Sensitive Quantitation of Residual Phenylhydrazine in Antipyrine by LC-ICP-MS with Iodo Derivatization

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Phenylhydrazine is a genotoxic impurity commonly used as a raw material in active pharmaceutical ingredients. In this study, a novel method, using inductively coupled plasma-mass spectrometry combined with two-dimensional liquid chromatography (LC-ICP-MS), was employed for the quantitation of residual phenylhydrazine in antipyrine. The compound 2,3,5-triiodobenzoyl chloride, which contains three iodine atoms, was investigated as a derivatization reagent. The DL 0.06 ppm was obtained, and the method was applied to the quantitation of residual phenylhydrazine in antipyrine. No residual phenylhydrazine was detected in five lots of antipyrine obtained from the commercial source.

Keywords Phenylhydrazine, LC-ICP-MS, 3-iodophenyl isocyanate, 2,3,5-triiodobenzoyl chloride, genotoxic impurity, antipyrine, active pharmaceutical ingredient

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Introduction

During the development of pharmaceutical products, residual impurities such as raw materials, intermediates, by-products, excipients, and degradation products are controlled according to the International Conference on Harmonization (ICH) Q3A/Q3B guidelines.¹ Control of the residual genotoxic impurities (GTIs) is one of the critical concerns in the development of active pharmaceutical ingredients (APIs). However, the toxicological assessment of GTIs is not addressed in sufficient detail in the ICH Q3A/Q3B guidelines. Recently, the ICH M7 regarding the limits of GTIs was discussed and is now under an ICH consensus process. The US Food and Drug Administration (FDA) and European Medicines Agency (EMA) have published guidelines (for the control of GTIs).²⁻⁵ These agencies set the threshold of toxicological concern at 1.5 µg/day for GTIs in APIs or drug products. Thus, the allowable limits of GTIs are generally at ppm levels, depending on the duration and daily exposure dose. The risk assessment of GTIs during clinical development has become a major liability.⁶⁻¹⁰ Therefore, in addition to the rigid manufacturing process controls, a sensitive and selective analytical method for the detection of GTIs at low ppm levels is required.¹¹⁻¹³

Phenylhydrazine is one of the GTIs commonly used as a raw

material for APIs. Recently, the use of phenylhydrazine in API synthesis has been avoided because of its genotoxicity.¹⁰ However, if it is unavoidable to use phenylhydrazine during the manufacturing of an API, an additional process step such as purification may be required to reduce the amount of phenylhydrazine in the APIs. In that case, the residual amount of phenylhydrazine in the final API must be monitored. Therefore, a sensitive and selective analytical method is required to confirm the amount of phenylhydrazine in the starting and intermediate materials and/or API to ensure compliance with safety needs.

Generally, liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) have been used for trace level analyses of GTIs in APIs.¹⁴⁻²⁰ However, in some cases, these techniques might not be applicable because of the properties (ionization efficiency, polarity, solubility, and purity, *etc.*) of the GTIs and/or APIs. To overcome this problem, it is preferable to have an alternative methodology for GTI analysis. Inductively coupled plasma-mass spectrometry (ICP-MS), which has been utilized for trace level elemental analysis in many industries, is one of the options for trace analysis because of the high sensitivity of the system.²¹⁻²³ Applications of ICP-MS to the control of residual metals and speciation studies concerning several metals in APIs have been successfully performed.^{24,25} Moreover, ICP-MS has been used for organic analyses because of its highly efficient ICP ionization and selective, sensitive MS detection.^{26,27} Based on these perspectives, we have developed a novel analytical method for one GTI, 4-chloro-1-butanol, using ICP-MS with iodo derivatization.²⁸ The use of iodine for sensitive detection by ICP-MS is advantageous because of the low background, which

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is a result of the slight interference of polyatomic ions at m/z 127 (iodine). The developed method can also be applied to other GTIs such as phenylhydrazine.

In this study, an LC-ICP-MS method with iodo derivatization for the analysis of residual phenylhydrazine in APIs was investigated. First, since phenylhydrazine has no sensitive element for an ICP-MS detector, the mono-iodo derivatization reagent (3-iodophenyl isocyanate) was utilized for the detection of phenylhydrazine. In the previous study, 3-iodobenzoyl chloride was utilized as an iodo derivatization reagent, and it can be applied to phenylhydrazine analysis.²⁸ However, in order to expand the range of options of the iodo derivatization reagents, 3-iodophenyl isocyanate was investigated. Incidentally, sensitivity for an ICP-MS analysis is considered to be almost the same between the reagents because both reagents have one iodine atom in the structure. Then, the tri-iodo derivatization reagent (2,3,5-triiodobenzoyl chloride) was utilized with the expectation that it would improve sensitivity. The detection limit (DL) obtained from the two reagents was evaluated with regard to the number of iodine atoms. Finally, since phenylhydrazine is widely used as a raw material for antipyrine, the developed LC-ICP-MS methods with 3-iodophenyl isocyanate or 2,3,5-triiodobenzoyl chloride were applied for the quantitation of residual phenylhydrazine in antipyrine.

Experimental

Chemicals and reagents

Purified water (18.2 M Ω -cm) from a Milli-Q water purification system connected to an Elix3 pre-system (Millipore, Billerica, MA) was used to prepare the mobile phase and sample solutions. Phenylhydrazine (>98% purity) and formic acid (LC-MS grade) were purchased from Tokyo Chemical Industry (Tokyo, Japan). A supply of 3-iodophenyl isocyanate (>97% purity) was purchased from Sigma-Aldrich (Milwaukee, WI). Supplies of 2,3,5-triiodobenzoyl chloride (>96% purity), HPLC-grade methanol and acetonitrile (ACN), and organic synthesis-grade pyridine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Special grade tetrahydrofuran (THF) and *N,N*-dimethylformamide (DMF) were purchased from Kanto Chemical (Tokyo, Japan). Argon (Ar) gas (>99.999% purity) and oxygen (O₂) gas (>99.999% purity) were purchased from Taiyo Nippon Sanso (Tokyo, Japan). Five lots of antipyrine (an analgesic antipyretic drug) were purchased as follows: one lot of extra pure grade (>98.0% purity) from Kanto Chemical, two lots of first grade (>99.0%) from Tokyo Chemical Industry, and two lots of biochemistry grade (>99.0%) from Wako Pure Chemical Industries.

Derivatization reaction

The derivatization schemes for the two derivatization reagents are shown in Fig. 1. For 3-iodophenyl isocyanate derivatization, 20 μ L of 3-iodophenyl isocyanate was diluted with THF to 10 mL, which was then used as the derivatization solution. The derivatization was carried out by adding 0.5 mL of the derivatization solution to 1 mL of THF containing approximately 25 mg of antipyrine. After sonication at room temperature, the solution was diluted with 2 mL of DMF and an 80% ACN aqueous solution to 5 mL.

For 2,3,5-triiodobenzoyl chloride derivatization, the derivatization solution was prepared using 20 mg of 2,3,5-triiodobenzoyl chloride in 10 mL of THF and 0.5 mL of pyridine. After sonication for 5 min at 50°C, this solution was diluted with THF to 20 mL. The derivatization was carried out

Fig. 1 Derivatization scheme for LC-ICP-MS analysis.

Table 1 ICP-MS conditions

Parameter	Value
Mass/Analyte	m/z 127 (iodine)
RF power	1600 W
Plasma gas (Ar) flow rate	17 L/min
Auxiliary gas (Ar) flow rate	1.2 L/min
Nebulizer gas (Ar) flow rate	0.98 L/min
Ion lens	7.8 (V)
Sample introduction gas (O ₂) flow rate	30 mL/min
Integration time	40 min

by adding 0.5 mL of the derivatization solution to 1 mL of THF containing approximately 25 mg of antipyrine. After sonication at 50°C, the solution was diluted with 2 mL of DMF and an 80% ACN aqueous solution to 5 mL.

LC-ICP-MS system

An ELAN DRC II ICP-MS instrument (PerkinElmer, Waltham, MA) was used in this study. A platinum cone, quartz injector (2.0 mm orifice), PC3 spray chamber, and MEINEHARD glass nebulizer were used with the instrument. The mobile phase was connected to the glass nebulizer. The PC3 spray chamber was cooled to 5°C, and an introduction gas (O₂) was used. The O₂ gas prevents buildups of carbon on the interface cones and leads to a stable ion intensity. The ICP-MS conditions are summarized in Table 1.

A two-dimensional LC (2D-LC) system (column switching system) was employed to separate the analyte from interferences. The HPLC gradient system (the first LC system for separation) consisted of two LC-20AD pumps, an SIL-20AC autosampler, and a CTO-20AC column oven (Shimadzu, Kyoto, Japan). In addition, two LC-20AD pumps were used for the second HPLC gradient system (the second LC system for analysis). The HPLC instrumental setup was the same as in the previous study.²⁸

An L-column C8 (3 μ m, 2.0 mm i.d. $\times$ 150 mm, CERI, Tokyo, Japan) (for the first LC system) and an InertSustain C18 (3 μ m, 2.1 mm i.d. $\times$ 250 mm, GL Sciences, Tokyo, Japan) (for the second LC system) column were used for the analysis of the 3-iodophenyl isocyanate derivative. An InertSustain C18 (3 μ m, 2.1 mm i.d. $\times$ 150 mm) (for the first LC system) and InertSustain C18 (3 μ m, 2.1 mm i.d. $\times$ 250 mm) columns (for the second LC system) were used for the analysis of the 2,3,5-triiodobenzoyl chloride derivative. The flow rate of the mobile phase was set at 0.2 mL/min. The column temperature was maintained at 40°C. A 10- μ L aliquot of either the sample solution or standard

solution was injected. Derivatized compounds were monitored by m/z 127 (iodine).

LC-MS system

An Orbitrap MS (Thermo Fisher Scientific, Waltham, MA) equipped with an LC-30AD two-dimensional LC system (Shimadzu) was used to confirm the structure of the derivatized compound of phenylhydrazine. The Orbitrap MS was operated in the ESI positive mode with a sheath gas flow of 50 arb, an aux gas flow of 10 arb, a spray voltage of 5.0 kV, a capillary temperature of 300°C, a capillary voltage of 30 V, and a tube lens voltage of 10 V.

Preparation of standard and sample solutions for method validation

The standard stock solution was prepared at approximately 50 ppm ($\mu\text{g/g}$ API) (relative to 25 mg of API), which corresponds to a 0.25 $\mu\text{g/mL}$ phenylhydrazine solution. The standard stock solution was diluted to 0.06, 0.08, 0.1, 0.2, 0.5, 1, and 10 ppm with an 80% ACN aqueous solution. These solutions were used to estimate the linearity, DL, and quantitation limit (QL) validation (0.06 and 0.08 ppm solutions were prepared only for the 2,3,5-triiodobenzoyl chloride derivative).

The sample solution was prepared using approximately 25 mg of antipyrine. Blank solutions were also prepared without antipyrine using the same procedure. The sample solutions spiked with 1, 10, and 50 ppm of phenylhydrazine were prepared in triplicate. The standard solution was prepared at 10 ppm. These solutions were used for method validation of specificity, accuracy, and precision.

Method validation

The linearity was validated using 0.1, 0.2, 0.5, 1, 10, and 50 ppm solutions. The DL and QL were based on the relative standard deviation (RSD) of the peak area obtained from six injections of the 3-iodophenyl isocyanate derivative (0.2 and 0.5 ppm) and 2,3,5-triiodobenzoyl chloride derivative (0.06 and 0.2 ppm). The specificity was validated using a blank solution, standard solution with 10 ppm phenylhydrazine, sample solution, and sample solution spiked with 1 ppm of phenylhydrazine. The accuracy (recovery) was validated using the standard solution (10 ppm) and sample solution spiked with three different concentrations (1, 10, and 50 ppm). The standard and sample solutions were analyzed in triplicate. The repeatability of the spike recovery (three repetitions at three concentrations) was confirmed for the precision validation. The range was validated by the results for linearity, accuracy, and precision.

Quantification of phenylhydrazine in antipyrine by LC-ICP-MS

For the 3-iodophenyl isocyanate derivatization method, approximately 25 mg of antipyrine was weighed and dissolved in 1 mL of THF, and a 0.5-mL aliquot of the derivatization solution was added to the solution. After incubation at room temperature for 15 min with sonication, the solution was diluted to 5 mL with 2 mL of DMF and an 80% ACN aqueous solution, and this was used as the sample solution. A standard solution was separately prepared following the same procedure using 1 mL of a 0.25- $\mu\text{g/mL}$ phenylhydrazine THF solution.

For the 2,3,5-triiodobenzoyl chloride derivatization method, approximately 25 mg of antipyrine was weighed and dissolved in 1 mL of THF, and a 0.5-mL aliquot of the derivatization solution was added to the solution. After incubation at 50°C for 15 min with sonication, the solution was diluted to 5 mL with 2 mL of DMF and an 80% ACN aqueous solution, and this was

used as the sample solution. A standard solution was separately prepared following the same procedure using 1 mL of a 0.25- $\mu\text{g/mL}$ phenylhydrazine THF solution.

Results and Discussion

2D-LC for ICP-MS

A two-dimensional LC system (column switching system) was employed to separate the analyte from the matrix, *i.e.*, API and the derivatization reagent in the sample solution. In the first LC system, the analyte was separated from the matrix, and the fraction including the analyte was directly introduced into the second LC system by switching the valves. Under optimized conditions, a baseline separation of the interferences and analyte could be achieved, and the analysis was conducted in 40 min. The optimized condition for 3-iodophenyl isocyanate and 2,3,5-triiodobenzoyl chloride are summarized in Tables 2 and 3, respectively.

Derivatization with 3-iodophenyl isocyanate (mono-iodo derivatization method)

The derivatization conditions were optimized in the following experiments. The progress of the reaction in ACN and THF was investigated to select the best 3-iodophenyl isocyanate reaction solvent. Since the reaction efficiencies were almost the same, both solvents could be used for the reaction. In this study, THF was selected as the reaction solvent and used in the following experiments. The sonication temperature was tested at room temperature and 50°C. The derivatization reaction was complete even at room temperature. Incubation times of 5, 15, and 30 min were tested, and the derivatization reaction was complete within 15 min. Therefore, incubation for 15 min at room temperature was selected for the derivatization reaction.

To confirm the structure of the 3-iodophenyl isocyanate derivative, structural analysis was conducted using LC-MS. The protonated ion $[M+H]^+$ at m/z 354.010 and fragment ions at m/z 336.984 and m/z 219.962 were detected, and the results suggested the structure of the phenylhydrazine derivatized with 3-iodophenyl isocyanate (Fig. 2).

Validation results of mono-iodo derivatization method

The specificity was confirmed using a blank solution, 10 ppm phenylhydrazine standard solution, sample solution, and sample solution spiked with 1 ppm of phenylhydrazine. In the chromatogram of the sample solution, no peak was observed around the retention time of the phenylhydrazine derivatized with 3-iodophenyl isocyanate. Linearity in the 0.5 to 50 ppm range was obtained, and the R^2 value was 0.9998. The relative standard deviations (RSDs) of the six injections of the 0.2 and 0.5 ppm solutions were 3.4 and 6.1%, respectively. Thus, the DL and QL were 0.2 and 0.5 ppm, respectively, which corresponded to 0.95 and 2.38 ng/mL of phenylhydrazine. The accuracy (recovery test) was in the range of 99.5 to 111.9% at 1 to 50 ppm. The repeatability of the spike recovery test was 3.7%, and good spike recovery and repeatability were obtained. The linearity, accuracy, and precision results support a range from 1 to 50 ppm. This LC-ICP-MS method using 3-iodophenyl isocyanate offers a method for the analysis of phenylhydrazine in antipyrine at sub-ppm levels.

Derivatization with 2,3,5-triiodobenzoyl chloride (tri-iodo derivatization method)

As described above, the LC-ICP-MS method using 3-iodophenyl isocyanate has sufficient sensitivity. However, if

Table 2 HPLC conditions for 3-iodophenyl isocyanate derivatization method

Separation condition (First LC system)			
Column	L-column C8 (3 μm, 2.0 mm i.d. × 150 mm, CERI)		
Column temperature	40°C		
Flow rate	0.2 mL/min		
Injection volume	10 μL		
Mobile phase A	0.05% Formic acid in water		
Mobile phase B	0.05% Formic acid in acetonitrile		
Gradient program	Time/min	A %	B %
	0	75	25
	30	10	90
Valve program	Time/min	Valve position	
	0	A	
	15.9	B	
	16.4	A	

Analytical condition (Second LC system)			
Analytical column	InertSustain C18 (3 μm, 2.1 mm i.d. × 250 mm, GL Sciences)		
Column temperature	40°C		
Flow rate	0.2 mL/min		
Mobile phase C	Water		
Mobile phase D	Methanol		
Gradient program	Time/min	A %	B %
	0	40	60
	16.4	40	60
	40	10	90

Table 3 HPLC conditions for 2,3,5-triodobenzoyl chloride derivatization method

Separation condition (First LC system)			
Column	InertSustain C18 (3 μm, 2.1 mm i.d. × 150 mm, GL Sciences)		
Column temperature	40°C		
Flow rate	0.2 mL/min		
Injection volume	10 μL		
Mobile phase A	0.05% Formic acid in water		
Mobile phase B	0.05% Formic acid in acetonitrile		
Gradient program	Time/min	A %	B %
	0	70	30
	30	10	90
Valve program	Time/min	Valve position	
	0	A	
	19.4	B	
	19.9	A	

Analytical condition (Second LC system)			
Analytical column	InertSustain C18 (3 μm, 2.1 mm i.d. × 250 mm, GL Sciences)		
Column temperature	40°C		
Flow rate	0.2 mL/min		
Mobile phase C	Water		
Mobile phase D	Methanol		
Gradient program	Time/min	A %	B %
	0	40	60
	19.9	40	60
	40	10	90

Mass Spectra

Mass/Mass spectra

Mass Spectra

Mass/Mass spectra

Fig. 2 Structural analysis of the iodo derivative by LC-MS. The structure of the iodo derivatized phenylhydrazine was confirmed by the protonated ion and the fragment ions. Mass and mass/mass spectra are shown in (a) 3-iodophenyl isocyanate derivative and (b) 2,3,5-triodobenzoyl chloride derivative.

derivatization reagents with multiple iodine atoms are employed, the DL and QL value might be improved because of the increase in the number of target atoms (iodine). In this respect,

2,3,5-triodobenzoyl chloride, which has three iodine atoms in its molecule, was also investigated as a derivatization reagent.

THF was used as the reaction solvent, and the reaction

Fig. 3 LC-ICP-MS chromatogram of phenylhydrazine derivatized with 2,3,5-triiodobenzoyl chloride for specificity. The specificity was confirmed using (a) a blank solution, (b) a standard solution (10 ppm), (c) a sample solution, and (d) a sample solution spiked with phenylhydrazine (1 ppm).

conditions were optimized in the following experiments. The sonication temperature was tested at room temperature, 50, and 70°C. Since the derivatization reaction was complete at 50°C, the reaction temperature was set at 50°C. Then, incubation times of 5, 15, 30, and 60 min were tested. Though the derivatization reaction was complete within 5 min, the incubation time was set to 15 min to guarantee a complete reaction.

To confirm the structure of the 2,3,5-triiodobenzoyl chloride derivative, structural analysis was conducted using LC-MS. The protonated ion $[M+H]^+$ at m/z 590.793 and the fragment ions at m/z 482.724 and m/z 464.896 were detected, and the results suggested the structure of the phenylhydrazine derivatized with 2,3,5-triiodobenzoyl chloride (Fig. 2).

Validation results of tri-iodo derivatization method

The specificity was confirmed using a blank solution, 10 ppm phenylhydrazine standard solution, sample solution, and sample solution spiked with 1 ppm of phenylhydrazine. In the chromatogram of the sample solution, no peak was observed around the retention time of the phenylhydrazine derivatized with 2,3,5-triiodobenzoyl chloride (Fig. 3). Linearity in the 0.2 to 50 ppm range was obtained, and the R^2 value was 0.9983. The RSD of the six injections of the 0.06 and 0.2 ppm solutions were 6.7 and 2.6%, respectively. Thus, the DL and QL were 0.06 and 0.2 ppm, respectively, which corresponds to 0.29 and 0.97 ng/mL of phenylhydrazine. The accuracy (recovery test) was in the range of 99.1 to 111.1% at 1 to 50 ppm (Table 4), and the repeatability of the spike recovery test was 4.0%. The linearity, accuracy, and precision results support a range from 1 to 50 ppm. The spike recovery rates were about 100% even at 1 ppm. Considering that the desired sensitivity for GTI analysis is at the sub-ppm level, the developed method was successfully applied for the phenylhydrazine analysis of antipyrine.

Quantification of phenylhydrazine in antipyrine by LC-ICP-MS

Because phenylhydrazine is commonly used as a raw material

Table 4 Accuracy for 2,3,5-triiodobenzoyl chloride derivatization method

Accuracy	Recovery, %		
	<i>n</i> = 1	<i>n</i> = 2	<i>n</i> = 3
Spiked with 1 ppm	100.9	100.9	99.1
Spiked with 10 ppm	106.8	105.7	106.3
Spiked with 50 ppm	109.6	109.0	111.1
Average, %	105.5		
Standard Deviation, %	4.27		
Relative Standard Deviation, %	4.0		

for antipyrine, residual phenylhydrazine may present in antipyrine. The residual amounts of phenylhydrazine in five different commercially available lots of antipyrine were analyzed by the developed methods using 3-iodophenyl isocyanate or 2,3,5-triiodobenzoyl chloride as the derivatization reagent. The results showed that the phenylhydrazine residues in all the lots were less than each DL of 0.2 and 0.06 ppm.

In this study, the acyl halide and an isocyanate compound containing iodine were employed as the derivatization reagents for phenylhydrazine analysis. First, 3-iodophenyl isocyanate, which has one iodine atom, was investigated as the derivatization reagent. The developed method gave excellent method validation results in the range of 1 to 50 ppm relative to the API. Then, 2,3,5-triiodobenzoyl chloride, which has three iodine atoms, was also investigated as a derivatization reagent to enhance the sensitivity of ICP-MS detection. As expected, the DL of the method was 0.29 ng/mL of phenylhydrazine solution, and this value was one third of that obtained from the 3-iodophenyl isocyanate method (0.95 ng/mL). This data strongly indicate the contribution of the number of iodine atoms to the sensitivity. This method gave excellent method validation results in the range of 1 to 50 ppm relative to the API.

Furthermore, five lots of antipyrine were analyzed by the developed methods using 3-iodophenyl isocyanate or 2,3,5-triodobenzoyl chloride, and all the results were less than each DL of 0.2 and 0.06 ppm.

Concerning the analysis of GTIs in APIs, David *et al.* have conducted some studies to develop a strategic approach for the analysis, and reported the decision tree on the method selection for GTI analysis.¹⁰ In their article, the LC-MS method using hexylchloroformate derivatization was developed as an analytical method for phenylhydrazine in APIs. The sample solution was prepared in a concentration of 100 mg/mL, and the linearity in the 0.25 to 5 ppm range was obtained with an R^2 value of >0.99. A comparison of the range of developed LC-ICP-MS methods to that of the LC-MS method showed that the sensitivity of the developed methods is either the same or better than that of the LC-MS method.

This proves that the LC-ICP-MS methods allow for the analysis of phenylhydrazine at sub-ppm levels. The methods can be used for batch release testing, and they are also applicable to the process control testing of API manufacturing. Moreover, the methods can be applied to the analysis of GTIs containing a hydrazino, amino and/or hydroxyl group in the molecule.

Conclusions

Sensitive quantitation methods using LC-ICP-MS with iodo-derivatization were developed. The methods allow for the trace level analysis of phenylhydrazine in antipyrine. The QL values of the methods were sufficient for sub-ppm analysis.

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