Use of a Reversed-Phase Evaporation Vesicle Formulation for a Homogeneous Liposome Immunoassay

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Complement-mediated release of enzyme molecules from reversed-phase evaporation vesicles serves as the basis of the sensitive homogeneous immunoassay reported here. We found it necessary to co-entrap the substrate glucose 6-phosphate with the bacterial enzyme glucose-6-phosphate dehydrogenase (EC 1.1.1.49) to protect enzyme activity during liposome preparation. Enzyme can be released specifically from these liposomes by incubation with antibody and complement. The enzyme is not merely available to substrate but is actually physically free of the liposomes. Inhibition of this complement-mediated lysis by theophylline is the basis for the homogeneous liposome immunoassay described. The assay results vary linearly with theophylline concentrations in plasma in the clinically relevant range, and serum components do not interfere. The reagents in the assay kit are stable for at least seven months when stored at 5 °C. No nontheophylline compounds reacted significantly with the antisera used. The assay can be run in a kinetic format, with either ultraviolet or colorimetric detection.

Additional Keyphrases: theophylline • complement • kinetic enzyme assay • encapsulated glucose-6-phosphate dehydrogenase

Phospholipids can be dispersed in aqueous media to yield closed vesicles that are composed of one or a series of compartments separated by concentric lipid bilayers (1). These vesicles, or liposomes, can carry receptor molecules e.g., antigens, on their exterior surfaces. In addition, one can entrap in these vesicles aqueous solutions containing various water-soluble components of a wide range of molecular masses. These characteristics of liposomes make them potentially useful for monitoring concentrations of therapeutic drugs, such as theophylline.

Theophylline is used to relax the smooth muscles in the bronchi, especially in asthmatic patients (2). However, at concentrations above the therapeutic range, 10–20 mg/L (3, 4), theophylline can produce life-threatening cardiac arrhythmias (5) or seizures (6).

Past efforts involving a liposome-based assay have included color changes induced by cation-responsive dyes (7), mellitin-mediated lysis (8), complement-mediated lysis (9), enhanced agglutination (10), and solid-phase liposome immunoassay (11). Immunoassay systems involving vesicles with lysable lipid membranes have advantages over more-traditional diagnostic tests. Not only can the ligand-binding reaction and the measurement of released "reporter" labels from the lysed vesicles be performed in the same assay mixture, as a homogeneous assay (9), but also vesicle-lysis immunoassays can be highly sensitive, because relatively few ligand-binding events on the vesicle surface will release a large number of reporter molecules.

Several types of encapsulated reporter compounds have been used in lipid vesicle reagents: fluorescent compounds (11), hemoglobin (12), spin-labeled molecules (13), and enzymes (9, 14). The latter offer a number of advantages over smaller chromogenic, fluorescent, or paramagnetic molecules. Enzymes exhibit very little leakage upon storage, and very little release by nonspecific lysis in the presence of complement. They can participate in reactions that can be followed spectrophotometrically, obviating the need for relatively expensive instruments. In addition, multiple sensitivity is possible because of the high turnover of each enzyme molecule.

In the assay we report here, the bacterial enzyme glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) is encapsulated in reversed-phase evaporation vesicles (REVs), prepared by forming inverted micelles in an organic solvent and subsequently evaporating this solvent (15). This formulation avoids two difficulties encountered in previously studied methods. The enzyme used in a previous method (9), alkaline phosphatase, has been replaced; its presence in relatively high amounts in serum makes it unsuitable for use in determinations of serum analytes in a homogeneous assay format. Secondly, we avoided the use of multilamellar vesicles to encapsulate the enzyme, which have seriously decreased the activity concentrations of encapsulated enzyme that could be released by a complement-mediated lysis (14). Not only did multilamellar vesicles entrap less enzyme, but also these vesicles had to be lysed layer after layer to release all of the enzyme, necessitating longer assay times and higher concentrations of complement and antibody. Encapsulating the enzyme in REVs produces an essentially unilamellar vesicle with a high entrapment and release efficiency.

Materials and Methods

Reagents. Egg phosphatidylcholine (PC), egg phosphatidylglycerol (PG), and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Avanti Polar Lipids, Birmingham, AL. α-Tocopherol was obtained from Henkel Corp., Minneapolis, MN, and cholesterol from Nu-Chek-Prep, Inc., Elysian, MN. Glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides) was supplied by Cooper Biomedical, Malvern, PA. Glucose 6-phosphate (G6P), nicotinamide

1 Nonstandard abbreviations: DPPE, dipalmitoylphosphatidylethanolamine; G6P, glucose 6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; HLIA, homogeneous liposome immunoassay; REVs, reversed-phase evaporation vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

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Received January 23, 1986; accepted April 30, 1986.
dinucleotide (NAD\textsuperscript{+}), phenazine methosulfate, and 2,6-dichlorophenolindophenol were from Sigma Chemical Co., St. Louis, MO. Rabbit anti-theophylline serum was obtained from Western Chemical Research, Fort Collins, CO, and guinea pig complement from Miles Scientific, Naperville, IL. The gel-filtration agarose beads, Bio-Gel A-15m (100–200 mesh), were purchased from Bio-Rad Laboratories, Richmond, CA. Other compounds were reagent grade.

Preparation of the DPPE derivative of 8-(3-carboxypropyl)-theophylline (I). The starting material, I, was prepared according to Cook et al. (16) from glutaric anhydride and 4,5-diamino-1,3-dimethylpyrimidine-2,6-dione, then reacted with a 10-fold molar excess of thionyl chloride to form the hydrochloride of I. The DPPE derivative was then prepared by slowly adding a 3.5-fold molar excess of the above hydrochloride to DPPE, dissolved in chloroform, in the presence of a 25-fold molar excess of triethylamine. The triethylamine salts were extracted into aqueous buffer, and the DPPE derivative in the chloroform phase was dried over anhydrous sodium sulfate and chromatographed on a silica gel column (yield, 65%). The structure is depicted in Figure 1.

Preparation of REVs. Liposomes of the following lipid composition—PC/PG/cholesterol/theophylline-DPPE/α-tocopherol (45.2/12.9/40.4/1.4/0.1, molar ratio)—were prepared as described by Szoka and Papahadjopoulos (15). In brief, the lipids were evaporated and the residues redissolved in diethyl ether. After addition of phosphate-buffered saline (pH 7.4) that contained approximately 1000 U of G6PD and 15 mmol of G6P per liter, the mixture was sonicated for 30 s. The diethyl ether was removed under reduced pressure, leaving a viscous gel, and subsequently an aqueous suspension. Unencapsulated enzyme was removed by gel filtration through a Bio-Gel A-15m column. The lipidosome fractions were pooled and NaN\textsubscript{3} preservative was added, to give a final concentration of 0.2 g/L.

Assay for G6PD. We measured enzyme activity by incubation in a final volume of 500 μL of the dialyzed preparation in 300 mM Tris buffer, pH 7.8, containing 4 mmol of G6P and 1 mmol of NAD\textsuperscript{+} per liter, for 20 min at room temperature. Total enzyme activity in the REV preparation was assayed after disrupting the liposomes with 5 mL/L Triton X-100 solution. We stopped the reaction by adding Na\textsubscript{2}CO\textsubscript{3} (280 mM, final concentration) and read the absorbance at 340 nm. One unit is defined as the amount of enzyme that can reduce 1 μmol of NAD\textsuperscript{+} per minute at room temperature in the above buffer.

Ultraviolet spectrophotometric assay of antibody–complement-mediated lysis of theophylline-DPPE liposomes. Complement-mediated lysis was measured by incubating the liposomes with 2.5 μL of serum, anti-theophylline antibody, and complement in 90 mM Tris buffer, pH 7.8, containing 150 mmol of NaCl, 0.5 mmol of MgCl\textsubscript{2}, and 0.2 g of NaN\textsubscript{3} per liter in the presence of G6P (6 mmol/L) and NAD\textsuperscript{+} (1.5 mmol/L) for 30 min at 37 °C (final reaction volume, 500 μL). The reaction was essentially stopped by adding Na\textsubscript{2}CO\textsubscript{3}, and the absorbance was measured at 340 nm.

Kinetic colorimetric assay (17) of antibody–complement-mediated lysis of theophylline-DPPE liposomes. We measured complement-mediated lysis by incubating the liposomes as in the ultraviolet spectrophotometric method except we also included phenazine methosulfate (1 mg/L) and dichlorindophenol (6 mg/L) in the medium. After the reaction proceeded for 12 min at 37 °C, we monitored it kinetically for the next 2 min.

Inhibition of antibody–complement-mediated lysis by theophylline was monitored by adding theophylline-containing serum samples, and assayed by either of the above two methods.

Results

Retention of G6PD activity during REV formation. Because the REV method entails exposure of the enzyme to be encapsulated to organic solvents and sonication, it was not surprising that a significant amount of the enzyme was inactivated during the procedure. G6PD encapsulated in the REVs in a buffer of low ionic strength lost 90% of its enzymatic activity. Raising the ionic strength led to a threefold greater recovery of catalytic activity. Regardless of ionic strength, however, the inactivation of enzyme could be largely prevented at total amounts of enzyme of 100 U or less by the addition of substrate at 8 mmol/L (Table 1). At total enzyme amounts near 1000 U, G6PD provided optimal protection at 8 to 15 mmol/L substrate concentrations increasing up to 80 mmol/L gave less protection. The enzyme cofactor NAD\textsuperscript{+} contributes to, rather than protects the enzyme from, inactivation.

Purification of REVs. After preparing the REVs, we removed the nonentrapped with Bio-Gel A-15m enzyme G6PD by column chromatography. As shown in Figure 2, REVs labeled with a trace amount of \textsuperscript{125}I-labeled PE were eluted in the void volume. The free enzyme activity was eluted in fractions 25 to 36; little or no free enzyme activity was demonstrated in the liposome fractions. The total enzyme activity was measured after addition of either Tween-20 or Triton X-100, 5 mL/L, to release the entrapped enzyme from REVs. Even after purification of the REVs by column chromatography, some free (nonlatent) enzyme activity is usually detected (3–5% of that measured from lysed liposomes), which may be due to a weak association of enzyme in the bilayer membrane.

Complement-mediated enzyme release. Purified liposomes

![Fig. 1. Structure of theophylline derivative of DPPE](image)

| Table 1. Analytical Recovery of G6PD Activity after Encapsulation Into REVs |
|-----------------------------|-----------------|-----------------|
| Protegant concn, mmol/L | Initial G6PD, acty, U | Recovery, %* |
| Glucose, 300 | 85 | 9 |
| NaCl, 80 | 57 | 27 |
| G6P, 8 | 31 | 94 |
| | 96 | 104 |
| | 306 | 81 |
| | 1809 | 64 |
| G6P, 15 | 1127 | 62 |
| G6P, 25 | 1127 | 44 |
| G6P, 80 | 778 | 17 |
| NAD\textsuperscript{+}, 8 | 136 | 18 |
| NAD\textsuperscript{+}, 20 | 118 | 0.1 |

*All the measurements determined in duplicate.
labeled with a trace amount of 125I-labeled PE were incubated with antibody and complement and the lysed vesicles were immediately passed through a Sephadex G-200 chromatographic column to separate the liposomes from released enzyme. A control reaction mixture in which buffer was substituted for complement was incubated and chromatographed under identical conditions. The G6PD activity and lipid content (expressed as 125I counts/min) for both the control and complement-treated preparations, were separately determined.

In the control, most of the vesicle lipid and enzyme activity were retained at the top of the column, possibly reflecting liposome aggregation (Table 2). In the complement-treated preparation, however, about 75% of the enzyme was released from liposomes and eluted in the free enzyme fraction. In the control, most of the radiolabeled lipid remained at the top of the column, again suggesting an aggregation of liposomes upon treatment with antibody only, whereas complement-mediated lysis appeared to disrupt the liposomes into fragments as shown by the appearance of 125I in all of the column fractions. Similar results were obtained for four replicates of this experiment, demonstrating that G6PD was actually released from liposomes upon specific lysis.

Theophylline homogenous liposome immunoassay model system. Liposomes encapsulating G6PD and G6P and carrying surface-bound theophylline antigen molecules were prepared as described in Materials and Methods. Treatment of these purified REVs with complement and specific antibody lysed the vesicles, with concomitant leakage of the entrapped enzyme. Table 3 indicates that increasing the amount of antibody led to a progressively greater lysis of the vesicles. Complement itself did not cause a nonspecific lysis of the REVs. The increase in absorbance noted (0.065 A) above that for antibody alone was due to the natural absorbance of the complement serum at 340 nm.

Inhibition of complement-mediated lysis by theophylline. The homogeneous liposome immunoassay (HLIA) involves the interaction of REVs, which contain a theophylline–lipid derivative incorporated into the membrane and a reporter molecule within the aqueous compartment, with exogenous complement and specific antibody. In the absence of theophylline in the surrounding medium, anti-theophylline binds to the REV-linked theophylline molecule and provides the necessary immune complex to activate the complement system. The REV bilayer is subsequently disrupted, making the G6PD molecule available to its substrate G6P and cofactor NAD+ present in the assay system. The addition of a patient's serum containing theophylline results in a decrease in enzymatic activity because of competition with the REV-linked theophylline for antibody. This inhibition is illustrated in Figure 3 (left) in the form of a standard curve. The curve is linear when plotted in a semi-logarithmic fashion, and the absorbance range in the clinically relevant portion of the assay is wide enough to allow for a precise assay. The CV associated with the measurement is usually about 5%.

Stability of assay components. Stored at 5 °C, the liposomes were stable with no loss of activity for at least seven months. At 37 °C the liposomes became unstable after 41 days. A lyophilized reagent containing antibody, complement, NAD+, and G6P in buffer has been stable for at least four months at 5 °C; reconstituted, this reagent is stable for seven to 10 days.

Cross reactivity of theophylline antiserum. We assessed the cross reactivity of the theophylline antiserum for purine derivatives that might interfere with an assay of theophylline: caffeine, often present in the diet; 3-methylxanthine, a major metabolite of it in plasma; and 1,3-dimethyluric acid, a metabolite in the plasma of renal-failure patients. Cross reactivities of these compounds tested in the assay were slight: 2.5%, 0.3%, and 1.4%, respectively.

Possible interference from serum components. Various theophylline-free sera, both normal and abnormal, were tested with the HLIA. Only two of the 255 serum samples tested gave positive values for theophylline; however, these samples were also positive by the IGRF assay (Syva Co., Palo Alto, CA).

A colorimetric method for the HLIA. Both human serum and guinea pig complement absorb at 340 nm and thus contribute to background. Measuring the change in absorbance in the visible region of the spectrum would circum-

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**Table 3. Complement-Mediated Lysis of REVs as a Function of Antibody Concentration**

<table>
<thead>
<tr>
<th>Antibody dilution</th>
<th>Complement added</th>
<th>A, 340 nm</th>
<th>Lysis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>0.220</td>
<td>0</td>
</tr>
<tr>
<td>—</td>
<td>+</td>
<td>0.285</td>
<td>0</td>
</tr>
<tr>
<td>1/2500</td>
<td>+</td>
<td>0.317</td>
<td>2</td>
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<tr>
<td>1/1000</td>
<td>+</td>
<td>0.671</td>
<td>25</td>
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<tr>
<td>1/500</td>
<td>+</td>
<td>0.935</td>
<td>44</td>
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<tr>
<td>1/167</td>
<td>+</td>
<td>1.354</td>
<td>69</td>
</tr>
<tr>
<td>1/100</td>
<td>+</td>
<td>1.466</td>
<td>76</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>1.780</td>
<td>100</td>
</tr>
</tbody>
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*All the measurements determined in duplicate. 
*Triton X-100 (5 mL/L) added instead of antibody.
vent this problem. The NADH formed by the enzyme reaction reduces the blue dye 2,6-dichlorophenolindophenol, which absorbs at 610 nm, to a colorless compound in the presence of phenazine methosulfate. In addition, the greater absorptivity of this dye, which results in a threefold greater absorbance signal, can lead to a more sensitive standard curve (Figure 3, right). We evaluated serum interference in this method with 21 serum samples. One of the sera gave positive theophylline values, but it was also positive by the ultraviolet spectrophotometric method and the Syva EMT assay.

Discussion

Although the assay we describe is not the first liposome-based immunoassay to be developed, it has definite advantages over earlier methods (8, 9, 13, 14). The nonspecific release of low-M₆ "reporters" by complement alone was avoided by encapsulating a larger molecule, an enzyme. To assure a virtually quantitative specific release, we used an active complement preparation from Miles. Release kinetics were improved by the use of essentially single-layer liposomes (REVs) instead of multilamellar vesicles. The assay format is simple, only two additions are required, and it can be automated. The lytic agent is the naturally occurring complement system, which eliminates any questions of encountering an interfering anti-lytic antibody in a patient's serum. The reagents are stable for extended periods.

The incorporation of bacterial G6PD as the enzyme circumvents the possibility of interferences from any endogenous serum enzymes. In addition, protection of the enzyme by its substrate allows for the encapsulation of more active enzyme into liposomes, which enhances the precision and sensitivity of the assay.

In summary, use of this HLIA for therapeutic drug monitoring is rapid and simple and does not require expensive sophisticated instrumentation. The cross reactivity with 1,3-dimethyluric acid is minimal, which is an advantage over the particle-enhanced turbidimetric (18) and other immunoassays (19). In addition, this procedure requires only 2.5 µL of serum, further reducing the possibility of serum interference.

This project is sponsored by Cooper Biomedical Inc. We thank Drs. Mike Fountain and Francis J. Martin for their valuable scientific advice.

References


