A Novel Assay to Determine the Hemolytic Activity of Drugs Incorporated in Colloidal Carrier Systems

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INTRODUCTION

Colloidal drug carrier systems serve to minimize the side effects of drugs used for parenteral application. Side effects often result from the destruction of corpuscles of blood or tissue cells at the site of injection, and these may be reduced by incorporating the drug in colloidal carriers (e.g., o/w emulsions). Many hemolytic tests are employed for drug testing *in vitro* (1,2). Usually the hemoglobin release of red blood cells is detected spectrophotometrically (1). In the case of colloidal dispersions, the photometric assay is distorted by the Tyndall effect. Therefore, all the particles must be removed to obtain reproducible results. However, emulsion droplets or liposomes for i.v. administration cannot be removed completely by centrifugation because of their small size and the low density difference between carriers and the aqueous dispersion medium. Removing the particles by other methods such as ultrafiltration or ultracentrifugation or the use of radioactive markers to detect hemolysis is time-consuming and requires special laboratory equipment (3,4). This paper describes a simple and reproducible assay which allows the direct measurement of hemoglobin release, i.e., without mechanical removal of drug carriers.

MATERIALS AND METHODS

Materials

Red Blood Cell Suspension. Red blood cells (RBC) were isolated by centrifugation (1000*g* at 20°C for 5 min) of heparinized human blood and redispersed in isotonic phosphate buffer, pH 7.4. The RBC were washed three times with buffer (redispersion followed by centrifugation) and, finally, redispersed to give a concentration of about 4 million cells/μL buffer. The RBC were stored at 4°C and used in the test for a maximum of 48 hr. Prior to the assay the cell suspension was diluted to obtain a fixed concentration of hemoglobin (RBC stock dispersion). Dilution was performed with buffer to yield an absorption of approx. 2.0 (398 nm; absorption cell thickness, 10 mm) after total hemolysis in the assay (control = 100% hemolysis). The hemoglobin concentration in the stock dispersion was about 4 m*M*.

Drug Solution. The drug solution was prepared by dissolving the drug in isotonic phosphate buffer, pH 7.4.

Emulsion System. Emulsions were prepared by high-pressure homogenization. The mean droplet size was 320 nm and the diameters of all emulsion droplets were below 1.8 μm. The drug free emulsion contained middle-chain triglycerides (200 mg/g), soybean lecithin (12 mg/g), sorbitol (50 mg/g), and water for injection (738 mg/g). Solutions of the hemolytic drug in different concentrations replaced parts of the water in the drug containing emulsions.

Raw Materials. The following raw materials were used: middle-chain triglycerides, Miglyol 812 (Hüls, Witten, Germany); soybean lecithin, Phospholipon 80 (Nattermann, Köln, Germany); sorbitol, Karion (Merck, Darmstadt, Germany); and ethanol (99%, v/v; Gebr. Möllgard, Kiel, Germany). Buffer substances and HCl were of analytical grade (Merck). The antineoplastic substance hexadecylphosphocholine (INN miltefosin, ASTA Medica, Frankfurt, Germany), an alkylphosphocholine, was used as a model drug with a high hemolytic activity.

Methods

One milliliter of each of the isotonic samples containing equidistantly increasing concentrations of the test agent was pipetted into an Eppendorf vial (2.0 mL). One hundred microliters of the RBC stock dispersion was added to each sample. After incubation under shaking at room temperature for 5 min, the samples were centrifuged to remove intact RBC and cell debris (750 g, 3 min). One hundred microliters of the supernatant was added to 2.0 mL of an ethanol/HCl mixture [1 part HCl (37%, w/w) + 39 parts ethanol (99%, v/v)]. The absorption of hemoglobin was determined at 398 nm by photometric monitoring against blank samples.

To get a concentration–response relationship, the fractional release caused by each test concentration was expressed as a relative percentage of the control (= 100% hemolysis). The concentrations of the tested agents were calculated after dilution of the samples (1.0 mL) with 100 μL RBC stock dispersion (total volume = 1.1 mL). The half-maximum effective concentration (H_{50}, concentration of hemolytic agent needed for 50% hemolysis) used to describe the hemolytic activity of the tested agent was computed from the concentration–response curve using a modified Hill equation.

Optimization of the Assay

Optimization of RBC Concentration. A dilution series of hemoglobin was prepared and processed like samples (100 μL added to 2.0 mL ethanol/HCl). The hemoglobin was obtained by lysis of RBC with distilled water. A linear relationship between hemoglobin concentration and resulting absorption exists for absorption values below 2.0.

Influence of RBC Concentration on the Half-Maximum Concentration H_{50} of Hexadecylphosphocholine. For many hemolytic agents no linear relationship exists between the concentration of the hemolytic agent needed to get 50% he-

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Table I. Relation Between H_{50} of Hexadecylphosphocholine (HDPC) and RBC Concentration [Expressed as Hemoglobin Absorption of a Completely Lysed Sample (E_{max})[a]

	Sample series no.				
	I	II	III	IV	V
H_{50} (μg HDPC/g)	5.26	7.02	8.03	9.93	10.66
Hemoglobin absorption (E_{max})	1.06	1.41	1.55	1.96	2.2
Ratio H_{50}/E_{max} (μg/g)	5.0	5.0	5.2	5.1	4.9

[a] The hemoglobin concentration is proportional to the RBC number (8).

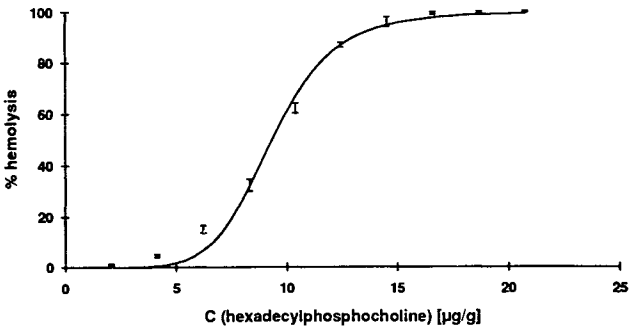

Fig. 1. Concentration–response curve of the solution of the model drug hexadecylphosphocholine (n = 6).

molysis (H_{50}) and the concentration of RBC used in the test (7). Therefore, usually the concentration of RBC has to be adjusted exactly to get comparable and reproducible results.

However, when using the model drug hexadecylphosphocholine, a linear relationship was found (Table I). This means that it is not necessary to adjust the RBC number in the samples by counting the cells in the stock dispersion. It is sufficient to calculate the ratio H_{50}/E_{max} (absorption after total hemolysis of the RBC in the sample) to correct for minor variations in the number of erythrocytes used in the test. The hemoglobin concentration after total hemolysis is proportional to the RBC concentration in the sample (8).

Aging Effects on RBC. The sensitivity of RBC to hemolytic agents may change with the aging time of the erythrocyte suspension (9). The sensitivity of RBC to hexadecylphosphocholine in isotonic aqueous solution was followed over a period of 4 days to assess aging effects. No change in sensitivity occurred over a period of 24 hr; after 2 days the ratio H_{50}/E_{max} of the hemolytic agent slowly decreased (Table II).

Variation of RBC Sensitivity Between Donors. RBC from different donors were studied with regard to differences in sensitivity. The differences were found to be negligible.

Reproducibility of the Assay. RBC from six donors were used to determine the H_{50}/E_{max} ratio of a solution of hexadecylphosphocholine. The RBC were used directly after donation or after 1 day of storage at 4°C. The relative standard deviation was found to be below 6% (n = 25).

Application of the Assay

Figure 1 shows the concentration–response relationship of the hemolytic model drug hexadecylphosphocholine. The sigmoidal response curve shows a relatively steep increase within a small concentration range of 10 μg/g solution.

The concentration–response curve of the emulsion system was shifted to the right by a concentration factor of about 300 compared to the curve of the solution (Fig. 2). Additionally, the increase in the sigmoidal curve is less steep. This demonstrates the ability of colloidal formulations to reduce hemolytic side effects of the chosen model drug.

Table II. Ratio of Half-Maximum Effective Concentration H_{50} of Hexadecylphosphocholine in Isotonic Aqueous Solution (μg/g) and E_{max} in Relation to the Age of the RBC

	Age of RBC stock dispersion				
	4 hr	24 hr	48 hr	72 hr	96 hr
Sample series I	5.1	5.2	4.9	4.8	4.5
Sample series II	5.1	5.2	4.9	4.7	4.5

DISCUSSION

The aim of the study was to develop an *in vitro* assay for detecting hemolysis caused by drugs incorporated in colloidal carrier systems like fat emulsions. After incubation with RBC and removal of intact cells and debris by simple centrifugation, the samples are simply dissolved in a mixture of ethanol and HCl. The dissolving allows the photometrical assay of the hemoglobin release without time-consuming treatments. The mixture of ethanol and HCl dissolves all components present (emulsion droplets, water, hemoglobin) and avoids the precipitation of hemoglobin. This simple treatment allows large numbers of samples to be handled simultaneously.

The test procedures described in this paper had to be standardized and optimized because of the different sample treatments, measurement settings, and test conditions compared with assays described elsewhere (1,2,10).

Usually the determination of the hemolytic activity is based on the preparation of a dilution series (1,2). Dilution of sample is possible for drug solutions but not for drugs incorporated in colloidal carriers. Dilution of these systems may lead to a redistribution of the drug between the lipophilic phase (oil core or emulsifier film of emulsions, phospholipid bilayer of liposomes) and the surrounding water phase according to the Nernst coefficient. The drug redistributed to

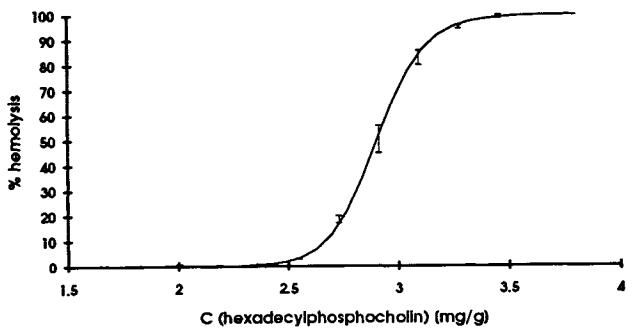

Fig. 2. Concentration–response curve of the model drug hexadecylphosphocholine incorporated in a fat emulsion (n = 3).

the water phase will be hemolytically active, leading to an apparent hemolytic activity which is distinctly higher than in the original dispersion. For this reason the presented assay is based on using very small volumes of highly concentrated RBC stock dispersion to minimize dilution of the samples. The assay presented is an *in vitro* assay using only corpuscles of blood. Therefore, the protection effect of the plasma against the lysis of erythrocytes (11,12) is not taken into consideration. However, the method can be used in routine screening to estimate the ability of different carrier systems to minimize the hemolytic side effect of incorporated drugs. The first results show that it is possible to decrease the hemolytic activity of the model drug used, hexadecylphosphocholine, by incorporating it in colloidal carrier systems such as emulsions for parenteral administration.

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