Determination of Estrogens in Dosage Forms by Fluorescence Using Dansyl Chloride

SEYMOUR FISHMAN

Abstract □ A sensitive, reproducible, fluorometric procedure for the determination of estrogens in pharmaceutical preparations is presented. The estrogens are determined fluorometrically following their reaction with dansyl chloride. The optimum conditions for the reaction such as pH, reaction solvent composition variations, and speed of reaction are discussed. In addition, a linearity study of the relationship between concentration and fluorescence intensity for estrone, estradiol, and ethinyl estradiol is reported. Solvent extraction procedures based on acid–base behavior or column chromatography are used when necessary to isolate the estrogen prior to reaction with dansyl chloride and fluorometric measurement. The recovery of estrogens from spiked samples indicated that the proposed method is efficient and reproducible. Comparison of the dansyl procedure with the official NF method in the analysis of estrone aqueous suspension showed the proposed method to be accurate and more precise than the NF assay. Estrone aqueous suspension, estradiol in sesame oil, estradiol valerate in castor oil and in sesame oil (in the latter case, in the presence of testosterone), estradiol benzoate in sesame oil, and ethinyl estradiol tablets (0.5 mg/tablet) from commercial sources were satisfactorily analyzed, with average results within compendial limits and no coefficient of variation greater than 2%.

Keyphrases □ Estrogens—determination in dosage forms, fluorescence using dansyl chloride, pH, reaction solvent composition, and speed of reaction, compared to compendial method □ Dansyl chloride—preparation of estrogen-dansyl derivatives, fluorometric determination of estrogens in pharmaceutical preparations, compared to compendial method □ Fluorometry—analysis, estrogens in dosage forms

Estrogens are important therapeutic agents that have been used as replacements or supplements for inadequate supplies of the natural hormone or in suppressive therapy to counter the effects of other hormones. Many analytical procedures have been described for the C_{18} estrogens, all of which are characterized by a phenolic substituent in the 3-position. Some methods are based on the modified iron–Kober colorimetric procedure (1–7), analyses that are notoriously laborious and time consuming, with the production of interfering background color as a result of the charring effect of the sulfuric acid on nonestrogenic impurities (7).

Analyses of estrogens have been developed that depend on the formation of derivatives. IR quantitative analysis has been used to measure estrone, equilin, and equilenin after formation of the benzenesulfonate phenolic esters (8). The reaction with Girard's reagent T is the basis of another IR spectrophotometric procedure that is somewhat nonspecific (9). Polarographic analysis of the Girard T (10) and nitrosophenol (11) derivatives of estrogens has been reported. Unfortunately, since the half-wave potentials for all of the different estrogens are the same, the polarographic analyses are also nonspecific.

The application of chromatographic methods to the separation and measurement of estrogens has been reported. Separations by GC have involved pure standards, pharmaceuticals, and steroids in biological media. For example, equine estrogens have been separated after silylation (12), conjugated estrogens have been analyzed after preliminary hydrolysis (13), estrone has been measured in dermatological dosage forms (14), estrogens have been monitored in human plasma (15), urinary estrogens have been determined after a preliminary TLC separation (16), and halogenated estrogens have been estimated by electron-capture behavior (17). A high-speed liquid chromatographic procedure has been described for the separation of equine estrogens (18). Conventional partition chromatography has been successful in the separation of most constituents of conjugated estrogens (19). An interesting application of the adsorptive properties of a cross-linked dextran gel[1] has been made to the separation of three common estrogenic steroids (20).

The phenomenon of fluorescence offers the clear advantage of sensitivity in estrogen analysis. Although estrogens do exhibit native fluorescence, the observed intensity is not great enough to be useful. Until this point, fluorescence procedures for estrogens were based on the Kober reaction that employs sulfuric acid to produce an analytically useful fluorophore (21–24). Simple solvent extraction could separate the estrogen from any interferences prior to the fluorogenic step (25, 26). Reaction of dansyl chloride, a substituted naphthalene sulfonyl chloride, with the phenol at the 3-position of all estrogens, is an excellent procedure that results in a fluorescent derivative. This reagent has been used in the TLC separation of estriol in pregnancy urine (27), the TLC separation of dansyl derivatives of estrone, estradiol, and estriol, and the quantitative measurement of these steroids by fluorodensitometry (28, 29). Other applications of dansyl derivative fluorescence have been made to primary and secondary amines (30–34), amino acids (30–34), and terminal amino group analysis in proteins and peptides (32–34) as well as to phenols (32–35).

This paper reports a study using the formation of the dansyl-estrogen followed by fluorescence mea-

[1] Sephadex.

surement for estrogen analysis in pharmaceutical dosage forms. Reaction conditions were investigated as to solvent composition, reagent concentration, and rate factors. The dosage form analyses were carried out under the determined conditions and yield results that were quite satisfactory.

EXPERIMENTAL[2]

Chemicals and Reagents—All reagents were of analytical reagent grade (ACS) purity and were used without further purification. Petroleum ether was ACS certified. Dansyl chloride[3] (1-dimethylaminonaphthalene-5-sulfonyl chloride) was stored in a desiccator over silica gel. Acid-washed siliceous earth[4] was used.

The following estrogen standards were used: estrone[5], 3-hydroxyestra-1,3,5(10)-trien-17-one, $C_{18}H_{22}O_2$, mp 254–256°, mol. wt. 270.4; estradiol[6] (β-estradiol), estra-1,3,5(10)-triene-3,17β-diol, $C_{18}H_{24}O_2$, mp 177–179°, mol. wt. 272.4; estradiol benzoate[6], estra-1,3,5(10)-triene-3,17β-diol 3-benzoate, $C_{25}H_{28}O_3$, mp 191–196°, mol. wt. 376.5; ethinyl estradiol[5], 17α-ethinyl-1,3,5(10)-estratriene-3,17α-diol, $C_{20}H_{24}O_2$, mp 141–143°, mol. wt. 296.4; and estradiol valerate[5], estra-1,3,5(10)-triene-3,17β-diol 17-valerate, $C_{23}H_{32}O_3$, mp 144–145°, mol. wt. 356.5. Commercial estrogen dosage forms were selected for analysis.

Preparation of Reagents—*0.01% (0.000371 M) or 0.02% (0.000742 M) Dansyl Chloride Reagents*—Dissolve 50 or 100 mg, respectively, of dansyl chloride in 500 ml of acetone. If necessary, filter the solution through fluted filter paper[7], 18.5 cm. Dansyl chloride is stable in acetone. However, the solution was prepared freshly approximately every 3 weeks.

Sodium Carbonate Aqueous Acetone Solution—Dissolve 1.00 g of sodium carbonate decahydrate in a solution of 300 ml of water and 150 ml of acetone.

Water-Washed Ether—Shake equal volumes of ether and water for 2 min in a separator. Discard the aqueous phase.

The following were all aqueous solutions: 2.5 *N* sodium hydroxide solution, 50% sulfuric acid solution (18 *N*), 1 *M* sodium bicarbonate solution, and dilute sulfuric acid (1 + 2).

Analytical Applications—Procedures are provided for the preparation of estrogen standard solutions and the treatment of the various dosage forms for the isolation of the estrogens prior to the application of the dansyl chloride reaction and fluorometric measurement.

Estrogen Standard Solutions—These solutions were prepared according to the following directions. Dissolve the appropriate quantity of estrogen, accurately weighed and dried as indicated, in about 25 ml of acetone and dilute to exactly 100 ml with acetone:

estrogen	drying conditions	quantity to be weighed, mg
estradiol	105°, 4 hr	2
estrone	105°, 4 hr	2
ethinyl estradiol	silica gel, room temperature, 4 hr	2
estradiol benzoate	105°, 3 hr	3
estradiol valerate	105°, 3 hr	2

Aqueous Suspensions for Injection—Vigorously shake the suspension to be sampled to obtain a homogeneous dispersion. Using a "to contain" pipet, transfer accurately a volume of the estradiol, estrone, or other estrogen suspension equivalent to about 4 mg of the estrogen into a 200-ml volumetric flask, wash the pipet inner bore with several increments of acetone and retain the washings in the flask, and add additional acetone in increments to the flask to a volume of about 125 ml, swirling after each addition and heating, if necessary, to aid in dissolving the estrogen. Dilute to volume, mix well, and filter the solution through filter paper[8] into a glass-stoppered conical flask, discarding the first 25 ml of filtrate. Using this sample solution, continue with the procedure *Fluorometric Determination Using Dansyl Chloride.*

Oleaginous Solutions for Injection—Using a "to contain" pipet, transfer a volume of estradiol, estrone, or other estrogen solution equivalent to about 5 mg of the estrogen into a 125-ml separator. To ensure complete transfer, wash the pipet inner bore with two 10-ml and a third 5-ml portion of petroleum ether[9], retaining the washings in the funnel. Add 15 ml of 2.5 *N* sodium hydroxide solution, shake vigorously for 2 min, and allow the phases to separate completely. Transfer the aqueous phase to a second 125-ml separator and repeat the extraction of the petroleum ether[9] with two additional 10-ml portions of 2.5 *N* sodium hydroxide solution, adding each extract to the second separator.

Wash the combined alkaline extracts with 25 ml of petroleum ether, and discard the petroleum ether phase. Add 50% sulfuric acid dropwise to acidify the combined alkaline portion to pH 1, using pH indicator paper, and cool the solution. Then add 25 ml of benzene and shake for 2 min. Transfer the acid layer to another 125-ml separator and extract with another 25-ml portion of benzene. Discard the aqueous layer. Wash the combined benzene solutions with two 5-ml portions of 1 *M* sodium bicarbonate solution and two 5-ml portions of water; discard the aqueous phases.

Pass the benzene solution through benzene-washed cotton into a beaker, and evaporate to dryness on a steam bath with the aid of a current of air. Dissolve the residue in acetone, warming if necessary to aid dissolution. Transfer the solution quantitatively to a volumetric flask of a size such that a final concentration of 0.020 mg/ml will result. Dilute to volume with acetone, and continue with the procedure *Fluorometric Determination Using Dansyl Chloride.*

Estradiol Benzoate Injectable Solution in Sesame Oil—Prepare a chromatographic column using acid-washed diatomaceous earth[4] as described by James (37).

Using a "to contain" pipet, transfer a volume of sample equivalent to 15.0 mg of estradiol benzoate in oil to a 100-ml volumetric flask. Wash the pipet inner bore with several portions of *n*-heptane, retaining the washings in the flask, and dilute to volume with *n*-heptane.

Transfer 2.0 ml of sample solution to the column, and wash the column with 75 ml of *n*-heptane, discarding the washings. Change receivers to a 250-ml glass-stoppered (TS 24/40) erlenmeyer flask, and elute the estrogen with approximately 200 ml of *n*-heptane. Evaporate the eluate to dryness, dissolve the residue in 10 ml of acetone, and add 20 ml of sodium carbonate aqueous acetone solution. Similarly, transfer 10.00 ml of the estradiol benzoate standard solution to a glass-stoppered (TS 24/40) erlenmeyer flask, and add 20 ml of sodium carbonate aqueous acetone solution. Equip both the sample and standard flask with water condensers (TS 24/40 joint), and heat at reflux for 45 min. Then quantitatively transfer the contents of the conical flasks to separate 50-ml volumetric flasks, wash each flask with two successive 3-ml portions of the sodium carbonate aqueous acetone solution, and transfer each portion quantitatively to the appropriate 50-ml volumetric flask. Add 10.00 ml of 0.02% dansyl chloride reagent, and dilute to volume with the sodium carbonate aqueous acetone solution. For a blank, add 10 ml of acetone and 10.00 ml of 0.02% dansyl chloride reagent to a 50-ml volumetric flask and dilute to volume with the sodium carbonate aqueous acetone solution. Continue with the procedure *Fluorometric Determination Using Dansyl Chloride,* beginning with "Then let the solutions stand . . . ."

Tablets—Weigh accurately 20 tablets, reduce them to a fine powder by trituration, and pass the powder through a 60-mesh sieve. Accurately weigh a portion of the sample equivalent to about 1.0 mg of ethinyl estradiol or other estrogen into a 125-ml separator. Add 10 ml of water and 0.5 ml of dilute sulfuric acid (1 + 2), extract with 25 ml of chloroform, separate the phases, and then extract with two additional 25-ml portions of chloroform.

Wash the combined chloroform solutions with two 5-ml portions of 1 *M* sodium bicarbonate solution and two 5-ml portions of water, and discard the aqueous phases. Pass the total chloroform

[2] A Perkin-Elmer model MPF-3 spectrofluorometer equipped with a xenon lamp and power source (model 150) and a Hitachi recorder (model QPD-33) were used.
[3] Aldrich Chemical Co., Cedar Knolls, NJ 07927
[4] Celite 545, Johns-Manville, Denver, CO 80217
[5] USP Reference Standard, United States Pharmacopeial Convention, Inc., Rockville, Md.
[6] NF Reference Standard, American Pharmaceutical Association, Washington, DC 20037
[7] Whatman, 2V.
[8] Whatman No. 42.
[9] For the castor oil-containing estradiol valerate dosage form, the solvent used was *n*-heptane.

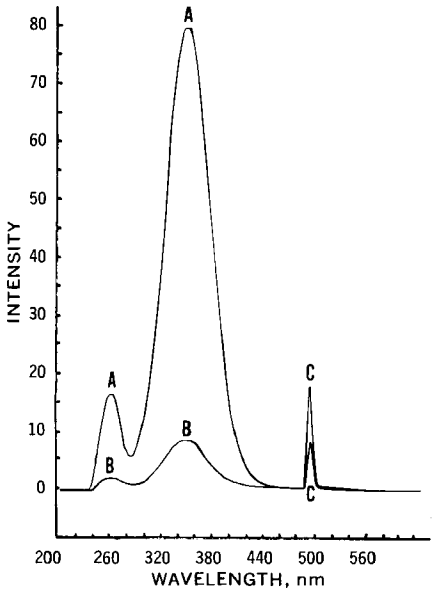


Figure 1—*Excitation spectra of dansyl-estrone* versus *the blank in chloroform; wavelengths of excitation are 264 and 355 nm (with emission at 502 nm). Key: A, dansyl-estrone; B, blank; and C, scattered radiation.*

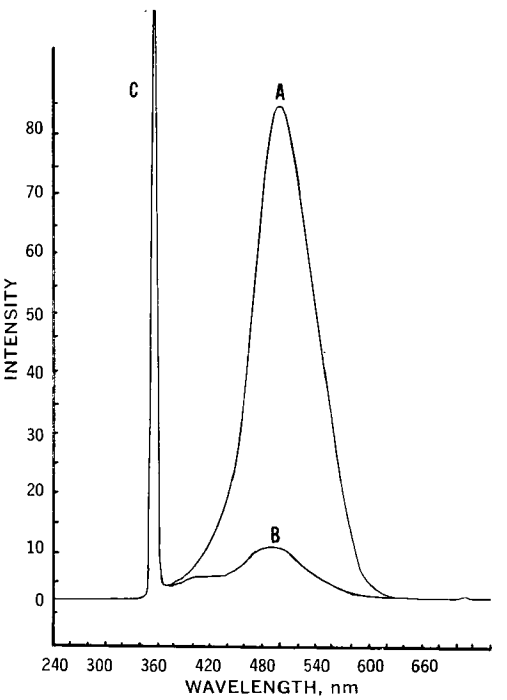


Figure 2—*Emission spectra of dansyl-estrone* versus *the blank in chloroform; wavelength of emission is 502 nm (with excitation at 355 nm). Key: A, dansyl-estrone; B, blank; and C, scattered radiation.*

extract through the chloroform-washed cotton. Evaporate the chloroform to dryness on a steam bath with the aid of a current of air. Dissolve the residue in acetone, warming if necessary to aid in dissolution, and dilute to a final concentration of 0.020 mg/ml with acetone. Continue with the procedure *Fluorometric Determination Using Dansyl Chloride.*

Fluorometric Determination Using Dansyl Chloride—Take exactly 10 ml of the sample solution, and transfer to a 50-ml volumetric flask. Add exactly 10 ml of 0.01% dansyl chloride reagent, and dilute to volume with sodium carbonate aqueous acetone solution (Solution A).

Similarly, transfer exactly 10 ml of the appropriate estrogen standard solution and exactly 10 ml of acetone to serve as a blank into separate 50-ml volumetric flasks. Add exactly 10.00 ml of 0.01% dansyl chloride reagent, and dilute to volume with the sodium carbonate aqueous acetone solution (Solution A). Then let the solutions stand in the dark for 60 min. When the time has elapsed, transfer exactly 10.00 ml from Solution A into a 125-ml separator. Extract with three 10-ml portions of water-washed ether, filter each fraction through ether-washed cotton into a 50-ml glass-stoppered conical flask, and evaporate the combined ether fractions to dryness on a steam bath under a stream of air. Dry the residue for 15 min in a vacuum desiccator containing silica gel. Transfer exactly 10.0 ml of chloroform to each flask (Solution B). Read the solutions in 1-cm quartz cells in a spectrofluorometer, setting the standard solution fluorescence to read 70% full-scale deflection; the excitation and emission wavelengths are 355 and 502 nm, respectively (Figs. 1 and 2).

Calculations—The fluorescence intensity *(F)* of the standard and sample is measured at their maximum at 502 nm. The fluorescence intensity of the blank, measured at the same wavelength, is subtracted from the readings of both the sample and standard. The concentration of estrogens *in injectables* is calculated as:

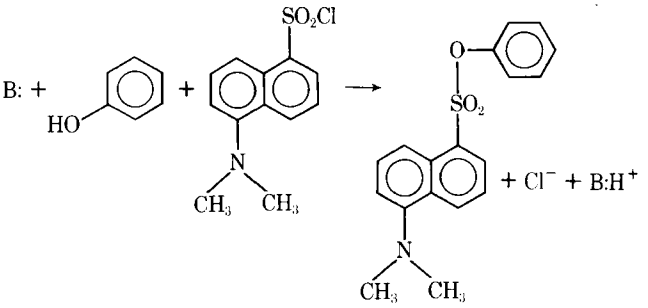
$$\frac{F_{spl} - F_{blank}}{F_{std} - F_{blank}} \times \frac{\text{weight of standard, mg}}{\text{volume of sample taken, ml}} \times \frac{\text{dilutions of standard}}{\text{dilutions of sample}} \times \frac{\text{volume of initial sample solution, ml}}{\text{volume of initial standard solution, ml}} = \frac{\text{mg of estrogen}}{\text{ml of injection taken}} \quad \text{(Eq. 1)}$$

Alternatively, the concentrations can be determined from standard curves. The concentration of estrogens *in tablets* is calculated as:

$$\frac{F_{spl} - F_{blank}}{F_{std} - F_{blank}} \times \frac{\text{weight of standard, mg}}{\text{dilutions of sample}} \times \frac{\text{dilutions of standard}}{\text{weight of sample}} \times \text{average tablet weight} = \frac{\text{mg of ethinyl estradiol}}{\text{tablet}} \quad \text{(Eq. 2)}$$

RESULTS AND DISCUSSION

Formation of Dansyl-Estrogen Derivatives—*Dansyl Chloride Reactions*—The chemical reaction of interest may be conveniently described by Scheme I.



Scheme I

The scheme is presented in a generic manner in that the base B: may be any base such as OH^- or CO_3^{-2}. It seems clear that the reaction should proceed well in an alkaline solution or in the presence of a base since the chemical change involves the displacement of the chloride ion by the phenolate species.

Solution conditions not only have an effect on the desired reaction (Scheme I) but promote a concomitant change since the base can also cause reaction of the dansyl chloride to yield an undesired product. Thus, if hydroxyl ion is the base, the reaction produces 1-dimethylaminonaphthalene-5-sulfonate. In addition, an unfortunate solvent choice could cause hydrolysis of the dansyl-estrogen derivative. The reaction conditions must permit complete formation of the dansyl-estrogen derivative and yet minimize the unde-

Table I—Recovery Study of Estrone, Estradiol, and Estradiol Benzoate in Dosage Forms

Sample	Replicates	Prepared Concentration, mg/ml	Percent Recovered (Average)	Range of Percent Recovered	*SD*	Coefficient of Variation, %
Estrone aqueous suspension	4	2[a]	100.1	99.8–100.3	±0.3	0.264
Estrone aqueous suspension	4	5[a]	102.6	101.9–103.8	±0.8	0.813
Estradiol in sesame oil solution (synthetic preparation)	7	1.018[b]	99.3	95.8–101.1	±1.9	1.92
Estradiol in sesame oil solution (synthetic preparation)	6	0.994[b]	100.6	98.6–102.9	±1.5	1.51
Estradiol benzoate in sesame oil (synthetic preparation)	4	2.99[b]	99.9	98.1–101.8	±2.0	2.03
Estradiol benzoate in sesame oil (synthetic preparation)	4	4.94[b]	99.2	97.1–101.5	±1.8	1.82

[a] Spiked with 2.892 μg of estrone. [b] 3% (v/v) benzyl alcohol.

sired reactions of the dansyl chloride and the dansyl-estrogen hydrolysis. The various reaction conditions studied are treated in the following sections.

Reaction Solvent Composition—Two factors must be considered when selecting a solvent system—*viz.*, the maintenance of a homogeneous system and the preservation at a reasonable rate of the reaction described by Scheme I. The dansyl chloride reagent is soluble in acetone, but a high proportion of acetone in the solvent reduces the velocity of the reaction (34). On the other hand, water increases the speed of the dansyl derivatization reaction but it can likewise accelerate the rate of the undesired hydrolytic conversion of the dansyl chloride to the corresponding sulfonic acid and of the desired dansyl-estrogen to the parent estrogen.

Experiments have indicated that acetone–water mixtures offer desirable advantages (27, 34). As the ratio of acetone to water is decreased from a 1:1 ratio, the solvent polarity increases and the effect is that there is a net loss of dansyl-estrogen by hydrolysis (with a resultantly diminished fluorescence) as well as an increase in the hydrolysis of dansyl chloride. The optimum solvent composition was an acetone–water system in a 2:3 volume ratio; this medium was used in the various estrogen analyses to be described.

Reagent and Base Concentrations and Reaction Rate—Study of Scheme I indicates that a 1:1 stoichiometric relationship exists between estrogen and dansyl chloride. However, to ensure the completeness of the reaction, a five- to 10-fold excess of dansyl chloride reagent was chosen and used satisfactorily.

Since the dansyl-estrogen reaction (Scheme I) proceeds by means of the estrogenic phenolate ion, the need for a base must be incorporated into the analytical solvent system. When using aqueous properties as a guide only, calculations show that there is very little phenolate species in solution at pH 8, the predominant form being the conjugate acid, phenol. At pH 9, 25% of the total phenol is present as the phenolate ion; at pH 12 the percentage rises to more than 99% phenolate ion. However, at very alkaline conditions the hydrolysis of dansyl chloride and the dansyl-estrogen derivative becomes significant, depleting the reagent and resultantly affecting the desired derivatization reaction (Scheme I).

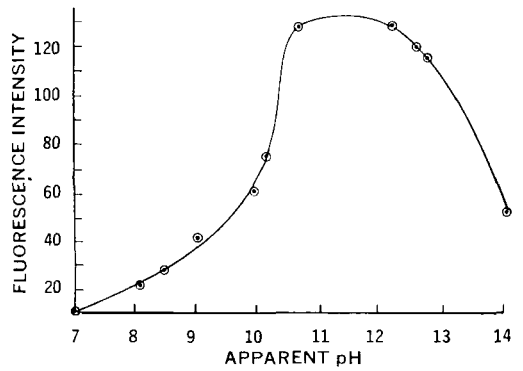


Figure 3—*Effect of apparent pH on the fluorescence intensity of dansyl-estrogen derivatives.*

The means to establish optimum alkaline conditions was taken as the production of maximum fluorescence when the dansyl-estrogen reaction was conducted under particular conditions. The results of such a study are presented in Fig. 3, and the plot indicates that an apparent pH range of 11–12 is optimum. Sodium carbonate was chosen as the base since it is convenient to use and is strong enough to achieve, but not exceed, the desired alkalinity.

To determine an effective sodium carbonate concentration, a series of studies (Fig. 4) was carried out. The concentration of sodium carbonate was varied, and the fluorescence intensity achieved by the dansyl chloride-estrogen reaction was measured as a function of time. The results indicate that in each case the maximum fluorescence is attained in about 30 min. The intensity of fluorescence is seen to be smallest when the largest quantity of 40 mg of sodium carbonate decahydrate is in solution. This phenomenon is probably ascribable to an increase in the reverse reaction (Scheme I) causing hydrolysis of the fluorescent dansyl-estrogen derivative and the dansyl chloride reagent. The intermediate concentration of sodium carbonate decahydrate results in a fluorescence that reaches a maximum within 30 min and is relatively stable for long periods. These results suggest that the best concentration of base is about 20 mg of sodium carbonate decahydrate in 30 ml of solution.

The described study that established the concentration of sodium carbonate to be used also permitted the selection of tempera-

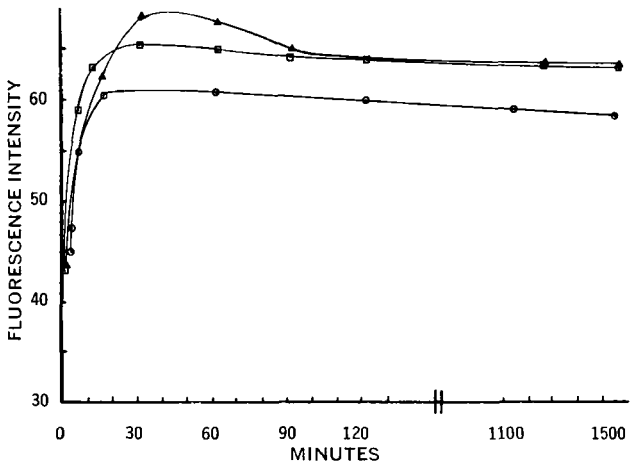


Figure 4—*Speed of reaction at various concentration levels of sodium carbonate decahydrate at 22 ± 2°. The symbols used indicate the weight of sodium carbonate decahydrate in milligrams dissolved in 30 ml of 40% H_2O in acetone for each study. Key: △, 10; □, 20; and ○, 40.*

Table II—Determination of Estrogens in Various Dosage Forms

Sample	Replicates	Declared, mg/ml or mg/Tablet	Average, %	Range	*SD*	Coefficient of Variation, %
Estrone aqueous suspension—1	8	1	1.06 (106)	1.04–1.07	±0.0119	1.12
Estrone aqueous suspension—2	8	2	1.98 (99)	1.91–2.02	±0.0389	1.97
Estrone aqueous suspension—3	5	2	2.00 (100)	1.98–2.05	±0.0306	1.53
Estrone aqueous suspension—4	6	5	4.82 (96.4)	4.80–4.85	±0.0177	0.367
Estradiol in sesame oil injection	10	1	0.955 (95.5)	0.940–0.970	±0.010	1.05
Estradiol valerate in castor oil—1	4	20	20.2 (101)	19.8–20.6	±0.370	1.83
Estradiol valerate in sesame oil—2	4	4[a]	3.91 (97.8)	3.87–3.98	±0.0497	1.27
Ethinyl estradiol tablets	8	0.5	0.487 (97.4)	0.475–0.499	±0.00998	2.04

[a] With testosterone enanthate, 90 mg/ml.

ture and time variables. From Fig. 4 it is seen that a constant fluorescence intensity is reached after 30 min at 22°. To ensure complete reaction, 60 min at room temperature was chosen as the reaction conditions for each estrogen analysis.

In this work, the manner of preparation of the reaction solution system is an important consideration, since the solvent is an acetone–water mixture with dissolved sodium carbonate. Since sodium carbonate is insoluble in acetone, aqueous solutions seem like a good way to introduce sodium carbonate. However, if an aqueous solution of sodium carbonate is added to acetone, an undesired two-phase system results. To have a homogeneous solvent, the solute sodium carbonate must be dissolved in the mixed acetone–water system.

Isolation of Dansyl-Estrogen Derivatives—Although the desirable analytical reaction may be expected to proceed to completion, the reaction solution may contain dansyl-estrogen derivative, excess dansyl chloride reagent, and dansyl hydroxide, formed by hydrolysis of the reagent. TLC monitoring of reaction solutions showed that any excess dansyl chloride is hydrolyzed to the corresponding sulfonic acid after 30 min, the analytical reaction time. Thus, to avoid interference, it becomes necessary to separate the dansyl hydroxide (a fluorescent sulfonic acid) from the dansyl-estrogen derivative.

Since the dansyl hydroxy is acidic, it forms a sodium salt and the dansyl-estrogen can be conveniently extracted from the alkaline reaction solution with a suitable organic solvent. Two of the organic solvents tried, isooctane and chloroform, were judged unsuitable because of turbidity. Ether did not give rise to turbidity and, in addition, the desired solute possessed a high partition coefficient in the ether–alkaline reaction solution. Calculations show that almost 99% of the dansyl-estrogen is extracted with the first 10-ml portion of ether. Thus, three 10-ml portions will extract virtually 100% of the dansyl-estrogen from the alkaline reaction solution.

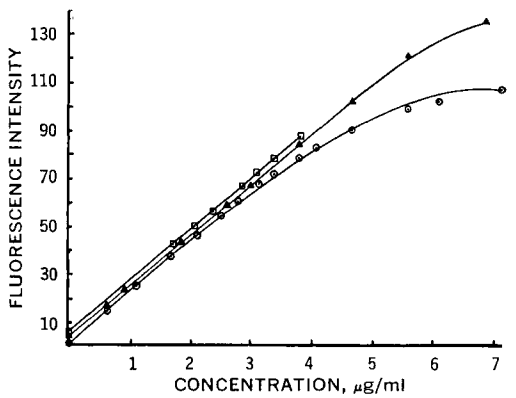


Figure 5—*Relationship between fluorescence intensity and concentration for three estrogens including a least-squares plot. Key:* □, *ethinyl estradiol;* △, *estradiol; and* ○, *estrone.*

Emission and Excitation Spectra of Dansyl-Estrogen Derivatives—The emission and excitation spectra (uncorrected) of dansyl-estrone and the blank in chloroform, the solvent to be used analytically, are shown in Figs. 1 and 2. For dansyl-estrone, two excitation maxima at 264 and 355 nm are observed. The intensity of the peak at 355 nm is approximately five times greater than the peak at 264 nm. Both excitation peaks produce an emission peak at 502 nm. Since all of the derivatives have the same fluorophore, all dansyl-estrogen derivatives have similar emission and excitation spectra.

The blank is run under identical reaction conditions utilizing the same reagents and solvents as the estrogen, except that the estrogen solution is replaced by an identical solvent volume (acetone). The blank has a different emission spectrum than dansyl-estrone, indicating that it is composed entirely of trace amounts of unextracted, intact and hydrolyzed dansyl chloride. The emission peak is at 490 nm, and the excitation peak is at 355 nm.

To minimize errors, it is highly recommended that a blank be run parallel to any estrogen determinations and that its fluorescence intensity be subtracted from that of the estrogen.

Fluorescence Intensity, Concentration, and Sensitivity—The relationship of the fluorescence intensity of the dansyl-estrogen to estrogen concentration was studied using estrone as a generic model. This type of class representation is possible since, in this instance, the fluorophore is the same for all estrogens—*viz.*, the aromatic ring A with a dansyl phenolic ester at the 3-position. Figure 5 is a least-squares plot of the fluorescence intensity of the dansyl-estrone derivative at 14 different concentrations with all solution components constant, estrone being the sole variable. The fluorescence intensity is linear with estrone concentration up to a concentration of 3 μg/ml; the correlation coefficient was calculated to be 0.996. The deviation from linearity at high concentrations is taken to be concentration quenching. Studies with estradiol and ethinyl estradiol showed linearity up to 6 and 4 μg/ml, respectively. These results indicate that the dansyl-estrogen system is amenable to analytical applications.

The sensitivity level was estimated to be 0.5 μg/ml for the final concentration of estrogens based on a minimum exhibited dansyl-estrogen fluorescence intensity of three times the blank. Below this concentration level, there are interferences due to residual and hydrolyzed dansyl chloride reagent. By using the USP XVIII procedure as a guide, it was estimated that the iron–Kober method has a sensitivity of about 1 μg/ml (36). Thus, the proposed method is about twice as sensitive.

APPLICATIONS

Isolation of Estrogens from Dosage Forms—As is usually the case, an analytical step cannot be applied until the material to be measured has been isolated from other dosage form components. The liquid–liquid extraction systems used in this work rely on the

obvious acid–base properties of estrogens. These compounds, being phenols, conveniently form water-soluble sodium salts whereas the phenol itself is soluble in a number of organic solvents.

Although the organic solvents used in the described laboratory procedures work well, some circumstances may require different solvents. This behavior is used where necessary to remove the estrogens from their dosage form matrixes. In those instances where the acid–base behavior cannot be used, chromatographic separations may be applied.

Dosage Form Analyses—To evaluate the proposed method in terms of its ability to isolate and measure estrogens, a recovery study was carried out on an aqueous suspension and an oil solution (Table I). In the case of the estrone aqueous suspension, a standard estrone was added to each sample of estrone suspension. The percentage recovery reported is based on the total estrogen present, *i.e.*, the quantity present in the original dosage form as well as the amount added. The recovery percentages and standard deviations (<1%) indicate the proposed procedure to be quite efficient and capable of measuring estrone in aqueous suspension.

The recovery and measurement of estradiol in an oleaginous medium were studied with solutions prepared in the laboratory and hence labeled "synthetic." To make the situation realistic, benzyl alcohol was included since, in some instances, it is added as a bacteriostatic agent as well as a local anesthetic. The results are excellent, with recoveries at 100.0% and standard deviations of less than 2%. These results demonstrate that the separation and measurement procedure used separates the estrogen from any interferences and that the benzyl alcohol does not interfere. Replicate analyses indicate the expected reproducibility.

It should be mentioned that phenol or phenolic compounds, *e.g.*, the parabens, used on occasion in estrogen dosage forms, would be expected to, and do in fact, interfere in the analysis. Thus, since the separation procedure presented here does not separate other phenols from the phenolic estrogens, another separation scheme would be required if nonestrogenic phenols are present or the interference is anticipated in some manner. Chlorobutanol, another preservative, did not interfere.

The use of estradiol benzoate in dosage forms in the recovery study introduces a problem heretofore not encountered since the benzoate is a phenolic ester. Since the benzoate ester does not offer a free phenolic function, the acid–base liquid extraction used for other estrogens could not be employed. A chromatographic column procedure was adopted (37, 38) to isolate the estrogen from the oil. Once the separation is complete, the ester needs to be hydrolyzed to make the phenol available for the dansyl reaction. By using the procedure described in the *Experimental* section, the analysis was carried out and the results are presented in Table I. These results are excellent (based on label statement) and are reproducible to within 2%.

The dansyl-estrogen proposed procedure was studied comparatively by using a common estrone aqueous suspension as the test sample. The latter sample was analyzed by the official NF XIII procedure, a gravimetric analysis based on reaction of the 17-keto group with a hydrazide, and the dansyl-estrogen method. The coefficient of variation for the analyses are as follows: NF XIII, 3.20%; and dansyl, 0.68%. Although the basis for each method is different, the results are comparable with a standard deviation for the NF XIII of 2.04 ± 0.065 and for dansyl of 2.06 ± 0.014, indicating that the dansyl procedure yields reliable results with acceptable reproducibility.

A number of commercial estrogen injectables and suspensions and one estrogen tablet were analyzed by this method (Table II). Inspection of these results indicates that, in each instance, the values are within acceptable limits, with the minimum percentage of 94.3%. The reproducibility is satisfactory, with only 1 *SD* slightly greater than 2%. For those products that are official compendial dosage forms, these analyses indicate them to be within rubric limits.

The analysis of estradiol valerate, although an ester, may be carried out by the general procedure since the ester is at the 17-position and does not interfere with the dansyl reaction. There is a complication, however, owing to the nature of the oil solvent used in the dosage form. In this study, the castor oil-based estradiol valerate dosage form required the use of *n*-heptane as the organic solvent. The remainder of the procedure is the same. As may be seen from Table II, one estradiol valerate sample analysis was conducted in the presence of testosterone enanthate. Since no interference is observed, as is evident from the assay values, the procedure would be expected to function satisfactorily in the presence of androgenic steroids.

One sample of ethinyl estradiol tablets (0.5 mg/tablet) was analyzed (Table II). The procedure was also applied to the analysis of single tablets satisfactorily. Although the results are more than adequate, lower potency ethinyl estradiol tablets, 0.05 mg/tablet, were difficult to analyze and results were inconsistent and nonreproducible. At this low estrogen level, the behavior may be due in part to the interference of tablet excipients and excess dansyl chloride reagent. The method of James (39) is suggested as an alternative.

At present, work is proceeding in the study of the application of the dansyl-estrogen system to the analysis of the components of conjugated estrogens.

REFERENCES

(1) S. Kober, *Biochem. Z.*, **239,** 209(1931).

(2) S. Kober, *Biochem. J.*, **32,** 357(1938).

(3) E. H. Venning, K. A. Evelyn, E. V. Harkness, and J. S. L. Browne, *J. Biol. Chem.*, **120,** 225(1937).

(4) C. Bachman, *ibid.*, **131,** 455(1939).

(5) J. Carol and J. C. Molitor, *J. Amer. Pharm. Ass., Sci. Ed.*, **36,** 208(1947).

(6) E. O. Haenni, J. Carol, and D. Banes, *ibid.*, **42,** 162(1953).

(7) E. O. Haenni, *ibid.*, **39,** 544(1950).

(8) J. Carol, J. C. Molitor, and E. O. Haenni, *ibid.*, **37,** 173(1948).

(9) J. Carol and F. A. Rotondaro, *ibid.*, **35,** 176(1946).

(10) O. Björnson and M. Ottesen, *Quart. J. Pharm. Pharmacol.*, **19,** 519(1946).

(11) C. Heusghem, *Bull. Soc. Chim. Biol.*, **31,** 1114(1949).

(12) K. M. McErlane, *J. Chromatogr. Sci.*, **12,** 97(1974).

(13) I. Schroeder, J. C. Medina-Acevedo, and G. López-Sánchez, *ibid.*, **10,** 183(1972).

(14) P. P. Karkhanis and J. R. Anfinsen, *J. Pharm. Sci.*, **59,** 535(1970).

(15) H. H. Wotiz, H. F. Martin, S. C. Chattoraj, G. Charransol, and I. N. Smith, in "Theory and Application of Gas Chromatography In Industry and Medicine," H. S. Kroman and S. R. Bender, Eds., Grune and Stratton, New York, N.Y., 1968, pp. 110–119.

(16) J. H. H. Thijssen and W. Veeman, *Steroids*, **11,** 369(1968).

(17) D. Exley and A. Dutton, *ibid.*, **14,** 575(1969).

(18) A. G. Butterfield, B. A. Lodge, and N. J. Pound, *J. Chromatogr. Sci.*, **11,** 401(1973).

(19) G. J. Krol, R. P. Masserano, J. F. Carney, and B. T. Kho, *J. Pharm. Sci.*, **59,** 1483(1970).

(20) H. Van Baelen, W. Heyns, and P. DeMoor, *J. Chromatogr.*, **30,** 226(1967).

(21) G. F. Marrian, *Biochem. J.*, **24,** 435(1930).

(22) R. W. Bates and H. Cohen, *Endocrinology*, **47,** 166, 182(1950).

(23) J. W. Goldzieher, J. M. Bodenchuk, and P. Nolan, *J. Biol. Chem.*, **199,** 621(1952).

(24) J. W. Jailer, *J. Clin. Endocrinol.*, **8,** 564(1948).

(25) G. Ittrich, *Z. Physiol. Chem.*, **312,** 1(1958).

(26) G. Ittrich, *Acta Endocrinol.*, **35,** 34(1960).

(27) R. Dvir and R. Chayan, *J. Chromatogr.*, **45,** 76(1969).

(28) L. P. Penzes and G. W. Oertel, *ibid.*, **51,** 325(1950).

(29) *Ibid.*, **74,** 359(1972).

(30) W. R. Gray and B. S. Hartley, *Biochem. J.*, **89,** 59p(1963).

(31) A. A. Boulton and I. E. Bush, *ibid.*, **92,** 11p(1964).

(32) N. Seiler and J. Wiechmann, *Experientia*, **20,** 559(1964).

(33) N. Seiler and M. Wiechmann, *Z. Anal. Chem.*, **220,** 109(1966).

(34) N. Seiler and M. Wiechmann, in "Progress In Thin Layer Chromatography," A. Niederwieser and G. Pataki, Eds., Ann-Arbor Humphrey, Ann Arbor, Mich., 1970, pp. 95–143.

(35) J. W. Wells, *Acta Endocrinol., Suppl.*, **119,** 119(1967).

(36) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 244.

(37) T. James, *J. Ass. Offic. Anal. Chem.*, **54,** 1192(1971).

(38) E. Smith, *J. Pharm. Sci.*, **56,** 630(1967).

(39) T. James, *ibid.*, **61,** 1306(1972).

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Determination of Acetaminophen in Pharmaceutical Preparations and Body Fluids by High-Performance Liquid Chromatography with Electrochemical Detection

RALPH M. RIGGIN, ALLEN L. SCHMIDT, and PETER T. KISSINGER ˣ

Abstract □ A sensitive and very rapid assay for acetaminophen was developed based on the combination of high-performance chromatographic columns with a thin-layer electrochemical detector. Application to liquid and solid dosage forms and body fluids has been demonstrated. Great advantage derives from the detector selectivity, which permits discrimination against many potentially interfering substances without need for extensive separations or formation of derivatives. As little as 0.005% of the hydrolysis product, *p*-aminophenol, can be detected in the presence of the intact drug following cation-exchange chromatography. Acetaminophen can be quantitatively determined in serum on the 50-ng/ml level by liquid chromatography using a pellicular polyamide packing.

Keyphrases □ Acetaminophen—analysis in dosage forms, serum, and urine, high-performance liquid chromatography with electrochemical detection □ Liquid chromatography, high performance—determination of acetaminophen in pharmaceutical preparations and body fluids, electrochemical detection □ *p*-Aminophenol—determination of trace amounts in acetaminophen preparations, high-performance liquid chromatography with electrochemical detection

Fundamental advances recently have been made in liquid chromatography and analytical electrochemistry. Both fields depend on heterogeneous processes, which are now sufficiently well understood to permit optimization of the controlling parameters. This knowledge has resulted in a new enthusiasm for methods that only a few years ago were thought to be outmoded. Liquid chromatography, while easy enough to use, continues to suffer from the lack of a completely satisfactory detector, particularly one with sufficient sensitivity to compete with GC. On the other hand, modern electrochemical techniques have attained considerable sensitivity but suffer from rather poor resolution, chronic irreproducibility, and poor human engineering.

It is not surprising that great advantages accrue from combining the two technologies. Strong parallels exist with the more established association of liquid chromatography and electronic spectroscopy (both absorption and emission). In both cases, two phenomena of modest specificity are combined into a single high-resolution device. The optical instrumentation presently used in liquid chromatography is often insufficiently sensitive or selective for quantitating body fluid levels for many clinically important compounds. Although less generally applicable than optical devices, electrochemical detectors can sometimes form the basis for successful assay procedures.

Recently, a thin-layer electrochemical transducer was developed that has significant advantages over conventional electrochemical cells when coupled to high-performance liquid chromatography (1, 2). The principles of operation of this detector and other developments in hydrodynamic electrochemistry have been described (2, 3). In brief, the column effluent is passed between two plates separated by a thin spacer (typically 50 μm). Electrodes imbedded in the walls of the channel efficiently convert sample molecules into the product due to the short diffusional pathway across the moving solution film. It is possible in this manner to detect as little as 1 pg of an electroactive material while maintaining a detector dead volume of less than 1 μl.

In view of the large number of electrochemically reactive pharmaceuticals, liquid chromatography with electrochemical detection could have wide utility in quality control and drug metabolism studies. The present paper describes several procedures developed for acetaminophen (*N*-acetyl-*p*-aminophenol) in dosage forms and body fluids.

Acetaminophen is most commonly assayed in dosage forms by spectrophotometry. The drug may be monitored directly (4, 5) or following conversion to 2-nitro-4-acetamidophenol (6). Many published procedures are based on acid hydrolysis to *p*-aminophe-