Laser Induced Fluorometric Analysis of Drugs

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Laser induction of molecular fluorescence was investigated for the analysis of intrinsically fluorescent compounds such as quinine sulfate, salicylic acid, carprofen (a carbazole), 2-methoxy-11-oxo-11*H*-pyrido-[2,1-*b*]quinazoline-8-carboxylic acid, and fluorescent derivatives of nonfluorescent compounds such as the quinazolinone produced by the photolysis of demoxepam, the 9-acridanone derivative of flurazepam, and the fluorescamine (Fluram) derivative of amphetamine. The sensitivity limit of reliable quantitation utilizing laser-induced fluorescence was equal to or better than that obtained by a conventional spectrofluorometer.

Laser-induced molecular luminescence is a rapidly advancing new frontier in analytical chemistry (*1*, *2*). The monochromaticity, coherence, and peak power output of tunable dye lasers (*3*) capable of exciting aromatic molecules over the wavelength range of 220–650 nm (ultraviolet–visible) is of particular interest and applicability to trace-level pharmaceutical analysis. Comparisons to conventional luminescence have shown sensitivity improvements using laser excitation (*4*, *5*). The feasibility and high sensitivity of the technique have been demonstrated in the analysis of rhodamine (*5*), aflatoxins (*6*), polynuclear aromatic hydrocarbons (*7–9*), phosphorimetric analysis of drugs (*8*, *9*), vitamins of the B complex (*10*, *11*), fluorescein (*4*, *11*), and several coumarin analogues (*12*). The improvements in sensitivity suggest the possibility of analyzing drugs in blood and plasma in the picogram (10^{-12} g) to femtogram (10^{-15} g) range required to quantitate high potency drugs administered at very low doses. Laser-induced luminescence has also been used as an ultrasensitive detection system in combination with high-performance liquid chromatography (HPLC) (*13–16*) and has proven to be an effective means of increasing the sensitivity and specificity of this versatile chromatographic technique.

In this study, laser-induced molecular fluorescence was investigated for the analysis of intrinsically fluorescent compounds such as quinine sulfate, salicylic acid, carprofen (a carbazole derivative under development as an antiinflammatory agent) (*17*, *18*), 2-methoxy-11-oxo-11*H*-pyrido[2,1-*b*]quinazoline-8-carboxylic acid, under development as an antiallergy agent (*19*), and fluorescent derivatives of nonfluorescent compounds such as the quinazolinone produced by the photolysis of demoxepam (a metabolite of the anxiolytic, chlordiazepoxide) (*20*), the 9-acridanone derivative of flurazepam (a hypnotic) (*21*), and the fluorescamine (Fluram) derivative of amphetamine (*22*). The sensitivity limit of laser-induced fluorescence was either equal to or up to tenfold greater than that obtained by conventional spectrofluorometry for the compounds studied.

EXPERIMENTAL SECTION

Conventional spectrofluorometry was performed by using a Farrand Mark I spectrofluorometer having a Xenon-arc source and an off-axis ellipsoidal focusing mirror. Each monochromator was equipped with slits allowing a 10-nm band-pass. A schematic of the different laser configurations used for both low- and high-power excitation is given in Figure 1.

In part I (low-power laser), the excitation source was a Molectron Spectroscan 10 (Molectron Corp., NJ), a continuously tunable dye laser pumped by an internal 50-kW nitrogen laser operated at 100 pps (7 kW peak power at 580 nm) and equipped with a second harmonic generation accessory (frequency doubling crystals) and associated apparatus. The beam splitter and associated photomultiplier and high-voltage supply were disconnected. The sample chamber cover of a Farrand Mark I spectrofluorometer was modified by the addition of a front-faced UV reflective mirror mounted at a 45° angle to deflect the horizontal laser beam through 90°, so as to pass vertically through the sample contained in the quartz cuvette in the sample chamber, as shown in Figure 2. This arrangement allows the laser beam to enter the sample without the scatter from the front surface of the cuvette which occurs in horizontal alignment. The fluorescence emission is collected over the full length of the entrance slit, resulting in higher sensitivity. The modified sample chamber cover enabled the use of the sample chamber, the beam intensifier assembly consisting of two front-faced mirrors set at 90° to each other (Farrand Catalog No. 144557), the emission monochromator (F/3.5 grating monochromator, modified Czerny-Turner Type, spectral range 220–700 nm, 14 400 lines/in.), and the photomultiplier tube (PMT) mount of a commercially available spectrofluorometer, for this work. A 5-mm entrance slit and a 2-mm exit slit were used in the fluorescence monochromator (3–4 nm band-pass) except as otherwise noted (Table I). The photomultiplier tube (RCA 1P28) was powered at 500–900 V by a high-voltage supply housed in a quantum photometer (Princeton Applied Research Corp., Princeton, NJ, Model 1140). A wide-band preamplifier [P.A.R.C. Model 115] was used to amplify weak fluorescence signals and was switched in or out of the circuit as required. The PMT output was analyzed by a gated integrator module [P.A.R.C. Model 164] mounted in a boxcar averager [Model 162] having a digital option (for slow repetition rates). The boxcar scan was triggered by a positive going signal from a synchronization circuit in the nitrogen laser. The boxcar integrator/averager output was monitored by an X-Y recorder (Houston Instrument Co., Omnigraphic 2000), Figure 1.

The data collected in part I (low-power laser) were obtained with a boxcar "gate" of 10–30 ns scanning from +0.35 to +0.50 μs after the syncronization pulse from the Spectroscan 10 with a 100-s sweep time for the full 0.50 μs range, a 1-μs time constant set on the Model 164 integrator, and a 0.1-s time constant on the Model 162 averager. The beam intensifier assembly was not used in part I.

In part II (high-power laser), Figure 1, the Molectron Spectroscan 10 was replaced by a Molectron UV22 nitrogen laser (600 kW peak power at 20 pps) pumping a Molectron DL14 dye laser with built-in dye laser amplifier (producing a 6–8-ns pulse having 90 kW peak power at 580 nm when operated at 20 pps) and equipped with a second harmonic generation accessory (frequency doubling crystals and associated apparatus). The photomultiplier tube (PMT) was powered by a high-voltage supply [PARC HVS-1] housed in a power supply bin [Model 1107]. The wide-band preamplifier [Model 115] was not required for any of the experiments with the high-power laser and was switched out of the circuit. The PMT output [RCA-1P28 tube] was analyzed by the same boxcar integrator/averager configuration as in part I. Due to differences in the lasers, the data for part II were obtained with a boxcar "gate" of 15–30 ns scanning with a 200–500-s sweep time for the 1.0-μs range and a 10-μs time constant being used in the gated integrator [Model 164]. The beam intensifier assembly was used for all measurements. The boxcar scan was again triggered from a synchronization circuit in the nitrogen laser.

In part III (high-power laser, ratio of fluorescence signal to laser intensity), a beam splitter was used to direct a small portion of the excitation radiation from the dye laser used in part II into

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Table I. Luminescence Behavior of Drug Compounds and/or Their Derivatives by Dye Laser Induced vs. Conventional Spectrofluorometry

compound (solvent for measurement)	conventional spectrofluorometry	dye laser induced fluorescence: part I (low power)	dye laser induced fluorescence: parts II + III (high power)
Quinine sulfate (0.1N H_2SO_4)	ex[a] = 350 nm em = 445 nm sens ~ 5 ng/mL range[b] to >1000 ng/mL	ex = 380 nm sens ~ 5 ng/mL range[b] to >100 ng/mL	ex = 360 nm 2 mm slits sens = 1.0 ng/mL range[b] to >25 ng/mL
Salicylic acid (0.01N NaOH)	ex = 295 nm em = 410 nm sens ~ 2 ng/mL range to >1 μg/mL	ex = 295 nm[c] 10 mm slits sens ~ 10 ng/mL range to >100 ng/mL	ex = 295 nm[c] em = 410 nm, plus >320 nm transmission filter sens ~ 1 ng/mL range to >20 ng/mL
Carprofen (acetic acid:ethanol) (1:99)	ex = 300 nm em = 370 nm sens ~ 2.5 ng/mL range to >1 μg/mL	not tested	ex = 300 nm em = 370 nm plus >320 nm transmission filter sens = 2.5 ng/mL range to >25 ng/mL
Pyrido-[2,1-b]-quinazoline (Methanol: 0.01M) (pH 7.5 potassium) (phosphate (1:1)	ex = 280 nm em = 475 nm sens = 2 ng/mL range = >1 μg/mL	not tested	ex = 280 nm[c] em = 475 nm plus 418 nm transmission filter sens = 0.5 ng/mL range = >10 ng/mL ex = 360 nm em = 475 nm plus 418 nm transmission filter, 2-mm slits sens = 1.0 ng/mL range = >10 ng/mL
Quinazolinone Demoxepam photolysis product (0.1N NaOH)	ex = 380 nm em = 460 nm sens = 50 ng/mL range to >5 μg/mL	ex = 380 nm 2 mm slits sens ~ 20 ng/mL range to >0.5 μg/mL	ex = 380 nm 2 mm slits sens <10 ng/mL range to >0.5 μg/mL
9-acridanone of flurazepam (acetic acid:ethanol) (1:99)	ex = 402 nm em = 440 nm sens = 2 ng/mL range to >1 μg/mL	ex = 402 nm 2 mm slits sens ~ 2 ng/mL range to >10 ng/mL	ex = 402 nm 2 mm slits sens ~ 0.5 ng/mL range to >10 ng/mL
Fluorescamine derivative of amphetamine (reaction mixture) (pH 9.5)	ex = 390 nm em = 480 nm sens ~ 5 μg/mL range to >100 μg/mL	ex = 390 nm sens = >10 μg/mL[d]	ex = 390 nm 2 mm slits sens 2.5 μg/mL range to >10 μg/mL

[a] Key: ex = excitation, em = emission, and sens = sensitivity limit of quantitation. [b] See text for explanation of experimental procedure. [c] Frequency-doubled energy using second harmonic generation with KDP crystals. [d] The sensitivity limit could not be determined due to high background fluorescence and instrumental limitations.

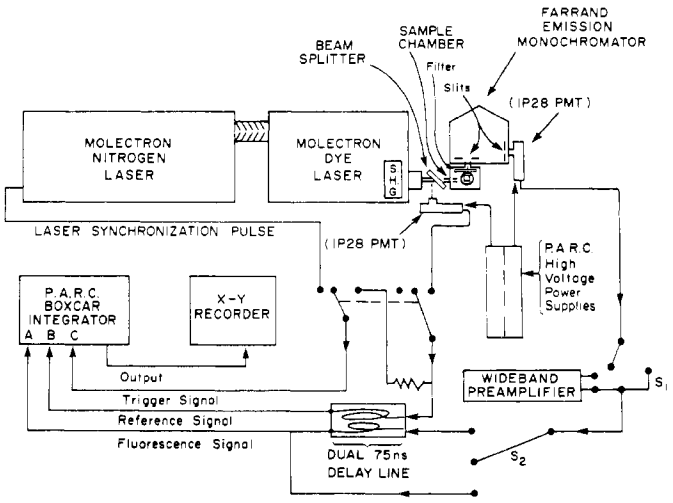

Figure 1. Instrumental schematic for dye laser induced fluorometry, parts I, II, and III.

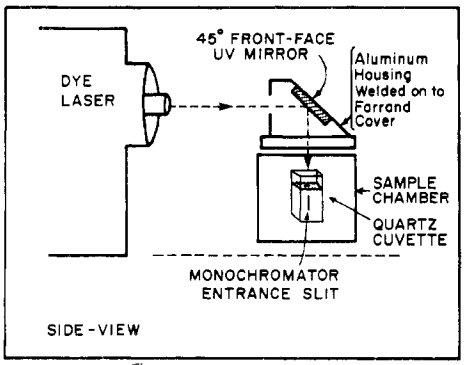

Figure 2. Schematic of sample chamber geometry for dye laser induced spectrofluorometry.

an RCA-1P28 PMT, powered by a second high-voltage supply (Figure 1). The laser reference signal and the sample fluorescence signal were individually routed through a dual passive time delay line [Tektronix 7M11]. A portion of the reference signal was split prior to the delay line to provide a negative going trigger signal to the boxcar averager [PARC Model 162], and the laser synchronization line was switched out of the circuit; the Tektronix 7M11 unit provided a 75-ns time delay for both signals (to compensate for an instrumental "deadtime", nominally of 75 ns,

between trigger detection and the effective beginning of the subsequent aperture scan of the aperture delay range) so that they might be analyzed in their entirety by the boxcar integrator/averager. The sample (fluorescence) signal (A) and the laser (reference) signal (B), Figure 1, were integrated in separate sampled integrators [P.A.R.C. Model 163] equipped with sampling heads (Tektronix Type S-1, $t_r \leq 350$ ps], and the output to the recorder represents the sample fluorescence signal intensity as a ratio of the laser excitation intensity (A/B). This ratio mode was used to compensate for laser intensity fluctuations.

Reagents. The laser dye solutions were obtained from Molectron Corp. All reagents used were ACS reagent grade or better. All aqueous solutions were prepared in distilled, carbon-filtered, deionized water filtered through a 0.2-μm membrane filter (Type DC System, Hydro Service and Supplies, Inc., Durham, NC).

The organic solvents used were diethyl ether, absolute ether (Mallinckrodt, Inc., St. Louis, MO), ethyl alcohol (anhydrous 200 proof) (Pharmaco Publicker Industries, Inc., Philadelphia, PA), methanol (Burdick and Jackson, Muskegon, MI), and acetic acid (J. T. Baker, Phillipsburg, NJ). All test compounds used were of pharmaceutical grade purity [>99%].

All glassware was thoroughly washed and rinsed successively with distilled deionized water, with methanol, and finally with the solution to be measured.

Compounds Examined. *Quinine sulfate dihydrate* [$(C_{20}H_{24}N_2O_2)_2 \cdot H_2SO_4 \cdot 2H_2O$, mol wt 782.9] was obtained from J. T. Baker Chemical Co. (Ultrex grade). Standard solutions were prepared in 0.1 N H_2SO_4. In part I, quinine sulfate was excited at 380 nm with 4,4′-bis(butylacetyloxy)quaterphenyl as the laser dye, whereas, in parts II and III, excitation was at 360 nm using 2-(4′-biphenylyl)-5-phenyl-1,3,4-oxadiazole as the laser dye. The fluorescence emission was monitored at 450 nm.

Salicylic acid [$C_7H_6O_3$; mol wt 138.12, mp 158–160 °C] was purchased from Aldrich Chemical Co. The stock solution (10 mg/100 mL) and all dilutions thereof were prepared in 0.01 N NaOH. Fundamental laser excitation was obtained with Rhodamine 6G at 590 nm which was then frequency doubled to 295 nm using an angle-tuned KDP crystal (Molectron: DL272-E) cut for 268–302 nm. The fluorescence emission was monitored at 410 nm.

Carprofen [*dl*-6-chloro-α-methylcarbazole-2-acetic acid, $C_{15}H_{12}ClNO_2$, mol wt 273.72, mp 192–194 °C] is a proprietary compound of Hoffmann-La Roche Inc. The stock solution (10 mg/100 mL) was prepared in methanol, and serial dilutions were made in 1% glacial acetic acid in ethanol. The laser dye, frequency doubling crystal, and excitation frequency (295 nm) used were as described for salicylic acid; the fluorescence emission was monitored at 370 nm in conjunction with an excitation cutoff filter (>300 nm).

Pyrido[2,1-b]quinazoline [2-methoxy-11-oxo-11*H*-pyrido[2,1-*b*]quinazoline-8-carboxylic acid, $C_{14}H_{10}N_2O_4$, mol wt 270.2, mp 300 °C] is a proprietary compound of Hoffmann-La Roche Inc. Standard solutions were prepared in methanol–0.01 M (pH 7.5) potassium phosphate buffer (1:1). Excitation at 360 nm was obtained by using 2-(4′-biphenylyl)-5-phenyl-1,3,4-oxadiazole as the laser dye, while excitation at 280 nm was obtained by using a proprietary fluorinated coumarin (C485) available from Molectron Corp., lasing at 560 nm, which was frequency doubled to 280 nm using an angle-tuned KDP crystal cut for 268–302 nm. The fluorescence emission was monitored at 475 nm.

Demoxepam [7-chloro-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one 4-oxide, $C_{15}H_{11}N_2O_2Cl$, mol wt 286.72, mp 236–236.5 °C] is a proprietary compound of Hoffmann-La Roche Inc. Standard solutions were prepared in 0.1 N NaOH in 50-mL Pyrex glass centrifuge tubes, and the fluorescent quinazolinone derivative was formed by exposing the tubes for 30 min to UV irradiation from a Pyro-Lux R-57 lamp (Luxor Corp., New York, NY) contained in an aluminum foil lined reflector box at a distance of 12 in. from the light source (*20*). The laser dye used for excitation at 380 nm was 4,4′-bis(butylacetyloxy)quaterphenyl, and the fluorescence emission was monitored at 460 nm.

9-Acridanone [2-chloro-10-(2-diethylaminoethyl)-9-acridanone hydrochloride, $C_{19}H_{21}ClN_2O \cdot HCl$, mol wt 365.32, mp 265–275 °C, 1.111 mg of HCl = 1.00 mg free base] is a fluorescent derivative of flurazepam (*21*), a hypnotic marketed by Hoffmann-La Roche Inc. A standard solution (10 mg free base equiv/100 mL) and several dilutions were prepared in 1% glacial acetic acid in ethanol. The laser dye used was 2-(4′-biphenylyl)benzoxazole exciting at 402 nm, and the fluorescence emission was monitored at 440 nm.

Amphetamine sulfate [$C_9H_{13}N_2H_2SO_4$, mol wt 368.49, mp >300 °C dec]. A stock solution (10 mg free base equiv/100 mL) and all dilutions were prepared in methanol. The formation of fluorescent derivatives of primary aliphatic and aromatic amines by reaction with fluorescamine (Fluram, Hoffmann-La Roche Inc., Nutley, NJ] has been reported in detail (*22*). Fluorescamine (100 mg) was dissolved in 100 mL of anhydrous reagent grade acetone and "aged" for 24 h (by allowing it to stand at room temperature) prior to use.

The fluorescent derivative was formed by the sequential addition of 10 mL of 0.1 M potassium phosphate buffer (pH 9.3) and 0.2 mL of fluorescamine (mg/mL) in acetone to the residue of the amphetamine standard, mixing, and then allowing the reaction to proceed for at least 15 min before fluorometric analysis. The laser dye used for excitation at 390 nm was 4,4′-bis(butylacetyloxy)quaterphenyl, and the fluorescence emission was monitored at 480 nm.

Analysis of Drugs in Biological Fluids. Seven drug compounds were investigated for feasibility of analysis by laser-induced luminescence spectroscopy, of which three compounds, carprofen, pyrido[2,1-*b*]quinazoline, and demoxepam, were also analyzed following extraction from human (drug free) plasma for comparison of the sensitivity attained with conventional fluorometry (Table II). Graded amounts of each authentic compound were added to human plasma and extracted using published procedures, viz., carprofen (*17*, *18*), pyrido[2,1-*b*]quinazoline (*19*), and demoxepam (*20*), respectively.

Carprofen. The residues of the extracts were chromatographed on silica gel thin-layer chromatoplates, and the separated drug was eluted with 5 mL of 1% glacial acetic acid in absolute ethanol and quantitated as described (*17*).

Pyrido[2,1-b]quinazoline. Human plasma spiked with the drug was buffered with an equal volume of 1 M potassium phosphate buffer (pH 2.7) and extracted twice with 2 × 5 mL of diethyl ether (*19*). The extracts were analyzed by two separate procedures: (i) following back-extraction into 5 mL of 0.1 M K_2HPO_4 (pH 9.0–9.3) as a "clean up" step, the extracts were reextracted into ether after acidification and analyzed; (ii) the residue of the ether extract was chromatographed on silica gel thin-layer chromatoplates, using benzene–ethanol–glacial acetic acid (90:10:10) as the developing solvent; the compound was eluted with 5 mL of (1:1) methanol–0.01 M potassium phosphate buffer (pH 7.5) and quantitated.

Demoxepam. Plasma was buffered to pH 7.0 and extracted into diethyl ether, the compound was selectively back-extracted into 5 mL of 0.1 N NaOH and irradiated for 30 min as previously described (*20*) for photochemical conversion to the fluorescent quinazolinone derivative which was quantitated.

The biological extracts were quantitated by conventional spectrofluorometry and by laser-induced fluorescence with the instrument configuration described in part III.

RESULTS AND DISCUSSION

The sensitivity limits of the compounds investigated by conventional spectrofluorometry, by low-power laser-induced fluorescence (part I) and by higher power laser-induced fluorescence (parts II and III) are summarized in Table I.

The linearity of laser-induced fluorescence was determined only as a demonstration of the utility of the technique. No attempt was made to extend the range to higher concentrations, approaching the quenching limits seen with conventional spectrofluorometry, due to instrumental limitations. The major goal of this study was in improving the sensitivity limits of detection within a linear response range (Table I), confined to 1 or 2 orders of magnitude.

The sensitivity limits obtained by exciting with a relatively low powered nitrogen laser pumped dye laser (part I) generally were equal to or better than those obtained by conventional spectrofluorometry when the excitation wavelength selected was within the fundamental lasing wavelengths of the dyes used. The sensitivity limits achieved by using the higher powered laser system (parts II and III) were generally better

Table II. Linearity and Sensitivity Limits of Fluorometric Detection of Drugs in Plasma: Conventional Spectrofluorometry vs. Dye Laser Induced Luminescence

compd	concn (ng) added to 1 mL of plasma	concn (ng) measd in 5 mL of final solution	relative fluorescence, arbitrary units: conventional fluorometry	relative fluorescence, arbitrary units: dye laser induced fluorometry [part III]
carprofen	1000	200	106	21.0
	500	100	64	12.5
	250	50	40[a]	9.0[a]
	100	20	27	7.0
	50	10	22	6.0
	blank	blank	20	5.0
pyrido[2,1-*b*]quinazoline				
(i) (back-extraction)	500	100	62.0	21.0
into pH 9.0 buffer	250	50	32.0	12.5
	100	20	17.0	6.7
	50	10	9.6	5.8[a]
	25	5	7.6[a]	5.0
	10	2	5.2	4.2
	blank	blank	3.5	3.3
(ii) TLC separation/elution	100	20	15.0	26.0
into methanolic phosphate	50	10	9.2[a]	16.5
buffer (pH 7.5)	25	5	8.2	10.2[a]
	10	2	7.0	8.8
	blank	blank	5.5	6.0
demoxepam	2500	500	32.0	25.0
	1000	200	16.7	11.0
	500	100	10.0	6.7
	250	50	8.2[a]	4.7
	100	20	4.0	3.0[a]
	blank	blank	4.0	1.5

[a] Sensitivity limit = 2 × blank reading.

than those obtained by using either the lower powered laser (part I) or conventional spectrofluorometry.

The fluorescence intensity data obtained for quinine sulfate using the lower powered laser (part I) is shown in Figure 3, and the calibration curve for quinine sulfate obtained by high power laser excitation (part II) was linear over the concentration range of 1–25 ng/mL of 0.1 N H_2SO_4. The sensitivity limit for quantitation of quinine sulfate by low-power laser (part I), conventional fluorometry, and high-power laser (part II) was 10, 5, and 1 ng/mL, respectively. The sensitivity limit is considerably improved (10×) by use of the high-power laser and is due to a greater sample/blank fluorescence ratio and an improvement in the reproducibility of response at low concentrations.

The energy output of the low-power dye laser (part I) was insufficient to provide frequency-doubled radiation of adequate intensity to excite salicylic acid at 295 nm without severe inner filter effects, resulting in lower sensitivity than that obtained in either part II or III, using the higher powered laser system. Carprofen (which was not tested in part I) also required frequency-doubled energy for excitation since its maxima are at 245 and 300 nm, with emission at 365 nm (*18*). The sensitivity for carprofen obtained in parts II and III confirmed that the high-power laser system was able to duplicate that obtained by conventional spectrofluorometry (*17*), for both pure standards and compound recovered from plasma, Table II. This was achieved by optimizing the resolution of the fluorescence emission at 365 nm from interference due to background light scatter.

The pyrido[2,1-*b*]quinazoline compound whose excitation maxima are at 230, 280 and 360 nm, with emission at 475 nm (Figure 4), presented an excellent opportunity for comparing the fluorescence emission obtained with fundamental radiation (360 nm) to that obtained with frequency-doubled radiation (280 nm). On the basis of the corrected excitation-emission spectrum of the compound (Figure 4), using a conventional Xenon-Hanovia 150-W lamp as the excitation source, the total

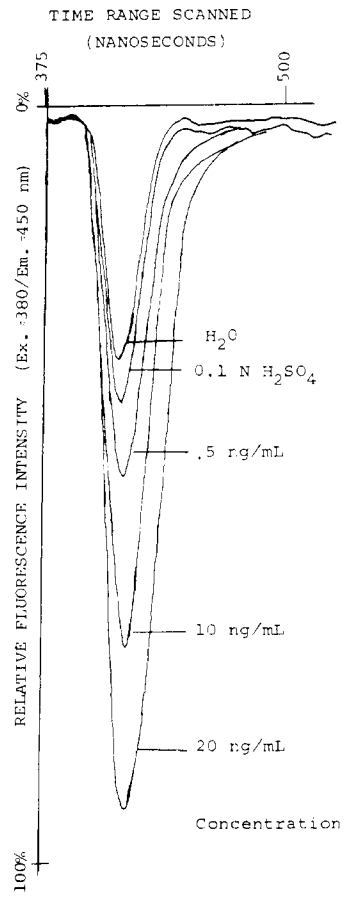

Figure 3. Dye laser induced fluorescence spectrum of quinine sulfate.

fluorescence yield at 475 nm (i.e., area under the fluorescence spectrum) obtained from excitation at either 230 or 280 nm should be at least twice that obtained from excitation at 360

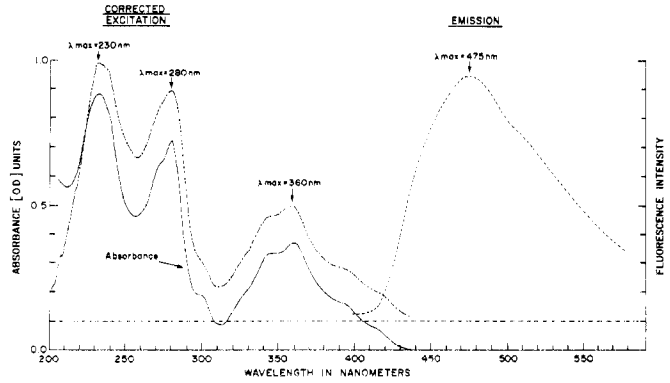

Figure 4. Corrected excitation-emission and absorbance spectra of 2-methoxy-11-oxo-11*H*-pyrido[2,1-*b*]quinazoline-8-carboxylic acid in methanolic:0.01 M (pH 7.5) K_2HPO_4 buffer (1:1).

nm. However, using dye lasers as excitation sources presents different problems in estimating the fluorescence yield of a compound, because each lasing dye has a different energy output which is dependent on its own specific quantum efficiency for lasing and the wavelength of excitation energy produced. Further complications are introduced by frequency doubling which has conversion efficiencies of only 2–20% depending on the energy output and wavelength of the primary lasing dye and the doubling crystal used. Consequently, the excitation intensity of fundamental radiation (360 nm) was more than tenfold greater and was expected to yield about a fivefold increase in sensitivity over that of frequency doubled radiation (280 nm). However, it resulted in only a twofold improvement of sensitivity over the latter (0.5 ng/mL vs. 1.0 ng/mL sensitivity limits). This is probably due to the fact that excitation at 280 nm yields a greater quantum efficiency of fluorescence with less interference due to scatter than excitation at 360 nm. For purposes of quantitation, however, the linear least-squares correlation of fluorescence intensity vs. concentration for this compound showed better correlation and a smaller average deviation for data obtained with fundamental radiation (360 nm) than for frequency-doubled radiation (280 nm), especially after attenuation of the 360-nm laser beam with an iris shutter device to limit light scatter. The quantitation of the pyrido[2,1-*b*]quinazoline compound after extraction from plasma and back-extraction into pH 9.0 buffer showed approximately equal sensitivity limits by either conventional spectrofluorometry or laser-induced fluorescence (Table II). A high background signal due to interfering fluorescent endogenous materials extracted from plasma limited improvement in sensitivity. The quantitation of this compound by thin-layer chromatographic separation showed some improvement in sensitivity of laser-induced fluorescence over conventional fluorometry of the same samples but no improvement in sensitivity of the TLC procedure over the back-extraction (clean up) procedure. The compound was analyzed with high sensitivity (5 ng/mL) by conventional fluorometric detection following high-performance liquid chromatographic analysis of plasma extracts (*19*), which may be improved by laser-induced luminescence detection.

Laser excitation of the fluorescent quinazolinone (photolysis product of demoxepam) produced at least a tenfold increase in its detection limits, compared to conventional spectrofluorometry. Demoxepam extracted from plasma showed a fivefold increase (Table II) in the detectable limit over that obtained by the published method (*20*).

The increased sensitivity limit for the 9-acridanone of flurazepam suggests that application of laser-induced fluorescence for its quantitation in biological fluids (*21*) could result in a fivefold improvement of sensitivity using the high-power mode, Table I.

Experiments with fluorescamine-labeled amphetamine showed limited sensitivity due to high fluorescence background from the excess reagent. These results indicate that high blank backgrounds can be a problem in any derivatization reaction where the fluorescent product is not separated from byproducts and excess reagents. The sensitivity limit of quantitation by spectrofluorometry was 5.0 μg/mL, while that obtained from the low-power laser system (part I) was approximately 10 μg/mL and that obtained by the high-power laser system (parts II and III) was about 2.5 μg/mL. The higher sensitivity in part II was probably due to the narrower slits used on the emission monochromater (thus limiting scatter), although the background fluorescence still remained problematic.

The sensitivities for quinine sulfate, the quinazolinone derivative of demoxepam, and the 9-acridanone of flurazepam were improved at least fivefold over conventional spectrofluorometric results. The deviation from the least-squares fit of a linear response vs. concentration regression line was less (indicating better linearity and reproducibility) for laser-induced fluorescence (part II, fundamental radiation only) than for conventional spectrofluorometry.

The sensitivity limits demonstrated in parts I and II (Table I) were limited by source-induced artifacts such as light scatter, source, detector, and systematic electronic noise and by limitations such as fluorescent solvent impurities. Blank fluorescence can be reduced by separation of interfering materials from the components of interest [e.g., selective extraction of the fluorescamine–amphetamine derivative from the reaction mixture (*22*)] and by using high-purity, spectral grade commercial solvents to reduce solvent contributions (*23*). Source-induced artifacts and some electronic noise may be minimized (though not eliminated) by temporal discrimination using short pulse lasers and fast response detectors to limit interferences due to the source and detection electronics (*24*, *25*).

Triggering of the boxcar integrator from the dye laser light pulse (part III) rather than from the nitrogen laser synchronization circuitry (part II), Figure 1, effectively eliminated a timing source jitter (±1 ns) where discrimination of the fluorescence signal from scatter, using a subnanosecond sampling head, would be possible for long-lived fluorophors. In addition, measuring the ratio of the fluorescence intensity to the laser intensity compensates for laser intensity fluctuations.

However, of the compounds tested, only quinine sulfate showed a fluorescence lifetime long enough to allow temporal discrimination of the fluorescence signal from scatter. A comparison of a scan of a 1.3×10^{-7} M solution (100 ng/mL) of quinine sulfate dihydrate in 0.1 N H_2SO_4 vs. the reagent blank is shown in Figure 5. The fluorescence decay lifetime was determined to be 19 ns and is in agreement with previously published data (*26*). The advantages of temporal discrimination are evident (Figure 5), in that measurements made at 30–40 ns show a high degree of resolution between the background signal from the solvent (0.1 N H_2SO_4) blank and the fluorescence signal due to quinine sulfate, thereby enhancing the sensitivity, precision, and accuracy of the measurement.

The 1P-28 photomultiplier tube used has too slow a rise time (~2 ns) for temporal resolution in the low nanosecond range. This limitation may be overcome by using either a more expensive, faster rise time photomultiplier, or a method of fluorescence detection other than analog measurement, such as photon counting (*11*, *27*). A number of papers dealing with the use of argon ion or kypton ion laser pumped dye laser systems have appeared together with some consideration of the handling of high repetition rate picosecond pulses with a minimum of distortion of the data (*5*, *27*, *28*). Most subnanosecond systems have been used in the determination of

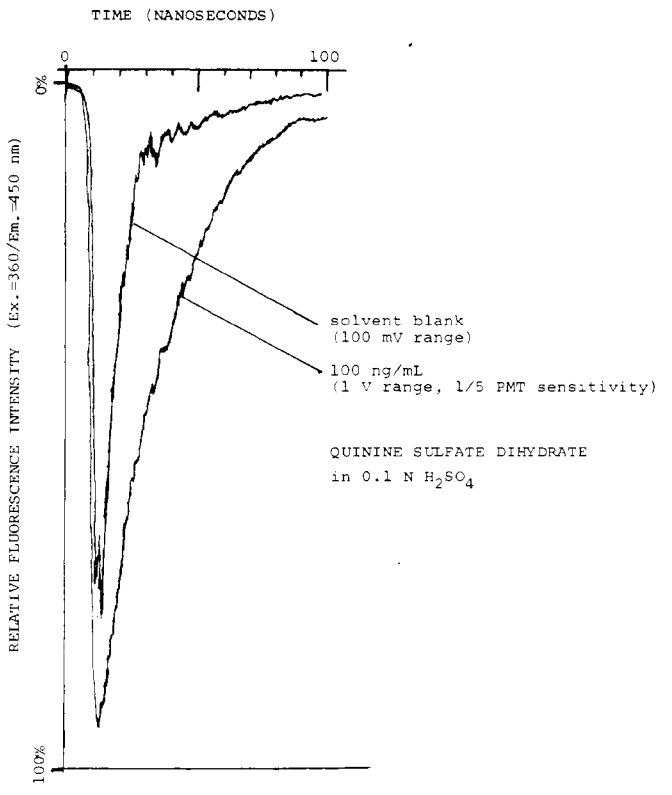

Figure 5. Comparison of time-resolved fluorescence signal to solvent background for quinine sulfate.

fluorescence lifetimes per se (*25*, *27*) with very little application to quantitative analysis. It is evident, however, that further gains in sensitivity beyond those obtained in this exploratory work should be possible through the use of time (temporal) resolution of fluorescence from scatter (*25*, *28*) and two-photon fluorescence techniques (*29*), which will be the subject of subsequent studies.

ACKNOWLEDGMENT

The authors thank Molectron Corp. and Princeton Applied Research Corp. for the loan of the Spectroscan 10 dye laser and the Model 162 boxcar averager and associated electronics, respectively, R. McGlynn for the drawings of Figures 1 and 2, and V. Waddell for the preparation of this manuscript.

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RECEIVED for review February 19, 1980. Accepted June 30, 1980.

Room Temperature Phosphorescence Characteristics of Substituted Arenes in Aqueous Thallium Lauryl Sulfate Micelles

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Micelle stabilized room temperature phosphorescence (MS-RTP) characteristics of functionally substituted aromatic molecules in aqueous Tl/Na lauryl sulfate micellar solution are reported for the first time. The compounds include ketones, aldehydes, alcohols, carboxylic acids, phenols, amines, and a large molecule of pharmaceutical interest. Limits of detection compare favorably in many cases with published 77 K conventional phosphorescence results. Some selectivity was observed in that electron-donating substituents produced larger MS-RTP intensities compared to electron-withdrawing groups substituted on the same lumiphor. Some selectivity was observed also for positional isomers. Triplet state lifetimes at room temperature and at low temperature (77 K) with relative standard deviations of ±10% are also reported.

Many organic unsaturated compounds can be induced to phosphoresce at room temperature when the radiative deactivational pathway is preserved by either isolating the excited molecules in a micellar interior (*1*) or immobilizing the molecules on solid surfaces (filter paper, silica gel, sodium acetate, etc.) (*2*, *3*). Room temperature phosphorimetry utilizing the micelle stabilized excited triplet state was first shown to be a feasible and potentially useful analytical method by Cline Love, et al. (*1*). The detection limits obtained at room temperature in a micellar environment were comparable to those obtained by the conventional low temperature phosphorimetry and by the technique of immobilizing molecules on solid surfaces. In the latter cases, heavy atoms are usually added to enhance the phosphorescence signal (*3*, *4*). Effects of the external heavy atoms on the detection limits and lifetimes at 77 K were investigated and their nature of interaction was discussed by Winefordner et al. (*5*, *6*).

Possible effects of the protective micellar environment and heavy atom counterions on the micelle stabilized room tem-

0003-2700/80/0352-1559$01.00/0