

Supplementary Table S1. Retention times and monitored ions of the analytes and internal standards (LC-HRMS).

Ret time [min]	Compound	Target ion	m/z	Internal standard	m/z
2.2	6 β -hydroxycortisol	[M+H] ⁺	379.2115	6 β -hydroxycortisol-d4	383.2366
4.7	18-hydroxycortisol	[M+H] ⁺	379.2115	18-hydroxycortisol-d4	383.2366
6.8	Cortisol	[M+H] ⁺	363.2166	Cortisol-d4	367.2417
6.9	Cortisone	[M+H] ⁺	361.2010	Cortisone-d8	369.2512

Supplementary Table S2. Recovery of added steroids in human urine (n=3), GC-MS method.

Steroid	Urine (µg/L)	Added (µg/L)	Obtained (µg/L)	Accuracy (%)
Cortisol	59±19	125	183±31	99±7
Cortisone	67±25	125	193±10	100±5
6β-hydroxycortisol	52±14	125	175±9	98±5
18-hydroxycortisol	219±15	125	353±28	107±8

Supplementary Table S3. Accuracy of added steroids in human urine (n=3), LC-HRMS method.

Steroid	Urine concentration range (µg/L)	Added (µg/L)	Mean recovery (%)
Cortisol	30-76	12.5	91
		25	98
Cortisone	12-34	12.5	107
		25	88
6β-hydroxycortisol	104-192	12.5	96
		25	94
18-hydroxycortisol	34-146	12.5	96
		25	96

Supplementary Table S4. Stability in the autosampler at 8°C (GC-MS method) and room temperature (LC-HRMS method) expressed as accuracy values of quality control and urine samples after 24h and 72 h. QC: quality control.

	GC-MS			LC-HRMS		
	µg/L	24h	72h	µg/L	24h	72h
Cortisol						
QC1	26	105.8	113.5	82	96.4	100.2
QC2	138	100.7	112.6	160	100.8	101.4
Urine	58	106.0	112.1	106	106.3	96.9
Cortisone						
QC1	26	98.1	101.9	82	105.4	102.9
QC2	130	101.2	104.2	164	100.6	99.5
Urine	70	103.6	108.6	134	100.2	97.1
6β-hydroxycortisol						
QC1	26	98.1	96.2	80	100.9	99.3
QC2	135	99.3	100.7	158	102.0	100.9
Urine	224	99.8	100.9	176	96.9	97.7
18-hydroxycortisol						
QC1	25	103.9	103.9	76	96.2	89.1
QC2	132	100.0	98.9	164	95.8	84.9
Urine	54	98.1	100.9	106	93.8	78.5

Supplementary Table S5. Efficiency of the extraction procedure (n=3).

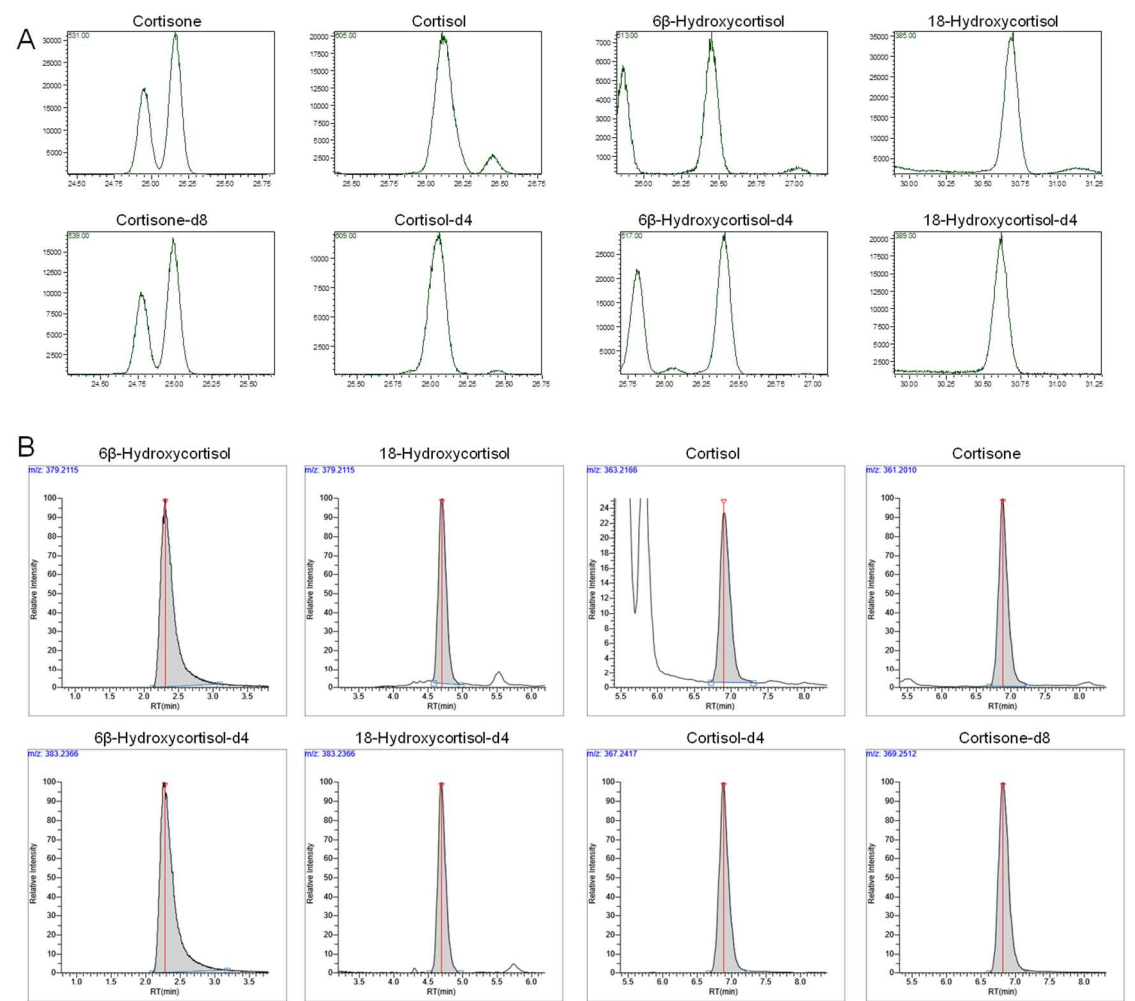
	$\mu\text{g/L}$	Process efficiency (%)
Cortisol		
	12.5	75 \pm 8
	100	51 \pm 6
	250	47 \pm 4
Cortisone		
	12.5	86 \pm 9
	100	53 \pm 4
	250	47 \pm 7
6β-hydroxycortisol		
	12.5	28 \pm 4
	100	10 \pm 8
	250	10 \pm 7
18-hydroxycortisol		
	12.5	42 \pm 5
	100	30 \pm 6
	250	25 \pm 5

Supplementary Table S6. Summary comparison of the characteristics of the LC-HRMS and the GC-MS method validated in this study and the GC-MS method published by Shackleton et al (31).

<u>Characteristic</u>	<u>LC-HRMS</u>	<u>GC-MS</u>	<u>GC-MS (31)</u>
<u>Urine volume</u>	<u>0.5 mL</u>	<u>2 mL</u>	<u>5 mL (*)</u>
<u>Urine extraction</u>	<u>Liquid:liquid with dichlorometane</u>	<u>Liquid:liquid with dichlorometane</u>	<u>C18 solid phase extraction with water and methanol</u>
<u>Internal standards</u>	<u>cortisol-d₄, cortisone-d₈</u> <u>6β-hydroxycortisol-d₄ 18-hydroxycortisol-d₄</u>	<u>cortisol-d₄ cortisone-d₈</u> <u>6β-hydroxycortisol-d₄ 18-hydroxycortisol-d₄</u>	<u>cortisol-d₄ cortisone-d₃ cortisone-d₂ (**)</u> <u>6β-hydroxycortisol-d₂ 18-hydroxycortisol-d₂ (**)</u>
<u>Derivatization</u>	<u>No</u>	<u>methoxyamine hydrochloride (55°C 60 min), BSTFA (2 min, microwave irradiation)</u>	<u>methoxyamine hydrochloride (60°C 60 min), BSTFA (16h, 100°C)</u>
<u>Time of chromatogram</u>	<u>16 minutes</u>	<u>35 minutes</u>	<u>40 minutes</u>
<u>Acquisition</u>	<u>Full scan</u>	<u>Single ion monitoring</u>	<u>Single ion monitoring</u>

(*) Free and total fraction analysis (**) Non commercial

Supplementary Figure S1. Chromatogram of urine samples measured by the GC-MS (A) and LC-HRMS (B) methods.



Supplementary Figure S2. A. Concentration results of the cortisol measurements of the human serum cortisol certified reference material ERM-DA192 by the GC-MS method. **B.** Cortisol spiked curves in human serums (n=3) compared with the respective curves in methanol measured by the GC-MS method.

