molecules

*Article*

# Fast and sensitive screening of oxandrolone and its major metabolite 17-epi-oxandrolone in human urine by UHPLC – MS/MS with on-line SPE sample pretreatment

## Supplementary Material

**Table of content**

**Table S1:** Optimization of the MS conditions. The highlighted data (in bold) represent optimal values.

| Cone voltage [V] | | Desolvation gas flow [L.h$^{-1}$] | | Desolvation gas temperature ($^{o}$C) $^{o}$C | | Capillary voltage [kV] | |
|---|---|---|---|---|---|---|---|
| *Tested range* | *Intensity %* | *Tested range* | *Intensity %* | *Tested range* | *Intensity %* | *Tested range* | *Intensity %* |
| 2 | 20 | 500 | 80 | 200 | 70 | 1.00 | 50.00 |
| 22 | 80 | 600 | 90 | 250 | 70 | 1.50 | 80.00 |
| **28** | **100** | **700** | **100** | 300 | 90 | 2.00 | 95.00 |
| 40 | 80 | 800 | 100 | 350 | 95 | 2.50 | 95 |
| 60 | 0 | 900 | 95 | 370 | 95 | 2.70 | 95 |
| 100 | 0 | 1000 | 95 | **400** | **100** | **3.00** | **100** |

**Table S2:** Optimization of UHPLC separation (stationary phase).

| Column type | Buffer | Temperature | % ACN | Retention time [min] | Width an half the height [min] | Column efficiency |
|---|---|---|---|---|---|---|
| Acquity UPLC BEH C18 (2.1X50, 1.7) | 0.1% formic acid | 40 °C | 40 | 1.31 | 0.0411 | 5628 |
| | | | 50 | 0.79 | 0.0371 | 2512 |
| | | | 60 | 0.59 | 0.0355 | 1530 |
| | | | 70 | 0.50 | 0.0322 | 1336 |
| | | | 80 | 0.46 | 0.0298 | 1320 |
| | | | 90 | 0.42 | 0.0297 | 1108 |
| Acquity UPLC BEH C8 (2.1X50, 1.7) | 0.1% formic acid | 40 °C | 40 | 1.19 | 0.0387 | 5238 |
| | | | 50 | 0.75 | 0.0381 | 2147 |
| | | | 60 | 0.59 | 0.0306 | 2060 |
| | | | 70 | 0.52 | 0.0300 | 1664 |
| | | | 80 | 0.45 | 0.0292 | 1316 |
| | | | 90 | 0.45 | 0.0288 | 1353 |
| Acquity UPLC CSH C18 (2.1 X50, 1.7) | 0.1% formic acid | 40 °C | 40 | 1.55 | 0.0436 | 7002 |
| | | | 50 | 0.90 | 0.0411 | 2657 |
| | | | 60 | 0.66 | 0.0405 | 1471 |
| | | | 70 | 0.55 | 0.0305 | 1802 |
| | | | 80 | 0.49 | 0.0284 | 1649 |
| | | | 90 | 0.46 | 0.0357 | 920 |
| Acquity UPLC HSS Cyano (2.1X50, 1.8) | 0.1% formic acid | 40 °C | 40 | 1.09 | 0.0440 | 3400 |
| | | | 50 | 0.73 | 0.0372 | 2133 |
| | | | 60 | 0.58 | 0.0306 | 1990 |
| | | | 70 | 0.50 | 0.0285 | 1705 |
| | | | 80 | 0.45 | 0.0305 | 1206 |
| | | | 90 | 0.45 | 0.0269 | 1550 |
| Acquity UPLC BEH Shield RP18 (2.1X50, 1.7) | 0.1% formic acid | 40 °C | 40 | 1.40 | 0.0372 | 7847 |
| | | | 50 | 0.84 | 0.0315 | 3940 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | 60 | 0.63 | 0.0283 | 2745 |
| | | | 70 | 0.52 | 0.0275 | 1981 |
| | | | 80 | 0.45 | 0.0251 | 1781 |
| | | | 90 | 0.45 | 0.0263 | 1622 |
| Acquity UPLC HSS T3 (2.1X50, 1.8) | 0.1% formic acid | 40 °C | 40 | 2.04 | 0.0431 | 12411 |
| | | | 50 | 1.11 | 0.0343 | 5802 |
| | | | 60 | 0.77 | 0.0290 | 3906 |
| | | | 70 | 0.61 | 0.0289 | 2468 |
| | | | 80 | 0.52 | 0.0259 | 2233 |
| | | | 90 | 0.49 | 0.0261 | 1953 |

**Table S3:** Optimization of UHPLC separation (mobile phase).

| Buffer | Temperature | % ACN | Retention time [min] | Width an half the height | Peak area | Column efficiency |
|---|---|---|---|---|---|---|
| 0.1% formic acid | 40 °C | 50 | 1.11 | 0.0329 | 100026 | 6306 |
| 10 mM AF | 40 °C | 50 | 1.09 | 0.0405 | 70657 | 4013 |
| 20 mM AF | 40 °C | 50 | 1.09 | 0.0427 | 55592 | 3610 |

AF – ammonium formate

**Table S4:** Optimization of the SPE procedure.

| Column type | Temperature | % ACN Load | Injection volume [µL] | Peak area – 2D | Recovery [%] |
|---|---|---|---|---|---|
| On-Line Extraction Column Xbridge C18 Direct Connect HP 10um (2.1X30) | 40 °C | 30 | 10 | 652562.6 | 92.36 |
| | | | | 653831.8 | 92.54 |
| | | 30 | 50 | 2987391 | 84.56 |
| | | | | 3009281 | 85.18 |
| | | 30 | 100 | 3581820 | 50.69 |
| | | | | 3935258.7 | 55.70 |
| | | 30 | 200 | 5372730 | 38.02 |
| | | | | 5020543.05 | 35.53 |
| On-Line Extraction Column Xbridge C8 Direct Connect HP 10um (2.1X30) | 40 °C | 30 | 10 | 650045.15 | 92.00 |
| | | | | 663446.7 | 93.90 |
| | | 30 | 50 | 2638867 | 74.70 |
| | | | | 2886090 | 81.69 |
| | | 30 | 100 | 1930680 | 27.32 |
| | | | | 2350128 | 33.26 |
| | | 30 | 200 | 2896020 | 20.49 |
| | | | | 2976828.8 | 21.07 |
| On-Line Extraction Column Oasis HLB Direct Connect HP 20um (2.1X30) | 40 °C | 30 | 10 | 627102.38 | 88.75 |
| | | | | 670818.33 | 94.94 |
| | | 30 | 50 | 2638867 | 74.70 |
| | | | | 2886090 | 81.69 |
| | | 30 | 100 | 1930680 | 27.32 |
| | | | | 2350128 | 33.26 |
| | | 30 | 200 | 2896020 | 20.49 |
| | | | | 2976828.8 | 21.07 |

| | | | | | |
|---|---|---|---|---|---|
| On-Line Extraction Column Acquity HSS T3 C18 Column (1.7 μm, 2.1 × 50 mm) | 40 °C | 30 | 10 | 726521.05 | 102.82 |
| | | | | 700304.85 | 99.11 |
| | | 30 | 50 | 3279735 | 92.84 |
| | | | | 3386346 | 95.85 |
| | | 30 | 100 | 6757380 | 95.64 |
| | | | | 6834955.6 | 96.73 |
| | | 30 | 200 | 12549420 | 88.81 |
| | | | | 13121548 | 92.85 |

**Table S5:** Stability of oxandrolone in urine matrix under different conditions.

| Conditions | Spiked concentration [pg.mL$^{-1}$] | Mean [pg.mL$^{-1}$] | Recovery % |
|---|---|---|---|
| **Autosampler stability at 6°C after 12h** | 75 | 72.10 | 96.13 |
| | 750 | 669.6 | 89.28 |
| | 5000 | 4436 | 88.72 |
| **Freeze–thaw cycle in urine (−70 °C, after the third cycle)** | 75 | 68.05 | 90.73 |
| | 750 | 641.9 | 85.58 |
| | 5000 | 4436 | 88.72 |

**Table S6:** Peak areas of oxandrolone in enzymatically hydrolyzed and non-hydrolyzed urine samples.

| | Area (sample1) | | Area (sample2) | |
|---|---|---|---|---|
| **Sampling time** | **Hydrolyzed** | **Non-hydrolyzed** | **Hydrolyzed** | **Non-hydrolyzed** |
| 10h after administration | 1368.2 | 1451.0 | 11050 | 1052.0 |
| 20h after administration | 1545.2 | 1559.9 | 1463.9 | 1566.0 |
| 48h after administration | 124.98 | 118.15 | 178.50 | 150.20 |

**Table S7:** Concentration of oxandrolone in urine taken after administration of one dose (10mg) of oxandrolone in tablet Oxandrix®.

| Time [hours] | c (OXA) Mean [pg.mL$^{-1}$] | SD [pg.mL$^{-1}$] | Creatinine [µmol.L$^{-1}$] | ng OXA /mmol creatinine | Area ratio Epi-oxandrolone/OXA |
|---|---|---|---|---|---|
| 4 | 86137 | 9475 | 13900 | 6.2 | 0.124 |
| 10 | 151063 | 16617 | 18300 | 8.3 | 0.123 |
| 20 | 163903 | 18029 | 10900 | 15 | 0.117 |
| 40 | 6903 | 759.3 | 531.0 | 13 | 0.222 |
| 48 | 9081 | 998.9 | 3200 | 2.8 | 0.269 |
| 87.5 | 4235 | 465.9 | 11100 | 0.382 | 0.616 |
| 96 | 3150 | 346.5 | 5800 | 0.543 | 0.824 |
| 120 | 1418 | 155.9 | 8700 | 0.163 | 0.800 |
| 144 | 529.3 | 58.2 | 7200 | 0.074 | 0.835 |
| 168 | 329.3 | 36.2 | 16900 | 0.019 | 0.921 |
| 192 | 267.7 | 29.4 | 8200 | 0.033 | - |
| 216 | 99.3 | 10.9 | 8190 | 0.0121 | - |
| 240 | 42.6 | 4.7 | 11520 | 0.0037 | - |

**Table S8:** Gradient of mobile phase and positions of the switching valves in SPE enrichment process.

| t [min] | B [%] | Flow [mL] | Left valve position | |
|---|---|---|---|---|
| 0 | 30 | 0.4 | Position 1 | SPE column-waste |
| 2.5 | 30 | 0.4 | | |
| 2.6 | 30 | 0.4 | Position 2 | SPE-Analytical column to MS |
| 5.5 | 90 | 0.4 | | |
| 8.9 | 90 | 0.4 | | |
| 9.0 | 30 | 0.4 | | |
| 10 | 30 | 0.4 | Position 1 | SPE column-waste |
| 11 | 30 | 0.4 | | |

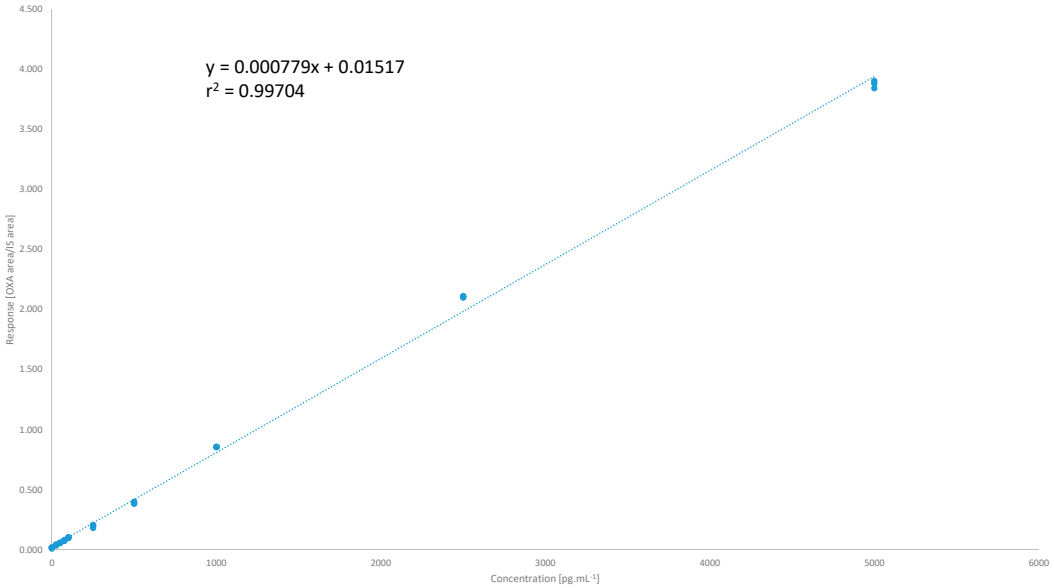


**Figure S1:** Calibration curve of oxandrolone.