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Biological *N*-oxidation of piperidine *in vitro*[1]

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Abstract The biological *N*-oxidation of piperidine, a pharmacologically active biogenic amine of mammals and human beings, was studied *in vitro*. After incubation of piperidine-HCl in a fortified rat liver microsomal preparation (9000 × *g* supernatant) at 37℃ for 30 min, 2 metabolites were detected. They were identified as *N*-hydroxy piperidine and 2, 3, 4, 5-tetrahydropyridine-1-oxide as evidenced by TLC, GLC, HPLC, GC-MS and MS.

Key words piperidine; *N*-hydroxy piperidine; 2, 3, 4, 5-tetrahydropyridine-1-oxide; liver; chromatography; mass spectrum analysis; biotransformation

Piperidine (Pip), a pharmacologically active biogenic amine, is a normal constituent in the brain and urine of mammals and humans[1]. It shows potent nicotine-like actions on the peripheral and central nervous systems[2]. It has also been suggested that Pip affects neural mechanisms governing the regulation of emotional behaviour, sleeping and extrapyramidal functions[3]. Although Pip plays an important role in mammal pharmacology, little information on the *N*-oxidation of Pip has been reported. On the other hand, Pip is a common structural moiety existing in many synthetic and natural compounds which have biological and pharmacological activities. Metabolic studies on Pip may also provide clues to the understanding of metabolism of these piperidine-containing drugs.

In this paper, we report the microsomal *N*-oxidation of Pip in order to explore the metabolic fate of Pip as well as the relationship between its metabolic *N*-oxidation and pharmacologic activities.

Materials and methods

N-Hydroxy piperidine (*N*-OH-Pip) was recrystallized from light petroleum ether (bp 40-60℃) to yield a white crystalline solid with a mp of 39-40℃. 2, 3, 4, 5-Tetrahydropyridine-1-oxide (THPO) was synthesized by oxidation of *N*-OH-Pip with yellow mercuric oxide in chloroform[4]. The crude oil obtained was purified using a silica gel-60 column. THPO was eluted with methanol to yield a white powder, mp 112-6℃ (decomp) uv max 230 nm (water), log E 3.77.

Pip-HCl, 3-hydroxy piperidine (3-OH-Pip), 4-hydroxy piperidine (4-OH-Pip) and piperidone-2 were purchased from Aldrich Chemical Co. Solvents such as methanol, chloroform, and acetonitrile were all HPLC grade.

Animals and microsomal preparations Male albino Wistar rats were sacrificed by cervical dislocation after fasting overnight and then liver 9000 × *g* supernatant fractions were prepared by ultracentrifugation[5]. One ml of microsomal preparation was equal to 0.5 g of liver.

Standard incubation techniques Incubations were carried out in 25 ml Erlen-

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meyer flasks at 37℃ using a shaking water bath for 30 min. The standard reaction mixture was composed of: 2 ml of phosphate buffer (0.2 mol/L, pH 7.4, containing NADP 1.57 mg; G6P 3.04 mg; G6PD 1.4 μl and 50% $MgCl_2$ 8 μl); 1 ml of microsomal preparation (suspended in Tris-KCl buffer pH 7.4) and 0.5 ml of substrate solution (Pip-HCl 10 μmol/ml in water). Controls or blank incubations were carried out either by using 1 ml of isotonic Tris-KCl buffer instead of tissue preparation or 0.5 ml of distilled water without the substrate.

Extraction of metabolites After incubation at 37℃ for 30 min, the incubation mixture was transferred to centrifuge tube with screwed cap, and then 1 g of NaCl and 4 ml of chloroform were added. After shaking for 10 min and centrifuging, the chloroform layer was collected. The aqueous layer was extracted with another 4 ml of fresh chloroform. The pooled chloroform extract was evaporated at 42℃ under nitrogen. The residue was dissolved in methanol. The controls and blanks were extracted in the same manner.

Detection and identification of metabolites

1 Thin-layer chromatography TLC was carried out on precoated silica gel-60 F_{254} plates. Solvent systems: A) methanol; B) chloroform:methanol:acetic acid 1:1:0.1 (vol:vol). Spots were visualized by a) uv 254 nm; b) ammoniacal silver nitrate (Tollen's reagent); c) exposure to iodine vapour; or d) 1% chloranil in toluene.

Primary and secondary amines can also be detected by converting to dinitrophenyl (DNP) derivatives according to the procedures described in literature[6]. The DNP derivatives thus produced were extracted with cyclohexane. After evaporation, the concentrate was spread on to a silica gel plate and developed by amyl acetate: acetic acid 50:1 (vol:vol). Yellow spots of DNP were detected visually.

For identifying metabolites, the authentic compounds and the extracts of control incubation (no substrate or no enzyme) were co-chromatographed.

2 Gas-liquid chromatography GLC analysis was carried out on a PYE series 104 gas chromatograph equipped with a flame ionization detector (FID) and linked to a Perkin-Elmer 56 recorder. A 2 m × 4 mm glass column packed with 7.5% Carbowax 20 M on Chrom. W (H. P.) was used. Carrier gas: nitrogen at a flow-rate of 40 ml/min. The oven temperature was varied to produce sharp peaks with a suitable *R*t.

In order to detect the putative metabolites in one injection, a temperature program was used, i. e. starting at 65℃ for 4 min, then increasing the temperature by 12℃/min, and finally maintaining it at 220℃ for 8 min.

3 High performance liquid chromatography HPLC analysis was performed on an instrument consisting of a M-45 solvent delivery system, a Lambda-Max model 480 L. C. spectrophotometer and a J J Instruments CR 650 A recorder. The column used was a 25-cm Spherisorb S 10 ODS. Acetonitrile, 6% in water, at a flow-rate of 1 ml/min was used as the mobile phase. The elutes were monitored at a wavelength of 230 nm.

4 Mass spectrometry Direct inlet mass spectra were obtained on a VG micromass spectrometer at 70 eV or 35 eV and a source temperature of 220℃. GC-MS: A WCOT capillary column of OV-101 was connected to the above instrument. Helium (2 ml/min) was used as the carrier gas. A temperature programme was starting at 40℃. After 2 min, the temperature was increased by 15℃/min to a maximum of 210℃.

Results

The TLC and GLC characteristics of

Tab 1. TLC characteristics of piperidine (Pip) and related compounds.

Compounds	Solvent system R_F values A	B	C	Detection (colour)
Pip	0.15	0.47		c(brown), d(blue)
N-OH-pip	0.65	0.80		b(black), c(brown)
THPO	0.40	0.60		a(violet), c(brown)
3-OH-pip	0.06	0.20		c(brown)
4-OH-pip	0.06	0.20		c(brown)
Piperidone-2	0.72	0.86		c(brown)
Pip incubate S-1	0.62	0.76		b(black), c(brown)
Pip incubate S-2	0.40	0.64		a(violet), c(brown)
DNP-Pip			0.85	yellow
DNP-3-OH Pip			0.31	yellow
DNP-4-OH Pip			0.20	yellow
DNP-Pip incubate			0.85	yellow

Solvent systems; A) Methanol, B) Chloroform:methanol:acetic acid 1:1:0.1(vol:vol) C) Amyl acetate: acetic acid 50:1 (vol:vol); Detection a)uv-254,b) Tollen's reagent; c)I_2 vapour; d) 1% Chloranil in toluene

Tab 2. GLC characteristics of piperidine (Pip) and related compounds.

Compounds	Retention time (min)	Oven temp (℃)
Piperidine	6.0	65
N-OH-pip	11.0	110
3-OH-pip	5.7	160
4-OH-pip	6.7	160
Piperidone-2	7.7	210
Pip-incubate	10.8	110

Column; 2 m×4 mm glass column packed with 7.5% Carbowax 20 M on Chrom. W (H P); Carrier gas; N_2 40 ml/min; Detector; FID

Pip, its potential metabolites, and some DNP-derivatives are presented in Tab 1 and 2.

Tab 2 shows that the compounds of interest for which different temperatures were required in order to obtain symmetrical peaks and good separation. Using the temperature programme, all compounds except THPO gave sharp peaks with R_T Pip 6.2, *N*-OH-Pip 10.2, 3-OH-Pip 11.6, 4-OH-Pip 12.1, piperidone-2 17.0.

THPO was nonvolatile, it did not elute on GLC under the conditions used in experimental part. Since it was the only compound which absorbs uv light, it could easily be detected by HPLC. Under our conditions, a peak with a R_T of 8.2 min was obtained which, however, did not appear on the chromatograms from control incubations.

Identification of *N*-oxidized products from piperidine after incubation of piperidine with fortified rat liver preparations After extraction of the incubation mixture at pH 7.4 with chloroform, the concentrated extract showed the presence of two new substances following TLC and subsequent exposure to I_2 vapor. These products had R_F values close to those of authentic *N*-OH-Pip and THPO using solvent systems A and B. One of these compounds produced immediate black area when sprayed with Tollen's reagent. GLC analysis produced a peak with R_T identical to that of *N*-OH-Pip. GC-MS produced a mass spectrum with molecular ion peak at m/z 101 and the main fragments at m/z 100 (M-1), 84 (diagnostic peak of hydroxylamines), 55 and 41. It was virtually identical to that of the authentic compound (Fig 1 A).

The formation of THPO as the second

metabolite was confirmed by the following observations : (1) HPLC analysis gave a new peak with R_T 8.2 min which was absent from the extracts of control incubations. (2) The TLC of a combined extract from ten incubations (solvent system: A, detection: uv 254 nm) showed identical R_F with the authentic THPO. This was further supported by the mass spectrum of the corresponding spot taken from the plate [m/z: 99 (M^+), 83 (M-16, typical for all *N*-oxides), 69, 55 and 41] which well conformed with that presented in Fig 1B.

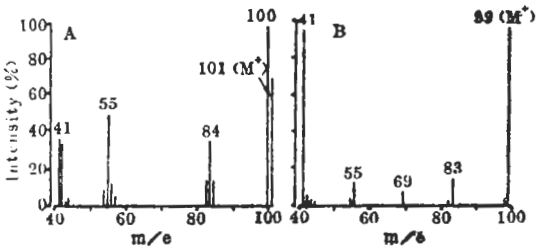

Fig 1. Mass spectra of synthetic *N*-hydroxy piperidine (A) and 2, 3, 4, 5-tetrahydropyridine-1-oxide (B).

Discussion

It has been well documented that many aromatic and aliphatic amines can be biologically *N*-oxidized to corresponding *N*-hydroxylamines and that some of them can be further oxidized to *N*-oxide[7-8]. For example, anabasine, a minor alkaloid of tabacco, gave *N'*-hydroxyanabasine and anabasine-*N'*-oxide as metabolites[9]. However, this paper is the first report concerning the *N*-oxidation of piperidine itself.

Okano *et al* [10] have studied the metabolites of piperidine in rat urine. They reported the detection and identification of 3-OH-Pip, 4-OH-Pip and their conjugates. In addtion, they found an unidentified metabolite marked M-1 which was neither *N*-acetyl, *N*-methyl piperidines nor piperidone-2. However, no attention was paid to the *N*-oxidized metabolites. In this study, no ring-hydroxylated piperidines were detected using DNP-derivatization technique. Okano also studied the pharmacological properties of 3-OH- and 4-OH-piperidines and demonstrated that piperidine of either an endogenous or exogenous origin was inactivated, at least in part, by being metabolized to its *C*-hydroxylated compounds and conjugats. Since *N*-hydroxylation can produce a change in the pharmacology of aliphatic amines [11], it would not be unexpected if these metabolites were implicated in the etiology of the side effects associated with piperidine-containing drugs.

The physical chemical properties of piperidine suggest that the *N*-oxidation found are rediated by the flavin containing mono oxygenase[12]. However, the finding of two stable *N*-oxidized metabolites from piperidine should allow the enzymology of both reactions to be delineated.

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哌啶的体外生物 *N*-氧化转化

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提要 以体外法研究哌啶的生物 *N*-氧化产物。将哌啶盐酸盐加至强化的大鼠肝匀浆 9000×*g* 上清液中，37℃温育 30 min 后，以氯仿提取，检出 2 个代谢产物。经薄层色谱、气-液色谱、高效液相色谱、薄层色谱-质谱和气相色谱-质谱鉴定，证实为：*N*-羟基哌啶和 2，3，4，5-四氢吡啶-*N*-氧化物。

关键词 哌啶；*N*-羟基哌啶；2，3，4，5-四氢吡啶-*N*-氧化物；肝；色谱法；质量光谱分析；生物转化

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