# Gas Phase Derivatization of Ammonia with 4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole and Its Application to Urease Assay

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**An ammonia-specific and rapid fluorometric method for determination of ammonia and urease activity was developed. The method is designed to assay ammonia levels or urease activity for the rapid diagnosis of *Helicobacter pylori* infection. 4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole was used to derivatize ammonia and 4-amino-7-nitrobenzo-2-oxa-1,3-diazole was analysed by high performance liquid chromatography at an excitation wavelength of 455 nm and an emission wavelength of 520 nm. Derivatization was designed to react with ammonia gas produced in a strong alkaline pH sample. The fluorescent intensity was linear in the range of 0.01—10 mM ammonia per tube when the reaction was carried out for 15 min at 37 °C. Urease activity, judged as the amount of ammonia production from urea, could be measured at 25 ng per tube ($S/N=1.5$) with Jack bean meal urease. Because of its rapidity, this assay is potentially superior to the current standard method in use in clinical settings.**

**Keywords** 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole; NBD-F; ammonia; urease; *Helicobacter pylori*; clinical methodology gastritis; peptic ulceration

## Introduction

4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) is known to react with amines and to give products with strong fluorescence.[2)] The reagent has been utilized for amino acid analysis[3–6)] because of the following merits: i) the reagent itself is not fluorescent, ii) it reacts rapidly with both amine and imine groups under mild conditions and produces a strongly fluorescent compound. The fluorescence of 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH), one of the major hydrolysis products of NBD-F, can be quenched in acidic pH. In this study, the advantages of NBD-F were utilized to develop a method for the determination of ammonia which in turn can be applied to a urease assay. The method is designed to assay ammonia levels or urease activity for the rapid diagnosis of *Helicobacter* (formerly *Campylobacter*) *pylori* (HP) infection, recognized to be a causative agent of gastritis and peptic ulceration.[7)]

Recently, Smoot *et al.*[8)] reported that the urease from HP may play an important role in its pathogenicity. A sensitive and rapid method described here fore the assay of urease activity may be valuable for both pathological study and diagnosis of HP infection.

## Methods and Results

A schematic reaction of this assay system is presented in Fig. 1.

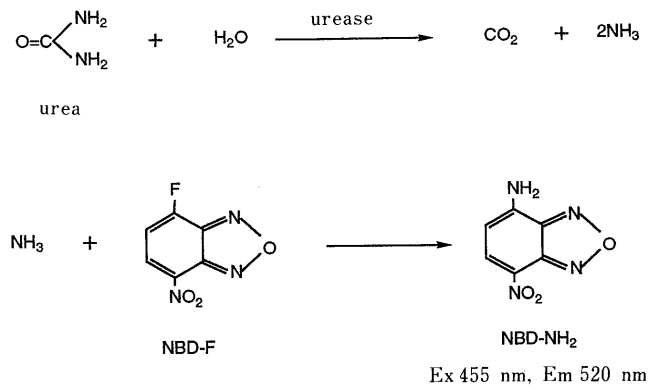


Fig. 1. A Schematic Reaction of Ammonia for Urease Assay

Ammonia produced from urea by urease is immediately derivatized with NBD-F, yielding 4-amino-7-nitrobenzo-2-oxa-1,3-diazole (NBD–$NH_2$) (Fig. 2). The product, NBD–$NH_2$, was separated and quantified by reverse phase high performance liquid chromatography (HPLC). NBD–$NH_2$ was eluted at 4.5 min (Fig. 3). Production of NBD–$NH_2$ increased linearly from 0 to 60 min of derivatization and an incubation time of 15 min at 37 °C was selected for derivatization. Under these conditions, the calibration curve with a standard ammonia solution in 50 mM phosphate buffer (pH 8.0) was linear in the range of 0.01—10 mM per tube (100 pmol—100 nmol per injection, $r=0.999$). Urease activity, judged as the amount of ammonia produced from urea, could be measured at 25 ng per tube ($S/N=1.5$) with Jack bean meal urease (Nacalai, Kyoto, specific activity of 3.5 unit/mg).

## Discussion

Detection limits can be lowered by a longer incubation

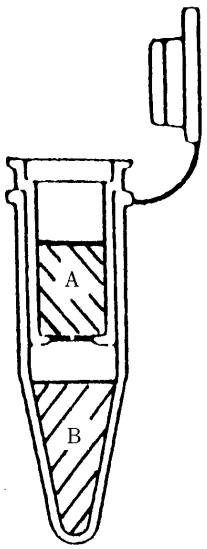


Fig. 2. Reaction Apparatus

A Millipore Ultrafree C3-HV tube with Durapore membrane filter (0.45 μm) was used as the reaction apparatus. Place 250 μl of a 1% urea solution in 50 mM phosphate buffer (pH 8.0) and 50 μl of urease solution (or gastric juice, or gastric biopsy) in the bottom of the polypropylene tube (B) and incubated for 15 min at 37 °C. Add 100 μl of 5 M NaOH to stop the enzymatic reaction. The cyclinder with a membrane filter containing 100 μl of 1 mM NBD-F in acetonitrile is placed in tube (A). The tubes are incubated for 15 min at 37 °C to allow the NBD-F to react with the $NH_3$ gas that permeates through the filter from the bottom of the tube. Add 100 μl of 1 M $HNO_3$ to terminate the derivatization. Inject 10 μl of the solution from the cylinder to the HPLC.

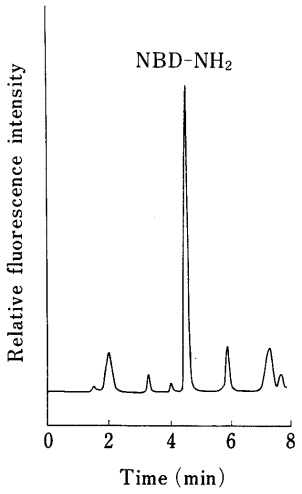


Fig. 3. A Typical HPLC Separation of Urease Analysis

HPLC system: Shimadzu model LC-6A; mobile phase, $CH_3CN$–$H_2O$ (2:3, v/v; isocratic); flow rate, 1.0 ml/min; detector, Shimadzu fluorescence monitor RF-550 (Ex 455 nm, Em 520 nm); column, Tosoh TSK-gel ODS-$80_{TM}$ (5 $\mu$m, 4.6 × 150 mm); column temperature, ambient. The peak of NBD–$NH_2$ was calculated to be 2.2 nmol.

time. However, for both the enzymatic reaction and NBD-F derivatization, 15 min is satisfactory for a urease assay and for measuring ammonia levels. Thus, this procedure is suited for the rapid diagnosis of HP infection. A further advantage of our assay is that blood contamination is not a problem because in order to selectively derivatize ammonia, the reaction is performed after converting ammonia in samples (gastric juice or biopsy) into gas by NaOH.

Currently, Mobley's method has been widely used for urease assay to identify HP infection in clinical practice.[9] This method is based on monitoring pH change caused by ammonia production by urease, and phenol-red is used as a pH indicator. Because of the patchy distribution of HP *in vivo*, several biopsy specimens from different parts of the stomach are necessary for the correct diagnosis. Moreover, except for heavy infection, the urease assay usually takes more than 3 h. Therefore, in order to diagnose a patient quickly, development of a more rapid method is necessary. On the other hand, the sampling of gastric juice is noninvasive and can be achieved without any endoscopic skills. The acidic pH in gastric juice does not allow for the application of Mobley's method to help in the diagnosis of HP infection, however, this fluorometric method by NBD-F derivatization is applicable to measure ammonia levels in gastric juice. Thus, it will be valuable if gastric juice can be used for the diagnosis of HP infection.

**References and Notes**

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