Low pg/mL Detection of Underivatized 17ß-Estradiol in Serum Through Increased Ion Sampling Efficiency Using LC-MS/MS

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Introduction

17ß-estradiol (E2) is the subject of significant clinical research. Low levels of 17ß-estradiol have presented several challenges for traditional analysis of the molecule. Due to a lack of highly ionizable functional groups, mass spectrometric approaches have relied on laborious derivatization methods to achieve a sufficient lower limit of quantitation (LLOQ). Through the use of dual ion funnel technology, ion sampling efficiency has been improved to the point that underivatized 17ß-estradiol can be quickly and accurately quantified at low levels using an LC-MS/MS approach.

Experimental

Sample preparation

Calibration standards ranging from 1 to 500 pg/ml were prepared by dissolving E2 in charcoal stripped human serum. 500 µL of spiked serum was extracted with diethyl ether. Samples were vortexed, centrifuged and 1 mL of the supernatant evaporated under nitrogen until dryness. Dried down extracts were reconstituted in water/methanol (70/30) and introduced to the LC-MS/MS system. In addition to these spiked serum samples, two sets of dried down extracts from the Mayo Clinic (Rochester, MN) were analyzed. The first set of samples consisted of phosphate buffer saline calibration standards in the range of 10 pg/mL to 600 pg/mL and patient samples. The second set consisted of calibration standards in the range of 0.1 pg/mL to 10 pg/mL.

Single LC Configuration: Agilent 1290 Infinity UHPLC

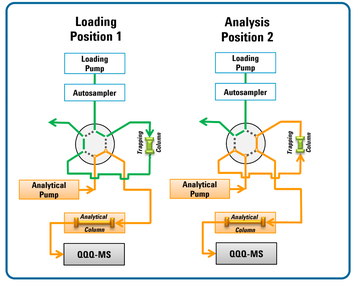
Column: Agilent Zorbax Extend-C18, 2.1 x 50 mm, 1.8 µm. **Mobile phase:** A= 0.05% Ammonium Hydroxide in water, B= Acetonitrile; **Injection volume:** 10 µL; **Flow rate:** 0.4 mL/min. **Gradient:** 15%B to 100%B in 1 min, 100%B during 1 min, 15%B at 2.01 min, stop time at 3.0 min. Autosampler with flexible cube

Matrix-Stripping LC Configuration: Agilent 1260 Infinity LC

Trapping Column: Agilent ZORBAX Extend-C18, 5um, 4.6 x 12.5mm. **Analytical Column:** Agilent Zorbax Extend-C18, 2.1 x 50 mm, 1.8 µm. **Injection V.:** 15 µL or 100 µL. **Loading:** 8%B. **Analytical Gradient:** 15%B to 100%B in 2 or 5 min, 100% B during 1 min. **Switching valve:** 0 min (Loading), 1 or 2 min. (Analysis), end of the gradient (Loading)

Experimental

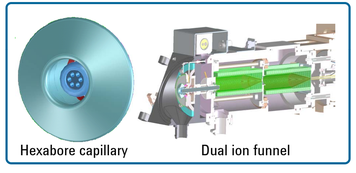
Column-switching hardware and valve positions



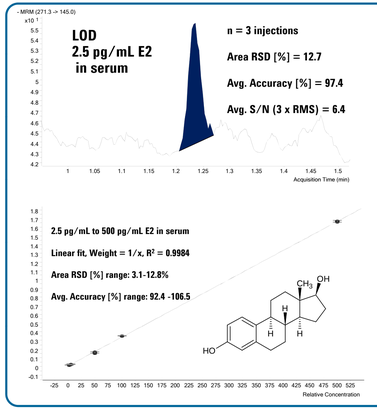
Agilent 6490 Triple Quadrupole MS:

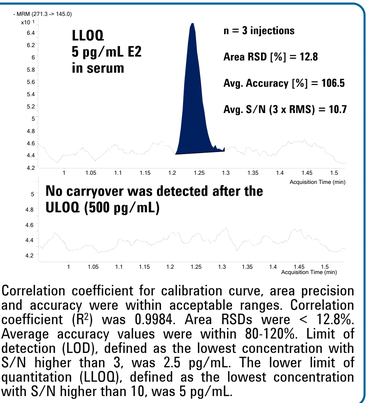
Scan type: MRM. **Polarity:** Negative. **MRM transitions:** 271.3 → 145.2 (Estradiol) and 276.3 → 147.2 (Estradiol-d_5). **Collision energy:** 41 V. **Dwell time:** 100 ms. **Resolution:** MS1/MS2: Unit/Unit (Q1: 0.7 m/z / Q2: 0.7 m/z). **Agilent Jet Stream Parameters:** Drying gas temperature: 180°C, Drying gas flow: 24 L/min, Sheath gas temperature: 350°C, Sheath gas flow: 12 L/min, Nebulizer pressure: 25 psi, Nozzle voltage: 2000 V, Capillary voltage: 3000 V.

iFunnel Technology: Agilent Jet Stream (AJS) technology consists of the addition of a concentric super-heated nitrogen sheath gas to the nebulizer. The super-heated sheath gas collimates the nebulizer spray producing efficient desolvation and ion generation. The use of an hexabore capillary increases the interface area of the capillary inlet within the AJS thermal ion rich zone. The bores spread across the central, ion rich part of the AJS thermal confinement zone. In this way the multibore capillary captures more ions but also more gas. This gas load would normally overwhelm the vacuum system and therefore a novel dual ion funnel was added to efficiently remove the gas, while focusing the ions into the entrace of the first quadrupole.



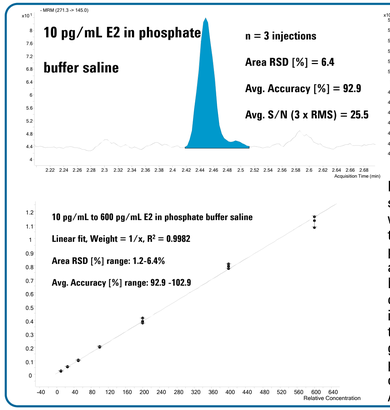
Single LC Configuration, Inj. V = 10 µL: Assay performance

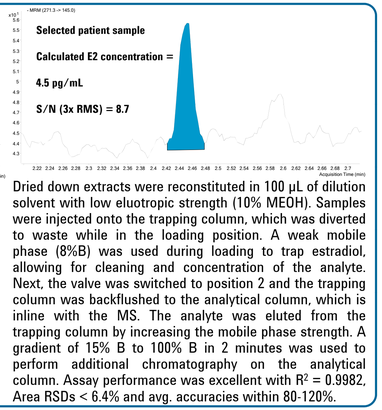




Correlation coefficient for calibration curve, area precision and accuracy were within acceptable ranges. Correlation coefficient (R^2) was 0.9984. Area RSDs were < 12.8%. Average accuracy values were within 80-120%. Limit of detection (LOD), defined as the lowest concentration with S/N higher than 3, was 2.5 pg/mL. The lower limit of quantitation (LLOQ), defined as the lowest concentration with S/N higher than 10, was 5 pg/mL.

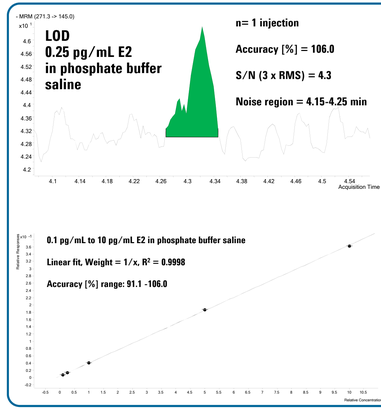
Matrix-Stripping LC Configuration, Inj. V = 15µL: Assay performance

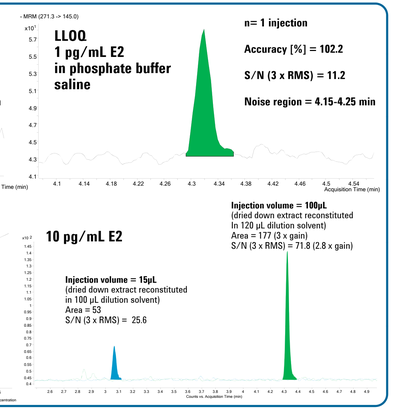




Dried down extracts were reconstituted in 100 µL of dilution solvent with low eluotropic strength (10% MEOH). Samples were injected onto the trapping column, which was diverted to waste while in the loading position. A weak mobile phase (8%B) was used during loading to trap estradiol, allowing for cleaning and concentration of the analyte. Next, the valve was switched to position 2 and the trapping column was backflushed to the analytical column, which is inline with the MS. The analyte was eluted from the trapping column by increasing the mobile phase strength. A gradient of 15% B to 100% B in 2 minutes was used to perform additional chromatography on the analytical column. Assay performance was excellent with R^2 = 0.9982, Area RSDs < 6.4% and avg. accuracies within 80-120%.

Matrix-Stripping LC Configuration, Inj. V = 100 µL: Assay performance





Although matrix-stripping LC involves more complicated column-switching hardware than is required for a single LC, it has several advantages. The setup allows for online sample cleanup, which reduces matrix effects, but it is also possible to inject large volumes of sample onto a narrow-bore sub-2µm particle size column without any band broadening effects. Therefore the possibility of achieving a LLOQ lower than 5 pg/mL was investigated by injecting 100 µL onto the matrix-stripping LC/MS system. A theoretical area response gain factor of 5.5 was expected in comparison to a 15 µL injection volume. Calibration standards in the range of 0.1 to 10 pg/mL E2 were prepared in phosphate buffer saline. Dried down extracts were reconstituted in 120 µL dilution solvent (10% MEOH) and 100 µL injected onto the LC/MS system. A longer loading time (2 min.) and longer gradient time (15%B to 100% in 5 min.) were used for the analysis of this set of samples. A factor 3.3 area response gain and factor 2.8 S/N (3 x RMS) gain were observed in comparison to the method using injection volume = 15µL. Correlation coefficient for calibration curve and accuracy were within acceptable ranges. Correlation coefficient (R^2) was 0.9998. Accuracy values were within 80-120%. The LOD was 0.25 pg/mL and the LLOQ was 1 pg/mL.

Conclusions

- Taking full advantage of dual ion funnel technology, it was possible to quantify underivatized estradiol at the low pg/mL level
- Sensitivity of the method was demonstrated with LLOQ at 1 pg/mL underivatized E2 using a matrix-stripping LC approach capable of concentrating a 100 µL injection volume to avoid band broadening effects
- This high quality estradiol assay with LLOQ = 1 pg/mL and excellent performance will be an important tool for clinical research where assay sensitivity is one of the most important factors