Contains Nonbinding Recommendations

Draft Guidance on Conjugated Estrogens

This draft guidance, once finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind the FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the Office of Generic Drugs.

Active Ingredient: Conjugated estrogens

Dosage Form; Route: Tablet; oral

Overview:

This draft guidance provides recommendations for the development of generic drug products for naturally-sourced Conjugated Estrogens Tablets derived from pregnant mares' urine. First, FDA provides recommendations for testing to support a demonstration of active pharmaceutical ingredient (API) sameness. Second, FDA provides recommendations for demonstrating bioequivalence of this product.

FDA encourages sponsors to contact Office of Generic Drugs (OGD) to obtain concurrence if an alternative approach is used to demonstrate API sameness or bioequivalence.

Recommendations for Demonstrating Sameness of Active Pharmaceutical Ingredient:

Conjugated Estrogens is an API obtained from a natural source. It contains a mixture of many steroidal and non-steroidal components derived from pregnant mares' urine. The Conjugated Estrogens USP monograph[1] defines 10 individual steroidal components and the acceptance criteria in the labeled content of Conjugated Estrogens. The Conjugated Estrogens Tablets USP drug product monograph[2] establishes the acceptance criteria of the two most abundant components (sodium estrone sulfate and sodium equilin sulfate) and their relative ratio in the tablets. The identification and quantification method in the USP monographs is a gas-chromatograph (GC) method. The sample preparation includes enzymatic cleavage of sulfate conjugation and chemical derivation using bis(trimethylsilyl)trifluoroacetamide before the GC analysis. Over the last decade, analytical separation techniques and mass spectrometer performance have improved significantly. In FDA laboratories, samples from 23 lots of the reference list drug product (RLD), Premarin® Tablets, manufactured over a two year period, were analyzed using liquid-chromatographic and mass spectrometry (LC-MS) methods. As the result of the analysis, FDA developed a LC-MS method that can identify the top 60 steroidal components consistently present in the RLD samples, as described below.

Sameness of a generic Conjugated Estrogens API obtained from the same natural source as the RLD API (i.e. pregnant mares' urine) can be established based on comparative physico-chemical characterizations. The sponsor is advised to use the USP GC method, the proposed FDA LC-MS

[1] Conjugated Estrogens monograph, USP 36, official from May 1, 2013

[2] Conjugated Estrogens Tablets monograph, USP 36-NF 31, official from December 1, 2013

method, and suitable in-house methods to analyze the RLD and the test API/drug product batches. The analysis should include both steroidal and non-steroidal components. A minimum of six different lots of each of the RLD drug product and the test API/drug product should be tested respectively as follows. For the RLD batches, three different lots of RLD tablets (0.625 mg) manufactured within a single year, based on expiration dates, should be studied to assess intra-year consistency. Similarly, three different lots of RLD tablets manufactured within a second year should be studied to assess inter-year consistency. This analysis will require six different lots of RLD tablets. The sponsor may study additional lots (more than six) of RLD tablets to assess variations of the RLD product. For test batches, three different batches of bulk API blended from pregnant mares' urine from a single collection year, and one lot of test tablets (0.625 mg) manufactured from each of the three API batches (three tablet lots total) within that year, should be studied to assess intra-year consistency of test bulk API batches and test tablet lots. Similarly, three different batches of test API and one lot of test drug product manufactured from each of the API batches from a second collection year should be studied to assess inter-year consistency. This process will yield six different batches of test bulk API and six different lots of test tablets.

I. FDA LC-MS ANALYTICAL PROCEDURE

It is recommended to use ultra-high performance liquid chromatography and high resolving power mass spectrometry (UHPLC-HRMS) for the chemical characterization of Conjugated Estrogens.

a. Materials:

Mass spectrometry (Optima) grade methanol, water and formic acid or equivalent
Waters Acquity UPLC BEH C_{18} 1.7 µm, 130 Å, 2.1 x 150 mm column or equivalent
Water Sep-Pak C_{18} cartridges, 500 mg, or equivalent
Estrone-3-sulfate sodium salt (E1-S), Equilin-3-sulfate sodium salt (EqS), $\Delta^{8,9}$-dehydroestrone-3-sulfate sodium salt (DHES) synthetic standards. Identity and purity of standards should be verified with orthogonal methods (e.g., HPLC, NMR and MS data).

Mobile Phases:
Mobile Phase A: Water, 0.1% formic acid
Mobile Phase B: Methanol, 0.1% formic acid

b. Qualifying Standards Preparation:

Estrone-3-sulfate sodium salt (E1-S), Equilin-3-sulfate sodium salt (EqS), $\Delta^{8,9}$-dehydrosterone-3-sulfate sodium salt (DHES)

Prepare 0.1 mg/mL solution in a 1:1 volume (v:v) of water:methanol for each steroid standard. Add 100 µL of each of the three standard solutions to a 1 mL vial and dilute with 700 µL of mobile phase A to a total volume of 1 mL.

c. Sample Preparation (tablets):

Depending on the tablet dosage, process enough tablets to have at least 3.6 mg of Conjugated Estrogens. For 0.625 mg dose, 6 tablets would be needed. Weigh the tablets to determine an average weight per tablet. Place the tablets in a 50 mL Erlenmeyer flask. Add 15 mL water. Shake the flask until the outer coating is dissolved, decant the water, and add another 15 mL of water for a quick rinse. Discard the rinse and transfer the tablets to a pre-weighted weigh boat. Dry the tablets in a vacuum oven for 45 minutes at room temperature. Weigh the boat with tablets and return to vacuum oven for another 30 minutes, repeat until constant weight. Determine the weight of an average washed tablet. Manually grind or pulverize the washed tablets. Transfer an equivalent of 0.6 mg Conjugated Estrogens of the powdered tablet into a 125 mL Erlenmeyer flask. The amount is calculated based on the weight of the washed and dried tablets. Add 50 mL water, stopper and shake at room temperature for two hours until total dissolution (no clumps or sticky particles). Centrifuge at 3000 × g for 15 minutes to remove any solid particulates. Perform Solid Phase Extraction (SPE) on the cleared supernatant.

SPE is performed on Waters Sep-Pak C_{18} cartridge, 500 mg, product number WAT020805. Condition the cartridge with 3 mL methanol followed by 3 mL 5% methanol solution (methanol:water, v:v). Solvents are mass spectrometry grade. Pass the sample solution through the cartridge and wash with an additional 3 mL of the 5% methanol solution. Elute the bound sample with 3 mL of methanol and dry under nitrogen to final volume of 1.0 mL. Syringe filter the resulting Conjugated Estrogens solution with a 0.45 µm nylon filter. For analysis, dilute the sample 1:5 with Mobile Phase A.

d. Sample Preparation (bulk):

For API without excipients, dissolve the substance in 15 mL water and perform the SPE purification as above.

e. Instrumentation:

An ultrahigh performance liquid chromatography high resolution mass spectrometer consisting of an ultra-high performance binary pump, vacuum degasser, autosampler, a thermostatted column compartment, an electrospray source, and high resolution mass spectrometer.

In order to distinguish between a monoisotopic mass of one species and the A+2 isotopic peak of another species, 2 m/z units below, a mass spectrometer with resolving power of 50,000 or more is preferred.

UHPLC conditions:
Column: Waters Acquity UPLC BEH C_{18} 1.7 µm, 130 Å, 2.1 x 150 mm, product number 186002353 or equivalent UHPLC USP L1 column
Flow: 0.35 mL/min
Total run time: 70 minutes
Injection volume: 1 µL, loop size 5-20 µL
Column oven temperature: 40° C

Gradient Program:

Time (min.)	%B
0	21
5	33
45	53
60	98
65	98
65.5	21
70	21

Mass Spectrometry (MS) Conditions:
Ionization method: Electrospray Ionization (ESI)
Source Conditions:
Scan type: MS, negative ion mode
Scan range: 250-700 m/z

System Suitability:

The elution order for the synthetic steroid standards should be DHES, EqS, E1-S. Masses to monitor are $C_{18}H_{19}O_5S$, m/z 347.0959 for DHES and EqS, and $C_{18}H_{21}O_5S$, m/z 349.1115 for E1-S. The retention time of E1-S should be between 22-26 minutes. The resolution (R) of the close pair, DHES and EqS, should be greater than or equal to 1.2 using Equation (1):

$$R = 2 \times \frac{t_{r2} - t_{r1}}{w_1 + w_2} \quad (1)$$

where t_r is the retention time and w is the width at the 5% peak height from baseline for compounds 1 and 2. Mass accuracy (A) should be within a mass tolerance of ± 5 ppm for each of the steroid standard based on Equation (2):

$$\text{Accuracy} = \frac{\text{Experimental m/z} - \text{Theoretical m/z}}{\text{Theoretical m/z}} \times 10^6 \quad (2)$$

f. Sample Analysis and Calculation:

Extracted ion chromatograms (EIC) for all masses in Table 1, with a mass tolerance of ± 5 ppm, should be created, and all peak areas should be recorded. Match the peak list with the 60 peaks in Table 2 using the mass and relative retention time (RRt). Relative retention time is calculated based on Equation (3):

$$RRt = Rt/Rt_0 \quad (3)$$

where Rt is the compound retention time, and Rt_0 is the retention time of E1-S which is the most abundant compound in the Conjugated Estrogens mixture at m/z 349.1115. If the retention time is longer than 37 minutes, calculate retention time using Equation (4):

$$RRt = 2.25 \times Rt/Rt_1 \quad (4)$$

where Rt_1 is the retention time of peak m/z 399.2215 (Rt_1 is approximately Rt_0 x 2.25).

Divide the peak area of each component in the EIC list by the sum all the peak areas of the 60 components to obtain relative peak area of each component.

g. Data Reporting:

For each peak detected, the RRt and exact mass should be reported. Sponsors should also match each peak with the appropriate peak in Table 3 (10 components identified in the Conjugated Estrogen USP monograph) based on the exact mass and RRt. In addition, each peak area should be reported in unit of area % relative to the sum of all 60 components as in Table 2. To aid in assignment, peaks are listed in decreasing order of relative peak area abundance.

Table 1. List of masses (m/z) to generate extracted ion mass chromatograms (EIC).

m/z Masses for EIC	
345.0802	379.1221
347.0959	381.1377
349.1115	385.1690
351.1272	387.1847
353.1428	395.1898
355.1585	397.2054
365.1064	399.2211
367.1585	413.2003
369.1741	415.2162
371.1898	465.2494
377.1064	511.2913

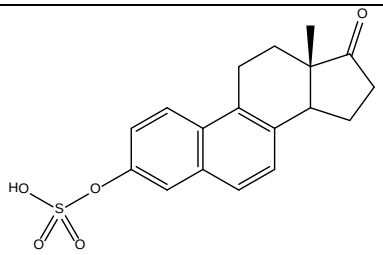
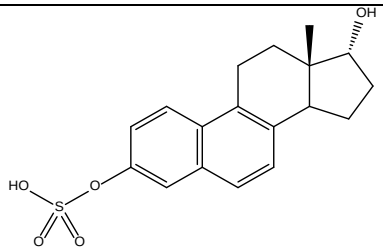
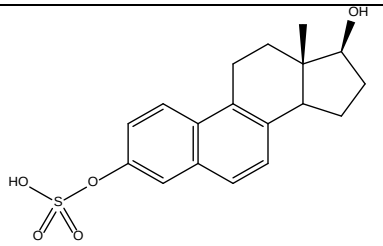
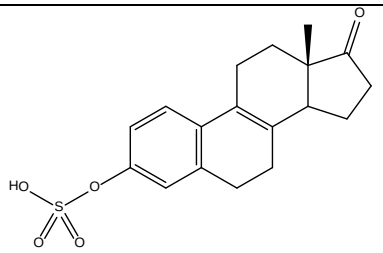
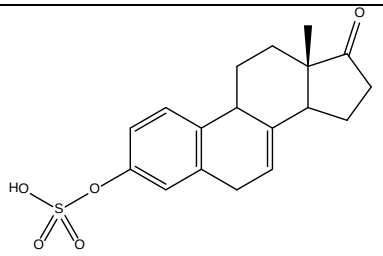
Table 2. List of 60 steroidal components identified in Conjugated Estrogens.

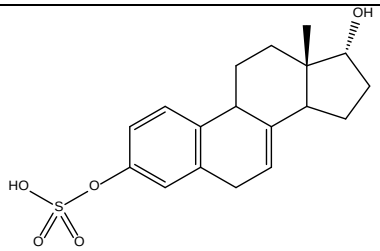
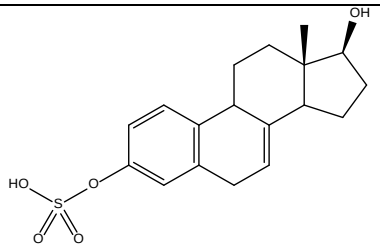
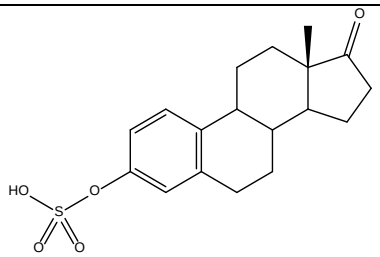
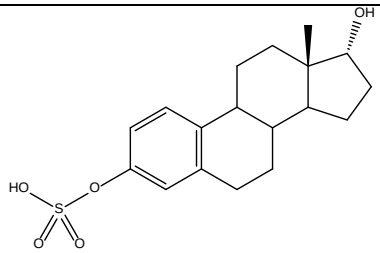
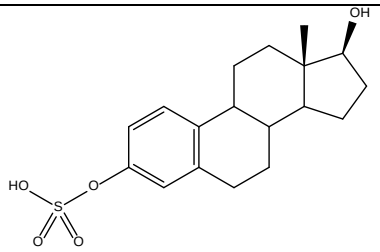
Peak #	Name	RRt	Mass m/z	Composition
1	E1-S[a] and DEq3S17a[a]	1.000	349.1115	$C_{18}H_{22}O_5S$
2	EqS[a]	0.937	347.0959	$C_{18}H_{20}O_5S$
3	413@1.28	1.280	413.2003	$C_{21}H_{34}O_6S$
4	399@2.25	2.250	399.2211	$C_{21}H_{36}O_5S$
5	351@1.17	1.172	351.1272	$C_{18}H_{24}O_5S$
6	E2-3S17a[a]	1.184	351.1272	$C_{18}H_{24}O_5S$
7	415@1.22	1.218	415.2162	$C_{21}H_{36}O_6S$
8	353@1.34	1.341	353.1428	$C_{18}H_{26}O_5S$
9	397@2.21	2.212	397.2054	$C_{21}H_{34}O_5S$
10	415@0.63	0.629	415.2162	$C_{21}H_{36}O_6S$
11	EqnS[a]	0.859	345.0802	$C_{18}H_{18}O_5S$
12	DEHS[a]	0.921	347.0959	$C_{18}H_{20}O_5S$
13	369@1.73	1.732	369.1741	$C_{19}H_{30}O_5S$
14	DEqn3S17a[a]	0.903	347.0959	$C_{18}H_{20}O_5S$
15	349@0.90	0.901	349.1115	$C_{18}H_{22}O_5S$
16	353@1.04	1.035	353.1428	$C_{18}H_{26}O_5S$
17	465@1.71	1.710	465.2494	$C_{25}H_{38}O_8$
18	511@1.18	1.182	511.2913	$C_{27}H_{44}O_9$
19	369@1.14	1.135	369.1741	$C_{19}H_{30}O_5S$
20	DEq3S17b[a]	0.928	349.1115	$C_{18}H_{22}O_5S$
21	385@0.56	0.562	385.1690	$C_{19}H_{30}O_6S$
22	371@1.48	1.476	371.1898	$C_{19}H_{32}O_5S$
23	379@1.02	1.021	379.1221	$C_{19}H_{24}O_6S$
24	413@0.86	0.855	413.2003	$C_{21}H_{34}O_6S$
25	355@1.13	1.132	355.1585	$C_{18}H_{28}O_5S$
26	413@1.90	1.896	413.2003	$C_{21}H_{34}O_6S$
27	397@2.28	2.276	397.2054	$C_{21}H_{34}O_5S$
28	413@1.05	1.045	413.2003	$C_{21}H_{34}O_6S$
29	351@0.83	0.833	351.1272	$C_{18}H_{24}O_5S$
30	365@0.91	0.906	365.1064	$C_{18}H_{22}O_6S$
31	415@0.79	0.789	415.2162	$C_{21}H_{36}O_6S$
32	415@1.10	1.102	415.2162	$C_{21}H_{36}O_6S$
33	353@1.29	1.285	353.1428	$C_{18}H_{26}O_5S$
34	415@0.72	0.723	415.2162	$C_{21}H_{36}O_6S$
35	E2-3S17b[a]	1.015	351.1272	$C_{18}H_{24}O_5S$
36	395@2.20	2.199	395.1898	$C_{21}H_{32}O_5S$
37	399@2.29	2.287	399.2211	$C_{21}H_{36}O_5S$

Peak #	Name	RRt	Mass m/z	Composition
38	385@0.59	0.593	385.1690	$C_{19}H_{30}O_6S$
39	351@0.89	0.891	351.1272	$C_{18}H_{24}O_5S$
40	353@1.24	1.242	353.1428	$C_{18}H_{26}O_5S$
41	385@0.55	0.546	385.1690	$C_{19}H_{30}O_6S$
42	367@1.45	1.450	367.1585	$C_{19}H_{28}O_5S$
43	DEqn3S17b[a]	0.790	347.0959	$C_{18}H_{20}O_5S$
44	387@0.73	0.725	387.1847	$C_{19}H_{32}O_6S$
45	387@0.68	0.682	387.1847	$C_{19}H_{32}O_6S$
46	395@2.11	2.113	395.1898	$C_{21}H_{32}O_5S$
47	413@1.11	1.111	413.2003	$C_{21}H_{34}O_6S$
48	413@2.07	2.067	413.2003	$C_{21}H_{34}O_6S$
49	413@1.14	1.138	413.2003	$C_{21}H_{34}O_6S$
50	413@1.01	1.010	413.2003	$C_{21}H_{34}O_6S$
51	351@1.10	1.105	351.1272	$C_{18}H_{24}O_5S$
52	511@1.86	1.863	511.2913	$C_{27}H_{44}O_9$
53	377@0.93	0.931	377.1064	$C_{19}H_{22}O_6S$
54	385@1.11	1.107	385.1690	$C_{19}H_{30}O_6S$
55	367@1.19	1.193	367.1585	$C_{19}H_{28}O_5S$
56	413@2.03	2.029	413.2003	$C_{21}H_{34}O_6S$
57	385@0.62	0.622	385.1690	$C_{19}H_{30}O_6S$
58	381@1.19	1.193	381.1377	$C_{19}H_{26}O_6S$
59	413@1.23	1.228	413.2003	$C_{21}H_{34}O_6S$
60	413@1.06	1.064	413.2003	$C_{21}H_{34}O_6S$

[a]These compounds are quantitated as part of the USP GC method

Table 3. List of steroidal components in the Conjugated Estrogens USP monograph.

Peak # from Table 2	Name	Shortened name	Structure	$[M-H]^-$ m/z
11	Equilenin-3-sulfate	EqnS		345.0802
14	Dihydroequilenin-17α-3-sulfate	DEqn3S17a		347.0959
43	Dihydroequilenin-17 β -3-sulfate	DEqn3S17b		347.0959
12	$\Delta^{8,9}$-dehydrostrone-3-sulfate	DHES		347.0959
2	Equilin-3-sulfate	EqS		347.0959

Peak # from Table 2	Name	Shortened name	Structure	$[M-H]^-$ m/z
1	Dihydroequilin-17 α -3-sulfate	DEq3S17a		349.1115
20	Dihydroequilin-17 β -3-sulfate	DEq3S17b		349.1115
1	Estrone-3-sulfate	E1-S		349.1115
6	Dihydroestrone-17 α -3-sulfate	E2-3S17a		351.1272
35	Dihydroestrone-17 β -3-sulfate	E2-3S17b		351.1272

II. SAMENESS OF API

The Agency's recommendation to establish sameness of the Conjugated Estrogens API obtained from pregnant mares' urine consists of qualitative and quantitative criteria. The sponsor should report all steroidal components consistently present at a level ≥ 0.1% (relative percentage of peak area, as described in Session **I(h)** Data Reporting) identified using the FDA LC-MS method.

a. Identification Test for Steroidal Components

All test API batches should contain the 60 steroidal components identified by the FDA LC-MS method (Table 2). All components should be identified by RRt and exact mass.

b. USP Quantification Test for 10 Steroidal Components

The components identified in the Conjugated Estrogens USP monograph should be present in all test API batches within the acceptance criteria specified in the USP monograph. Data obtained using the USP GC method or an appropriately validated in-house method is acceptable.

c. Control of Major non-USP Steroidal Components

All steroidal non-USP components consistently present at a level ≥ 1% (LC-MS method, relative percentage of peak area, as described in Session **I(h)** Data Reporting) in the RLD should be present in the test API batches at a level comparable to the RLD batches, otherwise should be justified or qualified.

d. Control of Additional Steroidal Components in the Test API Batches

Any steroidal components that present at a level ≥ 1% (LC-MS method, relative percentage of peak area, as described in Session **I(h)** Data Reporting) in any test API batches, but not consistently present at a level ≥ 1% in the RLD batches, should be justified or qualified.

e. Total Steroidal Components Content Test

The ratio of the sum of 10 USP steroidal components (LC-MS peak area) to the sum of the 60 steroidal components identified by the FDA LC-MS method (Table 2) should be calculated as the following:

$$\text{ratio} = \frac{\sum 10 \text{ USP steroidal components}}{\sum 60 \text{ steroidal components (Table 2)}}$$

The ratios obtained from the test API batches should be at a level comparable to the ratios from the RLD batches, otherwise should be justified.

f. Non-Steroidal Components in the Test API Batches

Non-steroidal components in test API batches should be treated as concomitant components, and should be analyzed in comparison to the RLD batches. Appropriate acceptance criteria should be set so that the levels of common non-steroidal components are comparable between the test and the RLD batches. Guidance for Industry ANDAs: Impurities in Drug Substances can be used to set acceptance criteria for non-steroidal components.

Recommendations for Demonstrating Bioequivalence:

Recommended Studies: Four studies

Bioequivalence (BE) may be established by conducting in vivo studies with pharmacokinetic endpoints. Four in vivo BE studies are recommended.

1. Type of study: Fasting
   Design: Single-dose, two-treatment, four-period, replicate crossover in vivo
   Strength: 1.25 mg
   Subjects: Normal, healthy, physiologically or surgically postmenopausal women.
   Additional comments: A replicate study design (TRTR and RTRT) is recommended to distinguish between unequal sequence and carryover effects in the single-dose fasting studies. The use of these two sequences (TRTR and RTRT) is the best design for balancing and minimizing bias should there be an unequal carryover effect in the study. A non-replicate (two-way crossover) study design may be conducted in lieu of the replicate study design, but the appearance of a statistically significant sequence effect may cast doubt on the conclusion of bioequivalence for the product.

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2. Type of study: Fed
   Design: Single-dose, two-treatment, two-period crossover in vivo
   Strength: 1.25 mg
   Subjects: Normal, healthy, physiologically or surgically postmenopausal women.
   Additional comments: If the initial submitted ANDA is for a dosage strength lower than 1.25 mg, this fed study should be conducted with the highest strength tablet submitted for approval. Any subsequent application for approval of any additional strength(s) will not necessitate the conduct of a second fed BE study.

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3. Type of study: Fasting
   Design: Single-dose, two-treatment, four-period replicate crossover *in vivo*
   Strength: 0.625 mg [Administer 0.625 mg x 2 tablets (Dose: 1.25 mg)]
   Subjects: Normal, healthy, physiologically or surgically postmenopausal women.
   Additional comments: A non-replicate study design for this study is acceptable providing no statistically significant carryover effect is observed on the initial fasting study (Study 1) conducted by the firm.

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4. Type of study: Fasting
   Design: Single-dose, two-treatment, four-period, replicate crossover *in vivo*
   Strength: 0.9 mg [Administer 0.9 mg x 2 tablets (Dose: 1.8 mg)]
   Subjects: Normal, healthy, physiologically or surgically postmenopausal women.
   Additional comments:
   a. Submission of a Bio Investigational New Drug Application (BioIND) is required prior to the conduct of a bioequivalence study [see 21 CFR § 320.31(b)(1)].

b. See additional comments for Study 3.

Analytes to measure (in appropriate biological fluid): Baseline-adjusted unconjugated estrone, baseline-adjusted total estrone, unconjugated equilin, and total equilin in plasma

Baseline (pre-dose) levels of unconjugated and total estrone (sum of unconjugated estrone, estrone sulfate and estrone glucuronide) in plasma determined at minus 48 hrs, minus 24 hrs, and pre-dose (time zero) should be averaged to obtain a single baseline value for each of unconjugated estrone and total estrone. Total equilin is the sum of unconjugated equilin, equilin sulfate and equilin glucuronide. Equilin is not endogenous in the human, thus baseline plasma levels are zero.

Bioequivalence based on (90% CI): Baseline-adjusted unconjugated estrone, baseline-adjusted total estrone, unconjugated equilin, and total equilin computed from blood sampling through 72 hours.

Waiver request of in vivo testing: 0.3 mg and 0.45 mg, based on (i) acceptable bioequivalence study on the 0.625 mg strength, (ii) acceptable dissolution testing of the 0.3 mg, 0.45 mg, and 0.625 mg strengths, and (iii) proportional similarity in the formulations of the 0.3 mg, 0.45 mg, and 0.625 mg strengths.

Dissolution test method and sampling times: The dissolution information for this drug product can be found on the FDA-Recommended Dissolution Methods website available to the public at the following location: http://www.accessdata.fda.gov/scripts/cder/dissolution/. Conduct comparative dissolution testing on 12 dosage units each of all strengths of the test and reference products. Specifications will be determined upon review of the abbreviated new drug application (ANDA).

In addition to the method above, dissolution profiles on 12 dosage units each of test and reference products generated using USP Apparatus I at 100 rpm and/or Apparatus II at 50 rpm in at least three dissolution media (water, pH 1.2 and 6.8 buffers) should be submitted in the application. Agitation speeds may have to be increased if appropriate. It is acceptable to add a small amount of surfactant, if necessary. Please include early sampling times of 1, 2, and 4 hours and continue every 2 hours until at least 80% of the drug is released, to provide assurance against premature release of drug (dose dumping) from the formulation.