Quantitative Determination of Diethylstilbestrol by Thin-Layer Chromatography

By LOUIS N. JONES, MURRY SEIDMAN, and B. C. SOUTHWORTH

A thin-layer chromatographic procedure has been developed for the analysis of diethylstilbestrol at the 0.01 percent level in a water-dispersible suppository base. Following a preliminary separation on an alumina column, the diethylstilbestrol is isolated on a silica gel chromatoplate. A color is then produced *via* the Folin-Ciocalteu procedure for phenols and the blue spot evaluated densitometrically directly on the chromatoplate. The standard deviation is 3.4 percent. Effects of variations in the operating parameters are discussed and evidence is presented to indicate that only intact diethylstilbestrol is measured.

WHILE BOTH biological and chemical methods are available for the determination of diethylstilbestrol [3,4-bis(*p*-hydroxyphenol)-3-hexene] in pharmaceutical preparations, no rapid assay procedure for this estrogen in combination with nitrofurazone in a water-dispersible suppository base has been reported. Biological procedures (1) require a minimum of 8 days to complete. Nitrofurazone interferes in the official USP XVII procedure (2) for diethylstilbestrol (DES) suppository, and any modification of this procedure would necessitate extensive purification before the irradiation analysis could be used.

Michels *et al.* (3) were able to separate DES from nitrofurazone and the suppository base in sufficiently pure form so that the Folin-Ciocalteu procedure could be used for its determination. Although the total procedure was fairly time consuming, their preliminary separation was rapid and served to separate the DES from the bulk of the suppository base. Lisboa *et al.* (4) have developed a thin-layer chromatographic classification system for a series of estrogens. After separating the DES from the bulk of the suppository base *via* the above-mentioned preliminary separation, Lisboa's system was found to be adequate for the purification and isolation of the DES. Having isolated the DES in pure form, densitometric determination directly on the chromatoplate was found to be feasible.

Lisboa *et al.* used the Folin-Ciocalteu reagent to visualize DES on a chromatoplate. Their techniques were designed for qualitative purposes so that refinements were necessary before a quantitative determination was possible. The stability of the color produced by the Folin-Ciocalteu reagent is very much dependent on the final pH adjustment. This pH adjustment may be obtained by observation of an indicator (thymolphthalein) color change as a base is sprayed on the plate. Alcoholic potassium hydroxide was chosen as this reagent, the base simply for its solubility characteristics; the alcohol for its volatility. After the pH adjustment has been completed, the alcohol evaporates and the color is "fixed." Attempts to use ammonia vapor (5) were not successful in that a wide background variation was noted in the final densitometric determination.

The recommended procedure calls for a sample size calculated to contain 0.5 mg. of DES. After an initial clean-up this 0.5 mg. is contained in 10 ml. of chloroform. The final determination requires four spots of 2 mcg. each for an accurate determination. The ultimate sensitivity of the procedure is about 0.1 mcg. of DES.

EXPERIMENTAL

Apparatus—Photovolt densitometer model 520 A, equipped with an automatic TLC stage, a Varicord recorder No. 42, and an Integraph integrator No. 49.

Reagents—Potassium hydroxide alcoholic solution, 0.5 *N*, V.S.; thymolphthalein solution, 0.1% in alcohol, T.S.; alcohol, 80%, R.

Developing Solvent—Ethyl acetate R., petroleum ether R., glacial acetic acid R., and alcohol R. (72:13.5:10:4.5, respectively).

Folin-Ciocalteu Reagent—Use the procedure according to Folin (6) or use Harleco item 2690.

Preparation of Standard DES Solution—Transfer exactly 100 mg. of USP reference standard diethylstilbestrol to a 200-ml. volumetric flask, dissolve it, and dilute to the mark with chloroform. Further dilute 10.0 ml. of this solution to 100 ml. with chloroform for use in the assay.

Preparation of Chromatoplates—Slurry together Silica Gel G and distilled water in the ratio of 1:2, apply the slurry uniformly to 20 × 20-cm. glass plates to a thickness of 750 μ and allow the plates to air-dry for 30 min. Activate the plates at 105° for 1 hr. and store under desiccation until used. Immediately before use, check the background of the chromatoplate according to the parameters to be used for the final spot measurement. Any variation should not exceed ±0.05 absorbance units.

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Preparation of Developing Chamber—Line the walls of the developing chamber (30.48 cm. × 10.16 cm. × 27.94 cm.) with filter paper. Add 100 ml. of the developing solvent, close the chamber tightly and allow at least 30 min. for complete saturation of the chamber atmosphere. Prepare this tank fresh daily.

Preparation of Column—Place a glass-wool pledget at the bottom of a 1-cm. i.d. chromatographic column and pack it to a height of 10 cm. with basic alumina. Add the adsorbent in small portions with horizontal tapping and gentle bouncing until no further settling occurs (7). Lot to lot variations in adsorptive capacity of the alumina have been noted. The following test is, therefore, recommended. Dissolve about 10 mg. of nitrofurazone in 100 ml. of dehydrated alcohol. Add 10 ml. of this solution to a prepared column. The nitrofurazone should remain at the top of the column in a narrow yellow band. Elute the band with 80% alcohol in water. The band should follow the solvent front. The characteristics of DES are similar to nitrofurazone under these conditions.

Assay Procedure—Melt and mix suppositories to be assayed and weigh accurately sufficient sample to contain 0.50 mg. of DES. Add 10 ml. of chloroform to the sample, warm gently to dissolve, then transfer with the aid of additional chloroform to the alumina column. Wash the column with 100 ml. of chloroform and discard the $CHCl_3$. Elute the DES from the column with 50 ml. of 80% alcohol collecting the eluate in a 150-ml. beaker. Evaporate the eluate to dryness on a steam bath using a stream of air to aid the evaporation. Dissolve the residue in chloroform, transfer it to a 10-ml. volumetric flask, and dilute to volume with chloroform. To an activated chromatoplate apply alternately 40-μl. spots of the reference standard DES solution and the sample solution. Repeat this operation three times (8 spots total). Transfer the plate to the developing chamber, chromatograph 100 mm., remove, and air-dry for 30 min. At a point in line with the 100-mm. mark, place a spot of thymolphthalein solution near each edge of the plate. Dilute the Folin-Ciocalteu reagent 1 to 4 with distilled water just prior to use and spray the plate evenly with about 5 ml. of the diluted reagent, using the spray procedure described by Stahl (8). In a similar manner, spray the plate with the alcoholic potassium hydroxide solution until the thymolphthalein spot remains a permanent blue color. The DES will be visualized as a blue spot (R_f approximately 0.9). Measure the density of the DES in the reference standard and the sample spots with the densitometer. Determine the areas under the recorded curves from the integrator pen traces. The following instrument parameters were used for the Photovolt densitometer: range setting 3; recorder response 4; 610 mμ filter (Photovolt No. 5408), and slit width, 1 × 7 mm.

Calculation—

$$\frac{\Sigma \text{ area of sample spots}}{\Sigma \text{ area of standard spots}} \times \frac{0.05}{\text{sample wt. (Gm.)}} = \% \text{ DES.}$$

RESULTS

Linearity of Results—Chloroform solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 mcg. of DES per 40 μl. of solution were prepared and treated according to *Experimental* starting at the thin-layer chromatographic step. Results are given in Table I.

TABLE I—LINEARITY OF SPOT DENSITY *Versus* CONCENTRATION

Plate	Regression Line	Correlation Coefficient (γ)	Standard Deviation (σ)
1	$y = 1.7 + 49.4x$	0.9990	1.7
2	$y = -0.3 + 41.0x$	0.9995	1.0
3	$y = -2.5 + 45.8x$	0.9997	0.8

The result of a given plate is given in the form $y = a + bx$ where y is the area of a spot in terms of integrator count, a is the y intercept, b is the slope of the line, and x is the quantity of DES in a spot. The remainder of the table is self-explanatory.

Recovery of Standard DES—To test the reproducibility of the above procedure, portions of standard DES equivalent to what would normally be found in a medicated suppository were treated as indicated in *Experimental*. Results are given in Table II.

The average areas of the sample spots and of the standard spots are given in columns $\bar{X}$ and $\bar{Y}$, respectively, as integrator counts. The average recovery was 98.4% with a standard deviation of 3.5%.

The statistical treatment is as follows: the area figures used in the calculations for the sample spots and for the standard spots are each an average of four essentially independent observations. The variance of an average result is:

$$\text{Var}\{\bar{X}\} = \sigma^2/n$$

or

$$\bar{\sigma}^2 = \frac{1}{n^2(n-1)}[n\Sigma X^2 - (\Sigma X)^2]$$

The value of σ^2 is then converted to a percentage coefficient of variance:

$$V^2 = (\sigma^2)\left[\frac{100}{\bar{X}}\right]^2$$

The variance for the result is:

$$V_T^2 = V_{\bar{X}}^2 + V_{\bar{Y}}^2$$

and the standard deviation is the square root of the variance.

Recovery of DES from a Suppository Mixture—A mixture of 0.3% nitrofurazone and 0.0125% DES in a water-dispersible base of glyceryl monolaurate and sorethytan (4) monostearate was prepared to simulate a commercial vaginal suppository.[1] Portions of the mixture were treated as indicated in *Experimental*.

Results are given in Table III. Data are treated as indicated above. The average recovery was 98.7% with a standard deviation of 3.4%.

Another suppository mixture[2] containing 0.2% nitrofurazone, 2% diperodon hydrochloride, and

[1] Furestrol Vaginal Suppository, Eaton Laboratories, Division of The Norwich Pharmacal Company, Norwich, N.Y.

[2] Furacin-E Urethral Inserts, Eaton Laboratories, Division of the Norwich Pharmacal Company, Norwich, N.Y.

TABLE II—RECOVERY OF STANDARD DIETHYLSTILBESTROL

Plate No.	Sample $\bar{X}$	Sample V_X^2	Standard $\bar{Y}$	Standard V_Y^2	Recovery %	Recovery V_T^2	Recovery σ
1	89.0	6.53	89.5	0.94	99.44	7.46	2.7
2	79.0	14.69	80.5	9.91	98.14	24.60	5.0
3	99.0	5.27	100.75	4.00	98.26	9.27	3.0
4	105.5	0.83	106.75	10.58	98.83	11.41	3.4
5	69.75	7.32	70.5	5.87	98.94	13.19	3.6
6	86.0	7.67	85.5	12.89	100.58	20.55	4.5
7	82.5	5.02	85.5	7.87	96.49	12.89	3.6
8	80.0	3.91	83.0	2.18	96.39	6.08	2.5

TABLE III—RECOVERY OF DIETHYLSTILBESTROL FROM A SUPPOSITORY MIXTURE

Plate No.	Sample $\bar{X}$	Sample V_X^2	Standard $\bar{Y}$	Standard V_Y^2	Recovery %	Recovery V_T^2	Recovery σ
1	123.2	1.48	123.2	2.31	100.0	3.78	1.9
2	82.8	1.95	83.4	5.26	99.28	7.22	2.7
3	85.75	8.47	86.25	17.34	99.42	25.81	5.1
4	92.6	5.90	94.6	1.85	97.89	7.76	2.8
5	93.25	7.16	95.0	5.73	98.16	12.89	3.6
6	82.0	1.00	83.25	5.14	98.50	6.13	2.5
7	79.5	17.22	81.5	13.17	97.55	30.40	5.5

TABLE IV—RECOVERY OF DIETHYLSTILBESTROL FROM COMMERCIAL SUPPOSITORIES

Mfr.	Label Claim, mg. DES/ Supp.	DES Added, mg./Supp.	DES Found, mg./Supp.	% of Label Claim Found
1	0.1	0	0.098	98
1	0.25	0	0.25	100
2	0.1	0	0.093	93
2	0.5	0	0.47	94
3	0.1	0	0.075	75
3	0.1	0.1	0.177	177

0.0077% DES in the above base was also studied. The diperodon hydrochloride was eluted in the preliminary chloroform wash and discarded. Otherwise, results were comparable to the above results.

Assay of Commercial Suppositories for DES—The DES present in five different dosage forms (commercially available units) from three different manufacturers was determined by the above procedure. Results are given in Table IV. The validity of the low recovery of DES from manufacturer 3 was confirmed by the addition of a known amount of DES. As an additional check, the insoluble portion of suppository base was extracted with chloroform *via* the Soxhlet procedure for 4 hr. No additional DES was found.

DISCUSSION

Effects of Variations in Column Parameters—It was ascertained (the alumina meeting the above criterion) that 75 ml. of chloroform was sufficient to elute interfering substances from the column. As much as 200 ml. of chloroform can be passed through the column without eluting any DES. The DES can be quantitatively eluted with 20 ml. of 80% alcohol. The DES can be satisfactorily eluted with 75–90% alcohol. Alcohol concentration of less than 70% or more than 95% is unsatisfactory for the elution. The column can handle a threefold increase in sample size without overloading.

Effects of Variations in the TLC Procedure—Using good technique, the only problem area in the TLC procedure is the spray pattern. A uniform spray must be laid down on the plate. The volume of the Folin-Ciocalteu reagent must be held to between 4 and 6 ml. The alkaline spray must also be uniform and close attention must be given to the indicator color change. The spots produced are then stable for about 4 hr.

Intact DES—In order to determine that the DES was not degraded by the assay procedure, DES was eluted from the plate and an infrared spectrum recorded. The spectrum compared favorably with that of standard DES.

Portions of a suppository mass were exposed to ultraviolet radiation from a germicidal lamp. The apparent decomposition approximated a first-order degradation. At 15 cm. from a 17-w. lamp 30% of the DES was decomposed in 10 min. The ketonic product formed was found to have an R_f of 0.7 and did not undergo the Folin-Ciocalteu reaction.

Similar Phenolic Compounds—Compounds with structures similar to DES can also be determined by the above procedure. Dienestrol, hexestrol, and 4,4′-dihydroxystilben will behave the same as DES. If present in admixture an additional separation would be necessary before the determination.

Common suppository base materials such as polyethylene glycol or theobroma oil do not interfere with the above procedure.

CONCLUSION

This method is sufficiently rapid for use as a routine quality control procedure and necessary technical skills can be readily learned. The high degree of selectivity demonstrated also indicates that the method is useful for a stability program. A further use of the method might be as a referee method or as an official procedure for the determination of diethylstilbestrol.

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Keyphrases

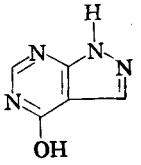
Diethylstilbestrol (DES)—analysis
Nitrofurazone–DES suppositories—analysis
Column chromatography—separation
TLC—identity
Densitometer—analysis

Qualitative and Quantitative Tests for Allopurinol

By EDWARD F. SALIM* and JAMES E. MURPHY†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

1H-PYRAZOLO [3,4-*d*]-pyrimidin-4-ol; $C_5H_4N_4O$; mol. wt. 136.11. The structural formula of allopurinol may be represented as



Physical Properties—Allopurinol occurs as a white to off-white, practically odorless powder and melts above 300°. It is very slightly soluble in water and in alcohol, and practically insoluble in chloroform and in ether. It is soluble in dimethylformamide and in dilute solutions of alkali hydroxides.

Identity Tests—A 1 in 100,000 solution of allopurinol in 0.1 *N* hydrochloric acid exhibits an ultraviolet absorbance maximum at about 250 mμ [absorptivity (*a*) about 56] and a minimum at about 231 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.3% dispersion of allopurinol in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Purity Tests—Dry about 1 Gm. of allopurinol, accurately weighed, in vacuum at 105° for 5 hr.: it loses not more than 1% of its weight.

Dissolve about 10 mg. of allopurinol in 1 ml. of 0.1 *N* sodium hydroxide. Arrange for ascending

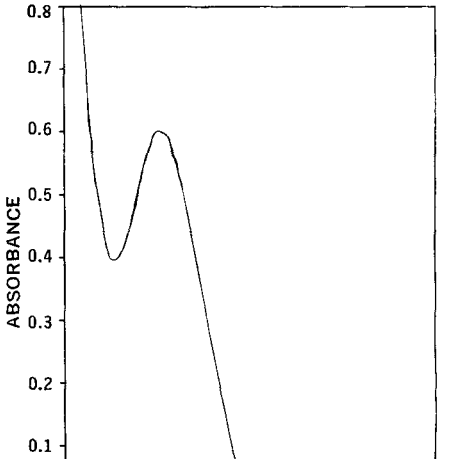


Fig. 1—Ultraviolet absorption spectrum of allopurinol in 0.1 N hydrochloric acid (10 mcg./ml.); Beckman model DK-2A spectrophotometer.

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† Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y. 10707.
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