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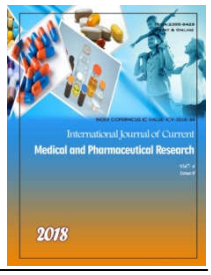

HEMOGLOBIN FRACTIONATION BY HPLC

Gireesh. V. Achalkar*

Department of Pathology, Raichur Institute of Medical Sciences, Raichur, Karnataka-584102

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ABSTRACT

Introduction: High performance liquid chromatography (HPLC) is a new technique which has demonstrated advantages in terms of accuracy and precision over conventional procedures employed in newborn and adult hemoglobinopathy screening programs for the identification of Hb variants. It has prompted the need to reassess our knowledge of hemoglobin fractions reference ranges as it relates to HPLC HB quantitation. **Materials and Methods**: Fifty microliter aliquots of fresh whole blood samples collected in ethylenediamine tetraacetic acid (EDTA) containing vacutainer tubes from normal newborn and adult individuals being screened at the were treated with a hemolyzate reagent, centrifuged at 12,000 rpm at 4°C for 15 minutes and passed through a 0.25 to 0.45 diameter syringe filter unit into a 1.5 ml tube. **Results**: In this study, the HPLC hemoglobin reference ranges derived from 100 normal adults (ages 18-50) and infants of upto 300 days of age were studied. And HPLC were chosen to establish the hemoglobin reference ranges.. The percentage values are as follows: Hb A mean of 92.3 percent; HbA1 mean 3.6 percent; Hb F mean of 3.2 percent; Hb A2 mean of 2.1 percent, respectively.
Conclusion: Application of the HPLC ranges to confront other abnormalities will prove most useful during blood screening processes. The HPLC hemoglobin ranges for normal adult and newborns have been established.

INTRODUCTION

Identification of human hemoglobin variants in blood from adults and new born babies are usually carried out by cellulose acetate and citrate agar electrophoresis and also by isoelectric focusing (IEF)[1]. In fact, these procedures are routinely employed in screening programs to detect sickle cell, thalassemia, and other hemoglobinopathies in affected individuals. Although, it is recognized that when used in combination, these techniques are capable of separating common hemoglobin variants like A, F, S, A2, Hb C, and Hb E; other variants are difficult to identify since they migrate together or very close. Quantification of the Hb variants by densitometry scanning of the gels is not recommended because of its sensitivity limits and inaccuracy. These limitations have prompted the use of a more versatile tool like high performance liquid chromatography (HPLC) not only to identify but to quantify a number of different and rare hemoglobins. The literature abounds with descriptive information about the use of HPLC, and others closely related like anion-exchange and reverse-phase chromatography to identify and characterize rare hemoglobin types. Reports on the identification and hemoglobin separation from newborn patients with severe alpha-thalassemia like Hb Bart's, Hb H, the embryonic Hb Portland I, Hb Portland II, Hb Portland III, and Hb Constant Spring (Hb CS) by HPLC using a weak ion exchanger and the work of other investigators have encouraged the use of this technique to overcome false positive diagnosis of sickle cell disease sometimes found with traditional electrophoretic determinations[2]. In spite of these advances, information on HPLC reference ranges is scarce or not readily available for the identification and quantification of hemoglobin variants.

Although it is recognized that HPLC reference ranges may vary from one laboratory to the next owing to materials, type of equipment and operating conditions employed, it is important that these parameters be established prior to its application in newborn/adult hemoglobin screening programs. The samples used in an high performance liquid chromatography machine is usually larger than that of a gas chromatography machine[3]. Despite the number of disadvantages it has, there are numerous advantages as well. The devices are more precise than other devices and can analyze a wider range of compounds, although the GC device can do better with more complex mixtures than the HPLC devices do. Our report deals with the development of normal hemoglobin reference range values for HPLC for a normal healthy adult population and the identification and quantification of several abnormal hemoglobins found in adult and newborn individuals[4].

Corresponding author:* **Gireesh. V. Achalkar
Department of Pathology, Raichur Institute of Medical Sciences, Raichur. Karnataka. 584102

There are more than 1,100 human hemoglobin variants[5]. The majority were discovered during population surveys and are not associated with clinical manifestations. OBJECTIVE: To establish normal baseline values for HB fractions.

MATERIALS AND METHODS

100 adults of age 18 to 50 and infants of 4 days to 300 days were selected for study. The clinical history and CBC findings were carefully studied. Subjects with normal CBC profile were included for study. Fifty microliter aliquots of fresh whole blood samples collected in ethylenediamine tetraacetic acid (EDTA) containing vacutainer tubes from normal newborn and adult individuals being screened at the were treated with a hemolyzate reagent centrifuged at 12,000 rpm at 4°C for 15 minutes and passed through a 0.25 to 0.45 diameter syringe filter unit into a 1.5 ml tube. In each case, 10ml of aliquot were transferred for hemoglobin variant analysis in a Hb Vario multisolvent HPLC system. The column used was a PolyCat A 300, stainless steel packed with hydrophilic cationic polymer; a gradient made up of mobile phase A containing 40 mM Bis Tris; 4 mM KCN at pH 6.5 and mobile phase B composed of 40 mM Bis tris; 4 mM KCN, 0.2 M NaCl at pH 6.8 and running conditions at 2.0 ml/min flow rate for 30 minutes, ultraviolet readings at 436 nm were observed. At the end of each run, qualitative and quantitative peak integration results were automatically obtained.

RESULTS

One hundred identified as normal adults (ages 18 to 50) by alkaline and acid electrophoresis and HPLC were chosen to establish the hemoglobin reference ranges. The corresponding hemoglobin ranges are shown in Table1. The percentage values are as follows: Hb A mean of 92.3 percent ; HbA1 mean 3.6 percent ; Hb F mean of 3.2 percent ; Hb A2 mean of 2.1 percent , respectively.

Hemoglobin Percentages of Normal New borns and Babies

Hemoglobin percentile ranges for normal newborns and babies from 4 days up to 10 months of age were determined , and the hemoglobin trend changes occurring during this time period were also statistically assessed. The HPLC results registered in table II show that the percentage mean values and the minimum-maximum ranges for Hb F vary from 77.3 (64.6 to 90.1) at 4 days to 13.4 mean (8.0 to 18.0 %) at 300 days. TABLE 3 for Hb A vary from 23.1 range 9.9 to 36.4% at 4 days to 86.6 (81.2 to 92.0) at 300 days after birth. The decreasing trend for Hb F and the corresponding increments for Hb A observed after 4, 8 , 14, 20, 30, 50, 150, 210, and 300 days after birth are shown in Tables 2 and 3.

Table I High Performance Liquid Chromatography Hemoglobin Reference Ranges in Normal Adults

HB types	Mean %	SD	Minimum%	Maximum%
HBA	92.3	1.2	88.0	96.7
HBA1	3.6	0.6	1.0	6.2
HBF	3.2	0.6	1.2	5.1
HBA2	2.1	0.5	0.6	3.7

Table II High Performance Liquid Chromatography Hemoglobin F Percentage Trend Variations in Normal Newborns and Babies Up to 10 Months

Age in days	Mean	SD	Minimum	Maximum
4	77.3	7.6	64.6	90.1
8	75.8	8.2	60.5	91.2
14	76.1	8.6	57.9	94.4
20	78.0	5.3	68.0	88.0
30	74.2	7.1	62.2	86.2
60	52.8	3.3	32.1	73.6
150	27.3	2.9	21.1	33.5
210	26.1	2.9	21.0	31.2
300	13.4	5.0	8.0	18.8

Table III High Performance Liquid Chromatography Hemoglobin A Percentage Trend Variations in Normal Newborns and Babies Up to 10 Months

Age in days	Mean	SD	Minimum	Maximum
4	23.1	7.6	9.9	36.4
8	24.1	8.2	8.7	39.5
14	23.8	8.6	5.6	42.1
20	22.0	5.3	12.0	32.0
30	25.8	7.1	13.8	37.8
60	47.1	3.2	26.4	67.9
150	72.7	2.4	66.5	78.9
210	73.9	3.0	68.8	79.0
300	86.6	5.2	81.2	92.0

DISCUSSION

Current hemoglobin screening for the identification of each of the numerous hemoglob in abno rmalities known requires the use of at least two procedures to reach a definite diagnosis; with th e more difficult cases, analysis of deoxyribonucleic acid (DNA) is necessary.[6,7] The use of cation exchange HPLC has added a tremendous value to hemoglobin variant detection for its ability to provide accurate qualitative data which is particularly important for the differential diagnosis of conditions such as SS and S(+ thal), CC and C(+ thal), and EE and E(+ thal) in newborns[8,9].

Because HPLC hemoglobin variant quantitative measurements vary significantly with respect to those compiled from other methodologies, ergo densitometric results from cellulose, citrate agar or isoelectrofocusing electrophoresis[10,11], the establishment of specific reference ranges to explore the quantitative capabilitie s of HPLC to differentiate the boundaries of a normal and abnormal hemoglobin conditions are being reported.[12]

High performance liquid chromatography hemoglobin ranges of Hb A, Hb A1; Hb F and Hb A2 encountered with a representative normal adult population fall within limits of normal controls derived from densitometric quantitation of cellulose acetate, citrate and/or isoelectrofocusing electrophoresis procedures[13,14]; *i.e.,* the latter showing that the approximate mean percentage for each of these hemoglobins is: Hb A, 90 percent; Hb A1; 6.5 percent; Hb F, 1.0 percent; and Hb A2, 2.5 percent[10,15,16]. The normal adult reference ranges shown in table 1 can be used with a certain degree of accuracy to detect the presence of an abnormal hemoglobin when the HPLC percentage results of a blood specimen does not correspond to these normal ranges. Hemoglobin statistical evaluation of normal newborns by HPLC varies with age, as expected[17,18]. Newborn percentage trends shift from Hb F of 77,3 percent and Hb A of 23.1 percent at 4 days to Hb F mean of 13.4 and Hb A mean of 86.6 percent at 300 days after birth.

The reported HPLC normal newborn Hb F percentages fall within the 70 to 85 percent values obtained by other procedures[19,20]; however, our HPLC results indicate that Hb F disappears in time at a much slower pace, while the Hb A percentage increments in the same fashion. In fact, after 150 days (5 months), 210 days (7 months), and 300 days (10 months), the mean percentage levels of Hb F were 27.3, 26.1, and 13.4, respectively. These results are in contrast with the Hb F rapid disappearing reports of 1 to 2 percent after 6 months[13,21,22]. The apparent relatively high levels of Hb F (15.0 percent) after 10 months obtained by the HPLC method should be taken into consideration when trying to interpret Hb F percentages of babies one year of age or older.[23,24,25]

Inherited abnormalities of hemoglobin synthesis may be due structural changes or due to reduced synthesis[26]. Many are functionally normal and therefore clinically silent [27,28]. Some form polymers (HBS) or crystals (HBC)[29,30]. Abnormal Hemoglobins in Newborn and adult sickle cell trait individuals, Hb FAS and Hb AS, are readily identified by cation exchange HPLC[12,31]. is in agreement with current hemoglobin detection protocols reported for adult Hb AS patients. However, when these same results were expressed in terms of Hb A/S ratios, Evaluation by HPLC of Hb FAS newborns at various age groups show a larger diminishing trend for Hb F and the corresponding increasing percentage of Hb A and Hb S with time when compared to normal babies, Hb FA or AA,. In all cases, the A/S ratios remaine constant with a mean of 1.5 (s.d. range 1.1 to 2.2) regardless of age group[32,33]. If this A/S ratio is compared with the adult Hb AS, there is practically no difference[34]. These results may suggest that HB A/S ratios reported may indicate the presence of other type of hemoglobin inherited disorder which will require additional confirmatory tests[35] , *i.e .,* thalassemias; otherwise, the Hb AS trait condition of the patient would prevail.

The purpose of newborn hemoglobinopathy screening is to detect sickle cell disease[36]. The most common types of sickle cell disease are sickle cell anemia (Hemoglobins SS), Hemoglobin SC disease, sickle beta thalassemia zero (Sβ°) and sickle beta thalassemia plus ($S\beta^+$)[37,38]. These conditions render infants susceptible to overwhelming pneumococcal infection and acute splenic sequestration[39,40]. These life-threatening complications may occur prior to other less severe complications that would lead to the routine diagnosis and institution of preventive measures.[41]

CONCLUSION

The HPLC hemoglobin ranges for normal adult and newborns have been established. Application of these ranges to confront abnormal hemoglobins like Hb AS, Hb FAS, Hb AC, and HB FAC has shown that the A/X ratio pattern remains constant, even if it is a newborn. Detection of small amounts of Hb A, Hb S, or Hb C in newborns who have large quantities of Hb F has been possible by HPLC owing to its high sensitivity and specificity. Usefulness of this approach will be further attested with time when HPLC analysis of blood specimens for newborn screening and identification of hemoglobin variants becomes widespread in this country.

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