COGENT
Bidentate C8™

Hormone Assay Methods by HPLC

Ethinylestradiol
Norethisterone acetate
Estriol
Estradiol
Progesterone

Extended Application Note

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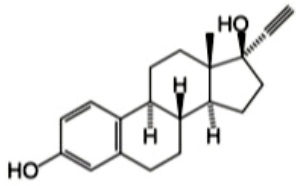
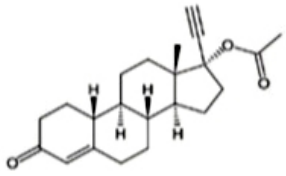


Ethinylestradiol



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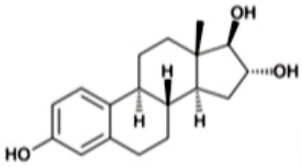

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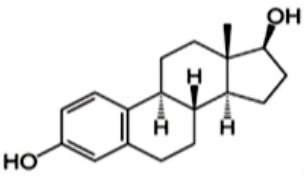
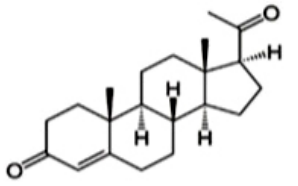


Estradiol



Progesterone

Introduction

Hormone replacement therapy is used to supplement naturally occurring hormones in the body or to use synthetic hormones in their place. The primary application of this therapeutic strategy is to treat symptoms in menopausal, perimenopausal, and postmenopausal women, although it can be used in some other situations as well.

These compounds are typically hydrophobic due to the steroid substructure. Therefore, reversed phase chromatography can be suitable for their routine analysis in pharmaceutical formulations. A variety of Cogent TYPE-C Silica™ columns are available for these applications. A Bidentate C8 or C18™ column may be chosen for traditional reversed phase selectivity. However, the Cogent UDC- Cholesterol™ column has the additional advantage of shape selectivity. This can be important if two structurally similar compounds co-elute using a standard alkyl column. The ligand is a liquid crystal and can differentiate based on shape, adding an additional element of separation to the separation.

Two different hormone replacement formulations were investigated in this study. The first is Femhrt®, which contains ethinylestradiol and norethisterone acetate. The second is Bi-Est®, which has estriol, estradiol, and progesterone components.

The structures of each compound are shown to the left. Note the similarities in structure among these analytes. Even so, reversed phase chromatography and the TYPE-C Silica™ Columns can be used to readily differentiate between them in the chromatographic runs.

Experimental

Materials

Femhrt® and Bi-Est® capsules were obtained. Formic acid LC- MS ultra-grade, ethinylestradiol, norethisterone acetate, estriol, estradiol, and progesterone were from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile and DI water were obtained from GFS (Powell, OH, USA).

Instrumentation

a Hewlett–Packard (Palo Alto, CA, USA) 1100 HPLC system consisting of an autosampler, degasser, binary pump, and variable wavelength UV detector was used. The system was interfaced with Agilent Chemstation (Santa Clara, CA, USA) software. The analytical columns were as follows:

Method 1: Cogent Bidentate C8TM 4µm 100Å, 4.6 x 75 mm
Method 2: Cogent UDC-CholesterolTM 4µm 100Å, 4.6 x 75 mm
Method 3: Cogent Bidentate C18TM 2.2µm 120Å, 2.1 x 50 mm

Method 1:

50% DI H_2O/ 50% Acetonitrile/ 0.1% formic acid

Flow Rate: 1.0 mL/minute
Injection Volume: 20µL
Detection: UV 240 nm

Method 2:

A: 50% DI H_2O + 0.1% formic acid
B: Acetonitrile + 0.1% formic acid formic acid

Time (min.)	%B
0	33
2	33
11	65
12	33

Flow Rate: 1.0 mL/min
Injection Volume: 10µL
Detection: UV 210 nm

Method 3:

A: 90% DI H2O/10 % acetonitrile/ 0.1% formic acid
B: Acetonitrile/ 0.1% formic acid

Time (min.)	%B
0	33
2	33
11	65
12	33

Flow Rate: 0.3 mL/minute
Injection Volume: 2µL
Detection: UV 210 nm

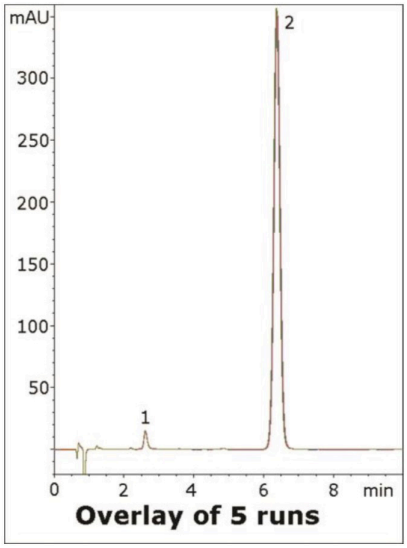


Figure 1

Peaks

1. Ethinylestradiol
2. Norethisterone acetate

Results and Discussion

The first method for the Femhrt® formulation shows excellent separation of the two active ingredients ethinylestradiol and norethisterone acetate (**Figure 1**). The presence of the ester group on norethisterone acetate contributes to its notably greater hydrophobicity, and therefore the two compounds can be well-resolved. Use of a Bidentate C8TM column was found to be adequate, as both hydrophobic compounds were significantly retained even at 50% aqueous content in the mobile phase. The simplicity of this isocratic method lends itself to ease of automation for routine QC assays.

The next method featured a different hormone replacement formulation, known under the trade name Bi- Est®. This capsule contains three components: estriol, estradiol, and progesterone. Progesterone is present at much higher concentration than the other two, and therefore there is a balance between obtaining adequate sensitivity of estriol and estradiol while also avoiding overload of progesterone. This was accomplished in the data shown in **Figure 2**.

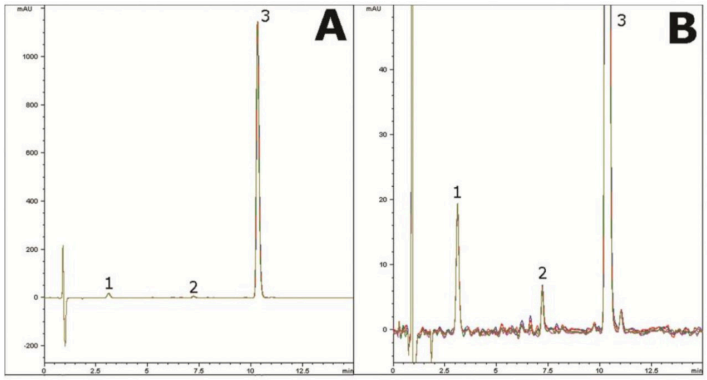


Figure 2

Peaks

1. Estriol
2. Estradiol
3. Progesterone

In **Figure 2A**, a full-scale chromatogram is shown in which the entire progesterone peak can be seen. The peak is sharp and symmetrical, indicating column overload of the analyte has not occurred. In order to see the other two peaks more clearly, a zoom-in view is shown in Figure 2B. Both peaks are well above the noise floor and can be readily quantitated in a QC assay method

Separation among the three peaks is also very good. Here, a Cogent UDC-Cholesterol™ column is used. The column is well-suited to separation of hormone compounds because these can often be distinguished by shape selectivity.

Peaks

1. Estriol
2. Estradiol
3. Progesterone

Figure 3 below shows chromatograms of the same formulation using the Cogent Bidentate C18™ columns.

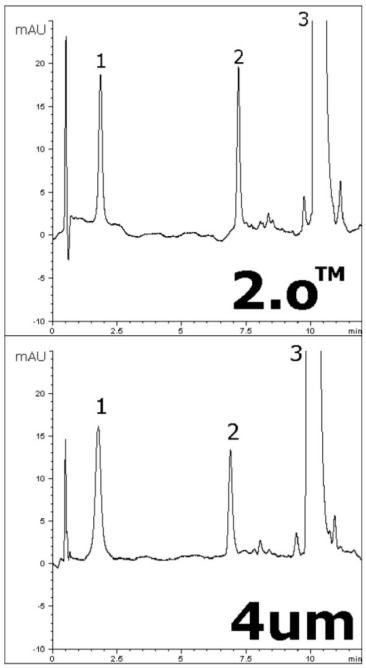


Figure 3

The top chromatogram in **Figure 3** uses a near-UHPLC 2.o™ column (2.2µm) while the bottom uses the standard 4µm phase. It is first noteworthy that retention times using the two columns are highly comparable. If an analyst has an existing method that uses a 4µm column, very little if any modification of the gradient will be required in order to transfer the method to the near-UHPLC phase. The second significant aspect of the data is that efficiency is higher on the 2.o™ phase. Compare, for instance, the peak shape of estriol (peak 1). The peak is somewhat broad when using the standard 4µm column but is much improved going to the smaller particle size. This can be advantageous in instances where good sensitivity is required, such as in this case.

Conclusion

Hormone replacement formulations can be analyzed reliably and routinely using Cogent TYPE-C Silica™ columns. Various columns can be successfully used in the analyses, including the Bidentate C8™, UDC-Cholesterol™, and Bidentate C18™. Use of a near-UHPLC column such as the Bidentate C18 2.o™ can result in increased efficiency and therefore higher sensitivity of low-level analytes. The UDC-Cholesterol™ can be useful in situations where two hormones are difficult to resolve by reversed phase selectivity alone.

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