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MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MUTAGEN, ETHYL N-ETHYL-N-NITROSOCARBAMATE, FORMED FROM URETHANE AND NITRITE

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The separation of mutagen, ethyl N-ethyl-N-nitrosocarbamate (EENC), from an acidic mixture (pH 1.2) of urethane and sodium nitrite is described, which employs micro high-performance liquid chromatography on a reversed-phase column (LiChrosorb RP-18). Ethyl N-nitrosocarbamate was characterized as an intermediate to EENC.

1 Introduction

Urethane, the simplest compound of the carbamates that have widely been used as herbicides, insecticides and fungicides, has been known to have no mutagenic activity on microorganisms and tissue cultures of cells, though it acted as a carcinogen on mice[1].

In the course of study on a new type of potent mutagen[2], we found that urethane showed a mutagenic activity to *E. coli* WP 2, *E. coli* WP 2 *uvr* A and *Salmonella Typhimurium* TA 100 in the presence of nitrite in an acidic medium, and isolated ethyl N-ethyl-N-nitrosocarbamate (EENC) as a mutagenic substance[3]. The biological assay for the mutagenic activity of EENC was time consuming[2], and the formation process of EENC has remained unknown. This paper describes the fundamental conditions for the rapid determination of EENC by micro high-performance liquid chromatography (micro HPLC) with UV detection and the separation of an intermediate to EENC from an acidic solution of urethane and sodium nitrite.

2 Experimental

2・1 Materials and procedure for the reaction

All chemicals and organic solvents used were of the reagent grade. Deionized and distilled water was used. EENC was prepared as previously described[3]. The reactions of urethane (0.01-0.4M) with $NaNO_2$ (0.01-4M) were carried out at 0°C, with a pH value of 1.2 for 30 min. The pH of the reaction mixture was adjusted to 1.2, corresponding to that of human gastric juice, with 1M HCl.

2・2 Apparatus and HPLC conditions

A JASCO FAMILIC 100N micro high-performance liquid chromatograph equipped with a JASCO UVIDEC 100-III UV/VIS spectrophotometer was used for the determination of EENC. The teflon tube (150 x 0.5 mm I.D.) was packed with LiChrosorb RP-18 (particle size: 10 μm; Japan Merck, Tokyo) using the slurry technique[4]. The column temperature was ambient. The mobile phase was methanol/water (1:1, v/v) and the

flow rate was 10 μl/min. A sample solution (0.3 μl) was injected. A JASCO LC/MS-100 system equipped with a Denshikagaku MMD-05A mass detector was connected in series with the UV monitor described above for the characterization of the intermediate. The m/e was set at 118 for a single ion monitor.

The fractionation of the intermediate was performed with a YANACO PN 101 high-performance liquid chromatograph equipped with a Hitachi wavelength tunable effluent monitor. A stainless-steel column (150 x 4 mm I.D.) was packed with LiChrosorb RP-18 (particle size: 5 μm) using the slurry technique[5]. The mobile phase was methanol/water (3:7, v/v) and the flow rate was 0.6 ml/min. A reaction mixture (100 μl) of 2M urethane in 1M HCl (2 ml) and 0.4M $NaNO_2$ (2 ml) was injected after it was allowed to react at 0°C for 30 min. The column temperature was ambient.

The absorption spectra were measured with a Shimadzu UV 200S spectrophotometer with 10-mm micro cells.

3 Results and discussion

3 · 1 The absorption spectra of EENC

EENC was soluble in water and normal organic solvents (*e.g.*, methanol, ethanol, *n*-buthanol, ethyl acetate, chloroform, ethylene chloride, ether and *n*-hexane). The aqueous solution of EENC (1.4×10^{-2}M) was acidic (pH 3.9) having an absorption band as shown in Fig. 1(a) with another band in the UV region {λmax nm (ε): 240 (5300)}. In the organic solvents described above, EENC showed a similar band shape to that dissolved in 95% ethanol shown in Fig. 1(b) with another band in the UV region {λmax nm (ε): 240 (6900)}. Since the molar absorptivity at 240 nm in each solvent was about 60 times larger than that of the visible region, the effluent in HPLC was monitored at 240 nm. EENC was stable for at least 24 hr at 25°C in the pH range of 3.5-6, but rapidly decomposed at pH higher than 8.

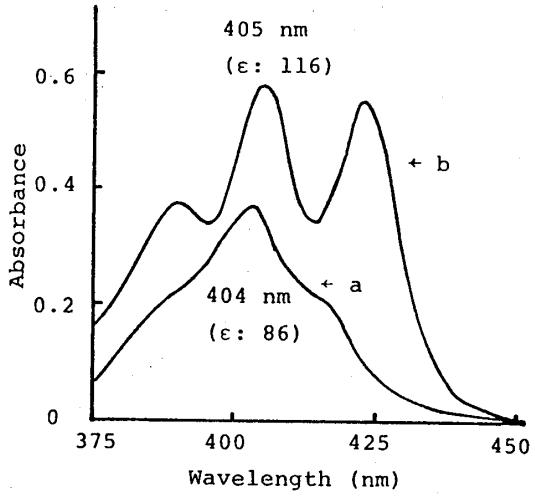


Fig. 1 Absorption spectra of EENC
a: in H_2O ; b: in 95% EtOH

3 · 2 Separation of EENC

The absorption spectrum of the reaction mixture of urethane (1M) and sodium nitrite (0.2M) in the acidic medium (pH 1.2) changed with time as shown in Fig. 2. Urethane showed no absorption band longer than 300 nm, while nitrite showed a characteristic spectrum chang-

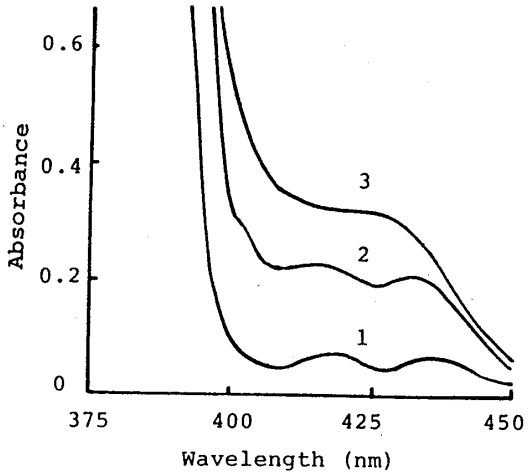


Fig. 2 Absorption spectral change of reaction mixture of urethane and nitrite
1, 15 : 2, 30 ; 3, 60 min

ing with time in the 320-400 nm range in a similar manner to that of the reaction mixture. The absorption at around 400-450 nm in Fig. 2 proved to be responsible for EENC (cf., Fig. 1) and an intermediate (cf., Fig. 5) formed in the reaction.

Fig. 3(a) shows a typical chromatogram of the reaction mixture obtained according to the procedure. EENC (peak 4) is completely separated from others. The peaks 1, 2 and 3 were assigned to nitric acid, the intermediate and nitrous acid, respectively.

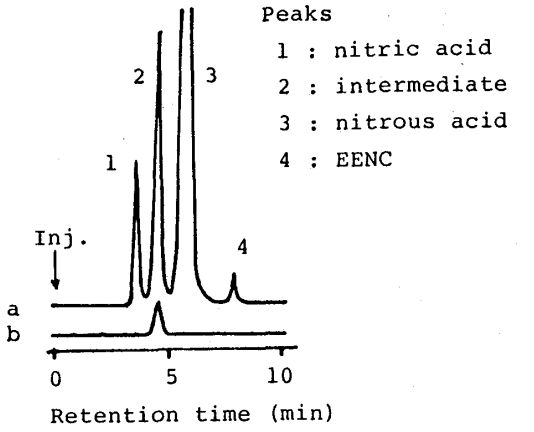


Fig. 3 Chromatograms of the reaction mixture of urethane and nitrite
a, UV detection (240 nm)
b, mass detection (m/e : 118)

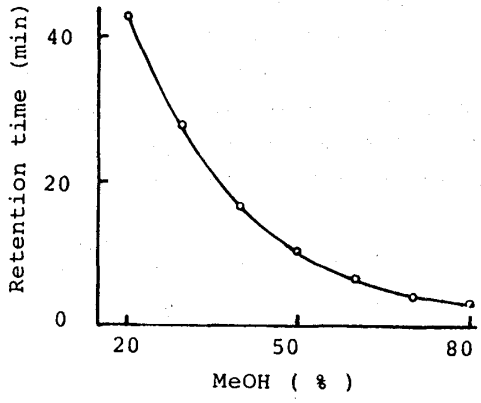


Fig. 4 Effect of concentration of methanol in mobile phase on the retention time of EENC

The methanol content in the mobile phase had an effect on the retention time of EENC as shown in Fig. 4. At methanol concentrations higher than 70%, the peaks closely overlapped, while a lower concentration of methanol (20%) caused a delay of the elution with broadening of the peaks; therefore, 50% methanol was employed as the adequate resolution.

The pH of the mobile phase did not affect the retention time of EENC in the pH range of 1-6. When the pH of the reaction mixture was higher than 3, EENC was not detected. However, the peak of EENC increased in intensity as the pH value of the reaction mixture decreased. The pH profile of the EENC formation agreed well with the reported data on the mutagenic activity of EENC[3)]

There was a linear relationship between the peak hight and the concen-

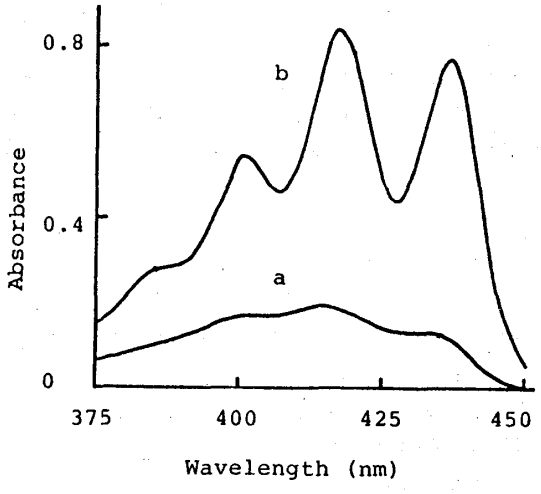


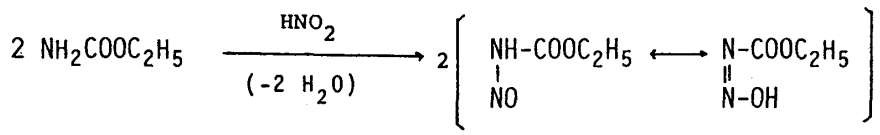
Fig. 5 Absorption spectra of the intermediate separated by HPLC
a, HPLC fraction ; b, ether extraction of the HPLC fraction

tration of EENC in the range of at least 0.1-50 nmol/0.3 µl(0.005 absorbance unit of full scale).

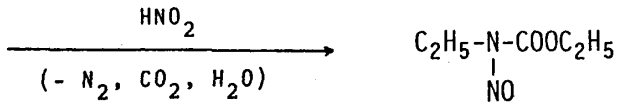
3 · 3 Fractionation and characterization of the intermediate

Fig. 5(a) shows the absorption spectrum of a HPLC fraction corresponding to peak 2 in Fig. 3(a). The ether extract of the fraction showed a band with fine structure due to the nitroso group in the 375-450 nm range (Fig. 5(b)) and another band in the UV region (λ_{max} nm: 224). The LC/MS chromatogram shown in Fig. 3(b) indicated that peak 2 in (a) was due to the compound of M^+=118, which could be assigned to ethyl N-nitrosocarbamate (EC).

The peak of EENC appeared as the peak intensity of EC decreased on the chromatogram, only when sodium nitrite was added to the acidic solution of EC. This indicated that EENC was formed *via* EC under the acidic reaction conditions in the presence of nitrite. The reaction is as follows:



EC



EENC

The micro HPLC technique is very suitable for the analysis of toxic substances such as mutagens and/or carcinogens because the detection and separation can be carried out on a small amount of the injection and elution volumes. The present micro HPLC may be applicable to the quantification of EENC and EC in biological samples. The mutagenic activity of EC has remained unknown.

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Keyword phrases

Micro high-performance liquid chromatography with UV detection; micro high-performance liquid chromatography with mass detection; mutagen; ethyl N-ethyl-N-nitrosocarbamate; ethyl N-nitrosocarbamate.