TECHNICAL NOTE 73553

Cortisol and cortisone in human urine by LC-MS

Quantification by high-resolution Orbitrap mass spectrometry for clinical research

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Application benefits

- Fast and simple quantitation of cortisol and cortisone in human urine
- Minimal offline sample preparation with protein precipitation followed by dilution and direct injection
- High-resolution mass spectrometry for improved selectivity

Goal

Implementation of an analytical method for the quantification of cortisol and cortisone in human urine for clinical research using a Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap™ mass spectrometer

Introduction

Clinical researchers are interested in investigating cortisol as a clinical marker that may indicate adrenal or pituitary disorders. Elevated cortisol levels can also be associated with exposure to stress. Cortisone, and the ratio of cortisol to cortisone, may add further value. A method for the

quantitation of cortisol and cortisone in human urine is reported in this study. The method is based on protein precipitation using three volumes of acetonitrile followed by injection onto a Thermo Scientific™ UltiMate™ 3000 UHPLC system. Detection was performed using a Q Exactive high-resolution, accurate-mass (HRAM) mass spectrometer operated in Full MS acquisition mode. Method performance was evaluated in terms of linearity of response, analytical accuracy, and intra- and inter-assay precision. A cross-validation of results was conducted against a method performed on a triple quadrupole LC-MS/MS system.

Experimental

Target analytes

Chemical structures of cortisone and cortisol are presented in Figure 1. D_4-cortisol and D_8-cortisone were used as internal standards. The chemical formulas and exact monoisotopic masses are given in Table 1.

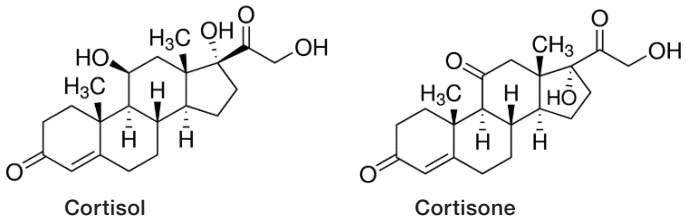

Figure 1. The chemical structure of cortisol and cortisone

Sample preparation

Certified standard solutions were obtained from commercially available sources. Calibrators were prepared at six concentration levels by spiking phosphate-buffered saline (PBS) with known amounts of pure standard solutions to cover a concentration range of 2–1000 nmol/L. PBS was used as surrogate matrix since it is impossible to obtain human urine that does not contain the analytes of interest. 100 µL of sample, calibrator, or control samples were diluted with 300 µL of acetonitrile containing the internal standards. This protein-precipitation-like approach for sample extraction was used to allow for easy adaptation to other matrices, such as serum and saliva. The samples were vortex-mixed for 10 seconds and centrifuged at 10,000 g RCF for 5 min. 200 µL of the supernatant were transferred to an HPLC clean vial and diluted with 600 µL of water. 100 µL was injected onto the LC system.

Liquid chromatography

Liquid chromatography was performed on a binary UltiMate 3000 RSLC system using the following mobile phases:

Mobile phase A: 10 mmol/L ammonium formate + 0.1% formic acid in water

Mobile phase B: Methanol

Chromatographic separation was achieved by isocratic elution on a Thermo Scientific™ Accucore™ C18 3.0 × 50 mm (2.6 µm) analytical column run at 40 °C at a flow rate of 1.0 mL/min using 47% of mobile phase B. After elution of the compounds of interest, at 1.3 min, the flow rate was raised to 2 mL/min and a wash step at 95% of mobile phase B for 0.7 min and an equilibration step at 47% mobile phase B for 0.5 min were performed. The total runtime was 2.5 min. The chromatographic conditions are given in Table 2.

Mass spectrometry

Detection was performed in full scan acquisition mode on a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer, equipped with an atmospheric pressure chemical ionization (APCI) ion source run in positive ion mode. The ion source conditions and mass spectrometry settings are presented in Table 3 and Table 4, respectively.

Table 1. Compounds, chemical composition, and monoisotopic masses.

Compound	Chemical formula	Exact mass $[M+H]^+$
Cortisol	$C_{21}H_{30}O_5$	363.2166
Cortisol-D_4	$C_{21}H_{26}D_4O_5$	367.2417
Cortisone	$C_{21}H_{28}O_5$	361.2010
Cortisone-D_8	$C_{21}H_{20}D_8O_5$	369.2512

Table 2. Gradient profile.

Time (min)	Flow rate (mL/min)	%A	%B
0	1.0	53	47
1.3	1.0	53	47
1.31	2.0	5	95
2	2.0	5	95
2.05	2.0	53	47
2.45	2.0	53	47
2.5	1.0	53	47

Table 3. Ion source settings

Parameter	Setting
Sheath gas flow rate	40 AU
Aux gas flow rate	15 AU
Sweep gas flow rate	3 AU
Discharge current	4.00 µA
Capillary temperature	350 °C
Vaporizer temperature	450 °C
S-lens RF level	50

Table 4. Mass spectrometer settings

General	
Polarity	Positive
In-source CID	—
Full MS	
Scan range	300–400 *m/z*
Resolution	140,000
AGC target	1E+06
Maximum IT	400
Microscans	1
Spectrum data type	Profile

Method evaluation

Method performance was evaluated in terms of linearity of response, accuracy, and intra- and inter-assay precision using test samples prepared by spiking PBS with known amounts of cortisol and cortisone and donor urine samples.

Intra- and inter-assay accuracy and precision results were evaluated from the concentrations determined in the test samples as percent of the nominal concentrations and as the coefficient of variation (%CV).

Intra-assay accuracy and precision results were evaluated in one batch at the concentration levels of the calibrators. Inter-assay accuracy and precision were evaluated in three batches (n=6) in test samples at two levels in the calibrated range.

Donor urine samples were used to investigate reproducibility and to cross-validate this method against a reference method based on LC-MS/MS.

Data analysis

Data was acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software. An extraction window of 5 ppm was used to extract the individual chromatograms. The exact masses of the analytes and internal standards are presented in Table 1.

Results and discussion

The method was linear in the range 2–1000 nmol/L. Results from back-calculated standards are presented in Tables 5 and 6, and calibration curves are presented in Figures 2 and 3 for cortisol and cortisone, respectively. The back-calculated accuracy was 90.5–105.8% for cortisol and 94.1–106.9% for cortisone.

Table 5. Back-calculated calibration standards for cortisol (nmol/L)

Nominal conc.	Back-calculated conc.		Accuracy%		
	Inj 1	Inj 2	Inj 1	Inj 2	Mean
2	2.12	1.99	105.8	99.7	102.7
6	5.47	5.43	91.1	90.5	90.8
20	20.7	20.8	103.5	104.0	103.8
80	79.9	78.5	99.9	98.1	99.0
300	307	304	102.4	101.3	101.9
1000	1013	1024	101.3	102.4	101.9

Table 6. Back-calculated calibration standards for cortisone (nmol/L)

Nominal conc.	Back-calculated conc.		Accuracy%		
	Inj 1	Inj 2	Inj 1	Inj 2	Mean
2	2.02	2.06	101.0	103.0	102.0
6	5.68	5.82	94.6	97.0	95.8
20	18.8	19.0	94.1	94.8	94.4
80	81.4	78.2	101.7	97.8	99.7
300	303	307	100.9	102.2	101.6
1000	1061	1069	106.1	106.9	106.5

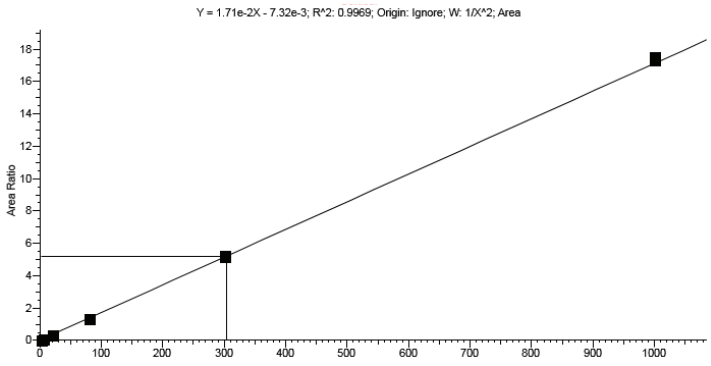

Figure 2. Calibration curve for cortisol

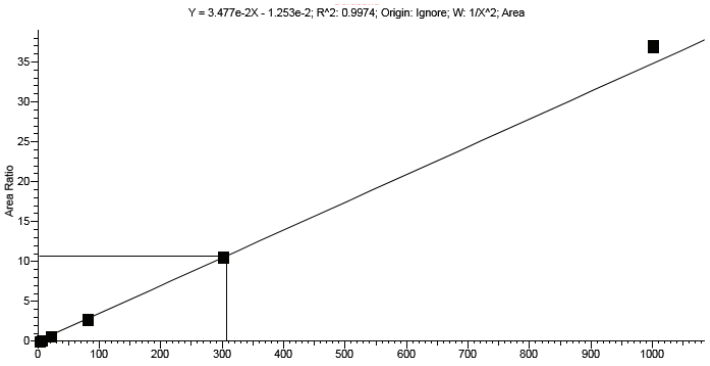

Figure 3. Calibration curve for cortisone

The intra-assay accuracy and precision (%CV) results are presented in Tables 7 and 8. The mean intra-assay accuracy (n=6) ranged from 95.2 to 114% for cortisol and 94.8 to 106% for cortisone. The % CV was 0.6–6.0% for cortisol and 1.2–2.7% for cortisone.

The inter-assay accuracy and precision results are presented in Table 9. The inter-accuracy was 102% for cortisol and 101–108% for cortisone. The inter-assay precision was 5.7–6.2% for cortisol and 3.4–5.4% for cortisone.

The results from the investigation of reproducibility of donor samples (n=3) are presented in Table 10. The %CV (n=6) was between 1.3 and 5.3% for cortisol and between 4.2 and 6.4% for cortisone. Chromatograms from a donor sample containing 42 nmol/L cortisol and 157 nmol/L cortisone are shown in Figures 4 and 5, respectively.

The results from cross-validation to another method based on triple quadrupole LC-MS/MS are presented in Figure 6. The results were in good correlation with a correlation coefficient of 0.9722.

Table 7. Intra-assay accuracy and precision for cortisol (nmol/L) (n=6)

QC level	2	6	20	80	300	1000
Mean	2.28	5.71	20.3	78.8	303	1024
Accuracy%	114	95.2	102	98.5	101	102
CV%	6.0	4.3	1.6	1.6	0.9	0.6

Table 8. Intra-assay accuracy and precision for cortisone (nmol/L) (n=6)

QC level	2	6	20	80	300	1000
Mean	2.1	5.69	19.3	78.5	304	1061
Accuracy%	105	94.8	96	98.1	101	106
CV%	2.6	2.7	1.7	1.2	1.4	1.3

Table 9. Inter-assay accuracy and precision

	Cortisol		Cortisone	
Nominal conc./nmol/L	100	600	70	250
n	18	18	18	18
Mean	102	615	75	254
Mean accuracy%	102	102	108	101
RSD%	6.2	5.7	5.4	3.4

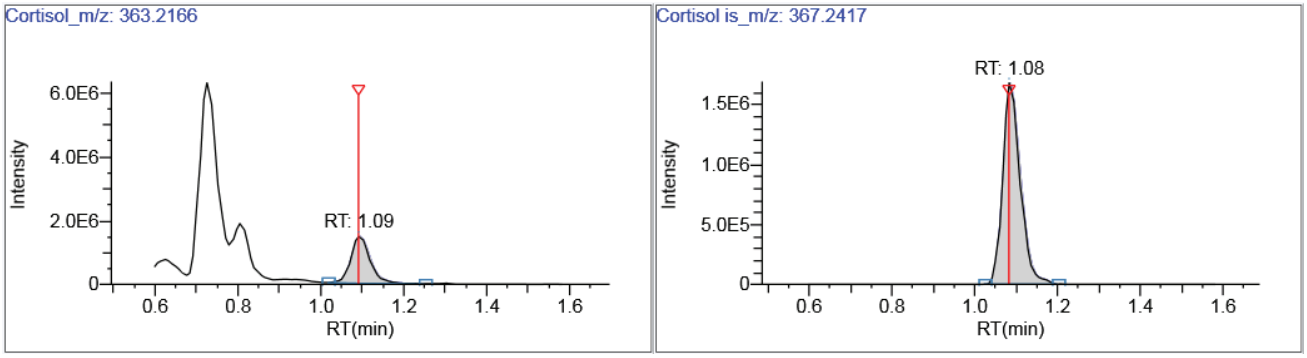

Figure 4. A representative chromatogram for cortisol (in the left trace) and the internal standard (in the right trace) from a donor sample at 56 nmol/L

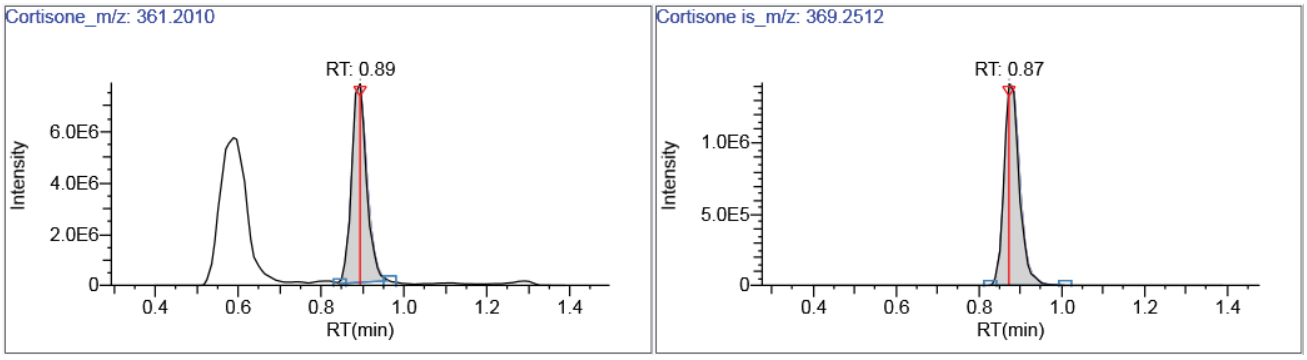

Figure 5. A representative chromatogram for cortisone (in the left trace) and the internal standard (in the right trace) from a donor sample at 157 nmol/L

Table 10. Reproducibility of donor samples

	Cortisol			Cortisone		
Replicate	Urine 1	Urine 2	Urine 3	Urine 1	Urine 2	Urine 3
1	54.6	38.1	82.2	161	149	126
2	56.2	41.7	80	164	157	133
3	56.3	44.2	76.2	173	165	123
4	56.7	44.1	83.5	176	174	137
5	56.2	42.7	78.7	164	166	137
6	55.6	42.0	77.5	158	165	117
Mean	**55.9**	**42.1**	**79.7**	**166**	**163**	**129**
CV%	**1.3**	**5.3**	**3.5**	**4.2**	**5.3**	**6.4**

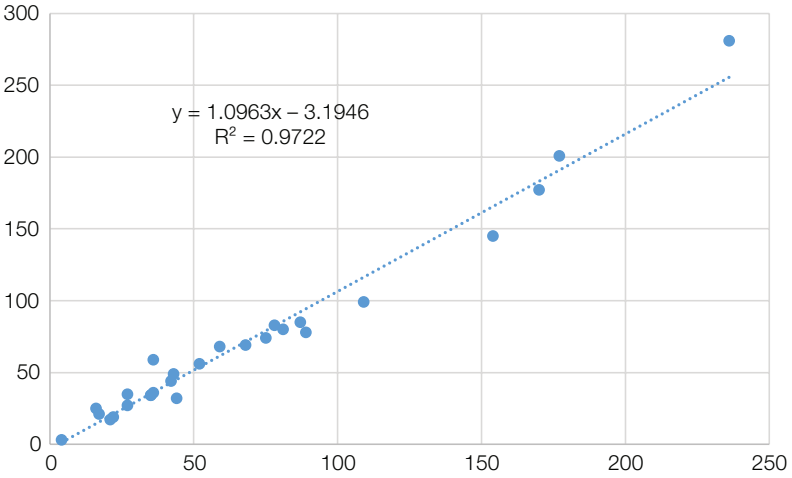

Figure 6. Results from cross-validation against a triple quadrupole LC-MS/MS method

Conclusion

A liquid chromatography-HRAM Orbitrap mass spectrometry method for clinical research was developed and implemented for the quantification of cortisol and cortisone in human urine. The method incorporates a quick and simple offline sample preparation procedure including internal standard addition. The described method meets research laboratory requirements in terms of sensitivity in the range 2–1000 nmol/L, linearity of response, accuracy, and precision.

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