Comparison of Acidic and Enzymatic Hydrolysis Procedures for Identification of Natural Estrogens in Pharmaceutical Preparations

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Abstract □ The usefulness of a distinct enzyme-hydrolyzed preparation for the identification of conjugated and esterified estrogens USP was studied. No significant differences were found when the GLC identification test was performed on the acid-hydrolyzed assay preparation.

Keyphrases □ Estrogens—identification of conjugated and esterified estrogens in pharmaceutical preparations, comparison of acidic and enzymatic hydrolysis procedures for sample preparation □ Enzymatic hydrolysis—procedure for sample preparation for identification of conjugated and esterified estrogens in pharmaceutical preparations, comparison with acidic hydrolysis procedure □ GLC—identification of conjugated and esterified estrogens in pharmaceutical preparations, comparison of acidic and enzymatic hydrolyses for sample preparation

Conjugated and esterified estrogens are two types of natural estrogenic substances. Both groups are described in USP XIX (1) as a mixture of the sodium salts of the sulfate esters of the estrogenic substances, principally estrone and equilin for conjugated estrogens and estrone for esterified estrogens, that are of the type excreted by pregnant mares.

Since the publication of USP XIX, the colorimetric identification test has been replaced by a more specific GLC identification test (2) to show the presence of other complementary estrogens, in particular, α-estradiol, β-estradiol, 17α-dihydroequilin, 17β-dihydroequilin, 17α-dihydroequilenin, 17β-dihydroequilenin, 9-dehydroestrone, and equilenin. However, one major result of this test has been a substantial increase in analysis time.

This report discusses the possibility of reducing the analysis time and avoiding many of the required manipulations by using a single sample preparation for both identification and assay. The colorimetric assay described in USP requires a more purified extract than the GLC identification test. This extract is obtained by acidic hydrolysis and subsequent purification. The GLC identification test introduced recently involves a long enzymatic hydrolysis procedure (2.5 hr), resulting in an extract that is not pure enough for use in the assay.

The milder acidic hydrolysis procedure (3), developed in these laboratories to replace the USP XIX acidic hydrolysis procedure, and the USP XIX acidic hydrolysis procedure were compared to an enzymatic hydrolysis procedure (4), which was very similar to the procedure used in the USP XIX GLC identification test. Qualitative and quantitative results were obtained by the GLC procedure of McErlane and Curran (4).

Table I—Comparison of the Enzymatic Hydrolysis and the Milder Acidic Hydrolysis of a Commercial Conjugated Estrogens Formulation

Steroid	Enzyme Hydrolysis Amount[a], %	σ	CV, %	Milder Acid Hydrolysis Amount[b], %	σ	CV, %
α-Estradiol	3.9	0.18	4.6	3.5	0.12	3.3
β-Estradiol/ 17α-dihydro-equilin	15.5	0.14	0.9	15.6	0.42	2.7
17β-Dihydro-equilin	2.0	0.11	5.7	1.0	0.04	4.2
17α-Dihydro-equilenin	1.5	0.14	9.4	1.4	0.26	18.8
17β-Dihydro-equilenin	0.4	0.05	11.8	0.3	0.08	30.0
Estrone	50.2	0.98	1.9	51.7	1.09	2.1
Equilin	23.0	0.25	1.1	23.1	0.83	3.6
9-Dehydro-estrone	1.7	0.16	9.3	1.4	0.13	9.6
Equilenin	2.3	0.84	36.0	2.3	0.32	13.8
Assay, % of label claim	105.3	2.6	2.4	103.1	5.2	5.0

[a] Average of five determinations. [b] Average of seven determinations.

EXPERIMENTAL

Apparatus—The gas chromatograph[1] was equipped with a U-shaped column and a flame-ionization detector. Peak retention times and areas were obtained using a reporting integrator[2].

Acidic Hydrolysis—The residue obtained from elution with dicyclohexylamine acetate solution was dissolved in 20 ml of methanol. A few antibumping granules were added as a boiling aid. A 6.0-ml portion of hydrochloric acid of a specified concentration was added. A water condenser was placed in the neck of the flask, and the flask was placed on a steam bath so that only the liquid was immersed. After the specified time, the flask was cooled in an ice bath. The hydrolysate was extracted and purified as in the USP procedure.

For milder acidic hydrolysis, 0.6 *N* HCl (1 in 20) was used for 12.0 min. For the USP acidic hydrolysis, 2 *N* HCl (1 in 6) was used for 10 min. The stronger acidic hydrolysis required 4 *N* HCl (1 in 3) for 12 min.

Enzyme Hydrolysis—An aliquot equivalent to ~1.5 mg of conjugated estrogens in a 50-ml centrifuge tube was shaken for 30 min with 15 ml of 0.02 *M* sodium acetate (pH 5.2) and then extracted twice with 10 ml of benzene (shaken for 20 min each time). The tube was centrifuged for 10 min, and the benzene was discarded. After all traces of residual benzene had been removed, 7500 units of sulfatase enzyme[3] was added, and the tube was incubated for 30 min at 45° with occasional shaking. The tube then was cooled to room temperature, 15 ml of chloroform was added, and the tube was shaken for 15 min. After centrifugation for 10 min, the chloroform layer was filtered through anhydrous sodium sulfate and stored in a well-closed container in the refrigerator until it was used for derivatization.

Sample Derivatization—Two portions of each hydrolysate equivalent to 300 μg of estrogens were transferred to separate 5-ml polytef-lined screw-capped conical vials. To one vial was added 50 μg of ethynyl estradiol[4] (internal standard). The samples were evaporated to dryness using low heat and a gentle nitrogen stream. To the vials containing the internal standard were added 0.2 ml of pyridine and 0.05 ml of silylating reagent[5]; the vial then was capped and heated for 15 min at 60°. To the other vial (no internal standard) was added 0.2 ml of a 2% solution of methoxamine in pyridine; this sample was heated for 3 hr at 60°. After

[1] Model 2500, Bendix Corp., Ronceverte, WV 24970.
[2] Model 3385A automation system, Hewlett-Packard, Avondale, PA 19311.
[3] Sigma Chemical Co., St. Louis, MO 63172.
[4] USP reference standard.
[5] TRISIL TBT, Pierce Chemical Co., Rockford, IL 61105.

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Table II—Comparison of the Enzymatic Hydrolysis and Hydrolysis Using Three Acid Concentrations of a Commercial Conjugated Estrogens Tablet

Steroid	Enzyme Hydrolysis[a], %	Acid Hydrolysis, %		
		1 in 20[b]	1 in 6[b]	1 in 3[b]
α-Estradiol	0.8	0.8	0.8	0.8
β-Estradiol/17α-dihydroequilin	4.3	4.3	4.3	4.9
17β-Dihydroequilin	1.0	0.8	1.0	1.4
17α-Dihydroequilenin	0.5	0.5	0.5	0.5
17β-Dihydroequilenin	0.3	0.4	0.2	—[c]
Estrone	59.9	60.3	60.3	60.3
Equilin	30.0	29.7	30.0	30.1
9-Dehydroestrone	1.4	1.1	1.1	—[c]
Equilenin	2.1	2.1	1.9	2.0
Assay, % of label claim	118.7	112.1	117.3	114.9

[a] Average of two determinations. [b] Sufficient materials to perform a single determination only. [c] Peaks were present but had an area less than the area reject setting.

this time, 0.05 ml of silylating reagent was added, and the solution was heated at 60° for 10 min.

A 2–4-μl aliquot was injected into a 1.8-m × 0.63-cm o.d. (6 ft × 0.25 in.) glass U-shaped column packed with 3% methyl phenyl cyanopropyl silicone[6] (OV-225) on 100–120-mesh Chromosorb WHP[7]. The temperature was maintained at 225°, and the helium flow rate was 50 ml/min.

RESULTS AND DISCUSSION

Results from the milder acidic hydrolysis (assay preparation) and the enzymatic hydrolysis (test preparation) in the identification of a commercial conjugated estrogens formulation, which fully met requirements of the previous colorimetric identification test, are shown in Table I. Average amounts, standard deviations, and coefficients of variation (CV) are given for each component.

The distinctive estrone and equilin peaks and the prominent β-estradiol/17α-dihydroequilin peak[8] showed the same relative amounts, within experimental error, with both hydrolysis procedures. Moreover, the relative amounts of other additional minor peaks (α-estradiol, 17β-dihydroequilin, 17α-dihydroequilenin, 17β-dihydroequilenin, 9-dehydroestrone, and equilenin) were very similar to each other.

Reproducibility was good for the major peaks ($CV \leq 3.6\%$) but decreased in proportion to the concentration of the minor peaks.

The robustness of the acidic hydrolysis procedure was studied. Table II shows a comparison of the enzymatic hydrolysis and three different acidic hydrolyses where the acid concentration was varied (0.6, 2, and 4 N) for the identification of conjugated estrogens tablets that were bor-

[6] Ohio Valley Specialties Chemical, Marietta, OH 45750.
[7] Chromatographic Specialties, Brockville, Ontario, K6V SW1, Canada.
[8] The procedure does not resolve these two components.

Table III—Comparison of the Enzymatic Hydrolysis and the Milder Acidic Hydrolysis of a Commercial Esterified Estrogens Product

Steroid	Enzyme Hydrolysis[a], %	Acid Hydrolysis[b], %
α-Estradiol	1.6	1.2
β-Estradiol/17α-dihydroequilin	5.8	5.4
17β-Dihydroequilin	0.4	0.3
17α-Dihydroequilenin	0.8	0.6
17β-Dihydroequilenin	0.2	—[b]
Estrone	78.1	79.4
Equilin	11.8	12.1
9-Dehydroestrone	0.5	0.3
Equilenin	0.7	0.7
Assay, % of label claim	114.2	112.3

[a] Single determination. [b] Peak was present but had an area less than the area reject setting.

derline in the colorimetric identification test. No major differences were seen for all of the individual components among the enzymatic, milder acidic, and USP acidic hydrolyses. The only differences were a small increase of β-estradiol/17α-dihydroequilin and a small decrease of 17β-dihydroequilenin and 9-dehydroestrone in the acidic procedures.

The results in Table III show that the two hydrolysis procedures also are applicable to the identification of commercial esterified estrogens products. The small differences between the results obtained with the two procedures are not important and do not lead to any misinterpretations. Several other benefits are derived from the use of the acidic hydrolysis procedure. The assay preparation is a much cleaner extract than the enzymatic identification test preparation. The presence of equol (5) is no longer a problem; it is removed in the early chromatographic column step by benzene washing. Chromatograms exhibit less tailing, the detector background signal is reduced, and column lifetime is much longer.

In summary, the assay preparation obtained by the milder hydrolysis or by the USP acid hydrolysis gave identification test results for conjugated and esterified estrogens formulations comparable to the new enzyme-hydrolyzed identification test preparation. Since it is unnecessary to prepare the latter, the analysis time is shortened and the expense of enzyme reagents is avoided.

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