



High Sensitivity Measurement of Underivatized Estradiol by LC/MS/MS using Titan C18 Monodisperse Silica Columns

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Introduction

The sex hormone estradiol is crucial for the development of female secondary sex characteristics, maintaining reproductive function, regulation of the menstrual cycle, and pregnancy. Postmenopausal loss of estrogen is a principal factor in the development of osteoporosis.

In contrast to the relatively high levels of estradiol measured at the time of ovulation induction or IVF, the more technically challenging task is the measurement of low level estradiol in serum, which can be of clinical value in a number of different patient populations. These include children with signs of early breast development and premature puberty, adolescents with pubertal delay or disorders of sexual differentiation, amenorrhoeic women, men with gynaecomastia, perimenopausal females, postmenopausal women, and patients receiving aromatase inhibitors. Measuring low levels of estradiol to establish the degree of ovarian function in patients undergoing therapy for breast cancer may be important in evaluating the anticancer efficacy of some of the hormonal therapies that are used to treat the disease.

Immunoassays have been found to perform poorly for the measurement of low level serum estradiol as they suffer from a lack of sensitivity, specificity, and precision.¹ The improvements in selectivity offered by LC/MS/MS have seen it increasingly become the method of choice for the measurement of sex steroids. Early LC/MS/MS methods suffered from a lack of sensitivity and employed dansyl chloride derivatization to overcome this.² However, these early methods were plagued by non-specific fragmentation of derivatives along with an increase in isobaric interferences. In this study we describe a rapid and sensitive LC/MS/MS method for the measurement of low level serum estradiol without derivatization.

Materials and Methods

Calibrators were created by spiking stripped human serum with 17 β -estradiol to 10, 25, 50, 500, 1,000, and 5,000 pmol/L. To ascertain trueness of measurement, certified reference sera (BCR 576, BCR 577 and BCR 578) developed by the European Commission Joint Research Centre were obtained. Excess patient serum from the routine hospital service was anonymized and used for the purpose of this study. A 200 μ L sample of calibrator or serum was mixed with 50 μ L of internal standard solution (1 nmol/L ¹³C₃-estradiol in 30% methanol) and vortexed. Liquid-liquid extraction was performed by addition of 1 mL of MTBE. Supernatants were dried down and reconstituted in 100 μ L of 50% methanol before being transferred to the LC/MS/MS system. Quantifier ions are shown in Table 1.

Table 1. Quantifier Ions

Transition	Ion
271.1 \rightarrow 145.1	Estradiol quantifier
271.1 \rightarrow 143.0	Estradiol qualifier
274.1 \rightarrow 148.1	¹³ C ₃ Estradiol Internal Standard

Results

Figure 1 shows the chromatograms of estradiol quantifier traces for a patient sample (estradiol, 56 pmol/L) on a 5 cm Titan™ C18 column (top chromatogram) and 10 cm Titan™ C18 column (bottom chromatogram). The longer column demonstrated an improved resolution between isobaric interferences present in human serum and has a lower limit of quantitation of 10 pmol/L. Calibration curves were linear over the entire calibration range from 10 to 5,000 pmol/L (Figure 2). The proposed assay demonstrated a high degree of trueness when measuring serum-based certified reference material developed by the European Commission Joint Research Centre (Table 2).

Table 2. Assay Trueness

Serum-Based Certified Reference Material	Calculated Estradiol Concentration (pmol/L)	Estradiol Concentrations Assigned by Reference Laboratories (pmol/L)	% Trueness*
BCR - 576	120	114	95%
BCR - 577	713	690	97%
BCR - 578	1283	1340	104%

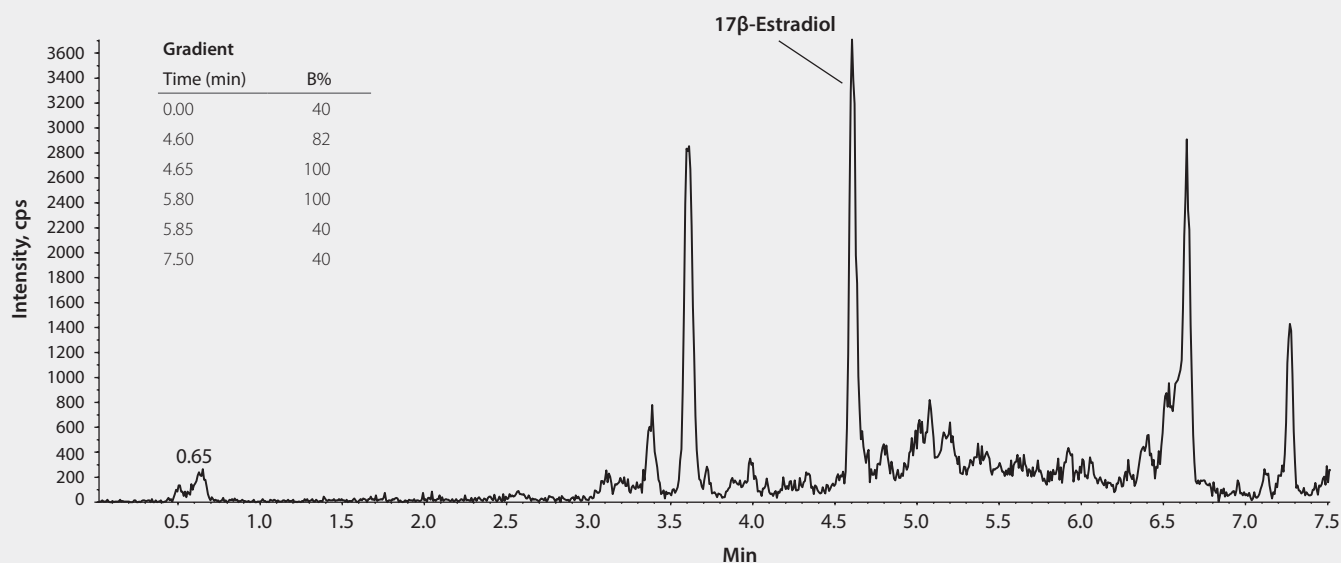
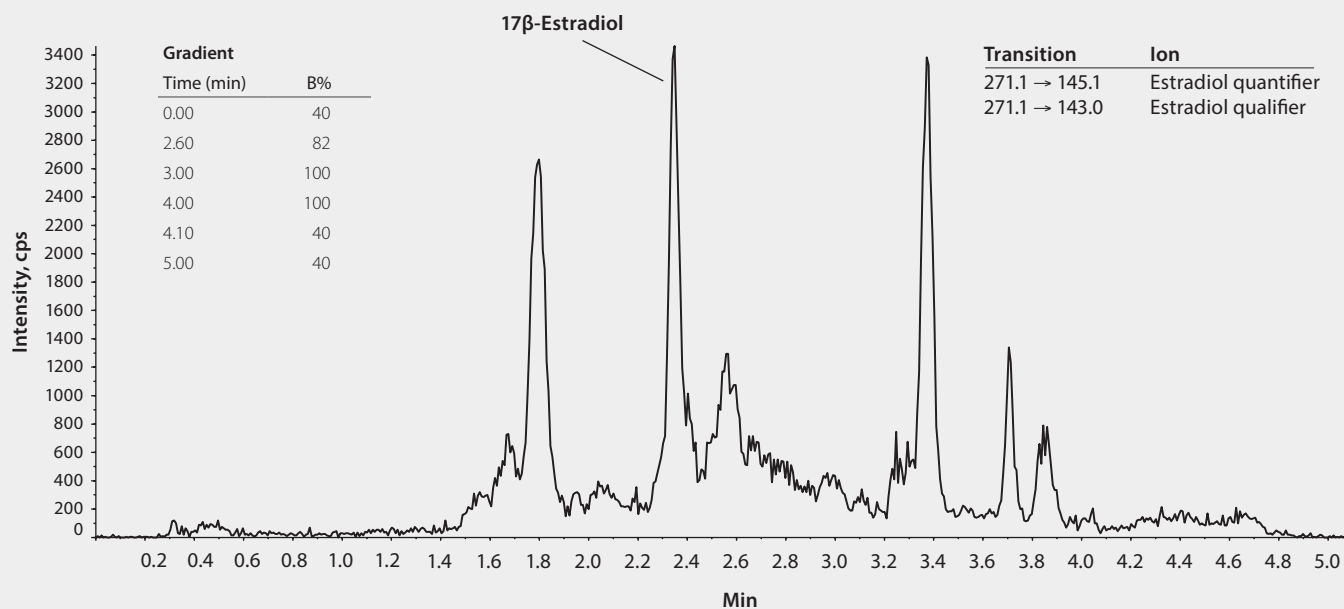
*Trueness (of measurement) – closeness of agreement between the average value obtained from a series of test results and an accepted reference value (ISO 5725-1).

Conclusions

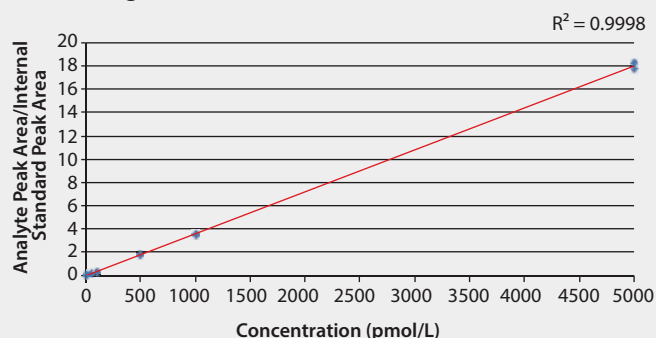
A fast and robust LC/MS/MS method was developed for the measurement of estradiol in human serum using Titan™ C18 1.9 μ m columns. The method requires a small patient sample volume of 200 μ L which is of particular interest to laboratories measuring infant samples. The separation power of the Titan column enabled the removal of isobaric interferences present in human serum, enabling reliable measurement in patient samples down to 50 pmol/L with a 5 cm column, and 10 pmol/L with a 10 cm column. Depending on the patient populations to be measured, this enables the user to decide between speed of the chromatographic run time and sensitivity of the assay. The method demonstrated good agreement with values determined by certified reference laboratories. This method is suitable for clinical laboratories and is currently in use for the investigation of ovarian function.

**Figure 1. LC/MS/MS Analysis of Estradiol (Underivatized) from Patient Serum on Titan™ C18. Comparison of Column Length**

column: Titan™ C18, 5 cm (top, 577122-U) or 10 cm (bottom, 577124-U) × 2.1 mm I.D., 1.9 μm
mobile phase: [A] 0.2 mM ammonium fluoride; [B] methanol
gradient: see figure
flow rate: 0.4 mL/min
column temp.: 40 °C
detector: MS, ESI(-)
injection: 20 μL
sample: patient serum sample containing estradiol, 56 pmol/L
instrument: Shimadzu™ Nexera UHPLC, AB SCIEX Triple Quad™ 6500 and QTRAP®



Data provided by Michael Wright, Department of Clinical Chemistry and Endocrinology, SEALS, Prince of Wales Hospital, Sydney, Australia

**Figure 2. Calibration Curves for Underivatized Estradiol in Serum using the Titan™ C18 Method****References**

- Middle, J. G.; Kane, J. W. Oestradiol assays: fitness for purpose? *Ann. Clin. Biochem.* **2009**, *46*, 441-456.
- Nelson, R. E.; Grebe, S. K.; O'Kane, D. J. et al. Liquid chromatography-tandem mass spectrometry assay for simultaneous measurement of oestradiol and oestrone in human plasma. *Clin Chem.* **2004**, *50*, 373-384.

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Description	Cat. No.
UHPLC Columns	
Titan™ C18, 5 cm × 2.1 mm I.D., 1.9 µm particles	577122-U
Titan™ C18, 10 cm × 2.1 mm I.D., 1.9 µm particles	577124-U
Mobile Phase Components	
Water, LC-MS Ultra CHROMASOLV®, tested for UHPLC-MS, 1 L, 2 L	14263
Methanol, LC-MS Ultra CHROMASOLV®, tested for UHPLC-MS, 1 L, 2 L	14262
Ammonium fluoride, eluent additive for LC-MS, 50 g	52481
CRMs (neat)	
17β-Estradiol, United States Pharmacopeia (USP) Reference Standard, 500 mg	1250008
17β-Estradiol, pharmaceutical secondary standard; traceable to USP, PhEur, and BP, 1 g	PHR1353
CRMs (solution)	
17β-Estradiol, 1.0 mg/mL in acetonitrile (Cerilliant® Certified Reference Material)	E-060
17β-Estradiol-2,3,4- ¹³ C ₃ , 100 µg/mL in acetonitrile (Cerilliant® Certified Reference Material)	E-073
Sample Prep	
MTBE (tert-Butyl methyl ether), CHROMASOLV® Plus, for HPLC, 99.9%, 1 L	650560

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