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DETERMINATION OF GLYCOSYLATED HAEMOGLOBIN BY ISOELECTRIC FOCUSING IN NON-LINEAR pH GRADIENTS

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SUMMARY

A new isoelectric focusing technique for the separation and quantitation of glycosylated haemoglobin (HbA_{1c}) is described. By using an equimolar mixture of two separators (0.2 *M* β-alanine + 0.2 *M* 6-aminocaproic acid) a 2-pH unit Ampholine range (pH 6—8) is transformed in a shallow, 0.6-pH unit span (pH 6.7—7.3). This brings about an increment of resolution between HbA and HbA_{1c} by a factor of about three, thus allowing proper densitometric evaluation of the trichloroacetic acid-fixed MetHb bands by conventional gel scanners. Excellent agreement is found among microchromatography, isoelectric focusing followed by densitometry in situ, and isoelectric focusing followed by band excision, elution and spectrophotometric determination. The present method also allows full resolution between HbA_{1c} and fetal haemoglobins (F and F_{ac} bands).

INTRODUCTION

Haemoglobin A_{1c} is the major component of the glycosylated haemoglobin pool [1]. It derives from HbA molecules through a post-translational modifica-

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tion: the $-NH_2$ group of $\beta^{1\,Valine}$, in fact, reacts with glucose residues to form an aldimine adduct which subsequently undergoes an Amadori rearrangement to form a more stable ketoamine linkage [2]. The rate of these non-enzymatic reactions is primarily affected by the glucose concentration in the medium [3]. Determination of the percentage of HbA_{1c} in a haemoglobin sample is thus important in some pathological alterations of the glycosidic metabolic pathways. In red cells of patients with overt diabetes mellitus there is a two- to threefold increase in percentage of HbA_{1c} (from 4—7% to 10—18% of total Hb) [4]. It has therefore been proposed that the percentage of HbA_{1c} could be used in assessing the degree of diabetes, by providing an integrated measurement of blood glucose according to the red cell life span.

HbA_{1c} can be separated from HbA by isoelectric focusing (IEF) in commercial pH gradients [5—7] but accurate quantitative determinations of HbA_{1c} under these conditions can be obtained only by high-resolution microdensitometry [8—11]. Improved separations are necessary for the densitometric evaluation of HbA_{1c} by conventional scanners. They can be obtained by the use of separators [12, 13] or by resorting to home-made, narrow pH cuts [14]. However, home-made, shallow pH gradients (< 1 pH unit) [14] and some separators (such as the dipeptide His-Gly of ref. 12) will never be available in most routine clinical laboratories, thus severely limiting the use of these methodologies.

In an extensive screening of umbilical cord blood for β-thalassemias in northern Sardinia, we have recently described the generation of very flat pH gradients, easily obtainable from commercial Ampholine pH 6—8 spans by the addition of an equimolar mixture of two amphoteric substances, β-alanine and 6-aminocaproic acid [15]. Such gradients were most satisfactory for the separation and unambiguous quantitation of the three major components of cord blood: fetal, adult and acetylated fetal Hbs.

We report here how the same type of gradient can be used, in adult blood, for resolution and densitometric evaluation of HbA_{1c} in diabetic screening.

MATERIALS AND METHODS

Acrylamide, N,N′-methylene bisacrylamide, ammonium persulphate and N,N,N,N′-tetramethylethylenediamine (TEMED) were from Bio-Rad, Richmond, CA, U.S.A. β-Alanine (β-Ala) and 6-aminocaproic acid (6-ACA) were from BDH, Poole, U.K. Ampholine, pH 6—8, pH 7—9 and pH 3.5—10 ranges, were from LKB Produkter, Bromma, Sweden, and Silane A-174 (organosilane ester) was from Union Carbide Silicones, Sisterville, WV, U.S.A.

Sample collection

Adult blood, from normal and diabetic patients, was washed three to four times in saline (10 ml/ml blood). Red blood cells (RBC) were lysed in distilled water containing 0.05% potassium cyanide (5 vols. lysis solution per 1 vol. packed RBC) and, if not immediately analysed, stored as such at −80°C. Prior to IEF, CCl_4 is added to the lysate (0.5 vol. of CCl_4 per vol. of packed RBC) to precipitate the ghosts. After centrifugation for 10 min at 10,000 *g*, the sample is diluted to about 1% content and analysed by IEF.

IEF fractionation

For routine IEF analysis, we have found a 0.5 mm gel thickness to be most suitable, since it does not require any particular skill from technical staff. The slab can be cast vertically in a cassette utilizing a U-gasket cut out from a 0.5-mm-thick rubber sheet, or horizontally utilizing the ready-made LKB glass plates. When only a few samples have to be analysed, we use square 12.5 × 12.5 cm glass plates, which accommodate 14—15 samples; otherwise, the 25-cm-long glass slab can be used, which allows analysis of 28—30 samples. For densitometric analysis, we prefer to fix the polyacrylamide gel covalently to the glass plate via a silane bridge [16], so that the gel can be dried directly onto the glass plate without peeling off. For this purpose, the glass is dipped for 30 sec in 0.2% Silane A-174 in anhydrous acetone and then left to dry in the air. As a cassette cover, we use a Lucite slab (8—10 cm thick, to prevent warping) modified to accommodate a pocket-forming device. For this purpose, a strip of Dymo label (200—250 μm thin) is glued 2.5 cm from the long edge of the Lucite cover and then short strips are removed with a surgical blade so as to form 5 × 3 mm^2 pockets, at 2-mm intervals. The sample is usually applied at the cathodic side, in a volume of about 3—5 μl (corresponding to about 20—30 μg total protein load). The gel contains 6% T and 4% C (for definition see ref. 17), 2% Ampholine pH 6—8 and 0.2% Ampholine pH 3.5—10. Anodic and cathodic solutions are 1 *M* orthophosphoric acid and 1 *M* sodium hydroxide, respectively. Conditions for a typical run (in a 12.5 × 12.5 cm gel slab) are as follows: 15 min at 400 V, then the sample is loaded and the run is continued for 90 min at 15 W (with a limiting voltage of 1500 V, which is reached after 20 min from sample application). The run is performed on an LKB 2117 Multiphor cell with a constant-wattage power supply (LKB 2103) and with the thermostat at 4° C.

Shallow pH gradients

For a proper resolution of HbA_{1c} from HbA, non-linear pH gradients, which have a much shallower slope around pH 7, have to be utilized. For this purpose, we have gelled plates, as above, containing a mixture of 0.2 *M* β-Ala and 0.2 *M* 6-ACA, as previously suggested [15], and the following amounts of carrier ampholytes (in a final gel volume of 10 ml): 0.5 ml of pH 6—8, 0.12 ml of pH 7—9 and 0.05 ml of pH 3.5—10 ranges. In this latter case, since the migration to equilibrium position is slower, the gels are run, after sample application, for somewhat longer periods (120 min). In non-linear pH gradients, it is imperative to apply the sample at the cathode, since anodic application would result in very long focusing periods.

Densitometry

At the end of the IEF run, the gels are dipped in 20% trichloroacetic acid (TCA), as previously suggested [18], whereby all Hbs are fixed and converted to the ferric form (MetHb). After washing the gel in distilled water to remove excess TCA, the polyacrylamide layer is desiccated with a hair-dryer and the sample tracks are read and integrated at 465 nm with a Cliniscan densitometer (Helena, Beaumont, TX, U.S.A.). For comparing the present method with the microchromatography of Trivelli et al. [19], at the end of the IEF run, prior

TABLE I

COMPARISON BETWEEN CHROMATOGRAPHIC AND ISOELECTRIC FOCUSING DETERMINATION OF HbA_{1c}

The figures reported are percentile values of HbA_{1c}, taking as 100% the sum of the HbA and HbA_{1c} peaks.

Sample No.	Microchromatography	IEF (densitometry)	IEF (elution)
1	15.68	16.40	17.02
2	12.50	13.31	13.42
3	6.67	7.02	7.93
4	11.23	12.04	12.91
5	14.33	15.29	15.06
6	8.04	8.61	9.00
7	21.41	22.87	23.02
8	19.04	18.43	19.56
9	6.03	6.60	7.05
10	13.81	14.69	14.13
11	11.82	12.67	12.92
12	14.07	14.76	15.20
13	18.23	17.22	18.40
14	12.17	13.09	13.63
15	15.10	16.29	15.61
16	13.56	14.67	15.03
17	20.23	21.86	20.78
18	13.64	14.43	14.39
19	5.71	6.80	6.53
20	16.04 a	15.36 c	16.41
	b	b	

[a] $r = 0.988$, $P < 0.001$.
[b] $r = 0.995$, $P < 0.001$.
[c] $r = 0.990$, $P < 0.001$.

to TCA fixation, the focused Hb zones are removed with a surgical blade, eluted overnight in 1.5 ml (HbA_{1c}) and 4.5 ml (HbA) of 0.05% potassium cyanide and read in a spectrophotometer at 415 nm. These data, compared with the IEF scan values and the eluate values from the Bio-Rex 70 column, are tabulated in Table I.

Microchromatography

For comparison, the same samples were also analysed by microchromatography, as modified after Schnek and Schroeder [20]. We have used the Combi-Test Kit for HbA_{1c} from Boehringer Mannheim (F.R.G.). The columns were thermostatted at 23°C and the eluates read at 415 nm.

RESULTS

Fig. 1 (left) shows the separation of adult blood lysates in conventional Ampholine gradients encompassing a 2-pH unit span (pH 6—8). It can be seen

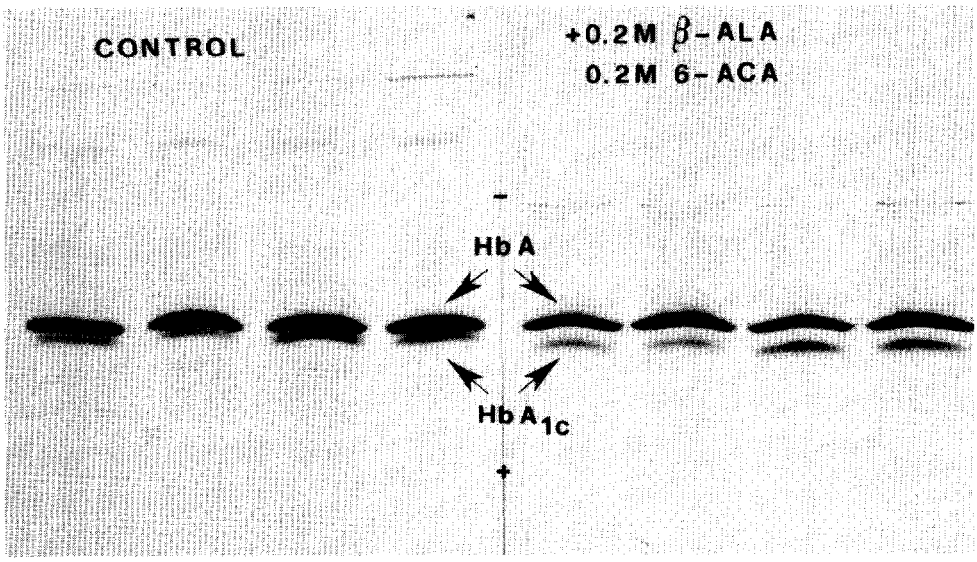

Fig. 1. Separation of normal human adult haemoglobin (HbA) from its glycosylated derivative (HbA_{1c}) by isolelectric focusing in polyacrylamide gel slabs. Left: control gel containing only 2% Ampholine pH 6—8, Right: the same gel with an equimolar (0.2 *M*) mixture of the two separators β-alanine and 6-aminocaproic acid. Experimental conditions are as described in Materials and methods.

that the glycosylated component (HbA_{1c}) is barely separated below the main HbA band: such a resolution is not generally enough for quantitation by the common scanners usually available in clinical laboratories. However, when the IEF run is repeated in a gel containing a mixture of separators (0.2 *M* β-Ala and 0.2 *M* 6-ACA), excellent resolution is obtained between HbA and its glycosylated form (Fig. 1, right). As shown in the densitometric scan of Fig. 2, in the latter case the tracing returns to the baseline before the onset of the second peak, thus allowing for meaningful integration; in the absence of separators, the HbA_{1c} peak is seen by the densitometer only as a shoulder of the main HbA zone.

What is happening in the presence of this mixture of "separators" is shown in Fig. 3: the 2-pH unit span of Ampholine pH 6—8 is converted into essentially a 0.6-pH unit interval (pH 6.7—7.3) which is centered on the isoelectric point of HbA (pI approximately 7.0). β-Ala flattens the portion of the pH gradient below HbA, while 6-ACA alters the pH course on the alkaline side of HbA. The combined action of the two "separators" produces a pH span which is optimal for separating not only HbA from HbA_{1c}, but also the fetal components, such as HbF and HbF_{ac} (the pI position of these four zones is shown by arrows in Fig. 3).

The reliability of this method is shown in Table I: in a double-blind experiment, twenty normal and diabetic samples were analysed by microchromatography, IEF followed by densitometry in situ and IEF followed by elution of excised bands and spectrophotometric quantitation. Excellent agreement was found among the three methods, as shown by the *r* and *P* values reported at the bottom of Table I, although there appears to be a trend, in both IEF

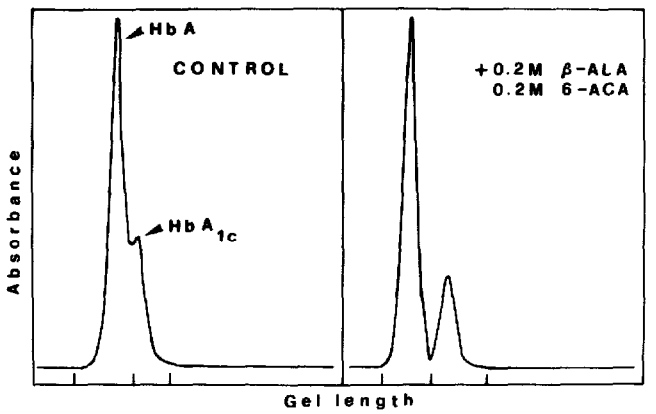

Fig. 2. Densitometric evaluation of the IEF-separated HbA and HbA_{1c} bands. Scans of TCA-precipitated, brown MetHb bands were performed at 465 nm with a Cliniscan densitometer. Left: scan of the Hb zones obtained by IEF in the control gel of Fig. 1. Right: scan of the Hb bands obtained by IEF in the presence of 0.2 *M* β-alanine and 0.2 *M* 6-amino caproic acid, as shown in Fig. 1 (right). The marks along the abscissa represent the integration intervals for each peak in the densitometric scan.

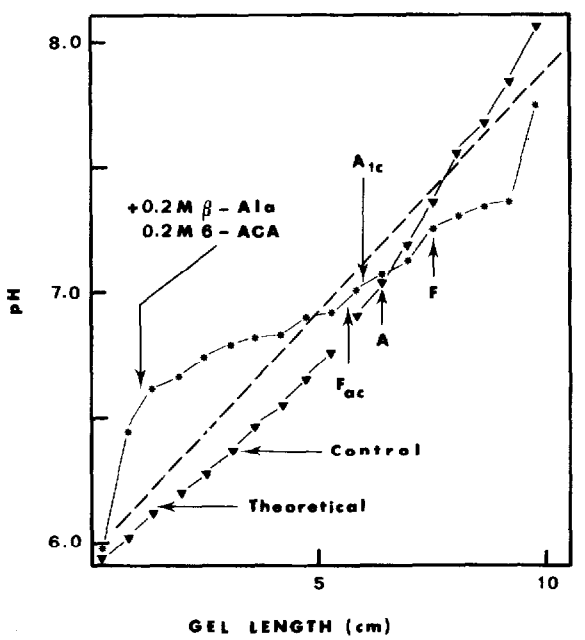

Fig. 3. Evaluation of the pH course in Ampholine gels. (- - -), Theoretical pH gradient in a gel containing only pH 6—8 ampholytes, assuming a linear pH course. (*—*), pH gradient obtained in a gel containing 2% Ampholine pH 6—8 and a mixture of 0.2 *M* β-alanine + 0.2 *M* 6-aminocaproic acid. (▾—▾), experimental pH gradient obtained with 2% Ampholine pH 6—8 in the absence of separators. In these last two cases, at the end of IEF, gel segments at 5-mm intervals were cut along the separation track, eluted with 300 μl of 10 m*M* potassium chloride and read in a pH meter. The arrows indicate the pI position, in the pH gradient with separators, of haemoglobins F, A, A_{1c} and F_{ac}.

analyses, to give somewhat higher amounts (5—10% higher). Four samples (Nos. 3, 6, 9 and 19) were correctly evaluated as normal.

DISCUSSION

In addition to the microchromatographic methods reported [19, 20], several other automatic techniques have been described for the separation and quantitation of HbA_{1c}. High-performance liquid chromatography [21, 22] has been adopted, but requires rather expensive equipment and still seems too sophisticated for non-specialized laboratories. Medium-pressure liquid chromatography [23] is also limited by cost factors in that it requires an expensive amino-acid analyser. Low-pressure liquid chromatography [14] uses simpler equipment and is cheaper to perform than the previous systems, but still has not found widespread application. The major drawback of all these chromatographic methods for assaying HbA_{1c}, is that this peak is eluted together with HbF [24]. Therefore when an abnormally elevated HbA_{1c} peak is found, the percentage of HbF has to be checked out by independent methods in order to rule out possible contamination of the HbA_{1c} peak. This problem is fully solved in our present technique: not only are HbA_{1c} and HbF widely separated (they focus on opposite sides as compared with the HbA zone) but the glycosylated Hb is also resolved from the acetylated HbF band (see Fig. 3 of the present report and Fig. 1B of ref. 15).

While several reports on IEF separation have been published [7—13] they do not seem to have found widespread application, possibly because of rather limited separation [7—10] or difficulties in synthesizing the right separator [12] or narrow pH range carrier ampholytes [14]. Even in what appears to be the simplest and most reliable method [9], the authors have, in a subsequent paper [25], resorted to band excision, overnight elution and spectrophotometric quantitation, thus rendering the technique no longer attractive for routine separations. Jeppsson et al. [25] have used 0.33 *M* β-Ala as the sole separator in their IEF gels: since β-Ala has a pI of 6.9, just below the pI of HbA_{1c} (6.95) and of HbA (pI 7.0), it is quite possible that, as the separation proceeds and the cathodic drift becomes more pronounced [26], the two protein zones roll down from the β-Ala plateau and fall in a pH region where the steep incline compresses the separation. In our present case, since we have made sure that the pH gradient is flattened both below and above the pI of the components of interest, the protein zones can drift along the wide plateau formed by the mixture of the two separators without hampering the separation obtained. The method is fast, accurate and requires only simple equipment. Moreover, several gels can be polymerized simultaneously and stored in the refrigerator before use for periods of at least two months.

We should like to emphasize that excellent separations of HbA_{1c} are obtained also in the recently invented technique of immobilized pH gradients [27, 28] but the gel casting technique is more complex and the focusing times, at present, are rather long (8—10 h).

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