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MICROCHROMATOGRAPHY OF HEMOGLOBINS

VII. DETECTION OF SOME UNCOMMON HEMOGLOBIN VARIANTS AND TWO RAPID METHODS FOR THE QUANTITATION OF Hb-A_2 IN THE PRESENCE OF Hb-C

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SUMMARY

Microchromatographic procedures on columns of DEAE- or CM-cellulose are described for the separation of the more common abnormal hemoglobins S and C from other, often uncommon, variants such as J, N, I, K-Woolwich, Hope, D, E, O, and Deer Lodge. Two procedures on conventionally sized columns of CM-cellulose permit the quantitative determination of Hb-A_2 in the presence of Hb-C in a day.

INTRODUCTION

Although certain microchromatographic procedures were devised for specific purposes such as the quantitative determination of Hb-A_2 [1] or the testing of cord blood for Hb-S and/or Hb-C [2], their utility is by no means limited to such usage. Thus, the method for the quantitative determination of Hb-A_2 is easily applicable to testing programs for hemoglobinopathies [3]. The present paper describes how minor modifications extend the applicability of these methods to the study of such fast moving hemoglobins as J and N or to the distinction of Hb-S from Hb-D and Hb-E or Hb-O from Hb-C. In addition, two rapid methods for the determination of Hb-A_2 in the presence of Hb-C on conventionally sized chromatograms are presented.

*Contribution No. 5288.

MATERIALS

Blood samples

Informed consent was obtained from individuals whose blood was examined. Specimens were obtained through the Sickle Cell Centers at Augusta and Los Angeles or had been sent to Augusta or Pasadena for further evaluation. The blood was collected in ethylene diaminetetraacetic acid or heparinized microhematocrit tubes.

Of the uncommon abnormal hemoglobins that were used in this investigation, hemoglobins N-Baltimore (β95, Lys→Glu), J-Baltimore (β16, Gly→Asp), K-Woolwich (β132, Lys→Gln), Hope (β136, Gly→Asp), I-Philadelphia (α16, Lys→Glu), and Deer Lodge (β2, His→Arg) were identified by chemical study of the aberration. Hemoglobins D-Los Angeles (β121, Glu→Gln), E (β26, Glu→Lys), and O-Arab (β121, Glu→Lys) were not so identified but had the proper electrophoretic behavior at both acid and alkaline pH and derived from the ethnic group at risk.

For most procedures, whole blood was used for the chromatograms. When hemolysates were used, they were prepared from washed cells by hemolysis with water equal to 1.5 times the packed cell volume plus 0.4 volume of carbon tetrachloride for 20 min at room temperature after which cellular debris was removed by centrifugation. In some instances, the sample was dialyzed against a large volume of the appropriate developer overnight at 4°.

Developers

The solutions for the several procedures to be described were made up with the quantities and to the molarities that are given in Table I. Some of these developers have been designated by letters or Roman numerals in other papers [1, 2, 4].

TABLE I

COMPOSITION OF DEVELOPERS*

Developer No.	Bis-tris		NaCl		Glycine		$NaH_2PO_4 \cdot H_2O$		pH
	M	g/l	*M*	g/l	*M*	g/l	*M*	g/l	
1					0.2	15			Unadjusted**
2			0.015	0.88	0.2	15			Unadjusted**
3			0.02	1.17	0.2	15			Unadjusted**
4	0.03	6.28	0.03	1.75					6.1***
5	0.03	6.28	0.12	7.01					6.1***
6	0.03	6.28	0.04	2.34					6.2***
7	0.03	6.28	0.05	2.92					6.2***
8							0.01	1.38	7.0§
9							0.01	1.38	8.0§

*All solutions contain 0.1 g KCN per liter (0.01%).
**The pH is about 7.6.
***pH adjusted with conc. HCl.
§ pH adjusted with 2 *N* NaOH.

Preparation of ion exchangers

CM-Cellulose or DEAE-cellulose as CM-52 or DE-52 (microgranular and preswollen) from Whatman (Clifton, N.J., U.S.A.) was used in all experiments.

DE-52 was prepared in developer No. 1 and equilibrated as described previously with the pH adjusted for optimal separation of Hb-A_2 and Hb-S [1]. CM-52 was prepared and equilibrated either with developer Nos. 4, 6, 7, or 8 as previously described for similar solutions [2, 4].

PROCEDURES AND RESULTS

Separation of Hb-N, Hb-J and similar hemoglobins

Procedure. A 0.5 × 8 cm column of DE-52 which has been equilibrated with developer No. 1 and adjusted in pH was poured in a Pasteur pipette. One drop of blood or undialyzed hemolysate was mixed with eight drops of water. If blood is used, hemolysis should proceed for at least 5 min before the sample is applied to the column. After the sample had been applied, development was made with developer No. 1 at a flow-rate of about 15 ml/h until the effluent equaled 5 ml. Developer No. 1 was then replaced by developer No. 3 and 15 ml were passed through the column.

Alternatively, a 0.5 × 15 cm column may be used at a flow-rate of about 10 ml/h. After development with 5 ml of developer No. 1, 60 ml of developer No. 2 was used.

Results. Development with developer No. 1 on the shorter column is the procedure for the quantitative determination of Hb-A_2 [1]. Therefore, Hb-A_2, as

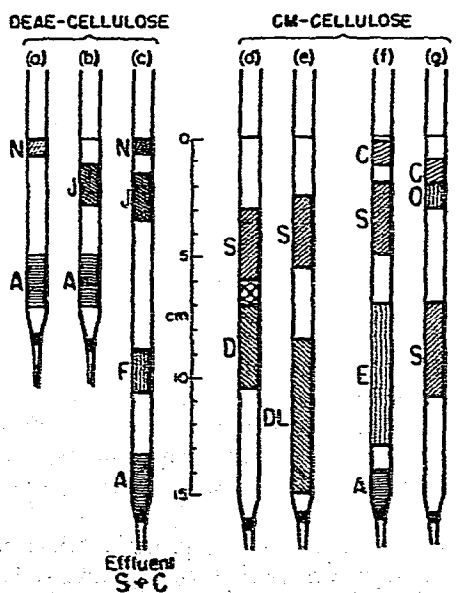

Fig. 1. The positions and separations of some common and uncommon hemoglobins on DEAE-cellulose and CM-cellulose under various conditions that are described in the text. The limits of the zones are depicted as sharp whereas in fact they are diffuse.

well as any Hb-C, passes through virtually unretarded. By the time that 5 ml of developer No. 1 has been used, Hb-S has moved to the middle of the column but any Hb-A, Hb-F, and electrophoretically fast moving hemoglobins at alkaline pH adhere to the top of the column. After development with developer No. 3 is complete, Hb-A and Hb-F are at the bottom, Hb-J in the middle and Hb-N still at the top of the column. Fig. 1a and b show the results when the method was applied to AN-Baltimore and AJ-Baltimore samples.

Experiments with other fast moving hemoglobins gave these results. Hb-K-Woolwich moved more rapidly down the microcolumn than N but more slowly than J. Hemoglobin Hope moves between A and J. On the other hand, Hb-N and Hb-I were indistinguishable. Hb-A and Hb-F did not separate.

When a complex artificial mixture of hemoglobins C, S, A, F, J, and N was applied to the longer column and developed as indicated, the hemoglobins emerged as well separated zones (Fig. 1c).

Comments. The procedures will clearly distinguish a wide variety of known or potential heterozygosities for hemoglobins. The study has included hemoglobins A, S, C, F, J, N, I, K-Woolwich, and Hope. Of the possible combinations, only that of N and I would not be identified, and Hb-F and Hb-Hope might be confused on microchromatographic evidence alone.

Samples of blood or hemolysate should be no older than three weeks or alteration products of Hb-A (that is, Hb-A_1) will interfere with identification of Hb-F or even possibly Hb-J.

Separation of Hb-S and Hb-C from electrophoretically similar hemoglobins

Procedure. A 0.5 × 15 cm column of CM-52 which has been equilibrated with developer No. 6 (or 7) was poured. The sample was prepared from 0.02 ml of blood, 0.2 ml of 0.004 *M* maleic acid, and 0.3 ml of 0.05% saponin (Calbiochem, Los Angeles, Calif., U.S.A.) in developer No. 6 (or 7). At least, 5 min was allowed for hemolysis. After the sample had been applied, the chromatogram was developed with developer No. 6 (or 7) at a flow-rate of about 10 ml/h. Developers Nos. 6 and 7 differ only in molarity of sodium chloride and were chosen in specific cases to provide a reasonable movement of the hemoglobins.

Results. When the blood of an individual with SD disease was chromatographed as above with 60 ml of developer No. 6, two zones were apparent on the column although not completely separated (Fig. 1d). The interzone was apparent but not clean.

Similarly, when blood of an S-Deer Lodge (DL) case [5] was chromatographed with 25 ml of developer No. 7, the hemoglobins occupied positions shown in Fig. 1e. On starch gel electrophoresis at pH 9, hemoglobins S and Deer Lodge produce a band that is broader in the anodal direction but distinct from Hb-S alone. This slight difference in charge is no doubt responsible for the better chromatographic separation of Hb-S and Hb-Deer Lodge as compared to that of Hb-S and Hb-D.

The separation of Hb-C and Hb-E is readily accomplished. When AC, AS, and AE samples were chromatographed on parallel columns and developed with 50 ml of developer No. 6, the final positions are shown in composite in Fig. 1f.

Hb-O-Arab can be distinguished from Hb-C but the separation is not as great as that of Hb-C and Hb-E. Thus, when 75 ml of developer No. 7 had been used

in parallel chromatograms, the results shown in composite in Fig. 1g were obtained.

Comments. The distinction between the SS and SD genotypes or between the CC and CE or CO genotypes is commonly done by citrate—agar electrophoresis [6] in which hemoglobins A, S, and C take distinctive positions but hemoglobins D and E behave like Hb-A, and Hb-O moves between the hemoglobins A and S. Microchromatography under these conditions provides more definite distinctions because Hb-D and Hb-Deer Lodge not only separate from Hb-S and, likewise, Hb-E and Hb-O-Arab from Hb-C but they do not mimic Hb-A.

The movement of hemoglobins in these developers as stressed previously [4] is very dependent upon sodium chloride concentration. The examples provided illustrate the altered movements that change in sodium chloride concentration brings about. No doubt by increase in sodium chloride concentration, Hb-C and Hb-O could be more completely separated. Because of the sensitivity to sodium chloride concentration, parallel chromatograms of known and unknown hemoglobins provide a more accurate comparison of chromatographic behavior.

Quantitative determination of Hb-A_2 in the presence of Hb-C

Procedure. Two procedures are available for this determination.

One procedure has been described [4] and uses the full gradient of sodium chloride in N,N-bis(2-hydroxymethyl)iminotris(hydroxymethyl)methane (Bis-tris) at pH 6.1 on CM-52. The chromatogram was complete in 24 h.

The second procedure is a modification of the method of Huisman [7]. CM-52 was equilibrated with developer No. 8 and poured into a column 1 × 32 cm in dimension which shrank to 30 cm in length during equilibration with the

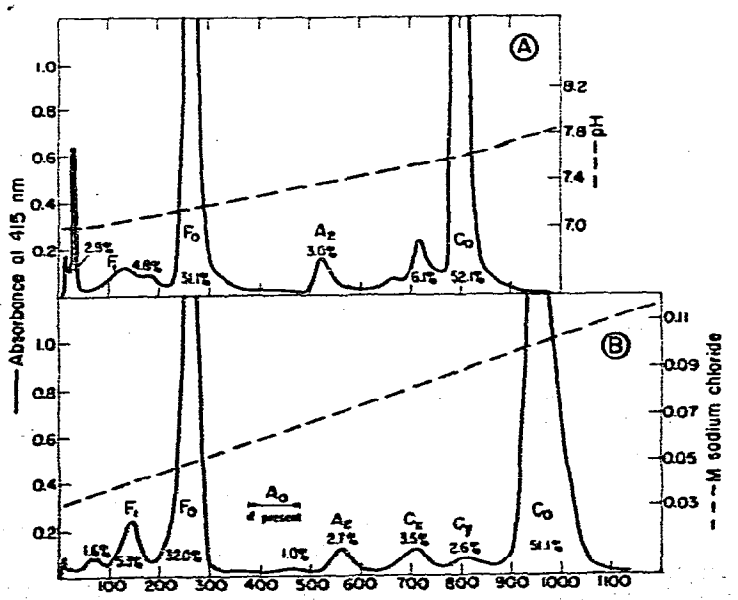

Fig. 2. The quantitative determination of Hb-A_2 in the presence of Hb-C by two methods on CM-cellulose.

same developer at 50 ml/h. A 40-mg sample which had been dialyzed against developer No. 8 was applied. Development was made with a two-vessel gradient system that contained 500 ml of developer No. 8 in the mixer and 500 ml of developer No. 9 in the second vessel. At a flow-rate of 50 ml/h, the chromatogram was complete in 20 h. The absorbance of the 5-ml fractions was read at 415 nm. The column may be used again after equilibration with 100 ml of developer No. 8.

Results. Fig. 2 depicts the application of these procedures to the hemoglobin of an individual with C-HPFH (hereditary persistence of fetal hemoglobin). The application of the Bis-tris procedure to an AC sample has been presented in Fig. 3B of ref. 4.

In order to test the validity of the new procedures, they have, in addition,

TABLE II

RESULTS OF THE APPLICATION OF SEVERAL METHODS FOR THE DETERMINATION OF Hb-A_2

Condition	Glycine micro A_2*	CM-Cellulose		DEAE-Sephadex**	Immuno***
		Bis-tris	Phosphate		
CC		4.6	4.8		4.5
C-β^+-Thal		6.8	6.8		
C-HPFH		2.7	3.0		
AC		3.7	3.3, 3.1		
AC		3.6	3.9		2.9
AC		4.0	3.9		3.0
AC		4.2	4.0		3.1
AC		3.9	4.1		
AC		3.9	4.4		
A-β-Thal	5.3		5.9		
A-β-Thal	4.9, 4.8	5.4	5.0	4.7	
AA	1.6, 1.6	2.8	2.3		
AA	2.0	2.8	2.4		
AA	2.9	3.1			
AA	2.5	4.4	2.7		
AA	2.5		3.1		
AA	2.5		3.5		
AA	2.5		2.9	3.0	
AA	2.7	4.2	2.6		2.3
AA	2.4, 2.3	3.5	2.0		2.1
AA	2.3, 2.5, 2.4, 2.6	3.6	3.3		
AA	2.5, 2.5, 2.4, 2.1	3.3	2.6		
AA	2.9	3.5	2.9		
AA	2.6	3.1	2.6		
AX§		3.1	2.6		2.9

*Procedure of ref. 1.
**Conventional chromatography [8, 9].
***Radioimmunoassay by Dr. Fred Garver [10].
§ An unidentified fast moving variant.

been applied to non-Hb-C-containing samples so that other chromatographic methods could be used for comparison [1, 8, 9]. Some samples with Hb-C where also tested by a newly developed radioimmunoassay [10]. The results are presented in Table II. Although some samples gave discrepant results for undetermined reasons, the data are generally concordant and show that the two methods here described provide means for the rapid determination of Hb-A_2 in the presence of Hb-C.

Comments. The prime consideration in the design of these procedures has been a good and rapid separation of Hb-A_2 from other hemoglobin components. The phosphate procedure has been started at a higher pH than originally [7] and hemoglobins that precede Hb-A_2 move out quickly. Hb-A will coincide with Hb-F in the phosphate method but will occupy the indicated position in the Bis-tris method. The separation of Hb-A and Hb-A_2, therefore, is markedly different in the two procedures. The fast flow-rate that is used reduces the time required to one-fourth that of the previously described chromatographic method [7].

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