Rapid, sensitive analysis of corticosteroids in plasma with reduced sample volume requirements

Using microflow chromatography

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Corticosteroids are a class of steroid hormones produced in the adrenal cortex of vertebrates, which are involved in a wide range of physiological processes, such as anti-inflammatory responses, vasoconstriction and electrolyte balance. The wide range of action of these molecules makes them of great interest to researchers working in the area of steroid biomarker quantification. A high-sensitivity workflow has been developed to quantify a group of five corticosteroid hormones: cortisol, corticosterone, 11-deoxycorticosterone (11-DOC), 11-deoxycortisol (11-DOCL) and aldosterone (Aldo).

Of these five corticosteroid hormones, aldosterone has the lowest concentration in human plasma. Due to this low concentration, many sample preparation workflows require a large volume of plasma (400 µL or greater) to achieve required detection limits of less than 10 pg/mL in serum. Obtaining and handling such large volumes of serum can often be problematic. In order to achieve required sensitivities on challenging compounds such as aldosterone, while maintaining the use of a reasonable sample volume, microflow chromatography was explored.

Using this lower flow chromatographic strategy, the sensitivity of the five corticosteroids was improved by approximately 5-15 times over that achievable by analytical flow chromatography. This enhanced sensitivity allows for the accurate quantification of these five corticosteroid compounds from a starting volume of only 50 µL plasma. As an example, the increase in sensitivity for aldosterone achieved by moving to a microflow LC approach is highlighted in Figure 1, where a 15x increase in sensitivity over analytical flow chromatography was observed.

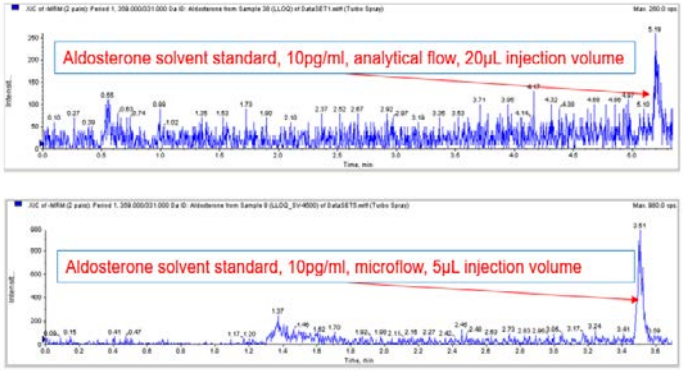

Figure 1. Comparison of analytical flow and microflow for aldosterone in solvent. Using the same concentration standard, aldosterone was analyzed by analytical flow chromatography (top, 20 µL injection) and microflow LC (bottom, 5 µL injection). A sensitivity gain of approximately 15x was observed for the microflow experiment.

Key features of microflow chromatography on the M5 MicroLC System

- Achieve the required sensitivities with microflow LC using much less sample than analytical flow. The higher sensitivity allows users to scale sample preparation to minimize sample volume requirements.
- Maintain throughput with runtimes comparable to analytical flow chromatography
- Accurate control of flow rates down to 1 µL/min for high-quality separations and excellent retention time reproducibility
- Lower flow rates require less solvent, reducing costs and reducing solvent waste
- Direct-injection and trap-elute capabilities for enhanced method development options. Simplify extractions with a wide range of online extraction options.

Methods

Sample preparation: Standards, QC's and blanks were prepared by spiking a known concentration of analytes and internal standard (corticosterone-d8) into 50 µL of human plasma. Samples were extracted by a liquid/liquid extraction (LLE) method, 300 µL of supernatant was evaporated to dryness and the residue was reconstituted in 100 µL of 20% methanol.

Microflow chromatography conditions: Chromatographic separation was performed using a Halo C18 column (0.5 x 50 mm, 2.7 µm, 90 Å, SCIEX). Mobile phase A consisted of ammonium acetate in water and mobile phase B consisted of methanol, applied with a short gradient from 20% to 90% B at a flow rate of 5 µL/min.

MS/MS conditions: A SCIEX Triple Quad™ 5500 LC-MS/MS System was used for acquisition, and was equipped with a Turbo V™ Ion Source with an electrospray ionization (ESI) probe fitted with a 50 µM ESI needle. The five analytes were detected using 2 MRM transitions per compound. Two time periods were employed, running aldosterone in negative ionization and the remaining five analytes in positive ionization. Compound specific parameters were optimized by infusion of a standard at an appropriate concentration. Source parameters were optimized to maximize the sensitivity of aldosterone using flow injection analysis and applied across both polarities and time windows.

Data acquisition and processing. Data was acquired using Analyst® Software 1.6.3, and processed using MultiQuant™ Software 3.0.3.

Improved chromatographic performance

A method was developed here for the quantification of five corticosteroid hormones in human plasma using liquid/liquid extraction from a sample volume of 50 µL plasma, followed by microflow chromatography LC-MS. The total ion chromatogram (TIC) for the 5 target analytes from an extracted prepared plasma calibrator is shown in Figure 2. This highlights the quality of the separation achieved by microflow LC, the very sharp peaks observed with fast runtimes. The 10-minute run time that was used was combined with multiple time windows and polarity switching to cover the target analytes.

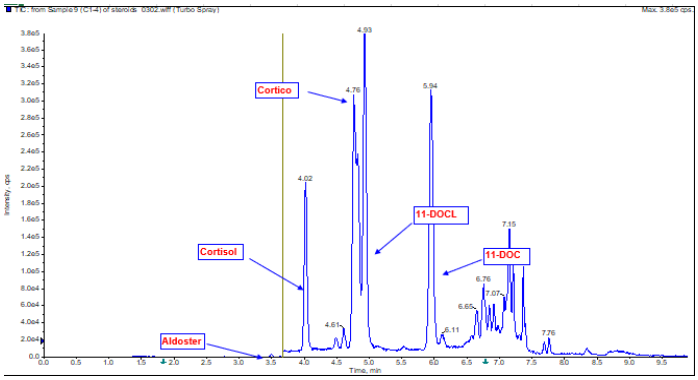

Figure 2. Total ion chromatogram of extracted mid-range plasma calibrator (see text for calibration ranges for individual analytes).

Analytical sensitivity and linearity

Using the workflow developed, a calibration curve was prepared in triplicate by spiking known concentrations of the five steroid hormones into commercial, double charcoal stripped human plasma. A concentration range of 0.01 to 100 ng/mL was interrogated.

Of the steroid hormones analyzed, aldosterone required the lowest limit of quantification (LLOQ) at 10 pg/mL (0.01 ng/ml, 27.8 pmol/L). The observed sensitivity of a plasma extract spiked with 10 pg/mL (27.8pmol/L) is shown in Figure 3 (bottom) compared with a plasma blank (top), again generated from a low sample volume of 50 µL. The calibration curve for aldosterone, run in negative mode, was linear from 0.01 to 10 ng/mL.

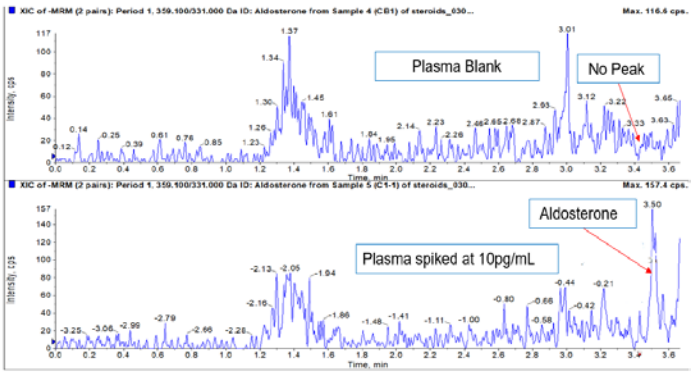

Figure 3. Comparison of extracted charcoal stripped plasma blank (top) with charcoal stripped plasma spiked at 10pg/mL (0.01ng/ml, 27.8pmol/L) (bottom) for aldosterone.

At the same time, the calibration curves of the other 4 steroid hormones run in positive mode was assessed and were found to be linear from 0.1-100 ng/mL with r values from 0.9912 to 0.9988. Figure 3 shows examples of calibration curves generated with this microflow LC-MS approach.

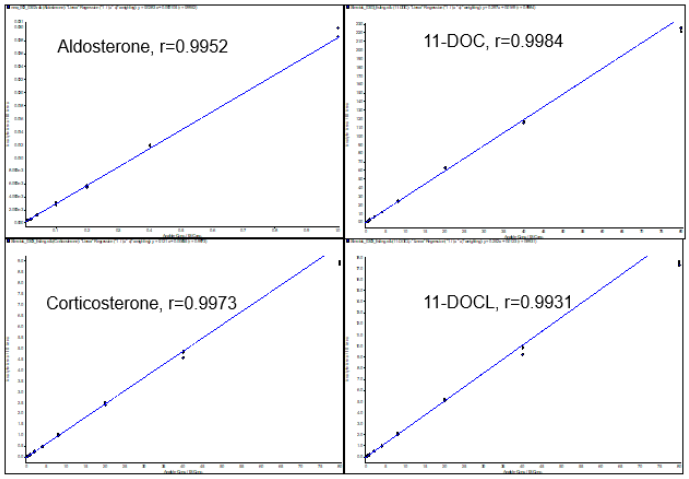

Figure 4. Examples of calibration curves generated in extracted plasma. The target analytes were analyzed across a concentration range in extracted plasma. Excellent linearity was observed for aldosterone in negative mode (0.01-10 ng/mL), and for all other analytes in positive mode (0.1-100 ng/mL).

Reproducibility

In order to determine the precision of this method, three replicate QC samples were prepared at each of the three low, medium and high concentrations, the plasma was extracted and quantified against the standard curves also run in extracted plasma. The precision of all analytes across all concentrations was determined to be ≤7.6%. Results from accuracy and precision experiments are shown in Table 1.

Table 1. Reproducibility results for three concentrations of QC samples, across three replicates. The low, medium and high concentration QC samples were analyzed and the concentrations were compared to the calibration curves. Very good agreement with the known concentration was observed (accuracies between 87% and 118%). Variance in calculated concentrations were all below 7.6%.

	Aldosterone		11-DOC		Corticosterone		11-DOCL		Cortisone	
	Conc (ng/mL)	Accuracy	Conc (ng/mL)	Accuracy	Conc (ng/mL)	Accuracy	Conc (ng/mL)	Accuracy	Conc (ng/mL)	Accuracy
QCL-01	0.0226	113	0.467	117	0.464	116	0.463	116	1.1	110
QCL-02	0.0209	104	0.432	108	0.458	114	0.421	105	1.01	101
QCL-03	0.0194	97.2	0.471	118	0.437	109	0.434	108	0.99	99
CV% (n=3)	**7.60**		**4.70**		**3.10**		**4.90**		**5.70**	
QCM-01	0.421	105	46.2	116	38.3	95.7	36.9	92.2	195	97.4
QCM-02	0.418	104	46.3	116	37.2	93	35.8	89.6	193	96.5
QCM-03	0.414	103	46.5	116	37.6	93.9	37.5	93.8	192	96.2
CV% (n=3)	**0.80**		**0.30**		**1.50**		**2.30**		**0.80**	
QCH-01	0.745	93.1	69.3	108	57.6	90	55.4	86.5	390	97.5
QCH-02	0.798	99.8	71.4	112	59.4	92.8	57.1	89.3	409	102
QCH-03	0.785	98.1	73.1	114	58.6	91.5	57.3	89.6	406	102
CV% (n=3)	**3.60**		**2.70**		**1.50**		**1.80**		**2.50**	

Conclusions

Here a microflow LC-MS method has been developed for the quantification of 5 corticosteroid hormones in plasma. The use of microflow chromatography allows the following performance improvements over analytical flow LC to be achieved:

- Up to 15x increase in analytical sensitivity
- Reduced sample volume requirements (400 µL to 50 µL)
- LLOQs (aldosterone) < 0.01 ng/mL (27.8 pmol/L)
- Linearity >0.99 for all compounds
- Reproducibility (%CV) ≤ 7.6 for all compounds

These results show the method provides an accurate and precise approach for the quantification of corticosteroids in plasma.

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