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Fluorometric Assay for *N*-Acetylprocainamide

Edward Matusik and Thomas P. Gibson

We describe a simple, rapid fluorometric assay for separate quantitative analysis of procainamide and *N*-acetylprocainamide in mixtures. The effective linear range (fluorescence vs. concentration) in serum is 0.1 to 10.0 mg/liter, regardless of the ratio (by weight) of the two drugs from 1:10 to 10:1. Analytical recoveries by the extraction method used were 100.0 ± 3.0% and 98.0 ± 4.0%, respectively. For determination of either compound, the maximum coefficient of variation was 10%.

Additional Keyphrases: *drug assay • pKa • quantum yield*

The therapeutic and toxic effects of procainamide (PA) are related to the concentration of the drug in the plasma of patients being treated (*1*). *N*-Acetylprocainamide (NAPA) has recently been identified as a metabolite of PA that has antiarrhythmic activity in mice, dogs, and man (*2, 3*). Because of this it is likely that NAPA contributes to the therapeutic efficacy and toxicity of PA.

Until now, NAPA in serum has been measured either by gas/liquid chromatography with use of capillary columns and flame-ionization detectors (*3, 4*) or by thin-layer chromatography with subsequent densitometry (*5*). The technology required for either of these methods is not readily available to many clinical laboratories. In contrast, spectrophotofluorometry is widely used in clinical laboratories and has been used to determine PA concentrations in serum and other biological fluids (*6*). The present study describes a spectrophotofluorometric method for the estimation of NAPA in serum.

Methods

N-Acetylprocainamide was prepared as previously described (*7*) and procainamide·HCl was obtained from K & K Laboratories, Inc., Plainview, N. Y. 11803.[1]

Samples for spectrophotofluorometry were analyzed on a Perkin-Elmer MPF-3 Fluorescence Spectrophotometer with a Corrected Spectrum accessory. The light source was an Osram Xenon lamp, the detector an R 446 photomultiplier tube, and the quantum counter a solution of Rhodamine B. The instrument was calibrated daily with triphenylene embedded in a plastic matrix (Precision Cells, Inc., Hicksville, N. Y. 11801). Suprasil quartz cuvettes with a 1-cm path length were used. Excitation (ex) and emission (em) slit widths of 10 nm were used throughout the entire study.

We prepared concentrated stock solutions of 200 mg each of PA and NAPA per liter in de-ionized distilled water. Aqueous solutions in concentrations ranging from 0.1 to 2.0 mg/liter in 0.1 mg/liter increments and from 2.0 to 10 mg/liter in 1.0 mg/liter increments were prepared by diluting the stock solutions with either 0.1 mol/liter HCl for the measurement of NAPA or 1 mmol/liter NaOH for the measurement of PA. Standard solutions in serum over the same concentration ranges were prepared by adding aliquots of the stock solution to acid-washed glass tubes and diluting to 10 ml with pooled specimens of fresh human serum. Aqueous and serum solutions of each compound alone and in a 1:1 ratio were prepared in the following concentrations: 0.1, 0.5, 1.0, 1.5, and 2.0 through 10.0 mg/liter in 1.0 mg/liter increments. Ratios of PA/NAPA of 10:1 and 1:10, with the concentration of the material in excess being 1.0, 5.0, 10.0, 15.0, and 20.0 mg/liter, were also prepared. Serum extracts were prepared for analysis as previously described (*7*), with the following modification. To minimize the rate of acid hydrolysis of NAPA to PA at room temperature (24 °C), the compounds were extracted from the organic phase into 3.0 ml of 0.1 mol/liter HCl. The concentration of NAPA was immediately measured in the acid phase (pH 1.0) with λ_{ex} 288 nm and λ_{em} 341 nm. Then the sample was alkalinized by the addition of 0.1 ml of 6 mol/liter NaOH and PA was measured at λ_{ex} 298 nm and λ_{em} 354 nm. Five samples at each concentration were analyzed. The mean fluorescent intensity after correction for background fluorescence was used to determine the linear regression equation for each experimental condition. Statistical differences between regression coefficients (slope and intercept) were calculated as described by Bliss (*8*). The null hypothesis, that no difference exists between the coefficients of regression of a compound present alone in serum versus the same compound in the presence of the second material at a given ratio, was tested. Results are expressed as a mean ± the standard error of the mean.

The relative quantum yields of PA and NAPA were determined as described by Chen (*9, 10*). Corrected emission spectra were integrated with a Perkin-Elmer Model 48 digital integrator. The quantum

Division of Biochemistry and Department of Nephrology, Walter Reed Army Institute of Research, Washington, D. C. 20012.

[1] The manufacturers' names and products are given as scientific information only and do not constitute an endorsement.

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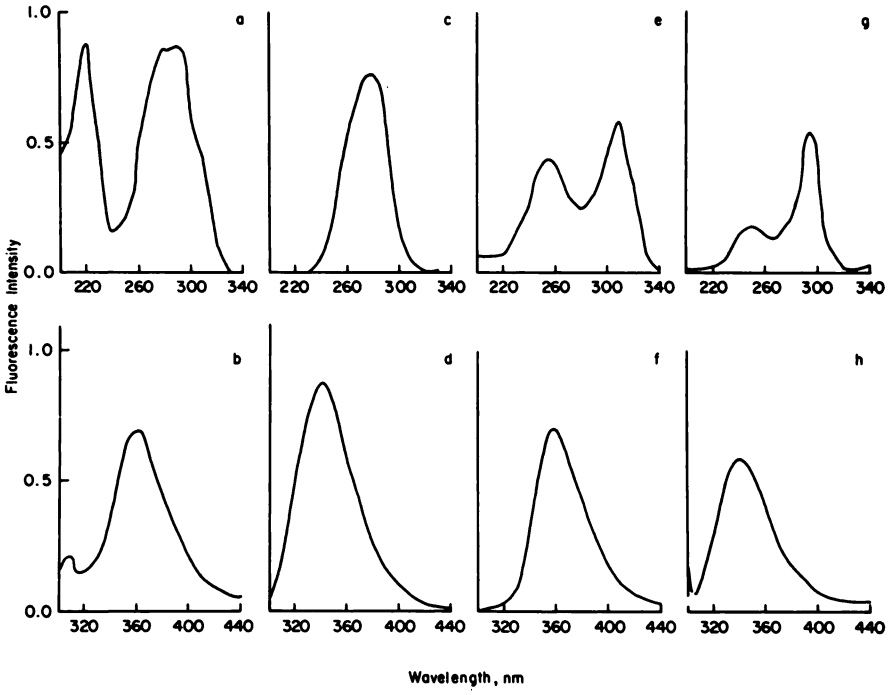

Fig. 1. Corrected excitation and emission spectra for PA and NAPA

1-*a*, excitation PA, pH 1; 1-*b*, emission PA, pH 1; 1-*c*, excitation NAPA, pH 1; 1-*d*, emission NAPA, pH 1; 1-*e*, excitation PA, pH 11; 1-*f*, emission PA, pH 11; 1-*g*, excitation NAPA, pH 11; 1-*h*, emission NAPA, pH 11

yield of 1-dimethylaminonaphthalene-5-sulfonate in 0.1 mol/liter $NaHCO_3$ was used for comparison (*10*). Absorption was measured with a Cary 14 UV-Vis-IR recording spectrophotometer.

The pKa of NAPA was estimated by titration with use of a Corning Model 10 pH meter equipped with a Corning combination glass electrode. Solutions of NAPA were prepared with de-ionized glass-distilled water, which had been boiled to remove excess carbonate and protected from the atmosphere by a gas trap.

Results

Figure 1 shows corrected fluorescence excitation and emission spectra for PA and NAPA. Ordinate values have not been normalized, and only band shapes and maximum wavelength positions can be determined. At pH 1, the excitation spectrum of PA (Figure 1-*a*) shows two well-resolved maxima, one at 220 nm and the other at 290 nm. The emission spectrum (Figure 1-*b*) has a maximum intensity at 360 nm. In a basic solution (pH 11) the peak intensity values in the excitation spectrum of PA are at 255 and 310 nm (Figure 1-*e*) and the emission maximum at 358 nm (Figure 1-*f*). Only one maximum, 279 nm, is found in the excitation of NAPA at pH 1 (Figure 1-*c*). The maximum emission intensity is found at 340 nm (Figure 1-*d*). Peak centers for excitation of NAPA at pH 11 were 250 and 295 nm (Figure 1-*g*) and for emission at 340 nm (Figure 1-*h*).

Quantum yield, ϕ, and log $A^{1\%}_{1cm}$ of PA and NAPA are presented in Table 1. The log $A^{1\%}_{1cm}$ value shown is that of the lowest energy-corrected excitation maximum for a given material at a specified pH. Note that at pH 11, the ϕ value for NAPA is a tenth that for PA, while at pH 1 the ϕ of NAPA is twice that of PA.

The pKa of NAPA was experimentally determined to be 8.3 ± 0.1.

Analytical recovery of PA from serum, correcting for dilution, was 100.0 ± 3.0%. Extraction of serum samples containing only NAPA gave a recovery of 98.0 ± 4.0%. The maximum coefficient of variation within-run (n = 5 at each concentration) and between runs (n = 15) was 10% for both compounds.

The empirical relationship used to calculate the fluorescence intensity is given in the appendix (Equation 1). Fluorescence intensity values for the concentration of PA and NAPA were calculated from the uncorrected excitation and emission peak values of 298 nm and 354 nm for PA and 288 nm and 341 nm for NAPA.

Procainamide

For a solution of PA the results calculated by using equation 1 are shown in the upper curve of Figure 2, where $A^{1\%}_{1cm}$ (298 nm) = 2.51×10^2 a·cm^{-1}·concn^{-1}, and ϕ = 0.01 (Table 1). The center curve was experimentally obtained by adding PA to an aqueous solution at pH 11. The addition of PA did not change the pH of the solutions. It can be seen for both curves that as the concentration (absorbance) increases, the curves best fit a parabolic equation. The lower curve represents experimental values obtained from serum extracts containing only PA. Here a linear dependence of fluorescence intensity on concentration over the entire concentration range is seen.

N-Acetylprocainamide

Departure from linearity of a plot of fluorescence intensity vs. concentration for NAPA at pH 1 (Figure 3) is similar to that observed for PA. Again using equation 1, we calculated the expected fluorescence intensity values, where $A^{1\%}_{1cm}$ (288 nm) = 2.82×10^2 a·cm^{-1}·concn^{-1}, and ϕ = 0.01 (Table 1). The lower curve of this figure again shows the range of linearity in serum extending from 0.1 to 10.0 mg/liter (μg/ml).

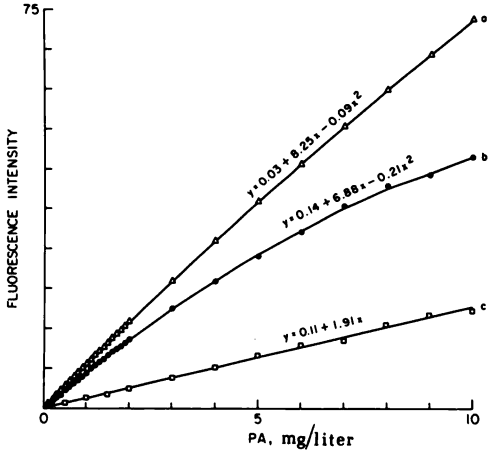

Fig. 2. Fluorescent intensity of PA vs. concentration, pH 11, excitation 298 nm, emission 354 nm, uncorrected

Curve *a*, intensity values calculated by using equation 1 (see text). Curve *b* is the experimentally determined curve at pH 11 in water. Curve *c* was obtained from serum

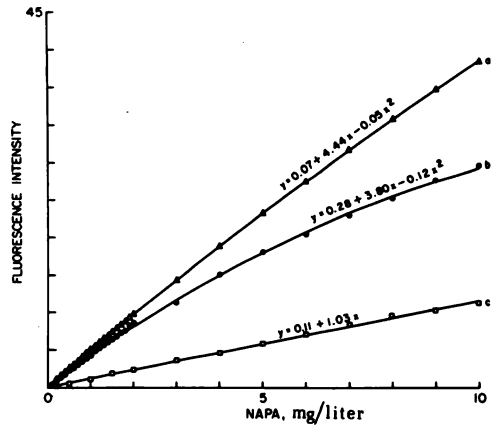

Fig. 3. Fluorescent intensity of NAPA vs. concentration, pH 1, excitation 288 nm, emission 341 nm, uncorrected

Curve *a*, intensity values calculated by using equation 1 (see text). Curve *b* is the experimentally determined curve in water. Curve *c* was obtained from serum

Table 1.

	Procainamide (pK_a = 9.4)[a]		*N*-Acetylprocainamide (pK_a = 8.3 ± 0.1)	
pH	$\log A^{1\%}_{1cm}$	$\phi \times 10^2 \pm (CL^{95\%}_{\bar{y}} \times 10^2)$	$\log A^{1\%}_{1cm}$	$\phi \times 10^2 \pm (CL^{95\%}_{\bar{y}} \times 10^2)$
1.0	1.30	0.5 ± 0.03	2.60	1.0 ± 0.05
3.0	2.55	1.0 ± 0.04	2.60	1.0 ± 0.05
8.5	2.58	1.0 ± 0.08	2.59	0.5 ± 0.01
11.0	2.72	1.0 ± 0.05	2.60	0.1 ± 0.03

ϕ = quantum yield; $CL^{95\%}_{\bar{y}}$ = 95% confidence limit of the mean; $\log A^{1\%}_{1cm}$ = value of log absorptivity at fluorescence excitation maximum.

[a] From data of Weily and Genton (*15*).

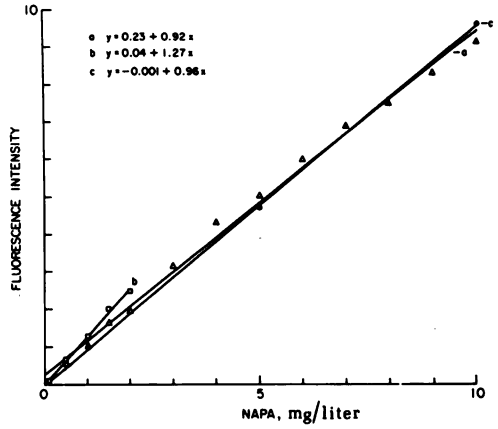

Fig. 4. Effect of various ratios of PA to NAPA on the measurement of NAPA at pH 1

Excitation 288 nm, emission 341 nm, uncorrected. *a*, 1:1 ratio; *b*, 10:1 PA excess; *c*, 10:1 NAPA excess

Because both PA and NAPA will be present in clinical samples of serum (*3*), the possibility of the interference of one with determination of the other was investigated. In Figure 4, observed fluorescence response for NAPA in the presence of PA in ratios of 1:1 (curve *a*), 10:1 (PA excess, curve *b*), and 10:1 (NAPA excess, curve *c*) are illustrated. There was no statistical difference between the slopes and intercepts of these lines when compared to those of NAPA alone from serum (lower curve, Figure 3).

Figure 5 is a similar plot for the determination of PA. Statistically, there again was no difference when the various ratios of PA to NAPA were compared to the values for PA alone in serum (Figure 2, lower curve).

Discussion

Our results indicate that NAPA can be accurately determined in serum samples by fluorometry. The effective linear range is 0.1 to 10.0 mg/liter regardless of the ratio of NAPA to PA from 1:10 to 10:1. Recoveries of PA and NAPA by the extraction method used were 100.0 ± 3.0% and 98.0 ± 4.0%, respectively. For determination of either compound the maximum coefficient of variation was 10%.

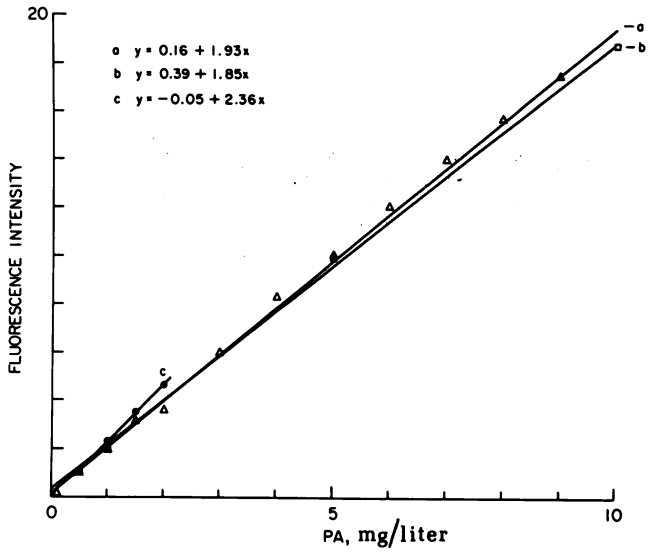

Fig. 5. Effect of various ratios of NAPA to PA on the measurement of PA at pH 11

Excitation 298 nm, emission 354 nm, uncorrected. *a*, 1:1 ratio; *b*, 10:1 PA excess; *c*, 10:1 NAPA excess

The quantum yield of PA was reduced when the aromatic amine was fully titrated at pH 1, and the absorptivity of the longest excitation wavelength was only 4% of that found at pH 11 (Table 1). Evidence that the excited-state singlet for PA is the same species regardless of pH can be seen in Figure 1-*b* and 1-*f*, where little or no change in bandshape or peak position of PA emission occurs. Clearly, the protonated excited state would have to be a stronger acid than ground state in order to yield some percentage of the unprotonated aromatic amine (*11*). A similar argument can be proposed for the excited-state species of NAPA (Figures 1-*d* and 1-*h*).

Reduction of the quantum yield of PA by formation of the *N*-acetyl metabolite would be expected because aniline (fluorescence λ_{max} = 345 nm), reacted with acetic anhydride, forms acetanilide, which is nonfluorescent (*11*).

As Chen and Hayes have shown (*12*) for a standard 1 × 1 cm cuvette, an appreciable percentage of the exciting beam is absorbed by even a dilute solution before the beam reaches the center effective area of the cuvette. Because of this, nonlinearity becomes very evident in fluorescence vs. concentration curves for a solution with an absorptivity greater than 0.1. This effect can be seen in Figures 2 and 3 for PA and NAPA. From our data an accurate estimate of the concentration of PA and NAPA by pH adjustment is possible as long as the solution total absorptivity does not exceed 0.08 absorbance unit. However, for serum extracts linearity exists over a wide concentration range because of dilution during extraction (lower curves, Figures 2 and 3).

Even when the ratio of PA/NAPA is 10:1 or 1:10, the concentration of either compound can be accurately estimated if the critical absorbance is not exceeded. Statistical comparison of the slopes and intercepts for the curves representing PA alone in serum (Figure 2, lower curve) and those representing PA/NAPA ratios of 1:1, 10:1, and 1:10 (Figure 5) shows no significant difference. The same is true for NAPA (Figure 4).

No interference with the determination of either compound was noted in serum from patients on the following medications: alpha-methyldopa, allopurinol, diazepam, flurazepam, folic acid, furosemide, heparin, hydralazine, prednisone, and propranolol.

In conclusion, the technical aspects of this method are far simpler than those required for gas chromatography with capillary columns or for thin-layer chromatography and densitometry. The availability of instrumentation for the fluorometric determination of PA and NAPA should allow many more investigators to monitor the concentrations of both compounds in serum.

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Appendix

The intensity of fluorescent radiation of a fluorophore is given by (*13, 14*):

$$F(\lambda) = f(\theta)g(\lambda)I_0\phi Abc \times \left[1 - \frac{(Abc)}{2!} + \frac{(Abc)^2}{3!} - \ldots + \frac{(Abc)^N}{(N+1)!}\right]$$

where:

$F(\lambda)$ = fluorescence intensity at wavelength λ
$f(\theta)$ = viewing angle of the instrument detector
$g(\lambda)$ = response characteristic of the detector
I_0 = intensity of the exciting radiation
ϕ = quantum yield of the fluorophore
A = absorptivity of the sample
b = sample pathlength along the axis of excitation
c = concentration of the sample

In calculations of $F(\lambda)$, only the first three terms of the power series were used.