Abnormal haemoglobins, Hb Takamatsu and Hb G-Szuhu, detected during the analysis of glycated haemoglobin (HbA\textsubscript{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\textsubscript{1c}) concentrations measured by high performance liquid chromatography (HPLC) were low, in spite of normal fasting plasma glucose concentrations. However, HbA\textsubscript{1c} concentrations measured by latex immunnoagglutination 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\textsubscript{1a+b}, which resulted in falsely low HbA\textsubscript{1c} concentrations. Isoelectric focusing analysis of the patients’ haemoglobin disclosed abnormal haemoglobins, which migrated faster than normal HbA\textsubscript{1c} 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 \(\beta 120 \text{Lys} \rightarrow \text{Gln}\) in the two female patients and \(\beta 80 \text{Asn} \rightarrow \text{Lys}\) in the male patient; that is, Hb Takamatsu and Hb G-Szuhu.

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


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

Glycated haemoglobin (HbA\textsubscript{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\textsubscript{1c} is performed by high performance liquid chromatography (HPLC), electrophoresis, immunnoassay, and colorimetry, as well as other methods. Among them, HPLC is generally used as the standard method for the determination of HbA\textsubscript{1c} concentrations. However, the HbA\textsubscript{1c} concentration is affected by several factors other than blood glucose concentrations, such as uraemia, alcohol abuse, high dose aspirin intake, hyperbilirubinaemia, and 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\textsubscript{1c} values when measured by means of HPLC. In this report, the detection of two abnormal haemoglobins, Hb Takamatsu (\(\beta 120 \text{Lys} \rightarrow \text{Gln}\)) and Hb G-Szuhu (\(\beta 80 \text{Asn} \rightarrow \text{Lys}\)), during HPLC analysis for HbA\textsubscript{1c} in two unrelated women and one man is described.

Patients, materials, and methods
Blood samples were obtained from two female outpatients during 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\textsubscript{1c} analyser, Model HA-8110; Daiichi, Kyoto, Japan; HbA\textsubscript{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\textsubscript{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.

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>Chain</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>(\alpha)-chain</td>
<td>Forward 5’ &gt; ACT CTT GTG TGC CCC ACA GA &lt; 3’</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; GTG TCT GAC ACA ACT GTG &lt; 3’</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 × 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.
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

Hba1c has been used as a routine clinical laboratory marker for evaluating long term glycaemic control. However, several factors are known to affect its concentration. They include uraemia, alcohol abuse, high dose aspirin intake, and massive bleeding, as well as various kinds of haemoglobinopathies. Uraemia, alcohol abuse, and high dose aspirin intake cause falsely high Hba1c concentrations, whereas bleeding, haemolytic anaemia, and pregnancy cause falsely low concentrations. However, in the present cases, the factors that typically cause low Hba1c concentrations were excluded, and it was shown that the disparity between low Hba1c concentrations and normal fasting blood sugar could be ascribed to abnormal concentrations of haemoglobins Hb Takamatsu and Hb G-Szuhu. Among the methods available for Hba1c determination, HPLC with a cation exchange column is widely used as the standard method. The haemoglobins (Hb Takamatsu and Hb G-Szuhu) from our patients caused falsely low Hba1c concentrations as estimated by HPLC. In HPLC, the peak of Hb Takamatsu and Hb G-Szuhu is included in the Hba1c peak calculation, leading to falsely low Hba1c results. In Hb Takamatsu, lysine at position 120 of the β-chain is substituted for glutamine, while in Hb G-Szuhu, asparagine at position 80 of the β-chain is substituted for lysine. However, these haemoglobinopathies disturb neither the oxygenation/deoxyxygenation function, nor the stability of the haemoglobin molecule because these mutations do not alter heme contacts or αβ1 contacts. In fact, one carrier of Hb Takamatsu complained of dyspnea on exertion because of mitral valvular disease, but the other was asymptomatic and showed no haematological abnormalities. The male patient with Hb G-Szuhu in our study suffered from cardiac failure as a result of aortic and mitral regurgitation. However, these valve dysfunctions were not considered to be symptoms of this haemoglobinopathy, although the patient described by Imai et al. showed mild polycythaemia. Therefore, both Hb Takamatsu and Hb G-Szuhu are considered to be asymptomatic haemoglobin variants.

Hb Takamatsu was first reported by Iuchi et al in 1980. The subject was a 38 year old woman who underwent a surgical operation for uterine cancer in 1979, and by 1987 16 families with Hb Takamatsu had been described in Japan. Hb G-Szuhu was first reported by Blackwell et al in 1969, and 24 cases of Hb G-Szuhu had been found in Japan by 1996 (T Harano, 1996, personal communication). Over 700 abnormal haemoglobins have been described throughout the world. One fifth of the haemoglobinopathies show symptoms such as cyanosis, polycythaemia, haemolysis and/or anaemia, whereas the remaining four fifths are asymptomatic. Most of the cases are accompanied by changes in electric charge because of the substitution of amino acids, resulting in an abnormal HPLC elution pattern. Hb Takamatsu and Hb G-Szuhu are not the only haemoglobinopathies that result in falsely low Hba1c concentrations. Others include HbC and HbS, as well as HbO.
Abnormal haemoglobins detected during HbA1c analysis

When abnormal haemoglobins are detected during HbA1c analysis, it seems necessary to investigate if the HbA1c concentration is seen because silent haemoglobinopathy is not rare in the elderly. In addition, it is necessary to investigate HbA1c concentrations by other methods, such as latex immunofluorescence 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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