Validated HPLC-MS/MS Method To Quantify Low Levels of Domoic Acid in Plasma and Urine after Subacute Exposure

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ABSTRACT: Domoic acid (DA) is a marine neurotoxin produced by several species of Pseudo-nitzschia. DA causes severe neurological toxicity in humans and animals. To address the current analytical need to quantify low levels of DA in human and animal body fluids, a sensitive and selective high performance liquid chromatography-tandem mass spectrometry method was developed to measure DA in plasma and urine. This method was fully validated to accurately and precisely quantify DA between 0.31 and 16 ng/mL in plasma and between 7.8 and 1000 ng/mL in urine. Our group introduced the use of a novel internal standard, tetrahydrodomoic acid to control for matrix effects and other sources of variability. This validated method will be useful to assess DA concentrations in biological samples of human or animal origin after suspected DA exposure from contaminated food. It will also be applicable to sentinel programs and research studies to analyze body fluids with low levels of DA.

INTRODUCTION

Domoic acid (DA) is a marine neurotoxin produced by several species of diatoms of Pseudo-nitzschia.1 DA is readily filtered and taken up by shellfish that is subsequently consumed by predators and humans. Exposure to DA via consumption of contaminated shellfish is a risk to human health. During a marked algal bloom in 1987, 107 people experienced acute neurological symptoms, and 3 died shortly after eating mussels contaminated with DA.2,3 To protect human health, DA monitoring programs have been established, and extensive efforts have been made to develop sensitive and selective methods to measure DA concentrations in seafood and seawater.4 The recently developed methods include indirect measurement of DA via competitive enzyme-linked immuno-sorbent assay (ELISA)5 and direct measurement of DA via liquid chromatography-tandem mass spectrometry (LC-MS/MS)6−14 or laser ablation-MS.15 These methods have been successful in monitoring DA in matrices that are available in large quantities, such as seawater and mussels. However, no methods have been published to determine DA concentrations in human blood or urine after potential exposures to subacute doses of DA, and plasma DA concentrations have not been reported in humans following contaminated shellfish consumption.16,17 DA exposures have, however, been measured using indirect competitive ELISA followed by confirmatory LC-MS/MS in marine mammals, such as California sea lions (CSTs),18 exposed to DA via food and showing neurological symptoms of acute or chronic toxicity. The DA concentrations ranged from undetectable to 200 ng/mL in serum and from undetectable to 3700 ng/mL in urine. On the basis of estimates of DA exposure and toxic effects observed therein, and from results of laboratory studies in nonhuman primates, researchers and environmental health agencies have proposed estimated tolerable daily intake (TDI) of DA from 0.075 to 0.1 mg/kg/day.19−21 However, recent studies have shown that some recreational harvesters consume DA in excess of the proposed TDI16 and that chronic low-level exposure to DA is associated with memory loss.22 On the basis of the dose−exposure relationship in nonhuman primates and physiologically based pharmacokinetic (PBPK) modeling and simulations,23 we predicted that a biochemical analysis with a lower limit of quantification (LLOQ) around 0.2−0.4 ng/mL is needed to detect DA in human or nonhuman primate plasma after exposure to DA at or above the proposed TDI. None of the currently available methods to detect DA in serum by LC-MS/MS reaches this sensitivity, and a single method has been reported to detect DA in urine at this level (Table 1). Hence, more sensitive, validated, LC-MS/MS methods are needed to assess human DA exposures.

DA is a hydrophilic molecule (clog P = −0.23, value obtained from Drug Bank on June 12, 2018, https://www.drugbank.ca/drugs/DB02852) with three carboxyl groups.

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(pK_a: 1.85, 4.47, 4.75) and an amine group (pK_a: 10.60), as shown in Figure 1A. The ionized and polar nature of DA makes extraction from biological matrices challenging. Solid-phase extraction (SPE) has been used to obtain DA from complex samples, but this step can introduce variability into the assay via sample recovery.

Several bioanalytical methods have been developed or optimized to measure DA concentrations directly or indirectly in serum and urine samples from various mammalian species, as listed in Table 1. However, validation data have not been provided for any of the LC-MS/MS methods for serum analysis and for only one of the urine analysis methods. Despite the extraction methods used in the published assays and the known variability in analyte ionization in LC-MS/MS methods, none of these published methods for serum and urine analyses includes an internal standard. In mussel and seawater analyses, the deuterated dansyl chloride derivative of DA, kainic acid (KA), and leucine-enkephalin (ENK) have been used as internal standards for LC-MS/MS methods. These internal standards have distinctly different chromatographic and spectrometric properties than DA, which decrease their usefulness. They have also not been applied to plasma, serum, or urine analysis. To address the lack of internal standards in published methods, we explored the feasibility to use fully reduced tetrahydrodomoic acid (THDA) (Figure 1B) as a suitable internal standard for DA measurement by LC-MS/MS.

The goal of this study was to develop and validate a high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for quantification of DA in urine and plasma with sufficient sensitivity to measure DA following low-level exposure to DA. The applicability of this method was confirmed in a cohort of Macaca fascicularis (cynomolgus) monkeys following a single oral dose of 0.075 mg/kg DA (proposed human TDI for DA). To further enhance the applicability, the method was cross-validated in human plasma.

### RESULTS

#### Fragmentation Patterns and HPLC-MS/MS Optimization
To characterize the ionization and fragmentation of DA and THDA, MS^2 scans of the precursor ions and MS^3 scans of the two product ions were collected. The enhanced product ion (EPI) mass spectra are shown in Figure 1B. The product ions observed in the MS^3 spectra for DA ([M + H]^+ m/z 312)
are consistent with published spectral data.\textsuperscript{31,32} The MS\textsuperscript{3} spectra of the two product ions of DA (\(m/z\) 312 > 294 and \(m/z\) 312 > 266) and THDA (\(m/z\) 316 > 298 and \(m/z\) 316 > 270) support the analogous fragmentation of the two compounds. HPLC-MS/MS multiple reaction monitoring (MRM) was optimized for quantification of DA. The three most sensitive MS\textsuperscript{2} product ions for DA (\(m/z\) 312 > 266, \(m/z\) 312 > 248, and \(m/z\) 312 > 220) and THDA (\(m/z\) 316 > 270, \(m/z\) 316 > 252, and \(m/z\) 316 > 224) were chosen for each compound. The final MS/MS conditions are described in Materials and Methods section. Representative chromatograms of DA and THDA are shown in Figure 3.

The on-column limit of detection (LOD) of DA was determined to be 0.52 pg (1.7 fmol) (Figure 4A). Signal-to-noise ratio (S/N) was greater than 3 for transition \(m/z\) 312 > 266, whereas the S/N was less than 3 for transitions \(m/z\) 312 > 248 and \(m/z\) 312 > 220 at LOD. The on-column lower limit of quantification (LLOQ) was 1.0 pg (3.3 fmol) (Figure 4B). The S/N was greater than 3 for all the three transitions at LLOQ.

**Method Validation.** Selectivity of the method was assessed by analyzing blank plasma and urine samples from six treatment-naive monkeys to determine potential matrix interference. An interference peak was observed at 2.4 min in both plasma and urine chromatograms with the MRM transition \(m/z\) 316 > 270 and hence this MRM transition was not used. No interference peak was observed in the chromatograms with the other MRM transitions (Figure 5A,B). Specificity of the method was assessed by analyzing plasma and urine samples with THDA as an internal standard and without DA to determine potential interference from the internal standard. No interference peak was observed at any of the DA MRM transitions. (Figure 5C,D). DA recovery was complete in all samples with measured recoveries of 105 ± 10, 108 ± 2, 107 ± 10, and 101 ± 2% at 0.31, 0.93, 7.8, and 12 ng/mL (1, 3, 25, and 40 nM), respectively, in plasma and 113 ± 9,
may include the following elements:

1. **Title**: The title should clearly state the main objective or finding of the research, making it clear what the study is about.

2. **Introduction**: This section should provide background information, state the research question, and outline the objectives of the study. It should also include a literature review to contextualize the research.

3. **Methods**: This section should detail the methods used in the research, including materials, procedures, and protocols. It should be clear and precise enough to allow others to replicate the study.

4. **Results**: This section should present the findings of the study, typically including tables, graphs, and statistical analyses. It should be concise and focused on presenting the data.

5. **Discussion**: This section should interpret the results, relate them to the literature, and discuss their implications. It should also address any limitations of the study.

6. **Conclusion**: This section should summarize the main findings and their implications, and may suggest areas for future research.

7. **References**: This section should list all the sources cited in the research, formatted according to a specific citation style (e.g., APA, MLA, Chicago).

For example, if the text contains a table, it should be formatted appropriately and include all necessary columns and rows. If the text contains a figure, it should be clearly labeled and accompanied by a description or caption. If the text contains a mathematical equation, it should be written in a clear and readable format, with all symbols and variables clearly defined.

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**Example Figure 4**

Figure 4. MRM chromatograms of DA at (A) on-column LOD at 0.52 pg (1.7 fmol) and (B) on-column LLOQ at 1.0 pg (3.3 fmol).

**Example Table 2**

| Compound | LOD (ng/mL) | LLOQ (ng/mL) |
|----------|-------------|--------------|
| DA       | 0.16        | 0.31         |
| THDA     | 0.5          | 0.31         |

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**Example Text**

The DA/THDA peak area ratio was linear between 0.31 and 16 ng/mL (1 and 50 nM) of DA in plasma and between 7.8 and 1000 ng/mL (25 and 3200 nM) of DA in urine. Extracted samples were stable for up to 5 days in the autosampler and up to a day on bench top. Plasma and urine samples were stable at −20 °C storage for at least five freeze–thaw cycles and for up to 28 weeks in plasma and 34 weeks in urine.

The DA/THDA peak area ratio was linear between 0.31 and 16 ng/mL (1 and 50 nM) of DA in plasma and between 7.8 and 1000 ng/mL (25 and 3200 nM) of DA in urine. All calibration curves satisfied the predefined acceptance criteria with greater than 80% nonzero calibration standards within 15% of nominal concentration. The LLOQ in plasma was 0.31 ng/mL (1 nM), at which concentration all three mass transitions (m/z 312 > 266, 248, 220) were detected with S/N > 3. The LOD in plasma was 0.16 ng/mL (0.5 nM), at which concentration only mass transition m/z 312 > 266 was detected with S/N > 3. The LLOQ in urine was 7.8 ng/mL (25 nM), at which concentration all three mass transitions were detected with S/N > 3. The representative LC-MS chromatograms at LLOQ in plasma and urine are shown in Figure 5E,F. The intraday and interday accuracy (% error) and precision (% CV) in plasma and urine are shown in Table 2.

Overall, the accuracy and precision parameters would have passed assay validation criteria if samples were quantified without an internal standard. However, the variability in the quantification of extracted samples nearly doubled in the absence of an internal standard, and in long LC-MS/MS run batches spanning more than a day (>150 samples), the variability exceeded 15% across the run without internal standard normalization. In addition, occasional samples prepared from plasma of individual animals had approximately 50% error in accuracy when quantified without internal standard normalization. This poor accuracy was corrected to 6% via the normalization to the internal standard. This error is likely due to matrix effects on DA and THDA. Based on an analysis of calibration curves of DA and THDA in solvent and matrix, matrix effects were present for both compounds, and the matrix effect was not different between the compounds.

This LC-MS method was also cross-validated in pooled human plasma and urine. Although different background peaks were detected in human plasma and urine when compared to monkey plasma and urine, none of these interfered with the quantification of DA or THDA. The human plasma LLOQ was 0.31 ng/mL (1 nM) and the urine LLOQ was 7.8 ng/mL (25 nM). Plasma and urine calibration standards satisfied the predefined acceptance criteria with 89 and 100% nonzero calibration standards of plasma and urine, respectively, falling within 15% of nominal concentration. The accuracy and precision data are shown in Table 2.

**Measuring DA in Monkey Plasma and Urine after Oral Exposure to DA.** Plasma and urine DA concentrations were determined in samples collected over a 24 h period from healthy female cynomolgus monkeys (n = 10) following single oral doses of DA at the human TDI of 0.075 mg/kg. Plasma and urine concentrations from these monkeys are shown in Figure 6. A total of 77 plasma samples were collected and analyzed to measure DA concentrations. Sixty three samples (82%) contained DA above the LLOQ at 0.31 ng/mL (1 nM). The range of determined plasma concentrations was between 0.31 and 11 ng/mL (1 and 35 nM). In the remaining 18% of samples (n = 14), DA was detected, indicating that the concentration of DA in these samples was between 0.16 ng/mL (0.5 nM, LOD) and 0.31 ng/mL (1 nM, LLOQ). A total of 60 urine samples were collected and analyzed to measure DA concentrations and DA excretion into urine. All 60 samples contained DA above the LLOQ of 7.8 ng/mL (25 nM). The range of determined urine concentrations was between 9.4 and 745 ng/mL (30 and 2400 nM). Pharmacokinetic parameters were estimated from nine monkeys (n = 9) because the AUC could not be reliably estimated from one monkey, as more than 50% of the plasma concentrations were below LLOQ. A geometric mean peak plasma concentration (Cmax) of 2.3 ng/mL (95% confidence interval (CI): 1.2–4.4) was observed between 1 and 12 h after the dose (tmax). The geometric mean area under the plasma concentration versus time curve (AUC0–24) was 19 ng·h/mL (95% CI: 13–28), the geometric mean amount of DA excreted unchanged in urine (A0–24) was 5.2 µg (95% CI: 3.6–7.4), and the geometric mean renal clearance (CLR) was 4.5 mL/min (95% CI: 2.5–8.2).

The potential presence of DA or any potential interference in the assay was also measured in plasma samples collected over a 24 h period from control healthy female cynomolgus monkeys (n = 10) following a single dose of 5% sucrose vehicle solution. A total of 20 plasma samples were analyzed, and none of the samples contained detectable DA.

**DISCUSSION**

Recent reports of DA consumption exceeding the proposed TDIs in recreational shellfish harvesters, and the health risks associated with chronic exposure to subacute levels of DA, highlight the need to investigate the exposure–effect relationship of DA. A sensitive method to detect low DA concentrations and DA excretion into urine. All 60 samples contained DA above the LLOQ of 7.8 ng/mL (25 nM). The range of determined urine concentrations was between 9.4 and 745 ng/mL (30 and 2400 nM). Pharmacokinetic parameters were estimated from nine monkeys (n = 9) because the AUC could not be reliably estimated from one monkey, as more than 50% of the plasma concentrations were below LLOQ. A geometric mean peak plasma concentration (Cmax) of 2.3 ng/mL (95% confidence interval (CI): 1.2–4.4) was observed between 1 and 12 h after the dose (tmax). The geometric mean area under the plasma concentration versus time curve (AUC0–24) was 19 ng·h/mL (95% CI: 13–28), the geometric mean amount of DA excreted unchanged in urine (A0–24) was 5.2 µg (95% CI: 3.6–7.4), and the geometric mean renal clearance (CLR) was 4.5 mL/min (95% CI: 2.5–8.2). The potential presence of DA or any potential interference in the assay was also measured in plasma samples collected over a 24 h period from control healthy female cynomolgus monkeys (n = 10) following a single dose of 5% sucrose vehicle solution. A total of 20 plasma samples were analyzed, and none of the samples contained detectable DA.
oral DA consumption at the TDI (0.075 mg/kg). This prediction set the required sensitivity threshold of analytical methods for DA quantification at about 5−10-fold below the predicted C_{max} (i.e., 0.2−0.4 ng/mL) to detect and quantify DA in samples from people with DA exposure near the proposed TDIs.

Despite many analytical methods having been developed for DA, only a few of them have been developed to determine DA in biological fluids, such as plasma and urine. Current published LC-MS/MS methods have largely been used to confirm the presence of DA in samples that were positive by ELISA detection, although the analytical sensitivity of most reported LC-MS/MS methods is lower than ELISA (Table 1). ELISA methods have been validated for DA quantification in serum and urine, whereas no validation data exist for LC-MS/MS methods used for DA quantification in plasma and only a single LC-MS/MS assay has been validated for measuring DA in urine samples. The method reported here is the first LC-MS/MS method for DA analysis that has been validated for measurement of DA in plasma samples. Importantly, the assay was validated to detect concentrations at 0.16 ng/mL (0.5 nM, LOD) and to reproducibly quantify concentrations at or above 0.3 ng/mL (1 nM, LLOQ) of DA in plasma, demonstrating sufficient sensitivity to assess subchronic exposures to DA. As shown by our study in cynomolgus monkeys, the sensitivity of this validated method was sufficient to quantify DA in plasma following a single oral dose at the proposed TDI. Similarly, our method was validated to detect and quantify DA at or above 7.8 ng/mL (25 nM, LLOQ) in urine. All urine samples collected from the monkeys dosed with DA contained quantifiable DA concentrations. Yet,
the LLOQ for urine samples can be further reduced with less dilution to increase the sensitivity of the assay if needed.

One of the major challenges when developing analytical methods to detect DA in biological samples is variable recovery following complex extraction or variable MS response due to matrix effects. The recovery of DA from different marine animal samples following SPE ranged between 20 and 100% resulting in challenging quantitation of DA. A few published methods used KA and ENK as internal standards in seawater and mussel analyses to control for assay variability, but the distinct chromatographic and mass spectrometric properties of KA and ENK may decrease their usefulness as internal standards for DA. Another recently published method introduced the use of the labeled dansyl chloride derivative of DA as an internal standard, but the sample and internal standard are derivatized separately. To address these issues with selection of an internal standard, we synthesized and validated THDA as an internal standard. THDA is structurally similar to DA and shares similar chromatographic and mass spectrometric properties. We demonstrated the appropriate chromatographic and mass spectrometric performance of THDA to control for assay variability. The use of THDA improved the reproducibility of the method as demonstrated by acceptable accuracy and precision when the internal standard was used, but inadequate accuracy and precision in the absence of an internal standard during long LC-MS/MS runs. Importantly, although this method uses a simple single methanol extraction step, THDA can easily be applied to other LC-MS/MS and sample preparation methods that quantify DA in more complex matrices.

ELISA-based methods to determine DA concentrations in samples from marine mammals are convenient to use with minimal lab equipment requirements. However, discrepancies between results from ELISA and LC-MS/MS methods have been reported. One of the possible reasons for these discrepancies is that marine mammals chronically exposed to DA may produce antibodies against DA, which may interfere with the ELISA assays, and hence ELISA assays would underestimate the DA content. Another possible reason is that complex biological matrices, like plasma or serum, may contain interfering components that bind to the anti-DA antibody used in the ELISA assay, which would overestimate the DA content. The validated HPLC-MS/MS method described here provides a key advantage over the ELISA by obviating the above issues and allowing direct detection of DA concentrations and exposure in chronically exposed marine animals. However, the

### Table 2. Accuracy and Precision Data for Monkey and Human Plasma and Urine

|          | LLOQ | LQC | MQC | HQC |
|----------|------|-----|-----|-----|
| Plasma   |      |     |     |     |
| 0.31 ng/mL |     |     |     |     |
| % error | −2.3 | 4.4 | 2.4 | −2.9 |
| % CV    | 5.6  | 2.6 | 3.7 | 3.1  |
| monkey intraday |      |     |     |     |
| monkey interday |      |     |     |     |
| human intraday |      |     |     |     |
| human interday |      |     |     |     |
| % error | 7.3  | 11  | 7.0 | 0.0  |
| % CV    | 13   | 7.3 | 3.5 | 1.9  |
| % error | 1.8  | 2.8 | 2.4 | 2.4  |
| % CV    | 13   | 7.7 | 4.7 | 4.7  |
| monkey intraday |      |     |     |     |
| monkey interday |      |     |     |     |
| human intraday |      |     |     |     |
| human interday |      |     |     |     |
| % error | 4.4  | 8.4 | 11.3| 11.4 |
| % CV    | 7.3  | 1.8 | 3.7 | 0.8  |
| % error | 1.8  | 2.3 | 3.4 | 0.5  |
| % CV    | 13   | 4.2 | 3.2 | 2.1  |
| monkey intraday |      |     |     |     |
| monkey interday |      |     |     |     |
| human intraday |      |     |     |     |
| human interday |      |     |     |     |
| % error | 4.4  | 2.8 | 2.4 | 0.0  |
| % CV    | 7.3  | 7.7 | 4.7 | 1.9  |
| % error | 5.6  | 6.9 | 7.0 | 0.8  |
| % CV    | 10   | 7.7 | 3.7 | 0.8  |
| monkey intraday |      |     |     |     |
| monkey interday |      |     |     |     |
| human intraday |      |     |     |     |
| human interday |      |     |     |     |

Intraday and interday accuracy (% error) and precision (% CV) of plasma and urine quality control (QC) samples. LQC, low QC; MQC, middle QC; HQC, high QC.

### Figure 6

DA pharmacokinetics following a single 0.075 mg/kg dose of DA to a group of 10 healthy adult female cynomolgus monkeys. (A) Geometric mean plasma concentration–time curve (black line) with individual data above the LLOQ (82%, n = 63) shown as symbols. Mean maximum plasma concentration (C\(_{\text{max}}\)) and area under the plasma concentration versus time curve (AUC) from 0 to 24 h are reported on the graph; (B) Individual urine concentration data measured above the LLOQ (100%, n = 60); and (C) individual cumulative % dose excreted unchanged in urine. Geometric mean amount of unchanged DA excreted (\(A_e\)) from 0 to 24 h and renal clearance (CL\(_r\)) are reported as inset in the graph.
The purity of the product was assessed by HPLC-MS/MS to determine on a Tecan Infinite 200 PRO spectrophotometer (Bogart, GA), and the UV spectra are shown in Figure 1.

**HPLC-MS/MS.** Aqueous stock solutions of the certified DA standard and THDA stock at 3.1 ng/mL (10 nM) and 0.63 ng/mL (2 nM), respectively, were used to characterize their fragmentation patterns and to optimize their MS/MS detection parameters on a Sciex 6500 QTRAP system (Foster City, CA) by positive ion electrospray ionization. The optimized MS parameters for both DA and THDA were ion source temperature at 550, curtain gas at 50, nebulizing gas (GS1) at 70, drying gas (GS2) at 70, collision activated dissociation gas at −2, ion-spray voltage at 5500, declustering potential at 15, and entrance potential at 10. Collision energy (CE) for MS2 analysis was set as 24, and excitation energy (AF2) for MS3 EPI scans was set at 0.05. For MS2 EPI scans, Q1 was set to filter for the precursor ions [M + H]+ m/z 312 for DA and m/z 316 for THDA. For MS3 EPI scans, the QTRAP function was used, and Q3 filter was set up to filter for m/z 312 > 294 (CE 15) and m/z 312 > 266 (CE 20) for DA and m/z 316 > 298 (CE 15) and m/z 316 > 270 (CE 20) for THDA.

The LC-MS method was developed and validated using a Shimadzu UFLC XR DGU-20AS (Kyoto, Japan) equipped with a Phenomenex Synergi Hydro-RP 100 Å (2.5 μm, 50 × 2 mm²) LC column and a guard cartridge (2 × 21 mm², sub 2 μm) (Torrance, CA) coupled to a Sciex 6500 QTRAP system (Foster City, CA). A 9 min gradient elution was employed using (A) water with 0.1% formic acid and (B) 95% acetonitrile with 0.1% formic acid at a flow rate of 0.5 mL/min. Sample injection volume was 10 μL. The gradient was initiated at 5% B for 1 min, increased to 100% B over the next 3 min, kept at 100% B for 30 s before decreasing to 5% B over 30 s, and re-equilibrating at 5% B for another 4 min. Three product ions for DA (m/z 312 > 266, m/z 312 > 248, and m/z 312 > 220) and for THDA (m/z 316 > 270, m/z 316 > 252, and m/z 316 > 224) were monitored to confirm the identity of the analytes. MRM conditions were optimized to collision energy (CE) at 24 eV, and collision cell exit potential at 10 eV.

The on-column LOD for DA was determined by injecting 10 μL of DA standard in water at concentrations ranging from 0.031 to 0.31 ng/mL (0.1–1 nM). The LOD was defined as the lowest amount of injected DA producing a S/N > 3 for the DA transition m/z 312 > 266. The on-column LLOQ was defined as the lowest amount of injected DA producing a S/N > 3 for all three DA mass transitions m/z 312 > 266, 248, 220. Calibration standards covering 4 orders of magnitude at concentrations ranging from 0.031 to 310 ng/mL (0.1–1000 nM) of DA were prepared in water to determine the linearity of response (peak area of DA mass transition m/z 312 > 266).

**DA Extraction from Plasma and Urine Samples.** DA was extracted from plasma by mixing 60 μL of plasma standards or samples with 120 μL LC/MS grade methanol containing 0.63 ng/mL (2 nM) THDA and vortexed for 30 s to precipitate proteins. The precipitated plasma standards and samples were centrifuged at 16 100g for 60 min, and the supernatant was removed for HPLC-MS/MS analysis. DA was extracted from urine by mixing 10 μL of urine standards or samples with 490 μL of LC/MS grade water containing 0.63 ng/mL (2 nM) THDA and subsequently 500 μL of methanol. The urine was vortexed for 30 s to precipitate proteins and centrifuged at 16 100g for 15 min. The supernatant was removed for HPLC-MS/MS analysis.

**Method Validation.** The method was validated according to the FDA Guidance for Industry Bioanalytical Method...
Validation using pooled cynomolgus monkey plasma and urine. The plasma calibration standards were prepared by spiking plasma with the DA certified standard and serially diluting with plasma to nine concentrations ranging between 0.16 and 16 ng/mL (0.5–50 nM). The urine calibration standards were prepared by spiking urine with DA certified standard and serially diluting with urine to nine concentrations ranging between 7.8 and 1000 ng/mL (25–3200 nM). The calibration standards were processed and analyzed by HPLC-MS/MS, as described above. The MRM chromatograms were integrated using AB Sciex MultiQuant software version 2.1.1 (Foster City, CA). DA was quantified using the peak area ratio of m/z 312 > 266 (DA) and m/z 316 > 252 (THDA), and a weighted (1/y²) calibration curve was fitted linearly to the data. The 1/y² weighing was used as the simplest weighing scheme providing sufficient accuracy over the concentration range used. The 1/y² weighing provided better accuracy over the concentration range studied in comparison to uniform weighing. The acceptance criteria for each calibration curve were defined as greater than 75% of all nonzero concentrations determined with less than 15% error from the nominal concentration, except at the LLOQ, which was accepted with less than 20% error.

Plasma QCs were prepared by spiking blank plasma with DA certified standard to 0.31 ng/mL (LLOQ), 0.93 ng/mL (LQC), 7.8 ng/mL (MQC), 12 ng/mL (HQC) (1, 3, 25, 40 nM). Urine QCs were prepared by spiking blank urine with DA certified standard to 7.8 ng/mL (LLOQ), 23 ng/mL (LQC), 50 ng/mL (MQC), 780 ng/mL (HQC) (25, 75, 1600, 2500 nM). Blank plasma and urine from six treatment-naive cynomolgus monkeys with and without internal standard were analyzed for potential interference. The accuracy and precision of the method were determined on three different days. Intraday variability was calculated with at least 12 replicates of each QC analyzed on 3 different days. Intraday variability was determined with five replicates analyzed on the same day. Calibration standards were analyzed in duplicates along with blanks and replicates of QCs (LLOQ, LQC, MQC, HQC) in each run. The LLOQ was defined as the lowest concentration in plasma or urine with S/N > 3 for DA transition m/z 312 > 266 that could be repeatedly determined with less than 20% error and within 20% CV. The % error and % CV were calculated according to eqs 1 and 2.

\[
\text{% error} = \frac{\text{measured concentration} - \text{nominal concentration}}{\text{nominal concentration}} \times 100
\]

\[
\text{% CV} = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2 / n - 1}{\bar{x}}} \times 100
\]

The LOD was defined as the lowest concentration in plasma and urine with S/N > 3 for the DA transition m/z 312 > 266, but did not meet the LLOQ acceptance criteria for reproducibility. QCs with less than 15% error from the nominal concentration or less than 20% error at LLOQ were accepted.

Stability of extracted samples was determined by repeated injections of QC samples (LQC, MQC, HQC) stored either in the autosampler at 4 °C for up to 5 days or on bench top at room temperature for a day. Acceptance criterion of extracted sample stability was defined as less than 15% CV from repeated injections. Long-term stability was determined by repeated analysis of spiked plasma controls at 0.93, 1.9, 3.7 ng/mL (3, 6, 12 nM) and spiked urine controls at 16, 160, 620 ng/mL (50, 500, 2000 nM) stored at –20 °C and that were subjected to at least five freeze–thaw cycles. Acceptance criterion of long-term stability was defined as less than 15% CV of measured concentration from repeated analysis.

Recovery of DA was determined at 0.31, 0.93, 7.8, 12 ng/mL (1, 3, 25, 40 nM) of DA in plasma and 7.8, 23, 500, 780 ng/mL (25, 75, 1600, 2500 nM) of DA in urine in triplicates. The recovery was calculated by comparing the DA/THDA peak area ratio of samples spiked with DA prior to methanol extraction to DA/THDA peak area ratio in samples extracted with methanol and subsequently spiked with the DA at matching amount. The THDA (0.32 ng/mL) was added to all samples following extraction. The percent recovery was reported as the percentage of peak area ratio of recovery samples divided by the mean peak area ratio of control samples.

Cross-validation using human plasma and urine was performed in a single accuracy and precision validation run. Plasma and urine calibration standards and QCs were prepared as described for monkey standards and QCs. Plasma and urine were pooled from banked tissue from six individuals. Potential matrix interferences were assessed by analyzing blank plasma and urine. Duplicates of calibration standards and five replicates of LQC, MQC, HQC, and four replicates of LLOQ were included in the cross-validation to assess variability.

Animal Study Samples and Application of the Method. Monkey plasma and urine samples from a DA toxicokinetic study were analyzed to evaluate the applicability of the assay to determine DA exposure after a single oral dose at the proposed TDI of 0.075 mg/kg. All animal procedures followed the guidelines set by the Animal Welfare Act and the Guide for Care and Use of Laboratory Animals of National Research Council. The research protocol was approved by the University of Washington Institutional Animal Care and Use Committee. Fresh blood and urine samples were obtained at 1, 2, 4, 6, 8, 10, 12, and 24 h from 20 healthy adult female cynomolgus monkeys following a single oral dose of 0.075 mg/kg DA in 5% sucrose in water (n = 10) or a single oral dose of 5% sucrose in water (n = 10). Training protocols using positive reinforcement were implemented to collect blood without sedation. Fresh blood was collected from the peripheral vein using sodium heparin-coated collection tubes and centrifuged at 3000g for 15 min within 1 h of collection to isolate plasma from blood cells. The plasma samples were stored at –20 °C until analysis. Urine was collected from the cage pans equipped with metabolic urine collection trays at the same time fresh blood was collected. The total volume of urine in the pan was measured, and a 2 mL sample was collected before discarding the rest of the urine. The urine samples were stored at –20 °C until analysis.

Pharmacokinetic Analysis. Pharmacokinetic parameters, including area under the plasma concentration–time curve (AUC) and maximum plasma concentration (Cₘₐₓ), were estimated by noncompartmental analysis using Phoenix WinNonlin (St. Louis, MO). The plasma concentration was assigned to be 0.2 ng/mL when DA was detected but was below the LLOQ for AUC calculation. The cumulative amount of unchanged DA excreted in urine (Aₑ) was calculated from the determined concentration and the volume of urine at all collection time intervals using eq 3. Renal clearance (CLₑ) was
estimated from the cumulative amount excreted unchanged in urine and the plasma AUC using eq 4

\[ A_u = \sum_{i=1}^{n} \text{volume, } \times \text{urine concentration, } \]

\[ CL_u = \frac{A_u}{AUC_{0-24}} \]

\[ (4) \]

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