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A New Excitation Method in Laser Fluorometry and Raman Spectroscopy Using an Optical Fiber——An Application to High Performance Liquid Chromatography

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The use of an optical fiber as a wave guide for a laser beam reduces the undesired scattered light in trace fluorometry and in Raman spectroscopy of microliter-size samples. It also permits effective sample excitation and easy optical alignment. In conjunction with high performance liquid chromatogaphy (HPLC), this excitation method has improved the detection limit by an order of magnitude without any other change in the conventional HPLC fluorometric detection system.

Keywords——laser fluorometry; Raman spectroscopy; optical fiber; HPLC; trace analysis; fluorescamine-labelled catecholamine; dansyl-alanine

In the past few years, lasers have been used extensively in analytical chemistry. The use of lasers as an excitation source has made it possible to obtain signals from weak and small emitting species, such as fluorescence from a fluorophore at low concentration or Raman scattering in a limited volume. However, as the excitation power becomes higher, the undesired scattered light becomes more intense. Owing to shortcomings in the optical arrangement, it often becomes more intense than the signal, and this factor limits the utility of the method. To reduce stray radiation, a multi-monochromator and/or selective filters are used for fluorometry and Raman spectroscopy according to the spectroscopic characteristics. In addition to instrumental improvements, the excitation method is one of the key points in obtaining a better signal-to-noise ratio (S/N). There are two major limiting factors in the highly sensitive detection of fluorescence or Raman scattering from samples in capillary cells, which are generally used for flowing liquid samples or for small amounts of static liquid sample. The first limiting factor is undesirable light scattering from the cell surface. The minimization of this scattered light is a rather laborious task and requires technical skill. Several types of flow cells have been designed with the aim of avoiding direct scattering of the laser light.[1-4] These have been used for laser fluorometric high-performance liquid chromatography (HPLC), and somewhat improved sensitivity was reported. The second limiting factor is the short path length. When the laser beam is focused into the sample perpendicularly to the capillary axis, it interacts with the sample only in a spatially small region, so that the emitted signal intensity is limited. The reported flow cells[1-4] have not overcome this second limiting factor.

We report here a new exciting technique using an optical fiber, which overcomes both the above-mentioned limiting factors in the highly sensitive detection of emission from samples.[5] An effective application of this method is laser fluorometry following HPLC. An ultratrace analysis of dansyl-alanine (DNS-Ala) and fluorescamine derivatives of dopamine and norepinephrine (FLA-DA and FLA-NE) has been carried out and compared to the results with a conventional HPLC fluorescence detector. Using this method, we have also observed

Raman spectra of microliter liquid samples without any difficulty of light arrangement.[6] Blank limitations in trace fluorometry are also discussed.

Experimental

Method of Sample Excitation——For the sample excitation an optical fiber was used as a wave guide to lead the laser beam axially into the capillary cell. A schematic diagram of the experimental set-up is shown in Fig. 1. The fiber was a silica core low-loss optical fiber with a core diameter of 200 μm and a numerical aperture (N.A.) of 0.20. A graded index type micro-lens (SELFOC LENS of Nippon Sheet Glass Co., Ltd.) of 1 mm diameter was attached to the exit end of the fiber to reduce the divergence of the transmitted beam. The Ar ion laser was focused onto the core of the entrance end of the fiber by a quartz lens of 3 cm focal length. The laser beam was emitted from the exit end of the fiber with a narrow diameter and small divergence. If the exit end of the fiber is inserted into a capillary cell, the laser beam thus irradiates the sample directly without irradiating the cell wall or the liquid surface, effectively exciting the sample along the capillary axis. The laser power was essentially not attenuated, but the polarization of the laser beam was almost entirely scrambled when the beam was transmitted along a fiber of 20 m in length. Light passed through the fiber contains weak Raman lines scattered from the fiber material, in addition to the monochromatic laser light. The intensity of the former obviously depends on the length of the fiber.

Reagents——The reagents were obtained from commercial sources: DNS–Ala from Sigma Inc., norepinephrine (NE)–HCl and dopamine (DA)–HCl from Nakarai Chemicals Co., Ltd., 3,4-dihydroxybenzylamine (DHBA)–HBr from Aldrich Co., Inc., and fluorescamine from Hoffmann-La Roche Inc. All other chemicals were of reagent grade, and were used without further purification.

Fluorometry Combined with HPLC——The magnified diagram (A) in Fig. 1 shows the schematics of the flow cell which we have used as the laser HPLC fluorometric detection system. The cell was made of quartz tubing, 1.5 mm in inner diameter and 20 cm in length. The optical fiber was inserted into the top of the cell to irradiate the sample, which flowed into the cell from a branched tube. The top of the tube was sealed tightly so that the liquid could not flow out. The branched tube (i.d. 0.5 mm, o.d. 3 mm) was connected to the outlet of an HPLC column through a stainless steel tube with the same inner diameter. This branched tube seemed to cause little disturbance in the flow from the chromatograph because the width of the peak obtained with this cell (Fig. 1(A)) was the same as that obtained with a conventional fluorometric HPLC cell. The simultaneous emission at 351.1 and 363.8 nm of an Ar ion laser (Coherent Radiation Model CR-4) were used for the excitation of fluorophores. The ultraviolet laser light passed through a short pass filter (Toshiba UV 33S) to remove visible background emission from the plasma tube and was then focused onto the optical fiber core. The laser light-induced fluorescence from the flowing sample was observed with the emission monochromator of a JASCO (Japan Spectroscopic Co., Ltd.) FP-110 fluorescence spectrometer through a slit of 1 mm wide and 10 mm high. Therefore, the effective cell volume was 17 μl. A seven cavity interference filter (Ditric Optics, half-bandwidth 40 nm) was provided to attenuate the Rayleigh and Raman scattering. A JASCO HPLC system, consisting of a model SP-240 pump, a model VL-611 injector and a model DM-101 damper was used. Operating conditions for HPLC are given in the legends to Figs. 2, 3, and 4.

Raman Spectroscopy——The magnified diagram (B) in Fig. 1 shows the schematics of the capillary cell which we have used for the measurement of Raman spectra of non-flowing micro-liter liquid sample. The cell was made of quartz tubing. The thin part of the cell was 0.5—1 mm in inner diameter and about 5 mm in length, holding 1—4 μl of liquid. It sucks up the sample liquid by capillarity. The exit end of the optical fiber was put into the thick part of the cell (i.d. > 1.5 mm) and fixed in such a way that the laser beam passed along the capillary without impinging on the cell wall. The scattered Raman light was collected by a collimating lens and focused onto the entrance slit of a JRS-400 Raman spectrometer (Japan Electron Optics Laboratory Co., Ltd.). The alignments of the exciting laser beam and the sample cell toward the spectrometer were very easy because the laser beam and the sample cell were always fixed. The laser used was a Coherent Radiation Model CR-2 Ar ion laser, operated to provide 150 mW of power at 488.0 nm.

Results and Discussion

Detection Limit of Laser Fluorometric HPLC

Figure 2 shows the chromatogram obtained with DNS–Ala (Ex_{max} = 350 nm, Em_{max} = 520 nm in MeOH) using the present fluorometric detector with a 0.85 mW laser, compared with that obtained using a conventional fluorometric HPLC detector equipped with a 130 W medium pressure mercury lamp. Both fluorometric measurements were performed with the same emission monochromator and the same detection and signal processing equipment. The detection limit of DNS–Ala by the laser detection system was 2 pg (S/N of 2), which is more

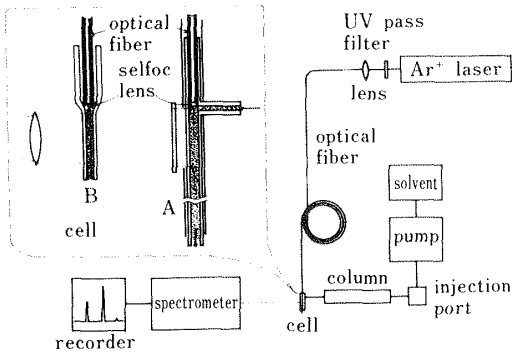

Fig. 1. The Configuration of the Laser Fluorescence Spectrometric System with an Optical Fiber and Schematic Diagrams of Microliter Size Cells

A: a flowing liquid sample cell, combined with HPLC. B: a static liquid sample cell.

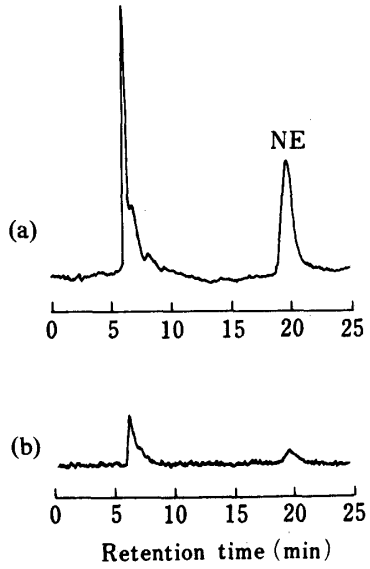

Fig. 3. Comparison of Fluorescence Response of FLA–NE with Two Fluorometric Detection Systems

Operating conditions of HPLC:[7] column, TSK LS-160 (10 μm), 600 mm × 4.0 mm i.d.; eluting solvent, acetonitrile–0.1 M Tris buffer, pH 8.4 (1 : 9); flow rate, 0.8 ml/min; column temperature, 56 °C. Detection conditions: (a) detection at 517 nm, excitation at 351 + 364 nm lines of Ar ion laser, laser power *ca.* 1.0 mW, (b) detection at 517 nm, excitation at 365 nm line of medium pressure mercury lamp of 130 W. 1 μl solution containing 1 ng of NE in 0.1 M borate buffer at pH 8.5 was injected.

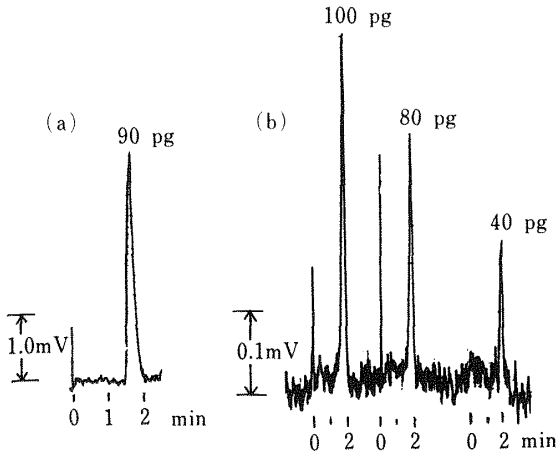

Fig. 2. Comparison of Fluorescence Response of DNS–Ala Using Two Fluorometric Detection Systems

Operating conditions for HPLC: column, JASCO ODS-10A (10 μm), 300 mm × 4.6 mm i.d.; eluting solvent, methanol; flow rate, 1.0 ml/min; column temperature, 25 °C. Detection conditions: (a) detection at 530 nm, excitation at 351 + 364 nm lines of Ar ion laser, laser power *ca.* 0.85 mW, (b) detection at 530 nm, excitation at 365 nm line of medium pressure mercury lamp (130 W).

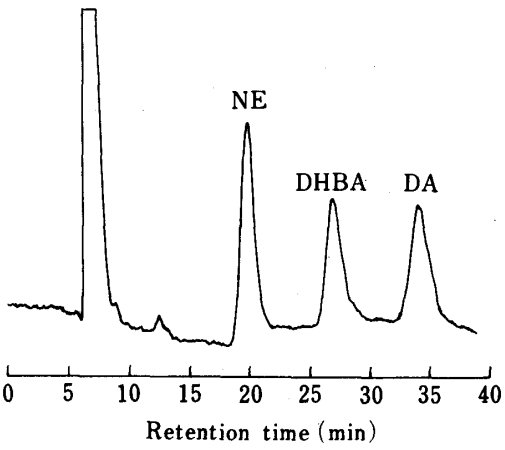

Fig. 4. High Performance Liquid Chromatogram Using Laser Fluorometric Detection System Obtained from Fluorescamine Derivatives of Authentic Catecholamines

70 μl of 0.1 M borate buffer solution (pH 8.5) containing NE (25 ng), DA (16 ng) and DHBA (20 ng) was mixed 30 μl of 0.02% fluorescamine in acetone and 10 μl of the reaction mixture was subjected to HPLC. Operating conditions were the same as that of Fig. 3.

sensitive by an order of magnitude than that of the conventional detection system. By injecting known quantities of appropriately diluted standards onto the column, we established a linear response in the range of 10^{-11}—10^{-7} g[5] (Fig. 6). By changing the laser power in the range of 0.1 to 1 mW, we confirmed that the response also increased linearly with the power of the laser. This result means that the laser power is far from the condition of saturation irradiance. We therefore expect that the detection limit could be improved, if the samples were

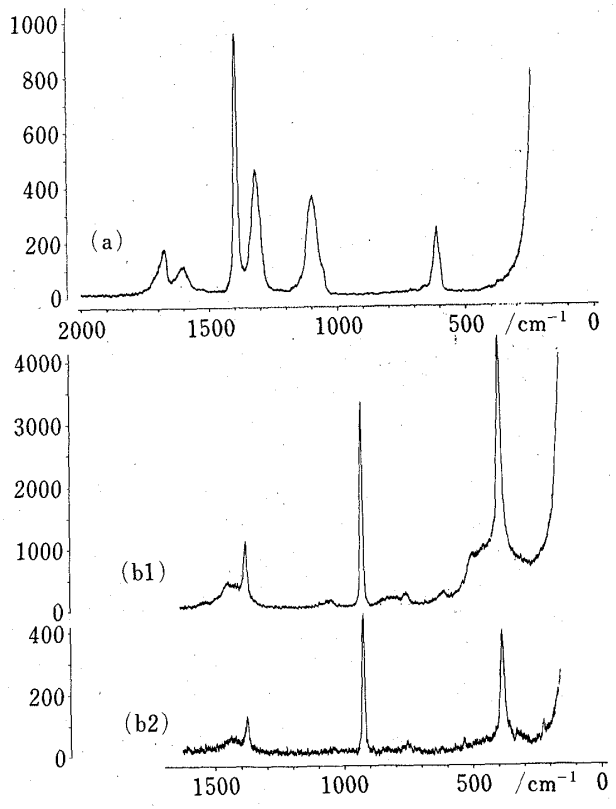

Fig. 5. Raman Spectra of (a) 1.5 μl Formamide and (b1) 4 μl Acetonitrile Excited by the Optical Fiber Method and (b2) 4 μl Acetonitrile Excited by the Conventional Capillary Method

The ordinate shows relative intensity. The spectral slit width of each spectrum is (a) 4 cm^{-1}, (b1) and (b2) 7 cm^{-1}

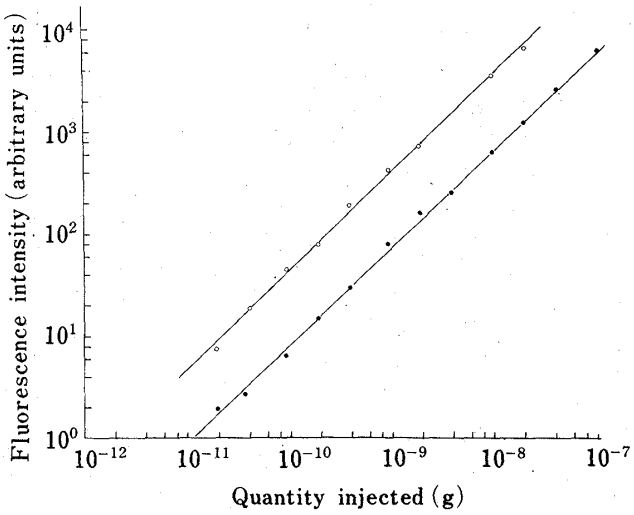

Fig. 6. Linear Quantitation of DNS–Ala Using Laser Detection System and Its Dependence on Laser Power

—○—, 0.80 mW; —●—, 0.15 mW.

excited by a higher power laser. However, high-power irradiation often inflicts photochemical damage on molecular samples. Our test showed that the rate of decrease in the fluorescence intensity of DNS–hydrazine was of the order of $10^{-3}\,s^{-1}$ or less, when it was irradiated with a 3 mW ultraviolet laser. The flowing sample was irradiated for 1 s when it passed through the cell (17 μl) at a flow rate of 1 ml/min, so that photochemical damage may be considered to be negligible (less than 10^{-3}) in the present case, but this might not be the case with higher power irradiation.

Figure 3 shows the chromatograms obtained from FLA–NE (Ex_{max} = 405 nm, Em_{max} = 480 nm in the eluting solvent) using the above two detection systems. The detection limit of NE (S/N of 2) with the laser detection system was 60 pg, whereas it was 800 pg with the conventional system. It was also shown that the laser fluorometric HPLC detector was more sensitive than the conventional one. A typical chromatogram of a mixture of authentic CA is shown in Fig. 4. Each CA was effectively separated by isocratic elution using the flow cell of Fig. 1(A). However, the present laser fluorometric HPLC is unfavorable for the highly sensitive detection of FLA–CA in two respects. The first point is the discrepancy between the wavelength of the laser lines (351 + 364 nm) and the absorption maximum of FLA–CA. The second point is the large background due to the eluting solvent, Tris buffer. The lower limit of detection is controlled in practice by the intensity of the optical background, which increases proportionally to the exciting laser power. For more sensitive fluorometric HPLC detection, we must use a lower-background mobile phase.[8] According to a detailed consideration of

blank luminescence by Matthews and Lytle,[9] on the other hand, the residual fluorescence for the better solvents corresponds to compounds having a concentration-quantum yield product ($C \times \phi_f$) of the order of 10^{-12}. Thus, the trace fluorometry of fluorescent species is blank-limited to the order of $C \times \phi_f = 10^{-12}$, for example, 2×10^{-12} M for $\phi_f = 0.5$ fluorophore. To achieve trace fluorometry at a lower limit concentration, the intensities of all the factors contributing to the background must be attenuated to the same order. Since the reduction of stray radiation has been achieved by the optical fiber method, the diminution of background due to the solvent is now crucial.

Raman Spectra

Figure 5 presents Raman spectra of formamide and acetonitrile excited by the optical fiber method. A Raman spectrum of acetonitrile obtained by the conventional capillary method is also shown for comparison. By the optical fiber method, the off-resonance Raman spectra of micro-liter size samples can be obtained without any difficulty of optical arrangement, and with better results in terms of the S/N ratio than can be obtained by the conventional capillary method. However, the optical fiber method is not very suitable for elaborate Raman spectra measurements, since the Raman bands of the fiber material are superimposed on the sample Raman spectrum in the regions of 300—500 cm^{-1} (strong, broad), 600 cm^{-1} (weak) and 800—850 cm^{-1} (weak, broad), and the slit of the Raman spectrometer must be opened wide for the effective detection of the Raman scattering light because the laser beam striking the sample is less concentrated compared to that in the conventional Raman excitation method.

Conclusion

The optical fiber method is effective in exciting an emitting species present in very small amounts, especially flowing in a capillary. The improvement of the detection limit of fluorometric HPLC by the present method results from the diminution of stray radiation as well as from the increase of signal radiation. It is noteworthy that the present method permits a highly sensitive laser fluorescence analysis with a simple general-purpose fluorometer for HPLC.

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