Homogeneous Fluorescent Immunoassay with Dry Reagents

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We describe an assay for theophylline in serum in which the reagents for the substrate-labeled fluorescent immunoassay procedure are dry reagents in a paper matrix. This assay format is both rapid and convenient. In one such paper strip assay, the competing drug conjugate is present in the diluting buffer (dilution-into-conjugate strip assay); in another, all necessary assay reagents are contained on the strip (integral strip assay). The latter strip can be prepared by using appropriate solvents in a "two-dip" procedure, which prevents premature reaction of the assay constituents. The dilution-into-conjugate strip assays yield results that compare well with those by the corresponding solution assay. Results of the strip assay are compared with those by an enzyme immunoassay for theophylline.

Additional Keyphrases: "kit" methods · theophylline · substrate-labeled fluorescent immunoassay

Homogeneous substrate-labeled fluorescent immunoassay (SLFIA) is one technique for measuring drug concentrations in serum. The assay depends upon a competitive protein binding reaction in which the drug of interest competes with a drug conjugate. The conjugate contains an enzyme substrate that produces a fluorescent product after reaction. The enzyme reaction is inhibited if the conjugate is bound to the antibody. Increasing the drug concentration increases the concentration of free drug-conjugate, which in turn results in a concentration-dependent increase in fluorescent signal (1). A solution assay for theophylline has been described recently that involves this principle (2).

Here we discuss the incorporation of the SLFIA assay into a paper strip format. Many tests have been adapted to a paper matrix for the detection of analytes in urine and, more recently, for the quantitation of analytes in serum (3). The reagents are incorporated onto paper strips and dried; only the addition of a sample is required to initiate the assay. For quantitation, changes in strip reflectance during reaction on the paper are measured.

The application to clinical assays of fluorescence measurements on solid surfaces has also been demonstrated (4, 5). Fluorescence measurements on strips are more sensitive than reflectance measurements, analogous to the increase in sensitivity afforded by fluorescence in solution, with conventional right-angle geometry, compared with spectrophotometric absorbance measurements.

The formulation of an SLFIA test on strips has been developed in two stages. In the first, the drug must be diluted into a solution containing the drug conjugate. In the second, the assay strip contains all assay constituents and requires only that a diluted specimen containing the drug be applied.

Materials and Methods

Apparatus

Excitation and emission spectra were obtained with a spectrophotometer (SLM 8000; SLM instruments, Inc., Urbana, IL 61801). For experiments on paper strips, front-face fluorescence was measured by holding the strip such that the excitation light was 30° from normal to the pad and the emission detector was 60° from normal to the pad. Solution SLFIA assays were performed with a Farrand Mark I (Farrand Optical Co., Inc., Valhalla, NY 10595). To measure fluorescence for immunoassay on strips, we used a strip-reading filter fluorimeter, which was built in-house and will be described in more detail elsewhere. In essence, the device consists of a 40-W mercury lamp source; a 405-nm, three-cavity interference filter at the excitation side; and a 450-nm, three-cavity interference filter (Ditric Optics, Hudson, MA) at the emission side. The common leg of a bifurcated fiber optic is mounted above horizontal paper strips in a light-tight chamber. One arm of the fiber optic brings excitation light to the strip; the second arm goes to the photomultiplier detector and associated electronics to measure the fluorescence signal.

Reagents

Antisera to theophylline and the theophylline conjugate, 8[3-(7-β-galactosylcoumarin-3-carboxamido)propy1]theophylline (β-galactosyl-umbelliferone-theophylline; GU-theophylline), were as previously described (2).β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23, from Escherichia coli) was purchased from Sigma Chemical Co., St. Louis, MO 63178 (Grade IV).

Sera from patients receiving theophylline were obtained from the South Bend Medical Foundation, South Bend, IN 46601.

Theophylline and o-nitrophenyl-β-D-galactopyranoside were also purchased from Sigma Chemical Co. Bicine [N,N-bis(2-hydroxyethyl)glycine] buffer was from Calbiochem-Behring Corp., San Diego, CA 92112.

Whatman 31ET paper was obtained from Whatman, Inc., Clifton, NJ 07014.

Procedures

The conjugate product, theophylline umbelliferone, was prepared by hydrolysis of a GU-theophylline stock solution in 50 mmol/L Bicine buffer, pH 8.5, in the presence of β-galactosidase, 3500 U/L. For spectral measurements of solutions, we prepared a 20 mmol/L solution of conjugate product. For spectral measurements of paper strips, we applied a 50-μL sample of a 1 μmol/L solution of conjugate product to a 1 × 1 cm Whatman 31ET paper pad. For measuring the emission spectra, we used an excitation wavelength of 400 nm. For measurement of the excitation spectra, the emission wavelength was 450 nm.
In initial titrating and kinetic experiments, the immunoassay strips were prepared by placing 20 &mu;L of impregnation solution onto 0.5 x 1.0 cm or 1 x 1 cm Whatman 31ET paper pads mounted with double-stick adhesive onto a 0.5 x 8.3 cm or 1 x 8.3 cm polystyrene "handle." The impregnation solution contained various concentrations of theophylline antisera (or normal rabbit sera) as specified, &beta;-galactosidase, and Bicine buffer (0.1 mol/L, pH 8.5). Paper strips were dried at 50 °C for 10 min in a forced-air oven. To prepare a double-reciprocal plot for kinetic studies, we determined the rate from the change in concentration of product from 0.5 to 1.5 min.

To prepare the paper strips for the theophylline immunoassay, we used two formats. In the first (dilution-into-conjugate strip assay), enzyme and antisera were incorporated and dried into paper, and the serum sample was diluted 40-fold into a solution containing the GU-theophylline conjugate before being applied to the paper pad. Whatman 31ET paper from rolls 2 or 4 in. in width, was passed through an impregnation solution consisting of 250 mL of theophylline antisera and 2500 U of &beta;-galactosidase per liter of 0.1 mol/L Bicine buffer, pH 8.5. The paper was passed between two scraper bars into a drying tunnel set at 50 °C.

In the second format (integral strip assay), all assay constituents were incorporated into the paper by use of a "two-dip" procedure. The first-dip impregnation solution contained 300 mL of theophylline antisera and 5000 U of &beta;-galactosidase per liter of 0.2 mol/L Bicine buffer, pH 8.5. After drying, the paper was passed through a second impregnation solution containing 2 &mu;mol of GU-theophylline per liter of acetone. These papers were then cut and mounted as 0.5 x 1.0 cm pads on the polystyrene handle as described above.

To start the reaction, we applied to the paper pads 35 &mu;L of samples diluted in 50 mmol/L Bicine buffer, pH 8.5. For strips lacking the drug conjugate, serum samples were diluted 40-fold with 50 mmol/L Bicine, pH 8.5, containing 1.5 &mu;mol of GU-theophylline per liter (final concentration). The strips were calibrated with five calibrators from a TDA™ theophylline test kit (Ames Division, Miles Laboratories, Inc.). Theophylline concentrations were determined from fluorescence values by referring to a standard curve.

For strips containing all assay components, serum samples were diluted 20-fold with the Bicine buffer. To prepare a standard curve for this strip, we used a two-point calibration at 0 and 40 mg of theophylline per liter.

Generally, the fluorescence signal was measured after 3 min unless otherwise specified.

We determined &beta;-galactosidase activity as described by Wong et al. (6). The solution assays for theophylline conjugate by use of the Ames TDA test kit, which follows the SLFIA principle, and an enzyme immunoassay test kit (EMIT™; Syva Co., Palo Alto, CA 94304) were performed as described in the product inserts.

A high-performance liquid chromatographic analysis for theophylline was done as described by Weidner et al. (7).

**Results**

**Fluorescence Measurements on Paper**

Figure 1 shows a comparison of the fluorescence excitation and emission spectra of the product of hydrolysis of GU-theophylline, by conventional right-angle measurements in solution and by a front-face measurement on paper strips. The excitation peaks for the product in solution and on paper are coincident at 398 nm. The emission peak for the product in solution is at 452 nm and on strips is at 450 nm. The emission spectrum on strips is shifted slightly toward shorter wavelengths.

Several concentrations of the GU-theophylline hydrolysis product were applied to paper strips and the fluorescence response was measured. The instrument response to fluorescence on paper was linear with concentration over the range utilized in the assay procedure (0–2 mmol/L) and up to at least 16 mmol/L.

**Kinetic Response on Paper**

Kinetic data for the hydrolysis of GU-theophylline on paper was used to prepare a double-reciprocal plot, to determine the Michaelis constant at 26 °C. The value we obtained, 0.33 mmol/L, compares well with the previously described value of 0.29 mmol/L (2) determined for the solution reaction.

The concentration of GU-theophylline in the assay on paper (as well as in solution) is well below the K_m of the enzyme. An example of the kinetic response of a dilution-into-conjugate strip for theophylline is shown in Figure 2. The drug conjugate is added with the sample. The development of fluorescent product is nonlinear and, as shown in the figure insert, the response conforms to a first-order reaction, which is the expected result for substrate concentrations that are well below the K_m.

For strips prepared by the "two-dip" procedure, the kinetic response (Figure 3) differs from that observed when the GU-theophylline is added in solution: after an initial rapid reaction, a much slower second-reaction component is observed. This response precludes a direct application of a first-order analysis. The same type of response is observed if
only the GU-theophylline conjugate is dried into the paper and the reaction is initiated by adding a solution containing β-galactosidase (not shown). The change in kinetic response from the previous strips is therefore a consequence of drying the GU-theophylline conjugate into the strip.

We also prepared strips by drying increasing quantities of antisera to theophylline into paper along with the buffer and β-galactosidase. As shown in Figure 4, as the quantity of antisera increased, there was a progressive decrease in the development of fluorescent signal, similar to results observed for solution.

Figure 5 depicts a standard curve for the initial dilution-into-conjugate strip assay, where relative fluorescence intensity is plotted vs the initial concentration of theophylline in serum. The results show that theophylline alleviates the antisera-dependent inhibition of GU-theophylline hydrolysis and that the extent of this alleviation depends upon the theophylline concentration. The strip response is very similar to the response previously described in solution (2). The shape of the dose–response curve can be adjusted from sigmoidal to hyperbolic as the ratio of GU-theophylline to antisera is increased.

We compared the performance of these theophylline immunoassay strips (y) with results from serum theophylline determination by the solution TDA assay and to a “high-performance” liquid-chromatographic method for theophylline. The results were as follows: y = 1.00·solution results + 0.77 (r = 0.975, SE = 1.67, n = 25) and y = 1.17·chromatographic results – 1.54 (r = 0.980, SE = 1.49, n = 25).

We extended the procedure for theophylline immunoassays on strips to permit the assembly of all reagents required in the assay into the paper strip (integral strip assay). Figure 6 illustrates a dose–response curve for this method, where relative fluorescence intensity is plotted vs the final concentration of theophylline on the strip. The conditions used in assembling the strip prevented interaction of GU-theophylline with antisera or β-galactosidase before addition of sample. The strip was tested over the concentration range in which response is essentially linear. Using a two-point standard curve for the strip assay, we compared within-run results for clinical serum samples containing theophylline by the integral strip assay and by the enzyme immunoassay for theophylline.

As shown in Figure 7, correlation between the two procedures was excellent. No background fluorescence was observed in any of the sera tested.

Discussion

These studies demonstrate the feasibility of preparing serum drug immunoassays in a dry-reagent format. The fluorescence assay performs in its intended manner in the paper matrix, producing a signal that is a linear function of product concentration. This is in contrast to the measurement of reflectance changes in paper strips, which are not linearly related to concentration and must be converted to the corresponding Kubelka-Munk ratio, K/S (8). Further, the dynamic
range available in reflectance measurements is much less than the range available in fluorescence.

$K_m$, the $\beta$-galactosidase reaction with GU-theophylline in the strips, 0.33 mmol/L, is very similar to the previously reported (2) value, 0.29 mmol/L, in solution and is similar to the solution value described for o-nitrophenyl-\(\beta\)-D-galactose, 0.14 mmol/L. Because the strip assay involving the dilution into-conjugate approach has a first-order reaction rate, the reaction rate could be used as a means of determining initial and final fluorescence values (9). However, for convenience, we used the fluorescence response at 3 min in these initial studies.

The reaction on these strips does approach an endpoint. Under conditions generally used here, this occurs at about 4 min, but this can be reduced to about 1 min by using high concentrations of enzyme.

In preparing strips containing all necessary assay elements, we had to identify procedures that would prevent premature reaction of these components. In particular, for the current assay, it was necessary to prevent formation of a GU-theophylline/antibody complex before the sample was applied, and that the competitive protein-binding reaction could occur. This was made possible by choosing a solvent that would allow incorporation of conjugate (GU-theophylline) in a second dip but that would not support interaction of GU-theophylline with antibody. It was also essential that the solvent not seriously affect performance of the antibody or $\beta$-galactosidase. Incorporation of conjugate into the strip does influence the kinetic response; under these conditions, an additional, more slowly developing fluorescent signal appears.

As noted in Results, assay conditions can be established that permit use of a linear standard curve over the relevant concentration range in the integral strip. This permits calibration of strips with use of two standards. It remains to be determined whether strip stability will permit the long-term application of a two-point standard curve, which would, of course, provide additional convenience in the assay. The requirements for reagent dilutions general to most commercial immunoassay procedures are obviated, and the assay requires only a single dilution. No separation steps are required, serum volumes of less than 2 $\mu$L are applied to the strip, and the assay is rapid and exceptionally easy to perform.

References