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Quantitative Analysis and evaluation of Free and Total Estrogens and their metabolites in serum using LC-MS/MS with and without derivatization for Clinical Research

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ABSTRACT

Introduction: Estrogens are the primary female sex hormones but are also found in men and are involved in many biological functions. The active estrogens are estrone (E1) and estradiol (E2), while estriol (E3) is the main pregnancy estrogen and the measurement of serum estrogens at ultra-sensitive levels are required as a verification of the sexual characteristics in men and women. A robust and sensitive liquid chromatography-tandem mass spectrometry method for the simultaneous analysis of the total and free fractionated estrogens in serum was developed. Sample preparation methods for the detection of free and total Estrogens with and without derivatization were developed and evaluated for their suitability for enhanced detection and ease of utilization in order to achieve the challenging sub pg/ml range.

Methods : A Thermo Scientific™ TSQ Altis™ tandem mass spectrometer in positive and negative Electrospray mode and a Thermo Scientific™ Vanquish™ HPLC system were utilized. 500 µl of human serum was used for the analysis of free and total estrogens and the sample preparation included ultracentrifugation using Amicon centrifugal units and liquid-liquid extraction. Derivatization agents used included Dansyl Chloride, 4-(Dimethylamino) benzoyl chloride and others to demonstrate which is the most appropriate and easiest to use in order to achieve pg/ml detection requirements. A Thermo Scientific™ Accucore™ C18 50 x 2.1 mm, 2.6 um column with a water:methanol mixture containing Ammonium Fluoride was used for a 6 minute run time. Quantitative analysis was performed using selective reaction monitoring (SRM) transition pairs for each analyte and internal standard. The accuracy of the analytical methods were verified using standardized reference materials.

Preliminary Data: The ultra-sensitive measurements of Estrogens are required since they are bound to sex hormone-binding globulin and albumin with approximately 2.21% free and biologically active. Good linearity and reproducibility were obtained with the concentration range of 0.5 pg/ml to 1000 pg/ml for free estrogens and 1 pg/ml to 10,000 pg/ml for measurements of total estrogens made in estrogen free human serum albumin. The lower limit of detection (LLOD) of free estrogens were achieved at 0.25 pg/ml for E2 and 1 pg/ml for E1 and E3 while the LLOD for total estrogen were 1 pg/ml for E2 and 2.5 pg/ml for E1 and E3 using dansyl chloride derivatization while the other derivatizing agents employed yielded comparable results. The intra- and inter-day CV's for the free and total estrogens have been shown to be <10% for all the calibrators and QC material. The analytical methods were compared using measurements from Standard reference material (SRM 971) from NIST and submitted samples. Extraction recovery was determined and matrix effect was evaluated in individual samples of non-stripped plasma by comparing the signal of the deuterated internal standards to neat solutions. Thus a sensitive, simple, specific and accurate liquid chromatography- tandem mass spectrometry method was developed and evaluated for the measurement of free and total Estrogens in human serum. The various derivatization agents have been evaluated and the initial data demonstrates that there is little difference between their capabilities and each experience matrix issues at the ultra-sensitive levels.

INTRODUCTION

Estrogens (Estrone-E1, Estradiol-E2 and Estriol-E3) are involved in the development and maintenance of the female sexual characteristics, germ cell maturation, and pregnancy as well as growth, nervous system maturation, bone metabolism/remodeling, and endothelial responsiveness. The active estrogens in non-pregnant humans are E1 and E2 while E3 is the main pregnancy estrogen only in women. Estrogens are produced primarily in ovaries, testes, the adrenal glands and some peripheral tissues. Measurement of serum estrogens are needed in the assessment of reproductive function in female and are used to monitor ovulation induction.

In this research study, we evaluated various columns and solvent combinations as well as simple and easy sample preparation techniques in order to develop an LC-MS/MS analytical method that can demonstrate the chromatographic separation, detection and quantification of the estrogens- Estrone, Estradiol and Estriol. The sample preparation choices were kept simple and included a one step liquid-liquid extraction for the analysis of total estrogens and ultracentrifugation followed by a one step liquid-liquid extraction for the analysis for free Estrogens. The need to achieve sub pg/ml detection capabilities resulting in various derivatization techniques of the estrogens to be determined as different techniques have been published recently. The methodologies were developed on a TSQ Altis tandem mass spectrometer in positive and negative Electrospray ionization modes with a Vanquish HPLC system for a 6 minute analytical gradient.

MATERIALS AND METHODS

Standards

The following analytical reference standards and Internal standards were obtained from Cerilliant, Inc. (Round Rock, TX)

Estriol	
Estrone	Estrone-13C6
17β-Estradiol	17β-Estradiol-13C6

Reagents

The following Fisher Scientific™ acids, reagents and solvents were used-

HPLC grade Water	Ammonium Fluoride
Methanol	Methyl-Tert-Butyl Ether (MTBE)
Sodium Bicarbonate	Dansyl Chloride
Pyridine-3-sulfonyl chloride	1,2-dimethyl-1H-imidazole-5-sulphonyl chloride
2-fluoro-1-methylpyridinium-p-toluenesulfonate	4-(Dimethylamino)benzoyl chloride
Acetone	

The standards and internal standards were made up in Methanol

Total Sample Preparation- Liquid-Liquid Extraction

- 500 µL of Serum/HSA mixture calibrators, controls and serum samples were added to a test tube and 50 µL of Estrogen ISTD mixture at 1 ng/mL were added to each and vortexed briefly
- 3 ml of MTBE was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The upper organic layer was transferred to a new test tube and dried down under nitrogen at room temperature
- The extract was reconstituted in 200 µL of 1:1 water and acetonitrile
- The supernatant was transferred to an MS vial and capped.

Free Sample Preparation- Ultracentrifugation

- 600 µl of serum sample, calibrator matrix, controls was added to a Millipore Amicon Ultra 0.5 ml, Ultracel 10 membrane, 10 KDa Centrifugal Filter unit prior to centrifugation for 60 min at 13200 rpm at 37˚C.
- 500 µl of the filtrate was removed to a new tube and 50 µl ISTD at 1 ng/ml were added to each tube and the calibrators were spiked with standards to the desired concentration and vortexed briefly
- The extraction process was continued as described above for the Total sample preparation- Liquid-Liquid Extraction protocol.

Derivatization Sample Preparation

- The dried extracts produced following liquid-liquid extraction were derivatized using 50 µL of Dansyl Chloride or Pyridine-3-sulfonyl chloride or 1,2-dimethyl-1H-imidazole-5-sulphonyl chloride and 50 µL of 100 mM Sodium Bicarbonate at 15 minutes at 65˚C
- Then 100 µL of 1:1 water:acetonitrile were added and the supernatant was transferred to an MS vial and capped
- The dried extracts were derivatized using 100 µL of 2-fluoro-1-methylpyridinium-p-toluenesulfonate or 4-(Dimethylamino)benzoyl chloride at 15 minutes at 65˚C and then dried under heated nitrogen at 40˚C
- The samples were reconstituted in 100 µL of 1:1 water:acetonitrile and the supernatant was transferred to an MS vial and capped.

The calibration curves ranged from 1 pg/mL to 1000 pg/mL and various pooled samples were used as control material.

Data Analysis

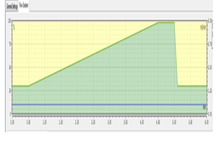
The software used included for this method included the Thermo Scientific™ Xcalibur™ 3.1 SW, Thermo Scientific™ TSQ Altis Tune™ 2.1 SW and Thermo Scientific™ Tracefinder™ 4.1 SW

METHOD

HPLC Conditions-

Vanquish Horizon HPLC binary pump, well plate, thermostatted column compartment

Column:	Accucore C18, 50 x 2.1 mm, 2.6 µm
Column Temperature:	50 °C
Injection Volume:	20 µL
Sampler Temperature:	4 °C
Needle Wash:	Flush port (50%Methanol:50%Water) 10 seconds
Mobile Phase A:	0.2mM Ammonium Fluoride
Mobile Phase B:	Methanol
Flow Rate:	0.5 ml/min
Gradient:	0.0 min- 70%A:30%B
	0.5 min- 70%A:30%B
	4.5 min- 2%A:98%B
	5.0 min- 2%A:98%B
	5.1 min- 70%A:30%B
Run time:	6 mins



MS and Ion Source Conditions-Underivatized

TSQ Altis triple quadrupole mass spectrometer

Ion mode:	Negative Electrospray (H-ESI) Mode
Vaporizer Temperature:	400 C
Ion Transfer Tube Temperature:	350 C
Sheath Gas:	36
Aux Gas:	25
Sweep Gas:	0
Spray Voltage: Negative Ion (V):	3500 V
Q1/Q2 Resolution: (FWHM)	0.7/0.7
Cycle time (sec):	0.6
CID Gas (mTorr):	2
Chromatographic Peak Width:	6 secs

MS and Ion Source Conditions-Derivatized

TSQ Altis triple quadrupole mass spectrometer

Ion mode:	Positive Electrospray (H-ESI) Mode				
Derivative:	DC	PS	DIS	FMP	DMAB
Vaporizer Temperature:	400 °C	400 °C	400 °C	400 °C	400 °C
Ion Transfer Tube Temperature:	350 °C	350 °C	350 °C	350°C	350°C
Sheath Gas:	43	46	41	43	36
Aux Gas:	20	25	25	25	25
Sweep Gas:	0	0	0	0	0
Spray Voltage: Positive Ion (V):	3750 V	3000 V	3000 V	4500 V	4500 V
Q1/Q2 Resolution: (FWHM)	0.7/0.7				
Cycle time (sec):	0.8				
CID Gas (mTorr):	2				
Chromatographic Peak Width:	6 secs				

Table 1- Scan Parameters- SRM table

Compound	Rt (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energies (V)	Rf Lens (V)
Estriol-None	1.6	Negative	287.12	171/145	37/41	125
Estrone-None	2.60	Negative	269.11	183/145	38/38	90
Estrone-13C3-None	2.60	Negative	272.12	186/148	37/38	89
Estradiol-None	2.59	Negative	271.13	183/145	40/39	100
Estradiol-13C3-None	2.59	Negative	274.12	186/146	41/40	94
Estriol-DC	3.60	Positive	552.16	171/156	36/54	101
Estrone-DC	3.91	Positive	504.16	171/156	35/54	98
Estrone-13C3-DC	3.91	Positive	507.12	171/156	38/38	100
Estradiol-DC	3.96	Positive	506.17	171/156	36/53	102
Estradiol-13C3-DC	3.96	Positive	509.17	171/156	36/52	100
Estriol-PS	2.72	Positive	430.16	366/288	29/24	88
Estrone-PS	3.15	Positive	412.16	348/270	27/23	82
Estrone-13C3-PS	3.15	Positive	415.16	351/273	27/23	79
Estradiol-PS	3.25	Positive	414.16	350/272	28/23	83
Estradiol-13C3-PS	3.25	Positive	417.16	353/275	29/23	77
Estriol-DIS	2.6	Positive	447.18	383/96	30/35	97
Estrone-DIS	3.04	Positive	429.16	365/96	29/33	91
Estrone-13C3-DIS	3.04	Positive	432.16	368/96	29/33	79
Estradiol-DIS	3.14	Positive	431.16	367/96	30/35	92
Estradiol-13C3-DIS	3.14	Positive	434.15	370/96	29/35	84
Estriol-FMP	2.19	Positive	380.19	128/110	55/43	112
Estrone=FMP	2.64	Positive	362.16	252/238	40/42	95
Estrone-13C3-FMP	2.64	Positive	365.19	255/241	41/42	85
Estradiol-FMP	2.68	Positive	364.21	128/110	55/43	105
Estradiol-13C3-FMP	2.68	Positive	367.21	131/110	55/43	87
Estriol-DMAB	3.32	Positive	436.18	16/148	19/36	66
Estrone-DMAB	3.94	Positive	418.15	166/151	28/41	85
Estrone-13C3-DMAB	3.94	Positive	421.15	166/151	28/43	87
Estradiol-DMAB	3.91	Positive	420.15	166/151	28/42	88
Estradiol-13C3-DMAB	3.91	Positive	423.16	166/151	28/42	89

RESULTS

Linearity/Sensitivity

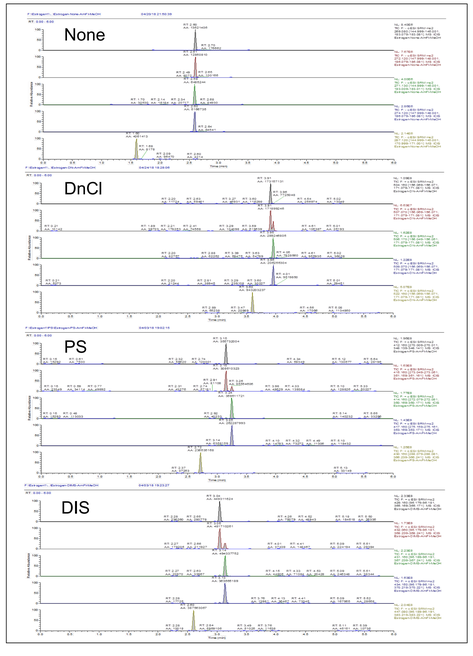
The assays were linear over the calibration curve for the underivatized and derivatized Estrogens in serum/HSA mixture from 1 – 1000 pg/ml for positive and negative mode. The linearity of each extraction was determined in triplicate over 3 days and the results are shown with the LOQ being determined as 10:1 of signal to noise. The mean coefficient of determination (R^2) > 0.98 for each sample extraction technique and the %CV for each calibration point were all <10% in order to be accepted. The analysis of total and free Estrogens derivatized by positive mode electrospray using the LC and source conditions shown were found to be more sensitive than negative mode with a 5 to 10 fold difference in mass spectral response.

Precision/Specificity

The inter–assay precision and accuracy for total and free Estrogens was determined by extracting and quantifying in-house control material resulting in %CV for T4, rT3, T3 of <10% deviation form the targeted mean.

Therefore, the analytical method was determined to work best for the dansyl chloride, pyridine and dimethylimidazole derivatives in positive mode for total and free estrogens. The fluoromethylpyridyl and dimethylaminobenzoyl derivatization proved difficult to replicate and gave inconsistent results and double peaks. The use of underivatized and proven derivatized analytical technique can achieve the laboratory required accuracy for the analysis of total and free estrogens in serum.

Figure 1: Chromatograms Underivatized, derivatized, negative and positive mode



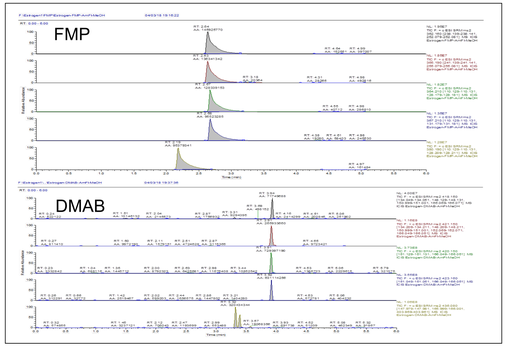


Table 2- Linearity and Sensitivity for the extraction methodology

Compound	LOQ-Total (pg/ml)	LOQ-Free (pg/ml)
Estriol-None	5	5
Estrone-None	2.5	1
Estradiol-None	1	1
Estriol-DC	0.5	0.5
Estrone-DC	0.25	0.25
Estradiol-DC	0.25	0.25
Estriol-PS	2.5	1
Estrone-PS	0.25	0.25
Estradiol-PS	0.25	0.25
Estriol-DIS	1	1
Estrone-DIS	0.5	0.5
Estradiol-DIS	0.5	0.5

CONCLUSIONS

- Baseline separation of estrogens in 6 minutes with good LOQ in positive and negative mode with the derivatized estrogens resulting in the better LOQ levels.
- A clean serum matrix is extremely important to achieve the desired calibration curve and LOQ as the their are many steroidic and lipid interferences at the pg/ml levels and further work will be carried out to improve this very sensitive methodology
- Excellent linearity of calibration curves with better accuracy, precision and reproducibility in positive mode than in negative mode by a factor of 10 fold and the dansylated, pyridylated and dimethylimidazole derivatization gave comparable results for both free and total estrogens
- The FMP and DMAB derivatives were not used as they did give consistent nor did they achieve the same degree of sensitivity as the other derivatives examined

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REFERENCES

1. Liquid chromatography-Tandem Mass Spectrometry assay for the simultaneous measurement of Estradiol and Estrone in Human Plasma Clinical Chemistry, 50:2, 373 – 384
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TRADEMARKS/LICENSING