BioCOMPUTING

Technical Report

A Simple Microassay for Computing the Hemolytic Potency of Drugs

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ABSTRACT

A simple microassay and computer program are described for determining the erythrocyte hemolytic potency of drugs in vitro. This microassay is sensitive for both micro as well as macro ranges of hemoglobin concentration. An ELISA reader has been adapted to read erythrocyte lysis (hemolysis), which reduces the number and culture of replicates. A computer program was developed that calculates parameters such as C_{50} (concentration of drug causing 50% hemolysis), C_{100} (concentration of drug causing 100% hemolysis) and β (slope of the curve) and graphically expresses the hemolytic patterns of various drugs simultaneously. The program can obtain optical densities directly from a 96-well plate ELISA reader by interfacing the microplate reader to the computer or by using a keyboard. This method is useful for screening a large number of hemolytic drugs and requires lower amounts of test compounds. It may also be applicable to quantitative functional assays, such as complement-mediated hemolysis and enumeration of antibody-secreting cells. The program can be obtained from the authors on request.

INTRODUCTION

The quantitative determination of erythrocyte hemolysis is of clinical significance and a comparison of the strength of various hemolytic agents is important for research in many areas such as drug screening, biochemistry and medicine. Certain drugs are known to distribute and/or bind to the erythrocytes and induce hemolysis (3–8). Various methods have been described for quantitative determination of hemolysis (3,7,8,10,20). Most of these methods are based on a macroassay where the drug is added to the tubes containing erythrocytes. Each tube is handled separately for determining the extent of hemolysis caused by each drug. This method is tedious when testing multiple samples and requires high drug amounts (3,13–16). To measure the hemolytic potency or the toxicity of the drug and to study the kinetics of hemolysis, it would be useful (i) to develop a simple and sensitive quantitative assay for determining hemolysis applicable to a wider range of erythrocyte concentrations and (ii) to express graphically the hemolysis caused by different drugs at varying drug concentrations (characteristics of the drug).

Here, we describe a sensitive microassay and a computer program for estimating the hemolysis caused by different drugs. In this method, optical density (OD) data obtained from a 96-well enzyme-linked immunosorbent assay (ELISA) plate are fed into a computer by either directly interfacing the microplate reader to the computer or using the keyboard (17,18). The program performs the data analysis and expresses the hemolytic patterns of drugs graphically.

MATERIALS AND METHODS

The hemolytic drugs indomethacin (IM), flufenamic acid (FA) and ibuprofen (IP) were purchased from Sigma Chemical (St. Louis, MO, USA). Dimethyl formamide (DMF) was obtained from Merck India Ltd. (Bombay, India). Stock solutions (100 mM) of the drugs were prepared in DMF and diluted in phosphate-buffered saline (PBS; 8 mM phosphate buffer pH 7.4, 2.7 mM KCl, 137 mM NaCl).

Mouse erythrocytes were prepared as described previously (23) and washed three times with PBS before use. Erythrocyte suspension (20 µL) was added to a 96-well vinyl assay plate (Corning Costar, Cambridge, MA, USA), and the cells were thoroughly suspended in 180 µL of drug-solubilizing buffer (PBS containing 10% DMF). For complete hemolysis, the cells were suspended in 200 µL of distilled water. Control wells were taken with PBS containing 10% DMF. Plates were incubated at 37°C for 90 min and centrifuged at 500× *g* for 5 min. Supernatants were directly transferred with a multipipet to a new plate, and the OD of each well was read at a wave-

length of 543 nm using an MPR-A4 Urogenetics EIA-reader (Model MPR-A4; Eurogenetics, Tessenderlo, Belgium). This reader can be interfaced to the serial communication port of any IBM®-compatible microcomputer by an RS-232 interface (2,17,18).

COMPUTATION

OD data obtained above were entered into a microcomputer using the keyboard, but the computer program also has an option to enter the data into the microcomputer directly from the microplate reader by using an RS-232 interface. The program uses the OD data to construct an 8 × 12 table of data points that can be printed (2,18). The percentage of hemolysis caused by the drug at a given concentration was calculated by following equation:

$$H = \frac{OD_s - OD_0}{OD_{100} - OD_0} \times 100 \qquad \text{[Eq. 1]}$$

where H, OD_s, OD_{100}, OD_0 are percentages of hemolysis, optical density in the presence of drugs, optical density in the presence of water, and optical density in the presence of drug-solubilizing buffer, respectively.

The computer program computes the percentage of hemolysis caused by different drugs at varying concentrations using equation 1. The percentage of hemolysis vs. drug concentration data was used to express the hemolytic patterns of drugs and to derive a linear interpolation formula for calculating different parameters as described below.

Hemolytic Pattern of Drugs

The computer program first calculates the percentage of hemolysis caused by different drugs at varying drug concentrations. It then draws the hemolytic pattern (percentage hemolysis vs. drug concentration) of different drugs on the same graph. Similarly, the hemolytic pattern of drugs for various parameters, such as incubation time and temperature, can be drawn on a single graph. The graphical presentation of the hemolytic pattern helps in studying the kinetics of hemolysis and for simultaneously screening the hemolytic drugs.

Linear Interpolation Formula

The graph of the percentage of hemolysis (dependent variable) vs. drug concentration (independent variable) of a known standard was used for calculating the linear interpolation formula. The linear range of the curve was depicted (17). Data in the linear range of the curve were fitted using the following linear equation:

$$H = A_0 + A_1 \times (C) \qquad \text{[Eq. 2]}$$

where H, C, A_0 and A_1 represent the percentage of hemolysis, drug concentration, the constant and slope of the curve, respectively. The values of A_0 and A_1 were calculated by fitting the data in the range using the least squares curve fitting method from the computer program. Equation 2 represents the linear interpolation formula obtained from the standard sample and was used to calculate C_{50} (concentration of the drug causing 50% hemolysis), C_{100} (concentration of the drug causing 100% hemolysis) and β (slope of the curve) (A_1). The concentration of the drug causing a specified percentage of hemolysis can also be calculated using equation 2.

RESULTS

Sensitivity and Validity of the Microassay

The samples with different erythrocyte concentrations (10^5 to 10^8) were completely hemolyzed with water (200 µL) into a 96-well plate. The OD of each well was measured and

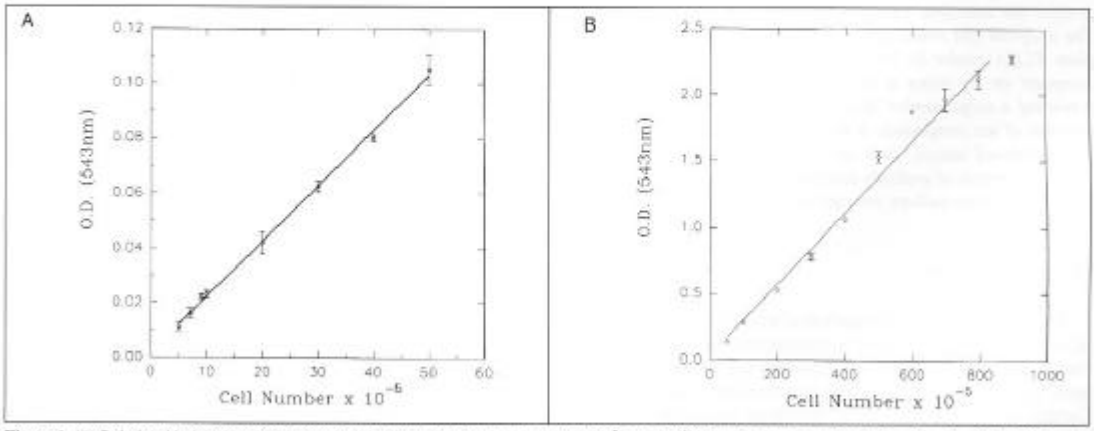

Figure 1. (A) Relationship between OD (543 nm) and cell number in micro range (5×10^5 to 5×10^6). Samples were completely hemolyzed with distilled water as described in Materials and Methods. (B) Relationship between OD (543 nm) and cell number in macro range (5×10^6 to 9×10^7). Samples were completely hemolyzed with distilled water as described in Materials and Methods.

Table 1. Hemolysis Caused by Different Drugs

Drugs	Our Method		Published Method (3)	
	C_{50}	C_{100}	C_{50}	C_{100}
Indomethacin	1.96 ± 0.052	3.76 ± 0.084	1.95	3.8
Flufenamic Acid	1.49 ± 0.076	2.32 ± 0.042	1.45	2.3
Ibuprofen	7.12 ± 0.142	10.18 ± 0.082	7.2	10.1

the data were analyzed using a computer program. OD vs. cell concentration was plotted and fitted with the linear regression equation (Figure 1, A and B). These curves showed linearity in the micro range (5×10^5 to 5×10^6) (Figure 1A) as well as in the macro range (5×10^6 to 8×10^7) (Figure 1B), thus showing that the assay developed was applicable to a wider range of erythrocyte concentrations.

This assay was checked for its validity in determining erythrocyte hemolytic potency of known hemolytic drugs. Hemolysis was monitored at various concentrations of IM, FA and IP. Values of C_{50} and C_{100} were calculated by a computer program for these drugs. These were then compared with the reported ones (Table 1). It is evident that the method had an excellent correlation, establishing the utility of the assay for calculating the hemolytic potency of various drugs.

Graphical Representation of the Hemolytic Pattern

The samples with erythrocyte concentrations (3×10^7) were hemolyzed by water, buffer and drugs (IM, FA, IP) at varying drug concentrations in a 96-well plate. The OD of each well was measured and entered into a computer. The program automates the computation of parameters C_{50}, C_{100}, β, etc., and expresses the hemolytic patterns graphically, which can be plotted on an HP-GL-compatible plotter. The hemolytic patterns of the drugs, IM, FA and IP, are shown in Figure 2. For each plate, the user has an option to print the average OD values and parameters (C_{50}, C_{100}, β) and to plot the hemolytic patterns of the drugs.

Kinetics of Hemolysis

The studies were further extended to test if the assay can also be used for performing kinetic studies of drug-induced hemolysis. For this, the effect of the incubation time was taken as one of the parameters. The percentage of hemolysis was measured at different incubation time intervals using various concentrations of IM at 37°C with different cell concentrations (Figure 3). This figure shows that lysis reached a plateau in about 50–90 min.

DISCUSSION

The objective of this study was to develop a simple and reliable microassay and to design a computer program to study the behavior of drugs based on their hemolytic potency. The analysis performed by the program is rapid (multiple samples) and automated (reading and analyzing data). It also displays graphically the hemolytic patterns of drugs and the

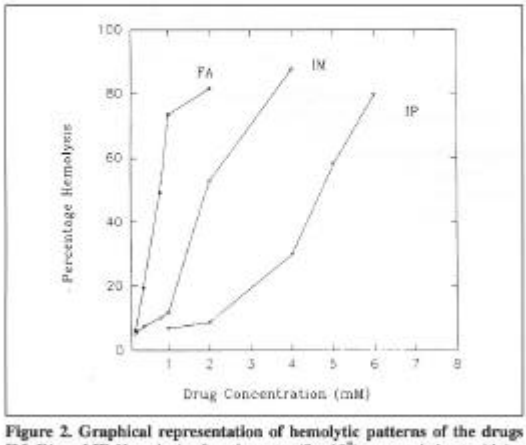

Figure 2. Graphical representation of hemolytic patterns of the drugs IM, FA and IP. Hemolysis of erythrocytes (3×10^7) was carried out with increasing concentrations of drugs. The percentage of hemolysis was computed using a computer program.

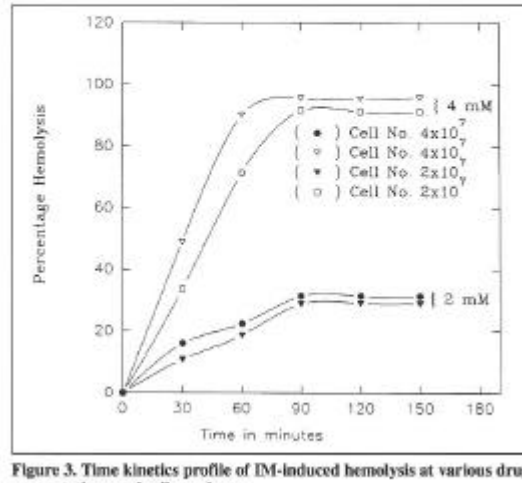

Figure 3. Time kinetics profile of IM-induced hemolysis at various drug concentations and cell numbers.

kinetics of hemolysis.

Various methods described so far for quantitative determination of hemolysis can be classified as the (i) coil planet centrifugation (CPC) method (8,9), (ii) radiolabeled chromium release method (10), (iii) and static method (macroassay) (3,12,13,15,16). The CPC method, originally developed to monitor osmotic hemolysis as an index of erythrocyte fragility, was later adapted to measure drug-induced lysis of erythrocytes (8). Although this method is rapid, it cannot measure biological effects (8). The radiolabeled chromium release method is sensitive at the micro range but is tedious to perform and also adds the additional burden of handling radioactivity. The widely used macroassay is easy to perform with a small number of samples and can measure biological effects. However, this method becomes more cumbersome and time-consuming for multiple samples. To overcome the above limitations, we have described a simple microassay that is (i) sensitive at the micro range; (ii) can handle multiple samples at a time by adapting the 96-well plate ELISA reader; (iii) requires less cell numbers or hemoglobin concentration; and (iv) utilizes lower amounts of test compound.

To our knowledge, no method has yet been described that can express the hemolytic patterns of drugs graphically, which is highly useful in studying the characteristics of drugs in terms of their hemolytic potency. In this direction we have developed a computer program that has the following options: (i) reading data directly from the ELISA plate reader; (ii) calculating the average OD of samples, which are in duplicate, triplicate, etc.; (iii) calculating parameters like C_{50}, C_{100}, β; (iv) displaying the hemolytic patterns of drugs; and (v) representing kinetics of hemolysis graphically. Thus, this program may find wide applications for quantitative analysis of drug-induced hemolysis and other functional assays such as complement-mediated erythrocyte lysis (1,19,21), carrier-specific antibody response (11) and enumeration of antibody-secreting cells (16,22).

In conclusion, this report describes (i) a simple and sensitive microassay for quantification of hemolysis that requires a lower amount of test compound and can handle a large number of samples simultaneously and (ii) a computer program that facilitates feeding of OD data directly from the ELISA reader by interfacing it to the computer for computing parameters like C_{50}, C_{100} and β and representing hemolytic patterns of drugs graphically.

The program for the analysis and calculation of the data is written in Q-BASIC, can be run on an IBM-compatible computer under MS-DOS® Version 2.0 or higher and requires a CGA, EGA or VGA card. The hard copy of the graphs can be

plotted on HP-GL-compatible plotters. This is a menu-driven computer program and provides a data storage facility on a disk. A copy of the source and executive version of the program can be obtained by sending a 5.25-in. floppy disk to the authors or by EMail: grish@imtech.ernet.in

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