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My Robot - can complete automation of sample preparation be achieved?

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Abstract

A major part of the time taken to prepare urine samples for sports drug testing analysis involves repetitive processes such as volumetric delivery of reagents and transfer of samples to a variety of different vial types. At present this is mostly done manually using a variety of handheld dispensing devices. Laboratory robots are now available which can be programmed to perform many of the tasks needed such as reagent addition, sample transfer, incubation and solid phase extraction (SPE). A study has been carried out to determine if a Tecan Freedom Evo robotic system can carry out all the sample preparation steps required to prepare urine samples for instrumental analysis. After some modifications to the existing semi-automated method it is possible to carry out all the steps required, apart from drying and the final derivatisation, using the robotic system. The system repeatability is of the order of 5% for a range of analytes and the results obtained including the steroid profile are similar to those found using our existing protocol. The robot takes two hours to carry out all the steps need to prepare 48 samples for instrumental analysis by Immulite, LC/MS and GC/MS including sample transfer, reagent addition, and SPE with an additional two hours required for enzyme incubation. The robotic procedure is faster and more reproducible than our existing method.

Introduction

There are an ever increasing number of WADA prohibited substances which must be detected in urine samples. Cost pressures mean that more staff are not available to cope with this increased workload. The reporting of quantitative steroid data for use in athlete profiling means that improved precision and reproducibility is required for these measurements. Despite recent advances in instrumental techniques it is still not possible to detect all the compounds required at the desired levels by direct injection of urine. Sample cleanup and concentration steps using liquid-liquid extraction or SPE are required along with sample pretreatment using enzymes. The urine must be transferred to a variety of vials along with the addition of reagents including internal standards. All these steps must be performed quantitatively and reproducibly. In most laboratories these steps are carried out manually using a variety of pipetting devices. The work is boring and repetitive and hence liable to human error. Such repetitive liquid handling tasks can be performed faster and more precisely by modern laboratory automation systems. The use of automation for detection of doping related substances has been reported (1) however a number of manual steps were still required for reagent addition. A second hand Tecan Freedom Evo was purchased to determine whether it was possible to automate most of the sample pretreatment and hence reduce cost whilst improving efficiency.

Experimental

The robotic system used was a Tecan Freedom Evo 200 with an 8 channel liquid handling arm and a robotic manipulator arm. The system was fitted with 5 mL syringes which meant the maximum volume that could be dispensed in a single step was 3.2 mL. It was also fitted with a Te-Vacs vacuum separation module for carrying out SPE using 96 well plates. The SPE plates were Agilent Bond Elut 96 square well Nexus 60 mg. A Torrey Pines Echotherm cooling heating dry bath was used for incubation. The deck of the Tecan was equipped with a variety of sample holders for 96 and 48 deep well plates, 12 x 32 mm glass vials, 11 x 25 mm plastic vials, Immulite cups, 12 x 75 mm glass tubes, and 23 x 90 mm plastic tubes. The configuration of the deck is shown in Figure 1.

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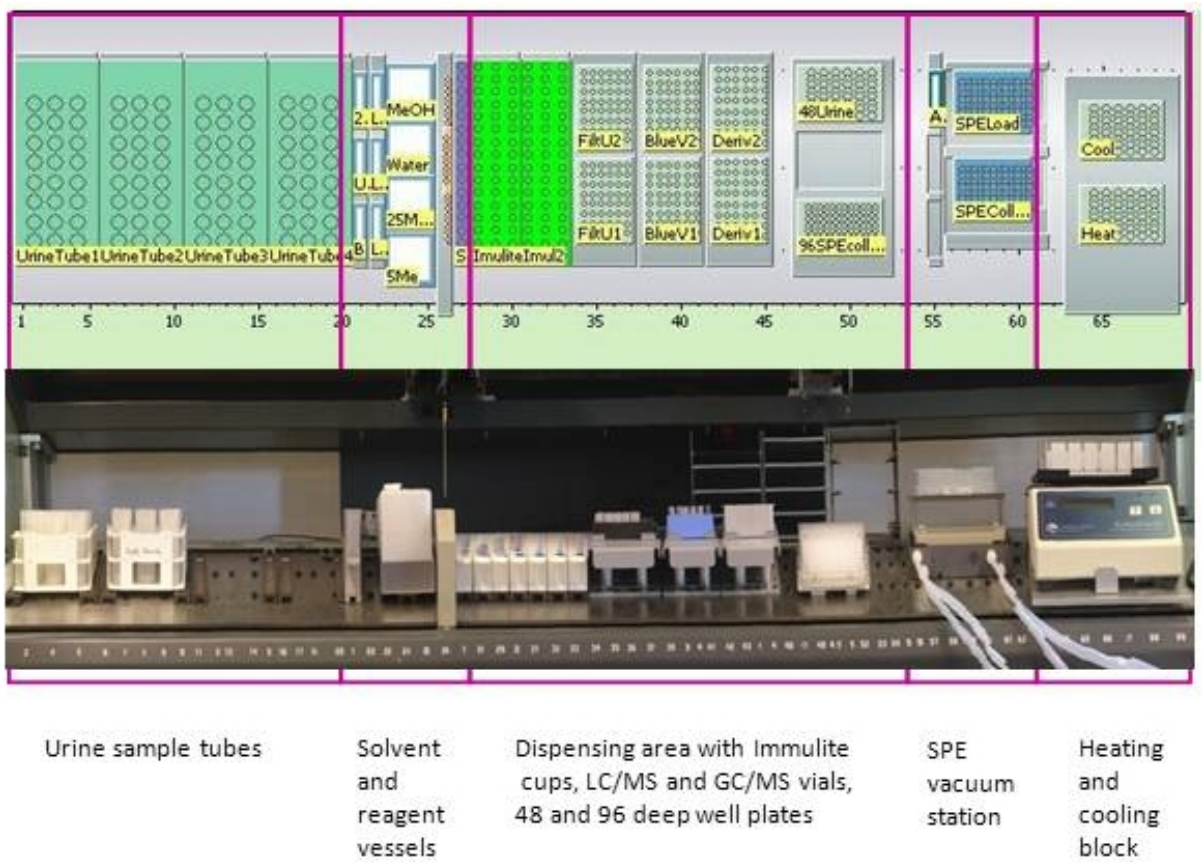

Figure 1: Layout of sample holders and devices on the Tecan deck.

The system has a maximum capacity of 96 urine samples but is currently only programmed to process batches of 48 samples. The program used was Freedom Evoware.

The urine samples (approximately 10 mL) were poured from the A bottles into 23 x 90 mm plastic tubes and placed in the appropriate sample holders on the left hand side of the deck. The robot was required to carry out all the liquid transfer operations involved in the preparation of urine samples for analysis by Immulite, LC/MS and GC/MS including enzymatic hydrolysis and SPE extraction. Two LC/MS samples were required, one for direct analysis and one for analysis after enzymatic hydrolysis and SPE extraction. The urine remaining in the 23 x 90 mm plastic tubes after the Tecan had finished dispensing was used for manual measurement of pH and SG. Sample drying and the addition of MSTFA based derivitisation agents were not possible as the Tecan was not in a fume hood. The enzymatic hydrolysis was carried out in a 48 deep well (DW) plate with a maximum capacity of 4 mL per well (Axygen Scientific, California USA P-4ML-SQ-C). The collection plate used for the SPE was a 96 DW plate with a maximum capacity of 2 mL per well (Axygen Scientific, California USA P-2ML-SQ-C).

A summary of the operations carried out by the Tecan are set out in Table 1.

All reagents were of analytical reagent grade or better. The water was from a Millipore Milli-Q Direct 16. The methanol was LiChrosolv from Merck (Darmstadt). The phosphate buffer was 0.85 M Na_2HPO_4/KH_2PO_4. The enzyme was ß-glucuronidase E. Coli K12 13707601001 from Roche (Mannheim). Reference standards of prohibited substances used in the preparation of the mixed excretion studies Kmix and Lmix were obtained from Sigma-Aldrich (Australia) and from the National Measurement Institute (Australia).

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Robot operation (needles used)	Volume uL	Source	Destination
Liquid transfer (1)	100	Surrogate IS in 16 x 100 mm glass tube	48 DW plate
Liquid transfer (8)	400	Phosphate buffer/enzyme in trough	48 DW plate
Liquid transfer (8)	200	LC/MS buffer/IS in trough	11 x 24 mm plastic tubes
Liquid transfer (8)	3200	Urine samples in 23 x 90 mm tubes	48 DW plate
Liquid transfer (8)	200	Urine samples in 23 x 90 mm tubes	11 x 24 mm plastic tubes
Liquid transfer (8)	300	Urine samples in 23 x 90 mm tubes	Immulite cups
Mix (8)			48 DW plate
Move 48 DW plate		Tecan deck	Heating block
Wait 110 minutes			
Move 48 DW plate		Heating block	Cooling block
Wait 5 minutes			
Liquid transfer (8)	1000	Methanol reservoir	SPE plate wash position
Liquid transfer (8)	1000	Water reservoir	SPE plate wash position
Move 48 DW plate		Cooling block	Tecan deck
Liquid transfer (8)	1800	Digested urine in 48 DW plate	SPE plate wash position
Liquid transfer (8)	1800	Digested urine in 48 DW plate	SPE plate wash position
Liquid transfer (8)	1800	Water reservoir	SPE plate wash position
Liquid transfer (8)	1500	25% methanol/water reservoir	SPE plate wash position
Vacuum 6 minutes			SPE plate wash position
Move 48 DW plate		SPE plate wash position	SPE plate collect position
Liquid transfer (8)	1000	Methanol reservoir	SPE plate collect position
Liquid transfer (8)	1000	Methanol reservoir	SPE plate collect position
Vacuum 6 minutes			SPE plate collect position
Move 96 DW plate		SPE plate collect position	Tecan deck
Mix (8)			96 DW collection plate
Liquid transfer (8)	250	96 DW collection plate	12 x 32 mm glass vials
Liquid transfer (8)	1750	96 DW collection plate	12 x 75 mm glass tubes
Liquid transfer (1)	100	MeT IS in 16 x 100 mm glass tube	12 x 75 mm glass tubes

Table 1: Sequence of operations carried out by the Tecan robot.

Results and Discussion

The first stage of determining whether the Tecan was capable of meeting our needs was to determine the system repeatability. Six aliquots from the same urine sample were prepared for our standard GC/MS screening protocol using the Tecan and injected once. One of these prepared samples was then injected seven times to determine the reproducibility of the final instrumental analysis. The results obtained are set out in Table 2. It can be seen that the robotic extraction process was highly repeatable as on average it only added less than 1% additional CV for the nine steroid measurands.

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Measureand	Mean Value (ng/mL)	Repeat Injection CV (n=7)	Repeat SPE CV (n=6)
Testosterone	29.8	3.6	6.1
Androsterone	1454	3.1	3.3
Etiocholanolone	1415	1.9	4.9
11OH-Androsterone	897	3.4	6.2
5a3a-Androstandiol	35.0	6.2	5.3
T/E by height	1.10	4.2	4.8
Andro/Etio	1.03	2.9	3.0
5a3a-Adiol/5b3a-Adiol	0.33	8.0	5.2
D5Andro/D5Etio	1.07	2.4	5.3

Table 2: System repeatability using six separate extracts from the same urine sample.

In order to determine whether the robotic system produced results that were essentially the same as those produced by our existing routine protocol seven urine samples which had previously been analysed routinely were each re-extracted six times using the Tecan. The samples (3 female and 4 male) had SGs ranging from 1.007 to 1.028. The reproducibility of the results was again comparable to that found when performing repeat injections of the same sample extract and the quantitative steroid values were similar to those found previously using the routine screen. The comparability of the steroid profiling data can be seen by comparing the printout obtained from the routine screen (Figure 2) with that from the same urine using the Tecan protocol (Figure 3).

Another aspect of concern in evaluating a new method is its ability to recover the analytes of interest. This was evaluated using two mixed excretion urines named Kmix and Lmix which are extracted and analysed in each batch of our routine steroid screening method. Kmix contains 19 compounds which are mostly steroid metabolites and Lmix contains 45 compounds. Samples of these urines were analysed using the Tecan protocol and the results compared. As the routine screen uses another solid phase (3M Empore) compared to the Tecan (Nexus) differences were expected. The relative recoveries for a subset of the compounds present in Kmix and Lmix are set out in Table 3. The mean relative recovery over all analytes was higher for the Tecan protocol than for the routine screen although there were some analytes that had lower recoveries. However all limit of detection requirements were met by both procedures.

One potential problem with a robotic system using fixed needles is the possibility of sample carryover from one sample to another due to incomplete needle washing. This was tested using a pregnancy urine having a high level of hCG and a blank urine. The pregnancy urine was dispensed with one needle and then the same needle was used for the blank urine after the standard wash cycle. There was no evidence of an increased hCG concentration in the sample from the blank urine.

Apart from the benefits of improved reproducibility and reliability a major consideration in choosing to use a robotic method is the potential for time and cost savings. The robot takes approximately two hours to carry out all the dispensing steps and SPE procedures for 48 samples with an additional two hours needed for sample incubation. The Tecan deck has space for 96 urine samples and the time taken to process all 96 samples in two batches of 48 would be about five hours. The time taken by an operator to set up the samples, tubes and reagents is less than one hour. A second batch of 96 urine samples could be loaded onto the deck after an hour of the first batch starting and all 192 samples would be complete in a little over seven hours. Once the Tecan has begun to operate it has the capability to process samples at the rate of approximately 25 per hour virtually indefinitely. The potential for human error is much reduced with the robotic system and analysts can now be used for more productive tasks than carrying out multiple manual dispensing of samples, reagents and solvents. The second hand Tecan Freedom Evo 200 cost under $100000 including installation.

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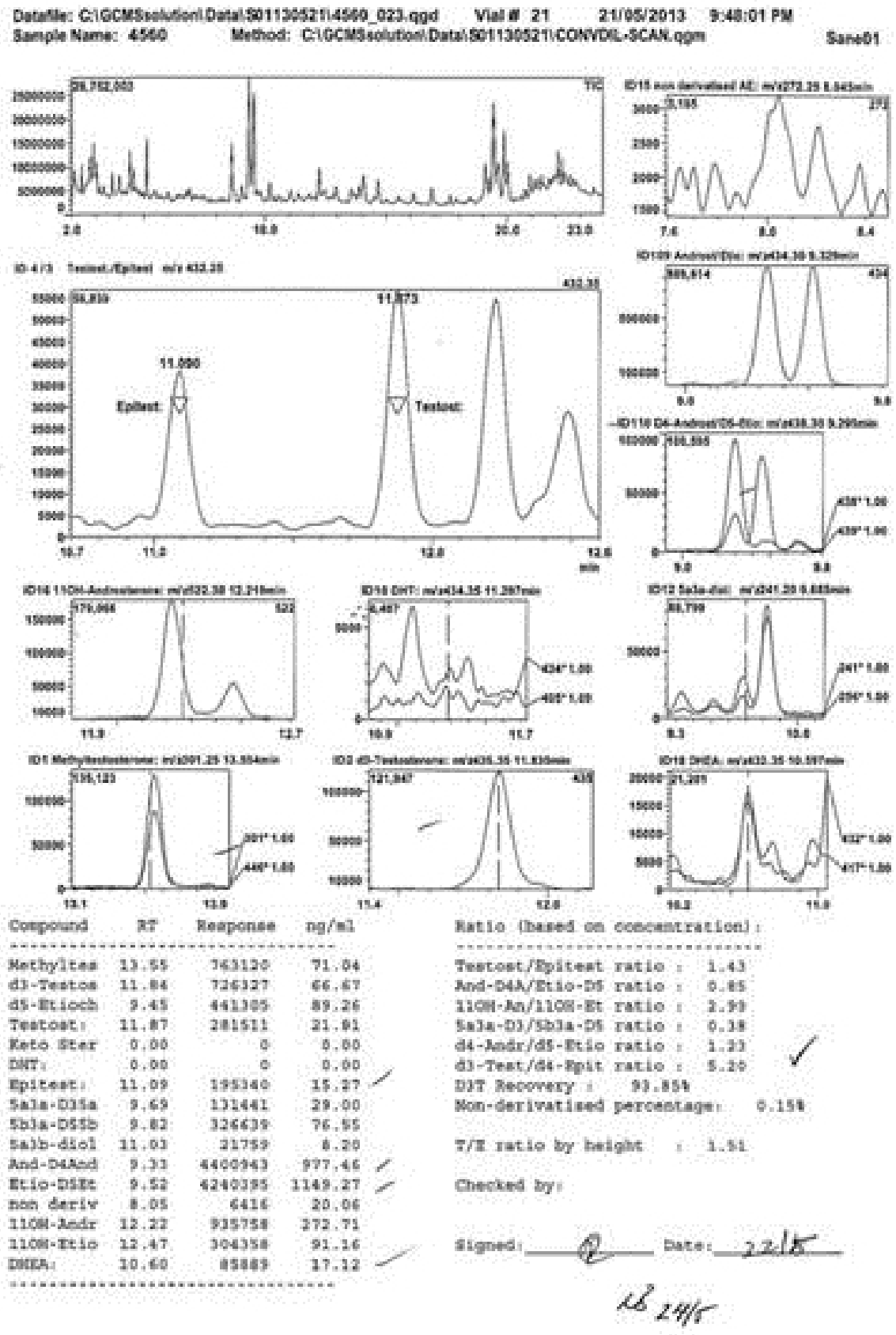

Figure 2: Steroid profiling data from a sample using our routine screening protocol.

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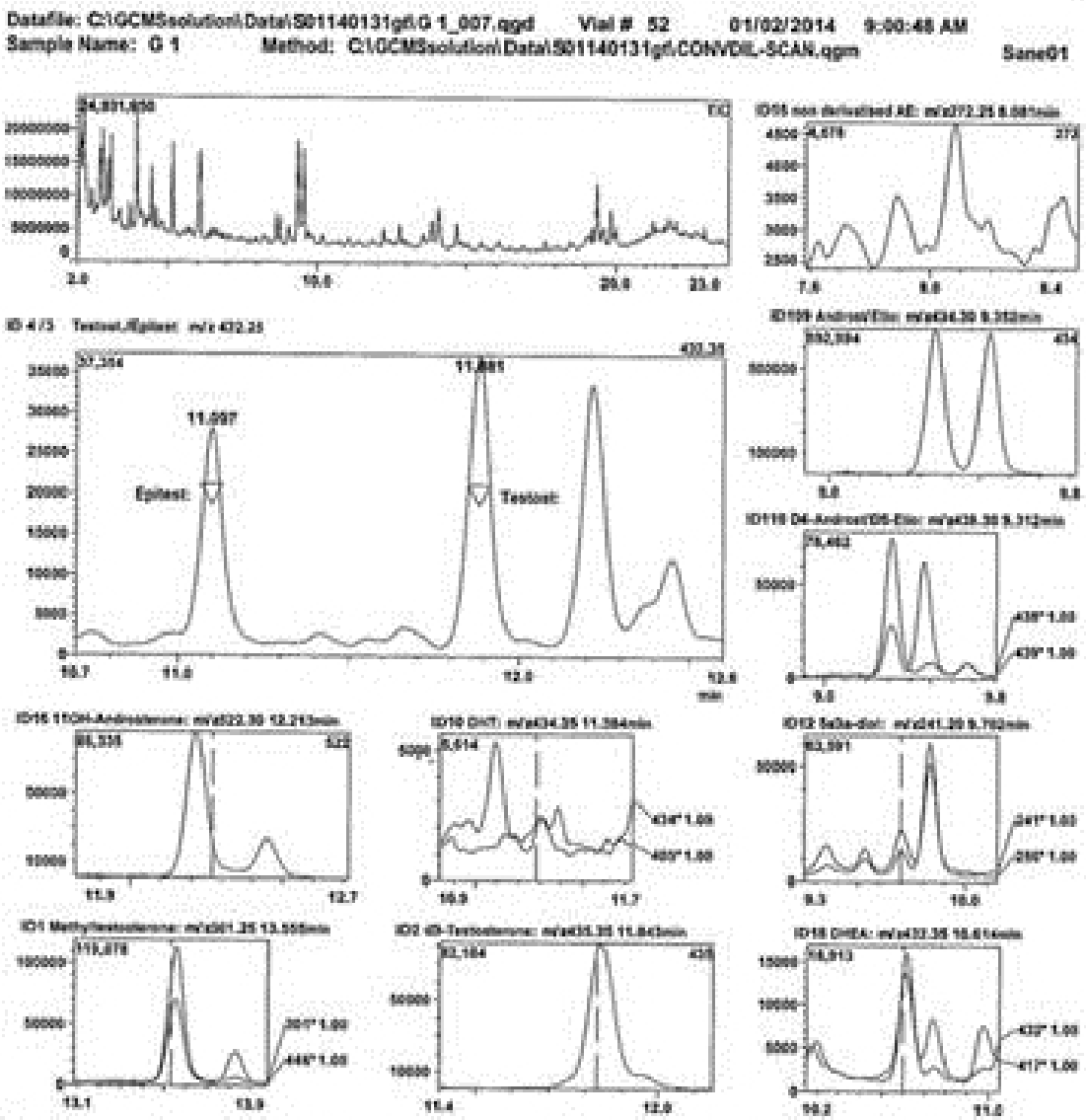

Compound	RT	Response	ng/ml
Methyltes	13.56	575695	102.02
d3-Testos	11.84	433975	66.67
d5-Etioch	9.46	283957	109.90
Testost:	11.88	140840	20.33
Keto Ster	0.00	0	0.00
DHT:	0.00	0	0.00
Epitest:	11.10	118413	13.60
5a3a-D3Sa	9.70	89523	30.53
5b3a-D5Sb	9.84	203644	74.18
5a3b-diol	11.06	10881	5.89
And-D4And	9.35	2930149	961.84
Etio-D5Et	9.53	2852650	1110.70
non deriv	8.08	5302	58.21
11OH-Andr	12.21	423081	199.26
11OH-Etio	12.45	119737	57.01
DHEA:	10.61	58762	19.78

Ratio (based on concentration):

Testost/Epitest ratio :	1.50
And-D4A/Etio-D5 ratio :	0.87
11OH-An/11OH-Et ratio :	3.49
5a3a-D3/5b3a-D5 ratio :	0.41
d4-Andr/d5-Etio ratio :	1.27
d3-Test/d4-Epit ratio :	5.08

D3T Recovery : 45.35%
Non-derivatised percentage: 0.18%

T/E ratio by height : 1.35

Checked by:

Signed:_____ Date:_____

Figure 3: Steroid profiling data from the same urine in Figure 2 using the new Tecan based method.

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Analyte	Relative recovery %	Analyte	Relative recovery %
19 nortestosterone metab	76	Oxymetholone metab	48
Drostanolone	70	Tamoxifen metab	111
Metenolone	126	Probenecid	490
Boldenone PC	110	Terbutaline	280
Boldenone metab	235	Salbutamol	360
Clostebol metab	91	Monoacetylmorphine	120
Mesterolone	52	Codeine	108
Methyltestosterone metab	54	Amiloride	189
Bolasterone PC	106	Triamterene	91
Furazabol metab	81	Clenbuterol	80
3OH Stanozolol	80	Tibolone metab	99
Mibolerone PC	363	Bromantane metab	64
Oxabolone metab	390	Benzoylecgonine	247
Oxandrolone	96	Pemoline	918
Fluoxtmesterone metab 1	182	Canrenone	156
Fluoxtmesterone metab 2	68	Butorphanol	88

Table 3: Recoveries of a range of analytes in Kmix and Lmix comparing the new Tecan method to the existing screening protocol.

Conclusions

It has been demonstrated that it is possible to use a robotic system to carry out all the sample dispensing and reagent addition required for the routine preparation of athlete urine samples for instrumental analysis. The Tecan system used is also capable of carrying out SPE and giving highly reproducible results. Despite the use of a different phase material in the SPE the GC/MS results used for steroid profiling were very similar to those obtained with the existing routine screening protocol. The analyte recoveries were in general better with the Tecan method than with the existing protocol. The system is capable of processing 192 urine samples in an eight hour day with less than two hours of operator time.

References

1. Cuervo, D., Diaz-Rodriguez, P. and Munoz-Guerra, J. (2014), An automated sample preparation for detection of 72 doping-related substances. *Drug Test Analysis*, 6, 516-527.