*Short Communications***Analysis of Short-Term Changes in Reversibly and Irreversibly Glycosylated Haemoglobin A₁: Relevance to Diabetes Mellitus**G. Bolli¹, P. Compagnucci², M. Giulia Cartechini², P. De Feo¹, F. Santeusano² and P. Brunetti¹Institutes of ¹Medical Pathology and ²Clinical Medicine, University of Perugia, Italy

Summary. We have determined the stable (irreversibly glycosylated) fraction of haemoglobin A₁ (HbA₁) on Bio-Rex 70 after incubation of red blood cells in 0.9% saline solution for 6 h at 37 °C. The total (reversibly + irreversibly glycosylated fractions) HbA₁ was determined before each incubation. Labile (reversibly glycosylated) HbA₁ represented the difference between total and stable HbA₁ fractions. Total and stable HbA₁ fractions were determined during insulin- or meal-induced blood glucose fluctuations in 24 insulin-dependent diabetics and in seven subjects with impaired glucose tolerance. In the diabetics, the maximal fluctuation of total HbA₁ was 1.47% over 2–12 h, while the simultaneous plasma glucose variation was 21.5 mmol/l. The stable HbA₁ fraction did not change significantly. In diabetics the differences between the maximal and minimal values of plasma glucose and total HbA₁ were significantly correlated. Plasma glucose correlated with simultaneously determined total and labile HbA₁ fractions, but not with stable HbA₁. In subjects with impaired glucose tolerance, similar changes in total but not in stable HbA₁ were observed during an oral glucose tolerance test. We conclude that, although rapid changes in chromatographically determined HbA₁ are relatively small, the determination of stable HbA₁ should be performed to circumvent this problem and to ensure a more accurate index of blood glucose control.

Key words: Glycosylated haemoglobin, stable HbA₁, labile HbA₁, diabetes mellitus

A number of recent papers have shown that chromatographically determined HbA₁ consists of two fractions, a labile one (Schiff base) which is

acutely responsive to changes in glucose concentration, and a stable one (ketoamine) which is not [1–4]. Because of the interference of the labile adduct on the HbA₁ concentration, the question has arisen whether or not this parameter may still be considered a valuable index of long-term blood glucose control in diabetics.

In the present study we have evaluated the quantitative importance of the rapid changes in labile HbA₁ during blood glucose fluctuations frequently observed in diabetics. Furthermore, in order to circumvent the problem of the rapid changes in HbA₁, we propose that the estimation of the stable HbA₁ fraction, which is not influenced by blood glucose fluctuations, is a better index of long-term glycaemic control in diabetics.

Methods*Chromatography*

The determination of HbA₁ was carried out in duplicate by a rapid chromatographic method (1 h) [5, 6], in thermostatically controlled macrocolumns (18 °C). Before chromatography, all haemolysates were gel-filtered with developer No. 6 [7] in macrocolumns (internal diameter 1.5 cm), filled with Sephadex G-25 (10 cm high) at 4 °C, thus ensuring that haemolysates had the same pH and ionic strength as the eluent buffer.

Our mean coefficients of intra-assay variation (from duplicate determination of each sample) ranged between 1.0% (HbA₁ > 8%) and 1.5% (HbA₁ < 8%).

Total and Stable HbA₁ Determination

Blood samples were collected in heparin and as soon as possible red blood cells (RBC) were washed threefold with 0.9% saline (50 ml, 4 °C) and haemolysed with distilled water (five volumes,

Table 1. Correlation coefficients (Kendall) between HbA_{1c} (labile, total and stable) and simultaneous plasma glucose in each group of subjects during blood glucose fluctuations

	Simultaneous plasma glucose			
	Group A	Group B	Group C	Group D
Labile HbA _{1c}	0.76 <i>p</i> <0.001	0.79 <i>p</i> <0.001	0.63 <i>p</i> <0.001	0.62 <i>p</i> <0.002
Total HbA _{1c}	0.36 <i>p</i> <0.05	0.48 <i>p</i> <0.005	0.40 <i>p</i> <0.025	0.30 NS
Stable HbA _{1c}	0.09 NS	0.14 NS	0.10 NS	0.07 NS

NS = non-significant

4°C). The haemolysate was chromatographed immediately, or after a short storage (up to 6 h, 4°C). This procedure ensured a constant recovery not only of the stable, but also of almost all the labile HbA_{1c} fraction and avoided the changes in the labile fraction which can occur during a long storage of RBC in glucose-rich plasma or of haemolysates in glucose-free solution. The HbA_{1c} concentration thus obtained was *total HbA_{1c}*, i. e. the labile and stable fractions estimated simultaneously.

It has been observed that the concentration of HbA_{1c} decreases rapidly when RBC are incubated in glucose-free solution [3, 4]. In our experience the decline is barely significant after 6 h at 4°C, while it is almost complete at 37°C. We define concentration of *stable HbA_{1c}* as that determined chromatographically after an incubation of RBC in 100 volumes of 0.9% saline at 37°C for 6 h. The concentration of *labile HbA_{1c}* is calculated as the difference between the concentration of HbA_{1c} obtained before (total) and after (stable) the incubation of RBC in glucose-free solution.

Plasma glucose was determined by means of a Beckman glucose analyzer. Statistics were by non-parametric tests (Wilcoxon signed rank-sum test and Kendall's correlation). All results are expressed as mean ± SEM.

Subjects and Protocols

Total and stable HbA_{1c} were determined in 24 insulin-dependent diabetics divided into three groups, and also in seven subjects with impaired glucose tolerance [8]. Group A subjects (*n* = 7), treated with a single dose of lente insulin at breakfast, were selected because they all showed marked hyperglycaemia after breakfast and hypoglycaemia before dinner. Groups B (*n* = 9) and C (*n* = 8) were in fair glycaemic control and received two daily insulin injections, at breakfast and at dinner (mixture of Actrapid and Lente, Novo Industri, Copenhagen, Denmark). Group D consisted of the subjects with impaired glucose tolerance. In the 31 subjects the mean percentage of ideal body weight was 105.1 ± 1.8% (range: 87–121%); age was 35 ± 2 years (range: 17–53 years); diabetes duration was 6 ± 1 years (range: 2–13 years).

Results

Group A: 90 min after breakfast the plasma glucose was 21.1 ± 1.1 mmol/l. The total HbA_{1c} was 12.60 ± 0.43% and the stable HbA_{1c} 11.14 ± 0.42%.

Half an hour before dinner plasma glucose fell to 4.7 ± 0.5 mmol/l (*p* < 0.01) and the total HbA_{1c} decreased to 11.74 ± 0.43% (*p* < 0.01), whereas the stable HbA_{1c} did not change significantly (11.16 ± 0.43%, NS). The labile HbA_{1c}, expressed as percentage of the stable was 9.2 ± 1.3% (range: 18.9–4.3%).

Group B: The fasting plasma glucose was 7.0 ± 0.5, 7.1 ± 0.2 and 7.3 ± 0.5 mmol/l on three consecutive days while simultaneously determined total HbA_{1c} was 9.57 ± 0.40, 9.61 ± 0.40 and 9.6 ± 0.4%. Twelve hours after acute insulin withdrawal, plasma glucose increased to 19.4 ± 1.2 mmol/l (*p* < 0.01) and total HbA_{1c} to 10.62 ± 0.38% (*p* < 0.01). Stable HbA_{1c} did not change significantly (8.90 ± 0.33 and 8.93 ± 0.33%, NS). The labile HbA_{1c} was 13.3 ± 1.4% (range: 5.3–22.4%).

Group C: The fasting plasma glucose was 8.6 ± 0.5 mmol/l, total HbA_{1c} 10.70 ± 0.28% and stable HbA_{1c} 9.99 ± 0.26%. Two hours after breakfast plasma glucose increased to 15.1 ± 0.7 mmol/l (*p* < 0.01) and total HbA_{1c} to 11.21 ± 0.30% (*p* < 0.01). Stable HbA_{1c} did not increase significantly (9.99 ± 0.25%, NS). The labile HbA_{1c} was 9.7 ± 0.8% (range: 4.7–16.6%).

A significant correlation was found in the above groups between delta values (calculated as difference between the maximal and the minimal value) of total HbA_{1c} and plasma glucose (group A: 0.62, *p* < 0.05; group B: 0.83, *p* < 0.01; group C: 0.57, *p* < 0.05). Significant correlations were also found in each group between plasma glucose and simultaneously determined total and labile HbA_{1c}, but not with stable HbA_{1c} (Table 1).

Group D: The fasting plasma glucose was 5.3 ± 0.4 mmol/l, total HbA_{1c} 7.95 ± 0.23% and stable HbA_{1c} 7.58 ± 0.21%. Two hours after the oral glucose tolerance test plasma glucose increased to 13.1 ± 1.6 mmol/l (*p* < 0.01) and total HbA_{1c} to 8.75 ± 0.31% (*p* < 0.01). Stable HbA_{1c} did not change significantly (7.59 ± 0.21%, NS).

A significant correlation was found between delta total HbA_{1c} and delta plasma glucose (0.71, *p* < 0.025). A close correlation existed between plasma glucose and labile HbA_{1c} determined in the same blood sample, but not with total or stable HbA_{1c} (Table 1).

In all subjects (*n* = 31) there was a significant correlation between total and labile HbA_{1c} determined in the same sample (0.46, *p* < 0.001).

Discussion

Our results show that the concentration of chromatographically determined HbA₁ significantly changes in diabetics over a few hours, depending on plasma glucose fluctuations immediately before sampling. The rapid increase in total HbA₁ was dependent on the simultaneous increase in labile HbA₁, while the stable HbA₁ did not change significantly during the blood glucose fluctuations observed. This suggests that the correlation which we have found in diabetics between total HbA₁ and plasma glucose determined in the same sample is entirely influenced by the rapid changes in the labile HbA₁ fraction. In fact, plasma glucose and labile HbA₁ are closely correlated. The increase in HbA₁ concentration which we observed in subjects with impaired glucose tolerance was consistent with our recent observations of rapid changes in total HbA₁ during transient hyperglycaemia in non-diabetics [9].

The changes in chromatographically determined HbA₁ which we have observed in diabetics are relatively small, since the maximal fluctuation of total HbA₁ (delta value) was 1.47%, while delta plasma glucose was 21.5 mmol/l. On the other hand, wider short-term changes in total HbA₁ have been reported in individual diabetics [1] suggesting that the determination of total HbA₁ is unreliable in order to establish glycaemic control on a long-term basis [10, 11].

Our results demonstrate that the stable HbA₁ concentration is totally unrelated to recent changes in plasma glucose levels and that it is independent of plasma glucose at the time of sampling. Stable HbA₁ determination is easy to perform for routine purposes and provides a more accurate assessment of control in diabetics showing wide fluctuations of plasma glucose.

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