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Photoluminescence and chemiluminescence methods of drug analysis*

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Abstract: The analysis of drugs in biological fluids requires sensitive, selective and convenient methods. Luminescence techniques provide extreme sensitivity and, alone or in conjunction with other methods, excellent selectivity. This paper assesses several recent developments in luminescence analysis, including the use of derivative and synchronous spectroscopy, luminescence immunoassays, room temperature phosphorimetry, and fluorescence excited by chemiluminescence energy transfer.

Keywords: *Derivative spectroscopy; synchronous scanning fluorimetry; total luminescence spectrometry; fluorescence immunoassays; chemiluminescence immunoassays; room temperature phosphorimetry; chemiluminescence energy transfer.*

Introduction

The analysis of drugs in biological fluids is probably among the most difficult tasks performed in an analytical laboratory. The analytes are often present at ng/ml, pg/ml or even lower concentrations, and sample volumes may be only a few microlitres: exceptional sensitivity is thus required. The sample matrices are immensely complex and contain hundreds of chemical species, including probably analogues and metabolites very similar in structure to the analyte: a highly selective method is thus essential. In addition, biochemical samples are dynamic systems — for example a drug may be protein-bound to an extent that depends on the identities and amounts of other materials present in the sample. If these essentially chemical problems can be overcome, a number of technical problems remain. A suitable method must be rapid, easily automated, moderate in cost, and free of hazards. Not surprisingly, a number of methods that are acceptable for less testing analyses are unsuitable for drug determinations, and only a relatively limited number of techniques are in widespread use.

Luminescence methods have found many applications in the field of drug analysis. Since the late 1950s, fluorescence (FL) techniques have been widely adopted, and more recently chemiluminescence (CL) and bioluminescence (BL) methods have become increasingly popular. The principal advantages of these methods lie in their excellent

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limits of detection (pg/ml and fg/ml for FL methods and even lower levels in CL/BL analyses), their spectroscopic selectivity, and the wide range of sampling procedures with which they can be combined, for example in conjunction with liquid and laminar chromatographic techniques.

In practice relatively few FL, CL or BL analyses are performed directly on the sample or a simple extract of the sample. Such methods are generally insufficiently selective and may in consequence show poor limits of detection (see below). Most luminescence determinations involve additional experimental steps or data-handling procedures to improve selectivity and sensitivity. Three major approaches are available in this context [1]: (A) *Selective spectroscopy* — in recent years several methods have been investigated with a view to increasing the selectivity of fluorescence (and phosphorescence) methods. Of particular interest are synchronous, derivative and contour spectroscopy; (B) *Immunoassay methods*, in which additional specificity is furnished by a biochemically-specific antigen–antibody reaction; (C) *Combined luminescence–chromatography methods*, particularly h.p.l.c. and t.l.c. with fluorescence and phosphorescence detection. In these hybrid techniques, the chromatography provides most of the selectivity, while the luminescence detection may provide extra selectivity as well as great sensitivity.

This paper reviews developments in the field of selective spectroscopy, giving particular attention to the problems caused by the fluorescence background signal from blood serum and other biological samples. It also discusses recent developments in fluorescence and chemiluminescence immunoassays, and assesses the present status of phosphorimetry as a practical analytical method. Finally some future developments in luminescence methods of drug analysis are outlined.

Limits of Detection in Fluorescence Methods — Effects of Background Signals

It is well known that luminescence methods are capable in theory of much better limits of detection than absorptiometric methods. Absorption spectrometry of a very dilute solution involves the detection of a small difference between two substantial light signals. This problem is much harder to solve than that in luminescence spectrometry, where trace analysis involves the detection of a small signal above a theoretically zero background. As already mentioned, however, the extraordinary capacity of luminescence methods in trace analysis is often confined in practice to pure solutions of luminescent solutes. In multicomponent samples substantial background signals occur, so that the small luminescence signal must after all be observed against a non-zero background: in other words the main theoretical advantage of fluorimetry and other luminescence methods is lost.

This background signal has two main causes. First, there is often a substantial contribution from scattered and stray light in the fluorescence spectrometer (this effect should not, of course, occur in well-designed chemiluminescence detectors). Scattered light signals from protein-containing samples are particularly strong, and their effects will be worst when a fluorophore has a small Stokes shift (i.e. difference between the excitation and emission wavelengths — fluorescein is a good example of such a fluorophore, its Stokes shift being only *ca* 30 nm). The stray light characteristics of many commercial fluorescence spectrometers are rather indifferent, exacerbating this source of background signal. The second source of background signals is the unwanted fluorescence arising from solvents, cuvettes and, most importantly, other fluorophores in

the sample matrix. This type of background signal is serious because of the large spectral bandwidths of many fluorophores. Bandwidths at half-maximum intensity of 70 nm or more are not uncommon. In practice, the result is that the fluorescence of an interfering endogenous compound may have a serious effect at wavelengths well removed from its excitation and emission maxima. It is thus apparent that any spectroscopic method by which fluorescence bandwidths can be reduced will be of considerable potential value, and a number of such methods are further discussed in subsequent sections.

In clinical analysis and related studies such as pharmacokinetics the principal biological sample used is blood serum (or plasma). Urine and (more recently) saliva are also of value. The fluorescence from normal human urine has been thoroughly studied [2], but is much less intense than the fluorescence of serum/plasma; the fluorescence of saliva has apparently not been investigated. Rather surprisingly, the fluorescence of normal human serum seems not to have been systematically studied either. A good deal of information is, however, available from various sources, and some methods for reducing the background signal have been suggested.

The principal fluorescence emission from serum is certainly that from the aromatic amino acids, particularly tryptophan. The latter compound has an excitation maximum at 280 nm and (in aqueous solutions) fluoresces at *ca* 350 nm as the free acid and at rather shorter wavelengths when incorporated into proteins. This fluorescence effectively prevents the direct fluorimetric analysis of drugs with absorption bands at 300 nm and below. Extraction (or chromatographic) techniques are needed to clean up the sample in such cases: extraction methods are tedious and may not remove all the problems of scattered light and fluorescence background interference [3]. The remaining fluorescence bands in blood serum (Table 1) are much weaker than the tryptophan emission. In practice, however, they may present more serious background problems because they occur at wavelengths close to those of many common fluorescent and fluorigenic labels. They interfere substantially, for example, with most homogeneous fluorescence immunoassays (see below), and with other important determinations such as the fluorescent staining of electrophoretic separations [4]. Several methods are available to reduce or minimize these interferences, in addition to the spectroscopic bandwidth approaches discussed in the next section. Chemical treatments have been described [5], and a series of simple adsorbent columns has been developed to remove different groups of proteins [6]. Since many low molecular weight endogenous fluorophores are at least partly protein-bound, and since proteins contribute much of the light scattering background from serum, the latter approach may be particularly effective.

Table 1
Serum fluorescence

λ_{ex} (nm)	λ_{fl} (nm)	I (nm)	Origin
285	340	100*	Proteins, amino acids
350	450	0.7	?
400	470	0.5	?
425	520	0.3	Albumin-bound bilirubin?
285 520	650	~0.1	Porphyrins?

*Standard.

Synchronous Fluorescence Spectrometry

Several methods with the important aim of reducing fluorescence spectral bandwidths have been studied. Some of them, such as the different types of matrix isolation spectrometry [7, 8], are too complex for everyday use, and may be restricted in the number of solutes that can be studied. By contrast, synchronous scanning fluorimetry [9] is a readily accessible technique with wide application to many types of analysis. In its simplest form it involves scanning both monochromators of fluorescence spectrometer simultaneously and at the same rate, but with a fixed wavelength difference, $\Delta\lambda$, between them. If the fluorescence properties of a compound are visualized as a 'contour' spectrum (also known as a 'total luminescence' or 'excitation–emission matrix' spectrum: Fig. 1) then a synchronous scan is depicted as a 45-degree section through the contours. The band-narrowing and simplifying effect this procedure has on the spectra is theoretically predictable [10] and easily observed in practice. Most applications of the synchronous method have involved aromatic hydrocarbons, whose sharply structured spectra are much simplified in synchronous mode, but work in the author's laboratory and elsewhere [11–13] has shown its value in the study of compounds with broad and featureless spectra. For example, the fluorescence bandwidth of tryptophan is about 65 nm in the conventional emission spectrum, but less than half this figure in the synchronous scanning mode when a $\Delta\lambda$ value of *ca* 70 nm is used.

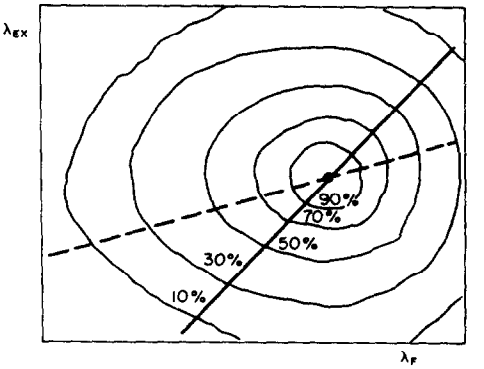

Figure 1
Total luminescence spectrum ('contour spectrum') of a hypothetical fluorophore. The diagonals represent conventional (– – –) and variable angle (— — —) synchronous scans.

The synchronous method can be used in two slightly different ways. If, for example, a two-component mixture of fluorophores is to be resolved, it may be possible to isolate the individual fluorophores by judicious choice of two $\Delta\lambda$ values. It may alternatively be possible to observe both components by using a single $\Delta\lambda$ value, their emissions then appearing at different positions in the spectrum. Many of the attributes of the synchronous method are shown by its application to the notoriously difficult resolution of the fluorescence of the amino acids tyrosine and tryptophan [14, 15]; it is particularly hard to detect a small tyrosine fluorescence signal in the presence of a strong tryptophan emission, using conventional spectra. Use of a $\Delta\lambda$ value of 10 nm allows tyrosine to be observed in synchronous mode without interference from tryptophan: a value of 70 nm shows the two fluorophores at two distinct positions in the spectrum, with excitation/emission wavelengths of *ca* 230/300 nm (tyrosine) and 280/350 nm (tryptophan).

For some mixtures of fluorophores the optimum selectivity might be found by scanning a section through the contours at an angle other than 45 degrees. Such scans can be obtained by scanning the two monochromators at different rates; or by determining the

whole of the contour diagram and calculating the required section using a microcomputer. The former method is simpler [16], and this further approach to selective fluorimetry, which has been christened 'variable angle scanning', already shows promise in multicomponent analysis. Synchronous scanning methods have not yet been applied regularly to the removal of background signals, but there is every reason to expect that they will be successful in such applications. Moreover the advent of microcomputer-controlled spectrometers will facilitate repeated scanning of a narrow wavelength interval without operator intervention, thus allowing synchronous methods to be applied to automatic analysis.

Derivative Fluorescence Spectrometry

Another approach to reducing spectral bandwidths that has recently been applied to many forms of instrumental analysis is the use of derivative methods. The even (i.e. second, fourth, etc.) derivatives of a spectrum can readily [17] be shown to become progressively narrower compared with the original spectrum. Moreover, second derivative spectra (the derivative most commonly used in quantitative analysis) can be obtained without difficulty by electronic or digital processing of the zero-order spectrum. Digital derivative methods may also include some smoothing of the spectrum. This helps to overcome one of the problems of derivative spectroscopy, *viz.* that each successive differentiation step involves a deterioration of the signal:noise ratio. Good second derivative spectra are thus obtained only from high quality zero order spectra, so it is probably unrealistic to expect the derivative approach to operate satisfactorily at ultra-trace solute concentrations. A further disadvantage of derivative methods is that increasing the order of the derivative introduces additional complexity in the form of the satellite bands (Fig. 2 provides a simple example). In the resolution of overlapping

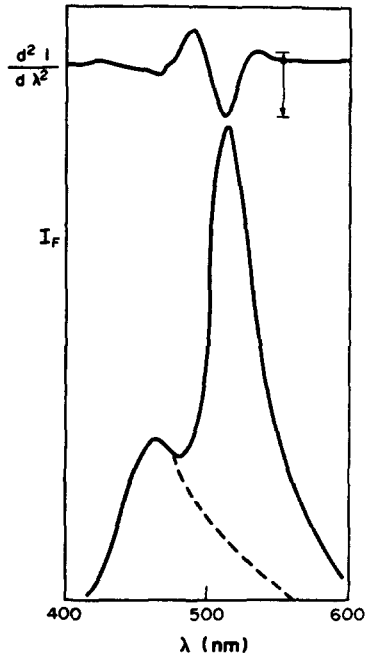

Figure 2
Zero order and second derivative fluorescence spectra of a fluorescein derivative in diluted blood plasma: the intensity of the derivative spectrum measured at the high wavelength satellite is independent of the background signal.

spectra these satellites complicate the picture and tend to nullify the value of the band-narrowing effect.

An essential feature of the derivative method is that it emphasizes zero order spectral bands that are sharp, compared with those that are broad. This attribute has both advantages and disadvantages [18]. It facilitates the determination of a compound whose fluorescence band is superimposed on a continuous background: but it also produces derivative spectra of (unwanted) scattered light peaks that are more intense than the derivative signals from the (desired) broader fluorescence peaks.

Derivative spectroscopy can be combined with synchronous scanning to produce exceptionally narrow bands [19]. This approach has not yet been thoroughly explored, but at present it seems likely that synchronous spectrometry alone will be sufficient for many analytical purposes. Indeed, the power of synchronous scanning methods is so great that, in practice, the application of derivative fluorescence spectrometry may be quite limited.

Fluorescence Immunoassays

Investigations on non-isotopic substitutes for the radiolabels used in radioimmunoassays have been in progress for over 20 years, and recently several fluorescence immunoassay systems have been successfully developed and commercially exploited. Fluorescent or fluorigenic labels are usually easily introduced, cheap, stable and hazard-free. They thus seem to have all the necessary attributes for a range of useful non-isotopic immunoassays. In practice, however, the familiar problems of background scattered light and the fluorescence of the sample matrix have retarded work in this field.

Immunoassays are of two general types. Heterogeneous assays include a physical separation step to separate antibody-bound from unbound analyte. This separation also removes much of the background signal and thus allows very sensitive assays to be developed: but it complicates the assay protocols and, in particular, makes the development of a completely automatic system extremely complex. Homogeneous assays take advantage of a change in the properties of a fluorescent-labelled material when combination with an antibody occurs: the results can be obtained without the need for a separation step, thus simplifying the protocols and expediting the development of fully automated assays. The price to be paid in such methods is the non-removal of the background signal, with the result that detection limits are usually in the ng/ml or μg/ml range. In either assay type the fluorophore may in principle be attached either to the analyte or the antibody: in practice, however, heterogeneous systems tend to be antibody-labelled systems, and homogeneous assays mostly use labelled analytes.

Although several ingenious and effective heterogeneous fluorescence immunoassay systems have been marketed [20–22], the ultimate goal must be the development of a very sensitive homogeneous assay method — one in which the background problems are minimized or abolished — and this section reviews the latest developments in homogeneous fluorescence immunoassays. Perhaps the earliest type of fluorescence immunoassays to be intensively studied was the fluorescence polarization immunoassay [23]. Fluorescence polarization [24] is defined by $p = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are respectively the fluorescence intensities of the sample when viewed between parallel and crossed polarizers. A solution of a low molecular weight fluorophore should have a p value close to zero, because in the 10 ns or so that elapse between the excitation of the molecules and their fluorescence emission (the 'fluorescence lifetime') the

molecules will freely tumble in solution, collide with other molecules, etc., and thus lose all trace of their original orientations. By contrast a solution containing macromolecules, or low molecular weight fluorophores bound to macromolecules, will have a polarization value >0, because the solute molecules will have retained some trace of their original orientations. Polarization measurements provide an elegant and simple method for distinguishing between antibody-bound and unbound antigens, and hence the basis for a homogeneous assay method. In theory, when polarized exciting light is used, $p < 0.5$, though in practice it seems that the polarization values change by only small amounts (*ca* 0.1–0.2 units). If small changes in p are detectable (use of a thermostatted sample compartment and a stable fluorescence spectrometer should allow changes of 0.001–0.002 to be measured) small proteins as well as low molecular weight analytes can be determined [25], so the fluorescence polarization immunoassay method is in principle a more or less universal technique for the analytes normally studied using immunoassays. A recently introduced commercial system, using electro-optic polarizers in a fully automatic spectrometer, shows the potential of the method [26]. In practice, however, the background problems will continue to be severe, until fluorophores can be developed whose excitation and emission wavelengths are more suitable than those presently available. Scattered light may be expected to be a problem in polarization immunoassays, since it is strongly polarized: the fluorophore used should thus have a large Stokes shift.

In addition to the search for more suitable fluorophores of the conventional type, several research groups have studied the feasibility of using delayed emitters as labels in immunoassays. This approach has the advantage that the background scattered light and fluorescence signals decay very rapidly (<*ca* 20 ns). A label with a much longer lifetime can thus be readily distinguished from the background using a fluorescence spectrometer with a pulsed light source and a gated detector [27]. Several such instruments are commercially available [28, 29], thus stimulating interest in this approach. Delayed emitters are of two types — lanthanide ions, and phosphorescent organic molecules. Both types give rise to emissions with millisecond lifetimes, and so combat the background problem very effectively, but both have disadvantages as well. The lanthanide ions (usually Tb(III) or Eu(III)) are only feebly luminescent unless they form part of a chelated organic complex, and the synthesis of bifunctional molecules that incorporate such chelates and can also be covalently bound to proteins and other molecules is difficult [30]. Even when such a complex is obtained, there is a risk that it will not be stable in physiological conditions, i.e. that the lanthanide ion might be displaced by an endogenous metal ion. In addition the luminescence properties of the lanthanides, which arise from orbitals in the interior of the electron clouds of the ions, are not susceptible to environmental factors, and thus do not change significantly when antigen–antibody combination occurs. The result is that assays based on lanthanides seem likely to continue to be heterogeneous. But, as indicated above, heterogeneous methods do not in any case suffer very severe background effects — the whole concept of using a lanthanide label system is thus questionable.

The phosphorescence of organic molecules provides a second approach to the development of time-resolved immunoassays. The measurement of phosphorescence at room temperature is discussed in more detail in a subsequent section. Here we merely note that such measurements are feasible, though the intensity of the emitted light is likely to be low because of collisional quenching. Oxygen quenching of the triplet state is a particularly severe problem, and appropriate precautions must be taken: these include

the addition of sodium sulphite or a similar oxygen scavenger, and/or purging the solution with oxygen-free nitrogen. Erythrosin isothiocyanate is a suitable phosphorescent label, and homogeneous assays based on the intensity changes accompanying antibody binding are feasible [31]. While time-resolved assays show some promise, it may be that in the long term better results will be obtained by the use of conventional fluorophores, but with luminescence properties superior to those in current use.

In recent years, chemiluminescence (CL) immunoassays have been intensively studied [32, 33]. They have several immediately apparent advantages — very low detection limits, absence of scattered light interference, and very simple detection equipment. Homogeneous assays, based on changes of luminescence intensity or changes of reaction rate, are feasible [34, 35]. Again, however, there are several problems. While the background signals may cause less interference than in fluorescence assays, most CL systems seem to suffer substantial quenching (the mechanism is perhaps free radical scavenging) in biological sample matrices: some progress in overcoming this problem has recently been reported [36]. Further difficulties are the problems of reproducible reagent mixing (the intensity of a chemiluminescence signal is proportional to the rate at which the excited state product is formed), and the difficulties of incorporating CL labels with high quantum yields into analyte molecules. Work in the author's laboratory and elsewhere has shown that these two last problems can be tackled by the use of flow injection analysis [37] equipment coupled with a luminometer, and by the use of a CL energy transfer system to excite a conventional fluorophore. In the latter connection the oxalate ester systems discovered several years ago, but only recently used in analytical work [38], are of particular interest (Fig. 3). Flow injection analysis provides

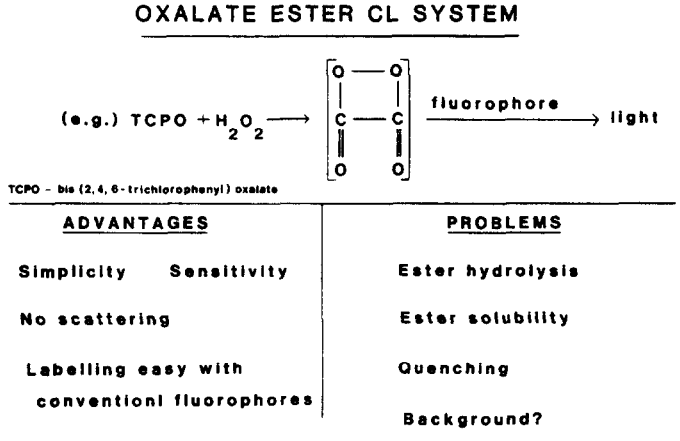

Figure 3
Use of the oxalate ester method to excite luminescence by energy transfer.

reproducible mixing and very precise control over the timing of the CL reaction, and the excitation of a fluorophore by the oxalate ester system gives a method with the sensitivity of a CL technique combined with the ease of labelling with a fluorophore such as fluorescein isothiocyanate or Lucifer Yellow VS [39]. While the problems of developing CL immunoassays are not entirely overcome, there seems every likelihood that they will eventually supplant fluorescence methods.

Phosphorescence Analysis Methods

Phosphorimetry was first used in analytical work in 1957, but was prevented from achieving widespread popularity by the belief that most organic molecules phosphoresce only in cryogenic conditions. About 1970, there was an upsurge of interest in room temperature phosphorescence (RTP) phenomena, and in recent years several new methods of obtaining quite intense RTP signals have been observed, drug analysis providing one of the major areas of application.

Phosphorimetry at liquid nitrogen temperature (77 K) is admittedly an inconvenient method; but it is capable of great sensitivity and selectivity. Its advantages include the absence of scattered light interference (cf. the previous section), the use of time resolution methods to distinguish the components of phosphorescent mixtures, and the ability to study many molecules that show little or no intrinsic fluorescence. Included in the latter category are many heterocyclic molecules, and species containing electron-withdrawing or 'heavy-atom' substituents (e.g. NO_2, Br, etc.). Phosphorimetry can be successfully combined with thin layer chromatography [40, 41]. An example of the application of the combined technique is given in Fig. 4.

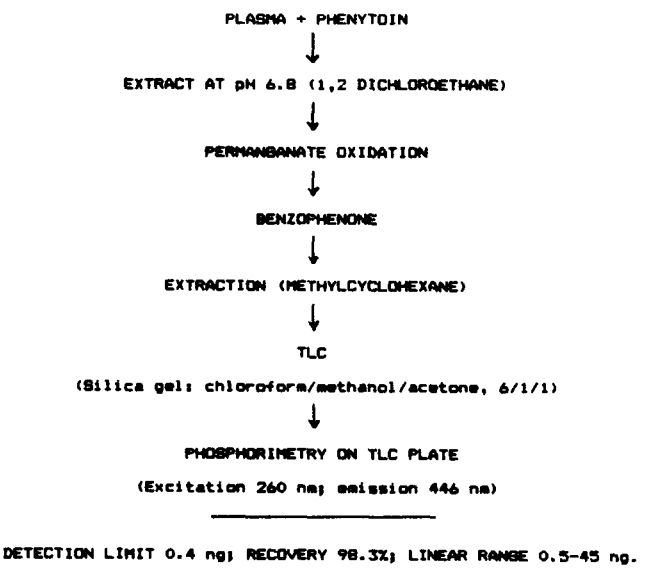

Figure 4
Determination of plasma phenytoin by combined thin layer chromatography and phosphorimetry at 77 K.

Several approaches to the detection of RTP are now available, and extraordinary ingenuity has been expended on the development of methods that provide the advantages of phosphorimetry without the need to cool the sample. Many organic compounds show appreciable RTP signals when adsorbed on solid (especially cellulosic) surfaces [42]. In this environment the molecules seem to be at least partly protected from the effects of oxygen quenching, but the RTP signal is vulnerable to the presence of water vapour, so thorough drying is necessary, and again inconvenient. The RTP intensity is usually in these circumstances much lower than the signal that would be obtained at 77 K. Further approaches to RTP measurements include the development of

sensitized RTP [43, 44] which can be used as a detection system for h.p.l.c. separations; the use of detergent micelles to protect the solute molecules from quenchers [45], and most recently and most promisingly, the use of cyclodextrins for the same purpose [46]. These advances strongly suggest that phosphorimetry is about to break through to the status of a major analytical technique in biological and environmental chemistry.

Conclusions

This review has of necessity been highly selective: but it is apparent that photoluminescence and chemiluminescence methods have great value in the field of drug analysis. They are capable of extreme sensitivity, selectivity, and harmonious combination with a great variety of other techniques. Moreover, the very substantial research effort now being invested in this area of analytical science is bringing corresponding benefits. The development of new fluorescent label molecules, the use of laser light sources, fibre optics and multichannel detectors, more detailed studies of the effects of detergents on luminescence phenomena, new microcomputer-based methods for the resolution of overlapping spectra, and the more widespread use of chemiluminescence and bioluminescence phenomena, are just some of the dynamic research areas that will ensure the continuing and expanding use of luminescence techniques.

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