

THE CHEMICAL ESTIMATION OF EPINEPHRINE AND NOR-
EPINEPHRINE IN HUMAN AND CANINE PLASMA. I. A
CRITIQUE OF THE ETHYLENEDIAMINE CON-
DENSATION METHOD¹

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The recovery of epinephrine (E) and norepinephrine (N) from plasma on activated alumina is reproducible and this adsorption technique can be made reasonably specific for catechols. The development of chemical methods for the assay of E and N in peripheral plasma has been hampered, however, by: 1) the low concentrations of E and N normally present (1 μ g. per L. or less); 2) the lack of specificity of the methods for E and N in the presence of other catechols which are excreted in urine (1, 2) and thus presumably are present in blood; and 3) difficulty in obtaining "blanks" from plasma extracts or eluates, a condition usually met in methods designed to measure endogenous substances.

In 1949 and 1950, Lund (3-5) described a method based on the conversion of E and N to their fluorescent trihydroxyindole derivatives. Only dihydroxyphenyl compounds possessing a side chain which contains both an hydroxyl and an amino group appear to be detected by this method, and "blanks" can be obtained by allowing oxidation of the trihydroxyindole derivatives of E and N to proceed to a non-fluorescent state. However, the sensitivity of the method appears insufficient to permit measurements of E and N in small quantities of normal peripheral plasma (5). Simultaneously, Natelson, Lugovoy, and Pincus (6) described the condensation of E with ethylenediamine (EDA) to form a highly fluorescent product. In 1952 and 1953, Weil-Malherbe and Bone (7, 8) extended the use of this procedure to the simultaneous estimation of E and N in blood by measuring the intensity of fluorescence at two different wave lengths. Modifications (9, 10) have been reported, and the method appears to be in fre-

quent use. It is less specific than the Lund (trihydroxyindole) method, and will detect the presence of dihydroxyphenyl compounds other than E and N (7).

The levels of E and N found in normal human plasma by Weil-Malherbe and Bone (1.3 and 5.2 micrograms per liter, respectively) differ from those observed by others (10-12) using essentially the same method, and from the levels obtained by bioassay (13). In view of the fact that a reliable method for estimation of E and N can assume importance in the interpretation of physiological changes in man, it was considered necessary to re-evaluate the EDA method and to compare the results obtained with this technique with those obtained by the trihydroxyindole method. Our results with both dog and human plasma indicate that the EDA method is not specific for N, and may not be so for E.

METHODS

Blood was collected in heparinized syringes, the cells separated by centrifugation, and to a measured amount of the plasma one-half its volume of sodium fluoride (2 per cent)-sodium thiosulfate (3 per cent) solution was added.

Removal of E and N from plasma was accomplished by adsorption of the catechol amines on activated alumina at pH 8.0 to 8.5, followed by elution from the alumina column with acetic acid as described by Weil-Malherbe and Bone (7). Woelm alumina, non-alkaline, Grade I (Alupharm Chemical Co., Elmont, L. I.) was found uniformly low in blank value and satisfactory for routine use. Pressure was used instead of suction on the columns in order to avoid vaporization of the solvent within the column bed.

A Farrand fluorometer with output connected to a Rubicon galvanometer was used to measure fluorescence. The primary filter used for the EDA method was an interference filter with a transmission peak at 436 m μ plus a Wratten gelatin filter No. 35; secondary filters were 1) a green filter pair composed of an interference filter

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with a peak at 500 $m\mu$ plus a Wratten gelatin filter No. 57, and 2) an interference filter with a peak at 580 $m\mu$.

The permanent standard used was a 10-ml. isobutyl alcohol extract of 10 ml. of water containing 20 micrograms of E which had been condensed with EDA; this was stored at 4°C and diluted 1:100 with isobutyl alcohol for use as needed to maintain instrument sensitivity at a preassigned value (90.0 divisions at diaphragm opening No. 3) for each secondary filter.

I. Ethylenediamine method. Two major modifications were necessary before the Weil-Malherbe and Bone method could be used. They were required because: 1) the fluorescence of E condensate in alumina eluate is roughly 150 per cent of its value in water or acid solution, and 2) the fluorescence of N condensate decays exponentially and irreversibly in strong blue or green light. Modifications were made as follows: 1) preparation, for use with each analysis of two standards of E and N (0.04 microgram of base per 10 ml.) and of two reagent blanks, each in 5 ml. of 0.33 M acetic acid which has been passed through a 0.7 gram column of alumina previously prepared in water, plus water to make a total volume of 10 ml.; 2) following the addition of EDA, all samples were exposed to light (dim room light only) for the same length of time. Readings were made through the secondary green filter pair first, noting the galvanometer deflection at a constant time interval (five seconds) after opening the shutter; the procedure was then repeated using the yellow secondary filter. This allowed for completion of galvanometer response, allowed equal decay-time of N condensate for all samples, and reduced the fluorescence by the least amount possible. (The blue-green fluorescence of ethylenediamine condensates of noradrenaline and certain other catechols decays rapidly when these substances are excited by light of 436 $m\mu$; the yellow emission is relatively stable.)

II. Trihydroxyindole method. The method of Lund (5) as modified by von Euler and Floding (14) was used essentially as described to assay E and N. Blanks were prepared by adding ascorbic acid to the plasma eluate before the ferricyanide, thus preventing conversion of E and N to their respective chromes. The intensities of fluorescence due to E and N were determined, using two primary interference filters (400 and 438 $m\mu$).

In both methods calculations of E and N levels were made by solving simultaneous equations involving the net galvanometer deflections (fluorescence above blank) observed using the two filters.

RESULTS

I. Specificity

Specificity of the EDA method was studied in three ways: a) by the addition of certain mono- and di-hydroxyphenyl compounds² to plasma, fol-

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lowed by estimation of their recovery by alumina column chromatography, b) by condensation of the substances with EDA and comparison of their fluorescence spectra with those of E and N condensates, and c) by comparison of apparent E and N levels in the same plasma eluates by both the EDA and trihydroxyindole methods. The compounds selected were some of those which might be expected to be present in blood, and which could interfere in the EDA method.

A. Recovery. The recoveries of dihydroxyphenylalanine (dopa), 3-OH tyramine (dopamine), 3,4-dihydroxyphenyl acetic acid ("dopac"), isopropylnoradrenaline, and 5-hydroxytryptamine (serotonin), after addition to plasma and assay by the EDA method, are shown in Table I, together with representative recoveries of E and N. Serotonin was the only compound tested which was recovered in negligible amounts, due to non-adsorption on alumina. Omission of the fluoride-thiosulfate preservative from the plasma did not reduce the recovery of E, reduced that of N by less than twenty per cent, but diminished that of dopac by more than fifty per cent.

B. Relative fluorescence of condensation products. Standard solutions of the mono- and dihydroxyphenyl compounds were made up in the same manner as the usual E and N standards, using EDA condensation. The fluorescence intensities of these compounds and of E and N are given in Table II. The intensity of fluorescence of added E obtained with the secondary yellow filter

TABLE I
Recovery of hydroxyphenyl compounds added to plasma
(EDA method)

Compound	Micrograms added	Per cent recovered
1-Epinephrine (3,4-dihydroxyphenyl-B-methyl aminoethanol)	0.001-0.20	78
1-Norepinephrine (3,4-dihydroxyphenyl-B-aminoethanol)	0.005-4.0	80
Dopac (3,4-dihydroxyphenyl acetic acid)	0.08-0.40	65
Dopamine (3,4-dihydroxyphenyl-B-aminoethane)	0.02-50	79
Dopa (3,4-dihydroxyphenyl-B-aminopropanoic acid)	50	94
Isopropylnoradrenaline (3,4-dihydroxyphenyl-B-isopropyl-aminoethanol)	0.25-0.50	72
Serotonin (5-hydroxyindole acetic acid)	150-250	0.17

has been arbitrarily assigned a value of 100 (actual value = 930 divisions per microgram).

C. Comparison of EDA and trihydroxyindole methods. Approximately 1.5 L. of arterial plasma was obtained by exsanguinating three dogs under light pentobarbital anesthesia (25 mg. per Kg. intravenously). The catechols were eluted from twenty 100-ml. lots of plasma-preservative mixture on two gram alumina columns, washed with sodium acetate solution (5 ml. of 0.2 M) and water (5 ml.), and eluted in 5 to 10 ml. of 0.3 M acetic acid. Aliquots of the combined eluates from each dog's plasma were analyzed using both the EDA and trihydroxyindole methods. Blanks and additions of E and N were prepared from each eluate for assay by the trihydroxyindole method. There was good agreement between the E levels estimated by the two methods (E = 1.78 $\mu\text{g. per L.}$ by EDA and 1.84 $\mu\text{g. per L.}$ by trihydroxyindole); however, N levels were invariably less by the trihydroxyindole method and averaged only 30 per cent of the level obtained by the EDA method (N = 0.85 by EDA and 0.29 by trihydroxyindole).

Smaller quantities of human plasma (25 to 50 ml.) were separated from blood drawn from the antecubital veins of four normal volunteers, and assayed for E and N by the trihydroxyindole method. The mean values were ($\mu\text{g. per L.}$) E = 0.00 ± 0.005 (S.E.) and N = 0.20 ± 0.097 (S.E.). A 10-ml. sample of plasma was obtained by similar means from each of 17 normal subjects and the E and N levels estimated by the EDA method. The mean values were ($\mu\text{g. per L.}$) E = 0.097 ± 0.034 (S.E.) and N = 2.74 ± 0.277 (S.E.); both are significantly ($p < 0.01$ by Fisher t test) greater than the levels obtained using the trihydroxyindole method.

Three 30-ml. samples of normal human plasma

TABLE II
Fluorescence of hydroxyphenyl compounds (EDA method)

Compound	Relative fluorescence intensity with filter No.		Fluorescence ratio (500/580)
	580 (yellow)	500 (green)	
Epinephrine	100	58	0.58
Norepinephrine	34	127	3.74
Dopac	5.1	21	4.04
Dopamine	30.9	35	1.13
Dopa	0.65	1.1	1.69
Isopropylnoradrenaline	59	43	0.73
Serotonin	2.2	4.9	2.22

were divided in half. Preservative was added to one half but not to the other, and both were analyzed by the EDA method. The apparent E level was increased by an average of 0.07 $\mu\text{g. per L.}$ when preservative was omitted, while that of N was reduced to from thirty to fifty per cent of that estimated in the preservative-treated fraction.

II. Sensitivity

The sensitivity of the EDA method for E and N is limited largely by variation in the blanks. Addition and recovery experiments indicate that as little as 0.001 to 0.002 $\mu\text{g.}$ E and 0.005 to 0.010 $\mu\text{g.}$ N can be detected in plasma eluates.

DISCUSSION

To facilitate discussion the E and N levels ($\mu\text{g. per L.}$) found in human peripheral plasma by various workers have been summarized in Table III. It is evident that the E levels estimated by most workers using the EDA method are in the same range (0.0 to 0.2 $\mu\text{g. per L.}$) as those estimated by bioassay or by the trihydroxyindole method, while the N levels measured by the latter are only a

TABLE III
Epinephrine and norepinephrine concentration in human peripheral plasma ($\mu\text{g. per L.}$)

Method	No. of subjects	E \pm S.D.	N \pm S.D.	Author
Guinea-pig uterus	1	<0.06	<1.0	Holzbauer and Vogt, 1954
Trihydroxyindole	4	0.00 ± 0.01	0.20 ± 0.19	Valk and Price, 1956*
EDA (with preservative)	17	0.097 ± 0.14	2.74 ± 1.14	Same
EDA (without preservative)	3	0.083 ± 0.16	1.26 ± 1.32	Same
EDA	7	0.14 ± 0.21	3.96 ± 1.7	Manger and co-workers, 1954
EDA	6	0.4 ± 0.2	2.1 ± 1.3	Aronow, 1956
EDA	22 males	1.18 ± 0.21	5.29 ± 1.10	Weil-Malherbe and Bone, 1953
	21 females	1.46 ± 0.38	5.16 ± 0.74	

* Uncorrected for losses in recovery (ten to twenty per cent).

tenth as great as by the EDA method. This is evidence for the presence in plasma of a substance which: 1) is adsorbed on alumina at pH 8.0 and eluted by dilute acid, 2) has a fluorescence ratio (net fluorescence read using green secondary filter divided by that read on yellow filter) similar to that of N by the EDA method, and 3) is not detected by the trihydroxyindole method. If the fluorescence ratio of the interfering substance differed markedly from that of N, solution of the simultaneous equations used to calculate E and N by the EDA method would yield a false value for E, and the apparent E levels calculated by the two methods would differ considerably. Comparison with the results obtained by Holzbauer and Vogt (13) further suggests that the substance interfering in the EDA method is either not recovered by their procedure or does not inhibit carbachol-induced contractions of the guinea pig uterus.

Among the catechol substances which have been reported present in human urine, tissue extracts, or blood are: 1) isopropylnoradrenaline, tentatively identified by Lockett (15) in the adrenal medulla; 2) dopamine, found in urine (1), and 3) dopac which has been tentatively identified in urine by von Euler, von Euler, and Floding (2). Dopa has not been shown to be present in tissue or biological fluids, nor has dihydroxyphenylserine, and isopropylnoradrenaline constitutes less than one per cent of the E and N in the human adrenal medulla (15). Therefore, the principal possibilities for the substances detected in plasma eluates by the EDA method (but not by the trihydroxyindole method) appear to be dopamine and its supposed metabolite dopac. Both substances are adsorbed from plasma by alumina and are eluted by dilute acetic acid; also, neither substance produces significant fluorescence by the trihydroxyindole method. However, the EDA condensate of dopamine has a fluorescence ratio different from that of either E or N, while the fluorescence ratio of dopac condensate differs from that of N by less than ten per cent. Furthermore, dopac is apparently biologically inactive (2). A substance present in plasma and interfering in the EDA method may therefore be dopac.

Further evidence for the presence of a dopac-like substance in plasma was obtained by omitting the addition of preservative to same plasmas, thereby reducing the recovery of authentic nor-

adrenaline from the plasma only slightly while diminishing that of dopac to approximately thirty per cent. This technique resulted in much better agreement between the "N" levels estimated by the EDA and trihydroxyindole methods than occurred when preservative was used.

Since the fluorescence ratios of N and dopac are slightly different the presence of dopac in plasma eluates will introduce a systematic error in the estimation of E by formulae involving the fluorescence ratio observed for N. Recalculation of the data obtained from normal human plasma indicates that the average E level of 0.1 micrograms per liter should be more nearly 0.2 to 0.3 micrograms per liter, assuming at least two-thirds of the "N" value to be due to the presence of dopac. This value thus exceeds the level calculated by the trihydroxyindole method by at least 0.2 micrograms per liter. This difference could have a number of explanations, among which are 1) the presence of a fourth catechol in plasma, or 2) a systematic error in either method.

The levels of "E" and "N" obtained by Aronow and Howard (10, 11) and Manger and his co-workers (12) using the EDA methods are in substantial agreement with our own, but differ greatly from those estimated by Weil-Malherbe and Bone (8). Further, we have been unable to confirm the finding of Weil-Malherbe and Bone that there is an association of "E" and "N" with platelets (16) and we have observed an increase in plasma "E" during insulin hypoglycemia in man (17) rather than a decrease which they (18) have reported. It may be concluded that differences in method other than those described must exist.

The sensitivity and precision of the EDA methods appear sufficient to permit detection of quantities of E and N in plasma of the order of 10^{-8} μ g. However, the specificity of the methods for N in plasma eluate is inadequate, while specificity for E is not established. If further effort is to be expended on methods to determine E and N levels in peripheral plasma by chemical means, it appears likely that procedures more specific than EDA condensation must be employed.

SUMMARY AND CONCLUSIONS

1. The ethylenediamine condensation method is capable of detecting amounts of epinephrine and

norepinephrine of the order of 10^{-8} micrograms after their addition to plasma.

2. The method is not suitable for estimating the norepinephrine concentration in either canine or human peripheral plasma.

3. Changes in epinephrine concentration can be measured provided that the concentrations of other catechols in plasma remain unchanged.

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