Automated Determination of Rat Adrenal Ascorbic Acid in the Bioassay of Corticotropin

By WILLIAM F. BEYER

An automated procedure for the determination of rat adrenal ascorbic acid used in the U.S.P. XVII bioassay of corticotropin is described. Ascorbic acid analyses of manually filtered, adrenal gland homogenates are performed with buffered 2,6-dichloro-indophenol sodium. A sample–rinse ratio of 1:2 is used and sample carry-over is not detectable. AutoAnalyzer Sampler II, proportioning pump, colorimeter, and recorder are used. The coefficient of variation for the automatic procedure is approximately 1 per cent at an effective sampling rate of 60/hr. An IBM 1620 computer is used for calculations of ascorbic acid determinations and for the bioassay of corticotropin using a 3 × 3 statistical design. Assay results using automated and manual ascorbic acid values compare favorably.

THE DEPLETION of ascorbic acid of rat adrenal glands is used as the measure of response in the bioassay of corticotropin as directed by U.S.P. XVII (1). For the subcutaneous assay, hypophysectomized rats are injected in random order with varying doses of standard or test preparations using a 3 × 3 design. Three hours after injection, the paired adrenals are removed, freed of excess tissue, weighed, and homogenized in 2.5% metaphosphoric acid. The ascorbic acid content of each pair of adrenal glands is then determined using buffered 2,6-dichloro-indophenol sodium. Statistical analyses are applied to ascorbic acid values to determine potency, precision, and validity of the assay. This report describes an automated procedure for the determination of rat adrenal ascorbic acid used in the bioassay of corticotropin. The automated method, based on the U.S.P. XVII procedure, requires manual filtration of adrenal gland homogenates. Automatic sampler,[1] proportioning pump, colorimeter, and recorder are used, permitting the analysis of 60 samples per hour, including wash between samples.

MATERIALS AND METHODS

Instruments—Automatic sampler, proportioning pump, colorimeter with 524 mμ filters and 1.5-cm. flow cell, recorder, and chart reader.

Reagents—(*a*) 2,6-Dichloro-indophenol sodium, 48.0 mg./1000 ml. Filter and store in a brown bottle under refrigeration. (*b*) Sodium acetate trihydrate, 45.3 Gm./1000 ml. Adjust to pH 7 with acetic acid. Store in a brown bottle under refrigeration. (*c*) Color reagent, equal parts of 2,6-dichloro-indophenol sodium and sodium acetate trihydrate solutions are mixed when cold. Store in a brown bottle under refrigeration. (*d*) Metaphosphoric acid, 2.5%. (*e*) Rinse solution for automatic sampler, 4.0 ml. of polysorbate 80 is dissolved in 1000 ml. 2.5% metaphosphoric acid.

Standards—Accurately weighed quantities of U.S.P. ascorbic acid reference standard or house standard are dissolved in 2.5% metaphosphoric acid within the range of 3 mcg./ml. to 11 mcg./ml. for the standard curve. Prepare just prior to use.

Test Samples—Paired adrenal glands of test rats are freed of excess tissue, weighed, and homogenized in 12.0 ml. of 2.5% metaphosphoric acid using a tapered glass tissue homogenizer.[2] Clear filtrates are then obtained using Whatman No. 2 filter paper placed in collection funnels.[3]

Procedure—Using the manifold as shown in the flow diagram of Fig. 1, the rinse line of the automatic sampler is placed in rinse solution for automatic sampler, and all other lines in water. With all instruments operating, a 99% transmission base line is established. The color reagent line is placed in iced color reagent and the sample line with probe is properly positioned in the rinse compartment of automatic sampler. Following a stable transmittance recording, the sampling rate is set at 60/hr. with a 1:2 sample–rinse ratio. Standards are placed in duplicate cups for the standard curve and test samples in single cups.

Calculations—The recorded transmittance peaks for each level of ascorbic acid standard are plotted, and a chart reader is used to determine the microgram per milliliter concentration of each test sample. A computer program is written in Fortran (formula translation) to calculate ascorbic acid in terms of milligrams ascorbic acid per 100 Gm. of adrenal tissue. The program also transforms the random order of samples into proper dosage groups, checks for outliers, and carries out statistics for the bioassay.

RESULTS AND DISCUSSION

Initial experiments with various manifold systems and sampling rates produced unreliable results and unsatisfactory recordings. Most of the difficulties were due to sample carry-over, erratic movement of the air bubble between sample and rinse, and pulsation from the pump action. Carry-over was eliminated by severing the 0.090-in. sample line at

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[1] AutoAnalyzer Sampler II, Technicon Controls, Chauncey, N. Y.

[2] Manufactured by Norman Erway, Oregon, Wis.

[3] Marketed as DiSPo by Scientific Products, Division of American Hospital Supply Corp., Evanston, Ill.

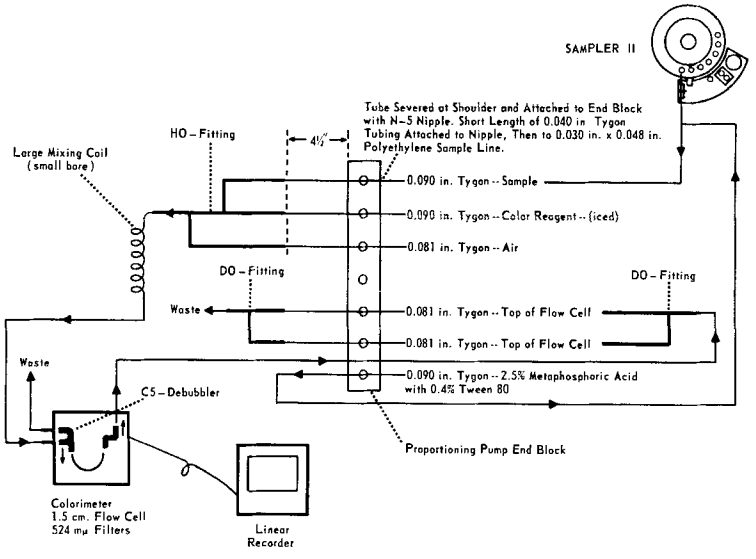

Fig. 1—Manifold flow diagram for the automated determination of ascorbic acid in rat adrenal gland homogenate. Analyses are made using Technicon AutoAnalyzer equipment: colorimeter, proportioning pump, recorder, and assorted tubing. Sampling rate is 60/hr. with a 1:2 sample–rinse ratio.

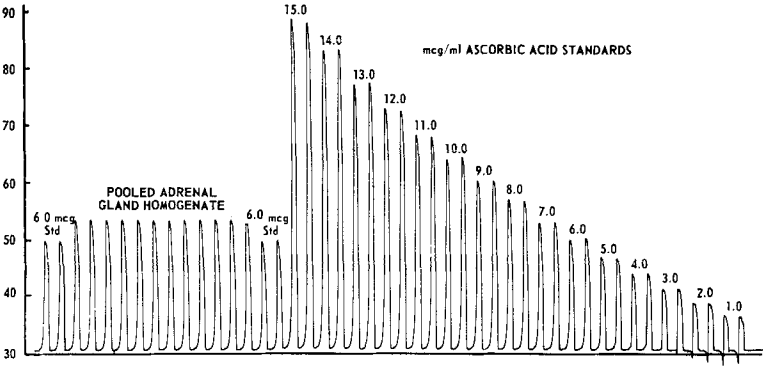

Fig. 2 — Transmittance-concentration recording of ascorbic acid standards and replicate sampling of pooled adrenal gland homogenate.

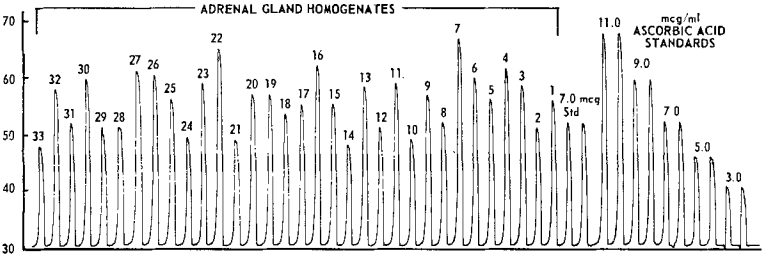

Fig. 3 — Representative transmittance recording of ascorbic acid standards and adrenal gland homogenates from a corticotropin bioassay.

the end block (Fig. 1) and attaching it to a 0.030 × 0.048-in. polyethylene sample line, and by using a 1:2 sample–rinse ratio. Satisfactory movement of the air bubble between samples and rinse occurred when polysorbate 80 was added to the metaphosphoric acid rinse. Polysorbate 80 also reduced the deposition of fatty material from adrenal gland homogenates on the walls of the sample line. The effect of pulsation was lessened by allowing approximately 4.5 in. of manifold tubing to extend beyond the exit end of the end block and by using an H-O cactus.

Twelve milliliters of 2.5% metaphosphoric acid was used in place of the 8.0 ml. as directed by U.S.P. XVII in order that adequate adrenal gland homogenate was available for both automated and manual ascorbic acid determinations. The concentration of 2,6-dichloro-indophenol was reduced to compensate for the greater volume of adrenal gland homogenate.

Attempts to filter the adrenal homogenate automatically with Technicon's continuous filter using various filter papers and a Millipore[4] nylon roll filter

[4] Millipore Filter Corp., Bedford, Mass.

TABLE I—PRECISION OF REPLICATE ASCORBIC ACID DETERMINATIONS

Ascorbic Acid Std., mcg./ml.						Adrenal Gland Homogenate, mcg./ml.
2.0	4.0	6.0	8.0	10.0	12.0	
2.00	3.90	6.00	7.90	9.85	12.05	7.10
2.00	4.00	5.95	7.95	10.00	12.00	7.10
2.00	4.10	6.00	8.00	10.00	12.00	7.15
2.00	4.05	5.95	8.05	10.00	12.25	7.15
2.05	4.00	6.00	8.05	9.90	12.00	7.15
2.05	4.00	6.05	8.05	10.00	12.00	7.15
2.00	4.00	6.05	8.05	9.90	12.05	7.10
2.00	3.95	6.00	8.00	10.00	12.00	7.10
						7.10
						7.15
						7.10
						7.10
			Coefficients of Variation, %			
1.15	1.49	0.60	0.67	0.62	0.72	0.36

TABLE II—RECOVERY OF ASCORBIC ACID FROM VARIOUS QUANTITIES OF ADRENAL GLAND HOMOGENATE ADDED TO ASCORBIC ACID STANDARD

Ascorbic Acid from Homogenate, mcg./ml.	Ascorbic Acid Std. Added, mcg./ml.	Theoretical Amt. of Ascorbic Acid, mcg./ml.	Ascorbic Acid Found, mcg./ml.	Recovery, %
0.45	4.85	5.30	5.25	99.1
0.95	4.85	5.80	5.80	100.0
1.90	4.85	6.75	6.75	100.0
2.85	4.85	7.70	7.65	99.4
3.80	4.85	8.65	8.65	100.0
4.75	4.85	9.60	9.70	101.0
				Av. 99.9

were unsatisfactory. Dialysis was not attempted in these studies; however, it is possible that newer membranes permitting more efficient dialysis and a flow cell with a longer light path would provide the necessary sensitivity.

Figure 2 gives a recorder tracing of ascorbic acid standards in the range of 1.0–15.0 mcg./ml. and replicated samples of pooled adrenal gland homogenates. The uniformity of replicate analyses and the return to base between samples can be noted. When the values for the standards of Fig. 2 are plotted, a linear relationship exists for the logarithm of transmittance and ascorbic acid concentration. Figure 3 gives transmittance recordings for standards and a portion of the recordings from adrenal gland homogenates in a typical corticotropin bioassay.

The precision of the automated method was determined by repetitively assaying 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 mcg./ml. ascorbic acid standards and pooled adrenal gland homogenate. In Table I, it can be seen that the coefficients of variation range from 0.36% to 1.49%, with less precision occurring at lower ascorbic acid concentrations.

Recovery of ascorbic acid with the automated procedure was determined by adding standard to various quantities of adrenal gland homogenate. An average recovery of 99.9% was obtained, varying from 99.1% to 101.0% (Table II). Similar ascorbic acid values for adrenal gland homogenates were obtained for both automated and manual methods of assay. Table III shows a representative adrenal ascorbic acid depletion assay for corticotropin with ascorbic acid determined on parallel samples of homogenate. It can be noted that statistical data for the corticotropin bioassay of Table III are in close agreement. Numerous additional bioassays have been carried out using both manual and automated procedures for adrenal ascorbic acid with satisfactory results.

TABLE III—PROTOCOL OF AN ADRENAL ASCORBIC ACID DEPLETION BIOASSAY FOR CORTICOTROPIN[a]

mg. Ascorbic Acid/100 Gm. Gland											
U.S.P. Corticotropin Std.						Corticotropin Extract					
40 mu/Rat		100 mu/Rat		250 mu/Rat		40 mu/Rat		100 mu/Rat		250 mu/Rat	
M	A	M	A	M	A	M	A	M	A	M	A
345.8	356	308.6	329	290.8	293	330.7	333	309.5	320	282.1	286
414.9	411	361.8	372	272.3	282	359.0	362	310.8	324	284.5	286
461.9	465	323.8	326	232.5	242	484.4	478	290.7	298	270.3	281
437.2	444	310.8	320	241.0	247	479.6	483	313.2	324	235.7	257
395.9	396	335.9	344	273.2	284	367.7	374	295.9	304	307.2	318
400.8	405	262.3	289	274.4	283	382.6	384	333.3	340	253.1	261
402.1	411	292.9	299	226.9	243	399.3	410	323.2	342	293.8	308
				M		A					
Potency (units/ml.)				44		44					
Variance				1216		1094					
Test for parallelism of slopes (F)				0.8580		1.1415					
Log-confidence interval (L)				0.2576		0.2554					

[a] Ascorbic acid determinations were made on each pair of rat adrenals by both manual (M) and automated (A) procedures. Statistical analysis carried out as directed by U.S.P. XVII.

An alternate procedure has been developed for calculations of adrenal gland ascorbic acid based on absorbance readings. Absorbance paper is used in conjunction with a single 8.0-mcg./ml. level of ascorbic acid standard. A computer program is used that provides for the subtraction of recorded absorbance readings of standard and test samples from the absorbance of the color reagent +2.5% metaphosphoric acid base line. The program also provides for the computation of milligrams ascorbic acid per 100 Gm. of paired adrenal glands based on the relative absorbances of the 8-mcg./ml. standard and test samples and on the weights of paired adrenal glands. The computer program places ascorbic acid values in their proper dosage groups and provides for statistical calculations for the assay as directed by U.S.P. XVII.

SUMMARY

An automated procedure is described for the determination of ascorbic acid in rat adrenal gland homogenates in the bioassay of corticotropin. The procedure, based on that of the U.S.P. XVII, permits the analysis of 60 samples/hr. with a coefficient of variation of approximately 1%. Ascorbic acid was recovered satisfactorily from standard samples spiked with varying amounts of adrenal gland homogenate. Corticotropin bioassays using ascorbic acid results obtained with manual and automated procedures compare favorably.

The automated ascorbic acid procedure and the computer program should have application in the Parlow ovarian ascorbic acid depletion bioassay of luteinizing hormone, chorionic gonadotropin, and pregnant mare's serum (2).

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.
(2) Parlow, A. F., *Federation Proc.*, **17**, sect. 1587(1958).

Notes

Estimation of Volume of Distribution and Half-Life of a Compound After Rapid Intravenous Injection

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It is common practice to plot the plasma or serum concentrations of a compound obtained following rapid intravenous injection on semilogarithmic graph paper, then estimate the rate constant ($\hat{\beta}$) for loss of compound from the body or half-life ($0.693/\hat{\beta}$) from the slope of the terminal linear segment, extrapolate the linear segment to time zero, and divide the intercept into the dose to obtain the apparent volume of distribution ($\hat{V}_d$). Under certain conditions the estimates obtained by this procedure may be valid. However, this report shows that if the model which applies is a two compartment open system, then the $\hat{V}_d$ estimated by this method is always an overestimate of the true total volume ($V_1 + V_2$), and the error depends upon the relative values of V_1/V_2 and K_1/K_2 where K_1 is the first-order rate constant for distribution and K_2 is the first-order rate constant for loss from the inner compartment. The half-life estimates ($0.693/\hat{\beta}$) will always be greater than the true half-life ($0.693/K_2$), and $\hat{\beta}$ as an estimator of K_2 also depends on the ratios V_1/V_2 and K_1/K_2.

IT IS COMMON practice[1] (1–5) to plot the plasma or serum concentration of a compound obtained following rapid intravenous injection on semilogarithmic graph paper, then estimate the rate constant for loss of compound from the body ($\hat{\beta}$) or the half-life ($0.693/\hat{\beta}$) from the slope ($\hat{\beta}/2.303$) of the terminal linear segment; and extrapolate the linear segment to time zero and divide the intercept ($\hat{B}_1$) into the dose (D) to obtain the apparent volume of distribution, pool size, or "space" ($\hat{V}_d$). If the model which applies to the particular system involves a single compartment and the initial nonlinearity of the semilogarithmic plot is assumed to be due to mixing, then the estimates $\hat{\beta}$ and $\hat{V}_d$ would be very close to those expected on the basis of the appropriate mathematical expression.

Nelson (6), however, pointed out that the error in the estimated volume of distribution may be substantial if there are two compartments with volumes V_1 and V_2 and the volume of the outer compartment (V_2) is large, and rate of attainment of equilibrium is slow. This report will show that if the model which applies is a two compartment open system, then $\hat{V}_d$ always overestimates the true total volume

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[1] The practice is so common that the authors considered it unnecessary to provide an extensive bibliography. However, *References 1–5* are a representative sample.