Absorption and Mass Spectrometry Measurements of Aqueous Ammonia after the Addition of Phenol and Sodium Hypochlorite Pentahydrate

Yasunori Tokuhara[1)*], Tatsuya Morinishi[1)], Yasuhiko Ota[1)], Satoshi Tada[1)]

1) Department of Medical Technology, Faculty of Health Sciences, Kagawa Prefectural University of Health Sciences

Abstract

Ammonia is a metabolite of amino acids in dietary proteins. Excess ammonia is toxic to the human body and causes central nervous system dysfunctions. Therefore, it is catabolized into urea, a non-toxic substance, by the urea cycle in the liver. Measurements of blood ammonia levels are used to clinically detect liver dysfunctions; however, the methods currently used to assess ammonia levels have a number of limitations and require complex technical methods as well as expensive analytical instruments. In the present study, we attempted to develop a quick and easy method to detect ammonia using a spectrophotometer. We observed color changes and analyzed the absorption spectrum of aqueous ammonia after the addition of phenol and sodium hypochlorite pentahydrate ($NaOCl{\cdot}5H_2O$), which is a strong oxidant. We also examined the oxidation reaction of ammonia using liquid chromatography time-of-flight mass spectrometry (LC/TOF-MS). The results obtained showed that aqueous ammonia turned blue in color and showed unique absorption and mass spectra following the addition of phenol and $NaOCl{\cdot}5H_2O$. This spectrophotometric method may enable ammonia to be detected in sample solutions without expensive analytical instruments or complex methods.

Key Words：ammonia, phenol, sodium hypochlorite pentahydrate ($NaOCl{\cdot}5H_2O$), spectrophotometric method

Introduction

Ammonia is generated from amino acids contained in dietary proteins through modifications to their metabolism by intestinal bacteria and several metabolic reactions, such as the glutamate dehydrogenase and purine nucleotide cycle pathways, in mammals[1-3]. Although ammonia contains nitrogen, an essential substrate for the biosynthesis of amino acids, proteins, and nucleic acids, a blood ammonia level higher than 50μM is toxic and causes central nervous system dysfunctions[4, 5]. Therefore, excess ammonia is detoxified and converted to urea by the urea cycle in the liver[6]. Accordingly, liver dysfunctions are associated with elevated ammonia levels and, ultimately, hyperammonemia[7].

A number of methods are currently available to measure ammonia, including enzyme-based assays, ion-selective electrodes (ISEs), and liquid chromatography coupled to mass spectrometry (LC-MS)[8-10]; however, these methods have various limitations. Enzyme-based assays are sensitive to several factors, such as salt concentrations and its metabolites[8], while ISEs and LC-MS involve complex technical methods and require expensive analytical instruments[9, 10].

In the present study, we attempted to develop a quick and easy method to detect ammonia using a spectrophotometer, which is used at many research

*Correspondence to : Yasunori Tokuhara, Department of Medical Technology, Faculty of Health Sciences, Kagawa Prefectural University of Health Sciences, 201-1 Hara, Mure-cho, Takamatsu, Kagawa 761-0123, Japan
E-mail: tokuhara-y@chs.pref.kagawa.jp

＜Received 15 September 2021＞　＜Accepted 10 November 2021＞

institutes and clinical laboratories. We measured the absorption and mass spectra of aqueous ammonia after the addition of phenol and sodium hypochlorite pentahydrate ($NaOCl{\cdot}5H_2O$), a strong oxidant with a solid (finely ground) form. The results obtained suggest that the blue color change observed in aqueous ammonia after the addition of phenol and $NaOCl{\cdot}5H_2O$ exhibited a characteristic spectrum, and this new method may be useful for detecting ammonia in sample solutions.

Materials and Methods

Reagents

Aqueous ammonia (28–30%), phenol (99%), and indophenol [N-(p-Hydroxyphenyl)-p-benzoquinone-Monoimine] were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). $NaOCl{\cdot}5H_2O$ was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Pure water was prepared using Barnstead Lab Tower EDI (Thermo Scientific, Langenselbold, Germany).

Transient absorption spectrum measurements

Sample solutions were measured using a model U-2900 spectrophotometer (Hitachi High-Tech Corp., Tokyo, Japan) with microcells with a 10-mm path length. One hundred microliters of aqueous ammonia was mixed with 400 μL of pure water, followed by 100μL of pure water or phenol and then 20 μL of 20 wt% $NaOCl{\cdot}5H_2O$ water solution. The solution was incubated on ice for 3 min prior to measurements. Each absorption measurement in the visible region from 500 to 700 nm was performed with a 1-nm bandwidth at a scan speed of 100 nm/min.

Liquid chromatography time-of-flight mass spectrometry (LC/TOF-MS)

Flow injection LC/TOF-MS analyses were performed using a Shimadzu Corporation Nexera X2 UHPLC System and Bruker Daltonics maXis 4 G. Regarding sample preparation, 100 μL of 20 wt% $NaOCl{\cdot}5H_2O$ water solution was added to a mixed solution of 300 μL of aqueous ammonia and 300 μL of phenol. The sample solution or 20 mg of indophenol dissolved in 500 μL of methanol was incubated on ice for 3 min and directly introduced into LC/TOF-MS without passing through the column. The mobile phase was 50% methanol aqueous solution and the flow rate was 0.2 mL/min. The ESI capillary voltage was set at 3000 V, fragmentor voltage at 150 V, gas temperature at 200 ℃ , gas flow at 2 mL/min, and nebulizer pressure at 1 bar. Mass spectra (m/z 50–1500) were acquired in the positive-ion mode.

Results

Color changes and absorption spectra of aqueous ammonia

We initially added pure water or phenol and then $NaOCl{\cdot}5H_2O$ to aqueous ammonia and observed changes in its color. Aqueous ammonia did not show a color change after the addition of pure water and $NaOCl{\cdot}5H_2O$ (Fig. 1A); however, it turned blue after the addition of phenol and $NaOCl{\cdot}5H_2O$ (Fig. 1A). We also analyzed the absorption spectra of aqueous ammonia in the visible region (500–700 nm). Although the absorbance spectrum of the aqueous ammonia did not show any absorption peaks following the addition of pure water and $NaOCl{\cdot}5H_2O$, a peak was detected at approximately 640 nm following the addition of phenol and $NaOCl{\cdot}5H_2O$ (Fig. 1B).

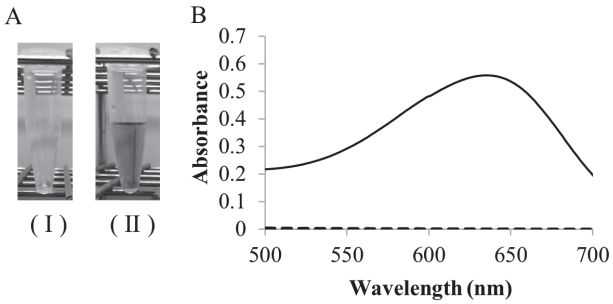

Figure 1. Color change and absorption spectra of aqueous ammonia.
(A) Color change in aqueous ammonia after the addition of pure water and $NaOCl{\cdot}5H_2O$ (I) or phenol and $NaOCl{\cdot}5H_2O$ (II).
(B) Absorption spectra of aqueous ammonia after the addition of pure water and $NaOCl{\cdot}5H_2O$ (dashed line) or phenol and $NaOCl{\cdot}5H_2O$ (solid line).

Analysis by LC/TOF-MS spectrometry

Indophenol[11], which is the oxidative coupling of an aromatic compound generated from phenol and nitrogen, is a well-known blue pigment (Fig. 2). To examine whether the blue pigment combined in aqueous ammonia after the addition of phenol and $NaOCl{\cdot}5H_2O$ was indophenol, we analyzed mass spectra using LC/TOF-MS and compared indophenol with the pigment combined by our method (Fig. 3). Indophenol was represented by a peak at m/z 200 $[M+H]^+$ (Fig. 3A). Aqueous ammonia, which turned blue in color after

the addition of phenol and NaOCl·5H$_2$O, showed a peak at m/z 292 (Fig. 3B).

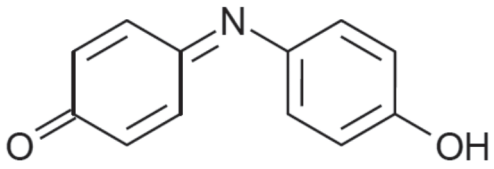

Figure 2. Structure of indophenol.

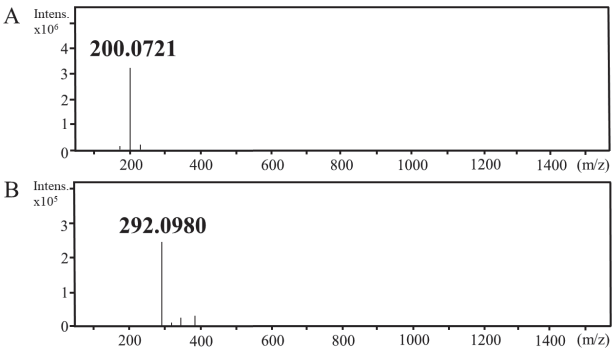

Figure 3. LC/TOF-MS spectra.
(A) MS spectrum of indophenol.
(B) MS spectrum of aqueous ammonia after the addition of phenol and NaOCl·5H$_2$O.

Discussion

In the present study, we observed changes in the color and absorption and mass spectra of aqueous ammonia after the addition of phenol and NaOCl·5H$_2$O. The results obtained revealed that aqueous ammonia turned blue in color following the addition of phenol and NaOCl·5H$_2$O and showed a characteristic absorption spectrum with a peak at approximately 640 nm. These results will contribute to the development of a novel detection method for ammonia using a spectrophotometer.

Ammonia, a nitrogen-containing compound, is mainly produced from amino acids in dietary proteins through modifications to their metabolism by intestinal bacteria in the human body[1, 2]. Since ammonia is very toxic to the brain, it is detoxified and converted to urea by the hepatic urea cycle[6, 12]. Therefore, blood ammonia levels are elevated in patients with hepatic disorders and used as a clinical test item[13]. Enzyme-based assays, ISEs, and LC-MS are currently used to quantify ammonia[8-10], but have a number of limitations, including an unstable color reaction, inconvenient sample treatments, time-consuming, or the need for expensive analytical instruments[8-10]. In an attempt to develop a quick and easy method that detects ammonia using a spectrophotometer, which is used at many research institutes and clinical laboratories, we measured the absorption spectrum of aqueous ammonia after the addition of phenol and NaOCl·5H$_2$O in order to examine the specificity of the characteristic peak. As shown in Fig. 1, aqueous ammonia showed a blue color change and a characteristic peak at approximately 640 nm after the incubation with phenol and NaOCl·5H$_2$O. These results suggest that our simple method will enable many researchers and laboratory technicians to detect ammonia in samples from patients with hepatic disorders. Since spectrophotometers are used at many research institutes and clinical laboratories, are very simple to operate, and rapidly provide results, ammonia may be easily detected using our method. Furthermore, it is simple to automate our method because the spectrophotometric method is installed in many automated biochemical analyzers.

A colorimetric assay is another method by which to detect ammonia[14]. In this assay, the oxidative coupling of ammonia and phenol generates indophenol, a blue pigment (Fig. 2)[11, 14]. As shown in Fig. 1A, the blue pigment combined in aqueous ammonia after the addition of phenol and NaOCl·5H$_2$O may have been indophenol because the blue color change required not only ammonia, but also phenol. Therefore, we compared indophenol with the blue pigment combined in aqueous ammonia after the addition of phenol and NaOCl·5H$_2$O using LC/TOF-MS (Fig. 3). Indophenol showed a peak at m/z 200 (Fig. 3A), whereas the blue pigment combined by our method showed a peak at m/z 292 (Fig. 3B). These results indicate that the blue pigment combined by our method differed from indophenol and, thus, a novel blue pigment appears to be generated. In present study, we used NaOCl·5H$_2$O for the oxidative coupling of phenol and ammonia. NaOCl·5H$_2$O is a strong oxidant that has been shown to accelerate the oxidation reaction of divalent phenolic acids[15, 16]. However, since NaOCl·5H$_2$O has only recently been developed[17], few studies have used it as an oxidizing agent[15, 16]. To develop a novel oxidative coupling reaction, we attempted to oxidize phenol and ammonia by the addition of NaOCl·5H$_2$O, and combined a blue pigment, which showed a different mass spectrum to that of indophenol (Fig. 3). Although further studies, such as those involving nuclear magnetic resonance spectrometry, are needed to elucidate the structural formula of the blue pigment showing a peak at m/z 292

(Fig. 3B), the present results suggest that NaOCl·5H_2O is useful for the development of a novel oxidative coupling reaction due to its strong oxidative potential.

In summary, we herein demonstrated that aqueous ammonia showed a blue color change and characteristic absorption spectrum at approximately 640 nm through an oxidation reaction following the addition of phenol and NaOCl·5H_2O. Moreover, we confirmed that the blue pigment combined by our method showed a peak at m/z 292 using LC/TOF-MS, which was not consistent with the mass spectrum of indophenol. Collectively, these results suggest that our spectrophotometric method is suitable for the development of a novel method to detect ammonia in sample solutions.

Acknowledgments

The authors would like to thank Keisuke Sogabe (Kaneka Techno Research Corporation) and Yasukazu Ejima (Kaneka Corporation) for their technical support and comments on LC/TOF-MS analyses. This work was supported by JSPS KAKENHI Grant Number JP21K07394.

References

1）Richardson AJ, McKain N, Wallace RJ. Ammonia production by human faecal bacteria, and the enumeration, isolation and characterization of bacteria capable of growth on peptides and amino acids. BMC Microbiol 13: 6, 2013.

2）Smith EA, Macfarlane GT. Formation of phenolic and indolic compounds by anaerobic bacteria in the human large intestine. Microb Ecol 33(3): 180-188, 1997.

3）Cooper AJ, Plum F. Biochemistry and physiology of brain ammonia. Physiol Rev 67(2): 440-519, 1987.

4）Meijer AJ, Lamers WH, Chamuleau RA. Nitrogen metabolism and ornithine cycle function. Physiol Rev 70(3): 701-748, 1990.

5）Cagnon L, Braissant O. Hyperammonemia-induced toxicity for the developing central nervous system. Brain Res Rev 56(1): 183-197, 2007.

6）Haussinger D. Nitrogen metabolism in liver: structural and functional organization and physiological relevance. Biochem J 267(2): 281-290, 1990.

7）Lockwood AH, McDonald JM, Reiman RE, Gelbard AS, Laughlin JS, et al. The dynamics of ammonia metabolism in man. Effects of liver disease and hyperammonemia. J Clin Invest 63(3): 449-460, 1979.

8）Ye H, Yang K, Tao J, Liu Y, Zhang Q, et al. An enzyme-free signal amplification technique for ultrasensitive colorimetric assay of disease biomarkers. ACS Nano 11(2): 2052-2059, 2017.

9）Mueller AV, Hemond HF. Statistical generation of training sets for measuring NO3(-), NH4(+) and major ions in natural waters using an ion selective electrode array. Environ Sci Process Impacts 18(5): 590-599, 2016.

10）Spinelli JB, Kelley LP, Haigis MC. An LC-MS approach to quantitative measurement of ammonia isotopologues. Sci Rep 7(1): 10304, 2017.

11）Searle PL. The berthelot or indophenol reaction and its use in the analytical chemistry of nitrogen. A review. Analyst 109: 549-568, 1984.

12）Braissant O. Ammonia toxicity to the brain: effects on creatine metabolism and transport and protective roles of creatine. Mol Genet Metab 100 Suppl 1: S53-58, 2010.

13）Tarantino G, Citro V, Esposito P, Giaquinto S, de Leone A, et al. Blood ammonia levels in liver cirrhosis: a clue for the presence of portosystemic collateral veins. BMC Gastroenterol 9: 21, 2009.

14）Bolleter WT, Bushman CJ, Tidwell PW. Spectrophotometric determination of ammonia as indophenol. Anal. Chem 33(4): 592–594, 1961.

15）Tokuhara Y, Shukuya K, Tanaka M, Sogabe K, Ejima Y, et al. Absorbance measurements of oxidation of homogentisic acid accelerated by the addition of alkaline solution with sodium hypochlorite pentahydrate. Sci Rep 8(1): 11364, 2018.

16）Hosokawa S, Shukuya K, Sogabe K, Ejima Y, Morinishi T, et al. Novel absorbance peak of gentisic acid following the oxidation reaction. PLoS One 15(4): e0232263, 2020.

17）Kirihara M, Okada T, Sugiyama Y, Akiyoshi M, Matsunaga T, et al. Sodium hypochlorite pentahydrate crystals (NaOCl·5H2O): a convenient and environmentally benign oxidant for organic synthesis. Org. Process Res. Dev. 21(12): 1925–1937, 2017.

フェノールおよび次亜塩素酸ナトリウム五水和物添加後のアンモニア水の吸光度やマススペクトルの測定

徳原 康哲[1)]*，森西 起也[1)]，太田 安彦[1)]，多田 達史[1)]

[1)] *香川県立保健医療大学保健医療学部臨床検査学科*

要旨

アンモニアは，飲食物中のタンパク質に含まれるアミノ酸が体内で代謝される過程で生成される．しかし，過剰なアンモニアは人体にとって毒性が高く，中枢神経系障害を引き起こす．そのため，アンモニアは肝臓における尿素サイクルの働きにより，毒性のない尿素に代謝され尿中に排泄される．このように，アンモニアは肝臓で代謝されるため，血中アンモニア濃度は肝機能を反映する指標の一つとして利用されているが，現在のアンモニア測定法は煩雑な手技や高価な分析装置を必要とする等の制限がある．本研究では，分光光度計を用いた簡易・迅速なアンモニア測定法の開発を目的とし，強力な酸化剤である次亜塩素酸ナトリウム五水和物（$NaOCl{\cdot}5H_2O$）およびフェノールをアンモニア水に添加後，色調変化の観察や分光法により吸光度を測定した．さらに，液体クロマトグラフィー飛行時間型質量分析（LC/TOF-MS）によりアンモニアの発色反応を解析した．本研究により，フェノールおよび $NaOCl{\cdot}5H_2O$ を添加したアンモニア水は青色に変化し，特徴的な吸光度およびマススペクトルを示すことが明らかとなった．以上の結果から，高価な分析装置や煩雑な手技を必要とすることなく，検体中のアンモニアを簡易に測定する検査法の開発には，本法が有用である可能性が示唆された．

Key Words：アンモニア，フェノール，次亜塩素酸ナトリウム五水和物（$NaOCl{\cdot}5H_2O$），分光法

*連絡先：〒761-0123 香川県高松市牟礼町原281-1 香川県立保健医療大学保健医療学部臨床検査学科 徳原 康哲
E-mail: tokuhara-y@chs.pref.kagawa.jp