Application Note

Environmental

Agilent

Trusted Answers

Mass Spectrometry Analysis of Hormones in Water by Direct Injection

Using the Agilent 6470 Triple Quadrupole Mass Spectrometer

Authors

Imma Ferrer,
E. Michael Thurman, and
Jerry A. Zweigenbaum
University of Colorado and
Agilent Technologies, Inc.

Abstract

Some hormones are included in the Contaminant Candidate List CCL4 of the Environmental Protection Agency (EPA) to be assessed for regulation in drinking water. These compounds are also of regulatory interest in the EU, China, and other countries. Therefore, the environmental community often desires the analysis of these compounds in water samples. This Application Note describes the methodology used for the determination of eight hormones (17-α-ethinylestradiol, 17-β-estradiol, estriol, 4-androstene-3,17-dione, equilin, estrone, progesterone, and testosterone) in tap water using an Agilent 6470 triple quadrupole mass spectrometer. A direct injection method using 100 µL of water sample was carried out. This method saves time, reduces handling errors and analytical variability, and is sensitive enough to detect hormones in surface and drinking water at ng/L levels.

Introduction

The presence of hormones in environmental waters has always been controversial because either none have been detected, or very low traces have been found in surface water systems[1]. Once the residual waters are treated in wastewater treatment plants, these compounds adsorb to sludge and sediments, and have removal rates of up to 100 %[2]. Their presence in drinking water is unlikely. However, these compounds have known endocrine effects at sub-ng/L levels in water. As a result, they have always been included in regulatory environmental protection lists, and there is a need for their determination in drinking water[3]. It is also possible that a regulatory action will be enacted to force water treatment facilities to look for these compounds in raw and finished waters.

Seven hormones (17-α-ethinylestradiol, 17-β-estradiol, estriol, 4-androstene-3,17-dione, equilin, estrone, and testosterone) were proposed for the third unregulated contaminant monitoring rule (UCMR3) under the EPA regulation of 2012. A method (EPA 539) was published for their determination in drinking waters. This method used solid phase extraction of a large volume of water (1 L), and targeted LC/MS/MS detection. In 2016, a Contaminant Candidate List (CCL4) was published, and only five of the seven hormones were included (17-α-ethinylestradiol, 17-β-estradiol, estriol, equilin, and estrone).

This Application Note develops a simple and rapid methodology for the detection of eight hormones (17-α-ethinylestradiol, 17-β-estradiol, estriol, 4-androstene-3,17-dione, equilin, estrone, progesterone, and testosterone) using a direct injection of 100 µL of tap water. A multiple reaction monitoring targeted method was used for the detection of the hormones.

Both positive and negative ion modes were used, depending on the specific compound. We also used a novel mobile phase approach that included ammonium fluoride to enhance the negative ion signal[4].

The work in this Application Note was completed using an Agilent 1290 Infinity II UHPLC system consisting of a binary pump, autosampler, thermostatted column compartment, and a 6470 triple quadrupole LC/MS.

Experimental

Reagents and standards

All target hormones were supplied by RESTEK (Bellefonte, PA). Ammonium fluoride was purchased from Sigma-Aldrich (St. Louis, MO). Reagent grade methanol, acetonitrile, and water were obtained from Burdick & Jackson (Muskegon, MI). Individual hormone stock solutions (approximately 1,000 µg/mL) were prepared in pure acetonitrile or methanol, depending on the solubility of each compound, and stored at −18 °C. A mix of all the hormones was prepared at a concentration of 1 µg/mL. Serial dilutions were prepared to obtain a calibration curve ranging from 1 to 500 ng/L.

Sample preparation

Tap water was chosen as a matrix for building calibration curves. Laboratory-fortified samples were prepared by adding known amounts of the hormone mix to the water. Fortification levels for the target hormones ranged from 1 to 500 ng/L in tap water.

Instrument and operational parameters

This method was developed on an Agilent 1290 Infinity II LC with an Infinity II multisampler, running an Agilent InfinityLab Poroshell 120 EC-C18 column (p/n 693775-902). The LC system was coupled to a 6470 triple quadrupole LC/MS. Table 1 lists the instrument conditions chosen for the analysis of the eight hormones studied.

Table 1. LC/MS/MS chromatographic and instrumental conditions used in this study.

LC conditions for the 1290 Infinity II LC	
Column	InfinityLab Poroshell 120 EC-C18, 150 mm × 2.1 mm, 2.7 µm (p/n 693775-902)
Column temperature	25 °C
Injection volume	100 µL
Mobile phase	A) Acetonitrile B) 1 mM NH_4F in water
Run time	11 minutes
Flow rate	0.3 mL/min
Gradient	30 % A at 0 minutes, gradient to 100 % A at 10 minutes, hold at 100 % A for 1 minute, then 4 minutes post run time
MS conditions with positive/negative switching	
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
Gas temperature	250 °C
Gas flow	10 L/min
Nebulizer pressure	45 psi
Capillary voltage	3,000 V in both positive and negative
Nozzle voltage	0 V in positive mode and 500 V in negative mode

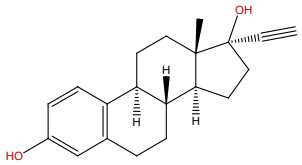
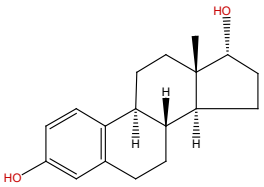
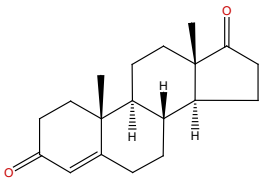
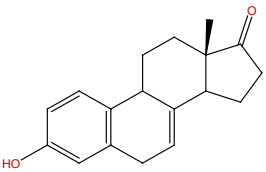
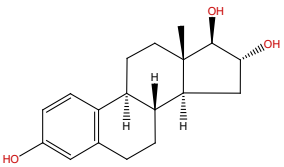
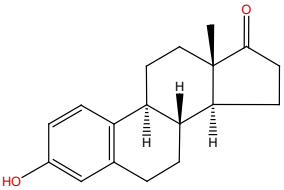
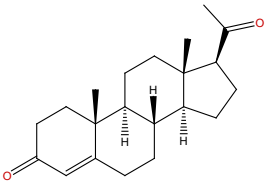
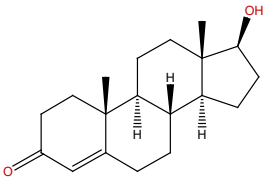
Results and discussion

Ionization requirements

Table 2 shows the names, CAS numbers, and chemical structures of the hormones studied. The list includes eight hormones (seven from the UCMR3 list plus progesterone, an important endogenous female hormone). These eight compounds were tested in both positive and negative ion modes. Compounds with a keto group in the benzylic ring (4-androstene-3,17-dione, progesterone, and testosterone) tend to ionize better in positive ion mode. In contrast, the rest of the compounds, with hydroxyl groups in the benzylic ring, ionize in negative ion mode.

Hormones do not exhibit great sensitivity under electrospray conditions compared to other pharmaceuticals or pesticides. Since most of the hormones ionize in negative ion mode, the deprotonation of the hydroxyl group is not favorable compared to other molecules that have stronger electronegative moieties in their chemical structures. For this reason, ammonium fluoride was used to enhance ionization in negative ion mode. It was found that a concentration of 1 mM in the aqueous mobile phase enhanced the sensitivity of most of the hormones.

Table 2. Names, CAS numbers, and chemical structures of the hormones studied.

Analyte	CAS Number	Chemical structure
17-α-Ethinylestradiol	57-63-6	
17-β-Estradiol	50-28-2	
4-Androstene-3,17-dione	63-05-8	
Equilin	474-86-2	
Estriol	50-27-1	
Estrone	53-16-7	
Progesterone	57-83-0	
Testosterone	58-22-0	

Source parameter optimization

The Source Optimizer tool was used to optimize all parameters (drying gas temperature and flow, sheath gas temperature and flow, nebulizer pressure, capillary voltage, and nozzle voltage). From all the parameters, the ones with the most variability were the capillary voltage and the nozzle voltage. Figure 1A shows the relative intensity versus the capillary voltage values used. A base value of 2,500 V was chosen for the optimization of the capillary voltage. Hormones that ionize in positive ion (4-androstene-3,17-dione, progesterone, and testosterone) see an increase in sensitivity from low capillary voltage values to approximately 3,000 V, then a decrease in signal is observed at higher voltage values. In contrast, hormones that ionize in negative ion do not see much variability with the voltage used. Therefore, a compromise value of 3,000 V was chosen for their determination in both positive and negative ion mode.

Figure 1B shows the relative intensity versus the nozzle voltage values used. A base value of 0 V was used for the optimization. In this case, a dramatic drop in sensitivity occurred when using higher nozzle voltages for hormones that ionize in positive ion mode. Similarly to the capillary voltage, not much variability was observed for negative ion compounds. A slight increase in sensitivity was observed for 17-β-estradiol, estrone, and estriol at 500 V compared to 0 V. This value was chosen for negative ion mode conditions.

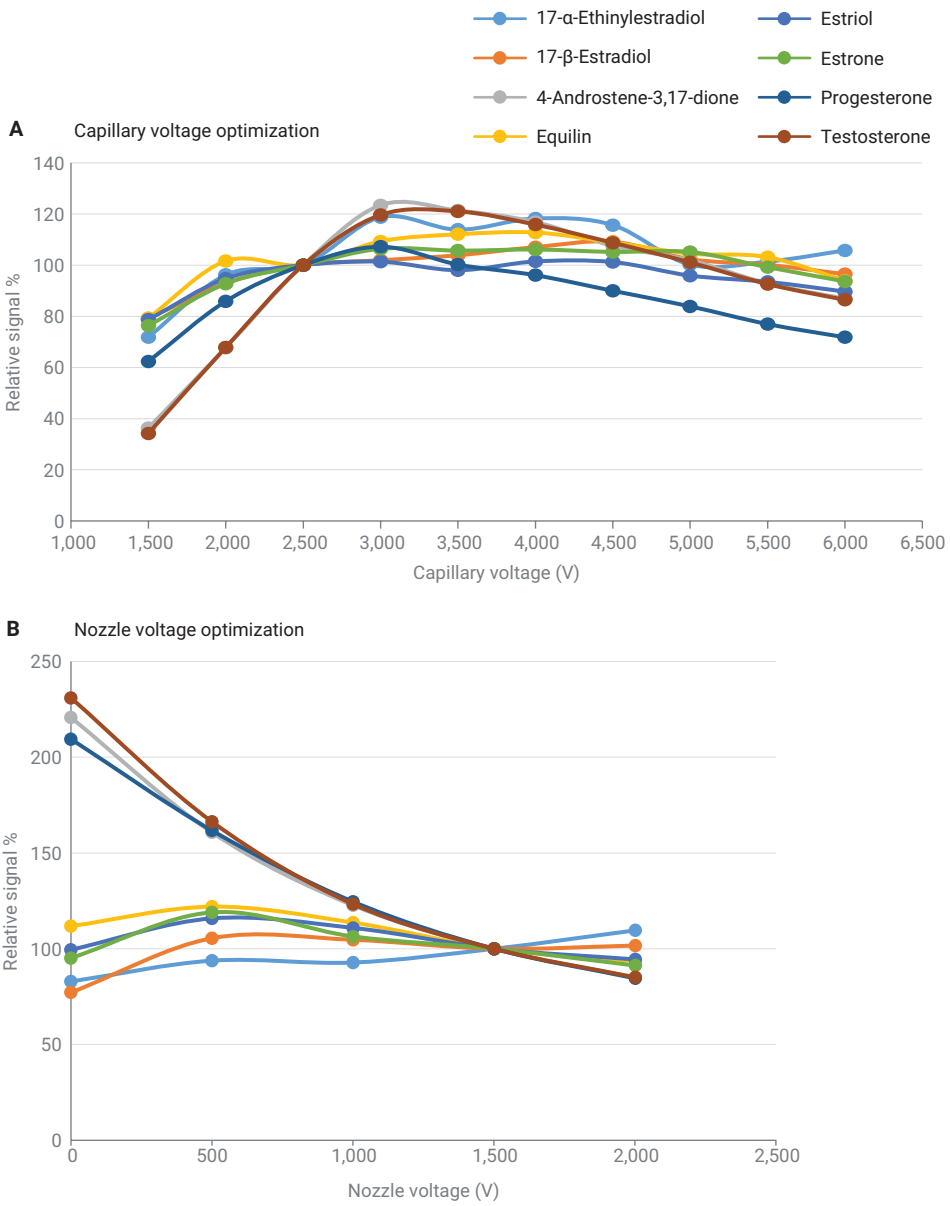


Figure 1. Optimization of capillary voltage (A) and nozzle voltage (B) for the hormones studied in this work.

Analysis parameters

The Optimizer tool was used to get the best fragmentor voltages and collision energies for the target hormones studied. The precursor ion was always either the protonated or the deprotonated molecule. First, the optimal fragmentor voltage for the largest production of precursor ion was found. Next, two product ions for each of the hormones were obtained, and the collision energy required to form the product ions were optimized. The settings included wide and unit mass to capture the most ion signal and yet retain low baseline background-noise from spurious ions in the matrix and solvents. Some common product ions included the *m/z* 145, 183, 109, and 97 ions.

Table 3 shows the 6470 triple quadrupole LC/MS dynamic multiple reaction monitoring (dMRM) analysis parameters. Some hormones exhibit better sensitivity by negative ion and others by positive ion, as stated previously.

Chromatographic separation

Separation of the hormones was successfully achieved using a slow gradient of acetonitrile (from 30 to 100 % in 10 minutes), while maintaining a reasonable analysis time. Figure 2 shows a chromatogram for the analysis of a 100 ng/L standard in tap water.

Table 3. dMRM analysis parameters for the target hormones. Cell acceleration voltage: 7 V; Delta EMV: 200 for positive ion and 400 for negative ion.

Compound	Precursor ion	Product ions	Fragmentor voltage (V)	Collision energy (V)	Retention time (min)	Polarity
17-α-Ethinylestradiol	295.2	183 145	150	40 40	6.0	Negative
17-β-Estradiol	271.2	183 145	160	45 45	5.5	Negative
4-Androstene-3,17-dione	287.2	109 97	110	25 20	6.3	Positive
Equilin	267.1	223 143	140	35 35	6.1	Negative
Estriol	287.1	171 145	180	40 45	2.8	Negative
Estrone	269.2	183 145	150	40 40	6.2	Negative
Progesterone	315.2	109 97	120	25 20	8	Positive
Testosterone	289.2	109 97	120	25 20	5.6	Positive

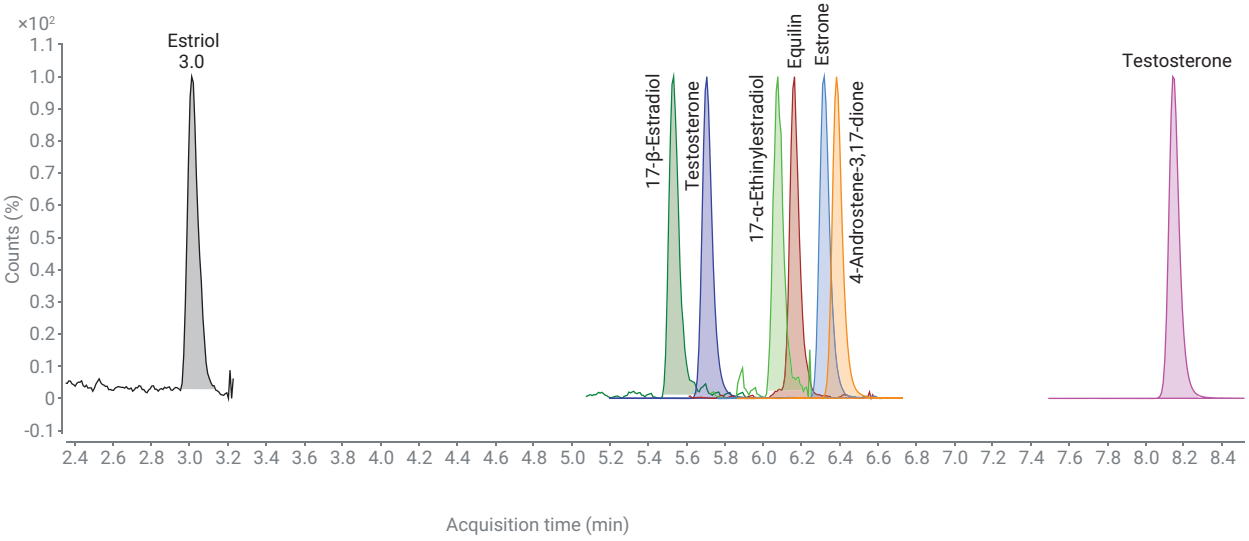


Figure 2. dMRM chromatogram for the eight studied hormones in tap water (100 ng/L). Chromatograms have been scaled to the largest in each chromatogram for clarity of view.

Analytical performance of the developed method

Good linearity was found for all eight hormones when standard curves were developed for each compound. Figure 3 shows the standard curve for estrone. Calibration standards included eight calibration points, at 1, 5, 10, 20, 50, 100, 300, and 500 ng/L. Excellent dynamic range was found between these concentration levels. Precision and accuracy were well below 15 % for intraday and interday analyses.

Table 4 shows the method limits of detection (LODs) for the eight hormones after direct injection of 100 µL of tap water. These LODs account for the fact that both MRM transitions for the two fragment ions (quantifier and qualifier) have to be observed at the right ratios. The LODs varied from 0.1 to 20 ng/L depending on the sensitivity of the compound.

Table 4. Method limits of detection for the hormones studied accounting for both MRM transitions.

Compound	LOD (ng/L)
17-α-Ethinylestradiol	20
17-β-Estradiol	5
4-Androstene-3,17-dione	0.5
Equilin	15
Estriol	15
Estrone	5
Progesterone	0.1
Testosterone	0.1

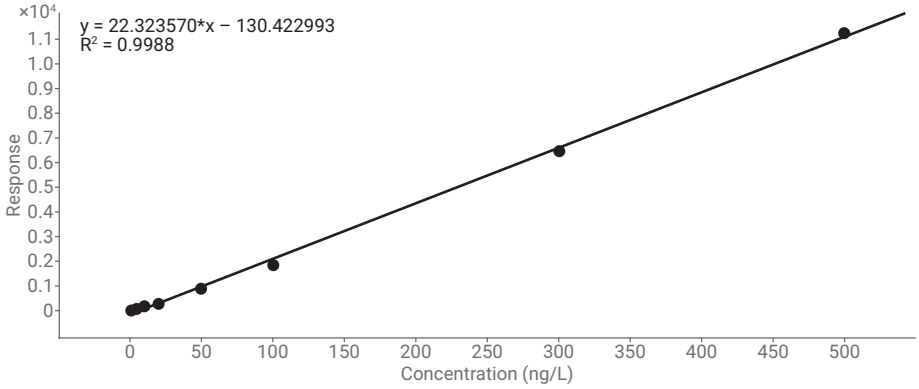


Figure 3. An example of the calibration curve for estrone showing good linearity and dynamic range.

Conclusions

A simple, rapid, and sensitive method was developed for the analytical determination of eight hormones in drinking water using direct aqueous injection. The method uses two MRM transitions for quantitation and confirmation of the target compounds. Low LODs were obtained for all the hormones, indicating that the determination of this class of compounds is feasible in environmental water samples. From our experience monitoring for these compounds in Colorado surface water samples, we have yet to see a detection of any of these compounds. Should lower LODs be needed, a larger volume could be injected, up to 900 µL. Alternatively, either an offline or online solid phase extraction step could be performed before injection into the LC/MS/MS system.

References

1. Kolpin, D. W.; *et al.* Response to Comment on "Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999–2000: A National Reconnaissance." *Environmental science and technology* **2002**, *36(18)*, 4004–4004.
2. Ting, Y. F.; Praveena, S. M. Sources, mechanisms, and fate of steroid estrogens in wastewater treatment plants: a mini review. *Environmental Monitoring and Assessment* **2017**, *189(4)*, 178.
3. Kidd, K. A.; *et al.* Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences* **2007**, *104(21)*, 8897.
4. Hindle, R. Improved Analysis of Trace Hormones in Drinking Water by LC/MS/MS (EPA 539) using the Agilent 6460 Triple Quadrupole LC/MS. *Agilent Technologies Application Note*, publication number 5991-2473EN, **2013**.

www.agilent.com/chem

Printed in the USA, October 3, 2018
5994-0317EN

Agilent
Trusted Answers