The Analysis of Estrogens Using Liquid Chromatography and Negative Electrospray Ionization Mass Spectrometry

Katie Estridge1,2, Carol Babyak2, Wendy Lewis2
1Environmental Science Program, Appalachian State University, Boone, NC
2A.R. Smith Department of Chemistry, Appalachian State University, Boone, NC
estridgeke@appstate.edu

Abstract

Many estrogenic compounds enter the environment via wastewater treatment plants (WWTPs) that discharge treated water into aquatic systems. Effluent from the Boone, NC WWTP into the South Fork of the New River is responsible for 23-30% of its total flow. To date, no field-testing has been conducted to determine if elevated concentrations of estrogenic compounds are being expelled from the WWTP when a larger student female population is living in Boone during Appalachian State University’s academic year. To investigate natural and synthetic estrogens sourced from the student population, including estrone, estradiol, and ethinylestradiol, we developed an analytical method for the identification and quantification of various estrogens using high-performance liquid chromatography coupled to an electrospray ionization mass spectrometer (HPLC-ESI-MS) operating in negative ion mode. To determine the appropriate cone voltage, probe temperature and mobile phase, a 100 part per million (ppm) multi-component standard solution containing each of the target estrogens was used. Optimal needle voltage was determined using three 100 ppm single component estrogen solutions, which were manually injected into the ESI-MS. With respect to these parameters, optimal results were obtained using a cone voltage of 120 V, a probe temperature of 450°C, a mobile phase of degassed deionized water, and a needle voltage of -2.0 kV.

1.0 Introduction

The presence of pharmaceuticals in environmental and drinking waters has recently been a growing concern internationally. Specifically, sex hormones may induce some of the most readily observable and daunting effects when they are present in elevated concentrations in nature [1]. While sex hormones occur naturally, an increased use of birth control drugs and the application of hormones to livestock likely exacerbate this problem [2]. The effects of pharmaceuticals in drinking water may have less of an immediate impact on humans compared to the immediacy of endocrine disruption on certain aquatic species. In a controlled exposure experiment by Metcalfe et al. (2001) [3], male Japanese medake fish were exposed to various estrogen compounds including estradiol, ethinylestradiol (EE2), estrone, and estriol. At a concentration of approximately 1.0 part per billion (ppb), intersex gonads were evident; at concentrations of 5-10 ppb, total sex reversal was observed [1]. Waste water treatment plants exist to collect and treat or “clean” human sewage before releasing it as a water effluent back into the environment. The major goal of such treatment facilities is to remove suspended solids, pathogenic microbes, chlorine compounds, excessive phosphorus and nitrogen nutrients, heavy metals, and pharmaceuticals prior to discharging the water back into the ecosystem. A typical WWTP effluent report may include levels of dissolved oxygen, suspended solids, pH, fecal coliform, metals, nitrogen, etc., but these reports are not likely to include information on pharmaceutical discharge content because measuring a vast number of pharmaceutical compounds is obviously impractical for most facilities. Due to the
lack of attention given to pharmaceutical content in WWTP effluent, some drugs may not be completely removed during the treatment process, and may exist in effluent at trace concentrations or higher.

In general, estrogens leave the body as conjugates (primarily glucuronides), which have the potential to dissociate into their original free estrogen form after being released into the environment or during treatment at a WWTP [2,4]. Recent research suggests that these estrogens, synthetic EE2, estradiol, and estrone (see Figure 1), were found to be the most potent estrogenic compounds in WWTP effluent [4]. Synthetic EE2, used in 90% of birth control pills, can pass through the body either un-metabolized or can be metabolized into estradiol or estrone [5]. The methods developed, as described herein, focus on the detection and quantification of the synthetic estrogen EE2, as well as two natural estrogens: estradiol and estrone.

The dilute estrogen concentrations in treated effluent are further reduced when they reach natural sources such as rivers or streams; as a result, it is necessary to employ the use of instrumentation with appropriate sensitivity [4]. Previously, an isocratic high-performance liquid chromatograph (HPLC) with a photo diode array (PDA) detector was used to achieve separation and detection of analytes [6]; however, due to a lack of sensitivity, this method was revised. In order to gain a second element of analyte detection, a gradient elution method described by Croley et al (2000) [1] was more desirable because it is compatible with HPLC coupled to electrospray ionization mass spectrometry (ESI-MS).

Unlike more traditional ionization sources used in mass spectrometry, which have pre-developed standardized conditions as well as standardized mass chromatograms, ESI is a relatively new technique without standardized conditions. Therefore, ESI operations have not been fully developed and are unique to a particular set of analytes for individual experiments. For example, electron impact (EI) ionization sources typically operate using 70 eV electron energy because it is under these conditions that ion yield is generally maximized. Also, under conditions of 70 eV, standardized EI mass spectra are readily available in databases such as the NIST Chemical WebBook (http://webbook.nist.gov/chemistry/). However, ESI parameters within the ionization source, such as cone voltage, needle voltage, and probe temperature, must be manipulated to achieve optimum volatilization and fragmentation for the target analytes.

The goal of this work is to describe the method development process for the detection of estrogenic compounds using HPLC-ESI-MS. With the addition of a mass spectrometry component, analyte detection may be improved with better detection limits. With the ability to achieve detection in two ways (via PDA detector and MS), one technique can be used to aid in validating the other. Because the high-performance liquid chromatograph - ultraviolet-visible spectroscopy (HPLC-UV-VIS) method has already been developed successfully [6], any mass spectral inconsistencies obtained during ESI method development were compared to LC results, which were generally more consistent.

2.0 Experimental Methods

2.1 Reagents & Laboratory Equipment

Estrogen stock solutions were prepared using powdered estrogens from Sigma-Aldrich (17-α-ethynylestradiol- minimum 98 % HPLC,

<table>
<thead>
<tr>
<th>Structure</th>
<th>Estrogen</th>
<th>Source</th>
<th>Molecular Weight (amu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>estrone</td>
<td>natural</td>
<td>270.3661</td>
</tr>
<tr>
<td>B</td>
<td>estradiol</td>
<td>natural</td>
<td>272.3820</td>
</tr>
<tr>
<td>C</td>
<td>EE2</td>
<td>synthetic</td>
<td>296.4043</td>
</tr>
</tbody>
</table>

Figure 1. Structures of estradiol, estrone, and EE2
β-estradiol- minimum 98%, estrone- minimum 99%). Mobile phases were prepared using deionized (DI) water (Nanopure Diamond, Barnsted Hollow fiber filter, gamma irradiated, 0.2 µm pore size rating), acetonitrile (OminiSolv, LC-MS grade), methanol (EMD, HPLC grade), acetic acid (glacial, EMD), and ammonium acetate (OminiPur, EMD).

Solid mass measurements were achieved using an analytical balance (Adventurer, Ohaus, service certified: 6-15-09) and liquid aliquots were measured using glass micro syringes (Hamilton micro syringes). Mobile phases were degassed (sparged) with helium gas (Airgas, He compressed, UHP) and filtered using Whatman filters (cellulose acetate membrane: 0.45 µm pore size, and nylon membrane: 0.2 µm pore size).

All glassware was washed with isopropyl alcohol three times, rinsed with DI water three times, and then solvent rinsed.

2.2 Mobile Phase & Standard Preparation

All mobile phases were filtered using vacuum filtration and then degassed. Degassing was initially accomplished using nitrogen gas; however, mobile phases are now degassed using helium gas, which is thought to be more effective because of its low mass. Mobile phase shelf life integrity was questionable because degradation and re-gassing became apparent with age; as a result, new mobile phases were prepared at the beginning of each week.

Single component estrogen stock solutions were prepared by dissolving appropriate weights of each estrogen in a 100.00 mL volumetric flask with methanol to achieve a final concentration of 2000 ppm. Eventually the estrone stock solution was prepared at a concentration of 1000 ppm because estrone would not dissolve at higher concentrations. Also, estrone would occasionally precipitate out of solution after being stored in the freezer. Multi-component estrogen standards were prepared by adding appropriate aliquots of stock solutions to five 10.00-ml volumetric flasks, and diluted with methanol. Concentrations of multi-component calibration standards included the following: 5, 10, 25, 50 and 100 ppm. Stock solutions were prepared no less than every six months and calibration standards were prepared according to how often calibrations were needed for experimental purposes. Stock solutions and standards were stored in volumetric flasks, capped and wrapped with parafilm and aluminum foil to minimize evaporation and light exposure, and placed in a freezer. Prior to their analysis, approximately 1 mL of the standard solutions were placed in amber HPLC vials and allowed to equilibrate to ambient temperature.

2.3 Instrumentation

Separations were achieved using a Dionex HPLC equipped with the following Ultimate 3000 components: pump (with Smart Flow), autosampler, column compartment, and photodiode array detector (with deuterium and tungsten lamps). A Phenomenex column was used in separations (Luna, 5µ, C18, 100A, 250mm x 4.6 mm), equipped with a guard column (Phenomenex, Security Guard HPLC, C18, 4 x 3.0 mm). Column oven temperature was set at 30ºC and a normal injection mode (20 µL injection volume) was employed. At a flow rate of 0.667 mL/min, a gradient elution was used to optimize estrogen separation: at time= 0 min (100 % mobile phase A, zero hold time), at time = 3 min (70% mobile phase B, hold 7 min), at time = 10 min (100% mobile phase A, hold 10 min) for a total separation time of 20 min. Mobile phase A is considered the primary mobile phase (DI water was determined best) and mobile phase B is an organic modifier solution of 1:3 methanol: acetonitrile. The LC separation chromatograms were obtained at wavelengths of 217 and 230 nm.

Instrumental interface was achieved between the HPLC and an ESI-MS also manufactured by Dionex (Surveyor MSQ Mass Spectrometer System, MSQ20826), and equipped with a single quadrupole mass analyzer and nitrogen generator (NitroGen, Peak Scientific, N418LA). Mass spectrometry was carried out in negative ion mode and the following parameters for optimum ionization were determined throughout the course of the experiment: probe temperature: 450ºC, needle voltage: -2.0 kV, selected ion monitoring (SIM) cone voltage: -120 V, and total ion count (full scan ion monitoring) cone voltage: -110 V. Because the ionization was carried out in negative ion mode, molecular ion peaks of [M-H]- were prominent.

Prior to interfacing the instruments, the PDA was allowed to equilibrate using wavelengths of 230 and 217 nm, and a basic function test (BFT) was performed on the MS. During a BFT, mass spectra are viewed in real time using the following conditions: 0.25 mL/min flow rate, positive
ion mode, probe temperature: 350°C, cone voltage: 70 V, needle voltage: +3.0 kV, and a mobile phase of 50/50 acetonitrile: water. These analyses were performed at the beginning of each day to ensure the MS was functioning properly, which was confirmed by the presence of two major mass-to-charge ratio (m/z) peaks (at 42 and 83).

3.0 Results and Discussion
For many of the experiments described, the objective was to achieve optimal and reproducible mass spectral data after altering parameters within the ESI-MS. Because analytes were not being quantified, rigorous calibrations were rarely conducted. A 100 ppm multi-component estrogen standard containing estradiol, estrone, and EE2 was used throughout each experiment. Subsequent experiments are listed in the chronological order in which they were performed.

3.1 Cone Voltage Parameterization
Electrospray ionization is used to fragment large molecules into smaller ions, so one of the parameters of ESI that influences the fragmentation of ions is the cone voltage; different cone voltages cause compounds to ionize and fragment in different ways. In order to determine the optimal cone voltage to use for the analysis of the target estrogenic compounds, varying cone voltages were evaluated by incrementally increasing 10 trial voltages ranging from 60 to 130 V.

In general, peaks from UV-VIS chromatograms were very distinguishable with consistent retention times for beta-estradiol near 11.0 minutes, EE2 around 11.5 minutes, and estrone around 12.4 minutes. Molecular ion peaks for beta-estradiol were observed at m/z = 271 and began to appear at -90 V and are present at highest abundances from -110 V to -120 V. For EE2, strong molecular ion peaks with m/z = 295 were apparent from -110 V to -130 V, with a cone voltage of -110 V showing the best fragmentation. Molecular ion peaks for estrone were seen at m/z = 269 and were present with more consistency, becoming observable starting at -80 V, but optimizing at cone voltages of -120 and -130 V. Based on these data alone, the optimal cone voltage to be used for these estrogenic compounds was determined to be around -110 to -120 V. For the rest of experimentation, a SIM cone voltage of 120 V and a full scan monitoring cone voltage of -110 V were used. These values are similar to work done by Crolely et al. (2000) [1] where a cone voltage of -110 V was used in the analysis of estrogens.

3.2 Probe Temperature Parameterization
Next, a series of experiments were conducted to examine the probe temperature effects on compound/estrogen fragmentation and ionization. Like cone voltage, probe temperature also affects fragmentation during the ionization process. Increasing in increments of 50°C, trial temperatures ranged from 250 to 500°C. In order to assess and ensure reproducibility in obtaining target analytes as molecular ion base peaks, a total of three trials were conducted for each temperature examined.

Upon analysis of the total ion chromatograms (TIC), probe temperatures of 400 ºC and 450°C produced the greatest ion yield for each estrogen (see Figure 2); however, mass spectra show the most desirable fragmentation when probe temperature was set to 450°C. It should be noted that at 500°C, mass spectra from each source were too noisy to effect molecular ion peaks. Also, estrone was consistently abundant in mass spectra for all temperatures, while the appearance and abundance of estradiol and EE2 fluctuated greatly between different temperatures. Based on these results, a probe temperature of 450°C was used for the remaining sets of experiments described in the following sections.

3.3 Mobile Phase Modification Experiment
In an effort to optimize the ESI response in negative ion mode, mobile phase modification was examined. Research/work conducted by Wu et al. (2004) [7] describes a phenomenon referred to as the "wrong way round" concept where the addition of weak acids to mobile phases actually aid in the deprotonation of analytes in negative ion ESI-MS. Up until this point, the mobile phase used in our experimentation was a solution of 10 mM ammonium acetate. Challenging Wu’s results, varying concentrations of acetic acid were used as mobile phase modifiers (in place of the original ammonium acetate solution). The modified mobile phase concentrations included 1uM, 10uM, 100uM, 1mM, 10mM, and 100mM acetic acid solutions, as well as a sample of pure DI water as a control. Acetic acid solutions were prepared in de-ionized (DI) water, and degassed before being run through the LC-MS.

Analysis of mass spectra indicated that the best results were obtained when the DI water control was used as mobile phase. As acetic acid
concentration gradually increased, so did the noise apparent in mass spectra (see Figures 3a, 3b, 3c). Results obtained using a DI water mobile phase are slightly better than results obtained previously when the ammonium acetate solution was used as the mobile phase.

3.4 Needle Voltage Experiments

Like cone voltage and probe temperature, needle voltage also influences the molecular fragmentation during the ionization process. When operating in negative ion mode, needle voltages were measured in negative kilovolts (-kV). Prior to the investigation of needle voltage, a needle voltage of -3.0 kV had been used, in accordance with the Croley method [1]. Single component estrogen standards (100 ppm) were injected manually into the ESI-MS in triplicate for the following needle voltages: -2.0, -2.5, -3.0, -3.5, -4.0 and -4.5 kV. Manually injecting single component estrogen solutions was a faster alternative to carrying out the entire LC-MS separation and detection. Also, using this method, mass spectra could be viewed in real time as estrogens were introduced to the detector. Mass spectral analysis indicated that increasing needle voltage resulted in an undesirable amount of molecular fragmentation (see Figure 4). Thus, for this work, a needle voltage of -2.0 kV (the lowest our instrument is capable of) provided optimal results. The same manual injection technique described above was used to confirm optimal cone voltage.

3.5 Adduct Formation

Occasionally the molecular ion peak [M-H]- for each estrogen was less reproducible as the base peak (if present at all) in mass spectra. Mass to charge ratios of the most abundant adducts observed include 61.9, 207.1, 297.1, 311.2 and 325.2. The source of these adducts is currently unknown; however, it is suspected that they are related to a structure (see Figure 5), which each of the three estrogens have in common.

Adducts only appear when samples are examined (apparent in spectra for each estrogen) and not when mobile phases are examined alone — with the exception of m/z= 62. A BFT modified to negative ion mode was performed on each mobile phase (freshly prepared) and in each test real time chromatograms displayed base peaks at m/z= 62. Since the adduct present at m/z=62 is present during sample analysis and mobile phase analysis, we believe that this structure may be related to nitrate (NO3-, 62.0049 g/mol) formation during the ionization process. Despite good BFTs, replacing the ESI capillary and following the manufacturers maintenance protocol for cleaning the entrance cone, cone wash, capillary sleeve and RF lens, mass spectral data continued to contain major peaks (often base peaks) at m/z=62.

The presence of m/z=62 often interfered

Figure 2. TIC Peak Areas vs. Probe Temperature for Estrone, β-estradiol, and EE2
Figure 3a. Mass spectra for each estrogen using the DI water mobile phase.
Figure 3b. Mass spectra for each estrogen using the 1uM acetic acid mobile phase.
Figure 3c. Mass spectra for each estrogen using the 100 mM acetic acid mobile phase.
with our ability to obtain mass spectra with target analytes as the most abundant molecular ion peaks (see Figure 6). To confirm the suspected identity of molecular ion peaks at 62, the MS carrier gas (nitrogen) was modified with the addition of helium gas. Because our instrument did not function using 100% helium gas, both gases were introduced to the MS at varying flow rates. To begin, nitrogen flow was highest with a slight helium flow, which was slowly adjusted such that nitrogen flow was minimized and helium flow was maximized. During carrier gas adjustments, mass spectra were observed in real time. As helium flow was increased and nitrogen flow was decreased, the abundance of m/z=62 decreased to the point where it was barely detectable. Based on these observations, and the fact that nitrate was often the most abundant molecular ion within our scan range, it was determined that nitrate (m/z=62) was responsible for consistent interference with analyte detection. Otherwise, the presence of nitrate is not interfering with our target analytes. For future work, the scan range will be adjusted such that values at or below m/z=62 are excluded.

4.0 Conclusions & Future Work
The purpose of this research was to develop an instrumental method suitable for the detection of estrogens in treated wastewater and in natural water systems receiving effluent. Presently, it has been determined that a primary mobile phase of DI water, a probe temperature of 450ºC, a SIM cone voltage of -120 V, a full scan monitoring cone voltage of -110 V, and a needle voltage of -2.0 kV are the optimal instrumental conditions for the ionization of estrogens of interest. A solid phase extraction method similar to the one used by Croley, et al. (2000) is currently being investigated and a sample collection method must also be established. A deuterium labeled internal standard has been purchased and is intended for use during actual sample analysis.

References
[2] Tso, Jerry, Sudarshan Dutta, Shreeram In-
Figure 4. Mass spectra for estrone at needle voltages of 3.0 kV (top), 3.5 kV (middle), 4.0 kV (bottom). The trend of increasing fragmentation with increasing needle voltage is also evident in estradiol and EE2.

Figure 5. Structure shared by estrogens identified in the text.
Figure 6. The mass spectra for estradiol (top), EE2 (middle) and estrone (bottom) with the interference of m/z=62. The presence of m/z=62 often interfered with our ability to obtain mass spectra with target analytes as the most abundant molecular ion peaks.