Steroid Hormone Analysis in Serum using Supel ™ Swift HLB DPX TIPS

INTRODUCTION

Routine hormone analysis is necessary for establishing and monitoring patient diseases. For example, the continuous monitoring of cortisol levels can help diagnose a patient with Cushing disease (high cortisol) or Addison disease (low cortisol). A robust method for steroid hormone determination in serum is imperative in diagnostics and treatment. The Supel™ Swift HLB DPX Tips (3 mg bed, Hamilton®) allow for reduced sample volume, sample evaporation mitigation, and offer a fully automated approach. In this method, a total of 9 steroids (cortisone, cortisol, 11-deoxycortisol, androstenedione, testosterone, dehydroepiandrosterone, 5-dihydrotestosterone, 17-hydroxyprogesterone, and progesterone) were analyzed as a panel to cover a variety of testing applications and disease diagnostics.

The Supel™ Swift HLB sorbent provides good selectivity and sensitivity for steroids in a neutral solution[0], allowing for dilution with water prior to injection. The sorbent has significant versatility in analyte binding due to the co-polymer phase containing both hydrophilic and lipophilic functional groups.[1]

This validated method used 100 µL of serum and the final volume available for injection was approximately 100 µL, allowing for a 1:1 concentration factor without solvent evaporation. Recoveries for the 9 analytes range from 65-86% **(Table 1)**. The LOQ's for all analytes fall below clinically relevant values, and possessed linear dynamic ranges between 0.025 ng/mL and 250 ng/mL. The automated extraction method allows up to 96 samples to be processed simultaneously in approximately 20 minutes prior to LC-MS/MS analysis.

Figure 1. *Supel™ Swift DPX HLB 3 mg (bed) Tips. The tips are being actively picked up by the automated liquid handler.*

METHODS

The solvents used include Methanol (Optima™ LC-MS Grade, Fisher Chemical™, Hampton, NH) and Acetonitrile (LC-MS Grade, Fisher Scientific, Hampton, NH). All standards and internal standards were from Cerilliant (Round Rock, TX). Formic Acid (Optima Grade, Fisher Scientific, Hampton, NH). For sample matrix, DDC Mass Spect Gold® Serum (MSG3000-100 mL) was used for calibration preparation and was provided by MilliporeSigma (Burlington, MA). The µXTR tips (The Supel™ Swift HLB DPX® Tip 3 mg Hamilton® 300 µL PK96, Catalog # DPX180338) are a patented DPX Technologies (Columbia, SC) product. A Hamilton MICROLAB® NIMBUS96 liquid handler was utilized to automate sample preparation. The analysis was performed on an Agilent 1290 LC system coupled with a SCIEX 6500+ tandem mass spectrometer. The LC Column used was an Ascentis® Express C18 (2.7 µm)

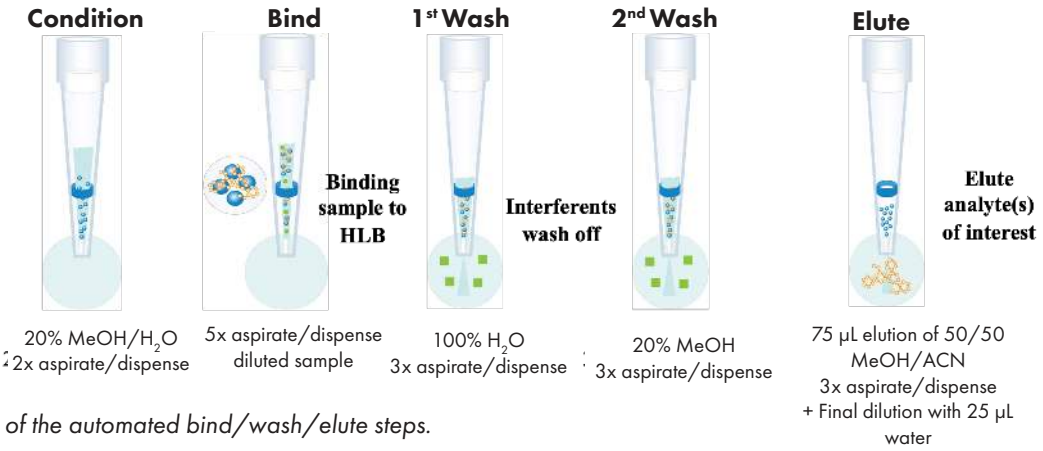

Figure 2. *Schematic of the automated bind/wash/elute steps.*

particle size, L × I.D. 10 cm × 3 mm) joined with an EXP® Pre-Column Filter cartridge in an Ascentis® Express Guard Cartridge Holder (Millipore Sigma, Burlington, MA); this combination allowed for optimal separation of all steroids. An injection volume of 15 µL was sufficient to hit required cut offs. The LC conditions are found in **Table 2**. Ammonium fluoride additives are common in steroid analysis but were found to decrease retention time stability. Mass spectrometer source parameters included a curtain gas of 35, collision gas of 8, and a positive mode IonSpray voltage of 4500. Source temperature operated at 600°C, and a setting of 60 was used for both ion source gases.

SAMPLE PREPRATION

Serum is aliquoted (100 µL) into a well plate. Internal standard is added (10 µL) and allowed to incubate for 1 hour. The well plate is then loaded onto the NIMBUS96 system for the rest of the automated protocol. The ALH picks up a set of standard transfer tips, adds 200 µL of 0.4% formic acid to the sample and mixes thoroughly; this solution is then incubated for 15 minutes prior to sample binding. While the protein dissociation step is occurring, the ALH picks up a second set of transfer tips for aliquoting the wash solvents into appropriate well plates. The ALH picks up t

This is done to avoid solvent evaporation of the low elution volume while the rest of the method is being performed. Finally, the ALH picks up the Supel Swift HLB DPX® Tips again and moves to the elution location (75 µL 50/50 MeOH/ACN), aspirating/dispensing three times. The DPX tips are ejected, and standard tips are picked up to dilute the eluent with 25 µL of water. The sample preparation is complete and samples are immediately ready for injection. (**Figure 1 & 2**)

Table 1. *Recovery and matrix effects. Values found using SWGTOX guidelines.*

Analyte	Average Recovery (n=9)	% RSD	Matrix Effects (n=8)*
Cortisone	72%	10%	29%
Cortisol	70%	10%	30%
11-Deoxycortisol	69%	9%	40%
Androstenedione	67%	9%	34%
Testosterone	69%	10%	30%
DHEA	86%	10%	12%
OH-Progesterone	67%	9%	41%
DHT	70%	12%	38%
Progesterone	65%	8%	46%

*Matrix effects are defined as: (+) positive indicates ion suppression, and a (-) negative indicates ion enhancement.

IONIZATION EFFECTS AND RECOVERIES

Ionization effects and recovery studies were performed as outlined by Scientific Working Group for Forensic Toxicology, SWGTOX[5]

Recoveries were evaluated by preparing two sets of serum samples; the first that were spiked with internal standards prior to extraction, and the second set were serum samples that were spiked with internal standards post-extraction.

Ion suppression and enhancement was evaluated by preparing two sets of samples. The first set being internal standards prepared in final solution composition and the second set being the internal standards spiked into the postextracted solution. All internal standard concentration were as described earlier (200 ng/mL, except for DHT and progesterone which were 500 ng/mL).

#	Step Performed
1	Sample Plate
2	Wash Plate
3	Wash Plate #2
4	Elution Plate
5	0.4% Formic Acid in Water
6	20% Methanol in Water
7	Water
8	50/50 Acetonitrile and Methanol
9	Standard Transfer Tips (300 µL)
10	Supel™ Swift HLB DPX Tips, 3 mg (bed)
11	Standard Transfer Tips (300 µL)

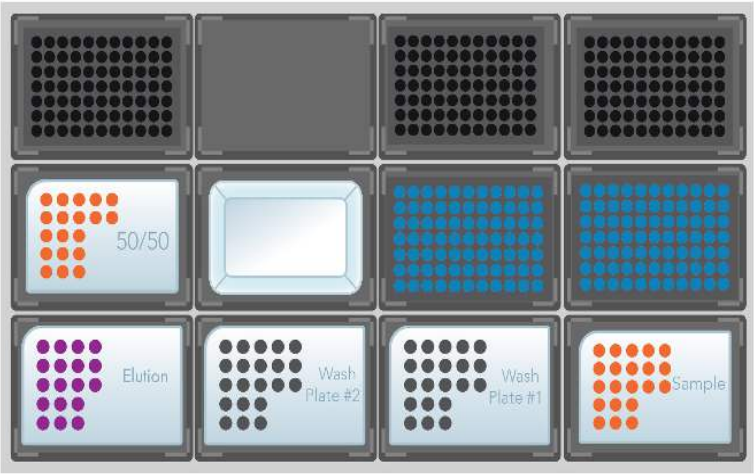

Figure 3. *Representative Hamilton® Microlab NIMBUS96 deck overview with designation of lab equipment and/or role in the method*

RESULTS AND DISCUSSION

Great sensitivity and chromatographic separations allowed for levels of detection in the trace ng/mL range. Limits of quantification (LOQ) range below the lowest calibrator level at 0.025 ng/L for all analytes except cortisone and cortisol, which had a lowest calibrator of 0.25 ng/mL. LOQ was calculated based on a signal-to-noise above 10 and LOD was based on a signal-to-noise above 3. **(Table 1)**

Furthermore, a three-day precision and accuracy study was performed for 8 analytes utilizing external quality control serum from UTAK and NIST-971a (UTAK Laboratories, Inc., Valencia, CA, USA and NIST, Gaithersburg, MD, USA). Neither source offered verified values for DHT, therefore a study could not be done. Ultimately, the inter-day precision of 8 analytes (DHT did not have a QC standard to use for comparison) varied from 0.30% to 12%. Intra-day precision ranges from 1.9% to 8.5%. **(Table 3)**

Table 2. *The LC Parameters Used*

Time (min)	%A	%B	Flow Rate (µL/min)
0	50	50	500
0.5	45	55	400*
3.7	45	55	500
3.8	20	80	500
6.0	10	90	500
9.5	0	100	500
12.0	0	100	500
12.1	50	50	500

Column Temp:	40°C
Detector:	MS/MS (see settings **Table 2 & 3**)
Injection Volume:	15 µL

*A slower flow rates was utilized to allow 11-deoxycortisol, 17-deoxycortisol, and 21-deoxycortisol separation.

METHOD REPEATABILITY

A three-day precision and accuracy study was performed for 8 analytes utilizing external quality control serum from UTAK and NIST-971a (UTAK Laboratories, Inc., Valencia, CA, USA and NIST, Gaithersburg, MD, USA). Neither source offered verified values for DHT, therefore it was omitted. Ultimately, the inter-day precision of the 8 analytes (excluding DHT) varied from 0.30% to 12%. Intra-day precision ranged from 1.9% to 8.5%. Samples were performed in triplicates over three days.

Table 3. *Real patient sample validation with UTAK Quality Controls. and NST Quality Controls. Conc. Range correspond to values from standards.*

UTAK Quality Control Samples		*Low (ng/mL)*	*High (ng/mL)*
Progesterone	Concentration Range	7.5-12	20-24
	Average Concentration (%CV)	6.55 (6.5%)	17.9 (6.4%)
OH-Progesterone	Concentration Range	3-4.49	8-9.2
	Average Concentration (%CV)	3.9 (3.3%)	9.37 (5.5%)
Testosterone	Concentration Range	3-3.99	8-8.37
	Average Concentration (%CV)	3.49 (5.2%)	9.23 (7.3%)
Androstenedione	Concentration Range	1.5-1.74	4-4.38
	Average Concentration (%CV)	1.42 (0.4%)	4.03 (4.7%)
Cortisone	Concentration Range	15	40
	Average Concentration (%CV)	17.6 (3.4%)	49.4 (6.8%)
Cortisol	Concentration Range	60-65	180-200
	Average Concentration (%CV)	66.3 (3.9%)	226 (6.4%)
11-Deoxycortisol	Concentration Range	1-1.06	3-3.61
	Average Concentration (%CV)	1.05 (6.1%)	3.17 (2.3%)
DHEA	Concentration Range	3-3.8	8-11
	Average Concentration (%CV)	3.55 (9.5%)	9.67 (0.39%)

Using the SWGTOX guidelines and a total of eight replicates for each analyte, the recoveries and the matrix effects for each analyte were determined.

CONCLUSION

The use of Supel Swift HLB DPX® Tips for the analysis of various steroids in blood serum is reproducible and an advantageous method for clinical testing laboratories. This method provides necessary sensitivity relevant to clinical values while also promoting high throughput sample processing for fast turnaround times. The accurate and sensitive method described here is a valuable tool for quantification of steroids in blood serum.

REFERENCES

1. Supel™ Swift DPX HLB Tips Datasheet T721005

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