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Conjugated Estrogens

DEFINITION

Conjugated Estrogens is a mixture of sodium estrone sulfate and sodium equilin sulfate, derived wholly or in part from equine urine or synthetically from estrone and equilin. It contains other conjugated estrogenic substances of the type excreted by pregnant mares. It is a dispersion of the estrogenic substances on a suitable powdered diluent.

Conjugated Estrogens contains NLT 52.5% and NMT 61.5% of sodium estrone sulfate and NLT 22.5% and NMT 30.5% of sodium equilin sulfate, and the total of sodium estrone sulfate and sodium equilin sulfate is NLT 79.5% and NMT 88.0% of the labeled content of Conjugated Estrogens. Conjugated Estrogens contains concomitant components as sodium sulfate conjugates NLT 13.5% and NMT 19.5% of 17α-dihydroequilin, NLT 2.5% and NMT 9.5% of 17α-estradiol, and NLT 0.5% and NMT 4.0% of 17β-dihydroequilin of the labeled content of Conjugated Estrogens.

IDENTIFICATION

• **A.** The relative retention times of the 17α-dihydroequilin peak, the estrone peak, and the equilin peak from the *Sample solution* correspond to those from the *Standard solution*, as obtained in the *Assay*.

• **B.** The chromatogram of Conjugated Estrogens from the *Sample solution* in the *Assay* exhibits additional peaks or shoulders, corresponding to 17α-estradiol and 17β-dihydroequilin at retention times of about 0.24 and 0.35, relative to that of 3-*O*-methylestrone.

ASSAY

• **Procedure**

Buffer: Sodium acetate TS, 1 N acetic acid, and water (79:21:400). Adjust with 1 N acetic acid or sodium acetate TS to a pH of 5.2 ± 0.1, if necessary.

Internal standard solution: 150 µg/mL of 3-*O*-methylestrone in methanol

Standard stock solution: 160 µg/mL, 70 µg/mL, and 50 µg/mL each of USP Estrone RS, USP Equilin RS, and USP 17α-Dihydroequilin RS, respectively, in alcohol

Standard solution: Pipet 1.0 mL of the *Internal standard solution* and 1.0 mL of the *Standard stock solution* into a suitable centrifuge tube fitted with a tight screw cap or stopper. Evaporate the mixture with the aid of a stream of nitrogen to dryness, maintaining the temperature below 50°. To the dry residue, add 15 µL of dried pyridine and 65 µL of bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane. Immediately cover the tube tightly, mix, and allow to stand for 15 min. Add 0.5 mL of toluene, and mix.

System suitability solution: Pipet 1.0 mL of a 2.0-µg/mL solution of USP Estradiol RS (17β-estradiol) in alcohol, 1.0 mL of *Internal standard solution*, and 1.0 mL of *Standard stock solution* into a centrifuge tube fitted with a tight screw cap or stopper. Proceed as directed for *Standard solution*, beginning with "Evaporate the mixture…".

Sample solution: Transfer a quantity of Conjugated Estrogens, equivalent to 2 mg of total conjugated estrogens, to a 50-mL centrifuge tube, fitted with a polytef-lined screw cap, containing 15 mL of *Buffer* and 1 g of barium chloride. Cap the tube tightly, and shake for 30 min. If necessary, adjust the solution with 1 N acetic acid or sodium acetate to a pH of 5.0 ± 0.5. Sonicate for 30 s, then shake for an additional 30 min. Add a suitable sulfatase enzyme solution equivalent to 2500 Units, and shake for 20 min in a water bath maintained at 50°. Add 15.0 mL of ethylene dichloride to the warm mixture, cap the tube again, and shake by mechanical means for 15 min. Centrifuge for 10 min or until the lower layer is clear. Transfer as much of the organic phase as possible, and dry by rapidly passing through a filter consisting of a pledget of dry glass wool and 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 for *Standard solution*, beginning with "Evaporate the mixture…".

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 15-m fused silica capillary column, bonded with a 0.25-µm layer of phase G19

Temperature

Column: 208°

Injector port: 260°

Detector: 260°

Carrier gas: Hydrogen

Flow rate: 2 mL/min

Injection mode: Split

Split flow rate: 40–60 mL/min

Injection size: 1 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—Adjust the operating conditions as necessary to maintain the elution time of the 3-*O*-methylestrone peak between 17 and 25 min.]

[NOTE—The relative retention times for 17β-estradiol, 17α-dihydroequilin, estrone, equilin, and 3-*O*-methylestrone are 0.29, 0.30, 0.80, 0.87, and 1.00, respectively.]

Suitability requirements

Resolution: NLT 1.2 between estrone and equilin, *System suitability solution*

Tailing factor: NMT 1.3 for the estrone peak, *System suitability solution*

Relative standard deviation: NMT 2.0% for the estrone peak ratios for NLT four injections of the *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of sodium estrone sulfate and sodium equilin sulfate in the portion of Conjugated Estrogens taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times F \times 100$$

R_U = ratio of the peak response of the appropriate analyte to that of the internal standard from the *Sample solution*

R_S = ratio of the peak response of the appropriate analyte to that of the internal standard from the *Standard solution*

C_S = concentration of USP Estrone RS or USP Equilin RS in the *Standard solution* (µg/mL)

C_U = concentration of the *Sample solution* (µg/mL)

F = conversion factor (ratio of molecular weight of sodium salts to free estrogen), 1.381

Acceptance criteria: 52.5%–61.5% of sodium estrone sulfate and 22.5%–30.5% of sodium equilin sulfate

OTHER COMPONENTS

• **CONTENT OF 17α-DIHYDROEQUILIN, 17β-DIHYDROEQUILIN, AND 17α-ESTRADIOL** (concomitant components)

Buffer, Internal standard solution, Standard stock solution, Standard solution, System suitability solution, Sample solution, and

Chromatographic system: Proceed as directed in the *Assay*.

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—The relative retention times for 17α-estradiol, 17α-dihydroequilin, and 17β-dihydroequilin are about 0.82, 1.00, and 1.11, respectively.]

Identify the peaks for 17α-estradiol, 17α-dihydroequilin, and 17β-dihydroequilin from the *Sample solution*.

Calculate the percentages of 17α-estradiol, 17α-dihydroequilin, and 17β-dihydroequilin as their sodium sulfate salts in the portion of Conjugated Estrogens taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times F \times 100$$

R_U = ratio of the peak response of the appropriate analyte to that of the internal standard from the *Sample solution*

R_S = ratio of the peak response of 17α-dihydroequilin to that of the internal standard from the *Standard solution*

C_S = concentration of USP 17α-Dihydroequilin RS in the *Standard solution* (µg/mL)

C_U = concentration of the *Sample solution* (µg/mL)

F = conversion factor (ratio of molecular weight of sodium salts to free estrogen), 1.381

Acceptance criteria: NLT 13.5% and NMT 19.5% of 17α-dihydroequilin, NLT 2.5% and NMT 9.5% of 17α-estradiol, and NLT 0.5% and NMT 4.0% of 17β-dihydroequilin, as their sodium sulfate conjugates

IMPURITIES

• **Limits of 17α-Dihydroequilenin, 17β-Dihydroequilenin, and Equilenin** (signal impurities)

Buffer, Internal standard solution, Standard stock solution, Standard solution, System suitability solution, Sample solution, and **Chromatographic system:** Proceed as directed in the *Assay*.

Analysis

Samples: *Standard solution* and *Sample solution*

[Note—The relative retention times for dihydroequilenin, 17β-dihydroequilenin, 3-*O*-methylestrone, and equilenin are 0.56, 0.64, 1.0, and 1.3, respectively.]

Identify any peaks for dihydroequilenin, 17β-dihydroequilenin, 3-*O*-methylestrone, and equilenin from the *Sample solution*. Calculate the percentages of 17α-dihydroequilenin, 17β-dihydroequilenin, and equilenin as their sodium sulfate salts in the portion of Conjugated Estrogens taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times F \times 100$$

R_U = ratio of the peak response of the appropriate analyte to that of the internal standard from the *Sample solution*

R_S = ratio of the peak response of estrone to that of the internal standard from the *Standard solution*

C_S = concentration of USP Estrone RS in the *Standard solution* (µg/mL)

C_U = concentration of the *Sample solution* (µg/mL)

F = conversion factor (ratio of molecular weight of sodium salts to free estrogen), 1.381

Acceptance criteria: NMT 3.25%, NMT 2.75%, and NMT 5.5% of the labeled content of Conjugated Estrogens for 17α-dihydroequilenin, 17β-dihydroequilenin, and equilenin, respectively, as their sodium sulfate salts

• **Limits of 17β-Estradiol and $\Delta^{8,9}$-Dehydroestrone**

Buffer, Internal standard solution, Standard stock solution, Standard solution, System suitability solution, Sample solution, and **Chromatographic system:** Proceed as directed in the *Assay*.

Analysis

Samples: *Standard solution* and *Sample solution*

[Note—The relative retention times of 17β-estradiol, 3-*O*-methylestrone, and $\Delta^{8,9}$-dehydroestrone are about 0.29, 1.0, and 0.9, respectively.]

Identify any peaks for 17β-estradiol, 3-*O*-methylestrone, and $\Delta^{8,9}$-dehydroestrone from the *Sample solution*.

Calculate the percentages of 17β-estradiol and $\Delta^{8,9}$-dehydroestrone as their sodium sulfate salts in the portion of Conjugated Estrogens taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times F \times 100$$

R_U = ratio of the peak response of the appropriate analyte to that of the internal standard from the *Sample solution*

R_S = ratio of the peak response of estrone to that of the internal standard from the *Standard solution*

C_S = concentration of USP Estrone RS in the *Standard solution* (µg/mL)

C_U = concentration of the *Sample solution* (µg/mL)

F = conversion factor (ratio of molecular weight of sodium salts to free estrogen), 1.381

Acceptance criteria: NMT 2.25% and NMT 6.25% of the labeled content of Conjugated Estrogens for 17β-estradiol and $\Delta^{8,9}$-dehydroestrone, respectively, as their sodium sulfate salts

• **Limit of Estrone, Equilin, and 17α-Dihydroequilin** (free steroids)

Buffer, Internal standard solution, Standard stock solution, System suitability solution, and **Chromatographic system:** Proceed as directed in the *Assay*.

Free steroids standard solution: Dilute the *Standard 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 solution* in the *Assay*, beginning with "Evaporate the mixture…".

Sample solution: Proceed as directed for *Sample solution* in the *Assay* with the following exceptions: do not add the sulfatase enzyme solution, and transfer 6.0 mL of the solution, instead of 3.0 mL, to the centrifuge tube.

Blank solution: Prepare a reagent blank in the same manner as the *Sample solution*.

System suitability: 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 NMT 5.5%, from NLT two replicate injections.

Analysis

Samples: *Free steroids standard solution* and *Sample solution*

Calculate the total percentage of estrone, equilin and 17α-dihydroequilin (free steroids) in the portion of Conjugated Estrogens taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = ratio of the sum of the estrone, equilin, and 17α-dihydroequilin peak areas (corrected for any peaks found in the *Blank solution*) to the internal standard peak area from the *Sample solution*

R_S = ratio of the estrone peak area to the internal standard peak area from the *Standard solution*

C_S = concentration of USP Estrone RS in the *Standard solution* (µg/mL)

C_U = concentration of the *Sample solution* (µg/mL)

Acceptance criteria: NMT 1.3% of free steroids

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers. Store at controlled room temperature, or under refrigeration.
- **Labeling:** Label it to state the content of Conjugated Estrogens on a weight-to-weight basis.
- **USP Reference Standards** ⟨11⟩

USP 17α-Dihydroequilin RS

Estra-1,3,5(10),7-tetraene-3,17α-diol.

$C_{18}H_{22}O_2$ 270.37

USP Equilin RS

USP Estradiol RS

USP Estrone RS

Auxiliary Information - Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
CONJUGATED ESTROGENS	Documentary Standards Support	SM52020 Small Molecules 5

Chromatographic Database Information: Chromatographic Database

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