



# OFFICIAL METHOD

## Conjugated Estrogens

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**Health Products and Food Branch**

## I. APPLICATION

This method shall be used for the preparation of dilute assay preparation A, assay preparations A and B, and the equilin reagent and for the testing of conjugated estrogens, conjugated estrogens for injection and conjugated estrogens tablets in accordance with Sections C.06.002, C.06.003 and C.06.004 respectively of the *Food and Drug Regulations*.

## II. PROCEDURE

The test shall be carried out in accordance with the following instructions:

### A. *Conjugated Estrogens*

#### 1. *Assay Preparation A*

- (1) Mix a quantity of Conjugated Estrogens, accurately weighed and equivalent to about 10 mg of Conjugated Estrogens, with 8 g of chromatographic siliceous earth;
- (2) add, with mixing, 3 mL of water;
- (3) add 3 g of chromatographic siliceous earth to a 25 x 175 mm chromatographic tube that contains a small pledget of glass wool and is fitted with a stopcock at the lower end, then transfer the mixture to it in two approximately equal portions, pressing each down moderately with the packing rod to a final height of about 8 cm;
- (4) dry rinse the emptied container with 2 g of chromatographic siliceous earth, add the latter to the tube, and tamp. Wipe the container and all the equipment with a small pledget of glass wool, transfer it to the column, sweeping the walls of the tube with it, and press it down lightly on the top of the column;
- (5) with the aid of reduced pressure, wash the column with 100 mL of benzene, and discard the washings (Flow rate 3-5 mL per minute; elute only to the top of the column);
- (6) elute the column with 150 mL of dicyclohexylamine acetate T.S. (Note 1) maintaining a flow rate of 3-5 mL per minute;
- (7) collect the eluate under reduced pressure in a round-bottom 250 mL flask;

- (8) evaporate the eluate cautiously on a rotary evaporator until the residue is almost dry;
- (9) add about 10 mL of methanol, and evaporate to dryness on a rotary evaporator;
- (10) dissolve the residue in 20 mL of methanol;
- (11) add a few anti-bumping granules as a boiling aid;
- (12) add 6.0 mL of dilute hydrochloric acid (1 in 20). Place a water condenser in the neck of the flask and place the flask on a steam bath, such that only the liquid is immersed for 12.0 min then cool in an ice bath;
- (13) transfer to a 250 mL separator with the aid of 60 mL of potassium hydroxide T.S. (6.5% w/v aqueous solution) and mix;
- (14) wash the solution with two 60 mL portions of carbon tetrachloride, and combine the washings;
- (15) extract the washings with a 40 mL portion of potassium hydroxide T.S., add the extract to the washed alkaline solution, and discard the washed carbon tetrachloride;
- (16) add sufficient dilute sulfuric acid (1 in 3) to adjust to about pH 1;
- (17) extract immediately with a 20 mL and then a 15 mL portion of benzene, shaking vigorously each time for 1 min;
- (18) discard the aqueous phase;
- (19) combine the benzene extracts;
- (20) wash the benzene extracts successively with one 10 mL portion of water, several 15 mL portions of sodium carbonate solution (1 in 50) - (until the last one is colorless), and one 10 mL portion of water;
- (21) backwash the combined aqueous washings with a 10 mL portion of benzene and discard the washings;

- (22) backwash this last benzene extract with a further 5 mL portion of water, and discard the washing;
- (23) transfer benzene extracts to a 100 mL volumetric flask through a filter consisting of a pledget of glass wool upon which 3 g to 4 g of anhydrous sodium sulfate has been placed;
- (24) rinse with several portions of fresh benzene;
- (25) add benzene to volume. Designate as *Assay Preparation A*;
- (26) dilute 20.0 mL portion of *Assay Preparation A* to 25.0 mL with benzene. Designate this as *Dilute Assay Preparation A*.

#### *Assay Preparation B*

- (1) Transfer 60.0 mL of *Assay Preparation A* in four 15.0 mL portions to a 25 mL conical flask, carefully evaporating each portion with the aid of low heat and a stream of nitrogen to dryness, so that all of the residue is deposited on the bottom of the flask. Rinse flask with 1-2 mL of fresh benzene and evaporate;
- (2) add 100 mg of trimethylacetylhydrazide ammonium chloride (Girard's Reagent T) and 0.5 mL of glacial acetic acid to the residue;
- (3) cover the flask with a small watchglass and heat at 80-100°C for five minutes with occasional swirling;
- (4) cool the solution, and transfer with the aid of 50 mL of ice-cold water to a 125 mL separator containing 10 mL of sodium acetate solution (1 in 20);
- (5) wash the solution immediately with four 10 mL portions of chloroform, and combine the washings in a second separator;
- (6) wash back the chloroform with 5 mL of water and discard the chloroform;
- (7) combine aqueous fractions;
- (8) add 7 mL of dilute sulfuric acid (1 in 3), immediately draw off and discard any chloroform that may separate and mix gently;

- (9) allow the solution to stand for 30 minutes, accurately timed;
- (10) then extract with a 20 mL and then with a 15 mL portion of benzene, shaking vigorously each time for 1 minute. Discard the aqueous phase;
- (11) combine the benzene extracts;
- (12) wash them successively with one 10 mL portion of water, two 15 mL portions of sodium carbonate solution (1 in 50), and one 10 mL portion of water;
- (13) backwash the combined aqueous washings with a 5 mL portion of benzene, and discard the washings;
- (14) wash back this last benzene extract with a further 5 mL portion of water, and discard the washing;
- (15) transfer the benzene extracts to 50 mL volumetric flask through a filter consisting of a pledget of glass wool upon which 3 g to 4 g of anhydrous sodium sulfate has been placed;
- (16) rinse with several portions of fresh benzene;
- (17) add benzene to volume. Designate as *Assay Preparation B*.

2. *Identification Test for Conjugated Estrogens*

- (1) Transfer 1 mL of *Dilute Assay Preparation A* to an 18 x 150 mm test tube;
- (2) Remove the solvent with the aid of low heat and a stream of nitrogen;
- (3) Add 0.5 mL of alcohol to the sample tube and to a second tube for use as a blank;
- (4) Place the tubes in a cold water bath at about 10°, add 5.0 mL of equilin reagent to each tube and mix;
- (5) Place the tubes in a boiling water bath, swirl them collectively after about 3 minutes, and continue heating for a total of 9 minutes;

- (6) Quickly transfer the tubes to the cold water bath, remove the tubes and allow to come to room temperature;
- (7) Determine the absorbance spectrum relative to the blank over the range 350 nm to 800 nm with a suitable recording spectrophotometer;
- (8) The spectrum exhibits maxima only at  $468 \pm 2$ ,  $528 \pm 2$ , and  $634 \pm 4$  nm, and minima at about 493 and 555 nm;
- (9) Read the absorbance from the recorded spectrum at the wavelength of maximum absorbance at about 468 nm and the wavelength of minimum at about 493 nm. The ratio of the absorbance at the maximum to that at the minimum is not less than 1.3.

### 3. *Equilin Standard Preparation*

Prepare a solution of USP Equilin Reference Standard in benzene having a known concentration of about 20 mcg per mL.

### 4. *Equilin Reagent*

- (1) To 145 g of cool purified phenol (Note 2), cautiously add 90 mL of sulfuric acid, mix until phenol is liquified. Mix cautiously until homogenous, and allow to stand in the dark overnight;
- (2) add 105 mL of the mixture to 350 mL of dilute sulphuric acid (50%, v/v);
- (3) add to this new mixture a separately prepared solution consisting of 7.0 mL of hydrochloric acid, 3.0 mL of cobalt nitrate solution (1 in 1000) and 25 mL of distilled water;
- (4) heat for 30 minutes in a boiling water bath after the temperature of the mixture has reached 95°C. Cool to room temperature, and store in low-actinic glass container;
- (5) to this mixture, add 3.0 mL of sodium hypochlorite 5% solution;
- (6) mix well and allow to stand for at least 40 hours;

- (7) the absorption spectrum of 1 mL of equilin standard preparation determined with this reagent as directed under Identification Test for Conjugated Estrogens exhibits a maximum only at about  $634 \text{ nm} \pm 4 \text{ nm}$  and does not exhibit any shoulder or maximum between 600 nm and 450 nm.

## **B. Conjugated Estrogens for Injection**

### *1. Assay Preparation A*

- (1) Dissolve the contents of 1 container of Conjugated Estrogens for Injection in 10.0 mL of water;
- (2) pipet a 4 mL portion of this solution into a round-bottom flask that contains a few pieces of silicon carbide as a boiling aid;
- (3) add 20 mL of methanol and 2 mL of dilute hydrochloric acid (3 in 20);
- (4) place a water condenser in the neck of the flask and place the flask on a steam bath, such that only the liquid is immersed for 12.0 min, then cool in ice bath;
- (5) transfer with the aid of 60 mL of water to a 250 mL separator;
- (6) extract immediately with a 20 mL and then with a 15 mL portion of benzene, shaking vigorously each time for 1 min;
- (7) continue as directed in Assay Preparation A under Conjugated Estrogens beginning at step (18).

### *2. Identification Test for Conjugated Estrogens for Injection*

- (1) The identification test for Conjugated Estrogens for Injection shall be that described for Conjugated Estrogens.

## **C. Conjugated Estrogens Tablets**

### *1. Assay Preparation A*

- (1) Mix a quantity of finely powdered Conjugated Estrogens Tablets, accurately weighed and equivalent to about 10 mg of Conjugated Estrogens, with 8 g of chromatographic siliceous earth;

- (2) continue as directed in Assay Preparation A under Conjugated Estrogens beginning step (2);

2. *Identification Test for Conjugated Estrogens Tablets*

- (1) The identification test for Conjugated Estrogens Tablets shall be that described for Conjugated Estrogens.

**III. NOTES**

1. *Dicyclohexylamine Acetate T.S. shall be prepared as follows:*

- (1) dissolve 50 g of dicyclohexylamine in 150 mL of acetone, cool in an ice bath, and add, with stirring, a solution consisting of 18 mL of glacial acetic acid in 150 mL of acetone;
- (2) recrystallize the precipitate that forms, by heating the mixture to boiling and allowing it to cool in an ice bath, then collect the crystals on a filtering funnel, wash with a small volume of acetone, and dry in air;
- (3) dissolve 300 mg of the dicyclohexylamine acetate so obtained in 200 mL of a mixture of 6 volumes of chloroform and 4 volumes of water-saturated ether;
- (4) use immediately.

2. *Phenol shall be purified as follows:*

- (1) add to a melted quantity of phenol 4% of its weight of ferric chloride hexahydrate, finely powdered;
- (2) reflux the mixture for at least 3 days;
- (3) separate the phenol by distillation;
- (4) add sodium hydroxide (pellets) 1% w/w to the phenol, distill and then redistill;
- (5) purified phenol shall produce no colour with sulfuric acid.

#### **IV. REFERENCE**

The Pharmacopeia of the United States of America, Eighteenth Revision, 1970, Mack Printing Company, Easton PA. 18042, U.S.A.

The method described above, being comprised of 8 pages and identified as DO-29, CONJUGATED ESTROGENS and dated October 15, 1981, is hereby designated the "official method" referred to in Sections C.06.002, C.06.003 and C.06.004 of the *Food and Drug Regulations*.