1. Report No. FAA-AM-71-15	2. Government Accession No.	3. Recipient's Catalog No.
4. Title and Subtitle AUTOMATED DIFFERENTIAL FLUOROMETRIC ANALYSIS OF NOREPINEPHRINE AND EPINEPHRINE IN BLOOD PLASMA AND URINE		5. Report Date April 1971
		6. Performing Organization Code
7. Author(s) Vincent Fiorica, Ph.D. Russell Moses, B.A.		8. Performing Organization Report No.
9. Performing Organization Name and Address FAA Civil Aeromedical Institute P. O. Box 25082 Oklahoma City, Oklahoma 73125		10. Work Unit No.
		11. Contract or Grant No.
12. Sponsoring Agency Name and Address Office of Aviation Medicine Federal Aviation Administration 800 Independence Avenue, S.W. Washington, D.C. 20590		13. Type of Report and Period Covered OAM Report
		14. Sponsoring Agency Code

15. Supplementary Notes

16. Abstract

An automated fluorometric trihydroxyindole procedure is described for the measurement of norepinephrine (NE) and epinephrine (E) in blood plasma or urine. The method employs conventional techniques for isolation of the catecholamines by alumina column chromatography. Column eluates are analyzed in an AutoAnalyzer system incorporating two fluorometers. Differentiation of the amines is based on differences in the fluorescence characteristics of the lutines of NE and E. The accuracy (as estimated by recoveries of added amines) and precision of the method are comparable to those reported for other trihydroxyindole techniques. Representative values for urinary excretion and for plasma levels of NE and E compare favorably with values stated in the literature.

17. Key Words Biochemical analysis, Catecholamines, Norepinephrine, Epinephrine, Urine, Blood Plasma, Fluorometry, Automated Analysis	18. Distribution Statement Availability is unlimited. Document may be released to the National Technical Information Service, Springfield, Virginia 22151, for sale to the public.		
19. Security Classif. (of this report) Unclassified	20. Security Classif. (of this page) Unclassified	21. No. of Pages 18	22. Price $3.00

Form DOT F 1700.7 (8-69)

ACKNOWLEDGMENTS

AUTOMATED DIFFERENTIAL FLUOROMETRIC ANALYSIS OF NOREPINEPHRINE AND EPINEPHRINE IN BLOOD PLASMA AND URINE

I. Introduction.

During the past several years an increasing number of reports has appeared in the literature describing the application of the AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N.Y.) system to the analysis of the catecholamines, norepinephrine (NE) and epinephrine (E). This trend seems to indicate a growing interest in modifying or adapting existing manual techniques for NE and E to the more routinely manageable AutoAnalyzer procedures. The development of such automated analytical systems has given this difficult analysis much broader appeal for general clinical usage or for studies involving large numbers of samples.

Aviation studies dealing with the problems of stress and fatigue frequently require measurements of adrenal catechol hormones in order to evaluate the magnitude of response to the stressor under study. Because this analysis is an intricate, demanding and time-consuming procedure, the additional requirement for large numbers of samples (as in work shift rotation studies, circadian rhythm studies and time-zone crossing studies) intensifies the problem of keeping chemical analysis time commensurate with the time required for the analysis of other data acquired. A practical solution seems to be the automation and simplification, to the extent possible, of this complex analysis to reduce excessive manipulation and to increase the speed and accuracy of the test. The method described in this paper was devised to meet those needs.

Of the automated techniques presently available (1–10) all are based, completely or in part, on the methodology proposed by Merrills in 1962.[1][2] The key feature of Merrills' differential scheme is the use of a solution (1% v/v) of thioglycolic (mercaptoacetic) acid as a stabilizing reagent. After oxidation of both NE and E in a sample eluate by ferricyanide at pH 6.0, only the fluorescent noradrenolutine is stabilized in the presence of thioglycolic acid. The NE in the sample is thereby measured directly. If thioglycolic acid is replaced by ascorbic acid (0.3% v/v) and a second aliquot of the sample eluate is analyzed, both the noradrenolutine *and* the adrenolutine are stabilized and the total NE + E in the sample is measured. The E content of the sample is then obtained by difference. Although several modifications of Merrills' procedure have been proposed, these have been concerned chiefly with improving the sensitivity of the assay.

The present method is based on the differential system of Cohen and Goldenberg[11][12] a procedure used successfully in its original form, but which, until now, has not been adapted for automated analysis. Differential analysis of the catecholamines is achieved in the Cohen-Goldenberg scheme by utilizing differences in the fluorescence characteristics of noradrenolutine and adrenolutine. By expanding our existing system for automated analysis of total catecholamines,[13] differential fluorometry could be achieved without further modification. This was accomplished by the simple expedient of adding, in series with the existing fluorometer (Turner, Model 111), a second fluorometer, differing from the first only in the wave lengths selected by the primary and secondary filter combinations.

The system described in this paper offers several advantages over existing automated procedures:

1. It permits the use of a smaller initial sample because it eliminates the need for dividing the eluate (from the adsorption step) for the two runs required on the AutoAnalyzer in the differential oxidation/pH system, the thioglycolic

1

acid/ascorbic acid system, or in differential fluorescence systems utilizing a single fluorometer.

2. It avoids the need for changing reagents for the AutoAnalyzer between sets of samples.

3. It is sufficiently sensitive for the analysis of NE and E in peripheral blood plasma, yet permits the analysis of NE and E in urine without requiring changes in either the AutoAnalyzer manifold or adjustments in fluorometer sensitivities.

As in the procedure upon which this method is based,[13] the technique of batch adsorption and column elution of NE and E has been retained in order to maximize the recovery of the isolated catecholamines in a minimum volume of eluate.

II. Materials and Methods.

Reagents. Reagents were prepared from chemicals of reagent grade or highest purity available. Solutions were prepared with twice-distilled water, the second distillation made in an all-glass apparatus.

1. Alumina (Woelm), neutral, activity grade 1 for chromatography. Alumina was treated by the procedure described by Anton and Sayre.[14] The purified alumina was stored in a desiccator.

2. Sodium acetate, 0.2 M, purified by passing the solution through a 25 x 300 mm column of Chelex-100 (Bio-Rad Laboratories, Richmond, California), 50–100 mesh, Na+ form. The pH of the purified buffer was adjusted to 8.4±0.1 (glass electrode) with 0.5 N sodium carbonate.

3. Ethylenediamine tetraacetate, disodium, 5% (w/v).

4. Ethylenediamine tetraacetate, disodium, 10% (w/v).

5. Sodium acetate, 1.5 M containing 0.01% (w/v) potassium ferricyanide. The pH of this solution was adjusted to 9.0±0.1. Traces of suspended material were removed by passing the solution through a medium porosity fritted glass filter.

6. Ascorbic acid, 0.3% (w/v). The Eastman product was used without additional purification.

7. Sodium hydroxide, 2.5 M.

8. Perchloric acid, 4.4 N.

9. Acetic acid, 0.3 M.

10. Acetic acid, 0.3 M, adjusted to pH 3.85.

11. Stabilizing solution, 0.01 N HCl containing 0.5 mg/ml sodium metabisulfite.

Standards. Stock standards for NE and E were prepared from dl-arterenol hydrochloride and l-epinephrine bitartrate (Winthrop Laboratories, New York) respectively. The primary standards for each were prepared in stabilizing solution at a concentration of 50 μg/ml (as the free base). All subsequent dilutions were also made with stabilizing solution. Another set of catecholamine stock standards containing *both* NE and E in the ratio 2:1 were prepared to contain a total of 50 μg/ml of both amines as free bases (33.3 μg NE and 16.7 μg E per ml). Working standards for NE, E and the combination of NE and E were prepared from dilutions of the respective stock standards. Appropriate dilutions were made to give working standards of 1 ng/ml of NE or E, or in the case of the combined standard, 1 ng total catecholamines per ml. In routine use, only the individual standards for NE and E are used. Under usual conditions 5.0 ml of each working standard were taken as the sample; the total quantity of each amine used as a standard was 5 ng.

Stock standards for either NE or E, prepared in stabilizing solution, are stable under refrigeration for at least 6 months. All working standards were prepared freshy before each run.

Apparatus.

1. Glass adsorption tubes for column chromatography were slightly modified from those described previously.[13] The tubes presently used (Figure 1) have the following dimensions; reservoir, 95 x 28 (I.D.) mm, stem 175 x 4 (I.D.) mm. A capillary tip, 10 x 2 (I.D.) mm is sealed to the stem. The wall thickness of both the reservoir and stem is 2 mm. Capacity of the reservoir is 50 ml.

The adsorption tubes are used in a negative pressure manifold which facilitates washing of the column. The flow through each tube is controlled independently so that uniformity of flow from tube to tube can be maintained. One analyst can conveniently handle 14 tubes in such a manifold system.

2. Automated analysis was achieved with a standard AutoAnalyzer system consisting of a sampler (II), pump, two fluorometers (Turner, Model 111) and a two-pen recorder. A schematic flow diagram of the system is shown in Figure 2. In this scheme, sample (column eluate, pH 3.85) is mixed with ferricyanide in acetate buffer (pH
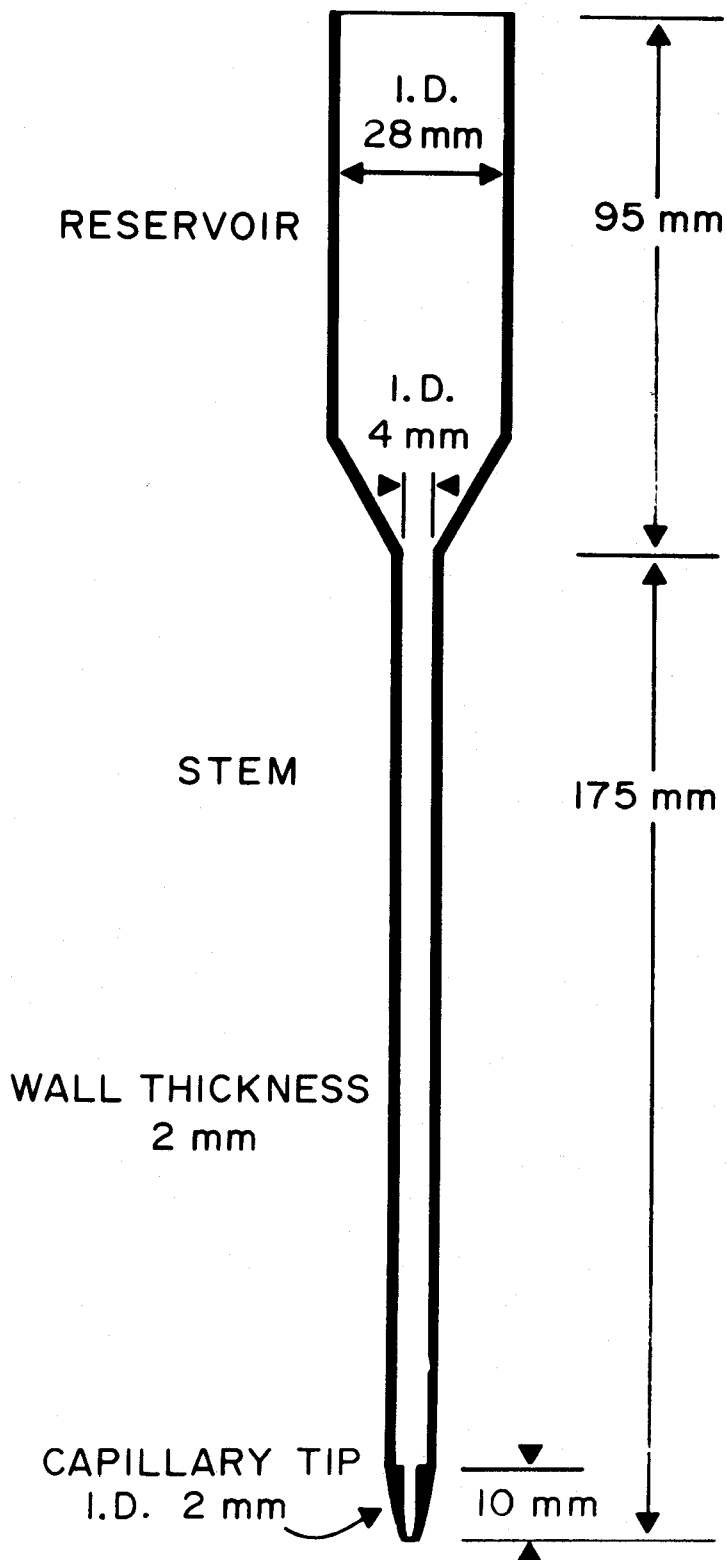
2

FIGURE 1. Diagram of glass adsorption tube used for alumina column chromatography. Capacity of the reservoir is 50 ml. Use of a capillary tip minimizes the dead volume after the elution step.

3

9.0) and segmented with air. Before its introduction into the liquid stream, air is pulled through a water scrubber to remove particulate material (e.g. smoke) which can be a factor influencing background fluorescence. Oxidation of the catecholamines by ferricyanide takes place in a double mixing coil at pH 6.0±0.1. The duration of the reaction is determined by the total flow rate and the length of the coil. In this system the time permitted for oxidation of NE and E to noradrenochrome and adrenochrome was 100 seconds. After the oxidation step this stream is joined by the alkaline ascorbate mixture. High background fluorescence, often encountered as the alkaline ascorbate reagent deteriorates during the course of a run, is avoided in this system as the mixture is made just before its addition to the ferricyanide stream. The oxidized catecholamines, in the form of noradrenochrome and adrenochrome, are rearranged in alkaline solution to the corresponding lutines. The presence of ascorbate in the alkaline mixture stabilizes the lutines by retarding their further oxidation. The rearrangement reaction also takes place in a double mixing coil and similarly takes about 100 seconds. Beyond this point air is removed from the stream and the stabilized lutines are pumped through the dual fluorometer system.

Because noradrenolutine and adrenolutine have slightly different fluorescence characteristics, this variance can be utilized in differentiating the proportion of the two in a mixture. The use of two fluorometers in series, each equipped with filters passing wave lengths near the fluorescence optima for excitation and emission of one or the other of the lutines, is the simplest means of achieving the differential analysis in a continuous flow system.

The first fluorometer, designated A in Figure 2, is equipped with primary filter Wratten 47B (Turner 110–813) passing light at wave lengths of 405 and 436 mu. The secondary combination consists of a Wratten 2A–12 (Turner 110–818) and a Corning 3–70. This set provides a sharp cut filter passing wave lengths longer than 510 mu.

The second fluorometer, B in Figure 2, uses primary filter RDUV #1, B–3(A), narrow pass 395–410 mu. This filter is available from Baird Atomic Corp., Cambridge, Mass., and, according to Hathaway, et al.,[5] has the following specifications: "Type B–3(A), Peak center of 395–410 mu, Min. transmission at 455 mu, Block to 690 mu. Make to provide maximum transmission, size 2 x 2 in." The secondary filter for this fluorometer is a Wratten #8 (Turner 110–817) passing wave lengths longer than 485 mu. Several minor changes in the fluorometers were necessary in order to achieve the sensitivity required for the assay. As light sources for excitation, continuous emission (400–520 mu) blue lamps (GE–F4T5–B) were installed in both fluorometers using the adapters supplied by Turner (110–856). These lamps replaced the general purpose lamps normally furnished with this instrument.

A second change made was the replacement of the standard cuvet holder in each fluorometer with a high-sensitivity temperature-stabilized sample holder. This holder as supplied by the manufacturer is designed to be used manually with round glass cuvets. For use with the flow system, the commercial sample holders were modified to accept a specially designed flow cuvet holder (Figure 3). The holder was fabricated from aluminum tubing stock and sprayed inside and out with flat black paint. The flow cuvet, also shown in Figure 3, was constructed from fused quartz tubing. The volume of the cuvet chamber, of the dimensions indicated, is 1.0±0.1 ml. Flow cuvets of this size permit the use of the maximum slit area of the commercial sample holder at the smallest practical cuvet chamber volume.

To provide uniform temperature conditions at the flow cuvets, water was circulated through the jackets of the sample holders. The temperature of the sample holders was maintained between 20 and 24 C.

For the conditions described, the fluorometer sensitivity settings were: A; 10 X excitation light aperture, span adjustment fully counterclockwise, (from the back of the instrument), B; 30 X excitation light aperture, span adjustment fully clockwise. The limiting factor in establishing fluorometer sensitivities is the response of epinephrine. With the filter combinations used and at the fluorometer settings indicated, the specific fluorescence (fluorescence per ng) of epinephrine under A conditions is nearly identical to that under B conditions. The specific fluorescence of norepinephrine under B conditions however, is about twice that under A conditions.
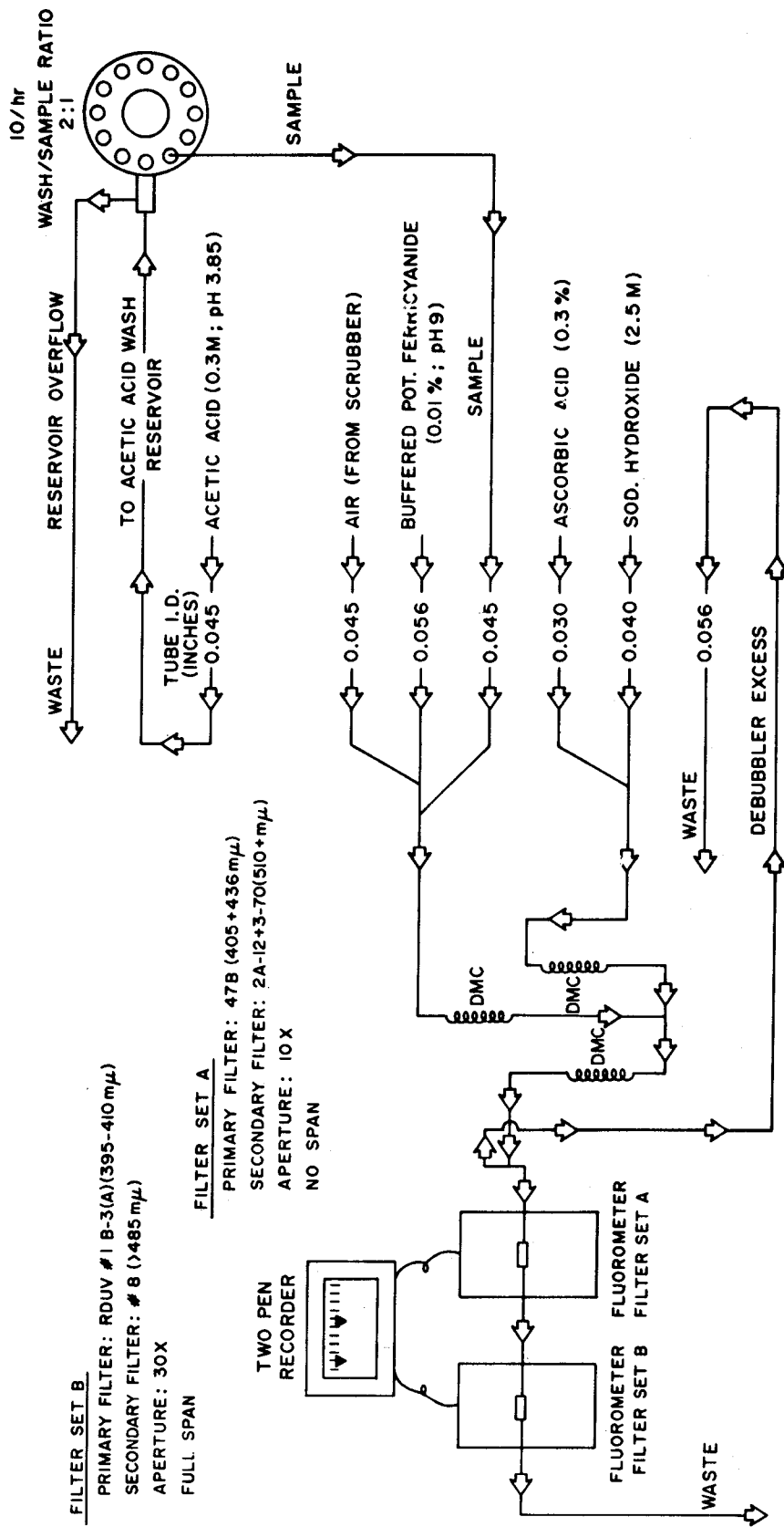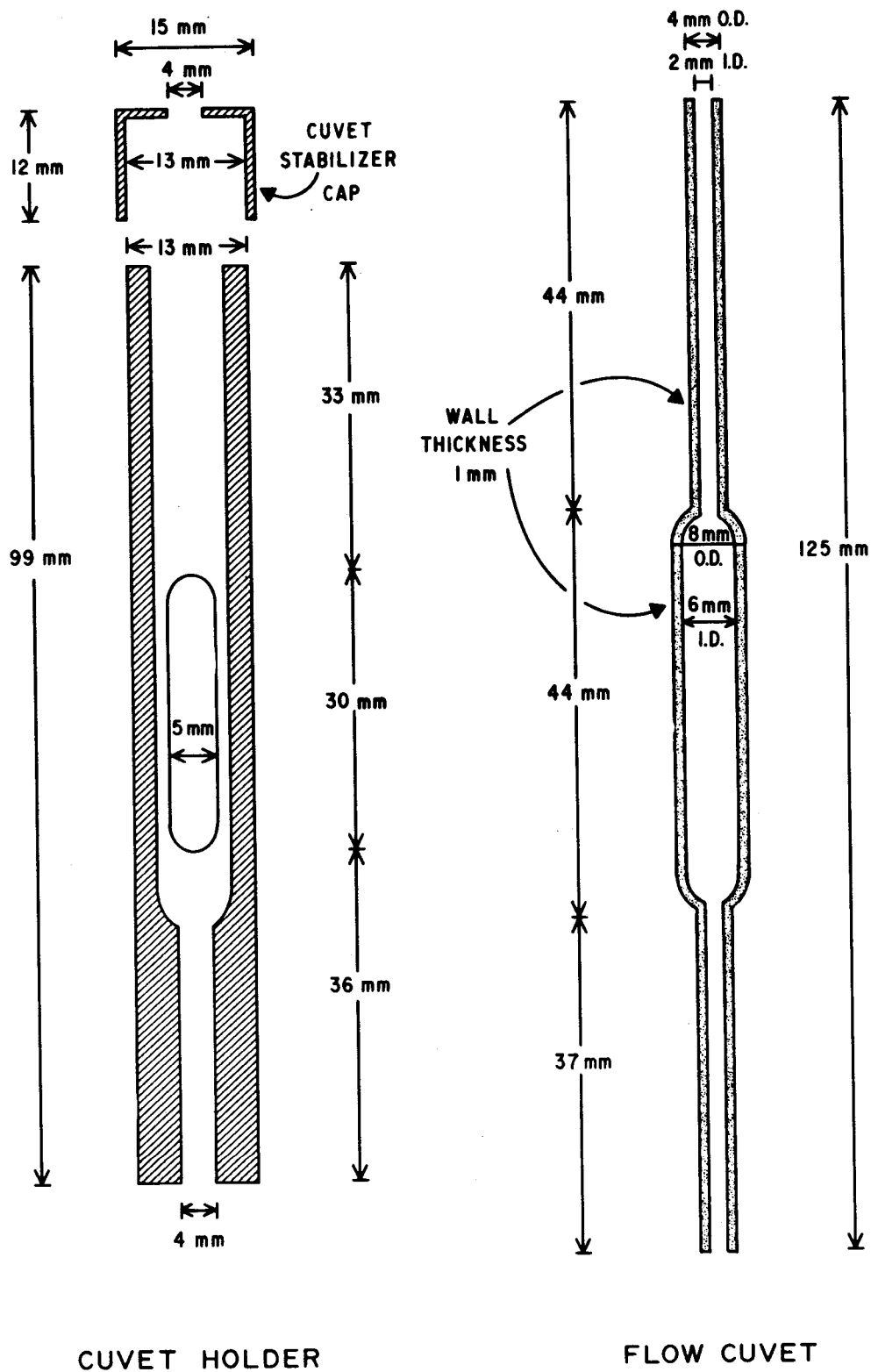
4

FIGURE 2. Schematic diagram of the AutoAnalyzer flow system for fluorometric differentiation of NE and E. The use of two fluorometers in series permits the fluorescence from two filter sets to be recorded from a single pass of sample through the system.

15 mm

4 mm

12 mm

13 mm

CUVET
STABILIZER
CAP

13 mm

99 mm

5 mm

4 mm

4 mm O.D.

2 mm I.D.

44 mm

WALL
THICKNESS
1 mm

8 mm
O.D.

6 mm
I.D.

44 mm

37 mm

125 mm

33 mm

30 mm

36 mm

CUVET HOLDER

FLOW CUVET

FIGURE 3. Diagram of the cuvet holder required for the jacketed high-sensitivity sample holder supplied by Turner for the Model 111 fluorometer. The dimensions given for the cuvet holder are specific to accommodate the fused quartz flow cuvet shown. The volume of the cuvet chamber is 1.0 ± 0.1 ml.

This difference in specific fluorescence forms the basis for the differential assay.

To maximize sample fluorescence, a sampling rate of 10 per hour was selected with a wash/sample ratio of 2:1. This rate and ratio were achieved by removing one of the lifters from a standard 20 per hour cam and using this modified cam to activate the sampler. In this system, sample pickup occurs over a two-minute period and wash (0.3 M acetic acid; pH 3.85) pickup over a four-minute period. Using a 2:1 wash/sample ratio permits near baseline conditions to be achieved between samples and minimizes sample-to-sample interference. At the sample pumping rate used (0.80 ml/min) two minutes of pickup required a minimum sample volume of 1.8 ml.

III. Procedure.

Sample volumes specified in the following description of procedure were selected by assuming concentrations of NE and E ordinarily encountered in plasma and urine. Generally, the total quantity of catecholamine in the sample should be between 2.5 and 25 ng.

Plasma. Blood is drawn in heparinized syringes. The sample is transferred to a centrifuge tube coated with dry sodium metabisulfite (0.5 mg/ml blood), chilled in ice and centrifuged. Exactly 5.0 ml of plasma are transferred to a round bottom polycarbonate centrifuge tube (16 x 100 mm; 12 ml capacity). To the sample are added 5.0 ml stabilizing solution and 1.0 ml perchloric acid. The tube is capped, vigorously agitated, and centrifuged at 30,000 x g (10 C) for ten minutes. The supernatant liquid is carefully decanted into a small beaker.

Urine. Urine is acidified as collected in the approximate proportion of 1 ml 9N H_2SO_4 per 200 ml urine. This schedule of acidification should give a final pH of 3 or less. Exactly 0.5 ml of the urine sample is transferred to a round bottom polycarbonate centrifuge tube as above. To the urine sample are added 9.5 ml stabilizing solution and 1.0 ml perchloric acid. The tube is capped, thorougly shaken and centrifuged at high speed as for plasma. The supernatant liquid is decanted.

Standards and Reagent Blanks. Standards and blanks are treated in the same manner as samples.

NE and E standards are prepared in duplicate. Exactly 5.0 ml of working standard (1 ng/ml) for NE or E are added to polycarbonate centrifuge tubes as above. To the standards are added 5.0 ml stabilizing solution and 1.0 ml perchloric acid. After the solutions are mixed, the tubes are centrifuged at high speed and the supernatant liquid decanted. The reagent blank is prepared similarly except that 1.0 ml perchloric acid is added to 10.0 ml stabilizing solution.

Adsorption and Elution. As the method is presently carried out, each set of unknowns is accompanied by one reagent blank (0 ng), two NE standards (5 ng) and two E standards (5 ng). In a full analytical run, nine unknowns can be processed along with the standards and blank.

To 50 ml beakers containing 500 mg acid-washed alumina and 10 ml 0.2 M sodium acetate, pH 8.4, are added exactly 9.0 ml aliquots of the supernatant liquid decanted after high speed centrifugation. If the unknown samples are urine, 4 ml 10% EDTA are added to each beaker; for plasma samples, 1 ml 5% EDTA is added. The pH of the alumina—sample mixture is adjusted to 8.4±0.1 (glass electrode) and maintained at that pH for 4 minutes by the dropwise addition of 5 N sodium carbonate. By using a small turntable (10 rpm) to rotate the beaker and a motorized glass stirring rod, the mixture can be agitated continuously through the pH adjustment so that the particles of alumina are evenly distributed throughout the solution. Using this arrangement for mixing avoids the problem of pulverizing the alumina, an annoyance generally encountered with the magnetic mixing bar. After the pH has been maintained for 4 minutes, the alumina is transferred as a slurry to the adsorption tubes. The alumina settles rapidly to form a uniform column in the stem of the tube. Presently we use a small piece of glass wool placed just above the capillary tip to support the column. (We have tried replacing the glass wool with 100 pore polyurethane filter material, Bellco Glass Co., Vineland, N.J. When this material is used to support the column, a smooth flow of liquid is achieved and the alumina column formed packs uniformly. Preliminary tests were encouraging and indicated no difference in the results obtained, whether the column support was polyurethane or glass wool). The buffer solution above the column is permitted to flow through the column at the rate of

1–1.5 ml/min. The maintenance of this rate of flow is facilitated by the negative pressure manifold. After the buffer solution has passed through the column, the column is washed with 40 ml glass-distilled water (pH 8.4±0.1) at a flow rate between 1.5 and 2 ml/min. The last ml of wash is permitted to flow through the column by gravity. The catecholamines are eluted from the column with 2.0 ml 0.3 M acetic acid also allowed to flow by gravity. The eluate is collected in a round bottom polycarbonate tube for high speed centrifugation (30,000 x g, 10 C). This is done to precipitate the fine suspension of alumina occasionally encountered after elution. The centrifuged eluate is finally decanted into standard sample cups and processed by the Auto-Analyzer system described. The fluorescence of standards (run through columns) and unknowns from filters sets A and B are recorded on a two-pen recorder. The fluorescence value of the reagent blank is subtracted from the fluorescence peak values for both standards and unknowns.

Calculations for Differential Fluorometry. Computation of the quantity of NE and E in a sample is based on the following consideration:[11]

let A = total fluorescence of unknown with filter set A

let B = total fluorescence of unknown with filter set B

let N_A = fluorescence contributed by NE with filter set A

let E_A = fluorescence contributed by E with filter set A

let N_B = fluorescence contributed by NE with filter set B

let E_B = fluorescence contributed by E with filter set B

Then: $A = N_A + E_A$ (1)

and $B = N_B + E_B$ (2)

Further:

let X = quantity (ng) of E in the unknown

let Y = quantity (ng) of NE in the unknown

let E_a = fluorescence of E with filter set A per ng E (standard)

let E_b = fluorescence of E with filter set B per ng E (standard)

let N_a = fluorescence of NE with filter set A per ng NE (standard)

let N_b = fluorescence of NE with filter set B per ng NE (standard)

Equation (1) and (2) then become

$A = YN_a + XE_a$ (3)

$B = YN_b + XE_b$ (4)

Solving equation (4) for X gives:

$$X = \frac{B - YN_b}{E_b} \quad (5)$$

Substituting (5) into (3) and solving for Y gives:

$$A = YN_a + \frac{(B - YN_b)\ E_a}{E_b}$$

$$= \frac{YN_a + BE_a - YN_bE_a}{E_b}$$

$$= \frac{YN_aE_b + BE_a - YN_bE_a}{E_b}$$

$$AE_b = YN_aE_b + BE_a - YN_bE_a$$

$$YN_aE_b - YN_bE_a = AE_b - BE_a$$

$$Y = \frac{AE_b - BE_a}{N_aE_b - N_bE_a}$$

$$= \frac{\dfrac{AE_b}{E_a} - B}{\dfrac{N_aE_b}{E_a} - N_b}$$

Normally, 5 ng standards for NE and E are run through the column procedure in duplicate and the mean values (corrected for reagent blank) used to compute N_a, N_b, E_a and E_b.

Linearity. The relationship between quantity of catecholamine per sample and sample fluorescence for NE, E and a mixture of the two (2NE : 1E) were examined over the range 0.0 to 20.0 ng for both filter sets A and B. Samples were taken through the entire procedure of adsorption, elution and automated analysis to arrive at the fluorescence per sample.

Recoveries. Studies were made of the recovery of NE and E from aqueous solution, canine plasma or human urine. Several levels of added amine were examined. Additions of NE and E were made from solutions containing the individual amines, or from solutions containing both the amines. In every instance the quantity of amine to be recovered was added to the original sample (urine or plasma) before the sample
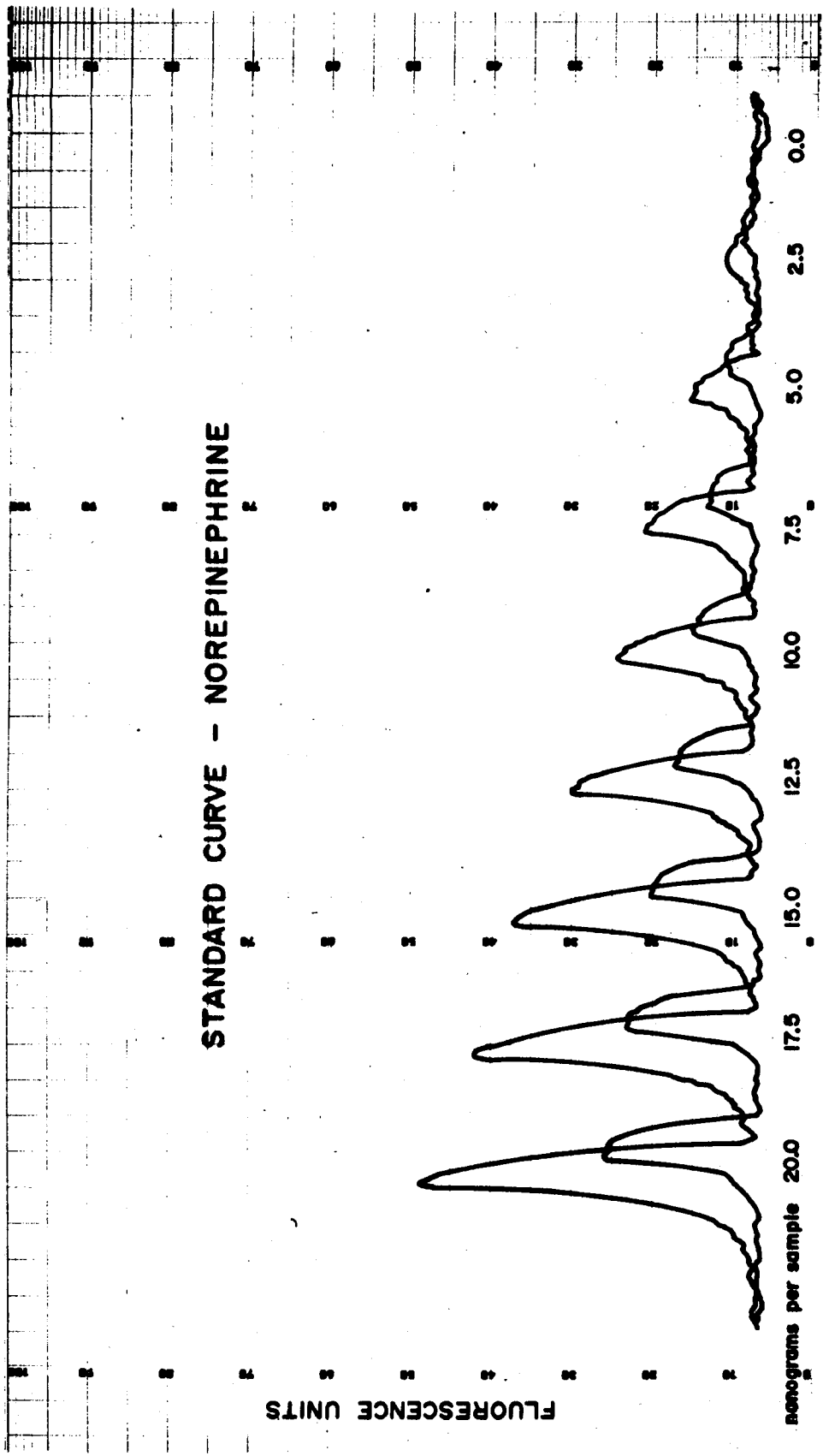
8

FIGURE 4. Tracing of a typical standard curve recording from the AutoAnalyzer system. Reading from right to left, the first peak of the pair represents the fluorescence of NE with filter set A (see text); the second peak represents the fluorescence of NE with filter set B. Sample quantities are the amounts of NE in the starting sample. All samples were carried through the entire procedure.
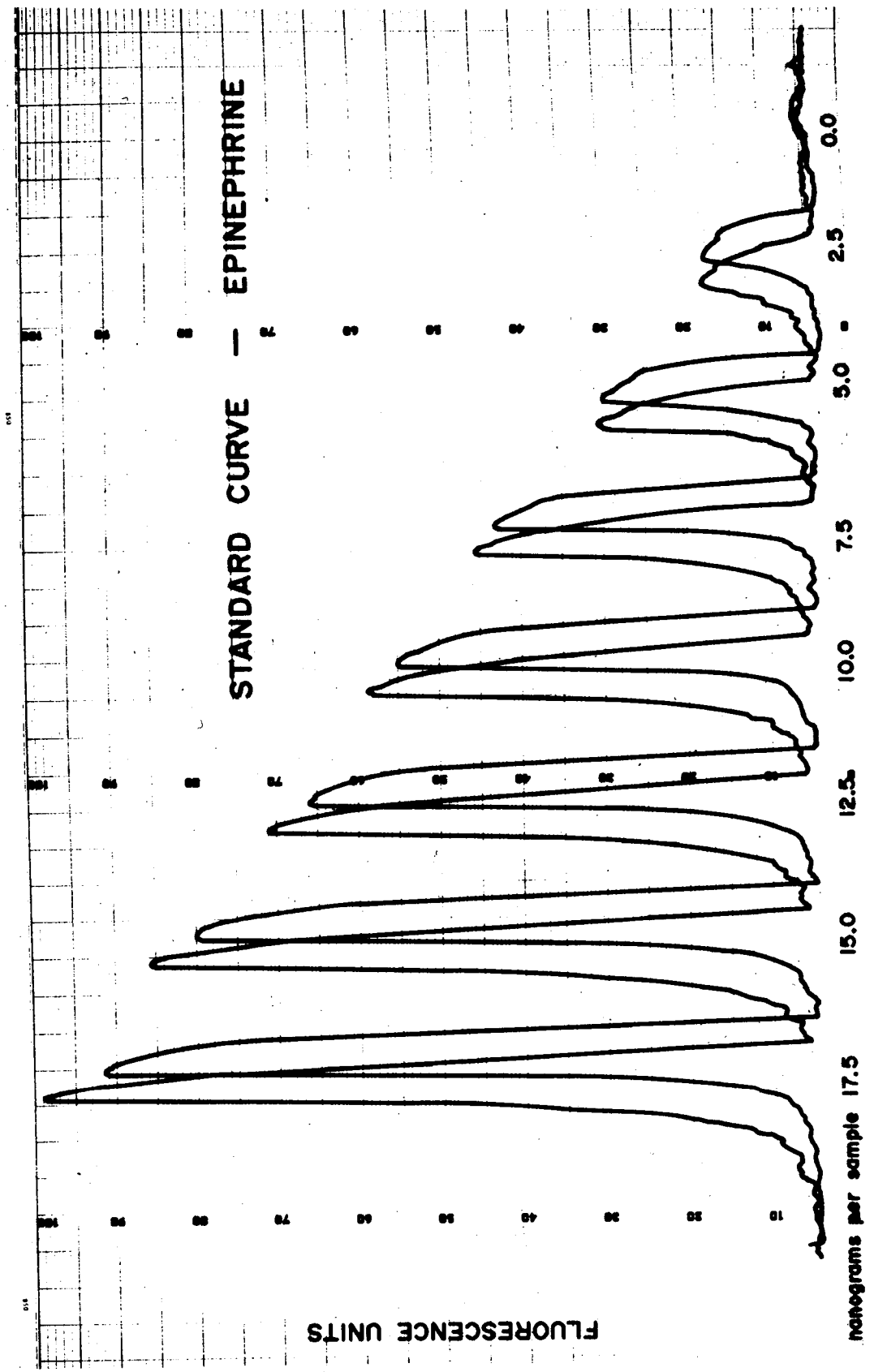
FIGURE 5. Tracing of a typical standard curve recording from the AutoAnalyzer system. Reading from right to left, the first peak represents the fluorescence of E with filter set A (see text) ; the second peak represents the fluorescence of E with filter set B. Sample quantities are the amounts of E in the starting sample. All samples were carried through the entire procedure.

10

was run through the full procedure. The recovery was stated as:

$$\% \text{ Recovery} = \frac{\begin{array}{c}\text{Quantity measured} \\ \text{in sample with} \\ \text{added amine}\end{array} - \begin{array}{c}\text{Quantity measured} \\ \text{in sample without} \\ \text{added amine}\end{array}}{\text{Quantity of amine added}} \times 100$$

IV. Results.

Typical recordings from the AutoAnalyzer system for graded sample quantities of NE and E are shown in Figures 4 and 5. The first peak (reading from right to left) of each pair represents sample fluorescence from the A filter set; the second peak represents sample fluorescence from the B filter set. In practice the two channels of recording are distinguished from each other by use of differently colored inks.

Standard curves for NE, E and a combined standard containing both catecholamines in the ratio of 2NE : 1E are shown in Figures 6, 7, and 8. In all of these curves each point represents the mean of five separate analyses of standard carried through the complete procedure. Over the range 0.0 to 20.0 ng/sample, fluorescence is linearly related to the amount of NE in the sample for both the A and B filter sets. With filter set B (and B fluorometer sensitivity) NE fluorescence is about twice that obtained with filter set A (and A fluorometer sensitivity) per ng NE. With standards containing E alone, fluorescence with A and B filter sets is also linearly related to the quantity per sample (Fig. 7). For E, however, the sensitivity (slope) under A conditions is approximately the same as under B conditions over the range 0.0 to 17.5 ng per sample.

With standards containing both catecholamines (2NE : 1E) fluorescence under A and B conditions is also a linear function of the total quantity of amine in the sample over the range 0.0 to 20.0 ng per sample. In the case of the combined
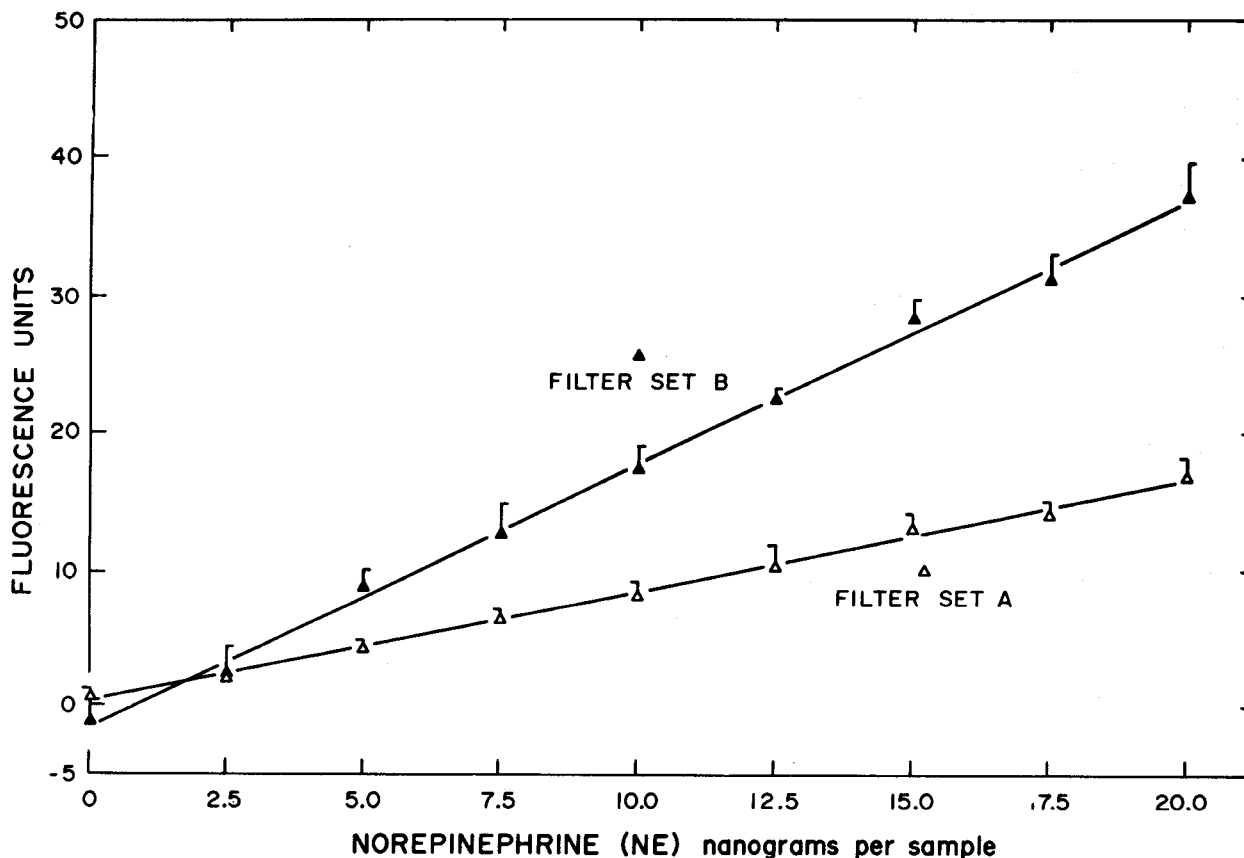

FIGURE 6. Relationship between amount of NE in starting sample and fluorescence intensity with filter sets A and B. The volume of sample was 5.0 ml in all instances and the samples were carried through the entire procedure. Points represent the means of five measurements; the flags represent the standard deviation.
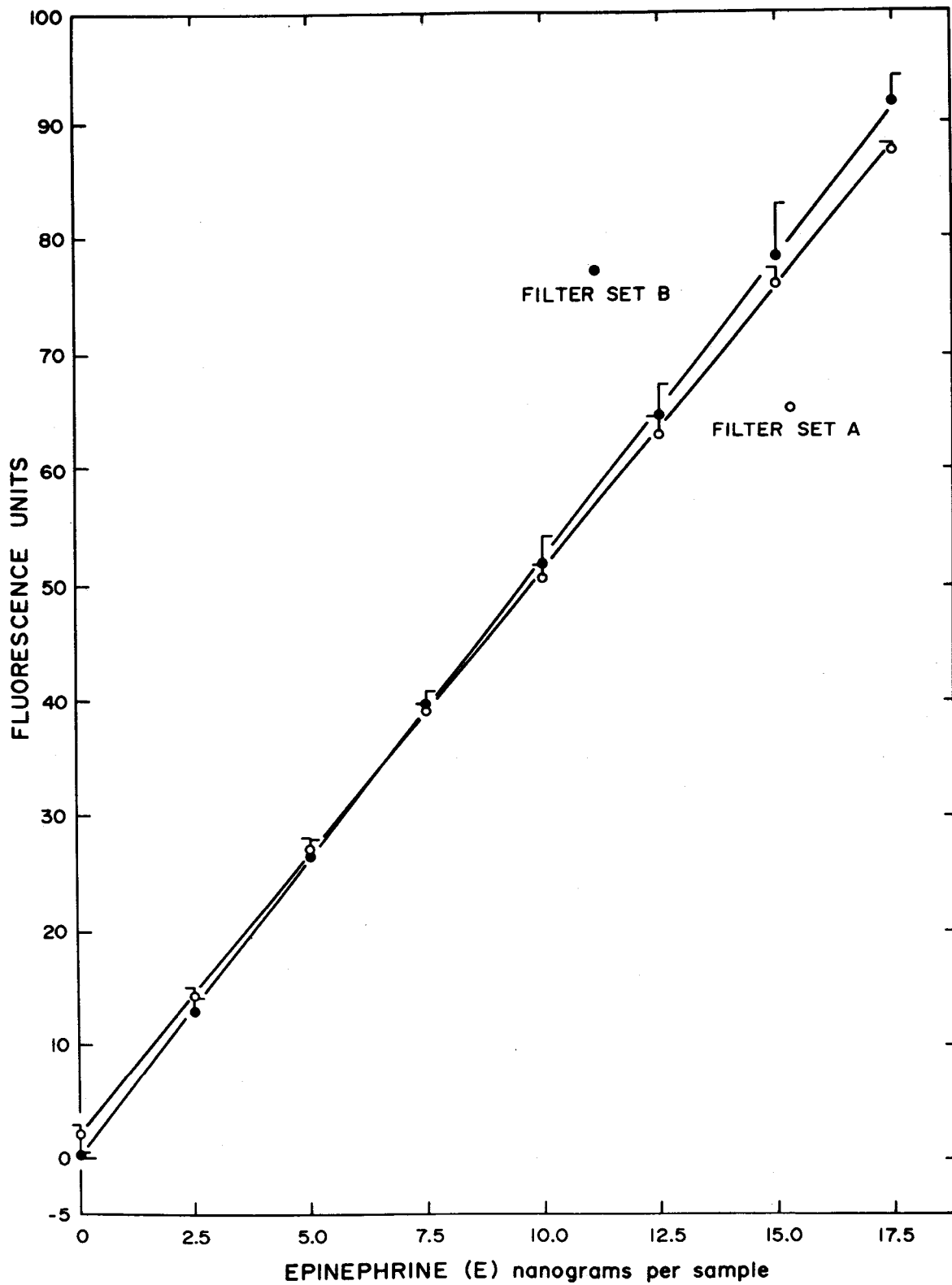
11

FIGURE 7. Relationship between amount of E in starting sample and fluorescence intensity with filter sets A and B. The volume of sample was 5.0 ml in all instances and the samples were carried through the entire procedure. Points represent the means of five measurements; the flags represent the standard deviation.
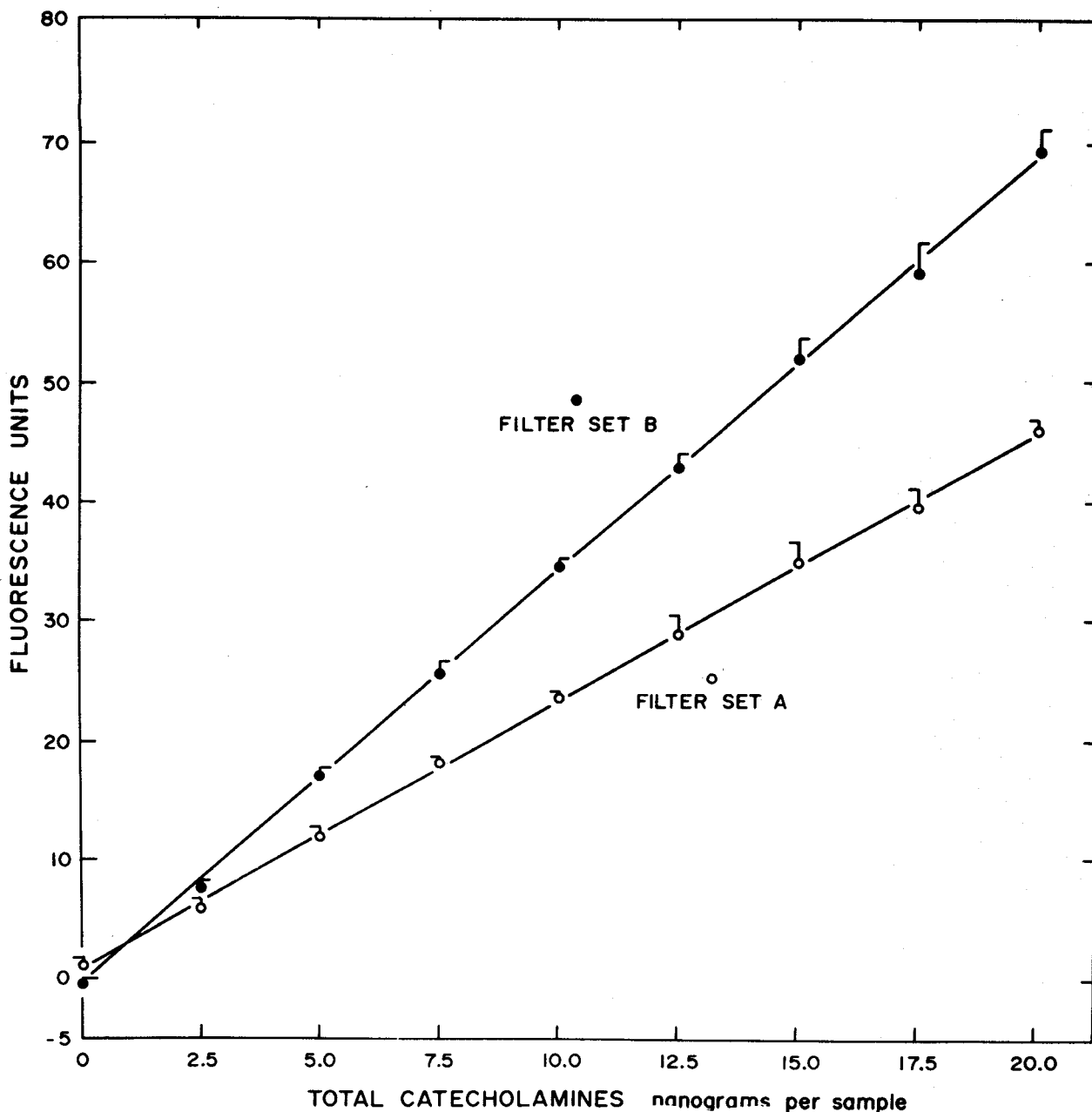
12

FIGURE 8. Relationship between amounts of total catecholamine (NE + E) in starting sample and fluorescence intensity with filter sets A and B. The volume of sample was 5.0 ml in all instances and the samples were carried through the entire procedure. The ratio of catecholamines in each sample was 2 NE : 1 E. Points represent the means of five measurements; the flags represent the standard deviation.

standard the sensitivities are intermediate to the sensitivities obtained under both A and B conditions with standards containing only NE or E.

Recovery data are given in Tables 1–3. Several aspects of the recovery of NE and E, added in physiological amounts to samples of plasma or urine, were examined in order to evaluate the accuracy of the procedure. To provide a point of reference, we first examined the simplest case, the recovery of added NE and E from an aqueous solution containing both amines in amounts likely to be found in peripheral plasma (Table 1). Under this set of conditions added NE and E were recovered to about the same extent (NE, 91.2%; E, 93.1%). In a second series of recovery experiments, we examined the effect of adding two levels of each amine and the effect of adding both amines together on recovery from canine

13

TABLE 1.—Recovery of a Mixture of NE and E from an Aqueous Sample (5.0 ml) Containing 3.33 ng NE and 1.67 ng E

Number of Recoveries		Quantity Added (ng)		*Quantity Recovered(ng)		*Recovery (%)	
NE	E	NE	E	NE	E	NE	E
15	15	3.33	1.67	3.12±0.64	1.47±0.27	91.2±23.6	93.1±19.2

*Mean and standard deviation

plasma (Table 2). Adding 5.0 or 10.0 ng of each amine to samples of canine plasma yielded recoveries of approximately 81% for NE and 95% for E. When the NE and E were added together, but in smaller quantities, the recovery of E remained at about 95% but the NE recovery decreased to about 70%. In a third series, we evaluated the recovery of the amines from urine (Table 3). NE or E added to urine at a single level (5.0 ng) was recovered to the extent of about 80% for NE and about 94% for E. When the amines were added to the urine sample together, the recoveries for NE and E respectively were 101% and 76% at the low level (5.0 ng total) and 85% and 98% at the high level (10.0 ng total). It appears that the greatest variability in recovery is encountered when attempting to recover small quantities from samples already containing relatively larger amounts of the amines. This is especially true for recoveries from urine. The overall means for recoveries of added NE and E from 130 separate analyses were 82.9 ± 22.1 (S.D.) % for NE and 93.1 ± 13.4 (S.D.) % for E.

TABLE 2.—Recovery of NE and E Added Individually or Together to Canine Plasma (5.0 ml)

Number of Recoveries		Quantity Added (ng)		*Quantity Recovered (ng)		*Recovery (%)	
NE	E	NE	E	NE	E	NE	E
32	32	5.0	5.0	4.03±1.11	4.77±0.60	80.7±22.2	94.8±11.1
16	16	10.0	10.0	8.06±0.99	9.63±0.32	80.6±9.9	96.3±3.2
20	20	**3.33	**1.67	2.23±0.77	1.61±0.20	67.1±23.1	96.4±11.8

*Mean and standard deviation
**Added together

To compare values for NE and E obtained by the present method with data reported in the literature, measurements were made of the 24-hour urinary excretion of NE and E in man and of the arterial plasma levels in the dog. Values reported were not corrected for recovery. Twenty four hour urine specimens were collected from 12 men of similiar work routines (laboratory personnel). The urine specimens were kept well-acidified through the collection period by the prior addition of 6 ml 9N H_2SO_4 to the collecting vessel. The final pH of the 24-hour speci-

TABLE 3.—Recovery of NE and E Added Individually or Together to Human Urine (1.0 ml)

Number of Recoveries		Quantity Added (ng)		*Quantity Recovered (ng)		*Recovery (%)	
NE	E	NE	E	NE	E	NE	E
16	16	5.0	5.0	4.01±0.78	4.69±0.41	80.2±15.5	93.8±8.4
15	15	**3.33	**1.67	3.37±0.79	1.26±0.27	101.1±23.8	75.5±15.9
16	16	**6.67	**3.33	5.82±1.23	3.28±0.28	87.2±18.5	98.4±8.4

*Mean and standard deviation
**Added together

mens ranged from 2.7 to 4.1 (mean 3.2). Values for the 24-hour excretion of the catecholamines in this sample of men are given in Table 4. So that comparisons may be made with the existing literature, the data for urinary excretion have been expressed in the three most common forms, μg/24 hours, ng/mg creatinine and ng/minute.

Results of multiple analyses for NE and E in canine arterial plasma are summarized in Table 5.

Although such data are not intended as an estimate of the population means for NE and E levels in canine plasma, they do indicate the plasma concentration of the catecholamines that can be anticipated and give an approximation of the variability encountered. For this study dogs were prepared with chronically indwelling arterial catheters. Blood samples were taken from the catheter in nonanesthetized animals,

TABLE 4.—24 Hour Urinary Excretion of NE and E of Twelve Male Laboratory Workers (Mean and Standard Deviation)

	Quantity μg/24 hours	Per Unit Creatinine ng/mg creatinine	Per Unit Time ng/min
NE	45.7±11.5	24.0±6.1	31.7±8.0
E	9.1± 1.7	4.8±1.2	6.3±1.2
NE+E	54.8±12.7	28.8±7.0	38.0±8.8

avoiding both the effects of anesthetics and the trauma of venipuncture on the catecholamine levels. The animals were permitted an adequate period of recovery (several days) between samplings. The red cell volume was measured daily (hematocrit) to monitor the effects of repeated blood sampling; no changes were detected.

The precision of the method was evaluated from multiple analyses on a single sample of urine or plasma. With urine, 18 measurements

of a single sample yielded the following information: NE; 14.9 ± 1.3 (mean and S.D.) ng/ml, C.V. (Coefficient of variation), 8.9%, E; 5.6 ± 0.5 ng/ml, C.V., 9.0%. Eighteen measurements on a single sample of plasma gave: NE, 0.80 ± 0.09 ng/ml, C.V., 11.8%, E; 0.35±0.06 ng/ml, C.V., 16.5%. As expected, measurements of sample containing smaller amounts of the amines showed a slightly greater variability.

TABLE 5.—Multiple Measurements of NE and E in Canine Arterial Plasma from Four Dogs (Mean and Standard Deviation)

Dog No.	No. of Observations N	NE ng/ml	E ng/ml	NE+E ng/ml
2767	8	0.53±0.13	0.15±0.08	0.68±0.09
2785	23	0.67±0.17	0.05±0.04	0.72±0.17
1567	43	0.51±0.22	0.08±0.05	0.59±0.20
2021	23	0.56±0.11	0.03±0.03	0.59±0.11
All Observations Combined	97	0.56±0.19	0.07±0.06	0.63±0.18

V. Discussion.

None of the automated methods that have been developed thus far for the differential estimation of NE and E in plasma or urine are as rapid or direct as one might wish for studies involving large numbers of samples or for the clinical laboratory. The technique outlined in this paper is no exception. One operator working alone

can analyze nine unknown samples (and five standards) per day. Two operators with equivalent training and working together can process six standards and 22 unknown samples per day. The merit of the scheme proposed resides in the reduction of the number of manipulations required to conduct the automated portion of the assay, and in the ease with which the procedure

accepts either urine or plasma samples without necessitating changes in the AutoAnalyzer system. We believe these points represent distinct advantages over those systems that permit the analysis of only urine or that require two analytical runs for differentiation.

In attempting to compare the characteristics of the present method with those of published techniques, certain inconsistencies were encountered which made a direct comparison difficult. For example, although most authors report data for recoveries, the techniques used to evaluate recovery of added NE or E were so varied as to preclude a comparison of the recovery values given . In some instances NE and E were added to the original sample, in other cases the amines were added to column eluates and in still other cases no information was presented about how the recoveries were performed. Further, in several reports the quantities of NE and E added to samples were several times the amounts that might be expected in the sample, biasing the result in the direction of a higher recovery.

In order to provide reference points for evaluating the present method in comparison with other techniques (where possible) the following conditions were observed:

1. The quantities of NE and E used for standards approximated the quantity of NE and E likely to be measured in the unknown sample.

2. The quantities of NE and E added to samples in studies of the recovery of the amines also approximated the amounts of the amines present in the sample from which the recovery was evaluated.

3. All recoveries were based on adding NE, E, or both to samples of plasma or urine before the deproteinizing step. In this way the estimate of recovery considered all of the manipulations to which the sample was subjected.

We recognize that adherence to the above criteria has the effect of apparently increasing the variability of measurement, but we also believe that such an estimate of the overall variability presents a more accurate appraisal of the technique than,

TABLE 6.—Comparison of Catecholamine Values in Urine and Blood Plasma Estimated by Automated (A) or Manual (M) Trihydroxyindole Techniques.

Author and Reference	Human Urine μg/24 hr		Human Plasma ng/ml		Other Plasma ng/ml	
	NE	E	NE	E	NE	E
(A) Sampson (4)	[1] 29.7±7.7	7.3±4.0				
(A) Robinson & Watts (3)	[2] 24.9±4.6	6.7±1.0				
(A) Martin & Harrison (8)	19.6±8.7	13.7±3.0	[3] 0.75	0.16	[4] 1.0±0.33	0.76±0.02
(M) Cohen & Goldenberg (12)			[5] 0.30±0.07	0.06±0.05		
(M) O'Hanlon et al. (15)			0.174±0.052	0.079±0.047		
(A) Hathaway et al. (5)	31.0	8.7				
	21.0	5.9				
(A) Mabry & Warth (9)	10–100	5–15				
(A) Viktora et al. (7)	[7] 30.7±2.23	1.7±0.06				
	[8] 15.8±1.62	1.4±0.17				
(M) Crout (16)	30±13	5.6±3.1				
(M) DuToit (17)	35.8	4.5				
(M) Anton & Sayre (14)	[6] [9] 25.2	8.4	0.97	0.48	[10] 1.05	0.41
(M) Becker & Kreuzer (18)	24.3±6.9	7.1±2.9	0.12	0.23		
(M) Hale et al. (19)	[6] [11] 33.4	7.0				
(M) Terry et al. (20)					[10] [12] [13] 0.21±0.14	0.14±0.08
(M) Manger et al. (21)					[10] [12] [13] 1.6±1.93	1.0±1.02
(A) Fiorica & Moses	45.7±11.5	9.1±1.7			[10] [14] 0.56±0.19	0.07±0.06
(This study)	[6] 24.0±6.1	4.8±1.2				

[1]All values are Means and SD unless indicated otherwise. [2]Means and SE. [3]Mean of 2 samples. [4]Calf plasma. [5]Mean and Average Deviation. [6]Nanograms per mg creatinine. [7]After acid hydrolysis. [8]Free catecholamines. [9]Restated from 0.63 μg NE and 0.21 μg E per 25 mg creatinine; 12–24 hr. urine. [10]Canine plasma. [11]28-hour collection interval. [12]Arterial sample. [13]Under pentobarbital anesthesia. [14]Unanesthetized.

for example, do methods that report recoveries from eluates alone.

Values for NE and E obtained by a number of different workers, using automated or manual trihydroxyindole procedures, are shown in Table 6. Although, it is apparent that the reported values differ widely, it should be realized that none of the data cited adequately represents a population sample. Rather than consider the data shown as "normal" values, it is probably more reasonable to view the levels given as representing an order of magnitude of levels that have been measured. The reasons for the variations encountered in reported values are many and may be the result of small-sample variations, nonstandard conditions surrounding the sampling interval, the fundamental lability of the sympathetic system and, of course, methodological differences in measurement techniques. From our evaluation, data derived by the procedure reported here appear comparable in accuracy, precision and order of magnitude to results obtained by other trihydroxyindole techniques, both automated and manual.

REFERENCES

1. Merrills, R. J.: An Autoanalytical Method for the Estimation of Adrenaline and Noradrenaline, NATURE 193:988, 1962.

2. Merrills, R. J.: A Semiautomatic Method for Determination of Catecholamines, ANAL. BIOCHEM., 6:272–282, 1963.

3. Robinson, R. L., and D. T. Watts: An Automated Trihydroxyindole Procedure for the Differential Analysis of Catecholamines, CLIN. CHEM., 11:986–997, 1965.

4. Sampson, P. A., Jr.: Automated Trihydroxyindole Procedure for Analysis of Epinephrine and Norepinephrine, Aerospace Medical Research Laboratories, Report No. AMRL–TR–67–96, Wright-Patterson AFB, Ohio, 1967.

5. Hathaway, P. W., L. Jakoi, W. G. Troyer, Jr., and M. D. Bogdonoff: A Method for Semiautomatic Differential Analysis of Urinary Catecholamines, ANAL. BIOCHEM., 20:466–476, 1967.

6. Sampson, P. A., Jr.: Automated Trihydroxyindole Procedure for Analysis of Epinephrine and Norepinephrine, AEROSPACE MED., 39:1123, 1968.

7. Viktora, J. K., A. Baukal, and F. W. Wolff: New Automated Fluorometric Methods for Estimation of Small Amounts of Adrenaline and Noradrenaline, ANAL. BIOCHEM., 23:513–528, 1968.

8. Martin, L. E., and C. Harrison: An Automated Method for Determination of Noradrenaline and Adrenaline in Tissues and Biological Fluids, ANAL. BIOCHEM., 23:529–545, 1968.

9. Mabry, C. C., and P. W. Warth: An Automated Technic for Separate Fluorometric Measurement of Epinephrine and Norepinephrine in Urine, AM. J. CLIN. PATH., 52:57–68, 1969.

10. Arnold, D. E.: A Sensitive Semiautomated Method for Determination of Urinary Epinephrine and Norepinephrine, USAF School of Aerospace Medicine Report No. SAM–TR–70–10, Brooks AFB, Texas, 1970.

11. Cohen, G., and M. Goldenberg: The Simultaneous Fluorometric Determination of Adrenaline and Noradrenaline in Plasma—I, J. NEUROCHEM., 2:58–70, 1957.

12. Cohen, G., and M. Goldenberg: The Simultaneous Fluorometric Determination of Adrenaline and Noradrenaline in Plasma—II, J. NEUROCHEM., 2:71–80, 1957.

13. Fiorica, V.: An Improved Semi-Automated Procedure for Fluorometric Determination of Plasma Catecholamines, CLIN. CHEM. ACTA., 12:191–197, 1965.

14. Anton, A. H., and D. F. Sayre: A Study of the Factors Affecting the Aluminum Oxide-Trihydroxyindole Procedure for the Analysis of Catecholamines, J. PHARMACOL. EXP. THER., 138:360–375, 1962.

15. O'Hanlon, J. F., H. C. Campuzano, and S. M. Horvath: A Fluorometric Assay for Subnanogram Concentrations of Adrenaline and Noradrenaline in Plasma, ANAL. BIOCHEM., 34:568–581, 1970.

16. Crout, J. R.: *Standard Methods of Clinical Chemistry Volume 3*, New York, Academic Press, 1961, pp 62–80.

17. DuToit, C. H.: A Study of Chemical Methods for Quantitative Measurements of Catechol Amines, Wright Air Development Center Report No. WADC–TR–59–175, Wright-Patterson AFB, Ohio, 1959.

18. Becker, E. J., and F. Kreuzer: Catecholamine Excretion by the Healthy Adult Human, PFLUGERS ARCH., 316:95–113, 1970.

19. Hale, H. B., E. W. Williams, and C. J. Buckley: Endocrine-Metabolic Effects of Transatlantic Helicopter Flight, USAF School of Aerospace Medicine Report No. SAM–TR–68–68, Brooks AFB, Texas, 1968.

20. Terry, E. N., R. H. Clauss, and W. Redisch: Determination of Catecholamines in Arterial Tissue and Plasma with Simultaneous Measurements of Peripheral Blood Flow in the Anesthetized Dog, J. CARDIVASC. SURGERY (Torino), 9:353–357, 1968.

21. Manger, W. M., O. S. Steinsland, G. G. Nahas, K. G. Wakim, and S. Sufton: Comparison of Improved Fluorometric Methods Used to Quantitate Plasma Catecholamines, CLIN. CHEM., 15:1101–1123, 1969.