Diabetologia (1984) 27: 596–598

Diabetologia

Short communications

Haemoglobinopathies: a pitfall in the assessment of glycosylated haemoglobin by ion-exchange chromatography

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Summary. Total glycosylated haemoglobin was determined by a minicolumn ion-exchange chromatography technique (Bio-Rad) and correlated with the mean of fasting and post-prandial blood glucose values for the preceding 6 weeks. In 360 diabetic subjects, free of congenital haemoglobinopathies and other detected causes of haemoglobin A_1 misinterpretation (reference diabetic group), a highly significant correlation was established between haemoglobin A_1 and glucose ($y = 0.54 \times + 4.91$; $r = 0.791$; $p < 0.01$). In 28 of the 29 patients with heterozygous haemoglobinopathies (HbS, C, D, E), the apparent haemoglobin A_1 values were lower than expected according to the 95% confidence limits of the diabetic reference group. The apparent haemoglobin A_1 value was above these limits in patient 29, with β thalassaemia. Patients with inappropriate glycosylated haemoglobin values should be investigated for causes of haemoglobin A_1 misinterpretation, in particular, haemoglobinopathies.

Key words. Glycosylated haemoglobin, haemoglobinopathies S, C, D, E, β thalassaemia, diabetes.

The determination of glycosylated haemoglobin (HbA_{1c}) has become a widely used index for the assessment of diabetic control [1]. The rapid determination by minicolumn chromatography of a fast migrating group of fractions, termed HbA_1, is convenient and readily available [2]. Erroneous interpretations may be due to various pathophysiological and pharmacological interferences [3].

The aim of the present study was to define how the measurement of HbA_1 is affected by the presence of congenitally abnormal haemoglobins [4–10].

Subjects and methods

Subjects

A total of 389 diabetic subjects were studied: 360 were free of congenitally abnormal haemoglobin (diabetic reference group); 29 others had congenital haemoglobinopathy, as described below. Both groups were similar in age (45 ± 5 versus 50 ± 5 years; mean ± SEM), sex ratio (190/170 versus 18/11) and the percentage of insulin dependence (50% versus 55%). Patients with either recurrent hypoglycaemia, shortened erythrocyte life-span or renal failure were excluded.

Laboratory methods

HbA_1. The HbA_1 fraction was determined by a rapid minicolumn chromatography technique (Bio-Rad, Richmond, California, USA). The heparinized venous blood samples were haemolyzed with polyoxyethylene ether 0.33% (v/v), put on the Biorex resin column (25 mm × 7 mm, Bio-Rad), then eluted at 22 ± 1 °C with phosphate buffer (pH 6.7, 0.01 mol/l). The optical density of eluates was read at 415 nm. The percentage of HbA_1 was calculated in comparison with total haemoglobin. The HbA_1 value, measured in 30 non-diabetic subjects, was 6.7 ± 0.7% of total haemoglobin.

Haemoglobin studies: Electrophoresis was performed by two different migrating systems [11], both from Helena, Beaumont, Texas: (1) on cellulose acetate plates using a Tris/EDTA/boric acid buffer (pH 8.6), and (2) on agar gel with a citrate/citric acid buffer (pH 6.0).

Further identification of HbS was made by a precipitation test [12]. Heterozygous thalassaemia was diagnosed by the typical haematological (microcytosis) and electrophoretic patterns: high HbA_2 (9%) and HbF (3%), and the alkaline denaturation test [13]. The variants HbD and HbE were identified by direct structural analysis according to established techniques [14–15]. In all cases the variant was present in the heterozygous state and the abnormal fraction amounted to 31–44% of the total haemoglobin.

Blood glucose. This was measured on heparinized venous blood samples by a glucose-oxidase technique (Boehringer, Mannheim, FRG).

Calculations. A mean blood glucose value was calculated in each patient from 6–15 fasting and post-prandial determinations for the preceding 6 weeks. The regression line of the HbA_1 versus glucose values and the corresponding 95% confidence limits were calculated from the data relative to the 360 reference diabetic patients.

Statistical analysis. The regression lines were calculated according to classical methods [16].

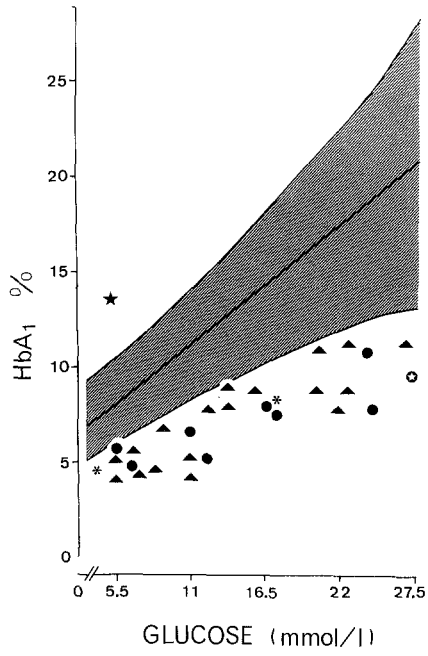

Fig. 1. Apparent HbA_1 values in the patients with haemoglobinopathies. The hatched area and regression line correspond to the diabetic reference group and its 95% confidence limits. The individual HbA_1 values are shown from patients with haemoglobin S (▲), C (●), D (○), E (*) and thalassaemia (★)

Results

HbA_1 and glucose correlation

In the reference diabetic group, there was a significant correlation between the HbA_1 and glucose values (Fig. 1; the equation of the regression line was: $y = 0.54 \times + 4.91$; $r = 0.791$; $n = 360$; $p < 0.01$). No significant change in slope coefficient appeared when this was calculated for successive 2.7 mmol/l glucose ranges.

In the patients with haemoglobinopathies (HbS, $n = 17$; HbC, $n = 8$; HbD Pundjab, $n = 1$; HbE, $n = 2$; β thalassaemia, $n = 1$), all apparent HbA_1 values were outside the 95% confidence limits constructed from the reference group. In the patients with C, D, E and S haemoglobins, the measured HbA_1 values fell below these limits. A good correlation was found between these apparent HbA_1 values and the corresponding glucose concentration. However, the slope of the regression line was shallower than for the reference group ($y = 0.30 \times + 2.50$). In contrast, in the patient with heterozygous β thalassaemia (with HbF and A_2), the HbA_1 value was above these limits.

The inspection of chromatography columns readily detected the abnormal haemoglobins C, D and S. The fractions D and S appeared as slow migrating bands with a r_f approximately half that of normal haemoglobin (HbA_0). The C fraction presented as a thin band which stuck to the top of the column. In the β-thalasaemic patient, HbF was not detected by the examination of the columns, since it moved faster than HbA_0, and co-eluted with the HbA_1 fraction.

Discussion

A highly significant correlation was found between HbA_1 and glucose concentration in the reference diabetic group. The patients with haemoglobinopathies had values outside the 95% confidence limits. The presence of haemoglobins S, C, D, E led to the under-estimation of HbA_1 and overestimation in a patient with thalassaemia. The haemoglobins S, C, D and E are more positively charged compared with HbA, hence elution from the cation exchange resin is retarded. The fast-moving fraction then corresponds only to the glycosylated HbA, which represents only a part of total haemoglobin [17–20]. Conversely, HbF is more negatively charged than HbA and co-eluates with HbA_{1c} on cation exchange chromatography, leading to an over-estimation of this fraction [21]. The same occurs with the rare, fast-moving varients HbK, I, J, with HbH in some β thalassaemia patients [20–22] and HbF in patients with β thalassaemia, hereditary persistence of fetal haemoglobin and acquired HbF [23].

The concept of haemoglobin variants interfering with the measurement of glycosylated haemoglobin by ion exchange chromatography is not new [1, 24]. However, this problem has not received wide attention. It was a potential cause of misinterpretation in 6.9% of our patients, due to the large number of patients from countries with a high prevalence of haemoglobinopathies. Since the HbA_1 assay has been proposed for the screening of diabetes among large population groups [19], the interference of haemoglobin variants with this determination must be known, particularly in ethnic groups with a high incidence of haemoglobin variants. It is of interest that HbA_1 measurements in diabetic patients led to the description of two hitherto undescribed haemoglobin variants [25, 26]. In diabetic subjects with congenitally abnormal haemoglobin, the percentage of a haemoglobin variant can be measured by electrophoresis, thus allowing corrections of HbA_1 values. However, it may be advisable, in such patients, to measure haemoglobin glycosylation by either chemical methods or affinity chromatography on aminophenyl boronic acid resins [27].

Acknowledgments. We thank Pr. J. Lellouch and Mrs E. Patois (INSERM U 169) for expert assistance in the statistical analysis; Mrs M. J. Clergé and the nursing staff, and Mrs I. Herckla and M. Th. Dalle for technical assistance and D. Boillot for editorial help. This work was supported by a grant from Université Xavier Bichat.

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Received: 25 May 1984
and in revised form: 8 October 1984

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