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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

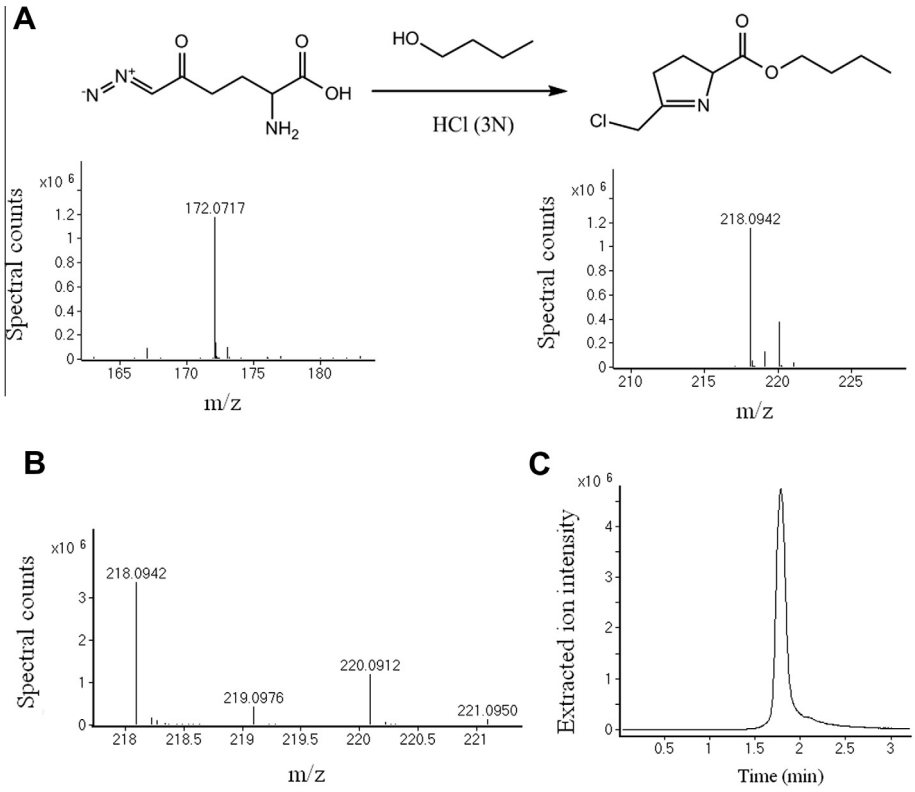


Fig.1. (A) Derivatization of DON in 3 N HCl +*n*-butanol followed by LC–MS analysis. DON was heated at 60 °C for 30 min in *n*-butanol-containing 3 N HCl to derivatize the molecule into a stable quantifiable analyte of molecular mass $[M+H]^+$ 218.0942. (B) The high-resolution mass and isotopic abundances were used to generate the molecular formula $C_{10}H_{16}ClNO_2$. The observed isotopic abundance closely matched the predicted isotopic abundance of the proposed chemical structure incorporating a chlorine atom. This is apparent from the 3:1 ratio of [M] (218.0942) and [M+2] (220.0912). (C) Ultra-performance liquid chromatography trace of derivatized DON. Derivatized DON was injected on an Agilent 1290 LC system equipped with a C18 column and detected with an Agilent 6520 QTOF mass spectrometer.

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. Brain samples obtained from mice treated with DON were treated in exactly the same manner except that exogenous DON was not added. DON concentrations in brain samples were determined by interpolation using the standard curve.

Animal studies

All protocols were approved by the animal care and use committee at Johns Hopkins University. C57BL/6 male mice (4–5 weeks old) were administered DON at different doses either intravenously (i.v.) or intraperitoneally (i.p.) as indicated. DON working solution was diluted in phosphate-buffered saline (PBS) each day from aliquots of a 100-mM stock solution in PBS stored at −80 °C. At the indicated time points after DON administration, mice were euthanized in a CO_2 chamber and blood was collected transcardially. When collecting brains, mice were perfused with PBS before brain collection. Samples were frozen immediately at −80 °C and kept frozen until bioanalysis. Plasma and brain samples were processed and analyzed as stated in the previous two sections.

Results

LC–MS analysis after DON derivatization in 3 N HCl ±n-butanol shows the presence of a chlorine-containing derivative

During DON derivatization using 3 N HCl +*n*-butanol, we found that DON reacted and rearranged to form a stable and quantifiable derivative (Fig. 1A). The high-resolution mass and isotopic abundances in the observed mass spectrum were used to generate the molecular formula $C_{10}H_{16}ClNO_2$. The isotopic abundances closely match the predicted isotopic abundance of the proposed chemical structure. The 3:1 isotopic abundance ratio between the molecular ion (218.0942) and the M+2 (220.0912) unambiguously indicated that a chlorine atom had been incorporated into the derivatized product (Fig. 1B). The corresponding chromatographic trace of the molecular ion of derivatized DON is shown in Fig. 1C. When DON was incubated with 3 N HCl in water in the absence of butanol, a peak with molecular mass *m*/*z* 162.0316 was observed in the mass spectrum (Fig. 2). The molecular formula generated included a chlorine-containing derivative, $C_6H_8ClNO_2$. The structure that fits the molecular mass and molecular formula is the same as the derivatized structure in Fig. 1 without the butyl moiety (Fig. 2). This is as expected given that butanol was not used in this derivatization reaction.

LC–MS/MS analysis of fragmentation pattern of ester-containing derivative confirms the presence of cyclic structure and chlorine atom

In a separate experiment, DON was derivatized with 3 N HCl ±*n*-butanol and subsequently analyzed by LC–MS/MS. The resulting product ions of 162.0318 and 116.0262 match the loss of the butyl ester and a radical formed after the loss of the entire carboxylate ester moiety, respectively (Fig. 3A). These product ions are consistent with the expected DON derivative structure. When using 3 N HCl during derivatization, the resulting product ion of 116.0258 is consistent with the expected DON derivative structure in the absence of butanol (Fig. 3B).

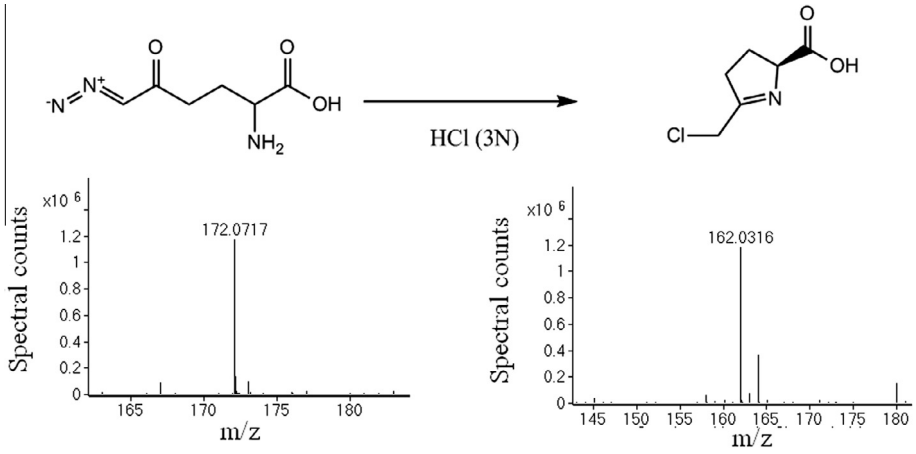


Fig.2. Derivatization of DON in 3 N HCl without butanol followed by LC–MS analysis. Derivatized product exhibited a mass consistent with the proposed methylene chlorine-substituted 1-pyrroline structure but lacking the butyl ester.

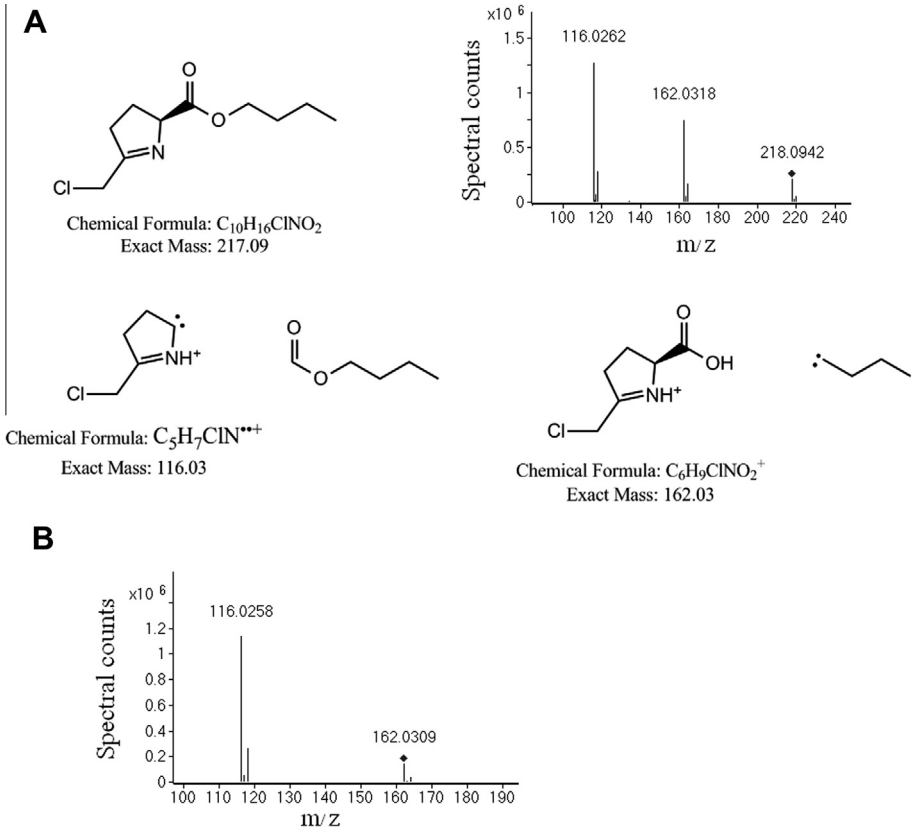


Fig.3. Tandem mass spectrometry of derivatized DON. (A) Collision-induced dissociation of DON after derivatization with acidified butanol. DON was derivatized with *n*-butanol-containing 3 N HCl and analyzed by LC–MS/MS. The resulting product ions of 162.03 and 116.03 match the loss of the butyl ester and radical formed after the loss of the entire carboxyl ester moiety. These product ions are consistent with the expected DON derivative structure. (B) Collision-induced dissociation of DON after derivatization with 3 N HCl. DON was derivatized with 3 N HCl without butanol and analyzed by LC–MS/MS. The resulting product ion of 116.03 is consistent with the expected DON derivative structure in the absence of butanol.

DON derivative was quantified from plasma and brain tissue using LC–MS

To verify that the DON derivatization protocol was adequate to use to determine DON concentrations in biological matrices, known concentrations of DON were added to mouse plasma and brain followed by derivatization using 3 N HCl in *n*-butanol. Derivatized samples were then analyzed by LC–MS and a standard curve for each matrix was generated. In each case, there was a linear correlation between signal response and the concentration of derivatized material. Standard curves were linear in the range of 30 nM to 100 μM for both plasma (Fig. 4A) and brain (Fig. 4B).

DON was measured in plasma and brain using the new bioanalysis procedure

The new derivatizing procedure was used to determine DON concentrations in plasma and brain following i.v. and i.p. administration. In the first study, mice were given DON (1.6 mg/kg, i.v.) and blood was collected at 0.25, 0.5, 1, 2, 4, and 6 h. The exposure of

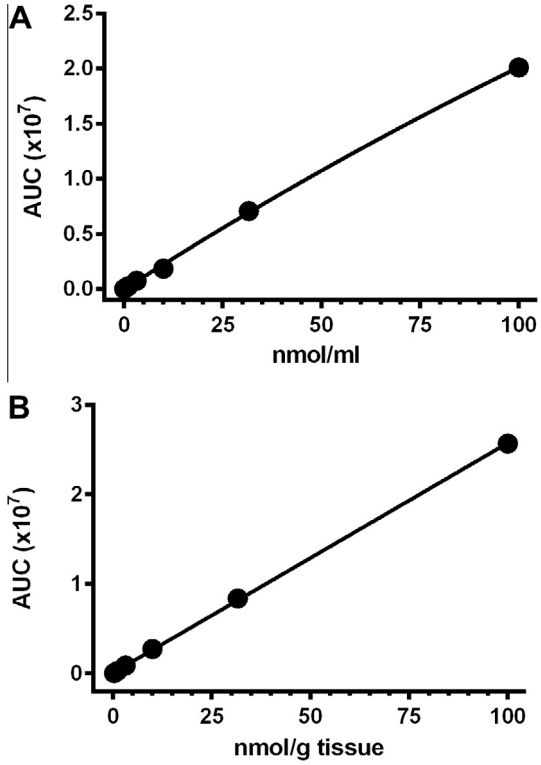


Fig.4. (A) Standard curve of DON in plasma after derivatization with acidified butanol and LC–MS analysis. DON was spiked into untreated mouse plasma to generate standards at various concentrations. DON derivatization was carried out with *n*-butanol-containing 3 N HCl. After centrifugation to separate denatured proteins, supernatant was incubated at 60 °C for 30 min. Derivatized DON was detected by LC–MS. Standards in the range of 30 nM to 100 μM were used to generate a standard curve. (B) Standard curve of DON in brain after derivatization with acidified butanol and LC–MS analysis. DON was spiked into untreated mouse brain to generate standards at various concentrations. DON derivatization was carried out with *n*-butanol-containing 3 N HCl. After centrifugation to separate denatured proteins, supernatant was incubated at 60 °C for 30 min. Derivatized DON was detected by LC–MS. Standards in the range of 30 nM to 100 μM were used to generate a standard curve.

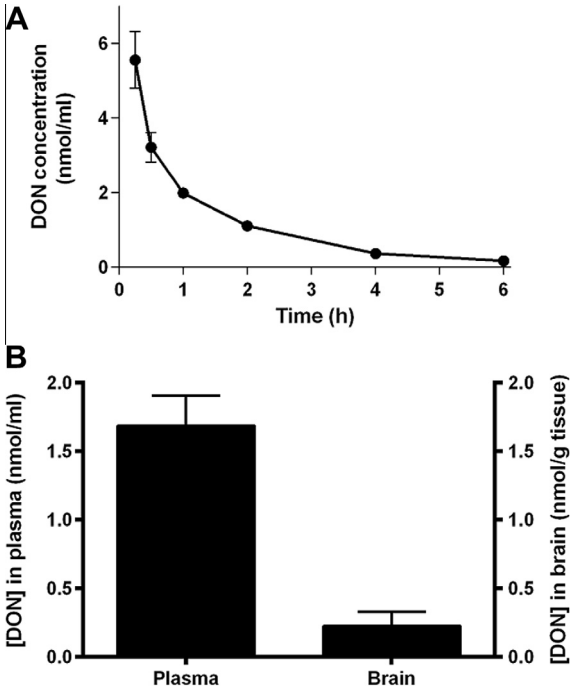


Fig.5. (A) DON concentrations in plasma over time. Mice were administered DON at 1.6 mg/kg (i.v.). Mice were euthanized, and blood was collected transcardially 0.25, 0.5, 1, 2, 4, and 6 h after dosing. Blood was centrifuged, and plasma was collected and stored at −80 °C. *n*-Butanol-containing 3 N HCl (250 μl) was added directly to samples (50 μl) and centrifuged at 16,000g for 5 min to precipitate proteins. An aliquot (200 μl) of the supernatant was incubated at 60 °C for 30 min. After derivatization, the samples were analyzed by LC–MS (see Materials and Methods). (B) DON plasma-to-brain ratio analysis. Mice were administered DON (0.6 mg/kg, i.p.), and blood and brain were collected 1 h after DON administration. Plasma was isolated from blood samples as described in panel A. Brains were collected following perfusion with PBS and frozen immediately at −80 °C. Before bioanalysis, brains were weighed and *n*-butanol-containing 3 N HCl was added to each sample (5 μl/mg tissue) and homogenized using a pestle. Resulting homogenates were centrifuged at 16,000g for 5 min to precipitate proteins and analyzed the same way as the plasma samples. All determinations in both panels A and B were carried out in triplicate. Error bars correspond to ±standard errors.

DON estimated from the AUC was 8 nmol h/ml with a plasma half-life of 1.2 h (Fig. 5A). In the second study, mice were given DON (0.6 mg/kg, i.p.) and both plasma and brain were harvested 1 h after DON administration. DON concentrations in plasma and brain were 1.7 ± 0.5 nmol/ml and 0.22 ± 0.18 nmol/g tissue, respectively (Fig. 5B), suggesting a brain-to-plasma ratio of approximately 0.1.

Analyses of derivatized DON and intact DON gave the same results

It is conceivable that when DON is used in vivo, it could form by-products that could also form the derivatized structure. To determine whether this was the case, DON concentration was measured both directly using a less sensitive method (limit of detection [LOD] > 1 μM) and through acidified butanol derivatization in plasma samples collected from mice 15 min after DON administration (1.6 mg/kg i.v.). The two methods gave the same DON concentration within experimental error: 3.9 ± 0.3 nmol/ml and 4.2 ± 0.8 nmol/ml when using the underivatized and derivatization methods, respectively (Fig. 6).

Discussion

Several quantification methods for DON have been described previously [26–29], all with LODs at the low micromolar level. HPLC/fluorescence, radiolabel, and microbiological assays all have the potential for nonspecific signals. DON is a polar amino acid that elutes unretained in the void volume with many other polar compounds during reverse phase chromatography (RPC). Due to ion suppression and poor chromatography that result in broad irregular peak shapes, DON cannot be successfully separated and quantified from complex matrices such as brain and plasma with ordinary RPC. Direct measurement of DON quantification by LC–MS has been possible by using a porous graphitic carbon-based chromatographic column (Hypercarb) that minimizes the ion suppression seen with the silica-based C18 column (unpublished observation); this measurement, however, also exhibits low sensitivity (LOD > 1 μM), so it is not an alternative for routine pharmacokinetic samples where low nanomolar levels are of interest. One alternative was to derivatize DON. Polar amino acids are often derivatized to make them more amenable to separation by RPC [34]. Esterification of the carboxylic acid on an amino acid improves RPC separation and increases the analyte mass, which enhances ionization at the electrospray source of the mass spectrometer. For example, derivatization with an *n*-butyl ester has been used to quantify plasma methylmalonic acid [35]. However, in the case of DON, in addition to the carboxylic acid moiety, there is the added complication of the diazo ketone moiety that lacks stability and is not expected to survive derivatization conditions.

In an effort to develop a reliable way to measure DON in complex biological matrices, we incubated DON with butanol in

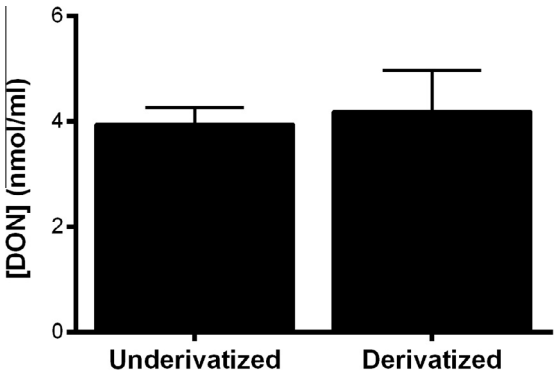


Fig.6. DON concentration in plasma from DON-treated mice using underivatized and derivatization protocols. Mouse plasma samples were obtained 15 min after DON administration (1.6 mg/kg, i.v.). For LC–MS bioanalysis of underivatized DON from plasma, DON was extracted from plasma with methanol, dried, and resuspended in H_2O and separated in a Hypercarb column (see Materials and Methods). For LC–MS bioanalysis of derivatized DON from plasma, DON was derivatized in butanol-containing 3 N HCl for 30 min at 60 °C, dried, reconstituted in 30% acetonitrile, and separated by RPC (see Materials and Methods). Analytes eluting after each chromatographic separation were detected by QTOF mass spectrometry. For each treatment, $n = 3$. Error bars correspond to ±standard deviations.

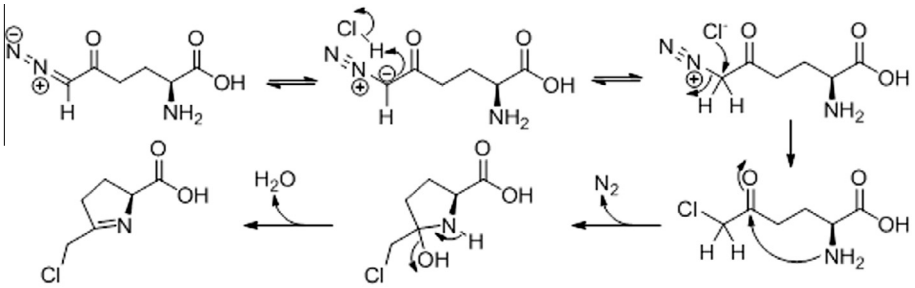
3 N HCl for 30 min at 60 °C, the same procedure used to derivatize carboxylic acids to make the corresponding *n*-butyl ester [35]. DON derivatization under these conditions produced a chlorine-containing derivative as supported by the 3:1 isotopic abundance ratio between the molecular ion (218.0942) and the M+2 (220.0912) (Fig. 1). Incorporation of a chloromethyl ketone into the diazo moiety of DON has been shown previously [36]. The molecular mass (218.0942) (Fig. 1A) and isotopic abundances (Fig. 1B) observed in the mass spectrum are consistent with cyclization to form butyl 5-(chloromethyl)-3,4-dihydro-2*H*-pyrrole-2-carboxylate (Fig. 1A). When derivatization was carried out in 3 N HCl in the absence of butanol, a chlorine-containing derivative that corresponds to the same cyclized product lacking the butyl group on the ester with molecular formula $C_6H_8ClNO_2$ (molecular mass m/z 162.0316) was formed (Fig. 2).

In a separate effort to confirm the structure of derivatized DON, the product of derivatization was analyzed after collision-induced dissociation (CID). The fragmentation pattern was consistent with the presence of an ester-containing derivative and a 1-pyrroline ring with a methylene chlorine substitution (Fig. 3A). The resulting product ions match the loss of the butyl ester and a radical formed after the loss of the carboxylate ester (Fig. 3A). Further confirmation was obtained when the 116.0258 product ion was also seen with CID of derivatized DON with 3 N HCl without butanol (Fig. 3B).

A possible mechanism of the derivatization reaction in the absence of butanol is illustrated in Scheme 1. At low pH (3 N HCl), the α-carbon of the carbonyl close to the diazo moiety will abstract a proton from the solvent, resulting in a diazonium ion. In the next step, the same α-carbon undergoes chlorine ion addition and concomitant N_2 loss. The chloromethyl ketone undergoes cyclization and dehydration to form the 1-pyrrolinedine derivative illustrated in Scheme 1. When the derivatization reaction is carried out in *n*-butanol containing 3 N HCl, the carboxylic acid moiety will also undergo standard acid-catalyzed esterification [37].

We used the new derivatizing procedure to determine DON concentrations in both plasma and brain from mice after DON administration. First, we generated standard curves of signal intensity versus known concentrations of DON that were added to both plasma and brain followed by derivatization, extraction, and bioanalysis. The resulting standard curves for plasma (Fig. 4A) and brain (Fig. 4B) were then used to determine unknown concentrations of DON in plasma and brain from mice that had been treated with DON. The results show that DON can be readily monitored using the new derivatizing procedure. We report i.v. pharmacokinetic parameters of $t_{1/2} = 1.2$ h and AUC = 8 nmol h/ml (Fig. 5A) and for the first time show DON brain penetration with a brain-to-plasma ratio of 10% at 1 h (Fig. 5B). The latter finding was surprising given DON's polar structure. Because the animals were perfused, DON found in brain at this level is unlikely to be due to blood contamination. DON could be actively transported by an amino acid carrier into the brain; previous reports have demonstrated active DON uptake systems that can be inhibited by glutamine in leukemia cells [38] and *Xenopus* oocytes [39].

One potential drawback of the bioanalysis procedure is that DON could cyclize in vivo to form a by-product that in turn could convert into our analyte during derivatization. This could give artificially high DON concentrations. To rule out this possibility, we performed a control experiment where we compared DON concentrations obtained when using the derivatizing protocol (3 N HCl +*n*-butanol) and when measuring DON directly. Even though measurement of underivatized DON is far less sensitive than when using the derivatizing procedure (LOD for underivatized method > 1 μM vs. LOD for derivatizing procedure = 30 nM), when measuring micromolar (μM) levels of DON, a side-by-side comparison of the two methods would unveil whether the derivatization method is measuring DON by-products. We found that the derivatization procedure gave the same DON concentrations within error as the direct measurements of underivatized DON (Fig. 6). Furthermore, a cyclized by-product, made by heating DON at 37 °C for 2 h and then adding to plasma, did not convert to the derivatized product when using the derivatization protocol. The result showed that the cyclized by-product of DON was impervious to the derivatization procedure. In a separate study, the cyclized by-product of



Scheme 1. Proposed mechanism for the derivatization reaction of DON in 3 N HCl in the absence of butanol: Reaction at the diazo moiety. At low pH, the α-carbon of the carbonyl close to the diazo moiety abstracts a proton from solvent, resulting in a diazonium ion. Subsequently, the same α-carbon undergoes chlorine ion addition and concomitant N_2 loss. The chloromethyl ketone undergoes cyclization and dehydration to form the five-member ring 1-pyrrolinedine with the methylene chlorine substitution. When butanol is present in the reaction mixture, the same derivatization occurs along with esterification of the carboxylate moiety with the *n*-butyl group.

DON was not observed by LC–MS analysis of plasma from DON-treated mice 2 h after intravenous treatment.

In summary, we have developed a simple robust method to quantify DON in complex biological matrices using ultra-performance liquid chromatography mass spectrometry after DON derivatization with acidified butanol. DON in the sample is made to react with *n*-butanol-containing 3 N HCl to form butyl 5-(chloromethyl)-3,4-dihydro-2*H*-pyrrole-2-carboxylate. A single solvent for extraction and derivatization solution simplifies sample processing and shortens analysis time. The derivatization LC–MS method is rapid, reproducible, and rigorous and has a lower limit of quantitation of 30 nM that is more than 30-fold more sensitive than methods reported in the literature. Mass spectrometry is able to reduce nonspecific signal because only one analyte with a specific molecular formula is quantified. This method was applied to monitor DON levels in plasma and brain and could readily be applied to other tissues as well.

Acknowledgments

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