SOME SPECTROPHOTOFLUORIMETRIC OBSERVATIONS ON BLOOD AND URINE CATECHOLAMINE ASSAYS

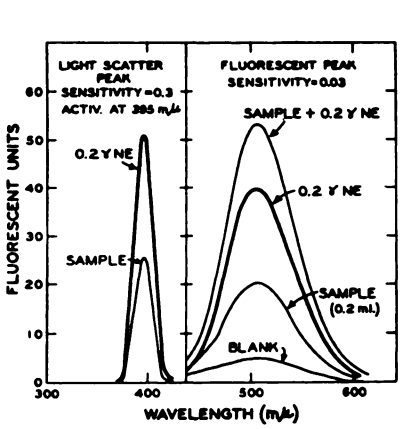
J. RICHARD CROUT

Section on Experimental Therapeutics, National Heart Institute, National Institutes of Health, Bethesda, Maryland

Any quantitative chemical assay must meet certain criteria before it can be accepted as specific and accurate. The most elementary of these criteria in a fluorimetric assay is that the activation and fluorescent spectra of the sample being analyzed approximate in all respects the corresponding spectra of the standard solution. The spectrophotofluorometer provides an opportunity to study these spectra which is not afforded by filter fluorometers, and the following curves obtained with the Aminco-Bowman instrument are presented to illustrate how precisely each of the assays under discussion at the conference fulfills this basic criterion.

Figure 1 illustrates the curve obtained from a urine eluate prepared by adsorbing 200 ml of normal urine on alumina at pH 8.4 and then eluting with 10 ml of 0.6 N oxalic acid; 0.2 ml of this eluate was then oxidized by the iodine technique, which we prefer over ferricyanide as we think less quenching occurs. The results with ferricyanide, however, would not have been grossly different. The activation light was held constant at 395 mμ, and the graph shows the emitted light during a scan from 300 mμ to 600 mμ. The peak on the left is the light scatter peak appearing at the wavelength of the activating light and is shown here at a sensitivity ten times less than that of the fluorescence peak on the right. Note that the light scatter peak from the urine eluate differs from that of the standard by no more than a factor of two. In our experience this degree of correlation between the light scatter peaks is quite sufficient to give good results. The fluorescence peak of the urine eluate on the right follows exactly the contour of the standard norepinephrine (NE) curve. Furthermore, the addition of norepinephrine to the eluate increases the fluorescence obtained by exactly that expected from the standard curve; that is, no quenching of fluorescence occurs. Thus, this urine assay satisfies the criteria of good fluorimetry: the light scatter peaks are similar in magnitude, the fluorescence peaks are similar in contour and no quenching occurs. This happens, however, only if the activating light is set at the activation peak for noradrenolutin. Minor distortion of the urine eluate curve occurs if the activation light is set up to 436 mμ and very major distortion occurs if it is moved down to 365 mμ, as the latter in particular highly activates a number of substances in the blank.

If the trihydroxyindole method is pushed to the limits of sensitivity, however, as must be done for blood analyses, a number of deviations from ideality appear. In the following example 40 ml of blood plasma collected in heparin were passed over 1 g of alumina and eluted with 10 ml of 0.2 N acetic acid; 3 ml of this eluate were then oxidized at pH 6 by the ferricyanide technique of von Euler and Floding. It should be emphasized that all of the recommended procedures for



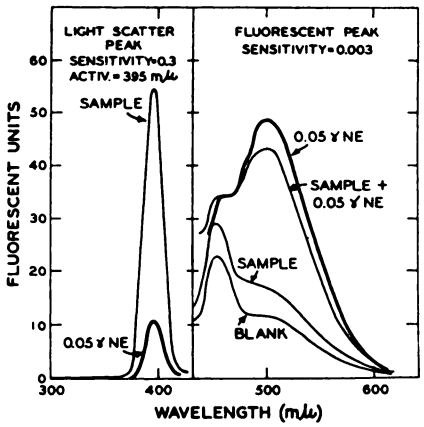


FIG. 1. (left) Determination of norepinephrine (NE) in urine. Iodine oxidation of urine eluate at pH 6.0.

FIG. 2. (right) Determination of norepinephrine (NE) in plasma. Ferricyanide oxidation of plasma at pH 6.0; eluate volume 3.0 ml.

purifying reagents were done here; that is, the alumina was washed repeatedly in HCl and water and dried, deionized water was used throughout and the ascorbic acid was recrystallized to minimum fluorescence. In addition, the standard solutions were prepared in 0.2 N acetic acid previously passed over alumina. Figure 2 shows the curve obtained. The light scatter peak was recorded at a sensitivity 100 times less than the fluorescence peak. By comparison the fluorescent emission is thus extremely low. The light scatter peak of this blood eluate is about five times that of the NE standard. This large amount of light scatter is the result of a fine precipitation of aluminum salts in the basic reaction mixture. This precipitation can be prevented by the addition of disodium versenate, but this increases blank fluorescence more than we find desirable. There was no visible turbidity in this solution and this amount of precipitation cannot be removed by ordinary clinical centrifugation. We have occasionally obtained precipitates heavy enough to be seen, and under these circumstances the light scatter peak is much higher than shown here and sometimes even overlaps the fluorescence peak. The NE standard on the right still shows a fluorescence peak; however, the eluate at these low concentrations demonstrates not a peak but only a sloping line running slightly higher than the reagent blank. Furthermore, the addition of a standard amount of norepinephrine produces less fluorescence in the eluate than it does in the standard. This marked quenching is the direct result of the high light scatter peak of the sample; the photons of scattered light become unavailable for the production of fluorescence, and the resultant fluorescence emission is thus less intense. In our experience a high light scatter peak is the most common cause of quenching in the trihydroxyindole method. As a sideline, the small peak shown in the fluorescence spectrum at 460 mμ is a Raman peak. It is characteristic of the solvent, not the fluorescent solute, and for water

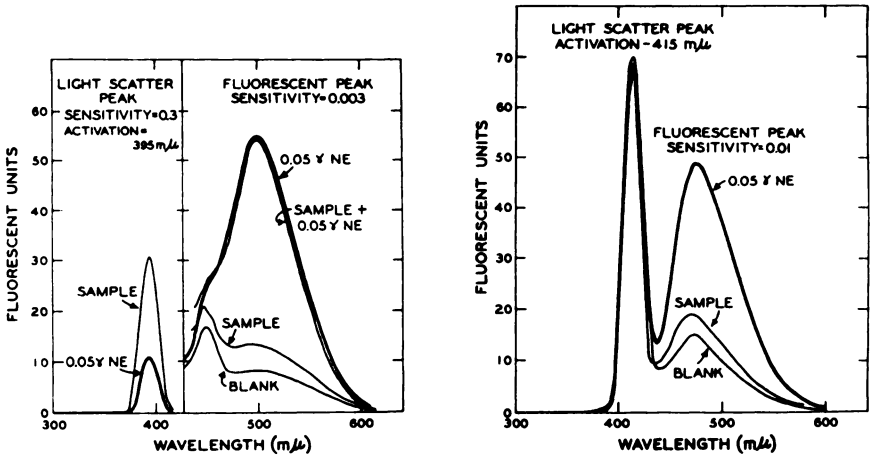


FIG. 3. (left) Determination of norepinephrine (NE) in plasma. Ferricyanide oxidation of plasma eluate at pH 6.0; eluate volume 1.25 ml.

FIG. 4. (right) Determination of norepinephrine (NE) in plasma. Ethylenediamine condensation of plasma eluate.

it occurs about 65 mμ above the activation wavelength in this region of the spectrum.

Figure 3 shows the curve obtained when 1.25 ml of the same blood eluate was oxidized with ferricyanide. Here the smaller amount of eluate produced less precipitation and the light scatter peak is correspondingly less. The sample reads almost the same as in the preceding figure, although the eluate volume taken was considerably less and the quenching of added norepinephrine is now less prominent. It is thus apparent that the degree of quenching is a function of the eluate volume taken. Even at constant eluate volume, however, we have been troubled with a variable amount of quenching from sample to sample. Figure 3 illustrates the least amount of quenching we have obtained, and many samples of the same eluate volume are less satisfactory than this particular one.

Figure 4 shows the curve we obtained when 1.25 ml of the same plasma eluate was assayed by the ethylenediamine technique of Weil-Malherbe. Here the light scatter peak is much less than that given by the trihydroxyindole method and the criteria of good fluorimetry are again achieved. Though not shown here, additions of norepinephrine to eluates assayed by the ethylenediamine method increased the fluorescence almost proportionally, indicating only minimum quenching. In our hands, better comparison of the eluate curve with the standard curve was achieved with the ethylenediamine method than with the trihydroxyindole method at these high sensitivities.

A second major problem we have encountered with the trihydroxyindole method has been the preparation of a stable sample blank. Whether we prepared these blanks by the "fading" technique of Lund or by the addition of ascorbic acid prior to the ferricyanide as suggested by Price, there was a marked tendency

of the blanks to rise with time faster than the samples. Thus, 30 seconds after preparation the sample may read 7 to 8 units above the blank, but 5 minutes later the difference may be only 2 to 3 units. The major contribution to the blank comes from the decomposition of ascorbic acid in alkali, and as yet we have found no satisfactory means of eliminating this problem.

We would like to suggest that the widely divergent results reported with the trihydroxyindole method may be related to individual variation between laboratories in the amount of quenching obtained and in the times at which the samples are read. We hope that a renewed appreciation of the light scatter problem in particular plus a determined effort to meet the strict criteria of a good fluorimetric assay will reconcile these differences in the future.