CHROM. 3769

PRECISE LOCATION OF STEROIDS IN THIN-LAYER CHROMATOPLATES WITH MARKER DYES*

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SUMMARY

A method is described whereby the location of steroids in developed chromatoplates can be determined to ± 1 mm without spraying, by the use of marker dyes which do not interfere in subsequent quantification of steroid extracted from the adsorbant. Applications to urinary steroids are discussed.

INTRODUCTION

A systematic method for the qualitative and quantitative analysis of steroids based on the combined use of thin-layer and gas–liquid chromatography (TLC–GLC) was established in this laboratory[1–8].

By submitting total steroids to TLC in a chloroform–methanol system[1,2], a first separation based mainly on differences in polarity yielded a series of distinct TLC zones characterized by molecules which contained specific numbers of hydroxyl and carbonyl groups. Quantitatively removed and eluted groups of steroids[8] could be successfully analyzed by GLC.

As shown in a series of recent articles[9–11] the development in this laboratory of highly efficient nonpolar columns permitted excellent complementary separations of TLC-zone components on the basis of molecular weight and stereoconfiguration. Segregation into TLC subzones of group components that otherwise would form mixtures difficultly separable by GLC was observed[9,10].

The systematic simplicity of the method is undoubtedly dependent on zone and subzone boundaries being consistently and precisely located on chromatoplates.

The use of so-called "nondestructive" sprays is considerably limited for this purpose if quantification of very small amounts of steroids is contemplated; indeed none of such sprays effectively fulfils both the following requisites: (1) ability to reveal the position of very small amounts of steroids, (2) complete absence of interference in subsequent quantification of small amounts of steroids by GLC, fluorometry, UV, or IR spectrophotometry.

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In addition, sprays will not permit unequivocal location of individual steroids when relatively large amounts of other steroids are present in the same TLC zone.

Undoubtedly the use of UV light-induced fluorescence is helpful in the case of urinary steroids[5]. However, required trace amounts of fluorescing impurities accompanying urinary steroids are generally lacking in steroid samples of different origin. Hence UV light visualization of thin-layer chromatograms is not generally possible.

The present article proposes the use of marker dyes which consistently permit the location of steroid band centers within ± 1 mm, and do not interfere in subsequent quantification. Relationships between dye- and steroid-band positions were established by examination of a large number of sprayed chromatoplates in the manner described below.

EXPERIMENTAL

Materials and solutions

Adsorbosil 4 (10 % $CaSO_4$); Applied Science, Inc., State College, Pa.

Sudan Blue: No. 3788, Harleco.

p-Aminoazobenzene (No. 3477); 2,4-diaminoazobenzene (HCl) No. 12087; 4,4'-dihydroxyazobenzene (No. 4736): K & K, Plainview, N.Y. 11803.

Steroids: Steraloids Inc., Flushing, N.Y.

Phosphomolybdic acid: Analar, British Drug House.

Methanol: redistilled, Analar grade, British Drug House.

Chloroform: redistilled, Fisher C-754 reagent.

Developing solvent: $CHCl_3$–MeOH–H_2O (200:9:0.75, v/v/v).

Dye solution: Sudan Blue, 100 mg; *p*-aminoazobenzene, 10 mg; 2,4-diaminoazobenzene, 20 mg; 4,4'-dihydroxyazobenzene, 20 mg per 100 ml of $CHCl_3$–MeOH (2:1, v/v).

Steroid stock solutions: 1 mg steroid per ml of $CHCl_3$–MeOH (1:2,v/v).

EQUIPMENT

Desaga adjustable spreader, coating template, and 200/200/3.8 mm selected[6] glass plates.

Shandon developing tanks and storage cabinets.

Semi-automatic TLC sample applicator[6], Supelco, Inc., P.O. Box 581, Bellefonte, Pa. 16823.

Hamilton microsyringes.

Desaga spotting template.

METHODS

Steroids test solutions

250 μl of each of the required steroid stock solutions were successively evaporated at 56° under a stream of nitrogen in a 1-ml tube; 500 μl of the dye solution were added to the dry residue. The upper section of the ground-glass stoppers were lightly smeared with silicone grease and the stoppered tubes kept at −5° between uses.

Test solutions containing from eight to twelve different steroids were prepared. Component steroids were selected on the basis of R_b values previously observed in a similar system[1,2] to obtain distinctly separated bands in developed plates. A total of about 100 steroids were included in 26 test solutions, most steroids in more than one solution. A sprayed chromatogram obtained with one of the mixtures is shown in Fig. 1. Fig. 2 shows chromatograms obtained with urinary steroids.

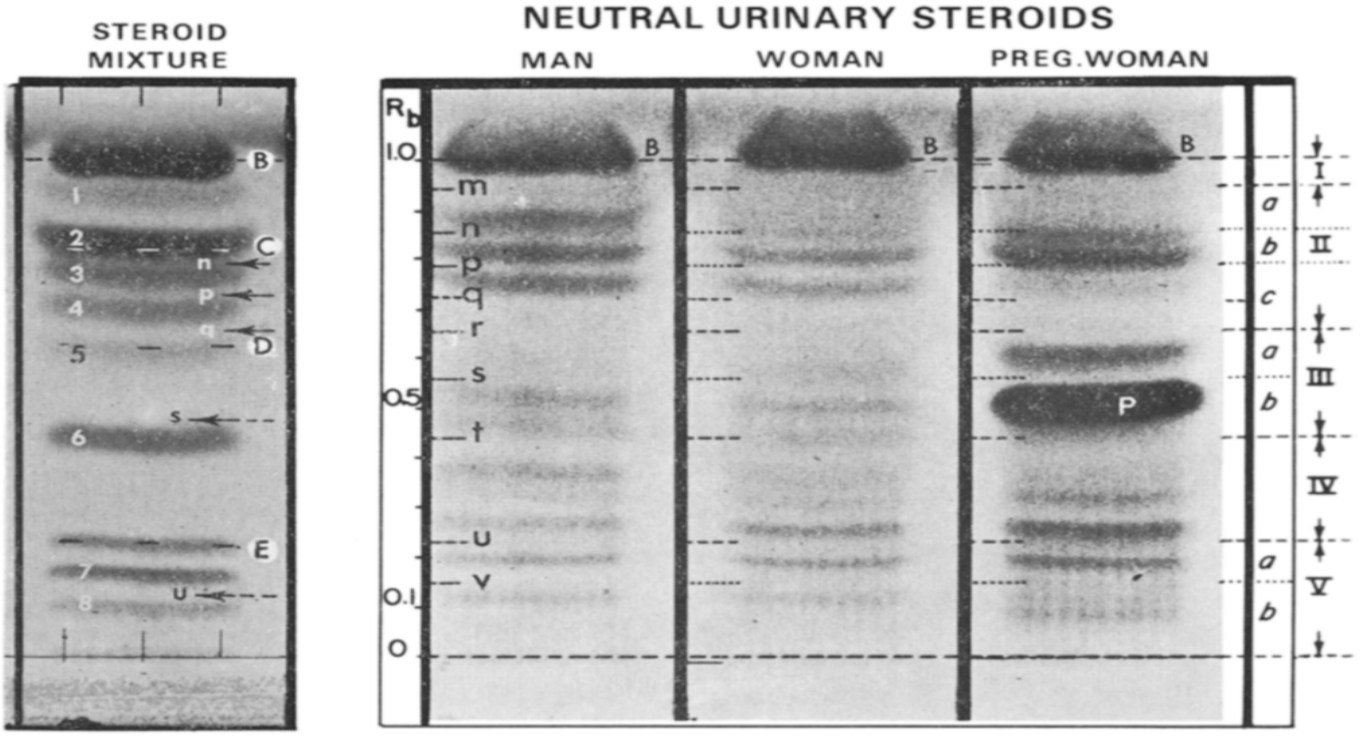

Fig. 1. Sprayed chromatogram of a mixture with the following composition; capital letters refer to marker dyes, numbers to steroids: (B) Sudan Blue; (1) progesterone (pregn-4-ene-3,20-dione); (2) cholesterol (3β-hydroxycholest-5-ene); (C) *p*-aminoazobenzene; (3) androsterone (3α-hydroxy-5α-androstan-17-one); (4) etiocholanolone (3α-hydroxy-5β-androstan-17-one); (D) 2,4-diaminoazobenzene (HCl); (5) 11-ketoandrosterone (3α-hydroxy-5α-androstane-11,17-dione); (6) pregnanediol (3α,20α-dihydroxy-5β-pregnane); (E) 4,4'-dihydroxyazobenzene; (7) pregnanetriol (3α,17α,20α-trihydroxy-5β-pregnane); (8) estriol (3,16α,17β-trihydroxyestra-1,3,5(10)-triene). For conditions: *cf.* text and caption of Fig. 4. Dye-band centers are indicated by a dashed line at both ends and middle of dye bands. Arrows indicate positions m, n, p... in intervals between steroid bands which correspond to zone and subzone boundaries indicated by the corresponding letter in Fig. 2 (*cf.* discussion). Dyes have the following colours in unsprayed chromatograms: (B) blue; (C, D, and E) pale yellow. In chromatograms sprayed with phosphomolybdic acid, then heated, B is very dark blue, C and E are bright red, D stays pale yellow; most steroid bands turn dark blue; only a few, progesterone, for example, are faint.

Fig. 2. Chromatograms of neutral urinary steroids obtained under conditions similar to those used for the chromatogram in Fig. 1; dyes were not included. Note correspondance of zone and subzone boundaries m, n, p... with points (arrows) within steroid-band intervals designated by the same letters in Fig. 1. The procedures used to obtain neutral urinary steroids from 5 ml of urine are described in ref. 5.

Measurement of migration distance

The dry developed plate was placed on a light box. A plastic Desaga spotting template (MNPQ, Fig. 3a) was placed over the plate, both as a protective cover and to provide a straight edge MN perpendicular to dye bands. By sliding the template

in a direction parallel to base line OO', MN was made to coincide successively with one end, the middle, and the other end of dye bands. In each of these positions the center of each dye band was carefully marked with a fine needle.

Distances OB, OC, and OE from base line OO' to the marked dye-band centers were measured to the nearest 0.25 of a millimeter or better by using the simple device shown in Fig. 3 b.

The plate was sprayed with 10% phosphomolybdic acid solution and heated to reveal steroid-band positions[1,2]. The positions of steroid-band centers, of zone and subzone boundaries (cut-lines) were marked and measured in the manner described for dye-band centers.

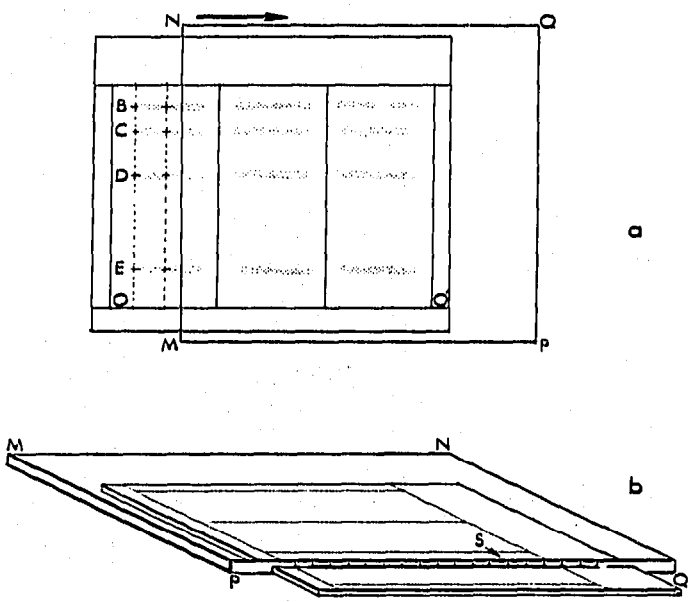

Fig. 3. Illustrating marking and measuring techniques. (a) *cf.* text. (b) Scale S is cemented to side PQ of Desaga spotting template MNPQ. Zero of scale graduated in 0.5 of a millimeter is made to coincide with plate origin.

Plotting data

R_b values. R_b values of dye-band centers C, D and E were calculated as the ratio of dye-band center distances OC, OD, and OE to the corresponding OB distance. R_b values for steroid-band centers, X, were similarly determined. The diagram in Fig. 4 shows a grouping of steroids according to average R_b values. Grouping was obviously dependent on specific numbers of hydroxyl and carbonyl groups as was previously observed in a similar solvent system[1-5].

Migration data for dyes C, D, and E, and steroids included in a typical test solution are given in Table I.

Steroid-dye positional relationship. From dye-band center and steroid-band center data, distances XY_n from steroid-band center, zone or subzone boundary, X, to nearest dye-band center Y_n, and distances BC, CD, DE, between dye-band centers were determined.

Plots relating XY_n to ratios of distances OB, OC, OD, OE, BC, CD, and DE were made with the purpose of finding that which expressed a consistent positional

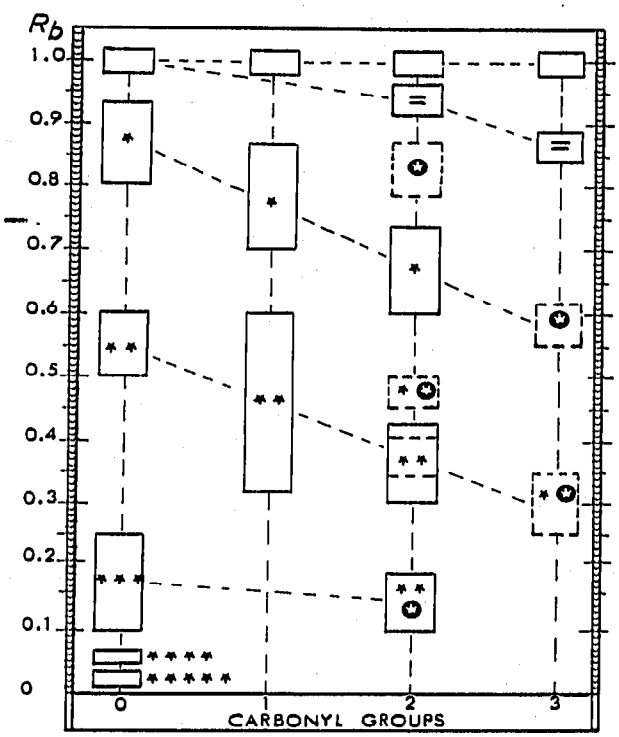

Fig. 4. Diagram showing relative migration distance R_b of steroids on TLC plates in relation to hydroxyl group (0 to 5) and carbonyl group (0, 1, 2 or 3) content. The 20 × 20 cm TLC plates were coated with a 0.25 mm layer of Adsorbosil 4 (Applied Science) and developed in $CHCl_3$–MeOH–H_2O (200:9:0.75, v/v/v). For details on coating, conditioning, loading, and developing, *cf.* refs. 3, 6, and 7. $R_b = 1$ is migration distance of Sudan Blue included in the samples. Rectangles indicate spread of band centers for components of specific groups of steroids; number of hydroxyl groups is indicated by number of stars, number of carbonyl groups by figure at bottom of relevant column. Dashed contours are used for corticosteroids; white star in black circle indicates 21-hydroxyl group. Two horizontal dashes indicate double bonds. Tetra- and pentahydroxysteroids (4 and 5 black stars at bottom of first column) also include 21-corticosteroids.

relationship of X to dye-band centers. Such a plot, characterized by an approximatively linear, tight grouping of points is exemplified by plots a and b in Fig. 5 for steroid-band centers.

In the combined TLC–GLC method of analysis, the positions of zone and subzone boundaries are required. These correspond to centers of characteristic intervals between zones and subzones observed, for instance, in sprayed chromatoplates obtained with urinary steroids exemplified in Fig. 2.

Plots relating the positions of zone and subzone boundaries to dye-band center positions were obtained. Examples of such plots are given in Fig. 6.

DISCUSSION

Difficulties in locating steroid bands and zone boundaries in unsprayed chromatoplates stem from the narrowness of such features; as seen in Figs. 1 and 2, they measure as little as 3 mm. Hence a method leading to points located within ± 1.5 mm of their actual center 99 % of the time, *i.e.*, a method corresponding to a standard deviation $\sigma < 0.58$, is required.

TABLE I

MIGRATION DATA[a] FOR A NUMBER OF STEROIDS[b] AND FOR DYES B, C, D, AND E

Dye or steroid[b]	*Mean XO* (*mm*)	$\Delta_{XO}max$[c] (± *mm*)	σ_{XO} (*mm*)	R_b (*av.*)	$\Delta_P max$[c] (± *mm*)	$\sigma\Delta_P$ (*mm*)	*Score*[d]
B	136.0	6.0	2.4	1.000	3.0	1.2	80
Progesterone	130.0	6.0	2.4	0.958	3.4	1.3	76
Cholesterol	112.5	6.0	2.4	0.827	3.6	1.4	72
C	111.5	6.0	2.4	0.819	4.0	1.6	64
Androsterone	106.0	7.5	3.0	0.778	4.8	1.9	57
Etiocholanolone	94.8	9.5	3.8	0.697	5.9	2.3	48
D	89.4	10.5	4.2	0.657	6.0	2.4	46
11-Ketoandrosterone	84.0	12.2	4.9	0.617	5.4	2.2	50
Pregnanediol	61.8	9.8	3.9	0.454	4.9	1.9	57
E	25.5	7.2	2.9	0.187	4.0	1.6	65
Pregnanetriol	19.6	6.4	2.6	0.144	2.7	1.1	82
Estriol	9.9	3.7	1.5	0.073	1.3	0.5	100

[a] Solvent front migrated 150 mm under conditions described in caption of Fig. 4.

[b] Chromatogram for this mixture is shown in Fig. 1; UPAC designations of compounds are given in caption of Fig. 1.

[c] $\Delta_{XO}max$ is maximum deviation from mean XO observed in a set of twenty chromatograms measuring from centers at both ends and middle of bands (60 values); $\Delta_P max$ is maximum difference observed for XO measured from the center at middle of bands for chromatograms in the same plate (usually three chromatograms per plate).

[d] Number of successful locations predicted from $\sigma\Delta_P$ for 100 independent experiments; *cf.* discussion.

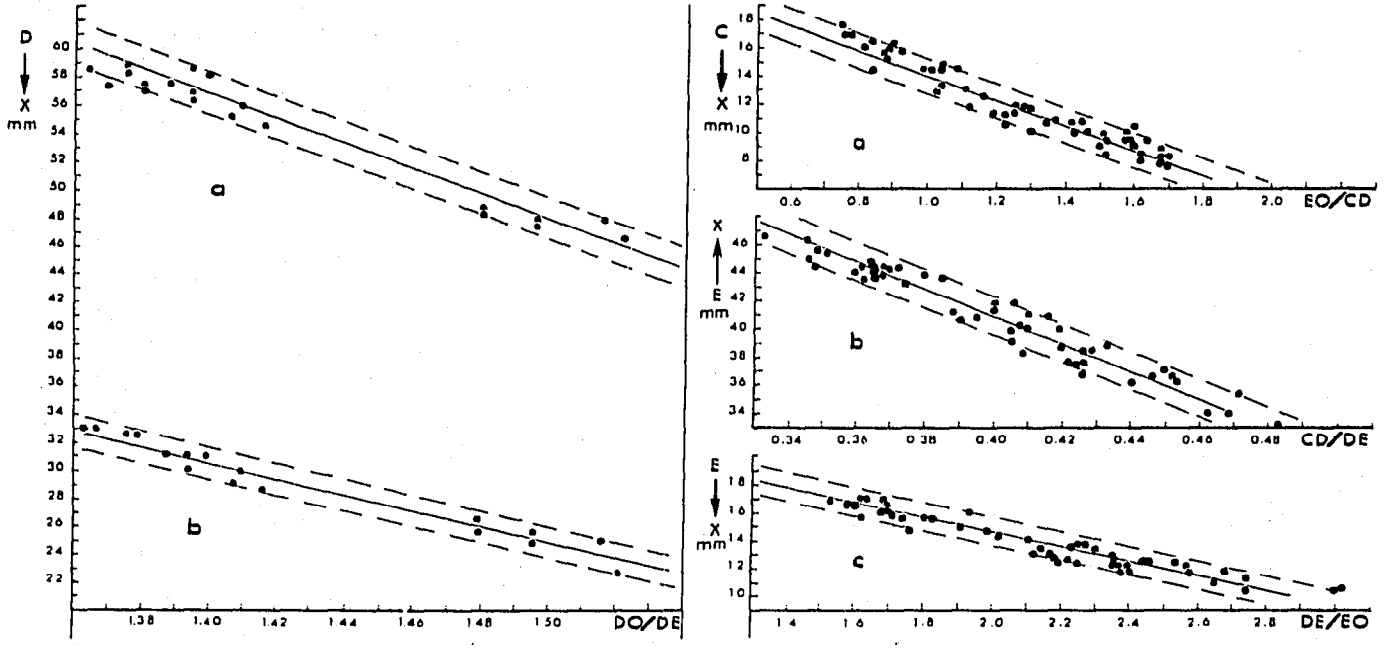

Fig. 5. DX *vs.* DO/DE plots obtained for single steroids. (a) Cortexolone (17α,21-dihydroxypregn-4-ene-3,20-dione); R_b value (average) = 0.471; σ_{DX} = 0.7. (b) Cortisone (17α,21-dihydroxypregn-4-ene-3,11,20-trione); R_b value (average) = 0.289; σ_{DX} = 0.55.

Fig. 6. (a) CX *vs.* EO/CD plot for interval *p* (Fig. 1) or subzone boundary *p* (Fig. 2); *cf.* discussion; σ = 0.6. (b) EX *vs.* CD/DE plot for interval *s* (Fig. 1) or subzone boundary *s* (Fig. 2); *cf.* discussion; σ = 0.67. (c) EX *vs.* DE/EO plot for interval *u* (Fig. 1) or subzone boundary *u* (Fig. 2); *cf.* discussion; σ = 0.5.

The XO data listed in columns 1, 2 and 3, Table I indicate remarkably little variation for migration data obtained with a multicomponent solvent system. Yet in all cases σ_{XO} was too large for a satisfactory prediction of X positions from average R_b values listed in column 4.

When use was made of the classical method consisting in spraying a chromatogram on one side of a plate, determining X positions for chromatograms on the unsprayed side from R_b data obtained from the sprayed one, chances of falling within $\pm$ 1.5 mm of actual centers were not considerably greater. This is evident from $\Delta_P$max (largest difference observed within a plate) and related $\sigma\Delta_P$ data given in columns 5 and 6, respectively. Except for estriol ($\sigma\Delta_P$ = 0.5 mm) scores shown in column 7 indicate as few as one successful positioning operation out of two trials by this method; yet $\Delta_P$max data concerned centers in the middle of bands, *i.e.*, centers for which Δ_P variation was least.

In plots shown in Fig. 5 and 6, solid lines represent regression lines of experimental points; broken lines indicate fiducial limits containing 95 % of points obtained from about 50 chromatograms; points actually shown resulted from plotting data from a series of chromatograms obtained in the following three months.

In plot a, Fig. 6, for example, the 95 % fiducial limits corresponds to a $\pm$ 1.25 mm variation of CX. Hence CX values determined from the regression line by using measured EO/CD ratios were within $\pm$ 1.5 mm of actual values 98 % of the time. In the case of plot c, Fig. 6, the score was even better: EX was within $\pm$ 1 mm of the actual center 95 % of the time.

The predictability of X positions by this method was lowest for positions which corresponded to $R_b \approx 0.65$; the variability of XO values (Table I) was also greatest in this case. However, X positions could still be predicted within $\pm$ 1.5 mm of the actual one 95 % of the time (Fig. 5a).

XY_n distances were represented by the general expression

$$XY_n = kR + h \qquad (1)$$

where k is the slope of the regression line, h a constant intercept, and R the ratio of two distances determined by three points; these were either three dye-band centers or two dye-band centers and O, the origin. Satisfactory vectors in ratio R were determined by trial and error; no rationale was found to guide this choice. However, reliable plots for X positions not too distant from each other were obtained with the same R (Fig. 5a and b). When the distance was less than 5 mm, parallel regression lines were obtained. Hence X positions in appreciably large sections of chromatoplates corresponded to the same R ratio.

In view of the overall variability of the TLC system indicated, for example, by the range of EO/CD values (a, Fig. 6) and DE/EO values (c, Fig. 6), a consistent relationship of X positions to such ratios is remarkable. Evidently, steroid migration is governed by laws whose complexity induces the apparent lack of coordination observed in the raw data (Table I, XO values.)

Properties indicated by plots exemplified in Fig. 5 and Fig. 6 lead to obvious applications. Since specific steroids can be located precisely, the adsorbant area which needs to be removed and eluted for subsequent analysis is smaller; hence, the mixture of accompanying steroids will be less complex and subsequent analysis

less difficult. Precise location of zone or zone boundaries likewise results in more easily analyzed steroid mixtures. Zone and subzone boundaries found in urinary steroid chromatograms, in Fig. 2, for example, result from a grouping of steroids according to rules already discussed[1,2,9,10]. In zone II, a subdivision in subzones a, b, and c is particularly apparent for male urinary steroids. Together with other minor components, the following steroids were found in these subzones[12]. Note GLC retention times* in square brackets.

Subzone II, a: 3β-hydroxy-5β-androstan-17-one [2.75]; 3α-hydroxy-5β-pregnan-20-one (epipregnanolone) [4.54]; 20β-hydroxy-5α-pregnan-3-one (6.44]; 5β-hydroxy-cholest-5-ene (cholesterol) [15.36].

Subzone II, b: 3α-hydroxy-5α-androstan-17-one (androsterone) [2.78]; 17β-hydroxy-5α-androstan-3-one (allodihydrotestosterone) [3.78]; 20β-hydroxypregn-4-en-3-one [7.99].

Subzone II, c: 3α-hydroxy-5β-androstan-17-one (etiocholanolone) [2.88]; 3β-hydroxyandrost-5-en-17-one (DHA) [3.37]; 17α-hydroxyandrost-4-en-3-one (epitestosterone) [3.87]; 17β-hydroxyandrost-4-en-3-one (testosterone) [4.59]; 3β-hydroxypregna-5,16-dien-20-one [4.96]; 3β-hydroxypregn-5-en-20-one (Δ^5-pregnenolone) [5.38].

As seen from their retention times, many of the above compounds would form difficultly separable pairs without a preliminary separation into subzones. On the other hand, retention times of compounds within each subzone differ by at least 6 %, *i.e.*, enough to be sufficiently separated and precisely quantified with our efficient 6.000-theoretical plate JXR columns[9].

Note that in the chromatogram shown in Fig. 1, compound 2, cholesterol, is characteristic of subzone II, a; compound 3, androsterone, is characteristic of subzone II, b; compound 4, etiocholanolone, is characteristic of subzone II, c. Hence band separations for these compounds correspond to subzone separations in Fig. 2. Indeed plots a, b, c, Fig. 6, and others obtained with simple mixtures of steroids, are applicable to the precise location of zone and subzone boundaries in chromatograms of urinary and other steroids.

Plot b, Fig. 6, for example, corresponds to boundary *s* between subzones III, a and b. Subzone III, b contains pregnanediol (P in Fig. 2) whereas subzone III, a contains a number of isomeric pregnanediols; among these is the 5α-epimer of pregnanediol which constitutes as much as 30 % of total pregnanediols in the urine of pregnant women. A preliminary TLC separation followed by analyses of subzones III, a and b allows separate estimations of pregnanediol and its isomers to be made[12].

Dyes, B, C, D or E do not interfere in the quantification of TMS derivatives of steroids by GLC. Addition of any of these dyes before TMS derivatization of many steroids did not alter the GLC response of the derivatives; relatively small peaks induced by dyes appeared well ahead of steroid peaks.

Analyses by fluorometry, UV or IR spectrophotometry of steroids eluted from TLC zones located between dye bands suffer no interference; if analyses by this method of steroids which migrate with or near dyes B, C, D or E are contemplated, other dyes of the same type with different R_b values should be used: *p*-hydroxyazobenzene (K. & K.), for example, migrates somewhat above dye D (R_b = 0.700).

* At 230°; *cf.* ref. 9.

While the present method could be viewed as a means of compensating for variations in migration distance of steroids, it should not be regarded as a corrective for poor TLC techniques. The described useful relationships undoubtedly depend on the consistent use of TLC procedures conducive to reproducibility[1-5], including the use of a sample applicator[6].

Presumably, somewhat different TLC procedures, if consistently applied, might lead to results equally useful in the present problem. In any event, the method is empirical and requires careful calibration.

Errors in the marking of band and zone-boundary centers, and errors in measuring distances between marks account for at least half the variability in XY distances computed by the present method. While measurements made on photostatic reproductions of marked plates are more accurate, the simple and expeditive techniques illustrated in Fig. 3 appear satisfactory for most purposes.

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