than 93% and not more than 107% for tablets of less than 300 mg.

Determinations of five potassium permanganate 300-mg. tablet samples were performed. The mean percent of the labeled amount was 99.65 with a *SD* of 0.43 when assayed spectrophotometrically and 99.89 with a *SD* of 0.59 when assayed titrimetrically.

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Quantitative Separation of Free Estrogens by Liquid Partition Chromatography

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Abstract □ A mixture of structurally related estrogens containing estrone, equilin, equilenin, 17α-estradiol, 17α-dihydroequilin, and 17α-dihydroequilenin was separated by a partition column chromatographic system based on a lipophilic polydextran stationary support and a composite organic solvent. The chromatographic column yielded 25 theoretical plates per centimeter of column height at 0.6 ml./min. flow rate; column efficiency was studied as a function of solvent flow rate, and an inverse relationship between the two parameters was observed. Since the complete separation of the six estrogens required 10 hr., the column was shortened to yield a 3-hr. elution, which separated completely four of the estrogens with only partial overlap between estrone and equilin. The overlap between estrone and equilin was resolved quantitatively by specific colorimetric and fluorometric determinations. The method may be scaled up for preparative purposes, it is applicable to other steroids that are too labile for gas chromatography, and the same chromatographic column can be used repeatedly.

Keyphrases □ Estrogens, free—quantitative separation □ Chromatography, liquid partition—separation □ Fluorometry—analysis □ Colorimetric analysis—spectrophotometer □ UV spectrophotometry—analysis

The separation and quantitative determination of estrogens were studied by a number of investigators. The problem was already approached by gas chromatography (1, 2), TLC (3), and liquid chromatography (4–6). However, no quantitative chromatographic method applicable to an estrogen mixture containing closely related structures such as estrone, equilin, equilenin, estradiol, dihydroequilin, and dihydroequilenin was reported in the literature. Although this study is based only on the analysis of an arbitrary mixture of these synthetic free estrogens, the sulfate esters of these free estrogens are the principal ingredients of the naturally occurring estrogenic hormones (7, 8).

Another consideration was the need for a chromatographic procedure that would be applicable to the separation of structurally related free estrogens for preparative purposes. Liquid column chromatography is ideally suited for this purpose. Furthermore, such a system may also be applicable to the analysis of other steroid structures that are too labile for gas chromatography. For example, Vandenheuvel and Horning (9) observed that gas chromatography of C-21 steroids containing an α-ketol side chain led to side-chain cleavage. Other internal rearrangements of C-21 steroids were observed by Brooks (10). Such rearrangements could lead to complications in the quantitation and the

Table I—Elution Volumes of Free Estrogens as a Function of the Solvent System

Solvent System[a]	Estrone N[e]	Estrone El. Vol.[f]	Estrone Fr. Vol.[g]	Estrone Ovl. Vol.[h]	Equilin El. Vol.	Equilin Rel. Vol.[i]	Equilin Fr. Vol.	Equilenin El. Vol.	Equilenin Rel. Vol.	Equilenin Fr. Vol.	Estradiol[b] El. Vol.	Estradiol[b] Fr. Vol.	DHEQ[c] El. Vol.	DHEQ[c] Fr. Vol.	DHEQN[d] El. Vol.	DHEQN[d] Fr. Vol.
A	1770	88	12	4.5	97	1.10	14	116	1.32	24						
B	1090	80	13	5.5	88	1.10	14	104	1.30	22						
C	910	75	17	8.5	83	1.11	16	96	1.28	20	161	24	185	26	230	32
D	1500	112	18	3.0	127	1.12	18	164	1.45	28	226	32	269	36		
E	700	71	18	9.0	80	1.13	18	99	1.40	19	138	31	159	33	211	46
F	950	62	16	6.0	70	1.13	12	84	1.36	20	111	22	132	26	160	40

[a] Solvent system: A = cyclohexane–xylene–methanol–triethylamine (400:400:75:5); B = cyclohexane–toluene–methanol–trimethylamine (400:400:75:5); C = cyclohexane–benzene–methanol–trimethylamine (400:400:75:5); D = cyclohexane–benzene–methanol (500:150:75); E = cyclohexane–benzene–methanol (500:150:75); and F = cyclohexane–benzene–methanol–trimethylamine (500:150:75:5). Column height for Systems A, B, C, and D = 65 ± 5 cm.; and column height for Systems E and F = 45 ± 2 cm. All elutions were carried out at 0.6 ± 0.1 ml./min. flow rate. All volumes except the relative volume are expressed in milliliters. [b] 17α- or 17β-Estradiol. [c] 17α- or 17β-Dihydroequilin. [d] 17α- or 17β-Dihydroequilenin. [e] Theoretical plate number; based on elution volume and peak width at half peak height. [f] The elution volume of a given estrogen at its maximum concentration. [g] The total volume of eluent containing a given estrogen fraction. [h] The overlap volume between estrone and equilin. [i] The elution volume of a given estrogen relative to the elution volume of estrone (estrone relative volume = 1).

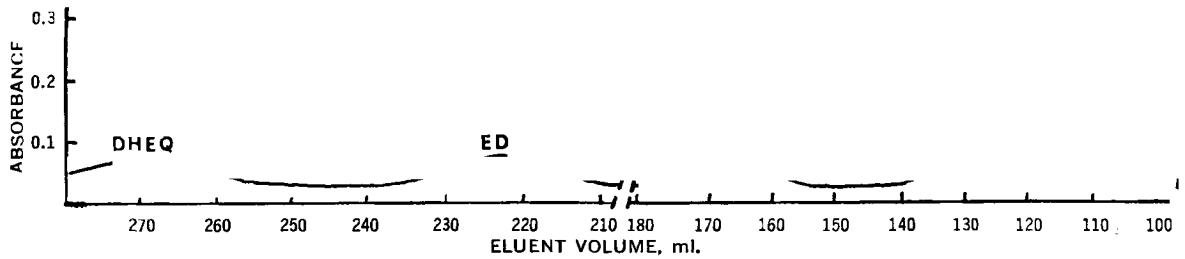

Figure 1—*Separation of estrone (ES), equilin (EQ), equilenin (EQN), estradiol (ED), and dihydroequilin (DHEQ) on Sephadex LH-20 (0.9 × 68-cm. column) with cyclohexane–benzene–methanol (500:150:75) solvent. Flow rate: 0.6 ± 0.1 ml./min.*

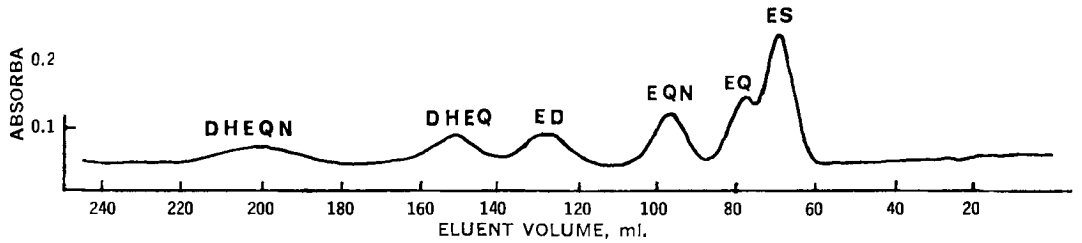

Figure 2—*Separation of estrone (ES), equilin (EQ), equilenin (EQN), estradiol (ED), dihydroequilin (DHEQ), and dihydroequilenin (DHEQN) on Sephadex LH-20 (0.9 × 45-cm. column) with cyclohexane–benzene–methanol (500:150:75) solvent. Flow rate: 1.6 ± 0.1 ml./min.*

degree of specificity of the gas chromatographic separation.

The chromatographic approach selected in this study was based on the need for a selective separation system which would be applicable to both analytical and preparative purposes. The choice of a partition chromatographic system involving a lipophilic polydextran gel support and a nonaqueous solvent system was suggested by the work of Nystrom and Sjovall (11) and Seki (12) with other steroids. However, the previous work (11, 12) was not quantitative and not directly applicable to the present requirements.

The chromatographic system reported in this paper yielded 3020 theoretical plates and separated the six estrogens. However, the complete separation required 10 hr. Since the rate-determining step is the separation of estrone and equilin, the authors decided, for practical considerations, to base the quantitative analytical method on a less time-consuming chromatographic system which separates all of the estrogens except estrone and equilin. The short column separation required 3 hr. Furthermore, the partial overlap between estrone and equilin was resolved quantitatively by three independent nonchromatographic procedures. One procedure depended on a colorimetric reaction which was specific for equilin (13). The other two depended on a relatively higher specific fluorometric (14) and colorimetric response of estrone as compared to equilin after heating in a sulfuric acid–methanol–water solution. The latter procedures were modified to enhance the selectivity for estrone.

Since the chromatographic solvent selected for the complete separation of the estrogen mixture was relatively volatile and inert, the separation may be adapted to preparative purposes. An analogous chromatographic system was also found applicable to C-21 steroid structures containing labile side chains and to steroids that are prone to dehydration under gas chromatographic conditions (15).

EXPERIMENTAL

Apparatus—All chromatographic separations were carried out in 0.9-cm. (i.d.) and 50–125-cm. columns.[1] Chromatograms were obtained with a flow-cell[2] and recording spectrophotometer[3] system. A pulseless pump[4] was used to vary the elution flow rate. A spectrofluorometer[5] was used for the fluorometric analyses. UV and colorimetric determinations were carried out with a recording spectrophotometer.[6]

Reagents and Materials—The following solvents were used: reagent grade glacial acetic acid, benzene, methanol, concentrated sulfuric acid, toluene, triethylamine, trimethylamine, xylene, and spectroquality cyclohexane;[7] reagent grade benzene was redistilled prior to use. Free estrogen samples were of reference standard purity.[8] The commercially available lipophilic gel[9] was used without pretreatment.

Preparation of Column—The gel was suspended in the chromatographic solvent (200 ml. of solvent/10 g. of gel), and the resulting slurry was equilibrated by shaking for at least 2 hr. The gel was allowed to settle, excess solvent was decanted, and a fresh portion of solvent was added. This procedure was repeated three times. The final slurry was transferred to the column by gravity feed and packed at elution pressure to the desired length.

Chromatography—An aliquot (0.5–1 ml.) containing a mixture of free estrogens (50–200-mcg. amounts) in a given chromatographic solvent was applied quantitatively to the column. The column was eluted with the aid of a pulseless pump at flow rates ranging from 0.4–2 ml./min. The elution patterns were recorded by the flow-cell spectrophotometer system, and fractions were collected at appropri-

[1] Obtained from Fisher and Porter Co., Warminster, Pa.
[2] Obtained from Arthur H. Thomas Co., Philadelphia, Pa.
[3] Beckman DB.
[4] Waters Associates, Inc., Framingham, Mass.
[5] Perkin-Elmer model 203.
[6] Cary model 14.
[7] Matheson, Coleman & Bell.
[8] Prepared by Ayerst Research Laboratories, Montreal, P.Q., Canada.
[9] Sephadex LH-20, Pharmacia Fine Chemicals Inc., New Market, N. J.

Table II—Elution Volumes of Estrogens as a Function of Column Height[a]

Col. Hgt., cm.	Estrone N[e]	Estrone El. Vol.[f]	Estrone Fr. Vol.[g]	Estrone Ovl. Vol.[h]	Equilin El. Vol.	Equilin Rel. Vol.[i]	Equilin Fr. Vol.	Equilenin El. Vol.	Equilenin Rel. Vol.	Equilenin Fr. Vol.	Estradiol[b] El. Vol.	Estradiol[b] Fr. Vol.	DHEQ[c] El. Vol.	DHEQ[c] Fr. Vol.	DHEQN[d] El. Vol.	DHEQN[d] Fr. Vol.
44	820	71	18	9.0	80	1.13	18	99	1.40	19	138	31	164	33	215	46
68	1500	112	18	3.0	127	1.12	18	164	1.45	28	223	32	269	36		
120	3020	196	22	0.0	222	1.13	24	280	1.43	30						

[a] Solvent system: cyclohexane–benzene–methanol (500:150:75); flow rate: 0.6 ± 0.1 ml./min. All volumes except the relative volume are expressed in milliliters. [b] 17α- or 17β-Estradiol. [c] 17α- or 17β-Dihydroequilin. [d] 17α- or 17β-Dihydroequilenin. [e] Theoretical plate number; based on elution volume and peak width at half peak height. [f] The elution volume of a given estrogen at its maximum concentration. [g] The total volume of eluent containing a given estrogen fraction. [h] The overlap volume between estrone and equilin. [i] The elution volume of a given estrogen at its maximum concentration relative to the elution volume of estrone (estrone relative volume = 1).

Table III—Elution Volumes of Free Estrogens as a Function of Flow Rate[a]

Flow Rate, ml./min.	Estrone N[e]	Estrone El. Vol.[f]	Estrone Fr. Vol.[g]	Estrone Ovl. Vol.[h]	Equilin El. Vol.	Equilin Rel. Vol.[i]	Equilin Fr. Vol.	Equilenin El. Vol.	Equilenin Rel. Vol.	Equilenin Fr. Vol.	Estradiol[b] El. Vol.	Estradiol[b] Fr. Vol.	DHEQ[c] El. Vol.	DHEQ[c] Fr. Vol.	DHEQN[d] El. Vol.	DHEQN[d] Fr. Vol.
0.3	1130	69	14	3.0	79	1.14	12	120	1.74	22						
0.6	800	70	16	6.0	78	1.13	16	97	1.40	19	132	26	161	33		
0.8	700	71	18	9.0	80	1.13	18	99	1.40	19	137	31	159	33	211	46
1.6	420	70	20	10.0	79	1.13	18	97	1.40	22	130	28	155	33	209	42

[a] Solvent system: cyclohexane–benzene–methanol (500:150:75). All elutions were carried out on the same (45-cm.) column. [b] 17α- or 17β-Estradiol. [c] 17α- or 17β-Dihydroequilin. [d] 17α- or 17β-Dihydroequilenin. [e] Theoretical plate number; based on peak width at half peak height. [f] The elution volume of a given estrogen at its maximum concentration. [g] The total volume of eluent containing a given estrogen fraction. [h] The overlap volume between estrone and equilin. [i] The elution volume of a given estrogen at its maximum concentration relative to the elution volume of estrone (estrone relative volume = 1).

ate elution volumes. A 100-ml. fraction free of any estrogens was collected for a blank in UV determinations.

Quantitation—Each fraction was evaporated to dryness at 50° under nitrogen. The estrogen fractions were reconstituted quantitatively with methanol in a 5-ml. volumetric flask. The 100-ml. blank fraction was reconstituted in a 10-ml. volumetric flask. Each fraction was scanned by a recording spectrophotometer against methanol and an appropriate blank, which was prepared from the 100-ml. fraction. Aliquots of each fraction were then withdrawn for the fluorometric and colorimetric analyses.

The fluorometric analysis was applied only to the fraction containing estrone and equilin. The determination required approximately 2–4 mcg. of estrone, which was contained in 50–100 μl. of methanol. The solution was pipeted into a 10-ml. volumetric flask, which was made up to volume with concentrated sulfuric acid, water, and methanol (8:1:1 parts by volume). The volumetric flasks were heated at 80° for 15 min. After cooling to room temperature, the fractions and standard reference solutions were analyzed spectrofluorometrically by activating at 273 mμ and reading the emission at 470 mμ.

By increasing the concentration range of the estrogens by a factor of 10–20, the fluorometric procedure was adapted to colorimetric analysis of estrone, equilin, equilenin, estradiol, 17α-dihydroequilin, and 17α-dihydroequilenin. After heating and cooling, the sulfuric acid solutions were scanned by a recording spectrophotometer from 700 to 420 mμ.

The chromatographic fractions containing estrone and equilin were also analyzed by a modified Kober test which is specific for equilin (13).

RESULTS AND DISCUSSION

Chromatography—The efficiency of the chromatographic system was studied as a function of solvent composition, column height, and flow rate. Table I presents the column plate numbers, the peak elution volumes, and the volumes of estrogen fractions as a function of the solvent system. Figures 1 and 2 illustrate representative elution patterns obtained with the selected solvent system.

These data and preliminary data indicated that a combination of a short-chain alcohol, cyclohexane, and an aromatic solvent yielded the most effective separation. Such a combination not only facilitated the miscibility of the first two components but also introduced selective interactions between the steroid molecules and the mobile phase. The selectivity of this system was apparently enhanced by the favorable partition coefficients resulting from marked contrast between the polarity of the methanol-solvated stationary phase and the relatively low polarity of the mobile phase. The structure and the relative ratio of the aromatic component to cyclohexane have been found to be rather critical. A somewhat favorable effect was also observed on addition of tertiary amines. This effect could be attributed to "masking" of the common phenolic group, which has a predominant effect on the polarity and solubility of estrogens, and subsequent enhancement of less significant structural variations between different estrogen structures. However, the somewhat favorable effect of tertiary amines was offset by the odor of the resulting solvent mixtures and complications in the quantitation procedure; thus, for quantitative analytical purposes, the amines were omitted from the solvent mixture.

Although the chromatographic system studied separated estrogens that differed only by one double bond, it did not separate the α- and β-isomers of estradiol, dihydroequilin, and dihydroequilenin. Table II reflects the results of the column height study. As anticipated, the degree of separation and the column plate number increased with increasing column height. However, due to practical considerations such as flow rate, there was a natural limitation to the height of the column. Increasing the pressure to increase the flow rate tended to compress the gel phase and thus increase the resistance to solvent flow.

Increasing the flow rate also led to decreasing column efficiency (increasing height of the theoretical plate). At higher flow rates, the operation is at nonequilibrium, and the diffusion-dependent mass transfer Cv term of the Van Deemter equation (16) is directly proportional to the square of the thickness of liquid film (d_f) on the support and inversely proportional to the diffusion coefficient of a solute in the solvent phase (D_e). Since in the chromatography system investigated, d_f is rather large while D_e is rather small, the Cv term made a relatively large contribution to the Van Deemter equation and thus led to the inverse relationship between column efficiency and solvent flow rate. These effects are apparent in Table III.

Quantitation—Since the system selected for quantitation did not yield complete separation between estrone and equilin, additional specificity was built into the quantitation procedure. The UV determination is suitable for quantitation of equilenin, estradiol, dihydroequilin, and dihydroequilenin which are separated from each other. However, the UV spectra of estrone and equilin are identical; thus the UV determination of the combined estrone and equilin fraction gives only the sum of their concentrations. Combining the result of the UV determination with a fluorometric and a colorimetric procedure, which is relatively selective for estrone,

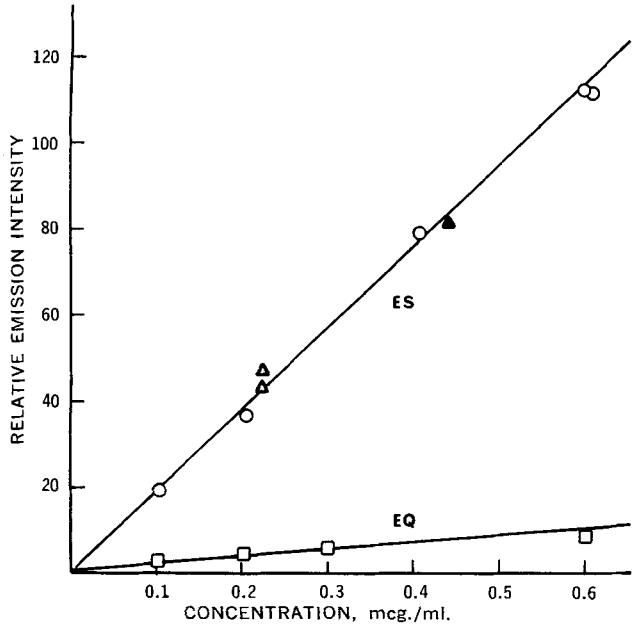

Figure 3—*Relative emission intensity of estrone (ES) and equilin (EQ) at 273-mμ activation wavelength and 470-mμ emission wavelength. Key: □, equilin (EQ); ○, estrone (ES); △, 0.2 mcg. of ES + 0.2 mcg. of EQ; and ▲, 0.4 mcg. of ES + 0.2 mcg. of EQ.*

and a modified Kober procedure, which is selective for equilin, gives three independent methods for individual determination of the estrone and equilin mixture.

The fluorometric procedure adopted in this study is based on a method developed by Bates and Cohen (14). However, the authors have observed that a mixture of sulfuric acid, methanol, and water yields a greater response than the sulfuric acid and water solution recommended by Bates and Cohen (14). By utilizing a spectrofluorometer rather than a fluorometer, a significant gain was also obtained in the specificity of estrone determination. The relative response ratio of estrone to equilin, observed by Bates and Cohen (14) with a filter fluorometer, was 4:1, while the ratio observed in the present study with the aid of a spectrofluorometer was 10.8:1 (Fig. 3). Given this relative response ratio and the total concentration of the two estrogens in an unknown mixture, one can postulate the following equations, which resolve the composition of the unknown mixture:

$$R = R'Es + R''Eq \qquad \text{(Eq. 1)}$$

$$R'' = \frac{R'}{10.8} \qquad \text{(Eq. 2)}$$

where R = observed fluorescence response of the estrone and equilin fraction; Es = mcg. of estrone in the sample; Eq = mcg. of equilin in the sample; R' = the fluorescence response for estrone (in response units per mcg. of estrone); and R'' = the fluorescence response for equilin (in response units per mcg. of equilin).

Note that concentrations are not involved since all samples are in the same volume.

Table IV—Observed Maximum Absorption Wavelength and Absorptivity in the Acid-Induced Colorimetric Determination

Estrogen	Maximum Absorption Wavelength, mμ	Absorptivity ($a \times 10^{-2}$)
Estrone	453	14.5
Equilin	453	7.0
Equilenin	480	11.6
Estradiol	455	4.0
17α-Dihydroequilin	490	19.0
17α-Dihydroequilenin	455	5.1

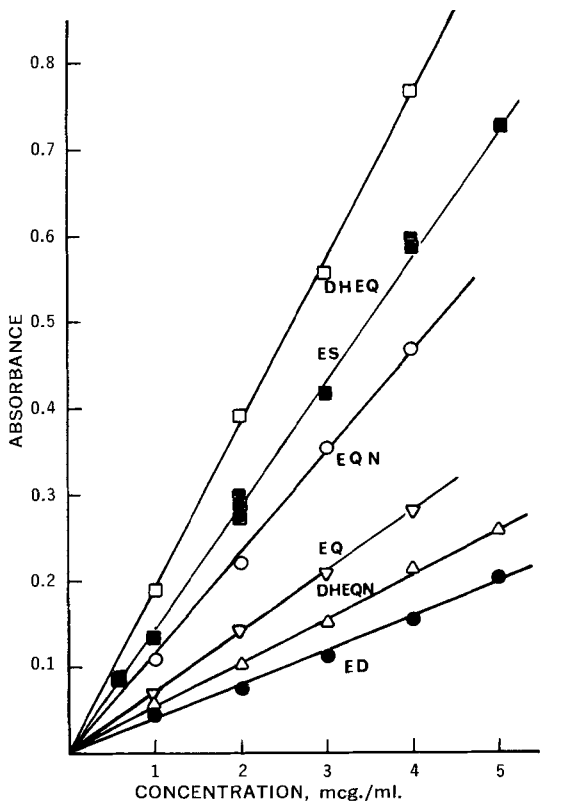

Figure 4—*Colorimetric absorbance of estrone (ES, at 453 mμ), equilin (EQ, at 453 mμ), equilenin (EQN, at 480 mμ), estradiol (ED, at 455 mμ), 17α-dihydroequilin (DHEQ, at 490 mμ), and 17α-dihydroequilenin (DHEQN, at 455 mμ).*

From UV determination:

$$Eq = C_T - Es \qquad \text{(Eq. 3)}$$

where C_T = total mcg. of estrone and equilin in the mixed fraction.

By substitution:

$$Es = 1.10\,\frac{R}{R'} - 0.102\,C_T \qquad \text{(Eq. 4)}$$

Equation 4 was applied to the analysis of several fractions containing estrone and equilin, and quantitative results were obtained. However, some chromatographic fractions, which apparently contained an unknown impurity that had a significant quenching effect, yielded values considerably lower than expected. These fractions were analyzed by the colorimetric procedure which, although less sensitive than the fluorometric analysis, was considerably more reliable. A set of equations analogous to those already listed was also applied to the colorimetric analysis of the fraction containing both estrone and equilin. The relative colorimetric response ratio of estrone to equilin was observed to be 2:1. As an independent check of the determination of equilin in the combined estrone and equilin fraction, a modified Kober test was utilized (13).

The colorimetric analysis was also applied to the determination of the remaining estrogen fractions. In this application, no blank correction was necessary; since there was no overlap between the remaining estrogens, the directly observed absorbances were used in the determination. Table IV summarizes the absorptivities and the wavelengths of maximum absorbance observed in the colorimetric determination. Figure 4 illustrates the relative colorimetric responses of the six estrogens. It is of interest to note the differences in the relative response of each of the estrogens. Jones and Hähnel (17) postulated a carbonium-ion mechanism for the formation of an acid-induced steroid chromophore. Since the double bond in Ring B of the equilin structure could affect adversely the carbonium-ion formation, this mechanism could explain the observed decrease in the absorptivity of equilin as compared to estrone. However, the

Table V—Comparative Recovery Data

Chromatographic Fraction	Theo. Input, mcg.	% Recovery Obtained by Different Methods: UV[a] A	UV[a] B	Fluorometry[a] A	Fluorometry[a] B	Colorimetry[a] A	Colorimetry[a] B	Modified Kober[a] A	Modified Kober[a] B
Estrone	210	97	100	94	103	96	95	100	104
Equilin	109			105	95	100	109	104	103
Equilenin	50.5	97	97			90	93		
Estradiol	99.3	100	106			102	110		
Dihydroequilin	104.0	98	110			97	99		
Dihydroequilenin	49.8	94	90			105	95		

[a] A and B values correspond to different elutions.

observed relative magnitude of absorptivities of other estrogens is not as readily explained and thus merits further investigation.

Table V summarizes the quantitative results observed in this study.

SUMMARY AND CONCLUSIONS

The partition chromatographic system investigated was found to be sufficiently selective to separate completely six estrogens which are characterized by only minor differences in the molecular structure. The chromatographic column used for this purpose yielded 3020 theoretical plates, 25 theoretical plates per centimeter of column height at 0.6 ml./min. flow rate. However, this separation required 10 hr. and, due to a relatively large contribution of the mass transfer factor to column efficiency, it was not possible to increase the efficiency of the system by increasing the solvent flow rate. This limitation was compensated by selecting a shorter column (800 theoretical plates) and resolving the partial overlap between estrone and equilin by specific fluorometric and colorimetric reactions. Since the elution volumes were reproducible, columns could be monitored manually without elaborate instrumentation. However, the separation procedure requires 3 hr. and thus is still relatively impractical. To reduce the elution time, the authors are now investigating the possibility of further optimization of their chromatographic system.

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