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A METHOD FOR THE ASSAY OF ANTIDIURETIC ACTIVITY

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Much recent work has been concerned with antidiuretic substances in body fluids. The method of Burn (1931) for the assay of antidiuretic activity is widely used, but has the disadvantages that it does not easily permit the estimation of the error of a single assay and that it cannot be completed in less than two to three days since the test involves a "cross-over." In the method to be described the error may be estimated from the results and the assay can be completed in four to five hours. It is based on a regimen of water administration described by Birnie, Jenkins, Eversole, and Gaunt (1949).

METHODS

Adult male albino rats weighing approximately 200 g. were used. Rats become conditioned to water administration by stomach tube. For this reason rats were given one dose of water (5 ml./100 g. body weight) on two occasions before being used in an assay. Once conditioned the rats were not used more frequently than once in 10 days and were discarded after being used five times. Groups of 18 rats, deprived of food for 18 hours but denied free access to water for the last hour only, were placed individually in small metabolism cages; urine was collected into graduated cylinders. Each rat was given two doses of tepid water by stomach tube (each dose=5 ml./100 g. body weight) with an interval of one hour between doses. One hour after the second administration of water the total volume of urine excreted in the preceding two hours was measured (V_2). At the same time a third dose of water (5 ml./100 g.) was administered and the animals injected subcutaneously with the test material. The urine output of each animal was measured thereafter at intervals of 30 minutes for 120 or 150 minutes. In order to ensure complete emptying of the bladder the animals were stimulated by prodding and handling before each administration of water and each measurement of urine volume.

The volume of urine, V_2, was expressed as a percentage of the amount of water administered in the first two doses. Those rats for which this value deviated from the mean for the whole group by more than 33 per cent of the mean were rejected. Since the rats were injected subcutaneously, the method of calculation adopted allowed 30 minutes for the complete absorption of the injected materials. The following formula was used:

$$\text{Percentage water excretion} = \frac{V_t - V_{30}}{3V_1 - (V_2 + V_{30})} \times \frac{100}{1}$$

where V_1 = the volume of water administered in each dose.
V_2 = the volume of urine excreted in the two hours before injection.
V_{30} = the volume of urine excreted in the first 30 minutes after injection.
V_t = the volume of urine excreted during the period of "t" minutes after injection.
(t=60, 90, 120, or 150 minutes.)

That is, the percentage water excretion at a given time is the volume of urine passed from 30 minutes after injection expressed as a percentage of the water administered but not excreted 30 minutes after injection.

The antidiuretic substance used in these experiments was vasopressin (Pitressin, Parke, Davis and Co.). All solutions for injection were prepared in 0.9 per cent (w/v) sodium chloride solution. The volume injected was usually 0.2 or 0.4 ml., but larger volumes may be used provided that the volumes of the unknown and standard injections are approximately equal.

In each assay two dose levels of standard and unknown are used ; the ratio, high to low dose, being the same for standard and unknown (the value of this ratio was either 2.0 or 3.0). Before the experiment, the rats were selected for treatment by a method of random sampling. In some assays, out of the 18 rats three were selected for each standard dose of vasopressin, three for each unknown, and three for injections of saline solution. Thus there were three extra rats for the replacement of those discarded owing to exceptional diuresis during the first two hours. In most assays there was no saline-treated group, four rats being selected for each treatment with vasopressin, leaving two extra rats.

TABLE I

Treatment		Percentage water excretion = Y			ΣY	Mean Y
		60 min.	90 min.	120 min.		
	Saline	36.0 25.5 35.1	67.1 48.1 69.3	85.1 72.5 96.4		
Standard	S_1 = 0.75 milliunits Vasopressin/100 g.	17.0 20.8 8.8	39.7 43.6 21.5	56.8 68.5 37.1	313.8	34.9
	S_2 = 1.5 milliunits Vasopressin/100 g.	5.0 14.5 2.8	14.0 26.6 8.3	31.7 47.9 25.0	175.8	19.4
Unknown	U_1	12.4 9.4 15.4	31.1 23.2 31.5	52.8 44.2 44.6	264.6	29.2
	U_2	3.4 4.3 3.2	6.8 9.3 13.4	18.1 23.5 29.6	111.6	12.4
	ΣY	117.0	269.0	479.8	865.8	

$$\text{Log potency ratio} = M = \frac{\Sigma Y_{\text{standard}} - \Sigma Y_{\text{unknown}}}{\Sigma Y_{\text{low doses}} - \Sigma Y_{\text{high doses}}} \times \log(\text{high dose/low dose})$$

$$= \frac{313.8 + 175.8 - 264.6 - 111.6}{313.8 + 264.6 - 175.8 - 111.6} \times 0.3010$$

$$= 0.1171$$

$$\text{Estimated potency ratio} = \frac{\text{Unknown}}{\text{Standard}} = R_E = \text{antilog } 0.1171 = 1.31$$

$$\text{Actual potency ratio} = \frac{\text{Unknown}}{\text{Standard}} = R_A \ldots\ldots\ldots\ldots = 1.20$$

Results and Discussion

Table I shows the results obtained in a typical assay of an unknown amount of vasopressin. The percentage water excretion was taken as the response and is inversely related to the antidiuretic potency. Regardless of the time of measurement, each percentage water excretion was taken as an independent response. This is not strictly true, since the urine volumes measured at, say, 120 minutes after injection are dependent on the amounts of urine voided and measured earlier. Thus the mean response for a given animal is weighted in favour of the urine excretion in the early periods.

TABLE II

Source of variance	Degrees of freedom	Sum of squares	Variance	Variance ratio=F	P
Deviations from parallelism	1	6.3	6.3	0.1	>0.2
Regression	1	2,352.2	2,352.2	36.8	<0.01
Standard and unknown ..	1	357.2	357.2	5.6	<0.05
Times of measurement ..	2	5,531.9	2,765.9	43.2	<0.01
Residual error	30	1,919.2	63.9	—	—
Total ..	35	10,166.8	—	—	—

Standard error of $M = s_M = \sqrt{\frac{4.sy^2}{n.b^2}\left(\frac{M^2}{[\log (\text{high dose/low dose})]^2} + 1\right)}$

where sy^2 = residual error = 63.9; n = number of responses = 36

b = slope of the log dose-response line $= \frac{\Sigma Y_{\text{low doses}} - \Sigma Y_{\text{high doses}}}{n/2.\log (\text{high dose/low dose})} = 53.8$

hence $s_M = \sqrt{\frac{4\times 63.9}{36\times 53.8^2}\left(\frac{0.1171^2}{0.3010^2}+1\right)} = 0.0609$

For 30 degrees of freedom $t = 2.042$ for P = 0.05

The fiducial range (P = 0.05) for $M = \pm s_M \times t = \pm 0.1243$

Since $M = 0.1171$, the fiducial limits for M are 1.9928 and 0.2414, and the fiducial limits for R_E are antilog $\bar{1}.9928$ and antilog 0.2414 = 0.984 and 1.74

Thus $R_E = 1.31$ with a fiducial range (P = 0.05) ± 0.38, approximately.

In the calculation of the potency ratio and error the methods described by Holton (1948) and Schild (1942) were used. The residual variance (error) was obtained through an analysis of variance (Table II) and the variance due to the different times of measurement of urine volume was eliminated. Fig. 1 shows the log dose-response lines based on the data in Table I.

Table III shows the results of 12 assays. The deviations of the estimated values of the potency of the unknown from the true values were never greater than the calculated fiducial limits (P = 0.05). In five assays (2, 4, 10, 11, and 12) the potency of the unknown was considerably different from that of the standard; in two of these assays (2 and 4) the good agreement between the true and estimated values must be regarded as fortuitous since the fiducial ranges were very wide. In practice, when an accurate assay is required and the ratio of the potencies exceeds 1.7 or is less than 0.6, a second assay should be performed in which the ratio is

TABLE III

Assay No.	S_1 milliunits vasopressin per 100 g.	S_2/S_1	R_A=actual potency ratio unknown/ standard	R_E=estimated potency ratio unknown/ standard	Approx. fiducial range (P=0.05)	Significance of deviations from parallelism P
1	0.75	3	1.20	1.20	±0.27	>0.2
2	0.75	2	0.53	0.56	0.28	>0.2
3	0.75	2	1.20	1.31	0.38	>0.2
4	0.75	2	2.53	2.51	0.86	>0.2
5	0.75	3	1.33	1.40	0.42	>0.2
6	0.75	3	0.80	0.72	0.11	>0.05
7	0.50	2	0.80	0.85	0.13	>0.2
8	0.75	3	1.07	0.97	0.23	>0.2
9	0.75	2	1.33	1.45	0.31	>0.05
10	0.75	3	0.53	0.48	0.09	<0.001
11	0.75	3	0.53	0.63	0.10	>0.05
12	0.75	3	0.53	0.57	0.10	<0.01

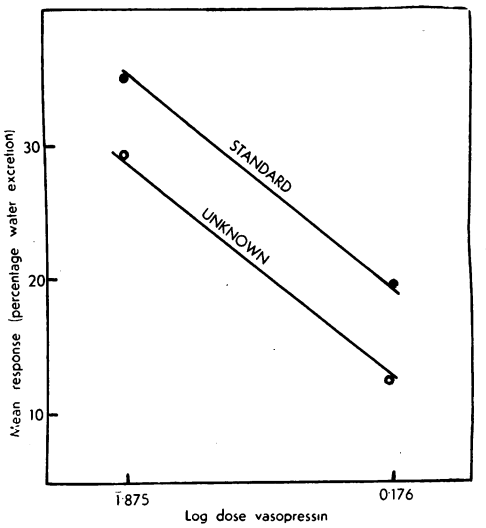

FIG. 1.—Regression lines relating response to log dose of standard and unknown. Open circles, unknown; closed circles, standard. Ordinates: Mean response (percentage water excretion). Abscissae: Log dose of vasopressin (milliunits per 100 g.).

closer to unity. The mean standard error of the method, calculated by Gaddum's (1938) method, is 8.9 per cent.

Fig. 2 shows the relationship between the log dose of vasopressin and the mean response in series of assays performed on three groups of animals. The points are fitted fairly well by straight lines, but there are significant changes in the slope and position of the lines obtained in assays performed on the same group of animals. There is a tendency for rats to become more sensitive in the later assays. Since the dose of vasopressin was related to the weight of the rat, this may be due partly to the increase in the weight of the rats and hence an increase in the absolute dose. The mean weight of the rats in the three groups at the time of the first assay was 209 g. and at the last assay 250 g. Changes in climate cannot be excluded as a possible explanation; the first assays were performed at the end of March and the last at the end of June.

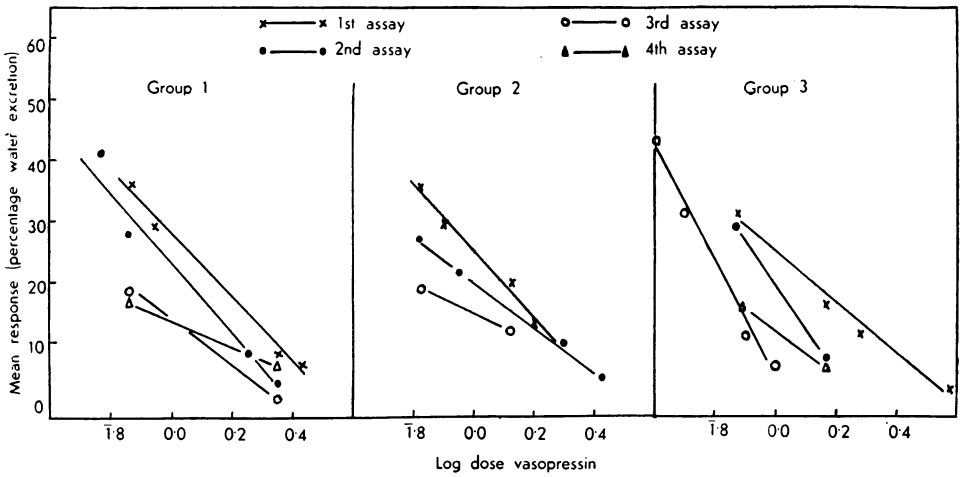

FIG. 2.—Log dose-response regression lines for 11 assays performed on three groups of rats. Ordinates: Mean response (percentage water excretion). Abscissae: Log dose of vasopressin (milliunits per 100 g.).

Sometimes the log dose-response relationship appears to show slight curvature. However, the deviations from parallelism of the standard and unknown log dose-response lines were significant in only two assays (see Table III). In these two assays the potency ratios, standard to unknown, were outside the range recommended for a precise assay; moreover, the lowest dose was 0.4 milliunits of vasopressin per 100 g. and the highest 2.25 milliunits/100 g. Over such an extended range of doses the approximation to a linear log dose-response relationship is no longer satisfactory, but log dose - log response lines are straight and parallel. According to Wood (1944) the four-point assay is still valid when the log dose-response relationship shows simple curvature: the difference between the actual and estimated potency ratios in the two assays referred to above was certainly not greater than usual.

If the variance is not independent of the magnitude of the response the analysis of variance loses its theoretical validity, but slight degrees of correlation particularly in balanced tests may be safely ignored (Emmens, 1948). In the first six assays performed the variance of the responses to each dose of vasopressin (after elimination of variance due to different times of measurement) is correlated with the magnitude of the mean response. The correlation coefficient, $r = +0.48$ ($P > 0.02$).

Though the sensitivity of the test varies, a satisfactory antidiuretic response is commonly obtained with a dose of 0.4 milliunits vasopressin per 100 g. rat; in most assays the lower dose of the standard was 0.75 milliunits, and the higher dose 1.5 or 2.25 milliunits vasopressin per 100 g. Thus the minimum total activity of the unknown required to give a satisfactory assay is about 10 milliunits; a satisfactory assay by Burn's method requires about 40 milliunits of activity. This increased sensitivity of the method is probably due to (1) a smaller number of animals being needed, (2) more complete suppression of endogenous posterior pituitary antidiuretic hormone secretion by the greater amount of water administered to the test animals,

and (3) the method of calculation in which greater weight is placed on the earlier measurements of urine volume, that is, when small doses show the greatest antidiuretic effect. The increased sensitivity of assays in which the area under the diuresis curve is taken as the response (Ham, 1943) is similarly due to greater weight being placed on the measurements of urine volume soon after injection of the antidiuretic substance.

Summary

1. A four-point assay procedure has been applied to the assay of antidiuretic activity.

2. One assay may be completed in four to four and a half hours.

3. " Unknown " amounts of vasopressin have been assayed against vasopressin standards. The mean standard error of 12 assays was 8.9 per cent.

4. A total " unknown " activity equivalent to 10 milliunits is sufficient for a satisfactory assay.

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