FAST TURNAROUND, HIGH THROUGHPUT SCREENING OF PRE-RACE SAMPLES IN CAMEL RACING

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ABSTRACT

There has been an increase in the demand for pre-race testing of camels in the UAE in recent years, particularly during major race carnivals where the numbers of participating animals can reach into the thousands. A rapid 96-well plate based solid phase extraction (SPE) system was developed for the screening of pre-race samples received by this laboratory. The system was designed to be fully automated to cope with a potential throughput of hundreds of samples per day without the need for manual sample handling. A fast instrument analysis protocol was also developed to identify suspect samples in a timely manner.

Blood samples were centrifuged upon arrival and loaded directly onto a Tecan Freedom EVO system configured for SPE of 96-well plates. Tube labels were automatically scanned using the system barcode reader. This information was used to track sample positions on the plates throughout the analysis and to automatically generate instrument acquisition sequences. Plasma was aspirated directly from the sample blood tubes using septum piercing needles. Aqueous buffer containing internal standards was added to the samples using a 96-pipette head and the diluted samples were extracted using Biotage EVOLUTE EXPRESS ABN 96-well plates. Following extraction of the samples, 2 µL of the eluate was spotted by the Tecan system onto a 96-LazWell plate for direct analysis by laser diode thermal desorption (LDTD) mass spectrometry. The remaining eluate was evaporated to dryness in the collection plate and dissolved by the Tecan system for fast LC/MS analysis.

Sample analysis consisted of LDTD/MS and fast isocratic elution LC/MS using triple quadrupole MS systems in SRM mode (two transitions per target). LDTD/MS covered higher concentration targets in positive ion APCI mode over a 12 second run time. Fast LC/MS was used for lower level targets in negative ion ESI mode over a 0.75 min run time. Samples flagged as suspect were re-analysed using the laboratory's standard confirmatory protocol. All screening results were confirmed. A sample turnaround of less than 48 hours was easily achievable and there is scope for the method to be scaled up for much larger sample numbers.

KEYWORDS

96-well plate, automation, camel, LDTD, pre-race

INTRODUCTION

Camel racing is an important traditional sport in the Arabian Gulf region and particularly in the United Arab Emirates. Public interest and racing camel ownership rates are high, and major race carnivals such as the Sheikh Zayed Camel Race Grand Prix Festival, Sheikh Mohammed Bin Zayed Arabian Camel Racing Festival and Al Wathba Annual Camel Race Festival attract literally thousands of competitors. The sport has developed rapidly over the last quarter of a century as modern training and husbandry techniques have crossed over from horse racing. This has necessitated the development of an anti-doping program, which commenced in 1994 with the establishment of testing facilities in the cities of Abu Dhabi and Dubai. The Racing Analytical Laboratory was established in the city of Al Ain in 2009 and has been the official provider of drug testing services to the United Arab Emirates Camel Race Association since 2012. During the 2015-2016 camel racing season the laboratory tested upwards of 6,500 camels over a 7 month period.

During the 2014-2015 racing season, the official testing program was expanded with the establishment of a pre-race testing program for the major race carnivals. Initial requirements were for 6 hour reporting, which severely restricted the scale and scope of testing. Individual batches of up to 20 samples were tested by solid phase extraction (SPE) followed by liquid chromatography-mass spectrometry (LC/MS) analysis on a single triple quadrupole instrument. Confirmatory analyses were not possible within the allowed timeframe and screening indications were applied in the form of a simple no-start rule with no further sanctions for offenders. For the 2015-2016 season the laboratory's LC/MS capacity was expanded and the required turnaround time was increased to 30 hours to accommodate higher sample loads. Overall capacity was

increased to a maximum of 100 samples per day with similar testing to the previous season. Again, confirmatory analyses were not possible and only screening indications were reported.

These initial experiences revealed some obvious limitations in our approach. The time taken to unpack and register samples delivered in tamper evident sample kits was identified as a major bottle neck. Also, the extension of the reporting deadline to the day after receipt meant that new sample consignments were arriving before work on the previous consignment was complete, putting a major strain on staff resources. The scope of testing was limited with sub-optimal sensitivity for some critical analytes. Finally, the inability to provide confirmed results within the required reporting timeframe limited the range of punitive measures available to the client. The client expressed a clear desire to further expand the program and to introduce heavier sanctions for positive findings, prompting a complete reassessment of the testing process.

The key objectives of the work described herein were the automation of as much of the process as possible and a reduction in instrumental analysis time for individual samples. The resulting system incorporates automated sample registration and 96-well SPE using a Tecan Freedom EVO platform with analysis by a combination of laser diode thermal desorption-mass spectrometry (LDTD/MS) and fast LC/MS. This approach has allowed the screening of up to 143 camels per day on a 48 hour turnaround with full confirmatory analysis of positive responses using one Freedom EVO system, one LDTD/MS system and two LC/MS systems. Scale up to several hundred samples per day using the same equipment is feasible.

MATERIALS AND METHOD

Chemicals and consumables

All chemicals and solvents were LC/MS grade unless otherwise stated. Methanol was purchased from Carlo Erba (Milan, Italy). Ammonium acetate and reagent grade ethylenediaminetetraacetic acid (EDTA) disodium salt were purchased from VWR International (Lutterworth, UK). Formic acid was purchased from Fluka (Missouri, USA). EVOLUTE EXPRESS ABN 96-well SPE plates (P/N 600-0030-PX01) were purchased from Biotage (Uppsala, Sweden). LazWell 96-well plates were purchased from Phytronix (Quebec, Canada) and were coated with EDTA before use (100 µg/mL EDTA disodium salt in 75:20:5 water:methanol:ammonium hydroxide solution; 5 µL spotted to each well and air dried).

Liquid handling

Sample registration, SPE eluate spotting and dissolution of samples extracts were performed using a Tecan (Männedorf, Switzerland) Freedom EVO-2 150 workstation configured for 192 sample capacity with a barcode reader, 8-channel pipetting head with Teflon coated liquid sensing tips (P/N 10612501.01), 96-channel pipetting head with fixed and disposable tip capability, 96-well plate SPE vacuum station and 96-well plate shaker.

Sample preparation

Blood samples were received in 10 mL Lithium-Heparin BD Vacutainer tubes and centrifuged at 3500 rpm for 10 min upon arrival. The tubes were loaded onto the Tecan Freedom EVO along with a plasma blank and two plasma QC spikes. SPE was performed using a Biotage EVOLUTE EXPRESS ABN 96-well plate conditioned with methanol (900 µL) followed by water (900 µL). Plasma (1 mL) was aspirated directly from the sample blood tubes using Teflon coated needles to a polypropylene 2 mL 96-well collection plate containing aqueous buffer (750 µL). After sample loading, the SPE plate was rinsed with water (2x1mL) before drying on full vacuum for 2 min. The samples were eluted with methanol (500 µL) and collected in a fresh polypropylene 2 mL round well 96-well collection plate. A portion (2 µL) of the methanol eluate was spotted by the Tecan system onto an EDTA coated 96-well LazWell plate for direct analysis by LDTD/MS. The remaining eluate was evaporated to dryness at 60 °C under nitrogen in a Biotage Turbovap 96 evaporator and was dissolved by the Tecan system in methanol (25 µL) and water (100 µL) for LC/MS analysis.

LC/MS analysis

LC/MS analysis was performed in negative ion ESI mode on a Waters (Elstree, UK) Xevo TQ-S mass spectrometer coupled to a Waters Acquity UPLC I Class liquid chromatograph. Separation was achieved on a Waters Acquity BEH C18 (50 mm x 2.1 mm x 1.7 µm) analytical column at 50 °C. The mobile phase consisted of methanol:water (75:25) containing 0.1% v/v formic acid and 2 mM ammonium acetate in isocratic flow at 0.5 mL/min.

LDTD/MS analysis

LDTD/MS analysis was performed using a Phytronix LDTD WX-960 ion source coupled to a Waters Xevo TQ-S mass spectrometer. The carrier gas was dehumidified air (>5 ppm humidity) at a flow rate of 3 L/min. The laser temperature was 20 °C. The MS was operated in APCI positive ion mode with a corona current of 3 µA, cone gas flow of 150 L/h and APCI probe temperature of 25 °C. Other MS settings were not optimised as they were not required for the operation of the LDTD ion source. Thermal desorption of the LazWell plate was achieved using a laser ramp with an initial delay of 1.5 s, ramping to 45% laser power after 5.5 s, held at 45% power until 8.5 s and returning to zero after 8.6 s. The total run time was 10.5 s.

RESULTS AND DISCUSSION

Sample preparation

The typical sample preparation approach for the LDTD/MS analysis of blood samples is protein precipitation. Protein is crashed using acetonitrile and a few microlitres is spotted onto the LazWell plate for analysis. In addition to classic protein precipitation, the use of phospholipid depletion plates affords added sample cleanup and the ability to automate using 96-well plate format. Initial experiments utilized this approach for typical concentrations of targets in our blood screen. However, the absence of a concentration effect as is achieved through SPE proved to be a major limiting factor and the protein crash approach was abandoned in favour of SPE. This worked well for most analytes, but two drug classes continued to be problematic: corticosteroids and thiazide diuretics. In the former case, the required sensitivity is normally achieved through the analysis of formate adducts in negative ion ESI mode. However, LDTD/MS is a solvent free system that imparts ionization using protonated water clusters in the gas phase and, unfortunately, formate adducts have thus far proven difficult to produce reliably. The main challenge is the introduction of a formic acid vapour into the carrier gas. Phytronix supplied a prototype T-piece connection into the carrier gas line for this purpose, but experiments showed the formation of adducts to be irregular and suggestive of sputtering at the carrier gas interface. It is possible that with more refinement the process could be made to work. In the latter case, the thiazide diuretics were poorly volatilised by the laser and displayed poor sensitivity and peak shape. This can be overcome to some extent using bovine serum albumin as a matrix additive, but this has not been implemented at the current stage of development.

Due to the aforementioned constraints, it was decided initially to focus on a two tiered analytical approach involving LDTD/MS for positive ion candidates (e.g. non-steroidal anti-inflammatory drugs) and LC/MS for negative ion candidates (e.g. corticosteroids, thiazide diuretics). The automated high capacity sample preparation was developed around this two level approach.

Sample extraction

The use of 96-well plate SPE format and automation has become more common for doping applications in recent years (Cuervo *et al.* (2014), Zahra *et al.* (2014), Steel (2016)). The Tecan EVO 150 was chosen as the liquid handling platform as it fulfilled most of our fast turnaround criteria. It is capable of sample and plate barcode reading, septum piercing, sample handling with liquid sensing, SPE in 96-well plate format and micro-spotting of the eluate onto the LazWell plate. It is able to pierce and aspirate 8 blood tubes simultaneously with the 8-channel head and then switch to 96-channel operation once the samples are in the plate. The result was that 96 samples could be registered, fully processed and spotted on a LazWell plate in less than 1 hour. Barcode reading information for the samples, collection plates and LazWell plates was exported as a text file which could be directly imported into the laboratory's LIMS system and Waters MassLynx software for LC/MS and LDTD/MS sequence generation. This enabled fully automated and reliable process control for the entire extraction and analysis procedure. Once set up, the system can be operated by a single analyst.

EVOLUTE EXPRESS ABN express plates were chosen for their ability to extract a wide range of acidic, neutral and basic analytes. Other sorbents were investigated, in particular the mixed mode cation exchange EVOLUTE EXPRESS CX as used in our normal post-race blood analysis, but ABN with a sample loading pH of 7 was found to provide the best compromise for LDTD/MS analysis. The sorbent's enhanced flow characteristics meant that sample blockage was rare. Load, wash and elution steps were all optimized, but the overall result remained very close to the recommended procedure from the Biotage package insert instructions.

LDTD/MS optimisation

The choice of solvent and the volume to spot onto a LazWell plate for LDTD/MS analysis are critical. The LDTD 'target' for spotting is a hexagonal recess set into the stainless steel base of the LazWell plate well. Ideally when dispensed into the well, the liquid remains in a small spot in the centre and is volatile enough to evaporate in a timely manner at room temperature. Solvents tested during development included ethyl acetate, isopropanol, methanol, acetonitrile and water. Combinations of these solvents were also investigated. Extraction eluates were evaporated to dryness and dissolved in 100 µL of water, acetonitrile, ethyl acetate, isopropanol, and methanol. Water had the best spotting properties but evaporation time took the longest. Acetonitrile had the second best spotting properties and evaporated in a relatively short time. Methanol, ethyl acetate and isopropanol all evaporated quickly but had a tendency to spread out to the edge of the well. Concentrating the eluate before spotting caused an increase in matrix background interference in the LDTD/MS analysis for all solvents investigated.

With regards to the volume of solvent that can be spotted, water and acetonitrile formed the smallest spots and therefore more solvent could be spotted (2-10 µL). For methanol, ethyl acetate and isopropanol their tendency to spread out more meant that lower volumes had to be spotted (2-5 µL). An increase in spotting volume generally showed an increase in signal. However, an increase in matrix background was also observed. 'Less is more' is

often the case with LDTD/MS in terms of sample loading. We investigated spotting the elution solvent (methanol) directly onto the LazWell plate following elution of the SPE plate. Peak area counts for a 10 ng/mL plasma spike showed good responses for all analytes and acceptable matrix backgrounds for blank plasma. 2 µL was determined to be the optimum spotting volume and the Tecan robot was able to spot the LazWell plate in a reliable manner with its 8-channel pipetting head. The LazWell plates were pre-coated with EDTA to promote the desorption of analytes containing carboxylic acid functional groups such as NSAIDs. The mechanism by which this occurs is unknown. Without the presence of EDTA these analytes demonstrated poor sensitivity and/or peak shape.

The rate and level of laser power required are compound dependent in LDTD/MS. At 45% of maximum laser power, the tests demonstrated effective desorption of all analytes with adequate peak shape and signal to noise. A lower 'heat' of 35% laser power resulted in wider, less Gaussian shaped peaks for some analytes and, consequently, poor signal to noise. A higher 'heat' of 65% laser power resulted in very sharp peak shapes for some analytes but also increased the matrix background, resulting in poor signal to noise for most analytes. A 4 s ramp time produced the best peak shape and, for some analytes, was able to partially resolve the target from matrix contributions. A delay of 1.5 s before the initial laser power ramping was necessary to observe a baseline signal prior to analyte desorption. Some analytes required very little laser power to evaporate from the plate. With this method, one 96-well plate can be analysed in 19.2 min.

Validation

Typical blank and spiked matrix chromatograms achieved by LDTD/MS in SRM mode are shown in Figure 1. A two-point calibration curve using the spike and blank matrix peak areas (normalized to the internal standard area) is used to determine approximate analyte concentration. This is necessary when using LDTD. In general, there is some background signal contribution from the matrix that requires normalization. Precursor and product ions, spike concentrations, collision energies (CE) and limits of detection (LOD) are given in Table 1. LODs were calculated based on Eurachem guidelines using matrix blanks under intermediate precision conditions (LOD = standard deviation × 3) (Magnusson *et al.*).

A rapid isocratic LC/MS method was developed to minimize analysis time for the non-LDTD compatible analytes with the desired lower detection limits (Figure 2). A sub-1 min method was developed with adequate resolution and sensitivity. The MassLynx software loop-ahead function enabled rapid throughput and minimized time between injections. No column pre-conditioning was required and more than 200 matrix injections could be performed before a column flush procedure was required. Precursor and product ions, spike concentrations, collision energies and limits of detection are given in Table 2. For LCMS, LOD evaluation was performed using plasma spikes at the concentrations deemed to be acceptable for the method (LOD = standard deviation × 3). Spikes were chosen over blanks in determining LOD as some analytes returned a zero response for matrix blanks. An example of this effect is shown for the blank trace monitoring hydrochlorothiazide in Figure 2.

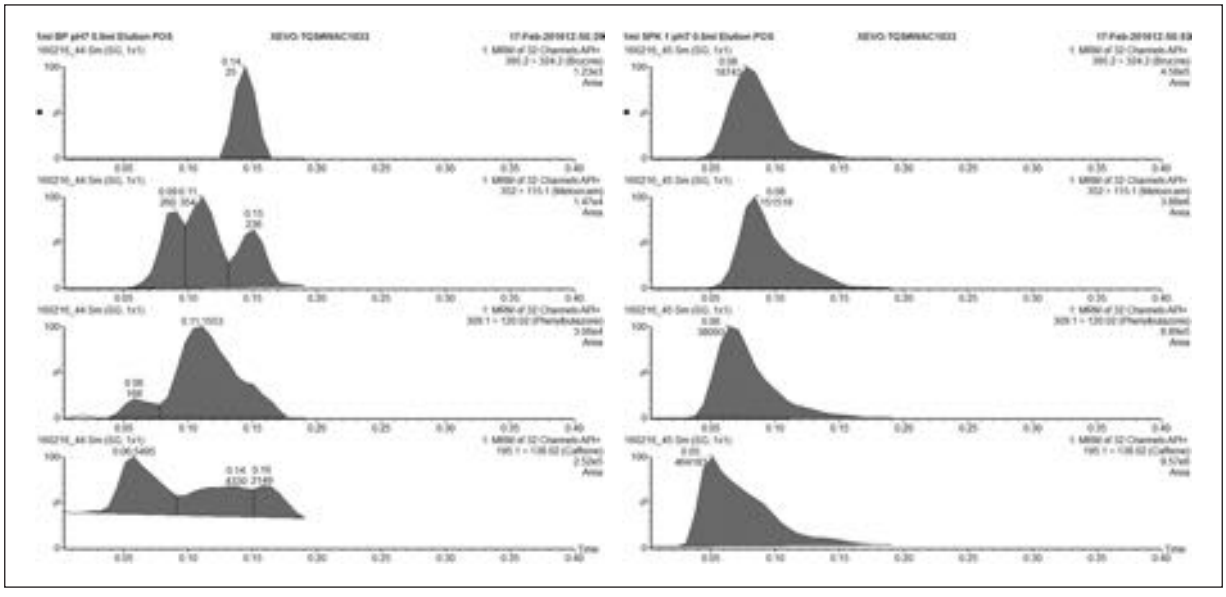

Figure 1: LDTD/MS transitions for brucine (5 ng/mL), meloxicam (10 ng/mL), phenylbutazone (10 ng/mL) and caffeine (50 ng/mL): Plasma blank (left) and plasma Spike (right)

Analyte	Concentration	Precursor Ion	Product Ion	CE (V)	LOD
Brucine	5 ng/mL	395.2	244.0	35	353 pg/mL (*n* = 608)
			324.2	35	
Caffeine	50 ng/mL	195.1	110.1	20	6 ng/mL (*n* = 656)
			138.02	20	
Meloxicam	10 ng/mL	352.0	115.1	18	214 pg/mL (*n* = 664)
			141.1	20	
Phenylbutazone	10 ng/mL	309.1	120.02	20	1 ng/mL (*n* = 649)
			160.2	20	

Table 1: Target analyte, spike concentration, precursor and product ions, collision energies and LOD values for some representative compounds in the LDTD/MS method.

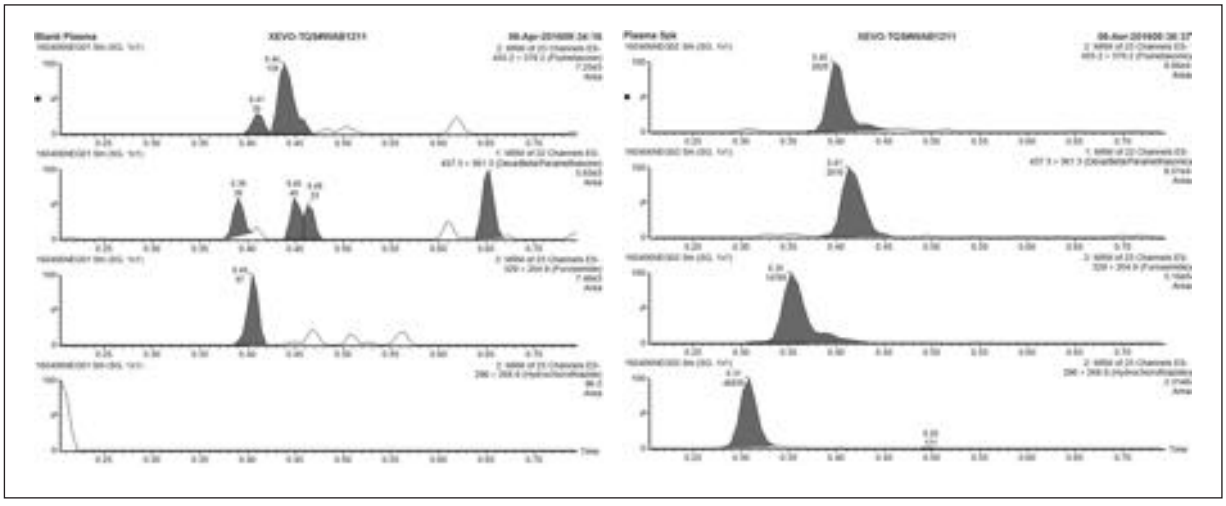

Figure 2: LC-SRM transitions for flumetasone (50 pg/mL), dexamethasone (50 pg/mL), furosemide (10 ng/mL) and hydrochlorothiazide (10 ng/mL); plasma blank (left) and plasma spike (right)

Analyte	Concentration	Precursor Ion	Product Ion	CE (V)	LOD
Dexamethasone	50 pg/mL	437.3	361.3	20	16 pg/mL (*n* = 10)
			307.2	30	
Flumetasone	50 pg/mL	455.2	379.2	18	28 pg/mL (*n* = 10)
			305.2	38	
Furosemide	10 ng/mL	329.0	204.9	20	5 ng/mL (*n* = 10)
		331.0	206.9	20	
Hydrochlorothiazide	10 ng/mL	296.0	268.9	20	5 ng/mL (*n* = 10)
			205.0	20	

Table 2: Target analyte, spike concentration, precursor and product ions, collision energies and LOD values for some representative compounds in the LC/MS method.

Analysis of Pre-Race Samples

During the Al Wathba Annual Camel Race Festival in 2016 the laboratory received 666 pre-race samples. 19 prohibited substances were detected using the LDTD/MS screen. These included phenylbutazone (5-900 ng/mL), brucine (1-2 ng/mL), caffeine (1 µg/mL), and meloxicam (25 ng/mL). All of these findings were confirmed using the laboratory's standard confirmatory procedure on a separate sample extract. All samples were reported within 48 hours of sample receipt. In order to establish confidence in the LDTD/MS screen the LC/MS portion was reinjected in positive ion ESI mode using our regular post-race LC/MS method. No false negatives or false positives were identified.

CONCLUSION

LDTD/MS offers chromatography-free MS that is easy to use, fast and capable of detecting many banned substances at appropriate concentrations in a racing anti-doping laboratory. Its limitations become apparent when dealing with extremely low concentration analytes (e.g. corticosteroids, inhaled bronchodilators, etc.) and in tailoring "compromise" sample preparations for multi-target analyses. The next stage of the project will involve an investigation of LDTD in conjunction with high resolution orbitrap MS, which has the potential to greatly expand the scope of the screen. The Tecan Freedom EVO platform is also under investigation for potential application to more routine laboratory tasks, including sample registration and preparation of post-race plasma and urine samples for LC/MS and GC/MS analysis.

AUTHORS' DECLARATION OF INTERESTS

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

The authors acknowledge the technical assistance of Dr. Pierre Picard of Phytronix and Mr. Marco Horstmann of Laboratory Automation Services Middle East (Dubai, UAE).

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