LC/MS/MS quantitation of β-estradiol 17-acetate using an Agilent 6460 Triple Quadrupole LC/MS working in ESI negative ion mode

Application Note

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Abstract

A sensitive, precise, and accurate quantitative liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the measurement of estradiol acetate in human plasma was developed. The sample preparation method employed solid phase extraction on Agilent SampliQ cartridges. The processed samples were chromatographed on an Agilent ZORBAX Eclipse Plus C18 analytical column and analyzed by triple-quadrupole tandem mass spectrometry in the multiple reaction monitoring (MRM) mode using negative electrospray ionization. The principal advantage of the LC/MS/MS method described in this application note is the simultaneous achievement of high absolute recovery (~ 80%), high sensitivity (LLOQ of 100 fg injected on-column or 28.6 pg/mL plasma), high precision (≤ 5.9% CV) and accuracy (81.5-118.1%), as well as excellent linearity over the concentration range 0.1 to 10000 pg/µL ($R^2 \geq 0.998$) with a short run time of only 1.2 min.

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Introduction

β-Estradiol 17-acetate is a derivative of the prime sex hormone estradiol. The molecular structure is shown in **Figure 1**. Highly sensitive analytical methods are necessary to determine these types of analytes in human body fluids. Because of its specificity and precision, LC/MS/MS in negative ion mode offers a better and faster high throughput technique to monitor these types of analytes in body fluids in comparison to radioimmunoassay and gas chromatography - mass spectrometry (GC/MS) methods. In addition to the usage of hazardous radioactive isotopes and the necessary extensive manual labor, immunoassays can be highly variable and inaccurate owing to cross reactivity issues. Purifying the analyte from a plasma sample is a critical segment in the whole sample preparation process because of sample loss issues. To overcome this, Agilent provides a wide range of SampliQ solid phase extraction (SPE) cartridges, which give excellent recovery for a wide range of compounds. The use of an Agilent ZORBAX column provided excellent separation of the analyte from biological matrix components.

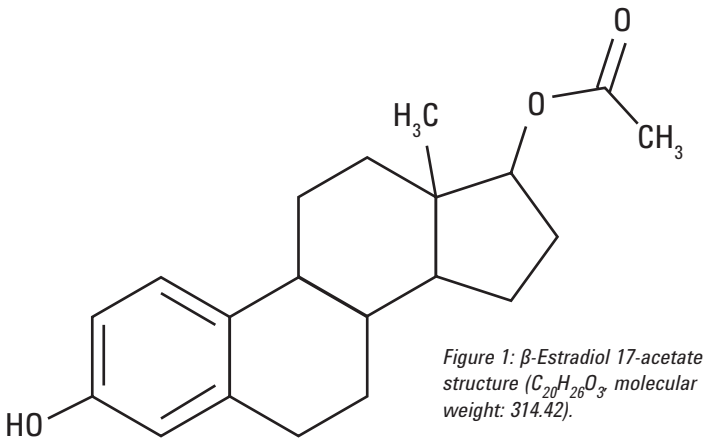

Figure 1: β-Estradiol 17-acetate structure ($C_{20}H_{26}O_3$, molecular weight: 314.42).

Experimental

Chemicals

ß-Estradiol 17-acetate, human plasma, formic acid and solvents for LC/MS/MS analysis were purchased from Sigma-Aldrich. HPLC grade water was freshly taken from a Milli-Q water purification system. Super gradient grade acetonitrile (ACN) and methanol were purchased from Lab-Scan (Bangkok, Thailand).

Preparation of the stock solution, calibration and quality control standards

A 0.2 mg/mL stock solution of ß-estradiol 17-acetate was prepared in acetonitrile (ACN)/water, 50/50 v/v (diluent). To prepare the various calibration levels in aqueous solutions, the stock solution was diluted using the diluent at seven concentration levels; the concentrations used for the linearity study were 0.1 pg/µL, 0.5 pg/µL, 1 pg/µL, 10 pg/µL, 100 pg/µL, 1000 pg/µL, and 10000 pg/µL.

In addition to the above concentration levels, a 0.2 pg/µL standard solution was also used for the analyte recovery study from human plasma to make a total of eight concentration levels. Calibration levels in plasma were prepared by spiking 100 µL of each standard solution to 250 µL of human plasma. A 500 µL aliquot of Milli-Q water was added followed by 150 µL of 0.5% formic acid. The samples were vortexed after each addition. Each sample was loaded onto SPE cartridges as described below. Three quality control (QC) samples were prepared at the following concentration levels to check for precision and accuracy: 20 pg/µL (low QC), 200 pg/µL (mid QC), and 2000 pg/µL (high QC).

Human plasma extraction procedure

Agilent SampliQ C18 end capped (100 mg) cartridges (part number: 5982-1311) were used for the extraction. The SPE cartridges were pre-conditioned with 2 mL methanol and conditioned with 5 mL acetonitrile. Equilibration was achieved using 2 mL of Milli-Q water. After loading the sample, SPE cartridges were washed with 2 mL of water (first wash) followed by 1 mL of ACN/water 20:80 v/v (second wash). After a three minute vacuum drying, the samples were eluted out from SPE cartridges using 1.5 mL acetonitrile (3x 500 µL aliquots). This organic fraction was collected separately and evaporated to dryness in a RapidVap Vacuum Evaporation System at 30°C. The sample was reconstituted with 100 µL of mobile phase (mobile phase A/B; 20:80), and the resulting sample was injected into the Agilent 6460 Triple Quadrupole LC/MS System coupled to an Agilent 1200 Series Rapid Resolution LC (RRLC) system. The SPE protocol is compiled in **Figure 2**.

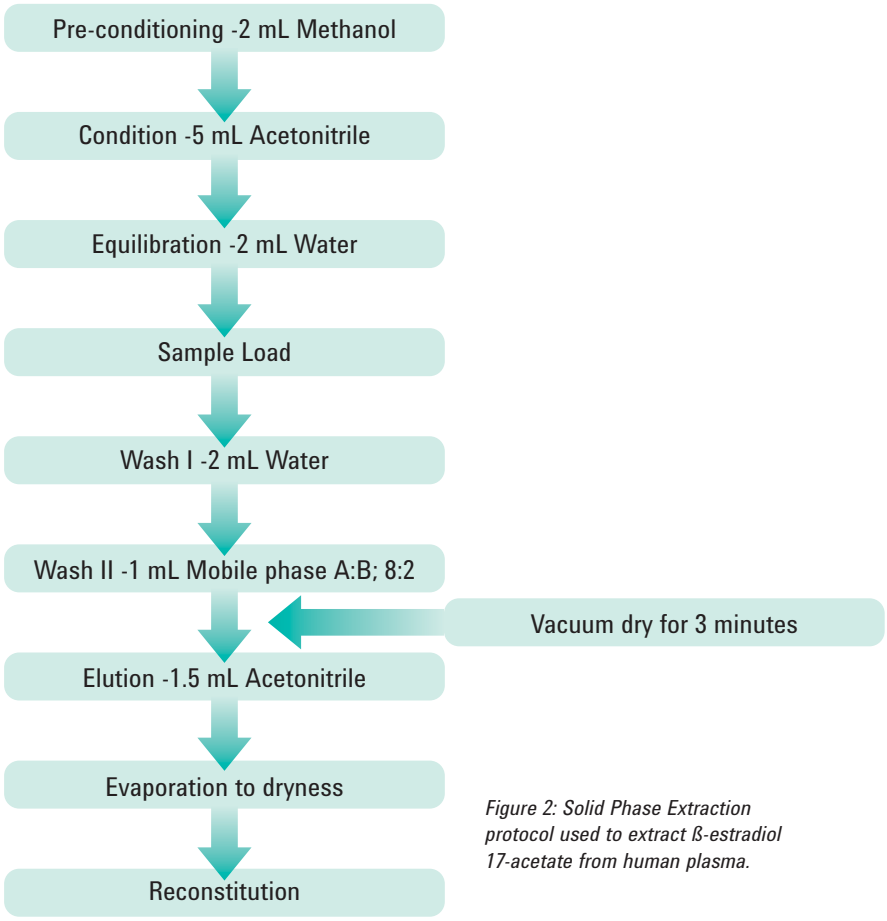

Figure 2: Solid Phase Extraction protocol used to extract ß-estradiol 17-acetate from human plasma.

Determination of extraction recovery

Extraction recoveries of ß-estradiol 17-acetate from spiked plasma samples were determined by comparing the peak areas obtained by extraction of freshly prepared plasma extracts, at different concentration levels, with those from an extraction of an aqueous standard solution at equivalent concentration (n = 3).

LC parameters

The Agilent 1200 Series RRLC system comprised a well plate autosampler with sample thermostat, degasser, column compartment, and pump. A 0.12 mm inner diameter stainless steel capillary was used for all tubing connections. The tubing length was minimized to reduce peak dispersion. The analytical column used for this experiment was ZORBAX Eclipse Plus C18 RRHT 2.1 x 100 mm 1.8 micron 600 bar (part number: 959764-902); the temperature was 40°C. Solvent A was 0.05% ammonium hydroxide (NH_4OH) in water and solvent B was 100% ACN. The pump delivered 80% B isocratic mobile phase at a flow rate of 0.5 mL/min. The run time was 1.2 min with no post run time. The flush port was activated for 5 sec with ACN/water 80:20 v/v. The autosampler was set for a sample injection volume of 1 µL.

Triple-quadrupole parameters

The Agilent MassHunter Workstation software suite incorporated LC/MS data acquisition, qualitative analysis, and quantitative data processing. A 100 pg/µL β-estradiol 17-acetate standard solution was used for the optimization of the MS parameters. The molecular ion mass of β-estradiol 17-acetate in negative mode was found to be 313 Da and in selective ion mode (SIM), an intense peak was observed with a fragmentor voltage value of 135 V. A collision energy (CE) study gave intense products ions at 253 Da (used as the quantifier ion) and 58.9 Da (used as the qualifier ion). The drying gas flow was optimized at 7 L/min at a constant temperature of 300°C. Data acquisition was performed in the negative ionization mode. Capillary voltage was -3500 V. Agilent Jet Stream Thermal Gradient Focusing was operated with a sheath gas flow rate of 11 L/min at a constant temperature of 350°C. A dwell time of 100 ms was used for each of the multiple reaction monitoring (MRM) transitions. Prior to the experiment, the instrument was autotuned in negative mode using default settings. The first quadrupole was operated at wide resolution (1.2 amu at FWHM) and the second one was at unit resolution (0.7 amu at FWHM). ΔEMV = 500 V. The signal-to-noise (S/N) ratio was calculated from the height RMS method found in the Method Editor of the MassHunter software. The parameters were as follows:

signal definition: height
noise definition: root-mean-square (RMS)
multiplication factor: 1
noise region: 0.6 - 0.8 minutes.

Table 1 gives the compiled triple-quadrupole parameters.

Parameter	Set value in 6460 Triple Quad (negative mode)
Fragmentor voltage [V]	135
Collision energy [V]	18
Sheath gas temperature [°C]	350
Sheath gas flow [L/min]	11
Nebulizer pressure [psi]	25
Cap voltage [V]	3500
Nozzle voltage [V]	2000
Drying gas temperature [°C]	300
Drying gas flow [L/min]	7
MS1/MS2 resolution	Wide/Unit
Dwell time per transition [ms]	100
Time filtering [min]	0.02
Delta EMV [V]	500

Table 1: Optimized triple-quadrupole parameters.

Data acquisition and processing

Data acquisition was performed with Agilent MassHunter Workstation Data Acquisition software. Data processing was performed with Agilent MassHunter Quantitative Analysis and Agilent MassHunter Qualitative Analysis software.

Results and Discussion

Quantitation of ß-estradiol 17-acetate in aqueous solutions

Figure 3 represents the precursor and product ions of β-estradiol 17-acetate. For quantitation of β-estradiol 17-acetate, the MRM transition 313 ⟶ 253 was monitored.

Figure 4 shows an overlay of a 100 fg ß-estradiol 17-acetate chromatogram in aqueous solution with a blank. 100 fg/µL standard solution yielded a S/N ratio of 53 (N = 4, Signal = Height, Noise = 1 x RMS).

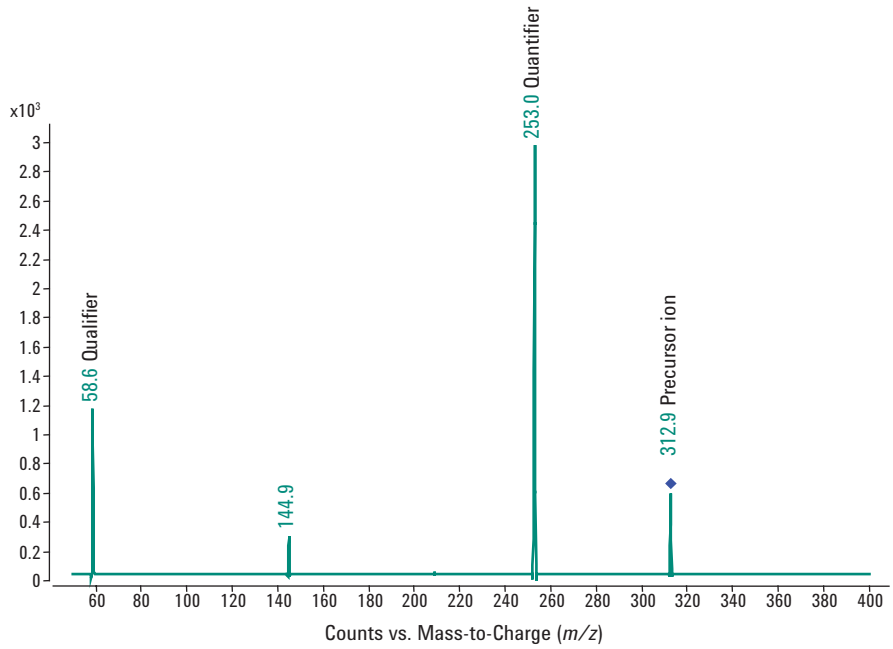

Figure 3: Fragmentation pattern for β-estradiol 17-acetate showing precursor and product ions at CE = 30 V.

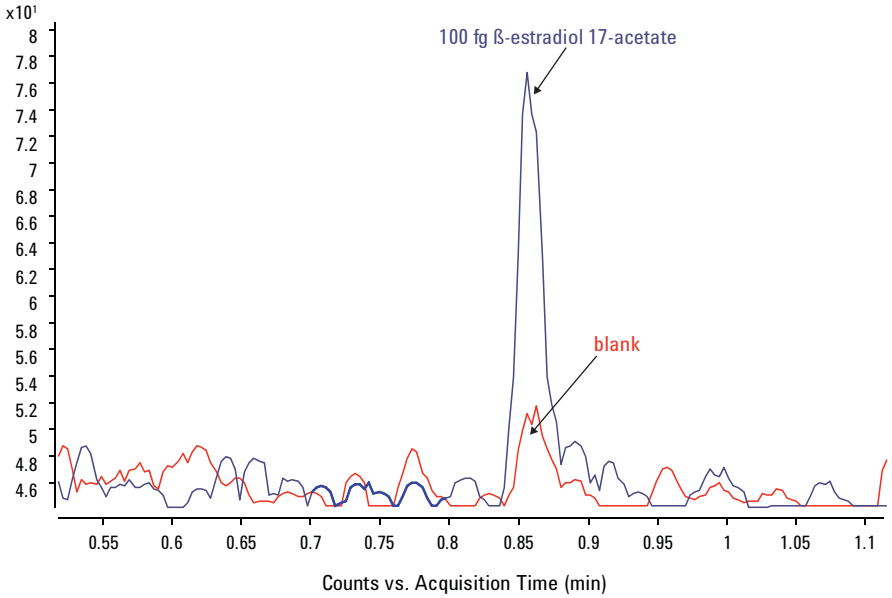

Figure 4: Overlay of a 100 fg β-estradiol 17-acetate chromatogram in aqueous solution (blue trace) with a blank (red trace).

Linearity tests were performed in the concentration range of 100 fg/µL to 10 ng/µL (7 levels, 4 replicates per level). A calibration curve was constructed by plotting the peak area of β-estradiol 17-acetate versus concentration (0.1 pg/µL, 0.5 pg/µL, 1 pg/µL, 10 pg/µL, 100 pg/µL, 1000 pg/µL, 10000 pg/µL, 5 orders of magnitude). The calibration plot was fitted by 1/x weighting. The linearity of the relationship between peak area and β-estradiol 17-acetate concentration is demonstrated by a correlation coefficient of $R^2 > 0.9999$.

The calibration curve for aqueous linearity is shown in **Figure 5** with an excellent correlation coefficient of $R^2 > 0.9999$.

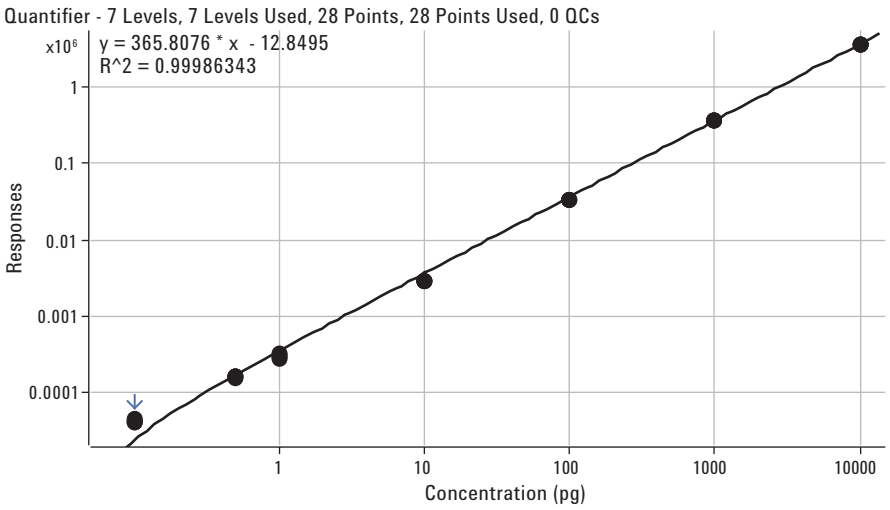

Figure 5: Linearity curve of β-estradiol 17-acetate from 0.1 pg to 10000 pg injected on-column (linear fit, weight 1/x).

Quantitation of ß-estradiol 17-acetate in human plasma

To assess the specificity of the analytical method, blank human plasma samples were extracted and analyzed for potential interferences. The obvious response at the retention time of β-estradiol 17-acetate was compared to the lower limit of quantification. **Figure 6** shows an overlay of a 100 fg β-estradiol 17-acetate chromatogram recovered from human plasma with a blank. 100 fg/µL in plasma yielded a S/N ratio > 38 (N = 4, Signal = Height, Noise = 1 x RMS).

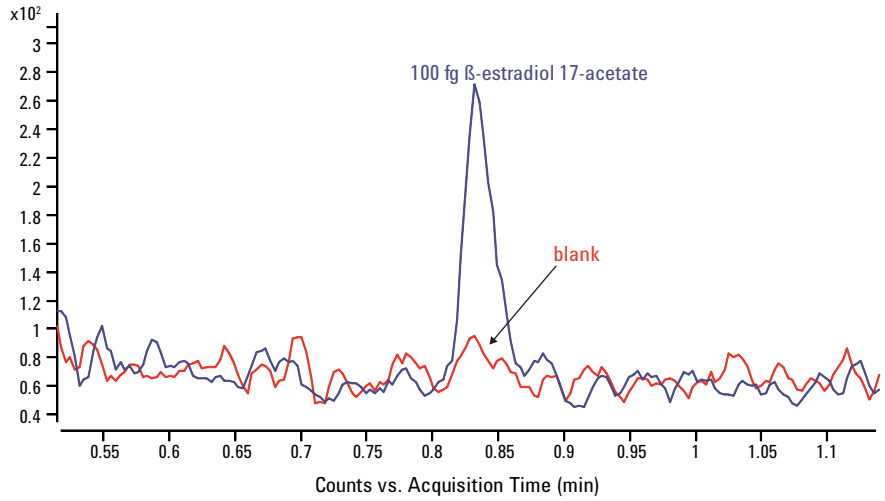

Figure 6: Overlay of a 100 fg β-estradiol 17-acetate chromatogram recovered from human plasma (blue trace) overlaid with a blank (red trace).

Linearity, precision, accuracy, and recovery

Linearity was studied over the concentration range of 100 fg/µL to 10000 pg/µL (eight levels, 4 replicates per level, 5 orders of magnitude). The correlation coefficient (R^2) was higher than 0.9985 (**Figure 7**). The calculation was performed using a linear weighted regression (1/x). The results obtained for linearity, precision, and accuracy are shown in **Table 2**. The method intra-day precision and accuracy values for low QC, mid QC, and high QC levels (20 pg/µL, 200 pg/µL, 2000 pg/µL, n = 4) are 1.25%, 0.42%, 0.92% and 85.0%, 103.2%, 100.9%, respectively. The injector precision and accuracy can be demonstrated using the RSD value of retention time at various linearity levels. Extraction recoveries of ß-estradiol 17-acetate from spiked plasma samples are found to be ~ 80% at 1 pg, 10 pg, and 100 pg concentration levels.

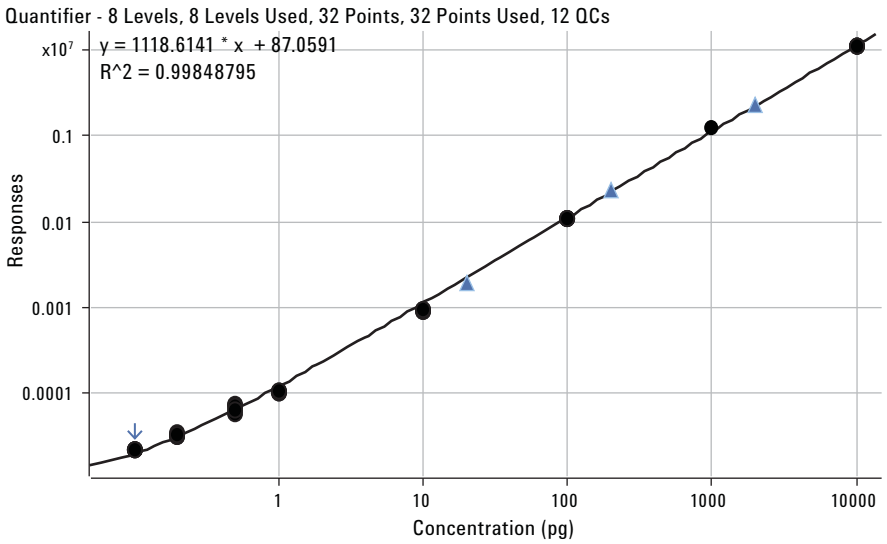

Figure 7: Linearity curve of β-estradiol 17-acetate recovered from human plasma from 0.1 pg to 10000 pg injected on-column (linear fit, weight 1/x). The blue triangles (▲) in the calibration line represent the QC samples.

Linearity level amount on-column	Retention time RSD [%]	Average response	Response RSD [%]	Average accuracy	Accuracy RSD [%]
0.1 pg	0.18	219	1.52	118.1	2.51
0.2 pg	0.34	325	5.24	106.5	7.14
0.5 pg	0.21	658	5.87	102.2	6.76
1 pg	0.29	1032	2.06	84.5	2.25
10 pg	0.00	9200	2.23	81.5	2.25
20 pg (L-QC)	0.00	19112	1.25	85.0	1.26
100 pg	0.00	107822	0.24	96.3	0.24
200 pg (M-QC)	0.18	230983	0.42	103.2	0.42
1000 pg	0.00	1254870	0.29	112.2	0.29
2000 pg (H-QC)	0.00	2257375	0.92	100.9	0.92
10000 pg	0.00	11056383	0.36	98.8	0.36

Table 2: RSD values for retention time, response, and accuracy from plasma linearity study (n = 4).

Conclusions

- Highly sensitive quantitation of β-estradiol 17-acetate from aqueous solutions and human plasma using an Agilent 1200 Series RRLC coupled to an Agilent 6460 Triple Quadrupole LC/MS in ESI negative ion mode is demonstrated.
- Extraction of spiked β-estradiol 17-acetate from human plasma down to a concentration of 100 fg injected on-column is demonstrated using Agilent SampliQ SPE cartridges.
- The linearity of the quantitation of β-estradiol 17-acetate in aqueous solution was demonstrated over five orders of magnitude (from 100 fg to 10000 pg on-column). The observed R^2 value for the calibration curve is > 0.9999.
- The linearity of the quantitation of β-estradiol 17-acetate in human plasma was demonstrated over five orders of magnitude (from 100 fg to 10000 pg on-column). The observed R^2 value for the calibration curve is > 0.998.
- The observed average S/N value for 100 fg β-estradiol 17-acetate in plasma (injected on-column) is > 33 (n = 4, Signal = Height, Noise = 1 x RMS).
- RSD of response for all the levels studied was < 5.9%.
- Accuracy RSD at LLOQ level in plasma is < 2.5% and for all other levels it is < 7.1%.

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