toluene, 108-88-3; xylene, 1330-20-7; phenol, 108-95-2; cresol, 1319-77-3; cyclohexane, 110-82-7; hexane, 110-54-3; indene, 95-13-6; pyridine, 110-86-1; pyrrole, 109-97-7; naphthalene, 91-20-3; azulene, 275-51-4; anthracene, 120-12-7; phenanthrene, 85-01-8; tetracene, 92-24-0; *p*-nitrotoluene, 99-99-0; 2,6-dinitrotoluene, 606-20-2; methanol, 67-56-1; ethanol, 64-17-5; 1-nonanol, 143-08-8; 1-octanol, 111-87-5; acetone, 67-64-1; benzophenone, 119-61-9; benzaldehyde, 100-52-7; *p*-dioxane, 123-91-1; ethyl formate, 109-94-4.

LITERATURE CITED

(1) Lubman, D. M.; Kronick, M. N. *Anal. Chem.* **1982**, *54*, 1546.
(2) Lubman, D. M.; Kronick, M. N. *Anal. Chem.* **1982**, *54*, 2289.
(3) Lubman, D. M.; Kronick, M. N., submitted for publication in *Anal. Chem.*
(4) Karasek, F. W. *Anal. Chem.* **1971**, *43*, 1982.
(5) Karasek, F. W.; Tatone, O. S. *Anal. Chem.* **1972**, *44*, 1758.
(6) Griffen, G. W.; Dzidic, I.; Carroll, D. I.; Stillwell, R. N.; Horning, E. C. *Anal. Chem.* **1973**, *45*, 1204.
(7) Karasek, F. W. *Anal. Chem.* **1974**, *40*, 710A.
(8) Karasek, F. W.; Denny, D. W. *Anal. Chem.* **1974**, *46*, 1312.
(9) Spangler, G. E.; Collins, C. I. *Anal. Chem.* **1975**, *47*, 393.
(10) Carr, T. W. *Anal. Chem.* **1974**, *51*, 705.
(11) Kim, S. H.; Betty, K. R.; Karasek, F. W. *Anal. Chem.* **1978**, *50*, 1784.
(12) Carr, T. W. *Anal. Chem.* **1977**, *49*, 828.
(13) Kim, S. H.; Betty, K. R.; Karasek, F. W. *Anal. Chem.* **1978**, *50*, 2006.
(14) Metro, M. M.; Keller, R. A. *J. Chromatogr. Sci.* **1973**, *11*, 520.
(15) Spangler, G. E.; Lawless, P. A. *Anal. Chem.* **1978**, *50*, 884.
(16) Cohen, M. J.; Karasek, F. W. *J. Chromatogr. Sci.* **1970**, *8*, 330.
(17) Karasek, F. W.; Cohen, M. J.; Carroll, D. I. *J. Chromatogr. Sci.* **1971**, *9*, 391.
(18) Karasek, F. W.; Kane, D. M. *Anal. Chem.* **1974**, *46*, 780.
(19) Karasek, F. W.; Kim, S. H.; Rokushika, S. *Anal. Chem.* **1978**, *50*, 2013.
(20) Carr, T. W. *J. Chromatogr. Sci.* **1977**, *15*, 85.
(21) Hagen, D. F. *Anal. Chem.* **1979**, *51*, 870.
(22) Baim, M. A.; Hill, H. H., Jr. *Anal. Chem.* **1982**, *54*, 38.
(23) Herndon, W. C. *J. Am. Chem. Soc.* **1976**, *98*, 887.
(24) Weast, R. C., Ed. "Handbook of Chemistry and Physics", 51st ed.; The Chemical Rubber Co.: Cleveland, OH, 1970–1971; pp E80–E84.
(25) Franklin, J. L.; et al. "Ionization Potentials, Appearance Potentials and Heats of Formation of Gaseous Positive Ions"; National Bureau of Standards: Washington, DC, 1969.
(26) Information provided courtesy of HNU Systems, Inc., Newton Upper Falls, MA, 1982.
(27) Information provided courtesy of Quantatec International, Inc., Chatsworth, CA, 1982.
(28) Altshuller, A. P.; Cohen, J. R. *Anal. Chem.* **1960**, *32*, 802.
(29) Seaver, M.; Hudgens, J. W.; DeCorpo, J. J. *Int. J. Mass Spectrom. Ion. Phys.* **1980**, *34*, 159.

RECEIVED for review September 27, 1982. Accepted January 20, 1983. This work received financial support from the U.S. Army Research Office, Contract No. DAAG-29-81-C-0023. The excimer laser was borrowed from the San Francisco Laser Center, supported by the National Science Foundation under Grant No. CHE79-16250 awarded to the University of California at Berkeley in collaboration with Stanford University.

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

Bert Walter,* Alfred C. Greenquist, and Willis E. Howard, III

Blood Chemistry Laboratory, Ames Division, Miles Laboratories, Inc., Elkhart, Indiana 46515

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 constituents 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 immunochemical assay, the substrate-labeled fluorescent immunoassay (*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)

$$\text{Ab–C} \rightarrow \text{Ab} + \text{C} \quad (1)$$

$$\text{Ab} + \text{C} + \text{D} \rightarrow \text{Ab–C} + \text{Ab–D} + \text{C} \quad (2)$$

$$\text{C} + (\text{E}) \rightarrow \text{C}_\text{f} \quad (3)$$

into a medium containing a preformed complex (Ab–C) between antibody (Ab) and a conjugate (C). The conjugate consists of a galactosylumbelliferone label attached to the drug (D) in question. Upon enzymatic hydrolysis of the conjugate with β-galactosidase (E), the conjugate can be monitored by fluoresence. 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 (C_f) is monitored by fluorescence after the enzymatic hydrolysis of galactose. The antibody bound conjugate is not susceptible to hydrolysis (*5*).

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

0003-2700/83/0355-0873$01.50/0

$$Ab + C + D \rightarrow Ab\text{–}C + Ab\text{–}D + C + D \quad (4)$$

$$C + \text{enzyme} \rightarrow C_f \quad (5)$$

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), sisomicin (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 Digital Voltmeter X-3 or as a voltage (millivolts) by 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.

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 meter or by connecting a Hewlett-Packard 3440A voltmeter to the instrument's recorder output after disconnecting the built-in digital meter. All studies were conducted with 5-nm slits at the excitation and emission monochromators.

All fluorescence work described in this report was conducted by excitation at 405 nm and measuring the fluorescence emission at 450 nm.

Preparation of β-Galactosidase. Ammonium sulfate suspensions of β-galactosidase were dialyzed, using an ultrafiltration device (Amicon Co.). The enzyme suspension was dissolved in 10 mL of a 0.1 M bicine buffer containing 0.05 M $MgCl_2$ and 0.1% NaN_3, 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 $MgCl_2$, pH 8.3, and 0.3 mL of 0.03 M ONPG. The rate of ONPG hydrolysis was monitored in 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*). 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 μL of gentamicin antisera and 0.1 mL of 0.84 μM 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 33 U/mL β-galactosidase was added. After an additional 15-min preincubation, the assay was initiated by introducing 0.1 mL of gentamicin in the range of 0–2 μg/mL. Fluorescence was measured with a Farrand MK-1.

Preparation of Solid-Phase Reagent Strips for Drug Detection by Reversible Complex Format. All reagent and control materials were prepared with Whatman 31ET paper as the carrier matrix. Sections of 1 × 1 cm were prepared by laminating reflective Mylar to one side of the paper followed by laminating the Mylar side to double adhesive tape. After the tape was cut into 1 cm ribbons, the composite was laminated onto one edge of a clear polystyrene ribbon 8.3 cm wide. This material was cut into 1 cm strips to give a 1 × 8.3 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 or 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 paper a 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 $MgCl_2$, 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 toluene solution containing 39.8 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.

RESULTS

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 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 was proportional to drug concentration. 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.

Solid-Phase Reagent Strips for Detection of Theophylline, Carbamazepine, Tobramycin, and Gentamicin

Table I. Strip Constituents for Reversible Complex Dissociation Assay

component	content/pad: theophylline strip	CBZ strip	tobramycin strip	gentamicin strip
1. antisera	10 μL	10 μL	10 μL	10 μL
2. conjugate	β-GUT	β-GUCBZ	β-GUTo	β-GUS
quantity	129.9 pmol	112.3 pmol	100 pmol	130 pmol
3. buffer (Bicine)	2 μmol	2 μmol	2 μmol	2 μmol
4. β-galactosidase	0.065 U	0.065 U	0.065 U	0.065 U
5. Me_2SO	0.17 μL	0.17 μL		

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

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

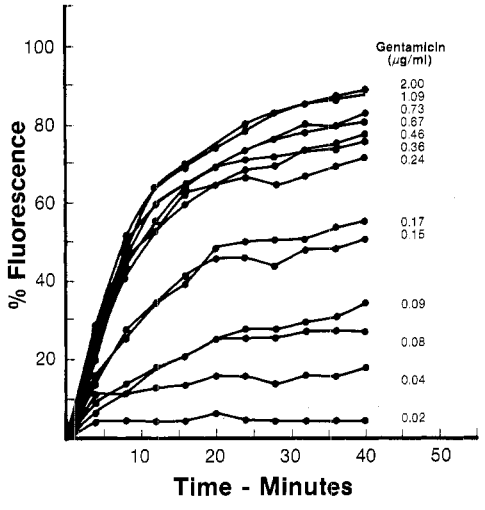

Figure 1. The extent of β-GUS displacement from the β-GUS antibody complex. Gentamicin in the range of 0.02–2 μg/mL was added to 3.0-mL cuvettes containing preformed complex. The released conjugate was monitored by fluorescence at 450 nm after the action of β-galactosidase. All rates were corrected for the blank reaction.

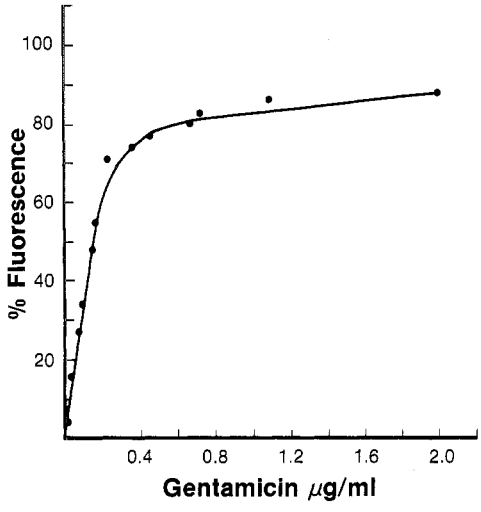

Figure 2. β-GUS released after 40 min as a function of gentamicin concentration.

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

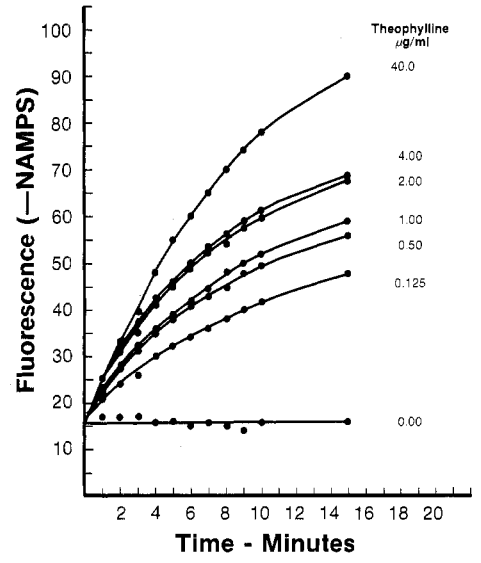

Figure 3. The rate of conjugate displacement from antibody conjugate complex of the theophylline strip on application of 0–40 μg/mL theophylline solutions.

solution onto 1 × 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

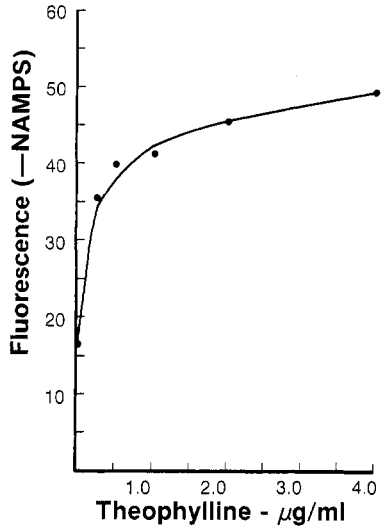

Figure 4. Dose response curves based on fluorescence released after 15 min as a function of theophylline concentration.

Table III. Content of Composite Model Strip for Theophylline Detection by SLFIA

component	quantity/strip analytical	control A	control B
antisera	10 μL		
normal Sera		10 μL	10 μL
buffer (Bicine)	2 μmol	2 μmol	2 μmol
enzyme (β-galactosidase)	0.126 U	0.126 U	
conjugate (β-GUT)	130 pmol	130 pmol	130 pmol

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 H_2O. 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 H_2O, 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.

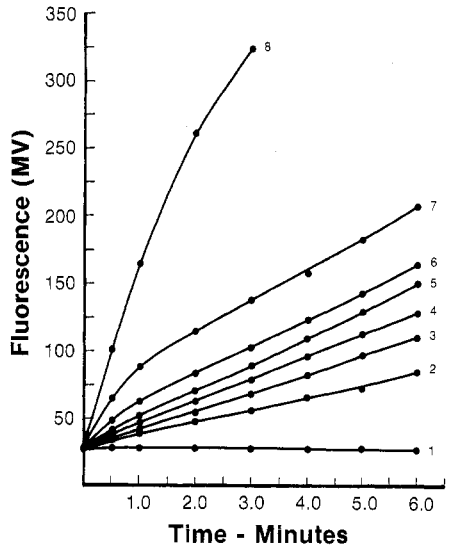

Figure 5. Response of model analytical and control strips for theophylline detection by competitive binding. Curves 1, 8, and 2 represent the response of control-B, control-A, and the detecting strip, respectively, after deposition of 35 μL of water. Curves 3–7 represent the response of the strips after deposition of 35 μL of 0.13, 0.25, 0.5, 1.0, and 40 μg/mL theophylline, respectively.

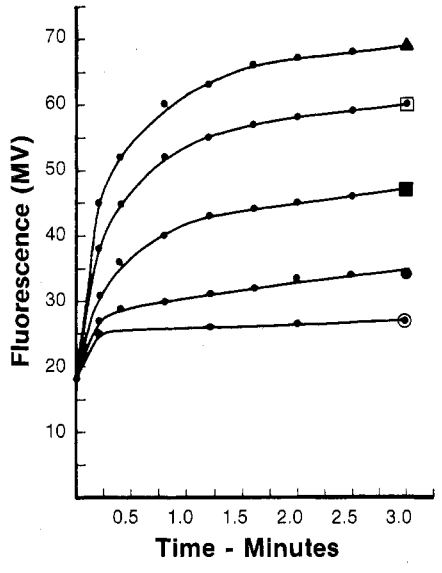

Figure 6. Response profile of prototype theophylline strip to increasing theophylline. Thirty-five microliters of 0 (○), 0.5 (●), 1.0 (■), 1.5 (□), and 2.0 (▲) μg/mL theophylline was applied to a given strip. Fluorescence was measured for 3 min.

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

component	strip theophylline	tobramycin	gentamicin	amikacin	CBZ	primidone	phenytoin
antiserum	7.7 μL	7.7 μL	15.4 μL	7.7 μL	7.7 μL	7.7 μL	7.7 μL
conjugate	β-GUT	β-GUTo	β-GUS	β-GUA	β-GUCBZ	β-GUPr	β-GUPh
(quantity)	76 pmol	59 pmol	130 pmol	77.8 pmol	65 pmol	67 pmol	70.6 pmol
buffer (Bicine)	10.8 μmol	10.8 μmol	21.6 μmol	10.8 μmol	10.8 μmol	10.8 μmol	10.8 μmol
magnesium chloride	1.0 μmol	1.0 μmol	2.0 μmol	1.0 μmol	1.0 μmol	1.0 μmol	1.0 μmol
β-galactosidase	0.33 U	0.33 U	0.66 U	0.33 U	0.33 U	0.33 U	0.33 U
sodium formate		0.5 μmol	0.2 μmol				
Me_2SO	0.17 μL			0.17 μL	0.12 μL	0.09 μL	0.14 μL

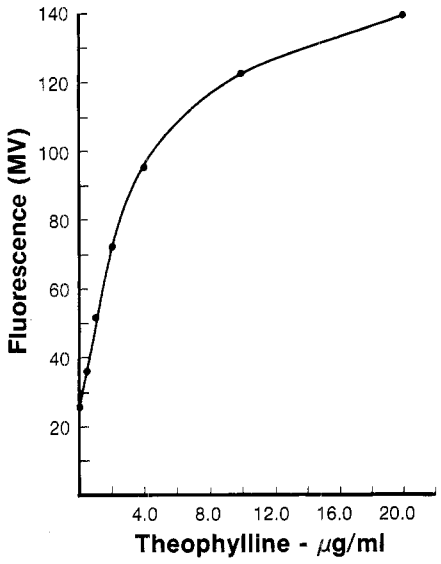

Figure 7. Dose response curve for theophylline. Fluorescence produced after 3 min was plotted as a function of theophylline.

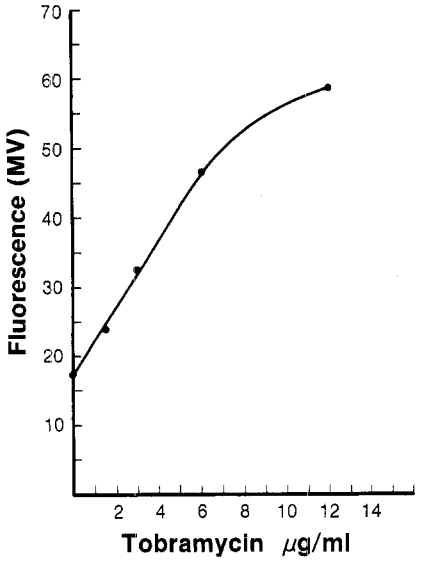

Figure 8. Dose response curve for the antibiotic tobramycin.

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 (Figure 9), 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 to conduct a correlation study between HPLC and the theophylline 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 (reagent strip) = 1.024x (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.

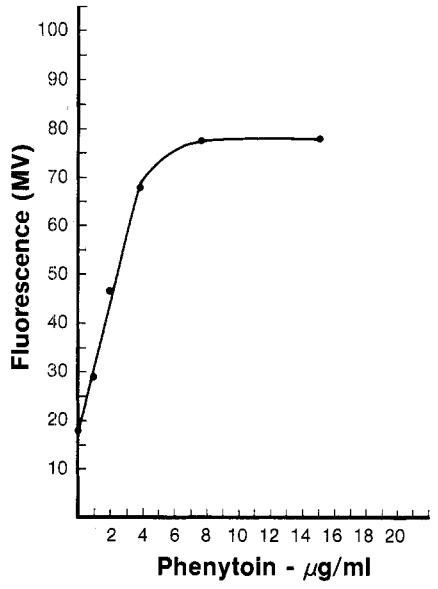

Figure 9. Dose response curve for strips detecting the anticonvulsant phenytoin.

Table V. Precision of Theophylline Reagent Strips

amt of theophylline, μg/mL	precision: within run (n = 30) std dev	within run (n = 30) % coeff of variation	among run (n = 10) std dev	among run (n = 10) % coeff of variation
5	0.42	8.4	0.42	8.4
15	0.57	3.8	0.56	3.7
25	0.90	3.6	0.90	3.6

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 immunoassays 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

concentration. The ratio of antibody binding sites to conjugate in the complex is adjusted to give a suitable dose response curve. The use of the immunoassay in the reversible complex format allows all reagents to be combined and stored in one compartment prior to use. This lends itself readily to constructing dry reagent analytical strips.

In preparing strips for drug detection by a competitive protein binding reaction (SLFIA), it was essential to utilize procedures that would prevent premature interaction of constituents. It was necessary to prevent conjugate binding to the antibody or conjugate hydrolysis by β-galactosidase prior to sample addition. This was accomplished by introducing the conjugate into the strip with a nonaqueous solvent. In this procedure, it was also important that the solvent did not adversely affect the antibody or the enzyme. This procedure was used to produce analytical strips for the quantitative detection of antiasthmatic drugs (theophylline), antibiotics (gentamicin, amikacin, and tobramycin), and antiepileptic drugs (primidone, phenytoin, and carbamazepine).

Of the two types of assay strips described, the competitive binding assay is the more rapid. Only 3 min are required per assay.

As described in the Results section, the use of the linear portion of the dose response curve allows calibration of a strip with two standards. A two-point standard curve was constructed for the theophylline strip and used for the correlation study. This eliminates the necessity for multiple calibrators required by many immunoassays. The availability of all assay constituents in one strip eliminates the requirement for reconstituting reagents, diluting of stock reagents, separating steps, and multiple additions to the assay medium. Only a single dilution of the sample is required to initiate the assay. This technology, therefore, has the potential to bring a level of convenience to immunochemistry not available with current methodology.

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

ACKNOWLEDGMENT

The authors are indebted to the Ames Immunochemistry 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 Osceola Skinner is also gratefully acknowledged.

Registry No. Theophylline, 58-55-9; tobramycin, 32986-56-4; gentamicin, 1403-66-3; amikacin, 37517-28-5; carbamazepine, 298-46-4; primidone, 125-33-7; phenytoin, 57-41-0.

LITERATURE CITED

(1) Booker, H. E.; Darcey, B. A. *Clin. Chem. (Winston-Salem, NC)* **1975**, *21*, 1766.
(2) Darcey, B. A.; Solow, E. B.; Pippenger, C. E. "Antiepileptic Drugs: Quantitative Analysis and Interpretation"; Raven Press: New York, 1976; pp 67–74.
(3) Kabra, P. M.; Stafford, B. E.; Marton, L. J. *Clin. Chem. (Winston-Salem, N.C.)* **1977**, *23*, 1284.
(4) Cook, C. E.; Christensen, H. D.; Amerson, E. W.; et al. "Quantitative Analytical Studies in Epilepsy"; Raven Press: New York, 1976; pp 39–58.
(5) Burd, J.; Carrico, R.; Fetter, M.; Buckler, R.; Johnson, R.; Boguslaski, R.; Christner, J. *Anal. Biochem.* **1977**, *77*, 56.
(6) Greenquist, A. L.; Walter, B.; Li, T. M. *Clin. Chem. (Winston-Salem, N.C.)* **1981**, *27*, 1614.
(7) Smith, T.; Skubilz, K. *Biochemistry* **1975**, *14*, 1496.
(8) Burd, J. F.; Carrico, R. J.; Kramer, H. M.; Denning, C. E. "Enzyme-Labelled Immunoassay of Hormones and Drugs"; Walter de Greyter and Co.: Berlin, 1978; pp 387.
(9) Li, T.; Benovic, J.; Buckler, R.; Burd, J. *Clin. Chem. (Winston-Salem, N.C.)* **1981**, *27*, 22.
(10) Howard, W. E.; Walter, B.; Greenquist, A. C. *Anal. Chem.* **1983**, *55*, 878.
(11) Craven, G. R.; Steers, E., Jr.; Anfinsen, C. B. *J. Biol. Chem.* **1965**, *240*, 2468.
(12) Wong, R. C.; Burd, J. F.; Carrico, R. J.; Buckler, R. T.; Thoma, J.; Boguslaski, R. *Clin. Chem. (Winston-Salem, N.C.)* **1979**, *25*, 686.
(13) Burd, J. F.; Wong, R. C.; Feeney, J. E.; Carrico, R. J.; Boguslaski, R. C. *Clin. Chem. (Winston-Salem, N.C.)* **1977**, *23*, 1402.
(14) Weidner, N.; Dietzler, D. N.; Landenson, J. H.; Kessler, G.; Larson, L.; Smith, C. H.; James, T.; McDonald, J. M. *Am. J. Clin. Pathol.* **1980**, *73*, 79.
(15) Dandliker, W. B.; Schapiro, H. C.; Mednski, J. W.; Alonso, R.; Feigen, G. A.; Humrick, J. P. *Immunochemistry* **1964**, *1*, 165.
(16) Portmann, A. J.; Levison, S. A.; Dandliker, W. B. *Biochem. Biophys. Res. Commun.* **1971**, *43*, 207.
(17) Lopatin, D. E.; Voss, E. W. *Biochemistry* **1971**, *10*, 208.

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

Automated Instrumentation for Fluorescence Assays on Reagent Strips

Willis E. Howard, III, Alfred Greenquist, Bert Walter,* and Frank Wogoman

Ames Division, Miles Laboratories, Inc., Elkhart, Indiana 46515

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 a reagent strip. For these strips, the sample is applied to the top of the paper and spectroscopic measurements are made from the top.

After a sample has been applied to a reagent strip, a reaction will occur. If it is to be followed spectroscopically, the reagent strip may be directly placed into an instrument made for that purpose. In the case of diffuse reflectance spectroscopy, a

0003-2700/83/0355-0878$01.50/0