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Note

Separation of traces of isomeric hydroxypyridines in aqueous solution by high-performance liquid chromatography

A comparison of UV absorption *versus* fluorescence detection

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In order to understand the reactions of hydroxy radical, generated radiolytically, with pyridine, it was necessary to develop a sensitive method for the detection and quantification of isomeric hydroxypyridines. We have reported earlier the separation of isomeric dihydroxyphenols on a μBondapak C_{18} column by high-performance liquid chromatography[1]. Due to extensive use of μBondapak C_{18} columns[2], we have employed this stationary phase for the separation of isomeric hydroxypyridines. The *meta*- and *ortho*-hydroxypyridines are strongly fluorescent, whereas *para*-hydroxypyridine is only weakly fluorescent[3]. The detection of hydroxypyridines was carried out optically by UV absorption at 270 and 250 nm and fluorimetrically by excitation at 305 nm.

EXPERIMENTAL

The main features of the liquid chromatograph used have been previously described[1-4]. The apparatus consists of the following components: Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 and 6000A pumps and a 150-μl sox-port injection valve. The chromatographic column used was a μBondapak C_{18} obtained from Waters Assoc. The optical detector was a Vari-Chrom UV-Vis variable wavelength detector obtained from Varian Instruments (Palo Alto, Calif., U.S.A.). The UV absorption spectra of isomeric hydroxypyridines were taken using a Cary Model 219 spectrophotometer. The fluorescence detector used was a Model FS970 LC fluorometer (Schoeffel Instruments). The fluorescence excitation was carried out at 305 nm whereas the emission was observed using a Corning 370 filter. The fluorescence emission spectra of hydroxypyridines were taken using a Spex Fluorolog fluorimeter.

RESULTS AND DISCUSSION

The chromatogram obtained at $\lambda = 250$ nm of a synthetic mixture of isomeric hydroxypyridines is shown in Fig. 1a. The three isomers are well separated but the sensitivity of detection of 2-hydroxypyridine is low due to its low extinction coefficient

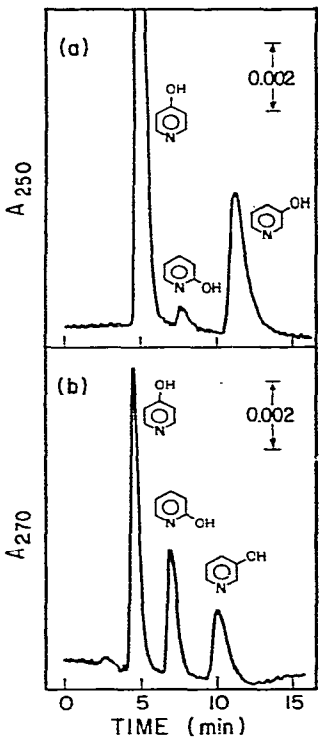

Fig. 1. (a) Separation of isomeric hydroxypyridines (10 μM each) on a μBondapak C_{18} column with 20% methanol as the mobile phase. The detection was carried out at 250 nm. (b) Experimental conditions are the same as in (a) except the detection was carried out at 270 nm.

(500 M^{-1}/cm). The *para* and *meta* isomers have absorption maxima at 250 nm with extinction coefficients of $1.3 \cdot 10^4$ and $3.0 \cdot 10^3$ M^{-1} /cm, respectively. All the three isomers have extinction coefficients ranging from 1500–3000 m^{-1}/cm at 270 nm and hence, the chromatogram obtained at this wavelength is shown in Fig. 1b. It is possible to detect $3 \cdot 10^{-7}$ M of 2 and 3 hydroxypyridines within $\pm 5\%$ accuracy. Due to higher extinction coefficient of 4-hydroxypyridine, the sensitivity of its detection can be enhanced by a factor of 4 compared to the other isomers.

The *ortho* and *meta* isomers are strongly fluorescent on excitation at 305 nm whereas 4-hydroxypyridine is only weakly fluorescent. Fluorimetric detection in general is considered to be more sensitive than optical detection[5]. Hence, fluorescent detection of isomeric hydroxypyridines was also carried out. The chromatogram of a synthetic mixture of isomeric hydroxypyridines is shown in Fig. 2. Since 4 hydroxypyridine is only weakly fluorescent, it was not detected. The fluorescent intensity of 3-hydroxypyridine increases at pH 9.0 whereas that of 2-hydroxypyridine remains the same. At pH 9.0, the detection limit of 3- hydroxypyridine can be enhanced by a factor of 10. The sensitivity of detection by fluorimetry 2 and 3 hydroxypyridines is at least an order of magnitude greater than optical detection. Since 4-hydroxypyridine is weakly fluorescent, only concentrations greater than $3 \cdot 10^{-4}$ M were detectable fluorimetrically.

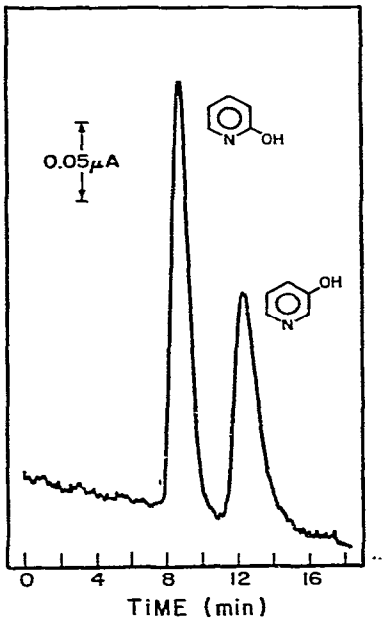

Fig. 2. Separation of isomeric hydroxypyridines (10 μM each) on a μBondapak C_{18} column with 20% methanol as the mobile phase. The detection was carried out fluorimetrically by excitation at 305 nm. Since 4-hydroxypyridine is weakly fluorescent, it was not detected under these experimental conditions.

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