Synchronous fluorescence studies of anthracycline anti-tumor drugs

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ABSTRACT

Synchronous fluorescence (SF) is used to study structurally and spectrally similar anthracycline anti-tumor drugs in biological fluids and model membranes. SF bandwidths at half maxima are typically 20-24 nm versus 75-80 nm for unresolved spectra acquired during conventional scans. This substantial band-narrowing effect permits excellent resolution between Adriamycin and an interferant, 5-iminodaunorubicin, in a two-component mixture. SF detection limits for Adriamycin are 7×10^{-7} M in serum and 1.5×10^{-7} M in buffered aqueous solution. Additional studies of structurally homologous drugs in liposomes and organic solvents indicate that SF may be useful in characterizing the influence of substituent groups on relative membrane permeation depth.

1. INTRODUCTION

The anthracycline antibiotics Adriamycin and daunomycin are highly versatile, widely prescribed antitumor medications.[1] Because of their powerful, broad-based antineoplastic and therapeutic activity, considerable effort has been directed towards identifying their mechanisms of biological activity. Many of these investigations have focused on determining drug dose- and toxicity-response relationships.[1,2] A significant consequence of this work is the observation that Adriamycin exhibits considerable healthy tissue (particularly cardiac tissue) toxicity.[3] On a cellular level, Adriamycin appears to function by binding to DNA and interfering with DNA-related activities.[1,2] Recently, however, Adriamycin has been observed to exert its cytotoxic effect without entering cells.[4,5] Although its actual mode of action may involve multi-location attack, membrane penetration and binding is clearly a pre-requisite for cytotoxicity. As such, detailed investigations of the influence of anthracycline structure on membrane location and dynamics have been pursued.[6,7].

In order to further facilitate both basic and clinical studies, simple, sensitive, and rapid analytical techniques must be developed. Currently, a complex, time consuming chromatographic separation procedure is used to evaluate anthracycline drug levels in tissues and biological fluids.[8] Although this technique is not generally employed in clinical management, anthracycline cardiotoxicity may, in fact, warrant its routine monitoring. Unfortunately, several factors including, 1) the heterogeneity of biological samples, 2) the broad anthracycline fluorescence emission bandwidth, and 3) the presence of spectral interferents among patients receiving multi-drug therapy, have hindered the development of simple, quantitative spectrofluorimetric methods for anthracycline detection. Techniques currently utilized to evaluate membrane binding and penetration depth, including determinations of iodide quench constants, fluorescence lifetimes and time-dependent decays of fluorescence anisotropy are similarly time-consuming and complex.[6,7]

In this work, we have attempted to overcome these analytical limitations by applying synchronous excitation fluorescence techniques (SF) to study structurally and spectrally similar anthracycline anti-tumor drugs in biological fluids and model membranes. SF detection can significantly enhance analytical

selectivity, particularly in the case of multi-component analyses of compounds with similar spectral characteristics.[9] It is performed by recording the fluorescence signal while simultaneously scanning both λ_{ex} and λ_{em}. A constant wavelength (or energy) interval, $\Delta\lambda$ or $\Delta\upsilon$, is maintained between excitation and emission wavelengths throughout the measurement. The resulting SF intensity is a function of the intensity of a compound's excitation and emission spectra and the extent to which these features overlap. By selecting a $\Delta\lambda$ or $\Delta\upsilon$ which approximately corresponds to a compound's Stokes' shift, δ_S, spectral reduction to a single narrow band can be achieved.[9,10]

In practical terms, three primary effects, bandwidth narrowing, spectral simplification, and spectral overlap reduction are observed. These phenomena can be fully exploited in the analysis of multi-component systems in complex matrices.[10-13] SF can be further utilized in order to provide information concerning a molecule's microenvironment (general solvent effects). This is due to the fact that, to a first approximation, the magnitude of the Stokes' shift is a function of the dielectric constant of the medium.[14] In polar solvents, reorientation of solvent dipoles decreases the energy difference between ground and excited states. Since fluorescence occurs following solvent relaxation, increasing solvent polarity will increase δ_S which in turn will alter the SF signal.

2. MATERIALS AND METHODS

2.1. Chemicals

Adriamycin (ADR) and N, N-dimethyldaunomycin (QD) were provided by Dr. Leonard Kedda of the Division of Cancer Treatment, National Cancer Institute. N-trifluoroacetyladramycin (NTFA) and N-benzyladriamycin (NBA) were generously contributed by Dr. Mervyn Israel of the University of Tennessee College of Medicine, Memphis, TN. 5-Iminodaunomycin (ID) was supplied as a gift from Dr. Edward M. Acton, SRI International, Menlo Park, CA. The structural and spectral characteristics of these molecules have been previously described.[5-7] The essential chromophore region of the anthracycline congeners used in this investigation did not vary. Structural analogs differed only in substitution at the amino sugar and at C-13 of the aglycon.[6,7]

Bovine serum albumin (BSA) and phosphate buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, MO). Reagent grade octanol, propanol, and methanol were purchased from Fisher Chemical Co. L-α-dimyristoylphosphatidycholine (DMPC) was purchased from Avanti Polar Lipid Co., Pelham, Alabama.

2.2. Preparation

Anthracycline stock solutions were prepared in methanol and stored in the dark at -20 °C. Unilamellar vesicle suspensions were freshly prepared prior to each experiment by sonicating well-vortexed 15 mg/ml lipid dispersions in PBS for 3-4 hours. Vesicles were annealed for 30 minutes at 37 °C prior to use. A detailed description of these techniques has been previously reported.[6]

Membrane-bound anthracyclines were prepared by incubating 5×10^{-6} M drug/liposome preparation for approximately 30 minutes at 37 °C. SF measurements were performed on fresh samples at 37 °C. Iodide quenching experiments, designed to evaluate the relative accessibilities of membrane-bound fluorophores to membrane-impermeable iodide,[6,7] were compared to SF results. Iodide-containing vesicles were prepared by adding up to 1.0 M salt and 2×10^{-3} M sodium thiosulfate (to prevent iodide oxidation) to 5×10^{-6} M drug/liposome preparations.

2.3. Measurements

Conventional and synchronously scanned fluorescence spectra (uncorrected) were obtained using an

SLM (Urbana-Champaign, IL) model 8000 spectrofluorimeter with a temperature-controlled sample chamber. SF scans were typically obtained at $\Delta\lambda$ = 10 nm, slits = 4 nm. Measurements were performed at room temperature (except for liposome studies which were conducted at 37 °C) in quartz cuvettes.

Iodide quenching studies were conducted, as previosly described,[6,7] using an SLM model 4800S lifetime fluorometer interfaced to a Compaq 286 computer. Modified Stern-Volmer plots constructed for each drug-lipsome system were linear, thus permitting determination of quench constants (k_q values) from plot slopes.[7]

3. RESULTS AND DISCUSSION

Figures 1-4 are results of SF investigations in PBS and multicomponent systems. Figure 1 shows the bandwidth narrowing and spectral simplification effects of SF. The full-width-half-maximum (FWHM) bandwidth of the single, sharp, SF peak is 24 nm ($\Delta\lambda$ = 10 nm). In contrast, the primary feature of the broad-band conventional emission curve has a 75-nm bandwidth (FWHM). SF and CF spectra were obtained, at different sensitivities, for 10^{-6} M ADR in PBS.

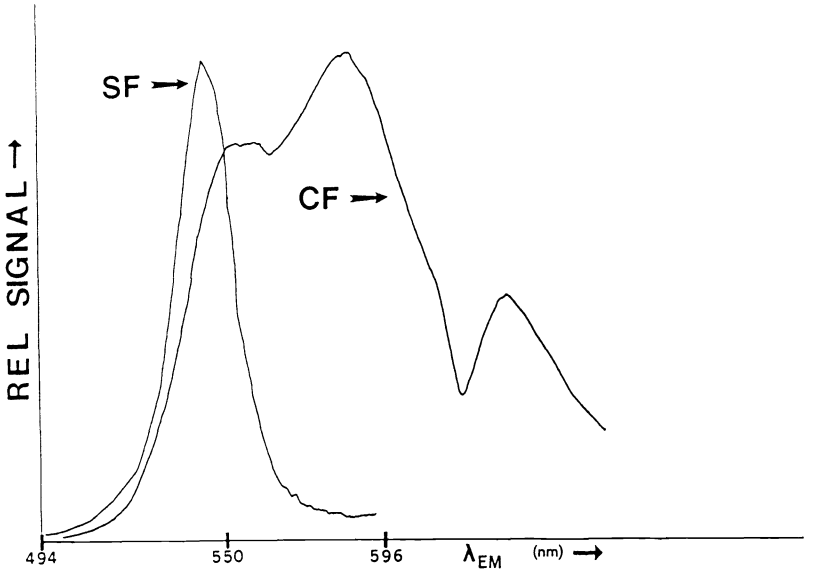

Figure 1. SF ($\Delta\lambda$ = 10 nm) and CF (λ_{ex} = 488 nm) scans of 1μM ADR in PBS.

Figure 2 illustrates three independently obtained synchronous scans of serum, ADR, and ID. Each scan exhibits sharp, non-overlapping features in the 420-700 nm range. From these results, one would expect that a mixture of drugs in serum could be spectrally resolved via SF. Figures 3 and 4 indicate that this is the case. Figure 3, a synchronous scan of 10^{-5} M ADR in serum, clearly shows the characteristic SF features of ADR as well as serum components. SF detection limits (based on S/N = 2) for ADR in PBS and serum were estimated to be 2 x 10^{-7} M and 7 x 10^{-7} M, respectively.

In Figure 4, SF is compared to CF for a mixture of ADR and ID. In this case, quantification is impossible due to spectral interference caused by overlap of ADR/ID emission curves. This overlap is responsible for a broad, poorly resolved feature extending between 450 and 675 nm. Significant simplification is achieved when this mixture is synchronously scanned, resulting in clear resolution of each component. The apparant sharp drop in relative CF signal (Figs. 1 and 4) at approximately 600 nm was later attributed to instrumental response, since it was not observed in corrected spectra.

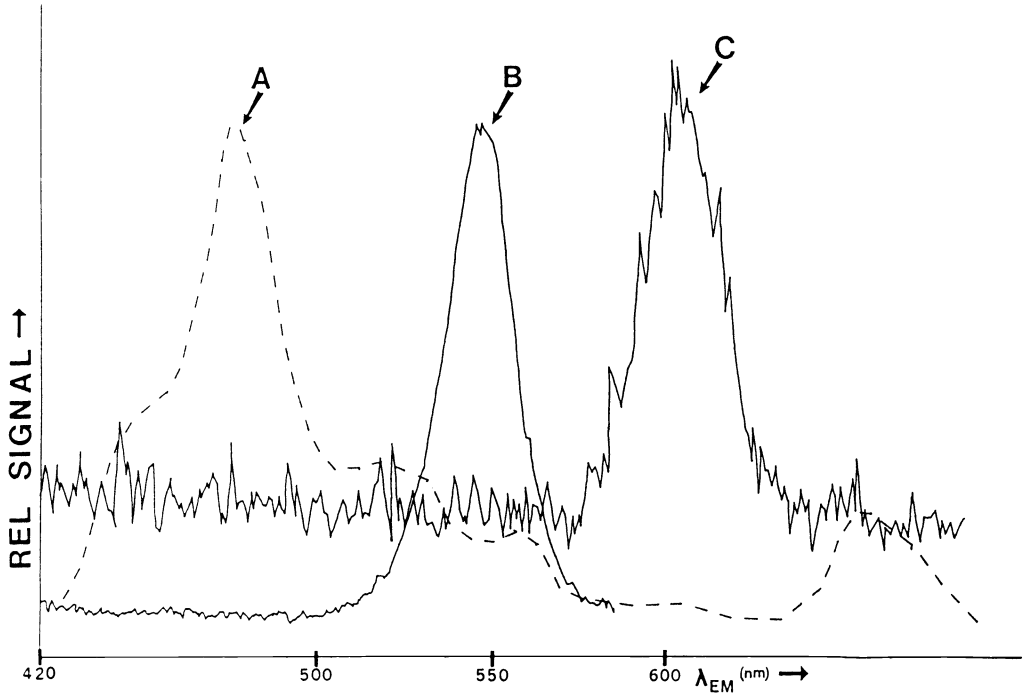

Figure 2. SF ($\Delta\lambda$ = 10 nm) of A, serum; B, 2 μM ADR/PBS; C, 0.56 μM ID/PBS.

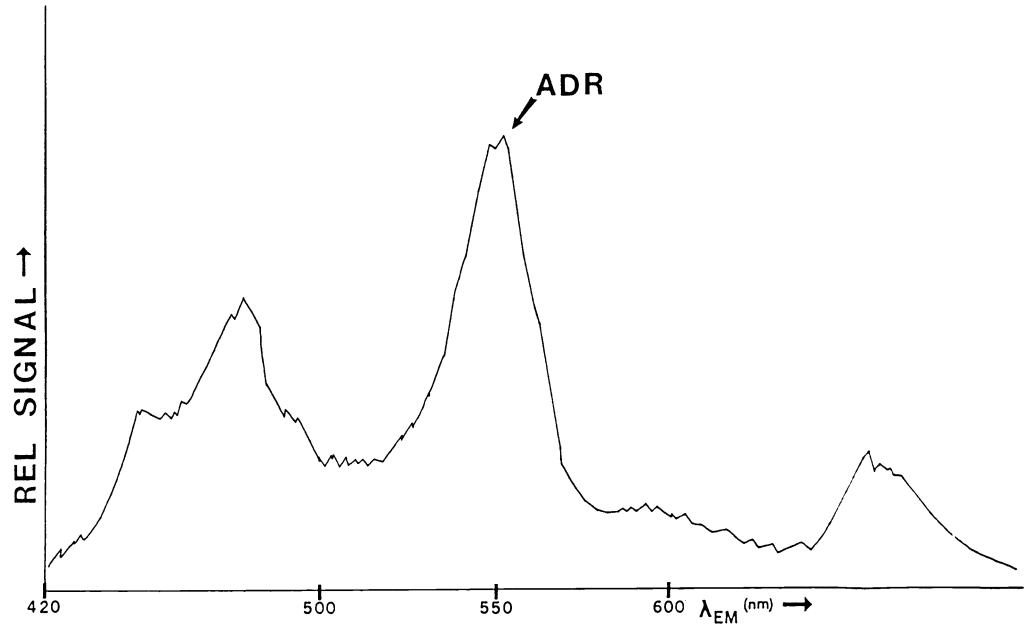

Figure 3. SF ($\Delta\lambda$ = 10 nm) of 10 μM ADR/serum.

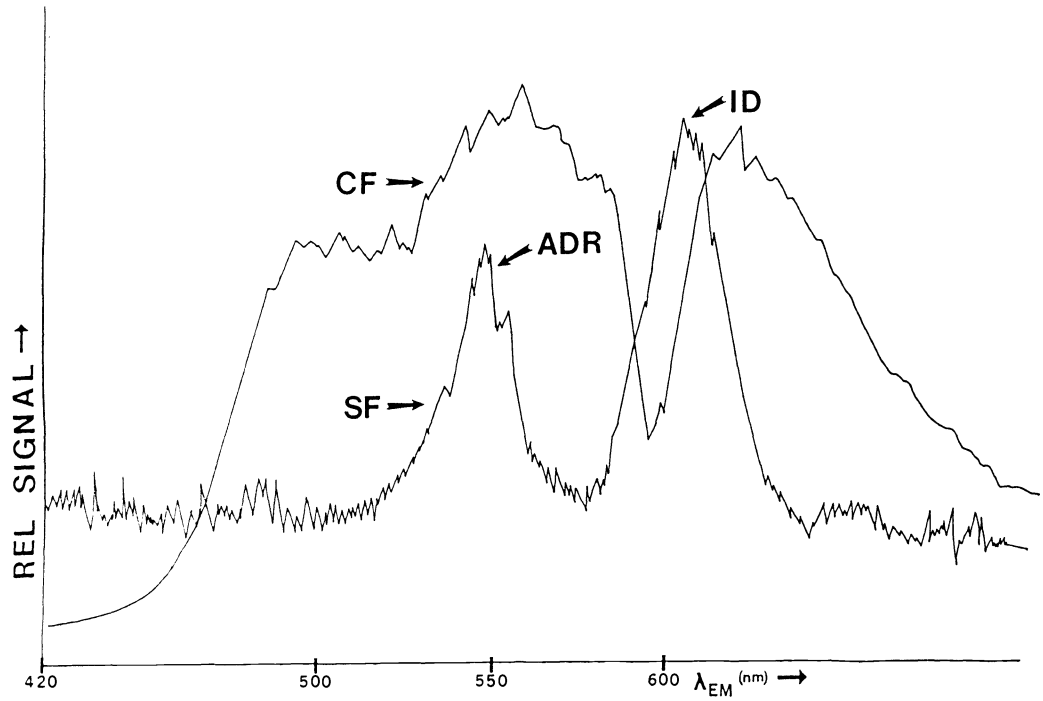

Figure 4. SF and CF of 0.9 μM ADR, 0.6 μM ID mixture in PBS.

Figures 5-8 illustrate SF and CF response in various solvents and DMPC bilayers. In Figure 5, the CF scans of 1μM ADR in water and octanol show an expected slight red shift in the aqueous solvent. With the exception of a slight positional change, the relative intensities of the 0-0 features (approximately 550 nm) are nearly identical. In contrast, the intensities of SF scans (at approximately 550 nm) of 1μM ADR in water, propanol, and octanol increase with increasing solvent hydrophobicity (Figure 6). The fact that the relative SF intensities of ADR are clearly distinguishable while the CF intensities (at 550 nm) are not, demonstrates the sensitivity of SF to $\delta\lambda_S$ variations.

These relationships were further explored using anthracyline structural analogs in DMPC bilayers. Figure 7 illustrates the resolution difficulties encountered when comparing CF scans for equivalent concentrations of NBA and QD in DMPC bilayers. In contrast, the relative SF intensities of NTFA, NBA, QD, and ADR in liposomes (Fig. 8) clearly vary. This difference is attributable to spectral simplification since SF permits consideration of a single (0-0 band) feature at 550 nm while the relative CF intensities at 550 and 585 nm alternate.

The SF behavior illustrated by Figure 8 may also be indicative of the influence of structural differences on bilayer permeation depth. Using modified Stern-Volmer plots, iodide quench constants (k_q) obtained for the anthracycline congeners NTFA, NBA, QD, and ADR were 0.4, 0.8, 1.3, and 1.4 x 10^9 M^{-1} s^{-1}, respectively.[7] Since iodide has an immeasureably small permeation into the bilayer, larger k_q values indicate greater proximity to the hydrophilic surface than the hydrophobic interior.[6] These results indicate that the relative membrane permeation depths are NTFA > NBA > QD > ADR. The observed order of SF relative signals (Figure 8) is, therefore, consistent with the iodide quenching data; provided that for a given chromophore, variations in membrane microenvironment influence SF response in the same manner as solvent hydrophobicity changes effect Figure 6.

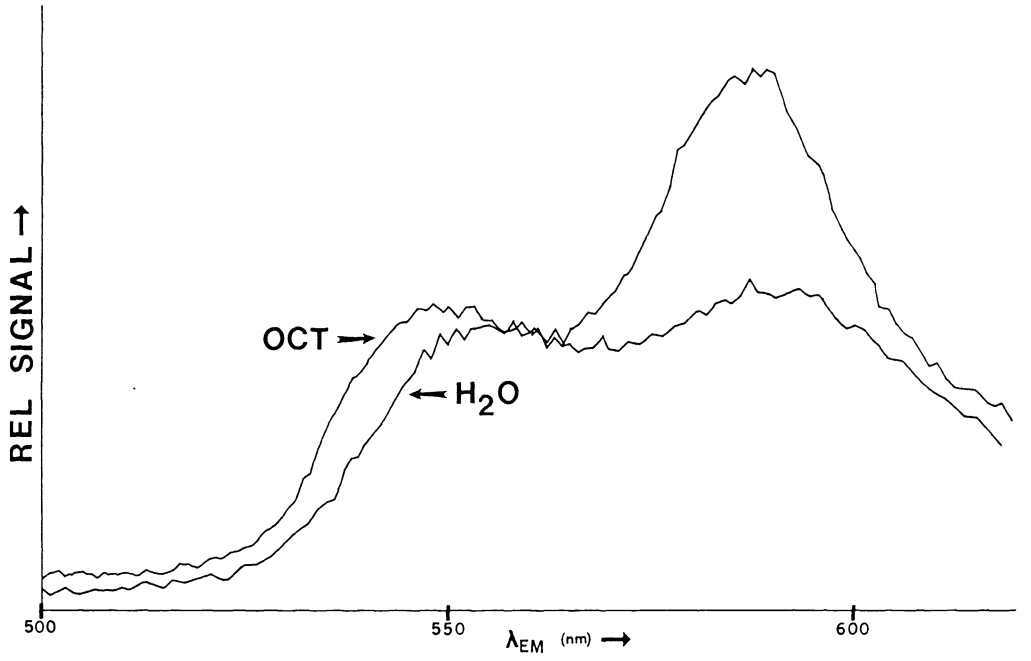

Figure 5. CF (λex = 488 nm) of 1 μM ADR in octanol and water.

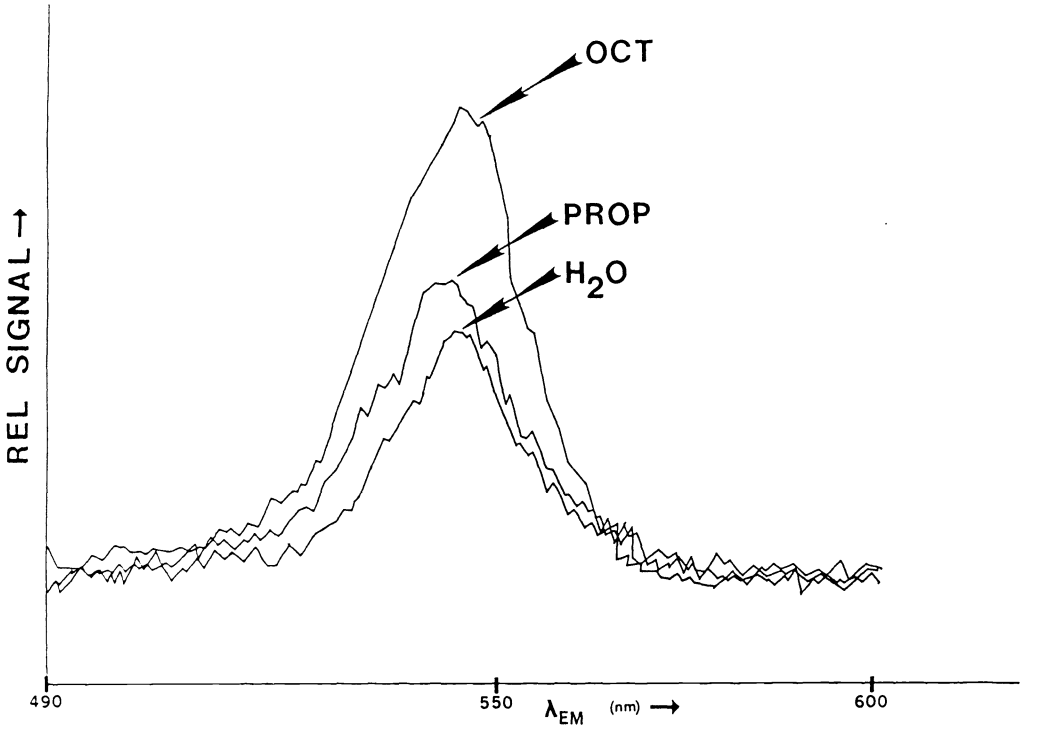

Figure 6. SF ($\Delta\lambda$ = 10 nm) of 1 μM ADR in octanol, propanol, and water.

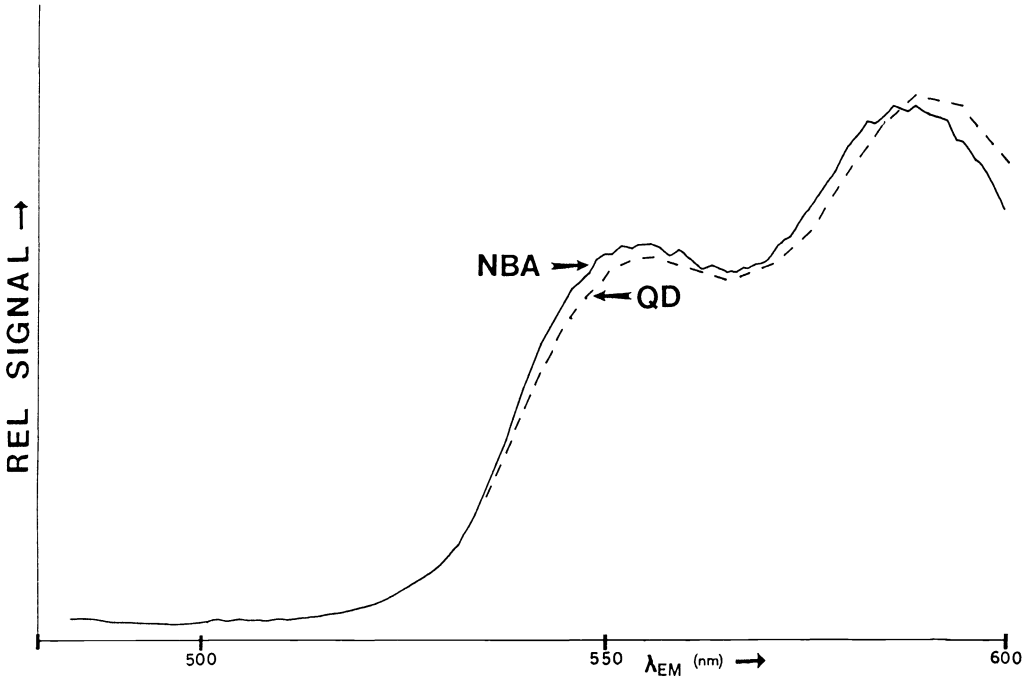

Figure 7. CF of 5 μM NBA and QD in DMPC bilayers.

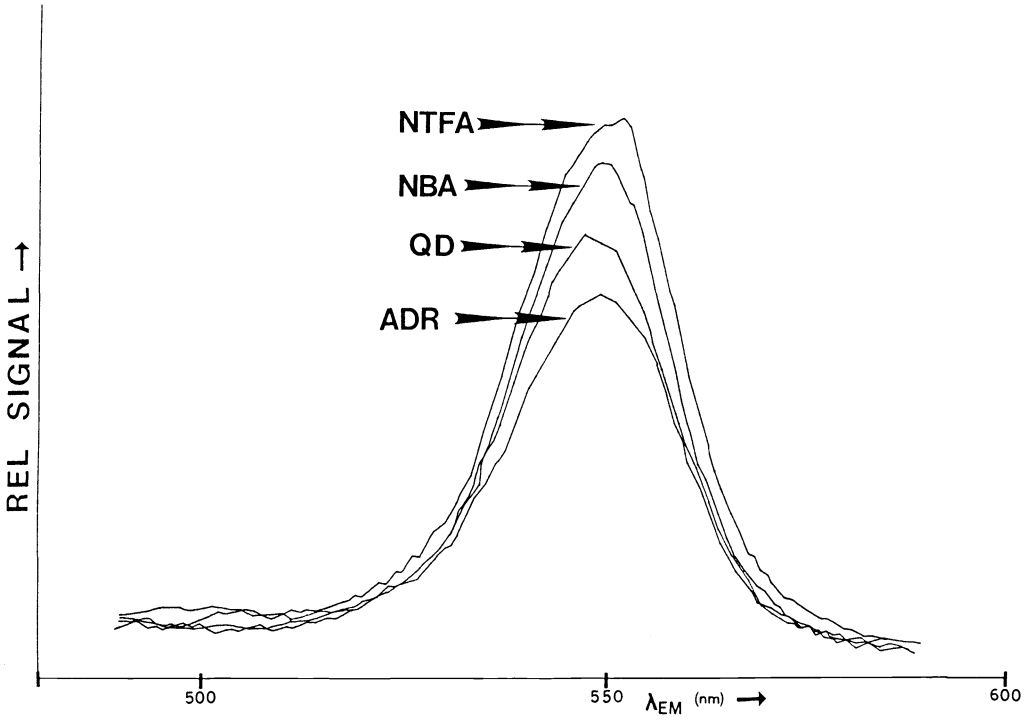

Figure 8. SF ($\Delta\lambda$ = 10 nm) of 5 μM anthracyclines in DMPC bilayers.

4. CONCLUSIONS

SF is a simple, rapid analytical technique which can be used to spectrally resolve fluorescent anti-tumor drugs from complex matrices, e.g. serum. In view of the unique information it provides, SF could be used as a screening technique for evaluating serum/plasma levels in patients receiving single or, more importantly, multiple drug therapy. These analyses could be performed in a matter of minutes (vs. hours to days for conventional HPLC-based techniques), allowing rapid correlation of circulating blood levels to clinical investigations of cardiotoxicity, tumorigenicity, and drug metabolism. In addition, SF features including Stokes' shift sensitivity and spectral simplification may prove to be useful in characterizing the influence of anthracycline structural variations on drug permeation in membranes.

5. ACKNOWLEDGEMENTS

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