Laser Fluorescence Immunoassay of Insulin

S. D. Lidofsky, T. Imasaka,[1] and R. N. Zare*

Department of Chemistry, Standford University, Stanford, California 94305

A new technique is described for a competitive binding assay. Bound and free fluorescent-labeled antigen are separated by high pressure liquid chromatography and detected by laser fluorimetry. This method is illustrated in the detection of insulin in well-characterized buffer solutions. Fluorescein isothiocyanate serves as the fluorescent tag, a gel filtration column permits a separation on the basis of molecular size, and the 488-nm line of a CW argon ion laser provides the excitation. The best detection limit of insulin is 0.4 ng/mL. The sensitivity of laser fluorescence immunoassay is found to be comparable to that of radioimmunoassay.

Radioammunoassay, originally developed by Yalow, Berson, and co-workers, is the method of choice in the analysis for a large number of hormones, drugs, and toxins (*1*). However, in some applications, it would be desirable to replace the radioisotopic tag because of its limited shelf life, the difficulties involved in handling and shipping radioactive materials, and the need for using expensive counting equipment. This has stimulated a search for fluorescent labels as substitutes (*2*).

Recently, the use of laser excitation in fluorescence analysis has opened up new possibilities for ultrasensitive detection in the liquid phase (*3–6*). It is our goal to combine laser fluorimetry with fluorescence immunoassay to achieve an attractive alternative to radioimmunoassay.

In immunoassay, unlabeled antigen (Ag) competes with labeled antigen (Ag*) for binding sites on an antibody (Ab), directed against the antigen:

$$\begin{array}{ccc} Ag^* + Ab & \rightleftarrows & Ag^*Ab \\ + & & \\ Ag & & \\ \downarrow\uparrow & & \\ AgAb & & \end{array}$$

In radioimmunoassay, the label is a radioisotope, usually ^{125}I or ^{131}I; in fluorescence immunoassay, the label is a small fluorescent molecule. In the latter, the fluorescence from the labeled antigen bound to the antibody (Ag*Ab) decreases as a function of increasing concentration of unlabeled antigen. This functional relationship is used to establish a calibration curve, called the standard curve, for the detection of a particular antigen.

In this procedure, it is essential to distinguish Ag*Ab fluorescence from Ag* fluorescence in solution. Sometimes, either Ag* or Ag*Ab dominates in fluorescence at a certain wavelength (*7, 8*) or the two species have such different polarization characteristics (*9, 10*), that a physical separation is unnecessary. In most cases, however, the fluorescence from from Ag* and Ag*Ab is not sufficiently different in polarization and/or wavelength, and the background fluorescence of the solvent overwhelms the signal of interest. Under such conditions, one must resort to a separation procedure for Ag* and Ag*Ab, e.g., chromatography, electrophoresis, or physical adsorption onto a solid phase.

The present work reports a method for the immunoassay of the protein hormone insulin in aqueous buffers. Fluorescein isothiocyanate (FITC) serves as the antigen label, and a CW argon ion laser is used as a fluorescence excitation source. FITC is a versatile compound for labeling proteins as it binds to free amino groups (*11*). It has a large absorption coefficient ($\epsilon_{490} \sim 7.66 \times 10^4$) (*12*), a high fluorescence quantum yield ($\Phi \sim 0.8$), and long term stability (*11*). Its excitation maximum occurs at 490 nm, which nearly coincides with the strong 488-nm line of the argon ion laser. The emission maximum of FITC occurs at 520 nm (*13*). Its visible spectrum does not appreciably change on binding to protein, but the fluorescent quantum yield is reduced (*13*).

We separate insulin bound in complex from free insulin on the basis of molecular size by using a gel filtration column in a high pressure liquid chromatography (HPLC) system. The molecular weight of the insulin–antibody complex is approximately 160 000; the molecular weight of insulin is approximately 6000. Thus, a separation of the two can be readily achieved. We can therefore measure the fluorescence from FITC–insulin–antibody complex as a function of the concentration of unlabeled insulin and generate a standard curve. In this study, we show that the detection of insulin by laser fluorescence immunoassay achieves a sensitivity comparable to that of radioimmunoassay.

EXPERIMENTAL

Figure 1 shows a schematic of the experimental setup. The sample is loaded onto the HPLC column by means of a microsyringe. The laser fluorimeter makes use of a flowing droplet described previously (*14*). The eluate from the column exits from a narrow bore stainless steel tube which has been notched to release possible trapped gas. A stainless steel rod of comparable outer diameter is coaxially positioned in proximity to the tube so that by liquid surface tension, a droplet forms between them. The droplet is stationary in space and constitutes, in effect, a windowless fluorescence cell of small volume (about 5 μL).

The 488-nm output (0.5 W) of an argon ion laser (model CR-2, Coherent Radiation) is focused inside the droplet by lens L_1. Although the laser beam is tightly focused, no saturation effect on the fluorescence output is observed. A 1-mm diameter portion of the droplet is imaged at right angles to the laser beam by lens L_2, the field lens L_3, and the aperture onto the bialkali cathode (1-cm diameter) of a low dark current photomultiplier (model Q4249B, Centronic). Three interference filters isolate the spectral region of interest. Two are broadband B5 fluorescein filters (Baird-Atomic), each of which has a 85% transmission between 520 and 580 nm. The other is a short pass filter (Ditric Optics) which has a 70% (average) transmission between 400 and 535 nm. This filter combination strongly suppresses scattered laser light and the ν_1 and ν_3 Raman bands of water (>10^3 rejection ratio), but the ν_2 Raman band at 529 nm is relatively unattenuated. Background fluorescence from impurities in the solvent are found to be comparable to the ν_2 Raman band of water. The output of the photomultiplier is displayed on a stripchart recorder. By connecting a capacitor (3-s RC time constant) between the recorder input and ground, high frequency noise on the signal is reduced.

Reagents Bovine insulin (Sigma; 24 IU/mg) was labeled with FITC (Sigma) according to the method of Hudson and Hay (*15*) but with the modification that 1.5 mg of FITC was added to 10 mg of insulin (in place of immunoglobulin) in 6 mL of carbonate–bicarbonate buffer (pH 9.5). The ratio of bound FITC molecules to insulin molecules (labeled and unlabeled) (F/I) was determined from the formula

$$\frac{F}{I} = \frac{(\text{OD } 495 \text{ nm}) \times (\epsilon_M{}^I \ 280 \text{ nm})}{[(\text{OD } 280 \text{ nm}) - 0.35 \ (\text{OD } 495 \text{ nm})] \times (\epsilon_M{}^F \ 495 \text{ nm})}$$

[1] Present address: Department of Applied Chemistry, Faculty of Engineering, Kyushu University, Hakozaki, Fukuoka, 812 Japan.

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Table I. Experimental Conditions

reaction condition	FITC-insulin concn, ng/mL	antiserum dilution	bovine serum albumin concn, %	incubation conditions	injection vol, μL	HPLC flow rate, mL/min	insulin detection limit, ng/mL
A	21	1:2000	1×10^{-4}	4 h at 37 °C followed by 18 h at 4 °C	1	1.0	2.0
B	2.4	1:40 000	2.5×10^{-5}	48 h at 4 °C	100	0.5	0.4

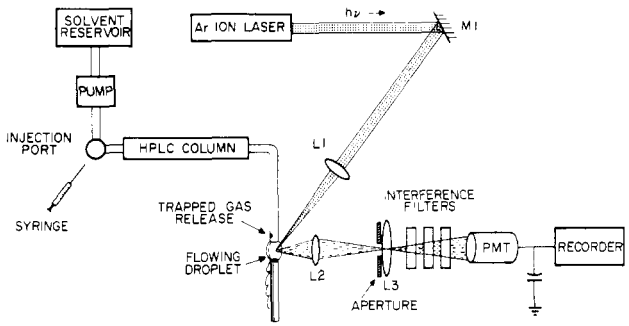

Figure 1. Experimental apparatus

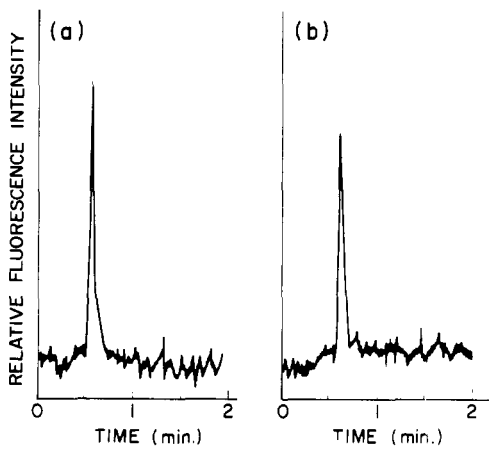

Figure 2. Chromatograms of samples of mixtures reacted under condition A in Table I. Injection volume: 1 μL. HPLC Flow Rate: 1 mL/min. (a) Insulin concentration in sample: 0 ng/mL. (b) Insulin concentration in sample: 5 ng/mL

where OD 280 nm and OD 495 nm represent the optical density of the solution at 280 nm and 495 nm, respectively, and $\epsilon_M{}^I$ 280 nm and $\epsilon_M{}^F$ 495 nm represent the molar extinction coefficient of insulin at 280 nm (taken to be 5.48×10^3) and of FITC at 495 nm (taken to be 7.66×10^4), respectively. Under our experimental conditions, F/I was 0.7 and seven molecules of FITC were bound per ten molecules of insulin. Unlabeled bovine insulin was added to a mixture (see Table I) of FITC-insulin, guinea pig antiserum to bovine insulin (Cappel Laboratories; Ab concentration = 1.08×10^{-3}M, determined by precipitin reaction), and bovine serum albumin (Boehringer-Mannheim) dissolved in sterile 0.07 M phosphate buffer (pH 7.4). The water used was doubly distilled. The reaction mixture (500 μL) was incubated at 4 or 37 °C for time periods indicated in Table I.

After incubation, 1–100 μL samples were injected onto a Bio-Sil GFC-10 gel filtration column (Bio-Rad), which was part of a HPLC system consisting of model 6000 A pump and a U6K injector (both Waters Associates). The elution solvent was 0.07 M phosphate buffer (pH 7.4), and the flow rate was 0.5 or 1.0 mL/min.

Some studies were also made with human serum supplied by G. M. Reaven of the Palo Alto VA Hospital.

RESULTS

When 10-μL buffer solution samples of FITC-insulin were injected onto the HPLC column, the detector response was linear over the range 0 to 10 ng. The detection limit was 0.8 ng, corresponding to 1.3×10^{-13} mol of labeled insulin. When 1-μL buffer solution samples containing antiserum to insulin in an excess of FITC-insulin (1 μg/mL) were injected onto the HPLC column, the peak corresponding to the FITC-insulin antibody complex was monitored. Under these conditions, the detector response was linear over the range of 0 to 28 ng of antibody. The detection limit was 2.8 ng (1.8×10^{-14} mol) of antibody. The noise was of the order of 0.5% of the background signal and is attributed to white noise from the photomultiplier.

Figure 2 shows chromatograms of 1-μL samples taken from reaction mixtures incubated under condition A of Table I. It should be noted that only one peak, which corresponds to the FITC-insulin-antibody complex, is present.

Initially, it was thought that the fluorescence from the complex was much stronger than that of FITC-insulin. However, subsequent experiments revealed that this was not the case, as antibody binding had no measurable effect on the fluorescence. It might then be inferred that FITC-insulin adsorbs onto the column support (*16*). Indeed, when we raised the FITC-insulin concentration in the reaction mixture to 1 μg/mL and held the antiserum dilution at 1:20 000 (Ab concentration = 5×10^{-8} M), a second peak appeared that corresponded to free FITC-insulin. At lower concentrations, the FITC-insulin peak is broadened to the point where it is undetectable. The adsorption process does not affect the background signal from sample to sample, nor does it affect the reproducibility of our measurements. This was carefully checked. The relative standard deviation in labeled complex peak heights corresponding to samples of reaction mixture prepared under identical conditions was typically less than 5%. Furthermore, in three months of use, the column performance has not deteriorated. Thus, if FITC-insulin is adsorbing onto the column, it is probably occurring in a reversible manner, which does not affect the overall results of the measurement.

Figure 2a presents a chromatogram of a sample that contained no unlabeled insulin. The peak height on this chromatogram is larger than that in Figure 2b, which was taken from a sample that contained 5 ng/mL (5 pg) of insulin. This is in accordance with the fact that the concentration of bound labeled insulin decreases as a function of increasing concentration of insulin in the reaction mixture.

The trend is borne out in Figure 3, which illustrates the standard curve for reaction conditions B in Table I. Under these particular conditions, the present limit of detection is 0.4 ng/mL. The total time needed to separate bound from free and measure fluorescence was less than 10 minutes. Further increases in injection volume did not increase the chromatogram peak heights and resulted only in a deterioration of resolution.

Figure 4 illustrates the combined effects of incubation time and temperature on the standard curve of reaction mixture A. It can be seen that the incubation time does not influence the shape of the curve. However, when mixtures were incubated for 4 h at 37 °C and then over 2 nights at 4 °C, slightly higher chromatograph peaks resulted. Under these conditions,

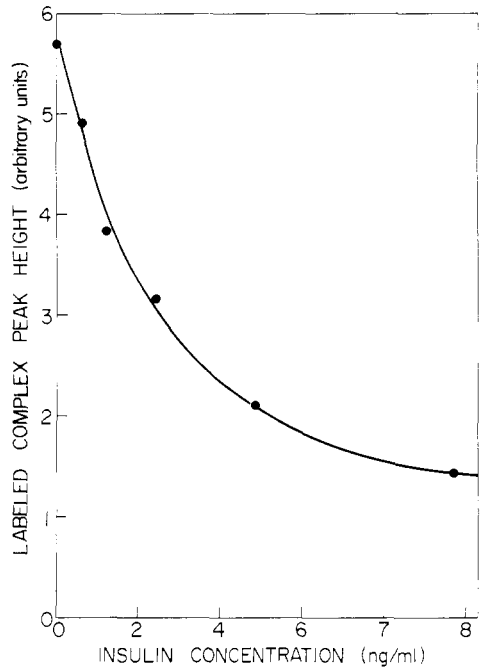

Figure 3. Immunoassay standard curve for reaction condition B. Detection limit: 0.4 ng/mL

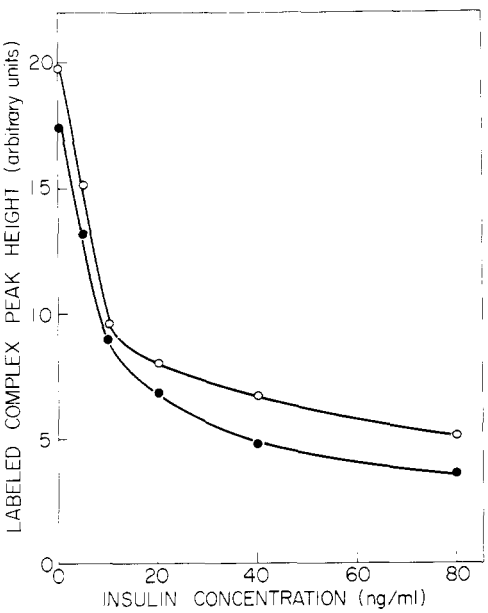

Figure 4. Dependence of standard curve for reaction condition A on incubation time and temperature. (O) 4 h (37 °C) + 44 h (4 °C). (●) 4 h (37 °C) + 18 h (4 °C)

the limit of detection was 2 ng/mL (2 pg) of insulin.

DISCUSSION

The use of laser fluorimetry is largely unknown in immunoassay. Kronick and Little (*17*) employ internally reflected laser light to excite fluorescence of an antigen–antibody complex bound to a quartz plate. Using a fluorescein labeled antibody, they report that morphine at a concentration of 2×10^{-7} M is readily detected. In this study we detect 7×10^{-11} M (0.4 ng/mL) insulin using laser fluorimetry in conjunction with HPLC. The limits of detection of insulin in various radioimmunoassays range from 0.1 ng/mL to 0.8 ng/mL (*18*, *19*). It must be noted that our detection limit represents the concentration of insulin in the reaction mixture. The detection limit of radioimmunoassay represents the concentration of insulin in the unknown sample introduced to the reaction mixture. Typically, the volume of this unknown sample is 100 μL. In our method, the maximum unknown sample volume is 400 μL. Under this condition, the detection limit of insulin in the unknown sample is equal to the detection limit of insulin in the reaction mixture multiplied by the ratio of the reaction mixture volume to the sample volume (0.4 ng/mL × 5/4), that is, 0.5 ng/mL. Thus, given the above qualification, the present method achieves a sensitivity similar to that of radioimmunoassay. In laser fluorescence immunoassay, reagents are stable, inexpensive, and easy to handle. Furthermore, separation and quantitation are achieved in a short time using standard chromatographic equipment on-line with a detector that incorporates a low power argon ion laser with proven reliability.

It is interesting to compare the present method with fluorescence polarization immunoassay. In the latter technique, the bound and free labeled insulin possess different degrees of polarization, the (lighter) free labeled insulin having a lower degree of polarization than the bound. Thus, a measurement of the degree of polarization can be related to the ratio of bound to free labeled insulin. Spencer et al. (*9*) report a detection of 60 ng/mL of insulin using this procedure. Our present detection limit is more than 100 times lower than that of fluorescence polarization immunoassay. Moreover, our method avoids some of the problems of background fluorescence from interfering components by the use of chromatographic separation.

Our detection limit is set by the ability to measure the change in the bound labeled insulin peak height as a function of added unlabeled insulin. Because this is a competitive binding assay, the concentrations of labeled to unlabeled insulin must be similar. Furthermore, the concentration of antibody must be such that the addition of unlabeled insulin competes with labeled insulin for binding sites. Thus, to improve our sensitivity, it is necessary to reduce the concentrations of both antiserum and FITC–insulin. By lowering these concentrations, however, one decreases the concentration of labeled insulin antibody complex, which depends on the association constant of complex formation. As the concentration of labeled complex decreases, the signal to noise ratio of the chromatogram decreases. This ultimately determines our limits of detection.

The sensitivity of our fluorimeter is limited by a number of factors. The most prominent of these is the background signal from solvent which originates from Raman scattering and from contaminant fluorescence. The former process is virtually synchronous with the excitation; the latter is uncharacterized. If one were to employ a label with a long fluorescence lifetime, such as a pyrene derivative (*20*), one could largely reject the background signal via temporal discrimination. This would require modifications in the excitation source and detection electronics.

Alternatively, one could improve the sensitivity by using an enzymatic label (*21*, *22*). If this enzyme has the property of catalyzing the conversion of a fluorogenic substrate to a fluorescent product, one could indirectly measure enzymatic activity with laser fluorimetry.

Finally, one could choose a solid phase separation scheme known as sandwich immunoassay. In radioimmunoassay, this technique is estimated to be ten times more sensitive than competitive binding assays (*23*). The method is sufficiently general to allow for the label to be a fluorescent molecule or an enzyme. Furthermore, the separation step removes fluorescent contaminants from the sample of interest.

Laser fluorescence immunoassay can sensitively and specifically quantitate the concentration of any antigen in a given buffer solution. In environmental analysis, it is of interest to detect small molecules which may not be antigenic. In these cases, it is still possible to analyze for them with

immunoassay if one couples them to a suitable carrier (*24*). Thus, our method can be applied to environmental analysis.

In clinical analysis, one commonly obtains samples taken from human serum. Serum contains a number of components which exhibit broadband fluorescence on visible excitation. When we injected a serum sample onto the HPLC column, the chromatogram contained strong peaks which overlapped the regions of both labeled insulin and labeled complex. There were no peaks, however, which corresponded to substances of molecular size greater than that of the labeled complex. Thus, at the present time, fluorescence from interfering components in serum prevents clinical applications of our technique.

If one could increase the molecular size of the labeled complex by linking the antibody to a nonfluorescent inert polymer, such as another antibody and separate on a column of larger pore diameter, one could access a region of the chromatogram which is free from fluorescence interference. Other possible routes toward circumventing interference problems include temporal discrimination, enzyme-linked immunoassay, and sandwich immunoassay. These approaches are currently under investigation.

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Determination of Sulfur by Vacuum Ultraviolet Atomic Emission Spectrometry with Hydrogen Sulfide Evolution

Paul D. Swaim* and Steve R. Ellebracht

Central Laboratory, Dow Chemical U.S.A. Freeport, Texas 77541

A method for the determination of sulfur has been developed by which sulfur is reduced to volatile hydrogen sulfide gas and then swept into a dc plasma where it is detected by vacuum ultraviolet atomic emission spectrometry at 180.7 nm. The method makes possible the determination of trace levels of sulfur in samples where the matrix would normally result in spectral and/or chemical interferences. The practical detection limit is 10 ng of sulfur and the linear working range is at least four orders of magnitude. Analysis time is rapid, with less than 2 min being typically required per sample. Analytical procedures are extremely simple, making the technique ideal for routine applications. Factors which govern the rate of hydrogen sulfide evolution were investigated, as were the effects of possible interferences.

Quick and reliable methods for the determination of trace levels of sulfur in a wide variety of sample matrices have long been pursued by the analytical chemist. As with many nonmetallic elements, sulfur has not proved easily amenable to detection of microgram and sub-microgram quantities. This has been further complicated by the fact that techniques for separating and/or concentrating sulfur at these levels are sometimes cumbersome. The result is that, at best, methods for traces of sulfur usually are tedious and require considerable operator time and skill.

We recently were in need of a method which could be used routinely to determine sulfur in salt samples in the range of 0.5 to 100 ppm. These samples consisted mainly of sodium chloride, potassium chloride, calcium chloride, and magnesium chloride in roughly equal proportions. Sulfur was present primarily as inorganic sulfate although lesser amounts of other inorganic sulfur species may also have been present. Existing titrimetric or colorimetric methods (*1–3*) were found to be either insufficiently sensitive or excessively time consuming. Turbidimetric and polarographic methods (*4*, *5*) for the determination of sulfate not only required prior conversion of other forms of sulfur but were found to suffer from lack of specificity and/or reproducibility. Instrumental techniques which have been used to determine sulfur include X-ray fluorescence (*6*, *7*), neutron activation (*8*), and charged-particle

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