Visual Drop Test of Hemoglobin in Whole Blood by Using Ring-like Concentration of Rhodamine B onto Octadecylsilanized Silica Plate

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It has been found that some dye solutes become concentrated along the perimeter of an aqueous drop sitting on an octadecylsilanized silica plate. The fluorescent ring of Rhodamine B was applied to a visual test of hemoglobin in whole blood. When a drop of diluted blood and the dye is set on the plate, the resulting fluorescent ring can be observed through the drop with an ultraviolet lamp in the dark only for the samples of low hemoglobin content.

There has been much theoretical and experimental interest recently in the formation of ring from dispersed micro-particles. Deegan et.al. reported on capillary flow as the cause of ring strains from dried liquid drops containing solids initially dispersed homogeneously over the entire drop such as a spilled drop of coffee.[1] The ring deposits also provide a potential means to print a fine pattern onto a surface. However, most experimental work has been directed at the behavior of solid particles, such as metals and metal oxides, and evaporation process.[1,2]

As part of our studies on sensitive visual determination,[3,4] the first application of ring-like deposits on a water-repellent surface to an analytical method was described.[5] The fluorescent aluminium chelate with 2,2'-dihydroxyazobenzene became concentrated in a ring zone on a hydrophobic filter paper when evaporated in an oven. The concentration into a tiny fraction enabled a novel method for the determination of aluminium ion at ppb level in a micro volume sample. Further, our work was extended to sharp ring formation induced by poly(vinyl alcohol) on a poly(vinyl chloride) plate by evaporation.[6] The ring formation in these methods were definitely different from conventional spot test. In Feigl's spot test, when a drop was placed on filter paper the solution spread through its coarse capillaries and the colored solute formed flecks or rings.[7] In Weisz's ring oven technique, a heating device was used to confine a spot size.[8]

In this study, it has been found that an octadecylsilanized (ODS) silica plate serves as an solid surface to concentrate some dye solutes along the perimeter of an aqueous drop without evaporation. In the discussion below, we describe an application of fluorescent ring of Rhodamine B to a visual test of hemoglobin in whole blood. Hemoglobin, a hydrophilic protein, in a sample drop is not adsorbed on an ODS plate. The deep red color affects the excitation and visual perception of the fluorescent ring through the drop with an ultraviolet (UV) lamp in the dark.

In spite of an urgent demand, there were no reports of a simple test method for hemoglobin in whole blood. Spectrophotometry of cyanomethemoglobin at 540 nm is the most frequently used method for the determination of hemoglobin. However, application of it to a rapid screening test for anemia (iron deficiency) is limited because the method requires a spectrophotometer and laboratory skill. Part of the reason for this is also due to biohazard during troublesome procedure and wastewater containing potassium cyanide. The object of this work is to develop a rapid, simple, and inexpensive method for hemoglobin measurement.

Rhodamin B was used as received from Wako Pure Chemical Industries, Ltd.. The dye solutions were prepared by dissolving the reagents in distilled water. The ODS silica plate, RP-18 HPTLC Art. 5914, was purchased from Merck. All other reagents used were of guaranteed reagent grade.

The visual detection of the fluorescent ring was carried out in the dark with a handy UV light, SPECTRONICS Model Q-12NF. For solid phase fluorometry and reflective spectrophotometry a Shimadzu Model CS-9300PC flying spot scanning densitometer was used.

A typical procedure for the drop test of hemoglobin is as follows : Add 58 μl of 3.5×10^{-6} mol dm^{-3} of Rhodamine B to 2 μl of whole blood sample. Apply a 50 μl drop of the mixture to the ODS plate. The tiny drop retains its semi-spherical shape dictated by the surface tension and the water repellency of the plate. After 5 min, look straight down through the drop at its plate interface under ultraviolet light in the dark. When the resulting fluorescent ring is observed through the drop, it indicates low hemoglobin content, that is less red in color and more transparent. Conversely, when the sample contains sufficient amount of hemoglobin, the fluorescent ring cannot be observed.

The characteristic pattern of the ring-like deposits of several dyes on the ODS plate were investigated in the following manner: A 50 μl drop of aqueous solution containing 10^{-6}-10^{-5} mol dm^{-3} of each dye was applied on the ODS plate and allowed to sit for 5 - 20 min. Then, the solution is removed with a filter paper and the residual stain with 4 mm diameter is observed. The measurement of the ring profile is also carried out with the densitometer.

When a drop of diluted blood and Rhodamine B is set on an ODS plate, the dye gives a fluorescent ring stain on the solid surface. Figure 1 shows a densitogram obtained by scanning the stain. The hydrophilic protein, hemoglobin, is not adsorbed on the plate. The fluorescent spectrum of Rhodamine B on the solid phase shows an excitation maximum at 550 nm and an emission maximum at 625 nm. The stain profile and fluorescent intensity were constant over the pH range 4 - 8. Taking the evaporation effect into consideration, the sitting time of 5 min was chosen for reproducible ring formation. The dye concentration of 3.5×10^{-6} mol dm^{-3} was chosen to distinguish a slight difference between 120 and 100 g L^{-1} hemoglobin, which corresponds to healthy and slightly anemic female subjects, respectively. Since the threshold for anemia varies with the subjects age and gender, it is necessary to adjust the dye concentration and dilution of the blood sample in order to obtain practical results. The hemoglobin disturbance on the excitation and emission of the dye, as well as human eye perception, can affect the outcome of the distinguishment. Table 1 shows the results of this visual test for hemoglobin

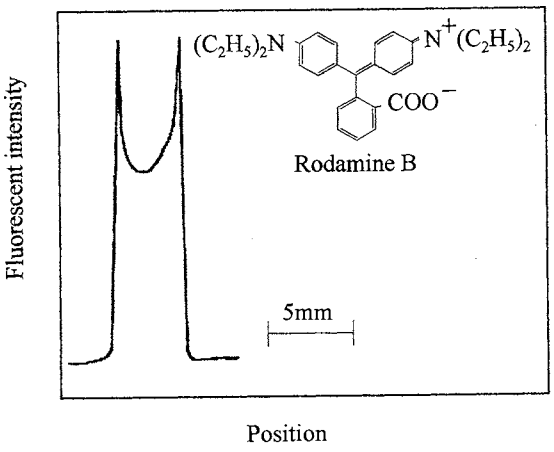

Figure1. Fluorescent densitogram of Rhodamine B stain. $[Rhodamine\ B]_{initial}$: 3.5×10^{-6} mol dm^{-3}; Volume of sample loading: 50 μ l; Substrate: RP-18 HPTLC Art. 5914; Excitation wavelength: 550 nm; Beam size: 0.4×0.4 mm.

Table 1. Result of visual hemoglobin test

Sample	Hemoglobin content /gL^{-1}	Detection of ring
Hemoglobin solution	120	No
Hemoglobin solution	100	Yes
Whole blood A[a]	145	No
Diluted whole blood A[b]	120	No
Diluted whole blood A[b]	100	Yes
Whole blood B[a]	160	No
Diluted whole blood B[b]	120	No
Diluted whole blood B[b]	100	Yes

[a] The hemoglobin content in the whole blood from male subjects were measured by conventional spectrophotometry. [b] Each sample was diluted to contain 120 and 100 g L^{-1} hemoglobin.

solution and whole blood samples.

In a separate experiment, it was found that the partition behavior of the triphenylmethane dyes on an ODS plate varied with the substituted groups on the three phenyl rings. The chemical structures and ring formation results of five triphenylmethane dyes are shown in Table 2. These results indicate that the ring formation would be attributed to the high hydrophobicity of the dye used. The initial growth of the ring apparently started at the outside edge. This is called three-phase interface, because this is where the aqueous phase, the solid surface, and the surrounding air meet. The initial ring growth indicates that the three-phase interface serves as a specific area around the drop. Thus, the first ring growth creates surface unevenness which augments the subsequent adsorbing effect. It

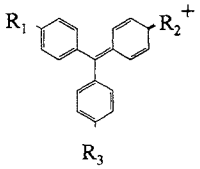

Table 2. Structure of triphenylmethane dye and stain profile

Dye	Substituted group			Result
	R_1	R_2	R_3	
Pararoseaniline	NH_2	NH_2	NH_2	fleck
Malachite Green	$N(CH_3)_2$	$N(CH_3)_2$	H	fleck
Crystal Violet	$N(CH_3)_2$	$N(CH_3)_2$	$N(CH_3)_2$	fleck
Brilliant Green	$N(C_2H_5)_2$	$N(C_2H_5)_2$	H	ring
Ethyl Violet	$N(C_2H_5)_2$	$N(C_2H_5)_2$	$N(C_2H_5)_2$	ring

leads to the final distribution of the solutes on the solid surface, that is ring-like concentration. On the interior interface between the overlaying aqueous solution and the plate, there is no immobilizing effect comparable to that at the three-phase interface. Only the much weaker interaction due to the two phase partition between water and ODS takes place. Consequently, the main points underlying this phenomenon are interpreted in light of the heterogeneity of the interface and hydrophobicity of the solutes.

Rhodamine B interacts strongly with the three-phase interface to form a significant ring, which provides an easy to distinguish visual result in this test. The advantages of the method reported here can be applied to the screening test of anemia, which is one of the most common diseases.

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