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Quantification of urinary *o,o′*-dityrosine, a biomarker for oxidative damage to proteins, by high performance liquid chromatography with triple quadrupole tandem mass spectrometry
A comparison with ion-trap tandem mass spectrometry

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Abstract

We recently described an isotope dilution reversed-phase liquid chromatography–atmospheric pressure chemical ionization–ion-trap-tandem mass spectrometry (HPLC–APCI–MS/MS) method for the quantitative determination of oxidized amino acids in human urine, including *o,o′*-dityrosine, a specific marker of protein oxidation. In the present study, we investigated the possibility to use a triple quadrupole instrument for the analysis of this biomarker in urine. The two instruments were compared in terms of sensitivity, specificity and reproducibility. Results showed that the triple quadrupole instrument reaches 2.5-fold higher sensitivity (LOD = 0.01 μM) compared to the previously used ion-trap instrument. Precision of the present assay is as follows: in-day variation is 4.6% and inter-day variation is 17%. The currently developed method was applied to a group of smoker urine samples. The mean urinary *o,o′*-dityrosine concentration was 0.08 ± 0.01 μM. Expressed per urinary creatinine concentration, this corresponds to 10.1 ± 0.4 μmol/mol creatinine. This is comparable to the previously reported values of 5.8 ± 0.3 μmol/mol creatinine in non-smokers night-time urines, and 12.3 ± 5 μmol/mol creatinine in day-time urines measured by the ion-trap instrument.

Keywords: *o,o′*-Dityrosine; Atmospheric pressure chemical ionisation; Triple quadrupole tandem mass spectrometry; Isotope dilution; Biomarker; Smoker

1. Introduction

o,o′-Dityrosine (dityrosine) cross-links naturally occur in proteins in many macro- and micro-organisms [1–7]. Vertebrate animal proteins known to contain dityrosine include elastin [8], collagen [9], and a storage form of thyroglobulin [10]. Interest in dityrosine is based on its potential as a specific marker for oxidatively damaged proteins and their selective proteolysis [11,12]. Thus, dityrosine may mark oxidatively damaged proteins in red blood cells exposed to hydrogen peroxide (H_2O_2) for proteolytic destruction [13]. Dityrosine has also been proposed as a biomarker of organismal oxidative stress, since its concentrations were found 100-fold higher in low-density lipoproteins isolated from atherosclerotic lesions than in normal ones [14]. Also, humans suffering from systemic bacterial infections had twice the concentration of dityrosine in urine than those of healthy individuals [14]. Beside these pathologies, endurance exercise, which is associated with increased oxygen consumption and subsequent increased reactive oxygen species (ROS) formation, caused elevation in urinary dityrosine concentrations for several days

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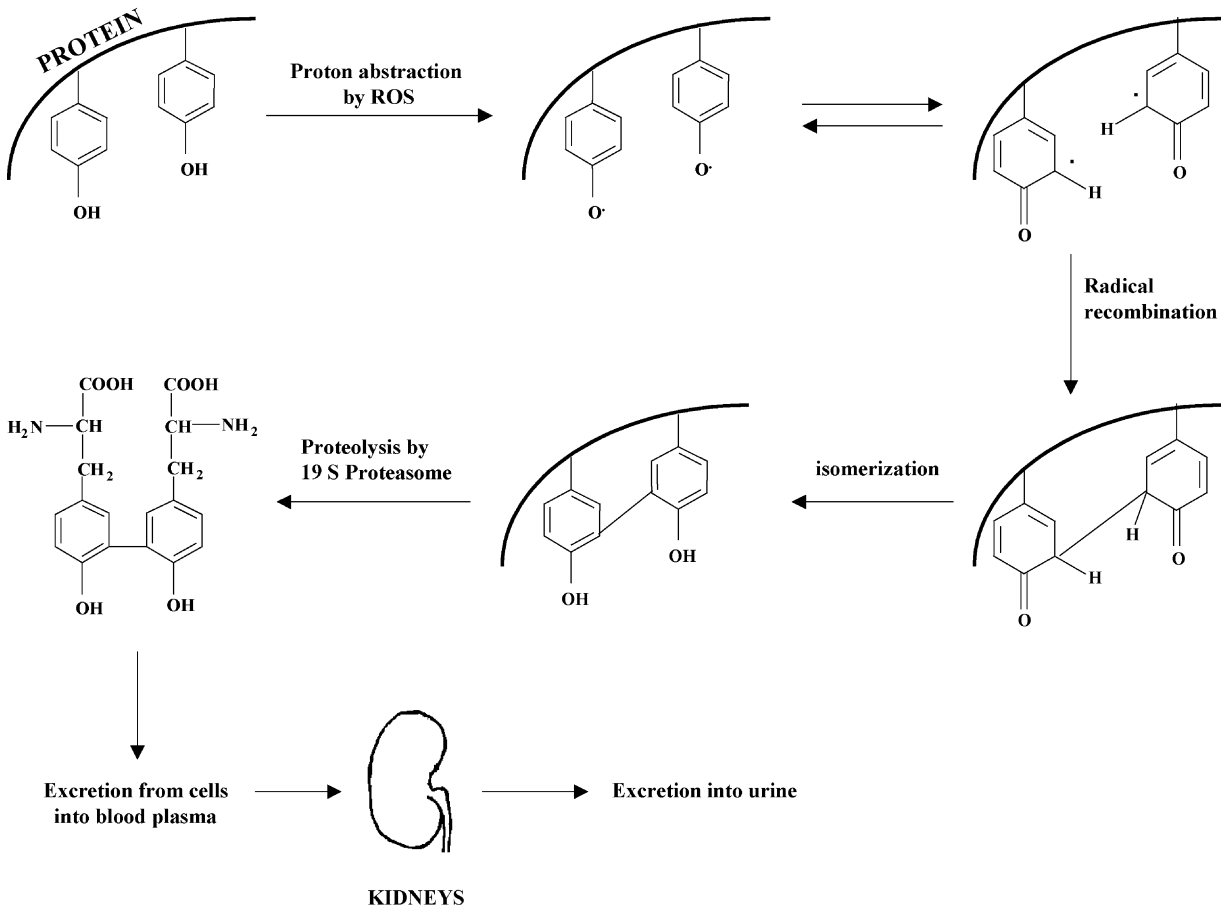

Fig. 1. Mechanism of dityrosine formation from tyrosine residues of proteins and its possible metabolic fate in the body. ROS, reactive oxygen species.

following the exercise [15]. Furthermore, tissue levels of this oxidized amino acid are elevated in prevalent diseases such as atherosclerosis, inflammatory lung disease, neurodegenerative disorders, and aging [16–20].

Dityrosine formation begins with the generation of tyrosyl radicals, followed by radical isomerization and diradical reaction, and finally enolization [21] (Fig. 1). Alternative pathways such as covalent binding of tyrosine to the heme group in myoglobin or addition of oxygen have also been proposed [22]. The reduction potential of the tyrosineO$^{\bullet}$/tyrosineOH couple is 0.88 V [23] indicating that the coupled reaction needs to be highly oxidative to be thermodynamically favourable. In this regard, hydroxyl radical, oxoferryl moiety, porphyrin π cation radical, peroxynitrite, nitrogen dioxide, and nitryl cation, are some of the species that fulfil this requirement [11]. Oxidants generated by activated phagocytes generate dityrosine cross-links in model proteins and lipoproteins in vitro [16,24–26]. NADPH oxidase of phagocytes, which plays a key role in host defences against microbial pathogens, converts oxygen into superoxide anion radical ($O_2^{\bullet -}$) [27,28]. The $O_2^{\bullet -}$ may then dismutase into H_2O_2, which is an oxidizing substrate for myeloperoxidase, a heme protein secreted by activated phagocytes [27,29]. Myeloperoxidase uses H_2O_2 to produce dityrosine cross-links from the tyrosine residues of target proteins [16,24,25]. This pathway was proved to exist also in vivo [30].

Despite the knowledge about their formation, little is known about the metabolic fate of oxidized amino acids released from tissue proteins. One possibility is that oxidized amino acids are excreted from cells and filtered from blood into urine [30]. Alternatively, they may be reabsorbed by the kidney, metabolized to other compounds, or reused for protein synthesis. This has recently been shown for *m*-tyrosine [31]. However, recent rat studies demonstrated that dityrosine is secreted into urine rather than being recycled into proteins [17,32]. As a biomarker, dityrosine has the advantage over other protein oxidation products of being a stable compound and once the 3′-3′ carbon–carbon bond is formed in dityrosine it is resistant to hydrolysis by lytic enzymes [12,13].

Because its universal formation and use as biomarker for oxidative (protein) damage, there is a need for sensitive and reliable analytical method(s) to determine dityrosine in biological media. Several analytical methods have been developed to quantify this product in vivo and in vitro. These include HPLC either with UV or fluorescence detections

[11,33,34], GC–MS analysis after derivatization [16], and LC–tandem MS analysis with APCI or electrospray ionization [35,36]. Unfortunately, HPLC technique with UV or fluorescence detections does not reach enough specificity for the analysis of urine or tissues extracts because several other protein modifications (e.g. the conversion of tryptophan to *N*-formylkynurenine, the conjugation of 4-hydroxynonenal with lysine residues [37], and the formation of retinoic acid adducts with proteins [11]) also yield products that exhibit similar fluorescence characteristics as dityrosine. Furthermore, derivatization-based methods may introduce artifactual oxidation product(s) formation, since they require extreme pH values and high temperature during derivatization. Theoretically, LC–MS based methods without derivatization may offer a solution to these problems. Therefore, we recently developed an isotope dilution HPLC–APCI–MS/MS for the quantitative determination of dityrosine in human urine with an ion-trap instrument [35]. In the present study, we explored the possibility to utilize a triple quadrupole LC–MS for the same task and compared its performance with that of the ion-trap instrument.

2. Materials and methods

2.1. Chemicals

Methanol (99.9%), acetic acid (99.8%), hydrochloric acid (37%) and ammonium acetate (98%) were obtained from Riedel-de-Haën (Seelze, Germany). Nanopure water was obtained from a Milli-Q-system (Millipore, Bedford, MA, USA). Dityrosine and [ring-$^2H_6$]dityrosine were synthesized and purified as described previously [35].

2.2. Sample preparation for analysis by HPLC–APCI–MS/MS

A stock solution of [$^2H_6$]dityrosine at a concentration 130 μM was prepared and 10 μl of this stock solution was added to 250 μl of either standard or urine samples (5 μM final concentration). After mixing, the samples were centrifuged at 14,000 × *g* for 15 min. The clear supernatant was used for the analysis. The stock solution of the internal standard was stored at −80 °C.

2.3. Analysis of dityrosine by triple quadrupole HPLC–APCI–MS/MS

Fifty microlitres of the clear supernatant was injected onto a Phenomenex Inertsil dp. 5 μm ODS-2 (150 mm × 4.6 mm) (Torrance, CA, USA), column using a HPLC system (Waters Alliance 2790, Etten-Leur, The Netherlands). A methanol gradient was applied as described previously [35]. Briefly, a gradient was formed from 10 mM ammonium acetate, adjusted to pH 4.5 with acetic acid, and methanol, starting with 1% methanol and increasing to 10% over 30 min. The flow rate was 0.8 ml/min. The HPLC system was coupled to the mass spectrometer (MicroMass Quatro Ultima; Waters, Etten-Leur, The Netherlands) was operated in the multiple reaction-monitoring (MRM) mode with positive ionization (APCI). Desolvation temperature was 650 °C. Corona discharge and cone voltage were 2 mV and 50 V, respectively. Cone gas nitrogen flow was 170 l/h, and desolvation gas nitrogen flow was 514 l/h. We monitored the precursor/product reactions of both labeled and unlabeled dityrosine between 2.5 and 8.5 min. The dwell time and pause time were 500 and 10 ms, respectively. After 8.50 min, the column was equilibrated for 40 min with 10 mM ammonium acetate containing 1% methanol.

2.4. Linearity, reproducibility and sensitivity of the HPLC–APCI–LC–MS/MS assay

[$^2H_6$]Dityrosine was added as internal standard to urine samples spiked with various concentrations of dityrosine (concentration range: 0.01, 0.025, 0.05, 0.10, 0.50, 1.0, 5.0 and 10.0 μM). These samples were used to determine the sensitivity and linearity of the method.

Information on reproducibility was obtained from non-spiked urine samples. For the determination of within-day variation, five individual samples were analysed in quintuplicate on the same day. For the determination of between-day variation, three urine samples were measured in quintuplicate on four different days At the beginning of the study, samples were aliquoted and stored at −80 °C between the runs.

2.5. Analysis of urine samples from smoker individuals

Spot urine samples were collected from 14 smokers (four men, 10 women; mean age ± S.D.: 60.8 ± 8.1years). The samples were stored at −35 °C before analysis. We have shown before that dityrosine under these conditions is stable for at least 3 months [35]. Urinary creatinine concentrations were determined by the Jaffee method [38].

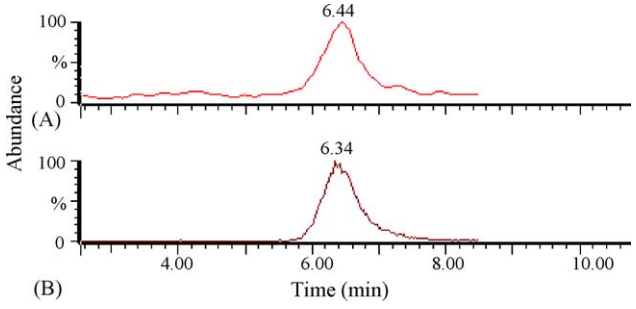

Fig. 2. Determination of *o,o′*-dityrosine in urine of a smoker individual by positive-ion atmospheric pressure chemical ionization tandem MS analysis in triple quadrupole instrument. (A) Multiple reaction monitoring (MRM) chromatogram of authentic dityrosine, *m/z* 361 → *m/z* 315. (B) MRM chromatogram of [$^2H_6$]-labelled dityrosine, *m/z* 367 → *m/z* 319.

Table 1
Validation data for urinary dityrosine

LOQ (μM)	Regression analysis	Precision (%)		Accuracy (%)[a]					
		Within-day	Between-day	0.1 μM	0.25 μM	0.5 μM	1 μM	5 μM	10 μM
0.03	$y = 0.222\ (\pm 0.028)x + 0.0036\ (\pm 0.003)$ $r^2 = 0.9999$	4.6	17	84.1	93.6	96.8	90.1	97.2	99.3

[a] Precision was determined with non-spiked urine samples and accuracy was determined with spiked urines. For the determination of within-day variation, five individual samples were analysed in quintuplicate on the same day. Only the highest CV is shown. For the determination of between-day variation, three urine samples were measured in quintuplicate on four different days.

3. Results

3.1. Analysis of dityrosine by triple quadrupole HPLC–APCI–MS/MS

The protonated molecular ion of dityrosine ($[M+H]^+$), *m/z* 361, was subjected to low energy collisionally activated tandem MS. It decomposed to several ions of which the most intense product ion is *m/z* 315 ($[M+H–HCOOH]^+$). The stable isotope labeled [$^2H_6$]dityrosine showed precursor/product ions at *m/z* 367 and 321, respectively. The retention times for dityrosine and for [$^2H_6$]dityrosine were 6.44 min and 6.34 min, respectively. A typical MRM chromatogram is represented in Fig. 2.

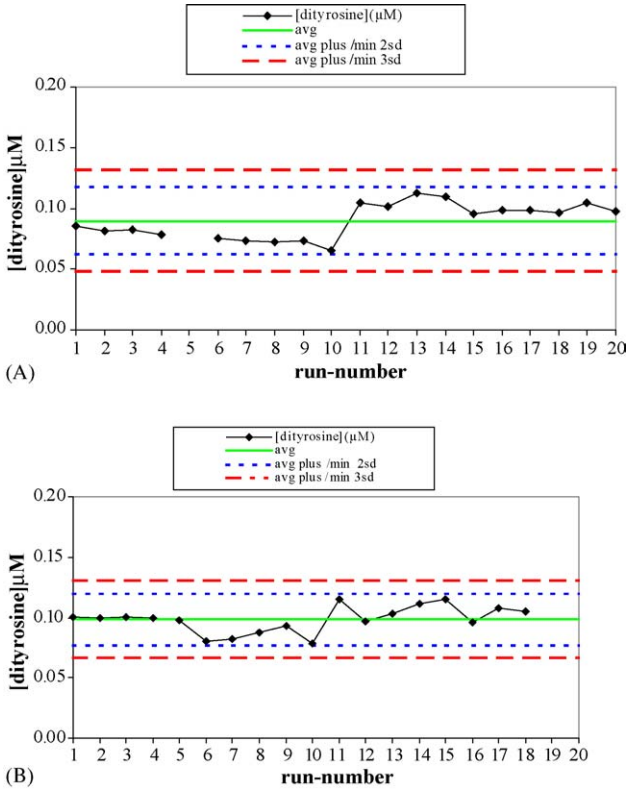

Fig. 3. Shewhart charts for quality assurance of urinary dityrosine analysis in pooled control human urine samples by liquid chromatography tandem–mass spectrometer. (A) Data derived from triple quadrupole instrument in the present study and (B) data derived from ion-trap instrument in the previous study.

3.2. Linearity, reproducibility and sensitivity of the HPLC–APCI–LC–MS/MS assay

Calibration curves of the HPLC–APCI–MS/MS method were linear ($r^2 = 0.9999$) over a concentration range of 0.01–10 μM. The LOD and LOQ were 0.01 and 0.03 μM, respectively. The analytical specifications of the assay are summarized in Table 1.

For analytical quality assurance, two control samples (non-spiked pooled urines from three individuals) were included in each assay, one after the standard series and one at the end of the assay row, and analysed with the samples. The data derived from these control samples were analysed by a Shewhart chart, which is represented in Fig. 3. For comparison to the previously developed ion-trap method [35], the Shewhart chart of that method is also represented. Both methods are reliable in a safe quantitation range (Fig. 2; 95% confidence interval).

3.3. Analysis of urine samples from smoker individuals

Urinary dityrosine concentration of smokers was 0.08 ± 0.01 μM (mean ± S.E.M.). Expressed per urinary creatinine concentration, this corresponds to 10.1 ± 0.4 μmol/mol creatinine (mean ± S.E.M.). Normalization to the concentration of creatinine corrects for differences in glomerular filtration rate between subjects.

4. Discussion

The main objective of the present study was to explore the applicability of a triple quadrupole MS for quantitative determination of urinary dityrosine, which is a promising biomarker of protein oxidation, and to compare its performance with the previously developed method for an ion-trap instrument.

Analysis of biomarkers in urine offers an important advantage over the analysis in other biological media e.g. such as blood, since the sampling is non-invasive. The present assay is more sensitive compared to the previously developed HPLC–ion-trap–tandem MS method [35]. The limit of quantitation (LOQ) with the present triple quadrupole tandem MS is 0.030 μM which is 2.5-fold lower than the previously reported value of 0.075 μM for the ion-trap tandem MS [35].

The present LOQ is approximately two-fold lower than the urinary dityrosine concentration in smokers of 0.08 μM. The signal-to-noise ratio of the lowest (unspiked) urinary concentration by the present triple quadrupole instrument was 33, whereas it was 14 by the ion-trap instrument. These detector responses explain the difference in sensitivity between two instruments.

The present method offers a great advantage over other described methods, as it does not require any pre-treatment other than centrifuging the urine sample and adding the labelled internal standard before injection. With this simple and rapid method, a large number of samples can be analysed in a short time period. The only limiting factor is the run time for one injection, which is 50 min. Although dityrosine elutes at 6.44 min the rather long run time is necessary to elute other compounds present in urine. With the described gradient elution and the conditions, there is no disturbing/interfering compound eluting in the same region with dityrosine. The use of six deuterium-labelled isotope as internal standard further increases the reliability of the method.

The present simple assay for urinary dityrosine has been found satisfactory reproducible (in-day C.V. = 4.6%). Inter-day variation is 17%, higher than in-day variation, however, it still allows quantifying dityrosine in a safe range. The Shewhart chart of the present assay is very similar to that of previously described ion-trap assay [35].

The presently developed method was applied to urine samples of smokers and our results show that urinary levels of dityrosine can easily be measured by this method. It has been previously reported that cigarette smoke converts tyrosine to dityrosine in vitro [39]. Although the presently measured urinary levels in smokers are comparable to the levels in non-smokers previously measured by the ion-trap instrument, a carefully designed, age- and sex-matched, time-controlled study is needed to establish whether smoking causes increased levels of urinary dityrosine or not.

In conclusion, a simple, rapid, sensitive and reliable method was developed for the quantitative analysis of urinary dityrosine. The triple quadrupole instrument reached 2.5-fold higher sensitivity compared to the ion-trap instrument-based assay we developed recently.

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