Steroid Hormone Analysis in Serum

XTR Tips with Supel™ Swift HLB

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. XTR tips with Supel™ Swift HLB 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.

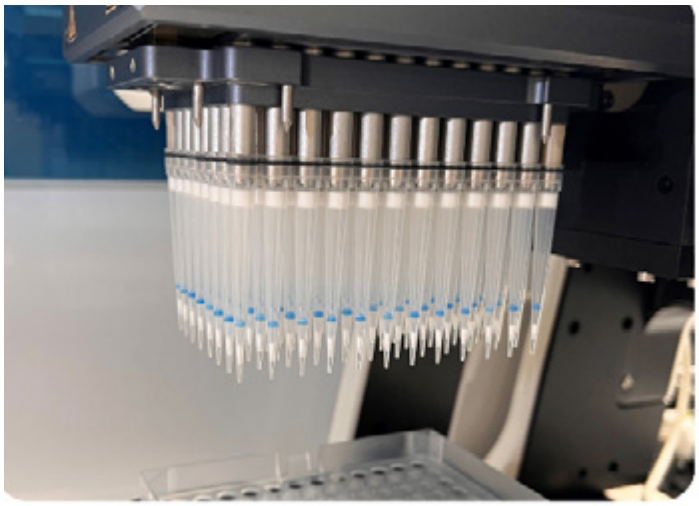


Figure 1. *XTR tips with Supel Swift HLB sorbent chemistry in Hamilton style 300 µL shown on a Microlab Nimbus96.*

The Supel™ Swift HLB sorbent provides good selectivity and sensitivity for steroids in a neutral solution[1], 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]

MATERIALS AND METHODS

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. The automated extraction method allows up to 96 samples to be processed simultaneously in approximately 20 minutes prior to LC-MS/MS analysis.

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). XTR tips with 3 mg of Supel™ Swift HLB in 300 µL Hamilton (DPX Technologies PK96, Catalog #DPX180338, Columbia, SC) were used on a Hamilton® Microlab NIMBUS96 liquid handler to automate sample preparation (shown in **Figure 1**).

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) 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 cutoffs. The LC conditions are found in **Table 1**. 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 PREPARATION

100 µL of serum and 10 µL of internal standard is added to a well plate and incubates for 1 hour. The well plate is then loaded onto the NIMBUS96 system for the rest of the automated protocol. The ALH picks up 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 the XTR tips with Supel Swift HLB and conditions the sorbent by aspirating and dispensing 300 µL of 20% methanol from a buffer reservoir two times. Once the protein dissociation timer is complete, the ALH moves to the sample well plate where it aspirates and dispenses the sample five times to bind analytes. The ALH moves to the first wash location (300 µL of 100% water) and aspirates/dispenses three times, and sequentially moves to the second wash location (300 µL of 20% methanol) and aspirates/dispenses three times. The ALH ejects the XTR tips with Supel Swift HLB back into the original deck position and picks up the transfer tips to aliquot the elution solvent into the appropriate well plate. This is done to avoid solvent evaporation of the low elution volume while the rest of the method is being performed. The ALH picks up the XTR tips with Supel Swift HLB again and moves to the elution location (75 µL 50/50 MeOH/ACN), aspirating/dispensing three times. The XTR tips are ejected, and standard tips are picked up to dilute the eluent with 25 µL of

Table 1. LC Parameters

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
Injection Volume: 15 µL

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

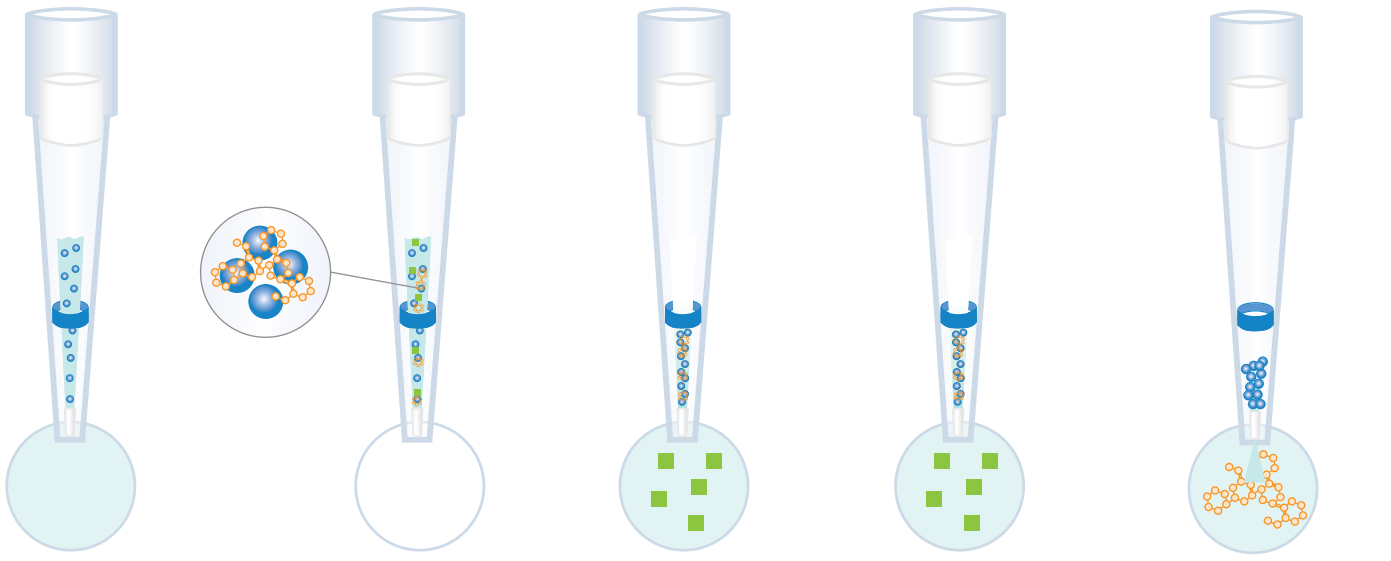
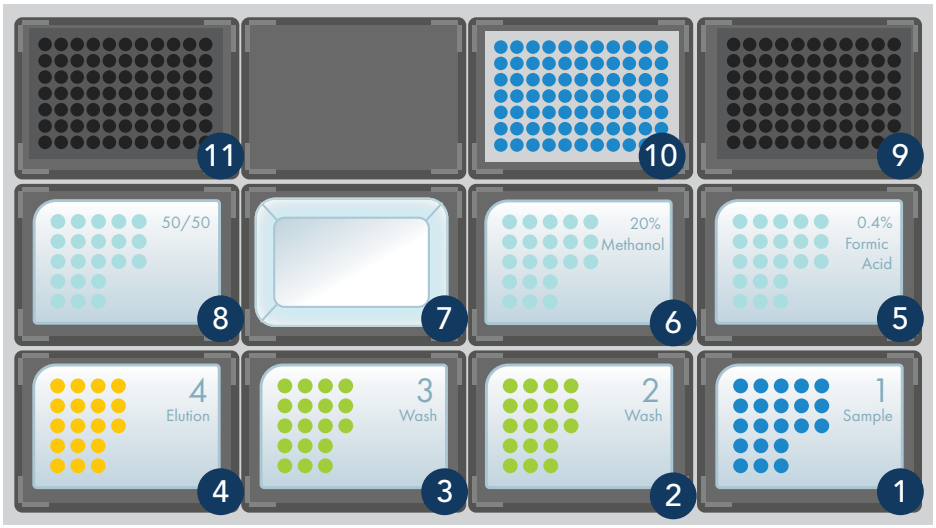


Figure 2. *Sample preparation with XTR tips*



1. Sample Plate
2. Wash 1 Plate
3. Wash 2 Plate
4. Elution Plate
5. 0.4% Formic Acid in Water
6. 20% Methanol in Water
7. Water
8. 50/50 ACN/ MeOH
9. Standard Transfer Tips (300 µL)
10. XTR tips with 3mg Supel Swift HLB
11. Standard Transfer Tips (300 µL)

Figure 3. *Hamilton Microlab NIMBUS deck layout and steps for the method.*

water. Samples are immediately ready for injection. Figure 2 shows a schematic of this method and Figure 3 shows the deck layout.

IONAZATION 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 post-extracted solution. All internal standard concentrations were as described earlier (200 ng/mL, except for DHT and progesterone which were 500 ng/mL).

RESULTS AND DISCUSSION

Recoveries for the 9 analytes range from 65-86% (Table 2). The limits of quantification's (LOQ) for all analytes fall below clinically relevant values, and possessed linear dynamic ranges between 0.025 ng/mL and 250 ng/mL. Great sensitivity and chromatographic separations allowed for levels of detection (LOD) in the trace ng/mL range. 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.

Furthermore, a three-day precision and accuracy

Table 2. Recovery and Matrix effect. Values found using SWGTOX guidelines.

Analyte	Avg. Recovery (n=9)	%RSD	Matrix Effects (n-8)*
Cortisone	72%	10%	29%
Cortisol	70%	10%	30%
11-Deoxycortisol	69%	9%	40%
Androstenedione	67%	9%	40%
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.*

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

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

CONCLUSIONS

The use of XTR tips with Supel Swift HLB 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.

REFERENCES:

1. *XTR tips with Supel™ Swift HLB, Datasheet T721005; Supel is a trademark of Sigma-Aldrich Co. LLC.*
2. *Nieman, Lynnette K et al. "The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline." The Journal of clinical endocrinology and metabolism vol. 93,5 (2008): 1526-40. doi:10.1210/jc.2008-0125*
3. *Munir S, Quintanilla Rodriguez BS, Waseem M. Addison Disease. [Updated 2022 May 15]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK441994/*
4. *Jia Y, Liu X, Xu L, Zhao J, Ni J, Zhang Y. Liquid chromatography tandem mass spectrometry measurement of 26 steroid hormones in human serum and plasma samples. J Sep Sci. 2021Jun;44(12):2358-2370. doi: 10.1002/jssc.202100091. Epub 2021 May 26. PMID: 33835710*
5. *Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology, Journal of Analytical Toxicology, Volume 37, Issue 7, September 2013, Pages 452-474, https://doi.org/10.1093/jat/bkt054*