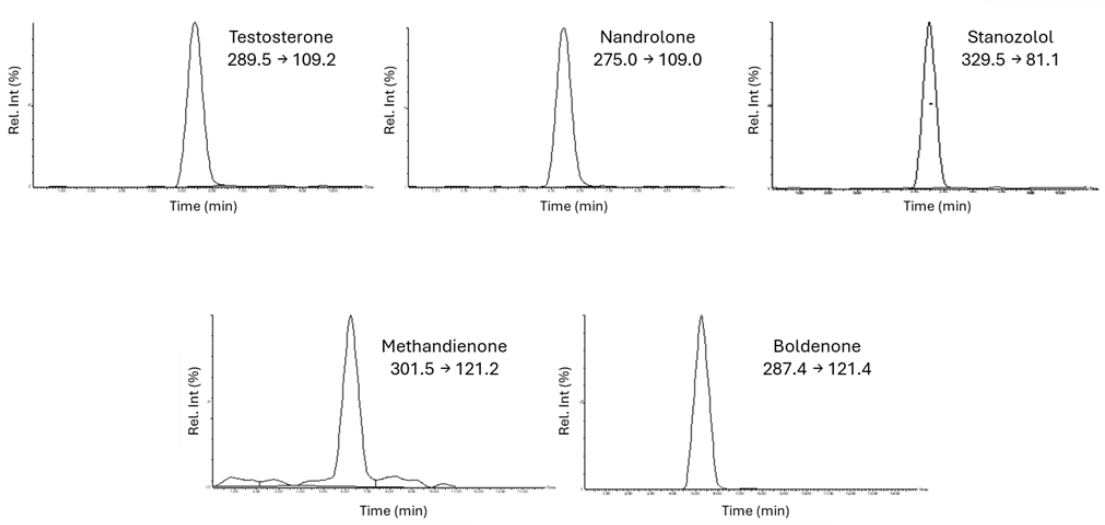

Figure 3 | Representative HPLC-MS/MS MRM chromatograms of a blood VAMS sample fortified with the analytes, showing clear peak separation and lack of interference.

2.1. Internal standard addition

Assays were carried out to find the best procedure for IS addition to VAMS. Three IS addition methods were tested, as described in the *'VAMS collection and pretreatment'* Section: IS addition by partial immersion (#1) or by pipetting (#2) before blood VAMS sampling, or by partial immersion (#3) after blood sampling. All three procedures provided reliable results, without significantly negatively impacting analyte determination; no significant differences were found in this respect. Adding ISs after blood sampling (procedure #3) is more convenient, since the subject could use a pristine, sealed VAMS and ISs would be added by the analytical laboratory personnel. While this also means that a potential source of variability (packaging and shipping to the lab) is not accounted for, no manipulation of the devices is needed before sampling, nor until analysis time. On the other hand, standard solution fortification before blood VAMS sampling (procedures #1 and #2) requires the un-sealing of devices before giving them to the subject, so it is suboptimal for anti-doping purposes. Finally, no significant differences were found between fortification by partial immersion (#1) and by pipetting (#2), however the latter requires some training on pipette use, while the former is easily doable by any subject who is also able to sample blood by VAMS.

2.2. Extraction yield and matrix effect

Three different concentrations were examined, representative of the entire linearity ranges for the different analytes. Average extraction yield values are shown in **Table 4**. These values are the average of six independent analyses.

As can be seen, extraction yields are satisfactory (87-95% range) and similar to what is expected from macroscopic samples. Thus, dried microsampling and extraction does not seem to impair analyte recovery. The combination of microsampling on the polymeric VAMS tip and solvent extraction by MeOH provided sufficient sample clean-up for the declared analytical purpose. In particular, matrix effect response was always in the 93-101% range for all analytes and all concentration levels, as summarised in **Table 5**.

Table 4 | Extraction yield assay results.

Compound	Average extraction yield (%)		
	Low conc. level	Middle conc. level	High conc. level
Testosterone	90	88	87
Nandrolone	89	92	91
Stanozolol	95	95	94
Methandienone	90	91	90
Boldenone	88	88	91

Table 5 | Matrix effect assay results.

Compound	Average matrix effect (%)		
	Low conc. level	Middle conc. level	High conc. level
Testosterone	94	95	94
Nandrolone	100	94	95
Stanozolol	97	93	99
Methandienone	95	95	93
Boldenone	98	100	101

2.3. Stability

Stability was tested for each analyte in VAMS stored for 30 days at RT for comparison with freshly prepared fortified blood VAMS. From the values shown in **Figure 4**, it can be surmised that short-to-medium-term stability of the analytes in the dried matrix is very good (mean analyte recovery ≥ 92%). The excellent analyte recovery values obtained underscore that neither analyte degradation nor "sample aging" happen within the studied timespan. The latter is a potential side effect of microsample drying, where the dried matrix progressively loses over time its capacity to release the analytes due to hardening and loss of wettability. These results confirm that VAMS samples maintain integrity for up to 30 days at room temperature, making them highly suitable for field-based anti-doping programs and remote sample collection in clinical studies.

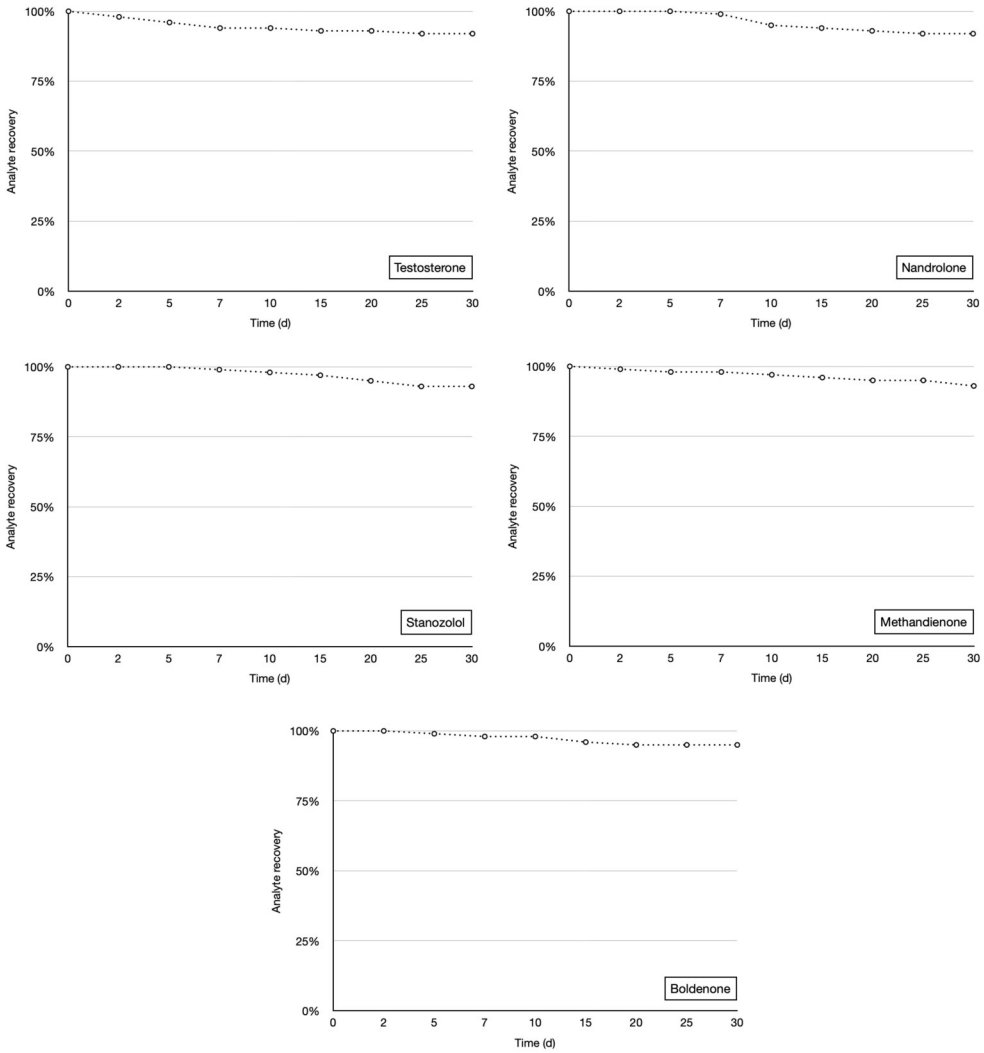

Figure 4 | Plots of stability assay results.

3. Comparison with existing sampling methods

The results above prove that VAMS is a mature platform, capable of outstanding volume accuracy and precision results, irrespective of HCT and other confounders. Compared to common venipuncture procedures used for AAS determination, VAMS is surely less invasive (being based on fingerpricking with disposable lancets) and much more error-resistant, leading to the possibility of self-sampling at home, or anyway far from healthcare facilities, by patients or athletes; sampling by non-trained personnel is also possible and feasible. Analyte stability is greatly enhanced in comparison to blood-based fluid matrices, since the latter need frozen storage, while AASs in blood VAMS are stable for at least 30 days at room temperature, also leading to reduced expenses for shipping and storage and reduced space requirements.

4. Real-world applications and limitations

The validated analytical workflow described in this study can be directly translated into real-world applications, especially in the therapeutic drug monitoring (TDM) area and anti-doping frameworks. Moreover, it represents a proof of concept for application to other therapeutic and doping agents in dried blood microsamples, leading the way for a great expansion of the VAMS applicability field. VAMS devices have been designed for straightforward use in automated analytical workflows, so it is conceivable that the procedure presented herein would be easily automated using existing liquid-handling automated instruments, however method performance should be confirmed and validation extended in this regard. The study has a few limitations; it has only been applied to fortified blank blood, thus samples from subjects undergoing pharmacotherapy with AASs, or taking them for any reason, should be analysed and results confirmed before full suitability can be claimed in the anti-doping and forensic spaces. Moreover, just a selection of endogenous and exogenous AASs has been quantified, and for this reason extension of the method to a wider variety of analytes would be advisable for increased applicability. Further assays are underway on both accounts.

Concluding remarks

An innovative method of capillary blood microsampling, based on VAMS, has been developed for the main purpose of anti-doping analysis, with possible applications to forensic and clinical analysis. Dried samples have been shown to be much more convenient than traditional venipuncture: they can be stored for up to 30 days at room temperature without losing more than 8% of their original analyte content. In addition, due to miniaturisation, samples take up much less shelf space and storage equipment and require only minimal amounts of solvents for extraction, making procedures much greener. Finally, analytical VAMS assays have provided ample assurance that this approach provides performances comparable to those typically associatd with classical peripheral blood drawing. This translational chemistry study has laid the groundwork for the forthcoming development and optimisation of standardised miniaturised sampling and extraction protocols with immediate applicability, both for preliminary screening and confirmatory analysis that can be easily implemented by both centralised and local laboratories. It has been carefully developed and optimised to provide possible immediate real-world applications, be they the monitoring and safeguarding of professional and amateur athletes or, in perspective, personalised medicine applications to patients undergoing treatment with AASs.

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