Fast Screening and Identification of WADA Prohibited Anabolic Steroids in Urine by UHPLC/MS/MS

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Introduction

The World Anti-Doping Agency (WADA) lists 5 endogenous anabolic steroids, 20 of their isomers, and 41 exogenous anabolic steroids prohibited for use in sports. For these compounds a minimum required performance level (MRPL) of 10 and 2 ng/mL for some hormones must be achieved by any laboratory doing this analysis. In addition, WADA requires that when precursor/product transition ions are monitored in MS/MS that the ratios of those ions be within a specified tolerance for identification. The advantage of fast analysis of athletes for events such as the Olympics with no false negatives or positives is obvious. This study examines the feasibility of using UHPLC /MSMS for urine samples to meet the above criteria with a 4.2 minute analysis time.

Experimental

Configuration:
Agilent 1290-Bin Pump Model G4220A ;
1290-ALS Model G4226A ;
Sampler Thermostat;
1290-Column Compartment Model G1316C

Method Conditions:
Column: Zorbax Eclipse Plus C18 HD, 2.1 x 150m (1.8µm), 40 °C, 0.5 ml/min
Injection volume: 2 uL
Autosampler temp: 4°C
Needle wash: Flushport (100% methanol), 5 seconds
Mobile phase: A = 0.1 % formic acid in water
B = Methanol

Gradient (no split at MS):

Time (min)	*%B*
0.00	*70*
3.0	*85*
3.5	*100*

MS/MS Agilent 6460 Triple Quadrupole:
Dry Gas Temp: 200 °C
Dry Gas Flow rate: 8 L/min
Nebulizer: 35psi
V_{cap}: 3000 V(pos)/2500(neg)
Frag.: 120V
Skimmer: 65V
OCT1 RFVpp 750V
SGF 11 L/min
SGT 275 °C
Nozzle Voltage 0 V(pos)/1000 V(neg)
Ion Source: ESI+Agilent Jet Stream
Scan Type: Dynamic MRM
Ion Mode: Positive/Negative switching (30 ms)
Cycle Time: 350 ms

Experimental

Compound Name	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Ret Time	Ret Window
6B-Hydroxyfluoxymesterone	353.2	Unit	149.1	Unit	170	28	1.169083	0.483
6B-Hydroxyfluoxymesterone	353.2	Unit	95.1	Unit	170	32	1.175783	0.483
6Beta-Hydroxymethandienone	317.2	Unit	281.3	Unit	77	4	1.2765	0.4337
6Beta-Hydroxymethandienone	317.2	Unit	147.1	Unit	77	20	1.277467	0.4337
formebolone metabolite	347.2	Unit	281.3	Unit	113	8	1.29185	0.6419
formebolone metabolite	347.2	Unit	147.1	Unit	113	20	1.2928	0.6419
Boldendione	285.2	Unit	147.2	Unit	116	8	1.702917	0.5583
Boldendione	285.2	Unit	121.1	Unit	116	16	1.70355	0.5583
Dehydroepiandrosterone	271.2	Unit	213	Unit	140	10	1.8516	0.4348
Androstenediol	273.2	Unit	159	Unit	130	17	1.85595	0.3973
19-norandrostendione	273.2	Unit	109	Unit	152	25	1.856317	0.5427
19-norandrostendione	273.2	Unit	83	Unit	152	25	1.8567	0.5427
Trenbolone	271.2	Unit	253.1	Unit	154	17	1.857067	0.4771
Trenbolone	271.2	Unit	199	Unit	154	21	1.857817	0.4771
Dehydroepiandrosterone	271.2	Unit	105	Unit	140	40	1.8582	0.4348
Epi-boldenone	287.2	Unit	121.1	Unit	95	20	1.934967	0.4014
Boldenone	287.2	Unit	121	Unit	113	22	1.934967	0.4014
Boldenone	287.2	Unit	135	Unit	113	10	1.940733	0.4014
Fluoxymesterone	337.2	Unit	91	Unit	178	60	1.979833	0.4947
Fluoxymesterone	337.2	Unit	131	Unit	178	33	1.98535	0.4947
Oxandrolone	307.2	Unit	289.1	Unit	86	5	2.041983	0.5678
Oxandrolone	307.2	Unit	271.1	Unit	86	9	2.042217	0.5678
Nandrolone	275.2	Unit	109	Unit	150	25	2.066667	0.5577
Nandrolone	275.2	Unit	79	Unit	150	49	2.0669	0.5577
4-Androstenedione	287.2	Unit	109	Unit	147	22	2.119333	0.4122
Androstenedione	287.2	Unit	109	Unit	154	25	2.119333	0.4122
4-Androstenedione	287.2	Unit	97	Unit	147	22	2.119917	0.4122
Androstenedione	287.2	Unit	97	Unit	154	21	2.119917	0.4122
Metandienone	301.2	Unit	149	Unit	124	13	2.16945	0.5072
Metandienone	301.2	Unit	121	Unit	124	25	2.16975	0.5072
Gestrinone	309.2	Unit	241.2	Unit	137	20	2.17355	0.4734
Gestrinone	309.2	Unit	199.1	Unit	137	36	2.173833	0.4734
4-hydroxytestosterone	305.2	Unit	125.1	Unit	161	24	2.276417	0.399
4-hydroxytestosterone	305.2	Unit	113.1	Unit	161	24	2.276683	0.399
Epi-boldenone	287.2	Unit	269.3	Unit	95	4	2.306067	0.4014
Epitestosterone	289.2	Unit	109	Unit	156	29	2.3224	0.4979
Epitestosterone	289.2	Unit	97	Unit	156	25	2.32265	0.4979
5a-dihydrotestosterone sulfate NEt2 salt	370.2	Unit	96.9	Unit	200	40	2.450217	0.4085
methyltestosterone	303.2	Unit	109	Unit	156	29	2.6058	0.5104
Norethandrolone	303.2	Unit	109	Unit	154	30	2.6058	0.5834
methyltestosterone	303.2	Unit	97	Unit	156	29	2.606433	0.5104
Methenolone	303.2	Unit	187	Unit	152	17	2.662283	0.5066
Methenolone	303.2	Unit	83	Unit	152	21	2.6642	0.5066
Testosterone	289.2	Unit	109	Unit	154	25	2.733483	0.4051
Testosterone	289.2	Unit	97	Unit	154	21	2.734217	0.4051
Dihydrotestosterone	291.2	Unit	159	Unit	150	19	2.8482	0.3459
Dihydrotestosterone	291.2	Unit	255.1	Unit	150	13	2.853367	0.3459
17a-methyl-1-testosterone	303.2	Unit	201.2	Unit	137	16	2.967317	0.485
17a-methyl-1-testosterone	303.2	Unit	145.1	Unit	137	24	2.9679	0.485
Norethandrolone	303.2	Unit	285.1	Unit	154	10	2.984017	0.5834
Mesterone	305.2	Unit	269	Unit	133	20	3.074933	0.4542
Mesterone	305.2	Unit	95	Unit	133	29	3.075517	0.4542
Stanazolol	329.3	Unit	95	Unit	204	45	3.079533	0.4928
Stanazolol	329.3	Unit	81	Unit	204	49	3.080117	0.4928
Oxymetholone	333.2	Unit	43	Unit	150	35	3.87335	0.388
Oxymetholone	333.2	Unit	99	Unit	150	33	3.876267	0.388

Sample Preparation

Human Control (100 mL), spiked 10 mL, 2 each at 0 ng/mL, 1 ng/mL, 5 ng/mL, and 25 ng/mL.
SPE Cartridge: Agilent SampliQ C_8 6 mL, 500 mg
Conditioning: 10 mL 60:40 ethylacetate:methanol
10 mL H_2O
Add 10 mL urine sample under vacuum, take to dryness 5 min
Elute with 3 mL 80: 20 ethylacetate:methanol
Blow down gently with N_2 to 100 µL and add 900 µL H_2O
Filter with 0.2 u PTFE 25 mm id membrane into 2.0 mL autosampler vials

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Results and Discussion

Chromatographic Results

The goal of this study is to show the feasibility of screening and confirming the World Anti-doping Agency (WADA) prohibited steroids in a rapid LC/MS/MS analysis. Fast separation is facilitated by the use of sub 2 micron particle size LC columns and an LC system that is both capable of high pressure and very low delay and dead volumes. Figure 1 shows the separation of 28 of the prohibited steroids and some of their metabolites. More would be included but the compounds are difficult to obtain and some are only available to WADA accredited laboratories. In addition, only steroids with non-saturated rings are ionized using electrospray and those with this chemical attribute are not included in this evaluation. Peaks are about 5 seconds at baseline and the unexpected dispersion is attributed to the hydrophobic nature of the compounds. The initial concentration of mobile phase of 65 % organic is selected because there was no gain in chromatographic resolution using a higher aqueous concentration. With 5 sec peaks a MS/MS cycle time of 500 ms or less is sufficient to obtain 10 points across a peak.

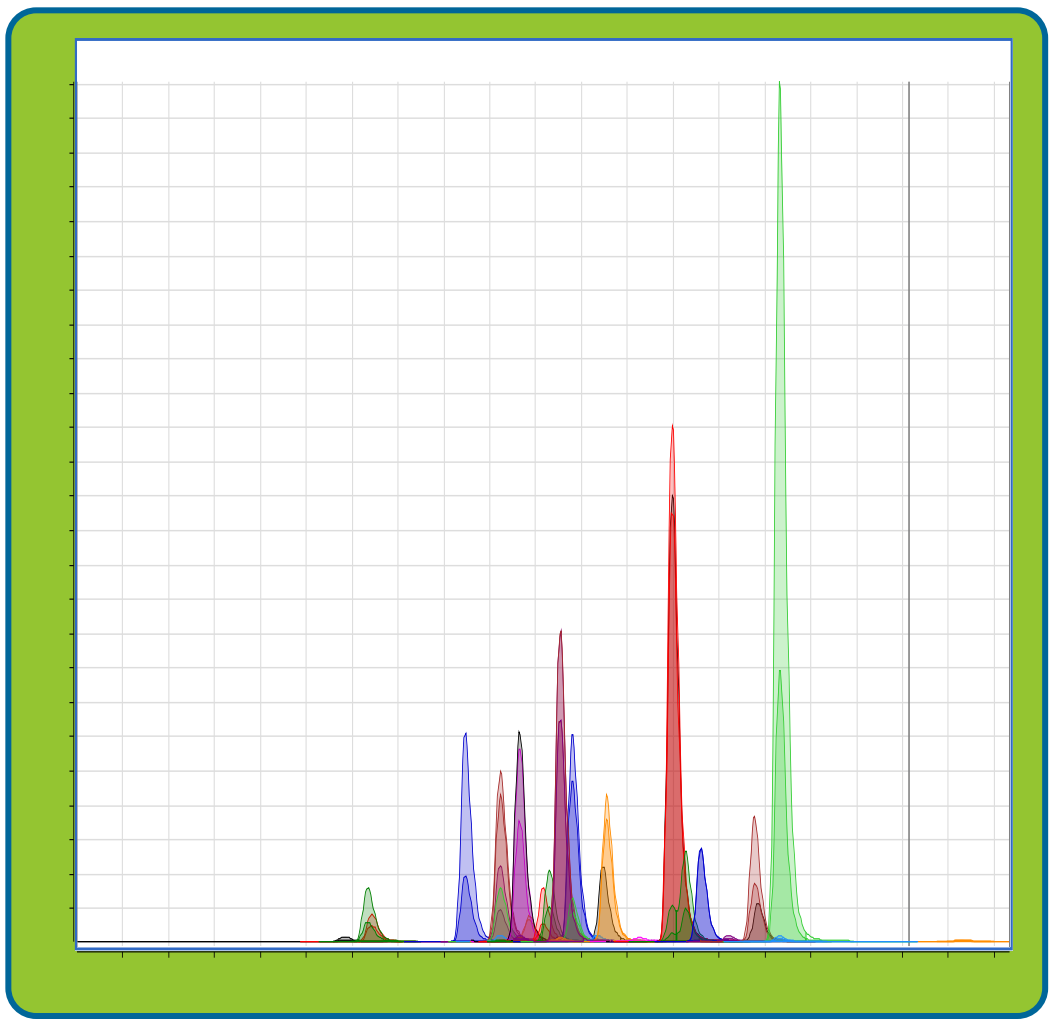

Figure 1. Separation and detection with two transitions each of 29 prohibited steroids in 4 min.

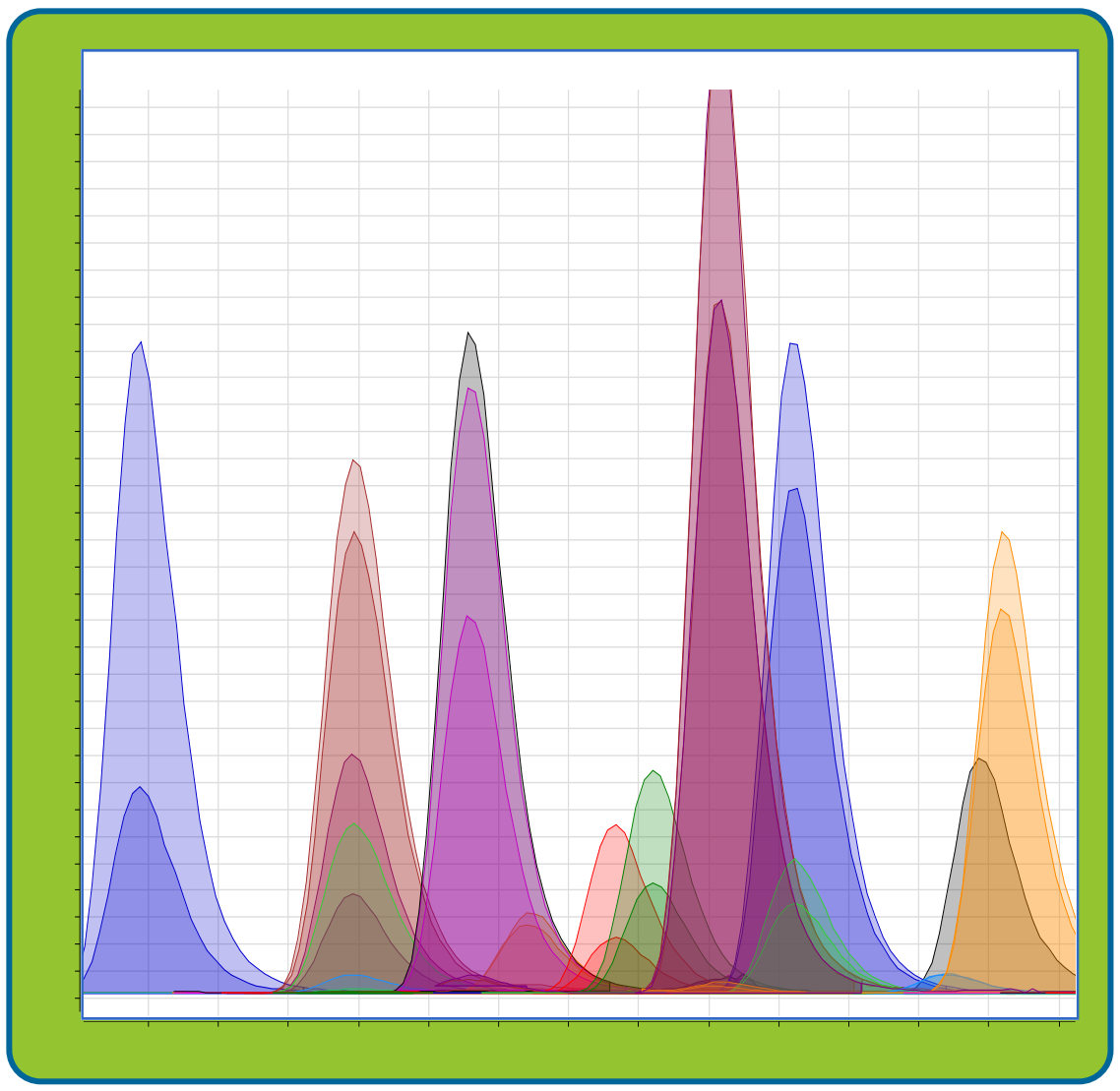

Figure 2. Region of separation where numerous transitions overlap. Dwell times are maximized by dynamic MRM with no degradation of peak integrity

MS/MS Results

The necessary cycle time is obtained using Dynamic multi-reaction monitoring (MRM) where each transition is only collected in a retention time window centered at the elution time of that transition. Dwell times are managed automatically by the number of co-eluting transitions and the selected cycle time. For this analysis a peak width of 1-2 sec at baseline would be desirable and if this could be obtained a faster cycle time and shorter retention time windows would be used. Figure 2 shows a section of the chromatogram where multiple transitions co-elute. Although dwell times are "dynamic" in this region, peak shapes and response are not affected. This demonstrates the power of the system (hardware, firmware, and software) working together to obtain high quality data. This extends the capability of LC/MS/MS in MRM mode to determine more transitions per unit time. Combining the high selectivity of MS/MS and the power of chromatography, co-eluting compounds are separated by the MRM transitions as shown with epi-boldinone and epi-testosterone in Figure 3. In the same figure the chromatographic separation of boldinone and testosterone with their epi forms shows the extensive power of the hyphenated technology.

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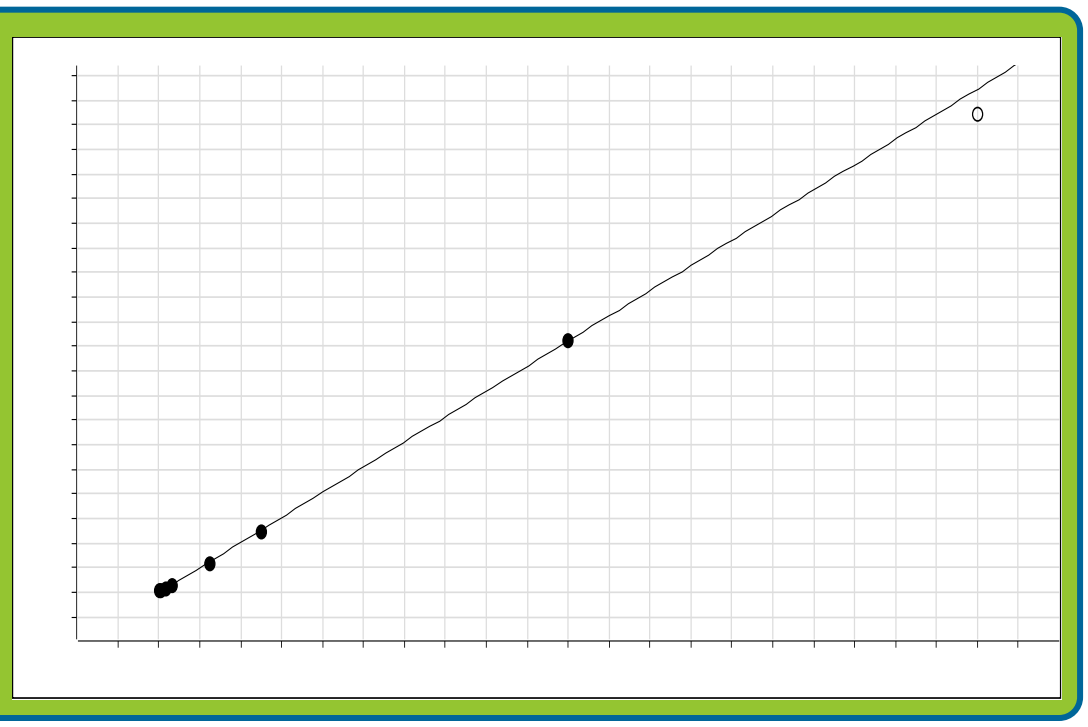

Figure 4. Calibration curve for boldenone showing that region of high transition overlap does not effect quantitative results.

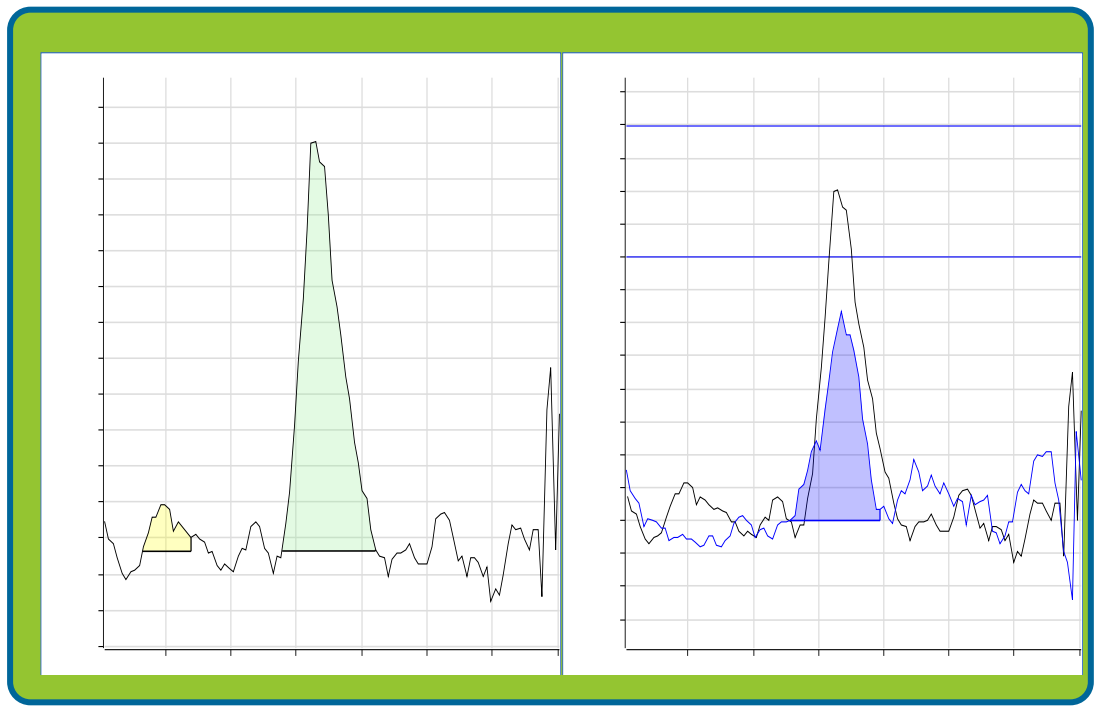

Figure 5. Quantifier and qualifier ion for boldenone at 0.1 ng/mL shows at this concentration the compound cannot be confirmed.

Quantitative Results

Using a 70 % initial organic mobile phase and all other conditions the same, quantitative results are evaluated. Figure 4 shows the linearity of boldinone in the transition dense region of the chromatogram. Figure 5 shows that this compound cannot be confirmed at 0.1 ng/mL using the ratios of the two selected transitions. Results of 20 steroids spiked into control urine demonstrate that most can be recovered and detected at 1 ng/mL as shown for norandrone in Figure 6.

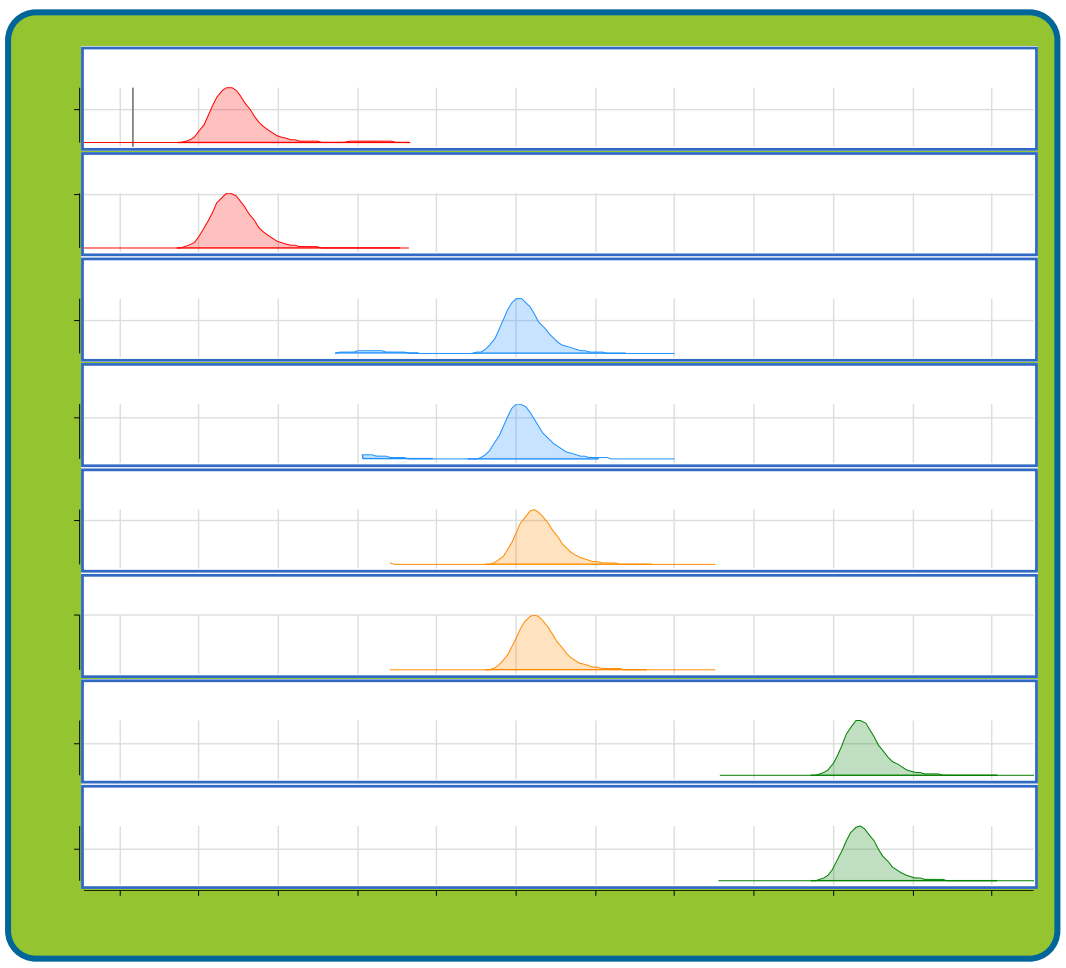

Figure 3. Separation of boldenone, epi-boldenone, testosterone and epi-testosterone, critical pairs that cannot be distinguished by MS/MS.

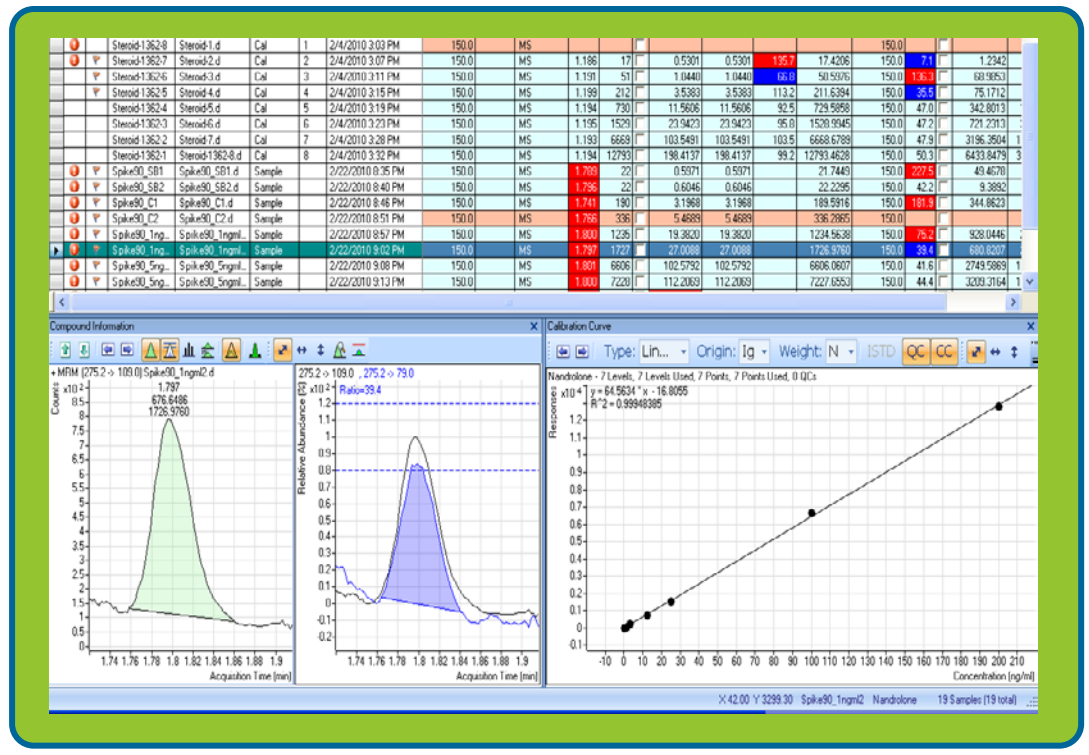

Figure 6. Norandrone spiked in urine at 1 ng/mL is both detected and confirmed with correct ion ratios of quantifier and qualifier and retention time match.

Conclusions

- *The ability to separate many steroids in a short analysis time with both chromatography and MS/MS is shown*
- *Dynamic MRM provides the ability to determine more transitions per unit time while maintaining chromatographic and quantitative fidelity*
- *With a simple SPE procedure most of the steroids examined can be detected, quantified, and confirmed at the 1 ng/mL level*
- *Future work will examine if peaks on the order of 1 to 2 sec at baseline can be obtained with C8 or C3 columns*
- *Future work will examine if APCI might ionize steroids with all unsaturated rings like mesterolone and norandrosterone.*