Optimization of Wash/Elution Conditions for Automated Solid-Phase Extraction (SPE) of Testosterone from Plasma

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Abstract
Neutral sorbents are preferred for the solid-phase extraction of testosterone, a hydrophobic anabolic steroid, since these maximize retention due to strong hydrophobic interactions. However, elimination of contaminants from plasma samples requires a strong organic wash, which also weakens and disrupts such hydrophobic interactions. By providing additional interactive features such as π-π and hydrogen bonding interactions, Strata™-X enhances the retention of testosterone and sustains a 70 % methanol wash. In contrast, the silica-based Strata® C18-E can tolerate only a 40 % methanol wash without loss of analyte. Such a wash is necessary to eliminate phospholipids and minimize ion suppression and other matrix effects, thereby improving analytical accuracy and precision.

Introduction
Steroids are fat soluble organic compounds of plant or animal origin and are characterized by their cyclopentanophenanthrene skeleton. They are classified as lipids. Hormones belong to the steroid family and are chemical messengers produced by the endocrine glands, which directly release them into the bloodstream for transportation to various body parts. Examples of hormones include androgens, estrogens, progesterone and corticosteroids. Hormones are crucial for body function ranging from anti-inflammatory effects to regulation of events during pregnancy. Defects in steroid metabolism lead to diseases such as cancer, diabetes, cartilage/bone damage and neurological problems.[1,2]

Testosterone (**Figure 1**) is an androgen, secreted by the glands of both male and female mammals that plays a vital role for energy enhancement, increased production of red blood cells and for protection against osteoporosis. It is an anabolic steroid and is the principal male sex hormone. Analysis of testosterone is crucial, not only in diagnosis and treatment, but also in endocrinal disruption due to environmental contaminants such as pesticides.

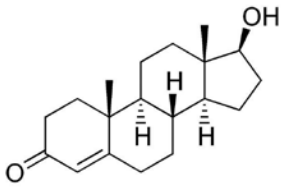

Figure 1.
TESTOSTERONE, (8R,9S,10R,13S,14S,17S)-17-hydroxy-10,13-dimethyl-1,2,6,7,8,9,11,12,14,15,16,17-dodecahydrocyclopenta[a] phenanthren-3-one [MW 288.43; log P 3.84; Molecular Formula $C_{19}H_{28}O_2$; M.P. 155 ºC, soluble in most organic solvents]

For the qualitative/quantitative analysis of testosterone in biological matrices, both GC/MS and LC/MS are commonly employed, the former analytical mode requiring derivatization.[3-6] In either case, sample preparation is essential to remove the proteins and other endogenous/exogenous materials from whole blood, serum, plasma, urine or tissue samples. Solid-phase extraction (SPE) is the most selective and popular method of sample clean-up and is used in the off-line tube or 96-well plate format; a few examples of dispersive SPE have also been reported. Silica-based neutral (C18) or mixed mode (cation exchange/C8) sorbents, as well as polar polymeric sorbents have been used for SPE of testosterone and other steroids in biological matrices. However, a major problem with these methods is the low organic wash which does not effectively remove contaminants. In this technical note, we present an automated SPE protocol based on the neutral polar polymeric SPE sorbent, Strata-X, that furnishes ultra-clean samples from plasma matrices. For comparison, results from a C18-E (hydrophobically end-capped) silica sorbent are also presented. It is inferred that this method will be applicable to steroids and hormones in general, or other similar small molecules in a complex biological matrix.

Experimental

Materials
Strata-X polymeric sorbent 96-well plates (30 mg/well, 33 µm particle size, 800 m^2/g surface area) and Strata C18-E 96-well plates (50 mg/well, 55 µm particle size and 500 m^2/g surface area) are from Phenomenex, Torrance, CA. Testosterone was obtained from Sigma and used as such. All solvents are ACS reagent grade and were also used as such.

Automated SPE protocol
The extractions were performed on a PerkinElmer® MultiPROBE II® automated liquid handler using a 96-well plate vacuum manifold deck accessory. Human plasma (500 µL), spiked with testosterone, was diluted with 1 mL of HPLC grade water. The following automated method was performed.

Condition:	400 µL methanol and subsequently 400 µL water
Load:	500 µL plasma diluted with 1 mL water (ULOQ concentration = 500 ng/mL)
Wash:	800 µL water
Wash:	800 µL of 10 % to 100 % methanol
Dry:	Reduce presence of residual wash solvents
Elute:	400 µL methanol (2 aliquots of 200 µL each)

The pooled eluent was evaporated to dryness under nitrogen and reconstituted in 500 µL of mobile phase prior to HPLC analysis.

Liquid Chromatography/Mass Spectrometry
Analysis was performed using an HP 1100 LC system from Agilent Technologies (Palo Alto, CA) equipped with a quaternary pump, in-line degasser, multi-wavelength detector and autosampler. HP Chemstation software was used to analyze the data. The HPLC column used was a Gemini® 5 µm, C18, 150 x 4.6 mm from Phenomenex (Torrance, CA).

HPLC Conditions

Flow rate: 0.5 mL/ min
Mobile phase: 0.1 % formic acid in water (pH 2.7, mobile phase A), 0.1 % formic acid in acetonitrile (mobile phase B); A/B 30/70; total run time 1 min; injection volume 5 µL
Autosampler: G1329A ALS
Pump: G1312A (binary)
MS detector: API 3000 LC/MS/MS with ESI (TurboIonSpray®)(www.AppliedBiosystems.com), positive ion mode in APCI; MRM, heater gas flow 7000 cc/ min; heater temperature 425 °C.

Results and Discussion

Figure 2 shows the set up of the 96-well plate for automated SPE on the MultiProbe II liquid handler system. This design enables the simultaneous screening of twelve different wash conditions after the initial loading of testosterone in plasma. Each wash condition is probed in quadruplicate for assessing reproducibility at a concentration arbitrarily termed ULOQ. Reference and blank plasma samples are also run, as well as a lower concentration (LLOQ) of testosterone. Methanol concentrations in increments of 5 % were studied from 10 % to 30 % and then in steps of 10 % up to 100 % methanol. The whole operation could be finished in about 35 minutes, in contrast to a similar manual cartridge based operation that would have taken 4 to 5 times longer, or about 2 to 3 hours.

ULOQ 10	ULOQ 15	ULOQ 20	ULOQ 25	ULOQ 30	ULOQ 40	ULOQ 50	ULOQ 60	ULOQ 70	ULOQ 80	ULOQ 90	ULOQ 100
ULOQ 10	ULOQ 15	ULOQ 20	ULOQ 25	ULOQ 30	ULOQ 40	ULOQ 50	ULOQ 60	ULOQ 70	ULOQ 80	ULOQ 90	ULOQ 100
ULOQ 10	ULOQ 15	ULOQ 20	ULOQ 25	ULOQ 30	ULOQ 40	ULOQ 50	ULOQ 60	ULOQ 70	ULOQ 80	ULOQ 90	ULOQ 100
ULOQ 10	ULOQ 15	ULOQ 20	ULOQ 25	ULOQ 30	ULOQ 40	ULOQ 50	ULOQ 60	ULOQ 70	ULOQ 80	ULOQ 90	ULOQ 100
E REF 10	E REF 15	E REF 20	E REF 25	E REF 30	E REF 40	E REF 50	E REF 60	E REF 70	E REF 80	E REF 90	E REF 100
EREF 10	EREF 15	EREF 20	EREF 25	EREF 30	EREF 40	EREF 50	EREF 60	EREF 70	EREF 80	EREF 90	EREF 100
BLK 10	BLK 15	BLK 20	BLK 25	BLK 30	BLK 40	BLK 50	BLK 60	BLK 70	BLK 80	BLK 90	BLK 100
LLOQ 10	LLOQ 15	LLOQ 20	LLOQ 25	LLOQ 30	LLOQ 40	LLOQ 50	LLOQ 60	LLOQ 70	LLOQ 80	LLOQ 90	LLOQ 100

Figure 2.
Layout of the 96-well plate (for both Strata™-X and Strata® C18-E) for the wash-elution studies used to determine optimal wash conditions.

Figure 3 summarizes the recovery yields of testosterone obtained from the SPE of the plasma samples on the silica-based Strata C18-E and the polymeric Strata-X sorbents, respectively. In the range of 10 % to 40 % methanol (by volume in water) used as the wash solvent, the recoveries for the two sorbents are comparable, around 70-80 %. However, when the concentration of methanol is increased further, the recoveries on C18E dramatically decreases and when the organic volume in the wash solvent exceeds 70 %, testosterone is completely removed in the wash step itself. On the other hand, with Strata-X, the recovery was still around 66 % with a 70 % methanol wash. From the data, it can be determined that a 60 % methanol wash could be used safely for Strata-X, while the corresponding methanol wash that would generate comparable recovery for Strata C18-E is lower, 40 %. A representative mass chromatogram is shown in **Figure 4**.

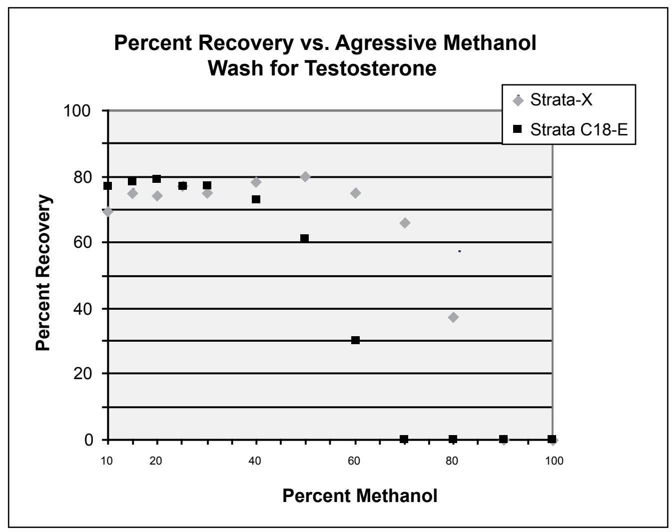

Figure 3.
Graphical representation of the recovery yields from Strata-X and Strata C18-E for testosterone in a plasma matrix for washes in the 10-100 % methanol range.

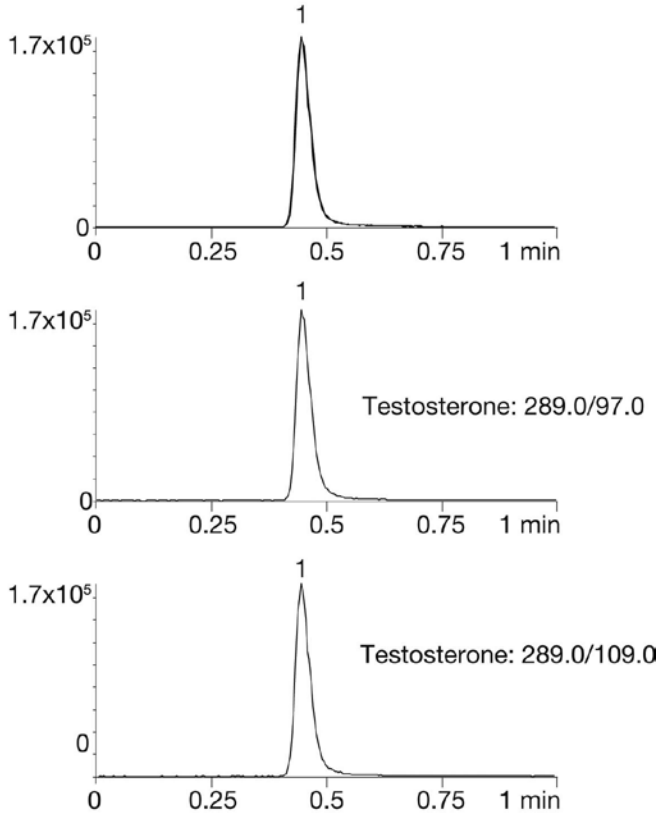

Figure 4.
LC/MS/MS mass chromatogram of testosterone from plasma after Strata™-X clean-up (fragment ion monitored: m/z 97 and 109).

A survey of literature indicates that a 70 % methanol wash can completely remove phospholipids from plasma samples, while a 40 % volume of the same solvent eliminates about 90 % of these lipid contaminants.[7] The recovery of phospholipids with 100 % methanol is about the same as with 70 % of the same solvent. This efficiency of methanol stems from its solubilization capability of phospholipids. It has also been noted that phospholipids can be selectively separated from neutral lipids and sterols on silica-based amino phases with about 80 % methanol mixed with chloroform, or water, under normal and reversed-phase conditions, respectively. This shows that the 70 % methanol wash, in our present work, is more than adequate to eliminate the phospholipids in plasma, which have been found to be the major source of ion suppression during ESI-MS.

In a recent study[8], a 30 % methanol wash was found to be optimal for extracting steroidal hormones from plasma using a C18 cartridge (500 mg bed mass). In other studies, only 10 % methanol wash over C18 was employed.[9] Polar functionalized polymers have also been used for extraction of testosterone and other hormones from different biological matrices and one report uses 60 % ethanol for eluting the steroids.[10] In the present study, the silica-based Strata® C18-E with a bed mass of 50 mg could sustain a 40 % methanol wash, which is much more efficient than protocols reported with other C18 cartridges using much larger sorbent bed masses.

The capacity of Strata-X to hold on to testosterone during 50-70 % methanol washes stems from its capacity to interact with testosterone not only through hydrophobic interactions, but also through π-π and hydrogen bonding interactions. With the Strata C18-E, optimized bonding and hydrophobic end-capping induces stronger hydrophobic interactions, which enables it to sustain 40 % methanol washes for testosterone.

Conclusions
An automated protocol incorporating a strong organic wash for the solid-phase extraction clean-up of testosterone from plasma employing Strata-X has been demonstrated to furnish very clean extracts with good recovery yields. Even the Strata C18-E withstands a stronger organic wash relative to similar sorbents, but the polymeric sorbent is the optimal choice for steroids from biological matrices for improved analytical accuracy and precision.

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Ordering Information - Strata™-X

Format	Sorbent Mass	Part Number	Unit
Tube			
	30 mg	8B-S100-TAK	1 mL (100/box)
	60 mg	8B-S100-UBJ	3 mL (50/box)
	200 mg	8B-S100-FBJ	3 mL (50/box)
	500 mg	8B-S100-HBJ	3 mL (50/box)
	100 mg	8B-S100-ECH	6 mL (30/box)
	200 mg	8B-S100-FCH	6 mL (30/box)
	500 mg	8B-S100-HCH	6 mL (30/box)
Giga™ Tube			
	500 mg	8B-S100-HDG	12 mL (20/box)
	1 g	8B-S100-JDG	12 mL (20/box)
	1 g	8B-S100-JEG	20 mL (20/box)
96-Well Plate			
	10 mg	8E-S100-AGB	2 Plates/Box
	30 mg	8E-S100-TGB	2 Plates/Box
	60 mg	8E-S100-UGB	2 Plates/Box

On-line Extraction Cartridge

Description	Part Number	Unit/Box
Strata on-line extraction cartridge, 20 x 2.0 mm	00M-S033-B0-CB	Ea
Catridge holder, 20 mm	CH0-5845	Ea

Ordering Information - Strata® C18-E

Format	Sorbent Mass	Part Number	Unit
Tube			
	50 mg	8B-S001-DAK	1 mL (100/box)
	100 mg	8B-S001-EAK	1 mL (100/box)
	100 mg	8B-S001-EBJ	3 mL (50/box)
	200 mg	8B-S001-FBJ	3 mL (50/box)
	500 mg	8B-S001-HBJ	3 mL (50/box)
	500 mg	8B-S001-HCH	6 mL (30/box)
Giga™ Tube			
	500 mg	8B-S001-HDG	12 mL (20/box)
	2 g	8B-S001-KDG	12 mL (20/box)
	5 g	8B-S001-LEG	20 mL (20/box)
	10 g	8B-S001-MFF	60 mL (16/box)
	20 g	8B-S001-VFF	60 mL (16/box)
	50 g	8B-S001-YSN	150 mL (8/box)
	70 g	8B-S001-ZSN	150 mL (8/box)
96-Well Plate			
	25 mg	8E-S001-CGB	2 Plates/Box
	50 mg	8E-S001-DGB	2 Plates/Box
	100 mg	8E-S001-EGB	2 Plates/Box

On-line Extraction Cartridge

Description	Part Number	Unit/Box
Strata on-line extraction cartridge, 20 x 2.0 mm	00M-S039-B0-CB	Ea
Catridge holder, 20 mm	CH0-5845	Ea

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