18. Einosuke Koshimura and Seiichi Okazaki: Studies on Follicular Hormones. VI[1]. Quantitative Analysis of Estrone and Estradiol by Paper Chromatography Measuring the Area of Colored Spot.

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Several reports[2~9] on the paper chromatography of steroids have been published recently. Zaffaroni, Burton, and Keutmann[10] separated keto-steroids as the hydrazones of Girard T reagent, using water-saturated butanol as the developer and they obtained more satisfactory results by the use of paper strips impregnated with propylene glycol or formamide prior to chromatography and toluene or benzene as a developer[11]. Heftman[12, 13] described the paper chromatography of follicular hormones as their azo dyes with *p*-nitrobenzene-azodimethoxyaniline, followed by developing with a mixture of toluene, petroleum ether, ethanol, and water (20 : 10 : 3 : 7). Bush[14] separated follicular or adrenocortical hormones using alumina-impregnated filter paper and benzene as a developer. Heusghem[15] described paper chromatography of follicular hormones using a mixture of chloroform, benzene, and *N* ammonium hydroxide solution (1 : 9 : 1) as a developer in an ammonia atmosphere. Kritchevsky and Calvin[16] used quilon-impregnated filter paper, Kritchevsky and Tisselius[17] described a paper chromatography of androgens by the silica-impregnated one.

On the quantitative analytical method by paper chromatography, several methods such as the measuring of the area of a spot[18~20], its color density[21~23], colorimetry of its eluate[24, 25], and retension analysis[26] have been described. Tennet, Whitla, *et al.*[21] and Axelorod[22] measured the color density of keto-steroids using Beckman DU spectrophotometer. The colorimetry of eluate[27] of 17-oxysteroids as α-ketol tetrazolium salt

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and other methods[28, 29] have also been described.

In our fourth report[30], we described colorimetric assay of estrone in pregnant mare urine by Kober's method, but the value of estrone was overestimated by the effect of other contaminated estrogens, such as estradiol. When the crude preparations are biologically assayed by smear method or uterine weight-increasing effect, estrone value is overestimated by the same reason. Therefore, both analytical methods, biological and colorimetric, do not give the accurate value of estrone. Especially, for materials of biological origin, such as pregnant mare urine, Kober's colorimetric assay gives unsatisfactory result.

In the present series of experiments, a mixture of estrone and estradiol was separated into each component and was quantitatively determined using alumina-impregnated filter paper described by Bush[14,31] and Kritchevsky, *et al.*[32] (cf. Experiment I). The minimum detectable amount of estrone was 2.5γ, 1.25γ of estradiol. Below this amount, no distinct coloration appeared, and the color reaction was negative by 1.25γ of estrone or 0.625γ of estradiol (cf. Experiment II). The relationship between areas of colored spots of estrone or estradiol and the logarithms of the quantity of the steroids presented a straight line (cf. Experiment III). The range of straight line of estrone was observed from 2.5 to 80.0γ (cf. Experiment IV). χ^2-Test of areas of the same quantity developed at the same time showed no significant difference (cf. Experiment V). The result of paper chromatography of several crude estrones prepared from pregnant mare urine, often showed existence of a relatively large amount of estradiol (cf. Experiment VI). Also, a successful result was obtained with crude material, which was extracted with benzene from the precipitate in the pregnant mare urine hydrolysed by the bacterial method described in the previous report[33]. Such precipitate was so crude that it could not be quantitatively determined by colorimetry but a satisfactory result was obtained by paper chromatography and the use of the following equation (cf. Experiment VII).

$$\log\frac{U}{S}=\frac{(U_1+U_2)-(S_1+S_2)}{(U_1-U_2)+(S_1+S_2)}\cdot\log K \quad \cdots\cdots(1)$$

U: γ of sample estrone
S: γ of standard estrone
U_1: Spot area of sample estrone (high quantity)
U_2: Spot area of sample estrone (low quantity)
S_1: Spot area of standard estrone (high quantity)
S_2: Spot area of standard estrone (low quantity)
$U_1/U_2=S_1/S_2=K$

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Experiment

I. Quantitative Analysis of Steroids by Paper Chromatography—Tôyô filter paper No. 50 (2×40 cm.) for chromatography was immersed for 30 seconds in 20% ammonium alum solution warmed to 60°. This paper was exposed to an ammonia atmosphere for 1 hour in a glass vessel, which contained 28% ammonium hydroxide solution in the bottom. The paper was then removed and continuously washed with tap water for 6 hours and dried at 19~20° overnight. The alumina-

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impregnated filter paper thus prepared must be used immediately. The alcoholic solution of steroids (0.001 cc. of the solution contains 2.5γ of estrone or estrodiol) was applied to a point 5 cm. from one end of the filter paper by a micropipet, applying less than 0.003 cc. at a time. Such amount as was further desired was applied repeatedly. The diameter of the original spot to which the solution is applied should not be greater than 7 mm. After drying, the paper was developed with benzene saturated with 10% sodium hydroxide solution in a glass chamber at 20° for 4.5 hours. In all the experiments, ascending method of development was used. After developing, the solvent front was marked and the filter paper was sprayed with iodine-saturated petroleum benzine reagent [iodine and petroleum benzine (b.p. 60~80°) being Special Analytical Grades.]. The filter paper colored violet-brown, the background iodine sublimed immediately, and the spot of estrone and estradiol retained yellow-brown color for a few minutes. The reagent was further sprayed on the spot and filter paper was turned over. The spot of estrone and estradiol, as shown in Fig. 1, consisted of two portions; deeper colored inside zone (B) and a lighter colored outside zone (A) which faded more rapidly than (B). (A) and (B) were outlined with a pencil. This color could be produced even after a few days and faded spot could be reproduced repeatedly but their sensitivity fell. The area of (A) and (B) was measured by a planimeter. The area was located on the ordinate and the logarithm of the quantity of estrone or estradiol as abscissa, by which a straight line was obtained. The Rf value of estrone and estradiol, developed at the same time was almost equal, but some differences were generally observed, comparing with other experiments. Estrone showed Rf 0.30~0.50 and estradiol, 0.08~0.30, from which it is learned that the two steroids were separated completely. As B zone was fairly clear but A zone was not, B zone was used for all the experiments.

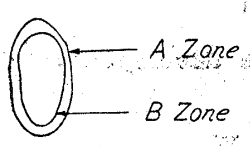

Fig. 1.

II. Limit of the Detectable Amount of Estrone and Estradiol—Various amounts of estrone and estradiol were examined by the method described above, and the results are shown in Table I.

III. Linear Relationship between Logarithm of Estrone and Estradiol, and the Area of Spots—The results obtained are shown in Tables II, III, and IV, and in Figs. 2, 3, and 4.

Table I.

Amount of steroid (γ)	Color reaction	
	Estrone	Estradiol
2.5	+	+
1.25	−	+
0.625	−	−
0.3125	−	−

+ colored, − not colored

Table II.

Estrone (γ)	B area* (mm²)	A area (mm²)
2.5	50	130
5.0	100	170
7.5	135	195
10.0	160	220
15.0	200	260
20.0	240	290

Rf=0.38, developed at 20° for 4.5 hrs.
*See Experiment I.

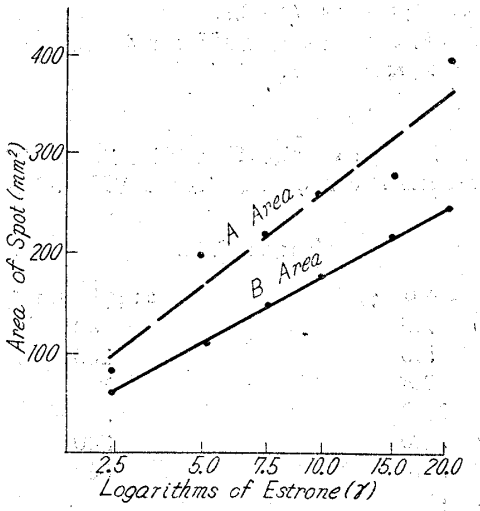

Fig. 2.

Table III.

Estrone (γ)	B area* (mm²)	A area (mm²)
2.5	60	80
5.0	110	200
7.5	150	220
10.0	180	260
15.0	220	280
20.0	250	400

Rf=0.45, developed at 20° for 4.5 hrs.
*See Experiment I.

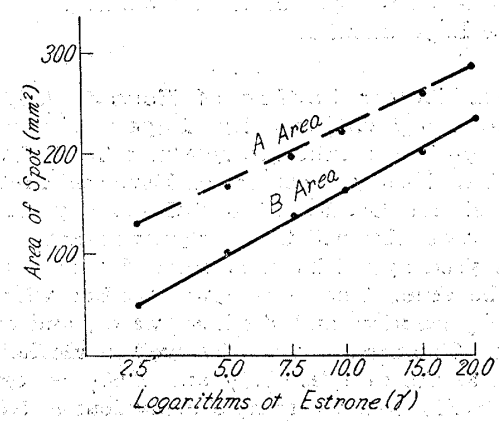

Fig. 3.

Table IV.

Estradiol (γ)	B area* (mm²)	A area (mm²)
1.25	10	30
2.5	30	55
5.0	65	115
7.5	95	145
10.0	120	185
15.0	145	235
20.0	170	260

Rf=0.11, developed at 22° for 5 hrs.
*See Experiment I.

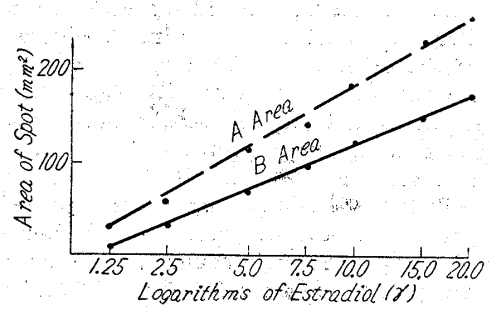

Fig 4.

IV. Difference of Area of Spots by the Same Amount of Estrone developed at the Same Time—The results are shown in Tables V and VI.

Table V.

No. of expt.	Estrone (γ)	B area* (mm²)	Difference	Square of difference
1	7.5	130	0	0
2	7.5	130	0	0
3	7.5	120	10	100
4	7.5	130	0	0
5	7.5	130	0	0

Expectation=130, mean=128, $n=5$,
$\phi=4$, $\chi^2=0.77$
$\phi=4$, $\chi^2=0.71$ ($p=0.95$)
Rf=0.48, developed at 19° for 4.5 hrs.
*See Experiment I.

Table VI.

No. of expt.	Estrone (γ)	B area* (mm²)	Difference	Square of difference
1	7.5	60	0	0
2	7.5	65	5	25
3	7.5	58	2	4
4	7.5	62	2	4
5	7.5	63	3	9
6	7.5	60	0	0

Expectation=60, mean=61.3, $n=6$,
$\phi=5$, $\chi^2=0.65$
$\phi=5$, $\chi^2=0.75$ ($p=0.98$)
Rf=0.43, developed at 23° for 4.5 hrs.
*See Experiment I.

V. Range of the Straight Line—The amount from 2.5γ to 80.0γ of estrone was examined. The results are shown in Table VII and Fig. 5.

Table VII.

Estrone (γ)	B area* (mm²)
2.5	43
5.0	100
7.5	130
10.0	200
20.0	340
40.0	520
80.0	750

Rf=0.49, developed at 19° for 4.5 hrs.
*See Experiment I.

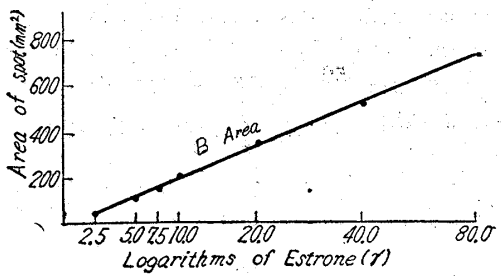

Fig. 5.

VI. Determination of Estradiol in Estrone Preparations by Paper Chromatography—The results are shown in Table VIII.

Table VIII.

No. of sample	m.p. (°C)	Color reaction of estradiol spot
1	225	−
2	248	−
3	195	+
4	234	−
5	255	−

− not colored, + colored

VII. Determination of Estrone and Estradiol in Crude Material—The precipitate which was obtained from pregnant mare's urine, hydrolysed by the bacterial method, was extracted with benzene. The benzene was washed with 10% Na_2CO_3 solution and distilled water, and evaporated. The residue was dissolved in alcohol and taken as the sample. 5.0γ and 20.0γ of estrone or estradiol and the sample equivalent to 5.0γ, 20.0γ and 80.0γ of the original residue were submitted to paper chromatography. The result is shown in Table IX.

TABLE IX.

	Standard				Sample				
	estrone		estradiol		for estrone		for estradiol		
Amount (γ)	5.0	20.0	5.0	20.0	5.0	20.0	5.0	20.0	80.0
B area* (mm^2)	110	250	70	190	80	180	none	none	110

*See Experiment I.

The amount of estrone was calculated by the equation (1), where the values of $S_1=250$, $S_2=110$, $U_1=180$, $U_2=80$, and $S_1/S_2=K=4$ were replaced.

$$\log\frac{U}{S}=\frac{(180+80)-(250+110)}{(180-80)+(250-110)}\times\log 4$$

$$=\frac{-100}{240}\times 0.6021=-0.2509$$

$$=1.7491=\text{anlog } 0.5610$$

The benzene extract contained about 56% of estrone.

The area of the spot of estradiol in the sample was located on the standard curve of estradiol and the amount of estradiol was obtained therefrom (cf. Fig. 6). The benzene extract contained about 10% of estradiol.

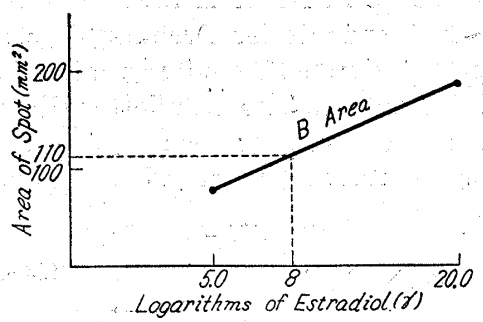

Fig. 6.

Summary

After developing estrone and estradiol with benzene which had been saturated with 10% sodium hydroxide solution on the alumina-impregnated filter paper, and spraying iodine-saturated petroleum benzine reagent on it, areas of the colored spots of estrone and estradiol were measured by a planimeter. The relation between logarithm of amount of the steroid and area of its spot showed a straight line. From this result, by the four-point assay or locating the area of steriods on the standard curve of estrone and estradiol, both steroids were quantitatively analyzed at the same time and this method was applied to the crude benzene extract from pregnant mare's urine prepared by the method reported previously. The result showed that the benzene extract contained about 56% of estrone and 10% of estradiol.

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