Quantification of Modified Tyrosines in Healthy and Diabetic Human Urine using Liquid Chromatography/Tandem Mass Spectrometry

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Received 9 June, 2008; Accepted 30 July, 2008

Summary  The quantification of urinary oxidized tyrosines, dityrosine (DiY), nitrotyrosine (NY), bromotyrosine (BrY), and dibromotyrosine (DiBrY), was accomplished by quadruple liquid chromatography-tandem mass spectrometry (LC/MS/MS). The sample was partially purified by solid phase extraction, and was then applied to the LC/MS/MS using multiple-reaction monitoring (MRM) methods. The analysis for the DiY quantification was done first. The residual samples were further butylated with n-butanol/HCl, and the other modified tyrosines were then quantified with isotopic dilution methods. MRM peaks of the modified tyrosines (DiY, NY, BrY, and DiBrY) from human urine were measured and the elution times coincided with the authentic and isotopic standards. The amounts of modified tyrosines in healthy human urine (n = 23) were 8.8 ± 0.6 (DiY), 1.4 ± 0.4 (NY), 3.8 ± 0.3 (BrY), and 0.7 ± 0.1 (DiBrY) µmol/mol of creatinine, respectively. A comparison of the modified tyrosines with urinary 8-oxo-deoxyguanosine, pentosidine, and Nε-(hexanoyl)lysine was also performed. Almost all products, except for NY, showed good correlations with each other. The amounts of the modified tyrosines (NY, BrY, and DiBrY) in the diabetic urine were higher than those in the urine from healthy people.

Key Words: dityrosine, nitrotyrosine, halotyrosines, urine, diabetes

Introduction

Oxidative stress may be involved in the initiation and/or progression of various diseases. For example, the pathogenesis of diabetic complications is known to involve oxidative stress [1]. Sources of reactive oxygen species (ROS) include the Maillard reaction products [2, 3] as well as the activated inflammatory system [4]. ROS cause modification of biomolecules such as proteins, lipids, and nucleic acids. The monitoring of oxidatively degraded biomolecules, which can become oxidative stress biomarkers, may act as an alarm/warning for the presence of diseases like diabetes. To monitor these markers, human urine is one of the most suitable sources for non-invasive sampling without inducing pain.

The oxidative modification of proteins often induces structural damage to amino acid residues [5, 6]. Of the twenty amino acids, tyrosine is a unique target for oxidizing species because the profiles of the tyrosine oxidation pro-

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products are different from ROS. For example, 3-nitrotyrosine (NY) is a marker for reactive nitrating species such as peroxynitrite and NO$^-$ [7, 8], which originate from excess nitric oxide (NO); NO should be generated from nitric oxide synthase. Therefore, NY may be a marker of activated macrophages. The halotyrosines, 3-chlorotyrosine (ClY) and 3-bromotyrosine (BrY), appear to be markers of inflammatory tissue damage because eosinophil peroxidase (EPO) and neutrophil myeloperoxidase (MPO) generate halogenating species such as HOCl/HOBr [9–13]. Dityrosine (DiY) is one of the cross-linkers of proteins. DiY can thus become one of the universal protein oxidation markers because DiY can be generated by various ROS, such as peroxynitrite (ONOO$^-$), metal-catalyzed oxidation, and UV-irradiation [14]. These products that originate from tyrosine may serve as oxidative stress biomarkers (Scheme I).

Immunochromatographic approaches have been used to detect the modified tyrosines NY, DiY, and ClY. Antibodies to NY have been prepared and are widely used for immunohistochemical staining, enzyme-linked immunosorbent assay (ELISA), and Western blot analysis [15]. DiY has been immunohistochemically identified in the lipofuscin of pyramidal neurons in the aged human brain [16] and in atherosclerotic lesions in Apo-E deficient mice [17]. Positive staining of DiY has been reported in models of Alzheimer and Parkinson diseases [18, 19]. The antibody to ClY was recently prepared and used in immunohistochemical staining [20, 21]. The preparation of an antibody recognizing dihalotyrosines (both dichloro- and dibromotyrosines) has also been done and the positive immunostaining of the liver of lipopolysaccharide (LPS)-treated mice has already been described [22]. Although these immunochromatographic approaches can visually show the localization of modified tyrosines, this method is not suitable for the “rigid” quantification and chemical identification of the modified tyrosines.

To estimate the exact amount of these tyrosine-derived products, gas-chromatography mass spectrometry (GC/MS) and high performance lipid chromatography (HPLC)-electrochemical detection (ECD) have been used [23–25]. Urinary DiY has also been quantified by GC/MS [26] and by tandem liquid chromatography-mass spectrometry (LC/MS/MS) [27]. A more recent study has reported the simultaneous quantification of DiY, including other modified tyrosines in urine using LC/MS/MS [28]. NY has also been quantified by GC-MS, HPLC-ECD, and LC/MS/MS [24, 25, 29, 30]. To avoid the artificial generation of NY, GC/MS in the negative ion chemical ionization mode has been applied [31]. Halotyrosines, such as ClY, BrY, 3,5-dichlorotyrosine (DiClY), and 3,5-dibromotyrosine (DiBrY), have also been detected in tissues using GC/MS [9, 13, 32–34]. ClY and BrY in human urine have been detected by GC/MS [35]. However, there are few reports on the simultaneous quantification of these several modified tyrosines [28].

In this study, we serially and simultaneously quantified the modified tyrosines and other oxidative stress markers (including advanced glycation end-products [AGEs] and an oxidized nucleoside) in the urine of healthy controls. Moreover, as a preliminary study, we compared the levels of modified tyrosines between healthy controls and people with diabetes.
Materials and Methods

Materials

3-Nitrotyrosine (NY) and 3-chlorotyrosine (C1Y) were purchased from Sigma Chemical Company. 3,5-Dibromotyrosine (DiBrY) was prepared as described below and also obtained from Aldrich. N-Bromosuccinimide (NBS), tyrosine, sodium hypochlorite (NaOCl), and other reagents were purchased from Wako Chemicals Co., Ltd. Stable isotopic tyrosines, \( ^{13}\text{C}_9\)tyrosine and \( ^{13}\text{C}_9\)tyrosine, were obtained from Cambridge Isotope Laboratories, Inc.

Synthesis of modified tyrosines

Synthesis of DiY was done as previously reported [36]. Briefly, L-tyrosine (5 mg) was incubated for 1 h with 75 μg of horseradish peroxidase (HRP) in the presence of 5 μl of 30% H2O2 in 5 ml of 0.1 M borate buffer (pH 9.0). The mixture was acidified by the addition of 500 μl of 6 N HCl and 100 μl of trifluoroacetic acid (TFA) and then applied to a solid phase extraction (SPE) column (Supelco Speclidean ENV1-18, 500 mg solid). The column was then washed with 2 ml of 0.1% TFA, and the sample was eluted with 1 ml of 0.1% TFA/CH3OH (1:3). The eluate was concentrated and further purified by reversed-phase HPLC [36]. BrY and DiBrY were simultaneously prepared by treating L-tyrosine (2 mM) with 2 mM of NBS in water. The reaction was terminated by the addition of L-methionine (final concentration 3 mM). The crude reaction mixture was acidified by the addition of a bolus of TFA solution. The reaction mixture was then applied to an SPE ENV1-18 column, which was washed with 2 ml of 0.1% TFA, and the sample was then eluted with 2 ml of CH3OH. BrY and DiBrY in the eluate were concentrated and further purified by reversed-phase HPLC using Develosil ODS-HG-5 (8 × 250 mm) equilibrated with 0.1% acetic acid/CH3CN at a flow rate of 2 ml/min. In addition, commercially-obtained DiBrY was also used. DiC1Y was prepared from N-acetyl-tyrosine (NAY) in several steps [22]. NAY (2 mM) was chlorinated by exposure to 2 mM NaOCl in 0.1 M phosphate buffer (pH 7.4) for 24 h at room temperature. The reaction was terminated by the addition of 5 mM L-methionine. The reaction mixture was concentrated by freeze-drying and N-acetyl-3,5-dichlorotyrosine (NADiC1Y) in the sample was purified by reversed-phase HPLC. The sample was applied to the ODS-HG-5 (8 × 250 mm) using 0.1% TFA/CH3OH (1:1) at a flow rate of 2.0 ml/min, and the peak was collected and further purified. The purified NADiC1Y was then hydrolyzed by 6 N HCl at 105°C for 24 h. The generated DiC1Y was purified by reversed phase HPLC using the ODS-HG-5 (8 × 250 mm) with 0.1% acetic acid/CH3OH (7:3) as the eluent at a flow rate of 2.0 ml/min. The concentration was estimated by quantification of the amino residues using trinitrobenzenesulfonic acid (TNBS) [37].

As internal standards, stable isotopic DiY \( \left( ^{13}\text{C}_9\right)\)DiY, was prepared from \( ^{13}\text{C}_9\)tyrosine by treatment with HRP and H2O2, and then purified as described above. To prepare the isotopic mono- and di-bromotyrosines, \( ^{13}\text{C}_9\)tyrosine was modified by NBS as described above and the formed bromotyrosines were purified by HPLC. To prepare the isotopic mono- and di-chlorotyrosines, N-acetyl-\( ^{13}\text{C}_9\)tyrosine was prepared first. Briefly, \( ^{13}\text{C}_9\)tyrosine (70 μM) was acetylated by acetic anhydrate (0.36 mM) in a saturated sodium acetate solution for 24 h at room temperature. The sample was partly purified using the SPE column (ENV1-18, 500 mg), which was then washed with 1 ml of 0.1% TFA and eluted with 3 ml of CH3OH. The eluate was concentrated and purified by reversed phase HPLC using an ODS-HG-5 (8 × 250 mm) with 0.1% TFA/CH3OH (75:25) at a flow rate of 2.0 ml/min by monitoring at UV 215 nm. The N-acetyl-O-acetyl-\( ^{13}\text{C}_9\)tyrosine obtained was treated for 24 h with 0.1 M borate buffer (pH 9.0) to remove the O-acetyl moiety and the resulting N-acetyl-\( ^{13}\text{C}_9\)tyrosine was purified by reversed phase HPLC using the ODS-HG-5 (8 × 250 mm) with a solvent of 0.1% acetic acid/CH3OH (7:3) at a flow rate of 2.0 ml/min. The purified stable NAY was further modified by NaOCl as described above. After purifying the chlorinated N-acetyl-\( ^{13}\text{C}_9\)tyrosines by HPLC, N-acetyl-\( ^{13}\text{Br}_9\)chlorotyrosine and N-acetyl-\( ^{13}\text{C}_9\)dichlorotyrosine were de-acetylated by acid hydrolysis, and further purified as described above. \( ^{13}\text{C}_9\)NY was synthesized by treating \( ^{13}\text{C}_9\)tyrosine with peroxynitrite. Briefly, peroxynitrite was prepared as previously described [38, 39]. The tyrosine (0.2 mg/ml) was reacted with 1 mM Fe/ethylenediaminetetraacetic acid (EDTA) (pH 7.4) [15]. The reaction mixture was acidified by the addition of TFA and then applied to a solid-phase extraction (SPE) column (ENV1-18). The SPE column was washed with 2 ml of 0.1% TFA and then eluted with 2 ml of 0.1% TFA/methanol (1:1). The eluate was collected and further purified by reversed-phase HPLC using the ODS-HG-5 (8 × 250 mm) with the solvent 0.1% acetic acid/CH3OH (7:3) at a flow rate of 2.0 ml/min by monitoring at UV 274 nm.

Sample preparation

Human urine was collected as a spot sample after approval by the Nakatsugawa Municipal Hospital committee. The urine was frozen at −70°C until use. The sample was dissolved and centrifuged to remove any insoluble materials. The supernatant was collected and 300 μl of the sample was used for the analysis of the modified tyrosines. An internal standard cocktail (containing 500 nM of \( ^{13}\text{C}_9\)tyrosine and 50 nM of \( ^{13}\text{C}_9\)DiY, \( ^{13}\text{C}_9\)C1Y, \( ^{13}\text{C}_9\)DiC1Y, \( ^{13}\text{C}_9\)NY, \( ^{13}\text{Br}_9\)Y, and \( ^{13}\text{C}_9\)DiBrY) was added to the sample and the proteins in the urine were precipitated by 0.1% TFA and 10% trichloroacetic acid (TCA). The sample was centri-
fuged to remove the proteins and the supernatant underwent solid phase extraction (ENVI-18 [500 mg]), which had been preconditioned by 2 ml of methanol, followed by 6 ml of 0.1% TFA. The SPE column was washed by 3 ml of 0.1% TFA and the sample was eluted in three 750 μl aliquots of 0.1% TFA/methanol (2:3). The combined eluate (approx. 2.25 ml) was concentrated by centrifugal evaporation, and the dried sample was dissolved in 250 μl of 2 mM ammonium formate and analyzed by LC/MS/MS as described below. As another method to remove urinary proteins, CH3CN (9:1) was added to the urine and the sample was centrifuged. The upper layer was evaporated using a centrifugal evaporator, dissolved in 0.1% TFA and applied to an ENVI-18 SPE column. While a previous report showed the possibility of an artifact generation of NY by acid plus nitrite [40], NY formation by TCA precipitation could not be confirmed in our urine samples.

First LC/MS/MS analysis for tyrosine and dityrosine (Method I)

The SPE-purified urine (10 μl) was analyzed twice using an electrospray-ionization quadrupole tandem mass spectrometer (API-3000, Applied Biosystems Co.) connected to an Agilent 1100 HPLC system. Data were calculated from the averages of two serial analyses of one sample. For the DiY and tyrosine analyses, HPLC was done by gradient systems using solvent A (0.1% acetic acid) and solvent B (CH3CN). The separation was performed using the ODS-HG-3 (2 × 50 mm). The gradient program is as follows: 0 min (A100%), 2 min (A100%), 7 min (A50%), 7.1 min (A100%), 15 min (A100%). The positive mode was used for the electrospray ionization (ESI). To reduce possible contamination of the ion source, the flow was separated by a Valco switching valve using the following program: 0 min (waste), 2 min (mass), 7 min (waste). The four multiple reaction monitoring (MRM) transitions used for the DiY were as follows: 379.2/332.1 (internal standard, [13C6]DiY-1), 379.2/253.1 (internal standard, [13C6]DiY-2), 361.2/315.1 (native DiY-1), and 361.2/237.1 (native DiY-2). The two MRM combinations for one molecule showed a similar sensitivity. The use of two MRMs on one molecule is meaningful for the identification of DiY. To estimate urinary tyrosine, the analyte was diluted 100-fold by a solution of 2 mM ammonium formate with an additional internal standard (500 nM of [13C6]tyrosine) and then analyzed separately. Two MRMs 182.2/136.1 (tyrosine) and 188.1/142.1 ([13C6]tyrosine) were used for the analysis. To check for artifact formation of DiY during sample preparation, the MRM of the [13C6]tyrosine-derived DiY was also determined regularly. A summary of the MRM scan settings is listed in Table 1. To avoid a decrease in the S/N ratio, the MRM scan program for the [13C6]tyrosine-derived DiY was omitted in routine analyses.

Table 1. Conditions for urinary dityrosine and tyrosine analyses by LC/MS/MS

| Sample   | Q1  | Q3  | Scan time (msec) |
|----------|-----|-----|------------------|
| Tyr      | 182.2 | 136.1 | 150              |
| [13C6]Tyr | 188.1 | 142.1 | 150              |
| DiY-1    | 361.2 | 237.1 | 150              |
| [13C6]DiY-1 | 379.2 | 253.1 | 150              |
| DiY-2    | 361.2 | 315.1 | 150              |
| [13C6]DiY-2 | 379.2 | 332.1 | 150              |
| [13C6]DiY-2* | 373.2 | 249.1 | 150              |

* Asterisks show omission of setting for actual routine analysis.

Second LC/MS/MS analysis for butylated modified tyrosines (Method II)

The rest of the DiY analysis was used for analysis of butylated modified tyrosine. The sample, which had already been enriched with the stable isotopes, was recovered in a glass tube and concentrated by centrifugal evaporation. The dried sample was butylated with 500 μl of n-butanol/HCl (2:1) at 65°C for 30 min. The sample was again evaporated by centrifugal evaporation and then dissolved with 200 μl of 0.05% formic acid/CH3CN (95:5). The HPLC was done by gradient systems using 0.05% formic acid (solvent A) and CH3CN (solvent B). The separation was performed using the ODS-SR-5 (2 × 150 mm), with a gradient program as follows: 0 min (A80%), 2 min (A 80%), 22 min (A70%), 23 min (A50%), 25 min (A50%), 25.1 min (A80%), and 35 min (A80%). The positive mode was used for the ionization. Twenty MRM transitions, which were derived from the liberation of the butyl moiety, were used for the analysis. The switching valve was set as follows: 0 min (waste), 3 min (mass), 20 min (waste). To check the artifact formation of the modified tyrosines during sample preparation, the MRM of the modified [13C6]tyrosines was also determined. A summary of the MRM scan settings for butylated samples is listed in Table 2.

Addition and recovery experiments

The coefficient of variance (CV) was estimated using 12 urine sample that were enriched with 50 nM of authentic modified tyrosines. The samples were processed and analyzed by LC/MS/MS as described above. Estimation of the recovery of the added modified tyrosines was done as follows. The authentic modified tyrosines (final concentration 0.325, 0.651, 1.30, 2.60, 5.21, 10.4, 20.8, 41.7, 83.3, 167, 333 nM) were added into the urine samples and then processed for the LC/MS/MS analysis as described above.

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Table 2. Conditions for butylated urinary NY, CIY, BrY, DiC1Y, and DiBrY analyses by LC/MS/MS

| Butylated Sample | Q1    | Q3    | Scan time (msec) |
|------------------|-------|-------|------------------|
| NY               | 283.2 | 181.0 | 30               |
| [13C6]NY         | 292.5 | 189.0 | 30               |
| [13C6]NY*        | 289.2 | 187.0 | 30               |
| 35CIY            | 272.2 | 170.0 | 30               |
| [13C6]35CIY      | 281.0 | 178.0 | 30               |
| [13C6]35CIY*     | 278.2 | 176.0 | 30               |
| 37CIY            | 274.2 | 172.0 | 30               |
| [13C6]37CIY      | 283.0 | 180.0 | 30               |
| 79BrY            | 316.0 | 214.0 | 30               |
| [13C6]79BrY      | 325.0 | 222.0 | 30               |
| [13C6]79BrY*     | 322.1 | 220.0 | 30               |
| 81BrY            | 318.0 | 216.0 | 30               |
| [13C6]81BrY      | 327.0 | 224.0 | 30               |
| [13C6]81BrY*     | 324.1 | 222.0 | 30               |
| 35Cl 35CIY       | 306.1 | 204.0 | 30               |
| [13C6]35Cl 35CIY| 315.0 | 212.0 | 30               |
| [13C6]35Cl 35CIY*| 312.1 | 210.0 | 30               |
| 79Br 79BrY       | 394.0 | 292.0 | 30               |
| [13C6]79Br 79BrY | 403.0 | 300.0 | 30               |
| [13C6]79Br 79BrY*| 400.0 | 298.0 | 30               |
| 81Br 81BrY       | 396.0 | 294.0 | 30               |
| [13C6]81Br 81BrY | 405.0 | 302.0 | 30               |
| [13C6]81Br 81BrY*| 402.0 | 300.0 | 30               |
| 81Br 81BrY       | 398.0 | 296.0 | 30               |
| [13C6]81Br 81BrY | 407.0 | 304.0 | 30               |
| [13C6]81Br 81BrY*| 404.0 | 302.0 | 30               |

* Asterisks (*) show omission of setting for actual routine analysis.

Estimation of oxidatively modified tyrosines in human urine

Urinary 8-oxo-deoxyguanosine (8-Oxo-dG) was determined using an ELISA kit (JAICA, Nikken Zeil Co.). It has been reported that the heating procedure of frozen urine improves the recovery of urinary 8-Oxo-dG [41]. Because we could not obtain constant results by the heating, the supernatant of centrifuged urine without heat was used for the estimation of urinary 8-Oxo-dG. MRX (Maillard Reaction Product X) and pentosidine in the urine were determined by an HPLC-fluorescence method [42]. Urinary creatinines were determined by the Creatinine Test Wako (WAKO Pure Chemicals). Urinary N-(hexanoyl)lysine (HEL) was estimated by LC/MS/MS as described elsewhere [43, 44].

Statistical analysis

Values (urinary biomarkers/creatinine) are expressed as means ± SEM. Spearman’s rank correlation was used to evaluate the relationship between the two biomarkers. Comparisons between two groups were made using the Mann-Whitney test.

Results

To develop the detection method of trace amounts of urinary modified tyrosines by LC/MS/MS with stable isotopic methods, the pattern of the collision-induced dissociation was investigated by infusion analysis of authentic modified tyrosines. To prevent the artificial conversion of the isotopic internal standard of tyrosine into the isotopic modified tyrosine, we used [13C6]tyrosine as the internal standard for the tyrosine analysis itself, and [13C6]tyrosine was used as the parent molecule for the synthesis of the six stable modified tyrosine isotopes. Several fragments, which occurred owing to the collision-induced fragmentation of DiY, were observed [45]. Of these, the combination of Q1/Q3 ions, 361/273 (for stable DiY) and 361/315 (for native DiY) are similar and have a good sensitivity. The detection limit of DiY was 1 nM and the calibration curve of DiY was linear (R = 0.999) over a concentration range of 1–300 nM. Using the selected MRM transitions, the DiY in human urine was estimated by the LC/MS/MS. DiY in some urine samples cannot be determined owing to contaminants in the urine as described elsewhere [28]. Therefore, before the LC/MS/MS analyses, the urine samples were uniformly purified by solid-phase extraction (SPE). The DiY in all the SPE-treated urines can be detected by the mass spectrometer (a typical chromatogram is shown in Fig. 1). The CV for supplemented 50 nM DiY into the urine was 2.4% and the recovery of authentic DiY in the urine was significantly high (R = 0.998). We called the determination methods as “Method I” and the MRM combination is shown in Table 1. Because we did not prepare the standards of the [13C6]tyrosine-derived modified tyrosines including [13C12]DiY, the accurate quantification of these modified products, which have a backbone of [13C6]tyrosine, could not be done. The artifact DiY ([13C12]DiY) was observed at very low levels on the MRM chart. For tyrosine and the other modified tyrosines, including stable isotopes, the estimated MRM combinations are derived from the liberation of HCOOH (−46) from the precursor ions. For example, the MRMs 182.1/136.1 for the native and 188.1/142.1 for the stable isotope tyrosine, [13C6]tyrosine, were used to quantify tyrosine levels. By checking the formation of the [13C6]tyrosine-derived modified tyrosine, artifact generation of the modified tyrosine can be examined during sample preparation. We attempted the quantification of NY and halogenated tyrosines (such as CIY and BrY) in urine by Method I. However, the free halotyrosines could not be determined in the SPE-treated human urine. NY cannot be clearly detected.
because of a co-eluting contaminating large unknown product, which has the same signal of MRM 227.2/181.2 for NY (data not shown).

To detect the NY and halotyrosines, the rest of DiY analyte, which included the stable internal standards mixture, was further derivatized by butanol/HCl, and the successive butylated tyrosines were analyzed by LC/MS/MS (Method II) as described in the Materials and Methods section. To evaluate the artifact formation of the modified tyrosines, butylated modified tyrosines with a $^{13}$C$_6$ ring were analyzed. The characteristic collision-induced fragmentations caused by the liberation of the butyl moiety (–102 for native, –103 for stable internal standard) were used for the MRM transmission of all the analyses for the butylated modified tyrosines [27]. The detection limits were approximately 0.1 nM (CIY and DiBrY), 0.3 nM (BrY, DiCIY), and 1 nM (NY). Calibration curves of Method II were linear (R>0.99) over a concentration range of 1–300 nM for NY, 0.1–300 nM for CIY, 0.3–300 nM for BrY, and 0.1–300 nM for DiBrY. Using Method II, NY, BrY, and DiBrY in the urine were successfully determined simultaneously (Figs. 2–4 and Table 2). Urinary NY was detected without interference from any unknown contaminants. Each MRM peak was co-eluted with the “partner” internal standard which have a $^{13}$C$_6$ backbone. Moreover, the difference in the natural existence ratio of isotopes for $^{79}$Br and $^{81}$Br (1:1) was also used for identification. Because BrY has one Br atom with an equal ratio of $^{79}$Br:$^{81}$Br, $^{79}$BrY and $^{81}$BrY show a similar intensity (Fig. 3). DiBrY shows a unique mass profile of three major isotopic peaks (triplet peak, 1:2:1) because DiBrY consists of $^{79}$Br$^{79}$BrY, $^{79}$Br$^{81}$BrY, and $^{81}$Br$^{81}$BrY. Whereas a similar analogy of the isotope ratio is applicable to the identification of chlorotyrosine (CIY), CIY could not be clearly detected from all samples, as successful detection of CIY was dependent on the behavior of the samples. DiCIY could not be detected in most samples. It seems likely that the urinary modified tyrosines are excreted as conjugates such as glucuronide conjugates. However, the treatment of a urine sample with β-glucuronidase/sulfatase had no effect on the level of the modified tyrosines (unpublished observation). The recovery of the additional authentic NY, CIY, DiCIY, BrY, and DiBrY was also estimated (NY, R = 0.999; CIY 0.991; DiCIY, 0.999; BrY, 0.995; DiBrY, 0.998). The CV
of the 50 nM standards into the urine were 4.9 (NY), 2.9 (ClY), 4.5 (DiClY), 4.7 (BrY), and 5.1 (DiBrY). The addition of phenol to the urine prior to sample preparation had no effects on the amount of the modified tyrosines (data not shown), suggesting that artifact generation during sample preparation is negligible.

First, we analyzed the urine from healthy adults (n = 23, Table 3) using Methods I and II, and correlations between several tyrosine-derived products were examined. We also determined the amounts of urinary 8-Oxo-dG and Nε-(hexanoyl)lysine (HEL) [43, 44]. Urinary pentosidine and MRX [47], which are advanced glycation end-products, were also determined. The levels of urinary 8-Oxo-dG, HEL, pentosidine, and MRX were 8.8 ± 0.9, 2.3 ± 0.2, 17.0 ± 0.9, and 539 ± 70 μmol/mol of creatinine, respectively. The amounts of the modified tyrosines in healthy human urine (n = 23) were 8.8 ± 0.6 (DiY), 1.4 ± 0.4 (NY), 3.8 ± 0.3 (BrY), and 0.7 ± 0.1 (DiBrY) μmol/mol of creatinine. The relationship between each modified tyrosine and the other biomarkers are summarized in Table 4. Of the modified tyrosines examined, DiY was significantly correlated with BrY, DiBrY, HEL, 8-Oxo-dG, pentosidine, and MRX (p < 0.01). NY was not correlated with the other markers, suggesting that the in vivo mechanism of NY production is quite different. Urinary 8-Oxo-dG was significantly correlated with urinary HEL, DiY, BrY, DiBrY, and pentosidine (p < 0.01), and urinary HEL was significantly correlated.
with urinary DiY \( (p<0.01) \), DiBrY \( (p<0.05) \), 8-Oxo-dG, and pentosidine \( (p<0.01) \). Pentosidine and MRX were also significantly correlated \( (p<0.01) \). In addition, there were no significant changes in the amounts of modified tyrosines in urines by aging or smoking status.

Next, we investigated the effects of diabetes on the generation of these modified tyrosines. People with diabetes (without nephropathy, \( n=12 \)) were selected for this pilot study because it is known that the disease is associated with oxidative stress. The concentration of the modified tyrosines was corrected for urinary creatinine. The three markers, NY, BrY, and DiBrY, were significantly increased in diabetic urine compared with the healthy controls (Fig. 5). When the modified tyrosine per tyrosine was calculated, the tendency was similar to the creatinine correction. However, the value from the tyrosine correlation varied widely compared with creatinine correction.

**Discussion**

The estimation of urinary modified tyrosines would be a useful tool for the evaluation of the oxidation/reduction balance in our body. Indeed, we found differences in the urinary modified tyrosines between healthy people and people with diabetes. Diabetic complications are commonly associated oxidative stress [1]. The simultaneous determination of DiY, halotyrosines, and NY should show a “profile” of oxidative stress. We have also examined the effect of cocoa drink on the contents of urinary modified tyrosines [46].

The simultaneous determination of modified tyrosines, DiY and NY, in human urine by HPLC-atmospheric pressure chemical ionization tandem mass spectrometry (HPLC-APCI-MS/MS) has been reported [28]. The determination was carried out without a pretreatment. However, DiY in some samples (2–3 of eight) could not be determined by this method. Moreover, NY and ClY could not be determined in intact urine. A recent report described an immunoaffinity LC/MS/MS method to measure 3-NY in various biological fluids at very low levels [47]. However, that method will be difficult to apply for simultaneous determination of several modified tyrosines because the method needs specific antibodies to each modified tyrosine. We have also found that modified tyrosines, except for DiY, could not be detected by LC/MS/MS using urine without the derivatization step in our Method I. Butylation of the samples resolved this problem, except for the detection of the urinary ClYs. Levels of DiY, NY, BrY, and DiBrY were successfully quantified by our serial and simultaneous Methods I–II in all of the urine samples examined. Our HPLC-ESI-LC/MS/MS methods required several processes including protein precipitation, solid phase extraction, and further additional
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We determined DiY separately without any derivatization because it is difficult to control the incorporation of the butyl moiety into a DiY, which has two COOH terminals [27]. It has been reported that DiY, NY, and α-Tyr in cat urine could be quantified by butylation of samples using HPLC-ESI-(quadruple)MS/MS and a quadruple/time of flight (Q-TOF) tandem MS [27]. Using our methods, CIY and DiCIY were not completely determined in human urine samples, but the BrYs were. The CIYs (especially DiCIY) were below the detection limit in some urine samples, probably because the amount of BrY in human urine is about 10-times higher than that of CIY [35].

In this research study, we detected urinary DiY, NY, BrY, and DiBrY and simultaneously quantified their levels using quadruple mass spectrometry with liquid chromatography. The determination of these modified tyrosines has been performed by GC/MS [24, 25, 31]. Recently, Mita et al. detected CIY and BrY in 2 ml of human urine by GC/MS [35]. They reported that the urinary BrY is 22.6 ± 10.8 ng/m mol of creatinine, which can be converted into about 8 μmol/mol of creatinine. On the other hand, we found that the urinary BrY is 3.8 ± 0.3 μmol/mol of creatinine. Our methods require only 300 μl of urine for determination of the four modified tyrosines. The concentration of (non-septic) human control urinary DiY has been shown to be approximately 3.9 ± 1.0 μmol/mol of creatinine by GC/MS [48]. It has also been shown that urinary DiY is 5.8 ± 0.3 μmol/mol of creatinine (morning urine) or 12.5 ± 5 μmol/mol of creatinine (first following urine) by HPLC-APCI-MS/MS [28]. The same group also showed that the mean urinary DiY concentration was 10.1 ± 0.4 μmol/mol of creatinine [49]. We detected DiY at a concentration of 8.8 ± 0.6 μmol/mol of creatinine in the control human urine. The quantification of DiY and NY were also reported as 22.0 to 49.1 DiY/creatinine (μmol/mol) and 0.1 to 0.4 NY/creatinine (μmol/mol) in cat urine by LC/ESI-MS/MS [27].

Peroxidasıes, such as MPO, can modify the tyrosine moiety to DiY [50]. Metal-catalyzed oxidation systems are also considered to be a plausible generator of DiY [51–57]. Peroxynitrite leads to the formation of DiY as well as 3-NY [14] and photo-oxidation is also a plausible source for DiY generation [36]. In this study, urinary DiY was significantly correlated with the various markers examined. These results may indicate that DiY is one of the important universal biomarkers of oxidative stress. The enhancement of DiY in urine samples in people from people with diabetes has been reported [58]. However, in our study, no statistical difference in DiY was observed but the urinary DiY tended to increase in people with diabetes. In addition, we found that the urinary DiY in people with diabetes (n = 12) is significantly higher than in healthy controls (n = 41) (p<0.05, unpublished observation). It has already been shown that the formation of DiY occurs in the kidneys of diabetic Akita mice [59].
Acknowledgements

We thank Fushimi Pharmaceutical Co., Ltd. for determining MRX and pentosidine. We thank Shinsuke Hisaka for his helpful technical assistance. We also thank Dr. Anthony J. Kettle for his critical reading and helpful advice for this manuscript. This work was supported in part by a Grant-in-Aid (Y. K.) for Encouragement of Young Scientists (No. 16780097) from the Japan Society for the Promotion of Science.

Abbreviations

ROS, reactive oxygen species; NY, 3-nitrotyrosine; CIY, 3-chlorotyrosine; BrY, 3-bromotyrosine; EPO, eosinophil peroxidase; MPO, myeloperoxidase; DIY, 3,3′-dityrosine; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; GC/MS, gas chromatography-mass spectrometry; ESI, electrospray ionization; Q-TOF, quadruple-time of flight tandem mass spectrometry; APCI, atmospheric pressure chemical ionization; LC/MS/MS, liquid chromatography-tandem mass spectrometry; SPE, solid phase extraction; NAY, N-acetyl-tyrosine; NBS, N-bromosuccinimide; HRP, horseradish peroxidase; MPR, myeloperoxidase and protein oxidation in cystic fibrosis. Am. J. Physiol. Lung Cell Mol. Physiol., 279, L537–546, 2000.

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