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Progress in the Simplification of Drug Screening

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Introduction

We like other WADA laboratories face an ongoing problem which relates to the need to continually expand the suite of compounds which we are able to detect. Each year more compounds are added to the WADA Prohibited List (WADA 2006) many of which are not amenable to our conventional GC/MS screening methods. In addition our clients expect us to detect all compounds on the List at no or minimal extra cost. Last year we reported some possible solutions to these problems (Goebel et al 2005) and this year we have instigated some major changes to our routine screening methods. The change was driven by the need to carry out the drug testing for the Melbourne Commonwealth Games which required the analysis of over 1000 samples in ten days with fast turnaround times. The maximum sample load was to be 120 samples per day. Thus we carried out an extensive reevaluation of our screening methods with a view to achieving maximum output with minimal extra human and physical resources.

In 2005 the major screens used were :-

Anabolic steroids - SPE extraction with C18 and analysis by GC/MSD and GC/HRMS.

Stimulants – L/L extraction and analysis by GC-NPD and GC/MSD.

Narcotics – Extractive alkylation and analysis by GC/MSD.

Diuretics – SPE extraction with Nexus and analysis by LC/MS/MS.

Corticosteroids – diuretics extract re-analysed by LC/MS/MS.

The instrument available within ASDTL to carry out this work were - 1 Finnigan MAT95S GC/HRMS, 5 Agilent 6890/5973 GC/MSD, 1 Agilent 6890 GC-NPD, 1 Waters Quattro Micro LC/MS/MS, 1 ABI 4000QTrap LC/MS/MS, 1 Thermo Delta Plus GC/CIRMS, 1 HP 1090 LC, 3 Gilson ASPEC XL4. Some additional instruments were available for the period of the Games from other sections of NMI. These were - 1 Finnigan MAT95XL GC/HRMS (only available part

time), 2 Agilent 6890/5973 GC/MSD, 1 Waters Quattro Micro LC/MS/MS (part time only), and 1 Gilson ASPEC XL4.

It was apparent that this instrumentation was not adequate to meet fast turnaround times for samples arriving at the rate of more than 100 samples per day over consecutive days. To meet turnaround times it was necessary to complete the extractions within 8 hours of sample arrival and have the instrumental analysis complete in another 10 to 12 hours. For steroids this required a minimum of 2 Gilson ASPEC XL4s, 4 GC/MSDs and 2 GC/HRMSs. In addition one GC/MSD needed to be reserved for T/E confirmations as with a cut off of 4 we anticipated at least two T/E confirmations per day. For diuretics we also needed 2 Gilson ASPECs as well as 2 LC/MS/MS for analysis. The corticosteroids needed another 2 LC/MS/MS. The stimulant screen needed 1 GC-NPD with dual injectors and detectors and 1 GC/MSD. Narcotics needed a Gilson ASPEC XL4 for sample preparation and 2 GC/MSDs for analysis. The requirements were greater than our resources. For example we needed 5 Gilson ASPECs but only 3 of the 4 we had could be relied on as the fourth ASPEC was at the end of its useful life having performed over 1 million cycles.

The proposals for the Games analyses were -

- Maintain the normal steroid analysis procedure
- Maintain the normal stimulants procedure
- Remove the GC/MSD narcotics screen and incorporate some of these analytes into the steroid screen and others into the corticosteroid screen.
- Develop a fast (less than 10 minute cycle time) LC/MS/MS method for analysing both corticosteroids and narcotics.
- Combine two urine samples prior to extraction on the Nexus cartridge for diuretic, corticosteroid, and narcotic analysis.

If all this could be achieved then the resources available were adequate for the Games testing.

Experimental

The diuretics method (Goebel et al 2004) uses Varian Nexus SPE cartridges for extraction and a Waters 2795/Quattro Micro. The method was revalidated to confirm that is capable of meeting the WADA MRPL of 250 ng/mL (WADA) when two samples are combined. A series of 20 2.5 mL urine samples were combined so that the total volume of urine to be extracted by the Nexus

cartridge was 5 mL. The urines were chosen to represent a range of specific gravities and combinations thereof. The ten combined urines were spiked at 125 ng/mL equivalent to 250 ng/mL in one sample.

The existing corticosteroids method (Goebel et al 2005) needed to be modified so that it had adequate sensitivity for combined samples, included the required narcotics and had a cycle time of less than 10 minutes. The existing corticosteroid analysis used a Waters 2795/Quattro Micro whilst it was proposed to use the faster scanning and more sensitive Agilent 1100 LC/ABI 4000 QTrap for the new method. A change in the extraction procedure was needed to incorporate the narcotics. Two 2.5 mL samples of urine were combined, and to this was added 0.5 mL of 1M phosphate buffer pH 7, 100 µL of mefruside/MeT internal standard (10/0.8 ug/mL) and 40 µL b-Glucuronidase E coli. The tubes were vortexed and incubated at 50°C for 30 mins and the analytes extracted by passage of the sample through a Varian ABS ELUT Nexus SPE column (60 mg, 3 mL), followed by a 1 mL water wash, a 1 mL wash with 25% methanol in water, and eluted with 2 mL 0.5% glycerol in methanol. Methanolic extracts were evaporated to dryness under nitrogen and reconstituted in 200 µL of 25% methanol in water. The glycerol was added to the methanol in the elution step as a keeper for the narcotics during the evaporation step. The evaporated samples were reconstituted in 25% MeOH in H_2O as we had observed some peak splitting in the LC chromatograms of the narcotics when higher methanol levels were used.

The LC separation was carried out using a C18 column (Alltech Prevail, 50 mm × 2.1 mm × 3 um) protected by a C18 guard column. The flow rate was 300 uL/min and 1 uL of sample was injected. The eluting solvents were water with 0.1% formic acid (A) and acetonitrile/water 90/10 with 0.1% formic acid (B). The gradient was 0 mins 100% A to 0.5 mins 67% A, 0.5 to 2.0 mins 67% A, 2.0 mins 67% A to 3.0 mins 0% A, 3.0 to 4.0 mins 0% A, 4.0 mins 0% to 4.5 mins 100% A & 4.5 to 6.5 mins 100% A.

The spray conditions of the QTrap 4000 with Turbo V Spray interface were: temperature 450°C, gas one flow 55 L/hr, and gas two flow 65 L/hr. The probe was set at 5.5 kV and the substance specific cone voltage and collision energy were optimised for each compound. Multiple reaction monitoring (MRM) was carried out at the optimum settings using positive ion mode. By using dwell times as low as 5 ms it was possible to have a single experimental MRM which contained all the transitions for the compounds monitored.

Results and Discussion

When two samples were combined in the "new" diuretics method all the desired analytes were readily detected. This was to be expected as it was normal to run spikes at 100 ng/mL for the "old" method and detect all analytes easily. The recoveries and standard deviations for a selection of analytes where some differences were observed between the "old" and "new" methods are shown in Table 1. The recoveries are lower for several analytes however the sensitivity is still more than adequate to meet the MRPL.

	Recovery %	S.D. (n=7)	Recovery %	S.D. (n=10)
Acetazolamide	84	14.8	37.9	11.9
Bendroflumethiazide	77.9	34.6	53.6	17.6
Benzthiazide	102.5	18.5	119.0	23.9
Bumetanide	98.1	18.6	62.6	13.6
Canrenone	69.3	20.6	122.6	35.5
Chlorexolone	107.9	19.8	69.3	4.6
Chlorothiazide	122.4	25.5	91.5	22.2
Chlorthalidone	113.1	18.7	86.8	16.8
Clopamide	110.2	19.4	72.8	12.3
Cyclopenthiazide	82.6	21.1	44.7	10.7
Cyclothiazide	84	18.1	38.6	10.8
Dichlorophenamide	116.2	18.6	73.4	11.5
Ethacrynic Acid	94.5	16.5	68.1	14.6
Hydrochlorothiazide	105	16.2	86.8	29.7
Hydrofluthiazide	108.8	24.3	88.5	23.2
Methazolamide	106.7	18.3	68.8	13.4
Pemoline	52.7	8.5	31.7	9.1
Piretanide	98.6	15	40.7	12.5
Torasemide	105.3	19.2	49.2	8.2
Triamterene	97.7	16	134.4	55.7
Trichlormethiazide	81.9	15.8	44.8	19.0
Xipamide	122.1	28.2	85.0	31.4
Trenbolone 199			99.3	21.0
TH Gestrinone 241.1			109.4	32.7
Gestrinone 241			102.6	26.7

Table 1. Recoveries and SDs of some compounds included in the "old" (columns 2 and 3) and "new" LC/MS/MS diuretics screen (columns 4 and 5).

The new corticosteroid/narcotic screen faced several problems with respect to the addition of the narcotics. One was the need to use only positive ion mode because of the slow polarity switching of the ABI 4000 compared to the Waters Quattro Micro. Also it was not known if the Nexus

cartridge would extract all of the desired analytes and problems had been encountered previously with some of the more volatile analytes such as etilefrin being lost on evaporation after extraction. These problems were seen with the new method but were overcome by the addition of a small amount of glycerol to act as a keeper. A comparison of the recoveries and standard deviations of the original corticosteroid method and the new corticosteroid/narcotic method for combined urines is shown in Table 2. The recoveries are comparable and above 70% for all the corticosteroids. However for two of the compounds added to the screen namely ritalinic acid and benzoylecgonine the recoveries were very poor indicating that they were not being extracted effectively. Despite this they were always readily detected at their MRPL as shown in Figure 1 as both had a very high MS/MS response. In addition these compounds are also detected in other screens and their inclusion in the corticosteroid/narcotic screen was merely a back up.
Figure 2 summarises the high volume screening methods currently in use at ASDTL. Separate methods are used for special analytes such as HES, EPO and HBOCs. Samples from male athletes are tested for hCG using a DPC Immulite. The changes mean that a full screen sample now only requires three extractions of which only one requires chemical derivatisation prior to analysis.

	Recovery %	S.D. (n=10)	Recovery %	S.D. (n=10)
Beclomethasone	100.5	17.3	70.6	25.5
Beta/Dexamethasone	94.0	17.5	71.7	25.3
Budesonide	81.8	14.0	83.1	18.5
Desonide	88.0	22.8	80.3	19.5
Flumethasone	92.9	27.6	75.1	22.3
Flunisolide	73.6	7.2	84.2	25.7
Fluocortolone	82.0	19.0	91.7	22.0
Methylprednisolone	88.8	20.1	80.2	18.0
Prednisone	89.4	24.1	92.5	26.3
Prednisolone	60.9	13.7	101.3	24.6
16a OH Prednisolone			89.5	28.1
Bamethan			63.7	9.2
Ritalinic acid			1.12	0.74
Pholedrine			30.4	2.9
Ethamivan			82.2	16.2
Etilefrin			62.4	2.9
Buprenorphine			99.9	13.8
Dextromoramide			90.8	12.0
Norbuprenorphine			86.0	7.9
Pentazocine			83.8	5.7
Oxycodon			56.0	7.2
Oxymorphone			45.8	2.0
Benzoylecgonine			2.37	0.69

Table 2. Recoveries and SDs of compounds in the original corticosteroid screen (columns 2 and 3) compared to those in the combined corticosteroid/narcotic screen (columns 4 and 5).

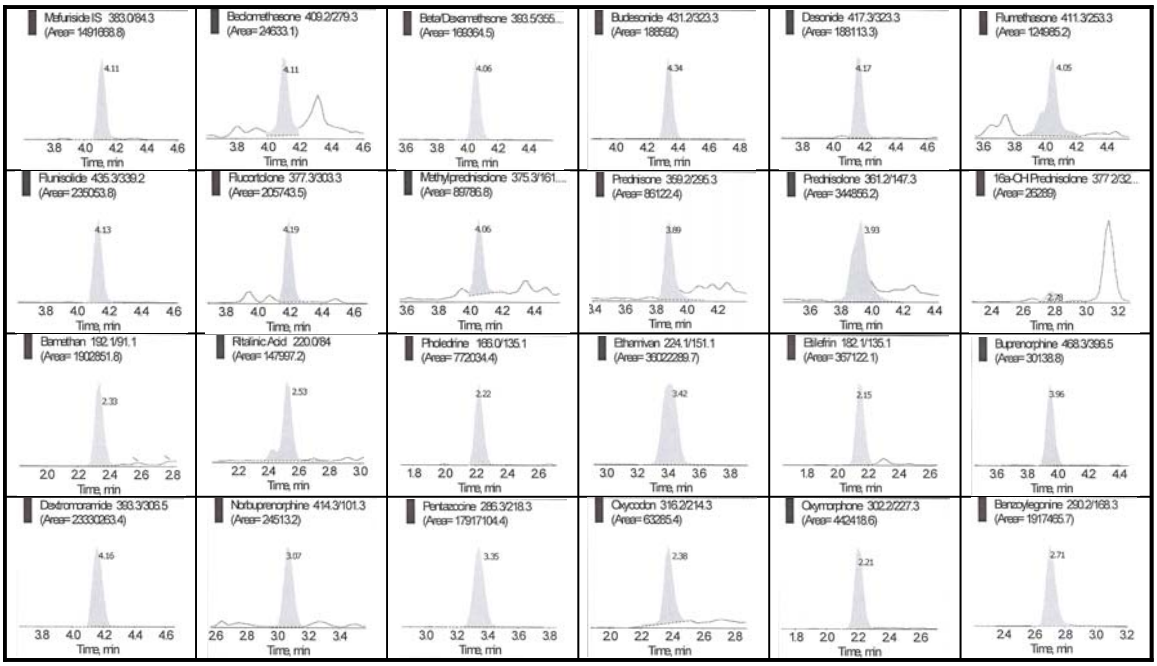

Figure 1. Results from urine spiked at the MRPL for each analyte.

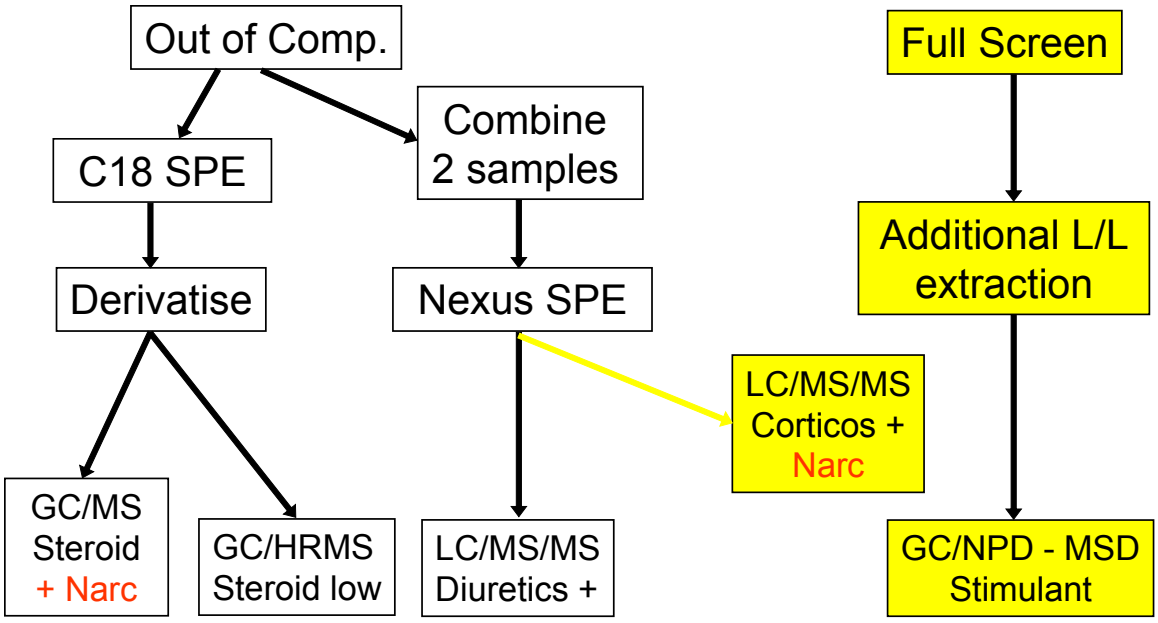

Figure 2. Schematic of 2006 high volume chromatographic screening methods.

Conclusions

The demand to test for more compounds can be met without increasing staff and by changing the way we screen for new and existing compounds. In this way the ongoing cost per sample based on staff time and consumables can be maintained or even reduced. However to achieve this the laboratory needs large capital injections to invest in new technology such as high sensitivity LC/MS/MS..

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