Pure & Appl. Chem., Vol. 63, No. 8, pp. 1083–1088, 1991.
Printed in Great Britain.

ADONIS 206922209100095Q

Modern aspects of fluorimetry as applied to clinical chemistry

A. Gómez-Hens

Department of Analytical Chemistry, Faculty of Sciences, University of Córdoba, 14004 Córdoba, Spain

Abstract - This paper deals with advanced aspects of fluorimetric techniques such as derivative synchronous fluorescence spectroscopy, laser-induced fluorescence spectroscopy, stopped-flow fluorescence spectroscopy, fluorescence polarization spectroscopy and time-resolved fluorescence spectroscopy. The potential of each of these techniques in the clinical laboratory is discussed. The advantages of modern fluorimetric techniques over conventional techniques in terms of sensitivity, selectivity, precision, automation and scope of application are also systematically discussed.

INTRODUCTION

The luminescence phenomenon, which involves the emission of electromagnetic radiation by chemical species, is one of the most active current research fields, as shown by the increasing number of papers, reviews and monographs published on this topic each year. Fluorescence is the luminescent phenomenon most frecuently used in the different fields of Analytical Chemistry including Clinical Analysis. In addition to conventional spectrofluorimetry, which involves measurement of an enhanced or quenched fluorescent signal, there is a large variety of fluorimetric techniques such as derivative and synchronous spectrofluorimetry, and the combination of both, fluorescence polarization spectroscopy, fluorescence line-narrowing spectroscopy (where a laser as excitation source and cryogenic conditions are used), and time-resolved techniques (where the fluorescence lifetime is measured).

Regarding fluorimetric methodologies, some methods involve chemical reactions, while others are based on the native fluorescence of the analytes. One can also distinguish between equilibrium or endpoint methods and kinetic methods; the latter include methods involving reaction-rate or fluorescence lifetime measurements. This paper presents an overview of some of the most recently reported fluorimetric techniques as applied to Clinical Chemistry, namely derivative synchronous fluorescence spectroscopy, laser induced fluorescence spectroscopy, stopped-flow fluorescence spectroscopy, fluorescence polarization spectroscopy and time-resolved fluorescence spectroscopy. A brief description of each is made and some of their main clinical applications are commented on.

DERIVATIVE SYNCHRONOUS FLUORESCENCE SPECTROSCOPY

The combination of derivative (ref. 1) and synchronous (ref. 2) fluorescence spectroscopy was first suggested by John and Soutar (rèf. 3) as a mean of improving the selectivity of analytical methods. In synchronous spectrofluorimetry, the excitation and emission monochromators are scanned simultaneously at a constant wavelength ($\Delta\mu$) (ref. 2) or energy ($\Delta\nu$) (ref. 4) difference. The resulting synchronous fluorescence spectra are simpler, narrower and hence more distinct than conventional excitation and emission fluorescence spectra (ref. 5). These features make this technique specially useful for the elimination of matrix effects and the resolution of mixtures. However, although synchronous spectra are often sufficiently resolved for analytical purposes, their derivatization to the first or second order spectra is also useful to resolve closely spaced bands. Thus, when conventional spectra are strongly overlapped, the synchronous technique reduces the extent of overlap but does not allow the mixture to be adequately resolved. However, complete resolution is still possible if the derivative synchronous spectrum is recorded.

The main advantages of derivative-synchronous fluorescence spectroscopy are selectivity, low cost, simplicity and rapidity, while its chief constrains are its not too high resolution, which is never comparable to that of chromatographic techniques, and the decreased signal-to-noise ratio.

Table 1 summarizes some applications of this technique to clinical analysis. It has been used for the direct determination of catecholamines (ref. 6) and porphyrins (ref. 7) in urine and pyridoxal and its derivatives (ref. 8) and cephalosporins (ref. 9) in serum. Thus, the determination of pyridoxal, pyridoxal-5-phosphate and pyridoxic acid in serum by conventional fluorimetry involves a series of steps including deproteination with trichloracetic acid (TCA), removal of excess TCA and serum interferences, and separation of the analytes by a chromatographic technique, usually HPLC, which is rather time-consuming for routine assays and generally calls for special, expensive instrumentation. However, derivative synchronous fluorescence spectroscopy allows the direct determination of these compounds in serum with only a prior deproteination step with TCA.

TABLE 1. Derivative-synchronous fluorescence spectroscopy applied to clinical analysis.

Mixtures	Matrix	Dynamic Range	Reference
Epinephrine +	Urine	2 - 50 ng/ml	6
Norepinephrine		2 - 50 ng/ml	
Coproporphyrins +	Urine	0.4 - 250 ng/ml	7
Uroporphyrins		0.4 - 300 ng/ml	
Pyridoxal +		4 - 32 ng/ml	
Pyridoxal-5-Phosphate +	Serum	4 - 36 ng/ml	8
Pyridoxic acid		1 - 1.500 ng/ml	
Cephradine +	Serum	0.025 - 5 μg/ml	9
Cephalexin		0.01 - 15 μg/ml	

LASER INDUCED FLUORESCENCE SPECTROSCOPY

Although laser instruments have not yet been widely accepted as routine instrumentation in the clinical laboratory, their unique properties (high output power, collimation, monochromaticity and temporal resolution) make them quite useful for the development of analytical methodologies based on fluorimetry and used in the clinical and biological fields (ref. 10).

Thus, regarding chromatographic separations and, particularly, those using liquid chromatography, excitation with intense laser radiation may improve analyses by lowering limits of detection and/or eliminating cumbersome concentration steps. This technique has also been used to develop both enzymatic and immunochemical methods of clinical analysis. The high power supplied by lasers allows the intensity of the fluorescence signal to be increased, while the monochromatic output minimizes background radiation, so the resulting limits of detection are generally lower than those obtained with conventional radiation sources. Thus, fluoroimmunoassays are usually less sensitive than radioimmunoassays by a few orders of magnitude, but the increased sensitivity afforded by laser excitation makes the former much more competitive with the latter.

Another interesting application of laser induced fluorescence spectroscopy involves the use of fiber optic devices. The small size of laser-based single-strand fiber-optic sensors enables in vivo measurements of solution fluorescence in body fluid reservoirs usually considered to be too small for analysis. This instrumentation has been used, among others, to monitor the fluorescence of doxorubicin in the intersticial fluids of human tumors transplanted into immune-deficient laboratory mice (ref. 11).

This technique has also been applied in cytology for diagnosing tumors and atherosclerosis (ref. 12). The ability to focus intense beams tighly on subcellular dimensions is important in obtaining measurable signals from weakly fluorescent cells, and essential when morphological information is required.

The clinical applications of laser induced fluorescence spectroscopy discussed so far involve condensed-phase fluorescence measurements at room temperature. Under these conditions, excitation and emission spectra are very broad and featureless, mainly because of spectral congestion and variations in the microenvironments of a given type of solute within its sample matrix. The resulting broad spectrum makes it impossible to exploit the enhanced analytical selectivity afforded by the monochromaticity of lasers. However, the use of certain procedures in sample preparation can result in narrow-line fluorescence spectra by reducing the effects of non-homogeneous broadening, thereby giving rise to high-resolution methods. One of the techniques developed for this purpose is fluorescence line-narrowing spectroscopy (ref. 13 - 15), in which a subset of species occupying similar lattice sites in the solid state is selectively excited. This technique has two main requirements: 1) a laser is used as excitation source to obtain a small bandwidth compared with that of the absorbing species in order to obtain line-narrowing fluorescence emission; 2) sample cooling equipment is required to render the effects of thermal broadering negligible. The resulting spectrum can be very sharp, thereby enabling highly selective measurements on complex samples. This technique has been applied to a wide variety of biomolecular systems including cellular macromolecular damage and chemical carcinogenesis (ref. 16).

STOPPED-FLOW FLUORESCENCE SPECTROSCOPY

The use of reaction-rate methods in chemical analysis has gained popularity in recent years, especially with laboratories where speed and selectivity are mandatory. Among the automatic approaches developed in this context, the stopped-flow technique is the most commonly used when fast reactions are involved. In applying this technique, two reactants are driven at a high speed by means of two drive syringes through a mixing chamber, the flow of the reactants is abruptly stopped by using a third, stop syringe, and the extent of reaction is monitored by measuring a given property of the solution such as its absorbance or fluorescence emission.

The main features of the stopped-flow technique are its ability to mix sample and reagent solutions automatically, the possibility of making measurements shortly after mixing, a high overall precision, the minimization of potential interferences, and its suitability for fast and slow reactions. These features make it particularly attractive to clinical laboratories, where automated instrumentation, high sample throughput and low solution volumes are required.

One limitation of this technique is the high cost of commercially available stopped-flow instruments, which somehow limits its use. To overcome this limitation, an inexpensive stopped-flow mixing module was designed by our research group (ref. 17). It can be readily fitted to any spectrophotometer or spectrofluorimeter and is controlled by means of a simple on-line computer and applied to routine analyses. The module consists of three parts: the mixing system, the propelling syringe system and the thermostated chamber.

We have used the stopped-flow fluorescence spectroscopy technique in clinical analyses for the determination of individual species and the resolution of mixtures. Thus, we developed a simple, direct method for the fluorimetric determination of uric acid in serum and urine samples based on the fluorescent reaction between uric acid and TRIAP (1,1,3-tricyano-2-amino-1-propene) in the presence of hydrogen peroxide (ref. 18). This method avoids the use of enzymatic reagents and, because of its low detection limit and high selectivity, is particularly useful when the blood sample volume is difficult to obtain (e.g. in paediatric work).

We also developed a stopped-flow method for the simultaneous fluorimetric determination of epinephrine and norepinephrine in urine samples (ref. 19). These catecholamines are usually determined fluorimetrically by the trihydroxyindole method (ref. 20) in two steps: oxidation of the catecholamines to their corresponding adrenochromes, and subsequent rearrangement to the fluorescent lutins in an alkaline solution. With our stopped-flow method, the overall reaction (oxidation and tautomerization) takes place in a single step at a pH intermediate between the optimum values required for the two processes. This simplified scheme cannot be applied by using slow mixing methods because the formation rate of the lutins is very high in the presence of boric acid, which is used as eluent in the prior ion-exchange separation of the catecholamines from the urine sample matrix. Thus, only the stopped-flow technique allows the simultaneous kinetic resolution of mixtures of the two catecholamines.

FLUORESCENCE POLARIZATION SPECTROSCOPY

This technique was developed about forty years ago (ref. 21) and was widely used in theoretical studies of fluorescent molecules. However, the main analytical interest of this technique today lies with immunoassays because of its suitability for homogeneous determinations in therapeutic drug monitoring.

When a fluorescent compound is excited with linearly polarized light produced by a polarizing filter placed between the light source and the sample, those molecules with their absorption dipoles aligned with the electric vector of the polarized light, have the highest excitation probability. The emission of polarized radiation from these molecules will mainly depend on the lifetime of the excited state compared with the time required for rotational motion, as well as on environmental variables such as viscosity and temperature. When substances with a small molecular volume are excited by polarized light, their molecules have a rotation rate that is much higher than the fluorescence decay rate, so little or no polarized fluorescence is obtained. However, molecules with very large molecular volumes rotate at a rate comparable to or lower than the rate at which their fluorescence decays, so the fluorescence will be partially polarized.

The degree of polarization can be calculated from the expression:

$$p = \frac{I_H - I_V}{I_H + I_V}$$

where I_H and I_V are the fluorescence intensities measured as the emission polarizer placed between the sample and the detector lies paralel or perpendicular, respectively, to the excitation polarizer.

Fluorescence polarization immunoassays (ref. 22) are based on the difference in molecular volume of a small fluorescent-labelled antigen or hapten, which acts as the tracer, when free and when bound to a bulky antibody. The polarized fluorescence emitted by the free tracer is low because of the small molecular volume and fast rotational motion, while that emitted by the antibody-bound labelled antigen is high as a result of the increased molecular volume.

This technique allows homogeneous determinations to be carried out in so far as permits one to discriminate between free and labelled antigens without the need for a prior separation step. This makes it readily adaptable for automated analyses. One other important feature is that it is only suitable for the assay of low-molecular weight antigens such as haptens, whereas it is not applicable to antigens with high molecular weights because only a relatively small change in polarization will then be observed.

The main problem encountered in applying this technique to biological samples, particularly serum samples, which are the most commonly encountered in immunoassays, is it low sensitivity, which is limited by three factors: 1) The background fluorescence of the serum samples, which arises from partly scattered light and partly from the intrinsic fluorescence resulting from the presence of fluorescent compounds such as NADH and bilirubin; 2) Non-specific binding of the tracer to serum proteins such as albumin, which is probably the chief agent responsible for this interaction because it has versatile ligand properties and shows affinity for many anionic dyes such as fluorescein, the most common label used in this technique; 3) Finally, the presence of the polarizers in the excitation and emission beams reduces the light intensity and the potential sensitivity by a factor of up to about 10.

In spite of these limitations, the advent of microprocessor technology, improvements in optics and detectors, and advances in tracer design and immunological techniques have turned fluorescence polarization immunoassay into a practical technique for use in the clinical laboratory (ref. 23). Some commercially available instruments allow one to implement this technique in an automatic fashion. It is widely used for the determination of therapeutic drugs such as anticonvulsants, antibiotics, antiarrhythmics, tricyclic antidepressants and other such as theophylline, acetylsalicylic acid, paracetamol, etc. Also, a number of methods for the determination of drugs of abuse and hormones have been reported.

TIME-RESOLVED FLUORESCENCE SPECTROSCOPY

This kinetic technique involves measuring the fluorescence lifetime (or fluorescence decay time) of a species (ref. 24). The instrumentation required includes a pulsed excitation source with an exciting pulse substantially shorter than the lifetime of the fluorescent species, and very responsive detection system and processing electronics. Because the fluorescence lifetimes of many conventional fluorescent probes fall in the nanosecond time range, this instrumentation is rather complex. However, there is a special

group of fluorescent compounds formed by a series of lanthanide chelates with fluorescence decay times from 10 to 1000 μs (ref. 25). Such long lifetimes allow the development of simple, inexpensive instrumentation, in which an N_2-laser (ref. 26) or simply a pulsed xenon discharge lamp (ref. 25) is used for excitation.

Lanthanide ions, particularly Eu^{3+} and Tb^{3+}, form fluorescent complexes which absorb radiation at the characteristic wavelength of the ligand and emit radiation with the characteristic wavelength of the lanthanide ion. Energy is absorbed by the organic ligand, which is promoted to an excited singlet state, after which it jumps to a triplet state, and finally its energy is transferred to the metal ion, which subsequently emits characteristic radiation. This is the reason for the long lifetime of the lanthanide complexes, which is also dependent on the temperature and solvent used.

The use of lanthanide chelates as labels has given rise to a new immunoassay technique, namely time-resolved fluoroimmunoassay (TRFIA) (ref. 27), which can advantageously replace other isotopic and non-isotopic immunoassays. Thus, the main problems posed by conventional fluorimetry as applied to immunoassays where fluorescein is used as a label include: 1) poor separation between fluorescence emission and excitation; 2) Rayleigh and Raman scattering; 3) background fluorescence from cuvettes, optics and sample; 4) nonspecific binding of the reagents; and 5) fluorescence quenching. Thus, the autofluorescence of serum, which is primarily due to bilirubin bound to albumin, overlaps significantly with the emission spectrum of fluorescein and contributes to a high background signal.

The following are some of the advantages of using lanthanide chelates as fluorescent probes for biological materials: high quantum yields, excepcionally large Stokes shifts, narrow emission peaks and optimal emission and excitation wavelengths. Thus, the hydrolyzed 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid-Eu^{3+} complex (BCPDA-Eu^{3+}) (ref. 27) has a Stokes shift of 290 nm as compared to 28 nm for fluorescein, which makes separation of excitation and emission wavelengths very easy. Also, the emission bandwidth is very narrow, which allows the use of narrow band-pass emission filters with no loss of energy.

A large number of applications have been developed by using time-resolved fluoroimmunoassays (ref. 28); many of them are now used in clinical routine analysis as commercially available kits. Some of the clinical fields where this technique has proved to be useful are: gynaecology, thyroid disease, cancer diagnosis, viral infections, citotoxicity and enzyme activity measurement. In terms of automation, there are currently in the market instruments suitable for performing this technique in a fully automated fashion and with random access capability.

CONCLUSIONS

The examples briefly outlined here allow us to conclude that modern fluorimetric techniques applied to clinical problems provide analytical methodologies which improve on one or more of the basic properties of the analytical process, namely sensitivity, selectivity, precision, rapidity and automation. Thus, the use of lasers as excitation sources improves the sensitivity of conventional fluorimetric methods; the features of derivative synchronous fluorescence spectroscopy allow the development of selective fluorimetric methods; stopped-flow fluorescence spectroscopy has given rise to the development of precise, fast and automatic methods; the use of fluorescence polarization enables development of automatic homogeneous immunoassay methods; finally, time-resolved fluorescence spectroscopy circumvents many of the shortcomings of conventional fluorescence immunoassays.

REFERENCES

1. G.L. Green and T.C. O'Haver, Anal. Chem., 46, 2191-2196 (1974).
2. J.B.F. Lloyd, Nature, 64 64-65, (1971).
3. P. John and I. Soutar, Anal.Chem., 48, 520-524 (1976).
4. E.L. Inman Jr. and J.D. Winefordner, Anal. Chem., 54, 2018-2022 (1982).
5. T. Vo-Dinh, Anal. Chem., 50, 396-401 (1978).
6. M. Valcárcel, A. Gómez-Hens and S. Rubio, Clin. Chem., 31, 1790-1794 (1985).
7. M. Valcárcel, A. Gómez-Hens, S. Rubio and A. Petidier, Clin. Chem., 33, 1826-1831 (1987).
8. A. Petidier, S. Rubio, A. Gómez-Hens and M. Valcárcel, Anal. Biochem., 157, 212-220 (1986).
9. P. Izquierdo, M.C. Gutiérrez, A. Gómez-Hens and D. Pérez-Bendito, Anal. Lett., 23, 487-505 (1990).
10. M.J. Sepaniak, Clin. Chem., 31, 671-678 (1985).

11. M.J. Sepaniak, Bj. Tromberg and J.F. Eastham, Clin. Chem., 29, 1678-1682 (1983).
12. S. Andersson-Engels, J. Johansson, K. Svanberg and S. Svanberg, Anal. Chem., 62, 19A-27A (1990).
13. M.J. Feld and A. Javan, Phys. Rev., 177, 549-554 (1969).
14. A. Szabo, Phys. Rev. Lett., 25, 924-928 (1970).
15. A. Szabo, Phys. Rev. Lett., 27, 323-328 (1971).
16. R. Jankowiak and G.J. Small, Anal. Chem., 61, 1023A-1032A (1989).
17. A. Loriguillo, M. Silva and D. Pérez-Bendito, Anal. Chim. Acta, 199, 29-40 (1987).
18. D. Pérez-Bendito, A. Gómez-Hens, M.C. Gutiérrez and S. Antón, Clin. Chem., 35, 230-233 (1989).
19. M.P. Llavero, S. Rubio, A. Gómez-Hens and D. Pérez-Bendito, Anal. Chim. Acta, 229, 27-33 (1990).
20. A. Lund, Acta Pharmacol. Toxicol., 6, 137-146 (1950).
21. G. Weber, Adv. Protein Chem., 8, 415-459 (1953).
22. W.B. Dandliker, R.J. Kelly, J. Dandliker, J. Farquhar and J. Levin, Immunochemistry, 10, 219-227 (1973).
23. M.C. Gutiérrez, A. Gómez-Hens and D. Pérez-Bendito, Talanta, 36, 1187-1201 (1989).
24. F.E. Lytle and M.S. Kelsey, Anal. Chem., 46, 855-860 (1974).
25. E. Soini and H. Kojola, Clin. Chem., 29, 65-68 (1983).
26. J.E. Kuo, K.H. Milby, W.D. Hinsberg III, P.R. Poole, V.L. Guffin and R.N. Zare, Clin. Chem., 31, 50-53 (1985).
27. E.P. Diamandis, Clin. Biochem., 21, 139-150 (1988).
28. E. Soini and T. Lövgren, Nonisotopic Immunoassay, p. 231, Plenum Press, New York (1988).