

Immunoassay of Theophylline by Fluorescence Polarization: Use of the T-format Sample Module and Automatic Polarizers

Abstract

Fluorescence immunoassay is applied to the detection of theophylline, a drug used for myocardial stimulation. Interference from other sample components is limited by use of polarization measurements, which discriminate between bound and unbound fluorophores. The detection system includes a T-format sampling module mounting two emission spectrometers and programmable polarizers. A calibration curve is generated and the technique demonstrated by running test samples in serum.

Introduction

Immunoassay methods are based on competitive-binding reactions between labeled and unlabeled ligand for receptor sites on a specific antibody.¹ Measurement of some physical property of the label permits the construction of a calibration curve of that property versus concentration of the unlabeled ligand. The selectivity of the antibodies for a specific antigen allows the analysis of the ligand (analyte) of interest in the presence of similar substances.

To date, the most popular immunoassay technique has been radioimmunoassay (RIA) because of its high sensitivity. There are a number of problems, however, associated with RIA.² A license is required to handle radioactive material, waste disposal is complicated, and reagents have short shelf-lives. Consequently, other techniques have been developed to supplant RIA. One of the most promising, generally applicable approaches, is fluorescence immunoassay (FIA). With FIA, the label is a small fluorescent molecule (the probe), which is bound covalently to the analyte. The intensity of fluorescence produced by this probe is related to the ligand concentration, to produce the calibration curve.

Ideally, a fluorescent label should satisfy certain criteria:

Chemical

- The labeled ligand should be water-soluble to match physiological conditions.
- The label should have at least one reactive functional group, such as isothiocyanate, acid chloride, or diazonium salt, to ease its conjugation to the ligand.
- The label should not alter the specificity of the ligand for its antibody by shielding the reactive sites for antibody recognition.
- The shelf-life and stability of the complex should be adequate for practical laboratory use.

Spectroscopic

- The tag should have a strong absorption at the excitation wavelength of the experiment.
- The quantum yield of the labeled species should be large.
- The absorption and emission bands should be in the visible range to avoid interference from native proteins.
- The separation between excitation and emission peaks should be large.

A further requirement of any immunoassay method is the possibility of distinguishing between free and bound labeled ligand, or the two species must be separated somehow. Here, FIA has a distinct advantage over RIA: by monitoring fluorescence polarization, the separation step is circumvented.

In fluorescence polarization, the excitation light is passed through a polarizing prism, and the emitted light is analyzed with another polarizer that is alternately oriented parallel and perpendicular to the excitation polarizer. The amount of polarization retained by the molecule depends upon its Brownian rotation between absorption and emission. A bound ligand will retain much of its polarization because the large molecular volume of the antibody gives the complex a long rotational relaxation time. The free ligand, on the other hand, has a

¹ Visor, G.C.; Schulman, S.G. *J. Pharm. Sci.* **1981**, *70*, 469.

² Smith, D.S.; Hassan, M.; Nargessi, R.D. In *Modern Fluorescence Spectroscopy*; Wehry, E.L., Ed.; vol. 3, 93, Plenum Press: New York, 1981.

much smaller volume, so the rotational relaxation time is shorter than its fluorescence lifetime. The emitted radiation, therefore, has a polarization that is totally randomized.

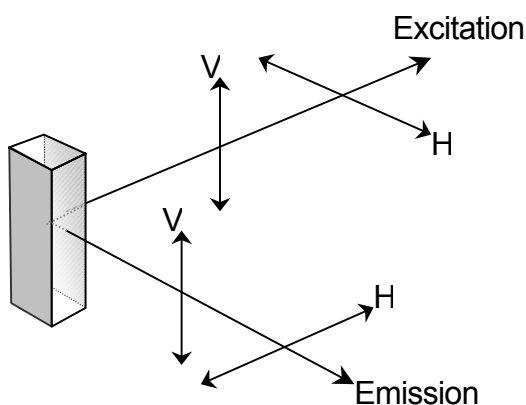


Figure 1. Relationship of polarization of exciting radiation (**V** and **H**) to that emitted by the sample (**V** and **H**).

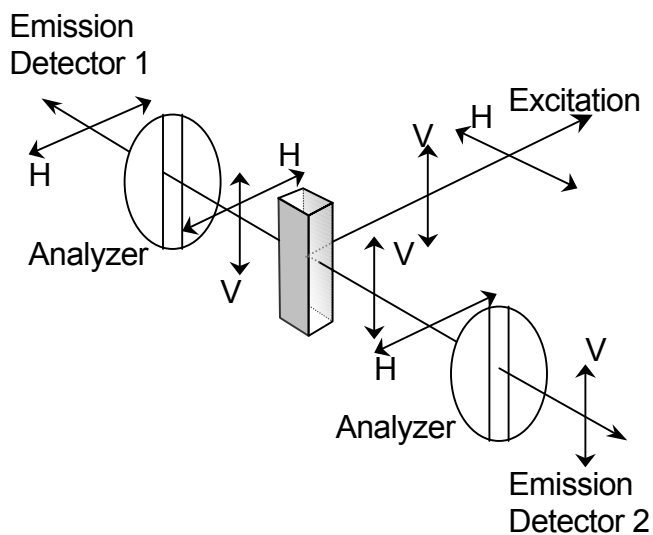


Figure 2. With separate detectors observing emission polarization through polarization analyzers in the **H** and **V** orientations, the number of measurements is reduced to two: excitation **V** and **H**.

The degree of polarization is defined as

$$P = \frac{P_r - 1}{P_r + 1}$$

where P_r is defined as $I_{\parallel} / I_{\perp}$, with I_{\parallel} the intensity of emitted light polarized in the same direction as the excitation beam, and I_{\perp} the intensity of emitted light polarized perpendicular to the excitation beam. In practice, $I_{\parallel} = I_{VV}$, where **V** is the polarization vector normal to the optical plane, and the double subscript refers to the orientation of excitation and emission polarizers (see Figure 1). I_{\perp} should be equivalent to I_{HH} , I_{HV} , and I_{VH} , where **H** is the polarization vector lying in the optical plane. Because of instrument artifacts, however, I_{HH} , I_{HV} , and I_{VH} are not equivalent. Measuring all four intensities is therefore necessary. The artifacts are then excluded by calculating P_p :

$$P_p = \frac{I_{VV}I_{HH}}{I_{HV}I_{VH}}$$

The required number of measurements may be reduced to two by observing the emission simultaneously through two polarizers, using two detectors (Figure 2). In this *T-format*, one emission polarizer is fixed in the **H** position, the other in the **V** position, while the excitation polarizer is rotated between the **V** and **H** positions. With the SPEX® Third Polarization Unit, the emission wavelength of one detector is selected by the emission monochromator, while the other detector has its wavelength selected by a filter.

Though convenient and economical, the filter method is insufficient when highly scattering samples are being analyzed, or scanning the emission spectrum is necessary, or when two wavelengths must be monitored simultaneously. In such cases, the T-format Sample Module must be employed, for it mounts two emission monochromators at 90° to the excitation beam.

Finally, polarization measurements can be performed completely under external control by adding an Autopolarization Assembly. This is a stepper-motor-driven polarizer mount that may be positioned in the excitation beam to rotate the polarizer between the **H** and **V** orientations under computer control. The host computer records the ratios I_{VV}/I_{VH} and I_{HV}/I_{HH} , then calculates either polariza-

tion or anisotropy, depending on the operator's choice of ACQUISITION MODE.

Experiment

The concentration of theophylline in serum or plasma is monitored by fluorescence polarization immunoassay. Theophylline (Figure 3) is a drug administered for myocardial stimulation, increased coronary blood flow, and smooth-muscle relaxation. It can be a bronchodilator in the control of asthma, for example. The observed fluorescence polarization of a fluorescein-labeled theophylline tracer is inversely related to the drug concentration. The drug level in a given sample therefore can be determined by comparison with a calibration curve.

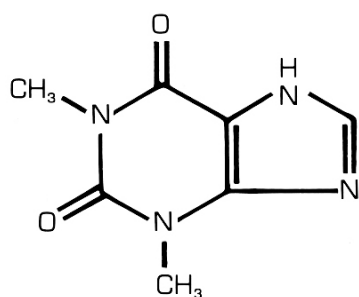


Figure 3. Theophylline.

A pre-treatment solution, the theophylline standard in a pH-7.2 buffer, the theophylline-fluorescein tracer, and antiserum were mixed together and incubated for three minutes before determining the fluorescence polarization. The theophylline samples were from General Diagnostics; Abbot Laboratories supplied the standards and other reagents.

Polarization measurements were performed on a SPEX® FLUOROLOG® spectrofluorometer consisting of three single-grating spectrometers, the T-format Sampling Module, a T-format Polarization Kit, the Autopolarization Assembly, and two Hamamatsu R928P detectors. A 450-W xenon lamp supplied the excitation.

Results and Discussion

Figure 4 shows excitation and emission spectra of bound, tagged theophylline and antibody taken with a bandpass of 7.2 nm on the excitation, and 3.6 nm on the emission spectrometers. The excitation maximum occurs at 485 nm, while the emission maximum occurs at 513 nm. The tagged drug shows virtually no polarization when not bound to

the antibody, as shown by the dashed curve in Figure 5. Upon binding, a polarization of about

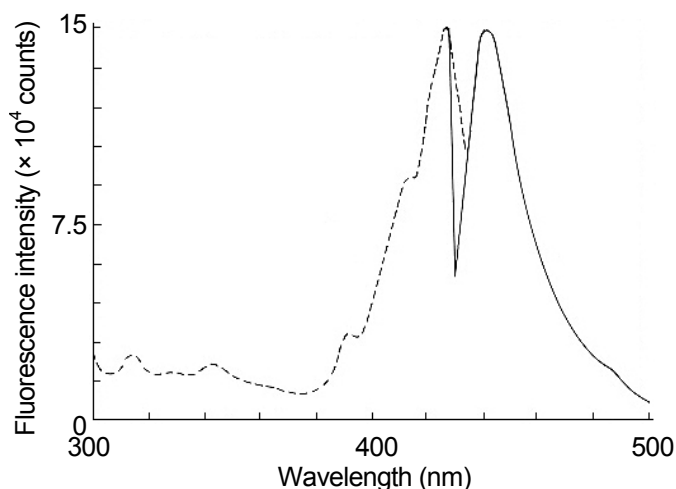


Figure 4. Excitation (dashed) and emission (solid) spectra of tagged theophylline plus antibody. The excitation and emission maxima are 485 and 513 nm, respectively. The integration time was 1 second.

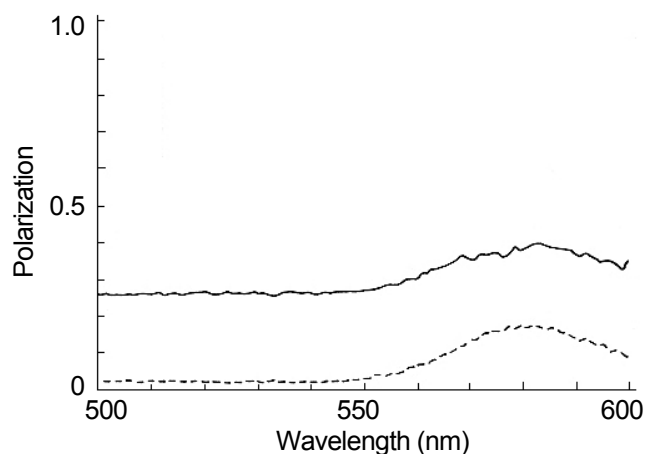


Figure 5. Polarization spectra of tagged theophylline with (solid) and without (dashed) antibody). The excitation wavelength was 485 nm. Tagged theophylline lacking antibody shows virtually no polarization, while complexed theophylline has a polarization of 0.27.

0.27 is observed (solid curve). This demonstrates that the complexed and uncomplexed theophylline can be distinguished readily by polarization without the need for chemical separation.

To generate a calibration curve, the spectrometers were set at the excitation and emission maxima, and the polarization was directly calculated. Eleven readings with a 5-second integration time were averaged at each of six concentrations (0, 2.5, 5, 10, 20, and 40 $\mu\text{g}/\text{mL}$ of theophylline in buffer) to produce the calibration curve (see Figure 6).

Finally, a trial sample was produced which contained the following concentrations of eleven different drugs:

- gentamicin at 5 $\mu\text{g}/\text{mL}$
- theophylline at 17.8 $\mu\text{g}/\text{mL}$
- tobramycin at 6.5 $\mu\text{g}/\text{mL}$
- quinidine at 2.6 $\mu\text{g}/\text{mL}$
- phenytoin at 12 $\mu\text{g}/\text{mL}$
- digoxin at 2.2 ng/mL
- salicylate at 220 $\mu\text{g}/\text{mL}$
- acetaminophen at 217 $\mu\text{g}/\text{mL}$
- lithium at 1.2 meq/L
- meclofenamic at 6.0 $\mu\text{g}/\text{mL}$
- phenobarbital at 51.4 $\mu\text{g}/\text{mL}$

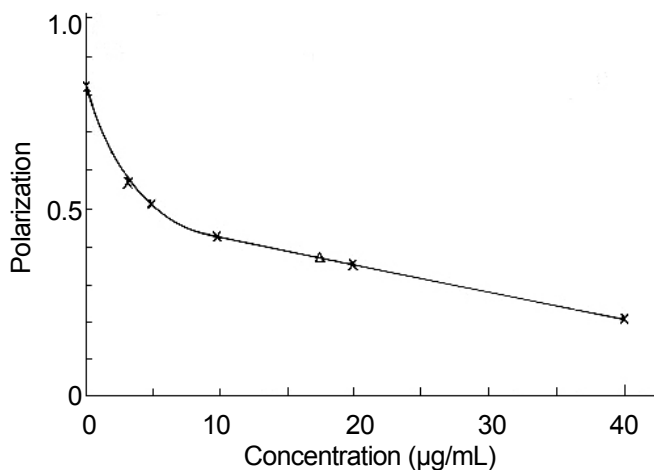


Figure 6. Calibration curve of theophylline concentration versus polarization. Concentrations used (x) for creating the curve were 0, 2.5, 5, 10, 20, and 40 $\mu\text{g}/\text{mL}$. The triangle indicates determination of the trial sample.

Three determinations of different aliquots of this sample produced an average polarization of 0.122 ± 0.002 , which corresponds to a theophylline concentration of $18.0 \pm 0.3 \mu\text{g}/\text{mL}$ on the calibration curve (see Figure 6). This agrees with the actual concentration listed above.

In a normal fluorescence experiment, interference would be anticipated from gentamicin, quinidine, salicylate, and acetaminophen. In this experiment, however, the antibody's specificity for a particular drug, combined with polarization measurements, eliminates interference from other fluorophores as well as unbound tags.

This example demonstrates that fluorescence immunoassay has, like radioimmunoassay, the specificity and sensitivity to perform at biologically relevant concentrations. The advantage of FIA, however, is that no separation step is required.

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