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Short communication

Sensitive determination of nitrotyrosine in human plasma by isocratic high-performance liquid chromatography

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Abstract

A highly sensitive and simple isocratic high-performance liquid chromatographic method was developed for determination of 3-nitrotyrosine in human plasma with precolumn derivatization with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole. The precision of the method was satisfactory (coefficient of variation 4.8%), and the detection limit was established at 0.1 pmol of 3-nitrotyrosine allowing the determination at the level of 6 pmol/ml in human plasma. The recoveries of 3-nitrotyrosine and α-methyltyrosine, an internal standard, were 89.3 ± 7.1 and 85.7 ± 7.6%, respectively. The 3-nitrotyrosine level was 31 ± 6 pmol/ml (mean±S.D., $n=9$) in plasma from healthy volunteers. Since 3-nitrotyrosine is a stable product of peroxynitrite, an oxidant formed by a reaction of nitric oxide and superoxide radicals, the measurement of its plasma concentration may be useful as a marker of nitric oxide-dependent oxidative damage.

Keywords: Nitrotyrosine

1. Introduction

Nitric oxide (NO), a diffusible gas with a short half-life, is produced by endothelial cells, macrophages and neurons and plays several important roles [1–3]. However, the interaction with superoxide (O_2^-) forms peroxynitrite ($ONOO^-$), a powerful oxidant capable of causing pathological damages in conditions such as inflammation, atherosclerosis, ischemia-reperfusion, and lung injury [4–9]. Peroxynitrite can decompose to give a range of products, including hydroxy radicals and nitronium ion, which results in nitration of aromatic amino acids. Nitration on the *ortho* position of tyrosine (Tyr) is a major reaction. Therefore, the determination of nitrotyrosine (NO_2-Tyr) may reflect the degree of peroxynitrite (nitric oxide and superoxide)-dependent tissue damage, since it is difficult to demonstrate the other oxidative products of peroxynitrite [2,4–8,10].

Immunohistochemical investigations have demonstrated the existence of NO_2-Tyr residues in proteins of damaged tissues, using polyclonal and monoclonal antibodies [6–8]. However, no sensitive method has been developed to detect NO_2-Tyr levels in soluble fractions. Although, in some pathological conditions, elevated NO_2-Tyr levels were demonstrated by reversed-phase high-performance liquid chromatography (HPLC) with a direct detection of its own ultraviolet absorbance (274 nm), the detection limit was 0.2 μM in this technique [4,5]. Serum NO_2-Tyr

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levels in range of 0.0–1.2 μM in patients with rheumatoid arthritis and undetectable in healthy controls were reported [5]. In addition, for the analysis of NO_2-Tyr and its metabolites in urine, the gas chromatographic system with a thermal energy analyser was reported [11]. Although the detection limit was ca. 2 pmol per injection, this method required a purification step with reversed-phase column before precolumn derivatization and chromatographic procedure at high temperature.

Several fluorogenic reactions of amino acids have been exploited in the HPLC analysis of amino acids in plasma [12–16]. However, the NO_2-Tyr levels in plasma of healthy controls have not been reported, because the NO_2-Tyr is predicted to exist at a concentration of less than one-thousandth of that of the other amino acids [5,11,13–16]. The precolumn derivatization with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) is one of the most sensitive methods that can detect several fmol levels of amino acids [15,16]. Therefore, in this paper, we adopted the precolumn derivatization with NBD-F and an isocratic separation with reversed-phase HPLC to detect sub-pmol levels of NO_2-Tyr in human plasma.

2. Experimental

2.1. Chemicals

3-Nitro-L-tyrosine (NO_2-Tyr), L-tyrosine (Tyr), α-methyl-L-p-tyrosine (Me-Tyr), glycine-p-nitroanilide, 3-methoxy-L-tyrosine, p-nitrophenethylamine, and p-nitro-L-phenylalanine were purchased from Sigma (St. Louis, MO, USA). Amino acids standard solution (L-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine, o-phosphoserine, o-phosphoethanolamine, taurine, L-serine, sarcosine, L-proline, hydroxy-L-proline, L-citrulline, glycine, L-alanine, L-α-aminoadipic acid, DL-α-amino-n-butyric acid, ethanolamine, L-valine, L-cystine, L-cystathionine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, β-alanine, DL-β-aminoisobutyric acid, L-threonine, γ-aminobutyric acid, L-ornithine, L-lysine, DL-hydroxylysine, L-histidine, L-1-methylhistidine, L-3-methylhistidine, L-arginine, L-anserine, and L-carnosine) and L-tryptophan were from Wako (Osaka, Japan). 4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) was from Dojindo (Kumamoto, Japan). Methanol (HPLC grade) was from Cica–Merck (Tokyo, Japan). Other chemicals were of analytical reagent grade and obtained from companies listed above.

2.2. Sample preparation

Heparinized blood samples were obtained from nine healthy volunteers (males, aged 20–44) with consent. The separated plasma (60 μl) was mixed with ethanol (140 μl) containing Me-Tyr (150 pmol), as an internal standard. After centrifugation at 10 000 g for 10 min, the resulting supernatant was kept at −80°C until the chromatographic analysis.

2.3. Chromatographic equipments and precolumn derivatization

The HPLC system consisted of solvent delivery pump, autosampler, column oven, fluorescence-spectrophotometer, and integrator (L-6200, AS-4000, L-5020, 650-10LC, and D-2500: Hitachi, Tokyo, Japan). The stored samples (100 μl) were derivatized with 20 μl of 0.1 M sodium borate buffer (pH 8.7) and 20 μl of NBD-F (50 mg/ml in ethanol), incubated at 60°C for 2 min, essentially as reported previously [15,16]. The reaction was terminated by addition of 30 μl of 0.1 M HCl and aliquot (100 μl) was injected onto the column, using a programmable autosampler. The chromatographic elution was carried out at 37°C with a reversed-phase column (Wakosil 5C18, 250×4.6 mm I.D., Wako, Osaka) and a mobile phase (pH 7.2) of 0.1 M sodium phosphate buffer–methanol (45:55, v/v) at a flow-rate of 1.0 ml/min. After 30 min elution, the mobile phase was switched to 80% methanol solution for wash. The fluorescence detector was set at 470 and 530 nm for excitation and emission wavelengths, respectively and slit widths were 5 nm for both. Calculation of NO_2-Tyr concentration in each sample was based on ratio to Me-Tyr, the internal standard.

3. Results and discussion

Fig. 1 shows typical chromatographic patterns of NBD-F derivatized amino acids by HPLC with the reversed-phase column, analyzing samples of human

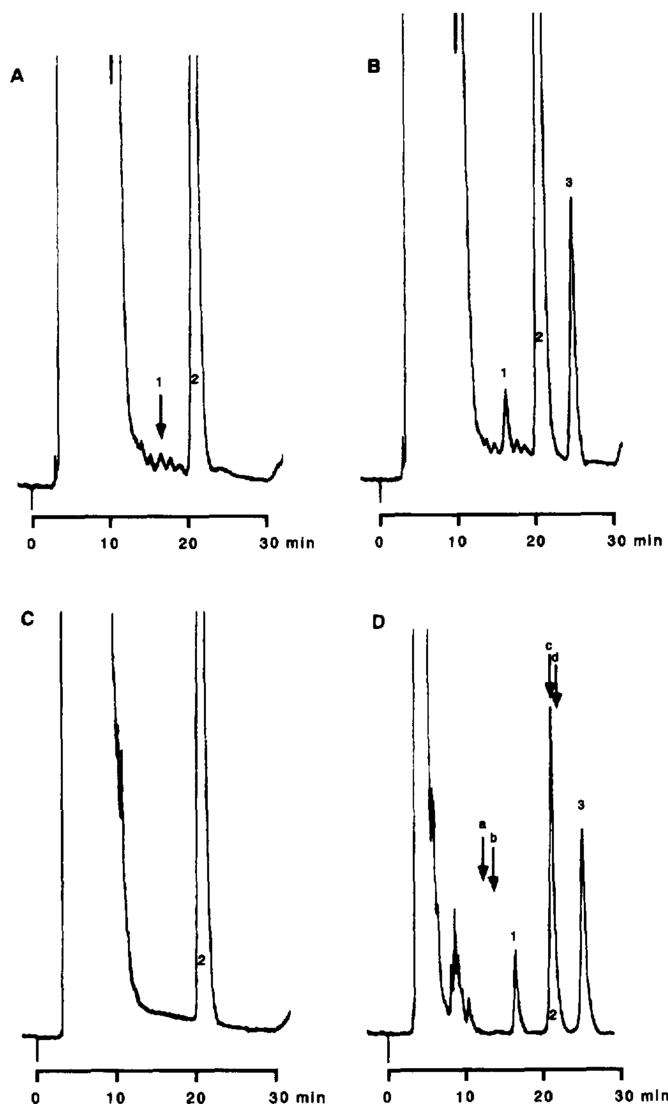

Fig. 1. Typical chromatograms of human plasma without (A) and with exogenous NO_2-Tyr (5 pmol) and Me-Tyr (50 pmol) (B), 39 amino acids standard solution (1–2 nmol, each) (C), and authentic standard sample containing NO_2-Tyr (10 pmol), Tyr (25 pmol), and Me-Tyr (40 pmol) (D). Peaks: 1=3-nitro-L-tyrosine (NO_2-Tyr), 2=L-tyrosine (Tyr), 3=α-methyl-L-p-tyrosine (Me-Tyr). Positions indicated by arrows: (a)=glycine-p-nitroanilide, (b)=3-methoxy-L-tyrosine, (c)=p-nitrophenethylamine, and (d)=L-tryptophan. Although all amino acid-related substances listed in Section 2.1 were analyzed under the same conditions, they were not eluted around peaks of 1–3 except (a)–(d) in Fig. 1D.

plasma with or without authentic NO_2-Tyr and Me-Tyr, 39 biological amino acid-related substance mixture, and a standard solution containing NO_2-Tyr, Tyr and Me-Tyr. The identification of NO_2-Tyr peak in plasma samples was carried out on the basis of the retention time of the authentic amino acid (Fig. 1D), the spiking experiment (Fig. 1A and B), and the comparison of fluorescence spectral analysis. In addition, we investigated various conditions to separate the peak of NO_2-Tyr from those of other biological amino acids by changing the mobile phase: pH, methanol concentration, and addition or replacement with acetonitrile or tetrahydrofuran. Although the present conditions gave the best separation and stable baseline, both peaks of endogenous and authentic NO_2-Tyr were eluted at the same

position as a single peak. The stepwise replacement of methanol by tetrahydrofuran up to 50% made the retention time of NO_2-Tyr longer than that of Tyr but failed to separate the peak of endogenous and authentic NO_2-Tyr. Therefore we considered that the peak indicated by Fig. 1A represents the concentration of NO_2-Tyr in human plasma.

When we examined the 39 amino acid-related materials, which are present in biological materials, and 6 amino acids related to Tyr or containing nitro-group listed in Section 2.1, no amino acid peaks interfered with the quantitation of peaks of NO_2-Tyr and Me-Tyr (Fig. 1C). However, some of them were eluted near these peaks (Fig. 1D). Although the analysis of plasma samples revealed several unknown peaks, there was no peak at the position corresponding to Me-Tyr (Fig. 1A). Therefore, we adopted Me-Tyr as an internal standard to analyze NO_2-Tyr because of the general fluorescence instability of NBD-derivatives of amino acids.

Since the precolumn derivatization of amino aids with NBD-F was reported to allow their analysis at the fmol level [15,16], we expected to determine the plasma concentration of NO_2-Tyr at the same sensitivity. However, a minute amount of unknown substances in plasma caused baseline noise. Therefore the detection limit was set 100 fmol (more than three-fold of the signal-to-noise ratio), which corresponded to 6 pmol/ml of plasma. As discussed above, because human plasma contains ca. 40 amino acid-related substances (each, 1.5–3 nmol/30 ml), we used 40–50 fold amount of NBD-F (5.5 mmol per reaction) to derivatize all amino-group-containing molecules in addition to NO_2-Tyr. This amount of NBD-F was considered sufficient because further increase failed to elevate the peaks. Since NBD-derivatives of amino acids were reported to be unstable to light [15,16], we analyzed standard samples that had been kept under room light for 1, 2, 3 and 4 h after the precolumn derivatization. However, there was no significant decrease in peaks of NO_2-Tyr, Tyr and Me-Tyr, compared to those analyzed immediately after the preparation. NBD-Tyr was reported to be eluted poorly by the solvent containing salts [15,16], unlike the elution profile of NBD-Tyr with 0.1 *M* sodium phosphate buffer in the present experiment. Although the reason is unclear, we could not elute any fluorescent substances when the mobile phase was changed to 55% methanol in water after 30 min analysis of NO_2-Tyr, Tyr and Me-Tyr (Fig. 1D) with the standard mobile phase. The different derivatizing conditions may not allow the conversion of NBD-Tyr to the N,O-bis-NBD-derivative that possesses strong affinity for the reversed-phase column.

Authentic NO_2-Tyr (2–25 pmol), Tyr (5–25 pmol) and Me-Tyr (40 pmol) were analyzed by HPLC and relative ratios to Me-Tyr (y) were plotted as a function of the amount of amino acids (x). Linear relationship was obtained for NO_2-Tyr and Tyr with $y=0.0447x-0.0148$ ($n=28$, $r=0.989$) and $y=0.0619x+0.0292$ ($n=24$, $r=0.988$), respectively, indicating that the fluorescence intensity of NO_2-Tyr was 72% of that of Tyr. When the reproducibility of the method was studied by injecting 18 samples containing NO_2-Tyr (20 pmol) and Me-Tyr (50 pmol), the coefficient of variation was 4.8%. Due to the detection limit, NO_2-Tyr concentrations in the spiked plasma samples were analyzed by both the present and reported methods of HPLC with ultraviolet absorption [5]. In the concentration range 400–2000 pmol/ml, NO_2-Tyr values were similar for both methods with the linear regression of $y=0.999x-11$ ($n=12$, $r=0.997$).

For the determination of normal levels in human plasma, plasma samples were deproteinized with ethanol, derivatized with NBD-F, and analyzed by isocratic HPLC. Fig. 1A and B show representative chromatograms of plasma NO_2-Tyr without and with authentic NO_2-Tyr and Me-Tyr. The recoveries of NO_2-Tyr and Me-Tyr were 89.3 ± 7.1 and 85.7 ± 7.6%, respectively. Both the within-day and the day-to-day coefficients of variation of NO_2-Tyr in separate determinations in plasma were less than 5% ($n=4$ and 5, respectively). The 3-nitrotyrosine level was 31 ± 6 pmol/ml (mean±S.D., ranging 23–40) in plasma from 9 healthy volunteers (males, aged 20–44). These values are approximately more than 4-fold of the detection limit (6 pmol/ml). To our knowledge, this is the first report on normal plasma levels of NO_2-Tyr. In the pathological condition of rheumatoid arthritis, serum levels were reported to be 490 ± 270 pmol/ml (mean±S.D., ranging 0–1200) with undetectable normal levels [5], because the detection limit was as high as 200 pmol/ml by direct absorption at 274 nm. Since our fluorescent de-

rivatization with NBD-F improved the sensitivity of NO_2-Tyr up to 6 pmol/ml of plasma, we were able to detect normal plasma values of less than one-tenth of those in the pathological condition.

According to the ratio of NO_2-Tyr level to Tyr level in normal plasma, 0.05±0.01% (mean±S.D.) of Tyr is present as the nitrated form in human plasma. Furthermore, when we analyzed NO_2-Tyr and Tyr contents in plasma proteins after acid-hydrolysis with 6 *M* HCl at 105°C for 24 h, 0.01–0.07% of Tyr residues were nitrated to NO_2-Tyr residues in proteins. Hydrolysis analysis of commercially available proteins (100 mg) revealed that endogenously nitrated Tyr (NO_2-Tyr) content was 0.04–0.38 nmol [11], corresponding to ca. 0.01–0.1% of Tyr residues in protein. These data were consistent with our present results of the nitration ratios of Tyr in plasma and its proteins. Since NO_2-Tyr level in normal human plasma has not been reported due to the high detection limit, it was predicted to be less than one-thousandth of that of other amino acids [5,11]. Our data support this prediction.

In this paper, we described a highly sensitive and simple isocratic HPLC method for determination of NO_2-Tyr in human plasma with fluorometric detection of the corresponding NBD-derivative. Although only NO_2-Tyr can be measured by this method, the other amino acids levels in plasma were detectable by any amino acid analyzing systems [12–16]. The normal level of NO_2-Tyr in human plasma was found to be 31±6 pmol/ml, which corresponded to 0.05% of Tyr level. Since peroxynitrite generated from superoxide and nitric oxide radicals is a strong and labile oxidant to cause various pathological damages, NO_2-Tyr is considered as the stable marker for peroxynitrite [2,4–10]. Therefore, the rapid HPLC method for determination of NO_2-Tyr in plasma may be one of the important diagnostic procedures for peroxynitrite (nitric oxide and superoxide)-dependent tissue damage.

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