from ethanol; mp 285–287° (dec.); IR: 3550 (OH), 3000–2700 (N^+H_2 stretch, CH), 1600 (N^+H_2 def.), 1105, and 1030 (CO stretch) cm^{-1}; mass spectrum: *m/e* 361 (4) and 316 (100).

Anal.—Calc. for $C_{23}H_{39}NO_2$·HCl: C, 69.40; H, 10.13; N, 3.51. Found: C, 69.31; H, 9.97; N, 3.61.

3β-Methoxy-5-androsten-17-one 17-Oxime (V)—A solution of II (5 g, 0.016 mole), sodium acetate (9 g), and hydroxylamine hydrochloride (3 g) in 5% (v/v) aqueous ethanol (80 ml) was refluxed for 4 hr. After sitting at room temperature overnight, the crystalline precipitate was collected and washed with water, giving 4 g (78%) of V. Recrystallization from methanol gave the analytical sample, mp 218–219°; $[\alpha]_D^{25}$ +11.6°; IR: 3500 (OH), 1660 (C=N), 1105, and 1030 (CO stretch) cm^{-1}; PMR ($CDCl_3$): δ 0.92 (s, 3H, C-13 methyl), 1.03 (s, 3H, C-10 methyl), 3.36 (s, 3H, C-3 methoxyl), and 5.35 (broad, 1H, C-6 proton) ppm.

Anal.—Calc. for $C_{20}H_{31}NO_2$: C, 75.66; H, 9.84; N, 4.42. Found: C, 75.69; H, 9.97; N, 4.51.

17β-Amino-3β-methoxy-5-androstene Hydrochloride (VI·HCl)—Sodium (12 g, freshly cut) was added (during 1 hr) in small increments to a refluxing soluton of V (2 g, 0.006 mole) in absolute ethanol (100 ml). After sodium addition, the reaction was stirred at the reflux temperature for 2 hr. The hot mixture was then diluted with warm (60°) water (1 liter) and allowed to stand at room temperature for 24 hr. The resultant solid was dissolved in dry ether, and hydrogen chloride gas was passed through the ethereal solution to give 1.2 g (45%) of VI·HCl. Recrystallizations from ethanol gave the analytical sample, mp 294–296° (dec.); $[\alpha]_D^{25}$ −19.1°; IR: 3200–2800 (N^+H_3 stretch, CH), 1610 (asym. N^+H_3 def.), 1515 (sym. N^+H_3 def.), 1105, and 1030 (CO stretch) cm^{-1}; mass spectrum: *m/e* calc. for $C_{20}H_{33}NO$ (M^+ − HCl): 303.2562. Found: *m/e* 303.2562.

Anal.—Calc. for $C_{20}H_{33}NO$·HCl·C_2H_5OH: C, 68.44; H, 10.44; N, 3.63. Found: C, 68.24; H, 10.40; N, 3.69.

REFERENCES

(1) W. R. Buckett, in "Advances in Steroid Biochemistry and Pharmacology," vol. 3, M. H. Briggs and G. A. Christie, Eds., Academic, London, England, 1972, p. 39.

(2) C. G. Bergstrom, *J. Med. Chem.*, **11**, 875 (1968).

(3) R. B. Filderman and B. A. Kovacs, *Br. J. Pharmacol.*, **37**, 748 (1969).

(4) Y. Nagai, *Chem. Pharm. Bull.*, **17**, 1749 (1969).

(5) P. D. Klimstra, U.S. pat. 3,627,756 (1971).

(6) P. D. Klimstra, U.S. pat. 3,639,598 (1972).

(7) P. D. Klimstra, U.S. pat. 3,636,014 (1972).

(8) R. E. Counsell, P. D. Klimstra, and R. E. Ranney, *J. Med. Chem.*, **5**, 1224 (1962).

(9) H. Budzikiewicz, C. Djerassi, and D. H. Williams, in "Interpretation of Mass Spectra of Organic Compounds," Holden-Day, San Francisco, Calif., 1965, p. 65.

(10) B. M. Regan and F. N. Hayes, *J. Am. Chem. Soc.*, **78**, 639 (1956).

(11) G. R. Pettit, R. L. Smith, A. K. Das Dupta, and J. L. Occolowitz, *Can. J. Chem.*, **45**, 501 (1967).

(12) P. F. D'Arcy, E. M. Howard, P. W. Muggleton, and S. B. Townsend, *J. Pharm. Pharmacol.*, **12**, 659 (1960).

(13) "Dictionary of Organic Compounds," vol. 3, I. Heilbron, A. H. Cook, H. Bunbury, and D. H. Hey, Eds., Eyre and Spottiswoode, London, England, 1965, p. 1642.

(14) F. W. Heyl, M. E. Herr, and A. P. Centolella, *J. Am. Chem. Soc.*, **71**, 247 (1949).

ACKNOWLEDGMENTS

The authors thank Professor and Mrs. P. F. D'Arcy for their valuable assistance with the animal experimentation and Mr. M. Jordan for assistance with the chemical syntheses. Gifts of steroid starting material from Allen and Hanburys Ltd. are also gratefully acknowledged. S. C. Griggs acknowledges the receipt of a Science Research Council postgraduate studentship.

Steady-State Urinary Excretion Method for Determining Bioequivalence of Conjugated Estrogen Products

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Received June 18, 1976, from the *Analytical Research and Development Laboratory and the Pharmacy Research and Development Laboratory, Ayerst Laboratories, Inc., Rouses Point, NY 12979.* Accepted for publication December 20, 1977.

Abstract □ The steady-state excretion of conjugated estrogens in the urine of postmenopausal women dosed with conjugated estrogens tablets was studied using a modification of a previously published method. The procedure was used to quantitate the estrogens both before and during conjugated estrogens replacement therapy. The method, which is relatively specific, involves enzyme hydrolysis of urine samples, a number of classical extraction and purification steps, and analysis of the silylated estrogens on a 2.7-m, 1.7% diethylene glycol succinate column using flame-ionization detection. The results indicate that steady-state urinary estrogen excretion levels were obtained within 17 days of dosing. Furthermore, the urinary estrogen excretion profile was significantly different from the composition of the estrogens in the dosage form.

Keyphrases □ Estrogens, conjugated—GLC analyses, steady-state urinary excretion in postmenopausal women □ Excretion, urinary—conjugated estrogens at steady state in postmenopausal women □ GLC—analyses, conjugated estrogens in urine of postmenopausal women

Conjugated estrogen mixtures have been used therapeutically since 1942. However, there is no detailed information concerning the human plasma or urinary levels of these estrogens after administration, primarily because of the lack of sufficiently sensitive and specific analytical methods.

BACKGROUND

Many recent reports focused on the analysis of estriol and other estrogens in pregnancy urine to follow or monitor fetal health (1–5). The urinary estrogen excretion levels in nonpregnant women (6, 7), in postmenopausal women (8–11), and in men (12, 13) were studied. Other investigators studied the types of conjugation of steroids and separated

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steroid conjugates into classes such as glucuronides, mixed glucuronide–sulfates, and sulfates (14–16).

Requirements for added specificity and sensitivity (picograms per milliliter of blood) brought about the use of newer techniques such as mass fragmentography (17, 18) and radioimmunoassay (19, 20). Mass fragmentography may possess the desirable properties required for a blood, plasma, or urine analysis, but major drawbacks are its expense and complexity. While radioimmunoassay may impart the necessary sensitivity required, it may not possess the necessary specificity to distinguish adequately between several structurally related steroids (21, 22). Attempts were made to improve radioimmunoassay specificity by coupling a chromatographic separation prior to quantitative radioimmunoassay (23, 24).

Estimates of bioavailability are conventionally determined from plasma level and/or urinary excretion data of the drug or its metabolite(s). The usual dose of conjugated estrogens is 2.50 mg or less daily on a cyclic schedule. Therefore, total plasma levels of each estrogen would be in the picogram per milliliter range following 2.50 mg po.

Conjugated estrogens prepared from natural sources contain at least nine estrogens (25). Extensive metabolic pathways, which are known only partially (26) or not at all, are possible for each estrogen component. These pathways include interconversion from one estrogen to another (26, 27). Thus, estrogen levels in the plasma or urine cannot be assumed to reflect only unchanged drug. For these reasons, it is believed that a true plasma bioavailability study of conjugated estrogens tablets would be extremely difficult.

An alternative approach, which has several advantages, is to examine the urinary excretion of the estrogens after dosing of subjects to the steady state. The estrogen concentrations in the urine are higher than in the blood, larger amounts of sample are available for analysis, and a direct quantitative comparison can be made between the daily dose and the 24-hr pooled urine samples. Furthermore, a previously developed specific GLC method for the analysis of conjugated estrogens in raw materials and finished dosage forms (25) was available for extensive modification and application.

The objectives of this investigation were to develop a sensitive and specific method to quantitate estrogens in the urine of postmenopausal subjects after dosing to the steady state and to relate the resultant urinary excretion profile to the composition of estrogens in the dosage form.

EXPERIMENTAL

Instrumentation—A research grade gas chromatograph[1] was equipped with a U-shaped racetrack glass column, a flame-ionization detector, and a recorder[2]. The 2.7-m × 3.5-mm i.d. column was silylated and packed with 1.7 ± 0.2% diethylene glycol succinate[3] on 100–120-mesh silanized Gas Chrom Z[3]. Thermogravimetric analysis was used to determine exactly the amount of liquid phase incorporated on the solid support. The column was conditioned with oxygen-free[4] helium carrier gas at 195 ± 5° overnight at ~60–80 ml/min.

Chromatographic Conditions—The following instrumental conditions were used: column oven temperature, 195 ± 5°; detector temperature, 240°; injection port temperature, 225°; helium flow rate, 60–80 ml/min; hydrogen flow rate, 40 ml/min; air flow rate, 1.8 standard cubic feet per hour; and attenuation, 4×10^{-11} amp.

Reagents and Standards—The following reagents and solvents were reagent grade quality or equivalent: absolute ethanol, ethylene dichloride, acetic acid, sodium hydroxide, sodium bicarbonate, anhydrous sodium sulfate, distilled dry chloroform, potassium hydroxide, carbon tetrachloride, and benzene. The nine free estrogens used to establish relative retention times and chromatographic response factors relative to 6-dehydroestrone[5] were house standards[6] that were assayed using phase solubility analysis. All were greater than 98.6% pure, except for 17α-dihydroequilenin, which was 97.2% pure.

Working Enzyme Solution—Sufficient enzyme concentrate[7] was diluted to 100 ml with distilled water to give a working solution containing about 500 ± 50 units of sulfatase/ml and about 2000 ± 200 units of glucuronidase/ml.

Standard Steroid Solutions—Standard solutions of the 10 steroids were prepared in absolute ethanol at the following approximate concentrations: 17α-estradiol, 1 μg/ml; 17β-estradiol, 0.8 μg/ml; 17α-dihydroequilin, 1.6 μg/ml; 17β-dihydroequilin, 1.6 μg/ml; 17α-dihydroequilenin, 1.3 μg/ml; 17β-dihydroequilenin, 1.3 μg/ml; estrone, 9.5 μg/ml; equilin, 3.5 μg/ml; equilenin, 4.0 μg/ml; and 6-dehydroestrone, 12.4 μg/ml.

Standard Solution—Aliquots of 1 ml of each of the 10 standards were pipetted into a 13-ml conical glass centrifuge tube equipped with a polytef-lined screw cap. Just before instrument standardization, the mixture was evaporated to dryness with nitrogen at 40° in a water bath. The residue was dissolved in 50 μl of anhydrous chloroform and 50 μl of silylating agent[8]. The standard was mixed well and allowed to stand for 15 min prior to injection. Silylated standards were prepared fresh daily.

(The very small amounts of the silylated estrogens required that special care be taken to avoid exposure of the sample to moisture.)

Preparation of Urine Extracts—*Hydrolysis*—A 24-hr urine collection was measured for total volume, and a sample was withdrawn for creatinine analysis (28, 29). Although interindividual urinary excretion of creatinine may vary over a wide concentration range, intraindividual excretion rates are relatively constant. By comparing a patient's creatinine levels over several 24-hr collection periods, an assessment of the completeness of a given 24-hr urine pool was made. All urines were refrigerated during the collection process and assayed as soon as possible after receipt. Urine samples were not frozen, and no bacteriostatic agents were added.

The urine pH was adjusted as necessary to pH 5.2 ± 0.2. Two 100.0-ml aliquots were removed from each urine sample and placed in separate 250-ml glass bottles fitted with polytef-lined screw caps. Working enzyme solution (5 ml) was added, and the bottles were flushed with nitrogen and capped immediately. Then the bottle contents were mixed and placed in a 50 ± 1° water bath for 48 hr.

Extraction—Into each bottle, 25.0 ml of ethylene dichloride[9] was pipetted, and the bottle was shaken mechanically for 30 min. The samples were centrifuged for 10 min at about 2200 rpm once, or twice if necessary, to obtain a relatively clear lower layer. The bulk of the urine was aspirated from the samples, and the organic extracts were transferred quantitatively into separate 60-ml glass bottles fitted with polytef-lined screw caps. A 10-ml aliquot of 5% sodium bicarbonate was added, and the bottle was shaken mechanically for 5 min and centrifuged, as already described, to break the emulsion. Most of the aqueous layer was aspirated carefully, and this bicarbonate wash sequence was repeated seven additional times.

The remaining ethylene dichloride extract was filtered through anhydrous sodium sulfate contained in a 4-cm diameter polyethylene funnel plugged with glass wool. Over 20 ml of extract was collected if possible. Then 20.0 ml of extract, or as much as possible, was pipetted into 60-ml glass bottles and evaporated to dryness with nitrogen in a 40° water bath. A 10-ml aliquot of 2 *N* KOH was added, and the mixture was shaken mechanically for 15 min. Then 10 ml of carbon tetrachloride[9] was added, and the bottle was shaken for 5 min and centrifuged for 5 min. Most of the lower organic layer was carefully removed and discarded. The washing sequence was repeated with a second 10-ml portion of carbon tetrachloride.

The remaining basic extract was chilled and, with swirling, brought to pH 1 with 3 *N* HCl. Then 15.0 ml of benzene[9] was added, and the bottle was shaken for 15 min and centrifuged. The lower layer was discarded. The organic layer was treated with three successive 10-ml bicarbonate washes, and then the benzene was filtered through anhydrous sodium sulfate. Over 12 ml was collected if possible.

Benzene extract, 12.0 ml, was pipetted into 13-ml conical glass centrifuge tubes fitted with polytef-lined screw caps. Then 1 ml of the internal standard solution was added, and the mixture was evaporated to dryness with nitrogen at 40°. To the dry residue, 50 μl of anhydrous chloroform and 50 μl of silylating reagent[8] were added. The solution was mixed carefully and allowed to stand for 15 min prior to injection.

(To prevent possible sample composition changes and degradation with time, samples were silylated only as needed. All silylated samples were carefully protected against exposure to moisture.)

Instrument Standardization—About 1–3 μl of silylated standard was injected onto a well-conditioned column, and the identity of the standard estrogen peaks was established by comparing their relative retention times (*RRT*) *versus* 6-dehydroestrone with those indicated in

[1] F&M model 400 equipped with a Keithley 417K electrometer.
[2] Honeywell Electronic 194.
[3] Applied Science Laboratories, State College, Pa.
[4] Oxy-Sorb Trap, Regis Chemical Co., Morton Grove, Ill.
[5] Searle Chemicals, Chicago, Ill.
[6] Ayerst Research Laboratories, Montreal, Quebec, Canada.
[7] Glusulase, Endo Laboratories, Garden City, N.Y.
[8] Regisil [bis(trimethylsilyl)trifluoroacetamide plus 10% trimethylchlorosilane], Regis Chemical Co., Morton Grove, Ill.
[9] Distilled-in-glass solvents, Burdick and Jackson Laboratories, Muskegon, Mich.

Table I—Recoveries of Estrogens from Urine Pool Aliquots Spiked within the Estrogen Concentration Range of Urine Samples after Dosing

Estrogen	Input[a], μg/100 ml	Mean Recovery[b], μg/100 ml	Mean Percentage Recovery	SD	CV, %
Sodium 17α-estradiol sulfate	0.69	0.80	115	0.06	7.4
	1.38	1.39	101	0.06	4.0
	2.76	2.56	92.9	0.33	12.7
	4.14	4.51	109	0.27	6.1
	6.21	6.68	108	0.13	1.9
			105 ± 8.5[c]		
17β-Estradiol	1.12	0.90	80.7	0.06	6.8
	2.24	1.89	84.4	0.13	6.9
	4.48	3.83	85.5	0.37	9.6
	6.72	5.99	89.2	0.30	5.0
	10.08	9.51	94.3	0.26	2.7
			86.8 ± 5.2		
17α-Dihydroequilin	0.54	0.38	71.0	0.10	26.6
	1.08	0.84	77.8	0.02	2.4
	2.16	1.67	77.2	0.06	3.4
	3.24	2.66	82.1	0.16	5.9
	4.86	3.91	80.5	0.10	2.5
			77.7 ● 4.3		
Sodium 17β-dihydroequilin sulfate	1.025	0.74	71.9	0.03	4.1
	2.05	1.45	70.9	0.06	4.4
	4.10	2.80	68.2	0.29	10.3
	6.15	4.83	78.5	0.31	6.3
	9.23	7.19	77.9	0.12	1.6
			73.5 ± 4.5		
17α-Dihydroequilenin	1.44	1.16	80.6	0.06	5.4
	2.88	2.43	84.3	0.04	1.7
	5.76	5.06	87.8	0.33	6.5
	8.64	8.09	93.6	0.34	4.2
	12.96	11.85	91.5	0.28	2.3
			87.6 ± 5.3		
Sodium estrone glucuronide	11.4	9.19	80.6	0.11	1.2
	22.8	18.78	82.4	0.12	0.6
	45.6	38.18	83.7	0.35	0.9
	68.4	55.64	81.3	1.34	2.4
	102.6	85.37	83.2	0.64	0.7
			82.2 ± 1.3		
Sodium equilin glucuronide	0.64	0.38	58.9	0.06	15.3
	1.28	0.79	61.7	0.06	7.7
	2.56	1.79	70.1	0.14	7.7
	3.84	2.70	70.2	0.09	3.4
	5.76	4.24	73.7	0.26	6.1
			66.9 ± 6.3		

[a] Three 100-ml urine aliquots from a male urine pool were spiked at each concentration level with aliquots from a stock solution containing all seven estrogens. [b] Results are corrected by subtraction of background peaks obtained from the corresponding blank urine samples. [c] The grand mean percentage recovery and its standard deviation are reported for each estrogen.

Fig. 1. The sample size and/or instrument attenuation were adjusted so that the larger estrogen peaks were more than 50% of full chart scale.

To determine the response factors, the peak heights were measured carefully. For peaks eluting early and on the tailing portion of the solvent front, an exponential decay of the front was assumed, and peak heights were measured to this drawn line. With the measured peak heights and known concentrations, an estrogen response factor (RF_e) was calculated for each component according to:

$$RF_e = \frac{P_d C_e}{C_d P_e} \qquad \text{(Eq. 1)}$$

where P_d is the peak height of 6-dehydroestrone in the standard, C_d is the micrograms of the internal standard (6-dehydroestrone) in the standard, C_e is the micrograms of estrogen in the standard, and P_e is the peak height of the estrogen peak in the standard. Initially with new columns, two standards were injected in succession to check for system stability and reproducibility. For well-conditioned columns, only single injections of the estrogen standard at the beginning and end of the assay day were required. These RF values were averaged for subsequent calculations.

Chromatographic Analysis—Following system standardization, 1–5 μl of the silylated urinary extracts was injected. The sample estrogen peaks were identified by comparing their RRT values *versus* 6-dehydroestrone with the RRT values generated for the standard. Typical standard and sample RRT values are given in Figs. 1–3. Standard and sample RRT values should agree to within ±2% for a given peak to establish the identity of a sample peak with some confidence. The peak heights of the identified estrogen peaks should then be measured.

The amounts of the individual estrogens present in the 24-hr urine pool, expressed as the sulfate conjugates, were calculated from:

$$\text{weight of conjugated estrogen } (\mu g/24 \text{ hr}) = 1.38\,(RF_e)\,(\text{dilution factor})\,(P_{es})\left[\frac{C_{ds}V_t}{P_d V_a}\right] \qquad \text{(Eq. 2)}$$

where 1.38 is a factor converting free estrogens into the corresponding sodium estrogen sulfate salts, dilution factor is (25 ml/20 ml) (15 ml/12 ml), P_{es} is the peak height of estrogen in the sample, C_{ds} is the micrograms of the internal standard (6-dehydroestrone) in the sample, P_{ds} is the peak height of 6-dehydroestrone in the sample, V_t is the total volume of the 24-hr urine pool in milliliters, and V_a is 100 ml (aliquot of urine taken into assay).

Clinical Study Protocol—Twelve postmenopausal female subjects[10] were selected. The postmenopausal syndrome was established by documented physiological or surgical menopause of not less than 2 years and anestrogenism established by colpocytogram. The average age of the eight physiologically menopausal subjects was 52 years (range of 50–57), and that of the four surgically menopausal subjects was 44 years (range of 37–54). The average weight of all subjects was 62 kg (range of 54–73). All subjects were in good health as established by physical examination, serum biochemistry tests[11], and urinalysis (pH, glucose, and protein)[12].

A 28-day washout period permitted urinary estrogen levels of those subjects previously taking estrogen medication to return to baseline

[10] Each subject signed a voluntary informed consent form.
[11] SMA 12/60.
[12] Combistix, Ames Co., Elkhart, Ind.

Table II—Elution Sequence and Separation of Estrogen Steroids

Component[a]	Relative Retention Time *versus* 6-Dehydroestrone
16-Estratetraene	0.094
17α-Estradiol[b]	0.130
17β-Estradiol[b]	0.154
Estradiol benzoate	0.160
17α-Dihydroequilin[b]	0.166
17β-Dihydroequilin[b]	0.195
Estriol	0.197
17α-Dihydroequilenin[b]	0.342
Methylestradiol methyl ether	0.378
17β-Dihydroequilenin[b]	0.389
3-Desoxyestrone	0.425
Estra-4-ene-3-ol-17-one	0.491
$\Delta^{5,7,9}$-Estratrien-3-ol-17-one	0.624
16-Hydroxyestrone	0.646
17α-Estradiol 17-acetate	0.657
Estrone[b]	0.781
16-Oxo-17β-estradiol	0.782
6-Oxoestradiol	0.805
Equilin[b]	0.875
9-Dehydroestrone	0.914
8-Dehydroestrone	0.953
6-Dehydroestrone	1.00
14-Isoequilenin	1.30
2-Methoxyestrone	1.46
1-Methylestrone	1.58
Equilenin[b]	1.65

[a] All components are eluted as the trimethylsilyl ethers. [b] Components tested for and quantitated in this study.

levels. At the end of this washout period, a 24-hr urine sample was collected from each subject. Each subject then received two 1.25-mg conjugated estrogens tablets[13] daily for 21 days. Each subject was instructed to select a time for dosing each morning and to take the tablets at the same time each morning. On Days 17–19 of the dosing period, three consecutive 24-hr urine samples were collected from each subject. The 24-hr collection period began at the time of dosing. Diet was unrestricted during the study, and each subject was instructed to take no additional medication.

Three subjects who participated in a study at the 2.50-mg dose level participated in a second study at the 1.25-mg dose level. The average age of the two surgically menopausal subjects and one physiologically menopausal subject was 42 years (range of 38–51), and their average weight was 62 kg (range of 55–71). The urinary estrogen levels of these subjects were determined for three consecutive 24-hr urine samples (Days 17–19) during cyclic dosing of one 1.25-mg conjugated estrogens tablet[13]

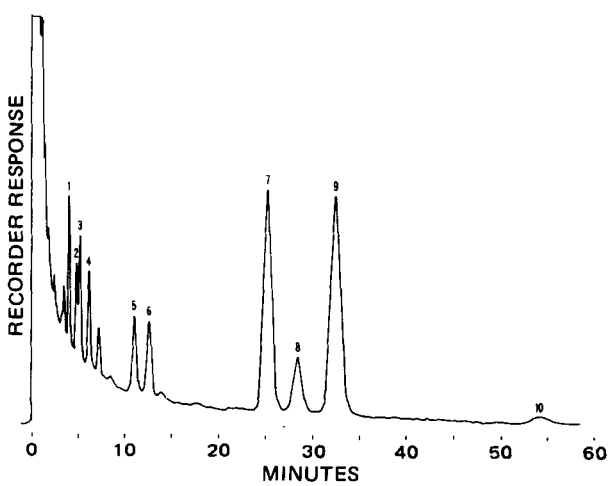

Figure 1—*Separation of the nine estrogen components and the internal standard, 6-dehydroestrone, as the trimethylsilyl ethers. The peaks, together with their relative retention times, are: 1, 17α-estradiol, 0.133; 2, 17β-estradiol, 0.157; 3, 17α-dihydroequilin, 0.167; 4, 17β-dihydroequilin, 0.197; 5, 17α-dihydroequilenin, 0.347; 6, 17β-dihydroequilenin, 0.394; 7, estrone, 0.780; 8, equilin, 0.878; 9, 6-dehydroestrone, 1.00; and 10, equilenin, 1.667.*

[13] Premarin, Ayerst Laboratories, New York, N.Y.

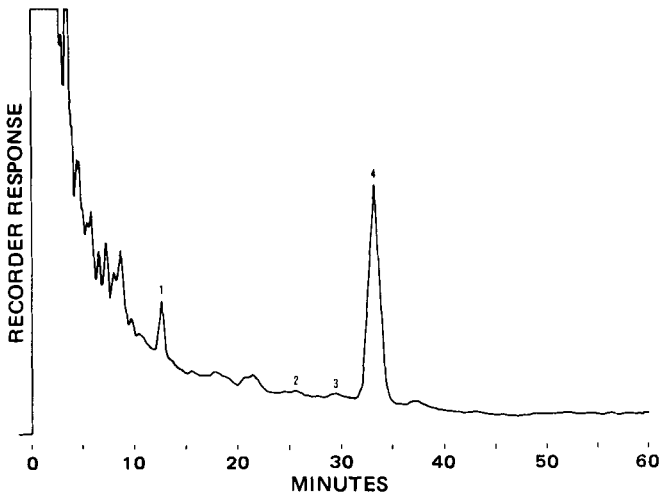

Figure 2—*Chromatogram of the trimethylsilyl ethers of the endogenous estrogens present in the urine of a postmenopausal female. The peaks, identified by comparison of their relative retention times with standards, are: 1, 17β-dihydroequilenin, 0.386; 2, estrone, 0.776; 3, equilin, 0.888; and 4, 6-dehydroestrone, 1.00.*

daily. This study was performed several months after a return from the 2.50-mg daily dose to the 1.25-mg daily dose.

RESULTS AND DISCUSSION

Sample Preparation—Documented steroid decomposition reactions, induced by acid hydrolysis of the conjugates (30, 31), led to the use of mild enzyme hydrolysis of urine samples coupled with controllable classical organic extraction and purification steps. The objective of this study was to determine the total daily amount of estrogens excreted in dosed patients at the steady state. Therefore, there was no need to differentiate between free and conjugated estrogens. The sample preparation used here produced an extract of free estrogens, regardless of the manner or form in which they were excreted.

For long-term storage of urine samples, freezing would be desirable. However, nonfrozen, fresh urine samples yielded higher precision and greater estrogen recoveries than did frozen samples. Therefore, during collection, all samples were stored at 4° without preservatives and were assayed as soon as possible.

The hydrolysis and extraction steps evolved as attempts were made to produce an extract containing reproducible and known recoveries of estrogens, sufficiently free from other urinary components, to allow their separation and quantitation. Following a 48-hr hydrolysis, the estrogen

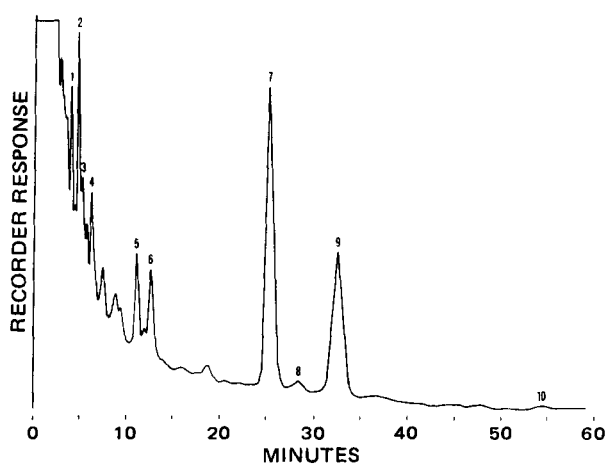

Figure 3—*Chromatogram of the trimethylsilyl ethers of the estrogens present in the urine of a postmenopausal female dosed to the steady state with conjugated estrogens tablets. The peaks, together with their relative retention times* versus *6-dehydroestrone, are: 1, 17α-estradiol, 0.133; 2, 17β-estradiol, 0.157; 3, 17α-dihydroequilin, 0.169; 4, 17β-dihydroequilin, 0.198; 5, 17α-dihydroequilenin, 0.345; 6, 17β-dihydroequilenin, 0.391; 7, estrone, 0.777; 8, equilin, 0.872; 9, 6-dehydroestrone, 1.00; and 10, equilenin, 1.664.*

Table III—Estimated Minimum Quantifiable Estrogen Levels in 24-hr Urine Pools

Sodium Estrogen Sulfate[a]	Minimum Quantifiable Amount[b], μg/24 hr
17α-Estradiol	0.9
17β-Estradiol	1.1
17α-Dihydroequilin	1.4
17β-Dihydroequilin	1.6
17α-Dihydroequilenin	2.8
17β-Dihydroequilenin	2.8
Estrone	5.9
Equilin	2.1
Equilenin	18.7

[a] Although the estrogens are excreted primarily as glucuronides, components are expressed as the sulfate conjugates to aid direct comparison with the tablet content. [b] Based on the amount of estrogen producing a peak 1% of chart scale at 4×10^{-11} amp full scale and an average daily urine volume of 1500 ml. Results represent an average of six separate determinations performed over 3 months.

steroids and other organics were partitioned into ethylene dichloride. This hydrolysis time was based on reported literature times of 24–48 hr (32, 33), together with our own experience and desire to ensure a complete conversion of the estrogen conjugates into the corresponding free steroids. This first partitioning left behind much of the water-soluble organics.

The remaining acids and phenolic acids in the extract, perhaps by weight the largest undesirable fraction, were removed by successive bicarbonate washes. This step also acted to control pH and to minimize steroid decomposition by removing the organic acids. Next, neutral steroids and other organic, nonphenolic by-products were removed by the carbon tetrachloride washes of the formed potassium phenolate salts. The regenerated phenols, washed again with bicarbonate solution, were then ready for GLC analysis. This combination of partitioning and extraction steps gave reproducible recoveries, and the peak patterns obtained for a given assay pair from a urine pool were almost superimposable.

Recovery data were generated for seven of the estrogen steroids (Table I). Ingested estrogen sulfates are excreted as the intact glucuronides, sulfates, free phenols, or mixed glucuronide–sulfate conjugates. Therefore, to show the broad utility of the method and its ability to measure total urinary estrogen excretion regardless of the manner in which the steroid may or may not be conjugated, the recovery studies utilized some of these different conjugate species.

A fresh urine pool was collected from an adult male. The estrogens to be spiked were added to the empty hydrolysis bottles, and the solvent was removed with nitrogen prior to the addition of 100-ml urine aliquots. Three aliquots of the urine pool were assayed at each of the five estrogen concentration ranges together with an unspiked or blank sample. The blank values were subtracted from the found amounts to obtain the recoveries indicated. Although there was a range of recoveries for the group of compounds as a whole, the variation of recoveries for each individual component was acceptable.

In the recovery study reported in Table I and in parallel with an earlier study, when 17α-dihydroequilin, 17β-dihydroequilin, and/or equilin were added to the urine aliquots, their corresponding oxidation products, 17α-dihydroequilenin, 17β-dihydroequilenin, and equilenin, respectively, could be detected and measured. For each estrogen, a relatively constant percentage of the oxidation product was detected. Thus, even with the temperature, nitrogen flushing, pH, and hydrolysis controls used in this method, approximately 12% of the equilin-type compounds was lost through oxidation. Other reported techniques utilizing more vigorous experimental steps, including acid hydrolysis, probably would cause even more significant composition shifts than those observed here.

Analysis—With the large number of known circulating estrogens and estrogen metabolites found in women (5, 34), a technique for resolving potentially complex mixtures of urinary estrogens was needed. For example, when 17β-estradiol was administered to pregnant women, seven individual metabolites were identified (35). Therefore, when patients are placed on conjugated estrogens therapy with drugs containing a mixture of estrogen steroids, the urinary profiles can be expected to contain numerous peaks. However, to keep the project and assay work within reasonable confines, only those estrogens present in the dosage form were quantitated and, without a gas chromatograph–mass spectrometer, no attempt was made to identify other metabolites.

The primary aim in this study was to quantitate the nine known estrogen steroids indicated in Fig. 1. The GLC method published earlier (25) was suitable for analyzing a minimum of nine separate estrogen steroids in dosage forms and appeared promising for use in analyzing urinary estrogen mixtures. The modifications discussed earlier evolved as the procedure was applied to more dilute and complex mixtures. A new internal standard was chosen to elute in one of the relatively few peak-free areas in the chromatograms to allow reproducible quantitation and peak identification.

Figure 1 illustrates the peak pattern obtained for a mixture of estrogen standards at a level approximating that required for the analysis of urine samples from dosed postmenopausal subjects. The amount of material on-column was 15–30 ng for the estrogen diols and 50–250 ng for the estrogen ketones. Because of the baseline obtained, normal disk and digital integration techniques were unsatisfactory for routinely and reproducibly measuring peak areas. Satisfactory quantitation was achieved using peak heights where operator judgment could quickly and reproducibly assign heights to the numerous peaks of interest.

To demonstrate method specificity, 26 different estrogen steroids were analyzed; their retention times were measured relative to 6-dehydroestrone. Except for two pairs whose *RRT* values were within 1% of each other, the procedure provided sufficient resolution for separation and identification of the remaining 22 estrogens. These estrogens, together with their relative retention times *versus* 6-dehydroestrone, are identified in Table II. Although the ultimate in specificity is probably a GLC–mass spectrometry system utilizing either electron impact or chemical ionization together with specific ion detection, such equipment is unavailable to most laboratories.

Method specificity can be expressed in several ways, and frequently a signal twice the noise level is used. However, in working with biological fluids, many unknown components and impurities in trace amounts are retained in the sample. If one reviews a scan of one of these samples, many minor background peaks emerge above the baseline that are of questionable significance. Therefore, a different, more stringent sensitivity estimate was made here to eliminate and identify false positives.

At the normal operating attenuation (4×10^{-11} amp full scale), all peaks less than 1% full scale were considered as background. The amount of estrogen required to produce a peak of this height was estimated from standards injected at the operating attenuation used in the analysis of samples from dosed females. With this quantity of estrogen standard known, an estimate could be made of the minimum amount that could be detected in a 24-hr urine pool. This estimate of method sensitivity was done for nine estrogens (Table III).

Table IV—Endogenous and Steady-State Urinary Estrogen Excretion Levels of 12 Postmenopausal Subjects

Sodium Estrogen Sulfate	Endogenous Level, μg/24 hr	Steady-State Level[a], μg/24 hr			
		Day 17	Day 18	Day 19	Mean
17α-Estradiol	10.0	32.7	30.3	32.0	31.7
17β-Estradiol	9.2	65.8	69.1	67.6	67.5
17α-Dihydroequilin	1.6	26.6	20.2	25.2	24.0
17β-Dihydroequilin	1.3	28.1	25.5	28.5	27.4
17α-Dihydroequilenin	3.9	72.2	66.5	69.4	69.4
17β-Dihydroequilenin	—	—[b]	—[b]	—[b]	—
Estrone	8.1	330.2	341.0	319.9	330.4
Equilin	3.4	15.0	13.3	14.5	14.3
Equilenin	—	—[c]	—[c]	—[c]	—
Total[d]	37.5	549.3	565.7	545.1	553.4

[a] Values are the means for 12 subjects with the exception of estrone and equilin for Days 18 and 19, which are the means for 11 subjects only. Steady-state levels are uncorrected for endogenous levels or the analytical losses reported in Table I. [b] Most peaks masked by urinary impurities. [c] Quantities are below the sensitivity limit of the method. [d] Totals represent the data of 11 subjects only.

Table V—USP XIX and GLC Analyses [a] of 1.25-mg Conjugated Estrogens Tablets [b] USP

Sodium Estrogen Sulfate	GLC Assay		USP XIX Colorimetric Assay [c]	
	mg/Tablet	%	mg/Tablet	%
Estrone	0.773	50.0	0.69	52.3
Equilin	0.403	26.1	0.35	26.4
Equilenin	0.037	2.4		
17α-Estradiol	0.048	3.1		
17β-Estradiol	0.010	0.6		
17α-Dihydroequilin	0.220	14.3		
17β-Dihydroequilin	0.027	1.7		
17α-Dihydroequilenin	0.020	1.3		
17β-Dihydroequilenin	0.008	0.5		
Total	1.546	100.0	1.31	

[a] The two methods give different assay values because of major differences in the analytical methods used. [b] D403 WXA. [c] USP XIX assay includes only the three individual quantitative tests shown.

Urinary Estrogen Levels—The steady-state urinary estrogen excretion after administration of 2.5 mg of conjugated estrogens was determined using the described method. Table IV presents the mean endogenous levels and the mean levels for each estrogen and their total obtained on Days 17, 18, and 19 of the dosing period. The mean of the 3-day excretion is also presented. The total endogenous levels observed were similar to values reported previously (10, 11).

A typical GLC scan of a washout urine sample is shown in Fig. 2. The dosed data clearly indicate the increased quantities of urinary estrogens expected from subjects receiving conjugated estrogens replacement therapy. The absence of an increasing trend in the excretion levels for these 3 successive days establishes that the study was performed under steady-state conditions. Figure 3 is an example of the estrogen excretion patterns found in the dosed subjects.

The USP and GLC assays of the conjugated estrogens tablets are shown in Table V. The mean total recovery of estrogens in the urine was about 17% of the daily ingested amount, based on the GLC assay. This value was obtained after subtraction of the mean endogenous amounts and the amounts of equilenin and 17β-dihydroequilenin sodium sulfates from the tablet content since these estrogens were not quantitated in the urine. The recovery values presented in Table I documented only the general completeness of extraction. Since the predominant conjugates excreted in the urine and their recoveries may vary from those presented, no correction for analytical losses was made to the recovery value of 17%.

Linear pharmacokinetic processes are often assumed in bioavailability and bioequivalence studies. To verify the linear elimination of the conjugated estrogens, the excretion of estrogens was compared in three subjects after dosing to the steady state with 1.25 and 2.50 mg daily. Table VI presents the mean amounts of each estrogen or estrogen metabolite in the urine as a percentage of the dosed amount. With the exception of 17α-estradiol, a close agreement was observed in the percentage excretion of each estrogen or estrogen metabolite at the two dose levels. This agreement indicates the absence of dose dependency within this dosing range, a result anticipated with drugs whose dose is very low. The reason for the decreased 17α-estradiol at the higher dose level is not apparent but may indicate a minor interfering peak in the urine sample of relatively greater importance at the lower dose. The period of time allowed to attain the steady state prohibits obtaining a blank urine immediately prior to sampling, which could eliminate this problem.

The inadequacy of a steady-state urinary excretion method to determine bioavailability is recognized. Neither the rate nor the absolute extent of absorption can be determined. As suggested by the low excretion levels of equilin and 17β-dihydroequilin and the high excretion levels of 17β-estradiol, the complexity in the metabolism of the estrogens results in a urinary estrogen excretion pattern substantially different from that of the composition of conjugated estrogens tablets. Nevertheless, the present method for the first time permits the extent of urinary excretion of seven estrogens to be determined after administration of conjugated estrogens tablets to the steady state.

Relative magnitudes of absorption and elimination half-lives of estrogens may be estimated from recent studies in which estrone sulfate (36) and 17β-estradiol (27) were administered orally and their plasma levels were determined. Estrone sulfate was administered as an oral solution; micronized 17β-estradiol was administered as a tablet. With a one-compartment model with first-order absorption and elimination, approximate ratios of the elimination half-life to the absorption half-life may be estimated at 14 for estrone sulfate and at eight for 17β-estradiol. It is generally recognized that for drugs with long elimination half-lives relative to their absorption half-lives, the steady-state plasma concentrations are relatively insensitive to the absorption rate but do depend on the extent of absorption. Thus, a comparison of the extent of urinary estrogen excretion after administration of conjugated estrogens products from multiple sources should provide one means of assessing their bioequivalence.

Table VI—Percentage Excretion of Individual Estrogens in Three Subjects at the Steady State as a Function of Conjugated Estrogens Dosed

Estrogen	Mean Excretion, % ± SD [a]	
	From 1.25-mg Dose of Conjugated Estrogens USP	From 2.50-mg Dose of Conjugated Estrogens USP
17α-Estradiol [b]	48.6 ± 7.4	36.1 ± 11.6
17β-Estradiol [c]	5.3 ± 0.9	5.1 ± 2.1
17α-Dihydroequilin [b]	5.8 ± 0.4	4.9 ± 3.2
17β-Dihydroequilin [c]	4.7 ± 1.2	5.0 ± 2.5
17α-Dihydroequilenin [c]	16.3 ± 3.9	18.9 ± 9.7
Estrone [b]	25.5 ± 7.2	26.8 ± 13.0
Equilin [b,d]	—	1.9 ± 1.2

[a] Values were calculated from the mean excretion during Days 17–19 for each subject and are uncorrected for endogenous levels or analytical losses. [b] Values are the percentages of the indicated estrogens excreted in the urine relative to the dosed amounts of those estrogens. [c] Values are the percentages of 17β-estradiol, 17β-dihydroequilin, and 17α-dihydroequilenin excreted in the urine relative to the dosed amounts of estrone, equilin, and 17α-dihydroequilin, respectively. A subsequent paper will present evidence suggesting that the major fraction of the former estrogens are derived from the latter. [d] The equilin peak was masked by an interfering peak in two of the three subjects at the 1.25-mg dose.

REFERENCES

(1) V. Ratanasopa, A. Schindler, T. Lee, and W. Hermann, *Am. J. Obstet. Gynecol.*, **99**, 295 (1967).

(2) W. Fischer-Rasmussen, *Dan. Med. Bull. Suppl. II*, **19**, 1 (1972).

(3) A. Howarth and D. Robertshaw, *Clin. Chem.*, **17**, 316 (1971).

(4) Y. Osawa and W. R. Slaunwhite, Jr., *Steroids*, **15**, 73 (1970).

(5) G. Adessi, D. Eichenberger, T. Nhuan, and M. Jayle, *ibid.*, **25**, 553 (1975).

(6) C. Longcope and K. Williams, *J. Clin. Endocrinol. Metab.*, **38**, 602 (1974).

(7) R. Hobkirk, *J. Steroid Biochem.*, **5**, 15 (1974).

(8) G. Goertz, O. Crepy, O. Judas, J. Longchampt, and M. Jayle, *Clin. Chim. Acta*, **51**, 277 (1974).

(9) V. Goebelsmann, *ibid.*, **43**, 285 (1973).

(10) M. Furuhjelm, *Acta Obstet. Gynecol. Scand. Suppl. 7*, **43**, 42 (1964).

(11) G. Dove, F. Morley, A. Batchelor, and S. Lunn, *J. Reprod. Fertil.*, **24**, 1 (1971).

(12) J. Fishman, I. Yoshizawa, and L. Hellman, *Steroids*, **22**, 401 (1973).

(13) H. Kaplan and M. Hreshchyshyn, *Am. J. Obstet. Gynecol.*, **111**, 386 (1971).

(14) M. Matsui, M. Hakozaki, and Y. Kinuyama, *J. Chromatogr.*, **115**, 625 (1975).

(15) M. Levitz and J. Katz, *J. Clin. Endocrinol. Metab.*, **28**, 862 (1968).

(16) R. Haehnel, *J. Steroid Biochem.*, **1**, 299 (1970).

(17) H. Adlercreutz and D. Hunneman, *ibid.*, **4**, 233 (1973).

(18) H. Adlercreutz, M. Tikkanen, and D. Hunneman, *ibid.*, **5**, 211 (1974).

(19) B. Lindberg, E. Johansson, and B. Nilsson, *Acta Obstet. Gynecol. Scand., Suppl.*, **32**, 21 (1974).

(20) B. Desfosses, S. DeLauzon, O. Judas, and M. Jayle, *Steroids Lipids Res.*, **4**, 86 (1973).

(21) A. Bolton and F. Rutherford, *J. Steroid Biochem.*, **7**, 71 (1976).

(22) E. Cerceo and C. Elloso, *Anal. Chem.*, **46**, 1578 (1974).

(23) D. Loriaux, H. Ruder, D. Knab, and M. Lipsett, *J. Clin. Endocrinol. Metab.*, **35**, 887 (1972).

(24) A. Jakobovits, *Kiserl. Orvostud.*, **25**, 275 (1973).

(25) R. Johnson, R. Masserano, R. Haring, B. Kho, and G. Schilling, *J. Pharm. Sci.*, **64**, 1007 (1975).

(26) R. I. Dorfman and F. Ungar, "Metabolism of Steroid Hormones," Academic, New York, N.Y., 1965, chap. 8.

(27) S. S. C. Yen, P. L. Martin, A. M. Burnier, N. M. Czekala, M. O.

Greaney, Jr., and M. R. Callantine, *J. Clin. Endocrinol. Metab.*, **40**, 518 (1975).
(28) S. Pino, J. Benotti, and H. Gardyna, *Clin. Chem.*, **11**, 664 (1965).
(29) M. Metcalf and E. Hunt, *Clin. Biochem.*, **9**, 75 (1976).
(30) C. Muehlbaecher and E. Smith, *Clin. Chem.*, **16**, 158 (1970).
(31) L. Cawley, B. Musser, W. Faucette, S. Beckloff, and H. Learned, *ibid.*, **11**, 1009 (1965).
(32) F. Vandenheuvel, *Can. J. Biochem.*, **45**, 191 (1967).
(33) H. C. Curtius and M. Muller, *J. Chromatogr.*, **30**, 410 (1967).
(34) N. Taylor and C. Shackleton, *Steroids*, **24**, 185 (1974).
(35) R. Hobkirk and M. Nilsen, *J. Clin. Endocrinol. Metab.*, **26**, 625 (1966).
(36) H. J. Ruder, L. Loriaux, and M. B. Lipsett, *J. Clin. Invest.*, **51**, 1020 (1972).

ACKNOWLEDGMENTS

The authors thank Dr. G. Myers for supplying the free estrogens and the estrogen conjugates used in the development of this procedure, Dr. J. Hasegawa for constructive comments and criticism, Mr. R. Haring for work in preparing the high quality column packings required for this work, and Dr. J. Southwick for medical supervision of the subjects.

Characterization of Pharmacologically Important Prototropic Species Derived from a Pyridinemethanol Antimalarial by Electronic Absorption and Fluorescence Spectroscopy

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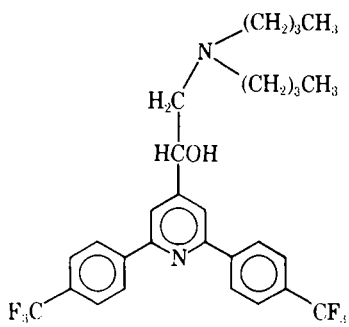
Received September 19, 1977, from the *School of Pharmacy, University of Georgia, Athens, GA 30602.* Accepted for publication December 21, 1977.

Abstract □ Variations of the absorption and fluorescence spectra of the experimental antimalarial drug, α-dibutylaminomethyl-2,6-bis(*p*-trifluoromethylphenyl)-4-pyridinemethanol, were investigated throughout the pH region in concentrated sulfuric acid media and in *n*-hexane. The predominant prototropic species at physiological pH is the singly charged cation. In the pH 6–12 region, the structured fluorescence of the monocation is quenched with the concomitant appearance of a diffuse, long wavelength emission while the corresponding absorption spectra shift only slightly to longer wavelengths. Furthermore, the dibutylamino group exhibits an unusually low basicity. This behavior is explained as due to the formation of an intramolecular hydrogen bond in the neutral molecule in the ground and lowest excited singlet states. A similar intramolecular hydrogen bond in the monocation is not spectroscopically visible.

Keyphrases □ Pyridinemethanol, substituted—prototropic species characterized from electronic absorption and fluorescence spectra at various pH values and in *n*-hexane □ Electronic absorption spectroscopy—used to characterize prototropic species of substituted pyridinemethanol at various pH values and in *n*-hexane □ Fluorescence spectroscopy—used to characterize prototropic species of substituted pyridinemethanol at various pH values and in *n*-hexane □ Antimalarial agents, potential—α-dibutylaminomethyl-2,6-bis(*p*-trifluoromethylphenyl)-4-pyridinemethanol, prototropic species characterized from electronic absorption and fluorescence spectra of various pH values and in *n*-hexane

A limited number of pyridinemethanol derivatives have been synthesized and screened for antimalarial activity (1–3). However, most of these compounds demonstrate either little activity or undesirable, toxic side effects (1). One of the few compounds of this class to exhibit antimalarial activity is the investigational drug α-dibutylaminomethyl-2,6-bis(trifluoromethylphenyl) -4- pyridinemethanol (I). It is 22 times more effective than quinine and is active against highly chloroquine-resistant *Plasmodium berghei* in mice (1). Although its specific mechanism of activity is unknown, it may be similar to, but not identical with, that of either chloroquine or quinine (1).



I

Most antimalarial compounds that exert their activity through a mechanism other than disruption of plasmodia nucleotide anabolism have at least one common feature. At physiological pH, these compounds exist as the singly charged cations or, as with chloroquine and quinacrine, as the doubly charged cation (4, 5). The cationic charge is believed to be involved in the binding of acridines, 9-aminoquinolines, and 8-aminoquinolines to parasitic DNA, and this binding is presumed to be related to antimalarial activity (6). Little is known about the mechanism of activity of quinine, alkaloids related to quinine, pyrimidines, quinolinemethanols, and pyridinemethanols. However, the singly charged cations of these agents may bind to plasmodia nuclear proteins, plasmodia DNA, or various enzymes in the metabolic network of these parasites (1, 7).

The present study of the dependence of the electronic spectra on the state of protonation and solvent properties of I was undertaken to elucidate the electronic structures of this compound and ultimately its interaction with native DNA, serum proteins, and selected enzymes.

0022-3549/78/0900-1224$01.00/0