of benzene were added. Unreacted *p*-phenylphenacyl bromide, a mixture of *p*-phenylphenacyl angelate and senecioate and *p*-phenylphenacyl senecioate (31 mg.), m.p. 136°, were obtained successively. The identity of the senecioate ester was established by thin-layer chromatography, infrared spectroscopy, and mixed melting point determination with an authentic sample of *p*-phenylphenacyl senecioate.

Synthesis of Lomatin Senecioate—Lomatin (200 mg.) was suspended in dry benzene (5 ml.). The suspension was added slowly to a solution of senecioyl chloride (b.p. 67°/39 mm. Hg) (230 mg.) in dry benzene (3 ml.). The mixture, protected by a calcium chloride drying tube, was refluxed on a steam bath for 16 hr. The reaction mixture was then cooled and washed with 5% sodium bicarbonate solution followed by distilled water. The benzene solution was dried over anhydrous magnesium sulfate, filtered, and the solvent removed under reduced pressure to yield a colorless, glassy substance (230 mg.). All attempts to crystallize the ester were unsuccessful. Thin-layer chromatography, however, showed only one spot with the same R_f value as noted for jatamansin and fractions 240–486. This compound was then further purified by silica gel chromatography and dried in a high vacuum to yield an amorphous, very hygroscopic substance with m.p. 58 ~ 62° $[\alpha]_D^{20}$ +75.0 (c 1.0, $CHCl_3$); ultraviolet spectrum: $\lambda_{max.}$ 326, 256, 246, and 219 mμ (log $\epsilon_{max.}$ 4.16, 3.73, 3.83, and 4.53, respectively); infrared spectrum (in $CHCl_3$) was almost identical with fractions 240–486, *i.e.*: 1724 (α-pyrone C=O), 1610, 1490 (aromatic C=C), 1140, 1115 (asymmetric and symmetric stretching of =C—O—C), and 834 cm.$^{-1}$ (1,2,3,4-aromatic substitution).

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Keyphrases

Coumarins—*Lomatium nuttallii*
Lomatium nuttallii—coumarins, isolated, identified
Nuttallin—new coumarin, isolated
Column chromatography—separation
TLC—identity
UV spectrophotometry—structure
IR spectrophotometry—structure
Optical rotation—identity
NMR spectrometry—identity

Gas-Liquid Chromatographic Determination of Dienestrol in the Presence of Methyltestosterone

By FRED L. FRICKE, STEPHEN M. WALTERS, and WILLIAM T. LAMPKIN

A gas chromatographic procedure has been developed for the determination of dienestrol in the presence of methyltestosterone. Dienestrol was extracted from tablets and quantitatively determined by gas chromatography of its bis-(trimethylsilyl)ether. The bis-(trimethylsilyl)ether of alizarin was used as an internal standard. Preliminary experiments indicate that the method is applicable in the presence of other androgens and corticosteroids.

THIS STUDY describes a rapid, specific, gas-liquid chromatographic procedure for the determination of dienestrol in the form of its bis(trimethylsilyl)ether derivative, with bis-(trimethylsilyl)acetamide as the silylating agent.

A number of analytical procedures have been reported for the determination of dienestrol. The USP XVI utilizes the Folin-Denis reagent in a colorimetric procedure. Cocking (1) also developed a colorimetric method based on a reaction of dienestrol with bromine in acetic acid, whereas Malpress (2) formed a polynitro derivative and measured the orange color produced. A method for the determination of dienestrol in biological samples, utilizing the formation and subsequent titration of the maleic acid adduct, was proposed by Smith (3). Gottlieb (4) formed nitrosophenols from various synthetic estrogens including dienestrol and measured the colors produced, while Gry (5) determined the nitroso derivative of dienestrol polarographically. Summa and Graham (6) determined dienestrol both by a polarograpic method, which is the basis of the NF XII assay, and by measurement of a yellow color [first observed by Banes (7)] that is formed with ultraviolet irradiation of dienestrol. In applications of gas chromatography as the determina-

Received August 23, 1967, from the Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Cincinnati, OH 45202
Accepted for publication October 24, 1967.

tive step, McGregor *et al.* (8) formed the dimethyl ether, and Luukainen *et al.* (9) prepared the trimethylsilyl ethers of steroids.

Advantages of the trimethylsilyl ethers are their easy and quantitative formation and the decrease in adsorption effects on the column. The trimethylsilyl ether derivatives have normally been prepared by the reaction of dienestrol with hexamethyldisilazane and trimethylchlorosilane in pyridine. However, complete formation of the derivative with these reagents often requires prolonged heating and removal of ammonia. Another disadvantage is that amine hydrochlorides are produced. Klebe *et al.* (10) have found that bis-(trimethylsilyl)acetamide as prepared by Birkofer *et al.* (11) is a superior silylating agent, in many respects, to the previously used reagents.

EXPERIMENTAL

Apparatus—Gas chromatograph, Packard series 7800 with flame-ionization detector and Minneapolis Honeywell recorder.

GLC Column—Glass coiled (1.8 m. × 4 mm.) packed with 5% SE-30 on Gas Chrom Q, 80/100 mesh. The column was conditioned for 8 hr. with a nitrogen flow of 60 ml./min.

Operating Conditions—Column temperature 230°; detector temperature 255°; injection port temperature 255°; hydrogen flow 90 ml./min.; air flow 750 ml./min.; nitrogen flow 100 ml./min.; sensitivity, 1×10^{-9} amp.

Reagents—Bis-(*trimethylsilyl*)*acetamide*[1]—This reagent must be stored under nitrogen to prevent destruction by atmospheric moisture. A dry box is convenient for storage.

Internal Standard Solution—Place 150 mg. of alizarin in a 50-ml. volumetric flask. Add approximately 40 ml. of ethyl acetate and shake until most of the alizarin is dissolved. Transfer to a dry box and add 2 ml. of bis-(trimethylsilyl)acetamide. Remove and heat at 60° for 5 min., cool, dilute to volume with ethyl acetate, and mix well.

Standard Dienestrol Solution—Place 4.0 mg. of NF dienestrol reference standard in a 10-ml. volumetric flask and dissolve in a small portion of ethyl acetate. Add 2.0 ml. of internal standard solution. Transfer to a dry box and add 10 drops of bis-(trimethylsilyl)acetamide. Remove the solution from the dry box and dilute to volume with ethyl acetate.

Extraction Procedure—Weigh not less than 20 tablets and grind to a fine powder. Weigh accurately a portion of the powder sufficient to give about 2 mg. of dienestrol for tablets containing 0.25 mg./tablet (or about 4 mg. for tablets containing more than 0.25 mg. of dienestrol/tablet) into a 250-ml. conical flask. Add 50 ml. of acetone, place on a hot plate with a magnetic stirrer, and boil gently with stirring for 10 min.; cool and let settle. Decant through a funnel fitted with a glass wool plug into a 250-ml. beaker. Repeat the extraction two more times with 50-ml. portions of acetone, combining the extracts in the 250-ml. beaker. Evaporate the extract on a steam bath with the aid of a current of dry air to approximately 10 ml. Transfer to a 50-ml. beaker with several small portions of acetone, using a stirring rod to break up any precipitate. Evaporate this extract just to dryness. Transfer the residue with several small portions of ethyl acetate to a 5-ml. volumetric flask if it contains 2 mg. of dienestrol (or to a 10-ml. volumetric flask if it contains 4 mg.). Then add 1.0 ml. of internal standard solution to the 5-ml. volumetric flask (or 2.0 ml. if a 10-ml. volumetric flask is used).

Etherification Step—Place the volumetric flask inside a nitrogen dry box and add an excess (about 0.5–1.0 ml.) of bis-(trimethylsilyl)acetamide. Mix

[1] Available from Aldrich Chemical Co., Milwaukee, Wis.

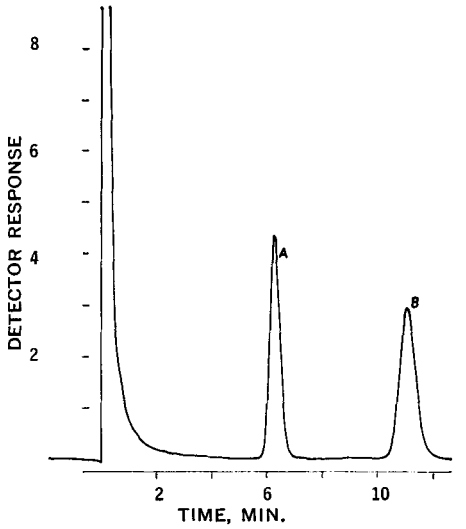

Fig. 1—A typical chromatogram. Key: A, bis-(trimethylsilyl)ether of dienestrol; B, bis-(trimethylsilyl)-ether of alizarin.

TABLE I—PRECISION DATA FOR THE CALIBRATION CURVE

Concn. of Dienestrol, mg./ml.	R			Mean	σ
0.1016	0.246	0.241	0.248	0.246	± 0.007
	0.244	0.250			
0.2032	0.497	0.495	0.491	0.494	± 0.003
	0.490	0.498			
0.4068	0.990	0.985	0.986	0.998	± 0.005
	0.987	0.990			
0.5080	1.249	1.251	1.245	1.247	± 0.008
	1.241	1.250			

TABLE II—RECOVERY STUDIES OF DIENESTROL ADDED TO A SIMULATED TABLET MIXTURE

Added, mg.	Recovered, mg.	% Recovery
3.95	4.01	102
4.08	4.10	100
3.97	3.97	100
4.59	4.55	99.1
4.13	4.06	98.3
4.06	4.20	103
4.10	4.13	101
3.92	3.92	100
2.21	2.19	99.1
2.29	2.24	97.8

Av. = 100% Standard deviation = 1.6

and remove from the dry box. (*Note*: If the volume is above the calibration mark, the excess may be removed with the aid of heat and a current of nitrogen.) Inject 6–7 μl. of this solution into the gas chromatograph. Make duplicate injections of both sample and standard.

Calculations—The peaks due to the bis-(trimethylsilyl)ethers of dienestrol and alizarin have retention times of approximately 6.6 min. and 11.1 min. (See Fig. 1.) Peak area is determined by multiplying the peak height times the width at half height.

The ratio of the peak areas is given by:

$$R = \frac{\text{area of bis-(trimethylsilyl)ether of dienestrol}}{\text{area of bis-(trimethylsilyl)ether of alizarin}}$$

mg. of dienestrol/tablet =

$$\frac{\text{R for sample} \times \text{standard concn.} \times \text{diln. factor} \times \text{av. wt. tablet}}{\text{R for standard} \times \text{wt. sample}}$$

RESULTS AND DISCUSSION

Table I shows the average peak area ratios (R) of the bis-(trimethylsilyl)ether of dienestrol to the bis-(trimethylsilyl)ether of alizarin of five injections of four standard solutions. These values indicate that dienestrol is linear over the concentration range used.

Simulated tablet mixtures containing all excipients including methyltestosterone and known quantities of dienestrol were taken through the entire procedure. These mixtures were chromatographed in duplicate. The results are given in Table II. These data indicate that the standard deviation for the method is 1.6. Table III shows results of the analyses of marketed products containing methyltestosterone and dienestrol.

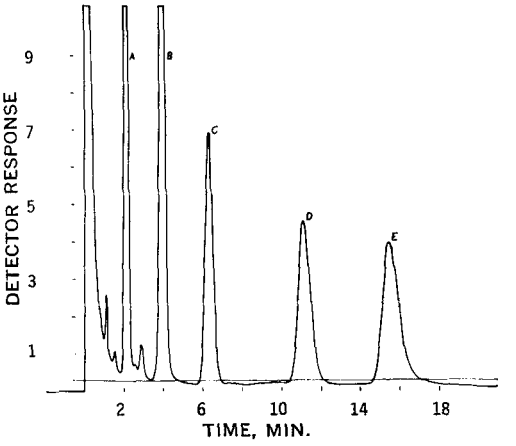

Fig. 2—A typical chromatogram of a sample. Key: A, (trimethylsilyl) ester of palmilic acid; B, (trimethylsilyl) ester of stearic acid; C, bis-(trimethylsilyl)ether of dienestrol; D, bis-(trimethylsilyl)ether of dienestrol; D, bis-(trimethylsilyl)ether of alizarin; E, methyltestosterone.

TABLE III—ANALYSIS OF DIENESTROL IN MARKETED PRODUCTS

Dienestrol found, % of Label Claim	
Preparation A: 0.5 mg./Tablet	Preparation B: 0.25 mg./Tablet
96.4	101
95.6	98.4
96.2	101
94.2	99.6
Av. 95.6	Av. 100
Std. Dev. 1.0	Std. Dev. 1.3

TABLE IV—PRELIMINARY STUDY OF SIMULATED TABLET MIXTURES CONTAINING ANDROGENS AND CORTICOSTEROIDS

Compound	Dienestrol Added, Mg.	% Recovery (Av. of 4)	Retention Time, min.
Corticosteroid			
Betamethasone acetate	3.84	98.5	133
Prednisone	3.84	99.4	13,31
Silylated prednisone	...	...	70
Prednisolone	3.84	100.0	20,44
Silylated prednisolone	...	...	107
Hydrocortisone	3.84	99.3	18,40
Silylated hydrocortisone	...	...	97
Desoxycortisone acetate	3.84	97.8	48
Androgen			
Testosterone	4.05	98.5	11.5
Silylated testosterone	...	...	14
Dromostanolone propionate	4.05	98.6	19

A typical chromatogram of a silylated mixture of stearates, dienestrol, alizarin, and methyltestosterone is shown in Fig. 2.

Preliminary experiments using simulated tablet mixtures indicate that the method is applicable in the presence of other androgens, corticosteroids, and stearates, which cause interference in many of the existing methods. The recovery data for dienestrol and the retention times of the various compounds are shown in Table IV.

The bis-(trimethylsilyl)ether of diethylstilbestrol has the same retention time as the bis-(trimethylsilyl)ether of dienestrol, but partially degraded upon injection in the chromatograph. Barbiturates were found to interfere in the silylation reaction.

In view of the results for both simulated mixtures and commercial samples, the proposed procedure appears to be suitable for determination of dienestrol in the presence of methyltestosterone.

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Keyphrases

Dienestrol analysis
Bis-(trimethylsilyl) ether, dienestrol—derivative
GLC—analysis
Bis-(trimethylsilyl) ether, alizarin—internal standard
Analysis—dienestrol–methyltestosterone combination

New Developments in Antibiotic Interference Thresholds of Microbial Assays

By B. ARRET and J. ECKERT

Interference thresholds have been determined for a number of antibiotics in the microbial assays of specific antibiotics. Two aspects were considered in order to facilitate the quantitative analysis of mixtures of antibiotics: the "interference threshold," which is the concentration of an antibiotic causing a significant interference in the presence of another antibiotic in a given assay, and the "sensitivity threshold," which is the concentration of an antibiotic causing a response in the assay for a different antibiotic.

In 1957, Arret *et al.* (1) described the problems encountered in assaying pharmaceutical preparations in which two or more antibiotics are combined, and proposed solutions to these problems. If the test organism used for the assay of one antibiotic (*a*) is not affected by a second antibiotic (*b*), the assay for (*a*) is uncomplicated. But if the test organism is affected by (*b*), erroneously high or low values for (*a*) may be obtained. Consequently, methods for eliminating the effect of (*b*) were developed. In general, one of the following five methods was used: (*1*) inactivating by biological or chemical means; (*2*) using a test organism which is sensitive to (*a*) and relatively resistant to (*b*); (*3*) artificially making the organism resistant to (*b*); (*4*) separating antibiotics by differential solubility techniques; or (*5*) compensating for the presence of (*b*) by adding it to every solution of (*a*) used for the standard curve.

The analyst needs definite quantitative knowledge of the interfering effects of various antibiotics in certain widely used assay procedures. He must be aware of such effects if confidence is to be maintained in the specificity of an assay.

Since the earlier publication (1), new antibiotics have been discovered, and combinations of these antibiotics together with the older ones make it necessary to update this publication. As before, all the pertinent data are in tabular form for easy reference.

The data obtained from this work simplify the problems of assaying antibiotic combinations Although combinations of two antibiotics have specifically been considered here, the approach can be used as a general guide to the assay of combinations of three or more antibiotics.

Received September 21, 1967, from the Division of Antibiotics and Insulin Certification, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, DC 20204
Accepted for publication November 6, 1967.

EXPERIMENTAL

The effects of 15 antibiotics were determined by assaying them according to the official microbiological assay methods found in the *Code of Federal Regulations* (2). The following antibiotics (assay organisms are given in parentheses) were studied: Bacitracin[1] (*Sarcina subflava*), chloramphenicol (*Escherichia coli*), colistin (*Bordetella bronchiseptica*), erythromycin (*Sarcina lutea*), kanamycin (*Staphylococcus aureus*), neomycin (*S. aureus*, *S. epidermidis*) oleandomycin (*S. epidermidis*), paromomycin (*S. epidermidis*), penicillin (*S. aureus*), polymyxin (*B. bronchiseptica*), streptomycin (*Klebsiella pneumoniae*, *Bacillus subtilis*), tetracycline (*S. aureus*), and viomycin (*K. pneumoniae*).

For each experiment, the antibiotic being assayed was regarded as (*a*). Solutions were prepared containing the reference concentration of (*a*) and various concentrations of the second antibiotic (*b*). The diluent in every case was that ordinarily used for the assay of (*a*). These solutions were assayed against the reference concentration of (*a*) alone as the standard of comparison. Solutions containing various concentrations of (*b*) alone were also prepared.

The lowest concentration of (*b*) which, in com-

[1] Since the previous publication, a new assay for bacitracin was reported (3) in which *Sarcina subflava* is used as the test organism instead of *Micrococcus flavus*. This method is now used routinely in our laboratory.