



Extraction, Derivatization and Ultra-Sensitive GC/Triple Quadrupole Analysis of Estrone and Estradiol in Human Serum

Technical Overview

Clinical Research

Authors

Anthony Macherone, Ph.D.
Agilent Technologies, Inc.
Visiting Scientist, Johns Hopkins
University School of Medicine

Joan Stevens, Ph.D.
Agilent Technologies, Inc.

Introduction

Estrone (E1) and estradiol (E2) are the two primary estrogens in females and males. We have previously reported on the development of a gas chromatograph/triple quadrupole mass spectrometry method for the 1.0 pg/mL or less detection of estrone/estradiol and a robust derivatization procedure [1-4]. This technical overview illustrates a newly developed solid phase extraction (SPE) procedure, an updated derivatization protocol, and new GC/MS method parameters. The addition of the SPE procedure offers a complete workflow from sample receipt to analysis.



Agilent Technologies

Sample Preparation for Extraction of E1 and E2

Sample preparation materials

- Agilent Bond Elut SI LRC SPE cartridges 500 mg, 10 mL (p/n 12113036)
- Agilent Vac Elut SPE 24 Manifold (p/n 12234041)
- VWR VX-2500 Multi-tube Vortexer
- Thermo Sorvall ST 16R Centrifuge
- Labconco CentriVap Concentrator

Solutions, calibrators, and standards

All chemicals, reagents, and solvents were ACS grade or above. Estrone, estradiol, 1-chlorobutane, and sodium acetate purchased from Sigma-Aldrich. Deuterated estrone and estradiol was purchased from Cerilliant. Ethyl acetate and hexanes from JT Baker were purchased through VWR International.

A 0.5 M sodium acetate (NaOAc) solution was made by weighing 68.04 g of NaOAc into a 1-L volumetric with the addition of nano pure water.

Working solutions (WS) of estrone and β -estradiol were made at 200 pg/mL and 2 ng/mL in acetonitrile.

Calibrators at 0.5, 10, 50, 100, 200, 400, and 600 pg/mL were made by diluting the appropriate WS: 200 pg/mL for 0.5, 10, and 50 pg/mL in nano pure water, and 2 ng/mL of WS for 100, 200, 400, and 600 pg/mL in nano pure water.

The internal standard working solution (IWS) was a mixture of d_4 -E1 and d_5 -E2 at 2 ng/mL in ACN.

Sample preparation procedure

Pretreatment of sample

1. Add 1.0 mL of 0.5 M NaOAc to sample tubes.
2. Add 0.5 mL of serum, or plasma, or 0.5 mL of calibrators to sample tubes.
3. Add 50 μ L of the IWS to sample tubes.
4. Vortex for 1–2 minutes.
5. Add 3 mL of 1-chlorobutane to all sample tubes.
6. Vortex for 3 minutes.
7. Centrifuge sample tubes at 5,000 rpm for 5 minutes.

SPE procedure

1. Condition a Silica SPE cartridge with 3 mL of ethyl acetate (EtOAc).
2. Condition the cartridge with 3 mL of hexanes.
3. Condition the cartridge with 3 mL of 1-chlorobutane.
4. Transfer the top layer from the sample tube to the conditioned cartridge with a glass Pasteur pipette, then allow the sample to pass through the cartridge under gravity.
5. Add 6 mL of EtOAc/hexanes (1:9, v:v) solution to the cartridges, and apply low vacuum; do not allow cartridges to dry out.
6. Elute the cartridges with 2 mL \times 2 of EtOAc/hexanes (1:1, v:v) solution, and apply low vacuum. Increase the vacuum once the second 2 mL of elution solution has been applied, and the eluent has passed through the cartridge.
7. Evaporate the collect eluent either by CentriVap concentrator, or under nitrogen in a Turbo Vap at 50 °C.

Derivatization of serum extracts containing E1 and E2

Derivatization materials

Sigma-Aldrich

1. Anhydrous ethyl acetate (p/n 270989)
2. Anhydrous pyridine (p/n 270970)
3. 2,3,4,5,6-Pentafluorobenzoyl chloride (p/n 76732)
4. Hexane (p/n 52767)
5. Sodium bicarbonate (p/n S8875)

Preparation of derivatization reagents

1. **1 % (v/v) pentafluorobenzoyl chloride in anhydrous EtOAc**
Add 1 mL fresh pentafluorobenzoyl chloride to 99 mL anhydrous EtOAc.
Prepare fresh daily.
2. **10 % (v/v) anhydrous pyridine in anhydrous EtOAc**
Add 1 mL anhydrous pyridine to 9 mL anhydrous EtOAc.
Prepare fresh daily.
3. **0.5 M (w/v) aqueous sodium bicarbonate**
Transfer 4.2 g NaHCO₃ to a 100-mL Class A volumetric flask.
qs to 100 mL and mix well.
Prepare every 14 days.

Derivatization procedure

To the dried extracts:

1. Add 0.5 mL of pyridine/EtOAc (10 %, v/v) solution.
2. Add 50 μ L of pentafluorobenzoylchloride/EtOAc solution (1 %, v/v).
3. Vortex.
4. Incubate at 60 $^{\circ}$ C for 30 minutes.
5. Evaporate under nitrogen in a Turbo Vap at 50 $^{\circ}$ C.
6. Add 1.0 mL of 0.5 M NaHCO₃ solution, and vortex for 1 minute.
7. Add 2.0 mL of hexanes, and vortex for 1 minute.
8. Centrifuge tubes at 3,000–5,000 rpm for 5 minutes.
9. Transfer the upper phase into 13 \times 100 mm test tubes.
10. Evaporate under nitrogen or in a Turbo Vap at 50 $^{\circ}$ C.
11. Reconstitute each tube with 50 μ L of dodecane, and vortex for 1 minute.
12. Transfer solution to a GC/MS vial containing a glass insert.

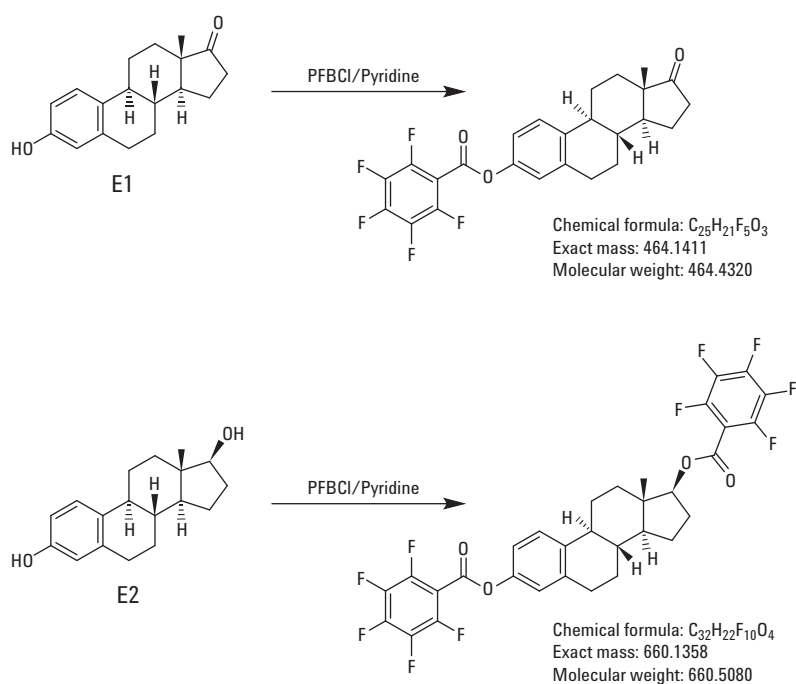


Figure 1. E1 and E2 derivatives.

GC/Triple Quadrupole method

GC

Oven program	210 °C for 0.9 minutes then 35 °C/min to 290 °C for 0 minutes then 2 °C/min to 305 °C for 0 minutes
Run time	10.686 minutes
Triple quadrupole collision cell	EPC
He quench gas	2.25 mL/min
N ₂ collision gas	1.5 mL/min
ALS injection volume	2 µL
Front MM inlet He	Pulsed splitless 290 °C
Transfer line temperature	295 °C
Column 1	Agilent 122-1801 DB-17ht, 5 m × 250 mm, 0.15 µm Flow 1.3 mL/min 0.5 minutes (Post run) 7.9111 mL/min
Column 2	Agilent 122-1811 DB-17ht, 15 m × 250 mm, 0.15 µm Flow 1.5 mL/min 0.5 minutes (Post run) 3.0647 mL/min

MSD

Negative Chemical Ionization mode

Ammonia reagent gas	40 %
Source temperature	150 °C

Table 1. MSD Configuration

Scan type	Compound name	ISTD	Precursor ion	MS1 resolution	Product ion	MS2 resolution	Dwell	Collision energy	Gain
MRM	E2-d ₅	TRUE	665	Wide	601	Wide	50	10	100
MRM	E2	FALSE	660	Wide	596	Wide	50	10	100
MRM	E1-d ₄	TRUE	468	Wide	404	Wide	50	10	100
MRM	E1	FALSE	464	Wide	400	Wide	50	10	100

Conclusion

A complete workflow for the analysis of E1 and E2 has been developed for use in clinical research settings. This workflow provides a quick reference for the establishment of ultra-low detection of estrogens in human serum, especially for samples obtained from subjects on aromatase inhibitors.

References

1. M. Churley, A. Macherone, Highly Repeatable Ultra Low Detection of Estradiol Using Triple Quadrupole GC/MS in NCI Mode, *Agilent Technologies Application Note*, publication number 5990-5513EN (2010).
2. A. Macherone, M. Churley, R. White "Ultralow detection of estrogenic compounds by GC–NCI–MS–MS" *Curr. Trends Mass Spectrom.* (May 2010), pp 10-15.
3. A. Macherone, Derivatization Procedure and Negative Chemical Ionization GC/MS/MS Conditions for the Analysis of Steroidal Analogs, *Agilent Technologies Application Note*, publication number 5990-9478EN (2012).
4. C. Williard "Application of GC/MS/MS in Monitoring Steroids as a Pharmacokinetic and Pharmacological Biomarker" (2013) Retrieved April 1, 2013 from, <http://xtalks.com/xto525pharmanet-i3thankyou.ashx>

www.agilent.com/chem

For Research Use only. Not for use in diagnostic procedures.

Information subject to change without notice.

© Agilent Technologies, Inc., 2014, 2015, 2016
Printed in the USA
November 29, 2016
5991-5289EN



Agilent Technologies