Journal of Pharmaceutical & Biomedical Analysis
Vol. 4, No. 6, pp. 771–775, 1986
Printed in Great Britain

0731–7085/86 $3.00 + 0.00

Use of luminescence spectroscopy for assay of pharmaceutical compounds*

GEORGE G. GUILBAULT

Keywords: *Fluorescence; immunoassay; enzyme immunoassay; solid-state fluoro-immunoassay.*

Introduction

Luminescence is one of the oldest and most well established analytical techniques, having been first observed by Monardes in 1565 from an extract of *Ligirium nephiticiem*. Sir David Brewster noted the red emission from chlorophyll in 1833, and Sir G. G. Stokes described the mechanism of the absorption and emission process in 1852. Stokes also named fluorescence after the mineral fluorspar (from the Latin: fluo, to flow; spar, rock), which exhibits a blue–white fluorescence.

Phosphorescence dates back to the early 1500s, being so named after the Greek word for "light bearing", also used for the element phosphorus since it was found to produce a bright light in a dark room.

The basic equation defining the relationship of fluorescence to concentration is:

$$F = K\Phi I_O (1 - e^{-\varepsilon bc}),$$

where Φ is the quantum efficiency, I_O is the incident radiant power, ε is the molar absorptivity, b is the path length of the cell, c is the molar concentration and K is a constant.

The basic fluorescence intensity–concentration equation indicates that there are three major factors, other than concentration, that affect the fluorescence intensity:

(1) the quantum efficiency, Φ. The greater the value of Φ, the greater will be the fluorescence;

(2) the intensity of incident radiation, I_O. Theoretically, the more intense source will yield the greater fluorescence. In practice a very intense source can cause photo-decomposition of the sample, so that a compromise is made in using a source of moderate intensity;

(3) The molar absorptivity of the compound, ε. In order to emit radiation a molecule must first absorb radiation. Hence, the higher the molar absorptivity, the higher the fluorescence intensity of the compound. It is for this reason that saturated nonaromatic compounds are nonfluorescent.

*Presented at the "Second International Symposium on Drug Analysis", May 1986, Brussels, Belgium.

For very dilute solutions ($A \leq 0.05$) the equation can be simplified:

$$F = K \Phi I_O \varepsilon bc.$$

Thus a plot of fluorescence versus concentration should be linear at low concentrations while at higher concentrations it is non-linear due to quenching and inner-filter effects.

Molecular emission (fluorescence and phosphorescence) is a particularly important analytical technique because of its high sensitivity and good specificity. Fluorimetric methods can detect concentrations of substances as low as one part in ten billion, a sensitivity 1000 times greater than that of most spectrophotometric methods. The main reason for this increased sensitivity is that in fluorescence the emitted radiation is measured directly and can be increased or decreased by altering the intensity of the exciting radiant energy, as indicated above. An increase in signal over a zero background signal is measured in fluorimetric methods. In absorptiometric methods the analogous quantity, absorbance, is measured indirectly as the logarithmic difference between the incident and the transmitted radiation leading to a loss in sensitivity.

The specificity of fluorescence results from two main factors: (a) there are fewer fluorescent compounds than absorbing ones, and (b) two wavelengths must be specified in fluorometry.

Materials that possess native fluorescence, those that can be converted to fluorescent compounds (fluorophores), and those that quench the fluorescence of other compounds can all be determined quantitatively by fluorimetry. This versatile technique thus has significant potential in the assay of pharmaceutical compounds.

Applications

Enzyme immunoassay

An increasingly popular assay for pharmaceutical products and for illicit drugs (cocaine, heroin, morphine, etc.), involves the technique of enzyme immunoassay. In this procedure, an antibody, specific for the drug to be assayed, is linked to an enzyme to form an inactive enzyme–antibody–antigen complex:

$$\underset{\text{(Active)}}{\text{E–Antibody}} + \text{Antigen} \rightarrow \underset{\text{(Inactive)}}{\text{E–Ab–An.}}$$

The enzyme activity can be followed radiometrically or by fluorescence spectroscopy. This latter technique has found widespread acceptance recently.

Energy transfer techniques

Several decades ago, Kosha showed that energy transfer, from one part of a molecule to another, could be effected if there is a close overlap of the two fluorescing parts of the molecule.

This principle can be utilized in fluorescent immunoassay, as described below:

$$\text{Fluorescein–Antibody} + \text{4-Methyl Umbelliferone–Antigen}$$
$$\downarrow$$
$$\text{Fluorescein–Ab–Ag–4-Methyl Umbelliferone.}$$

Thus, if the two fluorescing molecules are brought close together by antigen–antibody formation, then excitation of 4-methyl umbelliferone results in the emission spectrum of fluorescein being observed. Using a drug such as digoxin as antigen, competition occurs between the free digoxin in solution and the labelled digoxin for active sites on the labelled antibody. The fluorescence intensity observed is related to the concentration of the antigen, digoxin.

Fluorescence polarization methods

In one system available commercially (Abbott Co., Diagnostic Division, North Chicago, Ill.; TD_x system), the antigen, thyroxine in serum for example, competes with a thyroxine tracer for a limited number of binding sites on specific antibodies. The angle of polarization of the tracer, fluorescein, is changed and measured using a fibre optic detector. The changes in the angle of polarization reflect the degree of binding of the tracer to the antibodies. The concentrations of the samples measured are established using a calibration curve stored in the system.

Some of the tests possible using this system are presented in Table 1.

Solid-phase fluorescence immunoassay

The principle of solid-phase fluorescence immunoassay in drug assay was introduced with the Dade (Div. of American Hospital Supply Co.) Stratus analyser, illustrated in Fig. 1. The assay is based on a solid phase antibody, to which the sample is added. A specific antigen (e.g. digoxin) in the serum sample binds with the antibody, immobilized onto the paper support by physical adsorption. (In the case of competitive assays, both

Table 1
Methods based on fluorescence polarization immunoassay

Anticonvulsants	Antibiotics	Cardiac glycosides	Antiasthmatics
Phenobarbital	Gentamicin	Digoxin	Theophylline
Primidone	Streptomycin	Digitoxin	
Valproate	Tobramycin		

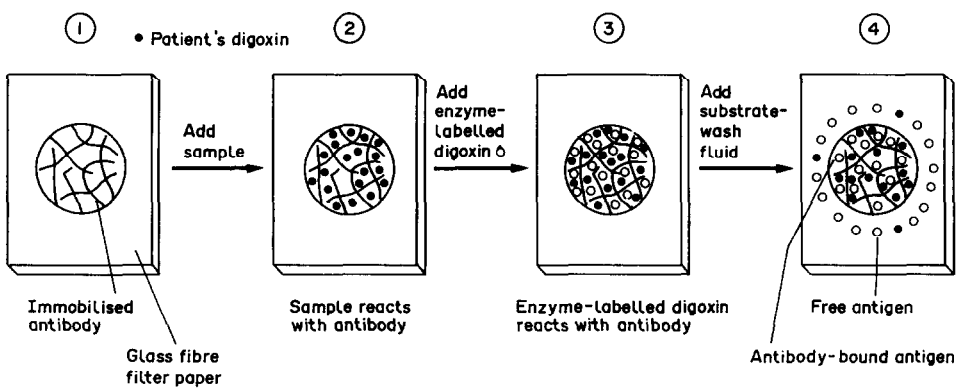

Figure 1
Schematic outline of radial separation immunomatrix assay.

enzyme-labelled conjugate and antigen in the patient serum are added at the first stage, where they compete for binding sites.) At the next stage an enzyme-labelled conjugate of digoxin is added, which binds with any unbound antibody during the incubation period. Finally, a substrate wash solution (4-methyl umbelliferone) is added, which elutes patient serum proteins and unbound conjugate from the reaction zone, while at the same time reacting with the bound enzyme-labelled conjugate to produce fluorescence at a linear rate. The fluorescence in the reaction zone is measured by a front surface fluorometer for 20 s, and compared to a stored calibration curve, to give a rapid print-out of antigen concentration. The performance of this system has been compared with radioimmunoassay, as illustrated in Fig. 2.

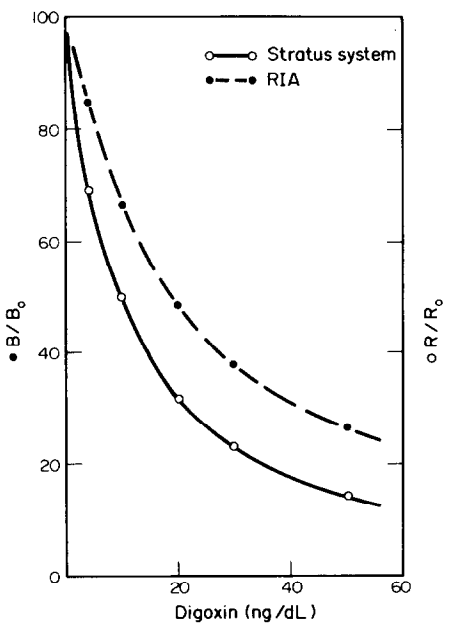

Figure 2
Comparison of solid-phase fluorescence immunoassay (stratus system) for digoxin with radioimmunoassay (RIA).

Table 2
Solid-phase fluorescence immunoassays

A.	Antiepileptics
	1. Primidone
	2. Carbamazepine
	3. Phenobarbital
	4. Phenytoin
B.	Aminoglycosides
	1. Gentamicin
	2. Tobramycin
	3. Amikacin
C.	Theophylline (bronchodilator)
D.	Digoxin (cardiac glycoside)
E.	Thyroid tests
	1. total thyroxine (T4)
	2. thyroxine – binding globulin = (TBG)

The system is based on antigen–alkaline phosphatase as conjugate, with 4-methyl umbelliferone phosphate as the substrate. The fluorescence of the free 4-methyl umbelliferone is then monitored.

A partial listing of the assays developed for this system is shown in Table 2. The system is reliable for the assay of a number of major drugs and pharmaceutical products.

Conclusion

Fluorescence technology offers a number of distinct advantages to the pharmaceutical analytical field: specificity, ultra-sensitivity and wide linear range. As applied to enzyme immunoassay techniques, especially linked to solid surface technology, the method appears to be most promising for applications in all fields of drug analysis.

[*Received for review 30 June 1986*]