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MICROCHROMATOGRAPHY OF HEMOGLOBINS

V.* THIN-LAYER CHROMATOGRAPHY OF SOME HEMOGLOBINS ON CM-CELLULOSE**

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SUMMARY

By means of thin-layer ion-exchange chromatography, human hemoglobins A, S, and C in various combinations can be distinctly separated. Also, hemoglobin F can be separated from the A, S, and C components of cord blood samples from infants and in adult blood samples with a relatively high percentage of F (15% or more). The procedure uses CM-cellulose and solutions of Tris–HCl, NaCl, and KCN. A 0.3-mg sample in 5–10 μl of solvent is required. Development can be completed in 30–40 min.

INTRODUCTION

Although the thin-layer chromatography (TLC) of hemoglobins on such supports as Sephadex[2,3] and Bio-Gel[3] has been reported, these procedures would not separate normal from abnormal hemoglobins because molecular weights are virtually identical. There seem to be no descriptions of the TLC of hemoglobins on ion-exchange substances despite the fact that many procedures are known for the ion-exchange separation of hemoglobins in columns[4–8]. Microchromatographic column methods have been described[1, 9–11]. The experiments to be reported here were designed to determine whether ion-exchange TLC of hemoglobins offered advantages in speed, reduced quantity of material required, etc., in the qualitative detection of common abnormal hemoglobins.

METHODS AND MATERIALS

Source of samples

Samples were obtained from the Los Angeles Sickle Cell Center and the Los Angeles County Health Department. They included both cord blood and adult specimens with various combinations of hemoglobins A, S, C, and F.

* For Part IV, see ref. 1.

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Preparation of hemolysates

Satisfactory solutions for chromatography may be prepared in one of the four ways given in Table I. Samples thus prepared were stored at 4° until use.

Although microchromatographic methods[1, 9–11] use hemolysates that are prepared from whole blood rather than washed cells, such hemolysates were unsatisfactory for TLC.

TABLE I

PROCEDURES FOR PREPARATION OF SAMPLES FOR TLC

Procedure	*Method 1*	*Method 2*	*Method 3*	*Method 4*
Washing of cells	Suspend cells in 10 vol.* of cold 0.9 % NaCl, centrifuge, and discard washings.			
Hemolysis	Add 1.5 vol.* water, and stir 1 min	Add 1.5 vol.* water and 0.4 vol. CCl_4, stir 1 min, and centrifuge cell debris	Add 1 vol.* water, stir 30 sec, and add 0.5 vol. 0.024 *M* maleic acid dropwise with much stirring	Add 1.5 vol.* water and dialyze against selected developer at 4° overnight

* Vol. refers to volume of packed red cells.

Preparation of solutions

Solutions for equilibration of the ion exchanger and for chromatographic development contained Tris–HCl, NaCl, and potassium cyanide. All solutions were 0.3 *M* in Tris (3.63 g/l) and 0.01 % KCN (0.1 g/l) with varying molarities of NaCl and were adjusted to pH 6.1 with 6 *N* HCl.

Preparation of resin

The carboxymethylcellulose was CM-52 (microgranular and preswollen, manufactured by Whatman, Maidstone, Great Britain, and marketed in the United States by Whatman, Clifton, N.J.). For equilibration of CM-cellulose, the desired developer is added to 100 g of ion exchanger until the total volume is 300 ml. After manual stirring for about 1 min, the pH is adjusted to 6.10 with 6 *N* HCl, the suspension is allowed to settle, and the supernatant liquid is decanted off. This equilibration procedure is then repeated three times (the pH need not be readjusted until the last equilibration). Supernatant fluid is then removed until the ratio of settled ion exchanger to supernatant volume is about 3:1. The slurry may be stored at room temperature in a covered container for as much as three weeks after preparation without any apparent change in its chromatographic properties.

Preparation of plates

Plate glass sections 5 × 20 cm in dimension were carefully cleaned by soaking in chromic–sulfuric acid solution for 1 h and, after rinsing, by scrubbing vigorously with an abrasive-type cleanser and a moderately stiff brush. After rinsing, the plate must be thoroughly dried before application of the slurry.

The slurry was applied by means of a Desaga/Brinkmann Standard Adjustable Applicator. In order to prepare four plates at one time, they are fastened with ion

sides adjacent by two-sided masking tape onto a plastic or glass base. A fifth plate is placed in contact with one of the short sides of the four plates. Its purpose is to catch the initial flow of slurry from the applicator and thus to allow a more even flow of the thin slurry to be established before the applicator reaches the edges of the four primary plates. Prior to application, 100 ml (which contains approximately 35 g of CM-52) of thoroughly suspended slurry is transferred into the applicator, and the plates are poured at a thickness setting of 2 mm. The plates may be dried overnight at room temperature or in 1–2 h in a stream of air. The thickness of ion exchanger on the dried plates is somewhat less than 1 mm. The plates can be stored at room temperature in the open air or wrapped in cellophane. Although the plates tend to crack on drying, this does not impair development or otherwise change their chromatographic properties.

Application of sample

About 0.3 mg of hemoglobin in 5–15 μl should be spotted. Consequently, it is convenient to adjust the concentration of hemolysates to about 30 mg/ml and to use 10 μl. A 10-μl portion of each of the samples to be spotted is drawn into a disposable micropipet and placed on the bench top. Shortly before all samples are ready for spotting, the plate to be used is placed in the development chamber (see below) and the developer is allowed to ascend about 3 cm because it is essential that the samples be spotted on a wet surface. When the developer reaches this point, the plate is removed from the chamber, placed flat on the lab bench, and the samples in the micropipets are spotted as quickly as possible about 1.5 cm below the developer front. The plate is then returned to the chamber.

Development

In these experiments, ascending development was carried out in a glass jar with a tightly fitting lid. The liquid level in the jar was about 5 mm. The rate of development which varied to a small extent from plate to plate averaged about 0.7 cm/min.

Washing off of plates

After a chromatogram has been completed, it may be kept as a permanent record, or photographed, etc. When a gentle stream of water is passed over the plate, the parts which have hemoglobin adhere firmly and the other CM-cellulose rinses off easily. This is, therefore, a method of detecting spots that might otherwise be difficult to see. Removal of these tightly adhering spots is achieved with a hard stream of water and gentle scrubbing with a soft brush. The plate is then ready for further use after cleansing with abrasive cleanser and brush, rinsing, and thorough drying.

RESULTS

Typical results of the ion-exchange TLC of hemoglobins by the methods described are depicted in Fig. 1. CM-cellulose was equilibrated with 0.03 *M* Tris–0.10 *M* NaCl–0.01 % KCN at pH 6.10 and the plates were developed with 0.03 *M* Tris–0.085 *M* NaCl–0.01 % KCN at pH 6.10.

The preparation of the sample is a critical step. The obvious hemoglobin at the solvent front in Figs. 1a and 1b is caused by the high pH of the hemolysate, which

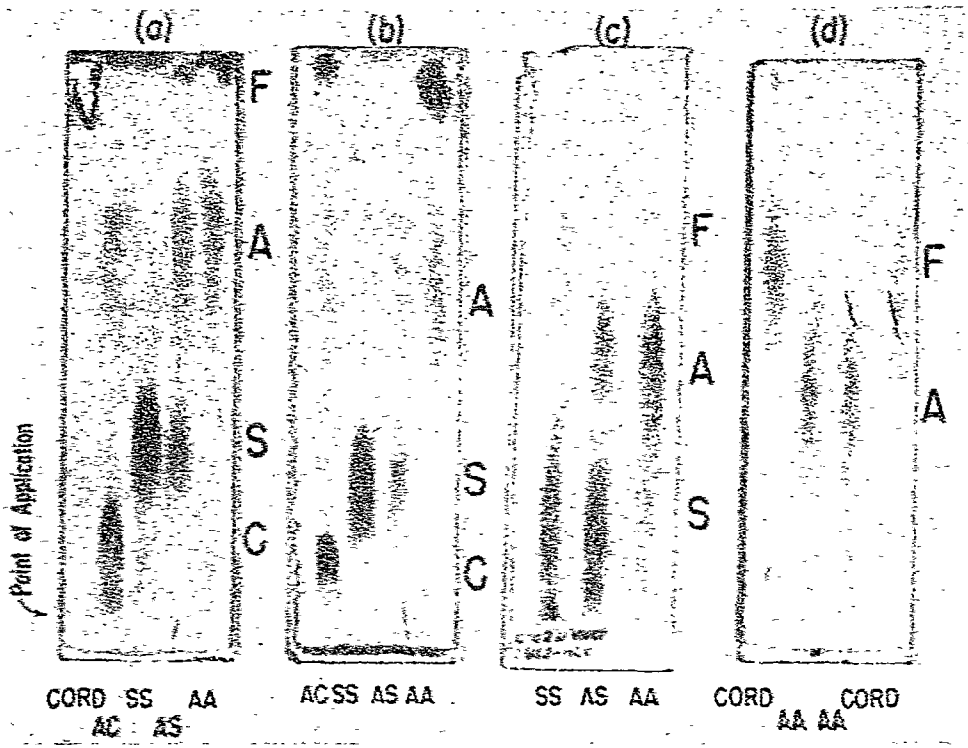

Fig. 1. Thin-layer ion-exchange chromatograms of human hemoglobins F, A, S, and C. See text for details.

is about 7 when Methods 1 and 2 are used. At this pH, hemoglobin is not strongly fixed on CM-cellulose in these Tris–NaCl–KCN solvents. Consequently, when hemolysates with unadjusted pH are used, the system is unable to compensate completely for the high pH, and a portion of sample apparently remains at the higher pH and chromatographs abnormally. Even when the pH of the sample is unadjusted, the movement of the main body of the hemoglobin is normal. However, if the pH of the sample is adjusted by dialysis against the developing solvent (Method 4) as it was for the chromatogram in Fig. 1d, hemoglobin is not present at the solvent front. Adjustment of pH can also be made by dropwise addition of acid (Method 3), which also eliminates the effect (Fig. 1c).

Figs. 1a and 1b show the distinct separation of hemoglobins F, A, S, and C. Hb-F moves with the front of solvent and has collected at the upper left-hand corne in Fig. 1a in addition to other hemoglobin that moved with the solvent front. Th amount of Hb-A in this sample of cord blood was so small that it was barely detec able on the original chromatogram and is not evident in the figure. The R_F value c Hb-A in the AA sample was 0.72, that of Hb-A in the AS sample 0.70, and that c Hb-A in the AC sample 0.68. The R_F values of Hb-S from the SS and AS sample were 0.31 and 0.29, respectively. The R_F values of Hb-C was 0.17.

The behavior of hemoglobins A and F is shown in Fig. 1d, which used Metho 4 for sample preparation. In this chromatogram, the sample was spotted 5 cm behin

the solvent front because of the rapid movement of hemoglobin F. Nevertheless, the R_F value of Hb-A was 0.68, which was in good agreement with values of 0.68–0.72 obtained in Fig. 1a. The R_F value of Hb-F was 0.93.

Figs. 1b–d permit a comparison between Methods 2, 3, and 4 of sample preparation. The separation of the hemoglobins is perhaps better by Method 2 although the extraneous hemoglobin at the solvent front is a disadvantage. The SS sample in Fig. 1c had about 25% of hemoglobin F, which is present in the indicated position. The R_F values of the hemoglobins in Fig. 1d are: Hb-S = 0.30; Hb-A = 0.70; Hb-F = 0.95.

The spots of the hemoglobins are diffuse and smeared in these chromatograms, and it has not been possible to devise conditions that produce more compact and distinct spots.

DISCUSSION

The literature provides surprisingly little guidance for devising suitable conditions for ion-exchange TLC. For column chromatography, the equilibrated ion exchanger is always in contact with solvent. However, if ascending TLC is to be used, the TLC plates must be dried. Consequently, many preliminary experiments were necessary before conditions of a satisfactory procedure were approached. In this, we were guided by data from microchromatographic procedures[1]. Some aspects of these experiments will be discussed.

Preparation of plates

The physical characteristics of CM-cellulose have required that the layers be formed from a rather thin slurry which, after application, tends to run off the edges of the plates. Thicker slurries produce uneven layers. The dried layers before use usually have cracks such as are visible in Fig. 1. However, these close during development and appear not to influence the chromatographic behavior.

Influence of salt concentration on movement of hemoglobins

The concentration of NaCl in equilibrating solvent for the ion exchanger and in the developer is of critical importance in controlling the motion of the hemoglobin. The significant value is the sum of the salt concentrations in the ion exchanger and in the developer. As this sum increases, the R_F values of the samples also increase. Once this optimal sum has been determined, the clearest and most distinct (least smeared) spots occur when the salt is divided fairly equally between the plate and the developer. For instance, if the optimal sum is 0.21 *M* NaCl, far better results are obtained if the salt concentration in the exchanger is 0.11 *M* and that in the developer 0.10 *M*, than if they are 0.17 and 0.04 *M*, respectively.

pH of sample

As already noted, the pH of the sample is important in preventing the movement of hemoglobin with the solvent front. In adult samples in which Hb-F is normally absent, this artifact could be ignored. However, it would certainly interfere with the detection of an electrophoretically fast-moving hemoglobin. Consequently, pH adjustment of the sample is desirable.

Diffuse spots

The diffuse smeared spots lead to less complete separations than are desirable. This could create problems of overlapping and difficulty of identification. Attempts to remedy this situation —by the use of carbonmonoxyhemoglobin to lessen any oxidation during chromatography, by reducing the temperature to 4° in order to lessen unwanted diffusion, by the use of multiple development with the same and different developers, by substituting phosphate for Tris buffers, etc.— have been unsuccessful.

Reproducibility

Tris is virtually without buffering capacity at pH 6.1. Variability in results in most instances probably is due to unexpected pH variation. Indeed, difference in pH meters and electrodes seems to be sufficient to introduce variation. Substitution of bis-Tris, which has a pK_a of about 6.5, should permit better control of pH. Although a system with bis-Tris buffers was devised, it was not as satisfactory as the Tris system. Reproducible results, therefore, require much care in the preparation of solutions and the equilibration of ion exchanger.

Applicability of method

These experiments were performed to ascertain the usefulness of the method for the qualitative detection of common abnormal hemoglobins. Indeed, the common abnormal hemoglobins can be successfully separated by ion-exchange TLC in less time than by any previously devised method. However, this method has disadvantages in comparison with microchromatographic methods[1,9–11]. For one, the required sample size is as large as or larger than that for microchromatographic separation. The preparation of TLC plates not only is more involved than the pouring of microchromatographic columns but also the amount of ion exchanger per sample is greater. Finally, the diffuse spots of TLC are more difficult to see than the compact zones on the microcolumns.

Therefore, the practicality of ion-exchange TLC for the separation of hemoglobins will depend largely upon the particular conditions under which the method is to be applied (speed, sample size, etc.).

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