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Detection of low level anabolic agents by LC/MS/MS with investigation of matrix effects

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Introduction

The detection of the five low level anabolic agents (Figure 1) by LC/MS/MS will require the investigation of matrix effects. Matrix effects are the Achilles heel of electrospray ionisation (ESI) quantification (Taylor, 2005). Ionisation suppression or enhancement can markedly affect quantitative results in ESI. In extreme cases the suppression can be sufficient to prevent detection of an analyte. If ESI is to be used to detect low level analytes then a means of ensuring that the analytes will be detected at required levels in all samples needs to be proven.

There are several proposed methods which can be used for the calculation of recoveries. Two will be examined, the addition of internal deuterated standards for each analyte added prior to extraction or post column infusion of internal standards. Three of the low level anabolic agents were already available commercially in deuterated form but 17ß-methyl-5ß-androst-1-ene-3a,17a-diol (EMD) and 17a-methyl-5ß-androstane-3a,17ß-diol (MethylT metabolite) needed to be synthesised by the reference standards group of NMI (Australia).

The post column infusion of a standard has been used by several investigators to examine matrix affects but it has also been suggested as a complete replacement for a spiked internal standard (Cheng and Tsai, 2008). The selected internal standard is added post column as a continual infusion into the HPLC column eluent. This can be used to correct for matrix effects by using either a single standard of similar structure to the group of analytes of interest, in this case progesterone was used, or a mixture of deuterated internal standards associated with the compounds of interest (deuterated mixture of 5 anabolic agents for this study).

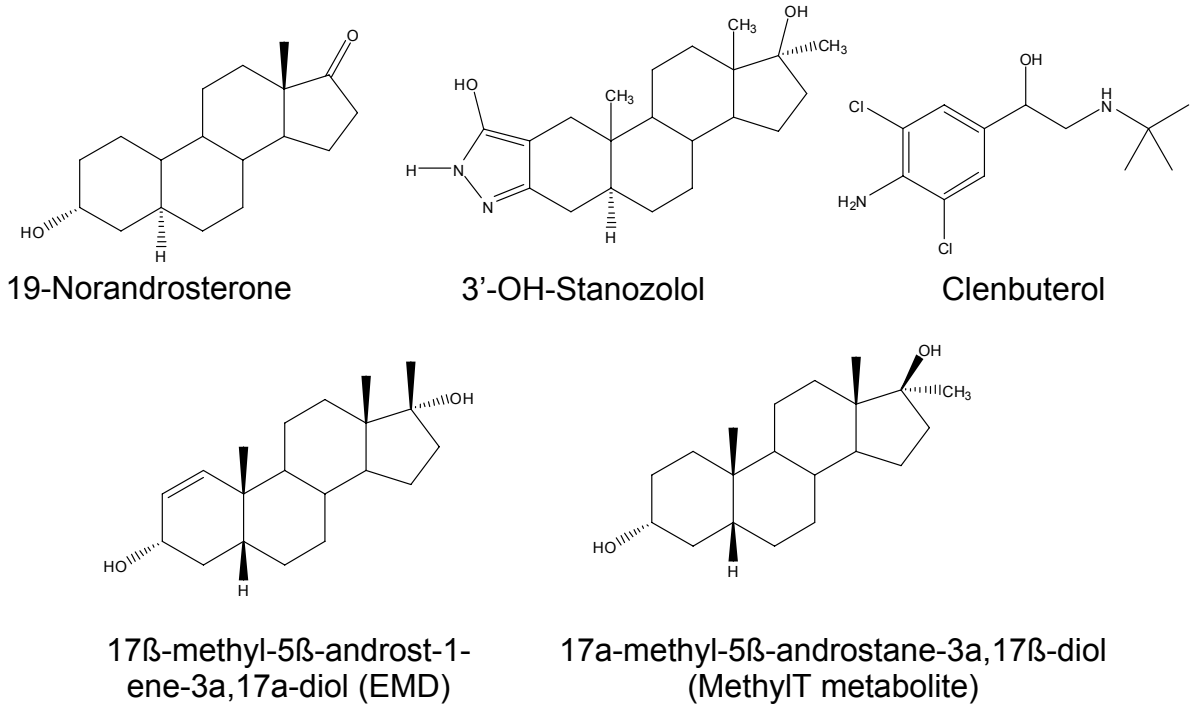

Figure 1 Structures for the five low level anabolic agents, 19-norandrosterone, 3'-OH-stanozolol, clenbuterol, EMD and methylT metabolite.

Materials and Methods

The anabolic agents and deuterated standards were sourced from the National Measurement Institute (Sydney, NSW, Australia). All reagents were AR or HPLC grade and water was purified with the laboratory's MilliQ system.

Sample Preparation

The extraction protocol for the NEXUS (Varian Inc.) cartridge as published Goebel *et al.* (2004) was followed with variation for the other SPE cartridges as described. The WCX (Waters Corp.) cartridge had samples loaded onto a conditioned SPE column (Condition: 3 mL methanol, 3 mL water) and then washed with 2 mL of 5% methanol in water. The samples were eluted with two aliquots of 1 mL methanol and two aliquots of 1 mL 2% formic acid in methanol. Chromabond HR-X (Macherey-Nagel GmbH & Co. KG) was conditioned with 5 mL of methanol and 5 mL of water. The prepared samples were loaded and then washed with 5% methanol. Samples are eluted with three aliquots of 2 mL methanol. The CUBCx3 (UCT Inc., C18 + Benzene sulfonic acid, UCT) had the sample acidified with 0.1 M HCl pH 2 to a range of pH 6 ± 0.5 (adjusted with 0.1 M monobasic or dibasic sodium

phosphate). The cartridges were conditioned with 3 mL methanol, 3 mL water and 1 mL 0.1 M phosphate buffer pH 6. The samples are loaded and then washed with 3 mL water and 1 mL 0.1 M acetic acid. The columns are dried and washed 2 mL with hexane. The columns are dried again and then the sample is eluted with 3 mL 98% methanol 2% ammonia.

Results and Discussion

Several LC/MS/MS instruments were evaluated to identify the most suitable for the anlysis of the five low level steroids, each was tested with a mixed solution. A solution of 0.5 ug/mL in acetonitrile:0.1% formic (1:1) of the five anabolic agents was given to an analyst familiar with each instrument. The instruments tested were ABI 4000 Q-Trap with Turbo V source, Thermo TSQ Vantage AM with HESI II source, Thermo Orbitrap with Ion Max source, Thermo Orbitrap with Michrom source, Waters Qmicro with Z-spray source, Waters Premier TQ with Z-spray source and Agilent 6510 QTOF with Nano flow source.

The signal to noise was calculated in comparison to a blank solution. In general the majority of the instruments have poor results for the two diol compounds, EMD and MethylT metabolite (Table 1). The ABI Q-Trap gave the best results across the board though there were some stand out results such as the 3'-OH-stanozolol on the TSQ Vantage with a signal to noise ratio of 835. Interestingly the 19-norandrosterone which is generally well ionised by ESI gave a very poor response on the Agilent 6510 QTOF.

Table 1 Signal to noise for five anabolic agents on six different instruments at 0.5ug/mL.

	19-Norandrosterone	3OH Stanozolol	Clenbuterol	EMD	MethylT metab.
ABI 4000 Q-Trap with Turbo V source 10 uL/min	52	111	214	41	37
Thermo TSQ Vantage AM HESI II source 10 uL/min	105	835	221	24	17
Thermo Orbitrap 10 uL/min	50	22	167	3	8
Thermo Orbitrap 4 uL/min	50	35	90	5	6
Thermo Orbitrap Michrom source 4 uL/min	11	30	310	17	4
Thermo Orbitrap Michrom source 0.5 uL/min	24	48	335	14	4
Waters Qmicro 10 uL/min	90	213	210	16	23
Waters TQ Detector 10 uL/min	108	263	49	Not Observed	Not Observed
Agilent 6510 QTOF	4.2	51	100	4	Not Observed

Matrix effects for anabolic agents

Matrix affects on each of the five compounds were examined for four extraction cartridges; NEXUS, WCX, Chromabond HRX and UCT. The identification of any possible contaminants that may affect the ionisation of a compound for these four cartridges needs to be examined as this could identify a possible problem for the SPE protocols. These analyses were completed on an ABI Q-Trap 4000 with an Agilent 1100 CapPump and Micro-WPS injector. An acquity BEH C18 1 × 100 mm column running at 50 µL/min with solvent A: 0.2% formic acid and B: 90% ACN, 10% H_2O with 0.2% formic using a gradient separation. Post column a mixed standard was infusion and an extracted urine sample was injected from each of the four different SPE cartridges. The infused standard consisted of 200 ng/mL at 1 µL/min into 50 µL/min from HPLC pump which gives an actual concentration of standards at the instrument of 4 ng/mL.

The results for the comparison of matrix affect for three of the low level compounds are represented in Figures 2 to 4. The solid line represents a blank methanol injection where no suppression or enhancement of ionisation should be observed. This solid line is then compared to the results for each cartridge. The 19-norandrosterone has no suppression for the NEXUS or UCT SPE cartridges at the expected retention time (dotted line, Figure 2), while there is some suppression observed for WCX and HRX.

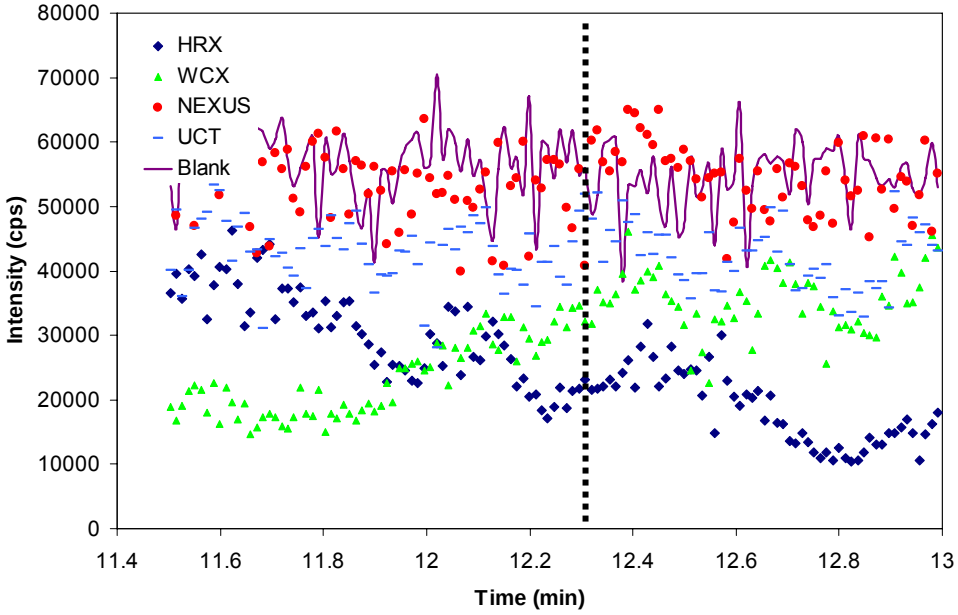

Figure 2 Comparison of ionisability for urine extracts from four SPE cartridges against a blank injection for 19-norandrosterone.

Clenbuterol has some enhancements in ionisation for the NEXUS, WCX and HRX extracts while the UCT extract is very similar to the blank (solid line, Figure 3). There is little difference observed in the ionisation for the urine extract from the four SPE cartridges for EMD as all of the results have about the same intensity as the blank injection (Figure 4).

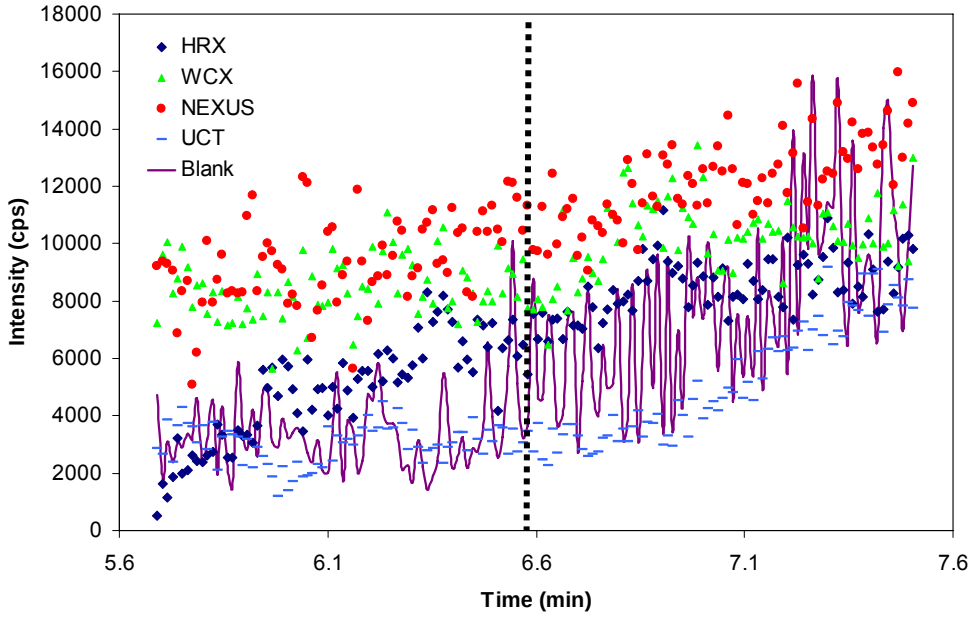

Figure 3 Comparison of ionisability for urine extracts from four SPE cartridges against a blank injection for Clenbuterol.

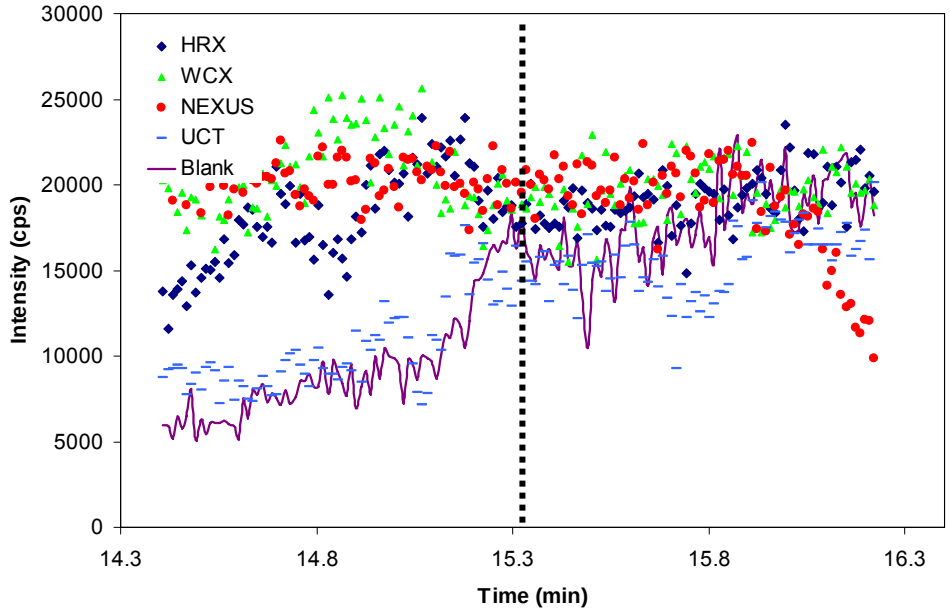

Figure 4 Comparison of ionisability for urine extracts from four SPE cartridges against a blank injection for EMD.

The methylT metabolite infusion was at a concentration too low to be able to determine any affects from the matrix. The NEXUS SPE cartridge shows substantial suppression for the 3'-OH-stanozolol, while the other three cartridges show only a small amount of suppression for the 3'-OH-stanozolol.

The matrix effects for SPE cartridges need to be taken into consideration the recoveries and standard deviations for all the compounds extracted. EMD and MethylT metabolite ionise the poorest (Table 1) and WCX, NEXUS and HRX all showed similar matrix affects (Table 2). It had previously been observed that the 3'-OH-stanozolol with NEXUS extract gave very poor GC/MS chromatograms and had significant suppression due to matrix affects (Table 2). The HRX cartridge had given high standard deviations of recoveries for the anabolic agents and substantial suppression for 19-norandrosterone. The results so far indicate that the WCX SPE cartridge gave the best recoveries and lowest standard deviations with overall least matrix affects.

Table 2 Summary of observed matrix effects for SPE cartridges. Highlights are an indication that there is little difference in suppression effects between these SPE cartridges

Increasing Suppression

19-norandrosterone	Clenbuterol	EMD	MethylT Metab	3'OH Stanozolol
NEXUS	NEXUS	WCX	WCX	UCT
UCT	WCX	NEXUS	NEXUS	WCX
WCX	HRX	HRX	HRX	HRX
HRX	UCT	UCT	UCT	NEXUS

A comparison of how the recoveries could be determined was also examined. The four SPE cartridges (UCT, WCX, NEXUS, HRX) were examined with the possible different ways for the determination of recoveries using internal and external standards. These included no internal standards, methyltestosterone added to sample after extraction, deuterated anabolic agents added to sample before extraction, post column infusion of progesterone and post column infusion of a mixture of deuterated anabolic agents.

The comparison was completed for all five anabolic agents and the results for 19-norandrosterone (Table 3) and EMD (Table 4) are shown as examples. The concentration that had been spiked into the urine samples before extraction was 10 ng/mL. In general the use of no internal standards, methyltestosterone added to sample after extraction, post column infusion of progesterone and post column infusion of a mixture of deuterated anabolic agents all gave very similar results. They were all within ±3 ng/mL of the expected result of 10 ng/mL, which is sufficient for a screening protocol. The use of the five deuterated compounds as the internal standard gave a result closest to the expected value for the four different cartridges.

Table 3 Comparison of internal standards for 19-norandrosterone. Expected result is 10 ng/mL

		No infusion No standard	No infusion Methyl T IS	No infusion 5 steroid IS	Infusion of Progesterone	Infusion of 5 steroid IS
HRX	Spiked with standards only	8.3	10.1	-	7.5	6.3
	Spiked with standards and deuterated standards	8.5	8.7	9.6	8.2	-
UCT	Spiked with standards only	8.2	13.8	-	8.1	6.5
	Spiked with standards and deuterated standards	8.5	12.2	11.7	12.6	-
WCX	Spiked with standards only	7.5	10.3	-	7.2	8.6
	Spiked with standards and deuterated standards	8.3	11.1	10.4	13.1	-
NEXUS	Spiked with standards only	6.8	9.1	-	7.1	12.6
	Spiked with standards and deuterated standards	7.5	12.7	10.7	9.2	-

Table 4 Comparison of internal standards for EMD. Expected result is 10 ng/mL

		No infusion No standard	No infusion Methyl T IS	No infusion 5 steroid IS	Infusion of Progesterone	Infusion of 5 steroid IS
HRX	Spiked with standards only	9.5	11.9	-	8.3	8.4
	Spiked with standards and deuterated standards	12.1	13.0	10.3	9.6	-
UCT	Spiked with standards only	8.8	14.1	-	7.4	14.0
	Spiked with standards and deuterated standards	8.4	12.8	9.9	8.3	-
WCX	Spiked with standards only	9.9	13.7	-	9.6	12.2
	Spiked with standards and deuterated standards	8.3	11.2	10.8	9.8	-
NEXUS	Spiked with standards only	7.1	9.6	-	6.6	7.8
	Spiked with standards and deuterated standards	7.8	9.7	9.8	7.1	-

In summary from the results so far, it is desirable to have the deuterated internal standards for each analyte available to obtain the best quantitative results. However for screening purposes it appears that the degree of suppression is not sufficient to require all five internal standards. The post column infusion of standards is not required for the extraction protocols tested as the results are not markedly better than those from external standardisation. Combining these results from the finding for the evaluation of the twelve different SPE cartridges the WCX cartridge from Waters has so far given the most consistent results.

The minimum required performance level (MRPL) is set at 2 ng/mL for 3'-OH-stanozolol, clenbuterol, EMD and methylT metabolite while it is 1 ng/mL for 19-norandrosterone. Calibrations curves with the use of the deuterated standards were analysed from 0.25 to 5 ng/mL (Figure 5). The standard had been spiked into urine samples which had been extracted with the WCX SPE cartridge. The calibration curves all had very low residuals and followed a linear curve in the range examined.

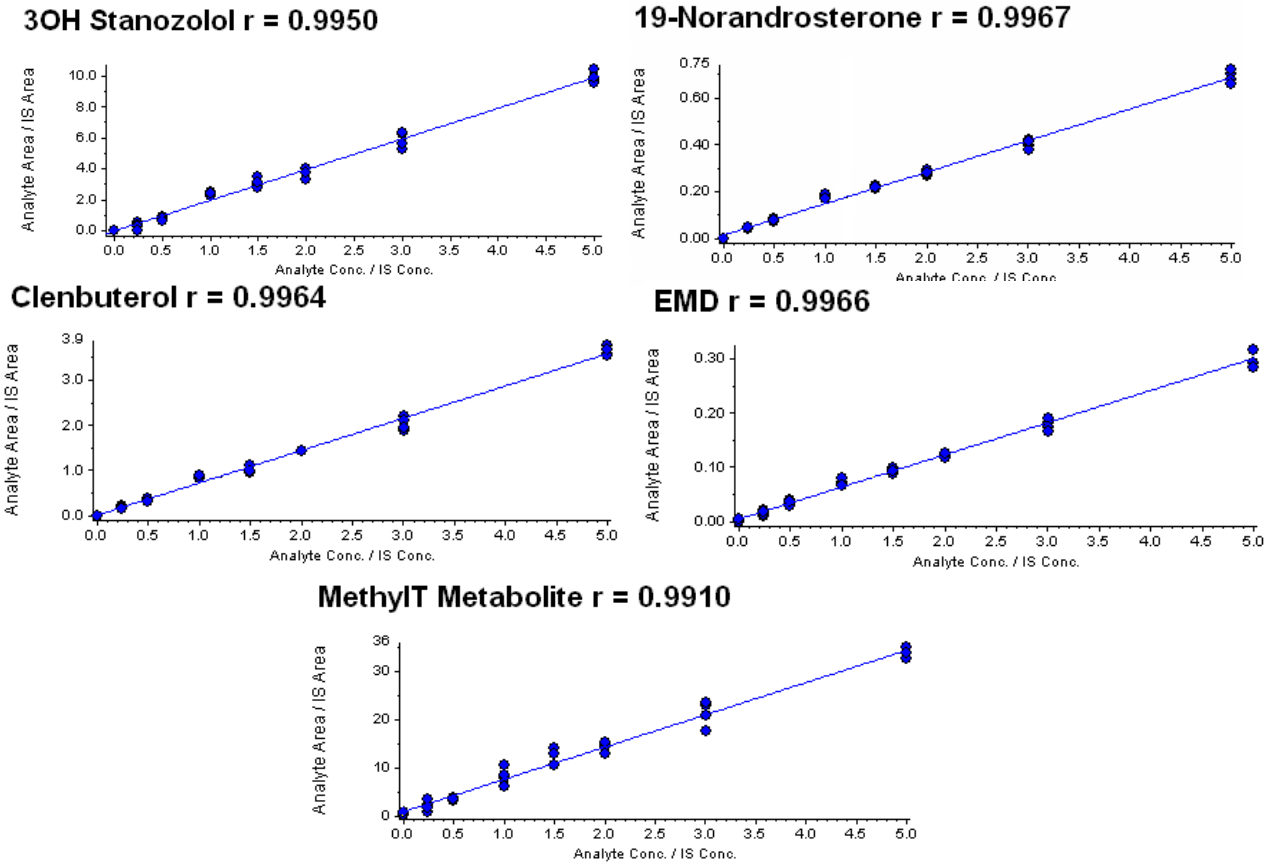

Figure 5 Calibration Curves with deuterated internal standards for 3'-OH-stanozolol, 19-norandrosterone, clenbuterol, EMD and MethylT metabolite.

In figure 6 a blank urine extract can be compared to a sample spiked at 2 ng/mL for all of the five anabolic agents. The blank chromatograms are generally very clean and a sample which had any of the anabolic agents present could be easily identified as the chromatograms of the spiked sample show.

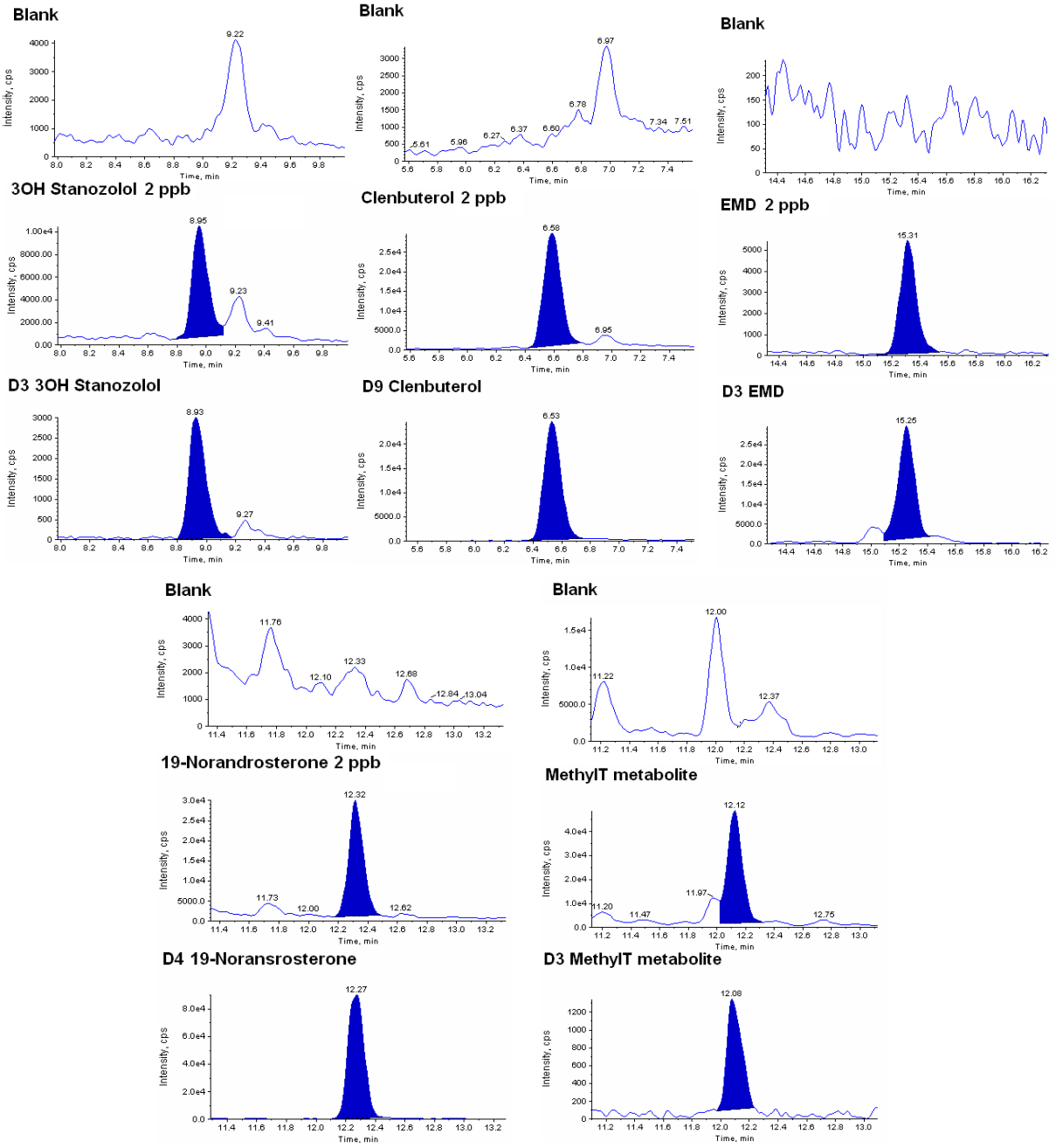

Figure 6 LC/MS/MS chromatograms of blank, spiked at 2 ng/mL, deuterated analytes for 3'-OH-stanozolol, clenbuterol, EMD, 19-norandrosterone, methylT metabolite and deuterated standards.

The recoveries when calculated using the deuterated internal standard added prior to extraction are all very good and the standard deviation from the seven replicates is excellent (Table 5). These results then prove the suitability of the LC/MS/MS analysis technique for the screening of the five low level anabolic agents. In case accurate quantification is required the addition of a suitable deuterated internal standards before the a extraction protocol is necessary.

Table 5 Recoveries and RSD at 2 ng/mL for seven replicates with the WCX Waters SPE cartridge.

	19-norandrosterone		Clenbuterol		EMD		MethylT		3'OH Stanozolol	
	Rec%	RSD %	Rec%	RSD %	Rec%	RSD %	Rec%	RSD %	Rec%	RSD %
Spiked after extraction	101.7	3.5	98.7	2.2	99.4	6.5	98.9	4.7	102.9	3.2
Spiked before extraction	98.7	5.9	101.0	4.7	97.9	5.5	100.1	3.5	89.0	4.4

Conclusions

LC/MS/MS with an appropriate instrument is suitable for the analysis of the five low level anabolic agents. The best results were obtained using deuterated internal standards added prior to extraction with the Waters WCX SPE cartridges. The use of the ABI Q-Trap 4000 has advantages and disadvantages. The newly released software package Analyst 1.5 for the operation of ABI instrumentation now has available scheduled MRM methods which make setting up and adding to methods with large number of analytes very easy. The slow switching between ionisation modes means it can only run in either positive or negative and there are a couple compounds which will not ionise in positive mode e.g. dichlorophenamide. The new Q-Trap 5500 has fast switching between positive and negative ionisation modes.

Acknowledgements

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References

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