Identification of Steroids in Water by Ion Trap LC/MS/MS

Application

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Abstract
The analytical boundaries for the rapid and sensitive identification and analysis of eight representative steroids were investigated using several LC/MS options including ion trap LC/MS/MS. Analysis was favored by direct large volume injections of aqueous sample and the appropriate choice of MS methodology.

Introduction
Synthetic steroids, potent hormones that can impact ecosystems at very low levels, are capable of causing significant and undesirable mutations in fauna. They can enter the environment from many real and potential sources and eventually enter into water drainage systems to lakes and seas. There is, therefore, a need to develop efficient and sensitive methods to monitor these compounds.

This paper summarizes an investigation to determine the analytical boundaries for the identification and analysis of these compounds in water using ion trap MS/MS methodology.

Experimental
Eight steroids, representing three different substituent classes, and diethylstilbestrol, were investigated and are shown in Figure 1. They were dissolved in water or selected solvents, injected into a liquid chromatograph for separation and characterized using several mass spectroscopic modes.
Sample Preparations

The experimental steroids used here were USP reference standards, and diethylstilbestrol was obtained from Aldrich. Experimental standards and their mixtures were prepared in isopropyl alcohol (IPA).

Instrument

Agilent 1100 Series LC/MSD Trap VL

LC Conditions

Column: Zorbax Eclipse XDB-C18, 5 cm long × 2.1 mm id, 3.5 µm particles
Mobile phase A: 0.25 mM ammonium acetate in water
Mobile phase B: Acetonitrile (ACN)
Gradient: Time, min % ACN
          0    20
          0.2  35
           8   90
          9    90
         9.7  20

Flow: 0.25 mL/min
Run time: 9.8 min
Post time: 8 min
Sample size injected: 1 to 100 µL, to deliver each at 5 to 50 ng on column

Binary pump with UV/VIS diode array detector (DAD)

Ion Trap Mass Spectrometry

Vaporizer: 475 °C average
Nebulizer: 30 psi
Dry gas: 8 L/min, 300 °C

V_{cap}^{+}: -3.0 KV for APCI positive ion mode
V_{cap}^{-}: +1.5 KV for APCI negative ion mode
V_{cap}^{-}: +2.0 KV for APPI negative ion mode

Figure 1. The compounds investigated.
Chromatography

A liquid chromatogram of the test compounds is shown in Figure 2. Both name and a peak number identify the steroid peaks. These same peak numbers will be used to identify the peaks in other figures.

Figure 2. Liquid chromatogram of target compounds, detected by ion trap MS.

Injection Volume

The effect of strong solvent injection, using IPA as an example, on the chromatography is shown in Figure 3. It is obvious that there is substantial loss of resolution as the injection volume increases.

Figure 3. DAD chromatograms using injection volumes of 1, 3, 5, and 10 µL on column, in IPA.
Solvent Choice

The effect of solvent choice on the sharpness of chromatographic peaks is shown in Figure 4. Here the reciprocal of peak width is plotted against injection volume for both water and IPA solutions. As much as 100 µL of an aqueous sample can be injected and still obtain the same peak width as a 1 µL IPA solution of the same concentration, which in this case is 5.0 µg/mL.

![Figure 4. The effect of solvent choice on the maximum allowable injection volume.](image)

Figure 5 compares the DAD chromatograms obtained from these solutions. Note that the water solution exhibits 10 times the signal intensity using a tenth of the concentration.

![Figure 5. Comparison of DAD chromatograms produced from water and IPA solutions of the target compounds.](image)
Vaporizer Temperature

Signal intensity is a function of both target compound and vaporizer temperature. The relationship for four steroids is shown in Figure 6. The signal intensity for estriol is greatest when the vaporizer temperature is about 450 °C, while for the others the signal intensity keeps increasing up to the experimental limit. When analyzing for all compounds in this set the vaporizer temperature must be a compromise, hence the 475 °C setting.

Figure 6. Signal intensity vs. vaporizer temperature relationship for estriol, norethindrone, norgestrel, and hydroxyprogesterone caproate.

Atmospheric Pressure Ionization and Mass Spectrometry

Several mass spectrometric options were evaluated and described. The major ions observed in these experiments are identified in Table 1. In the negative ion experiments the indicated solvents were added post column. We note that certain steroids were not detected by all modes. We also note that estriol and estradiol lose water during APCI positive ionization.

Table 1. Ions Observed Using Different Atmospheric Pressure Ionization Modes

<table>
<thead>
<tr>
<th>No.</th>
<th>Steroid</th>
<th>MW</th>
<th>APCI positive ion Expected m/z</th>
<th>Measured m/z</th>
<th>Ion</th>
<th>APCI negative ion, CH₂Cl₂ Expected m/z</th>
<th>Measured m/z</th>
<th>Ion</th>
<th>APPI negative ion, acetone Expected m/z</th>
<th>Measured m/z</th>
<th>Ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Norethindrone</td>
<td>298</td>
<td>299</td>
<td>299</td>
<td>[M+H]⁺</td>
<td>297</td>
<td>Not found</td>
<td></td>
<td>297</td>
<td>Not found</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Norgestrel</td>
<td>312</td>
<td>313</td>
<td>313</td>
<td>[M+H]⁺</td>
<td>311</td>
<td>Not found</td>
<td></td>
<td>311</td>
<td>Not found</td>
<td></td>
</tr>
</tbody>
</table>
The mass spectra for the APCI positive ions are stacked and shown in Figure 7. Arrows indicate the peaks chosen for additional dissociation to produce the second generation mass spectra shown in Figure 8.

**APCI Positive ion**

- Estriol
- Estradiol
- Estrone
- Norethindrone
- Norgestrel
- Progesterone
- Medroxyprogesterone acetate
- Hydroxyprogesterone caproate
- Diethylstilbestrol

**Figure 7.** Stacked APCI positive ion mass spectra for the target compounds.
**Auto MS/MS**

One major feature of the Agilent LC/MSD trap is its ability to automatically sense, from an initial scan of a chromatographic peak, the major masses worthy of MS². The compound mixture was processed this way and the resulting second generation mass spectra are shown in Figure 8. Here only one MS² spectrum is shown for each target compound, although many could have been automatically selected had the precursor ion exceeded threshold. Arrows in Figure 7 indicate the precursor ions chosen for further dissociation in the trap. Two precursor ions exceeding threshold were allowed for MS² in these Auto MS/MS experiments.

**APCI Positive ion MS/MS**

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**Estriol 271 m/z**

**Estrodiol 255 m/z**

**Estrone 271 m/z**

**Norethindrone 299 m/z**

**Norgestrel 313 m/z**

**Progesterone 315 m/z**

**Medroxyprogesterone acetate 387 m/z**

**Hydroxyprogesterone caproate 429 m/z**

**Diethylstilbestrol 269 m/z**

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Figure 8. Stacked second generation (MS²) mass spectra for one of each target compound’s precursor ions.
Chromatograms were generated using both first (MS) and second-generation (MS\(^2\)) data for each chromatographic peak at compound concentrations of 5 and 50 ng on column. These are compared in Figure 9. The separate chromatograms at each concentration represent different order MS data as indicated in the figure key. The MS/MS chromatograms represent sums of product ions from each parent. The ability to use multiple precursor ions is an analytical advantage when there are overlapping chromatographic peaks exhibiting similar primary dissociation ions.

Figure 9. MS and MS/MS chromatograms at 5 and 50 ng on column for the target compounds.
**Multiple Reaction Monitoring (MRM)**

MRM is an analytical option, which directly produces the desired second-generation mass spectrum. This is useful when the compound’s primary mass spectrum is already known and further analysis will only use second generation data from pre-selected primary ions.

Figure 10. MRM second generation chromatograms of target compounds at two concentration levels using two precursor ions per compound.
Another useful analytical option is the ability to add signal intensities derived from multiple secondary ions from the same chromatographic peak to further enhance sensitivity. This is shown in Figure 11. Note the dramatic signal intensity increase for peaks 4, 5, and 6 when compared to neighboring peaks 3 and 7. This is most advantageous for situations where the secondary dissociation produces many low intensity peaks and complex MS/MS spectra. (For example, MS/MS spectra of nordihydroguaiaretic acid, norgestrel, and progesterone in Figure 8.)

Figure 11. Comparison of MRM signal intensities using single and multiple product ion contributions.
Observations and Conclusions

- Chromatographic properties favor direct large volume aqueous sample injection. Rapid analysis is possible.

- Estriol, Estradiol and DES are best detected using negative ion APCI with 450–475 °C vaporizer temperature; all others are best detected in positive ion mode and at maximum temperature.

- APPI is very selective for some compounds under these conditions.

- Ionization in positive ion mode is usually simple. Some compounds lose water in the CID region.

- Positive ion APCI is best for analysis of weak ionic strength mobile phases.

- Auto MS/MS is possible in scan mode to 5 ng of compound on column. TRAP SL optics is expected to give significantly better sensitivity and speed [1].

- MRM allows product ion scans to better than 5 ng on column. TRAP SL optics is expected to give significantly better sensitivity and speed [1].

- Monitoring the sum of product ions is best for compounds with complex product ion spectra.

References

1. Supporting Agilent documents viewable via www.agilent.com
   a. Publication 5988-2870EN, June 1, 2001
   b. Publication 5988-3619EN, January 17, 2002
   c. Publication 5988-3620EN, October 31, 2001

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