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AUTOMATED EXTRACTION AND DERIVATISATION FOR THE DETECTION OF STEROIDS IN BLOOD USING MITRA® MICROSAMPLING DEVICES

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INTRODUCTION

Current anti-doping analytical methods are mainly performed using urine samples. However, collection of urine samples from athletes can be time consuming and intrusive due to the need for monitoring during sample collection to prevent the submission of counterfeit or adulterated sample. The alternative of blood sampling is less pursued due to its invasive nature and the need for specialists to collect the samples intravenously, adding to the expense of sample collection. Blood microsampling offers a very appealing alternative as it is significantly less invasive, does not require specialists for sample collection. Samples are easy to transport, are stable over extended periods of time and cheaper to both use and store.

In recent times, cards manufactured from treated papers have been developed and used for dried blood spot (DBS) sample collection to capture and store small amounts of blood samples from, for example, a finger prick sampling device. However, when using DBS, the blood hematocrit (HCT) level affects the viscosity and homogeneity of the collected blood spot (30-50 uL) as it dries onto the card, leading to high analytical variability from solvent extracted spots punched from the collection card. Recently, a new blood microsampling technology has emerged, the Mitra® microsampling device, driven by Volumetric Absorptive Microsampling (VAMS™) technology[1]. Using Mitra® devices, the "haematocrit effect" is avoided allowing collection of a fixed volume of blood regardless of HCT level, all of which can be solvent extracted to avoid sample inhomogeneity issues associated with paper based, spot collection methods.

The sample size collected using the Mitra® devices is ideal for high sensitivity automated sample preparation and analysis. When using automation, the accurate control of experimental variables and the consistency of task performance can significantly improve data quality and therefore offer a very attractive alternative to complex manual workflows.

This application note describes the optimisation of an automated workflow for the extraction, derivatisation and analysis of steroids from Mitra® devices for anti-doping purposes. The optimised automated method has been compared to the existing manual method to evaluate recoveries and assess method performance.

Figure 1: Mitra® microsampling device

MATERIALS

Steroids methanolic solution, blank blood and spiked blood both sampled on 20 µL Mitra devices were kindly provided by King's College London Drug Control Centre (DCC).

INSTRUMENTATION

Samples were extracted using a GERSTEL standalone Dual Head Robotic MultiPurpose Sampler (MPS) equipped with the following modules: Universal Syringe Modules (USM) with 1 mL and 50 µL syringes, Robotic Centrifuge CF200, VT40-2 mL trays, 180 mL solvent reservoirs, ultrasonic bath, QuickMix®, fast wash station. The full system configuration is shown in Figure 2.

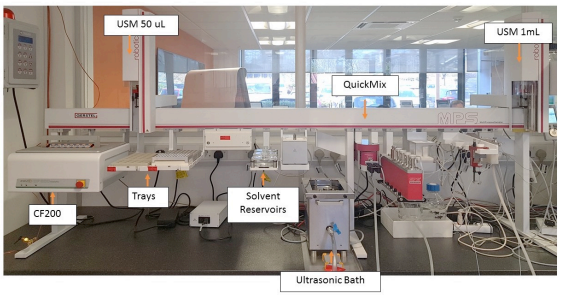

Figure 2: Automated sample preparation system for extraction of steroids from Mitra® microsampling device

Automated evaporation, derivatisation to the enol-trimethylsilyl (TMS) form and GC-MS-MS analysis were performed using a GERSTEL Dual Head Robotic MultiPurpose Sampler (MPS) mounted on an Agilent 7890 GC coupled to a 7010 Triple Quadrupole equipped with the High Efficiency Source (HES) in EI mode. The GERSTEL MPS was equipped with the following modules: Universal Syringe Modules (USM) with 100 µL and 10 µL syringes, VT40-2 mL trays, Large wash station, mVAP® evaporator and Agitator. Figure 3 shows this system configuration.

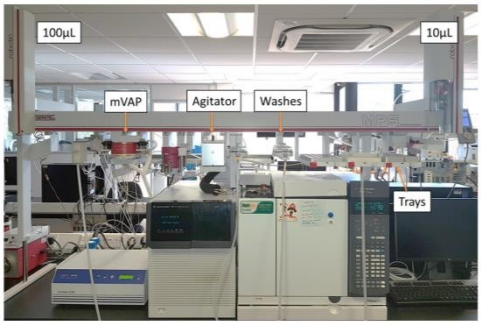

Figure 3: Online automated solution for the derivatisation and GC-MS-MS analysis of steroids from Mitra® microsampling device

METHODS

Method optimisation

As the method required optimisation of several parameters, a multivariable-at-a-time approach (MVAT) was taken. Design of Experiment (DoE) was applied to the extraction of the Mitra® tips using a Definitive Screening Design (DSD) to screen for dependant factors and identify nonlinear response effects. For more details regarding MVAT and DoE refer to AS198. Table 1 lists factors and ranges selected for the optimisation of the extraction conditions for the microsampling devices.

Table 1: Factors and ranges for DSD

Factors	Lowest	Highest
Solvent Volume [µL]	100	500
Solvent Type	MEOH	ACN
Sonication time [min]	5	30
Mixing time [min]	0.5	10

Peak area for each of the target analytes was selected as the response to be maximised. The DSD for the experimental factors (variables) required a total of 18 experiments which were performed under rigorously controlled experimental conditions and in randomised order. Automation provides the accuracy and rigour required to properly control the experimental variables but it can also efficiently maximise utilisation of the system thanks to the PrepAhead function within the MAESTRO software (for more details refer to AS189).

Briefly, the PrepAhead function allows overlap of sample preparation steps between samples for best possible system utilisation and highest sample throughput. Figure 4 shows the PrepAhead function applied to the preparation of the 18 DSD experiments (different coloured bars represent the different steps in the preparation workflow). The whole preparation required 3 hours and 30 minutes.

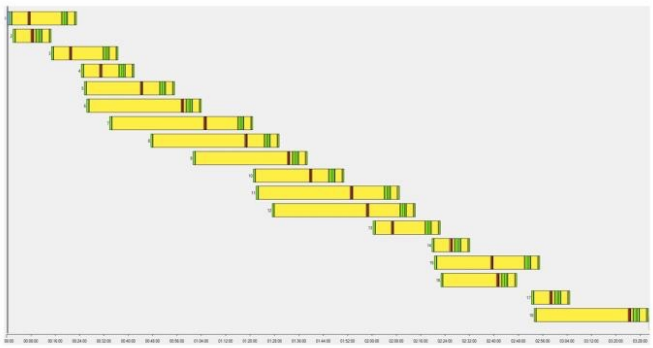

Figure 4: GERSTEL MAESTRO software PrepAhead function applied to the DoE experiments.

Experimental plan

6 spiked blood Mitra tips and 6 blank blood Mitra tips for post spiking were extracted using the established manual method (Drug Control Centre, Kings College, London, UK) and by the optimised automated method (Anatune), respectively. The blank blood samples were spiked after extraction with 20 µL of a 50 ng/mL methanolic solution of target steroids. Target analytes, expected retention time, MRM transitions and collision energy (CE) for the respective TMS derivatives are listed in Table 2.

Manual extraction method

The Mitra® tip was separated from the cartridge and placed into a glass tube. 1 mL of water and 2 mL of MTBE were added for liquid-liquid extraction (LLE). The mixture was extracted using an ultrasonic bath for 15 min and by rotary mixing for 15 min, followed by centrifugation at 3000 rpm for 5 min. The supernatant (organic portion) was transferred to a clean tube and dried under nitrogen at 60°C for 10 min before derivatisation and GC-MS analysis.

Table 2: Lowest, end point and actual concentration of formaldehyde, in ppmV, for results shown in Figure 4.

Compound	Retention time [min]	MRM Transitions	CE (eV)
Nandrolone	9.474	418>194	15
		418>182	15
		418>287	15
Mesterolone	9.550	448>141	20
		448>157	20
		448>156	20
Boldenone	9.629	206>191	10
		206>165	10
		430>206	10
Testosterone	9.700	432>209	15
		432>196	15
Testosterone d3	9.700	435>209	15
		435>196	15
Drostanolone	9.778	448>141	20
		448>157	20
		448>156	20
Metenolone	9.861	446>208	10
		446>195	10
		446>179	10
Metandienone	10.072	444>206	10
		444>191	10
		339>283	10
Oxandrolone	10.363	308>176	5
		363>161	5
		363>273	5
Dehydrochloromethyltestosterone	10.997	240>93	20

Optimised automated extraction method

The Mitra® tip was placed into a 2 mL screw cap glass vial for extraction. 500 µL of ACN were added and the device was sonicated for 30 min and mixed at 2000 rpm for 30 seconds. The extract was transferred to a high recovery vial whilst the tip was extracted a second time adding 500 µL water and 500 µL MTBE, respectively, mixing for 2 min at 2000 rpm and then centrifuging for 2 min at 4500 rpm to help separate any emulsions formed. The upper layer (MTBE) was then combined with the ACN extract and evaporated to dryness for 10 min at 45°C. The optimised automated workflow prep-sequence is shown in Figure 5. Duration for the extraction of the 12 samples was 2 hours and 34 minutes.

Action	Method / Value
PREP Vials 1-6	Ahead, Bounded
ADD	Add 500uL ACN
MOVE	
ULTRASONIC	30.00
MOVE	
WAIT	Wait to dry 30 s
MOVE	
MIX	Mix 0.5 min 2000 rpm
MOVE	
ADD	Prepierce vials
ADD	Transfer extract to clean vial
ADD	Add 500uL Water
ADD	Add 500uL MTBE
MOVE	
MIX	Mix 2 min 2000 rpm for LLE
MOVE	
MOVE	
CF200	Centrifuge 2 min 4500rpm
MOVE	
ADD	Combine MTBE with ACN extract
END	

Figure 5: GERSTEL Maestro software prep sequence for the automation of the Mitra® tip extraction

Automated derivatisation and Instrumental Analysis

Extracted sample residues were derivatised to enol-TMS form prior to analysis by GC-MS-MS. Derivatisation was fully automated and integrated into the GC method. 20 µL of derivatising reagent (MSTFA:ammonium iodide:ethanethiol 1000:3:9 v:w:v) and 20 µL dodecane were added to the samples and incubated at 80°C for 20 min. Once the derivatisation was complete, 1 µL was injected in splitless mode. A 15 mx 0.25 mm x 0.25 µm DB5MS column was used. The oven was programmed as follows: 130°C for 2 min, 32°C/min to 190°C then 9.6°C/min to 220°C then 16°C/min to 300°C for 3 min. The MS transfer line was held at 300°C and source temperature at 230°C. The triple quadrupole MS detector acquired data in MRM mode (transitions in Table 1) using a Gain setting of 10.

RESULTS AND DISCUSSION

Figure 6 shows the peaks for the Quantifier and Qualifiers transitions of each of the target analytes for the automated extraction of the spiked blood Mitra® tips.

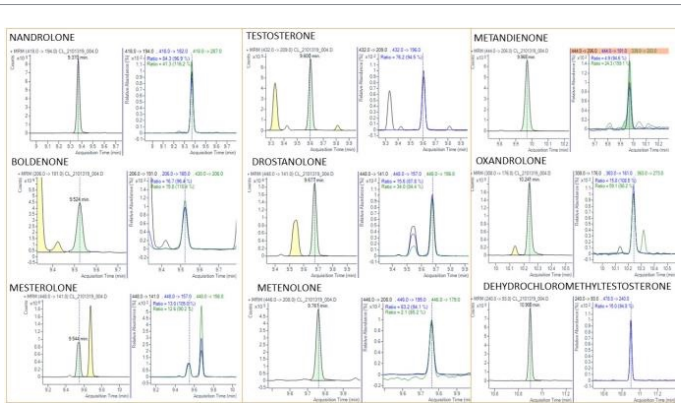

Figure 6: Quantifier (left hand side) and Qualifiers (right hand side) peaks of the target analytes for the automated extraction of the Mitra® devices

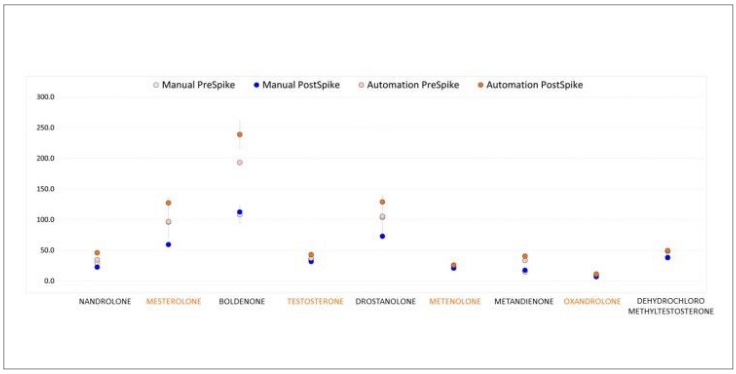

Figure 7: Peak are ratios (target area/IS area) for pre spiked and post spiked samples extracted with the manual (light blue/dark blue) and automated method (light orange/dark orange), respectively.

Retention time, peak symmetry and qualifiers ratios were consistent across all samples. The graph in Figure 8 summarises the trends of the peak area ratios (area target analyte/area IS) for both the manual and automated method, respectively, over the pre and post spike samples. Results for manual and automated approaches agree very nicely for all the analytes with the exception of mesterolone, boldenone and drostanolone where the automation gave higher results than the manual method.

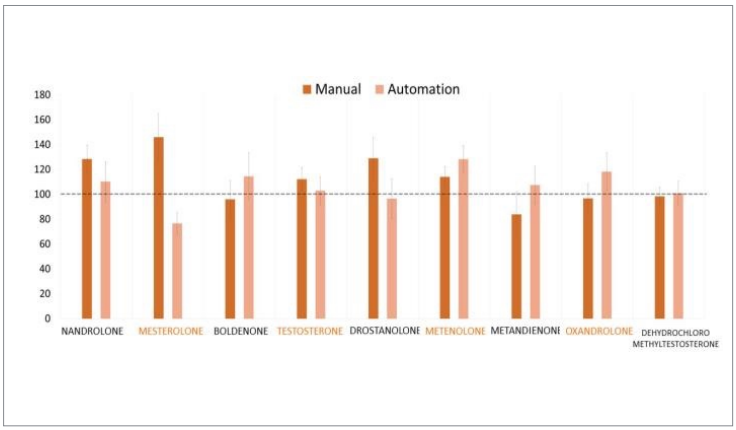

Figure 8: Comparison of the percent recoveries of the manual method and automation for the target analytes. Error bars represent the combined uncertainty.

Recoveries ranged between 84% -146% for the manual method and 77%-128% for automation. Good agreement was observed between the manual and automated approach. Percent relative combined uncertainties for the calculated recoveries were between 7%-21% for the manual method and between 9-17% for automation, respectively.

CONCLUSION

Mitra® devices are a very powerful tool which allow convenient blood sampling, in the field, for doping test purposes. The possibility to fully automate Mitra® device extraction and where required, analyte derivatisation, makes this approach even more appealing, offering improved robustness and reproducibility of sample preparation. This evaluation of the extraction of Mitra® tips for the detection of steroids in blood highlights the capability of automation to produce reproducible data consistent with the manual method performance for doping control testing using blood microsampling devices.

Whilst this paper describes the use of microsampling for doping control, microsampling is also extensively used in bioanalytical testing to support bioavailability and pharmacokinetics studies in all phases of pharmaceutical research and development. This approach is driven by the regulatory requirement to lower sampled blood volumes in both human and animal studies. Fully automated sample extraction of microsampling devices followed by high sensitivity analysis using HPLC with triple quadrupole MS detection for bioanalysis applications, is an exciting future prospect for this technology.

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