Article

Development of a Simultaneous Analytical Method for Amines Corresponding to 10 Typical Nitrosamines

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ABSTRACT: Regulatory authorities in various countries have successively issued notices and guidance to pharmaceutical manufacturers and distributors to evaluate the risk of contamination of nitrosamines in pharmaceutical products and to take appropriate measures. Analysis of nitrosamines in pharmaceutical products is not easy due to the large number of foreign substances, and the risk of contamination is determined by first conducting a desk investigation of the manufacturing process of the APIs or pharmaceutical products. However, a desk investigation may miss the risk since this method is not based on actual measurements. Therefore, in addition to conventional desk-based investigation, a new method is required to pick up risks that cannot be covered by a desk investigation. Nitrosamines are known to be formed by the reaction of amines with nitrosating agents such as nitrite. In the case of small alkyl nitrosamines such as NDMA and NDEA, the origin of the amines is mostly residual amines in the APIs. Residual amines in the APIs are a potential nitrosamine contamination risk, although the extent of that risk has rarely been reported. In this study, we developed and validated a simultaneous analytical method for amines corresponding to 10 typical small alkyl nitrosamines. Good linearity was obtained in the range of 0.003 to 10 μg/mL for MPA, 0.003 to 2 μg/mL for DIPA and DBA, 0.003 to 1 μg/mL for MeP, DEA, EIPA, and DPA, and 0.003 to 0.2 μg/mL for DMA, MOR, and MBA. The limits of quantitation and detection were 0.003 and 0.001−0.003 μg/mL, respectively. The recovery rates ranged from 70 to 130% for 121 APIs and were more than 40% for 83 APIs. Repeatability was also good, with %RSD < 15%. Although the correlation between the amount of amines detected in the APIs and the nitrosamines in the pharmaceutical products is under investigation, we expect that this analytical method will be used to determine the residual amine contents in APIs and contribute to the risk assessment of the nitrosamine contamination.
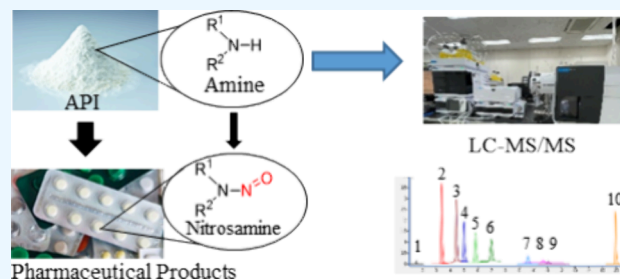
1. INTRODUCTION

Starting with the detection of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) in valsartan, one of the antihypertensive drugs in the sartan family, in 2018,[1,2] *N*-nitroso-*N*-methyl-4-aminobutyric acid (NMBA) contamination was reported in losartan, one of the sartan family, in 2019.[3] NDMA contamination was reported in ranitidine[4,5] and nizatidine[6] used to treat peptic ulcer disease in 2019, and metformin used to treat type 2 diabetes in 2020.[7] Since then, subsequent reports of the nitrosamine contamination of various other drugs have led to frequent voluntary recalls in many countries. When the nitrosamine contamination problem first came to light, there were only reports of contamination with small alkyl nitrosamines such as NDMA and NDEA, but after the contamination of varenicline, a smoking cessation drug, with *N*-nitrosovarenicline was reported in 2021,[8] contamination with nitrosamine drug substance-related impurities (NDSRIs), in which the active pharmaceutical ingredient (API) itself and its related substances are nitrosated, has been reported one after another. Against that background, the European Medicines Agency (EMA)[9] in 2019, the U.S. Food and Drug Administration (FDA)[10] in 2020, and the Japanese Ministry of Health, Labor

and Welfare (MHLW)[11] in 2021 issued a series of notices and guidance to pharmaceutical manufacturers and distributors, stating that they should assess the risk of nitrosamines contamination and take appropriate measures to reduce them.

Nitrosamines are known to be formed by the reaction of amines with nitrosating agents such as nitrite. The forming pathways of NDMA, NDEA, and NMBA in sartans such as valsartan and losartan are considered to be due to the reaction of dimethylamine (DMA) and *N*-methyl-4-aminobutyric acid (MBA), which are formed by the decomposition of the solvents *N*,*N*-dimethylformamide (DMF) and *N*-methylpyrrolidone (NMP),[12,13] respectively, with nitrous acid used to quench residual azide in the formation of a tetrazole ring.[12] In the case of ranitidine, intermolecular degradation reaction was reported to be responsible for NDMA formation.[14] In the case

Figure 1. Structures of target amines and nitrosamines.

of metformin, it was reported that NDMA was formed by the reaction of DMA remaining in the metformin API with nitrite in the excipients[15,16] and nitrogen oxide (NO_x) in the atmosphere[17] during the formulation process, and with nitrocellulose used in packaging materials[18] under storage.

As in the case of metformin, the contamination of pharmaceutical products with nitrosamines is not limited to contamination in the API, but it can also occur during the formation process and under storage conditions. Therefore, in assessing the risk of contamination of pharmaceutical products with nitrosamines, it is not sufficient to simply evaluate the risk of contamination of the API, but the risk of formation during the formulation process and under storage conditions should be considered. The most reliable method for evaluating nitrosamine contamination is to measure the amounts of nitrosamines contained in all pharmaceutical products one by one. However, this is not practical because pharmaceutical products contain a wide variety of excipients that interfere with analysis in addition to APIs, making it difficult to develop analytical methods and requiring an enormous amount of time for measurement. Therefore, instead of measuring the nitrosamine content in all pharmaceutical products, many pharmaceutical manufacturers and distributors first conducted a desk investigation to estimate the risk of nitrosamine contamination in the API manufacturing process and nitrosation in the formulation process, and for the high-risk pharmaceutical products, analytical methods for nitrosamines in pharmaceutical products will be developed, and the amount of contamination will be verified in practice. A desk investigation is an efficient and accurate risk assessment method to some extent. However, since it is an evaluation that is not based on actual measurements, omissions of risk pickup

may sometimes occur. Therefore, a new method is required to pick up what is missed during the desk investigation. In this study, we focused on small alkyl nitrosamines such as NDMA and NDEA, as described in the regulatory notices, since these are required to be evaluated for contamination risks in almost all pharmaceutical products, although the situation varies somewhat from country to country. For NDSRIs, risk assessment is more complex because the physical property of each pharmaceutical product has a significant impact on NDSRI contamination. Therefore, we believed that risk assessment for NDSRIs should be conducted in an appropriate method for each pharmaceutical product, and NDSRIs were not included in this study.

As mentioned above, there are many known sources of nitrosation, including nitrite in excipients[15,16] and nitro-cellulose used in packaging materials.[18] Moreover, we recently reported that a very small amount of NO_x in the atmosphere reacts with residual DMA in metformin API to form NDMA during the granulation process.[17] It indicated that even a very small amount of NO_x in the atmosphere can be a nitrosating agent. While it is very difficult to completely control and block contamination from nitrosating agents whose sources vary widely, most of the amine sources corresponding to small alkyl nitrosamines are residual amines in the API, which can be controlled. Although the extent of the risk of residual amines has rarely been reported, as the case of metformin, those in the APIs may pose a potential risk of nitrosamine contamination from the manufacturing of the API through the formulation process and during storage. In this study, we developed and validated an analytical method that can comprehensively analyze residual amines in APIs corresponding to 10 typical small alkyl nitrosamines by a simple way.

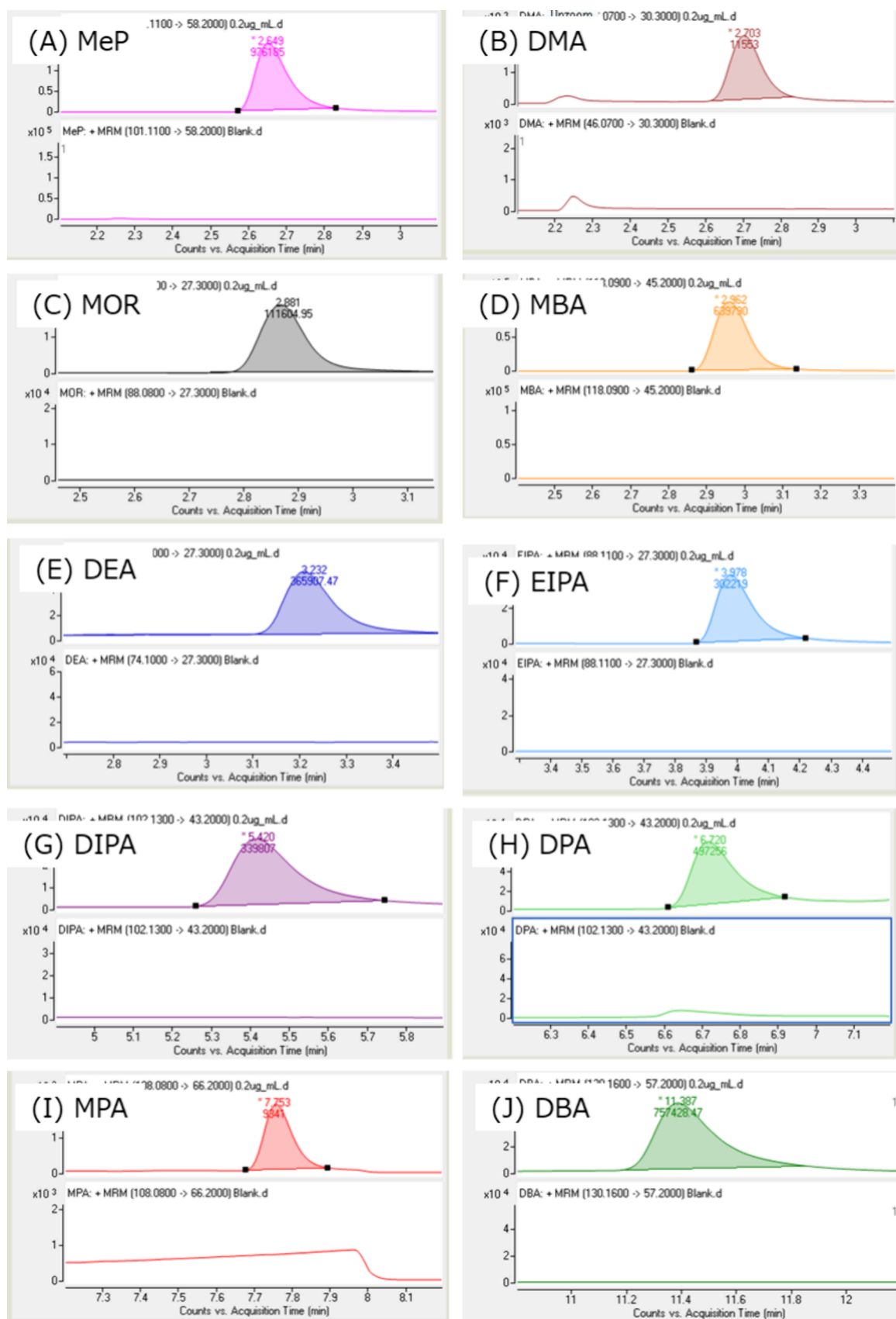**Figure 2.** MS chromatograms of 0.2 μg/mL of each amine standard solution (top) and the blank solution (bottom). (A), (B), (C), (D), (E), (F), (G), (H), (I), and (J) are chromatograms of MeP, DMA, MOR, MBA, DEA, EIPA, DIPA, DPA, MPA, and DBA, respectively.
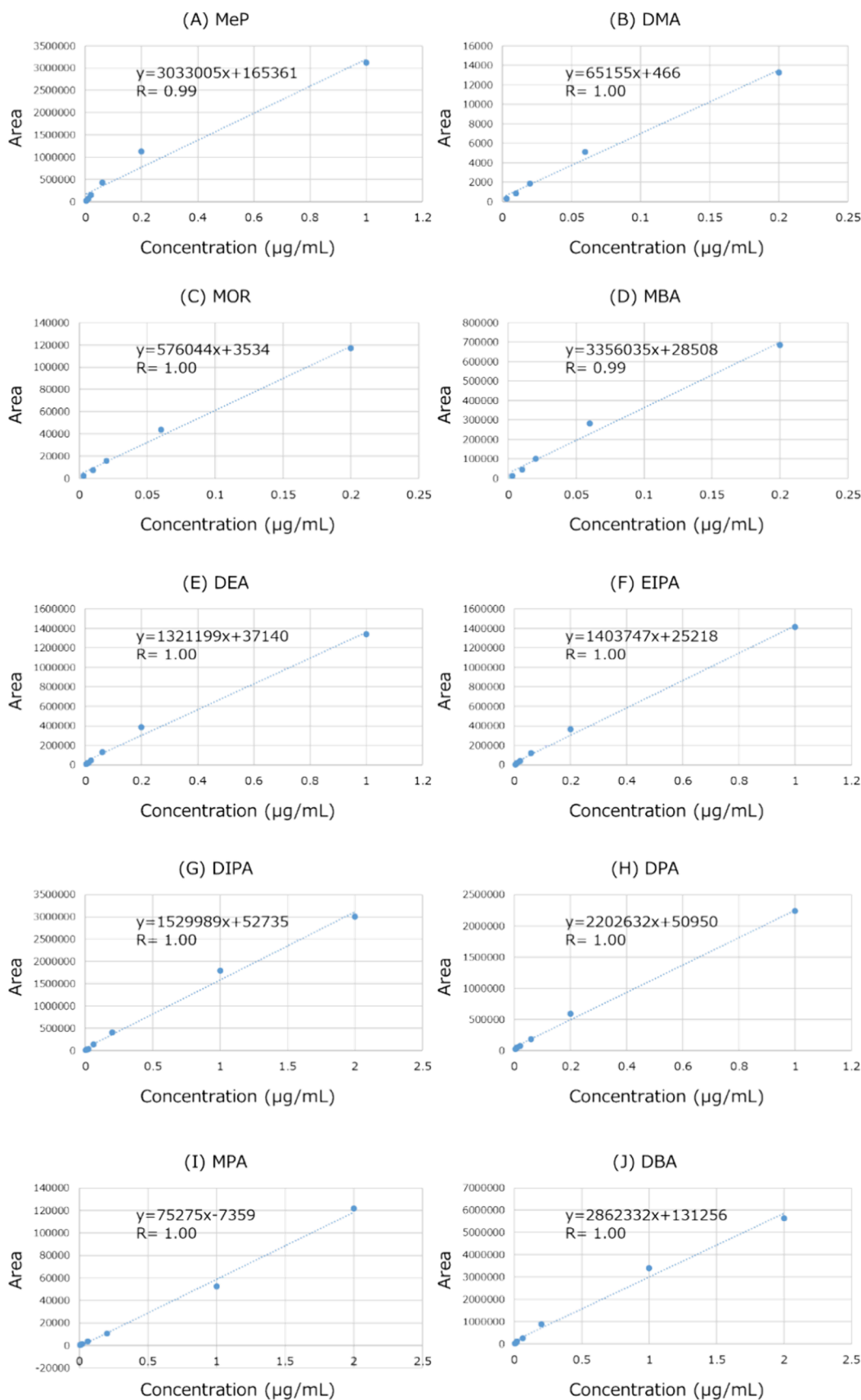
Figure 3. (A), (B), (C), (D), (E), (F), (G), (H), (I), and (J) are linearity of MeP, DMA, MOR, MBA, DEA, EIPA, DIPA, DPA, MPA, and DBA, respectively.

2. RESULTS AND DISCUSSION

2.1. Selection of Target Amines. Amines corresponding to the 10 small alkyl nitrosamines notified by the EMA, FDA, and MHLW[9−11] were included in the measurement (Figure 1). These are nitrosamines identified as possible contamination risks when the contamination of sartans with nitrosamines was first reported. Precursor amines are used as solvents or are produced by the degradation of the solvent used in the synthesis of APIs, placing them at high risk of reacting with nitrosating agents to form nitrosamines. In addition to the simple secondary amines shown in Figure 1, DMF and dimethylacetamide (DMAc), which are often used as solvents, and the tertiary amines trimethylamine (TMA) and triethylamine (TEA) are also known to be risk factors for nitrosamines through the formation of secondary amines by decomposition.[19,20] However, since solvents such as DMF and DMAc are evaluated as residual solvents, and since tertiary amines such as TMA and TEA have been reported to have very low ability to form nitrosamines compared to secondary amines,[21,22] these compounds were excluded from the assay.

2.2. Development Process of the Analytical Method. First, we tried to develop an analytical method using HS-GC/MS, which is relatively easy to apply to a wide variety of APIs, but highly polar amines were adsorbed in the GC system and good repeatability could not be obtained. Although there was a possibility that the use of an internal standard could improve the poor repeatability caused by adsorption, we did not adopt this approach, because it would have detracted the simplicity of the analysis and would have deviated from our goal of efficient evaluation in a short period of time.

Next, we employed LC-MS/MS with high specificity instead of GC-MS. The multiple reaction monitoring (MRM) mode with high specificity was utilized. First, a hydrophilic interaction liquid chromatography (HILIC) column capable of retaining highly polar compounds was experimented. Although the separation of 10 target amines was achieved, it was judged that this analytical method was not suitable for screening methods because it was necessary to confirm whether amines and APIs could be separated one by one when applying the method to a wide variety of APIs. Next, a column (SM-C18) that combines the ODS and cation exchange in one column was experimented. In general, APIs are retained on ODS columns because they are more hydrophobic than the amines with simple structures. On the other hand, highly polar amines are not retained on ODS columns but are retained on cation exchange columns. Since the APIs and amines can be retained by different principles, separation of a wide variety of APIs and amines was expected. As expected, a wide variety of APIs and amines could be separated, but a poor recovery rate for amines was frequently observed. The recovery rates were improved when the ODS column (UK-C18) was connected upstream of the SM-C18 column, so that the APIs were trapped in the upstream UK-C18 column.

The mobile phase and gradient conditions were then experimented. First, N-methylaniline (MPA) did not ionize in the mobile phase of aqueous ammonium formate and acetonitrile. We tried changing the pH of the aqueous ammonium formate to acidic or neutral to see if ionization would improve, but this did not improve the ionization of MPA. When the aqueous solutions were changed to aqueous formic acid, aqueous ammonium acetate, and aqueous acetic acid, all 10 amines ionized in aqueous formic acid and aqueous acetic acid, but MPA did not ionize in aqueous ammonium acetate. The aqueous formic acid was found to be more suitable than the aqueous acetic acid in terms of peak shape and sensitivity of amines, but some amines were hardly retained on the SM-C18 column in the aqueous formic acid. Since ammonium formate retains 10 amines but does not ionize MPA, and aqueous formate ionizes MPA but hardly retains some amines, we decided to use both of two aqueous mobile phases. The initial mobile phase was an aqueous ammonium formate, and all 10 amines are retained on the SM-C18 column, and then the mobile phase was changed to aqueous formate in order to ionize MPA. Acetonitrile was more sensitive to amines than methanol. The mobile phase of the organic solvent was acetonitrile/water mixture (9:1, v/v) because the peak shape was improved by adding a small amount of water to acetonitrile. Since the values of m/z for some amines in MRM mode were the same, the gradient conditions of the two aqueous mobile phases were adjusted to separate each of these amines. After all 10 amines were eluted, the composition of the organic solvent mobile phase was increased so that the APIs remaining on the UK-C18 and SM-C18 columns could be eluted.

The use of any ratio of water, methanol, and acetonitrile as the diluents had no effect on the analysis. DMSO at around 10% also had no effect on the analysis. In this study, a water/DMSO mixture (9:1, v/v) was selected as the diluent.

Glass and polypropylene (PP) were compared as materials for the volumetric flasks and vials used in the study. The vials and volumetric flasks made of glass caused poor linearity due to the adsorption of amines, and these made of PP were adopted.

2.3. Analytical Method Validation. As analytical method validation, specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and repeatability were evaluated. This analytical method was developed intended to be a screening analytical method rather than as a perfect analytical method for individual APIs. Therefore, linearity, LOD/LOQ, and repeatability were evaluated without an API matrix. The influence of the API matrix was evaluated in the accuracy section.

2.3.1. Specificity. Chromatograms of the amine standard solution (0.2 μg/mL) and the blank solution are shown in Figure 2. No blank-derived interference peaks were observed in the retention time of any of the target amines. The separations of 282 APIs from various amines were evaluated, and more than 90% of the APIs could be separated from these amines.

2.3.2. Linearity. Good linearities with correlation coefficients of 0.99 or more were obtained in the range of 0.003−10 μg/mL for MPA, 0.003−2 μg/mL for diisopropylamine (DIPA) and dibutylamine (DBA), 0.003−1 μg/mL for N-methylpiperazine (MeP), diethylamine (DEA), ethylisopropylamine (EIPA), and dipropylamine (DPA), and 0.003−0.2 μg/mL for DMA, morpholine (MOR), and MBA, respectively (Figure 3). Some amines have a narrow linear range, thought to be due to adsorption on columns and tubes at high concentrations.

2.3.3. LOD and LOQ. The LOD is the lowest detectable concentration and is not necessarily quantified. For DIPA, MeP, DEA, EIPA, DPA, MOR, and MBA, the LOD was defined as 0.001 μg/mL because these signal-to-noise ratios (S/N) were not less than 2. For DMA, DBA, and MPA, no peak could be detected at 0.001 μg/mL, so it was concluded
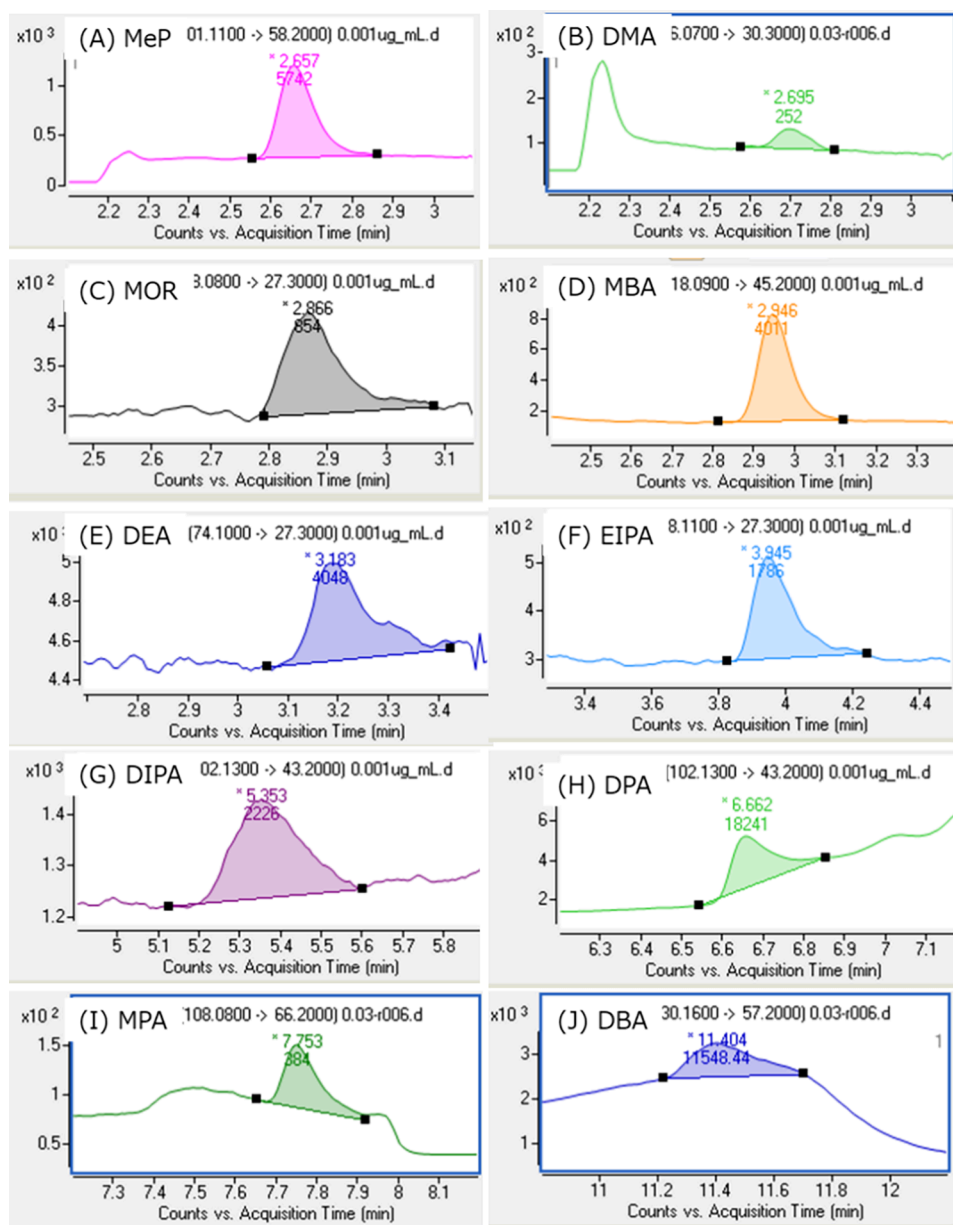
Figure 4. MS chromatograms of each amine at LOD concentrations. (A−J) Chromatograms of MeP, DMA, MOR, MBA, DEA, EIPA, DIPA, DPA, MPA, and DBA. MeP, MOR, MBA, DEA, EIPA, DIPA, and DPA are at 0.001 μg/mL, and DMA, MPA, and DBA are at 0.003 μg/mL.

Table 1. Repeatability Results of Each Amine Solution at the LOQ Concentration

	MeP	DMA	MOR	MBA	DEA	EIPA	DIPA	DPA	MPA	DBA
%RSD	1.1	13.2	2.8	0.8	11.6	1.0	5.3	4.5	6.5	8.4

that the LODs for them were in the range of 0.001−0.003 μg/mL. The chromatograms of each amine at the LOD concentration are shown in Figure 4. The LOQ is the lowest concentration that can be quantified with an acceptable precision and was defined as 0.003 μg/mL. Table 1 shows the results of the relative standard deviations (%RSDs) for six injections at the LOQ. The %RSDs were not more than 15%.

2.3.4. Accuracy. Since a wide variety of APIs are measured by this method, 0.2 μg/mL amines were spiked to each API (20 mg/mL), and the recovery rates of the spiked amines were evaluated as accuracy tests. Of the 282 APIs, 121 APIs showed recovery rates of 70−130% for all 10 amines. Of the other 161 APIs, 83 APIs had recovery rates of more than 40%. It was

thought that the presence or absence of amine contamination in these 83 APIs could be determined, although the quantitation of them may not be enough. For the rest of 78 APIs, at least one of the 10 amines had recovery rates of less than 40%, so it was thought that the presence of this amine contamination may not be determined. Many of the APIs with poor recovery rates have the common characteristics of forming salts with alkali and alkaline earth metals such as sodium and calcium or having acidic functional groups such as carboxyl and sulfo groups in their structural formula. These structural characteristics of these APIs may have caused them to react with spiked amines, which may have caused poor

Table 2. Repeatability Results of Each Amine Solution at 0.2 μg/mL Concentration

	MeP	DMA	MOR	MBA	DEA	EIPA	DIPA	DPA	MPA	DBA
%RSD	0.9	1.5	0.6	0.9	1.1	0.8	4.0	1.1	2.2	1.3

Table 3. Numbers of APIs with Each Amine Detected

amine content	MeP	DMA	MOR	MBA	DEA	EIPA	DIPA	DPA	MPA	DBA
>100 μg/g	1	0	0	0	0	0	0	0	0	0
10−100 μg/g	1	4	0	0	2	0	0	1	0	0
3−10 μg/g	0	9	1	0	3	0	8	0	0	1
<3 μg/g	254									

recovery rates. The results of the recovery rates for each API are listed as a Supporting Information.

2.3.5. Repeatability. The repeatability was evaluated using a standard solution of amines, and the results of the %RSDs for the six injections at 0.2 μg/mL amines are shown in Table 2. All %RSDs were less than 15%.

2.4. Stability of Sample and Standard Solutions. A standard solution of amines was stable for over 1 month at room temperature. On the other hand, the recovery rates of amines spiked to various APIs sometimes deteriorated when measured 1 week later. This reason was considered to be due to the reaction between the APIs and the amines in the diluent, and the sample solutions should be measured immediately after preparation.

2.5. Robustness of Each Analytical Parameter. Based on the knowledge obtained during the development of the analytical method, the influence of various parameters on the analysis is shown in this section. The ionization parameters of the MS are important because they affect the ionic efficiency of the amines or the sensitivity of the amine peaks. The HPLC gradient condition is an important analytical parameter and must ensure that ammonium ions are removed from the HPLC system before MPA elutes. The concentration of formic acid in aqueous ammonium formate should be as low as possible, so that ammonium ions are quickly removed from the HPLC system. The concentration of aqueous formic acid is tolerable, and changing the concentration does not significantly affect the analysis; comparing 0.1 and 0.5% concentrations, the peak shapes of amines improve slightly with the 0.5% concentration. As mentioned previously, this analytical method is flexible for the composition of the diluent. The API was diluted to 20 mg/mL, but lower concentrations should be acceptable. Differences between column lots slightly affected the DMA area values. The sensitivity of DMA should be checked in advance for each column lot used. For other amines, there was no effect of between-lot differences in the columns.

2.6. Results of Evaluation of Residual Amines in the APIs. Residual amines in 282 APIs were measured, and Table 3 shows how many APIs contained each amine. Only one API contained more than 100 μg/g of amine, and that amine species was MeP. Since the partial structure of MeP in this API is amide bonded, It is presumed to be unreacted MeP, or easily generated by the degradation of the API. For the eight APIs, one or more of the amines were detected at concentrations ranging from 10 to 100 μg/g, and for the 22 APIs, those were detected at concentration levels ranging from 3 to 10 μg/g. For the other APIs, all amines were less than 3 μg/g. The most frequently detected amine species was DMA, which was detected in 13 APIs. MBA, EIPA, and MPA were less than 3 μg/g for all APIs. The total number of APIs in the table does

not equal 282 since more than one species of amine was detected in some APIs, and these APIs were counted twice. These amines could be unreacted amines from the API manufacturing process or cross-contamination. The correlation between residual amines in the APIs and nitrosamines in pharmaceutical products is currently under investigation.

3. CONCLUSIONS

A simultaneous analytical method for amines corresponding to 10 typical small alkyl nitrosamines was developed and validated. Although the recovery rates were poor for some APIs, more than 200 APIs were evaluated for the amine contents by using this method. Although it is necessary to develop separate analytical methods for nitrosamines in pharmaceutical products whose APIs contain amines and to verify the amount of contamination in them in practice, we expect that this analytical method will be used to determine the residual amine content in APIs and contribute to the risk assessment of nitrosamine contamination.

4. MATERIALS AND METHODS

4.1. Reagents and Samples. DMA, EIPA, DIPA, MeP, and MOR were procured from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). DEA, DPA, DBA, and MPA were procured from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). MBA was procured from Combi-Blocks Inc. (California, USA). Acetonitrile (LC/MS grade), formic acid (LC/MS grade), and ammonium formate (GR-grade) were procured from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Ultrapure water was obtained from the Millipore Milli-Q purification system (Darmstadt, Germany). Each API was obtained from the actual API manufacturer used for each pharmaceutical product.

4.2. Instruments. Chromatographic analysis was carried out using a 1260 Infinity II LC system (Agilent Technologies, California, USA). Mass spectrometric detection was carried out using a 6495C triple quadrupole LC/MS instrument (Agilent Technologies, California, USA) operated in positive electrospray ionization and MRM mode. Hardware control and data acquisition were carried out using MassHunter Data Acquisition version 10.1 (Agilent Technologies, California, USA), and data treatment was carried out using MassHunter Qualitative Analysis version 10.0 and Quantitative Analysis (for QQQ) version 10.1 (Agilent Technologies, California, USA).

4.3. Liquid Chromatographic and Mass Spectrometric Conditions. Separations were carried out using a Unison UK-C18 column (3 μm, 3 × 75 mm) and a Scherzo SM-C18 column (3 μm, 3 × 100 mm) (Imtakt, Kyoto, Japan). The column oven was set at 40 °C. Mobile phases A, B, and C were

carried out using 5 mM aqueous ammonium formate, acetonitrile/water solution (9:1, v/v), and 0.5% aqueous formate, respectively. The gradient condition of the mobile phases is shown in Table 4. Flow rates of 0.3 mL/min and

Table 4. Gradient Condition of the Mobile Phases

time (min.)	mobile phase (vol %)		
	A	B	C
0−1	94	3	3
1−2	94−0	3−13	3−87
2−10	0	13	87
10−15	0	13−90	87−10
15−25	0	90	10

injection volumes of 1 μL were employed. The MS transitions of each amine are shown in Table 5. Nebulizer gas was injected

Table 5. MS Transitions of Each Amine

amines	m/z
DMA	46.1 > 30.3
DEA	74.1 > 27.3
EIPA, MOR	88.1 > 27.3
MeP	101.1 > 58.2
DIPA, DPA	102.1 > 43.2
MPA	108.1 > 66.2
MBA	118.1 > 45.2
DBA	130.2 > 57.2

at a pressure of 20 psi. Gas temperature and flow rate were set at 200 °C and 11 L/min, respectively. Sheath gas temperature and flow rate were set at 400 °C and 12 L/min, respectively. Capillary voltage and nozzle voltage were set at 2500 and 2000 V, respectively. High-pressure RF and low-pressure RF were set at 90 and 60, respectively.

4.4. Sample Preparation. DMA, MBA, DEA, EIPA, DIPA, MeP, and MOR were dissolved and diluted in water. DPA, DBA, and MPA were dissolved and diluted in DMSO since these amines did not mix well with water. Amine standard solution (0.2 μg/mL) was prepared by mixing each amine solution prepared by the above procedure and diluting in water/DMSO (9:1, v/v). 200 mg of each API was dissolved in 1 mL of DMSO and diluted in to 10 mL with water (final concentration 20 mg/mL). 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 the amines corresponding to nitrosamines in each API, and the following parameters were evaluated: specificity, linearity, LOD, LOQ, accuracy, and repeatability.

4.5.1. Specificity. The specificity was assessed using blank and amine standard solutions at one time each to confirm that no interfering peaks were present near each amine peak. In addition, the separation of each API was confirmed at the same time as for the actual measurement.

4.5.2. Linearity. The linearity was evaluated using the amine solutions covering the range of 0.003−10 μg/mL for MPA, 0.003−2 μg/mL for DIPA and DBA, 0.003−1 μg/mL for MeP, DEA, EIPA, and DPA, and 0.003−0.2 μg/mL for DMA, MOR, and MBA, respectively. The linearity was evaluated by the correlation coefficient, and the y-intercept and correlation coefficient should be not less than 0.99.

4.5.3. LOD and LOQ. LOD was evaluated as the lowest amount of analyte in a sample that can be detected but not necessarily quantitated. LOD was determined based on the S/N ratio and defined as the S/N ratio of 2 or higher. LOQ was evaluated as the lowest amount of analyte in a sample, which can be quantitated with suitable repeatability. The %RSDs of the area value of six injections at the LOQ concentration should be less than 15%.

4.5.4. Accuracy. Analyses were performed once for each API spiked with each amine at a concentration of 0.2 μg/mL. The peak area of each amine was measured, and the recovery rates were calculated. The recovery rate should be 70−130%. However, since this method was designed for screening, even recovery rates of 40% or more were acceptable for amine detection.

4.5.5. Repeatability. Amine standard solution (0.2 μg/mL) was analyzed six times, and the %RSDs of area values were calculated. The %RSDs should be less than 15%.

4.6. Stability and Robustness. Stability and robustness were discussed based on the findings during the development of the analytical method.

4.7. Laboratory Safety and Material Handling. Appropriate laboratory safety measures such as engineering controls, waste segregation, and personal protective equipment are recommended to minimize exposure and cross-contamination during the handling and analysis of amines and APIs.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c06293

     Accuracy result for each API (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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