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# 193. Michiko Takeuchi : Systematic Analysis of Steroids. I.*1 Systematic Analysis of Steroid Hormones by Thin Layer Chromatography.*2

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Thin layer chromatography has already been recognized as a valuable analytical tool. This technique was found to be far better with regard of sharpness of separation, minuteness of sample, rapidity of development, simplicity of apparatus and procedure, variety of detection reagents, possibility of heating, and applicability of adsorption and partition chromatography, from the known chromatographic techniques.

Microanalysis of steroid hormones has been conducted based on biological assay, elution column chromatography and paper chromatography which are all time-consuming, and lipophilic steroids required the use of a reversed phase partition chromatography. Although the recent development of gas chromatography[1,2] has effected a marked progress in rapid analysis, direct analysis by gas chromatography is impossible with a substance having many hydrophilic groups, which must be changed into such a lipophilic derivative as the acetate, and with a substance liable to be decomposed or undergo rearrangement by heat; *e.g.* a corticoid having an $\alpha$-ketol or 1,3-dihydroxy-2-propanone side-chain.[3] Furthermore, gas chromatography is disadvantageous because of difficulty in taking-out a minute amount of sample and uncertainty in identification merely by retention time. In thin layer chromatography extraction of each spot is possible and reliable identification is done with the Rf value and aid of a detection reagent.

Separation of steroid hormones by thin layer chromatography has been reported on androgens,[4] estrogens,[5~7] progestogens,[8] and corticoids,[9~11] but no systematic and simultaneous analyses of these steroid compounds have so far been made.*4

A systematic analysis of steroid hormones was carried out in the present series of work in order to apply thin layer chromatography to clinical analyses of hormones in urine and other biological materials. Attempts were especially focused on the relationship between structure and adsorptivity, and on the color reaction with twenty-six steroid hormones including androgens, estrogens, progestogens and corticoids.

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*1 This paper constitutes a part of a series entitled "Systematic Analysis of Steroids" by Shoji Hara.

*2 A part of this work was presented at the Kanto Branch Meeting of the Pharmaceutical Society of Japan in Tokyo, November 23, 1962, and Kanto Branch Meeting of the Biochemical Society of Japan in Tokyo, December 6, 1962. A brief summerized report of this paper appeared as a Letter in Endocrinologia Japonica, 1963.

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*4 Recently the following papers were appeared in literature, R. Neher's data in "Dünnschicht-Chromatographie" by K. Randerath (Verlag Chemie, Germany) p. 110 and D. Waldi's data in "Dünnshicht-Chromatographie" by E. Stahl (Springer-Verlag) p. 270.

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## Experimental

**Preparation of Plate**——A suspension was made from 30 g. of Wakogel B-5*5 and 60 ml. of $H_2O$ by triturating in a motar. This suspension was placed in an applicator (Yazawa Scientific Instrument Co.) and applied onto 4 pieces of glass plate, 20×20 cm., to make a thin layer of 250 μ in thickness. They were allowed to stand for about 10 min. in the atmosphere and dried at 130° for 60 min. to effect activation. The activity was verified by the Rf value of butter yellow (0.65) and indophenol (0.11), with benzene as the mobile phase. The plate was used immediately after being cooled to the room temperature. When the plate was to be preserved, it was placed in a tightly closed vessel and dried at 130° for 10 min. before use.

The sample in 0.1~1% MeOH or $Me_2CO$ solution was spotted with a capillary of ca. 0.02 cm. in diameter, at a position 1.5 cm. above the lower end of the plate. The size of cach spot was less than 0.2 cm. in diameter. The amount of each sample used was 0.1~1 μg. ($10^{-8}$~$10^{-9}$ mol.). The solvent of the sample solution was evaporated by irradiation of an IR lamp. At the position 15 cm. above the spotted level, the finish line of development was marked on the thin layer before starting development. For simultaneous analysis, 26 kinds of substance were spotted, each separately and as a mixture, on a 20×20 cm. plate.

**Development**——28 kinds of solvent systems were used (Fig. 1). The developing solvent was placed in the developing vessel which was saturated with the vapor of the mobile phase previous to the development. In order to facilitate saturation, a piece of filter paper soaked in the developing solvent

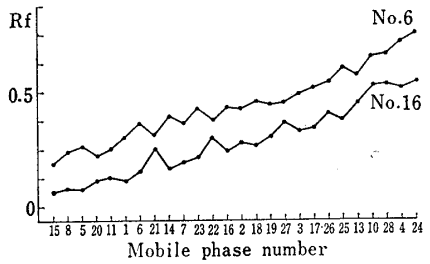


Fig. 1a. Androgens

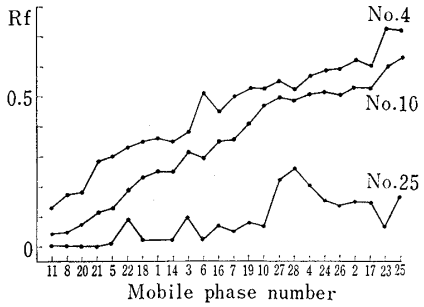


Fig. 1b. Estrogens

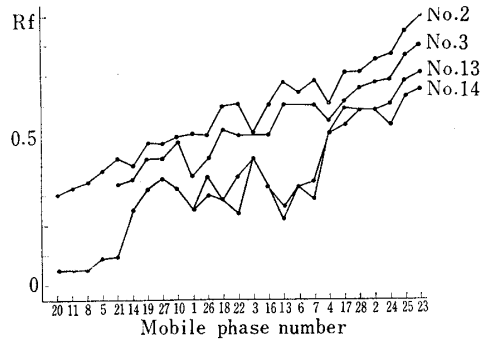


Fig. 1c. Progestogens

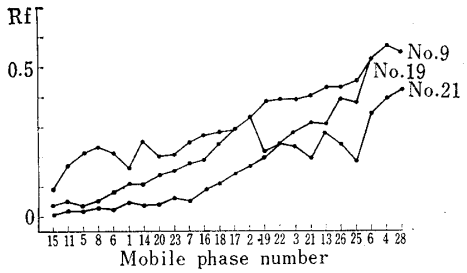


Fig. 1d. Corticoids

| | | | | | |
|---|---|---|---|---|---|
| 1 | Hexane-AcOEt (1:1) (v/v) | 11 | $CHCl_3$-MeOH (99:1) (v/v) | 21 | Benzene-MeOH (19:1) (v/v) |
| 2 | 〃 (1:4) | 12 | 〃 (97:3) | 22 | 〃 (9:1) |
| 3 | Hexane-$Me_2CO$ (3:2) | 13 | 〃 (19:1) | 23 | $Et_2O$ |
| 4 | 〃 (1:1) | 14 | Benzene-$Et_2O$ (2:3) | 24 | $Et_2O$-AcOEt (1:1) |
| 5 | $CHCl_3$-$Et_2O$ (4:1) | 15 | Benzene-AcOEt (4:1) | 25 | $Et_2O$-$Me_2CO$ (9:1) |
| 6 | 〃 (3:2) | 16 | 〃 (1:1) | 26 | $Et_2O$-MeOH (99:1) |
| 7 | $CHCl_3$-AcOEt (1:1) | 17 | 〃 (3:7) | 27 | AcOEt |
| 8 | $CHCl_3$-$Me_2CO$ (19:1) | 18 | Benzene-$Me_2CO$ (4:1) | 28 | AcOEt-MeOH (99:1) |
| 9 | 〃 (3:1) | 19 | 〃 (3:1) | | |
| 10 | 〃 (7:3) | 20 | Benzene-MeOH (49:1) | | |

*5 Silica gel for thin-layer chromatography containing 5% gypsum. A product of Wako Pure Chemical Co.

was placed inside the vessel. The plate with the sample spotted was vertically placed in the vessel so that the developing solvent reached 0.5 cm. below the spotted level. The development was done by ascending solvent chromatography at room temperature and completed when the solvent front reached the finish line; it took 30~60 min. The plate was taken out and dried with an IR lamp to evaporate the solvent.

**Detection**——The following 4 kinds of reagents were used for detection : conc. $H_2SO_4$, conc. $H_2SO_4$-$Ac_2O$ ($Ac_2O$ was sprayed on the plate, followed by conc. $H_2SO_4$ spray), $HSO_3Cl$-AcOH (1:2) and saturated $CHCl_3$ solution of $SbCl_3$. One of these reagents was sprayed on the plate which was then heated at 100° for 10~15 min. This heating procedure gave a color spot characteristic to each hormone on the plate. Some compounds required longer periods of heating to exhibit the characteristic colors. The colored spot was first examined under day light and then under a longer wave length UV ray.

Rf values were determined on the chromatogram obtained by developing a mixture of samples.

**Hydrolysis of Urine**——A solution consisting of 100 ml. of urine and 15 ml. of conc. HCl was heated at 100° for 60 min. and the hydrolysate thus obtained was extracted with $Et_2O$. The $Et_2O$ extract was treated with *N* NaOH. NaOH layer was acidified with dil. $H_2SO_4$ and re-extracted with $Et_2O$. The $Et_2O$ extract was washed with $H_2O$, dried over anhyd. $Na_2SO_4$, and evaporated to a small bulk and used as the sample of hydrolysis extract of urine.

## Result and Consideration

Various modifications have been devised in thin-layer chromatography but Stahl's method seems to be the best in the point of planarity of the stationary phase, fine structure of the capillary in the layer and reproducibility of the activity. Steroid hormones possess OH and =O groups which form hydrogen bondings with silica gel or alumina. Adsorptivity of these compounds seems to largely depend on distribution of these functional groups in the molecule. Thus, these adsorbents can afford satisfactory result in separating steroid hormones. However, activity of alumina is liable to vary under usual drying methods. On the other hand, constant activity of stationary phase can easily be obtained with silica gel and reproducibility of Rf values is high (variation of Rf values is less than $\pm 0.02$).

With the activity of silica gel used in the present series of experiments, such a solvent with the degree of polarity as chloroform has empirically found to be suitable for the mobile phase for separation of steroids based on the distinct adsorptivity due to OH and =O groups. In order to find out the mobile phase which possesses high degree of separability, twenty-eight kinds of solvent systems of a similar degree of polarity were newly prepared with hexane, benzene, chloroform, ether, ethylacetate, acetone, and methanol, either singly or as a mixture of polar and weak polar solvent.

Fig. 1 shows the variation in Rf values with change of the mobile phase, examied with each group of steroid hormones. The mode of variation in Rf values differs with each compound, but the difference in the Rf values are clearly shown with any solvent system when certain types of compounds are considered.

In general, system of solvent of small polarity containing a small amount of a solvent with a large polarity gives a better result. Separability was especially good with the solvent system of chloroform-methanol, chloroform-acetone, benzene-methanol, and benzene-acetone. A system containing ether gave a sequence of Rf values different from that with other types of mobile phase. Hexane-ethyl acetate system has been used for the separation of steroids, but it was found to be unsuitable in the present work, the spots lacking sharpness and often tailing.

Five solvent systems were selected on the basis of separability and utilized in simultaneous analysis of twenty-six steroid compounds. Fig. 2 shows Rf values obtained in these five types of analyses. Fig. 3 shows an example of the chromatogram obtained with benzene-acetone system.

As for the relationship between adsorptivity and chemical structure of steroids the following findings seem to generally hold with all solvent systems examined. Adsorptivity increases in the order of OAc, =O and OH, along with increase in the number

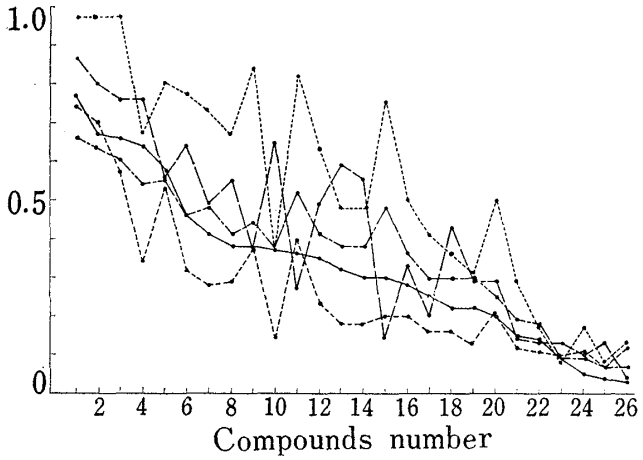


Fig. 2.

..... $CHCl_3$–MeOH (97:3)
–•—•– $CHCl_3$–$Me_2CO$ (3:1)
— — — $Et_2O$
——— Benzene–$Me_2CO$ (4:1)
– – – – – Benzene–MeOH

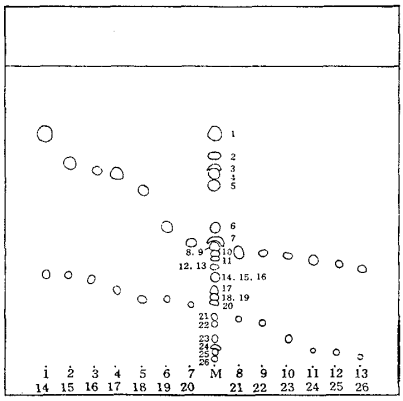

Fig. 3. Chromatogram of Steroid Hormones
Adsorbent: Wakogel B-5
Mobile phase: Benzene–$Me_2CO$ (4:1)
M: Mixture of 26 hormones

Compounds

1 3$\beta$-Acetoxy-5$\beta$-androstan-17-one
2 5$\alpha$-Pregnane-3,20-dione (allopregnanedione)
3 Pregn-4-ene-3,20-dione (progesterone)
4 3-Hydroxyestra-1,3,5(10)-trien-17-one (estrone)
5 Androst-4-ene-3,17-dione
6 3$\alpha$-Hydroxy-5$\alpha$-androstan-17-one (androsterone)
7 17$\beta$-Hydroxy-17$\alpha$-methylandrost-4-en-3-one (methyltestosterone)
8 3$\beta$-Hydroxy-5$\alpha$-androstan-17-one
9 21-Hydroxypregn-4-ene-3,20-dione (deoxycorticosterone)
10 Estra-1,3,5(10)-triene-3,17$\beta$-diol (estradiol)
11 Androst-4-ene-3,11,17-trione (adrenosterone)
12 17$\beta$-Hydroxyandrost-4-en-3-one (testosterone)
13 5$\alpha$-Pregnane-3$\beta$,20$\beta$-diol
14 5$\alpha$-Pregnane-3$\beta$,20$\alpha$-diol
15 5$\beta$-Androstane-3,12,17-trione
16 3$\alpha$-Hydroxy-5$\alpha$-androstane-11,17-dione
17 3$\alpha$-Hydroxy-5$\beta$-androstane-12,17-dione
18 5$\beta$-Pregnane-3$\alpha$,20$\alpha$-diol
19 17$\alpha$,21-Dihydroxypregn-4-ene-3,20-dione
20 12$\alpha$-Hydroxy-5$\beta$-androstane-3,17-dione
21 11$\beta$,21-Dihydroxypregn-4-ene-3,20-dione (corticosterone)
22 17$\alpha$,21-Dihydroxypregn-4-ene-3,11,20-trione (cortisone)
23 11$\beta$,17$\alpha$,21-Trihydroxypregn-4-ene-3,20-dione (hydrocortisone)
24 3$\alpha$,12$\alpha$-Dihydroxy-5$\beta$-androstan-17-one
25 Estra-1,3,5(10)-triene-3,16$\alpha$,17$\beta$-triol (estriol)
26 12$\alpha$-Acetoxy-3$\alpha$,7$\alpha$-dihydroxy-5$\beta$-pregnan-20-one

of these groups. Difference in the position (presence or absence, number) of a double bond in the steroidal skeleton is not usually recognized in adsorption chromatography but in compound with =O group in 3-position, the adsorptivity increases when a double bond is in 4-position ($\alpha,\beta$- conjugate system). Phenolic OH has less adsorptivity than alcoholic secondary OH. Effect of the position of a substituent upon adsorptivity is generally great, *e.g.*, adsorption due to =O group is greater at 12- than 11-position, and at 17- than 20-position. In the case of corticoids, 11$\beta$-OH (axial) has greater adsorptivity than 17$\alpha$-OH (axial). Equatorial orientation has the greater effect on adsorptivity than an axial one, *e.g.* eq. 3$\beta$-OH versus ax. 3$\alpha$-OH (5$\alpha$). Separation of 20-OH epimers is difficult but their separation can be effected by the use of a certain solvent system, especially that containing ether. Adsorptivity of 20$\alpha$-OH is greater than that of 20$\beta$-OH. Introduction of a methyl group to 17-C which has a OH decreases adsorptivity.

A steroid possessing 17-OH exerts greater adsorption than a corticoid possessing 20=O and 21-OH. This seeming paradox might suggest the formation of intramolecular hydrogen bonding between 20=O and 21-OH. The difference in the adsorptivity increases in the following order : =O and $\alpha,\beta$- unsaturated =O, phenolic OH and alcoholic secondary OH, =O and OH, one =O, and one OH. Accordingly, separation by the difference in the number of =O and OH, or difference between =O and OH can be effected by any of the solvent systems by the use of silica gel.

It should be emphasized that the pattern of chromatogram obtained in a simultaneous analysis of compounds with different substituents varies greatly when the solvent system is changed. Even complete reversal in the order of Rf values is observed with particular set of compounds. For example, as shown in Fig. 2, the order of Rf values of four compounds is : compound Nos. 9, 10, 11, and 12 when benzene-acetone is used as the mobile phase. The sequence, however, completely different when other solvent systems, particulary ether are employed. This fact indicates the liability to lead to an erroneous conclusion when elution column chromatography with different solvents is selected carelessly. Reversal of the order of adsorption is already known in the case of successive development of terpenes with different strengths of methanol,[12] but it is necessary to consider the change of adsorption by solvation in other solvents.

Some reports have been concerned with the use of conc. sulfuric acid[13] and few other reagents for color reaction in thin layer chromatography of steroid hormones, but no extensive examination has been made. The reagents used in this study were :

Table I.

| | $H_2SO_4$ | | $H_2SO_4$-$Ac_2O$ | | $HSO_3Cl$-AcOH | | $SbCl_3$ | |
|---|---|---|---|---|---|---|---|---|
| | D.L. | UV L. | D.L. | UV L. | D.L. | UV L. | D.L. | UV L. |
| 1 | P B | B | Y R | Y R | R P | R | B | R |
| 2 | lt. R | lt. Y R | Y | lt. P | lt. Y | B | — | B |
| 3 | Y R | B G | lt. Y | W | lt. Y | B G | — | B |
| 4 | R | R | Y R→R | Y R | Y R | Y R | Y R | R |
| 5 | B G | B | P | R | B G | G Y | Y | B |
| 6 | P B→G Y | B→R | R | R | R P | R | P B | R |
| 7 | Y R | G Y | dk. Y R | P B | Y R | Y | Y R | Y |
| 8 | P B | B | Y R | Y | R P | R | B | R |
| 9 | B | G | B | R | P | R | Y R | R |
| 10 | R | R | Y | Y | Y R | Y R | R | R |
| 11 | P B | P B | — | lt. B | lt. Y | P | Y | B |
| 12 | B G | G Y | B | W | P B→B G | Y | B | Y R |
| 13 | P B | B | P | B | R P | R | P B | Y R |
| 14 | P B | B | — | P | P | W | P B | R |
| 15 | Y | Y R | — | B | — | lt. B | — | — |
| 16 | Y | lt. Y R | Y R | Y R | Y | B | P | P |
| 17 | Y | Y R | Y R | Y R | Y R | B | B | P |
| 18 | P B | W | B G | R | Y R | R | P B | R |
| 19 | Y | Y R | R P | R | R P | R | Y R | R |
| 20 | B | B | R | B | lt. Y | B | — | B |
| 21 | G Y | B G | dk. Y R | G Y | G Y | G Y | Y | B |
| 22 | Y R | R | lt. Y R | B | R | W | Y | B |
| 23 | Y R | G | Y | B G | Y | G Y | Y R | R |
| 24 | G | Y R | P | R | R→B G | R | B | R |
| 25 | R P | R | R | R | R P | R | R | P |
| 26 | G | R | dk. Y | Y | Y R | G Y | G | Y |

B=blue, G=green, P=purple, R=red, Y=yellow, W=white, lt.=light, dk.=dark, —=no coloration.

12) H. J. Petrowitz : Angew. Chem., **72**, 921 (1960).
13) A. A. Akhrem, A. I. Kuzunetsova : Dokl. Akad. Nauk SSSR, **138**, 591 (1961).

conc. sulfuric acid, conc. sulfuric acid-acetic anhydride, chlorosulfonic acid-acetic acid, and chloroform solution of antimonous chloride, and were all found to be sensitive. Each of the steroid hormones gave a characteristic color reaction with all four reagents (detection limit, 0.1~1 μg.) and the spot showed distinct fluorescence under ultraviolet ray of a longer wave length (detection limit, 0.1~0.01 μg.). Additionary quantitative determination of steroid hormones is possible by densitometry on the colored spots.[14] The colors of the spots are given in Table I.

Attempt was made to apply this thin-layer chromatography to biological samples. A sample chromatogram obtained with different hydrolysate specimens prepared from human urines is shown in Fig. 4. It indicates that normal female urine contains estradiol as well as estrone while the urine of pregnant woman almost exclusively contains estriol. This finding is in good harmony with that obtained by gas chromatography.[15]

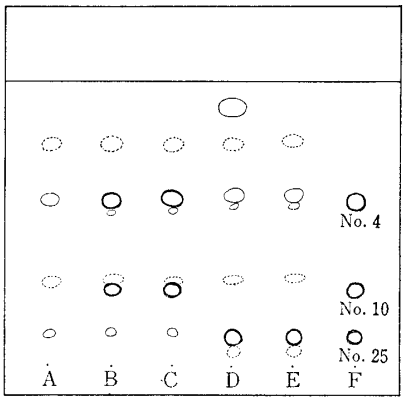


Fig. 4. Chromatogram of Hydrolysis Extracts of Human Urine

Adsorbent: Wakogel B-5
Mobile phase: Benzene-$Me_2CO$ (4:1)
A: Normal male, total extract
B: Normal female, total extract
C: Normal female, NaOH extract
D: Pregnant female, total extract (3-month)
E: Pregnant female, NaOH extract (3-month)
F: Pure substance, No. 4: estrone, No. 10: estradiol, No. 25: estriol.

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## Summary

Systematic and simultaneous analysis of twenty-six steroid hormones was carried out by thin layer chromatography. Twenty-eight solvent systems consisting of various organic solvents in various proportions were used as the mobile phase. The interrelationship among chemical structure and adsorptivity onto silica gel of steroid hormones is discussed. Characteristic color reaction of steroid hormones and four coloring reagents was examined.

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14) Unpublished result.
15) N. Ikekawa: Private communication.