Low Level Bioanalytical Quantitation Method for Analysis of Fludrocortisone Acetate in Human Plasma Using SCIEX Triple Quad™ 6500 System

A sensitive (5.0 pg/mL) and high-throughput method for quantifying low levels of a corticosteroid in human plasma using an AB SCIEX Triple Quad™ 6500 LC-MS/MS System and UHPLC Chromatography

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Key challenges of Fludrocortisone Acetate Quantitation

- **Removing or reducing matrix interference**– Presence of interfering endogenous steroids makes sensitive bioanalytical assay development challenging.
- **Poor Intrinsic Ionization** – Due to poor intrinsic ionization of corticosteroids the precision and accuracy can be compromised at low levels.

Key benefits of Steroid Quantitation on the SCIEX Triple Quad™ 6500 LC/MS/MS System

- **High Sensitivity and Reproducibility** – Low level corticosteroid detection (at pg/mL concentrations) in human plasma is enabled by IonDrive™ Technology.
- **Addressing regulatory concerns**– The combined solution of a robust, sensitive bioanalytical method enables the user to address USFDA bioanalytical method validation criteria.

Figure 1: Triple Quad™ 6500 System

Figure 2: IonDrive™ Components in SCIEX Triple Quad™ 6500 System

Unique features of the Triple Quad™ 6500 System for low-level steroid detection

- **IonDrive™ Turbo V™ Source** provides enhanced ionization and desolvation of analyte ions, while supporting high UHPLC flow rates of up to 3 mL/minute
- **IonDrive QJet Ion Guide** captures more ions as they enter the mass spectrometer orifice.
- **IonDrive High Energy Detector** technology boosts dynamic range and sensitivity.
- Compatible with **SelexION™ Ion Mobility Technology**, enabling an extra dimension of selectivity for challenging assays where inferences are high.

Introduction

Fludrocortisone, a corticosteroid, is used to treat Addison's disease where excessive amounts of sodium are lost in the urine. Fludrocortisone helps to control the amount of sodium that is lost in the urine.

The usual dose of fludrocortisone acetate for Addison's disease is 100µg tablets daily. In certain situations like transient hypertension or in pediatric treatments the dose of fludrocortisone is significantly reduced to a dose of 50ug daily thus require the need to do the quantitative analysis at low pg level in human plasma.

The chemical name for fludrocortisone acetate is 9-fluoro-11β,17,21-trihydroxypregn-4-ene-3,20-dione 21-acetate.

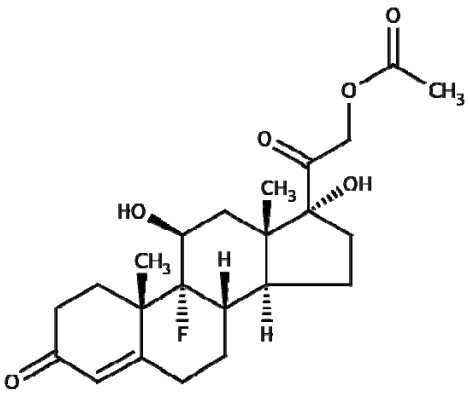

Figure 3: Fludrocortisone Acetate

Fluticasone propionate is used as internal standard for the assay of Fludrocortisone acetate in human plasma. Fluticasone also belongs to the category of corticosteroids. The chemical name of fluticasone propionate is S-(fluoromethyl) 6α,9-difluoro-11β,17-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate, 17-propionate.

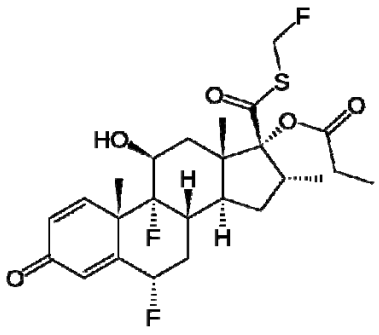

Figure 4: Fluticosone Propionate

Materials and Methods

Sample Preparation

500µL of plasma sample containing 2% fludrocortisone acetate standard and 10µL of internal standard were vortexed, followed by addition of 0.5mL water mixed by vortexing for 1 minute. Samples were extracted using Phenomenex Starta X cartridges conditioned with methanol followed by equilibration with Milli-Q-Water. After loading, cartridge was washed using 2mL of water followed by 2mL of 10% methanol in water. Analytes were eluted using dichloro methane followed by dry-down under nitrogen stream at 40 ºC and reconstitution of the samples with Acetonitrile: Water (50:50v/v) prior to analysis by mass spectrometry.

Chromatography

LC System: GL Sciences LC 800 System
Column: Inertsil ODS-4 (50 x 2.1, 2µ)
Column Temp: 40 °C
Injection: 50µL
Flow Rate: 0.500 ml/min
Mobile Phase: A) 0.1% v/v Formic Acid in Water
B) 0.1% v/v Formic Acid in Acetonitrile
Gradient Profile:

Time (Min)	% A	%B
0.00	80	20
1.00	80	20
1.50	40	60
2.00	40	60
2.25	20	80
4.00	20	80
4.10	80	20
5.00	80	20

Table 1: Gradient Elution Method

Mass Spectrometry

Analysis of Fludrocortisone acetate and Fluticasone propionate (IS) were carried out in APCI positive ionization mode and required different mass spectrometric parameter settings (Table 2). The MRM transition monitored for Fludrocortisone Acetate was 423.3/239.2 and 501.1/313.1 for Fluticasone propionate. Dwell time was kept 200ms.

Data System: SCIEX Triple Quad™ 6500 System

Interface: IonDrive™ Turbo V Source in APCI positive

Instrument Parameter	Fludrocortisone Acetate	Fluticasone Propionate
DP	100	60
EP	10	10
CE	35	19
CXP	5	15
CUR	30	
TEM	600°C	
NC	4	
CAD	10	
GS1	60	

Table 2: Mass spectrometry conditions

Data processing

All SCIEX Triple Quad™ 6500 System data was processed using MultiQuant™ Software. The concentration curves were analyzed using a linear fit with a $1/x^2$ weighting. Data acquired on the Triple Quad 6500 System was processed using the quantitation tools within Analyst® 1.6 Software.

Results and Discussion

Method Analysis and Data Quality

The Fludrocortisone acetate quantitative assay was developed and validated by using Fluticasone propionate as an internal standard spiked into human plasma. Figure 5 and Figure 6 shows representative chromatograms for plasma blank extract and plasma spiked lower limit of quantification with 5pg/mL Fludrocortisone acetate in Plasma. Linearity range covered plasma concentrations varied from 5.0 pg/mL to 2500.0 pg/mL in plasma. Lower limit of quantitation (LLOQ) in human plasma of 5pg/mL resulted in an excellent signal to noise ratio of 109.9 showed in the figure 6. Reproducibility of the assay was assessed by multiple replicate injections of quality control samples (Three Precision Accuracy batches, n=18) at LLOQ QC, LQC, MQC and HQC concentration levels. The calibration curve in plasma extracted samples showed excellent linearity over 2.5 orders of magnitude concentration range with an r value of >0.99.

Three precision accuracy batches were processed and data was compared in Table. 3 shows the data for calibration curve concentrations, Table. 4 and Table. 5 for intraday and between batch accuracy and precision for quality control samples. All the three batches were within the acceptance criteria of %CV ±20% at LLOQ level and ±15% at other levels.

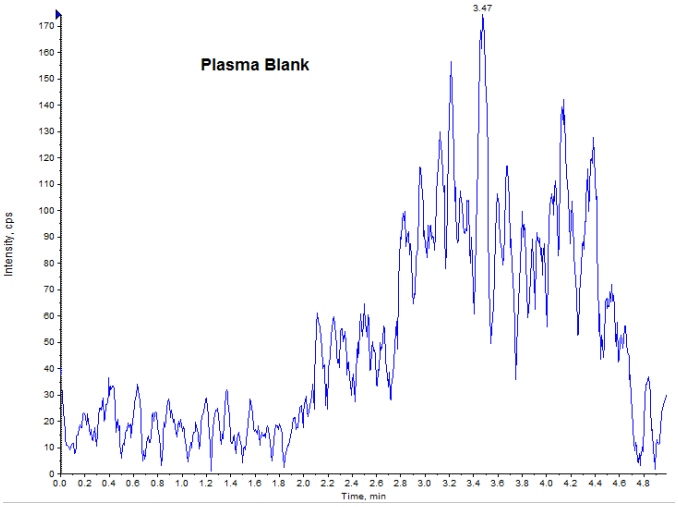

Figure 5: Chromatogram of Extracted Plasma Blank

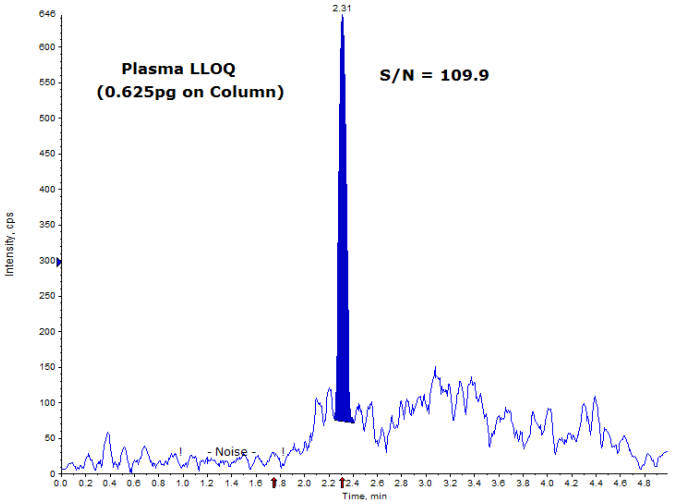

Figure 6: Chromatogram of Extracted Plasma LLOQ (5 pg/mL)

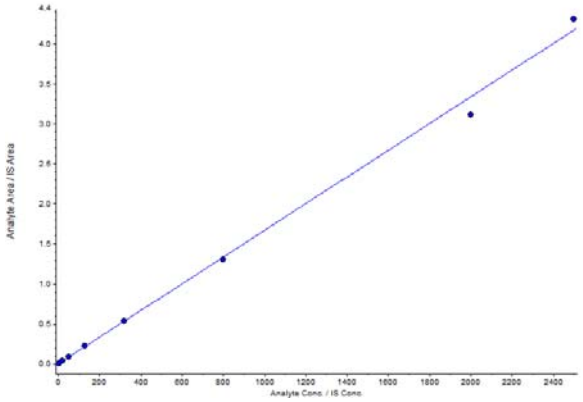

Figure 7: Calibration Curve (PA - 03) showing linearity range from 5.0 pg/mL to 2500.0 pg/mL with r value of 0.9991

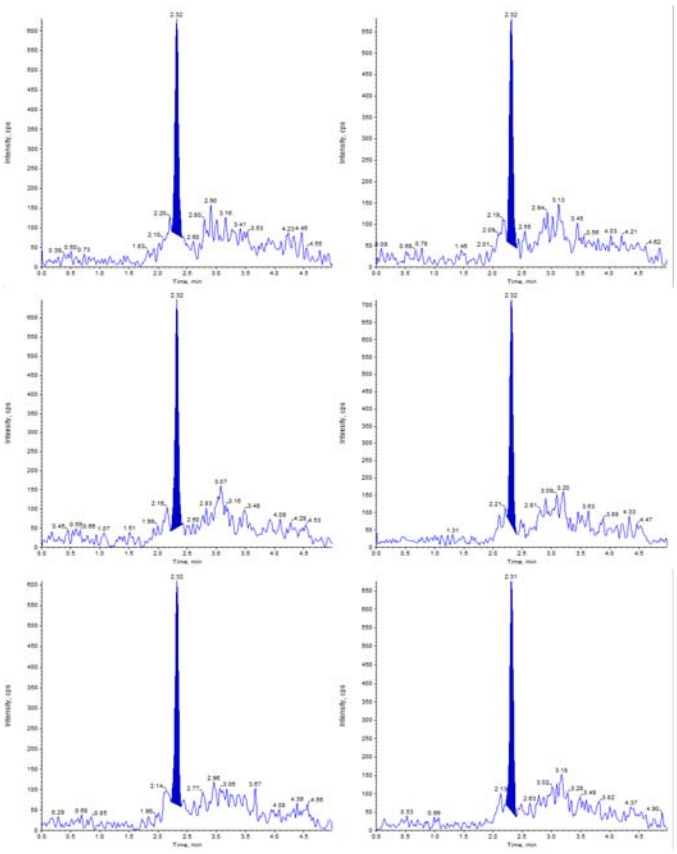

Figure 8: Chromatograms of Six LLOQ QC (5.023 pg/ml) samples

Percent recovery of the fludrocortisone acetate from the extracted samples were calculated at three different levels (LQC, MQC and HQC) using peak area and compared with the 6 replicates of the unextracted samples at all the three levels. Percent recovery of the Fluticasone propionate internal standard was calculated using 18 samples from all concentration levels. Extracted sample data from PA Batch 03 was used for the recovery calculations. Percent recovery for Fludrocortisone acetate was found to be 81.26% and for Fluticasone it was 86.98%. Table 6 shows the recovery calculations for fludrocortisone acetate and fluticasone.

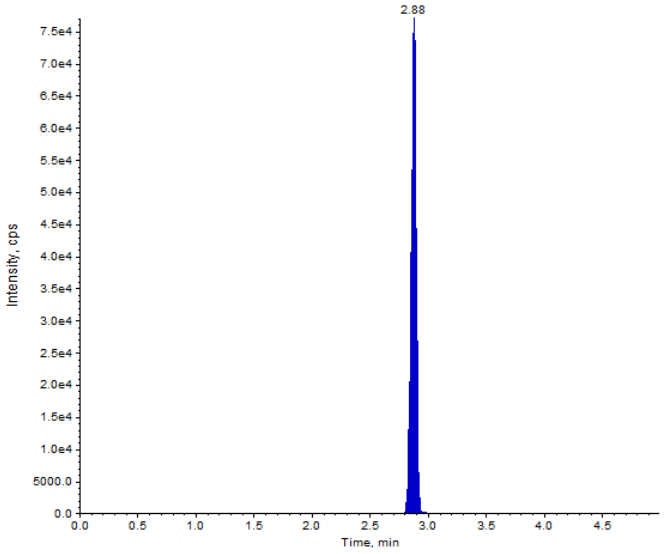

Figure 9: Chromatograms of Fluticasone (Internal Standard)

Calibration Curve Data for Three Precision Accuracy Batches							
Sample	**Nominal Concentration (pg/mL)**	**PA 01**	**PA 02**	**PA 03**	**Mean**	**C.V. (%)**	**% Nominal**
STD A	5.018	5.073	5.162	5.002	5.079	**1.58**	101.22
STD B	20.480	20.094	17.824	20.659	19.5257	**7.68**	95.34
STD C	51.200	50.422	52.744	50.471	51.2123	**2.59**	100.02
STD D	128.000	112.143	130.304	136.981	126.476	**10.16**	98.81
STD E	320.000	306.653	308.482	320.274	311.803	**2.37**	97.44
STD F	800.000	834.742	764.568	779.329	792.8797	**4.67**	99.11
STD G	2000.000	2114.056	2068.759	1863.443	2015.4193	**6.63**	100.77
STD H	2500.000	2720.357	2746.925	2579.615	2682.299	**3.35**	107.29
r value		**0.9975**	**0.9973**	**0.9991**			

Table 3: Calibration Curve Data for Three Precision Accuracy Batches

Intra Day Precision and Accuracy				
S.No	Nominal Concentration (pg/mL)			
	LLOQ QC	LQC	MQC	HQC
	5.023	15.040	800.000	2000.000
N	12	12	12	12
Mean	4.8945	14.4069	806.3593	2005.4965
% Nominal	97.44	95.79	100.79	100.27
S.D (+/-)	0.54102	0.93703	34.46636	125.5767
C.V. (%)	11.05	6.5	4.27	6.26

Table 4: Intra Day Precision and Accuracy for Fludrocortisone Acetate

Between Batch Precision and Accuracy				
Statistics	Nominal Concentration (pg/mL)			
	LLOQ QC	LQC	MQC	HQC
	5.023	15.040	800.000	2000.000
N	18	18	18	18
Mean	5.1146	14.8122	815.9908	2029.8186
% Nominal	101.73	98.47	102.00	101.49
S.D (+/-)	0.56096	1.02121	35.58725	125.74866
C.V. (%)	10.98	6.89	4.36	6.2

Table 5: Between Batch / Inter Day Precision and Accuracy for Fludrocortisone Acetate

% Recovery								
Statistics	Fludrocortisone Acetate						Fluticasone Propionate	
	LQC Response		MQC Response		HQC Response		Internal Standard	
	Extracted	Unextracted	Extracted	Unextracted	Extracted	Unextracted	Extracted	Unextracted
N	6	6	6	6	6	6	18	18
Mean	6181.5	7576.8	332188.7	396264.8	809585.0	1033056.3	244955.8	281625.7
S.D	99.62	269.99	10844.18	7968.99	31070.56	34395.02	5313.43	20334.70
% C.V	1.61	3.56	3.26	2.01	3.84	3.33	2.17	7.22
% Recovery	81.58		83.83		78.37		86.98	
Mean % Recovery	81.26							

Table 6: % Recovery Table of Fludrocortisone Acetate at Three Concentration Levels and Fluticasone (Internal Standard)

Conclusions

- A highly sensitive and high-throughput bioanalytical method was developed and validated for the detection of Low levels of the corticosteroid fludrocortisones acetate, in human plasma on the SCIEX Triple Quad™ 6500 LC/MS/MS System.
- The unique geometry of IonDrive™ Turbo V™ source provides excellent sensitivity and reproducibilty for Bioanalytical methods.
- Method sensitivity for fludrocortisones acetate detection was exceptional, and demonstrated high-reproducibility and cost effectiveness with good precision and accuracy.
- The achieved LLOQ for Fludrocortisones in plasma was 5.0 pg/ml (0.625 pg on column) with signal to noise ratio 109.9 using SCIEX Triple Quad™ 6500 LC/ MS/MS System.
- Analyte recovery is 81.26% for Fludrocortisone acetate and 86.98% for Fluticosone propionate.
- Developed and validated method for Fludrocortisones in human plasma is simple, easy to use, cost effective, high-throughput, sensitive and reproducible.

Reference

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