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Note

High-performance liquid chromatographic analysis of the *in vivo* metabolites of [^{14}C]pyridine

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Nitrogen heteroaromatic ring systems may undergo metabolic reactions at the heteroatom (*e.g.* N-oxidation or N-methylation) and at ring carbons (C-oxidation)[1]. The simple heterocycle, pyridine, has often been used as a model compound for studying the occurrence and enzymology of each of these metabolic pathways[2–9]. N-Oxidation of pyridine has previously been studied using gas–liquid chromatography (GLC)[10] and recently by high-performance liquid chromatography (HPLC)[9]. The N-methylation product, N-methylpyridinium hydroxide, has been isolated and identified by traditional chemical methods[2] (m.p., CHN analysis, colour reactions) and quantitatively determined by paper chromatography (PC)[4], by ultraviolet (UV) spectroscopy[4] and by radiochemical techniques (reverse isotope dilution)[8]. Data are available in the literature[11] on thin-layer chromatography (TLC) of 2-pyridone, 4-pyridone and 3-hydroxypyridine, but reports on their metabolic formation and quantitative determination are lacking.

In order to understand the relative importance of oxidative and methylation reactions in the metabolism of drugs and xenobiotics containing nitrogen heteroaromatic ring systems, we have chosen to carry out detailed *in vivo* metabolic studies with [^{14}C]pyridine. To this end we have developed an analytical procedure which allows the simultaneous determination of six potential *in vivo* metabolites of [^{14}C]pyridine by utilising a combination of cation-exchange and reversed-phase HPLC. The details of this assay procedure are described in this paper.

MATERIALS AND METHODS

[2,6-^{14}C]Pyridine (250 μCi; specific activity 27.1 mCi/mmole) was obtained from The Radiochemical Centre, Amersham, Great Britain and administered to various laboratory animal species. Urine samples were collected over a 24-h period as previously described[8]. Pyridine, phosphoric acid, glacial acetic acid, and HPLC-grade methanol and acetonitrile were purchased from Fisons, Loughborough, Great Britain. 3-Hydroxypyridine was from Fluka, Glossop, Great Britain. Potassium dihydrogen orthophosphate and disodium hydrogen orthophosphate (anhydrous)

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were obtained from BDH, Poole, Great Britain, and a batch selected which gave minimal absorption at 260 nm. Luma gel (liquid scintillation cocktail) was purchased from Lumac Systems, Basel, Switzerland.

Pyridine N-oxide[12], 2-pyridone[13], 4-pyridone[14], N-methyl-2-pyridone[15] and N-methylpyridinium iodide[16] were all synthesised by literature methods. All chromatographic standards were prepared as 10 m*M* solutions in methanol, where solubility permitted. Analyses were carried out on a modular unit constructed in our laboratory consisting of a HPLC Technology pump (Model RR/015) and a Pye Unicam LC3 variable-wavelength UV detector operating at 254 or 260 nm. Samples were introduced via a Rheodyne® loop injector. All eluent buffers used were filtered and degassed *in vacuo* before use. A Partisil-10 SCX microparticle column (Whatman), 25 cm × 4.6 mm I.D., was used for cation-exchange chromatography, whereas a Partisil-10 ODS microparticle column (Whatman), 25 × 0.50 cm I.D., was used for reversed-phase chromatography. Essential chromatographic operating parameters are to be found in the legends to the tables and figures.

Urine samples (20–100 μl) from animals dosed with [^{14}C]pyridine were injected, either directly or after protein precipitation using an equal volume of acetonitrile, on the HPLC columns. Radioactive effluent was monitored by collecting fractions of known volume on a LKB 7000 Ultrorac® fraction collector. Sufficient scintillation cocktail (Luma Gel, 4 ml) was added to each fraction to form a clear, homogenous gel or solution. A Packard Tri-Carb liquid scintillation spectrometer was used to measure the radioactivity in the samples. Radiochromatograms were constructed by plotting the radioactivity of the fractions against their retention times. To determine the recovery of radioisotope from analytical columns, an identical volume of radioisotopic urine applied to the column was added directly to a scintillation vial containing an appropriate volume of column effluent and treated as described above.

RESULTS AND DISCUSSION

Initial investigations were carried out on a Partisil-10 SCX cation-exchange

TABLE I

THE INFLUENCE OF ELUENT pH ON THE RETENTION OF POTENTIAL PYRIDINE METABOLITES BY PARTISIL-10 SCX CATION-EXCHANGE CHROMATOGRAPHY

Column, Partisil-10 SCX 25 cm × 4.6 mm I.D.; eluent, methanol–0.3 *M* ammonium acetate buffer (30:70, v/v): the ammonium acetate solution was adjusted to the pH shown before dilution with methanol; flow-rate, 1.5 ml/min at 1800 p.s.i.g.; detection, 254 nm.

Compound	*Retention time (min)**				
	pH 3.7	*pH 3.9*	*pH 4.9*	*pH 6.0*	*pH 7.0*
N-Methylpyridinium ion	10.3	12.6	14.9	17.5	21.0
Pyridine	3.6	4.5	4.4	3.0	2.3
Pyridine N-oxide	1.5	2.0	2.3	2.1	1.9
3-Hydroxypyridine	1.3	1.3	1.3	1.2	1.1
4-Pyridone	1.3	1.3	1.2	1.1	1.1
2-Pyridone	0.8	0.8	1.1	0.9	0.8

* Corrected retention time ($t_R - t_0$); t_0 = 1.8 min.

TABLE II

THE INFLUENCE OF ELUENT IONIC STRENGTH ON THE RETENTION OF POTENTIAL PYRIDINE METABOLITES BY PARTISIL-10 SCX CATION-EXCHANGE CHROMATOGRAPHY

Column, Partisil-10 SCX 25 cm × 4.6 mm I.D.; eluent, methanol–ammonium acetate, pH 3.7 (30:70, v/v); flow-rate, 1.5 ml/min at 1800 p.s.i.g.; detection, 254 nm.

Compound	*Retention time (min)**				
	0.1 M	*0.3 M*	*0.5 M*	*0.8 M*	*1.0 M*
N-Methylpyridinium ion	20.4	10.3	7.9	6.4	5.9
Pyridine	7.8	3.6	2.8	2.1	1.7
Pyridine N-oxide	2.1	1.5	1.4	1.2	1.2
3-Hydroxypyridine	1.1	1.0	0.9	0.9	0.8
4-Pyridone	1.0	0.9	0.9	0.8	0.8
2-Pyridone	0.7	0.6	0.6	0.7	0.7

* Corrected retention time ($t_R - t_0$); t_0 = 1.8 min.

column using a mixture of methanol and ammonium acetate buffer as the eluent (see Table I). Variation of hydrogen ion concentration has a marked effect on the retention times of the more basic solutes over the pH range 3.7–7.0, whereas the neutral pyridone derivatives, as expected, elute early and are relatively unaffected by change in pH. Optimum pH for separation of pyridine, pyridine N-oxide and N-methylpyridinium ion is in the range 3.7–4.9. As would be expected of an ion-exchange process, a decrease in the ionic strength of the buffer leads to longer retention times of all the three basic solutes (see Table II). The effect of varying the methanol content of the eluent (Table III) is a complex phenomenon, leading to a considerable change in the retention time of N-methylpyridinium ion accompanied by extensive peak broadening, while not affecting to any great degree the retention behaviour of pyridine, pyridine N-oxide or the neutral pyridones. These results in part, may reflect the

TABLE III

THE INFLUENCE OF METHANOL CONTENT OF THE ELUENT ON THE RETENTION OF POTENTIAL PYRIDINE METABOLITES BY PARTISIL-10 SCX CATION-EXCHANGE CHROMATOGRAPHY

Column, Partisil-10 SCX 25 cm × 4.6 mm I.D.; eluent, various proportions of methanol with 0.3 *M* ammonium acetate, pH 3.7; flow-rate: 1.5 ml/min at 1800 p.s.i.g.; detection: 254 nm.

Compound	*Retention time (min)**			
	20%	*30%*	*40%*	*60%*
N-Methylpyridinium ion	9.0	9.0	9.4	14.4
Pyridine	3.4	3.2	3.1	2.3
Pyridine N-oxide	1.6	1.6	1.6	1.6
3-Hydroxypyridine	0.8	1.0	1.1	1.2
4-Pyridone	0.9	0.9	1.0	1.1
2-Pyridone	0.5	0.6	0.7	0.8

* Corrected retention time ($t_R - t_0$); t_0 = 1.8 min.

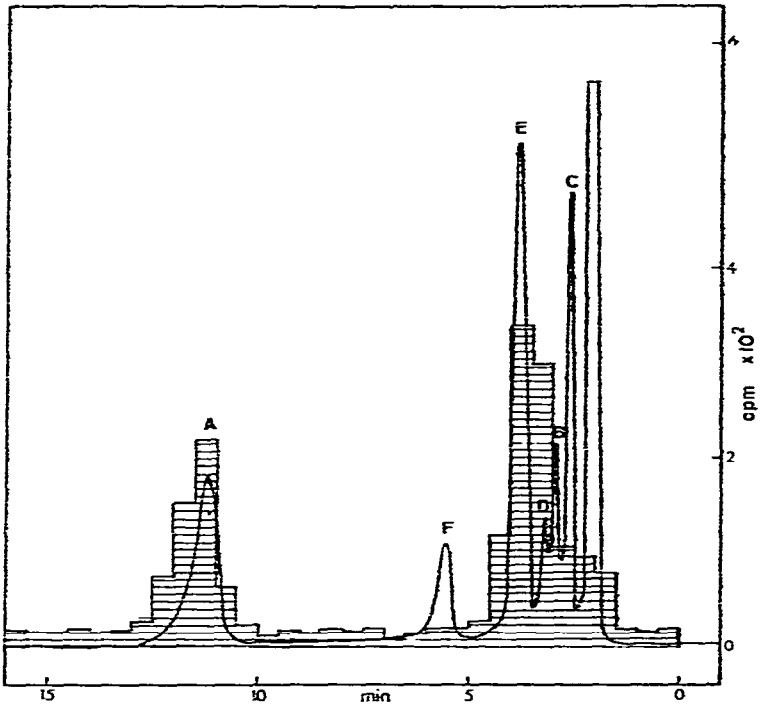

Fig. 1. Radiochromatogram of 24-h hamster urine, after intraperitoneal administration of [^{14}C]pyridine, using cation-exchange HPLC. Column, Partisil-10 SCX (25 cm × 4.6 mm I.D.); eluent, methanol–0.3 *M* ammonium acetate buffer (30:70, v/v); flow-rate, 1.5 ml/min at 1800 p.s.i.g.; detection, 254 nm; fractions collected every 30 sec for determination of ^{14}C (see text for details). Peaks: A = N-methylpyridinium ion; B = 4-pyridone; C = 2-pyridone; D = 3-hydroxypyridine; E = pyridine N-oxide; F = pyridine. R_t = Retention time.

relative solubilities of N-methylpyridinium ion, pyridine and pyridine N-oxide in water and methanol. Because of the many variables investigated, choosing optimum conditions was almost impossible. For practical purposes, a methanol–ammonium acetate buffer (0.3 *M*, pH 3.7) (30:70, v/v) was utilised for the analysis of radioactive urine samples (see Fig. 1). Under these conditions, the N-methylpyridinium ion, pyridine (protonated at pH 3.7), and pyridine N-oxide are well separated over a short elution period. However, 3-hydroxypyridine and the neutral pyridone derivatives co-elute early, almost in the void volume.

Reversed-phase chromatography was investigated as a method for analysis of the C-oxidation metabolites of [^{14}C]pyridine which co-elute early by cation-exchange chromatography. Table IV shows results obtained using a Partisil-10 ODS column and a 0.067 *M* phosphate buffer (pH 7.4) as eluent with acetonitrile concentrations varying from 0 to 10% (v/v). As expected, the very water-soluble N-methylpyridinium ion elutes in the void volume in this system, whereas pyridine, because of its lipophilic character, has a high affinity and a long retention time. Baseline separation of 3-hydroxypyridine, 2- and 4-pyridones and pyridine N-oxide was achieved using this method of analysis with no organic modifier. Incorporation of even small amounts of acetonitrile or methanol produce a marked improvement in the chroma-

TABLE IV

THE INFLUENCE OF ACETONITRILE CONTENT OF THE ELUENT ON THE RETENTION OF POTENTIAL PYRIDINE METABOLITES BY PARTISIL-10 ODS REVERSED-PHASE CHROMATOGRAPHY

Column, Partisil-10 ODS 25 × 0.5 cm I.D.; eluent, various proportions of acetonitrile with 0.067 *M* phosphate buffer, pH 7.4; flow-rate, 2 ml/min at 1700 p.s.i.g.; detection, 260 nm.

Compound	*Retention time (min)**				
	0.0%	*2.0%*	*2.5%*	*5.0%*	*10.0%*
Pyridine	36.8	31.6	26.5	16.2	11.5
Pyridine N-oxide	10.1	6.7	6.2	5.7	4.7
3-Hydroxypyridine	6.0	5.6	5.5	5.3	4.3
2-Pyridone	5.0	4.3	4.2	4.1	3.5
4-Pyridone	3.4	3.3	3.1	3.1	2.9
N-Methyl-2-pyridone	10.6	10.8	10.7	7.9	5.9
N-Methylpyridinium ion	0.0	0.0	0.0	0.0	0.0

* Corrected retention time ($t_R - t_0$); t_0 = 0.5 min.

tography of pyridine (*i.e.* retention time and peak shape), but lead to unstatisfactory separation of the C- and N-oxidation metabolites. Varying the ionic strength of the buffer appears to have little effect on the retention of any of the solutes examined (Table V), whereas varying the pH of the eluent, as expected, had a marked effect on the retention for pyridine, but little effect on that of the other solutes (Table VI). The eluent composition chosen for the reversed-phase analysis of the urinary metabolites of [^{14}C]pyridine was 0.067 *M* phosphate buffer, pH 7.4 without any organic modifier at a fast flow-rate of 4.2 ml/min at 2750 p.s.i.g. to achieve a relatively short analysis time (see Fig. 2). This analytical system, in combination with the Partisil-10 SCX chromatographic system described above, allows the analysis of six potential metabolites of [^{14}C]pyridine.

TABLE V

THE INFLUENCE OF ELUENT IONIC STRENGTH ON THE RETENTION OF POTENTAL PYRIDINE METABOLITES BY PARTISIL-10 ODS REVERSED-PHASE CHROMATOGRAPHY

Column, Partisil-10 ODS 25 × 0.5 cm I.D.; eluent, Acetonitrile–phosphate buffer, pH 7.4 (2.5:97.5 v/v); flow-rate, 2 ml/min at 1700 p.s.i.g.; detection, 260 nm.

Compound	*Retention time (min)**			
	0.034 M	*0.067 M*	*0.134 M*	*0.200 M*
Pyridine	24.4	26.5	26.0	25.7
Pyridine N-oxide	6.0	6.2	7.6	7.1
3-Hydroxypyridine	5.5	5.5	5.8	5.3
2-Pyridone	4.1	4.2	4.6	4.1
4-Pyridone	3.1	3.1	3.3	2.6
N-Methyl-2-pyridone	10.5	10.7	12.2	11.5
N-Methylpyridinium ion	0.0	0.0	0.0	0.0

* Corrected retention time ($t_R - t_0$); t_0 = 0.5 min.

TABLE VI

THE INFLUENCE OF ELUENT pH ON THE RETENTION OF POTENTIAL PYRIDINE METABOLITES BY PARTISIL-10 ODS REVERSED-PHASE CHROMATOGRAPHY

Column, Partisil-10 ODS 25 cm × 5.0 mm I.D.; eluent, acetonitrile–0.067 *M* phosphate buffer (2.5:97.5, v/v): the phosphate buffer was adjusted to the pH shown before dilution with acetonitrile; flow-rate, 2 ml/min at 1700 p.s.i.g.; detection, 260 nm.

Compound	*Retention time (min)**				
	pH 6.0	*pH 6.8*	*pH 7.0*	*pH 7.4*	*pH 8.0*
Pyridine	34.0	32.5	31.5	26.5	27.0
Pyridine N-oxide	7.5	8.2	7.9	6.2	7.7
3-Hydroxypyridine	5.7	6.3	6.2	5.5	5.9
2-Pyridone	4.7	4.9	4.8	4.2	4.7
4-Pyridone	3.2	3.4	3.4	3.1	3.3
N-Methyl-2-pyridone	12.5	13.5	12.1	10.7	12.3
N-Methylpyridinium ion	0.0	0.0	0.0	0.0	0.0

* Corrected retention time ($t_R - t_0$); t_0 = 0.5 min.

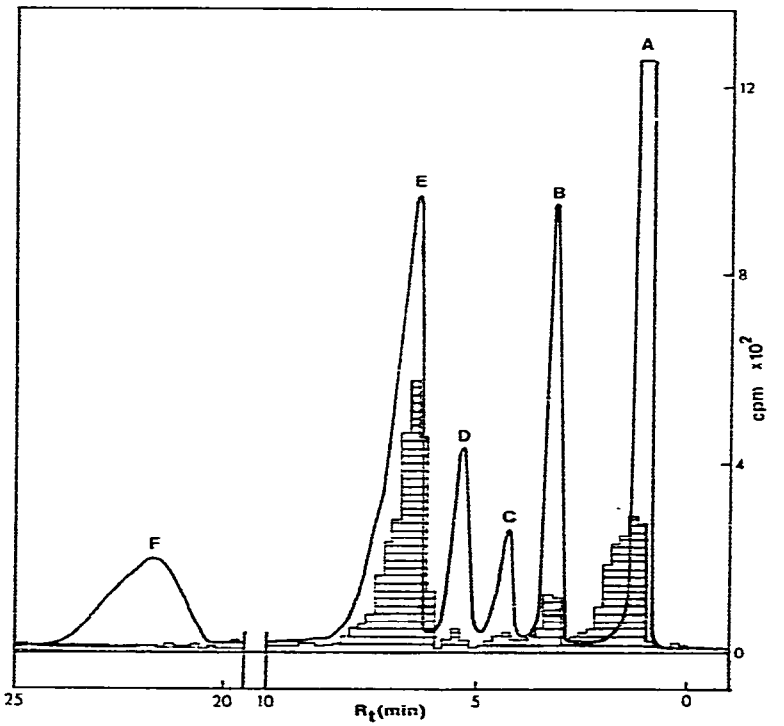

Fig. 2. Radiochromatogram of 24-h hamster urine, after intraperitoneal administration of [^{14}C]pyridine, using reversed-phase HPLC. Column, Partisil-10 ODS (25 cm × 5.0 mm I.D.); eluent, 0.067 *M* phosphate buffer; flow-rate, 4.2 ml/min at 2750 p.s.i.g.; detections, 260 nm; fractions collected every 12 sec for determination of ^{14}C (see text for details). Peaks: A = N-methylpyridinium ion; B = 4-pyridone, C = 2-pyridone; D = 3-hydroxypyridine; E = pyridine N-oxide; F = pyridine.

The very low dose of [^{14}C]pyridine utilised in this study (7 mg/kg body weight, *ca.* 10 μCi), and the consequent low activity of the urine samples collected, necessitated both the injection of the intact urine samples (20–100 μl, *ca.* 1000–2000 cpm) on to the column, and the monitoring of column effluent for ^{14}C by liquid scintillation counting for detecting and quantitating the small amount of metabolites present. The radiochromatograms obtained from analysis of a 24-h urine sample from a female Golden Syrian hamster dosed with [^{14}C]pyridine are illustrated in Fig. 1 and 2, and are typical of results obtained from analyses of urine samples from several other animal species. With each system, the amount of radioactivity applied to the column was almost totally recovered in the effluent. The cation-exchange column allows the estimation of the percentage urinary radioactivity present as N-methylpyridinium ion (*ca.* 25%) and as pyridine (*ca.* 0%). Analysis of an identical sample by reversed-phase chromatography shows that *ca.* 25% of the applied radioactivity elutes in the void volume (N-methylpyridinium ion), *ca.* 60% of ^{14}C co-elutes with the pyridine N-oxide peak and *ca.* 6% with the 4-pyridone peak, whereas 2-pyridone and 3-hydroxypyridine appear to be only minor urinary metabolites. The results from both analyses complement each other well and allow the quantitative determination of the five *in vivo* metabolites of pyridine present in hamster urine.

The above analytical procedure is being used for the analysis of urine samples obtained from several other animal species dosed with [^{14}C]pyridine, and these results will be the subject of future communications.

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