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Derivatization of estrogens enhances specificity and sensitivity of analysis of human plasma and serum by liquid chromatography tandem mass spectrometry

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ABSTRACT

Estrogens circulate at concentrations less than 20 pg/mL in men and postmenopausal women, presenting analytical challenges. Quantitation by immunoassay is unreliable at these low concentrations. Liquid chromatography tandem mass spectrometry (LC–MS/MS) offers greater specificity and sometimes greater sensitivity, but ionization of estrogens is inefficient. Introduction of charged moieties may enhance ionization, but many such derivatives of estrogens generate non-specific product ions originating from the "reagent" group. Therefore an approach generating derivatives with product ions specific to individual estrogens was sought.

Estrogens were extracted from human plasma and serum using solid phase extraction and derivatized using 2-fluoro-1-methylpyridinium-*p*-toluenesulfonate (FMP-TS). Electrospray in positive mode with multiple reaction monitoring using a QTrap 5500 mass spectrometer was used to quantify "FMP" derivatives of estrogens, following LC separation.

Transitions for the FMP derivatives of estrone (E1) and estradiol (E2) were compound specific (*m/z* 362→238 and *m/z* 364→128, respectively). The limits of detection and quantitation were 0.2 pg on-column and the method was linear from 1–400 pg/sample. Measures of intra- and inter-assay variability, precision and accuracy were acceptable (< 20%). The derivatives were stable over 24 h at 10 °C (7–9% degradation). Using this approach, E1 and E2, respectively were detected in human plasma and serum: pre-menopausal female serum (0.5 mL) 135–473, 193–722 pmol/L; male plasma (1 mL) 25–111, 60–180 pmol/L and post-menopausal female plasma (2 mL), 22–78, 29–50 pmol/L.

Thus FMP derivatization, in conjunction with LC–MS/MS, is suitable for quantitative analysis of estrogens in low abundance in plasma and serum, offering advantages in specificity over immunoassay and existing MS techniques.

Abbreviations: APCI, atmospheric pressure chemical ionization; CID, collision-induced dissociation; cps, counts per second; D, difference; E1, estrone; 3,4-[$^{13}C_2$E1, 3,4-[$^{13}C]_2$-estrone; d_4E1, 2,4,16,16-[$^2H]_4$-estrone; 17αE2, 17α-estradiol; E2, estradiol; $^{13}C_2$E2, 3,4-[$^{13}C]_2$-estradiol; d_4E2, 2,4,16,16-[$^2H]_4$-estradiol; ESI, electrospray ionization; FA, formic acid; FMP-TS, 2-fluoro-1-methylpyridinium-*p*-toluenesulfonate; HPLC, high performance liquid chromatography; IS, internal standards; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; LODs, limits of detection; LOQs, limits of quantitation; MD, mean difference; MRM, multiple reactions monitoring; RME, relative mean error; RSD, relative standard deviation; SD, standard deviation; SNR, signal/noise; SPE, solid phase extraction; TEA, triethylamine; UHPLC, ultra high performance liquid chromatography

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1. Introduction

Analysis of endogenous estrogens is challenging due to their extremely low concentrations, e.g. estradiol (E2) can circulate in concentrations less than 20 pg/mL. Concentrations are commonly <50 pg/mL (<184 pmol/L) in men [1] and 2–21 pg/mL (7–77 pmol/L) in postmenopausal women [2]. Moreover their levels reduce with age and in some diseases such as cardiovascular disease [3] and type 2 diabetes mellitus [4]. Although immunoassays have been widely used to quantify across the range of circulating concentrations of estrone (E1; 55–740 pmol/L) [1] and estradiol (E2; 118–915 pmol/L) [5], specificity is challenging due to interference from endogenous isomers and other steroids in the biological matrix [6]. In addition, none of the commercial methods have satisfactory sensitivity and selectivity for use in older female subjects, in whom concentrations are lower [6–8].

Liquid chromatography–mass spectrometry (LC–MS) provides an alternative analytical method of high specificity and indeed is already replacing immunoassays in the analysis of other steroids routinely monitored in clinical practice, e.g. testosterone and cortisol [6]. However, even with tandem MS coupled to ultra high performance liquid chromatography (UHPLC) [7,8], estrogens are often present in concentrations below the limit of detection of analysis in human plasma and serum, particularly in samples from post-menopausal women [9].

While ultra-sensitive methods have been developed using state-of-the art tandem MS [10], derivatization improves sensitivity by endowing the analyte with a chargeable or permanently charged group for analysis by MS, changing efficiency of ionization, fragmentation and retention [6,11]. LC–MS methods have been reported to measure underivatized and derivatized estrogens, with the latter demonstrating improved limits of quantitation (LOQs): for example LOQs of underivatized steroids (E1; 15, E2; 20 pg/mL) [1] versus those of derivatized steroids (E1; 1, E2; 0.5 pg/mL) [2]. Endogenous estrogens have ketone, hydroxyl and phenolic functional groups suitable for targeting by derivatization reagents.

Previous derivatization reagents employed include dansyl chloride [11–16], pyridine-3-sulfonyl chloride [17,18], 4-(1H-pyrazol-1-yl)benzenesulfonyl chloride [17], *N*-methyl-nicotinic acid *N*-hydroxysuccinimide ester [19] and isomers of 1,2-dimethylimidazole-sulfonyl chloride [17,20]. Reaction of the phenolic hydroxyl with a secondary amine moiety of dansyl chloride via nucleophilic aromatic substitution has been the most common approach [11–16], but a disadvantage for tandem MS is that the product ion is generated by the derivative moiety and hence is not specific for the analyte by mass. In particular the natural mass+2 isotopomers of estrone may generate a background signal within the mass transition of the estradiol derivative [12].

Some of these problems can be averted by chromatographic separation but confounding interference can still arise from unknown estrogen metabolites or from stable isotope labeled estrogens used as *in vivo* tracers or internal standards. Therefore a derivative generating analyte-specific precursor and product ions is desirable to improve specificity [6,13,17,20] and permit more rapid chromatography. This may be achieved by using 2-fluoro-1-methylpyridinium-*p*-toluenesulfonate (FMP-TS) (Fig. 1), which has been used previously to detect estrogens in water [13] and human and bovine serum [21].

We aimed to establish an analytical approach using a methylpyridinium derivative of estrogens to detect these hormones in low abundance in human plasma and, in particular, to evaluate if the approach could quantify circulating steroids in post-menopausal females. A highly sensitive method was validated using ion-exchange solid phase extraction, in conjunction with LC–MS/MS.

2. Materials and methods

2.1. Standards and solvents

Estrone (E1), 17β-estradiol (E2), 17α-estradiol (17αE2), formic acid (FA; ≥98%), triethylamine (TEA; ≥99.5%) and 2-fluoro-1-methylpyridinium-*p*-toluenesulfonate (FMP-TS) were from Sigma-Aldrich, Inc. (St. Louis, USA). 3,4-[^{13}C]$_2$-estrone ($^{13}C_2$E1), 2,4,16,16-[^2H]$_4$-estrone (95-97%) (d_4E1), 3,4-[^{13}C]$_2$-estradiol ($^{13}C_2$E2) and 2,4,16,16-[^2H]$_4$-estradiol (d_4E2) were from Cambridge Isotope laboratories Inc. (Andover, USA). HPLC grade glass distilled solvents (methanol, acetone, hexane and water) were from Fisher Scientific UK Limited (Leicestershire, UK).

2.2. Instrumentation

Structural elucidation of derivatives of steroids in solutions of high concentration was performed on a LC triple quadrupole mass spectrometer, a TSQ Quantum Discovery MS (Thermofisher, Waltham, USA) and operated using Xcalibur software version 2.0. Confirmation of accurate mass was performed by direct infusion into a 12 T SolariX dual source Fourier Transform Ion Cyclotron Resonance MS (FT-ICR MS; Bruker Daltonics, MA, US), operated with SolariX control v1.5.0 (build 42.8) software. High-sensitivity quantification was performed on a QTrap 5500 (AB Sciex, Warrington, UK) coupled to an Acquity™ Ultra Performance LC (Waters Corporation, Milford, USA), operated using Analyst software v1.5.1.

2.3. Plasma and serum samples

Male and female human plasma and post-menopausal female human serum for method development and validation were from TCS Biosciences (Buckingham, UK), stored at −20 °C. This was prepared from human blood from healthy donors in approved blood collection centers. Plasma was collected into anticoagulant (citrate phosphate dextrose adenine), whereas serum was collected without anticoagulant and allowed to clot naturally.

For method application, serum was collected from 27 pre-menopausal women undergoing investigations for menstrual disorders (24–52 years old); these patient samples were anonymised prior to analysis and ethical approval was thus not required for this method development study. Further plasma samples were obtained from subjects participating in experimental medicine studies for which local ethical approval had been obtained; 20 post-menopausal women (58–60 years old) and 48 men (21–85 years old) men [22].

2.4. Standard solutions

Estrogens and internal standards (IS; 5 mg) were dissolved in acetone (5 mL) and stored at −20 °C. Working solutions (1 pg/mL to 100 μg/mL) were prepared by serial dilution on the day of use.

2.5. Generation of FMP derivatives

FMP-TS (50 μL; 5 mg/mL in acetonitrile, containing TEA (1%)) was freshly prepared prior to reaction and added to the standard/extract. The mixture was vortexed (10 s) then incubated (40 °C, 15 min). Mobile phase (water: methanol 65:35), containing FA (0.1%, 50 μL) was added to quench the reaction.

2.6. Fragmentation analysis of FMP derivatives of estrogens

For product ion characterization by tandem MS at nominal mass, molecular ions of derivatives were isolated by Q1 and