**Supplemental Table 1.** The HarmoSter Consortium and major features of the LC-MS/MS methods.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Laboratory code (role)** | **Laboratory, Department, Institution** | **Laboratory**  **purpose** | **Method**  **development** | **Reference** | **Accreditatioa** | **Participation in EQA surveya, b** |
| **A**  **(Recruiting Center)** | Unit of Endocrinology and Prevention and Care of Diabetes, Department of Medical and Surgical Science, S.Orsola Hospital, University of Bologna, Bologna, Italy | n.a. | n.a. | n.a. | n.a. |  |
| **B**  **(Measuring Center)** | Center for Applied Biomedical Research, Department of Medical and Surgical Science, S.Orsola Hospital, University of Bologna, Bologna, Italy | Research | LDT | 38; 39 | ISO 9001c | no |
| **C**  **(Measuring Center)** | Institute for Laboratory Medicine, Ludwig Maximilians University Hospital, Munich, Germany | Research | LDT | 40 | ISO 17025 and 15189c | no |
| **D**  **(Measuring Center)** | Institute for Clinical Chemistry and Laboratory Medicine, University Hospital and Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany | Research | LDT | 41 | ISO 17189c | no |
| **E**  **(Measuring Center)** | Department of Pediatrics and Adolescent Medicine, Erlangen University Hospital, Erlangen, Germany | Research and clinic | LDT | 42 | ISO 15189 | SKML for serum corticosterone and 11-deoxycortisol |
| **Fd**  **(Measuring Center)** | Department of Clinical Biochemistry, University Hospital South Manchester, Manchester, United Kingdom | Research and clinic | n.a. | n.a. | n.a. | n.a. |
| **G**  **(Measuring Center)** | Endocrine Laboratory, Department of Clinical Chemistry, Amsterdam UMC, Amsterdam, The Netherlands | Research and clinic | LDT | n.a. | ISO 15189 | SKML for serum corticosterone and 11-deoxycortisol |
| **H**  **(Measuring Center)** | Department of Clinical Chemistry, Canisius-Wilhelmina Hospital, Nijmegen, The Netherlands | Research | LDT | n.a. | ISO 15189c | no |
| **I**  **(Measuring Center)** | Clinical Chemistry Laboratory, Lausanne University Hospital CHUV, Lausanne, Switzerland | Clinic | LDT | n.a. | ISO 17189 | no |
| **L**  **(Measuring Center)** | Institute of Laboratory Medicine EOLAB, Laboratory of Clinical Biochemistry & Pharmacology, Ente Ospedaliero Cantonale, Bellinzona, Switzerland | Clinic | MassChrom®, panel 1, Chromsystems | e | ISO 15189c | no |

N.a.: not applicable. LDT: laboratory-developed test. a Information referred to the time period of the HarmoSter measurements, late 2018 – beginning 2019; b information limited to the three analytes under investigation; c The LC-MS/MS steroid measurement under investigation is not listed in the accredited measurement procedures; d Not involved in the present study; e https://chromsystems.com/en/products/steroids/masschromr-steroids-in-serum-plasma-with-96-spe-well-plate-lc-ms-ms-72072-96%20&%2072072-480.html

**Supplemental Table 2-4.** These are shown in a separate excel file.

**Supplemental Table 5**. Passing-Bablok analyses of steroid measurements from each laboratory *vs* the medians of all laboratories as a function of the calibration system.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Analyte** | **Lab** | ***In house* calibration** | | | | **External calibration** | | | |
|  | **N** | **r (95CI)** | **Slope (95CI)** | **Intercept (95CI)** | **N** | **r (95CI)** | **Slope (95CI)** | **Intercept (95CI)** |
| Corticosterone | B | 78 | 0.998 (0.997 – 0.999) | 1.000 (0.993 - 1.006) | -0.090 (-0.164 - -0.024) | 78 | 0.998 (0.997 – 0.999) | 0.989 (0.982 - 1.000) | 0.173 (0.045 - 0.262) |
|  | C | 78 | 0.929 (0.891 - 0.955) | 1.000 (0.986 - 1.025) | -0.115 (-0.442 - 0.065) | 78 | 0.924 (0.884 - 0.951) | 0.930 (0.907 - 0.955) | 0.055 (-0.298 - 0.391) |
|  | D | 78 | 0.994 (0.991 - 0.996) | 0.968 (0.948 - 0.985) | 0.777 (0.560 – 0.989) | 78 | 0.994 (0.990 - 0.996) | 0.867 (0.852 - 0.882) | 0.423 (0.274 – 0.568) |
|  | E | 78 | 0.996 (0.993 - 0.997) | 1.072 (1.058 - 1.093) | 0.427 (0.646 – 0.256) | 78 | 0.994 (0.991 - 0.996) | 1.067 (1.048 - 1.084) | -0.197 (0.398 – 0.009) |
|  | G | 78 | 0.997 (0.995 - 0.998) | 0.996 (0.981 - 1.009) | -0.051 (-0.239 – 0.094) | 78 | 0.995 (0.992 - 0.997) | 0.914 (0.894 - 0.939) | 0.037 (-0.177 – 0.281) |
|  | H | 78 | 0.977 (0.964 - 0.985) | 0.993 (0.958 - 1.039) | -0.634 (-1.039 - -0.214) | 78 | 0.981 (0.970 - 0.988) | 1.071 (1.037 - 1.120) | -0.572 (-1.029 - -0.294) |
|  | I | 75 | 0.993 (0.988 - 0.995) | 0.957 (0.944 - 0.969) | -0.163 (-0.294 - -0.002) | 75 | 0.995 (0.992 - 0.997) | 1.024 (1.015 - 1.038) | -0.116 (-0.256 - 0.038) |
|  | L | 78 | 0.992 (0.987 - 0.995) | 1.153 (1.131 - 1.180) | -0.394 (-0.708 – 0.117) | 78 | 0.992 (0.987 - 0.995) | 1.239 (1.203 - 1.262) | -0.351 (-0.667 – -0.061) |
| 11-  Deoxycortisol | B | 72 | 0.990 (0.984 – 0.994) | 1.028 (1.000 - 1.074) | 0.022 (-0.005 - 0.040) | 72 | 0.989 (0.982 – 0.993) | 1.136 (1.113 - 1.167) | -0.014 (-0.035 - 0.006) |
| C | 63 | 0.984 (0.974 - 0.990) | 1.035 (1.003 - 1.076) | -0.031 (-0.063 - -0.003) | 63 | 0.965 (0.942 - 0.979) | 0.891 (0.854 - 0.930) | 0.023 (-0.019 - 0.043) |
| D | 69 | 0.977 (0.963 - 0.986) | 0.918 (0.868 - 0.957) | 0.025 (-0.005 – 0.055) | 69 | 0.979 (0.965 - 0.987) | 1.000 (0.954 - 1.026) | -0.040 (-0.064 – -0.014) |
|  | E | 67 | 0.964 (0.941 - 0.978) | 0.917 (0.856 - 0.968) | -0.033 (-0.066 – 0.008) | 67 | 0.962 (0.938 - 0.976) | 0.939 (0.877 - 1.000) | -0.039 (-0.080 – -0.005) |
|  | G | 13 | 0.933 (0.785 - 0.980) | 1.083 (1.000 - 1.206) | -0.171 (-0.452 – 0.010) | 13 | 0.940 (0.806 - 0.982) | 0.993 (0.958 - 1.077) | -0.042 (-0.220 – 0.058) |
|  | H | 72 | 0.992 (0.986 - 0.995) | 0.918 (0.901 - 0.938) | 0.026 (0.013 - 0.041) | 72 | 0.990 (0.985 - 0.994) | 1.000 (1.000 - 1.015) | 0.000 (-0.006 - 0.000) |
|  | I | 70 | 0.993 (0.989 - 0.996) | 1.121 (1.100 - 1.152) | -0.063 (-0.083 - -0.049) | 70 | 0.993 (0.989 - 0.996) | 1.032 (1.009 - 1.057) | -0.025 (-0.040 - -0.007) |
|  | L | 72 | 0.959 (0.936 - 0.974) | 1.080 (1.027 - 1.132) | 0.029 (-0.010 – 0.066) | 72 | 0.968 (0.949 - 0.980) | 1.067 (1.017 - 1.118) | 0.027 (-0.016 – 0.059) |
| Cortisone | B | 78 | 0.986 (0.979 – 0.991) | 1.028 (1.000 - 1.074) | -2.63 (-4.65 - -0.34) | 78 | 0.990 (0.985 – 0.994) | 1.032 (1.009 - 1.059) | -1.25 (-2.88 - -0.00) |
| C | 78 | 0.961 (0.939 - 0.975) | 1.011 (0.962 - 1.063) | -1.26 (-4.14 - 1.44) | 78 | 0.960 (0.938 - 0.974) | 1.047 (0.998 - 1.104) | 0.79 (-2.40 - 3.60) |
|  | D | 78 | 0.971 (0.954 - 0.981) | 0.916 (0.875 - 0.958) | 3.38 (1.05 – 5.69) | 78 | 0.967 (0.949 - 0.979) | 0.869 (0.829 - 0.920) | 4.68 (1.94 – 6.72) |
|  | E | 78 | 0.984 (0.975 - 0.990) | 1.035 (1.000 - 1.069) | -2.84 (-4.59 – -1.01) | 78 | 0.990 (0.984 - 0.994) | 1.004 (0.974 - 1.032) | -1.58 (-3.14 – 0.02) |
|  | G | 78 | 0.973 (0.957 - 0.983) | 1.124 (1.067 - 1.176) | -3.43 (-6.29 – 0.18) | 78 | 0.983 (0.974 - 0.989) | 1.045 (1.009 - 1.084) | -1.14 (-3.32 – 0.56) |
|  | H | 78 | 0.913 (0.867 - 0.944) | 1.264 (1.149 - 1.395) | -13.12 (-20.7 - -6.90) | 78 | 0.873 (0.808 - 0.918) | 1.017 (0.907 - 1.136) | -9.19 (-16.65 - -3.18) |
|  | I | 75 | 0.977 (0.964 - 0.985) | 1.118 (1.060 - 1.173) | -4.17 (-7.11 - -0.97) | 75 | 0.988 (0.981 - 0.993) | 1.030 (1.002 - 1.064) | -1.67 (-3.58 - 0.11) |
|  | L | 78 | 0.889 (0.831 - 0.928) | 0.997 (0.899 - 1.105) | 2.41 (-3.36 – 7.60) | 78 | 0.883 (0.822 - 0.924) | 0.993 (0.892 - 1.110) | 3.96 (-2.62 – 9.36) |

**Supplemental Table 6.** Distribution of bias *vs* median of all laboratories within each laboratory as function of the calibration system.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Analyte** | **Laboratory** | **N** | **% Bias with *in house* calibration** | | **% Bias with external calibration** | | **Wilcoxon** | **F test** |
|  |  |  | **Median**  **(2.5 - 97.5 c)** | **Variance** | **Median**  **(2.5 - 97.5 c)** | **Variance** | **P value** | **P value** |
| **Corticosterone** | B | 78 | -1.0 (-6.4 - 3.1) | 5.3 | 0.6 (-3.5 - 5.8) | 6.7 | < 0.001 | 0.305 |
|  | C | 78 | -0.9 (-57.7 - 8.3) | 277.5 | -6.7 (-58.6 - 7.1) | 263.1 | < 0.001 | 0.815 |
|  | D | 78 | 3.0 (-7.0 - 22.2) | 59.9 | -10.1 (-17.6 - 3.4) | 33.0 | < 0.001 | 0.010 |
|  | E | 78 | 3.9 (-2.5 - 16.4) | 22.0 | 5.0 (-2.2 - 15.4) | 21.8 | 0.004 | 0.971 |
|  | G | 78 | -0.9 (-10.0 - 5.7) | 16.3 | -8.2 (-18.1 - -0.1) | 23.9 | < 0.001 | 0.096 |
|  | H | 78 | -4.9 (-20.8 - 20.9) | 137.2 | 2.4 (-12.0 - 33.4) | 149.9 | < 0.001 | 0.698 |
|  | I | 75 | -5.6 (-17.4 - 0.8) | 20.1 | 1.7 (-8.4 - 11.0) | 17.7 | < 0.001 | 0.584 |
|  | L | 78 | 12.3 (-0.6 - 37.1) | 90.2 | 19.5 (3.3 - 47.2) | 102.1 | < 0.001 | 0.587 |
| **11-Deoxycortisol** | B | 72 | 6.9 (-0.3 - 19.3) | 31.1 | 12.0 (0.4 - 23.3) | 39.4 | < 0.001 | 0.325 |
| C | 63 | 0.0 (-32.6 - 11.5) | 87.7 | -9.4 (-29.3 - 5.9) | 72.6 | < 0.001 | 0.458 |
| D | 69 | -4.0 (-21.4 – 10.0) | 57.5 | -6.1 (-23.9 - 2.8) | 59.0 | 0.003 | 0.918 |
|  | E | 64 | -14.6 (-29.5 – 0.0) | 58.2 | -13.9 (-26.7 – 2.5) | 60.4 | < 0.001 | 0.886 |
|  | G | 13 | 0.2 (-10.9 - 19.3) | 51.3 | -2.0 (-7.8 - 13.4) | 26.5 | 0.017 | 0.266 |
|  | H | 72 | -3.8 (-12.4 - 7.2) | 19.7 | 0.6 (-4.1 - 13.1) | 13.3 | < 0.001 | 0.101 |
|  | I | 70 | 2.6 (-20.2 – 20.0) | 64.5 | 0.0 (-19.0 - 10.4) | 35.1 | < 0.001 | 0.012 |
|  | L | 72 | 12.4 (-3.4 - 55.1) | 188.3 | 10.2 (-3.5 - 38.9) | 144.4 | < 0.001 | 0.265 |
| **Cortisone** | B | 78 | -4.0 (-9.4 - 0.9) | 8.9 | 0.9 (-2.6 - 6.7) | 5.3 | < 0.001 | 0.025 |
| C | 78 | -1.1 (-9.5 - 7.0) | 22. 3 | 5.8 (-0.2 - 19.0) | 29.8 | < 0.001 | 0.202 |
|  | D | 78 | -2.0 (-8.8 - 7.9) | 15.0 | -4.7 (-11.4 - 5.9) | 17.9 | < 0.001 | 0.449 |
|  | E | 78 | -2.0 (-10.0 - 1.9) | 9.3 | -2.6 (-8.1 - 0.8) | 5.6 | 0.170 | 0.029 |
|  | G | 78 | 6.5 (-0.9 - 14.3) | 16.0 | 1.9 (-3.0 - 10.0) | 8.8 | < 0.001 | 0.009 |
|  | H | 75 | 1.6 (-17.1 - 18.2) | 89.5 | -14.8 (-32.8 - -0.7) | 75.7 | < 0.001 | 0.464 |
|  | I | 78 | 4.2 (-6.8 - 12.2) | 20.9 | 0.0 (-11. 3 - 4.7) | 14.4 | < 0.001 | 0.111 |
|  | L | 78 | 3.9 (-15.6 - 22.7) | 77.5 | 6.6 (-13.4 - 28.0) | 94.2 | < 0.001 | 0.395 |

N: number of samples.

**Supplemental Discussion**

A contribute to intra-assay imprecision may come from the number of data points used for peak definition, which, when below 15-20, may cause poor peak definition and inaccurate quantification [30]. Unfortunately, this aspect has not directly been evaluated in the present study.

IS labelling can influence analyte quantitation in different ways. ISs with only 2 amu gain are prone to cause inaccuracy at high analyte levels due to isotopic distribution. Among methods under investigation, the two using D2-isotopes did not show linearity loss or relevant proportional bias, suggesting that a careful optimization of IS concentration can minimize this problem. Furthermore, depending on LC conditions, the deuterium count can slightly alter the retention time of the IS, so that matrix interferences might differentially impact IS and analyte. In our methods, the retention time of analyte and respective IS differed of 0.02 to 0.20 min; however, no relationship can be inferred with bias reported for individual laboratories. In these regards, whether the different elution affects the analyte quantitation, depends on the type of sample preparation and on sample-specific factors, both potentially determining the systematic or occasional presence of interferences. Finally, another possible issue may derive by deuterium label stability in experimental conditions. Indeed, deuterium in certain locations may be prone to exchange with hydrogen, thereby altering the IS m/z and the final quantitation [33].