HEALTH SERVICES LABORATORIES

Development and Validation of an LC-MS/MS Method for Quantification of Serum Cortisol and Cortisone in the Clinical Assessment of Adrenal Insufficiency

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Introduction

Adrenal Insufficiency (AI)

AI is a rare disorder affecting approximately 300 per million people in western Europe and the US [1]. The disorder is caused by insufficient cortisol production by the adrenal glands. AI can be further classified as primary (adrenal gland dysfunction), secondary (pituitary dysfunction) or tertiary (hypothalamic dysfunction) [2].

Cortisol Regulation

Cortisol, the primary glucocorticoid produced in the zona fasciculata of the adrenal cortex, is essential for stress response and immune function.

LC-MS/MS

The Short Synacthen Test (SST), measuring serum cortisol response to ACTH, is often used to diagnose AI. Current diagnostic methods rely on immunoassays (IAs), which are prone to interference. Liquid chromatography - tandem mass spectrometry (LC-MS/MS) is a more specific method.

Keywords: Adrenal Insufficiency, Liquid Chromatography-Tandem Mass Spectrometry, Short Synacthen Test

Methods

LC-MS/MS Development

Sample Preparation: Samples were prepared using liquid – liquid extraction (LLE). Protein precipitation (PP) was used for method optimisation.

Instrument Conditions:

Reverse-phase liquid chromatography enabled chromatographic separation. The mobile phase conditions were optimised for cortisol and cortisone separation.

Mass spectrometry (MS) conditions included electrospray ionisation (ESI) with targeted multiple reaction monitoring (MRM) for precise cortisol and cortisone compound detection.

Multiple Reaction Monitoring (MRM) for Specificity: MRM enhanced the specificity of detection and minimised cross reactivity, improving the reliability of detection compared to IA methods.

LC-MS/MS Validation

Precision and Accuracy:

Intra-assay Precision: Determined by analysing three QC levels (low, medium and high) across five consecutive days, with samples prepared in duplicate for each level in a single run (n=5).

Inter-assay Precision: Evaluated by repeating measurements of the low, medium and high IQC levels on different plates over non-consecutive days (n=5) to assess between-assay consistency.

Accuracy: Three samples, which contained cortisol and cortisone were spiked with certified reference material (CRM) standard solutions for cortisol and cortisone.

Recovery: The samples underwent the PP method. All samples were assayed in triplicate. Recovery was calculated by comparing the observed concentration and the expected concentration.

Sensitivity: The lowest cortisol/cortisone concentration, with an acceptable coefficient of variance (CV) of <20% and signal to noise (S/N) ratio (>10), was established by serially diluting a serum sample with a low cortisol/cortisone concentration five times, with water. Sample aliquots were injected four times to calculate the CV.

Linearity and Carryover: A highly concentrated cortisol sample was diluted to solutions of 1:3, 1:5, 1:9 and 1:11, with 50:50 methanol.

Samples were pipetted in a random order to monitor the carryover between highly concentrated and blank samples.

Method Comparison: Serum samples that had previously been analysed on the Roche Cobas® e602 (IA) analyser at The Halo Building , were anonymised and analysed by the developed LC-MS/MS method (n=40).

Results & Discussion

LC-MS/MS Development

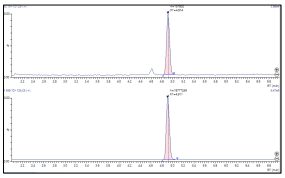


Figure 1| Cortisol LC-MS/MS Conditions
Chromatogram of cortisol (top) and its internal standard (bottom), showing retention times. Pre-peak interference present for cortisol.

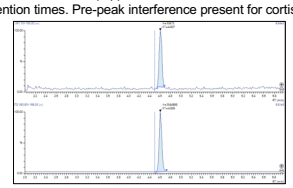


Figure 2| Cortisone LC-MS/MS Conditions
Chromatogram of cortisone (top) and its internal standard (bottom), showing retention times.

LC-MS/MS Validation

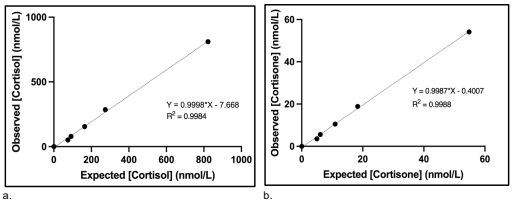
Precision and Accuracy (Recovery):

Intra-assay precision was acceptable for cortisol and cortisone QC samples.

High intra-/inter- assay precision was demonstrated, with coefficients of variation (CV%) between 3.7% - 7.8% for cortisol and cortisone. Cortisol and cortisone recoveries were between 90.3% - 110.5%.

Sensitivity: The lower limit of quantification (LLOQ) was determined to be 11 nmol/L for cortisol and 3 nmol/L for cortisone.

Linearity: Linearity was r^2 = 0.998 and 0.999, for cortisol and cortisone, respectively (Fig. 3).



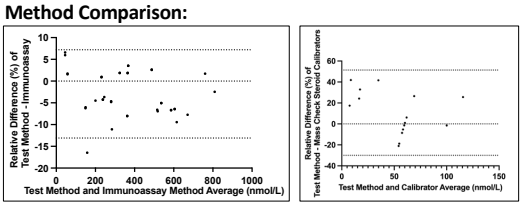
a. b.

Figure 3| Linearity Studies
Samples containing high concentrations of (a) cortisol and (b) cortisone were diluted with steroid depleted human processed serum, to determine the linearity. The samples were analysed in duplicate.

Carry Over:

No significant carryover of cortisol, cortisone or their internal standards were observed when serum spiked with high concentrations of analyte and IS were analysed with blank samples.

Method Comparison:



a. b.

Figure 4| Method Comparison
Bland-Altman difference plots for cortisol and cortisone. Demonstrated differences between the test method and Roche Cobas® vs. the mean of the two measurements (cortisol) and the test method and MassCheck Calibrator vs. mean of the two measurements (cortisone).

Summary

The developed method demonstrated good comparison with the Roche® method and superior specificity to IA, addressing long-standing cross-reactivity issues.

Acknowledgments

I would like to thank Mr Simon Salter and Dr Daniel Hills for their guidance and support. Emanuela Orlandi, Elisa Tammaro, Thomas Ng and the Mass Spectrometry Team for mass spectrometry training.

References

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2. Hahner S, Ross RJ, Arlt W, Bancos I, Burger-Stritt S, Torpy DJ, et al. Adrenal insufficiency. Nature Reviews Disease Primers. 2021 Mar 11;7(1).