27. Kakuma Nagasawa, Einosuke Koshimura, and Seiichi Okazaki: Studies on Follicular Hormones. VIII.[1] Quantitative Analysis of Estrone and Estradiol in Pregnant Mare and Stallion Urine by Paper Chromatography, measuring the Area of Colored Spots.

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The authors had reported[2] a quantitative analysis of estrone in pregnant mare and stallion urine by the Kober's color reaction[3] but its result gave an over-estimate of estrone because the contaminating estradiol appeared in the same color, and they later published a chromatographic analysis[4] measuring the area of colored spots on the alumina-impregnated filter paper.[5]

In the present series of experiments, the statistical examination of the chromatographic analysis by two-and-two dose assay, described in the biological assay method of the British Pharmacopoeia,[6] was applied to the known amounts of estrone and estradiol. The fiducial limits of error of the present experiments (cf. Experiment I) were less than that of bioassay, e.g., 80~125% with p=0.95 of smear test[7] or 81~124% with p=0.95 of rats' uterine weight increasing method,[8] therefore chromatographic method was fairly satisfactory. This method was applied to the quantitative analysis of estrone and estradiol in the urine of pregnant mare and stallion. Only very small amounts of free estrogens were found in the pregnant mare urine after it was ice-cooled as soon as the mare urinated, brought to the laboratory, and extracted rapidly by the method previously reported,[9] except the treatment of the benzene extract with 0.1 *N* NaOH (cf. Experiment II). In stallion urine stored for about 3 weeks at room temperature, some bacterial hydrolysis[9] might have occured, about equal amounts of free and conjugate estrone and estradiol were found, and besides these, two other spots were also obtained, whose nature could not be identified.

Levin[10] reported that stallion urine contained an average 54,000 R.U./L. of estrogens, 42~90% of which depended on estradiol. In the present experiment, about equal amounts in weight of estrone and estradiol were found, i.e., 75% of total estrogenic activity depended on estradiol when it was assumed that the activity of estradiol was three times more potent than that of estrone (cf. Experiment II). The authors presumed that the reason why some[11] reported finding rather large amounts of free steroids in urine was due to a bacterial or enzymatic hydrolysis of the conjugate during its storage.

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Experimental

I. Statistical Examination of the Quantitative Analysis of Estrone and Estradiol by Paper Chromatography, measuring the Area of Colored Spot—Two amounts, 7.50 γ and 15.00 γ of A-series (standard) estrone (2.50 mg./cc.) and two amounts, 6.75 γ and 13.50 γ of B-series (sample) estrone (2.25 mg./cc.), were applied to the alumina-impregnated Toyo Filter Paper No. 50 (2×40 cm.). The ratios of the high amount to the low amount should be equal in both standard and sample. Four strips were used for each amount. After developing and coloring, the area of the spots was measured (these methods were described in the previous report[4]). The results are shown in Table I.

Table I.

A-Series (Stadard)					B-Series (Sample)				
Strip No.	Amount of estrone γ	Log amount of estrone (x)	Area of spot mm²	Deviation from mean (d)	Strip No.	Amount of estrone γ	Log amount of estrone (x)	Area of spot mm²	Deviation from mean (d)
1	7.50	0.8751	52	0	1	6.75	0.8293	36	0
2	7.50	0.8751	48	−4	2	6.75	0.8293	40	4
3	7.50	0.8751	52	0	3	6.75	0.8293	32	−4
4	7.50	0.8751	56	4	4	6.75	0.8293	36	0
Mean (y)			52 (S_1)		Mean (y)			36 (T_1)	
1	15.00	1.1761	120	4	1	13.50	1.1303	104	−2
2	15.00	1.1761	112	−4	2	13.50	1.1303	100	−6
3	15.00	1.1761	116	0	3	13.50	1.1303	116	10
4	15.00	1.1761	116	0	4	13.50	1.1303	104	−2
Mean (y)			116 (S_2)		Mean (y)			106 (T_2)	

From the above values, potency ratio (R) and fiducial limits of error (f.l.e.) were calculated.

$$I=\log 2=0.3010;\quad E=(T_1-T_2+S_2-S_1)/2=67;\quad F=(T_1+T_2-S_1-S_2)/2=-13;$$
$$b=E/I=222.6;\quad M=F/b=-0.0584=\bar{1}.9416$$

R=antilog M=0.874, which was 97.1% of the theoretical value.

$$s^2=\sum(d^2)/\sum(n-1)=20.0;\quad V=s^2/n=5.0$$

at p=0.05 with 12 degrees of freedom, t=2.18.

t(From data)$=(T_1+S_2-S_1-S_2)/2\sqrt{V}=1.22$, which was less than 2.18.

Therefore, the slopes were not significantly different (t=1.22 was between p=0.3 and 0.2).

$A=V=5.0$; $B=A/I^2=55.2$; $g=Bt^2/b^2=0.005$, which was less than 0.1. Therefore, the calculation was made as $g=0$

$$\log \text{f.l.e.}=2\pm t\sqrt{(A+BM^2)}/b=\text{f.l.e.}=95.0\sim105.9\%$$

The regression line, $Y=\bar{y}+b(x-\bar{x})$, was calculated as follows:

	x	y	n	nx	ny	nx^2	nxy	ny^2
	0.8293	36	4	3.3172	144	2.75095	119.419	5184
	0.8751	52	4	3.5004	208	3.06320	182.021	10816
	1.1303	106	4	4.5212	424	5.11031	479.247	44520
	1.1761	116	4	4.7044	464	5.53284	545.709	53824
Total	4.0108	310	16	16.0432	1240	16.45730	1326.396	114344
Mean	$\bar{x}$ 1.0027	$\bar{y}$ 77.5						

	$(\sum nx)^2/\sum n$	$(\sum nx)(\sum ny)/\sum n$	$(\sum ny)^2/\sum n$
	16.064	1243.348	96100
b=83.048/0.3933=211.16	0.3933	83.048	18244

Therefore, the regression equation was $Y=77.5+211.16(x-1.0027)$. The linearity of the regression line was examined as follows:

Adjustment for mean·····96100

Nature of variation	Degrees of freedom	Sum of squares	Mean square
Regression	1	17536.15	
Deviation from regression	2	94731.8	47365.9
Between amounts	3	112268.0	
Within amounts	12	336726.5	28060.54
Total	15	448994.5	

F=4.67 (5%) with 1 and 13 degrees of freedom.

F(From data)=47365.9/28060.54=1.69, which was less than 4.67.

Therefore, there was no serious evidence of inequality of the regression coefficients.

The results of estrone are shown in Table II and those of estradiol in Table III.

Table II.

Ept. No.	Strip No.	Standard High amount 15.00 γ	Standard Low amount 7.50 γ	Sample High amount 12.00 γ	Sample Low amount 6.00 γ	t (p)	R*	f.l.e. % (p=0.95)
		Area of spot (mm²)						
1	1	128	76	100	72	1.14(0.3~0.2)	97.8	92.2
	2	124	72	108	60			136.5
	3	112	80	104	64			
	4	120	80	104	64			
2	1	200	80	164	68	4.07(0.01~0.001)	106.1	95.4
	2	188	76	160	68			104.8
	3	188	72	160	60			
	4	184	72	152	60			
3	1	88	60	80	48	1.16(0.3~0.2)	97.4	98.9
	2	88	60	80	48			101.1
	3	92	60	80	48			
	4	88	64	80	52			
4	1	120	76	116	64	0.82(0.5~0.4)	108.3	90.6
	2	120	72	108	60			120.4
5	1	124	72	108	68	1.00(0.4~0.3)	105.1	91.3
	2	128	76	112	64			108.6
6	1	108	72	96	64	1.73(0.2~0.1)	101.6	91.2
	2	112	68	96	60			109.6
		15.00 γ	7.50 γ	13.50 γ	6.75 γ			
7	1	120	52	104	36	1.23(0.3~0.2)	97.1	94.8
	2	112	52	104	40			105.4
	3	116	48	100	32			
	4	116	56	116	36			
8	1	148	72	136	60	1.60(0.2~0.1)	104.1	95.3
	2	144	64	128	60			104.7
	3	140	64	128	64			
	4	140	64	140	68			
9	1	100	72	88	68	1.00(0.4~0.3)	93.2	81.5
	2	100	64	92	60			122.8

* ……% of the theoretical value.

Table III.

Ept. No.	Strip No.	Standard High amount 15.00 γ	Standard Low amount 7.50 γ	Sample High amount 12.00 γ	Sample Low amount 6.00 γ	t (p)	R*	f.l.e. % (p=0.95)
		Area of spot (mm²)						
1	1	92	68	80	60	0.08	94.2	83.0~
	2	88	68	88	64	(0.9<)		120.4
	3	92	72	84	64			
	4	84	64	84	64			
		15.00 γ	7.50 γ	13.50 γ	6.75 γ			
2	1	92	68	100	44	1.16(0.4~0.3)	104.3	85.5~
	2	100	68	92	52			116.5

* ……% of theoretical value.

II. Quantitative Analysis of Estrone and Estradiol in Pregnant Mare and Stallion Urine—Chromatographic method was the same as for Experiment I. The results are summarized in Table IV.

Table IV.

Material	Estrone Free γ/cc	Estrone Free %*	Estrone Conjugate γ/cc	Estrone Conjugate %*	Estrone Total γ/cc	Estradiol Free γ/cc	Estradiol Free %**	Estradiol Conjugate γ/cc	Estradiol Conjugate %**	Estradiol Total γ/cc
P.M.U.	0.7†	0.9	81.0(95.2~105.0)	99.1	81.7	0.4†	1.9	20.5(86.6~115.5)	98.1	20.9

〃	1.9(94.7 ～104.7)	1.4	138.1(95.5 ～104.7)	98.6	140.0	1.4(82.6 ～121.0)	3.8	35.5(91.2～ 110.1)	96.2	36.9
〃	4.3(85.5 ～117.0)	4.1	100.7(82.6 ～118.2)	95.9	105.0	2.2(86.4 ～115.6)	13.2	15.0(83.5～ 119.9)	87.2	17.2
〃	0.6†	1.5	39.5(94.4～ 106.0)	98.5	40.1	0.3†	13.6	1.9(91.1～ 109.7)	86.4	2.2
Average	3.2	2.1	89.8	97.9	91.9	1.1	5.7	18.2	94.3	19.3
S. U.	17.6	44.9	21.6	55.1	39.2	19.6	46.6	19.9	50.4	39.5

P. M. U.……Pregnant mare urine.
S. U.……Stallion urine.
† ……Dilution Method.
( )……f.l.e. % with degrees of freedom and $p=0.95$.
* ……Free estrone+estrone after hydrolysis were 100%.
**……Free estradiol+estradiol after hydrolysis were 100%.

Summary

Two-and-two dose assay method was applied for the quantitative analysis of estrone and estradiol by paper chromatographic analysis, measuring the area of spots, and fairly satisfactory results were obtained. In a fresh urine of a pregnant mare, an average 2% of free estrone and 6% of free estradiol, 98% of conjugate estrone, and 94% of conjugate estradiol were found by this method. About equal amounts of estrone (39 γ/cc.) and estradiol (40 γ/cc.) were determined in the stored stallion urine.

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28. **Morizo Ishidate and Takeichi Sakaguchi**: Metal Chelate Compounds of Tetracycline Derivatives. I. Aureomycin.

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A patent[1] of American Cyanamid Co. claims that Aureomycin (chlorotetracycline) can be precipitated with calcium or magnesium hydroxide at pH 8.5. Albert[2] reported that Aureomycin and Terramycin (oxytetracycline) combine strongly with ions of heavy metals, particularly with ferric ion. Weidenheimer *et al.*[3] prepared Aureomycin-metal complexes having therapeutic properties superior to those of the free base or its acid salts, and Kämmerer[4] reported that the presence of Co^{++} diminished the activity of Aureomycin.

During our investigation of color reactions between Aureomycin or tetracycline and metal ions, especially with Zr, Th, and UO_2, it became apparent that the reaction represented a specific instance of a very general reaction involving those compounds which contain hydroxyl and carbonyl groups in the *peri*-position or an enolized hydroxyl group of 1,3-diketone, and that the form of metal complexes is similar to that of 1-hydroxyanthraquinone and its derivatives.

The present paper deals with the application of this chelation to the determination

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