Analytical & Preparative Separation Methods of Biomacromolecules

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edited by

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Analytical and Preparative Separation Methods of Biomacromolecules

To my beloved Parent and the most special people in my life,
Nagla, Youssef, Faisal, and Basil

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Preface

It is my pleasure to be an editor of this book, which gave me a chance to be exposed to this fascinating field of separation technology of biomacromolecules which represent, medically and biochemically, an important class of compounds.

This book consists of sixteen chapters representing the latest research results and recent developments in the area of high-performance liquid chromatography and capillary electrophoresis. Also, the book includes chapters discussing in detail: biochromatography, displacement chromatography, affinity capillary electrophoresis, and the new technologies of slalom chromatography. The scope of the topics presented are broad and therefore give a comprehensive view of the current status of the established and newly developed separation techniques that are useful for biomacromolecule isolation and purification.

I would like to thank the contributors, internationally renowned experts in their respective research fields, who have made this book possible. Also, my thanks are extended to Marcel Dekker, Inc., and, in particular, Mr. Russell Dekker for his encouragement to publish this book.

As we approach the next millennium and reflect on the rapid developments that have been achieved in the area of biotechnology, I certainly hope that this book will be a useful reference for scientists and researchers dealing with isolation, purification, and analysis of biomolecules.

Hassan Y. Aboul-Enein

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Analysis of HbA_{1C} and Some Hb Variants by HPLC

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I. INTRODUCTION

This chapter deals with selected aspects of high-performance liquid chromatography (HPLC) methods for quantitating of glycohemoglobin (HbA_{1C}) and characterizing some Hb variants. Determination of HbA_{1C} by ion exchange HPLC is used for monitoring of glycemic control in diabetic patients. The percentage of HbA_{1C} reflects blood glucose concentrations of the previous 2–3 months. It is therefore considered a valuable indicator of long-term diabetic control [1]. However, the methods currently used for its measurement in clinical chemistry laboratories show large differences between reported values, and comparison of results from different laboratories is difficult [2]. Lack of standardization of glycohemoglobin measurements remains the major source of interlaboratory variation. At present there is no acknowledged reference method or an accepted standard. This fact is well recognized and an IFCC (International Federation of Clinical Chemistry) working group is developing a reference method based on HbA_{1C} as the biochemically well-defined major glycohemoglobin component. Recently, the use of calibration based on a cation exchange HPLC method has been shown to increase the comparability between various analytical methods [3,4].

The classical method for the rapid determination of HbA_{1C} by chromatography on the weak cation exchange resin Bio-Rex 70 was introduced by Trivelli et al. in 1971 [5] based on the method of Allen et al. on Amberlite IRC-50 [6]. Several approaches, including minicolumns [7] and HPLC [8], were used to make ion exchange chromatography acceptable for use in clinical laboratories. Though acceptable resolution can be obtained with Bio-Rex 70, it has lower resolution

and is more time consuming than HPLC methods. Flow rate is limited by the compressibility of the resin and its nonuniform particle size. These drawbacks stimulated development of several methods using ion exchange resins for HPLC, such as Synchropak CM 300 [9], silica-based carboxymethylpolyamide [10], Mono S [8,11], and the Glycopak resin of the Diamat analyzer (Bio-Rad). These methods can usually also be used to measure fetal hemoglobin (HbF). They differ in their sensitivity to interferences such as adducts formed between Hb and urea, aldehydes, or acetylsalicylic acid and other hemoglobin (Hb) variants [12,13].

Glycohemoglobin can also be assayed by affinity chromatography using boronate agarose columns [14]. This method measures HbA_{1C} together with glycohemoglobins, which elute in the HbA_0 fraction in ion exchange chromatography.

In chromatographic methods, abnormal Hb variants may cause erroneous HbA_{1C} results [15,16], but at the same time they permit identification of individuals with such variants. This information is of importance for the interpretation of the HbA_{1C} results. Therefore it is an advantage if an HPLC method used for clinical purposes separates not only HbA_{1C} but also other hemoglobin components to permit identification of abnormal hemoglobins. The good resolution of more than 35 frequently encountered human hemoglobins and HbA_{1C} on PolyCAT A [17] makes it a useful method for preliminary characterization of Hb variants. The Diamat method has been shown to separate seven variants [18], but many overlap partially with the HbA_{1C} peak.

II. CURRENT HPLC METHODS FOR THE DETERMINATION OF HbA_{1C} BY CATION EXCHANGE CHROMATOGRAPHY

A. Mono S

The Mono S system was introduced by Stenman et al. and Jeppsson et al. [8,11]. The Mono S method has been widely used in clinical laboratories. It is precise, accurate, and easily operated [11]. The monodisperse cation resin, Mono S, does not shrink, swell, or leak functional groups with a LiCl gradient in malonate buffers at pH 5.7. The acetaldehyde adduct of Hb in alcoholics may interfere with HbA_{1C} determinations since it coelutes with HbA_{1C}. In uremic patients, carbamylated Hb may increase the HbA_{1C} values by as much as 1%. The separation has been further optimized by using a smaller column load, decreased flow rate, and a steeper LiCl gradient [19]. We have also used Mono S extensively for HbA_{1C} analysis by slightly modifying the original method [8]. Phosphate buffers with 10% acetonitrile and a higher pH of 6.85 were used at 30°C. These changes

improved the separation and the column life increased up to several thousand samples with constant resolution.

B. PolyCAT A

PolyCAT A is a weak cation exchanger with polyaspartic acid linked to silica [20,21]. This resin has been used for the determination of HbA_{1c} [22,23], of several minor molecular forms of hemoglobin [24], and for identification of hemoglobin variants [17]. The separation of HbA_{1c} from other Hb components such as acetylated Hb [13] and HbF depends critically on buffer composition, particularly on the pH and salt gradient. It is often necessary to make minor variations in the steepness of the salt gradient in order to get a good separation. The assay is precise [22] and it depends more on the quality of the chromatographic separation, the level of noise in the spectrophotometer, and the mode of integration than on volumes injected.

PolyCAT A with bis-tris buffers and sodium acetate gradients has been used to separate and quantitate many normal and abnormal Hb components [23,24]. Minor fetal Hb forms can be separated and HbA_{1c} can simultaneously be quantitated. We have compared the performance of PolyCAT A chromatography with a boronate affinity binding assay and the automated Diamat system for the determination of HbA_{1c} [23]. Elution was achieved with a linear gradient consisting of buffers A and B at a flow rate of 1.1 ml/min at room temperature. Buffer A (pH 6.87) contained 10 mmol of bis-tris and 1.0 mmol of KCN per liter. Buffer B (pH 6.57) contained buffer A plus 200 mmol of NaCl per liter. The gradient was: time 0–2 min, 21% B; time 16 min, 47% B; time 22 min, 100% B; time 24 min, 100% B; and time 26 min, 21% B. Integration was performed by the valley-to-valley method.

When we compared the two cation exchange methods, PolyCAT A and Diamat, we obtained an acceptable correlation (Fig. 1). The correlation coefficient was 0.90 but the regression equation (PolyCAT A = 1.06 × Diamat − 3.06) showed that much lower results were obtained by PolyCAT A. This suggests that the HbA_{1c} value measured by the Diamat assay includes a background of 2–3% of the total Hb. This might be due to the fact that the Diamat method also measures carbamylated and acetylated forms of Hb [12] and possibly some other derivatives formed in blood during storage, which can be separated from the HbA_{1c} peak by the use of methods with higher resolution. The PolyCAT A assay has been optimized to separate different Hb variants from HbA_{1c}. However, the difference between the Diamat method and the PolyCAT A assay cannot be explained only by carbamylated and acetylated Hbs, for which levels below 0.4% have been reported [12]. The slope of the regression line, 1.06, shows that the PolyCAT A method reflects differences in glycated Hb in the same way

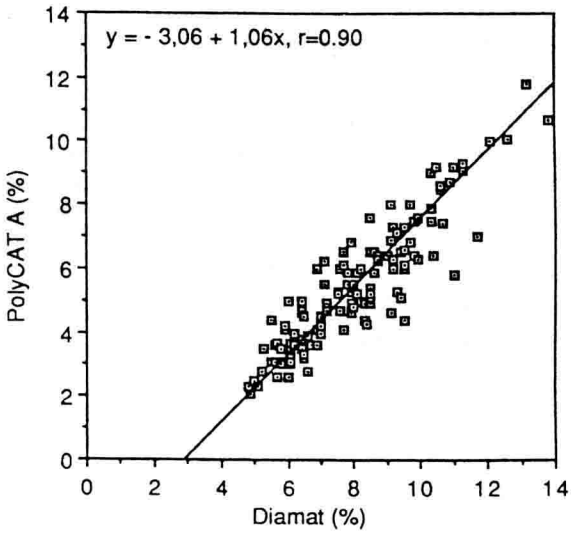

Figure 1 Correlation between the HbA_{1C} values obtained by PolyCAT A (y) and Diamat (x). Corresponding regression equation calculated by the standardized principal component is $y = 1.06x - 3.06$, $r = 0.90$.

as the Diamat method. The negative bias of about 2–3% of total Hb is present at all levels of HbA_{1C} when PolyCAT A is compared to Diamat.

C. Diamat and Variant Analyzers

The Diamat analyzer of Bio-Rad Laboratories is an automated HPLC instrument using a weak cation exchange column. The support material is silica with carboxymethyl functional groups. The analyzer forms a stepwise gradient of three phosphate buffers of increasing ionic strength to separate the HbA_{1C} from other Hbs. The system has been optimized for the quantitation of HbA_{1C}, and HbA_2 coelutes with HbA_0. Quantitation is based on the absorbance at 415 and 690 nm. Before analysis with the Diamat analyzer 5 μl of the whole-blood sample is diluted with 1.25 ml of hemolysis reagent containing 0.1% (v/v) polyoxyethylene ether in a borate buffer. The tubes are incubated at 37°C for 30 min to remove the labile HbA_{1C} fraction.

The Variant analyzer of Bio-Rad Laboratories offers a broader test selection of methods than the Diamat system. Although the separation principle is the same, with various programs it is possible to separate and quantitate, in addition

to HbA_{1c}, also HbF and HbA_2. The method has been calibrated against the Diamat method. In practice, however, there seems to be a slight difference between these systems, with the Variant giving slightly higher results than the Diamat [25].

III. DETERMINATION OF HbA_{1c} BY AFFINITY CHROMATOGRAPHY

The boronate affinity chromatography method involving minicolumns has gained wide acceptance because it is not affected by Hb variants, slight temperature changes, carbamylated Hb, and the labile glycated fraction [26]. The application of HPLC to affinity methods has the same advantages as the minicolumns. An automatic affinity HPLC system is now available and has been evaluated for the monitoring of glycohemoglobin [27].

A considerable bias has been observed between cation exchange and affinity chromatography. In two studies the correlations between the methods were: affinity chromatography = 1.40 × ion exchange − 2.19 [28] and affinity chromatography = 1.45 × ion exchange + 0.04 [24]. The difference in slope is explained by the fact that affinity methods measure glycated Hbs other than HbA_{1c}. The negative y intercept may be explained by the fact that nonglycated components of HbA_{1c} are measured by the ion exchange chromatography [23]. Methods based on boronate affinity detection of the glycated amino terminus of the β chains of Hb together with glycosylated Hbs eluting in the HbA_0 fraction in ion exchange chromatography may be expected to reflect blood glucose control more accurately than ion exchange chromatography methods, which also measure coeluting nonglycated Hb. However, this has to be determined in a true clinical setting.

IV. REFERENCE VALUES FOR HbA_{1c}

Determination of a method-specific reference range is important in glycohemoglobin analysis, due to differences between methods. We have estimated the reference values of HbA_{1c} which are lower with the PolyCAT A method than with the Diamat method, apparently because of better separation of HbA_{1c} from nonglycated coeluting forms of Hb with PolyCAT A. The reference range for HbA_{1c} is 4.5–5.8% for the Diamat and 2.5–4.4% for the PolyCAT A method [23]. The difference in mean values between the PolyCAT A (3.4%) and Diamat (5.1%) methods was 1.7%.

The reference range with PolyCAT A is lower than that observed with our original HPLC method, 4.2–6.6%, using the Mono S column [8] and that reported by Jeppsson et al., 3.9–5.3%, using the same column but a different gradient [11]. With PolyCAT A and other gradients, reference ranges of 2.8–5.5% [24]

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