basket and rotating paddle were relatively easy to use, reliable, and amenable for routine use in a quality control environment. The rotating filter–stationary basket apparatus was more difficult to operate, required more time per test, and generally gave greater variability.

Serum Level Data—Dissolution times measured with all three apparatus correlated equally well with serum drug levels in dogs for the four experimental hypoglycemic lots. None of these three apparatus gave substantially better correlations when other test conditions, such as the dissolution medium and relative agitation levels, were kept constant. These data cannot be used, of course, to prove or disprove ability to predict a relation between dissolution and blood levels in other species. On the contrary, since there is a high degree of correlation of dissolution results between apparatus when experimental conditions are kept as similar as possible, claims that one apparatus or another is *a priori* superior for prediction of *in vivo* behavior should be critically assessed.

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High-Pressure Liquid Chromatographic Analysis of Estrogens in Pharmaceuticals by Measurement of Their Dansyl Derivatives

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Received February 17, 1978, from the *Food and Drug Administration, Department of Health, Education, and Welfare, Brooklyn, NY 11232.* Accepted for publication April 27, 1978.

Abstract □ A high-pressure liquid chromatographic method is described for the analysis of estrogens in pharmaceutical tablet and injectable dosage forms. In general, the estrogens are isolated, an internal standard is added, the dansyl derivatives are formed, and the dansyl estrogen solution is injected into a liquid chromatograph. Linear response is experienced between the mass of estrogen and the ratio of the estrogen peak height to the internal standard peak height, using a microparticle silica column and chloroform–*n*-heptane mobile phases. With fluorometric measurement, limits of detectability for ethinyl estradiol and estradiol were 0.04 and 0.05 ng, respectively. Methyltestosterone, an androgen in combination with ethinyl estradiol, was analyzed simultaneously. Commercial pharmaceutical preparations containing estrone, ethinyl estradiol, and estradiol were analyzed by the proposed method. The results indicate the method to be sensitive, reasonably precise (<2%), and accurate in the analysis of estrogen in dosage forms.

Keyphrases □ Estrogens, various—high-pressure liquid chromatographic analyses of dansyl derivatives, pharmaceutical preparations □ High-pressure liquid chromatography—analyses, dansyl derivatives of various estrogens, pharmaceutical preparations □ Dansyl derivatives—various estrogens, high-pressure liquid chromatographic analyses, pharmaceutical preparations

Dansyl chloride, 5-(dimethylamino)-1-naphthalenesulfonyl chloride (I), is a useful reagent for the production of fluorescent derivatives (fluorogenic labeling) with several functional groups, including primary and secondary amines, imidazoles, and phenols. However, since I can decompose to yield dansyl hydroxide (actually a sulfonic acid), dansyl dimethylamide, and other compounds (1) under the conditions used for derivatization, analyses involving I usually include a procedure to separate the dansyl derivative from any other fluorescent compounds present in the solution.

The ability of various classes of compounds to form dansyl derivatives that can be detected at low levels is advantageous. Some analytical applications were reviewed by Seiler and Wiechmann (1). Recently, high-pressure liquid chromatographic (HPLC) methods for the analysis of carbamate insecticides (2), hydroxybiphenyls (3), and barbiturates (4) used dansyl derivatives and demonstrated the value of this approach.

Better methods of analysis for the determination of estrogens in pharmaceutical dosage forms are needed (5, 6). Penzes and Oertel (7, 8) described the TLC separation of the dansyl derivatives of estrone, estradiol, and estriol, and Fishman (5) introduced a conventional fluorescence method for some estrogens using dansyl estrogen derivatives.

This study was conducted to determine the utility of the formation of dansyl derivatives of estrogens in an HPLC analytical procedure. The procedure was adapted satisfactorily to the analysis of estradiol, ethinyl estradiol, and estrone in pharmaceutical dosage forms. Ethinyl estradiol, the estrogen receiving the most attention owing to its small dose, is frequently found in combination with a progestin (oral contraceptive) and an androgen such as methyltestosterone. Fortunately, some nonestrogen steroids are separated from the dansyl estrogens, so they can be analyzed with the same column. A simultaneous method for

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ethinyl estradiol and methyltestosterone also was developed.

EXPERIMENTAL

Apparatus—The liquid chromatograph[1] was equipped with a UV (254 nm) absorption photometer and, in series, a fluorescence detector[2] containing a broad band (240–420 nm) excitation filter and a 440-nm cutoff emission filter. Injections were made with a 10-μl injection valve.

Column and Mobile Phases—The column was a 25-cm × 3.2-mm i.d. × 0.63-cm o.d. stainless steel tube, slurry-packed with LiChrosorb Si 60[3] (5 μm). Mobile phases were simple combinations of *n*-heptane[4] and chloroform[5]. Prior to use, the mobile phases were degassed by ultrasonics for 10 min.

Chemicals, Solvents, and Standards—Unless otherwise specified, all chemicals were reagent grade. Certified ACS grade solvents were used, except where mobile phase requirements dictated otherwise. Standard estrogens were laboratory working grade materials with conventional assay values established above 99.0%. Prior to use in quantitative analysis, estradiol and estrone were dried at 105° for 4 hr; ethinyl estradiol was dried over silica gel for 4 hr at room temperature.

Assay of Estradiol and Ethinyl Estradiol in Commercial Preparations—*Estradiol Standard Solution A*—Accurately weigh and quantitatively transfer approximately 50 mg of estradiol to a 250-ml volumetric flask and bring to volume with acetone.

Estradiol Standard Solution B—Dilute 10.0 ml of estradiol standard solution A to 100.0 ml with acetone.

Ethinyl Estradiol Standard Solution A—Accurately weigh and quantitatively transfer approximately 40 mg of ethinyl estradiol to a 250-ml volumetric flask and bring to volume with acetone.

Ethinyl Estradiol Standard Solution B—Dilute 10.0 ml of ethinyl estradiol standard solution A to 100.0 ml with acetone.

Preparation of Standard Mixture Solution—Transfer 10.0 ml of estradiol standard solution A and 10.0 ml of ethinyl estradiol standard solution A to a 100-ml volumetric flask and bring to volume with acetone. Transfer 1.25 ml to a 125-ml glass-stoppered flask and evaporate just to dryness on a steam bath with an air current to obtain the standard estradiol–ethinyl estradiol residue. Continue with the procedure entitled *Dansylation and Extraction of Dansyl Estrogens.*

Estradiol in Sesame Oil (1.0 mg/ml)—Accurately measure and quantitatively transfer 5.0 ml of the estradiol in sesame oil sample to a 100-ml volumetric flask, add 25.0 ml of ethinyl estradiol standard solution A, and bring to volume with acetone. Transfer 20.0 ml of the latter solution to a 100-ml volumetric flask and bring to volume with methanol.

Centrifuge a portion of this solution at 2000 rpm for 10 min to separate most of the sesame oil. Then transfer 2.50 ml of the acetone–methanol solution (upper layer after centrifugation) to a 125-ml glass-stoppered flask and evaporate just to dryness on a steam bath with an air current to obtain the estradiol residue. Continue with the procedure entitled *Dansylation and Extraction of Dansyl Estrogens.*

Estradiol in Aqueous Suspension (1.0 mg/ml)—Accurately measure and quantitatively transfer 5.0 ml of estradiol in an aqueous suspension to a 250-ml volumetric flask, add 25.0 ml of ethinyl estradiol standard solution A, mix, bring to volume with acetone, and filter. Transfer 1.25 ml to a 125-ml glass-stoppered flask and evaporate just to dryness on a steam bath with an air current to obtain the estradiol residue. Continue with the procedure entitled *Dansylation and Extraction of Dansyl Estrogens.*

Estradiol in Tablets (0.5 mg/Tablet)—Accurately weigh and finely powder a representative number (usually 20) of tablets to pass through a 60-mesh sieve. Accurately weigh a portion of the powder, estimated to contain 1.0 mg of estradiol, and quantitatively transfer it to a 100-ml volumetric flask using methanol. Add 5.0 ml of ethinyl estradiol standard solution A and bring to approximately 50 ml with methanol.

Mix on a laboratory shaker for 30 min, bring to volume with acetone, and filter. Then transfer 2.50 ml of the filtrate to a 125-ml glass-stoppered flask and evaporate just to dryness on a steam bath with an air current to obtain the estradiol residue. Continue with the procedure entitled *Dansylation and Extraction of Dansyl Estrogens.*

Ethinyl Estradiol in Tablets (0.5 mg/Tablet)—Proceed as directed for estradiol in tablets (0.5 mg/tablet) but use the sample and internal standard as given here. Accurately weigh a portion of ethinyl estradiol in tablets sample powder, estimated to contain 0.8 mg of ethinyl estradiol, and add 5.0 ml of estradiol standard solution A. Label the final residue as ethinyl estradiol residue.

Ethinyl Estradiol in Tablets (0.01–0.05 mg/Tablet)—Just prior to use, prepare a conventional partition column in the following manner. To a 250 × 25-mm glass column containing a glass wool plug, add 8 g of anhydrous sodium sulfate, 2 g of diatomaceous earth impregnated with 1.0 ml of water (pack tightly), and 3 g of diatomaceous earth impregnated with 2.0 ml of 10% aqueous anhydrous sodium carbonate (pack tightly).

Accurately weigh and finely powder a representative number (usually 20) of tablets to pass through a 60-mesh sieve. Accurately weigh a portion of the powder, estimated to contain 80 μg of ethinyl estradiol, and quantitatively transfer it to a 100-ml beaker containing 4 g of diatomaceous earth. Dry mix thoroughly, add 3.0 ml of water, and mix until uniform. Quantitatively transfer the mixture to the prepared column, packing tightly after each one-third addition.

Dry wash the beaker with 1 g of diatomaceous earth, transfer to the column, and wipe the spatula, tamping rod, and beaker with glass wool. Then transfer the glass wool to the column. Elute the ethinyl estradiol with 175 ml of benzene, collecting the eluate in a 200-ml volumetric flask. Add 5.0 ml of estradiol standard solution B to the volumetric flask, mix, and bring to volume with acetone.

Transfer 50 ml of this solution to a 125-ml glass-stoppered flask and add 30 ml of acetone. Mix, evaporate on a steam bath using an air current to approximately 10 ml, add 10 ml of acetone, and continue evaporation just to dryness to obtain the ethinyl estradiol residue. Continue with the procedure entitled *Dansylation and Extraction of Dansyl Estrogens.*

Assay of Estrone in Aqueous Suspensions (5 mg/ml)—*Preparation of Standard Mixture Residue*—Accurately weigh and quantitatively transfer approximately 25 mg of estrone and 50 mg of ethinyl estradiol to a 250-ml volumetric flask and dilute to volume with acetone. Transfer 10.0 ml of this solution to a 100-ml volumetric flask and dilute to volume with acetone. Transfer 8.0 ml of the latter solution to a 125-ml glass-stoppered flask and evaporate just to dryness on a steam bath with an air current to obtain the standard mixture residue. Continue with the procedure entitled *Dansylation and Extraction of Dansyl Estrogens.*

Sample Preparation—Accurately weigh about 50 mg of ethinyl estradiol, quantitatively transfer to a 250-ml volumetric flask containing 25 ml of acetone, and mix until the solute dissolves. Transfer 5.0 ml of the estrone aqueous suspension, using an appropriate sampling technique, to the 250-ml volumetric flask; then swirl, dilute to volume with acetone, and filter.

Dilute 10.0 ml of the filtrate to volume with acetone in a 100.0-ml volumetric flask. Transfer 8.0 ml of this solution to a 125-ml glass-stoppered flask and evaporate just to dryness on a steam bath with an air current to obtain the estrone residue. Continue with the procedure entitled *Dansylation and Extraction of Dansyl Estrogens.*

Dansylation and Extraction of Dansyl Estrogens—*Preparation of Reagents*—Prepare a 0.10-mg/ml acetone solution of I and filter. To prepare the base solution, add 0.3667 g of anhydrous sodium carbonate to 300 ml of water, mix to dissolve, add 150 ml of acetone, and mix well.

Dansylation Reaction—To the 125-ml glass-stoppered flask containing the appropriate estrogen residue, add 5.0 ml of I solution and swirl to dissolve. Add 7.5 ml of base solution, mix, stopper, and allow to stand in the dark for 30 min.

Extraction of Dansyl Estrogen Derivatives—Transfer the dansylation reaction solution to a 60-ml separator with 25 ml of ether. Shake for several minutes, transfer the lower aqueous layer to a second 60-ml separator containing 25 ml of ether, shake, and discard the aqueous phase. Wash both ether extracts consecutively with 15- and 10-ml portions of water and discard the washings.

Transfer the ether extracts in both separators to a 50-g anhydrous sodium sulfate column, collecting the eluate in a 125-ml glass-stoppered flask. Wash both separators with 10 ml of ether, transfer the washings to the column, and wash the column with 25 ml of ether. Evaporate the total ether eluate on a steam bath with an air current just to dryness and then continue the evaporation for an additional 2 min[6]. Cool, add 2.0 ml of chloroform, swirl to dissolve the residue, and transfer to a 15-ml glass-stoppered centrifuge tube (label dansyl estrogen solution).

HPLC Measurement of Estradiol and Ethinyl Estradiol—Inject

[1] DuPont model 841.
[2] DuPont model 836.
[3] Altex Scientific, Berkeley, CA 94710.
[4] Spectranalyzed grade, Fisher Scientific Co., Fair Lawn, NJ 07410.
[5] ACS reagent, Eastman Kodak Co., Rochester, NY 14650.

[6] Additional evaporation causes I decomposition products to vaporize.

Table I—Chromatographic Behavior of Some Estrogens, Dansyl Estrogen Derivatives, Progestins, and Androgens [a]

Compound	Relative Retention Time [b]
Dansyl estradiol cypionate	—[c]
Dansyl estradiol valerate	—[c] (1.16)[d]
Dansyl estrone	1.60 (1.92)[d]
Testosterone enanthate	1.78
Dansyl ethinyl estradiol	2.26 (3.56)[d]
Norethindrone acetate	2.28
Estradiol cypionate	2.35
Estradiol valerate	2.70
Dansyl α-estradiol	3.00 (4.92)[d]
Estradiol benzoate	3.07
Dansyl estradiol	3.92 (6.72)[d]
Estrone	4.24
Norethindrone	6.08
Ethinyl estradiol	6.71
Methyltestosterone	7.12
α-Estradiol	8.71
Testosterone	9.48
Estradiol	11.25
Dansyl estriol	—[e]
Chloroform	1.00 (1.00)[d]

[a] Chromatography conditions were: column, LiChrosorb Si 60 (5 μm), 25 cm × 3.2 mm; mobile phase, 20% (v/v) *n*-heptane in chloroform; column temperature, ambient; inlet pressure, 1500 psig; flow rate, 0.75 ml/min; and detection, UV (254 nm) and fluorescence. [b] Retention times are expressed relative to chloroform (UV detection) with an absolute retention of 1.97 min in both mobile phases. [c] Less than 1.05. [d] Mobile phase of 30% (v/v) *n*-heptane in chloroform. [e] Requires a 6–10% absolute ethanol in chloroform mobile phase for elution.

the standard dansyl estrogen mixture solution onto a LiChrosorb Si 60 column, using a 20% (v/v) *n*-heptane in chloroform mobile phase (0.75-ml/min flow rate). Adjust the fluorescence settings to provide a 50–80% full-scale response.

Once reproducible behavior is observed, inject the sample dansyl estrogen solution. Calculate the quantity of estrogen present in the dosage

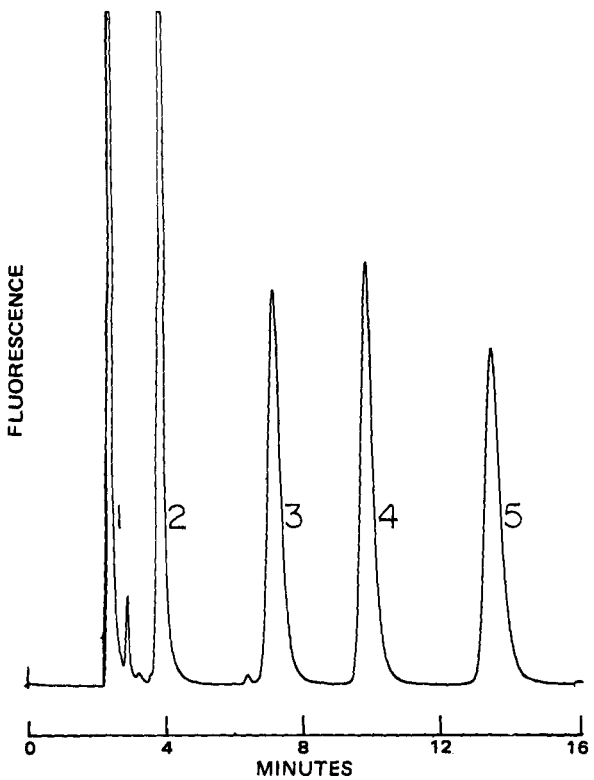

Figure 1—*Separation of five dansyl estrogen derivatives on a LiChrosorb Si 60 (5-μm microparticle silica) column with a 30% (v/v) n-heptane in chloroform mobile phase. Chromatography conditions were: temperature, ambient; pressure at inlet, 1500 psig; flow rate, 0.75 ml/min; and fluorescence sensitivity, 128 setting with a 7-54 excitation filter (broad band) and a 3-72 emission filter. Key (dansyl derivatives): 1, estradiol valerate; 2, estrone; 3, ethinyl estradiol; 4, α-estradiol; and 5, estradiol.*

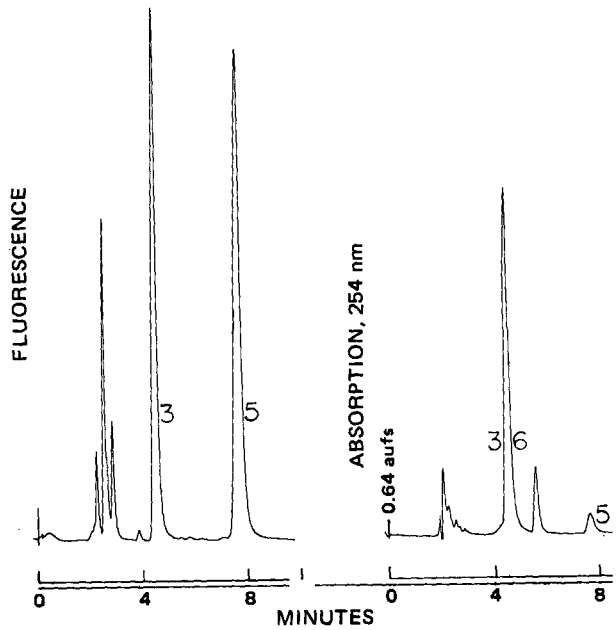

Figure 2—*Chromatograms recorded in the assay for ethinyl estradiol in norethindrone acetate and ethinyl estradiol tablets using fluorescence and UV (254 nm) detection. Chromatography conditions were: column, LiChrosorb Si 60 (5 μm); mobile phase, 20% (v/v) n-heptane in chloroform; temperature, ambient; pressure at inlet, 1500 psig; flow rate, 0.75 ml/min; and fluorescence sensitivity, 128 setting with a 7-54 excitation filter and a 3-72 emission filter. Key: same as Fig. 1; and 6, norethindrone acetate.*

form with standard peak height equations.

HPLC Measurement of Estrone—Proceed as directed for the measurement of estradiol and ethinyl estradiol; however, use a 30% (v/v) *n*-heptane in chloroform mobile phase.

RESULTS AND DISCUSSION

The conditions used for the formation of the dansyl estrogen derivatives are based on the work of Fishman (5). The solvent composition, as well as the base and its concentration, were described by Fishman; however, the I reagent concentration at the time of reaction was twice that used by Fishman, and the reaction time of 30 min was one-half that described (5). These conditions were satisfactory and ensured completed dansyl estrogen reaction as indicated by the following criteria.

1. Fishman found that 30 min would be adequate even with a smaller I reagent concentration than that used in this work.
2. The yellow color of I in the reaction medium was decreased to a constant intensity in about 15 min, indicating that the reaction was at a constant level.
3. Analysis of estrogens using the dansylation procedure to produce the dansyl estrogen and including the selected internal standard showed results that demonstrated a linear relationship (going through the origin) between the ratio of the estrogen analyte and the estrogen internal standard concentration.

The dansyl estrogen derivatives exhibited both strong UV absorption at 254 nm and useful fluorescent properties, as observed by Fishman. The fluorescence detector was sensitive to about 0.05 ng of estrogen (0.04 ng of ethinyl estradiol and 0.05 ng of estradiol), measured as the appropriate dansyl derivative; this sensitivity makes fluorescence detection the preferred method.

The elution behavior of seven estrogens, seven dansyl estrogens, two progestins, and three androgens on a microparticle silica adsorption column with 20% (v/v) *n*-heptane in chloroform as the mobile phase is summarized in Table I. The inclusion of progestins and androgens was based on their presence in pharmaceutical dosage forms in combination with ethinyl estradiol or as potential internal standards. The free estrogens, detectable owing to their UV absorption, were included to demonstrate their separability from their respective dansyl derivatives and to provide data useful for identifying the parent estrogen.

With dansyl estrone, the use of the 20% *n*-heptane in chloroform mobile phase did not yield satisfactory separation from the solvent; 30% (v/v)

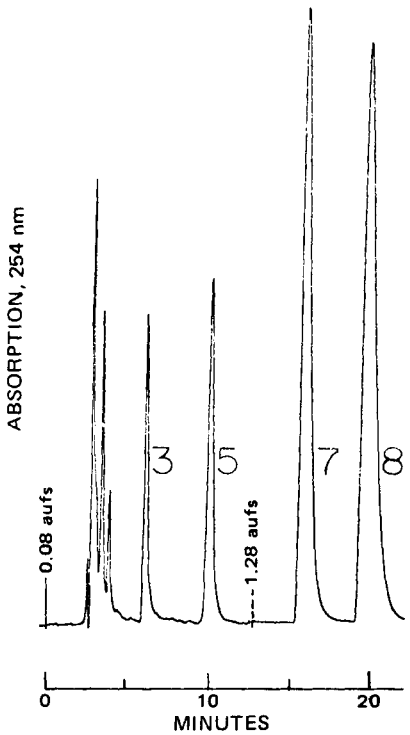

Figure 3—*Chromatogram recorded in the simultaneous assay for ethinyl estradiol and methyltestosterone in a commercial capsule dosage form. Chromatography conditions were: column, LiChrosorb Si 60 (5 μm); mobile phase, 20% (v/v) n-heptane in chloroform; temperature, ambient; pressure at inlet, 1500 psig; and flow rate, 0.75 ml/min. Key: same as Fig. 1; 7, norethindrone; and 8, methyltestosterone.*

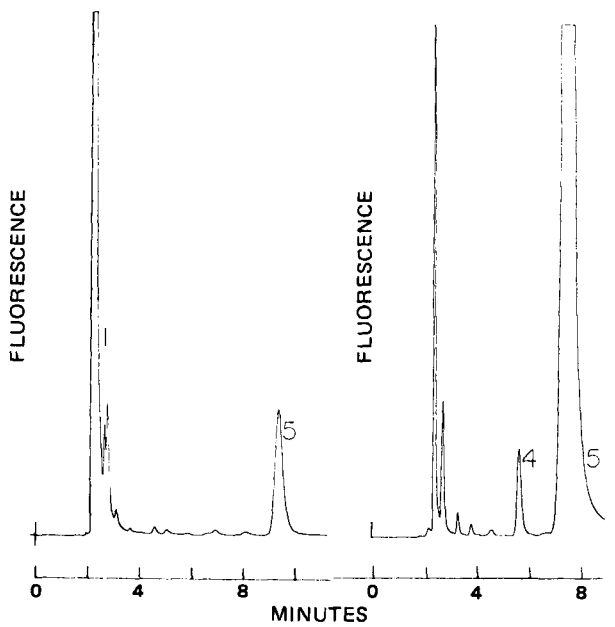

Figure 4—*Chromatograms recorded in the detection of estradiol and α-estradiol as impurities at the 1% level in estradiol valerate and estradiol, respectively. Chromatography conditions were: column, LiChrosorb Si 60 (5 μm); mobile phase, 20% (v/v) n-heptane in chloroform; temperature, ambient; inlet pressure, 1500 psig; flow rate, 0.75 ml/min (approximately ±20%); and fluorescence sensitivity, 128 setting with a 7-54 excitation filter and a 3-72 emission filter. Key: same as Fig. 1.*

n-heptane in chloroform as the mobile phase resulted in an improved mobility. The value of this mobile phase is demonstrated in Fig. 1. This chromatogram indicates that a useful separation of five different dansyl estrogens takes place with the 30% (v/v) *n*-heptane in chloroform mobile solvent.

The general approach to the analysis of estrogens in dosage forms is relatively simple. HPLC analysis of the estrogen is undertaken after the estrogen is separated from the dosage form matrix by dissolution in acetone and methanol followed by filtration or centrifugation. With the lower dosage ethinyl estradiol tablets (0.01 and 0.05 mg), the isolation step was a partition chromatographic procedure. This variation was useful since a large quantity of powdered tablet sample was necessary, making the simpler "dissolve and filter" approach unreliable. In each case, a steroid was chosen as the internal standard; not only did such a choice respect the usual guideline for structural similarity between analyte and internal standard, but it also made the dansyl estrogen formation possible for the internal standard, allowing for a straightforward quantitative measurement.

The chromatograms demonstrate the reasons for some of the experimental choices made in planning the procedures. Figure 2 is a pair of chromatograms recorded during the assay of ethinyl estradiol in norethindrone acetate and ethinyl estradiol tablets. Fluorescence is the detection method of choice owing to its greater sensitivity. The ability of fluorescence to detect selectively and to measure fluorescent compounds is important because, although dansyl ethinyl estradiol and norethindrone acetate have the same chromatographic mobility, only the dansyl estrogen is measured using the fluorescence detector; the UV detector would measure both steroids. Furthermore, if fluorescence detection is to be used, then the chosen internal standards must be estrogens to ensure formation of the dansyl estrogen for analytical comparison.

The UV absorption properties do have value when no fluorescence is present. Figure 3 is a chromatogram observed during the simultaneous analysis of ethinyl estradiol and methyltestosterone in a capsule. The analysis of the ethinyl estradiol would be carried out first with the fluo-

Table II—Analysis of Estrogens in Commercial Dosage Forms

Sample	Estrogen Declared	Percent Found: Dansyl Estrogen HPLC[a,b]	Percent Found: Alternative Method
Estradiol in sesame oil	1.0 mg/ml	95.8, 96.1	—[c]
Estradiol in aqueous suspension[d]	1.0 mg/ml	92.3, 90.6	—[e]
Estradiol tablets	0.5 mg/tablet	103.6, 103.3	102.4, 103.2[f]
Ethinyl estradiol tablets	0.5 mg/tablet	102.6, 101.2	100.2, 100.3[g]
Ethinyl estradiol tablets (coated) (Manufacturer 1)	0.05 mg/tablet	108.0, 110.7	104.1, 105.5[h] 107.9, 107.2[i]
Ethinyl estradiol tablets (coated) (Manufacturer 2)	0.05 mg/tablet	97.8, 97.5	91.5, 88.2[h] 90.9, 92.5[i]
Norethindrone acetate and ethinyl estradiol tablets NF[j]	0.05 mg/tablet	96.6, 95.8	94.5, 96.7[h]
Ethinyl estradiol and methyltestosterone slow-release capsules[k]	0.03 mg/capsule	103.9, 102.6	98.1, 96.0[h]
Ethinyl estradiol tablets	0.02 mg/tablet	101.5, 102.4	97.3, 95.8[h]
Ethinyl estradiol tablets	0.01 mg/tablet	94.3, 95.9	90.7, 87.5[h]
Estrone in aqueous suspension	5.0 mg/ml	97.9, 96.0	97.3, 96.7[l]

[a] Chromatography conditions were: column, LiChrosorb Si 60 (5 μm), 25 cm × 3.2 mm; mobile phase for estradiol and ethinyl estradiol, 20% (v/v) *n*-heptane in chloroform; mobile phase for estrone, 30% (v/v) *n*-heptane in chloroform; column temperature, ambient; pressure at inlet, 1500 psig; flow rate, 0.75 ml/min; and detection, fluoresence. [b] Internal standards were estradiol for ethinyl estradiol and ethinyl estradiol for estradiol and estrone. [c] Study showed 99.3% recovery of a synthetic preparation. [d] Contained declared 5.0 mg/ml of phenol. [e] No alternative assay performed. [f] Extraction and UV. [g] USP XIX, pp. 186, 187. [h] AOAC 12th ed., 39.031–39.034. [i] Modified AOAC 12th ed., 39.031–39.034. [j] Contained 1.0 mg of norethindrone acetate/tablet. [k] Contained 3.5 mg of methyltestosterone/capsule. [l] HPLC analysis without derivatization.

rescence detector. Then norethindrone would be added as an internal standard for methyltestosterone, followed by HPLC, which permits detection of all components and quantitative measurement of methyltestosterone. This procedure was carried out using a commercial capsule dosage form and yielded results of 100.4 and 100.1% of methyltestosterone.

The two chromatograms in Fig. 4 demonstrate another use of the analytical system described in this work. The high sensitivity of the fluorescence detector in measuring dansyl estrogens allows the monitoring of impurities of one estrogen in another. The chromatograms in Fig. 4 are measurements of estradiol (at the 1% level) in estradiol valerate and α-estradiol (1% added as an impurity) in estradiol. The USP (9) has a limit of 1% estradiol in estradiol valerate, whereas the NF (10) has a specification of 3% foreign steroids and other impurities in estradiol.

The results of the analysis of 11 different pharmaceutical dosage forms by the proposed procedure are presented in Table II. The values obtained are within 2% of each other, except for the ethinyl estradiol coated tablets. As a test of the accuracy of the method, a sesame oil solution of estradiol was prepared and assayed. Results of 99.0 and 99.6% estradiol indicated that the procedure was accurate to within 1% for this dosage form.

For comparison, analyses of all but two of the dosage forms were carried out by alternative procedures (given in the footnotes to Table II). The agreement between members in pairs of results was within an average of less than 2%, but three pairs of values differed by more than 2%. In each case, the alternative method average values were lower than the HPLC method. The greatest difference was observed for the lower dosage ethinyl estradiol (0.01 and 0.05 mg) dosage forms.

The AOAC method (footnote *h* of Table II) was collaboratively studied for antifertility tablets and gave results comparable to the HPLC method with norethindrone acetate and ethinyl estradiol tablets. With the lower dosage ethinyl estradiol dosage forms, the variation was wider. In two instances (footnote *i* in Table II), the AOAC method was modified in that chloroform was replaced by benzene and the chromogenic reaction was carried out in benzene. In both cases, somewhat higher results, closer to the HPLC results, were achieved. The alternative method (footnote *l* in Table II) consisted of extracting the estrone aqueous suspension with a chloroform solution of estradiol valerate (internal standard), followed by filtration and direct injection for HPLC (UV detection) analysis. Good agreement was observed with the HPLC procedure.

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Synthesis and Evaluation of Sulfur-Containing Steroids against Methylmercuric Chloride Toxicity

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Abstract □ Sulfur-containing steroids, analogs, and derivatives were synthesized for evaluation in mice suffering acute toxicity from methylmercuric chloride. Steroids were administered by intraperitoneal injection, by stomach tube feeding, or by absorption through the tail skin. Thiocholesterol and the thiocholanoic acids were effective if given prior to poisoning. The thiosteroids were significantly more effective than penicillamine or dimercaprol under these conditions.

Keyphrases □ Sulfur-containing steroids—synthesized, evaluated in treatment of methylmercuric chloride toxicity in mice □ Steroids, sulfur containing—synthesized, evaluated in treatment of methylmercuric chloride toxicity in mice □ Methylmercuric chloride toxicity—treatment by various sulfur-containing steroids evaluated in mice □ Chelating agents—various sulfur-containing steroids synthesized, evaluated in treatment of methylmercuric chloride toxicity in mice □ Structure–activity relationships—various sulfur-containing steroids evaluated in treatment of methylmercuric chloride toxicity in mice

Heavy metal poisoning traditionally is treated with chelating agents such as penicillamine, dimercaprol, and ethylenediamine tetraacetic acid, which are thought to combine with the metal and facilitate rapid elimination through the kidneys. Because the effectiveness of these agents frequently is limited by toxicity, novel chemotherapeutic agents of low toxicity are needed.

To minimize the burden placed on the kidney by heavy metal poisoning, such agents could include sulfhydryl-type chelators with lipophilic character that might form more nearly irreversible complexes with the heavy metal and be eliminated by other than the urinary route. Sulfhydryl analogs of bile components, specifically mercapto analogs of cholesterol and the bile acids, are such candidates.

The protective action of thiocholesterol (I*a*) and 3β-mercapto-5β-cholanic acid (VII*a*) against lethal doses of methylmercuric chloride was reported recently (1). Spironolactone (III) protects against mercuric chloride toxicity in rats (2) and mice (3). The present report describes continuing efforts to test other sulfur-containing steroids and their esters and analogs as therapy for methylmercuric chloride intoxication.

EXPERIMENTAL

Steroids—Thiocholesterol was converted to its acetate (I*b*) (4) and

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