METABOLIC INTERCONVERSION OF ACID METABOLITES OF COTININE *IN VITRO*

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SUMMARY

The *in vitro* metabolism of 4-(3-pyridyl)-4-oxo-butyric acid (POBA), 4-(3-pyridyl)-4-hydroxy-butyric acid (PHBA) and 5-(3-pyridyl)-tetrahydrofuranone-2 (PTHF) by hepatic fractions from different experimental animal species (rat, rabbit, hamster, guinea-pig) was studied. The results show that POBA can be reduced to PHBA followed by cyclisation to PTHF by enzymes present in the cell soluble fraction. Conversely PHBA can be oxidised to POBA *in vitro* by microsomal enzymes. The metabolic transformations observed are predominantly catalysed by enzymes. PTHF is transformed to PHBA by a chemical hydrolytic reaction which is enhanced by both microsomal and soluble enzymes. Under our HPLC analytical conditions, neither 3-pyridylacetic acid nor 4-(3-pyridyl)-butyric acid were detected. Among the four species studied, there was a significant difference in the reduction of POBA to PHBA, with rabbit and hamster having the stronger activity. When the three compounds were incubated with hepatic tissue from rats induced separately with phenobarbitone (PB), β-naphthoflavone (βNF) or arochlor-1254 (A-1254), no difference in interconversion of the metabolites was observed when compared to non-induced rats. Therefore, it is suggested that these interconversions of the compounds are probably not mediated by cytochrome-P450 isoenzymes. A consideration of the ratios of individual processes leads us to believe that the conversion of POBA to PHBA is on the major pathway in the degradation of cotinine to 3-pyridylacetic acid.

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KEY WORDS

3-pyridylacetic acid, 4-(3-pyridyl)-4-oxo-butyric acid, 4-(3-pyridyl)-4-hydroxy-butyric acid, 4-(3-pyridyl)-butyric acid, 5-(3-pyridyl)-tetrahydrofuranone-2, hepatic *in vitro* metabolism

INTRODUCTION

4-(3-Pyridyl)-4-oxo-butyric acid (POBA) has been suggested as an intermediate in nicotine metabolism both *in vitro* and *in vivo* since the work of McKennis /1/. A route from 4-(3-pyridyl)-4-oxo-butyric acid (POBA) to 3-pyridylacetic acid has been proposed (Fig. 1). It is suggested that 4-(3-pyridyl)-4-oxo-butyric acid is metabolised by keto reduction to 4-(3-pyridyl)-4-hydroxy-butyric acid (PHBA) followed by dehydrogenation 4-(3-pyridyl)-4-butenoic acid, subsequent reduction

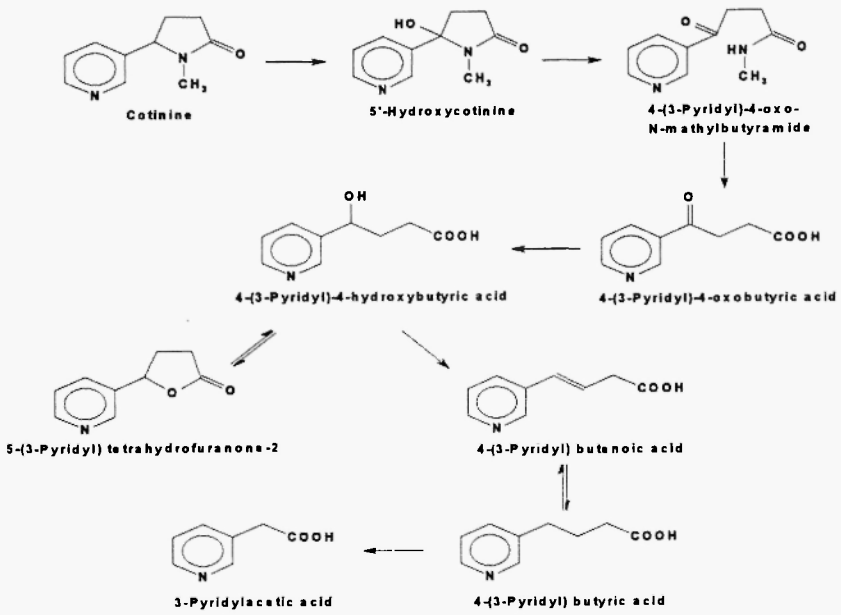

Fig. 1: Proposed metabolic pathway of cotinine to 3-pyridylacetic acid.

to 4-(3-pyridyl)-butyric acid and finally β-oxidation to give 3-pyridylacetic acid /1/. 4-(3-Pyridyl)-4-hydroxy-butyric acid (PHBA) can also form the cyclic lactone, 5-(3-pyridyl)-tetrahydrofuranone-2 (PTHF). The recent recognition of 3-pyridylcarbinol as a putative metabolite of nicotine suggests that 3-pyridylcarbinol, rather than 3-pyridylacetic acid, may be the terminal degradation product of nicotine /2/. The *in vitro* metabolism of 4-(3-pyridyl)-4-oxo-butyric acid, 4-(3-pyridyl)-4-hydroxy-butyric acid and 5-(3-pyridyl)-tetrahydrofuranone-2 has not been reported. Therefore the aim of the present study was to determine the metabolites formed *in vitro* during incubation of certain proposed intermediates of cotinine metabolism with hepatic fractions from different experimental animal species (rat, rabbit, hamster, guinea-pig).

MATERIALS AND METHODS

Preparation of some intermediates related to cotinine metabolism

4-(3-Pyridyl)-4-oxo-butyric acid (POBA), 4-(3-pyridyl)-4-hydroxy-butyric acid (PHBA), 4-(3-pyridyl)-butyric acid (PBA) and 5-(3-pyridyl)-tetrahydrofuranone-2 (PTHF) were prepared and purified using the methods of McKennis *et al.* /3,4/. All these compounds gave single discrete peaks when examined by high performance liquid chromatography (HPLC), and one area when monitored for purity by thin layer chromatography (TLC) (details not given). 3-Pyridylacetic acid (PAA) was purchased from Aldrich Chemical Company (Gillingham, Dorset).

Analytical method

The gradient HPLC system used consisted of a Waters pump (Model M45, solution delivery system), Waters UV detector (Model 480) and a reverse phase analytical column (Spherisorb ODS2, 5 μm). The gradient system parameters are shown in Table 1. The mobile phase consisted of two solvent systems, Solvent A: 0.1 M ammonium acetate and 0.5% triethylamine, adjusted with acetic acid to pH 4.8 and Solvent B: Solvent A and acetonitrile (10:1 v/v). The proposed metabolites were detected and well separated with this HPLC system (Fig. 2). Using the gradient HPLC system, the retention time of PAA is 9.28 min, PHBA 15.03 min, POBA 20.83 min, PBA 27.50 min and

TABLE 1

Gradient HPLC system

Time (min)	Flow rate (ml/min)	Solvent A (%)	Solvent B (%)	Curve #
Initial	1	95	5	6
3	1	95	5	6
10	1	70	30	6
35	1	30	70	7
45	1	95	5	11

See Waters Instrument Manual for gradient system

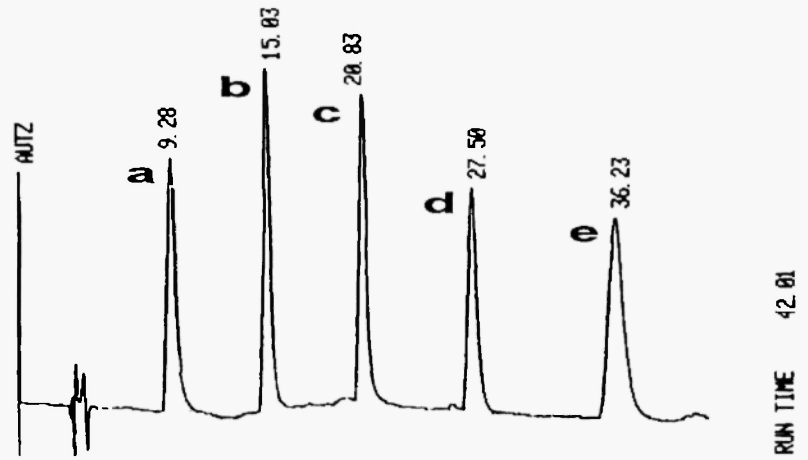

Fig. 2: Chromatographic separation of acid metabolites of cotinine using a gradient HPLC system. a) 3-Pyridylacetic acid (9.28 min); b) 4-(3-pyridyl)-4-hydroxy-butyric acid (15.03 min); c) 4-(3-pyridyl)-4-oxo-butyric acid (20.83 min); d) 4-(3-pyridyl)-butyric acid 27.5 min); e) 5-(3-pyridyl)-tetrahydrofuranone-2 (36.23 min). For details see text and Table 1.

PTHF 36.23 min. Metabolites were quantified by a peak area method which gave a linear response for each metabolite in the range 0-150 μmol/incubate.

Animal tissues and induction

Male rabbits (New Zealand, 2-3 kg), rats (Wistar, 200-300 g), hamsters (Syrian, 80-100 g) and guinea-pigs (Dunkin-Hartley, 600-

800 g) were provided by King's College Animal Facility. The animals were deprived of food for 12 h before being sacrificed by cervical dislocation. Hepatic microsomes, 10,000 *g* (microsome plus soluble fraction) and 27,000 *g* fraction (soluble supernatant) were prepared at 0-4°C using the calcium chloride precipitation method /5/.

Induction was carried out in rats which were injected daily with phenobarbitone (PB, 80 mg/kg in 0.5 ml saline), β-naphthoflavone (βNF, 80 mg/kg in 2 ml corn oil), arochlor-1254 (A-1254, 500 mg/kg in corn oil) or corn oil (as control, 2 ml) for three days. On the fourth day, the induced rats were sacrificed and the livers were removed for preparation of hepatic tissues as previously described.

Experimental incubations and extraction method

The incubation mixtures consisted of cofactor solution (1 ml) [NADPH-generating system composed of $NADP^+$ (2 μmol), glucose-6-phosphate (G-6-P, 1 unit), magnesium chloride solution (20 μmol)]; hepatic tissue (equivalent to 0.5 g original liver/ml, 0.5 ml); substrate (2.5 μmol/0.2 ml), and phosphate buffer (0.2M, pH 7.4) to a final volume of 2 ml. The cofactor solution was pre-incubated for 5 min prior to the addition of hepatic tissue and substrate. The incubations were carried out at 37°C in a shaking water-bath for 20 min. The reaction was terminated by adding acetonitrile (5 ml) and extraction with acetonitrile (5 ml) four times. The resultant organic phases were evaporated to dryness under a stream of nitrogen in a 40°C water-bath. The dried extracts were kept in a refrigerator at 4°C for later analysis. For HPLC analysis, the dried extract was reconstituted with solvent A (200 μl), and 20 μl was injected onto the HPLC column. Control experiments in which double distilled water (0.2 ml) instead of substrate was used in the incubation mixture or in which phosphate buffer (0.2 M, pH 7.4) replaced tissue preparations were also carried out.

RESULTS AND DISCUSSION

Species differences in the *in vitro* metabolism of potential intermediates of cotinine metabolism

4-(3-Pyridyl)-4-oxo-butyric acid (POBA): When POBA was incubated with various hepatic preparations from rats, hamsters, guinea-pigs and

rabbits, differences in the formation of PHBA and PTHF were observed (Table 2). Using microsomes, 10,000 *g* and 27,000 *g* supernatant from rats, neither PHBA nor PTHF was detected as a metabolite. In the case of guinea-pig, rabbit and hamster hepatic preparations, PHBA was observed with 10,000 *g* and 27,000 *g* supernatants but not with microsomes. Only traces of PTHF were detected in these experiments and only when PHBA was formed; PHBA was always the major product. The results are summarised in Table 2. In the case of rabbit 10,000 *g* and 27,000 *g* experiments, PTHF was not detected even though PHBA was present. Therefore, the formation of PTHF is probably mediated by enzymatic action. The generation of PHBA appears to be associated with the reduction of POBA by enzymes present in the soluble fraction.

4-(3-Pyridyl)-4-hydroxy-butyric acid (PHBA): When 4-(3-pyridyl)-4-hydroxy-butyric acid was incubated with hepatic preparations from rats, guinea-pigs, hamsters and rabbits, PTHF was always observed as a metabolite with all cell fractions, the amount of PTHF formed by various fractions depending upon the species. Thus in rats the activity is greatest in microsomes, whereas in hamsters this activity seems to be associated with the soluble fraction. This lactone was not present in control incubates devoid of tissue, therefore we assume that a non-specific enzyme, in regard to localisation, must be involved.

Using guinea-pig hepatic preparations, POBA was formed with 10,000 *g* supernatant, but not microsomes or 27,000 *g* soluble fraction. in the case of hamsters, rats and rabbits, formation of POBA was observed after incubation of PHBA with 10,000 *g* supernatant and microsomes. The results shown in Table 2 indicate that POBA is probably formed from PHBA by a microsomal oxidase.

5-(3-Pyridyl)-tetrahydrofuranone-2 (PTHF): Following the incubation of PTHF with tissue fractions prepared from all four species, PHBA and POBA were detected as metabolites. However, PHBA was also found in control incubates with buffer devoid of tissue; this was not the case with the formation of POBA. The results, shown in Table 2, indicate that the *in vitro* metabolism of PTHF to POBA can be mediated by enzymes present in the microsomes but not in the soluble fraction, and probably proceeds via PHBA as intermediate. The conversion of PTHF to PHBA is predominantly a chemical hydrolytic reaction which was enhanced in the presence of tissue preparation.

TABLE 2

The interconversion of cotinine metabolites with hepatic cell fractions from various species

Species	Substrate	4-(3-Pyridyl)-4-oxo-butyric acid (pmol/incub.)		4-(3-Pyridyl)-4-hydroxy-butyric acid (pmol/incub.)		5-(3-Pyridyl)-tetrahydro-furanone-2 (pmol/incub.)	
	Incubation with	PTHF	PHBA	PTHF	POBA	PHBA	POBA
Rat	10,000g	ND	ND	9.0 ± 1.1	21.1 ± 5.1	9.2 ± 1.2	21.0 ± 5.1
	microsomes	ND	ND	19.0 ± 4.0	5.0 ± 1.4	19.1 ± 4.3	5.2 ± 1.3
	27,000g	ND	ND	15.0 ± 3.1	ND	15.3 ± 3.1	ND
Guinea pig	10,000g	1.0 ± 0.1	8.0 ± 1.0	9.0 ± 1.2	8.1 ± 3.1	9.1 ± 1.1	8.1 ± 3.2
	microsomes	ND	ND	5.0 ± 1.1	ND	5.0 ± 1.0	ND
	27,000g	4.0 ± 0.8	14.0 ± 1.0	1.0 ± 0.2	ND	1.1 ± 0.2	ND
Rabbit	10,000g	ND	21.0 ± 3.0	21.0 ± 9.0	5.0 ± 1.2	21.3 ± 9.2	5.2 ± 0.8
	microsomes	ND	ND	14.0 ± 3.1	54.3 ± 25.3	14.1 ± 3.1	54.4 ± 25.1
	27,000g	ND	16.0 ± 5.2	16.1 ± 5.2	ND	16.3 ± 5.3	ND
Hamster	10,000g	1.0 ± 0.1	77.1 ± 12.2	41.0 ± 1.1	3.2 ± 0.9	41.2 ± 1.3	3.0 ± 1.0
	microsomes	ND	ND	1.1 ± 0.2	1.0 ± 0.4	1.3 ± 0.4	1.2 ± 0.4
	27,000g	1.0 ± 0.9	130.0 ± 33.3	35.3 ± 8.4	ND	35.2 ± 8.1	ND

Results are expressed as mean ±SD (n=3).
ND= not detected. Incubation conditions are described in the text.

PHBA was generally the major product formed during these incubations.

Effect of different enzyme inducers on *in vitro* metabolism

When 4-(3-pyridyl)-4-oxo-butyric acid was incubated with hepatic microsomes, 10,000 *g* supernatant or 27,000 *g* soluble fraction prepared from rats induced by PB, βNF or A-1254, no metabolites were observed as was the situation in the previous experiment with non-induced rat tissue. In the case of PHBA, POBA (Fig. 3A) and PTHF (Fig. 3B) were formed by all the preparations from induced rats. Surprisingly, the only significant enhancement of activity with this substrate occurred in the formation of PTHF by 27,000 *g* preparations from rats pre-treated with either phenobarbitone or arochlor. As the 27,000 *g* fraction represents the cytosolic fraction of the cell, which is not usually affected by inducing agents, this observation will need to be verified in further experiments.

In addition POBA (Fig. 3C) and PHBA (Fig. 3D) were detected as metabolites of PTHF, using all the preparations from rats treated with the various inducing compounds. In these experiments only arochlor pre-treatment had an enhancing effect on the formation of PHBA and POBA; no effect was observed with the 27,000 *g* cytosol fraction. This could be expected if the enzymes associated with these reactions were located in the microsomes as proposed earlier, rather than the cytosol. No qualitative differences were observed between hepatic preparations from induced and non-induced rats.

The preliminary results summarised in Fig. 4 indicate that POBA can be reduced to PHBA and thence to PTHF by cyclisation; PHBA can be reconverted to POBA by oxidation; and PTHF is probably transformed to PHBA by lactone hydrolysis *in vitro*. As the hydroxy acid was the major metabolite from all these substrates, it is probable that this compound is on the direct pathway between cotinine and pyridylacetic acid. 3-Pyridylacetic acid and 4-(3-pyridyl)-butyric acid were not detected as metabolites from these proposed intermediates by any hepatic fractions from any species. The metabolic transformations among the investigated compounds are catalysed by enzymes, except the hydrolysis of PTHF which seems to be predominantly a chemical non-enzymatic process. The further metabolism of POBA and PHBA to 4-(3-pyridyl)-butyric acid, 3-pyridylacetic acid and any intermediates is currently under investigation.

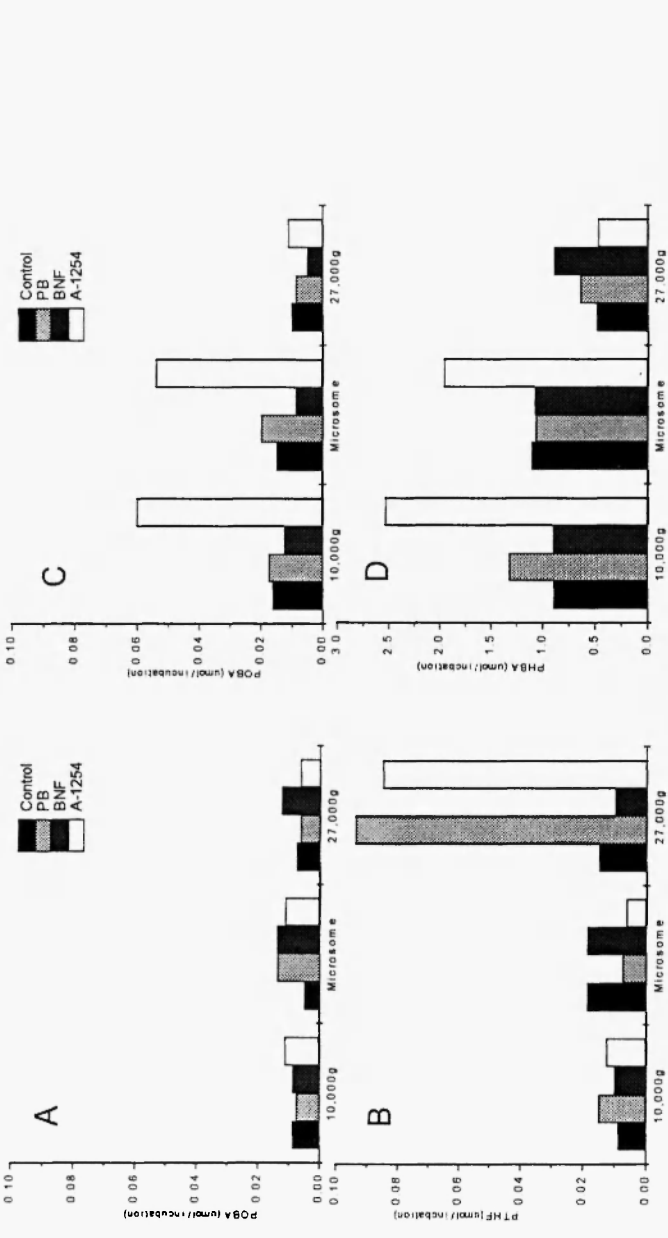

Fig. 3: *In vitro* metabolism of 4-(3-pyridyl)-4-hydroxy-butyric acid (PHBA) to 4-(3-pyridyl)-4-oxo-butyric acid (POBA) (A) and 5-(3-pyridyl)-tetrahydrofuranone-2- (PTHF) (B) by hepatic preparations from induced and non-induced rats; *in vitro* metabolism of 5-(3-pyridyl)-tetrahydrofuranone-2 to 4-(3-pyridyl)-4-oxo-butyric acid (POBA) (C) and 4-(3-pyridyl)-4-hydroxy-butyric acid (PHBA) (D) by hepatic preparations from induced and non-induced rats. Control: non-induced; PB: induced with phenobarbitone; BNF: induced with β-naphthoflavone; A-1254: induced with arochlor 1254.

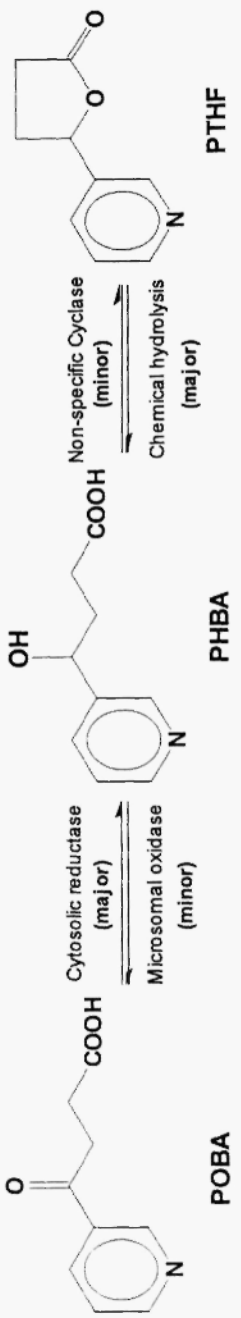

Fig. 4: The metabolic transformation of the cotinine derived acidic metabolites.

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