Improved sensitivity for aldosterone using the unique MRM^3 quantification workflow

Using the QTRAP® 6500+ LC-MS/MS System

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Aldosterone, an important mineralocorticoid hormone, is a steroid hormone produced by the zona glomerulosa of the adrenal cortex in the adrenal gland. It plays a central role in the homeostatic regulation of blood pressure, primarily by acting on the mineralocorticoid receptors in the distal tubules and collecting ducts of the nephron and influencing the reabsorption of sodium and excretion of potassium in the kidney, thereby indirectly influencing water retention or loss, blood pressure and blood volume. Researchers are interested in monitoring serum levels of aldosterone for several reasons, including developing potential novel therapies for the treatment of fluctuating or dysregulated blood pressure.

Steroids such as aldosterone that are present in the bloodstream at very low concentrations are traditionally analyzed by radioimmunoassay (RIA). RIA approaches are known to suffer from various issues, including cross-reactivity, leading to a lack of specificity and therefore inaccuracies at lower concentrations. LC-MS/MS analysis overcomes a number of these issues. The measurement of aldosterone by LC-MS/MS, however, poses some specific analytical challenges due to the low concentrations of this compound, interferences caused by endogenous steroids, and the compound displaying a relatively poor ionization efficiency. While it is possible to achieve required limits of detection utilizing a standard quantitative LC-MS/MS workflow, it is sometimes necessary to use a large sample volume or employ a costly consumable-heavy preparation workflow to do so.

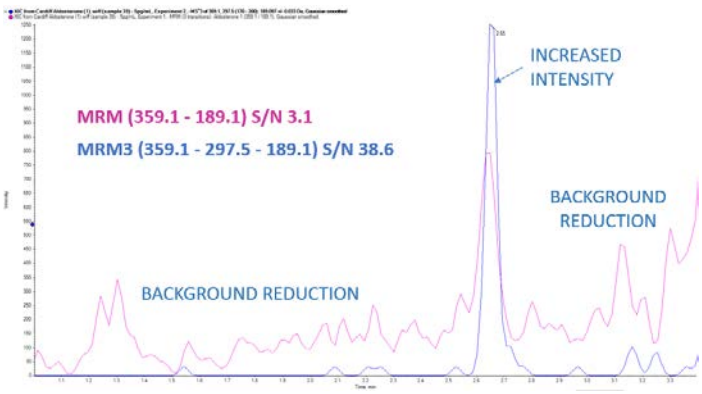

Figure 1. Significant improvement in specificity. Aldosterone sensitivity was compared between MRM and MRM^3 for a serum extract containing 5pg/ml aldosterone.

In order to reduce sample volume to an acceptable level and still maintain a fast workflow with minimal consumable cost, the unique MRM^3 scan available only on QTRAP® systems was investigated with a goal of improving sensitivity and reducing background interferences. This addition of a third MS stage has been shown to greatly increase selectivity and eliminate high baselines or other chromatographic interferences.[1] A comparison between MRM and MRM^3 for low level aldosterone is shown in Figure 1.

Key features of MRM^3 for quantification

- MRM^3 quantification—an MS/MS/MS scan is performed with a fast cycle time and using a narrow scan range centered at the second-generation product ion m/z used for quantification
- Fast linear ion trap scan speeds—scan speeds up to 20,000 Da/sec enable MRM^3 analysis within an UHPLC compatible cycle time with enough data points across the chromatographic peak
- Because of the multiple fragmentation steps used in MRM^3, higher selectivity is typically achieved
- Detection limits in very complex matrices can often be improved using MRM^3 analysis by removing interferences at the low end of the concentration range, improving signal/noise

Methods

Sample preparation: Calibrators and QCs were prepared by spiking known concentrations of aldosterone into 200 µL of human serum. Samples were spiked with internal standards at working concentrations, vortexed and extracted by a liquid-liquid extraction (LLE) method using methyl-tertiary-butyl ether (MTBE). Following mixing and centrifugation, the organic layer was separated by snap-freezing and evaporated to dryness. It was reconstituted in 200 µL mobile phase and then 25 µL was injected on the LC-MS/MS system.

UHPLC conditions: Chromatographic separation was achieved on a 50mm Phenomenex Kinetex column. A gradient of water and methanol (both containing ammonium fluoride) was used at a flow rate of 600 µL/min. The injection volume was set to 25 µL. The total run time for all compounds, including column equilibration time, was 4 minutes.

MS/MS conditions: A SCIEX QTRAP® 6500+ LC-MS/MS System, operated in low mass mode, was configured with the IonDrive™ Turbo V Ion Source. Two experiments were run in a looped configuration. The first MRM experiment included three MRM transitions (two for Aldosterone and one for the deuterated internal standard). The second experiment was the MRM^3 experiment with a mass window of 30 amu around the 2nd product ion of aldosterone. MRM and MRM^3 transitions and voltages were determined by infusion of a standard. Source and gas conditions were optimized by flow injection of a low solvent standard.

***Data acquisition and processing*:** Data was acquired using Analyst® Software 1.7.1 and processed using SCIEX OS Software 1.7.

Reduced sample volume requirements

Existing sample preparation methodologies for challenging steroids such as aldosterone often rely on a large sample volume (4 - 500 µL) and a significant concentration step in the extraction workflow in order to achieve the sensitivities required. Such a large sample volume is a limiting factor in itself for many reasons, but an additional downside to this approach is that not only is the compound itself concentrated during the extraction process, so is the matrix debris and other unwanted components of the extraction. This can lead to reduced reproducibility and adversely affect other aspects of assay performance.

With the increase in specificity leading to an increase in sensitivity of the MRM^3 approach, it has been demonstrated that the utilization of a sample volume of 200 µL and an identical reconstitution volume of 200 µL (no concentration) showed a significant sensitivity increase over MRM quantification. As shown in Figure 2, levels of aldosterone undetectable by MRM can be seen and quantified by MRM^3 using the proposed extraction workflow.

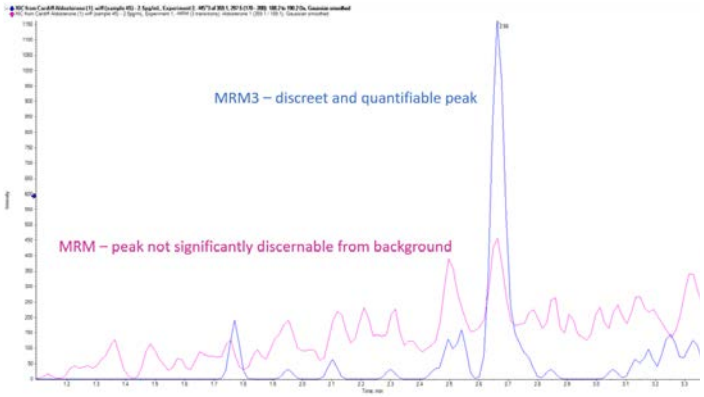

Figure 2. Comparison between MRM and MRM^3 for a serum extract containing 2.5pg/ml aldosterone

Analytical sensitivity

Figure 3 shows an extracted serum sample spiked with aldosterone at 2.5 pg/mL (approximately 7 pmol/L) with a clear, distinct and quantifiable peak. Signal/noise and number of data points across the peak are sufficient for reproducible quantification.

Analytical linearity

A series of calibrators were prepared in serum and processed by the proposed methodology. Figure 4 shows the linearity for aldosterone by the MRM^3 approach, with a concentration range of 2.5 – 1000 pg/mL (6.9 - 2778 pmol/L). Linearity (r^2) was calculated to be 0.9922.

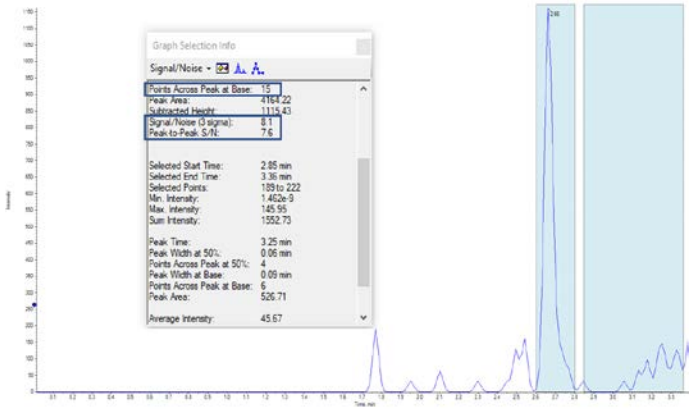

Figure 3. Analytical sensitivity in serum for aldosterone by MRM^3. Six points across the peak at base was obtained with this method providing a S/N ratio of 8.1, sufficient for good quantification.

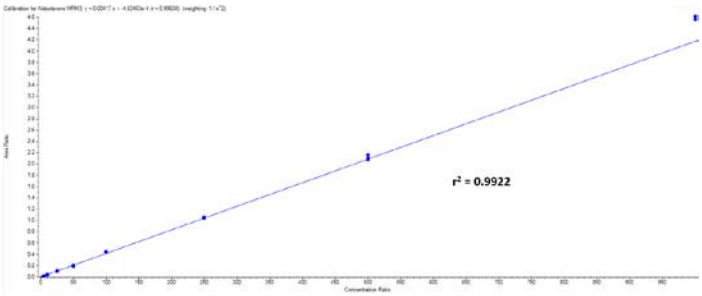

Figure 4. Linearity of aldosterone in serum by MRM³. A calibration curve was run across the range of 2.5 – 1000 pg/mL (6.9 - 2778 pmol/L). Very good linearity was observed with a r^2 value of 0.9922.

Reproducibility

In order to determine the precision of this method, the calibration standards were processed and analyzed in triplicate. The intra-day precision of aldosterone by MRM³ across all concentrations analyzed was determined to be ≤6.7%, with accuracies ranging from 88 - 110%. Results from accuracy and precision experiments are summarized in Table 1.

Conclusions

This improved methodology for the analysis of aldosterone using an MRM³ approach has shown the following benefits over a standard MRM approach:

- Ability to use a significantly lower sample volume
- Simplified sample preparation procedure
- Reduced chromatographic background and interferences
- Increased sensitivity
- Good linearity
- Potential to increase throughput through faster chromatography

The proposed methodology shows potential in areas where research into alternative methodologies is currently active, such as where sample volume is limited and improved sensitivity is critical.

Table 1. Accuracy and precision values from pooled serum.

Concentration (pg/mL)	n	Accuracy (%)	CV (%)
2.5	*3*	*107*	*6.6*
5	*3*	*92*	*5.0*
10	*3*	*88*	*5.2*
25	*3*	*100*	*2.3*
50	*3*	*96*	*4.4*
10	*3*	*105*	*1.0*
250	*3*	*101*	*0.8*
500	*3*	*101*	*1.9*
1000	*3*	*110*	*0.7*

References

1. MRM³ quantitation for highest selectivity in complex matrices. SCIEX technical note RUO-MKT-02-2739-A.

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