Differential Fluorometry in Catecholamine Determination: A Simplified Method of Calculation

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A method for calculating the results of differential fluorometry in the determination of epinephrine and norepinephrine is described. A form which outlines the calculations in steps is illustrated. This simplified method is applicable to differential fluorometry and other procedures which involve solving two equations simultaneously.

Two substances with a marked difference in their excitation and fluorescence spectra can be readily measured independently. However, when the spectra of the two substances overlap, one cannot be measured independent of the other. Then the principle of differential fluorometry is utilized. Measurements are made with two different sets of excitation and fluorescence wavelengths chosen to maximize the difference between the relative readings of the two substances while the absolute readings are kept relatively large. The concentration of each substance is then determined by solving two equations simultaneously. This principle may be utilized in analyzing mixtures of substances by measuring light absorption, radioactivity, and the like, as well as fluorescence.

In the determination of epinephrine and norepinephrine, both Price and Price (3) and Cohen and Goldenberg (2) independently suggested the utilization of slight differences in fluorescent properties of the indoles formed from these compounds for their differential determination. They indicated the equations involved and stated that the two

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equations are solved simultaneously. The basic assumption is that at a given excitation and fluorescence frequency, after subtraction of the appropriate blank, the fluorescence of a mixture of two or more substances is equal to the sum of the products of the concentration of each substance present times the specific fluorescence of that substance.

"Fluorescence constants" (external standards) may be determined and utilized for all calculations. However, we have found that the fluorescence of added epinephrine and norepinephrine is variable. For accurate differentiation, especially when it is desirable to measure accurately the presence of small amounts of epinephrine in the presence of large amounts of norepinephrine, internal standards are needed with each determination. Two equations must be solved simultaneously for each determination if internal standards are used. These calculations are tedious unless done in a systematic way. Described here is a simplified method for doing these calculations.

Method

The appropriate excitation and fluorescence wavelengths are selected utilizing curves (Fig. 1 and 2) from a spectrophotofluorometer such as the Aminco-Bowman instrument. The actual measurements

![Fig. 1 (left). Excitation curves of indoles formed from epinephrine and norepinephrine with fluorescence measured at a wavelength of 500 m.](image1)

![Fig. 2 (right). Excitation curves of indoles formed from epinephrine and norepinephrine with fluorescence measured at a wavelength of 520 m.](image2)
are made with a filter fluorometer such as the Farrand using interference filters. With the "A" filters (390 mµ excitation and 500 mµ fluorescence), the indoles formed from epinephrine and norepinephrine read approximately equal. With the "B" filters (430 mµ excitation and 520 mµ fluorescence), the indole formed from epinephrine reads approximately three times as much as that from norepinephrine. Fluorescence measurements are made on four samples. One is a blank (B), one is the "unknown," one the "unknown" with added epinephrine, and one an "unknown" with added norepinephrine. The blank is subtracted from the "unknown," and the result is referred to as "U." The "unknown" is subtracted from the "unknown" with added epinephrine and again from the "unknown" with added norepinephrine, and these results are referred to as "E" and "N."

The values obtained with the "A" filters are given the subscript "a," and those obtained with the "B" set of filters, the subscript "b." Uₐ and Uₜ are the net readings of the "unknown" less the blank at the "A" and "B" frequencies, respectively. Eₐ, Nₐ, Eₜ, and Nₜ represent the specific fluorescence of added epinephrine and norepinephrine at the "a" and "b" frequencies. If x equals the concentration of epinephrine in the unknown solution and y equals the concentration of norepinephrine in the unknown solution, the equations are: Uₐ = xEₐ + yNₐ, and Uₜ = xEₜ + yNₜ.

These equations can be readily solved for x and y separately, and their values summed to determine the total catecholamines. However, for convenience of calculation when these determinations are to be made repeatedly, the formula has been revised to solve for the amount of total catecholamines (x + y) and for the percentage of the total catecholamines which is epinephrine (%x). The derivation of the formula for total catecholamines and for the percentage of epinephrine is as follows:

\[ t = x + y \]
\[ y = t - x \]

By definition:
\[ Uₐ = xEₐ + yNₐ \]
\[ Uₜ = xEₜ + yNₜ \]

Solving for x:
\[ Uₐ = xEₐ + (t-x)Nₐ \]
\[ Uₜ = xEₜ + (t-x)Nₜ \]
\[ x = \frac{Uₐ - tNₐ}{Eₐ - Nₐ} \]
\[ x = \frac{Uₜ - tNₜ}{Eₜ - Nₜ} \]
**Fig. 3.** Form used for routine calculation of urinary epinephrine and norepinephrine excretion.
\[ x = x \]
\[ \frac{U_a - tN_a}{E_a - N_a} = \frac{U_b - tN_b}{E_b - N_b} \]
\[ U_aE_b - U_aN_b - tN_aE_b + tN_aN_b = U_bE_a - U_bN_a - tN_bE_a + tN_bN_b \]

Changing all signs and solving for \( t \):
\[ t(N_aE_b - E_aN_b) = U_aE_b - U_aN_b + U_bN_a - U_bE_a \]
\[ t = \frac{U_a(E_b - N_b) + U_bN_a - U_bE_a}{N_aE_b - E_aN_b} \]
\[ \frac{x}{x + y} = \frac{U_b - tN_b/t}{E_b - N_b} = \frac{U_b/t - N_b}{E_b - N_b} \]
\[ \% x = \frac{U_b/t - N_b}{E_b - N_b} (100) \]

To make these calculations in a convenient and reproducible way, we use a standardized form which makes it possible for a relatively inexperienced person to calculate these values readily, using a calculator (Fig. 3). After entering the basic measured values on the form (circled), the operations to be performed on these numbers are indicated by lines indicating the type of calculations, such as multiply (mult.), enter dividend (entr. divid.), repeat (rep.), and divide. The lines extend from the indicated operation to the specific values on the table. For example, the first value to be calculated is \( N_aE_b - E_aN_b \). The instruction is “mult.” and lines extend to \( N_a \) and \( E_b \). The product of \( N_a \) and \( E_b \) is not cleared from the machine. Lines extend from “neg. mult.” to \( E_b \) and \( N_a \). By using negative multiplication, this product is automatically subtracted from the preceding one. The difference between the two products is entered in the space to the right of the indicated operations. The remaining steps are carried out by following the lines from the indicated operations to the numbers to be used.

The results are in terms of a factor \( t \), and the actual values of epinephrine and norepinephrine in micrograms per 100 ml. depend on the amount of urine used and the concentration of the internal standards. As these factors vary with the technic used, no constant will be given. We also use the same calculation procedure in the determination of metanephrine and normetanephrine (1). The specific form illustrated here was designed for use with a Friden calculator.

References