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# Gas chromatographic–mass spectrometric determination of erythrocyte 3-deoxyglucosone in diabetic patients

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**Abstract**

To determine if the erythrocyte levels of 3-deoxyglucosone (3-DG) are increased in diabetic patients, and if they correlate with glycemic status, they were measured in diabetic patients without renal disease as well as in healthy subjects. The erythrocyte levels of 3-DG were measured by a selected ion monitoring method of gas chromatography–chemical ionization mass spectrometry using [$^{13}C_6$]-3-DG as an internal standard. The erythrocyte levels of 3-DG were significantly higher in diabetic patients than in healthy subjects. The erythrocyte concentration of 3-DG was significantly and positively correlated with HbA1c ($r=0.84$, $P<0.001$). However, no significant correlation could be found between erythrocyte 3-DG and age, onset age of diabetes, or duration of diabetes in our group of diabetic patients. In diabetes, the production of 3-DG in the erythrocytes is increased via the polyol pathway and/or the Maillard reaction due to hyperglycemia.


*Keywords:* 3-Deoxyglucosone

## 1. Introduction

The Maillard reaction is nonenzymatic glycation between reducing sugars and protein amino groups. In the early stage, glucose binding to protein forms Schiff base adducts which are then converted to relatively stable Amadori products. It further undergoes a series of reaction to form advanced glycation end products (AGEs). AGEs show a number of biological activities, and are involved in the pathogenesis of diseases such as aging, diabetes mellitus, uremia and Alzheimer's disease [1–4].

Hyperglycemia has been thought to be responsible for increased AGEs in diabetes. Some patients with insufficient glycemic control suffer from severe complications. AGEs are supposed to be produced more rapidly and directly from some highly reactive dicarbonyl compounds such as 3-deoxyglucosone (3-deoxy-D-erythro-hexos-2-ulose: 3-DG). 3-DG not only stimulates the formation of AGEs, but also shows some biological activities [4–8]. 3-DG is generated from degradation and rearrangement of the Amadori compound in the intermediate stage of the Maillard reaction [9]. 3-DG is also generated from fructose and fructose-3-phosphate via the polyol pathway. We consider that 3-DG is a clinically more

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relevant precursor for AGEs responsible for the occurrence of diabetic and uremic complications [3–5].

We studied the effect of an aldose reductase inhibitor (ARI) on erythrocyte 3-DG in diabetic hemodialysis (HD) patients by using gas chromatography–mass spectrometry (GC–MS) with a stable isotope-labeled 3-DG ([$^{13}C_6$]-3-DG) [10,11]. We demonstrated that the polyol pathway is at least partly responsible for intracellular production of 3-DG, because the administration of ARI which suppresses the polyol pathway, successfully decreased erythrocyte 3-DG. In the diabetic HD patients, however, 3-DG before ARI treatment did not correlate with HbA1c. Intracellular accumulation of 3-DG depends on the balance between the speed of generation and the capacity of detoxifying or excreting system. In uremic patients, the capacity of detoxifying or excreting system may be decreased. Thus, it is only natural that 3-DG level did not correlate with glycemic status in diabetic HD patients.

To determine whether hyperglycemia is responsible for production of erythrocyte 3-DG, we measured erythrocyte 3-DG in diabetic patients without renal disease using GC–MS, and analyzed the correlation with a glycemic parameter, hemoglobin A1c (HbA1c). HbA1c is glycated hemoglobin, produced by nonenzymatic conjugation of glucose with N-terminal valine in β-chain of HbA.

## 2. Experimental

### 2.1. Patients

Blood samples were obtained from thirteen diabetic patients (10.1±3.6 years old, mean±SD; five males, eight females), and eleven healthy subjects (20.7±3.3 years old; six males, five females). Eleven out of the patients had suffered from insulin-dependent diabetes mellitus (IDDM), and two from non-insulin-dependent diabetes (NIDDM). The onset age of diabetes was 7.0±3.9 years (mean±SD). They had been treated for 3.0±3.6 years (mean±SD), and were free from proteinuria or renal disease. The heparinized blood was centrifuged to separate erythrocytes from plasma. After removing the plasma and the buffy coat, the erythrocyte fraction was kept at −30 °C until sample preparation. HbA1c was measured using cation-exchange high-performance liquid chromatography with an automated glycohemoglobin analyzer (Hi-Auto HA8150, Arkray, Kyoto Daiichikagaku, Kyoto, Japan).

### 2.2. Sample preparation and GC–MS analysis

Samples for measurement of 3-DG levels in erythrocytes were prepared according to a previous report [12]. In short, after addition of [$^{13}C_6$]-3-DG (1.19 nmol) as an internal standard, hemolyzed erythrocytes (100 μl) were diluted with distilled water (900 μl). The mixture was deproteinized by adding ethanol (2 ml), and subsequently centrifuged at 1000 *g* for 10 min at 4 °C. The supernatant was applied to a Bond Elut SCX cartridge (cation exchange, 100 mg in 1 ml; Analytichem, Harbor City, CA, USA), and eluted with distilled water (3 ml). The collected eluate was then applied to a Bond Elut SAX cartridge (anion exchange, 100 mg in 1 ml; Analytichem), and eluted with distilled water (3 ml). The eluate was collected, and lyophilized. The carbonyl groups of the residue were transformed to their methoxime (MO) derivatives at 70 °C for 30 min with 1% methoxylamine hydrochloride (Sigma, St. Louis, MO, USA) in pyridine (200 μl). After evaporation over a nitrogen stream, the residues were kept at −20 °C until GC–MS analysis. The hydroxyl groups in the residues were converted to their trimethylsilyl (TMS) derivatives at 60 °C for 20 min with N,O-bis(trimethylsilyl)trifluoroacetoamide (BSTFA) (20 μl) containing 1% trimethylchlorosilane (TMCS) (Pierce, Rockford, IL, USA). After cooling to room temperature, the sample (2 μl) was subjected to GC–MS (GCMS-QP5000; Shimadzu, Kyoto, Japan) equipped with a capillary column (30 m×0.25 mm I.D., 0.23 μm thick membrane; J&W Scientific, Folsom, CA, USA). The column temperature was programmed at 10 °C/min from 100 to 250 °C, and the entrance pressure was 80 kPa with 20 ml/min of helium. For chemical ionization (CI), isobutane was used as a reactant gas. Quantitation of 3-DG was performed by selected ion monitoring (SIM) of *m/z* 337 for 3-DG and *m/z* 343 for [$^{13}C_6$]-3-DG as an internal standard.

### 2.3. Calibration for quantification of 3-DG

To quantify 3-DG in erythrocyte samples, calibration lines were obtained from SIM chromatograms. Amounts of 3-DG ranging from 80 to 400 ng were added to 200 ng of [$^{13}C_6$]-3-DG as an internal standard, these mixtures were processed as described above and analyzed by GC–MS. Calibration lines relating the concentrations of 3-DG to its peak area ratios at $m/z$ 437 (3-DG) to the internal standard at $m/z$ 443 ([$^{13}C_6$]-3-DG) were obtained from the SIM chromatograms. The correlation coefficient of the calibration line for 3-DG was 0.99987 ($y = 1.037x - 0.108$: $y$, concentration ratio; $x$, peak area ratio).

### 2.4. Statistics

A non-paired $t$ test was applied to the comparison between healthy subjects and diabetic patients. Significance was found when the $P$ value was less than 0.05.

## 3. Results

Fig. 1 shows total ion chromatogram (TIC) and SIM chromatograms of 3-DG and [$^{13}C_6$]-3-DG in a standard sample (upper figure) and an erythrocyte sample from a diabetic patient (lower figure). Two peaks, which consist of 3-DG isomers, are recognized between 12 and 13 min in the chromatograms. The major peak is detected at a retention time of about 12.1 min, and the minor peak at about 12.5 min. The base ions at $m/z$ 437, $(M+H)^+$, for 3-DG, and at $m/z$ 443, $(M+H)^+$, for [$^{13}C_6$]-3-DG were monitored for SIM. The peak area ratio of the peaks at $m/z$ 437–443 at a retention time of 12.1 min was used for quantification of 3-DG. Fig. 2 shows the calibration line for the quantification of 3-DG.

Table 1 lists the erythrocyte levels of 3-DG and HbA1c in diabetic patients and healthy subjects. HbA1c levels were significantly increased in the diabetic patients as compared with the healthy subjects. Erythrocyte levels of 3-DG in the diabetic patients (1680±320 ng/ml, mean±SE) were significantly ($P<0.01$) elevated as compared with healthy subjects (600±70 ng/ml). As shown in Fig. 3, a good positive correlation was observed between erythrocyte 3-DG and HbA1c in diabetic patients ($r=0.84$, $P<0.001$). However, no significant correlation could be found between erythrocyte 3-DG and age, onset age of diabetes, or duration of diabetes in our group of diabetic patients.

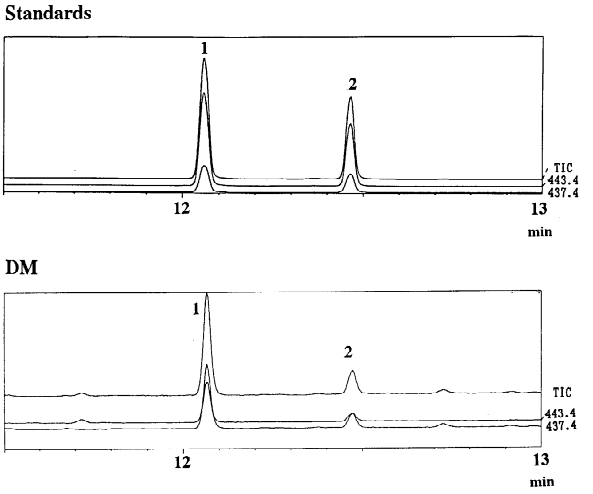


Fig. 1. Total ion chromatogram (TIC) and selected ion monitoring (SIM) chromatograms of 3-DG and [$^{13}C_6$]-3-DG in a standard sample (upper figure) and an erythrocyte sample from a diabetic patient (lower figure) obtained using gas chromatography–chemical ionization mass spectrometry (GC–CIMS). The base ions at $m/z$ 437, $(M+H)^+$, for 3-DG, and at $m/z$ 443, $(M+H)^+$, for [$^{13}C_6$]-3-DG are monitored for SIM.

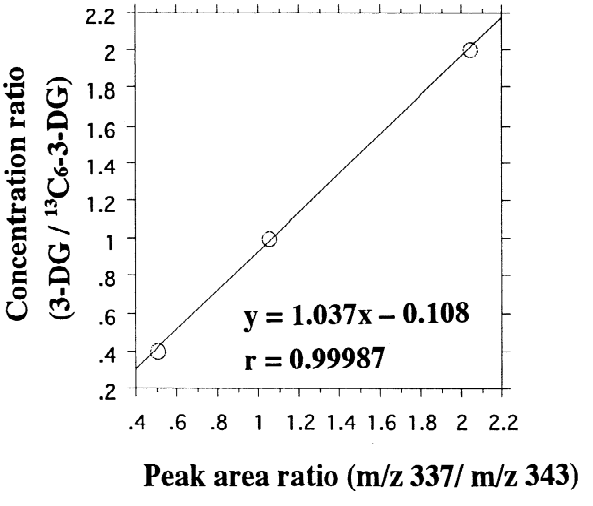


Fig. 2. Calibration line for quantification of 3-DG.

Table 1
Erythrocyte levels of 3-DG and HbA1c in diabetic patients and healthy subjects

| | Normal ($n=11$) | Diabetes ($n=13$) |
|---|---|---|
| HbA1c (%) | 4.8±0.03 | 8.5±0.5** |
| Erythrocyte 3-DG (ng/ml) | 600±70 | 1680±320** |

Values are expressed as mean±SE.
**, $P<0.01$ as compared with diabetic patients by non-paired $t$ test.

## 4. Discussion

3-DG rapidly reacts with arginine or lysine residues of proteins to form AGEs such as imidazolone or pyrraline, which are involved in the pathogenesis of aging, diabetes and uremia [3–5]. 3-DG also shows some biological activities, including induction of heparin-binding epidermal growth factor-like growth factor in rat aortic smooth muscle cells [6], promotion of apoptotic cell death in macrophage-derived cell lines [7], suppression of cell-cycle progression during the S phase of rat fibroblasts [8], and inhibition of glutathione peroxidase [4]. Thus, 3-DG is thought to be a glycotoxin or a uremic toxin.

3-DG is nonenzymatically produced from degradation of Amadori intermediate in the Maillard reaction, and from fructose and fructose-3-phosphate in the polyol pathway [13]. Erythrocyte fructose is phosphorylated to fructose-3-phosphate in an incubation solution containing glucose [8]. A specific phosphorylase converts fructose primarily to fructose-3-phosphate [14]. Thus, fructose and fructose-3-phosphate play an important role in the formation of intracellular 3-DG in diabetic patients, because the polyol pathway is enhanced due to hyperglycemia in diabetes.

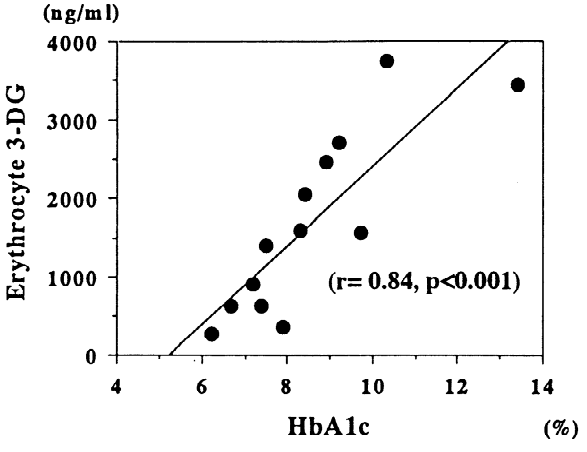


Fig. 3. Correlation between erythrocyte 3-DG and HbA1c in diabetic patients and healthy subjects. Erythrocyte 3-DG in diabetic patients showed significantly positive correlation ($r=0.84$, $P<0.001$) with HbA1c.

3-DG is detoxified mainly to 3-deoxyfructose by 3-DG-reducing enzymes, or to 2-keto-3-deoxy-gluconic acid by oxoaldehyde dehydrogenase [15]. The 3-DG-reducing enzymes were identified as aldehyde reductases [16,17], aldose reductases [17,18] and dihydrodiol dehydrogenases [19]. 3-Deoxyfructose has been detected in human urine and plasma [20]. The activities to detoxify 3-DG by these enzymes may provide a genetic basis for the differences in the severity of age-related pathologies or diabetic complications. Oxidative stress may impair detoxifying ability of 3-DG, and contribute to 3-DG accumulation [21].

In conclusion, we first demonstrated that the erythrocyte levels of 3-DG in diabetic patients were markedly elevated as compared with healthy subjects, and their levels depend on glycemic control. Thus, the production of 3-DG in the erythrocytes is increased via the polyol pathway and/or the Maillard reaction due to hyperglycemia.

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