SUITABILITY OF THE ETHYLENEDIAMINE METHOD OF WEIL-MALHERBE AND BONE AND MODIFICATIONS FOR QUANTITATING PLASMA PRESSOR AMINES

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In 1952 we modified the original method of Weil-Malherbe and Bone (37) in order to determine both epinephrine and norepinephrine in mixtures. The essential element of our modification consists in the use of sodium thiosulfate ($Na_2S_2O_3$), which in acid solution affects differentially the production of fluorescence when epinephrine and norepinephrine are condensed with ethylenediamine. A preliminary report of this modification was published in 1953 (22) and will appear in detail in a forthcoming monograph (23).

In 1953 Weil-Malherbe and Bone (38) reported a modification of their original method which made use of differences in fluorescent spectra of the condensation product of epinephrine and of norepinephrine and made possible differential identification of these substances in mixtures. Highly accurate results using the ethylenediamine method and differential secondary filters were reported by a number of investigators (1, 21, 28, 38). Methods employing differential secondary filters have the advantage of necessitating analysis of only one sample. There is no doubt that the fluorimetric methods of Weil-Malherbe and Bone, and modifications, are very reliable for quantitation of plasma pressor amines if these substances are present in high concentration and interfering substance is present in small proportion. These methods are unquestionably suitable for diagnosis of actively secreting pheochromocytoma, and appear suitable for investigation of certain types of physiologic stress and for study of the rates of disappearance of epinephrine and norepinephrine from the blood during infusion of these substances and during perfusion of organs. However, a significant increase of interfering substances, accompanying an increased secretion of pressor amines, would result in erroneous quantitation of epinephrine and norepinephrine.

According to Weil-Malherbe and Bone (37, 38), the fluorescent substances estimated in plasma of normal human subjects are "identical with adrenergic amines in their affinity to amine oxidase, in R_f values, and in the fluorescence spectra of their derivatives." Mangan and Mason have also demonstrated that the spectral distribution of fluorescence of ethylenediamine derivatives of catechols isolated from plasma closely approximated emission spectra of derivatives of synthetic epinephrine and norepinephrine (19, 21). In addition, Weil-Malherbe and Bone (41) found "the reactive material in plasma has the same electrophoretic mobility as adrenaline and noradrenaline." Finally, Weil-Malherbe obtained similar results whether he used his method or the trihydroxyindole methods to quantitate plasma pressor amines (34, 36, 41).

Blaschko and co-workers found satisfactory agreement in the results obtained

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using a bioassay technique and a modification of the ethylenediamine procedure for determination of the ratio of epinephrine to norepinephrine in homogenates of medullary fractions of bovine adrenals (5). Montagu (26) found similar results when she quantitated epinephrine and norepinephrine in extracts of rat tissue by the method of Weil-Malherbe and Bone, paper chromatography and bioassay. Richardson and Woods (29) have reported that bioassays of perfusates of isolated hearts were in good agreement with the values determined by their modification of the method of Weil-Malherbe and Bone.

We have not investigated extensively the specificity of the ethylenediamine procedure; however, by the method of paper chromatography followed by elution and fluorimetric quantitation, it appears that less than 20% of fluorescence in normal plasma may be due to substances other than epinephrine and norepinephrine. We have evaluated the specificity of the method further by treating various compounds with ethylenediamine or by adding these compounds to plasma and then performing the analytic procedure. If fluorescence did not increase after addition of a substance to plasma, it might be concluded that these substances do not interfere with quantitation of pressor amines. However, such conclusions are not justified unless these compounds have been administered to patients or animals without affecting the quantity of fluorescent substance in the plasma. Neither serotonin nor adrenochrome was adsorbed from plasma by alumina and therefore did not interfere with quantitation of epinephrine and norepinephrine.

There is other indirect evidence suggesting that the ethylenediamine procedure quantitates primarily epinephrine and norepinephrine in plasma. For example, we observed a significant decrease in the fluorescence of extracts of plasma obtained from dogs after ganglionic blockade of the autonomic nervous system. Within 2 to 11 minutes after hexamethonium bromide was administered intravenously, the concentration of epinephrine-like substance decreased an average of 64% of the control concentration. The concentration of norepinephrine invariably decreased, and once no norepinephrine was detectable. The change in concentration of epinephrine was variable.

Burger pointed out that Carlsson and Hillarp were unable to detect epinephrine in the adrenal medulla of rabbits after administration of large amounts of reserpine (6). Burger, using a modification of the method of Weil-Malherbe and Bone, found that 3 days after administration of reserpine to patients, there was a decrease in the plasma concentration of epinephrine and norepinephrine to 73 and 35% respectively of their initial concentrations. If reserpine prevents synthesis of epinephrine and norepinephrine in the central and peripheral nervous system (11), then decrease of fluorescent substance in blood after administration of reserpine further suggests a considerable percentage of fluorescence is due to pressor amines.

Richardson and Woods (27, 30) found that, following prolonged total epidural preganglionic sympathetic block, plasma pressor amine levels were either markedly reduced or no longer detectable. They stated that fluorescent characteristics

and biological activities of 3,4-dihydroxyphenylalanine and 3,4-dihydroxyphenylethylamine (dopamine) indicated these compounds do not contribute appreciably to responses involving sympathetico-adrenal activity (42).

Von Euler has demonstrated the presence of dihydroxyphenylacetic acid (dopac) and dihydroxymandelic acid (doma) and norepinephrine in beef plasma (9); and Lund has demonstrated the conversion of epinephrine to doma during perfusion of the liver (14).

Both dopac and doma produce fluorescent compounds when condensed with ethylenediamine.[2] However, we found the fluorescent intensities of dopac and doma condensates after extraction with isobutanol were, respectively, only about 6 and 8% of the intensities of similarly treated epinephrine. No fluorescence was detected after adding as much as the equivalent of 100 μg of dopac or doma per liter to a solution of sodium acetate and antioxidant ($Na_2S_2O_3$) which was then analyzed by the method of Weil-Malherbe and Bone (*i.e.*, adsorption on alumina, followed by elution, condensation and extraction). It is unlikely, unless present in relatively huge concentration, that dopac and doma interfere very significantly with the accuracy of quantitating epinephrine and norepinephrine in plasma when the method of Weil-Malherbe and Bone or modifications are used. Weil-Malherbe (32) and von Euler (9) have stated that acidic catechols are not extracted to any significant degree by isobutanol.

It is noteworthy that dopamine has never been demonstrated in plasma or in any significant amount in normal human adrenal medulla. However, we (23, 24) and others (25, 35) have very rarely identified it in pheochromocytomas. Blaschko stated: "Dopamine may differ from adrenaline and noradrenaline in that it is chiefly an intermediate and not released either as a mediator or a hormone" (4). Goodall's demonstration of radioactive dopamine and norepinephrine (and only a slight amount of epinephrine) when bovine and canine sympathetic nerves were incubated with labeled tyrosine or labeled dopa (12) supports the concept that norepinephrine is the chemical transmitter in the sympathetic nervous system and that dopamine may be its principal precursor. Dopamine, dopac, doma and other similar compounds are probably present in blood. However, we do not believe these interfering substances are in concentrations great enough to preclude fairly accurate estimation of epinephrine and norepinephrine in plasma by utilization of the ethylenediamine procedure.

Holzbauer and Vogt (13) have criticized the specificity of the method of Weil-Malherbe and Bone since the latter investigators reported a decrease in the concentration of plasma epinephrine during insulin-induced hypoglycemia (33, 39, 40), a finding contrary to the *increased* concentration of plasma epinephrine demonstrated by biologic assay. However, Valk and Price (31) and Bethune and co-workers (3) have found an increase in concentration of plasma epinephrine (and sometimes norepinephrine) during hypoglycemia when they

[2] We are indebted to Drs. H. H. Weissbach and E. O. Titus for supplying us with 3,4-dihydroxyphenylacetic acid and to Dr. M. D. Armstrong for furnishing 3,4-dihydroxymandelic acid.

used modifications of the fluorimetric method of Weil-Malherbe and Bone. Therefore, factors other than lack of specificity of the ethylenediamine method probably accounted for these divergent results.

It is not clear why the trihydroxyindole methods reported by some investigators (8, 31) have yielded lower plasma concentrations for epinephrine and norepinephrine than the ethylenediamine method. It is conceivable that use of an antioxidant (*e.g.*, $Na_2S_2O_3$) may be responsible for some of the differences in results. In the methods of Lund (15–18), von Euler and Floding (10), and Cohen and Goldenberg (7, 8), antioxidant is not added immediately to blood samples to prevent deterioration of pressor amines. Lund has indicated that there may be loss of pressor amines due to oxidation during adsorption on alumina (17). Adsorption without the use of an antioxidant could be a source of error. Furthermore, Lund has stated "adrenolutine in solution is very easily oxidizable irrespective of acidity" (17). He observed that fluorescent intensity of an alkaline epinephrine solution depends not only on the intensity of irradiating light but also on the amount of oxygen and concentration of sodium hydroxide in the solution (15); he also pointed out that a large concentration of ascorbic acid has a quenching effect on fluorescence of adrenolutine in alkaline solution (17). All these observations of Lund must be kept in mind when any of the trihydroxyindole methods are used to quantitate epinephrine and norepinephrine. A highly desirable feature of the ethylenediamine procedure is the relative stability of the fluorescent condensation products.

We studied the effect $Na_2S_2O_3$ has on preventing the disappearance from plasma of epinephrine which was added to blood. Canine blood, with a small amount of heparin to prevent coagulation, was placed in a beaker and sufficient synthetic epinephrine was added to give a concentration of between 10 and 20 μg per liter of plasma. The blood was allowed to stand at room temperature (72°F) and samples were removed immediately, and at 5, 15, 30, 60 and 120 minutes, respectively, thereafter and were analyzed in the usual way (after addition of $Na_2S_2O_3$ to each sample). As seen in Table 1, by the end of 1 hour a 36% loss of epinephrine-like substance from plasma had occurred with a 46% loss by the end of 2 hours. The percentages recorded are averages obtained after performance of several similar experiments. It is evident that at room temperature without $Na_2S_2O_3$, significant quantities of epinephrine can be lost during the time required for centrifugation and adsorption. If $Na_2S_2O_3$ was added to blood, as recommended by Weil-Malherbe and Bone, only about 15% of epinephrine-like substance disappeared from plasma in 24 hours. Mangan and Mason found

TABLE 1

Decrease in concentration of epinephrine-like substance in plasma after Suprarenin (synthetic epinephrine) is added to whole blood without an antioxidant (e.g., $Na_2S_2O_3$)

Minutes after Suprarenin added to blood...	5	15	30	60	120
Per cent decrease in concentration of epinephrine-like substance.....	7	10	15	36	46

that more than 11% of plasma epinephrine disappeared within 10 minutes after addition of epinephrine to whole blood maintained at body temperature *in vitro* (20). Weil-Malherbe and Bone demonstrated that if plasma (to which epinephrine had been added) was diluted with an equal volume of sodium acetate, adjusted to pH 8.4 and shaken in air for 30 minutes, about 57% of the epinephrine was lost (37).

It is noteworthy that Bain and co-workers (2), using a bioassay, found the approximate time required for inactivating 25% of epinephrine added to a blood sample was 40 minutes (in good agreement with the results of Table 1). However, they found the periods necessary to inactivate the same percentage in serum and plasma to be 80 and 180 minutes, respectively. They indicated that the more rapid early inactivation of epinephrine added to whole blood as compared with serum and plasma is due primarily to the development of an equilibrium of epinephrine between blood cells and plasma. The disappearance of the portion of epinephrine not in association with blood cells may be partially due to oxidation.

The findings of Bain and co-workers are very significant since they indicate that the concentration of epinephrine which can be detected in plasma will depend on the degree of equilibrium between epinephrine in plasma and that in association with cells which has been reached at the time analysis is made. Recognition of such an equilibrium is of obvious importance, especially in experiments where increased concentrations of pressor amines suddenly enter the circulation.

Whether $Na_2S_2O_3$ exerts its effect mainly by preventing oxidation of epinephrine or by preventing association of epinephrine and blood cells, or both, or by some other action, has not been determined. The results strongly suggest that use of $Na_2S_2O_3$ will minimize the rate of disappearance of epinephrine from the plasma of a sample of whole blood and thus insure consistent and accurate results.

Comments and conclusions

We have employed extensively the ethylenediamine procedure and believe this method is highly sensitive, fairly specific, quite accurate, relatively simple from the technical viewpoint and especially suitable for quantitating plasma pressor amines.

The ethylenediamine procedure has distinct advantages over other fluorimetric methods used for quantitating minute concentrations of epinephrine and norepinephrine in plasma. Only in this procedure is an antioxidant mixed immediately with freshly collected blood samples to prevent a decrease of plasma pressor amines. We believe this part of the procedure is highly important; otherwise, loss of pressor amines through autoxidation and/or other mechanisms may be considerable before and during the analytical procedures. Also, in accordance with the observations of Weil-Malherbe and Bone, we found the intensity of the fluorescent products resulting from condensation of ethylenediamine with epinephrine or norepinephrine remained unchanged for several hours. Such stability has not been reported in the trihydroxyindole methods and suggests ethylenediamine condensates of epinephrine and norepinephrine are relatively resistant to

auto-oxidation or other deterioration. On the other hand, oxidation of epinephrine and norepinephrine carried out in the trihydroxyindole methods must be cautiously controlled to avoid excessive loss of pressor amines.

A few investigators have criticized the specificity of the ethylenediamine procedure for two particular reasons. First, they stated that ethylenediamine forms fluorescent compounds with catechols and some substances other than epinephrine and norepinephrine. However, despite numerous attempts, no one has demonstrated any substance in human or animal plasma in sufficient concentration to interfere very significantly with accurate quantitation of pressor amines when the ethylenediamine method is used.

Second, some investigators criticized the ethylenediamine procedure on the basis that their own methods are more specific, and that the concentrations of epinephrine and norepinephrine they found in plasma were considerably less than concentrations found when methods employing ethylenediamine were used. To be justified, the second criticism requires that the methods of the critics be valid, which has not been proved. It is possible that significant loss of epinephrine and norepinephrine from the plasma of blood samples quantitated for these substances may occur without use of $Na_2S_2O_3$ and the ethylenediamine method. It is conceivable that analysis by the procedure of Weil-Malherbe and Bone may result in quantitating not only plasma pressor amines but some of the pressor amines which may be associated with blood cells or platelets. This might explain in part the greater concentrations of pressor amines obtained when employing the method of Weil-Malherbe and Bone as compared with the trihydroxyindole methods. Results of analyzing large quantities of plasma by paper chromatography and reports of studies on spectral characteristics of fluorescent substances produced by condensation of ethylenediamine with plasma extracts support the view that the ethylenediamine procedure is quite specific.

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