DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville MD 20857

MAR 8 2000

Joseph G. Valentino, J.D.
Senior Vice President and General Counsel
The United States Pharmacopeial
Convention, Inc.
12601 Twinbrook Parkway
Rockville, MD 20852

REF: 3-00-002-O

Dear Dr. Valentino:

This letter proposes revisions to the current USP 24 monograph for Conjugated Estrogens. The proposed changes are summarized below and a revised version of the monograph is enclosed for your convenience.

In the general definition, second sentence, immediately after the phrase, "It contains other conjugated estrogenic substances" we propose to add the phrase, "as well as conjugated pregnanes and conjugated androstanes." This addition will clarify that the name "Conjugated Estrogens" is a "term of art" that defines an array of substances, including but not limited to conjugated estrogens. We also propose to delete the term "concomitant components" and the current reference to synthetically-derived conjugated estrogens, and, as explained below, to modify the labeled content claims so that they no longer include 17β-dihydroequilin or 17α-estradiol.

To provide added assurance of the appropriate "natural source" origin of the product, we propose to add HPLC and GC "fingerprint" identification tests with allowable ranges to the monograph. The HPLC procedure is performed directly on the bulk drug substance. The GC procedure is a slightly modified version of the current USP *Assay*[1] and also is to be performed on the bulk drug substance. We propose to use the GC procedure both as an identification test and simultaneously as the assay procedure. We have removed the statements regarding the 17β-dihydroequilin, 17α-estradiol, 17β-estradiol, and $\Delta^{8,9}$-dehydroestrone components because their responses are included in the GC "fingerprint" procedure, and because USP Reference Standards for their assessment are not available. We propose the establishment of a USP Conjugated Estrogens Reference Standard, which is to be a round, white-coated tablet. Please note that many of the compounds included in the specified "fingerprint" peaks have not been identified yet, and may include non-steroidal as well as steroidal compounds.

1 The proposed procedure uses Helium as the carrier gas in lieu of Hydrogen and a longer, narrower capillary column with a thinner coating to improve resolution. Also, methylene chloride was substituted for ethylene dichloride because it is less noxious.

The procedures were developed and the repeatability demonstrated by our St. Louis laboratory, and their results were corroborated by our Seattle laboratory. Ranges were established for those components consistently present, on average, at 2% or above (based on peak area) with respect to the estrone peak. The allowable ranges are set at plus-or-minus four standard deviations about the average value obtained from analyzing numerous lots of Premarin®, with the exception of estrone, equilin, and 17α-dihydroequilin. Estrone does not have a range, and the ranges for equilin and 17α-dihydroequilin are the same as are in the existing USP monograph.

We hope these comments will be helpful to the USP and the Committee of Revision in their deliberations. Please feel free to contact Larry Ouderkirk on my staff if you have any questions. If you have extensive questions, we will be happy to arrange for your representatives to meet with the CDER scientists responsible for the draft monographs. Please use the reference number provided above on any ensuing correspondence.

As a final note, we would like to advise you that due to public interest in this matter, we will be placing a copy of this letter and the accompanying proposed monograph on the following CDER Internet website:

http://www.fda.gov/cder/regulatory/initiatives/cestrogens/

Sincerely,

Yana Ruth Mille

Yana Ruth Mille
Chief
Compendial Operations Staff, HFD-354
Office of Pharmaceutical Science
Center for Drug Evaluation & Research

Enclosures

"CONJUGATED ESTROGENS, USP" MONOGRAPH – PROPOSED REVISIONS

Conjugated Estrogens is a mixture of sodium estrone sulfate and sodium equilin sulfate derived from equine urine. It contains other conjugated estrogenic substances as well as conjugated pregnanes and conjugated androstanes excreted by pregnant mares. It is a dispersion of these steroidal substances on a suitable powdered diluent. Conjugated Estrogens contains, as the sodium sulfate conjugates, expressed in terms of the labeled content of Conjugated Estrogens, not less than 52.5 percent and not more than 61.5 percent of estrone, not less than 22.5 percent and not more than 30.5 percent of equilin, and not less than 13.5 percent and not more than 19.5 percent of 17α-dihydroequilin. The total of sodium estrone sulfate and sodium equilin sulfate is not less than 79.5 percent and not more than 88.0 percent of the labeled content of Conjugated Estrogens. The total of the sodium sulfate conjugates of estrone, equilin, and 17α-dihydroequilin is not less than 90.0 percent and not more than 110.0 percent of the labeled content of Conjugated Estrogens.

Packaging and Storage -- Preserve in well-closed containers.

Labeling -- Label it to state the content of Conjugated Estrogens on a weight-to-weight basis.

USP Reference Standards <11> -- *USP Estrone RS. USP Equilin RS. USP 17α-Dihydroequilin RS. USP Estradiol RS. USP Conjugated Estrogens RS.*

Identification –

A: Gas Chromatography --

Internal standard solution, pH 5.2 acetate buffer, Stock solution, System suitability solution, and *Chromatographic system*-- Proceed as directed in the *Assay.*

Standard preparation -- The *USP Conjugated Estrogens RS* is a round, white-coated tablet. Carefully remove the sugar coatings from the tablets with water, leaving the shellac coating intact, and dry under nitrogen. Finely powder a sufficient number of tablets and proceed as for the *Assay preparation*, below, except substitute the *RS* powder for the sample bulk powder.

Test preparation – Prepare as directed for *Assay preparation* in the *Assay.*

Procedure- Separately inject equal volumes (about 1 µL) of the *Standard preparation* and of the *Test preparation* into the chromatograph. After initial integration of the chromatograms, re-integrate the two chromatograms using an area reject value equivalent to 0.4 % of the area of the estrone peak. The re-integrated chromatogram of the *Test preparation* contains the same peaks as those in the chromatogram of the *Standard preparation*, as shown in the following table beginning with relative retention

times 0.15 through 1.3. Peaks with a mean content greater than 2 % of estrone (equivalent to about 1 % of label claim) must be present at the levels shown in the following table:

Peak #	RRT (to IS)	Average Area Range (% of estrone)
1	0.20	2.6-5.8
2	0.21	1.6-3.4
3	0.22	5.8-8.6
4	0.28	0.1-8.8
5	0.28	0.8-3.5
6	0.30	8.0-11.7
7	0.32	2.4-4.0
8	0.33	1.9-2.7
9	0.34	23.7-34.2
10	0.36	2.4-3.4
11	0.38	3.4-4.6
12	0.39	5.9-8.1
13	0.43	1.8-5.0
14	0.52	1.9-3.9
15	0.61	4.2-9.5
16	0.63	2.8-4.3
17	0.79	100
18	0.81	39.5-53.5
19	0.89	5.7-7.2
20	1.20	2.3-5.3

In addition, the chromatogram may exhibit approximately 40 other peaks with mean content greater than 0.4 % but less than 2 %.

B: HPLC --

Stock solution – Accurately weigh about 2 mg each of the sodium salts of equilenin sulfate, equilin sulfate, and estrone sulfate. Dissolve each in 10.0 mL of water.

System suitability solution – Pipet 1.0 mL each of equilin sulfate, estrone sulfate, and 0.5 mL of equilenin sulfate *stock solutions* into a 5.0 mL volumetric flask. Dilute to volume with water and mix well.

Standard preparation – Prepare as directed for *Standard preparation* in *Identification – A Gas Chromatography*.

Test preparation – Transfer an accurately weighed quantity of sample bulk Conjugated Estrogens powder, equivalent to about 0.625 mg conjugated estrogens, to a suitable centrifuge tube fitted with a polytef–lined screw-cap. Add 5.0 mL water and place the solution on a vortex mixer (full speed) for 5 minutes. Centrifuge at 2500 rpm for 10 minutes. Filter the solution through a 0.45-µm cellulose nitrate membrane filter into a LC injection vial.

Mobile phase A - Mix 1 mL of trifluoroacetic acid with HPLC grade water to give a final volume of 1000 mL.

Mobile phase B - Mix 1 mL of trifluoroacetic acid with HPLC grade acetonitrile to give a final volume of 1000 mL.

Chromatographic system (see Chromatography <621>) - The liquid chromatograph is equipped with a 215-nm detector and a 4.6 mm x 25 cm column that contains 5-µm packing L1. The flow rate is about 1 mL per minute. The column is maintained at a temperature of 30^0 C. The chromatograph is programmed as follows:

Time (minutes)	Solution A (%)	Solution B (%)	Elution
0	90	10	Initial conditions
75	60	40	Linear gradient
80	20	80	Linear gradient
85	20	80	Isocratic hold
90	90	10	Linear gradient to initial conditions
120	90	10	Equilibration time

Procedure -- Inject 30 µL of the *System suitability solution* into the liquid chromatograph. The retention times are about 52, 54, and 56 minutes for equilenin sulfate, equilin sulfate, and estrone sulfate, respectively. The tailing factor for the equilin sulfate peak is not greater than 2.0. The resolution, R, between equilenin sulfate and equilin sulfate is not less than 2.0.

Separately inject equal volumes (about 30 µL) of the *Standard preparation* and of the *Test preparation* into the chromatograph. The chromatogram of the *Test preparation* contains the same peaks as shown in the following table beginning with relative retention time 0.35 through 1.3 as those in the standard chromatogram. Peaks with a mean content

greater than 2 % of estrone sulfate (equivalent to about 1 % of label claim) must be present at the levels shown in the following table:

Peak #	RRT (to estrone sulfate)	Avg. Height Range (% of estrone sulfate)
1	0.37	3.1-5.2
2	0.37	1.8-2.4
3	0.52	24.9-46.2
4	0.54	0.1-7.4
5	0.57	1.7-4.1
6	0.58	0.1-4.9
7	0.75	2.0-3.4
8	0.79	2.5-3.6
9	0.80	3.2-6.8
10	0.81	1.3-6.3
11	0.83	11.0-12.2
12	0.85	1.4-3.9
13	0.87	3.3-4.4
14	0.88	26.0-38.1
15	0.92	3.5-5.4
16	0.93	10.3-11.1
17	0.94	14.1-18.0
18	0.97	44.1-61.0
19	0.98	2.1-5.3
20	1.00	100.00
21	1.17	3.3-4.9

Limit of estrone, equilin, and 17α- dihydroequilin (free steroids) --

Internal standard solution, pH 5.2 acetate buffer, Stock solution, and *System suitability solution* -- Proceed as directed in *Assay*.

Free steroids standard solution -- Dilute the *Stock solution* tenfold. Pipet 1.0 mL of the resulting solution and 1.0 mL of the *Internal standard solution* into a suitable centrifuge tube fitted with a tight screw-cap or stopper. Proceed as directed for *Standard preparation* in the *Assay*, beginning with ``Evaporate the mixture."

Test solution -- Proceed as directed for the *Assay preparation* in the *Assay* with the following exceptions: do not add the sulfatase enzyme preparation, and transfer 6.0 mL of the filtrate instead of 3.0 mL in the preparation of the test specimen. Prepare a reagent blank in the same manner.

Chromatographic system -- Proceed as directed in the *Assay* with the additional requirement that the relative standard deviation for the ratio of the peak response of estrone to that of the internal standard in the *Free steroids standard solution* is not greater than 5.5%, on the basis of not less than two replicate injections.

Procedure -- Separately inject equal volumes (about 1 µL) of the *Free steroids standard solution* and the *Test solution* into the chromatograph, record the chromatograms, and measure the peak responses. Calculate the ratio, R_U, of the combined peak areas of estrone, equilin, and 17α-dihydroequilin relative to the area of the internal standard in the *Test solution*, correcting for any reagent blank peaks. The ratio, R_U/R_S, where R_S is the peak response ratio of estrone to that of the internal standard obtained from the *Free steroids standard solution*, is not more than 0.65 (1.3% of free steroids).

Organic volatile impurities, *Method V* <467>: meets the requirements.
Solvent -- Use dimethyl sulfoxide as the solvent.

Assay --
Internal standard solution- Prepare a solution of 3-O-methylestrone in methanol containing about 150 µg per mL.

Stock solution- Using accurately weighed quantities of *USP Estrone RS, USP Equilin RS,* and *USP 17α-Dihydroequilin RS,* prepare, by quantitative and stepwise dilution, a solution in alcohol having known concentrations of about 160, 70, and 50 µg per mL, respectively.

pH 5.2 Acetate buffer- Mix 79 mL of sodium acetate TS with 21 mL of 1 *N* acetic acid, dilute with water to 500 mL, and mix. Adjust to a pH of 5.2 ± 0.1 by the addition of 1 *N* acetic acid, or sodium acetate TS, if necessary.

System suitability solution- Dissolve a quantity of USP Estradiol RS (17β-estradiol) in alcohol to obtain a solution containing about 10 µg per mL. Pipet 1.0 mL of this solution, 1.0 mL of *Stock solution* and 1.0 mL of *Internal standard solution* into a centrifuge tube fitted with a tight screw-cap or stopper. Proceed as directed for *Standard preparation* beginning with "Evaporate the mixture."

Standard preparation -- Pipet 1.0 mL of the *Stock solution* and 1.0 mL of the *Internal standard solution* into a suitable centrifuge tube fitted with a tight screw-cap or stopper. Evaporate the mixture to dryness with the aid of a stream of nitrogen, maintaining the temperature below 50 degrees. To the dry residue, add 25µL of dried pyridine and 75 µL of bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane. Immediately cover the tube tightly, mix, and allow to stand for 15 minutes.

Assay preparation- Transfer an accurately weighed quantity of the sample bulk Conjugated Estrogens equivalent to about 2 mg of total conjugated estrogens, to a 50-mL centrifuge tube, fitted with a polytef-lined screw-cap, containing 15 mL of *pH 5.2 acetate buffer* and 1 g of barium chloride. Cap the tube tightly, and shake for 30 minutes. If necessary, adjust the solution with 1 *N* acetic acid or sodium acetate to a pH of 5.0 ± 0.5. Place in a sonic bath for 30 seconds, then shake for an additional 30 minutes. Add a suitable sulfatase enzyme preparation equivalent to 2500 Units, and place in a water bath maintained at 50 ^{0} for 20 minutes, shaking occasionally. Add 15.0 mL of methylene chloride to the warm mixture, again cap the tube, and shake by mechanical means for 15 minutes. Centrifuge for 10 minutes at approximately 2500 rpm or until the lower layer is clear. Transfer as much of the organic phase as possible, and dry by filtering rapidly through a filter consisting of a pledget of dry glass wool and about 5 g of anhydrous sodium sulfate in a small funnel. Protect from loss by evaporation. Transfer 3.0 mL of the solution to a suitable centrifuge tube fitted with a tight screw-cap or stopper. Add 1.0 mL of *Internal standard solution*. Proceed as directed under *Standard preparation*, beginning with "Evaporate the mixture."

*Chromatographic system (*see *Chromatography <621>)*- The gas chromatograph is equipped with a flame-ionization detector held at 240°, a 0.18-mm x 20-m fused-silica capillary column bonded with a 0.20 µm layer of phase G19, and a split injection system. The column is held at 200° for 5 minutes and then programmed to rise at 5 ° per minute to a final temperature of 220° and held for an additional 60 minutes. The carrier gas is helium flowing at the rate of 0.7 mL per minute, and the split flow rate is 50 mL per minute. The injection temperature is held at 230°. Inject about 1 µL of the *System suitability solution* into the gas chromatograph. Adjust the operating conditions as necessary to maintain the elution time of the 3-O-methylestrone peak at about 55 minutes. The relative retention times are about 0.33, 0.34, 0.79, 0.81, and 1.00 for 17β-estradiol, 17α-dihydroequilin, estrone, equilin, and 3-O-methylestrone, respectively. The tailing factor for the equilin peak is not more than 1.3, the resolution, R, between 17β-estradiol and 17α-dihydroequilin is not less than 1.0, and the relative standard deviation of the estrone peak ratios is not greater than 2.0% for not fewer than four injections of the *Standard preparation*.

Procedure- Separately inject equal volumes (about 1 µL) of the *Standard preparation* and of the *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Separately calculate the quantities, in mg, of estrone, equilin, and 17α-dihydroequilin as their sodium sulfate salts in the portion of Conjugated Estrogens taken by the formula:

$$0.005(1.381C_S)(R_U/R_S),$$

in which 1.381 is the factor converting free estrogen to the conjugate sodium salt, C_S is the concentration, in µg per mL, of USP Estrone RS or USP Equilin RS or USP 17α-

Dihydroequilin in the *Stock solution*, and R_U and R_S are the ratios of the peak response of the appropriate analyte to that of the internal standard obtained from the *Assay preparation* and the *Standard preparation*, respectively.