**Substrate multispecificity among 20-hydroxysteroid dehydrogenase type 2 members**

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**Supplementary Materials:**

**Supplementary Table 1:** Steroid concentrations in calibrator stock solutions.

**Supplementary Table 2:** HPLC and mass spectrometric parameters for scheduled multiple reaction monitoring (sMRM) transitions.

**Supplementary Table 3:** Absolute and relative solid phase extraction recoveries at different calibrator concentrations.

**Supplementary Table 4:** Absolute intra- and inter-day variations in determination of steroid concentrations at different calibrator concentrations.

**Supplementary Table 5:** Relative intra- and inter-day variation in determination of steroid concentrations at different calibrator concentrations.

**Supplementary Table 6:** Important parameters for steroid quantification: individual linear equations and linearity resulting from calibrator’s linear regression, as well as LOD, and LLOQ values for all analyzed steroids.

**Supplementary Table 7:** Absolute and relative matrix effects at three different calibrator concentrations.

**Supplementary Figure 1:** Extracted ion chromatograms for cortisone, 20-dihydrocortisone, and the internal standard 13C-cortisol from exemplary enzyme assays using cortisone as substrate.

**Supplementary Table 1:** Steroid concentrations in calibrator stock solutions.

|  |  |
| --- | --- |
|  | Steroid concentration (ng/mL) |
| Steroid | **Cal 1** | **Cal 2** | **Cal 3** | **Cal 4** | **Cal 5** | **Cal 6** | **Cal 7** | **Cal 8** |
| 20-Dihydrocortisol | 14 | 28 | 140 | 280 | 1000 | 1400 | 2000 | 2800 |
| 20-Dihydrocortisone | 2 | 4 | 20 | 40 | 150 | 200 | 300 | 400 |
| 20-Dihydrocortisone | 2.4 | 4.8 | 24 | 48 | 170 | 240 | 340 | 480 |
| Aldosterone | 110 | 220 | 1100 | 2200 | 7700 | 11000 | 15400 | 22000 |
| Cortisol | 13.6 | 27.2 | 136 | 272 | 960 | 1360 | 1920 | 2720 |
| Cortisone | 2 | 4 | 20 | 40 | 150 | 200 | 300 | 400 |
| 20-Dihydrocorticosterone | 11.2 | 22.4 | 112 | 224 | 780 | 1120 | 1560 | 2240 |
| 20-Dihydro-11-desoxycortisol | 4 | 8 | 40 | 80 | 280 | 400 | 560 | 800 |
| Corticosterone | 5.6 | 11.2 | 56 | 112 | 400 | 560 | 800 | 1120 |
| 11-Desoxycortisol | 2 | 4 | 20 | 40 | 150 | 200 | 300 | 400 |
| 20-Dihydro-11-desoxycorticosterone | 2.4 | 4.8 | 24 | 48 | 170 | 240 | 340 | 480 |
| 17,20-Dihydroxyprogesterone | 2.4 | 4.8 | 24 | 48 | 170 | 240 | 340 | 480 |
| Testosterone | 0.8 | 1.6 | 8 | 16 | 60 | 80 | 120 | 160 |
| 11-Desoxycorticosterone | 3 | 6 | 30 | 60 | 200 | 300 | 400 | 600 |
| Androstenedione | 2 | 4 | 20 | 40 | 150 | 200 | 300 | 400 |
| 17-Hydroxyprogesterone | 3.4 | 6.8 | 34 | 68 | 240 | 340 | 480 | 680 |
| 20-Hydroxyprogesterone | 2.8 | 5.6 | 28 | 56 | 200 | 280 | 400 | 560 |
| Pregnenolone | 70 | 140 | 700 | 1400 | 5000 | 7000 | 10000 | 14000 |
| Progesterone | 1.2 | 2.4 | 12 | 24 | 80 | 120 | 160 | 240 |

**Supplementary Table 2:** HPLC and mass spectrometric parameters for scheduled multiple reaction monitoring (sMRM) transitions.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Steroid | RT (min) | sMRM window (min) | Q1(m/z) | Q3(m/z) | DP(eV) | CXP(eV) | CE(eV) | EP(eV) |
| 20-Dihydrocortisol | 6.7 | 3.0-9.0 | 365.3 | 121.4 | 56 | 7 | 33 | 10 |
| 20-Dihydrocortisone | 6.8 | 3.0-9.0 | 363.2 | 163.2 | 86 | 8 | 33 | 10 |
| 20-Dihydrocortisone | 7.3 | 3.0-9.0 | 363.2 | 163.2 | 86 | 8 | 33 | 10 |
| Aldosterone | 7.6 | 3.0-9.0 | 361.1 | 343.5 | 80 | 10 | 33 | 10 |
| Cortisol | 9.9 | 6.0-12.0 | 363.224 | 121.1 | 86 | 6 | 33 | 10 |
| Cortisone | 10.6 | 6.0-12.0 | 361.21 | 163.2 | 76 | 10 | 33 | 10 |
| 20-Dihydrocorticosterone | 12.1 | 8.0-14.0 | 349.3 | 121.2 | 40 | 10 | 41 | 10 |
| 20-Dihydro-11-desoxycortisol | 12.6 | 8.0-14.0 | 349.3 | 109.1 | 52 | 7 | 43 | 10 |
| Corticosterone | 14.2 | 10.0-16.0 | 347.2 | 121.1 | 76 | 8 | 33 | 10 |
| 11-Desoxycortisol | 14.6 | 10.0-16.0 | 347.3 | 109.2 | 70 | 8 | 40 | 10 |
| 20-Dihydro-11-desoxycorticosterone | 15.7 | 12.0-18.0 | 349.3 | 121.2 | 40 | 10 | 41 | 10 |
| 17,20-Dihydroxyprogesterone | 17.1 | 12.0-18.0 | 333.4 | 97.1 | 56 | 7 | 44 | 10 |
| Testosterone | 17.2 | 14.5-20.5 | 289.2 | 97.2 | 81 | 6 | 33 | 10 |
| 11-Desoxycorticosterone | 18.1 | 14.5-20.5 | 331.1 | 109.1 | 80 | 8 | 33 | 10 |
| Androstenedione | 19.1 | 14.5-20.5 | 287.1 | 97.1 | 101 | 14 | 33 | 10 |
| 17-Hydroxyprogesterone | 19.3 | 14.5-20.5 | 331.3 | 97.1 | 50 | 6 | 33 | 10 |
| 20-Hydroxyprogesterone | 21.9 | 17.5-23.5 | 317.4 | 109.2 | 72 | 8 | 30 | 10 |
| Pregnenolone | 22.0 | 17.5-23.5 | 299.4 | 105.1 | 70 | 14 | 52 | 10 |
| Progesterone | 22.2 | 17.5-23.5 | 315.2 | 109.1 | 51 | 8 | 33 | 10 |
| 13C-Cortisol | 9.9 | 0.0-24.0 | 366.224 | 124.1 | 86 | 6 | 33 | 10 |

RT – retention time; sMRM – scheduled multiple reaction monitoring; Q1 – quadrupole 1; Q3 – quadrupole 3; m/z – mass to charge ratio; DP – declustering potential; CXP – collision cell exit potential; CE – collision energy; EP – entrance potential

**Supplementary Table 3:** Absolute and relative solid phase extraction recoveries at different calibrator concentrations. Each calibrator solution was analyzed in triplicate (n=3). Relative recoveries were corrected by the internal standard. For input steroid concentrations, refer to Supplementary Table 1.

|  |  |
| --- | --- |
|  | Calibrator 2 |
|  | **Matrix 1 (reaction buffer + stop solution)** | **Matrix 2 (HEK293 cells in reaction buffer + stop solution)** |
| Steroid | **Absolute recovery (%)** | **Relative recovery (%)** | **Absolute recovery (%)** | **Relative recovery (%)** |
| 20-Dihydrocortisol | 92.49 ± 1.63 | 100.27 ± 2.00 | 83.40 ± 6.26 | 88.61 ± 5.79 |
| 20-Dihydrocortisone | 91.03 ± 1.13 | 97.91 ± 2.98 | 92.31 ± 2.37 | 98.01 ± 0.89 |
| 20-Dihydrocortisone | 85.43 ± 0.99 | 91.89 ± 3.87 | 90.06 ± 4.08 | 95.69 ± 4.51 |
| Aldosterone | 92.88 ± 3.46 | 99.83 ± 2.09 | 94.16 ± 1.97 | 100.04 ± 1.81 |
| Cortisol | 92.13 ± 3.96 | 98.98 ± 1.61 | 94.46 ± 1.11 | 100.37 ± 2.35 |
| Cortisone | 91.63 ± 4.04 | 98.44 ± 1.41 | 93.72 ± 1.78 | 99.58 ± 2.88 |
| 20-Dihydrocorticosterone | 91.55 ± 5.46 | 98.34 ± 3.82 | 94.28 ± 3.76 | 100.22 ± 4.81 |
| 20-Dihydro-11-desoxycortisol | 91.84 ± 4.35 | 98.76 ± 4.68 | 95.16 ± 1.35 | 101.11 ± 1.52 |
| Corticosterone | 100.79 ± 5.20 | 108.30 ± 5.16 | 100.53 ± 1.42 | 106.74 ± 1.23 |
| 11-Desoxycortisol | 94.27 ± 2.94 | 101.33 ± 0.73 | 97.61 ± 7.42 | 103.64 ± 6.37 |
| 20-Dihydro-11-desoxycorticosterone | 92.05 ± 3.12 | 98.93 ± 0.92 | 93.86 ± 2.51 | 99.75 ± 3.20 |
| 17,20-Dihydroxyprogesterone | 98.26 ± 2.82 | 105.78 ± 6.06 | 95.84 ± 2.78 | 101.88 ± 4.85 |
| Testosterone | 99.96 ± 1.13 | 107.54 ± 3.55 | 98.40 ± 1.10 | 104.53 ± 1.33 |
| 11-Desoxycorticosterone | 92.79 ± 1.28 | 99.87 ± 4.72 | 98.00 ± 2.29 | 104.07 ± 1.15 |
| Androstenedione | 99.16 ± 3.04 | 106.56 ± 1.60 | 102.02 ± 1.52 | 108.37 ± 1.68 |
| 17-Hydroxyprogesterone | 98.16 ± 2.69 | 105.50 ± 2.18 | 100.87 ± 0.94 | 107.17 ± 1.33 |
| 20-Hydroxyprogesterone | 97.66 ± 1.30 | 105.05 ± 3.28 | 96.88 ± 3.72 | 103.01 ± 5.62 |
| Pregnenolone | 96.93 ± 2.88 | 104.16 ± 0.92 | 103.01 ± 0.96 | 109.43 ± 1.78 |
| Progesterone | 95.78 ± 3.85 | 102.88 ± 0.24 | 100.89 ± 3.35 | 107.26 ± 5.03 |
| 13C-Cortisol | 93.07 ± 3.61 | 100.00 ± 0.00 | 94.16 ± 2.13 | 100.00 ± 0.00 |

**Supplementary Table 3:** (continued)

|  |  |
| --- | --- |
|  | Calibrator 3 |
|  | **Matrix 1 (reaction buffer + stop solution)** | **Matrix 2 (HEK293 cells in reaction buffer + stop solution)** |
| Steroid | **Absolute recovery (%)** | **Relative recovery (%)** | **Absolute recovery (%)** | **Relative recovery (%)** |
| 20-Dihydrocortisol | 94.21 ± 1.04 | 99.35 ± 1.13 | 96.87 ± 1.43 | 102.55 ± 3.83 |
| 20-Dihydrocortisone | 98.00 ± 0.32 | 101.55 ± 2.49 | 98.70 ± 1.65 | 104.59 ± 5.84 |
| 20-Dihydrocortisone | 95.90 ± 1.80 | 97.63 ± 3.62 | 96.47 ± 1.06 | 102.14 ± 3.51 |
| Aldosterone | 97.16 ± 1.54 | 100.72 ± 3.86 | 99.16 ± 0.66 | 105.03 ± 4.35 |
| Cortisol | 98.69 ± 2.10 | 102.27 ± 3.34 | 98.02 ± 0.67 | 103.79 ± 3.57 |
| Cortisone | 96.18 ± 0.29 | 99.68 ± 2.91 | 97.85 ± 1.32 | 103.66 ± 4.86 |
| 20-Dihydrocorticosterone | 93.83 ± 1.50 | 97.27 ± 4.25 | 92.35 ± 0.47 | 97.80 ± 4.50 |
| 20-Dihydro-11-desoxycortisol | 94.19 ± 3.12 | 97.70 ± 5.70 | 95.08 ± 2.14 | 100.76 ± 5.86 |
| Corticosterone | 97.67 ± 2.33 | 101.28 ± 5.08 | 96.93 ± 1.45 | 102.60 ± 2.76 |
| 11-Desoxycortisol | 94.36 ± 1.62 | 97.74 ± 2.11 | 96.44 ± 0.78 | 102.09 ± 3.36 |
| 20-Dihydro-11-desoxycorticosterone | 93.29 ± 2.66 | 96.67 ± 3.85 | 92.37 ± 1.22 | 97.84 ± 5.08 |
| 17,20-Dihydroxyprogesterone | 96.01 ± 1.29 | 99.50 ± 3.22 | 94.70 ± 1.19 | 100.33 ± 5.12 |
| Testosterone | 97.34 ± 2.12 | 100.82 ± 1.17 | 96.24 ± 0.38 | 101.91 ± 3.80 |
| 11-Desoxycorticosterone | 98.53 ± 1.72 | 102.07 ± 1.16 | 95.53 ± 0.44 | 101.15 ± 3.73 |
| Androstenedione | 98.04 ± 0.95 | 101.62 ± 3.68 | 96.12 ± 0.34 | 101.77 ± 3.80 |
| 17-Hydroxyprogesterone | 96.02 ± 2.69 | 99.49 ± 3.07 | 96.53 ± 1.31 | 102.24 ± 4.65 |
| 20-Hydroxyprogesterone | 94.71 ± 1.06 | 98.14 ± 2.57 | 91.85 ± 1.05 | 97.31 ± 5.16 |
| Pregnenolone | 96.45 ± 0.71 | 99.98 ± 3.38 | 95.57 ± 1.32 | 101.25 ± 4.95 |
| Progesterone | 95.03 ± 2.14 | 98.43 ± 1.63 | 93.26 ± 2.06 | 98.68 ± 2.35 |
| 13C-Cortisol | 96.57 ± 2.14 | 100.00 ± 0.00 | 94.58 ± 4.05 | 100.00 ± 0.00 |

**Supplementary Table 3:** (continued)

|  |  |
| --- | --- |
|  | Calibrator 4 |
|  | **Matrix 1 (reaction buffer + stop solution)** | **Matrix 2 (HEK293 cells in reaction buffer + stop solution)** |
| Steroid | **Absolute recovery (%)** | **Relative recovery (%)** | **Absolute recovery (%)** | **Relative recovery (%)** |
| 20-Dihydrocortisol | 97.75 ± 2.56 | 103.14 ± 3.19 | 95.31 ± 2.56 | 105.73 ± 1.06 |
| 20-Dihydrocortisone | 94.50 ± 0.53 | 99.70 ± 1.17 | 94.07 ± 1.91 | 104.37 ± 0.68 |
| 20-Dihydrocortisone | 94.90 ± 1.53 | 100.15 ± 1.45 | 91.43 ± 0.08 | 101.46 ± 1.49 |
| Aldosterone | 92.81 ± 2.36 | 97.91 ± 1.32 | 89.70 ± 2.49 | 99.50 ± 1.20 |
| Cortisol | 95.52 ± 0.65 | 100.78 ± 0.88 | 94.21 ± 1.14 | 104.52 ± 0.84 |
| Cortisone | 96.52 ± 1.58 | 101.82 ± 0.20 | 93.06 ± 2.63 | 103.23 ± 1.07 |
| 20-Dihydrocorticosterone | 94.48 ± 2.21 | 99.68 ± 0.85 | 91.60 ± 0.96 | 101.64 ± 1.17 |
| 20-Dihydro-11-desoxycortisol | 92.24 ± 0.24 | 97.35 ± 1.52 | 89.97 ± 0.56 | 99.83 ± 1.28 |
| Corticosterone | 93.05 ± 1.16 | 98.20 ± 0.39 | 91.22 ± 1.86 | 101.23 ± 0.39 |
| 11-Desoxycortisol | 93.46 ± 1.81 | 98.62 ± 2.38 | 90.45 ± 3.13 | 100.35 ± 1.58 |
| 20-Dihydro-11-desoxycorticosterone | 92.87 ± 1.25 | 97.97 ± 0.49 | 90.40 ± 1.12 | 100.31 ± 1.93 |
| 17,20-Dihydroxyprogesterone | 94.03 ± 2.09 | 99.26 ± 3.42 | 91.54 ± 1.64 | 101.56 ± 0.08 |
| Testosterone | 97.75 ± 5.31 | 103.08 ± 4.75 | 93.91 ± 2.99 | 104.19 ± 3.78 |
| 11-Desoxycorticosterone | 95.34 ± 3.98 | 100.54 ± 2.81 | 88.15 ± 4.20 | 97.74 ± 3.08 |
| Androstenedione | 94.22 ± 2.93 | 99.37 ± 1.79 | 91.61 ± 0.76 | 101.62 ± 1.22 |
| 17-Hydroxyprogesterone | 95.37 ± 1.02 | 100.59 ± 0.60 | 91.10 ± 0.28 | 101.07 ± 1.22 |
| 20-Hydroxyprogesterone | 91.92 ± 2.07 | 96.95 ± 0.70 | 90.58 ± 0.80 | 100.49 ± 2.04 |
| Pregnenolone | 88.37 ± 2.87 | 93.27 ± 3.70 | 82.72 ± 2.77 | 91.79 ± 2.65 |
| Progesterone | 93.68 ± 3.13 | 98.89 ± 4.53 | 87.85 ± 1.70 | 97.47 ± 1.387 |
| 13C-Cortisol | 94.78 ± 1.43 | 100.00 ± 0.00 | 90.13 ± 1.52 | 100.00 ± 0.00 |

**Supplementary Table 3:** (continued)

|  |  |
| --- | --- |
|  | Calibrator 6 |
|  | **Matrix 1 (reaction buffer + stop solution)** | **Matrix 2 (HEK293 cells in reaction buffer + stop solution)** |
| Steroid | **Absolute recovery (%)** | **Relative recovery (%)** | **Absolute recovery (%)** | **Relative recovery (%)** |
| 20-Dihydrocortisol | 96.24 ± 0.91 | 103.06 ± 1.06 | 94.32 ± 0.58 | 102.27 ± 2.24 |
| 20-Dihydrocortisone | 96.13 ± 0.51 | 102.95 ± 0.83 | 94.66 ± 0.39 | 102.67 ± 3.07 |
| 20-Dihydrocortisone | 94.68 ± 1.50 | 101.39 ± 1.79 | 93.50 ± 1.17 | 101.36 ± 1.61 |
| Aldosterone | 95.64 ± 0.42 | 102.42 ± 0.38 | 94.49 ± 0.18 | 102.47 ± 2.67 |
| Cortisol | 95.99 ± 0.46 | 102.78 ± 0.77 | 94.38 ± 0.53 | 102.33 ± 2.64 |
| Cortisone | 94.67 ± 0.37 | 101.38 ±0.23 | 94.08 ± 2.09 | 102.01 ± 2.78 |
| 20-Dihydrocorticosterone | 93.32 ± 0.90 | 99.93 ±0.60 | 90.41 ± 1.15 | 98.01 ± 1.74 |
| 20-Dihydro-11-desoxycortisol | 93.07 ± 1.02 | 99.67 ± 1.21 | 90.18 ± 1.68 | 97.75 ± 1.51 |
| Corticosterone | 93.52 ± 1.35 | 100.14 ± 1.63 | 91.78 ± 0.39 | 99.52 ± 3.09 |
| 11-Desoxycortisol | 95.62 ± 1.96 | 102.42 ± 2.28 | 93.48 ± 2.18 | 101.44 ± 4.84 |
| 20-Dihydro-11-desoxycorticosterone | 91.38 ± 0.62 | 97.85 ± 0.53 | 89.44 ± 1.52 | 96.97 ± 2.74 |
| 17,20-Dihydroxyprogesterone | 92.45 ± 2.80 | 98.99 ± 2.80 | 90.70 ± 0.49 | 98.36 ± 3.11 |
| Testosterone | 94.97 ± 0.31 | 101.71 ± 0.56 | 92.79 ± 0.49 | 100.64 ± 3.00 |
| 11-Desoxycorticosterone | 95.51 ± 0.01 | 102.29 ± 0.32 | 94.14 ± 1.52 | 102.12 ± 3.50 |
| Androstenedione | 93.89 ± 1.18 | 100.55 ± 1.00 | 92.02 ± 1.54 | 99.76 ± 1.34 |
| 17-Hydroxyprogesterone | 92.08 ± 0.67 | 99.68 ± 0.90 | 91.15 ± 1.15 | 98.83 ± 1.60 |
| 20-Hydroxyprogesterone | 90.11 ± 0.33 | 96.49 ± 0.56 | 87.69 ± 0.98 | 95.06 ± 1.86 |
| Pregnenolone | 85.21 ± 2.05 | 91.25 ± 2.34 | 84.00 ± 1.11 | 91.10 ± 3.69 |
| Progesterone | 93.63 ± 0.95 | 100.27 ± 1.22 | 91.04 ± 0.66 | 98.71 ± 2.85 |
| 13C-Cortisol | 93.38 ± 0.33 | 100.00 ± 0.00 | 92.28 ± 2.71 | 100.00 ± 0.00 |

**Supplementary Table 3:** (continued)

|  |  |
| --- | --- |
|  | Calibrator 8 |
|  | **Matrix 1 (reaction buffer + stop solution)** | **Matrix 2 (HEK293 cells in reaction buffer + stop solution)** |
| Steroid | **Absolute recovery (%)** | **Relative recovery (%)** | **Absolute recovery (%)** | **Relative recovery (%)** |
| 20-Dihydrocortisol | 96.61 ± 1.02 | 105.76 ± 1.50 | 94.50 ± 0.96 | 105.27 ± 2.65 |
| 20-Dihydrocortisone | 95.63 ± 1.89 | 104.67 ± 1.48 | 94.41 ± 2.17 | 105.14 ± 2.86 |
| 20-Dihydrocortisone | 93.36 ± 0.58 | 102.21 ± 0.71 | 92.15 ± 1.56 | 102.62 ± 2.11 |
| Aldosterone | 91.94 ± 0.56 | 100.64 ± 0.12 | 93.28 ± 2.26 | 103.84 ± 1.29 |
| Cortisol | 94.90 ± 0.61 | 103.90 ± 1.11 | 92.11 ± 3.06 | 102.52 ± 0.79 |
| Cortisone | 94.26 ± 0.82 | 103.19 ± 0.41 | 92.89 ± 1.88 | 103.43 ± 1.65 |
| 20-Dihydrocorticosterone | 94.26 ± 1.05 | 103.21 ± 1.53 | 91.90 ± 1.09 | 102.37 ± 2.48 |
| 20-Dihydro-11-desoxycortisol | 92.93 ± 1.34 | 101.73 ± 1.83 | 90.94 ± 1.08 | 101.30 ± 2.66 |
| Corticosterone | 93.56 ± 0.39 | 102.41 ± 0.37 | 90.86 ± 3.05 | 101.14 ± 0.96 |
| 11-Desoxycortisol | 95.24 ± 1.27 | 104.26 ± 1.54 | 92.91 ± 3.03 | 103.39 ± 0.53 |
| 20-Dihydro-11-desoxycorticosterone | 92.16 ± 0.38 | 100.90 ± 0.25 | 89.41 ± 2.40 | 99.55 ± 1.59 |
| 17,20-Dihydroxyprogesterone | 96.14 ± 1.05 | 105.24 ± 0.76 | 93.67 ± 2.85 | 104.27 ± 1.49 |
| Testosterone | 92.92 ± 0.57 | 101.73 ± 0.77 | 89.90 ± 1.62 | 100.13 ± 2.02 |
| 11-Desoxycorticosterone | 90.48 ± 1.25 | 99.05 ± 0.75 | 89.96 ± 3.52 | 100.12 ± 0.19 |
| Androstenedione | 92.25 ± 1.96 | 100.98 ± 1.52 | 90.20 ± 4.50 | 100.36 ± 2.38 |
| 17-Hydroxyprogesterone | 94.56 ± 0.28 | 103.51 ± 0.30 | 90.81 ± 3.24 | 101.08 ± 1.35 |
| 20-Hydroxyprogesterone | 90.79 ± 0.83 | 99.40 ± 1.10 | 87.41 ± 2.26 | 97.32 ± 1.36 |
| Pregnenolone | 85.92 ± 3.86 | 94.05 ± 3.43 | 83.32 ± 4.31 | 92.71 ± 2.37 |
| Progesterone | 90.76 ± 0.98 | 99.36 ± 0.54 | 88.08 ± 1.37 | 98.12 ± 2.34 |
| 13C-Cortisol | 91.35 ± 0.50 | 100.00 ± 0.00 | 89.86 ± 3.57 | 100.00 ± 0.00 |

**Supplementary Table 4:** Absolute intra- and inter-day variations in determination of steroid concentrations at different calibrator concentrations. For steroid concentrations, refer to Supplementary Table 1. n – number of repetitions. For the determination of intra-day variation, the same calibrator samples were injected 9 times at day one of the analysis series (n=9). For the determination of the inter-day variation, the same calibrator samples were analyzed 9 times at day one and at three subsequent days of the analyses series (n=36).

|  |  |  |  |
| --- | --- | --- | --- |
|  | Cal 3 | Cal 5 | Cal 7 |
| Steroid | **Intra-day** **variation (%)****n=9** | **Inter-day** **variation (%)****n=36** | **Intra-day** **variation (%)****n=9** | **Inter-day** **variation (%)****n=36** | **Intra-day** **variation (%)****n=9** | **Inter-day** **variation (%)****n=36** |
| 20-Dihydrocortisol | 3.49 | 5.24 | 1.90 | 1.51 | 0.78 | 1.29 |
| 20-Dihydrocortisone | 4.73 | 5.14 | 1.65 | 1.78 | 0.79 | 1.21 |
| 20-Dihydrocortisone | 4.42 | 4.98 | 2.14 | 1.49 | 1.05 | 1.49 |
| Aldosterone | 4.10 | 5.71 | 2.13 | 3.23 | 0.93 | 3.59 |
| Cortisol | 2.76 | 3.75 | 2.15 | 1.96 | 0.98 | 2.77 |
| Cortisone | 3.01 | 3.82 | 2.26 | 1.85 | 1.29 | 2.50 |
| 20-Dihydrocorticosterone | 1.73 | 3.20 | 1.12 | 1.34 | 1.46 | 2.37 |
| 20-Dihydro-11-desoxycortisol | 3.20 | 3.79 | 1.53 | 1.47 | 1.34 | 1.91 |
| Corticosterone | 2.63 | 3.00 | 2.92 | 2.49 | 1.85 | 2.65 |
| 11-Desoxycortisol | 4.49 | 4.00 | 1.35 | 1.62 | 1.94 | 3.27 |
| 20-Dihydro-11-desoxycorticosterone | 2.33 | 4.10 | 2.13 | 2.27 | 1.52 | 3.13 |
| 17,20-Dihydroxyprogesterone | 2.72 | 2.94 | 1.70 | 1.70 | 1.79 | 2.56 |
| Testosterone | 4.15 | 6.24 | 2.13 | 3.17 | 2.16 | 4.23 |
| 11-Desoxycorticosterone | 2.34 | 3.46 | 4.75 | 3.39 | 1.62 | 4.26 |
| Androstenedione | 4.33 | 6.25 | 4.47 | 3.61 | 1.85 | 3.76 |
| 17-Hydroxyprogesterone | 2.96 | 3.67 | 2.11 | 2.25 | 1.30 | 2.76 |
| 20-Hydroxyprogesterone | 4.75 | 4.08 | 1.69 | 1.76 | 0.95 | 3.04 |
| Pregnenolone | 5.84 | 5.94 | 5.40 | 4.57 | 0.97 | 5.52 |
| Progesterone | 3.68 | 5.58 | 2.42 | 2.77 | 2.05 | 3.65 |
| 13C-Cortisol | 2.92 | 3.78 | 2.13 | 2.24 | 1.22 | 2.81 |

**Supplementary Table 5:** Relative intra- and inter-day variation in the determination of steroid concentrations at different calibrator concentrations. Relative intra- and inter-assays variation were obtained by correcting by the internal standard. For steroid concentrations, refer to Supplementary Table 1. n – number of repetitions. For the determination of intra-day variation, the same calibrator samples were injected 9 times at day one of the analysis series (n=9). For the determination of the inter-day variation, the same calibrator samples were analyzed 9 times at day one and at three subsequent days of the analyses series (n=36).

|  |  |  |  |
| --- | --- | --- | --- |
|  | Cal 3 | Cal 5 | Cal 7 |
| Steroid | **Intra-day** **variation (%)****n=9** | **Inter-day** **variation (%)****n=36** | **Intra-day** **variation (%)****n=9** | **Inter-day** **variation (%)****n=36** | **Intra-day** **variation (%)****n=9** | **Inter-day** **variation (%)****n=36** |
| 20-Dihydrocortisol | 2.20 | 3.23 | 2.85 | 2.47 | 1.28 | 2.33 |
| 20-Dihydrocortisone | 3.86 | 4.45 | 2.18 | 2.37 | 1.50 | 2.41 |
| 20-Dihydrocortisone | 3.42 | 3.17 | 1.92 | 2.55 | 1.38 | 2.61 |
| Aldosterone | 2.77 | 2.82 | 2.17 | 2.36 | 0.95 | 2.36 |
| Cortisol | 1.65 | 1.48 | 2.06 | 1.68 | 0.81 | 1.63 |
| Cortisone | 2.25 | 2.29 | 1.99 | 2.21 | 1.81 | 2.39 |
| 20-Dihydrocorticosterone | 4.16 | 3.71 | 2.28 | 2.26 | 1.26 | 2.26 |
| 20-Dihydro-11-desoxycortisol | 3.75 | 3.04 | 1.67 | 2.30 | 0.82 | 2.72 |
| Corticosterone | 3.59 | 3.46 | 2.81 | 2.61 | 1.73 | 2.42 |
| 11-Desoxycortisol | 4.86 | 4.09 | 2.16 | 2.37 | 1.93 | 2.56 |
| 20-Dihydro-11-desoxycorticosterone | 2.83 | 3.76 | 1.56 | 2.76 | 1.53 | 2.92 |
| 17,20-Dihydroxyprogesterone | 2.98 | 4.63 | 1.94 | 2.24 | 1.26 | 2.65 |
| Testosterone | 3.93 | 4.22 | 2.15 | 2.68 | 1.59 | 2.74 |
| 11-Desoxycorticosterone | 2.77 | 3.49 | 4.26 | 2.82 | 1.63 | 3.04 |
| Androstenedione | 5.04 | 4.57 | 3.95 | 3.84 | 2.24 | 2.76 |
| 17-Hydroxyprogesterone | 3.01 | 4.35 | 2.83 | 2.63 | 1.55 | 2.92 |
| 20-Hydroxyprogesterone | 3.67 | 4.37 | 2.33 | 2.51 | 0.69 | 2.94 |
| Pregnenolone | 5.52 | 6.66 | 4.53 | 1.95 | 1.36 | 5.20 |
| Progesterone | 3.70 | 3.98 | 3.33 | 3.03 | 1.89 | 2.82 |
| 13C-Cortisol | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

**Supplementary Table 6:** Important parameters for steroid quantification: individual linear equations and linearity resulting from calibrator’s linear regression, as well as LOD, and LLOQ values for all analyzed steroids.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Steroid | Linear regressiony = ax + c | Linearityr | LOD[ng/mL] | LLOQ[ng/mL] |
| 20-Dihydrocortisol | y = 0.0101x + 0.000069 | 0.9998 | 0.35 | 1.4 |
| 20-Dihydrocortisone | y = 0.19x + 0.000335 | 0.9998 | 0.006 | 0.05 |
| 20-Dihydrocortisone | y = 0.241x + 0.000683 | 0.9998 | 0.008 | 0.03 |
| Aldosterone | y = 0.03x + 0.00334 | 0.9998 | 0.344 | 1.375 |
| Cortisol | y = 0.0799x + 0.00339 | 0.9998 | n.d. | 0.043 |
| Cortisone | y = 0.286x + 0.000886 | 0.9999 | 0.006 | 0.025 |
| 20-Dihydrocorticosterone | y = 0.0465x + 0.000325 | 0.9999 | 0.07 | 0.28 |
| 20-Dihydro-11-desoxycortisol | y = 0.13x – 0.00078 | 0.9994 | 0.025 | 0.1 |
| Corticosterone | y = 0.057x + 0.000805 | 0.9998 | 0.018 | 0.07 |
| 11-Desoxycortisol | y = 0.145x + 0.00147 | 0.9998 | 0.006 | 0.05 |
| 20-Dihydro-11-desoxycorticosterone | y = 0.198x + 0.000746 | 0.9998 | 0.015 | 0.06 |
| 17,20-Dihydroxyprogesterone | y = 0.18x + 0.000767 | 0.9998 | 0.008 | 0.06 |
| Testosterone | y = 0.459x + 0.00112 | 0.9998 | 0.003 | 0.04 |
| 11-Desoxycorticosterone | y = 0.166x + 0.00084 | 0.9998 | 0.009 | 0.075 |
| Androstenedione | y = 0.24x + 0.0018 | 0.9995 | 0.006 | 0.05 |
| 17-Hydroxyprogesterone | y = 0.167x + 0.000735 | 0.9998 | 0.021 | 0.085 |
| 20-Hydroxyprogesterone | y = 0.212x + 0.0083 | 0.9998 | 0.009 | 0.035 |
| Pregnenolone | y = 0.00613x + 0.00203 | 0.9998 | 0.219 | 1.75 |
| Progesterone | y = 0.286x + 0.00377 | 0.9999 | 0.004 | 0.06 |

n.d., not determined. LOD was not reached in the experimental set-up.

**Supplementary Table 7:** Absolute and relative matrix effects at three different calibrator concentrations. Per calibrator, three replicates were analyzed (n=3). Relative matrix influence was obtained by correcting by the internal standard. For steroid concentrations, refer to Supplementary Table 1.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Calibrator 3 | Calibrator 4 | Calibrator 6 |
| Steroid | **Absolute peak****area (%)** | **Relative peak** **area (%)** | **Absolute Peak****area (%)** | **Relative peak****area (%)** | **Absolute peak****area (%)** | **Relative peak****area (%)** |
| 20-Dihydrocortisol | 97.93 ± 1.63 | 98.83 ± 2.03 | 103.89 ± 4.77 | 103.46 ± 3.24 | 103.93 ± 0.37 | 102.84 ± 2.18 |
| 20-Dihydrocortisone | 101.64 ± 0.68 | 102.56 ± 0.34 | 99.73 ± 1.44 | 99.36 ± 0.67 | 101.47 ± 0.82 | 100.39 ± 1.75 |
| 20-Dihydrocortisone | 98.85 ± 1.25 | 99.77 ± 2.26 | 100.01 ± 1.50 | 99.64 ± 0.81 | 100.75 ± 0.67 | 99.69 ± 2.33 |
| Aldosterone | 99.54 ± 0.85 | 100.45 ± 0.36 | 98.17 ± 1.44 | 97.81 ± 0.77 | 100.81 ± 1.14 | 99.75 ± 2.24 |
| Cortisol | 96.88 ± 0.19 | 97.77 ± 0.88 | 100.21 ± 2.54 | 99.82 ± 0.51 | 101.36 ± 0.37 | 100.28 ± 2.05 |
| Cortisone | 101.30 ± 2.10 | 102.24 ± 2.83 | 99.69 ± 1.62 | 99.33 ± 0.98 | 99.87 ± 0.25 | 98.82 ± 2.16 |
| 20-Dihydrocorticosterone | 98.20 ± 2.46 | 99.12 ± 3.30 | 99.03 ± 0.80 | 98.68 ± 1.60 | 100.42 ± 0.45 | 99.35 ± 1.61 |
| 20-Dihydro-11-desoxycortisol | 98.76 ± 1.48 | 99.66 ± 1.31 | 98.91 ± 1.49 | 98.55 ± 1.68 | 99.43 ± 0.20 | 98.38 ± 1.71 |
| Corticosterone | 101.64 ± 3.40 | 102.60 ± 4.20 | 100.29 ± 0.99 | 99.95 ± 2.37 | 100.24 ± 1.10 | 99.17 ± 1.93 |
| 11-Desoxycortisol | 101.67 ± 2.96 | 102.61 ± 3.35 | 98.80 ± 1.73 | 98.45 ± 2.17 | 100.89 ± 1.43 | 99.84 ± 2.80 |
| 20-Dihydro-11-desoxycorticosterone | 99.64 ± 3.40 | 100.59 ± 4.38 | 99.97 ± 0.97 | 99.64 ± 2.36 | 100.15 ± 0.76 | 99.10 ± 2.32 |
| 17,20-Dihydroxyprogesterone | 100.08 ± 0.62 | 101.00 ± 1.62 | 97.78 ± 0.26 | 97.45 ± 1.89 | 99.75 ± 0.36 | 98.69 ± 2.15 |
| Testosterone | 100.79 ± 2.32 | 101.74 ± 3.32 | 100.48 ± 0.49 | 100.15 ± 2.37 | 100.40 ± 0.37 | 99.33 ± 1.69 |
| 11-Desoxycorticosterone | 98.58 ± 1.53 | 99.50 ± 2.35 | 100.22 ± 1.28 | 99.87 ± 1.68 | 103.75 ± 0.96 | 102.66 ± 2.27 |
| Androstenedione | 100.31 ± 1.37 | 101.24 ± 2.15 | 100.93 ± 3.32 | 100.53 ± 2.07 | 99.63 ± 0.46 | 98.58 ± 1.86 |
| 17-Hydroxyprogesterone | 100.45 ± 0.63 | 101.37 ± 1.62 | 99.37 ± 1.63 | 99.03 ± 2.59 | 99.88 ± 1.45 | 98.80 ± 0.46 |
| 20-Hydroxyprogesterone | 100.13 ± 1.68 | 101.03 ± 0.73 | 100.59 ± 1.76 | 100.22 ± 0.86 | 100.95 ± 1.02 | 99.88 ± 2.12 |
| Pregnenolone | 100.82 ± 0.32 | 101.75 ± 1.13 | 98.89 ± 0.78 | 98.55 ± 2.12 | 102.09 ± 0.67 | 101.00 ± 1.69 |
| Progesterone | 100.22 ± 1.42 | 101.15 ± 2.42 | 100.36 ± 1.16 | 100.00 ± 1.43 | 100.31 ± 0.67 | 99.26 ± 2.36 |
| 13C-Cortisol | 99.10 ± 1.03 | 100.00 ± 0.00 | 100.39 ± 2.11 | 100.00 ± 0.00 | 101.11 ± 1.89 | 100.00 ± 0.00 |



**Supplementary Figure 1:** Extracted ion chromatograms for cortisone, 20-dihydrocortisone, and the internal standard 13C-cortisol from exemplary enzyme assays using cortisone as substrate. Representative single ion chromatograms for the steroids 20-dihydrocortisone, cortisone, and the internal standard 13C-cortisol from the dataset of the enzyme assay with the substrate cortisone (Fig. 3 G of the main text) are shown for the following samples: Calibrator 4 (Panel A), zebrafish 20-HSD type 2 (B), tilapia 20-HSD type 2 (C), and untransfected HEK293 cells (D). Peaks identifiers: 1 – 20-dihydrocortisone, 2 – 20-dihydrocortisone, 3 – isobaric interference in the MRM of cortisone by aldosterone, 4 – internal standard 13C-cortisol, 5 – cortisone. The asterisk indicates the product peak of the zebrafish 20-HSD type 2 assays, which had the same retention time and peak shape as the reference peak of 20-dihydrocortisone in Calibrator 4. However, the product peak produced by zebrafish 20-HSD type 2 was in all experiments larger than expected and harbors most probably a second enzymatic product beside 20-dihydrocortisone.