Comparison of Potency Estimates for Glucocorticoids Using Two Thymolytic Assay Procedures. (27787)

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Since the classical work by Ingle(1), and Wells and Kendall(2), involution of the thymus gland has been used as a criterion of glucocorticoid activity(3,4,5). Experimentation has shown that the relative potency of a steroid varies with injection vehicle(3), route of administration and animal species(5). This communication presents results of testing glucocorticoids for thymolytic activity by 2 methods: (a) a single injection 48-hour assay using intact immature female rats and (b) a 5-day multiple injection assay using adrenalectomized immature male rats.

Materials and methods. 48-hour assay(6). Intact immature female rats (40-60 g, Sherman strain) were randomly distributed into 6 dosage groups (4 rats/group). Animals were permitted free access to drinking water and Purina chow pellets. Rats received a single subcutaneous injection of steroid suspended in 0.2 ml carboxymethylcellulose vehicle(7), excluding benzyl alcohol, and were autopsied 48 hours later. Control animals received vehicle only. Body and thymus weights were determined and results expressed as mg thymus/100 g final body weight.

5-day assay(8). The 5-day combination assay employed 2 criteria, liver glycogen deposition and thymus involution, measured in immature adrenalectomized male rats (40-60 g, Sherman strain), randomly distributed into 6 dosage groups (4 rats/group). Animals were maintained with Purina Lab Chow and 1% NaCl drinking fluid *ad libitum*. Twenty-four hours after adrenalectomy rats were injected subcutaneously with steroid suspended in 0.2 ml carboxymethylcellulose vehicle(7), excluding benzyl alcohol. Control animals received vehicle only. Steroid was administered daily for an additional 4 days; the rats were fasted 15 hours before and to insure maximum depletion of liver glycogen 7 hours after last injection. Only the relative thymus weights (mg thymus/100 g final body weight) are considered here.

Statistics. Data were analyzed by fitting straight lines by the method of least squares and computing the corresponding equation. Potencies were combined by the method of Wilcoxon and Haynes.* Slopes of dose-response regression lines were combined using the reciprocals of slope variances(9). Assay precision (λ) was calculated by dividing within assay standard deviation (s) by the slope (b) of the line(10). Minimum standard error of the potency estimates was calculated by the method of Sheps and Hendrie (11). The regression line in Fig. 1 was calculated by the maximum-likelihood method of Acton(12).

Results. Seventeen analogs of cortisone or hydrocortisone were tested in a single injection 48-hour assay and a multiple injection 5-day assay. The relative potencies are presented in Table I.

Regression of steroid thymolytic potencies determined in the 48-hour assay on the potencies obtained in the 5-day assay is presented in Fig. 1. Computation of the regression line allowed for variability in the potency estimates and, therefore, uncertainty in steroid potencies from each assay procedure. It was found that steroids were 0.82 times as active in the 48-hour assay as in the 5-day assay. For the relative potencies of the 17 steroids analyzed the relationship was as follows:

ρ48-hour assay $= 0.876$ (ρ5-day assay)$^{0.970}$

The 95% confidence limits for the exponent 0.970 are 0.915 and 1.026 which permits the following equation to be used.

ρ48-hour assay $= 0.82$ (ρ5-day assay)

This line differs significantly from the line ρ48-hour assay $= \rho$5-day assay ($P = 0.03$).

The 95% confidence limits on 0.82 are 0.69

* Unpublished.

TABLE I. A Comparison of Potency Estimates for Glucocorticoids Using Two Thymolytic Assay Procedures.

Steroid	No. of assays	48-hr assay	No. of assays	5-day assay
Hydrocortisone	21	1.0	33	1.0
9α-fluoro-16α-hydroxyhydrocortisone	1	.5 (.2- 1.5)*	2	2.4 (1.7- 3.2)*
16α-hydroxyhydrocortisone	1	.6 (.3- 1.0)	4	.3 (.2- .3)
2α-methyl-9α-fluorocortisone-16α,17α-acetonide	1	.9 (.6- 1.4)	1	1.3 (1.0- 1.7)
6α-methylcortisone-20-ethylene acetal	1	1.1 (.6- 1.8)	1	1.4 (1.1- 1.7)
Δ¹-9α-fluoro-16α-hydroxycortisone	3	1.8 (1.3- 2.5)	1	3.6 (2.8- 4.7)
Δ¹-9α-fluorocortisone-16α,17α-acetonide	3	2.0 (1.6- 2.6)	1	.6 (.4- .7)
Δ¹-hydrocortisone	2	2.1 (1.5- 3.0)	2	2.3 (1.7- 3.2)
Δ¹-9α-fluoro-16α-hydroxyhydrocortisone	4	3.9 (3.2- 4.7)	11	4.2 (3.9- 4.5)
9α-fluorohydrocortisone	2	5.9 (4.1- 8.6)	2	5.6 (4.5- 6.9)
Δ¹-6α-methylhydrocortisone	2	7.8 (5.5-11.1)	2	10.5 (8.2-13.6)
9α-fluorohydrocortisone-16α,17α-acetonide	2	10.4 (8.3-13.1)	2	14.4 (11.7-17.7)
9α-fluorocortisone	1	11.0 (6.4-18.7)	1	8.6 (5.4-13.7)
Δ¹-9α-fluorohydrocortisone	2	11.9 (9.3-15.2)	2	16.0 (12.9-19.8)
Δ¹-9α-fluorohydrocortisone-16α,17α-acetonide	2	26.3 (21.9-31.6)	5	32.3 (28.3-36.8)
9α-fluorohydrocortisone-16α,17α-acetonide-21-chloro	2	34.3 (23.3-50.4)	3	24.0 (21.1-27.3)
Δ¹-6α-methyl-9α-fluorohydrocortisone-16α,17α-acetonide	1	46.9 (30.9-71.0)	3	70.0 (60.3-81.2)
Δ¹-9α-fluoro-16α-methylhydrocortisone	1	54.9 (34.1-88.6)	3	55.1 (46.8-65.0)

* Relative potency and (95% confidence limits).

and 0.98, thus as indicated above, 0.82 differs significantly from 1.00. However, the difference between 0.82 and 1.00 is small and not considered by the authors to be of practical importance. This is especially true when one considers the simplicity and economy in time and steroid inherent in the 48-hour bioassay. Such a difference was measurable statistically only because of the large assemblage of data. Consideration of the data in Table I illustrates that with the exception of 4 steroids,

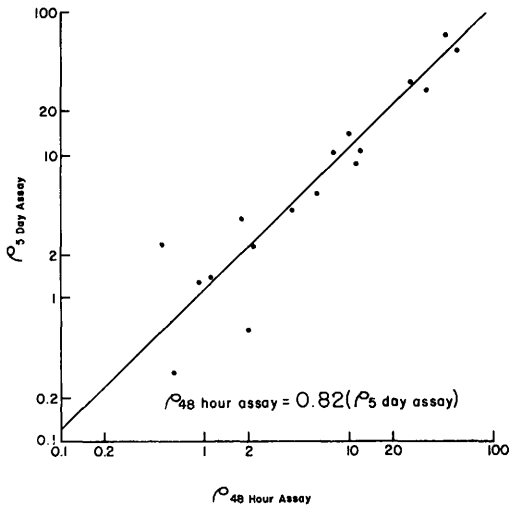

FIG. 1. Relative potencies from two assay methods.

potencies estimated by the two methods varied no more than 33%. Moreover, the mean difference in potency estimates for all steroids was only 30%.

Statistical calculations of the data from 2 assay procedures are compared in Table II. The weighted mean slope and standard deviation of the regression line for the 48-hour assay was −0.401 ± .002 and −0.743 ± .002 for the 5-day assay. The precision (λ = s/b) calculated for the 48-hour assay was 0.239 compared with 0.150 for the 5-day assay. The minimum standard errors of the potency estimates(11) were calculated by formula $S_M = \lambda \sqrt{1/Ns + 1/Nu}$ where S_M = standard error of the minimum variance of a log potency estimate (M) and Ns and Nu are, respectively, the number of animals on the standard and the unknown. S_M expressed as a percentage of the potency estimates was

TABLE II. A Comparison of Quantitative Values for a 48-Hour Assay with Those for a 5-Day Assay.

	48-hr assay	5-day assay
Slope ± S.E.M.	−.401 ±.002	−.743 ±.002
Precision (λ = s/b)	.239	.150
Minimum stand. error of potency estimates	17%	10%

TABLE III. A Comparison of Potency Estimates for Glucocorticoids from Different Laboratories.

Steroid	Potency relative to hydrocortisone			Order of activity		
	48-hr assay*	Stephenson†	5-day assay‡	48-hr assay*	Stephenson†	5-day assay‡
9α-fluoro-16α-hydroxyhydrocortisone	.5	1.8	2.4	1	2	3
16α-hydroxyhydrocortisone	.6	.13	.3	2	1	1
Δ^1-hydrocortisone	2.1	4.3	2.3	3	4	2
Δ^1-9α-fluoro-16α-hydroxyhydrocortisone	3.9	3.8	4.2	4	3	4
9α-fluorohydrocortisone	5.9	8.8§	5.6	5	5	5
Δ^1-6α-methylhydrocortisone	7.8	15.2	10.5	6	6	6
9α-fluorohydrocortisone-16α,17α-acetonide	10.4	40.2	14.4	7	8	7
Δ^1-9α-fluorohydrocortisone	11.9	17.1§	16.0	8	7	8
Δ^1-9α-fluorohydrocortisone-16α,17α-acetonide	26.3	76.6	32.3	9	10	9
Δ^1-6α-methyl-9α-fluorohydrocortisone-16α,17α-acetonide	46.9	115.5	70.0	10	11	11
Δ^1-9α-fluoro-16α-methylhydrocortisone	54.9	72.7	55.1	11	9	10

* Single injection 48-hr assay used in this laboratory.
† Injected 3 times daily for 2 days(3,4,15).
‡ Multiple injection 5-day assay used in this laboratory.
§ Acetate esters of the free alcohol were tested.

17% in the 48-hour assay and 10% in the 5-day assay.

Dorfman, *et al.*(13,14) reported the index of precision for a 48-hour multiple injection assay was 0.26 compared to 0.17 for a 6-day multiple injection test, with mean slopes of −1.90 and −2.23, respectively. The slopes for our assays, expressed in similar terms, were −1.20 for the 48-hour assay and −2.46 for the 5-day assay.

The thymolytic potencies of 11 steroids previously reported(3,4,15) assayed in intact female rats receiving multiple injections over 48 hours, were compared with potency values derived from the presently described thymus assays (Table III). Although the order of activity for the steroids evaluated by different assay methods compared favorably, the quantitative values did not agree. Greater agreement was observed between Stephenson's(3, 4,15) values and those recorded from the 5-day multiple injection assay than potency estimates from the 48-hour single injection method. Discrepancies between potency ratios were most pronounced for steroids possessing a 16α,17α-acetonide function. Earlier work(3) has shown that injection medium influences the relative potency of some corticosteroid analogs. Perhaps use of corn oil as steroid vehicle by Stephenson(3,4,15) and carboxymethylcellulose vehicle by present authors has contributed to the disparity in steroid ketal potencies.

Though potency estimates derived from different thymolytic assay procedures appear to differ quantitatively, the significance and explanation of these disparities will require additional experimentation. However, when one views the glucocorticoid bioassay literature, it is apparent that the quantitative discrepancies in potency estimates observed in thymolytic assays are far less than those recorded by other assay technics, *e.g.*, liver glycogen deposition(16).

Summary. A comparison of the thymolytic activity for glucocorticoids using a single injection 48-hour assay and a 5-day multiple injection assay is presented. Regression of steroid thymolytic potencies obtained by the 2 technics indicated that potencies estimated from the 48-hour assay were 0.82 times those from the 5-day assay. The weighted mean slope and standard deviation for the 48-hour assay was −0.401 ± .002 with an index of

precision of 0.239. The minimum standard error of the potency estimates from the 48-hour assay was 17%. Potency estimates from the single injection assay were also compared with those from a multiple injection 48-hour assay. The order of thymolytic activity agreed favorably regardless of the assay employed. Quantitative values, however, did not agree. Greatest discrepancies between potency ratios were noted for steroids possessing the 16α,17α-acetonide function. Nevertheless, quantitative differences observed for relative potencies of steroids from thymolytic assays appear to be far less than those recorded using other parameters of glucocorticoid activity.

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Development of a Hydrocortisone-Resistant Sub-Line of Mouse Lymphoma *in vitro*.* (27788)

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Anti-inflammatory steroids are known to inhibit the growth of some experimental transplanted mouse tumors, and there have been reports of the *in vivo* appearance of variants of these tumors which are steroid-resistant (1). We have recently described the properties of a mouse lymphoma cell line (ML-388), growing *in vitro,* which is extraordinarily sensitive to anti-inflammatory steroids (2). The present report describes the isolation, after several weeks cultivation in sublethal concentrations of hydrocortisone, of a sub-line of ML-388 which is no longer sensitive to the steroid. The resistance extends to all other steroids tested, and has remained characteristic of this sub-line even after cultivation for several months in the absence of any added steroid.

Methods and results. ML-388 cells were grown as adherent cultures in 8-oz prescription bottles using methods previously described(2). At 5-day intervals, the spent culture medium was poured off, and the cells suspended in 10 ml of fresh, sterile culture medium. The number of cells present in an aliquot of this suspension was determined, utilizing an electronic cell counter, and fresh bottles were then inoculated with 3×10^5 cells. In this manner, multiplication factors

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