Review

Solid-State Diode Laser-Induced Fluorescence Detection in High-Performance Liquid Chromatography

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The need to measure ultralow levels of pharmaceuticals in biological matrices at femtogram and attogram levels presents a significant challenge to bioanalysts. Liquid chromatography has proven to be a versatile and valuable tool for separating analytes from complex biological matrices and fluorescence detection provides both the sensitivity and the selectivity required to measure femtogram and attogram levels of analytes. Solid-state diode lasers have been used primarily in printers, compact disc recorders, and bar code scanners but have recently been adapted for use as light sources for fluorescence detection. Excellent spectral features in the visible and near-infrared regions of the spectrum, together with low cost and ruggedness, make diode lasers an attractive alternative for use as light sources in analytical measurement. Biological matrices demonstrate minimal background signals in the far-red regions of the spectrum which diode lasers emit and diode lasers are among the most stable light sources available. These facts along with expected developments in labeling systems make the potential for use of diode lasers in LC-detection quite promising. This paper reviews characteristics of diode lasers, the properties of potential visible and near-infrared fluorescent probes, instrumental aspects of diode laser fluorometers, and future trends that can be expected in this exciting field of bioanalytical research.

KEY WORDS: solid-state diode laser; fluorescence detection; high-performance liquid chromatography; derivitization.

INTRODUCTION

In recent years the trend in the pharmaceutical development has been to produce highly potent drug entities that are present at ultratrace levels in biological matrices. The ability to measure ultralow levels of these drugs presents a unique challenge to bioanalysts. This challenge can be met through the application of a liquid chromatographic separation with a suitable detection system. Absorbance methods of detection often do not provide the sensitivity for ultratrace analysis, while electrochemical and mass detectors are often not rugged enough for high throughput of the analytical process. Fluorescence represents a means of increasing both sensitivity and selectivity in high-performance liquid chromatography (HPLC) while maintaining reliability. Sensitivity is improved in fluorescence because of minimal background, and selectivity is enhanced due to the limited number of compounds that have fluorescent properties (1,2).

Through tagging an analyte of interest covalently to a fluorophore, the applicability of fluorescence detection can be extended to nonfluorescent compounds. These derivatization reactions can be performed, either before or after the chromatographic separation. In the prechromatographic method, derivatization can be performed in either an on-line or an off-line mode (3). The off-line mode is the more widely used because the solvent in which the prechromatographic reaction takes place need not be compatible with the chromatographic system. Also, side products formed during the derivatization, degradation products of the reagent, and excess reagent may be separable from the derivative on the chromatographic system. An alternative to chemical derivatization for extending fluorescence detection to nonfluorescent compounds is the utilization of indirect fluorescence (4). In this technique, a nonfluorescent analyte reduces the signal from a fluorophore present in the mobile phase as an additive. The fluorescence signal that is obtained is a result of the fluorophore added to the mobile phase. As the band of analyte passes through the detector, a decrease in fluorescence signal is observed which may be due to either quenching or displacement mechanisms. This decrease in signal is proportional to the concentration of the analyte. The analyte is represented on the chromatogram as a negative peak, while the baseline represents the steady-state signal from the mobile phase addition.

Although application of fluorescence detection using conventional light sources (e.g., high-pressure DC xenon arc lamps and low-pressure mercury vapor lamps) allows detection of pg/ml concentrations, it is difficult to obtain lower levels of detection because of limited radiant power of the exciting light, background interferences or noise associated with the light source (1). There is a direct relationship between the intensity of exciting light and the intensity of flu-

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orescence, and detection sensitivity can therefore be increased by high excitation intensities, which are common to laser light sources (5). Detection limits may also be improved if other factors such as the stability of the source and the lifetime of the fluorophore are optimal. Laser light sources have been used with fluorescence to obtain detection limits at femtogram and attogram per milliliter concentrations for compounds in biological matrices (6,7). In addition to having high intensities, laser beams are both monochromatic and coherent. The use of lasers in chromatographic detection is attractive because there is limited influence of Raman and Raleigh scattered light, due to the monochromaticity of the beam. Argon ion/krypton ion, Nd:YAG, and He:Cd lasers have been used as light sources in detection devices for liquid chromatographic systems. These lasers are chosen because of high power outputs (about 1–5 W/emission line) and appropriate emission wavelengths for commonly used labels. These techniques have been successful in ultralow-level analysis, although the high cost, lack of stability, and difficulties associated with operation and maintenance limit the routine use of gas and crystal lasers (8).

Solid-state diode lasers have been developed primarily for use in telecommunications and data processing. One million diode lasers are manufactured each month for use in compact disc recorders, bar code scanners, and laser printers (9,10). Ishibashi *et al.* and Winefordner *et al.*, in separate works, have explored the use of diode lasers for molecular fluorometry, reporting the technique to be efficient, compact, rugged, and inexpensive (7,11). Diode lasers also provide attractive alternatives as light sources by virtue of their long lifetimes, low noise characteristics, low power consumption, and excellent spectral characteristics. This paper discusses characteristics of diode lasers and reviews various aspects of their application to molecular fluorescence spectrometry as it pertains to liquid chromatographic detection in biopharmaceutical analysis.

SOLID-STATE DIODE LASERS (SEMICONDUCTOR DIODE LASERS)

Diode lasers are solid-state devices that utilize semiconductor materials to provide laser emission. They differ from other solid-state lasers, e.g., Nd:YAG, in that laser emission is a result of conduction energy bands rather than discrete electronic energy levels. In a Nd:YAG laser, the YAG crystal is doped with neodymium ions (Nd^+) at very low concentration. The dopant atoms are sufficiently isolated from one another in the crystal to allow energy levels to remain discrete, resulting in line spectrum emission (12). In diode lasers, the dopant atoms are present at high concentrations and are not isolated from one another. The energy levels of the entire crystal lattice must therefore be taken into account, which include the valence and conduction bands of the semiconductor (9,13). The property that distinguishes semiconductors, insulators, and conductors is the energy gap between the valence and the conduction bands (Fig. 1).

Solid-state diode lasers are constructed from insulator materials which are converted to semiconductors by incorporation of dopant atoms (Fig. 2). In the "n-type semiconductor" the dopant has one extra valence electron per atom than the host (insulator) and contributes a band of filled levels "D" in close proximity to the conduction band of the host (insulator). In the "p-type semiconductor" the dopant has one valence electron less per atom than the host (insulator) material, thereby contributing a band "F" that is devoid of electrons but easily accessible to electrons from the valence band. A solid-state diode laser utilizes the properties of a junction between a p-type and an n-type semiconductor made from the same host material. If enough current is applied to the p–n junction with the negative and positive terminals attached to the n and p regions, respectively, electrons flow from the n to the p region. This results in a displacement of the n and p energy levels, causing population inversion in the region of the junction (Fig. 3). By enclosing the junction in an optical cavity aligned along the junction, laser action is achieved (14). Since the optical cavity is very short and is formed from the junction, the resulting laser beam is highly divergent. The divergence of the beam is corrected using a collimating lens with a high numerical aperture determined by the radiation patterns of individual laser diodes.

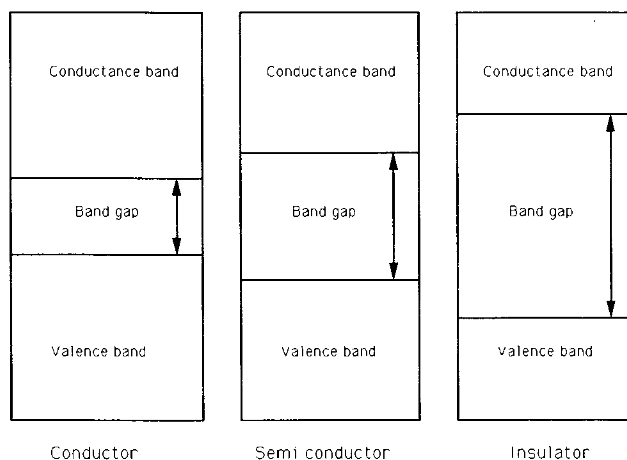

Fig. 1. Schematic indicating differences in band gap among conductors, semiconductors, and insulators.

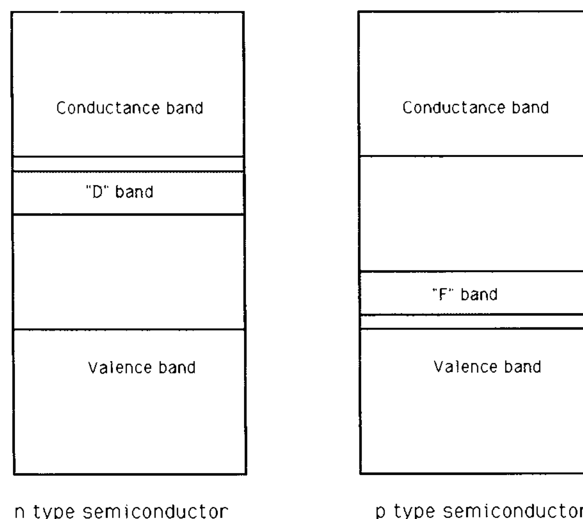

Fig. 2. Schematic of an n-type and a p-type semiconductor.

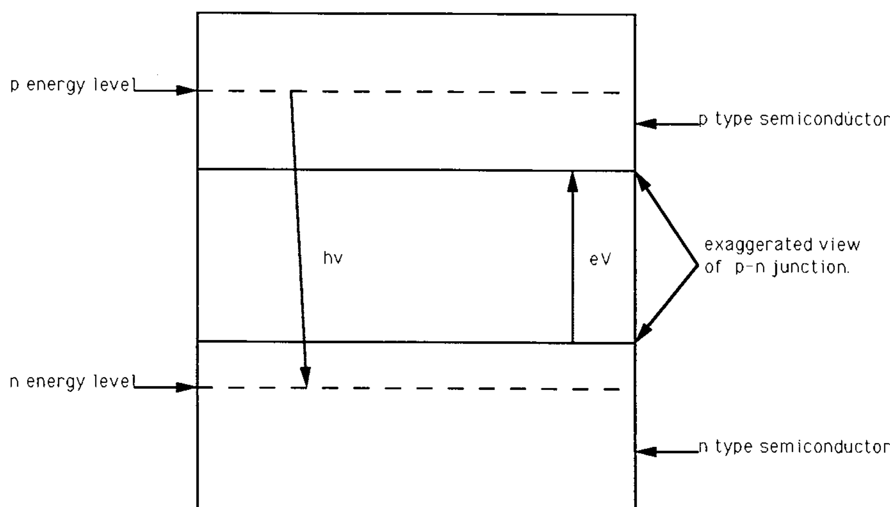

Fig. 3. Schematic depicting the result of applying a voltage across the p–n junction.

The band gap of the materials used in the construction of the semiconductor dictates the output wavelength of the diode laser. Diodes in use presently provide laser emission in the far-red to infrared (600-nm to 2.0-μm) regions of the spectrum (12,13,15–17). The semiconductor materials available are presently optimized for long wavelength emission and therefore lasers emitting at longer wavelengths have a higher output power than diodes lasing at shorter wavelengths. Second harmonic emissions (λ = 415 nm) with output powers ranging from 50 μW to 40 mW are now commercially available (18). The output wavelength of the diode is strongly dependent on both temperature and drive current. Solid-state diode lasers can therefore be tuned by varying the current supplied to the junction or by changing the temperature of the junction (19). A stable output wavelength from a diode laser is maintained by keeping the temperature of the diode constant using a thermocooling unit, a thermocouple sensor, and a feedback circuit (9). The tuning range of a particular laser is small, such that a series of diodes is necessary to cover an appreciable wavelength range (14). Recently, a continuous wave diode laser with an output power ranging from 25 mW to 1 W has been reported with a tuning range that extends from 600 to 1600 nm (20).

Solid-state diode lasers are very stable because they are output stabilized via a built-in PIN diode. They also do not require alignment as do gas or dye lasers (21). Their characteristic emission in the far-red and near-infrared regions of the spectrum provide for minimal background interferences in bioanalytical HPLC. Table I shows a comparison of some of the properties of solid-state diode lasers as compared to the argon ion gas laser (9,22).

Although, solid-state diode lasers have a limited spectral range, the use of solid-state diode lasers as sources in fluorescence detection should provide enhanced detection because of favorable spectral characteristics, high output power, and amplitude stability. The compact size of diode lasers is also a benefit as shown by Patonay *et al.*, who replaced the broadband light source in the source compartment of a SLM/Aminco 8000C fluorometer with a near-infrared laser diode for the analysis of cyanine dyes (23).

FLUORESCENT PROBES IN LIQUID CHROMATOGRAPHIC DETECTION

Classically, fluorescence detection in liquid chromatography has been carried out in the UV–visible region of the spectrum (24). One of the advantages of this is the availability of a large number of well-characterized fluorophores having high molar absorptivities, high quantum efficiencies, and functional groups for derivatization. A major drawback associated with detecting compounds in the UV–visible region is that endogenous compounds from biological matrices luminesce in that region. This results in a large background signal and the ability to detect trace quantities of analytes is limited because of background interference (8,10). In addition, high excitation energies at shorter wavelengths may result in photodegradation.

Imasaka and Ishibashi reported a group of molecules, the polymethine dyes, which luminesce in the near-infrared region (8). These dyes show minimal background when detected in biological matrices, which result in detector- rather than matrix background-limited determinations (8). These dyes have high molar absorptivities (greater than 100,000 M^{-1} cm^{-1}), and over 3000 dyes have been synthesized, many of which are commercially available (10).

Ishibashi *et al.* reported the chromatographic detection

Table I. Comparison of Solid-State Diode Lasers and a Gas Laser (Argon Ion) as Light Sources for Detectors in Liquid Chromatography

Parameter	Diode lasers	Gas lasers
Cost ($)	25–2,500	7,000–40,000
Dimensions (mm^2)	88 × 31.8	1,063 × 132
Efficiency[a] (%)	29–32	<1
Power stability of beam (%)	0.1	1.0–2.0
Life of laser (hr)	80,000–100,000	10,000–12,000
Output power[b] (W)	0.003–15	0.2–100

[a] Efficiency indicates the conversion of electrical current to laser emission.
[b] Output power indicated is for continuous wave lasers.

of 3,3′-diethyl-2,2′-(4,5;4′,5′-dibenzo)thiatricarbocyanine iodide (DDTC; λ_{ex} = 786 nm, λ_{em} = 840 nm) in methanol with a laboratory-constructed solid-state diode laser fluorometer. A preamplifier and a photon counter were used for sensitive detection and the detection limit was reported to be 5×10^{-12} *M* DDTC. The limiting factors for detectability were the output power of the diode laser and the dark current noise (6).

Sauda *et al.* chromatographically separated three polymethine dyes, 3,3′-dimethyl-2,2′-(4,5,4′,5′-dibenzo) thiatricarbocyanine iodide (NK 427), 3,3,3′,3′-tetramethyl-1,1′-dimethyl-4,5,4′,5′-dibenzoindotricarbocyanine perchlorate (NK2014), and anhydro-3,3,3′,3′-tetramethyl-1,1′-bis(4-sulfomethyl)-4,5,4′,5′-dibenzoindotricarbocyanine hydroxide sodium salt (NK2611), using a laboratory-constructed 780-nm diode laser as a light source for fluorescence detection (25). Fluorescence was measured with a red-sensitive PMT equipped with a cooling system. The three polymethine dyes had absorption maxima at or near 780 nm, which coincided with the emission of the diode laser. They reported a detection limit of 0.3 pg on column for NK2611, which was three orders of magnitude better than that obtained by a similarly equipped fluorimeter with a conventional light source (25).

Winefordner and co-workers detected 46,000 molecules of a polymethine dye, IR-140 (λ_{ex} = 780, λ_{em} = 830), in methanol using a diode laser fluorimeter in the near-infrared region (11). These studies indicate that the use of diode lasers in the near-infrared region has great potential for detection at ultratrace levels. Application to real samples has been limited because polymethine dyes generally lack functional groups necessary for chemical derivatization (10). An exception to this is indocyanine green. This compound has negatively charged sulfonic groups and is soluble in aqueous solutions (8). Imasaka and colleagues labeled protein in human serum with indocyanine green and were able to detect 1.3 pmol of albumin using a diode laser equipped fluorescence detector lasing at 780 nm. The radiation was measured using a cooled red-sensitive PMT and stable output from the diode laser was maintained using a current feedback circuit from the diode to a lock-in amplifier (26). Using a similar diode fluorometer Imasaka *et al.* applied the instrument to an enzymatic assay using indocyanine green as a fluorescent marker (27).

A problem associated with the use of indocyanine green as a fluorescent probe is chemical instability. March and Karnes reported that indocyanine green in human serum stored at −20 and −70° C, respectively, was unstable after 2 weeks (28). This and other studies show that use of indocyanine green in practical analysis may be limited (29–31).

Patonay and co-workers reported the synthesis of a number of near-infrared carbocyanine dyes containing functional groups for derivatization to amine, sulfhydryl, and hydroxyl analyte moieties (32–36). Boyer and co-workers reported the configuration of a near-infrared cyanine dye to goat anti-human immunoglobulins. The near-infrared fluorescence was read at 820 nm using laser diode excitation at 780 nm. The limit of detection was reported to be in the subpicomolar range (37).

In addition to near-infrared fluorometry, a number of fluorescent dyes which can be excited by diode lasers in the far-red visible region of the spectrum are available. These have functional groups which can be used to label analytes and are stable with relatively high molar absorptivities (Table II) (10,38). Thiazine, oxazine, rhodamine, Texas Red, and phycobiliprotein analogues have reactive sites that can be exploited for labeling of analytes (39,40). With the use of a bifunctional reagent employing water-soluble carbodiimide, a number of these analogues have been shown to bind covalently to albumin (10,41,42). It may also be possible to convert the cyano functionality (CN^-) on rhodamine 800 to an acid chloride, which could then be derivatized to an analyte containing nucleophilic substituents such as amino groups.

A very simple fluorometer using a light-emitting diode (λ = 540 nm) with a photodiode as a detector has been constructed (43). The system was evaluated using a fluorescent dye, oxazine 720 (λ_{ex} = 618 nm, λ_{em} = 640 nm), in methanol. The system exhibited an excellent linear dynamic range, although the limit of detection was only 20 ng/L.

The use of a simple semiconductor laser spectrometer as a detector for slab gel electrophoresis has also been reported. Methylene blue (λ_{ex} = 668 nm, λ_{em} = 683 nm) was tagged to protein and excited with a visible diode laser (λ = 670 nm) in an attempt to measure fluorescence. It was reported that the fluorescence was weak, which was ascribed to strong quenching of methylene blue by the proteins (44). In another study, methylene blue was used as a fluorescent marker to measure alcohol dehydrogenase activity and hence ethanol levels in serum samples (29). A flowing system with an LC pump was used and the concentration–response curve was reported to be linear over the range 0–40 nmol. Imasaka and colleagues constructed an analytical calibration curve for rhodamine 800 in an effort to determine the detectability of a visible diode laser fluorometric system employing a red-sensitive PMT. The authors reported a detection limit of 4×10^{-12} *M* and expressed that comparable detection limits in near-infrared fluorometry could be obtained only using a proton counter. They attributed this to increased fluorescence collection and more sensitive PMT's which are available in the far-red spectral region (10). Imasaka *et al.* reported the use of a visible diode laser spectrometer (λ_{ex} = 668 nm, λ_{em} = 683 nm) for indirect fluorescence detection. Methylene blue was used as the mobile-

Table II. Fluorescent Dyes in the Visible Region of the Spectrum Capable of Excitation by Solid-State Diode Lasers

Dye	λ_{ex} (nm)	λ_{em} (nm)	Molar absorptivity (M^{-1} cm^{-1})	Functionality
Methylene blue	668	683	66,600	Tertiary amine
Rhodamine 800	685	700	89,500	Cyano
Nile blue	640	672	77,500	Primary amine
Thionine	600	623	12,000	Primary amine
Oxazine 750	673	691	82,500	Secondary amine
DMOTC	682	718	198,000	Tertiary amine
Cresyl violet	601	632	67,400	Primary amine
Texas Red	596	615	85,000	Sulfonyl
Allophycocyanin	650	670	1,000,000	Primary amine (lysine)

phase additive and a detection limit of 1 pmol was reported. The authors expressed that detection limits could be improved substantially by optimizing the experimental conditions, such as the concentration of the mobile-phase additive and the pH of the mobile phase (18). Visible solid-state diode lasers used along with well-characterized organic probes possessing functional groups for derivatization will likely enhance the capability for sensitive and selective detection of analytes in biological matrices.

INSTRUMENTAL ASPECTS OF DIODE LASER FLUOROESCENCE DETECTORS

Parameters that characterize detectability in HPLC/fluorescence depend on a number of factors. These factors include the spectral characteristics of the excitation source such as linewidth, amplitude stability, and power output, the design of the flow cell, the efficiency of the collection of fluorescence, and transformation into an electrical signal. To obtain low levels of detection, it is essential to limit background fluorescence and stray light effects. Stray light is due to Raman scattering, Raleigh scattering, refraction and reflection. These optical phenomena are almost entirely determined by the flow cell design that is used in a liquid chromatographic system. A number of flow cell designs have been proposed to reduce scatter of the excitation light (21,44–46). When a conventional square flow cell is used, stray light due to Raman scatter can be minimized by exciting the sample at wavelengths that are far removed spectrally from the wavelength of fluorescence. This is accomplished by virtue of a large energy difference between absorbance and fluorescence maxima and is a characteristic of the fluorescing species. Raleigh scatter can be reduced considerably when fluorescence is collected at a 90° angle relative to the excitation beam (13).

In an effort to reduce reflection and refraction owing to the walls of the flow cell, researchers have designed a number of windowless flow cells (21,47). Winefordner *et al.* reported a study in which various configurations of diode lasers and detectors were used for obtaining the lowest detection limits in various sample introduction schemes (10). In the first configuration, a square cuvette was used. In another configuration, a flowing liquid jet was used as a windowless flow cell. A compact filter fluorometer was constructed by placing a laser diode as close as possible to the square quartz cuvette. Scatter was minimized by the use of a filter, and fluorescence was directed onto a low-noise, red-sensitive photodiode. The lowest limit of detection for the fluorescence of IR-140 was obtained using the liquid jet design (10).

Kawabata and colleagues constructed an ultramicro flow cell from a fused silica capillary with a diode laser fluorometric detector. A near-infrared fluorophor (NK 427) was excited using a diode laser (λ = 780 nm). The authors evaluated the flow cell by utilizing two different optical arrangements. In the first configuration, an optical fiber was introduced as a waveguide into the flow cell for excitation and fluorescence was measured through the wall of the flow cell. In the second optical arrangement, the diode laser was focused onto the flow cell and fluorescence was collected through the optical fiber. The authors reported detection limits of 12 and 90 fg of the dye in the two optical configurations, respectively (7). A solid-state diode laser fluorescence detector for capillary zone electrophoresis (18) has been constructed in which a portion of the polyimide coating on the capillary was etched off to create a window through which the separated analytes could be detected. A solid-state diode laser emitting second harmonic radiation (λ_{em} = 415 nm) was focused onto the flow cell and fluorescence was collected with a lens. The authors reported detection limits of ~100 amol for amino acids derivatized to a coumarin dye (DCCS; λ_{ex} = 437 nm). Flow cells may be constructed or adapted from commercial fluorescence detectors.

The ability of the optics to discriminate fluorescence from scattered light increases the detectability of the system by lowering background. In order to select emission wavelengths, a filter or a monochromator may be used. Monochromators generally possess appropriate light throughput although this depends on the optimal wavelength of the monochromator. Maximum transmission, for interference filters is usually between 20 and 60% and the spectral band width ranges from 10 to 80 nm. When using filters or monochromators, sensitivity can be traded for spectral selectivity by increasing the band width. Cutoff filters can also be combined with interference filters to enhance selectivity at the expense of sensitivity (5).

An ideal photomultiplier is one that has high conversion efficiency (i.e., the ability of the photocathode to convert light to electrical current), high gain, and low noise characteristics. A factor of primary importance when choosing a photomultiplier tube for diode laser fluorometry is the match of fluorescence emission from the sample and the spectral response maximum from the photomultiplier. For detection in the far-red and near-infrared regions of the spectrum, the photocathode materials most commonly used are gallium arsenide activated with cesium or a multialkali material, usually consisting of a combination of sodium–potassium–antimony–cesium (42). A major problem associated with the use of photomultiplier tubes is the effect of dark current, which increases the noise level. The most effective method of limiting dark current is cooling the photomultiplier tube (5,48,49).

A diode laser fluorescence detector can be constructed from a variety of off-the-shelf components available through a number of vendors. A schematic of such an instrument constructed in our laboratory is shown in Fig. 4. An infrared or visible diode laser requires both a heat sink and a laser driver to maintain stable output emission. Optics are used to collimate and accurately direct the laser beam onto a flow cell and a filter is placed in front of the laser to eliminate background fluorescence. A diode laser is also very sensitive to vibrational noise. To increase the detectability of the instrument, it is advisable to mount the laser and optics to an optical bench and enclose the diode laser, optics, and PMT with a nonreflective encasement. Calibration and optimization methods for the diode laser may be obtained from a variety of sources (50,51).

CONCLUSION

The use of solid-state diode lasers as light sources in LC-fluorescence detectors enhances the ability to measure ultralow levels of analytes in biological matrices. The output of diode lasers in the far-red and near-infrared regions of the

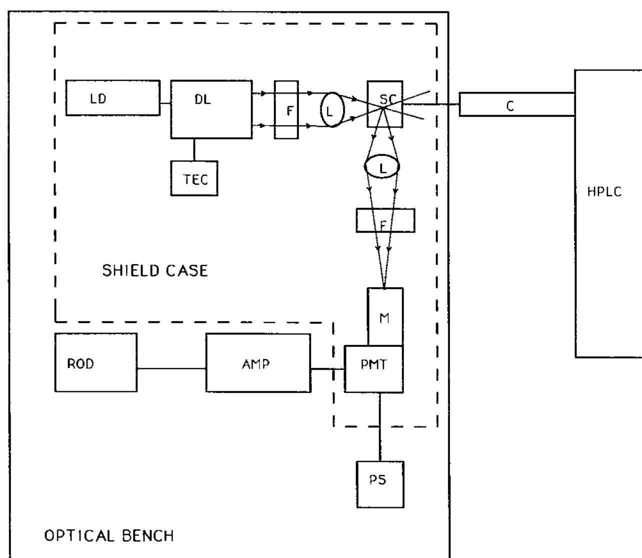

Fig. 4. Schematic of a LC-diode laser fluorescence detector. LD, Laser driver; DL, diode laser; TEC, thermoelectric cooler; F, filter; l, lens; SC, sample cell; M, monochromator; PMT, photomultiplier tube; PS, power supply; AMP, amplifer; ROD, Readout device; C, column; HPLC, high-performance liquid chromatography.

spectrum ensures minimal background interference due to the biological matrix and provides for methods that are instrument rather than matrix limited. Rapid improvements in diode laser technology have resulted in solid-state diode lasers oscillating with narrow linewidths, high amplitude stabilities, and high output powers. These characteristics of diode lasers enhance instrumental detectability beyond what can be achieved with conventional fluorometry. Problems associated with sample degradation are also reduced considerably because excitation is performed at low energy wavelengths. Aggressive research into the synthesis and characterization of water-soluble far-red and near-infrared probes with derivatizable functional groups is required for the technique to develop fully.

REFERENCES

1. J. Van der Greef, H. Lingeman, W. M. A. Niessen, and U. R. Tjaden. Future perspectives in bioanalysis. In D. D. Breimer and P. Speiser (eds.), *Topics in Pharmaceutical Sciences*, Elsevier, B.V., 1987, pp. 137–147.
2. R. Weinberger. Fluorescence detection for liquid chromatography. *Lab. Pract.* 36:65–67 (1987).
3. J. Goto. Fluorescence detection. In H. Lingeman and W. J. M. Underberg (eds.), *Detection Oriented Derivatization Techniques in Liquid Chromatography,* Marcel Dekker, New York, 1990, pp. 323–330.
4. E. S. Yeung and W. A. Kuhr. Indirect detection methods for capillary separations. *Anal. Chem.* **63**:275A–282A (1991).
5. H. H. Willard, L. L. Merrit Jr., J. A. Dean, and F. A. Settle Jr. *Instrumental Methods of Analysis,* Wadsworth, Belmont, CA, 1988.
6. T. Imasaka, A. Yoshitake, and N. Ishibashi. Semiconductor laser fluorimetry in the near infrared region. *Anal. Chem.* 56:1077–1079 (1984).
7. Y. Kawabata, T. Imasaka, and N. Ishibashi. Ultramicro flow cell for semiconductor laser fluorimetry. *Talanta* 33:281–283 (1986).
8. T. Imasaka and N. Ishibashi. Diode lasers and practical trace analysis. *Anal. Chem.* **62**:363A–371A (1990).
9. J. A. Melles. *Optics Guide 5,* Melles Griot, Irvine, CA, 1990.
10. T. Imasaka, A. Tsukamoto, and N. Ishibashi. Visible semiconductor laser fluorometry. *Anal. Chem.* **61**:2285–2288 (1989).
11. P. A. Johnson, T. E. Barber, B. W. Smith, and J. D. Winefordner. Ultralow detection limits for an organic dye determined by fluorescence spectroscopy with laser diode excitation. *Anal. Chem.* **61**: 861–863 (1989).
12. D. L. Andrews. *Lasers in Chemistry,* Springer Verlag, Heidelberg, 1986.
13. K. Seeger. *Semiconductor Physics,* Springer Verlag, Heidelberg, 1989.
14. J. M. Hollas. *Modern Spectroscopy,* J. Wiley and Sons, Chichester, 1987.
15. M. Jaros. *Physics and Applications of Semiconductor Microstructures,* Oxford University Press, New York, 1989.
16. D. A. Fraser. *The Physics of Semiconductor Devices,* 2nd ed. Oxford Physics Series, Oxford University Press, Oxford, 1979.
17. The 1992 Buyers Guide. *Laser Focus World* **27**:117–125 (1991).
18. T. Higashijima, T. Fuchigami, T. Imasaka, and N. Ishibashi. Determination of amino acids by capillary zone electrophoresis based on semiconductor laser fluorescence detection. *Anal. Chem.* **64**:711–714 (1992).
19. J. Lawrenz and K. Niemax. A semiconductor diode laser spectrometer for laser spectrochemistry. *Spectrochim Acta* **44B**:155–164 (1989).
20. NewsBreaks. *Laser Focus World* 28:13 (1992).
21. R. N. Zare and G. J. Diebold. Laser fluorimetry: Subpicogram detection of aflatoxins using high pressure liquid chromatography. *Science* **196**:1439–1441 (1977).
22. Inova 318 Ion Laser System. Coherent Laser Group Specifications, Palo Alto, CA, 1990.
23. J. Hicks, D. A. Wilberforce, and G. Patonay. A near infrared diode for an SLM 8000c fluorometer. *Anal. Instrum.* **19**:29–47 (1990).
24. C. M. B. Van den Beld and H. Lingeman. Laser based detection in liquid chromatography with emphasis on laser induced fluorescence detection. In W. R. G. Baeyens, D. D. Keukeleire, and K. Korkidis (eds.), *Luminescence Techniques in Chemical and Biochemical Analysis,* Marcel Dekker, New York, 1991, pp. 237–316.
25. K. Sauda, T. Imasaka, and N. Ishibashi. High performance liquid chromatographic detection based on near infrared semiconductor laser fluorimetry. *Anal. Chem. Acta* 187:353–356 (1986).
26. K. Sauda, T. Imasaka, and N. Ishibashi. Determination of protein in human serum by high performance liquid chromatography with semiconductor laser fluorimetric detection. *Anal. Chem.* 58:2649–2653 (1986).
27. T. Imasaka, T. Okazaki, and N. Ishibashi. Semiconductor laser fluorimetry for enzyme and enzymatic essays. *Anal. Chim. Acta* 208:325–329 (1988).
28. C. March and H. T. Karnes. The stability of indocyanine given in human serum at −20 and −70 degrees celcius. Poster (APQ 1067) presented at the 5th Annual AAPS Conference in Washington, DC, 1991.
29. T. Imasaka, T. Higashijima, and N. Ishibashi. Dehydrogenase and ethanol assay based on visible semiconductor laser spectrometry. *Anal. Chem. Acta* **251**:191–195 (1991).
30. J. Gathje, R. R. Steuer, and K. R. K. Nicholes. Stability studies on indocyanine green. *J. Appl. Physiol.* **29**:181–185 (1970).
31. B. Hollins, B. Noe, and J. M. Henderson. Fluorometric determination of indocyanine green in plasma. *Clin. Chem.* 33(6): 765–768 (1987).
32. J. Zena and G. Patonay. Near infrared fluorescence probe for pH determination. *Anal. Chem.* **63**:2934–2938 (1991).
33. D. A. Willberforce and G. Patonay. Fluoresence quenching studies of near infrared fluorophores. *Appl. Spectrosc.* 43(8): 1450–1455 (1989).
34. M. D. Antoine, S. Devananthan, and G. Patonay. Determination of hydrophobicity of albumins and other proteins using a near infrared probe. *Spectrochim. Acta* **47A**:501–508 (1991).
35. A. E. Boyer, S. Devananthan, D. Hamilton, and G. Patonay. Spectroscopic studies of a near infrared absorbing pH sensitive carbocyanine dye. *Talanta* 39(5):505–510 (1992).
36. G. Patonay and M. D. Antoine. Near infrared fluorogenic la-

bels. New approach to an old problem. *Anal. Chem.* **63**(6): 321A–327A (1991).

37. A. E. Boyer, M. Lipowska, J. Zen, and G. Patonay. Evaluation of near infrared dyes as labels for immunoassays utilizing laser diode detection: Development of near infrared dye immunoassay (NIRDIA). *Anal. Lett.* **25**(3):415–428 (1992).
38. T. G. Parlopoulos and J. H. Boyer. Quasi-aromatic heterocyclics as laser dyes. *SPIE* **1054**:217–223 (1989).
39. S. W. Yeh, L. J. Dng, J. H. Clark, and A. N. Glazer. Fluorescence properties of allophycocyanin and a cross linked allophycocyanin trimer. *Cytometry* 8:91–95 (1987).
40. J. A. Titus, R. P. Haughland, S. O. Sharrow, and D. M. Segal. Texas Red, a hydrophilic, red emitting fluorophore for use with fluorescein in dual parameter flow microfluorometric and fluorescence microscopic studies. *J. Immunol. Methods* **50**:193 (1982).
41. U. Brackmann. *Lambdachrome Laser Dyes: Data Sheets,* Lambda Physic Gmbh, D-3400 Gottingen, Germany, 1986.
42. R. P. Haughland. *Handbook of Fluorescent Probes and Research Chemicals,* Molecular Probes, Eugene, OR, 1989.
43. B. W. Smith, B. T. Jones, and J. D. Winefordner. High precision fluorimetry with a light emitting diode source. *Appl. Spectrosc.* 42(8):1469–1472 (1988).
44. T. Imasaka, T. Fuchigami, and N. Ishibashi. Determination of proteins by gel electrophoresis combined with visible semiconductor laser spectrometry. *Anal. Sci.* 7:491–493 (1991).
45. L. W. Hershberger, J. B. Callis, and G. D. Christian. Submicroliter flow through cuvette for fluorescence monitoring of high performance liquid chromatographic effluents. *Anal. Chem.* **51**:1444–1446 (1979).
46. M. J. Sepaniak and E. S. Yeung. Determination of adriamycin and daunorubicin in urine by high performance liquid chromatography with laser fluorometric detection. *J. Chromatogr.* **190**:377–383 (1980).
47. N. J. Dovichi, J. C. Martin, J. H. Jett, M. Trkula, and R. A. Keller. Laser induced fluorescence of flowing samples as an approach to single molecule detection in liquids. *Anal. Chem.* **56**:348–354 (1984).
48. Photomultiplier Tubes. Hamamatsu Photonics K. K., Electron Tube Center, Shimokanzo, Shizuoka-Ken, 438-01, Japan, 1990.
49. T. M. Rossi. Laser based detectors in chromatographic analysis. *J. Pharm. Biomed. Anal.* 8(6):469–476 (1990).
50. G. G. Guilbault. *Practical Fluorescence: Theory, Methods and Techniques,* Marcel Dekker, New York, 1973.
51. A. J. Pesce, C. G. Rosen, and T. L. Pasby (eds.). *Fluorescence Spectroscopy: An Introduction for Biology and Medicine,* Marcel Dekker, New York, 1971.