Abnormal haemoglobins, Hb Takamatsu and Hb G-Szuhu, detected during the analysis of glycated haemoglobin (HbA$_{1C}$) by high performance liquid chromatography

Y Moriwaki, T Yamamoto, Y Shibutani, T Harano, S Takahashi, T Hada

Abstract
Background—During medical checkups of two unrelated female outpatients during their annual health examination and one male inpatient suffering from cardiac failure the glycated haemoglobin (HbA$_{1C}$) concentrations measured by high performance liquid chromatography (HPLC) were low, in spite of normal fasting plasma glucose concentrations. However, HbA$_{1C}$ concentrations measured by latex immunoagglutination and fructosamine concentrations were within the normal range.

Method—Investigations were performed to elucidate the reasons for these discrepancies.

Results—Abnormal haemoglobins, Hb Takamatsu and Hb G-Szuhu, were found. The HPLC chromatogram showed an additional peak near HbA$_{1C}$, which resulted in falsely low HbA$_{1C}$ concentrations. Isoelectric focusing analysis of the patient's haemoglobin disclosed abnormal haemoglobins, which migrated faster than normal HbA, in the two female patients and slower in the male patient. The cDNA sequence and amino acid analyses of the haemoglobin $\alpha$-chains and $\beta$-chains indicated the presence of the haemoglobin variant $\beta$ 120 Lys $\rightarrow$ Glu (Hb Takamatsu) and $\beta$ 80 Asn $\rightarrow$ Lys (Hb G-Szuhu).

Conclusions—These cases show how these silent haemoglobin variants can result in falsely low HbA$_{1C}$ concentration readings when using HPLC.

Keywords: abnormal haemoglobin; high performance liquid chromatography; glycated haemoglobin

Glycated haemoglobin (HbA$_{1C}$) has been shown to be increased in diabetes mellitus and is widely used as a marker for long term glycaemic status. Analysis of HbA$_{1C}$ is performed by high performance liquid chromatography (HPLC), electrophoresis, immunooassay, and colorimetry, as well as other methods. Among them, HPLC is generally used as the standard method for the determination of HbA$_{1C}$ concentrations. However, the HbA$_{1C}$ concentration is affected by several factors other than blood glucose concentrations, such as uraemia, alcohol abuse, high dose aspirin intake, hyperbilirubinaemia, and the decreased life span of erythrocytes (massive bleeding, haemolytic anaemia, and anaemia in pregnancy). Moreover, several kinds of haemoglobinopathy are known to result in falsely high or low HbA$_{1C}$ values when measured by means of HPLC. In this report, the detection of two abnormal haemoglobins, Hb Takamatsu ($\beta$120 Lys $\rightarrow$Gln) and Hb G-Szuhu ($\beta$80 (EF4) Asn $\rightarrow$Lys), during HPLC analysis for HbA$_{1C}$ in two unrelated women and one man is described.

Patients, materials, and methods
Blood samples were obtained from two female outpatients during an annual medical checkup and one male inpatient. Haematological data were obtained by means of an automated analyser. The subjects were KM, a 60 year old woman whose blood pressure was 200/100 mm Hg; TY, a 65 year old woman with mitral valvular disease; and ES, a 76 year old man admitted for the treatment of cardiac failure. KM and TY are not related, although their parents are from the Kagawa prefecture. Automated HPLC (HbA$_{1C}$ analyser, Model HA-8110; Daiichi, Kyoto, Japan; HbA$_{1C}$ reference range, 4.0–5.4%, coefficient of variation (%CV), ± 0.2%) with a cation exchange column was used for the measurement of the HbA$_{1C}$ concentrations. Isoelectric focusing gel electrophoresis using a commercially available kit (haemoglobin IEF gel; Joko, Tokyo, Japan) was performed to detect abnormal haemoglobin in KM and TY. In ES, isoelectric focusing gel electrophoresis was performed using the method of Righetti et al.\textsuperscript{9}

Direct sequencing of haemoglobin cDNA in KM and TY was performed according to the following method. Total RNA was obtained from peripheral blood cells by guanidium thiocyanate/phenol/chloroform extraction and then cDNA was reverse transcribed from the total RNA. Table 1 shows the primers for the polymerase chain reaction (PCR), which were used under the following conditions: cDNA was denatured at 94°C for five minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for one minute, with a final extension step of seven minutes.

Table 1 Primers used in direct sequencing of Hb cDNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-chain</td>
<td>Forward 5' &gt; ACT CTT CTG GTC CCC ACA GA &lt; 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5' &gt; TTC AAA GAC CAG GAA GGG GC &lt; 3'</td>
</tr>
<tr>
<td>$\beta$-chain</td>
<td>Forward 5' &gt; GCT TCT GAC ACA ACT GTG &lt; 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5' &gt; CCC CAG TTT AGT AGT TGG AC &lt; 3'</td>
</tr>
</tbody>
</table>

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PCR was carried out in a volume of 25 µl containing 50 mM KCl, 10 mM Tris/HCl (pH 8.8), 1.5 mM MgCl₂, 0.1% Triton X-100, 200 µM each of dATP, dCTP, dGTP, and dTTP, 0.1 µg of cDNA, 10 pmol of each primer, and 1.5 U of Taq polymerase in a DNA thermal cycler (Perkin Elmer, Foster City, California, USA). Amplified DNA products were sequenced by the dye termination method using a DNA sequencing kit (Perkin Elmer) with the same primers as for the PCR, and analysed by means of an ABI PRISM 310 (Applied Biosystems, Foster City, California, USA).

Amino acid structural analysis of abnormal haemoglobin in ES was performed as follows. Isolation of the abnormal haemoglobin β-chain was carried out by means of CM-52 cellulose column chromatography, and peptide obtained by tryptic digestion of the aminoethylated abnormal haemoglobin was analysed by reversed phase HPLC (TSK gel 80Ts, 4.6 x 250 mm, Tosoh; elution buffer, 0.1% TFA/50% acetonitril-0.1% TFA/50 min; flow rate, 0.7 ml/min; detection wavelength, 214 nm). The amino acid composition of the hydrolysates of the abnormal peptide was analysed using an automatic amino acid analyser.

Results

Laboratory data revealed normal fasting blood sugar (850 mg/litre in KM, 950 mg/litre in TY, and 990 mg/litre in ES), although the concentrations of glycated haemoglobin were low: 1.2% in KM, 0.9% in TY, and 2.7% in ES. HbA₁c concentrations measured by latex immunooagglutination were normal, as were routine haematological data. Indices suggesting haemolysis, such as indirect bilirubin or lactate dehydrogenase, were normal. HPLC chromatograms showed an additional peak near HbA₂ (fig 1, Hb Takamatsu; fig 2, Hb G-Szuhu). Isoelectric focusing gel electrophoresis demonstrated an additional haemoglobin migrating faster than HbA₁ in KM and TY (fig 3), and an additional haemoglobin migrating slower than HbA₁ in ES (fig 4). Abnormal haemoglobin accounted for 43% (KM), 45% (TY), and 44% (ES) of the total haemoglobin. cDNA sequence and amino acid analyses of the patients’ haemoglobin disclosed that lysine at position 120 of the β-chain was replaced by glutamine in KM and TY (fig 5), and asparagine at position 80 of the β-chain was replaced by glutamine in ES.
Abnormal Tp-9: Lys


Table 2  Amino acid composition of abnormal peptide and amino acid sequence of the Tp-9 peptide

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Analytical value (molar ratio)</th>
<th>Theoretical value (Tp-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1.907</td>
<td>3</td>
</tr>
<tr>
<td>Ser</td>
<td>1.09</td>
<td>1</td>
</tr>
<tr>
<td>Gly</td>
<td>2.08</td>
<td>2</td>
</tr>
<tr>
<td>Ala</td>
<td>1.98</td>
<td>2</td>
</tr>
<tr>
<td>Val</td>
<td>1.06</td>
<td>1</td>
</tr>
<tr>
<td>Leu</td>
<td>2.93?</td>
<td>4</td>
</tr>
<tr>
<td>Phe</td>
<td>0.93</td>
<td>1</td>
</tr>
<tr>
<td>Lys</td>
<td>1.04</td>
<td>1</td>
</tr>
<tr>
<td>His</td>
<td>0.92</td>
<td>1</td>
</tr>
</tbody>
</table>


Abnormal Tp-9: Lys
Abnormal haemoglobins detected during HbA1c analysis

The abnormal haemoglobins detected during HbA1c analysis should be taken into consideration when a disparity between normal fasting blood sugar and HbA1c concentration is seen because silent haemoglobinopathy is not rare in the elderly. In addition, it seems necessary to investigate HbA1c concentrations by other methods, such as latex immunooagglutination and isoelectric focusing gel electrophoresis, in cases showing discrepant HbA1c and blood sugar concentrations, especially in patients with diabetes whose expected HbA1c concentration is disproportionately low compared with their blood sugar concentrations, to avoid misinterpretation of the glycaemic state.

Some medical checkups in Japan include HbA1c measurement routinely. Therefore, HbA1c measurement by HPLC can serve as an aid to detecting some additional silent haemoglobinopathies.

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