Solid-Phase Reagent Strips for Detection of Therapeutic Drugs in Serum by Substrate-Labeled Fluorescent Immunoassay

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Solid-phase reagent strips for therapeutic drug detection are described. The chemistries are based on the substrate-labeled fluorescent immunoassay (SLFIA) technique. The procedures described here permit the incorporation of all assay components in dry reagent format. Two procedures are described which use either a "reversible complex formation" assay or a direct competitive protein binding assay. These solid phase reagents provide a one-step immunoassay after appropriate dilution of the sample. Correlation studies between HPLC and the reagent strip for theophylline on clinical sera containing 4–40 μg/mL theophylline yield a correlation coefficient of 0.98, a standard error that is less than 1.72 μg/mL, and a slope of 1.024. In the decision range of 15–25 μg/mL, the reagent strip shows a within-run precision of less than 4% with a bias of less than 1 μg/mL.

In recent years, great emphasis has been placed on monitoring therapeutic drugs in serum. Development of various analytical techniques (1–4) made it possible to achieve and maintain drug therapeutic levels more efficiently. These techniques ensure that therapeutic levels of a drug in serum can be effectively monitored so that toxic levels are not reached. This paper describes a convenient approach for rapid drug determination by use of solid phase reagent strips. These dry reagent strips contain all the components essential for the quantitative detection of a specific drug.

The solid phase reagents are constructed by using chromatography paper as a carrier matrix containing the components of a homogeneous immunochromatographic assay, the substrate-labeled fluorescent immunooassay (5). Initial results on homogeneous immunoassays on paper matrices have been described previously (6). In this paper, two approaches are described which permit combination of all the assay components. The reaction format of one solid phase reagent strip is based on a "reversible complex formation" assay (7), whereas the reaction format of the other is based on a competitive protein binding assay (8).

The reversible complex formation assay is summarized by eq 1–3. The assay involves introducing a specific drug (D)

\[ \text{Ab-C} \rightarrow \text{Ab} + \text{C} \]  

\[ \text{Ab} + \text{C} + \text{D} \rightarrow \text{Ab-C} + \text{Ab-D} + \text{C} \]  

\[ \text{C} + (\text{E}) \rightarrow \text{C}_\text{f} \]  

into a medium containing a preformed complex (Ab-C) between antibody (Ab) and a conjugate (C). The conjugate consists of a galactosylumbelliferylone label attached to the drug (D) in question. Upon enzymatic hydrolysis of the conjugate with β-galactosidase (E), the conjugate can be monitored by fluorescence. As the complex dissociates, the drug and its respective conjugate compete for the antibody binding sites. The displaced conjugate is proportional to the bound drug and the extent of displacement is a function of drug concentration. The displaced conjugate product (Cf) is monitored by fluorescence after the enzymatic hydrolysis of galactose. The antibody bound conjugate is not susceptible to hydrolysis (9).

The protein competitive binding assay is summarized by eq 4 and 5.

**LITERATURE CITED**

Ab + C + D → Ab-C + Ab-D + C + D

C + enzyme → C_t

Upon introduction of a specific drug and its respective conjugate, competition for antibody binding sites takes place. The unbound conjugate is proportional to the bound drug. The conjugate is monitored by fluorescence measurement after enzymatic hydrolysis of galactose.

In constructing a single step reagent strip for drug detection based on the competitive protein binding procedure, it is necessary to avoid premature reaction of the conjugate with antibody or enzyme. This is accomplished by segregating assay components during assembly by appropriate selection of solvents.

MATERIAL AND METHODS

Materials. Ammonium sulfate suspensions of β-galactosidase and o-nitrophenyl-β-D-galactopyranoside (ONPG) were obtained from Sigma. The various antisera and β-galactosylumbelliferone conjugates of amikacin (β-GUA), carbamazepine (β-GUCBZ), phenytoin (β-GUPH), primidone (β-GUPR), isonicotinic (β-GUS), theophylline (β-GUT), and tobramycin (β-GUTo) were obtained as previously described (9). Bicine (N,N-bis(2-hydroxyethyl)-glycine) was obtained from Calbiochem. Whatman 31ET filter paper was obtained from Whatman, Inc., and reflective Mylar was obtained from 3M Co.

Instrumentation. Fluorescence readings were made by front-face analysis of horizontally mounted dry reagent strips using a filter fluorometer described in detail in the companion paper (10). The detector (PM-tube) output was measured as a current (nanoamperes) by a Hewlett-Packard 11490A digital voltmeter interfaced to the detector by a current-to-voltage amplifier. A Hewlett-Packard 17500A chart recorder was also interfaced to either voltmeter for kinetic studies.

Fluorescence studies in solution were made in 3.0-mL cuvettes using the Farrand MK-1. The instrument detector was monitored either by the use of the instrument's built-in digital voltmeter or by connecting a Hewlett-Packard 3440A digital voltmeter interfaced to the detector by a current-to-voltage amplifier. A Hewlett-Packard 17500A chart recorder was also interfaced to either voltmeter for kinetic studies.

RESULTS

Preparation of β-Galactosidase. Ammonium sulfate suspensions of β-galactosidase were dialyzed, using an ultracentrifugation device (Amicon Co.). The enzyme suspension was dissolved in 10 mL of a 0.1 M bicarbonate buffer containing 0.05 M MgCl₂ and 0.1% NaN₃, pH 8.3. Dialysis was carried out against the same buffer and stopped after 100 mL of buffer has passed through the system.

The enzyme was assayed with ONPG as a substrate (11, 12). The assay consisted of adding 0.1 mL of enzyme solution (0.2–5 U/mL) to a 3-mL cuvette containing 2.6 mL of 0.1 M Bicine buffer + 0.05 M MgCl₂, pH 8.3, and 0.3 mL of 0.03 M ONPG. The rate of ONPG hydrolysis was monitored on a Beckman DU-8 at 415 nm and 25 °C.

Determination of Antisera Titer. The titer of the antisera to various drugs was determined as previously described (8). The conjugate concentrations were determined by absorbance measurements at 340 nm (8, 12). Reversible Complex Dissociation Studies in Solution. Dissociation studies in solution on preformed antibody conjugate complex were conducted with the gentamicin SLFIA chemistry (13). Antibody–conjugate complexes were preformed by adding 6.5 mL of gentamicin antisera and 0.1 mL of 0.84 mM β-GUS conjugate to 2.7 mL of 0.1 M Bicine buffer, pH 8.3, in a 3.0-mL cuvette. After 15 min of preincubation, 0.1 mL of 53 U/mL β-galactosidase was added. After an additional 15-min preincubation, the resulting 0.1 mL of gentamicin complex was added to one edge of a clear polystyrene ribbon 8.3 cm wide. The material was cut into 1 cm strips to give a 1 × 3 cm handle with a 1 × 1 cm reagent pad.

Solutions for generating reagent and control pads were prepared by incubating a given conjugate with its respective antisera and control sera for 15 min. After addition of β-galactosidase, the solution was allowed to incubate an additional 15 min. Twenty-microliter aliquots of a given solution were deposited on 1 × 1 cm Whatman 31ET reagent pads and were dried for 15 min at 50 °C.

Reversible Complex Dissociation Assay on Analytical Strips. Assays were conducted by depositing 70 µL of a given drug solution onto solid phase reagent strips, and the reactions were allowed to proceed for 15 min in a humidified chamber. Each point on a dose response curve represents the mean of five replicates.

Preparation of Solid-Phase Reagent Strips for Drug Detection by Competitive Protein Binding. Solid-phase reagent strips for detection of theophylline, carbamazepine, tobramycin, amikacin, gentamicin, primidone, and phenytoin were prepared by a two-step impregnation process, using Whatman 31ET chromatography paper. The first step consisted of saturating the paper with an aqueous solution containing a given antisera, enzyme, and buffer followed by drying the paper at 50 °C in a drying tunnel. The second step consisted of saturating the papera second time with a solution of acetone containing the respective conjugates. The paper was again dried at 50 °C. The resulting reagent papers were made into 0.5 × 1.0 cm reagent strips. The quantity of constituents per reagent strip was calculated from the area of paper saturated by a given volume of impregnation solution.

Solid-phase reagent strips for gentamicin detection were prepared in smaller quantities in the following fashion. The first step consisted of depositing onto 1 × 1 cm Whatman 31ET reagent strip 20 µL of an aqueous solution containing 5 µL of antisera, 0.2 units of β-galactosidase, 7.2 µmol of bicine, and 1 µmol of MgCl₂, pH 8.3. The pads were dried at 50 °C for 15 min in a convection oven. The second step involved depositing onto the dried reagent strip 20 µL of a tolune solution containing 38.9 pmol of conjugate. The material was dried in the same manner.

Competitive Binding Drug Assay on Solid-Phase Reagent Strips. Drug assays using the solid-phase reagent strip were conducted by depositing 35 µL or 70 µL of a drug solution onto 0.5 × 1 cm or 1 × 1 cm reagent strips, respectively. The increase in fluorescence was monitored for 3 min. Each point on a dose response curve represents the mean of five replicates.

DISCUSSION

The feasibility of "reversible complex formation" approach as an analytical procedure for drug detection was first examined in solution by using the SLFIA constituents for gentamicin. The assay was initiated by introducing gentamicin to a 3-mL cuvette containing preformed antibody: conjugate complex and β-galactosidase. Figure 1 illustrates the extent of fluorescence increase as a function of increasing gentamicin concentration. Fluorescence that occurs in the absence of antisera is given a value of 100%. The displacement approach approaches a plateau value at about 40 min. A plot of fluorescence produced after 40 min against gentamicin concentration is shown in Figure 2. The extent of conjugate release from the complex is limited to one reagent strip. The response curve saturates at gentamicin concentrations greater than 0.4 µg/mL (74 pmol). This accounts for about 88% of the available 84 pmol of conjugate in the assay. These results demonstrate that reversible complex formation can be used as an analytical procedure for drug detection.
Table I. Strip Constituents for Reversible Complex Dissociation Assay

<table>
<thead>
<tr>
<th>component</th>
<th>theophylline strip</th>
<th>CBZ strip</th>
<th>tobramycin strip</th>
<th>gentamicin strip</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. antisera</td>
<td>10 µL</td>
<td>10 µL</td>
<td>10 µL</td>
<td>10 µL</td>
</tr>
<tr>
<td>2. conjugate</td>
<td>β-GUT</td>
<td>β-GUCBZ</td>
<td>β-GUTO</td>
<td>β-GUS</td>
</tr>
<tr>
<td>quantity</td>
<td>129.9 pmol</td>
<td>112.3 pmol</td>
<td>100 pmol</td>
<td>130 pmol</td>
</tr>
<tr>
<td>3. buffer (Bicine)</td>
<td>2 µmol</td>
<td>2 µmol</td>
<td>2 µmol</td>
<td>2 µmol</td>
</tr>
<tr>
<td>4. β-galactosidase</td>
<td>0.065 U</td>
<td>0.065 U</td>
<td>0.065 U</td>
<td>0.065 U</td>
</tr>
<tr>
<td>5. Me₆SO</td>
<td>0.17 µL</td>
<td>0.17 µL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table II. Summary of Therapeutic Range, Dilution Factors, and Dose Response Range for Each Drug

<table>
<thead>
<tr>
<th></th>
<th>theophylline</th>
<th>CBZ</th>
<th>tobramycin</th>
<th>gentamicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>clinical range of interest</td>
<td>0-40 µg/mL</td>
<td>0-20 µg/mL</td>
<td>1-12 µg/mL</td>
<td>1-12 µg/mL</td>
</tr>
<tr>
<td>(0-222 µM)</td>
<td>(0-84.6 µM)</td>
<td>(0-25.7 µM)</td>
<td>(0-22.7 µM)</td>
<td>(0-22.7 µM)</td>
</tr>
<tr>
<td>dilution factor for assay</td>
<td>1/40</td>
<td>1/50</td>
<td>1/20</td>
<td>1/10</td>
</tr>
<tr>
<td>range after dilution</td>
<td>0-1.0 µg/mL</td>
<td>0-0.4 µg/mL</td>
<td>0-0.8 µg/mL</td>
<td>0-1.2 µg/mL</td>
</tr>
<tr>
<td>(0-5.6 µM)</td>
<td>(0-1.7 µM)</td>
<td>(0-1.3 µM)</td>
<td>(0-2.2 µM)</td>
<td>(0-3.7 µM)</td>
</tr>
<tr>
<td>range examined</td>
<td>0-4 µg/mL</td>
<td>0-0.4 µg/mL</td>
<td>0-0.6 µg/mL</td>
<td>0-2 µg/mL</td>
</tr>
<tr>
<td>(0-22.2 µM)</td>
<td>(0-1.7 µM)</td>
<td>(0-1.3 µM)</td>
<td>(0-5.6 µM)</td>
<td></td>
</tr>
</tbody>
</table>

by Reversible Complex Formation. Table I summarizes the contents of solid-phase reagent strips for detection of theophylline, carbamazepine (CBZ), tobramycin, or gentamicin. Upon deposition of a drug sample onto a given reagent strip, the displacement of conjugate is initiated. The kinetics of the displacement is illustrated in Figures 3 for theophylline strips. Increasing the drug concentration results in increased conjugate displacement. After 15 min, approximately 20% of the conjugate is displaced at the highest drug concentration based on the total available conjugate of control strips. Dose response curves were generated by plotting fluorescence produced after 15 min as a function of drug concentration as illustrated in Figure 4. Similar observations were made with reagent strips designed to detect carbamazepine, tobramycin, and gentamicin. In all cases, the therapeutic range of the drug after appropriate dilution was covered by the dose response curve. Table II summarizes the clinical range of interest, the dilution factors, and the range examined for each drug.

Assembly of a Model Reagent Strip for Theophylline Detection Based on a Competitive Binding Assay. The SLFIA assay constituents for theophylline were incorporated into a paper matrix to generate a model dry reagent strip for a competitive binding assay. The antibody to theophylline, β-galactosidase, and the buffer were introduced in an aqueous solution onto 1 x 1 cm Whatman 31ET chromatography paper pads. After the pads were dried, the conjugate was introduced in an organic solution to prevent its interaction with the
antibody and the enzyme. To ascertain if the conjugate remained unreacted, two additional solid-phase reagent strips were prepared that served as controls. The analytical strips for drug detection had all the SLFIA components for a theophylline assay. Control-A strips were prepared by replacing the antisera with control sera. In addition to replacement of the antisera, control-B strips were prepared by deleting β-galactosidase. Table III summarizes the contents of each strip. Figure 5 illustrates the response curves of control strip A (curve 8), control strip B (curve 1) and the analytical strip (curve 2) after applying 70 µL of H2O. Curves 3–7 represent analytical strip response to increasing concentrations of aqueous theophylline (0.125–40 µg/mL). At zero time, all analytical strips and control-A strips showed a background fluorescence that was not significantly different from that of the control-B strips. This indicates that negligible hydrolysis of conjugate occurred during strip preparation. Upon application of H2O, the control-A strips showed an increase in fluorescence due to β-galactosidase action on the conjugate. The analytical strips showed a considerable lower rate of fluorescence release due to conjugate binding to the antibody.

This indicated that both enzyme activity and antibody binding capacity were present in the strip. These studies also demonstrated that the conjugate did not interact with the enzyme and antibody during and after preparation of the strips until the addition of an aqueous sample.

Table IV. Strip Constituents for Competitive Protein Binding

<table>
<thead>
<tr>
<th>component</th>
<th>strip</th>
<th>theophylline</th>
<th>tobramycin</th>
<th>gentamicin</th>
<th>amikacin</th>
<th>CBZ</th>
<th>primidone</th>
<th>phenytoin</th>
</tr>
</thead>
<tbody>
<tr>
<td>antiserum</td>
<td></td>
<td>7.7 µL</td>
<td>7.7 µL</td>
<td>15.4 µL</td>
<td>7.7 µL</td>
<td>7.7 µL</td>
<td>7.7 µL</td>
<td>7.7 µL</td>
</tr>
<tr>
<td>conjugate (quantity)</td>
<td></td>
<td>β-GUT</td>
<td>β-GUTTo</td>
<td>β-GUS</td>
<td>β-GUA</td>
<td>β-GUCBZ</td>
<td>β-GUPr</td>
<td>β-GUph</td>
</tr>
<tr>
<td>buffer (Bicine)</td>
<td></td>
<td>78 pmol</td>
<td>59 pmol</td>
<td>130 pmol</td>
<td>77.8 pmol</td>
<td>65 pmol</td>
<td>67 pmol</td>
<td>70.6 µmol</td>
</tr>
<tr>
<td>magnesium chloride</td>
<td></td>
<td>1.9 µmol</td>
<td>1.9 µmol</td>
<td>2.0 µmol</td>
<td>1.0 µmol</td>
<td>1.0 µmol</td>
<td>1.0 µmol</td>
<td>1.0 µmol</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td></td>
<td>0.33 U</td>
<td>0.33 U</td>
<td>0.66 U</td>
<td>0.33 U</td>
<td>0.33 U</td>
<td>0.33 U</td>
<td>0.33 U</td>
</tr>
<tr>
<td>sodium formate</td>
<td></td>
<td>0.17 µL</td>
<td>0.17 µL</td>
<td>0.12 µL</td>
<td>0.09 µL</td>
<td>0.14 µL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Solid-Phase Reagent Strips for Detection of Therapeutic Drugs by Competitive Protein Binding. Prototype analytical strips have been prepared for detecting antiasthmatic, antibiotic, and anticonvulsant drugs. The contents of each reagent strip are summarized in Table IV. The analysis time was reduced by increasing the β-galactosidase level. This is shown in Figure 6 by the response of the theophylline detecting strip to increasing drug concentration. At 3 min, nearly all the unbound conjugate is hydrolyzed. With a 3-min fixed time point, a dose response curve for theophylline concentrations of 0-20 µg/mL was generated as shown in Figure 7. In a similar fashion, dose response curves were generated with strips designed for detection of the antibiotics tobramycin (Figure 8), gentamicin, and amikacin. Similar dose response curves were generated for strips designed to detect the anticonvulsant drugs phenytoin, carbamazepine, and primidone. Each dose response curve covers the therapeutic range of the respective drug after an appropriate dilution. In addition, each curve has a linear region that lends itself to the application of a two-point standard curve.

Correlation Study between High-Pressure Liquid Chromatography (HPLC) and the Theophylline Detecting Strip. Clinical sera containing theophylline were used as reagent strip. Theophylline detection by HPLC was conducted as previously described (14). Calibrators and samples were diluted 1:20 with water before application. The system was calibrated with 5 and 30 µg/mL serum based theophylline calibrators to construct a two-point standard curve. This makes use of the linear portion of the dose response curve (0.25-1.5 µg/mL theophylline of Figure 7). A typical correlation study against HPLC consists of 30 clinical sera having a theophylline range of 4-40 µg/mL. The correlation results gave a line, $y_{(\text{reagent strip})} = 1.024x_{(\text{HPLC})} + 0.088$ µg/mL + a standard error of 1.72 µg/mL, and a correlation coefficient of 0.9830. Table V summarizes the precision of the strip at three levels of theophylline. The bias at all levels is less than 1 µg/mL.

**Table V. Precision of Theophylline Reagent Strips**

<table>
<thead>
<tr>
<th>Amount of Theophylline, µg/mL</th>
<th>Precision within run ($n=30$)</th>
<th>Precision among run ($n=10$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% coeff std dev variation</td>
<td>% coeff std dev variation</td>
</tr>
<tr>
<td>5</td>
<td>0.42</td>
<td>8.4</td>
</tr>
<tr>
<td>15</td>
<td>0.57</td>
<td>3.8</td>
</tr>
<tr>
<td>25</td>
<td>0.90</td>
<td>3.6</td>
</tr>
</tbody>
</table>

DISCUSSION

These studies demonstrate that solid-phase reagent strips for therapeutic drug detection can be prepared. The detection chemistry is a homogeneous immunoassay utilizing the constituents of substrate-labeled fluorescent immunosassays for therapeutic drugs. Each strip contains all the chemical constituents required to make a specific drug determination. After an appropriate dilution, the assay is initiated by a single step addition of the sample to the strip. In the case of the reversible complex formation assay, results can be obtained within 15 min. In the case of the competitive protein binding assay, results are obtained within 3 min.

The use of the reversible complex formation as an analytical procedure for therapeutic drug detection has been demonstrated. This approach depends upon dissociation of the conjugate antibody complex (15-17) and reequilibration of antibody with the conjugate and drug. Hence, the apparent displacement of conjugate is proportional to the analyte.
Automated Instrumentation for Fluorescence Assays on Reagent Strips

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An automated instrument has been constructed for measurement of fluorescence and reflectance from reagent strips. Examples of front face fluorescence measurements for the detection of fluorescent products on reagent strips are described. These reagent strips can be automatically loaded, samples automatically sequenced and dispensed, and reflectance and fluorescence measurements automatically made and stored on magnetic media for subsequent analyses.

Traditional wet chemistry has generally been performed by using some sort of glass or quartz container within which solutions are placed, both for separate reaction steps and also for a possible analysis which may be spectroscopic in nature. Recently, new blood chemistry products (1) have been introduced in the field of clinical chemistry which incorporate all reagents within a single solid matrix such as paper or thin films. The format which uses the paper matrix will be denoted as the "Reagent Strip" and used for the correlation of the presence of drugs.

In summary, it was demonstrated that paper matrices are acceptable for conducting immunoassays. Dry reagent strips containing all assay constituents can be prepared for the specific detection of therapeuti c drugs. These assays are rapid and require minimal technical skill.

ACKNOWLEDGMENT

The authors are indebted to the Ames Immunoochemistry Laboratory for supplying essential reagents for this study and, in particular, to John Burd and Thomas Li for helpful discussions. The excellent technical assistance of Christine Nelson and Oseola Skinner is also gratefully acknowledged.

Registry No. Theophylline, 58-55-9; tobramycin, 32986-56-4; gentamicin, 1403-65-3; amikacin, 37517-28-5; carbamazepine, 298-46-4; primidone, 125-33-7; phenytoin, 57-41-0.

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Received for review March 1, 1982. Resubmitted and accepted January 12, 1983.