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Environmentally Shifted Fluorescence Lifetimes for Multicomponent Phase-Modulation Fluorimetric Analysis

F. García Sanchez,[1,2] A. Navas Díaz,[1] and J. Lovillo Ramírez[1]

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Several cyclodextrins, surfactants, and a series of three organic solvents—methanol, isoamylic alcohol, and hexanol–with different polarities and viscosities, were used to study the influence of the microenvironment on the fluorescent behavior of three diuretics–furosemide, triamterene, and piretanide. We concluded that on going from methanol to hexanol, the fluorescence lifetime of furosemide from 0.91 to 2.10 ns, that of triamterene from 4.54 to 4.44 ns, and that of piretanide from 5.24 to 10.37 ns. At 40 MHz, the phase shifts (excitation/emission) produced by furosemide, triamterene, and piretanide were 12.0, 29.6, and 30.2° in methanol and 27.8, 48.1, and 69.0° in hexanol. A three-factor, three-level factorial design allowed us to establish a calibration matrix of the three diuretics that covered the three ranges from 10 to 40, 1.5 to 6, and 0.1 to 0.4 μM for furosemide, piretanide, and triamterene, respectively. Data processing incorporated PLS adjustment values. The function was fitted to a phase-resolved fluorescence intensity at the three phase angles selected. Percentages recoveries were from 88 to 115%.

KEY WORDS: Fluorescence lifetimes; solvent effects; diuretic drugs; simultaneous analysis.

INTRODUCTION

The spectral position and shape of emission bands and fluorescence lifetimes often reflect the excited-state interactions between solute and solvent that occur in fluorescent molecules. Also, changes in the microenvironment promoted by micelle-forming surfactant solutions and cyclodextrin-forming inclusion compounds sometimes alter fluorescence lifetimes and cause spectral shifts.

Although the correlations between solvent polarity changes and spectral shifts have been studied and are well documented in the literature [1–3], relaxation fluorescence lifetimes and the changes which occur in the microenvironment have received relatively little attention in the past few years. In this respect, changes in fluorescence lifetimes are frequently associated with the solvent viscosity, which can inhibit molecular motions [4], and also with the kind of structure and the arrangement of the solute substituents [5].

In several instances, appropriate selection of the solvent polarity can modify excitation/emission wavelengths, and in this way fluorophore mixtures can be resolved by selective excitation in the steady-state phase.

When wavelength-based selectivity does not work, fluorescence phase-modulation spectroscopy can sometimes be a useful technique to discriminate between compounds with closely similar fluorescence lifetime profiles [6]. However, the precision of the measurements depends on the phase-shift difference between the fluorophores. Changing lifetimes and phase shifts by modifying the environment can improve analysis.

In this work, we have selected three diuretic drugs, piretanide, furosemide, and triamterene, which have similar excitation-emission profiles and fluorescence

[1] Departamento de Química Analítica, Facultad de Ciencias, Universidad de Málaga. 29071-Málaga; Spain.
[2] To whom correspondence should be addressed.

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lifetimes. They showed little wavelength displacement but their fluorescence lifetimes changed considerably when the surrounding microenvironment was changed.

Several attempts to obtain calibration functions in simultaneous analysis of two- or three-component fluorescent compounds by using phase-resolved fluorescence intensity (PRFI) measurements have been made [6–8]. We used a three-factor, three-level factorial design to obtain first-grade equations that fit as closely as possible the experimental PRFI responses to concentrations.

THEORY

The theory and instrumentation of PRFS, described elsewhere [9,10], are based on the phase-modulation technique for determination of fluorescence lifetimes. The sample is excited with light with a time-dependent intensity $E(t)$ that has the form

$$E(t) = A\ (1 \pm m_{ex} \sin \omega t) \tag{1}$$

where A represents the dc intensity and m_{ex} is the degree of modulation. The resulting fluorescence $F(t)$ of a single-component sample with exponential decay will be phase-shifted by angle ϕ and is expressed as

$$F(t) = A'\ [1 + m_{ex} \cos\phi \sin (\omega t - \phi)] \tag{2}$$

where A' represents the dc component of the fluorescence intensity. For heterogeneous (multicomponent) systems, the observed emission (assuming independent uncorrelated emitters) is the sum of the individual emissions for each of the lifetime species.

For a solution containing j independent emitters,

$$F(t) = \sum_i A_i' [1 + m_{ex}\, m_i \sin (\omega t - \phi_i)] \tag{3}$$

Adjusting the phase sensitivity of the modulated component of the fluorescence signal gives the PRFI, expresses as

$$\text{PRFI} = A_\lambda'\, m_{ex} m \cos (\phi_D - \phi) \tag{4}$$

The total PRFI for a multicomponent sample at a given ϕ_{D_j} and ω is the sum of the PRFIs of the individual components and a three-component system,

$$\text{PRFI}_{\phi_{Dj}{}^i} = a_1 C_{i1} + a_2 C_{i2} + a_3 C_{i3} \tag{5}$$

where a_1, a_2, and a_3 are scalars that reflect the relative contributions for each component, and C_{i1}, C_{i2}, and C_{i3} are the concentrations for each component at the ith mixture.

This can be expressed in matrix notation as

$$\begin{matrix} PRFI_{\Phi_{Dj}{}^1} \\ PRFI_{\Phi_{Dj}{}^2} \\ \cdot \\ \cdot \\ \cdot \\ PRFI_{\Phi_{Dj}{}^n} \end{matrix} = \begin{matrix} C_{11} & C_{12} & C_{13} \\ C_{21} & C_{22} & C_{23} \\ \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot \\ C_{n1} & C_{n2} & C_{n3} \end{matrix} \begin{matrix} a_1 \\ a_2 \\ a_3 \end{matrix}$$

$$S = C \cdot A \tag{6}$$

Here S is a $(n \times 1)$ matrix that contains the measured PRFI data for each mixture, C is a $(n \times 3)$ matrix that contains the known concentrations, and A is a (3×1) matrix that contains the scalars. A simple least-squares solution for A is

$$A = (C^t \cdot C)^{-1} \cdot (C^t \cdot S) \tag{7}$$

A three-component system needs phase-resolved measurements at three detector-phase angles and these, in turn, generate a series of simultaneous linear equations,

$$\begin{aligned} PRFI_{\Phi D1} &= a_1 C_1 + a_2 C_2 + a_3 C_3 \\ PRFI_{\Phi D2} &= b_1 C_1 + b_2 C_2 + b_3 C_3 \\ PRFI_{\Phi D3} &= p_1 C_1 + p_2 C_2 + p_3 C_3 \end{aligned} \tag{8}$$

in which a_i, b_i, and p_i are scalars that were obtained by resolving Eqs. (7) for each phase angle chosen. To determine the concentrations in an known sample, the general matrix is solved for C_1, C_2, and C_3 using Cramer's rule.

Adding the equivalent of a second least-squares analysis to the procedure increases the complexity of the calculations and also the possibility of round-off errors by the computer.

The problem can be simplified by using PRFIs as independent variables. Here the system is expressed as

$$C_{il} = b_0 + b_1 \text{PRFI}_{\phi_{D1}{}^i} + b_2 \text{PRFI}_{\phi_{D2}{}^i} + b_3 \text{PRFI}_{\phi_{D3}{}^i} \tag{9}$$

where C_{ik} is the concentration at the kth component in the ith mixture, $\text{PRFI}_{\phi_{Dj}{}^i}$ is the phase-resolved intensity measured at ϕ_{Dj} for the ith mixture, and b_0, b_1, b_2, and b_3 are proportionality coefficients.

This, expressed in matrix notation, is

$$C = S \cdot A \tag{10}$$

in which C is a $(n \times 1)$ matrix that contains the known concentrations, S is a $(n \times 4)$ matrix that contains the measured PRFI at three different detector-phase angles plus an extra row of 1's to create a nonzero intercept, and A is a (4×1) matrix that contains the scalars. A simple least-squares solution for A is

$$A = (S^t \cdot S)^{-1} \cdot (S^t \cdot C) \qquad (11)$$

A three-component system gives three independent equations that can be used directly to analyze an unknown sample.

EXPERIMENTAL

Reagents and Solvents. Furosemide {5-(aminosulfonyl)-4-chloro-2-[(2-furanylmethyl)amino] benzoic acid}, triamterene (6-phenyl-2,4,7-pteridinetriamine), γ-cyclodextrin (cyclooctaamylose), β-cyclodextrin (cycloheptaamylose), cholic acid sodium salt (3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid), CTAB (*N,N,N*-trimethyl-1-hexadecanamonium bromide), Triton X-100, and piretanide [3-(aminosulfonyl)-4-phenoxy-5-(1-pyrrolidinyl) benzoic acid] were provided by Sigma. Methyl-β-cyclodextrin from Aldrich. Methanol, 1-hexanol, and isoamylic alcohol were obtained from Merck R.A.

Instruments. Fluorescence was measured with an Aminco SLM 48000S spectrofluorimeter equipped with a 450-W xenon lamp source, a Hamamatsu R928 photomultiplier detector tube, and a Pockel cell electrooptic modulator. The photocathode emission was minimized by cooling with a Peltier-cooled PMT housing. An IBM AT microcomputer was used for on-line data acquisition and data processing. Fluorescence lifetimes were measured by using multifrequency-modulated excitation beams; a silica gel scattering solution was the reference. Phase and modulation measurements used the "100 average" mode, in which each measurement value is the average of 100 samplings, carried out automatically by the instrument circuitry in approximately 25 s. The excitation monochromator was set at 350 nm and the slits were set at 16 nm, with a 8-nm bandpass entrance. To select the emission wavelength, instead of a monochromator, a cutoff filter (418 nm) was placed in the sample emission receiving channel and a bandpass filter (300–400 nm) in the stop channel to receive the scatter reference.

All PRFI measurements were made at 40 MHz in the delta-phase mode. We made multifrequency measurements of fluorescence lifetimes at several modulations between 10 and 90 MHz.

RESULTS AND DISCUSSION

Selection of Environment. Although fluorescence phase-modulation spectroscopy is a useful technique to discriminate between compounds that have closely similar fluorescence lifetimes, the precision of the measurements is determined by the difference between the phase shifts of the fluorophores. Changing lifetimes and the phase shift by modifying the microenvironment improvements precision and accuracy. Steady-state fluorescence excitations, emission spectra, and fluorescence lifetimes of triamterene, furosemide, and piretanide (Fig. 1) were obtained for several cyclodextrins, surfactants, and a series of three organic solvents of different viscosities and polarities. Table I summarizes the fluorescence lifetimes and wavelength data for each compound in the different types of environment. Small or no wavelength displacements, but considerable lifetime shifts were observed when the surrounding microenvironment was changed. Our data suggest that on going from methanol to hexanol the fluorescence lifetime of furosemide changed from 0.91 to 2.10 ns; that of triamterene, from 4.54 to 4.44 ns; and that of piretanide, from 5.24 to 10.37 ns.

The effect of the solvent on solute excitation and emission spectra has received much attention [3], however, the influence of the liquid environment on intramolecular dynamics in solutions is not yet understood. There are "more static" interactions, modifying intramolecular potential energy surfaces, and "more dynamical" interactions, influencing intramolecular motions via intramolecular exchanges of energy and momentum. In the first case, the observed spectral shifts appeared to be associated with dipolar and hydrogen-bonding solute–solvent interactions [16], and in the second case decay-relaxation lifetimes were closely associated with vis-

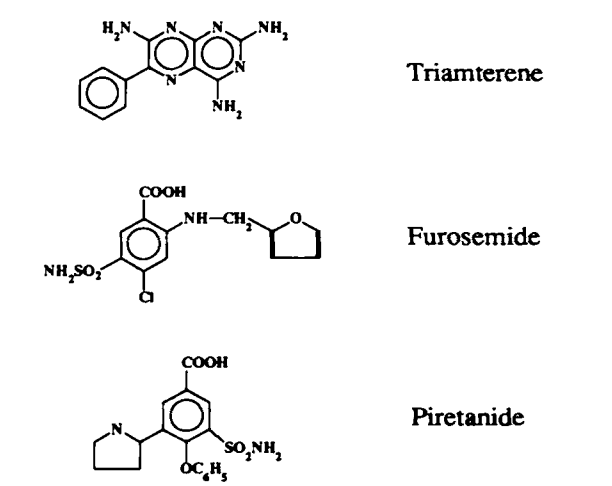

Fig. 1. Chemical structure of the compounds tested.

Table I. Microenvironment Effects on Fluorescent Properties of the Diuretic Drugs

Solvent	Viscosity (cP)[a]	$E_T(30)$ (kcal mol^{-1})[b]	Furosemide		Triamterene		Piretanide	
			τ (ns)	λ_{exc}–λ_{em} (nm)	τ (ns)	λ_{exc}–λ_{em} (nm)	τ (ns)	λ_{exc}–λ_{em} (nm)
H_2O	0.89[11]	63[3]	1.78	350–410	4.95	360–430	5.70	345–430
γ-CD[c]	100[12]	≈55	1.60	350–405	5.20	365–435	5.95	350–440
γ-CD (pH 3.0)	—	—	1.84	350–405	4.65	365–430	5.10	345–435
γ-CD (pH 8.5)	—	—	2.90	350–407	5.33	365–430	5.89	350–440
β-CD[d]	150[12]	54[13]	3.82	355–410	5.50	365–435	5.21	345–430
β-CD (pH 3.0)	—	—	1.55	350–410	4.87	355–435	5.14	345–430
β-CD (pH 8.5)	—	—	4.11	355–405	5.63	365–435	5.32	345–435
Methyl-β-CD[e]	>100	≈55	1.82	350–405	5.58	365–435	6.14	350–440
pH 3.0	—	—	1.41	350–405	4.81	360–435	5.47	345–435
pH 8.5	—	—	1.78	350–405	5.72	365–435	6.18	345–440
Cholic acid	—	—	3.49	355–405	5.60	360–430	6.27	345–440
Triton X-100	—	42.5[14]	1.86	355–410	3.99	355–435	5.05	350–445
CTAB[f]	30[15]	51.5[14]	1.93	355–410	5.05	365–440	5.35	355–440
Methanol	0.55[11]	55.5[14]	0.91	350–405	4.54	365–435	5.24	350–445
Isoamylic alcohol	4.50[11]	49.0[3]	1.93	355–415	4.19	360–440	10.04	355–445
1-Hexanol	4.93[3]	48.8[3]	2.10	350–410	4.44	365–440	10.37	350–440

[a] At 25°.
[b] Polarity.
[c] γ-Cyclodextrin.
[d] β-Cyclodextrin.
[e] Methyl-β-cyclodextrin.
[f] *N,N,N*-Trimethyl-1-hexadecanamonium bromide.

cosity inhibited molecular motions [4]. In the absence of strong solute-solvent interactions, reorientation time is a function of solution viscosity. Nikowa *et al.* [4] have recently explored these phenomena and show that changing the viscosity of the solvent by increasing the pressure (at 295 K) increased the decay of electrically excited *cis*-stilbene, and this was closed associated with viscosity.

In Table I, it can be seen that furosemide and piretanide showed long fluorescence lifetimes in hexanol and isoamylic alcohol [$\eta \approx 4.5$ cP, $E_T(30) \approx 49$ kcal mol^{-1}], while the lifetimes in methanol [$\eta = 0.5$ cP, $E_T(30) = 55$ kcal mol^{-1}] were short.

The lifetime shifts of triamterene appeared to be less sensitive to viscosity variations, probably because the large and relatively rigid molecular structure inhibited the influence of viscosity during the fluorescence lifetimes. The symmetry of molecules such as triamterene means that their dipolar moment should be zero or very small and, also, that the spectroscopic displacements caused by dipolar moments are also small [17].

Selection of Modulation Frequency. Curves of relative PRFI as a function of τ [18] pass through a maximum at $\tau = 1/\omega$, so that higher frequencies appeared more selective for the shorter lifetimes, and vice versa. The relative PRFI of furosemide (2.1 ns) was maximum at 75 MHz; that of piretanide (10.37 ns), at 15 MHz; and that of triamterene (4.44 ns), at 36 MHz. At 40 MHz, each component contributed high and individually similar PRFIs.

Selection of Detector Phase Angle. When differences in phase shift between components in the mixture are long enough (lifetime sufficient long), the null-phase method can be applied. The calculated lifetimes of furosemide, triamterene, and piretanide in hexanol were 2.10, 4.44, and 10.37 ns, respectively. In this case it is possible to employ the null-phase method, but when we choose the null phase angles of each component, we observed that only one component was nulled and the other components contributed to the total phase-resolved intensity. Consequently, the non-null phase method must be used. In this situation one must use the phase-resolved measurements at several detector phase angles, and these generate a series of simultaneous linear equations [Eq. (9)]. Two criteria were used to choose the nonnull detector phase angles: They had to give a high reproducibility of measurements obtained with high fluorescence intensity and detector angles, and the generated equations had to be clearly different.

The PRFIs plotted as a function of the detector phase

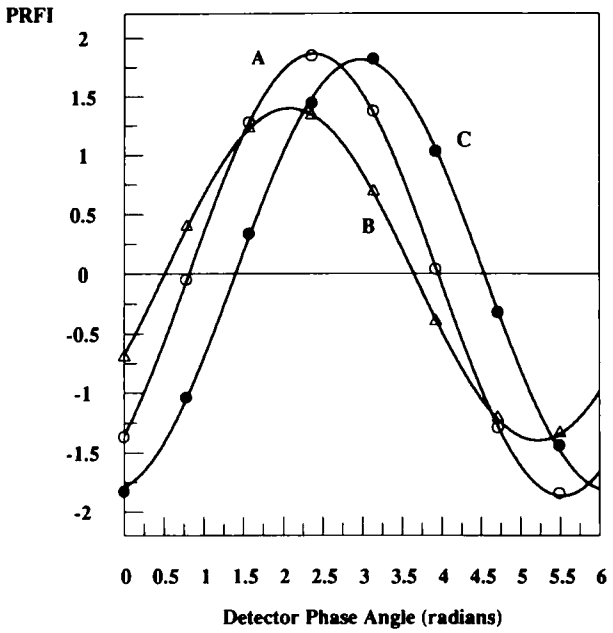

Fig. 2. Relative PRFI as a function of detector phase angle. (A) Triamterene; (B) piretanide; (C) furosemide.

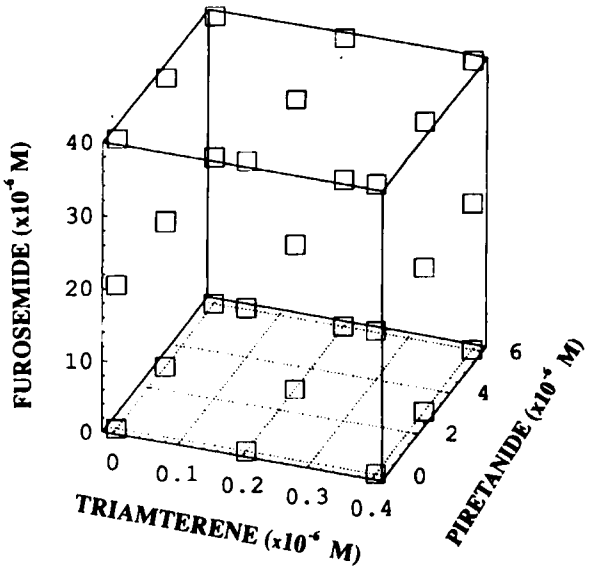

Fig. 3. A three-factor, three-level factorial design showing the set of 27 samples tested.

angle for the standard solutions of furosemide (10^{-4} *M*), triamterene (10^{-6} *M*), and piretanide (10^{-5} *M*) in hexanol (Fig. 2) were obtained at a modulation frequency of 40 MHz. The three detector phase angles were 90, 135, and 180° (1.57, 2.35, and 3.14 radians, respectively).

Data Processing. A three-factor, three-level factorial design for the multicomponent analysis was used to obtain experimental responses. To maximize statistically the information content in the calibration matrix, a calibration set of 27 samples with different composition was tested. The design of the composites used a factorial response surface design [19]. Figure 3 shows the distribution of the concentration domains of the three-component sample composites. The concentration ranges were selected according to two criteria: Each compound had a linear relationship with the PRFIs generated at each phase angle. For furosemide, piretanide, and triamterene, respectively, these were from 10 to 40, 1.5 to 6, and 0.1 to 0.4 μ*M*. The concentrations had to be selected according to the instrument settings so that no dc values greater than 9.0 V were obtained, because dc readings are the sum of contribution for each component.

The samples were measured at three detector phase angles (90, 135, and 180°). The known concentrations of each component alone, and also the PRFIs measured for each sample, were substituted in Eq. (9), to give a combination of 27 equations, which were solved by employing Eq. (11), in which *A* is a (3 × 1) matrix that contains the proportionality coefficient, and *S* is a (4 × 27) matrix that contains the measured PRFIs and an extra row of 1's, and *C* is a (27 × 1) matrix; this component includes all the known concentrations. In this way, the proportionality coefficients for each component were calculated. These generated three independent equations:

$$C_T = 0.164855\ PRFI_{90°} + 0.404429 PRFI_{135°} - 0.771519 PRFI_{180°}$$

$$C_P = 0.359972 - 2.749064 PRFI_{90°} + 13.48725 PRFI_{135°} + 17.53116 PRFI_{180°}$$

$$C_F = 1.651268 - 36.381861 PRFI_{90°} + 48.283108 PRFI_{135°} - 1.428944 PRFI_{180°}$$

Table II. Recovery Assay of Symthetic Mixtures

Mixture	Compound	Taken (μ*M*)	Found (μ*M*)	Recovery (%)
1	Triamterene	0.1	0.115 ± 0.005[a]	115
	Furosemide	30	29.99 ± 0.080	99.97
	Piretanide	1.5	1.68 ± 0.130	112
2	Triamterene	0.3	0.294 ± 0.004	98
	Furosemide	10	9.65 ± 0.301	96.5
	Piretanide	4.5	4.50 ± 0.110	100
3	Triamterene	0.1	0.089 ± 0.002	89
	Furosemide	10	11.35 ± 0.310	113.5
	Piretanide	1.5	1.51 ± 0.070	100.7
4	Triamterene	0.3	0.32 ± 0.006	106.7
	Furosemide	30	29.87 ± 0.190	99.6
	Piretanide	5	4.58 ± 0.071	91.6

[a] Standard deviation for three determinations.

C_T, C_P, and C_F are the concentrations of triamterene, piretanide, and furosemide, respectively; $PRFI_{90°}$, $PRFI_{135°}$, and $PRFI_{180°}$ are the PRFIs obtained at the corresponding phase-angle settings.

These equations resolved the three-component mixtures and covered the three concentration ranges within the composite cube used in the factorial design. To test the fit of the model we assayed "unknown" samples and carried out a recovery assay of four different mixtures of the three diuretics. The results are summarized in Table II, in which it can be seen that good recovery percentages and standard deviations were obtained in every case.

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