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Article

# Simple and Practical Method for the Quantitative High-Sensitivity Analysis of *N*-Nitroso Duloxetine in Duloxetine Drug Products Utilizing LC-MS/MS

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Cite This: *ACS Omega* 2024, 9, 13440−13446

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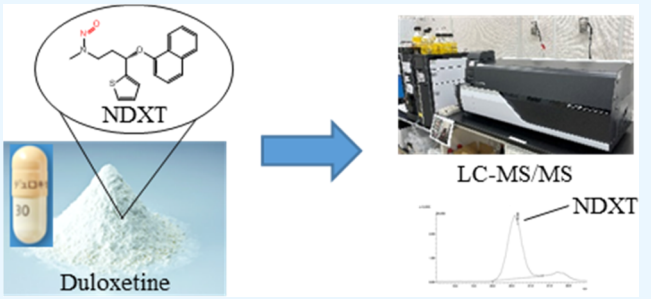
**ABSTRACT:** The high-sensitivity analytical method for the determination of *N*-nitroso duloxetine (NDXT), which can be carcinogenic and harmful in duloxetine drug products, was successfully developed utilizing liquid chromatography-tandem mass spectrometry (LC-MS/MS). Tandem mass spectrometric detection at positive electrospray ionization in multiple reaction monitoring (MRM) mode was then employed for the determination of NDXT. The quantitative range for NDXT was found in 0.075−3.75 ng/mL in terms of concentration in the dilution solvent for duloxetine active pharmaceutical ingredient (API) and capsules and 0.075−1.875 ng/mL for duloxetine tablets, and the recovery rates were in the range of 82.5−91.6% for the API, 91.0−113.4% for capsules, and 70.6−109.1% for tablets, respectively. The repeatability was 6.9% with a %RSD of $n$ = 9 for the API, 10.9% with a %RSD of $n$ = 9 for capsules, and 21.6% with a %RSD of $n$ = 9 for tablets, respectively. For reproducibility, the %RSD of the $n$ = 6 measurements between the two sites was 3.5%. The calibration curve of NDXT in the concentration range of 0.075−3.75 ng/mL was carried out, and the correlation coefficient ($R$) was found to be 1.000. The sample solution was stable for 7 days. The applicability of the determination of the content of NDXT in a variety of duloxetine drug products was demonstrated. This manuscript seeks to aid the risk assessment process of NDXT in duloxetine drug products through providing a fast and reliable quantitative LC-MS/MS analytical method.



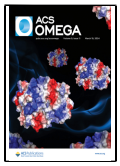

## 1. INTRODUCTION

In 2018, starting with the detection of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) contamination, which can be carcinogenic and harmful, in the angiotensin II receptor antagonist valsartan for the treatment of hypertension,[1] the European Medicines Agency (EMA) in 2019,[2] the U.S. Food and Drug Administration in 2021,[3] and the Japanese Ministry of Health, Labor, and Welfare in 2021[4] issued a series of notices and guidance to pharmaceutical manufacturers and distributors, stating that they should assess the risk of nitrosamine contamination and take appropriate measures to reduce it. The forming pathway of NDMA/NDEA in sartans such as valsartan is considered due to the reaction of the solvent *N*,*N*-dimethylformamide with nitrous acid, which is used to quench the residual azide used in the formation of the tetrazole ring.[5] In 2019, NDMA was detected in metformin drug products for the treatment of type 2 diabetes, and many metformin manufacturers voluntarily recalled their products.[6] Although the root cause is not clear, it is suspected that the dimethylamine impurity remaining in metformin API may react with nitrosating agents such as nitrite in excipients[7,8] and nitrocellulose in packaging materials[9] to form NDMA.

Under such circumstances, we have raised questions about commonly accepted beliefs on NDMA contamination in metformin drug products based on our findings that the amount of NDMA contamination varies among our manufacturing sites, even when the same raw materials were used. We successfully demonstrated the correlation between the concentration of atmospheric $NO_2$ around our factories and the NDMA content in metformin drug products,[10] suggesting that $NO_X$, an air pollutant, may be closely related to the formation and contamination of NDMA.

The problem of contamination of nitrosamines in drug products is spreading out rapidly not only to the previously mentioned nitrosamines but also to nitrosamine drug substance-related impurities (NDSRIs), drug-specific nitros-

Received: January 4, 2024
Revised: February 20, 2024
Accepted: February 26, 2024
Published: March 8, 2024



amine impurities whose chemical structure is similar to the API. Contamination of NDSRIs is inevitable because the nitroso group is bound to the API itself. Furthermore, there are also no carcinogenicity or mutagenicity test data for most NDSRIs, making it difficult to determine the acceptable intakes (AIs). In July 2023, the EMA proposed a new evaluation method called CPCA (Carcinogenic Potency Categorization Approach for *N*-nitrosamines) for the establishment of AIs for nitrosamines, which has eased the challenges related to AIs.[11] However, analytical methods for most NDSRIs have not been established, and there are still many challenges in the risk assessment of contamination with NDSRIs.

Recently, a very informative paper on a nitrite in excipient to assess nitrosamine risk for drug products was published. This paper refers that one risk of occurrence of nitrosamines is the interaction between nitrosating agents, derived from nitrites in excipients, and reactive amines, either present as moieties of the active molecule or as impurities/degradants.[15] Therefore, the results of the analysis of nitrosamines in APIs are not sufficient to evaluate nitrosamine contamination in pharmaceutical products, and the development of analytical methods for nitrosamines in pharmaceutical drug products is urgently needed.

Duloxetine is a selective serotonin and a norepinephrine reuptake inhibitor (SSNRI) antidepressant, which is used for the treatment of major depressive disorder.[12] In 2020, it was the 27th most commonly prescribed medication in the United States, with more than 22 million prescriptions.[13,14] The EMA defined an AI of 100 ng/day for *N*-nitroso duloxetine (NDXT) in October 2022,[11] requiring duloxetine manufacturers to assess the risk of NDXT contamination. The structural formulas for duloxetine and NDXT are shown in Figure 1.

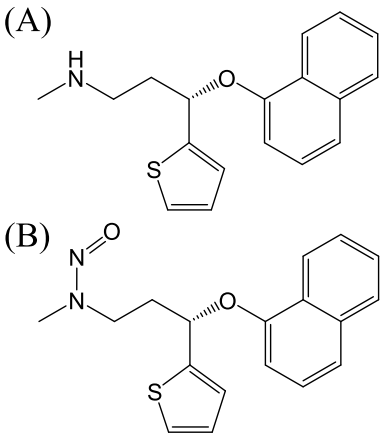



**Figure 1.** Chemical structures of (A) duloxetine and (B) NDXT.

Maruthapillai et al. recently reported the first example of an analytical method for the determination of NDXT in duloxetine hydrochloride utilizing LC-MS/MS.[16] The proposed analytical method was illustrated as a specific and sensitive assessment, but the sample preparation procedure was complicated because of the use of hexane/water liquid−liquid extraction. Furthermore, there is no mention of its application to duloxetine tablets or capsules containing various excipients, such as talc. Excipients often interfere with the analysis, and there are many cases in which the analytical method for the API cannot be directly applied to the analytical method for the drug products. Because of concerns about the formation of NDXT by reaction with duloxetine API and nitrosating agents in excipients, it is necessary to develop not only analytical methods for NDXT in duloxetine API but also highly practical analytical methods for NDXT in drug products in order to conduct a comprehensive NDXT contamination risk assessment.

Here, we describe that a new practical high-sensitivity analytical method to quantitatively determine the amount of NDXT contained in duloxetine API and drug products with a simple operation was developed and validated.

## 2. RESULTS AND DISCUSSION

**2.1. Development of the Extraction and Measurement Procedure for NDXT in Duloxetine Drug Products.** Coupling of LC with MS/MS detection is a highly selective technique that results in minimal interference from impurities in duloxetine drug products. Initially, several trials using different columns (many types of ODS, biphenyl, and phenyl columns) were conducted, and the Luna Omega Polar C18 column was adopted. Next, mobile phase compositions using different ratios of methanol and acetonitrile (70, 60, 50, and 40%) and flow type of gradient or isocratic elution were tried in order to separate the NDXT peak and impurity peaks derived from excipients. Optimum performance was obtained using 0.1% formic acid and 2 mM ammonium acetate aqueous solution:acetonitrile (11:9, v/v) as the mobile phase with isocratic elution. The flow rate of 0.2 mL/min was employed in order to maximize the signal intensity for NDXT over a run time. The precursor ions $[M + NH_4]^+$ of NDXT were detected in the full-scan mass spectra at $m/z$ 344. The MS/MS parameters were optimized, and the following MS/MS transitions were selected (344 > 183) for the determination of NDXT. The MS and MS/MS spectra of NDXT are shown in Figures 2 and 3, respectively. Various sample preparation techniques were tried, such as liquid−liquid extraction using hexane and solid-phase extraction of Oasis HLB to remove the excipients and other impurities. Liquid−liquid extraction using hexane was not suitable because NDXT could not be extracted, and solid-phase extraction was also not suitable because of the low recovery rate of NDXT. Extraction in duloxetine drug products was also employed using methanol, acetonitrile, water, and mixtures of them. For the extraction with acetonitrile, there was concern about the stability of NDXT in the sample solution. On the other hand, the solution extraction with methanol was adopted because NDXT was extracted more quickly in methanol than in acetonitrile and there were no problems with stability in the sample solution. Finally, a simple extraction procedure using methanol was established.

**2.2. Analytical Method Validation.** *2.2.1. Specificity.* Comparison of the results obtained from the analysis of duloxetine API and duloxetine drug products with NDXT standard solution indicated that endogenous substances and excipients do not interfere with the peak of NDXT. Chromatogram results for specificity are shown in Figure 4. This confirmed the high specificity of the analytical method toward the test compound in the presence of endogenous impurities and excipients.

*2.2.2. Linearity.* The result of the linearity is shown in Figure 5. The regression equation was $Y = 909\,309X + 10\,650$, $r = 1.000$, for NDXT, where $Y$ was the peak area of NDXT and $X$ was the concentration of NDXT in the diluent solvent (ng/


https://doi.org/10.1021/acsomega.4c00136
ACS Omega 2024, 9, 13440−13446

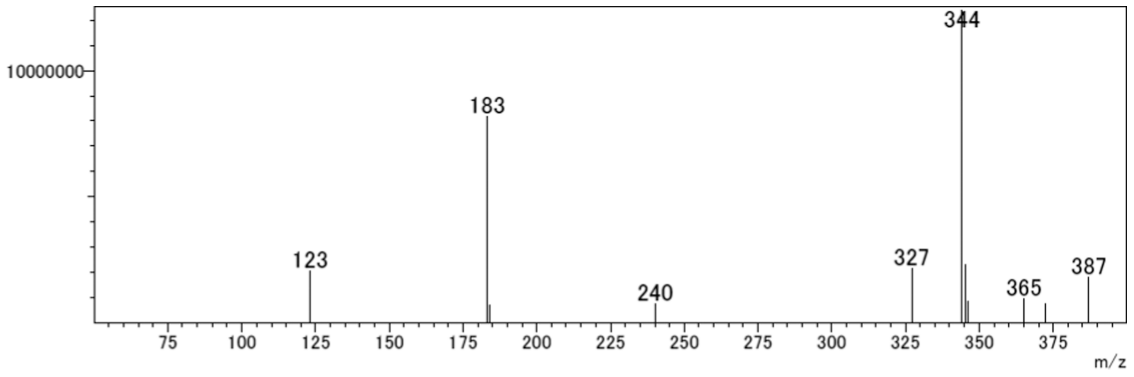



**Figure 2.** MS spectrum of NDXT.

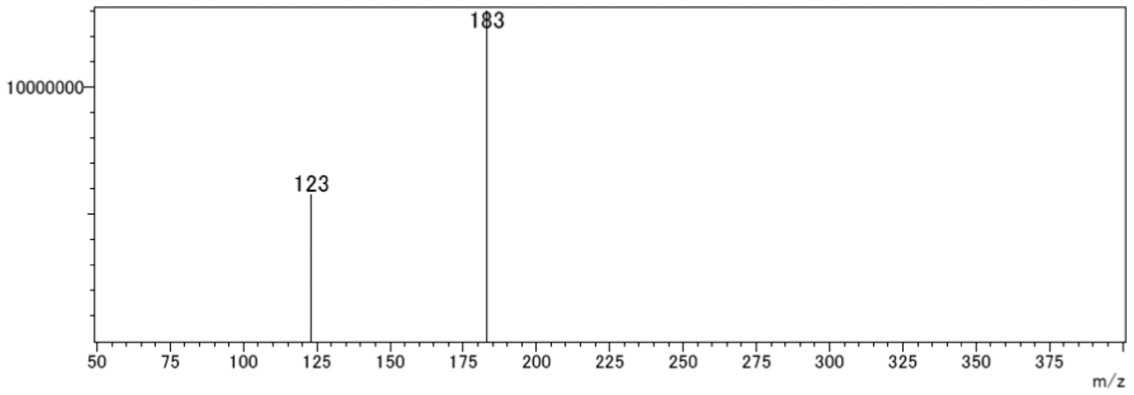



**Figure 3.** MS/MS spectrum of NDXT.

mL). A good linearity was shown between the NDXT concentration and the peak area.

*2.2.3. Limit of Detection (LOD) and Limit of Quantitation (LOQ).* The LOD was determined at 0.02 ng/mL for NDXT by evaluating the signal-to-noise (S/N) ratio. The MS chromatogram of LOD solution is shown in Figure 6 and the S/N ratio was 2.5. The LOQ was determined as the lowest concentration of NDXT (0.075 ng/mL) that could be quantitatively determined with acceptable precision and accuracy as described and was included in the study of various assay validation parameters.

*2.2.4. Accuracy and Repeatability.* A list of the results for accuracy and repeatability is shown in Tables 1−3. For duloxetine API, good results were obtained for accuracy and repeatability when NDXT solutions at 0.075 to 3.75 ng/mL concentrations were spiked. For the duloxetine capsules, initially, the results of recovery rates and repeatability were poor. It was supposed that this is due to the high content of NDXT in the capsules and the fact that the content varies in each capsule. In response to these factors, sample solutions were prepared by mixing the contents of several capsules in advance and the corresponding weight of each capsule was measured with good results for accuracy and repeatability. For duloxetine tablets, recovery rates were evaluated at concentrations of 0.075, 0.75, and 3.75 ng/mL at first, but the recovery rates at the concentration of 3.75 ng/mL were poor. Duloxetine tablets contain a higher percentage of excipients than capsules, and a longer time is required for disintegration. Therefore, excess NDXT which had been added was adsorbed by the excipients and it may have caused the poor recovery rates. Since good results for accuracy and repeatability were obtained when NDXT solutions at concentrations of 0.75 and 1.875 ng/mL were spiked, it was concluded that the matrix had no effect on the results in this range. Based on the above, it was concluded that the developed analytical method has extreme accuracy without affecting the measurement of each duloxetine drug product.

*2.2.5. Reproducibility.* The content of NDXT in the same lot of duloxetine capsules was determined by the developed analytical method in two different laboratories at Towa Pharmaceutical and Daichi Kasei. The quantitative values of NDXT obtained in the two laboratories were comparable, as shown in Table 4. This result indicates that our analytical method has high reproducibility with small variation.

*2.2.6. Range.* The linearity, repeatability, reproducibility, and accuracy results indicate that the validation range is from 0.075 to 3.75 ng/mL for NDXT in duloxetine API and capsules and from 0.075 to 1.875 ng/mL for NDXT in tablets.

*2.2.7. Stability and Robustness.* The sample solutions at room temperature were analyzed at several time points to check for variations from the initial values for the stability study. The results showed that the maximum fluctuation up to 1 week was 16%, confirming the stability of the sample solutions. Robustness of the sample preparation procedure, especially the extraction procedure, had been a concern during analytical method development and was evaluated. When the shaking time was varied, the deviations from the basic conditions were not more than 16%, indicating that this analytical method is tolerant of the shaking time.

## 3. CONCLUSIONS

A fast and accurate LC−MS/MS analytical method with a simple pretreatment sample preparation was developed and validated for the determination of NDXT in duloxetine drug



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ACS Omega 2024, 9, 13440−13446

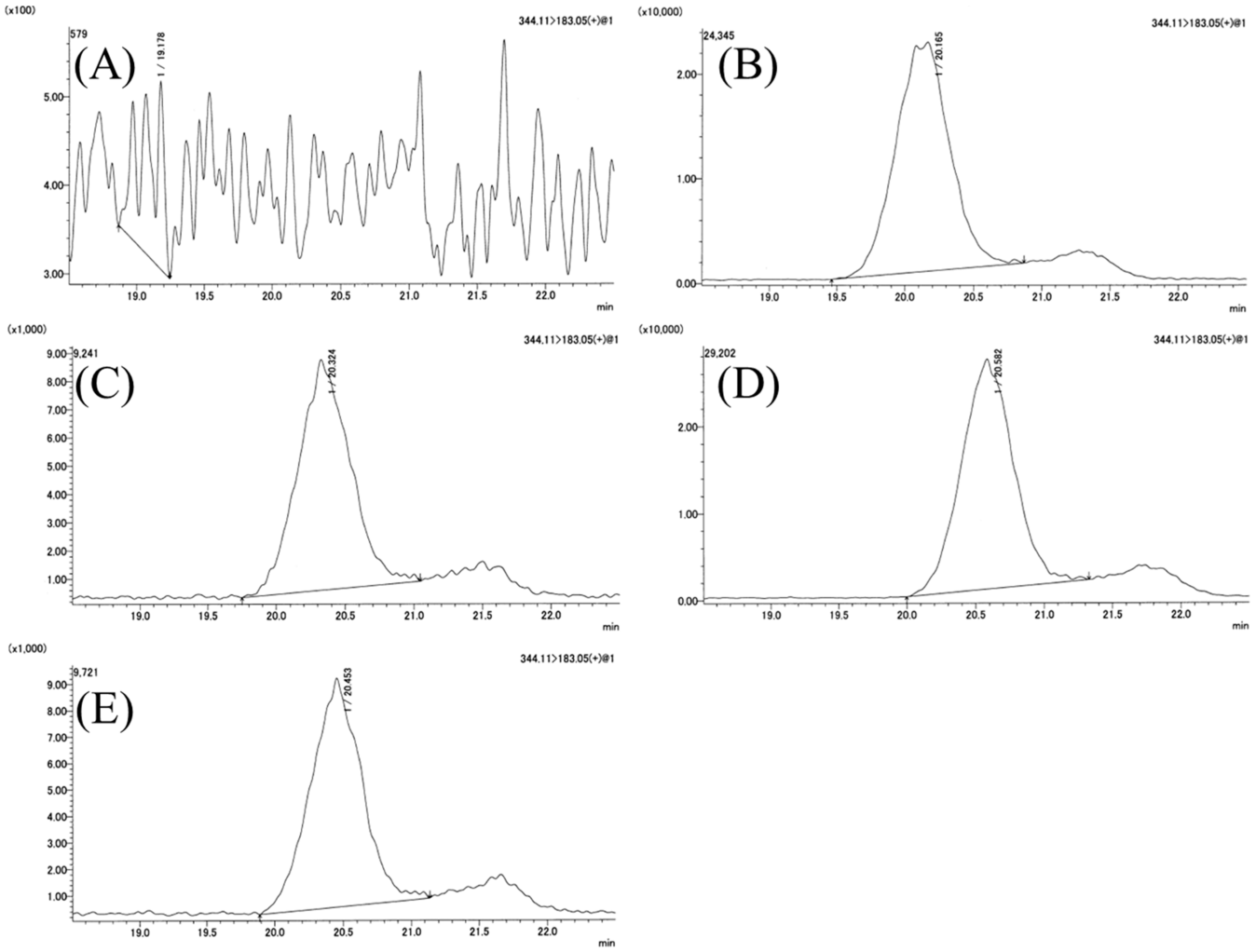


**Figure 4.** MS/MS chromatograms of (A) the blank sample, (B) the standard solution of NDXT (0.075 ng/mL), (C) duloxetine API, (D) duloxetine capsules, and (E) duloxetine tablets.

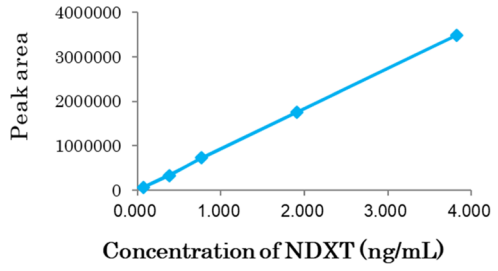


**Figure 5.** Calibration curve for NDXT in standard solution.

products. Results of the validation studies showed that the developed analytical method demonstrated specificity, accuracy, and repeatability in a concentration range that covers from 0.075 to 3.75 ng/mL for NDXT in duloxetine API and capsules and from 0.075 to 1.875 ng/mL for NDXT in tablets. The results also confirmed an appropriate extraction recovery, lack of endogenous interference and impurities, and the small interlaboratory variation in quantitative values. It should be emphasized here that our method can apply not only to duloxetine API but also to duloxetine capsules and tablets. Therefore, our novel analytical method provides a powerful tool to accelerate the risk assessment for NDXT.

## 4. MATERIALS AND METHODS

**4.1. Reagents and Samples.** NDXT and duloxetine API as reference standards were obtained from Toronto Research Chemicals, Inc. (Toronto, Canada) and Moehs BCN, S.L. (Barcelona, Spain). Cymbalta capsules 30 mg were purchased from Eli Lilly Japan K.K. (Kobe, Japan). Duloxetine capsules 30 mg and tablets 30 mg were obtained from Towa Pharmaceutical Co., Ltd. (Osaka, Japan). Acetonitrile (HPLC-grade), methanol (HPLC-grade), formic acid (LC/MS-grade), and ammonium acetate (GR-grade) were procured from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Ultrapure water was obtained from Millipore: Milli-Q purification system (Darmstadt, Germany).

**4.2. Instruments.** Chromatographic analysis was carried out using a Shimadzu HPLC LC-40 series system (Shimadzu Corp., Kyoto, Japan). Mass spectrometric detection was carried out using a triple quadrupole MS-8060NX mass spectrometer (Shimadzu Corporation, Kyoto, Japan) operated in positive electrospray ionization and multiple reaction monitoring (MRM) mode. Hardware control and data acquisition and treatment were carried out using LabSolutions Version 5.113 (Shimadzu Corp., Kyoto, Japan).

**4.3. Liquid Chromatographic and Mass Spectrometric Conditions.** Separations were carried out using a Luna


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ACS Omega 2024, 9, 13440−13446

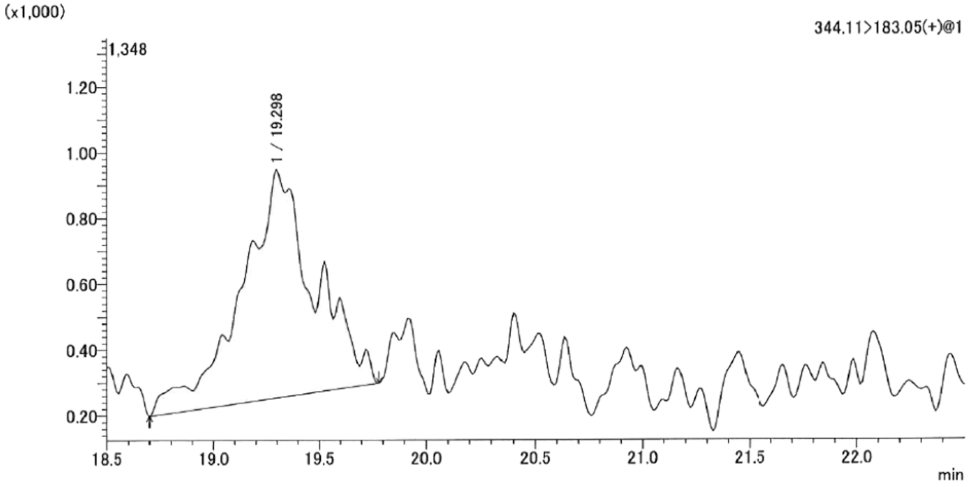


**Figure 6.** MS chromatogram of the LOD solution.

**Table 1. Results of Accuracy and Repeatability for the Determination of NDXT in Duloxetine API**

| | spiked level of NDXT | | |
|---|---|---|---|
| | 0.075 ng/mL | 0.75 ng/mL | 3.75 ng/mL |
| recovery rate (%) | 80.3 | 89.4 | 89.0 |
| | 91.6 | 93.8 | 90.4 |
| | 75.4 | 91.6 | 91.0 |
| average recovery rate (%)[a] | 82.5 | 91.6 | 90.1 |
| SD[a] | 8.3 | 2.2 | 1.0 |
| SD[b] | 6.1 | | |
| %RSD[b] | 6.9 | | |

[a]$n = 3$. [b]$n = 9$.

**Table 2. Results of Accuracy and Repeatability for the Determination of NDXT in Duloxetine Capsules**

| | spiked level of NDXT | | |
|---|---|---|---|
| | 0.075 ng/mL | 0.75 ng/mL | 3.75 ng/mL |
| recovery rate (%) | 115.4 | 113.9 | 93.4 |
| | 116.5 | 114.5 | 88.3 |
| | 108.2 | 111.8 | 91.4 |
| average recovery rate (%)[a] | 113.4 | 113.4 | 91.0 |
| SD[a] | 4.5 | 1.4 | 2.6 |
| %RSD[a] | 4.0 | 1.2 | 2.8 |
| SD[b] | 11.5 | | |
| %RSD[b] | 10.9 | | |

[a]$n = 3$. [b]$n = 9$.

**Table 3. Results of Accuracy and Repeatability for the Determination of NDXT in Duloxetine Tablets**

| | spiked level of NDXT | | |
|---|---|---|---|
| | 0.075 ng/mL | 0.75 ng/mL | 1.875 ng/mL |
| recovery rate (%) | 121.8 | 86.0 | 69.6 |
| | 102.6 | 84.3 | 71.5 |
| | 102.9 | 70.7 | 70.6 |
| average recovery rate (%)[a] | 109.1 | 80.3 | 70.6 |
| SD[a] | 11.0 | 8.4 | 1.0 |
| %RSD[a] | 10.1 | 10.5 | 1.4 |
| SD[b] | 18.7 | | |
| %RSD[b] | 21.6 | | |

[a]$n = 3$. [b]$n = 9$.

**Table 4. Results of Reproducibility for the Determination of NDXT in Duloxetine Capsules**

| | laboratory site | |
|---|---|---|
| | Towa Pharmaceutical | Daichi Kasei |
| value of NDXT (μg/g) | 0.4227 | 0.4580 |
| | 0.4545 | 0.4545 |
| | 0.4699 | 0.4346 |
| average value of NDXT (μg/g) | 0.4490 | 0.4490 |
| SD[a] | 0.0157 | |
| %RSD[a] | 3.5 | |

[a]$n = 6$.

Omega Polar C18 column (1.6 μm, 2.1 × 100 mm) and a mobile phase of 0.1% formic acid/2 mM ammonium acetate aqueous solution and acetonitrile (11/9, v/v). Isocratic elution with a flow rate of 0.2 mL/min for 25 min and injection volumes of 10 μL were employed. The following transitions $m/z$ 344 > 183 were used to monitor NDXT. The nebulizer gas with a flow rate, the drying gas with a flow rate, and the interface temperature were 3 L/min, 3 L/min, and 280 °C, respectively. The collision energy was −10.0 V.

**4.4. Sample Preparation.** NDXT standard solution (0.75 ng/mL) was prepared in methanol/Milli-Q water (1/1, v/v). Duloxetine API solution (0.5 mg/mL) was prepared in methanol/Milli-Q water (1/1, v/v). For duloxetine 30 mg capsules and tablets, an extraction operation was required. Thirty mL of methanol was added per capsule or tablet, and the capsules were extracted by shaking in a shaker at 300 rpm for 20 min and tablets for 40 min. The supernatant samples were centrifuged at 3000 rpm at room temperature for 10 min. The upper clear layer was carefully separated, and the supernatant was filtered through a 0.2 μm PTFE membrane filter. 0.5 mL of the filtrate was diluted by 0.5 mL of Milli-Q water, and this solution was used as the sample solution. All prepared samples were stored at room temperature until analysis.

**4.5. Analytical Method Validation.** Validation was carried out for the analytical performance parameters for the determination of NDXT in duloxetine API, duloxetine capsules, and duloxetine tablets, and the following parameters were evaluated: specificity, linearity, LOD, LOQ, range, accuracy, repeatability, and reproducibility. Stability in solution and robustness were also evaluated. From the AI and the


https://doi.org/10.1021/acsomega.4c00136
ACS Omega 2024, 9, 13440−13446

maximum daily dose of duloxetine, the specification limit for NDXT is calculated. Because the maximum daily doses of duloxetine are different in Europe (120 mg/day) and Japan (60 mg/day), the specification limits for NDXT are different. The specification limits are 0.83 μg/g in Europe and 1.67 μg/g in Japan. The developed analytical method was validated to cover both specification limits. The concentrations of NDXT in the validation test are expressed as the concentrations in the diluent solvent (ng/mL).

*4.5.1. Specificity.* Specificity was assessed using different samples of blank, standard solution, duloxetine API, duloxetine capsules, and duloxetine tablets at one time each to evaluate the peak resolution and confirm that these peaks were not affecting the NDXT peak.

*4.5.2. Linearity and Range.* The linearity was evaluated using the NDXT samples covering a range of 0.075−3.75 ng/mL for standard solution. The peak area of NDXT was plotted against the standard concentrations. The linearity was evaluated by least-squares regression analysis, the correlation coefficient, and the y-intercept, and the correlation coefficient should be not less than 0.99. The range was evaluated based on linearity, accuracy, and repeatability data.

*4.5.3. Limit of Detection and Limit of Quantitation.* The LOD was evaluated as the lowest amount of analyte in a sample that can be detected but not necessarily quantitated. The LOD was determined based on the S/N ratio and defined as an S/N ratio of 2 or higher. The LOQ was evaluated as the lowest amount of analyte in a sample that can be quantitated with suitable accuracy and repeatability.

*4.5.4. Accuracy and Repeatability.* Analyses were performed in triplicate using the samples of duloxetine API, duloxetine capsules 30 mg, and duloxetine tablets 30 mg spiked with NDXT at concentrations of 0.075, 0.75, and 1.875 ng/mL (tablets) or 3.75 ng/mL (duloxetine API and capsules) The peak areas of NDXT were determined, and the recovery rates and their %RSDs were calculated. The acceptance criteria for the recovery rate were determined as 70−130%. The repeatability was calculated by the %RSDs using triplicate at each concentration level and nine replicates at all concentration levels. The acceptance criteria for repeatability were determined as %RSDs of 25% or less.

*4.5.5. Reproducibility.* Analyses were performed in the laboratories of Towa Pharmaceutical and our group company Daichi Kasei (Hyogo, Japan) using duloxetine capsules 30 mg. Analyses were performed in triplicate in each laboratory, and the %RSD was calculated by analysis of variance. The acceptance criteria for reproducibility were determined as % RSD of 25% or less.

*4.5.6. Stability and Robustness.* For stability study, the percent deviations of the analyte at each time point (from 1 day to 7 days) compared to the initial time point were calculated and should be within 20%. Robustness was conducted in order to evaluate the tolerance of the analytical method to analytical errors that may occur in routine experimental operations. The shaking time of the duloxetine capsule was changed from the basic 20 min to 10 and 30 min, and the shaking time of the tablet was changed from the basic 40 min to 20 and 60 min, and the NDXT values under the basic conditions were compared.

**4.6. Laboratory Safety and Material Handling.** NDXT may have a potential carcinogenic risk. Therefore, appropriate laboratory safety measures, such as engineering controls, waste segregation, and personal protective equipment, are recommended to minimize exposure and cross-contamination during the synthesis, handling, and analysis of NDXT.

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### Author Contributions

#S.F. and Y.N. contributed equally to this work.


### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

The authors thank Makoto Hirata, Nanako Taniguchi, and Miyuki Hamada for NDXT measurement and Takehiro Hoshiyama for analysis of the data.

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https://doi.org/10.1021/acsomega.4c00136
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