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DIRECT GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF SOME PYRIDINE-N-OXIDES

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SUMMARY

A method is described for the direct gas–liquid chromatography of some pyridine-N-oxides. A method for the selective extraction of pyridine-N-oxides from biological materials is also described. The use of these techniques has permitted the development of sensitive methods for the detection and quantitation of alkyl- and halogen-substituted pyridine-N-oxides formed as metabolites in *in vitro* or *in vivo* metabolic studies.

INTRODUCTION

Tertiary aliphatic and aromatic N-oxides are thermolabile and have been shown to break down or rearrange during gas–liquid chromatography (GLC). Chlorpromazine-N-oxide is degraded to chlorpromazine, desmonomethylchlorpromazine and N-allyl-2-chlorophenothiazine[1]. Biological samples are usually examined for the presence of thermolabile N-oxides by the method of Beckett *et al.*[2]. This involves exhaustive extraction of the parent tertiary amine using an organic solvent in which the N-oxide is insoluble. The N-oxide, which is left in the aqueous phase, can then be assayed by GLC after reduction to the tertiary amine using titanium(III) chloride.

Heteroaromatic N-oxides are usually more stable than aliphatic or aromatic N-oxides[3]. Metabolic studies in which aromatic heterocyclic N-oxidation has been studied have almost always used radiochemical techniques[4,5]. The N-oxide group in pyridine-N-oxide and related compounds is quantitatively reduced by titanium(III) chloride[6] and the assay method for N-oxides developed by Beckett *et al.*[2] has been used in a study on the *in vivo* metabolism of pyrazines and some alkylpyridines[7]. Because of the inherent stability of aromatic heterocyclic N-oxides, the use of direct analysis by GLC was attempted in this study.

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MATERIALS AND METHODS

The substituted pyridines were commercial products from Aldrich (Gillingham, Great Britain) and Koch-Light (Colnbrook, Great Britain). Pyridine-N-oxides were synthesised by methods previously described[8]. Solutions of the compounds were prepared in redistilled ethanol (10 mg per 10 ml of ethanol) and stored at 0° until required. The compounds were stable under these conditions, giving one spot when examined by thin-layer chromatography at intervals.

N-Oxide solutions (1–2 μl) were examined by GLC, at different oven and injection port temperatures, using different nitrogen flow-rates, and using a Hamilton (10-μl) syringe equipped with either a short (5 cm) or a long (7 cm) syringe needle. Peak shapes were noted and the retention times of the peak or peaks were recorded. If more than one peak was obtained after the injection of an N-oxide solution, breakdown of the N-oxide to the parent pyridine was presumed. This was confirmed by injecting the parent pyridine and comparing its retention time (R_T) with the second peak (breakdown peak) obtained during N-oxide chromatography. Attempts were made to reduce breakdown to a minimum by lowering the injection port temperature, using a 7-cm long syringe needle and performing quick on-column injections. These procedures were adopted in order to minimize contact of hot metal with the pyridine-N-oxides.

Gas–liquid chromatography

A Perkin-Elmer F33 gas chromatograph, equipped with a flame-ionization detector and a 0–2.5-mV Perkin-Elmer 56 chart recorder, was used. Pre-coiled glass columns, 0.63 cm O.D., were packed as follows: column A, 1 m, 2% Carbowax 20M + 5% potassium hydroxide on 80–100-mesh AW, DMDCS-treated Chromosorb W; column B, 1 m, 3% OV-17 on 80–100-mesh AW, DMDCS-treated Chromosorb G. The columns were conditioned for 48 h at a temperature 10° higher than the proposed maximal operating temperature. Column B was silanized *in situ* with 3 × 5 μl of hexamethyldisilazane before use. Gas pressures were nitrogen 20 p.s.i. (140 kN/m^2), hydrogen 17 p.s.i. (119 kN/m^2) and air 25 p.s.i. (175 kN/m^2).

Gas–liquid chromatography–mass spectrometry (GLC–MS)

Combined GLC–MS was performed with a VG 12F mass spectrometer linked to a Pye 104 gas–liquid chromatograph, with a glass column (1 m × 0.64 cm O.D.) packed with 2% Carbowax 20M + 5% potassium hydroxide on 80–100-mesh AW, DMDCS-treated Chromosorb W; helium was used as the carrier gas (20–30 ml/min), the oven temperature was 200° and the ionization potential was 70 eV.

Extraction of pyridine-N-oxides from biological materials

Typical microsomal incubates[9] (3.5 ml) or urine samples[13] (3.5-ml aliquots) were rendered alkaline (0.5 ml of 1.0 *N* sodium hydroxide solution) and made up to 5.5 ml by the addition of water (1 ml) and the appropriate internal standard (another N-oxide) (0.5 ml). Aliquots (4 ml) were transferred on to sodium chloride (1 g) in screw-capped tubes (10 ml, Sovirel SVL) and extracted with three 5-ml volumes of freshly double-distilled diethyl ether, the organic phases being discarded. The aqueous phases were extracted with three 5-ml volumes of dichloromethane. The combined dichloro-

methane extracts were collected in evaporating tubes[10] and concentrated to about 10 μl in a water-bath (45°). Aliquots (1–2 μl) were injected on to GLC column A with the oven temperature at 170° and the injection port temperature at 225°, using a syringe equipped with a 7-cm needle. Peak-height ratios of the test compound to the internal standard were measured, and this allowed the determination of pyridine-N-oxide in the original 3.5-ml sample, using previously constructed calibration graphs (see below).

Calibration graphs

Alkyl- or halogen-substituted pyridine-N-oxides (10–100 nmole) in water (1 ml) were added to typical microsomal incubates (3.5 ml) or urine (3.5 ml). Any enzymic activity was terminated by the addition of 0.5 ml of 1.0 *N* sodium hydroxide solution. The appropriate internal standard (50 nmole of another pyridine-N-oxide in 0.5 ml of water) was then added and 4-ml aliquots were processed as described above. Peak-height ratios of the pyridine-N-oxide to the internal standard were plotted against the concentration of the pyridine-N-oxide.

RESULTS AND DISCUSSION

The pyridine-N-oxides showed partial decomposition to the parent pyridines using column A or B when the oven and injection port temperatures were high (>250°), and when the injection was performed using a syringe equipped with a short (5-cm) needle. The pyridines were eluted very close to the solvent front and their identities were confirmed by comparison of their retentions times with those of authentic compounds, and by combined GLC–MS. However, careful control of the various parameters, for example, a low injection port temperature and performing quick on-column injections on to an all-glass-lined inlet system in the gas–liquid chromatograph, reduced the breakdown of these N-oxides to a minimum, as shown in Fig. 1.

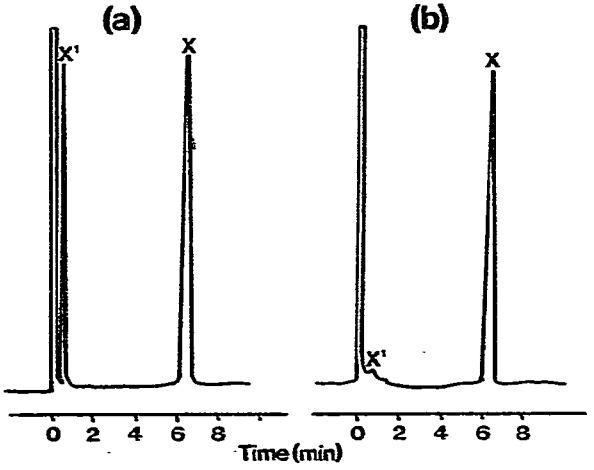

Fig. 1. Chromatograms of 3-ethylpyridine-N-oxide (X) using column A. X^1 represents the thermal breakdown product 3-ethylpyridine. (a) Oven temperature 170°, injection port temperature 300°; (b) oven temperature 170°, injection port temperature 225°.

Column B was found to produce tailing peaks, although it was useful for the direct GLC of several alkyl-, halogen-, acetyl- and cyano-substituted pyridine-N-oxides. This column was also useful for the direct GLC examination of quinoline- and isoquinoline-N-oxides[11]. The identities of the peaks were confirmed by combined GLC–MS (see below).

Column A was found to be of most use in the direct GLC analysis of alkyl- and halogen-substituted pyridine-N-oxides. This column was operated at lower oven and injection port temperatures, and the peaks did not show any significant tailing (see Fig. 2). The identities of the GLC peaks of pyridine-N-oxides were confirmed by GLC–MS, as shown in Fig. 2. For example, GLC–MS of peak 2 (pyridine-N-oxide) gave the molecular ion (*m/e* 95) as the base peak and the diagnostic $M^{\dagger}$ — 16 ion at *m/e* 79. Similarly, GLC–MS of peak 5 (3-ethylpyridine-N-oxide) gave the molecular ion (*m/e* 123) as the base peak and the $M^{\dagger}$ — 16 ion at *m/e* 107. Details of the MS and GLC–MS of pyridine-N-oxides have been described elsewhere[9,11].

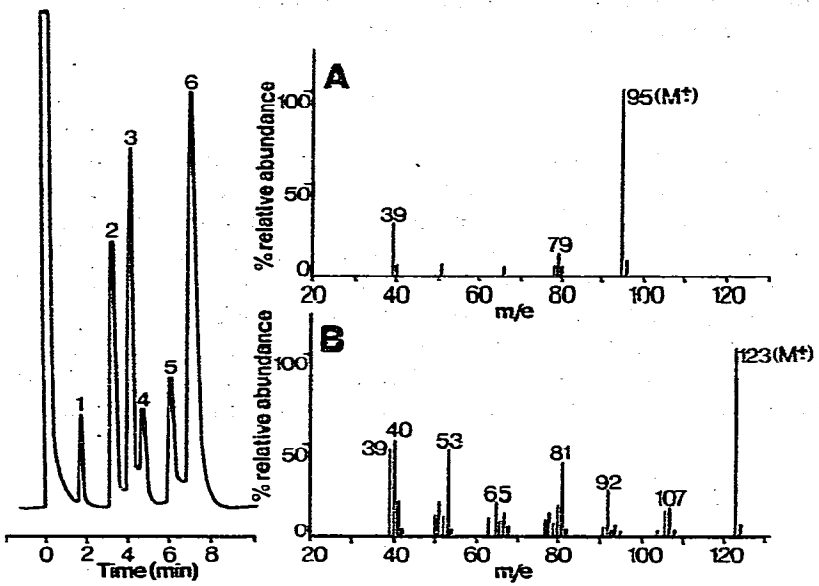

Fig. 2. GLC of 3-fluoropyridine-N-oxide (1), pyridine-N-oxide (2), 3-chloropyridine-N-oxide (3), 3-methylpyridine-N-oxide (4), 3-ethylpyridine-N-oxide (5) and 3-bromopyridine-N-oxide (6). A = GLC–MS of pyridine-N-oxide (peak 2); B = GLC–MS of 3-ethylpyridine-N-oxide (peak 5).

When pyridine-N-oxides were extracted from microsomal incubates or from urine from different animal species and analysed on column A, the normally occurring constituents of microsomes or urine did not interfere in the analysis. However, these constituents interfered with pyridine-N-oxide assay on column B.

Linear and reproducible calibration graphs were obtained in the range 10–500 nmole; regression analysis of the data gave correlation coefficients of not less than 0.999. The retention times of some 3-substituted pyridine-N-oxides are recorded in Table I; column A is also useful for the direct GLC analysis of 2- and 4-alkyl- and halogen-substituted pyridine-N-oxides.

The direct GLC of N-oxides is obviously desirable. This work shows that this

TABLE I

GLC SEPARATION OF SOME 3-SUBSTITUTED PYRIDINES AND THEIR N-OXIDES

Column A: Carbowax 20M–KOH–Chromosorb W (2:5:93); 1-m glass column, N_2 = 20 p.s.i. (140 kN/m²); oven temperature = 170°; injection temperature = 225°. Column B: 3% OV-17 on Chromosorb G (80–100 mesh); 1-m glass column; N_2 = 20 p.s.i. (140 kN/m²); oven temperature = 195°; injection temperature = 250°.

Parent compound	*Retention time (min)*			
	Base		*N-Oxide*	
	Column A	*Column B*	*Column A*	*Column B*
Pyridine	0.3	—	3.5	1
3-Methylpyridine	0.5	—	4.5	1.5
3-Ethylpyridine	0.6	—	6.0	2.0
3-Fluoropyridine	0.2	—	2.0	0.7
3-Chloropyridine	0.5	—	4.0	1.0
3-Bromopyridine	0.7	—	7.5	1.5
3-Acetylpyridine	1.0	—	—	3.5
3-Cyanopyridine	0.6	—	—	2.5

is possible if they are sufficiently thermostable and volatile, and if they can be extracted from aqueous media with a suitable organic solvent. The direct GLC of nicotine-1′-N-oxide using capillary columns has been described[12]. However, this allows only qualitative analysis as some thermal breakdown to nicotine and other products also occurs.

The work reported here has allowed the development of methods for the direct detection and quantitation of pyridine-N-oxides formed in biological systems. Our experience indicates that careful manipulation of instrumental conditions may be necessary to ensure minimal decomposition.

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