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

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Introduction

- Analysis of endogenous estrogens is a very difficult task due to their extremely low concentrations (~50 pg/mL in men and 30-50 pg/mL in postmenopausal women)
- Immunoassays have been widely used to quantify estrogens but suffer from interference due to similarity in chemical structure (Figure 1)

![Figure 1](https://example.com/figure1.png)

**Estrone (E1)** Estradiol (E2) 17α-Estradiol

- Liquid chromatography-mass spectrometry (LC-MS) provides an alternative analytical method with derivatisation to boost sensitivity
- Disadvantage for tandem MS is that often the product ion of derivatised species is formed from the derivatising group and is not specific for the analyte by mass
- A derivative generating a specific product ion is desirable as it allows greater distinction from background isotope interference, particularly in conjunction with rapid chromatography
- **Aims:**
  - To establish a derivative suitable for analysis of estrogens in human plasma, which yields precursor and product transitions specific to estradiol and estrone
  - To develop a validated method using ion-exchange solid-phase extraction in order to detect low levels of estrogens in human biological fluids by tandem MS

Derivatisation and MS/MS fragmentation

- Formation of methylypyridinium ether derivative of phenolic estrogens was achieved and optimised using 2-fluoro-1-methylypyridinium-triethanesulphonate (FMP-TS) in the presence of triethylamine (TEA) (Figure 2A)
- Derivatives for estrogens were tuned on a triple quadrupole mass spectrometer in MS/MS using ESI mode (Figure 2B,C)

![Figure 2](https://example.com/figure2.png)

**A) Estrogen** + **FMP-TS** → (CH3CH2)3N

**B) m/z 252**

**C) m/z 110**

Chromatographic separation

- Baseline resolution of all analytes were achieved (Figure 3)
- Limit of quantitation (LOQ) for estrogens derivatives were determined and compared favourably to the biological levels (Table 1)

![Figure 3](https://example.com/figure3.png)

**Table 1**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOQ (pg)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>5</td>
<td>35-50</td>
<td>150-250</td>
</tr>
<tr>
<td>E2</td>
<td>5</td>
<td>25-50</td>
<td>100-250</td>
</tr>
</tbody>
</table>

Method validation

- Linear standard curve of estrogen derivatives were generated in the range 0.01-1000 pg/mL (Figure 4 e.g. estrone calibration curve)
- The values of intra- and inter-assay precision and accuracy were acceptable (<20% RSD for precision and ±20% accuracy) (Table 2)
- Stability following storage in the auto-sampler (10°C) and freezer (-20 and -80°C) were assessed by injection after 24 and 48 hours and 28 days (Table 3)

![Figure 4](https://example.com/figure4.png)

**Table 2**

<table>
<thead>
<tr>
<th>Precision (%RSD)</th>
<th>Degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td>Time</td>
</tr>
<tr>
<td>Intra-assay</td>
<td>13</td>
</tr>
<tr>
<td>Inter-assay</td>
<td>15</td>
</tr>
<tr>
<td>Accuracy (RME)</td>
<td>18</td>
</tr>
<tr>
<td>Inter-assay</td>
<td>19</td>
</tr>
</tbody>
</table>

Extraction

- The optimised method using ion-exchange solid-phase extraction (Figure 5) was achieved with high recoveries (Mean ±RSD; E1 (95±5%) and E2 (92±3%))

![Figure 5](https://example.com/figure5.png)

**Condition:** 2mL methanol, 2mL water

**Loading:** 2mL sample

**Washing:** 2mL 5% methanol/water

**Elution:** 2mL methanol

Conclusion

- Derivatisation to form FMP ethers of phenolic estrogens, and analysis by LC-MS/MS, is suitable for quantitative analysis of these steroids in low abundance in biological fluids which improved specificity over previously reported derivatisation approaches
- Use of this approach allows robust measurement across typical physiological ranges found in men and premenopausal women
- Care should be taken to store derivatised samples at -80°C for periods greater than 24 h
- The derivatisation reaction may be applicable to other naturally occurring phenolic steroids