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Bioanalysis of 6-diazo-5-oxo-L-norleucine in plasma and brain by ultra-performance liquid chromatography mass spectrometry

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ABSTRACT

Glutamine is an abundant amino acid that plays pivotal roles in cell growth, cell metabolism, and neurotransmission. Dysregulation of glutamine-using pathways has been associated with pathological conditions such as cancer and neurodegenerative diseases. 6-Diazo-5-oxo-L-norleucine (DON) is a reactive glutamine analog that inhibits enzymes affecting glutamine metabolism such as glutaminase, 2-*N*-amidotransferase, L-asparaginase, and several enzymes involved in pyrimidine and purine de novo synthesis. As a result, DON is actively used in preclinical models of cancer and neurodegenerative disease. Moreover, there have been several clinical trials using DON to treat a variety of cancers. Considerations of dose and exposure are especially important with DON treatment due to its narrow therapeutic window and significant side effects. Consequently, a robust quantification bioassay is of interest. DON is a polar unstable molecule that has made quantification challenging. Here we report on the characterization of a bioanalytical method to quantify DON in tissue samples involving DON derivatization with 3 N HCl in butanol. The derivatized product is lipophilic and stable. Detection of this analyte by mass spectrometry is fast and specific and can be used to quantify DON in plasma and brain tissue with a limit of detection at the low nanomolar level.

Glutamine is one of the most abundant amino acids in the human body, playing a critical role in cell growth, cell metabolism, and neurotransmission. Dysregulation of glutamine-using pathways has been associated with a variety of pathologies, including cancer and neurodegenerative disease.

Glutamine serves as a nitrogen donor for purine and pyrimidine production, which is required for de novo nucleotide synthesis [1]. Because de novo synthesis of nucleotides is upregulated to support DNA replication and RNA expression for rapid growth and division of cancer cells [2], inhibition of amidotransferases, the enzymes involved in the transfer of the amide group of glutamine to other molecules to initiate nucleotide synthesis, has been suggested as potential cancer therapy. Glutamine is also a major source of energy for neoplastic cells via glutaminolysis where glutaminase converts glutamine to glutamate, which is further converted to α-ketoglutarate to enter the citric acid cycle [3]. In support of this hypothesis, glutaminase inhibition has been shown to be efficacious in models of cancer [4,5].

In addition, glutaminase-catalyzed hydrolysis of glutamine to glutamate is a major source of glutamate in the brain [6]. Normal synaptic transmission in the central nervous system (CNS)[1] involves the use of glutamate as the major excitatory amino acid neurotransmitter. Under certain pathological conditions, excessive glutamatergic signaling, termed excitotoxicity [7], is postulated to cause CNS damage in several neurodegenerative diseases such as stroke [8], amyotrophic lateral sclerosis [9], Huntington's disease [10], Alzheimer's disease [11], and HIV-associated dementia [12]. Consequently, inhibition of glutaminase has been suggested as a possible way to ameliorate high levels of glutamate in neurodegenerative diseases. In support of this hypothesis, glutaminase inhibition has been efficacious in models of CNS neurodegeneration [12–17].

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[1] *Abbreviations used:* CNS, central nervous system; DON, 6-diazo-5-oxo-L-norleucine; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography mass spectrometry; LC–MS/MS, liquid chromatography tandem mass spectrometry; QTOF, quadrupole time-of-flight; AUC, area under the curve; i.v., intravenously; i.p., intraperiotoneally; PBS, phosphate-buffered saline; LOD, limit of detection; RPC, reverse phase chromatography; CID, collision-induced dissociation.

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6-Diazo-5-oxo-L-norleucine (DON) is an amino acid analog of glutamine that is an inhibitor of glutamine-using enzymes. DON inhibits 2-*N*-amidotransferase to block purine synthesis [4]. DON was one of the earliest inhibitors to be identified for glutaminase [18]. It binds to the active site of glutaminase in an irreversible manner [18–20]. As an inhibitor of glutamine-metabolizing pathways, DON has been used both as a tool compound in preclinical in vivo models and as a clinical candidate. There have been several clinical trials using DON [21–26]; unfortunately it was not well tolerated at efficacious doses. Recently, phase II clinical trials were reported for DON in combination with PEGylated glutaminase with the goal of improving efficacy by coadministration with the glutamine-depleting enzyme [27]. DON is still commonly used as a tool compound in glutamine-related research due to its solubility and efficacy in various in vivo models [28,29]. However, DON, with its polar structure and reactive moiety, would be expected to have difficulty in reaching its target. Thus, a quantification assay for DON is of interest when using DON in animal models. DON quantification has been carried out in the past by several methods that include high-performance liquid chromatography (HPLC) of derivatized DON followed by absorbance and fluorescence detection [30], ion-paired HPLC followed by absorbance detection [31], radioisotope-labeled DON [32], and a microbiological assay [33]. HPLC analysis of DON suffers from interference from other materials in the sample; analysis may require boiling samples to confirm results [26], and often assays are not sensitive. Using radiolabeled DON has the issues of working with radioactivity, but more important is that the assay does not differentiate intact DON from degraded DON or from metabolized or covalently bound DON that retains the radiolabel. Microbiological assays are time-consuming and labor-intensive, could suffer from nonspecific effects, and might not differentiate between DON and its metabolites [33].

Here we report a novel robust bioanalytical method to quantify DON in tissue samples. Derivatization of DON forms a stable analyte that is detected unambiguously by mass spectrometry. In this study, we used this bioanalytical assay to quantify DON exposure following systemic administration in rodent plasma and, for the first time, also in brain tissue.

Materials and methods

DON derivatization

DON was derivatized in the presence of 3 N HCl ±*n*-butanol. DON (Sigma–Aldrich) was first dissolved in water at a concentration of 10 mM. An aliquot (10 µl) of this stock solution was added to 3 N HCl ±*n*-butanol (250 µi) in a low-retention microcentrifuge tube. The solution was then heated at 60 °C for 30 min in a shaking water bath. After heating, the sample was dried at 45 °C under a nitrogen stream, resuspended in 50 µl of water/acetonitrile (70:30), vortexed, and centrifuged at 16,000*g*. Supernatants were transferred to LC vials, and an aliquot (2 µl) was used for liquid chromatography mass spectrometry (LC–MS) or liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis.

Analysis of derivatized DON by LC–MS

Derivatized DON samples (2 µl) prepared as described above were injected and separated on an Agilent 1290 LC system equipped with an Agilent Eclipse Plus Rapid Resolution C18 column (2.1 × 100 mm, 1.8 µm) over a 5.5-min gradient from 30 to 70% acetonitrile +0.1% formic acid. Analytes were detected with an Agilent 6520 quadrupole time-of-flight (QTOF) mass spectrometer in positive mode with drying gas at 350 °C, 11 L/min, and 40 psi. The fragmenter was set at 70 V, and the capillary voltage (VCAP) was set at 4000 V.

Analysis of derivatized DON by LC–MS/MS

Analysis of derivatized DON after 3 N HCl ±*n*-butanol by LC–MS/MS was carried out in the same manner as for LC–MS except that the precursor mass (*m*/*z* 218.0942) was selected in the first quadrupole and the compound was made to collide with nitrogen gas with a collision energy of 15 V in MS/MS mode to afford the daughter ions with *m*/*z* 162.032 and 116.026.

Analysis of underivatized DON by LC–MS

Methanol (250 µl) was added to plasma samples containing DON (50 µl) and vortexed; samples were centrifuged for 5 min at 16,000*g* to precipitate proteins. An aliquot of the supernatant (200 µl) was dried and subsequently reconstituted in H_2O (50 µl). An aliquot (20 µl) was then injected and separated on an Agilent 1290 LC system equipped with a Thermo Hypercarb column (2.1 × 100 mm) with isocratic 2.5% acetonitrile +0.1% formic acid mobile phase. Analytes were detected with an Agilent 6520 QTOF mass spectrometer in MS mode as when analyzing derivatized DON by LC–MS.

Bioanalysis of DON in plasma

When using plasma, DON was derivatized only using 3 N HCl plus *n*-butanol and subsequently analyzed by LC–MS. To generate the standard curve to determine DON concentrations in plasma, DON (10 µl of 1 mM water solution) was added to untreated mouse plasma (90 µl) in a low-retention microcentrifuge tube. Standard solutions (100 µl) were then prepared by serial dilution to generate concentrations from 10 nM to 100 µM at half-log intervals. Prior to extraction, frozen plasma samples were thawed on ice. *n*-Butanol (250 µl) containing 3 N HCl was added directly to standards (50 µl), vortexed, and centrifuged at 16,000*g* for 5 min in low-retention microcentrifuge tubes to precipitate proteins. An aliquot (200 µl) of the supernatant was transferred to a new tube and incubated at 60 °C for 30 min in a shaking water bath to carry out the derivatization reaction. After derivatization, an aliquot of the reaction mixture (2 µl) was injected and analyzed by LC–MS as stated above. The area under the curve (AUC) representing the signal intensity of the extracted ion (*m*/*z* 218.0942) for each sample was used to generate the standard curve using Agilent MassHunter quantitative analysis software. Plasma samples obtained from mice treated with DON were treated in exactly the same manner except that exogenous DON was not added. DON concentrations in plasma samples were determined by interpolation using the standard curve.

Bioanalysis of DON in brain

When using brain, DON was derivatized only using 3 N HCl +*n*-butanol and subsequently analyzed by LC–MS. To generate the standard curve to determine DON concentrations in brain, frozen brain samples from untreated mice were thawed on ice. Tissue was weighed in low-retention microcentrifuge tubes, to which 5 µl of *n*-butanol-containing 3 N HCl was added per milligram tissue. Tissue was then homogenized with a pestle and vortexed. Known amounts of DON from a 1-mM stock solution in water were mixed with *n*-butanol-containing 3 N HCl and spiked to brain tissue to prepare standards at concentrations from 10 nM to 100 µM at half-log intervals. Samples were centrifuged at 16,000*g* for 5 min in low-retention microcentrifuge tubes to precipitate proteins. An aliquot (200 µl) of the supernatant was transferred to a new tube and