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Development and evaluation of a liquid chromatography–mass spectrometry method for rapid, accurate quantitation of malondialdehyde in human plasma

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ABSTRACT

Malondialdhyde (MDA) is a commonly used marker of lipid peroxidation in oxidative stress. To provide a sensitive analytical method that is compatible with high throughput, we developed a multiple reaction monitoring-mass spectrometry (MRM-MS) approach using 3-nitrophenylhydrazine chemical derivatization, isotope-labeling, and liquid chromatography (LC) with electrospray ionization (ESI)–tandem mass spectrometry assay to accurately quantify MDA in human plasma.

A stable isotope-labeled internal standard was used to compensate for ESI matrix effects. The assay is linear ($R^2 = 0.9999$) over a 20,000-fold concentration range with a lower limit of quantitation of 30 fmol (on-column). Intra- and inter-run coefficients of variation (CVs) were <2% and ~10% respectively. The derivative was stable for >36 h at 5 °C. Standards spiked into plasma had recoveries of 92–98%. When compared to a common LC-UV method, the LC–MS method found near-identical MDA concentrations. A pilot project to quantify MDA in patient plasma samples (n = 26) in a study of major depressive disorder with winter-type seasonal pattern (MDD-s) confirmed known associations between MDA concentrations and obesity ($p < 0.02$). The LC–MS method provides high sensitivity and high reproducibility for quantifying MDA in human plasma. The simple sample preparation and rapid analysis time (5x faster than LC-UV) offers high throughput for large-scale clinical applications.

1. Introduction

Oxidative stress occurs when the body is unable to detoxify reactive oxygen species (ROS) as quickly as they are produced. It is a known feature of many health conditions, including a predisposition to or manifestation of cardiovascular disease, diabetes, cancer, and acute tissue injury [1–11], and may be a mechanism of pathogenesis for some neurodegenerative and gastrointestinal diseases [12–14]. The ROS produced in oxidative stress cause cellular damage through non-specific reactions with lipids, proteins, and DNA/RNA, producing a variety of low-molecular weight (LMW) compound markers. LMW aldehydes, such as maldondialdehyde (MDA), result from the peroxidation of lipids and, as reactive compounds themselves, form part of a positive feedback cycle that causes further cellular damage. Extensive research has been published on these aldehyde biomarkers, which can be found at high concentrations in peripheral biofluids such as blood.

MDA's wide usage as a marker of oxidative stress is reflected in the large number of methods that have been published describing its quantitation. However, despite significant development efforts, there continue to be challenges associated with its measurement by available methods. There are several analytical methods that have been published for the determination of LMW aldehydes in biological and other samples. The thiobarbituric acid (TBA) assay by liquid chromatography (LC) with ultraviolet (UV) detection [15,16] is the method of choice to measure MDA but it has very low analytical specificity and often overestimates MDA concentrations [17].

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Alternatively, MDA and other LMW aldehydes are sometimes measured by chemical derivatization with 2,4-dinitrophenylhydrazine (DNPH) followed by LC-UV at a detection wavelength of 310 nm [18] or by LC/atmospheric pressure chemical ionization tandem mass spectrometry (APCI-MS/MS) [19].

While the DNPH derivatization LC-UV method is considered to be very reliable and highly specific, it requires >100 μL of plasma and has limited analytical sensitivity, typically achieving lower limits of quantitation (LLOQs) of >0.25 μM [18,20]. Its throughput is also limited because the assay's specificity depends on an extended gradient elution to separate the DNPH-MDA derivative from interfering compounds, requiring analysis times of 15–35 min per sample [18,20,21]. A method has been published using TBA derivatization with visible light detection and a significantly shorter gradient (8 min per sample) [22], but it requires additional extraction steps to remove interfering compounds, which prolongs and complicates sample preparation. Moreover, the visible light detection method has a limited linear range (0.28–6.6 μM) that does not allow for accurate measurement of plasma MDA concentrations of >6.6 μM, which are commonly reported within the physiological range [17,21]. Other LC methods for quantitation of LMW aldehydes still include chemical derivatization with 1,3-cyclohexanedione (CHD) with detection by fluorimetry [23]. Eggink et al. reported the use of 4-(2-(trimethylammonio)ethoxy) benzeneaminium dibromide (4-APC) [24], and later on, a modified version of this reagent, 4-(2-((4-bromophenethyl)dimethylammonio)ethoxy) benzenaminium dibromide (4-APEBA) [25] as the preanalytical derivatizing reagents for LC/electrospray ionization (ESI)-MS of LWM aliphatic aldehydes. The Guo group also published a method using a hydrazine group-containing Girard's reagent T for the analysis of up to six LMW aldehydes in cigarette smoke with detection by matrix assisted laser desorption/ionization MS [26,27]. However, there is no evidence to show these derivatization reagents can be used for quantitative measurements of dialdehydes such as MDA. Methods for measuring MDA using gas chromatography (GC)–MS do offer increased specificity and modest increases in sensitivity, but their linear range may be limited (0.156–5.0 μM), run times exceed 20 min, and extensive sample preparation is often required [28,29].

In order to overcome limitations in the existing methods for quantitation of MDA, we developed a new method with simple sample preparation using chemical derivatization with 3-nitrophenylhydrazine (3NPH) which does not need subsequent sample clean-up, and which utilizes separation by on-line reversed-phase ultrahigh-performance liquid chromatography (UPLC), and detection with multiple reaction monitoring-mass spectrometry (MRM-MS). Isotopically-labeled $^{13}C_6$-3NPH is used to generate an internal standard ($^{13}C_6$-3NPH-MDA) to ensure precise and accurate quantitation.

2. Materials and methods

2.1. Standards and reagents

Maldondialdehyde (MDA), analytical reagent-grade 3-nitrophenylhyrdrazine (3NPH).HCl, 2,4-dinitrophenylhydrazine (DNPH), trichloroacetic acid (TCA), trifluoroacetic acid (TFA), sodium hydroxide (NaOH), and LC–MS grade acetonitrile (ACN), water, and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of MDA in H_2O was prepared fresh daily at a concentration of 10 mM. Standard calibration solutions were prepared from this stock solution using a dilution series to obtain concentrations of 2000, 400, 80, 16, 3.2, 0.64, 0.12, and 0.024 μM of MDA in H_2O·. 3NPH was prepared at a concentration of 100 mM in 75% ACN containing 0.2% TFA.

$^{13}C_6$-3NPH·HCl was custom-synthesized by IsoSciences Inc. (King of Prussia, PA, USA). According to the accompanying certificate of analysis, the compound was structurally confirmed by ^{11}H-NMR spectroscopy on a Varian Mercury 300 MHz spectrometer (Palo Alto, California, USA) and by MS/MS on a Waters TQD triple-quadruple mass spectrometer (Billerica, MA, USA). IsoSciences determined the chemical purity of the compound to be 98.2% by LC-UV, confirmed the incorporation of six ^{13}C atoms in the benzyl ring of 3NPH by ^{13}C NMR spectroscopy, and determined the isotopic purity to be 99.0% by flow-injection MS.

The human plasma sample used for assay development was prepared by pooling 1 mL volumes of 10 human plasma samples, obtained from BioreclamationIVT (Westbury, NY, USA).

2.2. Optimization of chemical derivatization

A standard solution of 5 μM of MDA in water was used to optimize reaction conditions. 100 μL aliquots of MDA standard solution were mixed with 100 μL of 50 mM 3NPH in 3-mL borosilicate-glass test tubes. The mixtures were individually reacted at different temperatures (0, 10, 20, 30, 40, 50, 60, 70, and 80 °C) and for different time periods (0, 10, 20, 30, 40, 60, and 70 min). After reaction, the mixtures were cooled on ice for 1 min before being diluted with 600 μL of water. LC–MS/MS analysis was performed by injecting 10 μL aliquots of each sample.

2.3. Isotope-labeled internal standard (IS)

The $^{13}C_6$-3NPH-MDA derivative was prepared at a concentration of 2.5 μM by adding 200 μL of 1.25 mM MDA in 75% ACN-0.2%TFA to a 3-mL borosilicate-glass test tube containing 1 mg of $^{13}C_6$-3NPH·HCl. The mixture was reacted at 50 °C for 30 min, and then transferred to a volumetric flask and diluted with 20% aqueous acetonitrile to 100 mL. This solution was used as the internal standard (IS) for subsequent experiments. The $^{13}C_6$-3NPH-MDA derivative did not show any observable degradation over more than four months when stored at −20 °C.

2.4. Sample preparation and derivatization

Aliquots of 50 μL thawed spun-down human plasma were transferred to 3-mL glass test tubes. When measuring "free" unbound MDA, proteins were precipitated by adding 150 μL of ACN, followed by centrifugation in a Beckman X–22 R centrifuge for 15 min at 2782 x *g*, after which the clear supernatant was collected. When measuring "total" MDA in plasma, hydrolysis was performed on plasma samples prior to derivatization to liberate the bound MDA that has modified proteins, lipids, and DNA/RNA. In this case, 25 μL of 6 M NaOH was added to each 50 μL aliquot of plasma and diluted with 50 μL of water, after which the tubes were sealed, shaken at 60 °C for 30 min and then cooled on ice. The solutions were acidified and proteins were precipitated by adding 250 μL of 20% TCA and centrifuging for 15 min at 2782 x *g*.

For derivatization, 50 μL aliquots of the supernatant from hydrolyzed plasma or calibration standard or water (blank) were added to 100 μL of 3NPH in 3-mL tubes, which were then capped and sealed with parafilm. The reaction proceeded for 30 min at 50 °C, after which the tubes were cooled on ice and then mixed with 100 μL of 30% ACN. A 50 μL aliquot of the resulting reaction mixture was mixed with 50 μL of the IS solution in LC/MS certified sample micro-vials, and 10 μL of this solution was injected for LC–MS/MS analysis.