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PII: S0021-9673(20)30721-4
DOI: https://doi.org/10.1016/j.chroma.2020.461445
Reference: CHROMA 461445

To appear in: *Journal of Chromatography A*

Received date: 17 June 2020
Revised date: 28 July 2020
Accepted date: 31 July 2020

Please cite this article as: William Chih-Wei Chang , David A. Cowan , Christopher J. Walker , Nick Wojek , Alan D. Brailsford , Determination of anabolic steroids in dried blood using microsampling and gas chromatography-tandem mass spectrometry: Application to a testosterone gel administration study, *Journal of Chromatography A* (2020), doi: https://doi.org/10.1016/j.chroma.2020.461445

Highlights

- Nine anabolic steroids are separated within 6.4 min and are detectable at 50 fg
- VAMS dried blood exhibits good stability and better recovery over spotting card
- Quantification of testosterone between serum and VAMS dried blood is in agreement
- Doping with micro-dose testosterone can be caught by using 20 μL of dried blood

Determination of anabolic steroids in dried blood using microsampling and gas chromatography-tandem mass spectrometry: Application to a testosterone gel administration study

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Abstract

Anabolic androgenic steroids (AAS) have been the most commonly abused substances taken by not only professional sportsmen but also recreational bodybuilders. The detection of micro-dose testosterone (T) misuse is particularly challenging as it possesses pseudo-endogenous origin and is sometimes impossible to be identified in urine samples. Dried blood (DB) obtained by finger pricking has been proven to be an alternative matrix for better correlating to physiological responses. Moreover, the introduction of the volumetric absorptive microsampling (VAMS) technology allows overcoming some major limitations of spotting blood onto a filter paper card. In this work, a fast and sensitive GC-MS/MS method was developed and validated for the quantification of AAS in DB collected by means of VAMS. T and the eight top abused synthetic AAS, namely nandrolone, boldenone, mesterolone, drostanolone, metenolone, metandienone, oxandrolone, and dehydrochloromethyl T were selected as the target analytes. The method based on VAMS exhibited good precision, accuracy as well as stability, and superior extraction recoveries over the punched DB spots reported in the literature. The chromatographic separation was achieved within 6.4 min and the detection limit is as little as 50 fg (i.e. able to detect 0.10 ng mL^{-1} in 20 μL of DB). Confirmed by forty real blood samples, the Deming regression and Bland-Altman analysis revealed that the VAMS DB could be employed for quantifying blood T level in agreement with using the serum specimen. The feasibility of the method was then successfully proven by the analysis of samples collected from a three-arm T administration trial. Our results highlighted that DB total T was a sensitive indicator for identifying transdermal micro-dosing of T. In the groups of receiving T gel administration, T concentrations could rise up to ten times higher than the baseline at 9 h after the application. As a future step, this approach is being expanded to a large cohort screening of

bodybuilders at gym and ultimately may allow universal applications on monitoring sports drug misuse.

Keywords: sports doping; drug misuse; dried blood spot; alternative matrix; GC-MS/MS; volumetric absorptive microsampling

1. Introduction

For decades, anabolic androgenic steroids (AAS) have been the most common choice of abused substances within sports, which account for 44% of the adverse analytical findings according to a World Anti-Doping Agency (WADA) report [1]. The detection of xenobiotic AAS is currently the utmost priority for anti-doping scientists. So far, many androgen-derived steroids have been synthesised and display enhanced anabolic activity coupled with reduced androgenic activity. Although most of them are not currently approved for clinical use and are generally illicit, their availability on the black market is increasing due to a higher demand not only by professional sportsmen but also by recreational bodybuilders [2]. Nonetheless, the usage of exogenous testosterone (T) products on the black market retains the highest popularity among all AAS according to a survey [3]. The detection of the micro-dosing of such pseudo-endogenous origin compounds is particularly challenging as they are naturally present in the human body. For instance, transdermal T doping is sometimes impossible to be identified due to the mild variations in urinary parameters [4-7].

The preferred biological specimen for the quantitative analysis of a hormone is either whole blood, plasma or serum because its concentration found in blood gives the best correlation with the physiological responses [8]. However, the majority of the anti-doping testing relies on urine samples due to its non-invasive collection [9]. Yet the nature of urine collection is intrusive, time-consuming and in need of large space for long-term storage. Venous blood sampling is relatively invasive and hence normally conducted by health-care personnel. Specific shipping conditions are often required for conventional specimens. However, dried blood (DB) samples obtained from finger pricks have been proven to be a

cost-effective choice as an alternative matrix. Dried matrices increase analytes' stability and subsequently benefits the ease of handling, transportation and storage.

DB microsampling, collecting a very small volume of capillary blood with a minimally-invasive technique, has been successfully adopted for neonatal screening since 1963 [10] and has further been applied to some other clinical practices, such as therapeutic drug monitoring [11] and toxicology [12]. Despite the increasing popularity of DB, spotting blood onto a filter paper card suffers from some inevitable limitations. Unacceptable layering and insufficient or multiple application commonly cause a sample to become invalid. The blood may be spread inhomogeneously across the paper and produce serum rings via chromatographic-like effects ascribed to the haematocrit bias [13]. Volumetric absorptive microsampling (VAMS) technology was developed to overcome these limitations. VAMS allows the accurate and precise uptake of a fixed volume of blood independent of the haematocrit and is considered a superior sampling method for quantitative purposes [14].

Screening for xenobiotic AAS was first approached by means of radioimmunoassays (RIA), albeit the method's low specificity was an issue. Gas chromatography-mass spectrometry (GC-MS) later entirely substituted RIA because it offers superior specificity and robustness when dealing with complex biological matrices [15]. Currently, a standard runtime for GC-MS analysis can range from 13 minutes [16] up to 30 minutes [17, 18]. Given that there is considerable interest in improving the maximum sample throughput of anti-doping laboratories, scientists are continuously searching for ways to reduce analytical runtimes without sacrificing analytical performance.

This work aims to develop a fast, sensitive, and validated GC-MS/MS method for the quantitative analysis of major AAS in 20 microlitre volumes of DB samples collected by VAMS tips. T and eight synthetic steroids listed in the top ten abused AAS were

investigated, namely nandrolone, boldenone, mesterolone, drostanolone, metenolone, metandienone, oxandrolone, and dehydrochloromethyl T (DHCMT) [1]. Stanozolol and trenbolone were not selected as they possess marginal GC properties even after derivatisation [19]. The feasibility of this approach was further proven by a three-arm micro-dose T gel administration study.

2. Materials and methods

2.1. Chemicals, reagents, and materials

All reagents and solvents were of analytical grade. Methanol, ethyl acetate, and methyl-tertbutyl-ether (MTBE) were purchased from Fisher Scientific (Loughborough, UK). Dodecane, N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), ammonia iodide, and ethanethiol were purchased from Sigma-Aldrich (Poole, UK). Reference materials nandrolone, mesterolone, and T-d_3 were purchased from Cerilliant (Round Rock, Texas, USA). Drostanolone and oxandrolone were purchased from Grace (Columbia, Maryland, USA). Boldenone and T were purchased from Sigma-Aldrich (Poole, UK). Metenolone was purchased from Schering AG (Berlin, Germany). Metandienone and DHCMT were purchased from NMI National Measurement Institute (West Lindfield, NSW, Australia). Purified water was prepared from Purelab flex (ELGA LabWater, High Wycombe, UK). Mitra® VAMS devices (20 µL) were purchased from Neoteryx (Torrance, CA, USA).

2.2. Steroid-free blood

The preparation of steroid-free blood was according to the previous study [20]. A pooled fresh venous blood specimen was collected from the anonymous athlete biological

passport (ABP) samples. The serum and the red blood cells (RBCs) were separated via centrifugation at 2,000 rpm (at 4°C, 15 min). The serum was then discarded. RBCs were washed with 0.01 M phosphate-buffered saline, centrifuged, followed by discarding the supernatant; this step was repeated four times. The steroid-free serum (Stratech, Cambridgeshire, UK) was added to the washed RBCs to obtain a haematocrit of 45%. The prepared steroid-free blood was further confirmed with no detectable T signal.

2.3. Dried blood sample preparation

Sampling 20-µL (about 1/180th of a teaspoonful) DB was conducted by dipping one VAMS tip into whole blood until it was fully saturated, representing completely red. The tip was air-dried for a minimum of 2 h. The tip was then separated from the cartridge and placed into a glass tube. Upon extraction process, the tip was first fortified with the ISTD (an absolute amount of 200 pg of T-d_3), then 1 mL of water and 2 mL of MTBE were added for ultrasound-assisted extraction (UAE) followed by liquid-liquid extraction (LLE). The mixture was extracted by the ultrasonic bath for 15 min and by rotary mixing for 15 min, followed by centrifugation at 3,000 rpm for 5 min. The supernatant (organic portion) was transferred to another tube and dried under nitrogen at 60°C for 10 min. The extract was then flushed with nitrogen, added with 20 µL of derivatising reagent (MSTFA:NH_4I:ethanethiol solution, 1000:3:9, v/w/v) and 20 µL of dodecane, capped immediately, and heated at 80°C for 15 min. Finally, the solution was submitted to GC-MS/MS analysis.

2.4. GC-MS/MS analysis

Data were acquired using an Agilent Intuvo 9000 GC system interfaced with a 7010B triple quad and a 7693 autosampler. The inlet (260°C) was fitted with a 4-mm internal diameter single taper liner with glass wool. Each sample (1 µL) was injected in splitless mode, and chromatographic separation was achieved on a DB-1ms Ultra Inert Intuvo (10 m × 0.18 mm × 0.18 µm) with helium carrier gas flowing at 1 mL min^{-1}. The column temperature was programmed as follows: the initial temperature of 130°C at 50°C min^{-1} to 190°C, then at 15°C min^{-1} to 220°C, and finally 25°C min^{-1} to 300°C. The solvent delay was 4 min and the runtime was 6.4 min.

Nitrogen was used as collision gas at a flow rate of 1.5 mL min^{-1}, and helium was purged into the collision cell at a flow rate of 2.25 mL min^{-1} in order to eliminate the effect of metastable He ions in the mass spectrometer. The transfer line and source temperature were set at 280°C and 230°C, respectively. The MS was operated in a dynamic multiple reaction monitoring mode, acquiring the transition in a ±0.25 min window from the retention time of the analyte. The detection parameters are listed in **Table 1**.

2.5. Method validation

The prepared steroid-free blood was utilised for method validation. Method validation was according to the ICH guidelines. Specificity, precision, accuracy, linearity, limit of detection (LOD), lower limit of quantification (LLOQ), recovery, matrix effect, and stability of the method was examined. Validation was carried out by spiking the analytes, namely testosterone, nandrolone, boldenone, mesterolone, drostanolone, metenolone, metandienone, oxandrolone, and DHCMT (**Fig S1**), into the steroid-free blood prior VAMS tips absorption. The amount of standard solution in the blood sample was no more than 1% to avoid the dilution effects of blood components.

2.5.1. Specificity

Prepared steroid-free blood was analysed for the presence of interfering signals at the expected retention time of all target analytes. Particularly, the specificity for eight exogenous steroids was also tested with real finger pricking DB samples obtained from at least ten volunteers.

2.5.2. Precision and accuracy

To measure precision and accuracy of the assay, the blood samples were fortified with low (2 ng mL^{-1}), medium (10 ng mL^{-1}), and high (25 ng mL^{-1}) concentrations of the target analytes. For intra-day precision and accuracy, one batch of six specimens (n = 6) for each concentration were processed and tested on the same day. For inter-day precision and accuracy, three batches of three specimens (n = 3) for each concentration were respectively processed and tested on three different days. Precision was expressed as the relative standard deviation (RSD) in per cent and accuracy was determined as the bias in per cent as the relative difference between the actual and the nominal concentration.

2.5.3. Linearity

The target analytes were spiked into the blood samples to attain final concentrations of 0.02–200 ng mL^{-1} by 13-fold serial dilution. The calibration curve was established at least six concentration points. The range was considered to be linear if the square of the regression coefficient was greater than 0.995 and the accuracy of all points was less than ±10% and ±15% for the lowest concentration.

2.5.4. LOD and LLOQ

LOD was estimated by a measurable signal produced by a signal-to-noise ratio of 3:1. LLOQ was defined as the lowest concentration of the calibration curve.

2.5.5. Extraction recovery

Six blood samples were fortified with 20 ng mL^{-1} of target analytes and absorbed by VAMS tips. Another six blank samples were prepared and the respective amount of the analytes was added directly after the extraction process. The recovery was determined as the ratio of the peak areas of the samples spiked before and after processing (absorption and extraction).

2.5.6. Matrix effect

Matrix effects were investigated by comparing the peak areas of spiked blood matrix extract to the peak areas of spiked solvent (methanol) extract.

2.5.7. Stability

Blood samples fortified with 20 ng mL^{-1} of target analytes were collected with VAMS tips. The tips were packaged into a foil pouch with a desiccant. Three storage conditions were studied (room temperature, 4°C, and −20°C). For each condition, six samples were used. The tips were tested after storing for two months. Statistical differences were analysed by one-way analysis of variance with Bonferroni post hoc using IBM SPSS 22.

2.5.8. Comparison of two GC-MS/MS systems

To measure the potential improvement of this newly developed method, the comparison was made between the new system (Intuvo 9000 GC-7010 TQ) and the current system (7890 GC-7000 TQ) routinely used in the Drug Control Centre, a WADA-accredited anti-doping lab. Sensitivity was tested on both instruments using the same batch of calibration samples.

2.6. Comparison of serum specimen and VAMS dried blood

Forty anonymous ABP venous blood samples regardless their sex were used for comparing the T concentrations between serum specimen and VAMS DB. Each blood sample (0.5 mL) was first absorbed by a 20-μL VAMS tip. Then it was centrifugated to obtain the equal amount of serum specimen. Both specimens underwent the same extraction process, yet serum was not absorbed by the VAMS tip. The haematocrit level of each sample was processed by Sysmex XT-2000i and recorded before this experiment. The following equation was used to generate the 'calculated level' from VAMS DB. Comparison of 'serum level' and 'calculated level' was examined by using Deming regression and Bland-Altman analysis.

Calculated level = (Dried blood testosterone level)/(1 − Haematocrit)

2.7. Application study

2.7.1. Dried blood microsampling

An alcohol pad was used to cleanse the sampling area. After drying, a finger pricker device (Accu-Chek® FastClix) induced a small amount of blood from the finger by rapidly puncturing the skin with a sterile needle. A single VAMS tip was used to collect 20 µL of DB sample. The tips were packaged in a foil pouch with a desiccant before analysis (**Fig. 1**).

2.7.2. Testosterone gel administration study

The protocol of this study was reviewed and approved by the BDM Research Ethics Subcommittee, King's College London (reference: HR-17/18-6483). Healthy male adults, aged 18-45 years (representing the most prevalent age range for competing athletes), were recruited. The volunteers were screened by a questionnaire to ensure they were not participating in competitive sporting events or were registered in the testing pool for sporting organisations; they were not taking any medication that may interfere with the study or be contra-indicated with T administration (e.g. anabolic agents, chorionic gonadotropin, growth hormone, aromatase inhibitors, selective estrogen receptor modulators, ketoconazole, finasteride, probenecid, or diuretics); they did not have a history of liver, kidney, lung or heart disease, epilepsy, alcoholism or psychosis. With the volunteers' consent, their samples were also tested using the method routinely run for drug control purpose to guarantee that they were not currently taking any recreational drug such as morphine, cocaine, or cannabinoid mimetics.

Tostran® 2% Gel (Kyowa Kirin Ltd., Galashiels, UK) was applied as the T gel preparation and a hand gel (Cuticura, London, UK) was served as the placebo gel. All participants (age of 28.2 ± 4.2 y; height of 175.5 ± 6.1 cm; weight of 74.6 ± 11.6 kg) signed the informed consent forms before entering into the study. They were randomly assigned to receive single dose of placebo (n = 7), 100 mg of T (n = 7), and 50 mg of T (n = 7). The

participants were allowed to take part several times between different treatments, for instance, first receiving placebo then T gel or vice versa, but at least a one-week wash-out period was implemented. The gel was instructed to spread onto the skin of the abdomen and/or both upper arms. DB samples were collected before the administration (0 h) and 9, 24, 48, 72, and 96 h after the administration. The 0 h time was to obtain basal concentrations and 9 h was the time that we considered the blood T concentration to be maximally affected. The time 24, 48, 72, and 96 h were chosen as washout times to determine the maximum detection window of an elevated T concentration.

3. Results and discussion

3.1. Steroid-free blood and sample preparation

Although the steroid-free serum had already applied in some studies where the removal of endogenous steroids from the collected whole blood is not practical [20, 21], the concern of whether it is absent of steroids had been raised and required confirmation. Our results demonstrated no detectable T signal (S/N < 3) was found in the purchased steroid-free serum as well as the prepared non-spiked steroid-free blood (**Fig. 2A**). However, if the washing process of the RBCs collected from the ABP samples was not performed adequately, certain amounts of T would exist in the extract. This issue indicated the importance of confirmation prior to usage.

To simplify the DB collection and minimise athletes' discomfort, only one VAMS tip collecting 20 µL of blood was employed in this study. The whole blood sample is allowed to saturate the VAMS tip and exclude water during the drying process, as such the haemolysis of RBCs might have occurred [22]. Before a successful analysis of AAS in DB sample was

realised, numerous preliminary tests were conducted. Solid phase extraction (SPE) and LLE are two common ways of biological matrix pretreatment. From a practical consideration, we preferred UAE followed by LLE because for the SPE, the VAMS tip must undergo a proper liquid extraction before submitting the extracted solution to the SPE cartridge. In the LLE procedure, water could remove the undesirable matrix such as haemoglobin that colouring the extract and giving more background in the chromatograms, and the targeted steroids partition into the MTBE phase for analysis (**Fig S2**).

3.2. GC-MS/MS analysis and method validation

Rapid separation and sensitive detection of AAS in a limited volume of sample were accomplished by exploiting the Intuvo 9000 GC-7010 TQ MS. Compared to the conventional system, this system allows heat up and cool off much quicker (contact conduction heating in GC) and offers higher sensitivity (high-efficiency EI source in TQ MS). Nine target AAS were successfully identified within 6.4 min and quantified at the ng mL^{-1} level (**Fig 3**).

For specificity in DB matrix obtained from at least ten volunteers, no interfering signals were observed at the retention times of the target analytes. Precision and accuracy at low, medium and high levels are given in **Table 2**. Most of their RSD or bias were within the interval of −20% to 20%, but the low level of drostanolone amounted 23.1% of RSD and the high level of DHCMT amounted 25.5% of RSD at the inter-day precision.

The calibration curves fitting to a quadratic model were found to have excellent linearity for all compounds with a coefficient of correlation (r^2) of larger than 0.999 within the respective concentration ranges (**Table 3**). The accuracies of the calibration curves met the criteria of all points less than ±10% bias and the lowest concentration less than ±15% bias.

The LOD that determined as S/N > 3 ranged from 0.1 to 0.78 ng mL^{-1} (**Table 3**). Concerning the sample volume of 20 µL, the final solution volume of 40 µL, and the injection volume of 1 µL, the amount of analyte on the column is as little as 50 fg in the method developed here. The LLOQ that defined as the lowest point of the calibration curve ranged from 0.78 to 1.56 ng mL^{-1}. The validated GC-MS/MS method could confidently be used to measure the T in healthy men as their serum levels ranging from 2.8 to 13.2 ng mL^{-1} [23], whereas normal females' plasma with a range of 0.1−0.7 ng mL^{-1} may not be quantifiable [24].

Recently, Renterghem and colleagues [25] developed an ultra-sensitive chemical ionisation GC-MS/MS method for the detection of steroid esters in human plasma. From 500 µL of plasma sample, the LODs were 10−200 pg mL^{-1} where T acetate, T valerate, and T enanthate had the lowest LOD of 10 pg mL^{-1}. However, considering our method with the LOD of T at 0.1 ng mL^{-1} but only requires 25 times lesser sample volume (20 µL of DB), the detection limit of our method is comparable to that of the method proposed by Renterghem et al.

Satisfactory extraction recoveries for the steroids were noted, varying from 43% to 89% (**Table 3**). The sampling method based on the VAMS technique yielded much higher on recovery than that observed in the punched DB spots with a significant pre-analytic loss reported in the literature (recoveries ~20%) [26, 27]. In addition, a range of enhancement of matrix-induced response was found (113% to 234%). This could be owing to the matrix components block the active sites of GC injector (primarily inside the liner) and protect the target analytes from thermal degradation or adsorption [28].

Since steroids have been reported to be stable in plasma stored at freezer for up to a decade [29], their unconjugated forms are believed to be even stabler particularly in the dry

matrices. The DB samples stored at room temperature, 4°C, and −20°C were analysed after a two-month storage period. The storage condition of each analyte with the highest peak area was presented as 100%. Good stability of AAS was observed in DB collected with VAMS tips (**Fig S3**). Results showed the stabilities all of the target AAS varied between 80% to 100%; nonetheless, nandrolone level storing at room temperature was significantly lower than that storing at −20°C ($p < 0.05$). Hence, our results suggested 4°C and −20°C could be the proper condition for the long-term storage of DB specimens.

Finally, the newly developed method on the Intuvo 9000 GC-7010 TQ system was compared with the method routinely used on the 7890 GC-7000 TQ system in our laboratory. Their configurations and conditions were outlined in **Table S1**. Although two instruments were applied with different chromatographic column and oven temperature program, the analytes were well separated and identified unambiguously within a short period (6.4 min vs 13.0 min; **Fig S4**). The new system was demonstrated to have remarkable improvement in sensitivity by comparing the LOD for nandrolone (0.20 vs 0.78 ng mL^{-1}), boldenone (0.20 vs 0.78 ng mL^{-1}), mesterolone (0.20 vs 1.56 ng mL^{-1}); T (0.10 vs 1.56 ng mL^{-1}), drostanolone (0.20 vs 1.56 ng mL^{-1}), metenolone (0.10 vs 1.56 ng mL^{-1}), metandienone (0.10 vs 1.56 ng mL^{-1}), oxandrolone (0.78 vs 25 ng mL^{-1}), and DHCMT (0.78 vs 50 ng mL^{-1}). Interestingly, the sensitivities of the later two analytes, oxandrolone and DHCMT, steroid structures with the addition of heteroatoms are strikingly enhanced. On the other hand, two systems exhibited similar performance on the peak shape that generally was symmetrical, ranging within 0.9−1.2. The chromatograms obtained from the Intuvo 9000 GC-7010 TQ system displayed a slight fronting in mesterolone (0.71 vs 1.15) yet an amelioration of the tailing in oxandrolone (1.90 vs 2.53).

3.3. Comparison of serum specimen and VAMS dried blood

Good agreement in T levels of two specimens was revealed by the Deming regression and Bland-Altman analysis. The Deming slopes (**Fig. 4A**) ranged between 0.9664 and 1.1349 and the intercepts ranged between –1.7640 and 0.3533 (y = 1.0506x – 0.7053). The data correlation coefficient was 0.9718. According to the 95% confidence interval, the hypothesis of slope =1 and intercept = 0 was accepted as the confidence interval for slope contains the value 1 and intercept contains the value 0. It is concluded that the two methods did not have a proportional difference (slope) nor differ by a constant amount (intercept).

The Bland–Altman plot (**Fig. 4B**) showed that the mean (SD) difference between the two methods was −3.5% (14.2%). The mean ratio of T concentrations measured by the serum specimen to those obtained by the VAMS DB was 0.97, with variation in the ratio of 0.71–1.27. The distribution of variation indicated that one method was not constantly higher or lower than the other one. These observations have concluded that DB specimens obtained from the VAMS device could be employed for performing T determinations as suitable as using the serum specimens.

3.4. Testosterone gel administration study

Regular AAS dopers were reported to take up to seven AAS agents per cycle via intramuscular injection or oral ingestion [30]. In some cases, the average weekly AAS dose could amount larger than 1,000 mg and such dose was dozens of times of the therapeutic dose [31]. However, the T gel is typically administered in a dose of 50−100 mg. Given that transdermal T delivery has the bioavailability of merely 10% [32], the detection of its usage is exceptionally difficult using urine samples as reported in the literature [4-7]. To pursue

WADA's zero-tolerance policy against doping despite micro-dose being considered, the feasibility of using VAMS DB to identify T gel administration was evaluated by the present three-arm study.

The first group received placebo (**Fig. 5A**). We could observe the daily fluctuation of T level within the 5 days. The small amount of alcohol content from the hand gel applied topically was not able to influence the blood T level. The data generated from this group could thus be regarded as the baseline T concentration. The mean concentration of all DB samples was 6.1 ng mL^{-1} and the coefficient of variations (CV) were generally within 50%. Based on this evidence, the T responses in VAMS DB were fairly stable and the normal T concentrations in 'clean' samples would not exceed 30 ng mL^{-1}.

The second group received 100 mg of T gel (**Fig. 5B**). After the administration, the T concentrations dramatically increased to more than ten times higher than the baseline and such remarkable responses were found in all participants. The C_{max} was identified at 9 h post-administration and all above 100 ng mL^{-1} with the mean value of 234.9 ng mL^{-1}. Thereafter, T levels gradually declined and some even did not return to the baseline after 96 h. If applying the cut-off threshold of 30 ng mL^{-1}, the detection windows for single-dose 100 mg could last up to 48 h after termination of the application.

The third group received 50 mg of T gel (**Fig. 5C**). Likewise, the T levels reached a peak at 9 h post-administration. The mean C_{max} (91.2 ng mL^{-1}) was expected to be lower than that of taking 100 mg. Surprisingly, some participants' levels at 9 h (e.g. TGel50-D, TGel50-E and TGel50-F) barely attained 30 ng mL^{-1}, albeit the elevation is statistically significant compared with the baseline. This phenomenon highlighted the inter-individual variability of the T gel application. The possible reasons are the differences in terms of the ability to absorb the drug through the skin, distribute to the bloodstream, and metabolite via

conjugation or into other 17-ketosteroids. The blood T responses have also been shown to have the *UGT2B17* genotype dependence, the del/del displays smaller fluctuations compared with the ins/del and the ins/ins after T undecanoate administration [33]. Besides, the pharmacokinetic pattern may further vary due to different application sites [34], clothing barriers [35], and gel preparations [36]. A solution for these circumstances is to establish individual and longitudinal profiles as the principle of the ABP. By monitoring the T levels over time, any abnormal fluctuations may indirectly reveal the use of exogenous drugs.

This is not the first study inquiring into the detection of micro-dosing T gel by monitoring the blood variations, but unfortunately, most studies failed to observe appreciable changes in the blood T concentrations [5, 37-40]. Mullen et al. [40] proposed that the most sensitive biomarkers could either be the urinary 5α-androstane-3α,17β-diol/epitestosterone ratio (5αAdiol/E) or the serum dihydrotestosterone (DHT). After administering 100 mg of T gel, the urinary 5αAdiol/E increased up to 4-fold at 12 h and the serum DHT increased up to 2-fold at 24 h. Recently, some studies [4, 5, 39] have demonstrated that the salivary T concentration possesses a superior sensitivity for the detection of using transdermal T. The salivary T could rise ten times higher than the basal level and certainly became one of the most promising matrices in doping analysis. However, some problems have also been noted, which include, but are not limited to the concerns of poor stability at room temperature, low correlation to blood T, and cross-contamination by bleeding in the buccal mucosa.

One study investigated the responses of free and glucuronide-conjugated T in both DB and plasma samples after a single-dose T undecanoate ingestion [41]. The T glucuronide concentrations were subsequently increased to a hundredfold higher than the baseline, yet the free T concentrations had barely varied. As a consequence, making use of

the total fractions of T (free fraction plus the conjugated fraction) for identifying exogenous T usage is critical and generally accepted in doping screening [42].

We observed that the steroids were deconjugated after the sample preparation. The amounts of undeconjugated steroids were determined to less than 10% in the samples collected after the administrations (**Fig S5**). The utilisation of ultrasonication might assist both extraction and deconjugation process. Ultrasonication has been established to be an alternative as effective as the traditional assay (i.e. incubation at 55°C for 1 h with the β-glucuronidase enzyme from *E. coli*) for steroids glucuronide [43].

At this point, this study is being expanded to the screening of bodybuilders at gym. We are seeing the detection of drostanalone and nandrolone in some of the real samples which is in strong agreement with the urinary data. The intriguing findings will be addressed in the other paper.

4. Conclusions

In this work, we developed a promising and reliable approach for the determination of 9 AAS in 20 µL of DB using VAMS and GC-MS/MS. The GC-MS/MS method was able to detect 50 fg of AAS (i.e. 0.10 ng mL^{-1} in 20 µL of DB) within a separation time of 6.4 min. The extraction recoveries of the VAMS DB were found to be superior to that of the punched DB spots. Furthermore, good agreement between serum and VAMS DB was established for the AAS quantification via analysing forty real samples. A proof-of-concept study was carried out with a three-arm T administration trial. We demonstrated that the DB total T could be a sensitive indicator for the identification of micro-dosing pseudo-endogenous T. In the forthcoming work, this approach is being expanded to a large cohort screening of

bodybuilders at gym and ultimately may allow universal applications on monitoring sports drug misuse.

CRediT authorship contribution statement

William Chih-Wei Chang: Conceptualization, Formal analysis, Investigation, Validation, Visualization, Writing - original draft. **David A Cowan:** Conceptualization, Investigation, Resources, Supervision. **Christopher J Walker:** Conceptualization, Resources, Supervision. **Nick Wojek:** Conceptualization, Resources, Supervision. **Alan D Brailsford:** Conceptualization, Investigation, Resources, Funding acquisition, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was funded by the UK Anti-Doping. C.W. Chang wishes to acknowledge the Ministry of Science and Technology, Taiwan for supporting his fellowship program [grant number 107-2917-I-037-004].

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Figure captions

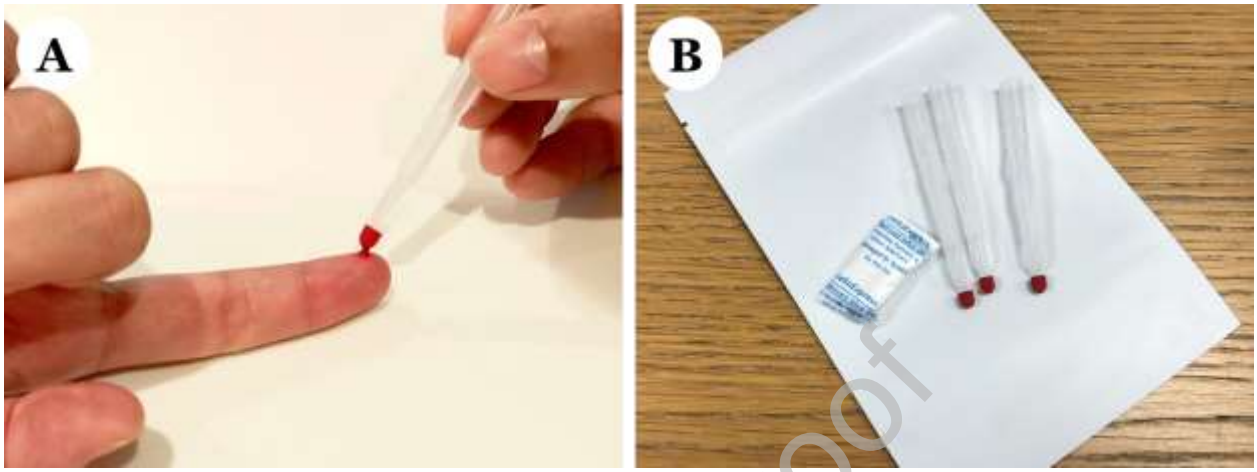

Fig. 1. Illustration of collection and storage of dried blood samples obtained by the VAMS tips. (A) a finger pricker was utilised to induce a small amount of blood from the finger followed by the collection of dried blood sample using VAMS tips. (B) The tips were packaged in a foil pouch with a desiccant.

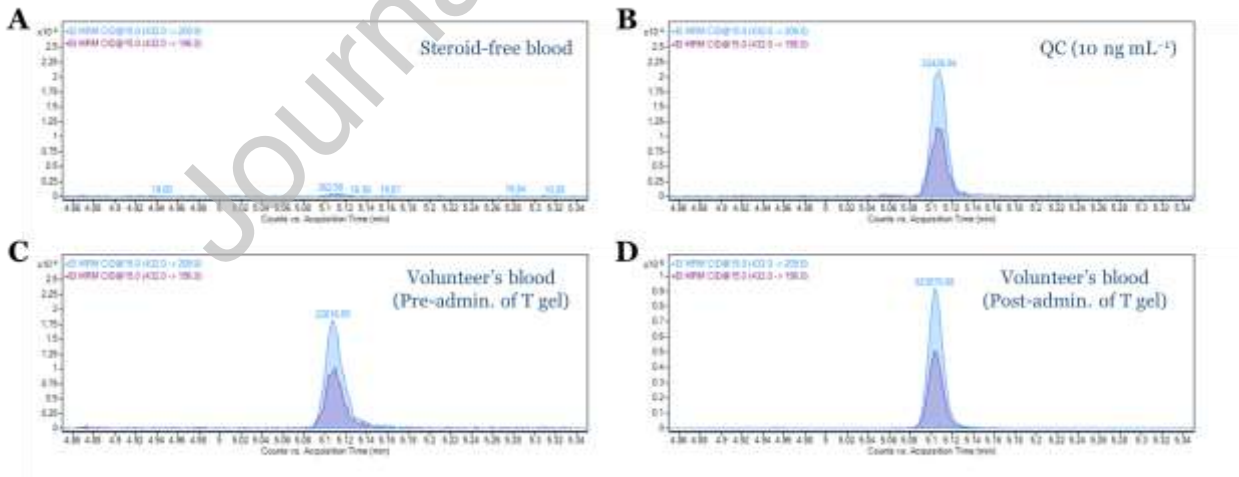

Fig. 2. The chromatograms of testosterone with the quantifier (m/z 432→209, presented in blue colour) and the qualifier (m/z 432→196, presented in purple colour) in DB samples obtained from (A) steroid-free blood, (B) QC spiked with 10 ng mL^{-1} of testosterone

standard, (C) finger-pricking within pre-administration of testosterone gel, and (D) finger-pricking within post-administration of testosterone gel.

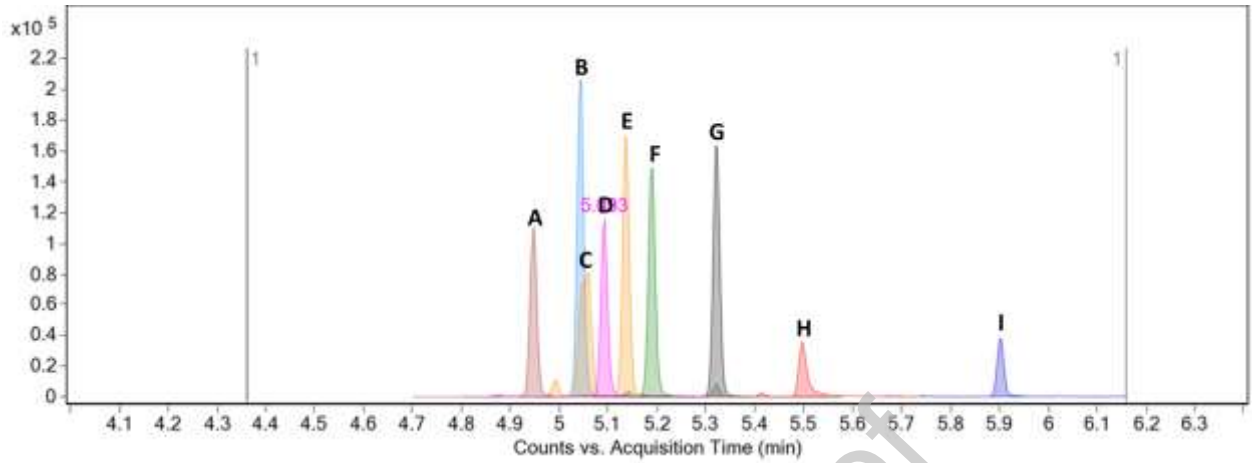

Fig. 3. The extracted MRM chromatograms of AAS in 20 µL of dried blood samples (12.5 ng mL^{-1}) analysed by GC-MS/MS with the total run time of 6.4 min. A; nandrolone (m/z 418→194), B; boldenone (m/z 430→206), C; mesterolone (m/z 448→141), D; testosterone (m/z 432→209), E; drostanolone (m/z 448→141), F; metenolone (m/z 446→208), G; metandienone (m/z 444→206), H; oxandrolone (m/z 308→176), I; DHCMT (m/z 240→93).

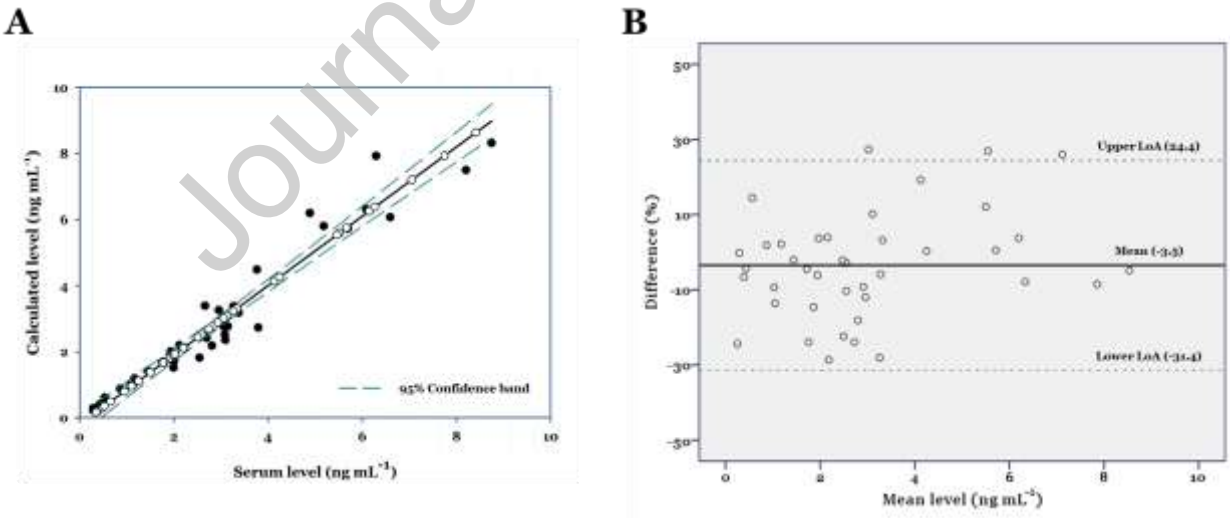

Fig. 4. Comparison of testosterone levels in serum specimen and VAMS dried blood using forty ABP venous blood samples. (A) Deming regression and (B) Bland-Altman plot.

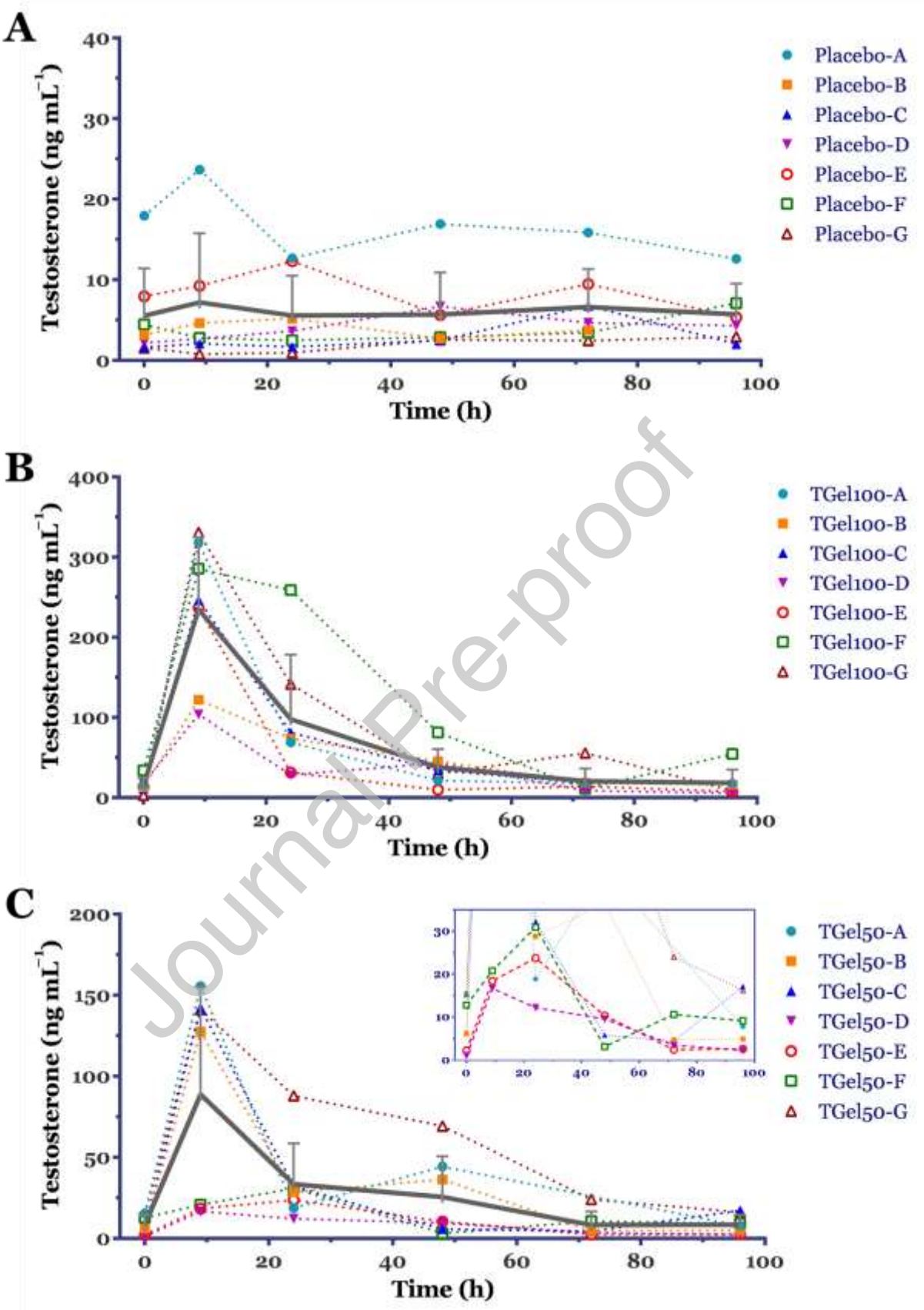

Fig. 5. Dried blood testosterone responses in the healthy male volunteers receiving the transdermal administration of (A) placebo, (B) Tostran gel containing 100 mg of testosterone, and (C) Tostran gel containing 50 mg of testosterone. The gray lines represent the mean concentrations with standard deviations.

Table 1. MS parameters for the analysed steroids.

Analyte	Precursor ion (m/z)	Product ion (m/z)		CE (eV)	Dwell time (ms)	Retention time (min)
Nandrolone	418	194	Quantifier	15	9.8	4.95
	418	287		15		
	418	182		15		
Boldenone	430	206	Quantifier	10	6.9	5.05
	206	165		10		
	206	191		10		
Mesterolone	448	141	Quantifier	20	6.8	5.06
	448	156		20		
	448	157		20		
Testosterone	432	209	Quantifier	15	6.3	5.09
	432	196		15		
Testosterone-d_3 (ISTD)	435	209	Quantifier	15	6.3	5.09
	435	196		15		
Drostanolone	448	141	Quantifier	20	6.2	5.14
	448	156		20		
	448	157		20		
Metenolone	446	208	Quantifier	10	7.1	5.19
	446	179		10		
	446	195		10		
Metandienone	444	206	Quantifier	10	9.1	5.32
	339	283		10		
	444	191		10		
Oxandrolone	308	176	Quantifier	5	16.8	5.50
	363	273		5		
	363	161		5		
DHCMT	240	93	Quantifier	20	42.6	5.90
	478	240		20		

Table 2. Precision and accuracy for the analysed steroids.

Analyte	Intra-day precision (%RSD)			Inter-day precision (%RSD)			Intra-day accuracy (%bias)			Inter-day accuracy (%bias)		
	High	Med	Low	High	Med	Low	High	Med	Low	High	Med	Low
Nandrolone	3.0	3.9	9.2	8.0	3.8	7.0	4.6	10.5	3.4	10.5	9.0	6.7
Boldenone	3.5	4.5	6.2	6.8	6.4	7.4	14.1	10.5	−4.1	17.9	7.8	3.9
Mesterolone	5.3	7.2	8.6	11.6	10.0	15.0	−6.5	−7.2	−4.2	2.7	−2.9	9.9
Testosterone	3.6	5.2	10.4	8.1	4.5	11.6	1.6	0.9	3.8	7.7	2.2	3.5
Drostanolone	7.0	10.8	13.7	14.1	3.8	23.1	−7.8	−7.3	−6.5	3.5	6.9	13.4
Metenolone	7.9	4.5	10.8	11.1	6.0	12.7	7.9	8.3	−0.9	19.9	16.1	16.2
Metandienone	3.0	3.0	9.7	6.2	6.6	5.4	−2.6	7.7	4.6	1.8	1.7	2.1
Oxandrolone	2.8	2.9	12.7	4.5	8.5	5.4	11.4	11.1	−6.5	17.5	15.0	11.1
DHCMT	9.2	15.7	7.7	25.5	16.3	7.1	−1.0	−3.0	−6.6	−17.1	−1.6	11.7

Low, medium, and high concentrations were 2, 10, and 25 ng/mL, respectively. One batch of six specimens (*n* = 6) was tested on the same day for intra-day validation. Three batches of three specimens (*n* = 3) were respectively tested on three different days for inter-day validation.

Table 3. Linearity, LOD, recovery, and matrix effect for the analysed steroids.

Analyte	Linear range (ng mL^{-1})	r^2	LOD (ng mL^{-1})	Recovery (%)	Matrix effect (%)
Nandrolone	1.56−200	0.9993	0.20	74	160
Boldenone	1.56−200	0.9998	0.20	73	161
Mesterolone	1.56−200	0.9997	0.20	55	113
Testosterone	0.78−200	0.9995	0.10	89	116
Drostanolone	1.56−200	0.9997	0.20	51	120
Metenolone	1.56−200	0.9995	0.10	60	152
Metandienone	0.78−200	0.9998	0.10	57	234
Oxandrolone	1.56−200	0.9997	0.78	72	159
DHCMT	0.78−200	0.9999	0.78	43	206

Calibration curves were generated by at least 6 points level. The bias of accuracy of all points was less than ±10% bias and ±15% bias for the lowest concentration. LOD was estimated by a signal-to-noise ratio (S/N) >3.