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Rapid and sensitive determination of the intermediates of advanced glycation end products in the human nail by ultra-performance liquid chromatography with electrospray ionization time-of-flight mass spectrometry

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ABSTRACT

The resolution of the intermediate advanced glycation end products (AGEs) in the human nail was carried out by the combination of 4,5-dimethyl-1,2-phenylenediamine (DMPD) derivatives and ultra-performance liquid chromatography with electrospray ionization time-of-flight mass spectrometry (UPLC–ESI–TOF–MS). The reaction of the reagent with 3-deoxyglucosone (3-DG), methylglyoxal (MG), and glyoxal (GO) effectively proceeds at 60 °C for 2 h. The resulting derivatives were efficiently separated by a gradient program (a mixture of water and acetonitrile containing 0.1% formic acid) using a reversed-phase ACQUITY UPLC BEH C_{18} column (1.7 μm, 50 × 2.1 mm i.d.) and sensitively detected by TOF–MS. The detection limits (signal-to-noise ratio = 5) of the TOF–MS were 10 to 50 fmol. A good linearity was achieved from the calibration curve, which was obtained by plotting the peak area ratios of the analytes relative to the internal standard (IS) (i.e., 2,3-hexanedione) versus the injected amounts of 3-DG, MG, and GO ($r^2 > 0.999$), and the intra- and interday assay precisions were less than 6.89%. The derivatives of the compounds in the human nail were successfully identified by the proposed procedure. As we know, these three kinds of dicarbonyl intermediates in the formation of AGEs—3-DG, MG, and GO—were first found in human nail samples. Using these methods, the amounts of compound in the nails of healthy volunteers and diabetic patients were determined. When comparing the index from the diabetic patients with that from healthy volunteers, there is no significant difference in the content of the MG and GO in the nails. However, a statistically significant ($P < 0.001$) correlation was observed between the 3-DG concentrations. Because the proposed method provides a good mass accuracy and the trace detection of the dicarbonyl intermediates of AGEs in the human nail, this analytical technique could be a noninvasive technique to assist in the diagnosis and assessment of disease activity in diabetic patients. Here we present a novel, sensitive, and simple method for the simultaneous determination of dicarbonyl compounds in the human nail.

The 3-deoxyglucosone (3-DG),[1] methylglyoxal (MG), and glyoxal (GO) possessing a reactive dicarbonyl group is an important intermediate in the formation of advanced glycation end products (AGEs) (Fig. 1). The AGEs are particularly important in diabetes because they have been correlated with the development of diabetic complications. Patients with diabetes have a higher concentration of Amadori products because their formation is directly related to the concentration of glucose. In that plasma, 3-DG was significantly more increased in diabetic patients than in nondiabetic control subjects, and 3-DG levels were well correlated with plasma glucose and HbA1c levels in diabetic patients [1–4]. Glycated proteins can fragment into reactive species such as 3-DG, MG, and GO [5–9]. These dicarbonyl compounds are potent protein cross-linkers and precursors of AGEs. It is these ultimate AGEs that have been implicated in the development of complications of diabetes mellitus. Consequently, measurements of 3-DG, MG, and GO were likely to provide valuable insights into the role of this metabolite in the etiology of diabetic complications as well as the aging process [10–12].

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[1] *Abbreviations used:* 3-DG, 3-deoxyglucosone; MG, methylglyoxal; GO, glyoxal; AGE, advanced glycation end product; FL, fluorescence; UV, ultraviolet; GC, gas chromatography; MS, mass spectrometry; LC, liquid chromatography; OPD, *o*-phenylenediamine; DAN, 2,3-diaminonaphthalene; UPLC–ESI–TOF–MS, ultra-performance liquid chromatography with electrospray ionization time-of-flight mass spectrometry; IS, internal standard; DMPD, 4,5-dimethyl-1,2-phenylenediamine; HP, 2-hydrazinopyridine; HMP, 2-hydrazinol-methylpyridine; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; MeOH, methanol; ESI, electrospray ionization; CV, coefficient of variation; LOD, limit of detection; S/N, signal-to-noise ratio; HPLC, high-performance liquid chromatography; DAD, diode array detection.

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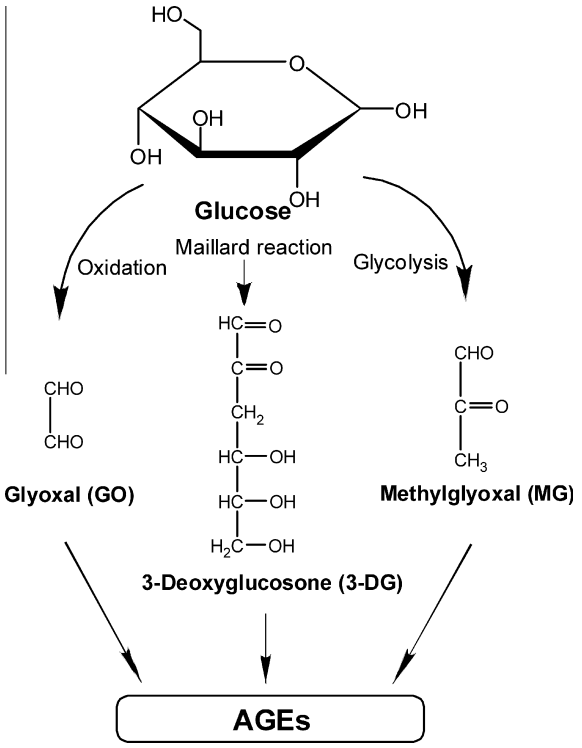

Fig.1. Pathway of AGE formation.

Tissue homogenates, peritoneal dialysis fluids, and plasma samples have been investigated extensively for the dicarbonyl intermediates of advanced glycation end product (AGE) assay in biological specimens [5,13–21]. The inherent problems of plasma and tissue homogenates, such as the fluctuation in composition during the day, should be considered. Hygienic practice during collection and handling is also another consideration. In contrast, the human nail is relatively clean and the samples can be quickly and noninvasively collected and easily stored. Analyzing the components of the human nail provides an important means for determining the individual past history of long-term chemical exposures because many substances have been detected in the nail [22–25]. Due to the stability of drugs in nails, many studies concerning nail analysis have dealt with drugs of abuse such as cocaine, itraconazole, and amphetamines [26,27]. During the past decade, interest in nail analysis has gradually shifted to other drug species such as doping agents and therapeutic drugs. According to recent reports, human nails may be used to obtain physiological information and may serve as a noninvasive biosample for the diagnosis of chronic disease. Certain kinds of endogenous biogenic amino acids have been detected in the human nail [23–25]. However, a method for determining the dicarbonyl intermediates of AGEs of the human nail has not been reported.

Various detection methods concerning the dicarbonyl intermediates of AGE analysis have been developed due to the importance of understanding diabetic complications in biological systems. However, the analysis of the dicarbonyl intermediates of AGEs in a real sample is very difficult due to no fluorescence (FL) and no effective absorption in the ultraviolet–visible (UV–vis) region. Therefore, derivatization using a suitable labeling reagent is a key step in the gas chromatography–mass spectrometry (GC–MS) [28–30] and liquid chromatography–mass spectrometry (LC–MS) [31–35] analyses of the dicarbonyl intermediates of AGEs. There are a significant number of precolumn labeling methods using fluorogenic and chromophoric reagents—such as *o*-phenylenediamine (OPD) [7,14,31,36], 2,3-diaminonaphthalene (DAN) [5,37], and 1,2-diamino-4,5-methylenedioxybenzene (DMB) [38]—for the analysis of the dicarbonyl intermediates of AGEs. The methods possessing excellent UV absorption or FL properties are certainly good, and subpicomole sensitivities can be achieved. However, the resolution of the dicarbonyl intermediates of AGEs in the human nail was very difficult even using the highly sensitive LC–FL and GC–MS. All of these reactions are usually the derivatization procedure and are time-consuming. The current study was undertaken to develop a reliable and sensitive method for the absolute quantitation of the dicarbonyl intermediates of AGEs in the human nail. Preliminary testing indicated that the detection of 3-DG, MG, and GO was difficult because the substance was unstable and existed in a very minute amount in the human nail. A higher sensitivity is essential in any assay to detect the dicarbonyl intermediates of AGEs. To achieve this goal, a new method was developed by substituting OPD with DAN [37,39].

According to this strategy, the aim of the current study was to inspect the usefulness of the human nail as a new noninvasive biological sample for the diagnosis of chronic disease and also to develop a reliable determination method to measure the free dicarbonyl intermediates of AGEs in the nail by ultra-performance liquid chromatography with electrospray ionization time-of-flight mass spectrometry (UPLC–ESI–TOF–MS). Therefore, this article describes the resolution of the dicarbonyl intermediates in the nail from diabetic patients.

Materials and methods

Materials and reagents

3-DG, MG, and GO were obtained from Dojindo (Kumamoto, Japan), Sigma–Aldrich (St. Louis, MO, USA), and Kanto Chemicals (Tokyo, Japan). 2,3-Hexanedione (Sigma–Aldrich) was used as the internal standard (IS). 4,5-Dimethyl-1,2-phenylenediamine (DMPD), 2-hydrazinopyridine (HP), 2-hydrazinol-methylpyridine (HMP), and *o*-phenylenediamine (OPD) were purchased from Tokyo Kasei (Tokyo, Japan). Trifluoroacetic acid (TFA), formic acid, hydrochloric acid, sodium dodecyl sulfate (SDS), methanol (MeOH), ethanol, and acetonitrile were of special reagent grade (Wako Pure Chemicals, Osaka, Japan). All other chemicals were of analytical reagent grade and were used without further purification. Deionized and distilled water was used throughout the study (Aquarius PWU-200 automatic water distillation apparatus, Advantec, Tokyo, Japan).

UPLC–ESI–TOF–MS

The UPLC–ESI–TOF–MS systems consisted of an ACQUITY Ultra-Performance Liquid Chromatography and Micromass LCT Premier XE Mass Spectrometer (high-sensitivity orthogonal TOF instrument, Waters, Milford, MA, USA). An ACQUITY UPLC BEH C_{18} column (1.7 μm, 50 × 2.1 mm i.d., Waters) was used as the analytical column. The column was maintained at 40 °C. The flow rate of the mobile phase was 0.6 ml/min. The TOF–MS was operated in the positive and negative ion modes using an electrospray ionization (ESI) source. The optimized conditions for the UPLC separation and TOF–MS detections are shown in Table 1.

Derivatizing dicarbonyl intermediates of AGEs with DMPD

3-DG, MG, GO, and 2,3-hexanedione (IS) were dissolved in water (2 μM each concentration). The solutions (100 μl each) and 100 μl of the DMPD (2 mM) in acetonitrile were mixed in 1.5-ml mini-vials. The vials were tightly capped and heated at 60 °C for 480 min using a dry heat block. The reaction mixture was

Table 1
UPLC–ESI–TOF–MS conditions.

UPLC (*Waters*)	
Column	ACQUITY UPLC BEH C_{18} (1.7 μm, 50 × 2.1 mm i.d.)
Mobile phase A_1	0.1% HCOOH in water
Mobile phase B_1	0.1% HCOOH in acetonitrile
Gradient	B_1% = 18:18:60% (0:4:7 min)
Column temperature	40 °C
Flow rate	0.6 ml/min
Injection volume	4 μl
TOF–MS (*Micromass LCT Premier XE Mass Spectrometer*)	
Ion polarity	ESI^+ (V mode)
Capillary voltage	3000 V
Sample cone voltage	5 V
Desolvation gas flow	650 L/h
Cone gas flow	50 L/h
Source temperature	120 °C
Desolvation temperature	300 °C
MS range (*m*/*z*)	100 to 1000

adequately diluted with acetonitrile, and then 4 μl of the solution was injected into the UPLC–ESI–TOF–MS system.

Human nail samples

Nail samples were collected from 20 healthy volunteers (30–69 years of age, 10 men and 10 women) and 20 diabetic patients (40–68 years of age, 10 men and 10 women) treated at the Fengxian Branch of Shanghai Sixth People's Hospital from January 2009 to February 2010. All patients provided written informed consent before entry into the study. The nail samples were rinsed with 1 ml of 0.1% SDS for 1 min by ultrasonication. The procedure was repeated two more times. After rinsing, the SDS on the nail samples was removed by three washings with distilled water. The nails were then dried in a desiccator under reduced pressure. The dried nails were crushed into a powder using a Shake Master (Bio Medical Science, Tokyo, Japan).

Validation of the method

Calibration curve preparation

Each 100 μl of the dicarbonyl intermediates of AGEs (3-DG, MG, and GO) in water (0.125–10 μM each) was mixed with 100 μl of 2.5 μM IS in water. The solution was reacted at 60 °C for 120 min with 100 μl of 2 mM DMPD. After the reaction, 4 μl of each solution was subjected to the UPLC–ESI–TOF–MS system. The amounts corresponding to an injection of 4 μl were 0.1 to 8 pmol. The calibration curves were obtained by plotting the peak area ratios of the analytes relative to the IS versus the injected amounts of the dicarbonyl intermediates of AGEs. The precision (coefficient of variation [CV]) for each concentration was also calculated from five replicated determinations.

Accuracy and precision of intra- and interday assays

The accuracy and precision (CV) of the intra- and interday assays were determined using the standard dicarbonyl intermediates of AGEs (3-DG, MG, and GO) described in the preceding subsection. These parameters were evaluated using three different concentrations in the range of 0.5 to 8 pmol for the dicarbonyl intermediates of AGEs. The determinations were repeated five times within 1 day and between days. Each 100 μl of the dicarbonyl intermediates of AGEs (3-DG, MG, and GO) was reacted with DMPD and then subjected to UPLC–TOF–MS, as described in the preceding subsection. The accuracy at each concentration was calculated from the calibration curves obtained from the preceding subsection. The precision (CV) for each concentration was also calculated from the standard deviations of five replicated determinations.

Limit of detection

The limit of detection (LOD) was defined as the calculated concentration at a signal-to-noise ratio (S/N) of 5. The standard solutions of the dicarbonyl intermediates of AGEs (3-DG, MG, and GO) were diluted to a series of concentrations (12.5–62.5 nM). Each 100-μl solution was reacted with DMPD and then subjected to the UPLC–ESI–TOF–MS system, as described in "calibration curve preparation" subsection. The LODs of the dicarbonyl intermediates of AGEs (3-DG, MG, and GO) were calculated from a comparison of the noise level and the peak height on the suitable mass chromatogram that had detected the target and the dicarbonyl intermediates of AGEs.

Standard addition calibration of dicarbonyl intermediates of AGEs spiked into human nail

Each 100 μl of the dicarbonyl intermediates of AGEs (3-DG, MG, and GO) in water (2.5–350 μM each) and 700 μl of MeOH were poured into glass vials containing 5.0 mg of human nail ($n = 7$). The mixture was kept at 50 °C for 16 h to extract the dicarbonyl intermediates of AGEs, vortex-mixed for 30 s, and centrifuged at 3000*g* for 10 min. After the extraction, the nail samples were washed with MeOH (200 μl, two times), and all of the supernatant fluids were collected and dried under a gentle stream of nitrogen gas. The resulting residues were redissolved in 100 μl of 2.5 μM IS in water, reacted with 100 μl of 2 mM DMPD in acetonitrile at 60 °C for 120 min, and determined with UPLC–ESI–TOF–MS, as described above. The recovery and precision (CV) of the three concentration sets ($n = 3$) were calculated from the calibration curve obtained by the method described in the preceding subsections. It was used as a standard addition calibration of the quantitation of the dicarbonyl intermediates of AGEs in the human nail.

Determination and quantitation of dicarbonyl intermediates of AGEs in human nail

Methanol (1000 μl) was added to 5.0 mg of human nail from each of the 20 diabetic patients and healthy volunteers. The extraction was performed as described in the dicarbonyl intermediates of AGEs spiked into human nail of preceding section. Furthermore, the amounts of dicarbonyl intermediates of AGEs in the nails of healthy volunteers and diabetic patients were calculated from the standard addition calibration curve obtained by the method described above.

Statistical analysis

The statistical analyses were performed using Welch's t test or Mann–Whitney's U test. A P value of <0.05 (0.01) was considered to be statistically significant.

Results and discussion

Optimization of DMPD labeling reaction and separation conditions

The dicarbonyl intermediates of AGEs are hydrophilic compounds. Therefore, the simultaneous separation of the dicarbonyl intermediates of AGEs by reversed-phase chromatography using an ODS column is very difficult due to the adsorption on the resins. In a conventional study, the 3-DG in rat and human plasma samples was successfully determined by high-performance liquid chromatography (HPLC) separation and FL and diode array detection (DAD) [5,17,37]. Derivatization with chromophoric agents such as OPD and DAN is a convenient method that allows separation and detection of dicarbonyl compounds by HPLC/DAD. However, these derivatization reagents require a long derivatization

time (e.g., 5-h reaction in OPD and 24-h reaction in DAN) and produce many unknown peaks. The derivatization conditions must be carefully controlled because incomplete derivatization or de novo formation of the analyte during the process may lead to incorrect quantification. The FL labeling is usually recommended for the determination of a real sample due to its high sensitivity and selectivity. However, the determination in complex matrices, such as hair and nails, seems to be fairly difficult by FL detection. Indeed, the determination of several dicarbonyl intermediates of AGEs in human nails by FL detection was interfered with by any of the peaks based on the endogenous substances. Although it seemed to be evitable through the optimization of the elution conditions, the determination within a short run time failed. Furthermore, no structural information can be obtained from FL detection. On the other hand, MS has recently become a popular technique for the determination of trace quantities of chemicals in real samples such as blood and urine. Among the various types of available MS instruments, ESI–TOF–MS is recommended for the selective determination of target compounds because of its excellent accuracy and the precision of the resulting m/z values. Thus, the simultaneous determination of dicarbonyl intermediates of AGEs by UPLC–ESI–TOF–MS was attempted in this study.

Several MS labeling reagents (OPD, HP, HMP, and DMPD) were first used for labeling the three dicarbonyl intermediates of AGEs. Conventional OPD had a low reaction rate with GO, and the product was not confirmed. In addition, sensitivity in the MS of the product of 3DG and MG was only half of the peak of the DMPD derivatization product. Although these reagents were essentially usable for the labeling, only DMPD efficiently labeled all of the tested dicarbonyl intermediates of AGEs. Therefore, DMPD was selected for the labeling of the dicarbonyl intermediates of AGEs in the current study. The reaction scheme of DMPD with 3-DG as a representative dicarbonyl intermediate of AGEs is shown in Fig. 2. The MS labeling effectively proceeds in a basic medium due to the electrophilic substitution reaction of DMPD for the dicarbonyl compounds. Higher temperatures are also important for the reaction to occur. Therefore, the reaction solution was heated at 60 °C. Fig. 3 shows the time courses of the labeling reaction of the three dicarbonyl intermediates of AGEs with DMPD. The reactions seemed to be completed within 2 h. Hence, the reaction condition (60 °C for 2 h) was selected for the MS labeling of the tested dicarbonyl intermediates of AGEs.

In the separation, an antipressurized column packed with small porous resins, ACQUITY UPLC BEH C_{18} (1.7 μm, 50 × 2.1 mm i.d.), was used for the rapid separation of the DMPD-labeled dicarbonyl intermediates of AGEs by UPLC. For the detection of the derivatives, the ESI–TOF–MS instruments were directly connected to the outlet of the column in this order. Separation of the DMPD-labeled dicarbonyl intermediates of AGEs was studied by gradient elution with water–acetonitrile containing 0.1% formic acid. Fig. 4 shows the typical mass chromatograms and mass spectra of the

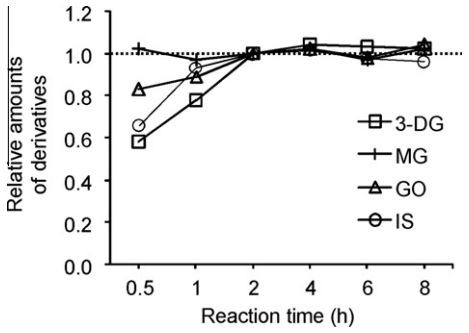

Fig.3. Time courses of reactions of dicarbonyl compounds with DMPD. The amounts of the derivatives formed by heating at 60 °C for 2 h were taken as 1.0.

DMPD-labeled 3-DG, GO, MG, and 2,3-hexanedione (IS). The peaks corresponding to the dicarbonyl intermediate derivatives were completely separated. Furthermore, a rapid separation within 7 min was performed by the combination of the antipressurized column and the UPLC instrument.

Validation of proposed method

Table 2 shows the calibration curves of the DMPD-labeled 3-DG, GO, and MG. The calibration curves were obtained by plotting the peak area ratios of the DMPD-labeled 3-DG, GO, and MG relative to the IS versus the injected amounts of the DMPD-labeled 3-DG, GO, and MG (0.1–8 pmol, $r^2 > 0.9997$) with five different concentrations for each substance. The determination at each concentration was repeated five times. A good calibration curve was obtained for each DL-amino acid. The CV values for each injected amount were in the range of 0.46 to 5.77% ($n = 5$). The detection limits (S/N = 5) in the MS were 10 to 50 fmol. To evaluate the current method, the accuracy and precision (CV) were determined. The accuracies and precisions for three different concentrations were evaluated using the intra- and interday assays. As shown in Table 3, the accuracies of the intra- and interday determinations were 93.96 to 108.0% and 93.06 to 105.67%, respectively. The CVs of the intra- and interday determinations were 0.882 to 5.28% and 2.02 to 6.89%, respectively.

Extraction of dicarbonyl intermediates of AGEs in human nail

For human nail analysis, extracting a nail sample is a necessary preprocess step as a solid sample. Because the dicarbonyl intermediates of AGEs are hydrophilic compounds, water-soluble solvents (MeOH) were tried as the extraction solutions at 4, 25, and 50 °C. Among the tested solutions, the dicarbonyl intermediates of AGEs were efficiently extracted by the MeOH at 50 °C for 16 h. As shown in Fig. 5, the extracting amounts of the dicarbonyl intermediates of

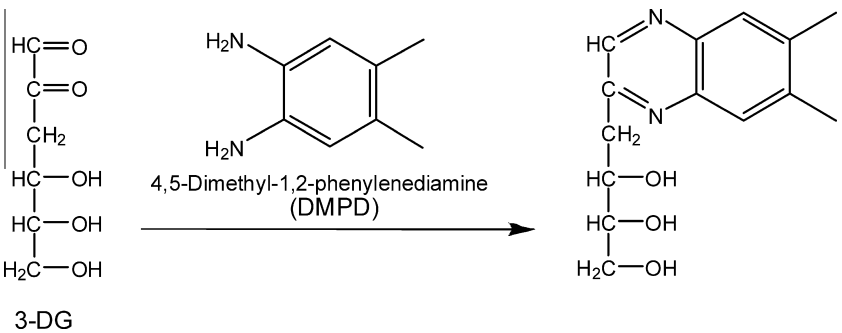

Fig.2. Formation of 3-DG derivative using DMPD reaction.

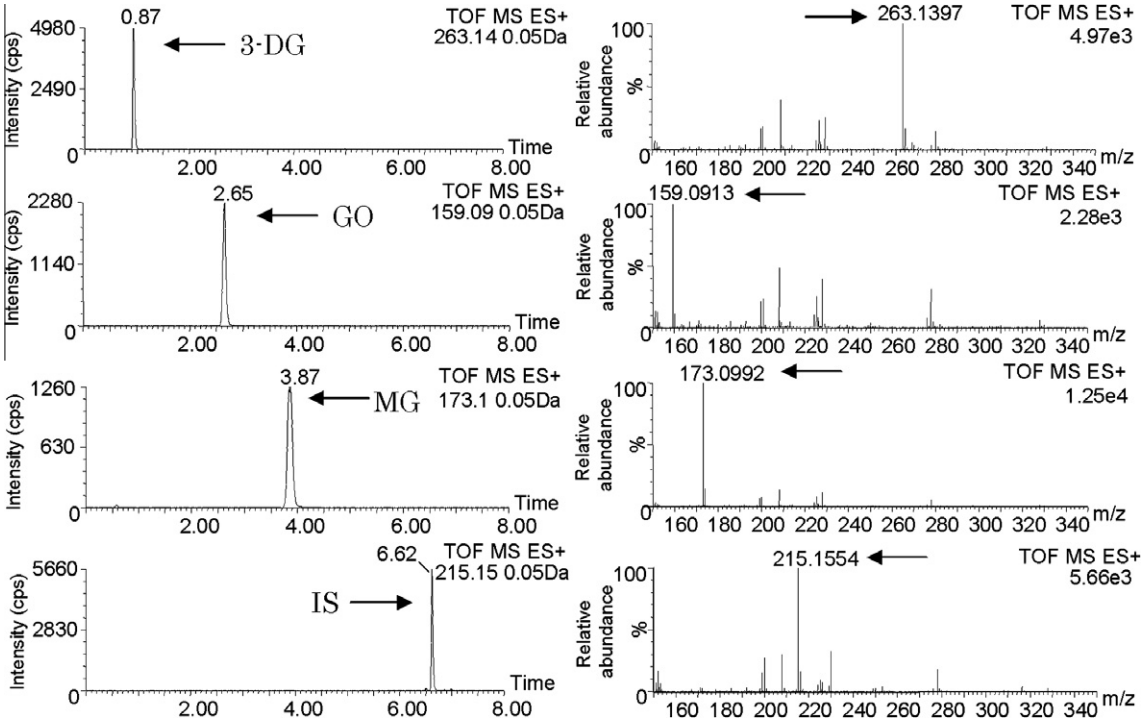

Fig.4. Mass chromatograms and spectrum for DMPD-labeled dicarbonyl compounds and IS in the positive ionization. The UPLC–TOF–MS conditions are described in Table 1.

Table 2
Calibration curves of dicarbonyl compounds by proposed method.

Dicarbonyl compound	Calibration range (pmol)	Linear equation	Linearity (R^2)	CV (%) ($n = 5$)	LOD (fmol)
3-DG	0.1–8.0	$y = 0.183x + 0.0097$	0.9997	0.96–4.51	30
MG	0.1–3.0	$y = 0.6065x + 0.0605$	0.9997	0.46–1.23	10
GO	0.1–8.0	$y = 0.1327x + 0.0079$	0.9998	4.44–5.77	50

Table 3
Accuracy and precision of proposed method by intra- and interday assays.

Dicarbonyl compound	Amount (pmol)	Intraday assay			Interday assay		
		Mean ± SD	CV % ($n = 5$)	Accuracy (%)	Mean ± SD	CV% ($n = 5$	Accuracy (%)
3-DG	0.5	0.47 ± 0.043	2.00	93.96	0.48 ± 0.014	3.91	95.66
	2.0	2.1 ± 0.024	1.36	103.6	2.0 ± 0.071	5.08	101.3
	8.0	8.0 ± 0.051	1.30	99.63	8.0 ± 0.21	3.04	99.90
MG	0.1	0.097 ± 0.098	1.11	97.36	0.093 ± 0.095	2.66	93.06
	0.5	0.53 ± 0.094	0.882	106.1	0.53 ± 0.087	2.02	105.67
	2.0	2.0 ± 0.071	1.36	100.7	2.0 ± 0.062	3.51	100.7
GO	0.5	0.54 ± 0.029	5.13	108.0	0.50 ± 0.015	5.94	99.03
	2.0	2.0 ± 0.038	4.96	95.43	2.0 ± 0.094	6.89	100.2
	8.0	7.9 ± 0.36	5.28	98.17	8.0 ± 0.30	4.37	99.99

AGEs increased with the extraction time, and the dicarbonyl intermediates of AGEs of human nail are almost completely extracted after one extraction. Based on these observations, the MeOH extraction at 50 °C for 16 h was performed for the sample preparation in this study.

Determination of free dicarbonyl intermediates of AGEs in nails of diabetic patients and healthy volunteers

The extracted dicarbonyl intermediates of AGEs from the nails of diabetic patients (40–68 years of age, 10 men and 10 women) and healthy volunteers (30–69 years of age, 10 men and 10 women) were then labeled with DMPD. Fig. 6 shows the typical mass chromatograms obtained from the dicarbonyl intermediates of AGEs in nails from healthy volunteers and diabetic patients by the UPLC–ESI–TOF–MS system. The peaks corresponding to the 3-DG, MG, and GO derivatives were completely separated without any interference from the endogenous substances in the nails. Furthermore, a rapid separation within 7 min was performed by the combination of the antipressurized column and the UPLC instrument. Of course, the structures of the derivatives were identified from a comparison of the positive ion mode MS of the authentic dicarbonyl intermediates of AGEs. These three kinds of dicarbonyl intermediates of AGEs (3-DG, GO, and MG) were detected from the human nail samples.

Through these methods, the amounts of dicarbonyl intermediates of AGEs in the nails of healthy volunteers and diabetic patients were calculated from the standard addition calibration curve

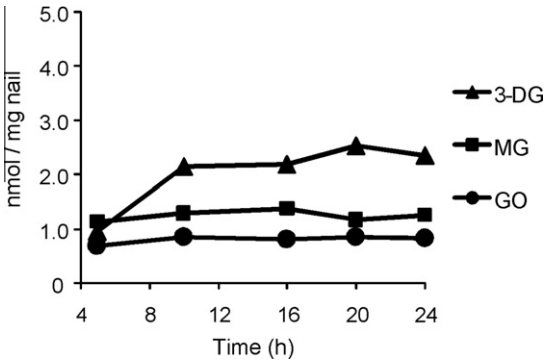

Fig.5. Time courses of extracting with methanol solution at 50 °C.

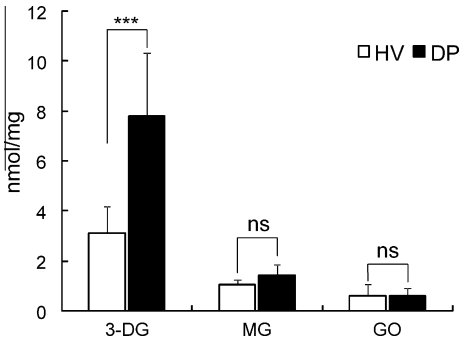

Fig.7. Statistical analysis of dicarbonyl compounds in healthy volunteers ($n = 20$) and diabetic patients ($n = 20$). The UPLC–ESI–TOF–MS conditions are described in Table 1. HV, healthy volunteers; DP, diabetic patients. $^{***}P < 0.001$; ns, not significant.

obtained by the method described in the "standard addition calibration of the dicarbonyl intermediates of AGEs spiked into human nail" section in Materials and Methods. This procedure uses 2,3-hexanedione as an IS, which might not be able to adequately compensate for the reactivity of the dicarbonyl intermediates of AGEs. In addition to this, 2,3-hexanedione (IS) was used to compensate for the reactivity of dicarbonyl intermediates of AGEs in the human nail. Fig. 7 shows the concentrations and a statistical analysis of the dicarbonyl intermediates of AGEs in the healthy volunteers and diabetic patients. The mean amounts of 3.11 pmol (3-DG), 1.03 pmol (MG), and 0.59 pmol (GO) in 1 mg of nail in the healthy men ($n = 10$) and women ($n = 10$) were calculated from each calibration curve. On the other hand, the diabetic patient amounts were 7.80 pmol (3-DG), 1.41 pmol (MG), and 0.60 pmol (GO) in 1 mg of nail. The MG and GO concentrations were similar in the healthy volunteers and diabetic patients. Furthermore, in the diabetic patients, the MG and GO concentrations were not statistically different from those in the healthy volunteers. In contrast, 3-DG concentrations were higher in the diabetic patients than in the healthy volunteers. Significant differences ($P < 0.001$) were observed between 3-DG in the healthy volunteers and that in the diabetic patients.

Furthermore, Fig. 8 shows a comparison of the concentrations of the men's and women's separate dicarbonyl intermediates of AGEs in the nails of healthy volunteers and the diabetic patients. When comparing the dicarbonyl intermediates of AGE concentrations, a significant difference was observed between 3-DG ($P < 0.001$) in the men and that in the women. MG and GO were not statistically significant in the men and women. A strong correlation was observed between the 3-DG concentrations. Although the biochemical mechanisms responsible for these peculiar diabetic 3-DG profiles are unclear, the 3-DG concentration ratios might serve as a potential marker for diabetes.

The 3-DG concentration was reported to increase with aging. Fig. 9 shows the concentration of 3-DG in the nail by ages of

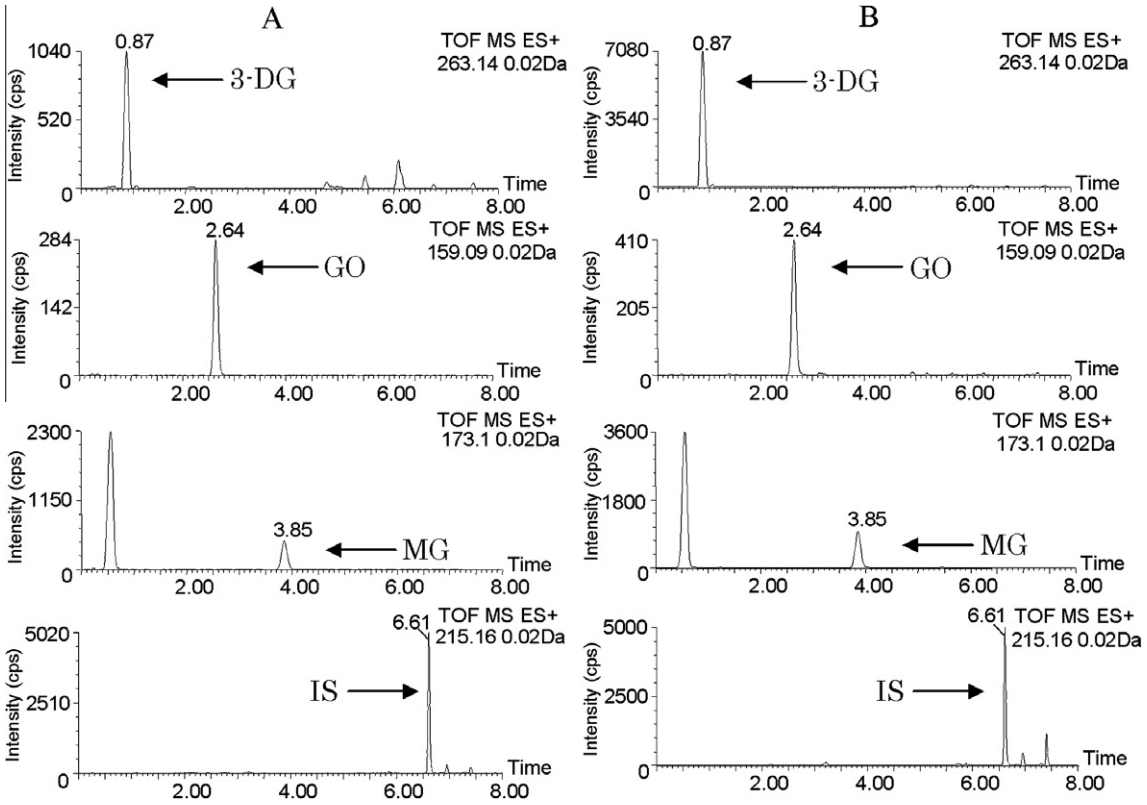

Fig.6. Mass chromatograms obtained from DMPD-labeled dicarbonyl compounds and IS in nails from healthy volunteers and diabetic patients: (A) healthy volunteers; (B) diabetic patients. The UPLC–TOF–MS conditions are described in Table 1.

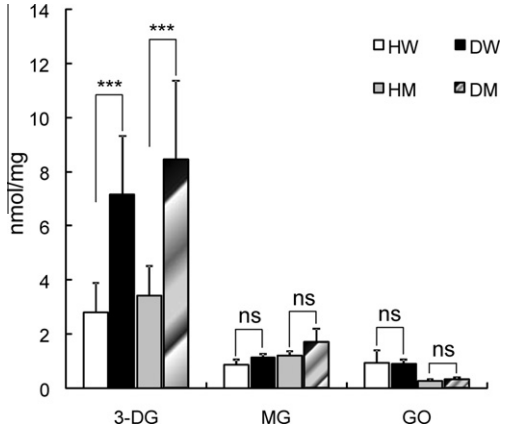

Fig.8. Statistical analysis of dicarbonyl compounds of men ($n = 10$) and women ($n = 10$) in nails of healthy volunteers and diabetic patients. HM, healthy men; DM, diabetic men; HW, healthy women; DW, diabetic women. The UPLC–ESI–TOF–MS conditions are described in Table 1. $^{***}P < 0.001$; ns, not significant.

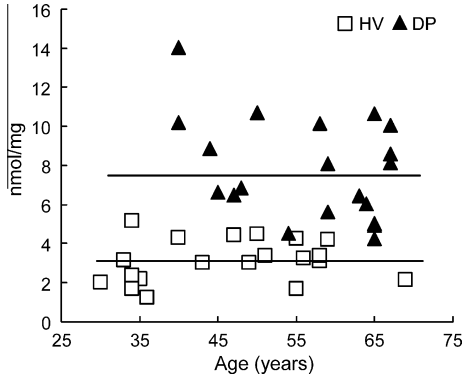

Fig.9. Relation between nail 3-DG amounts and age in healthy volunteers and diabetic patients. HV, healthy volunteers; DP, diabetic patients.

healthy volunteers and diabetic patients. Judging from the figure for the concentration of the 3-DG of human nails, the concentration for the diabetic patients was higher than that for the healthy volunteers but did not increase with age. Therefore, this analytical technique could be used as a noninvasive procedure to assist in the diagnosis and assessment of disease activity in diabetic patients.

Conclusion

In this study, the resolution of dicarbonyl intermediates of AGEs in the human nail was carried out by a combination of DMPD and UPLC–ESI–TOF–MS. Because the proposed method provides trace detection of the dicarbonyl intermediates of AGEs in the human nail, this study was useful for rapidly detecting 3-DG, MG, and GO from the nails of diabetic patients and healthy volunteers. The current study identifies, for the first time, the importance of three kinds of dicarbonyl intermediates of AGEs in the human nail. There was no significant difference in the content of the MG and GO in nails. However, a statistically significant ($P < 0.001$) and strong correlation was observed between the 3-DG concentrations. 3-DG may be useful as a potential diagnostic marker in patients with diabetes. The procedure reported here is reliable and sensitive; the current assay is a useful tool for assessing the relationship between the Maillard reaction and diabetic complications through the determination of 3-DG concentrations in the human nail.

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