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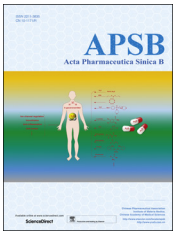

SHORT COMMUNICATION

Simultaneous determination of 3-chlorotyrosine and 3-nitrotyrosine in human plasma by direct analysis in real time–tandem mass spectrometry

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Abstract A novel method for the simultaneous determination of 3-nitrotyrosine (NT) and 3-chlorotyrosine (CT) in human plasma has been developed based on direct analysis in real time–tandem mass spectrometry (DART–MS/MS). Analysis was performed in the positive ionization mode using multiple reaction monitoring (MRM) of the ion transitions at *m/z* 216.2/170.1 for CT, *m/z* 227.2/181.1 for NT and *m/z* 230.2/184.2 for the internal standard, d^3-NT. The assay was linear in the ranges 0.5–100 μg/mL for CT and 4–100 μg/mL for NT with corresponding limits of detection of 0.2 and 2 μg/mL. Intra- and inter-day precisions and accuracies were respectively $<15\%$ and $\pm 15\%$. Matrix effects were also evaluated. The method is potentially useful for high throughput analysis although sensitivity needs to be improved before it can be applied in clinical research.

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1. Introduction

3-Nitrotyrosine (NT) and 3-chlorotyrosin (CT) are oxidation products of reactive oxygen species and other radicals formed under inflammatory conditions. CT is formed when neutrophil- and monocyte-derived myeloperoxidase catalyzes the formation of hypochlorous acid which then chlorinates tyrosine residues in proteins[1–4]. NT is formed when the superoxide anion $\left(O_2^{\bullet-}\right)$ reacts with nitric oxide (NO•) to produce the powerful oxidant peroxynitrite ($ONOO^-$), which nitrates tyrosine residues[3,5–7]. Many researchers have reported that NT and CT are associated with many disorders such as lung and cardiovascular pathologies, atherosclerosis, autoimmune diseases, type 2 diabetes mellitus, and other inflammatory conditions[7–16]. Because of this, the determination of NT and/or CT is of great importance in understanding the etiology of these disorders.

To date, a number of analytical methods have been developed to determine plasma NT and CT including ELISA[17], surface plasmon resonance immunoassay[18], high performance liquid chromatography (HPLC) after derivatization[19], HPLC with electrochemical[20,21] and fluorescence[22] detection, gas chromatography (GC) with electrochemical (GC–ECD) detection[23], GC with mass spectrometric (GC–MS) detection with[24] and without[25] derivatization, GC tandem mass spectrometry (GC–MS/MS)[26], liquid chromatography mass spectrometry (LC–MS)[27] and LC–MS/MS [25,28]. The application of MS to the determination of NT has been recently reviewed[29]. However, since these methods require sample preparation and, in the case of the chromatographic methods, retention and separation, they are limited for high throughput bioanalysis. Accordingly we have investigated the application of direct analysis in real time-tandem mass spectrometry (DART–MS/MS), a technique which requires minimal or no sample preparation.

Direct analysis in real time (DART)[30–32] is a novel ionization technique which relies on the fundamental principles of atmospheric pressure chemical ionization (APCI). The DART ion source consists of a tube containing a chamber through which helium or nitrogen flows at atmospheric pressure. A glow discharge is initiated by applying a kilovolt potential between a needle electrode and a grounded counter electrode. The gas exiting the chamber then passes through a tube containing a perforated intermediate electrode, an optional gas heater, and a grid electrode positioned at the exit behind an insulating cap. Ionization occurs when the gas makes contact with a sample in the open air gap between the DART outlet and the mass spectrometer sampling orifice[30]. The technique has been successfully employed for the analysis of human tissues and body fluids without sample preparation[31–32]. This paper reports the application of DART–MS/MS to the determination of NT and CT in human plasma.

2. Materials and methods

2.1. Materials

CT and NT were purchased from Sigma-Aldrich. Deuterium-labeled NT (d^3-NT) for use as internal standard (IS) was purchased from CDN Isotopes, Inc. Acetonitrile was HPLC grade. Ultrapure water was obtained using a Milli-Q RG unit (Millipore, Bedford, USA). Dip-it samplers were purchased from Aspec Technologies Ltd. (Beijing). Plasma samples for analysis were prepared from blood samples immediately after collection for diagnostic tests by centrifugation at 3000 rpm for 10 min at 4 °C and kept frozen at −20 °C until required. All procedures were performed in accordance with the local Ethics Committee guidelines.

2.2. Instrumentation and experimental conditions

A DART 100 source (IonSense Inc, Saugus, USA) with Control Software (Version 2.03) was coupled to a 5500 triple quadrupole tandem mass spectrometer (Applied Biosystems, AB Sciex, Toronto Canada) using Analyst 1.5 software (AB Sciex). The DART orifice, the ceramic tube (4 mm i.d. × 7.3 cm length) and the mass spectrometer orifice were aligned so that the stream of helium from the DART source was introduced into the mass spectrometer orifice. Introduction of samples into the DART gas stream was controlled by an acquiring module with dip-it samplers inserted into the DART source.

Analysis was performed in the positive ionization mode with multiple reaction monitoring (MRM) of the ion transitions at *m/z* 227.2/181.1 for NT, *m/z* 216.2/170.1 for CT and *m/z* 230.2/184.1 for the IS. Curtain gas was nitrogen (purity ≥ 99.999%) set at 20 psi, declustering potential (DP) +80 V and collision energies (CEs) +16, +18 V and +16 V for NT, CT and IS, respectively. DART parameters were as follows: ionizing gas helium (purity ≥ 99.999%) at 2.8 L/min and 350 °C; grid voltage +150 V; discharge needle voltage +350 V; distance between the DART orifice and the ceramic tube 4.5 cm; and sliding speed of the sample acquiring module 0.4 mm/s.

2.3. Sample preparation

Mixtures of 50 µL of plasma, 50 µL of the d^3-NT solution, and 100 µL of the standard solutions were vortexed and injected directly into the DART–MS system. Concentration of analytes was calculated using calibration curves prepared using calibration standards prepared freshly on each assay day.

2.4. Assay validation

2.4.1. Preparation of calibration standards and quality control (QC) samples

Stock solutions (1 mg/mL) of CT, NT and IS were prepared with 5% acetonitrile. Standard solutions of analytes were prepared at concentrations of 0.5, 5, 10, 20, 40, 80, 100 µg/mL for CT and 4, 8, 10, 20, 40, 80, 100 µg/mL for NT. Calibration standards were prepared from plasma samples thawed at room temperature by mixing 50 µL plasma with 50 µL IS solution (200 µg/mL) and 100 µL CT and NT standard solutions. Low, medium and high QC samples were prepared in the same way at concentrations of 7.8, 25 and 78 µg/mL respectively.

2.4.2. Specificity

A number of blank plasma samples were vortexed and injected directly into the DART–MS system.

2.4.3. Linearity and sensitivity

Linearity of calibration curves based on ratios of peak areas of analyte to IS was assessed by linear regression. The limit of detection (LOD) and lower limit of quantitation (LLOQ) were calculated as 3.3 × SD/slope and 10 × SD/slope, respectively, where SD is the standard deviation of the analyte response at a