IN VITRO METABOLISM OF (S)-(-)-[2'-^{14}C]NICOTINE, USING VARIOUS TISSUE PREPARATIONS OF MARMOSET

Mui-Chiung Tsai and John W. Gorrod*

Toxicology Unit, John Tabor Laboratories, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, Essex, UK

SUMMARY

The nicotine metabolite profile produced by marmoset liver, lung and kidney preparations was investigated after 30 minutes incubation of (S)-(-)-[2'-^{14}C]nicotine. Cation-exchange high performance liquid radiochromatography was employed to separate and quantify nicotine and its metabolites. Cotinine-*N*-oxide (CNO, 0.7%), 3′-hydroxy-cotinine (3′-OH-C, 0.2%), norcotinine (NORC, 0.9%) and nornicotine (NORN, 0.4%) were formed in the incubates of marmoset lung homogenates; when marmoset kidney homogenates were used, CNO, 0.4%; 3′-OH-C, 0.2%; NORC, 0.7%; NORN, 0.7%; and cotinine (COT, 0.4%) were detected in the incubates. These nicotine metabolites constituted only approximately 2.2% and 2.4% of the original nicotine substrate used by lung and kidney homogenates respectively. When marmoset hepatic homogenates and microsomes were used, both COT and NORN were detected as the major nicotine metabolites. In addition, traces of CNO and 3′-OH-C were also detected in both incubates. The amounts of COT (6.4%) and NORN (1.8%) in the hepatic homogenates were approximately twice that of those formed by hepatic microsomes (3.8% and 0.9%, respectively). Nicotine-1'-*N*-oxide (NNO, 1.1%) was only detected in the latter preparation. Under the experimental conditions, these nicotine metabolites constituted only 8.2% and 5.8% of the substrate nicotine used in the respective incubates. The present results showed that both primary *C*-oxidation pathways, i.e. cotinine formation and *N*-

* Author for correspondence
e-mail: jgorr@essex.ac.uk

demethylation of nicotine, occurred in the lung, kidney and liver of marmoset *in vitro*. However, *N*-oxidation of nicotine was only observed when a marmoset hepatic microsomal preparation was used.

KEY WORDS

nicotine, *in vitro*, marmoset, microsomes

INTRODUCTION

Nicotine (NIC, Fig. 1), a simple dibasic alkaloid, is a major constituent of tobacco and tobacco smoke. The metabolism of nicotine has been previously reviewed /1-3/. Nicotine is extensively metabolised by mammals to give a large number of metabolites; most of their structures and the metabolic pathways involved have been elucidated by McKennis and coworkers /1/. More recently, 3′-hydroxycotinine glucuronide and demethylcotinine $\Delta^{2,3'}$-enamine, which is probably derived from 5-hydroxynorcotinine (Li, Castagnoli & Gorrod, unpublished observations), both with long elimination half-lives, were identified in smokers' urine /4/. Previous studies using hepatocytes from various species, i.e. rat, mouse, guinea-pig and hamster, failed to obtain a species that closely resembled humans with respect to high yields of these latter two metabolites /5/. Furthermore, primates such as macaques, *Macaca arctoides*, have been reported to show a close similarity to humans in nicotine metabolism /6/ and have been used as an *in vivo* model /7/.

In the present study, the *in vitro* metabolism of nicotine using marmoset hepatic homogenates and microsomes, as well as marmoset homogenates from kidney and lung were investigated.

MATERIALS AND METHODS

Chemicals

(S)-(-)-Nicotine, (S)-(-)-cotinine, glucose-6-phosphate disodium salt (G6P), glucose-6-phosphate dehydrogenase (G6PD), and nicotinamide adenine dinucleotide monosodium salt (NADP) were purchased from Sigma Chem. Co., Dorset, U.K. *Trans*-3′-hydroxycotinine and

cotinine-*N*-oxide were prepared using reported methods /8,9/. Nicotine-1′-*N*-oxide was prepared by the method of Craig and Purushothaman /10/. (S)-(-)-Nornicotine was prepared using the method of Jacob /11/. (S)-(-)-Norcotinine was prepared according to the modified method of Glenn and Edwards /12/. Quickszint flow 302 was purchased from Zinsser Analytic, UK.

S-(-)-[Pyrrolidine-2'-^{14}C]nicotine-di-(+)-tartrate salt, specific activity 58.8 mCi/mmol and radiochemical purity ≥ 98%, was purchased from Chemsyn Science Laboratories, Kansas, USA.

HPLC instrumentation

The isocratic HPLC system used consisted of a Beckman pump (model 110A) coupled to a gradient controller (Beckman 420), a Rheodyne injector (7120) fitted with a 20 µl sample loop and a UV detector (Philips Pye Unicam, PU4020). The UV detector, which monitored at 260 nm and with a flow rate of 1 ml/min, was further connected to a Flow One Canberra Packard radiochemical detector (model A280), fitted with a 0.5 ml capacity radioactive flow cell. The mobile phase was mixed with Quickszint flow 302 cocktail (1:2 v/v) to produce a flow rate of 3 ml/min which was used for radioactivity detection. HPLC analysis was performed using a strong cation-exchange HPLC analytical column (Nucleosil SA 10 µm, 250 x 4.6 mm). The mobile phase consisted of a mixture of sodium acetate buffer (0.2 M) and methanol (70:30 v/v) and triethylamine (0.02%). The final pH of the mobile phase was adjusted to pH 4.5 using glacial acetic acid. The efficiency for the HPLC-radiodetector was determined to be 85%, and this value was taken into account in all quantitative work.

Animals and tissue preparations

Marmosets were obtained from The Parkinson's Disease Research Centre, King's College London, UK. Lungs, livers and kidneys were removed from two male marmosets, washed with isotonic potassium chloride solution, connective tissue removed, cut into small cubes, to prepare 10,000 *g* homogenates using the reported ultra-centrifugation method /13/. The concentration of homogenates was adjusted to approximately 0.5 g original tissue/ml in Tris buffer (0.25 M, pH 7.4). A portion of the liver homogenate was then used to prepare microsomes using the previously reported method /13/. The concentration of

marmoset microsomal preparations was adjusted to approximately 0.5 g original liver/ml with Tris-buffer (0.25 M, pH 7.4). Prior to incubation, the concentrations of cytochrome P-450 and protein in each tissue preparation were determined using standard methods /14,15/.

Incubation procedures

Each stoppered Erlenmeyer flask containing the standard cofactor solution (NADP, 1.57 mg; G6P, 3.04 mg; G6PD, 1 unit, 1.4 µl; $MgCl_2$, 20 µmol, 6 µl and 0.2 M phosphate buffer, pH 7.4 to a final volume of 2 ml) was pre-incubated for 5 min before addition of radiolabelled nicotine (5 µCi, 88 µl) and tissue preparations. Different marmoset tissue preparations, i.e. lung, kidney, and hepatic homogenates (0.5 g original tissue/ml, 1 ml each) and hepatic microsomes (0.5 g original liver/ml, 1 ml), were used (in duplicate). The complete reaction mixtures were incubated in a shaking water bath, at 37°C, for 30 min. Metabolic reactions were terminated by placing the flasks on ice. Subsequently, zinc sulphate solution (10% w/v, 0.5 ml) was added to the incubates which were centrifuged at 4000 rpm to precipitate protein. Aliquots (20 µl) of the supernatants were injected onto the chromatograph for direct analysis.

RESULTS AND DISCUSSION

The levels of cytochrome P-450 and protein in lung homogenates, kidney homogenates, liver homogenates and liver microsomes are shown in Table 1. It can be seen that the level of total cytochrome P-450 is highest in the hepatic microsomes, and lowest in the lung homogenates. Therefore, it is not surprising that the metabolism of nicotine is higher in the marmoset hepatic preparations than in the lung or kidney preparations. Moreover, this level of cytochrome P-450 in marmoset hepatic microsomes (2.9 nmol/g) was almost the same as that of the rat (unpublished data).

The structures of nicotine metabolites that were detected in the marmoset liver, lung and kidney incubates are shown in Figure 1.

The overall results of the *in vitro* metabolic studies of (S)-(-)-[2'-^{14}C]nicotine using various marmoset tissue preparations are shown in Figure 2. The HPLC analysis of incubation mixtures with marmoset

TABLE 1

The levels of cytochrome P-450 and protein content in varous marmoset tissue preparations

Tissue Preparation	Cytochrome P-450 (nmol/g)	Protein content (mg/g)
Lung (H)	0.5	13.4
Kidney (H)	0.8	19.8
Hepatic (H)	2.1	16.5
Hepatic (M)	2.9	6.5

(H) = 10,000 *g* homogenates, (M) = microsomes.

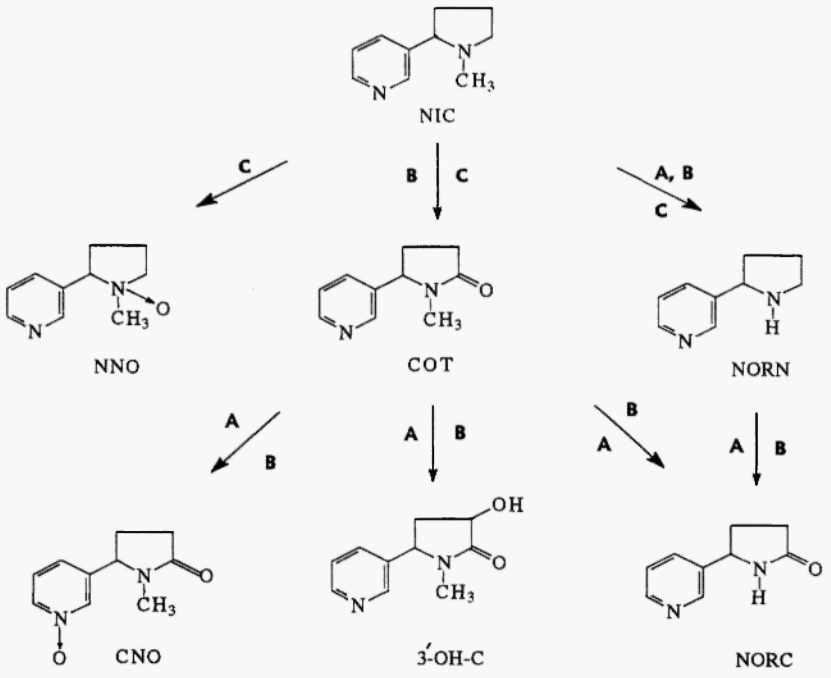

Fig. 1: The structures of nicotine metabolites detected in the marmoset lung (A), kidney (B) and hepatic (C) incubates.

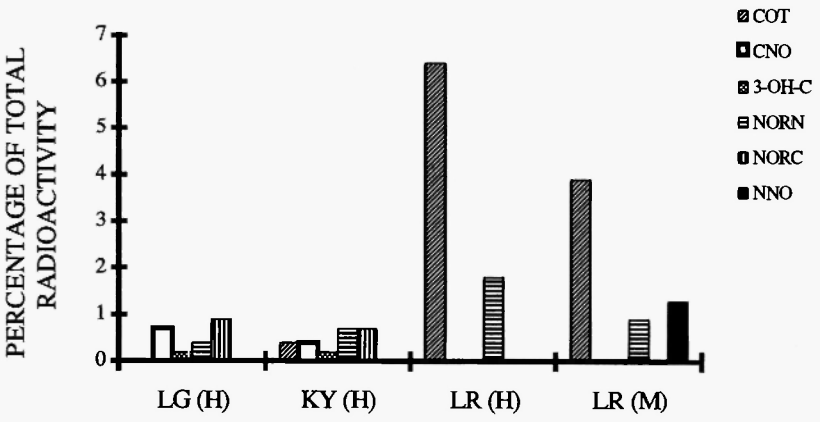

Fig. 2: The overall results of the *in vitro* metabolism of [2'-^{14}C]nicotine, showing the nicotine metabolites formed, using various marmoset tissue preparations. CNO = cotinine-*N*-oxide, 3′-OH-C = 3′-hydroxycotinine, NORC = norcotinine, COT = cotinine, NORN = nornicotine, NNO = nicotine-1'-*N*-oxide, LG = lung, KY = kidney, LR = liver, (H) = 10,000 *g* homogenates, (M) = microsomes.

lung and kidney homogenates demonstrated that cotinine-*N*-oxide (CNO), 3′-hydroxycotinine (3′-OH-C), nornicotine (NORN) and norcotinine (NORC) were formed from nicotine (NIC) *in vitro*. These four nicotine metabolites constituted only a total of approximately 2.2% and 2.4% of the nicotine used as substrate, when marmoset lung and kidney homogenates were used, respectively. The average percentage of nicotine unchanged in the lung and kidney incubates was 94.7% and 94.5%, respectively.

Cotinine (COT) was only detected in the incubates containing kidney homogenates but not those containing lung homogenates. However, since CNO, 3′-OH-C and NORC (primary metabolites of COT) were detected in both lung and kidney incubates, this suggests that COT was formed and then rapidly further completely metabolised to CNO, 3′-OH-C and NORC by enzymes present in lung. Furthermore, Gorrod /2/ postulated that *N*-demethylation of cotinine could proceed via the corresponding hydroxymethyl intermediate to produce norcotinine. This *N*-hydroxymethylnorcotinine has been detected as a new primary metabolites of cotinine *in vitro*, using

hamster hepatic microsomes /16/. However, in the present study this intermediate was not detected in the incubates of either lung or kidney, whereas norcotinine was formed. This may suggest that the norcotinine was derived from nornicotine /17/.

The formation of CNO from cotinine has been reported to be mediated by cytochrome P-450 /18/. Although CNO was detected in the incubates of kidney and lung homogenates, its level was higher in the lung incubates. Since cotinine was still present in the kidney incubates whereas it was absent in the lung, this indicates that the cytochrome P-450 activity for CNO formation must be higher in the lung than in the kidney homogenates. 3′-Hydroxycotinine was formed to the same extent in both marmoset lung and kidney incubates, thus, the catalytic activity for secondary *C*-oxidation of cotinine by the cytochrome P-450 isozymes in the two tissues was essentially identical under the present conditions.

Although NORN and NORC were both formed in lung and kidney incubates, a larger amount (approximately twice) of NORN was detected in the kidney incubates than in the lung incubates. However, in the case of NORC the situation was reversed, but to a lesser extent. Since NORC is a metabolite of NORN /17/, NORN may have been further metabolised to NORC in these homogenates.

When marmoset hepatic homogenates or microsomes were used, both COT and NORN were detected, whereas nicotine-1′-*N*-oxide (NNO) was also formed in the hepatic microsomal incubates. These three nicotine metabolites formed (COT, NORN and NNO) constituted approximately 5.8% of the original nicotine in microsomal incubates, whereas the nicotine metabolites (COT and NORN) formed when homogenates were used constituted 8.2% of total nicotine utilised (Fig. 2). Traces of CNO were also detected in incubates of hepatic homogenates and microsomes. However, traces of 3′-OH-C were only detected in the microsomal incubates.

This observation indicated that nicotine was metabolised to COT and NORN less extensively in the marmoset microsomal incubates than in the incubates using 10,000 *g* homogenates. The amount of COT formed was greater than that of NORN in both incubates. The ratio of COT to NORN in the hepatic homogenates and microsomes was 3.6 and 4.2, respectively. That the formation of COT was greater in the homogenates than in the microsomes can be explained by the presence of cytosolic aldehyde oxidase in the homogenates, as this enzyme is

involved in the oxidation of intermediate nicotine-$\Delta^{1',5}$iminium ion to cotinine /19/. COT and NORN were formed in much greater amounts in incubates of hepatic homogenates or microsomes, compared to those of lung or kidney.

NORN was detected in marmoset homogenates from lung, kidney and liver, although this metabolite was not detected in nicotine incubates when rabbit liver homogenates (9,000 *g*), phenobarbitone-induced purified rabbit cytochrome P-450 isozymes and microsomes or postmitochondrial fractions from hamsters were used /20,21/. Recent studies using hepatocytes from various species claimed that the intact cell may be required to demonstrate nornicotine formation *in vitro* /5/. However, our present study shows that NORN was formed in the fractionated cells from the liver, kidney and lung of marmoset.

Interestingly, NNO was only detected in microsomal incubates and not in the incubates with 10,000 *g* homogenates. The formation of NNO from nicotine is mediated by the FMO system /22/. The failure to detect NNO with 10,000 *g* homogenates could be due to the inaccessibility of the active sites of FMO to nicotine in the preparation, whereas in microsomes the active sites of FMO may be more exposed, due to the further purification of the liver subcellular fraction. Another possibility for this observation may be due to the back-reduction of formed NNO to nicotine by reductases present in the homogenates but absent from microsomal preparations.

ACKNOWLEDGEMENTS

This work was supported by grants from the Fabriques du Tabac Reunies SA (FTR), Neuchatel, Switzerland.

REFERENCES

1. Gorrod JW, Jenner P. The metabolism of tobacco alkaloids. In: Hayes WJ Jr, ed. Essays in Toxicology. Orlando,FL: Academic Press, 1975; 6: 35-78.
2. Gorrod JW. The mammalian metabolism of nicotine: an overview. In: Gorrod JW, Wahren J, eds. Nicotine and Related Alkaloids. London: Chapman & Hall, 1993; 31-59.
3. Kyerematen GA, Vesell ES. Metabolism of nicotine. Drug Metab Rev 1991; 23: 3-41.
4. Kyerematen GA, Morgan M, Chattopadhyay B, deBethizy JD, Vesell ES. Disposition of nicotine and eight metabolites in smokers and non-smokers:

identification in smokers of two metabolites that are longer lived than cotinine. Clin Pharmacol Ther 1990; 48: 641-651.
5. Kyerematen GA, Morgan M, Warner G, Martin LF, Vesell ES. Metabolism of nicotine by hepatocytes. Biochem Pharmacol 1990; 40: 1747-1756.
6. Benowitz NL, Jacob P. Daily intake of nicotine during cigarette smoking. Clin Pharmacol Ther 1984; 35: 499-504.
7. Seaton M, Kyerematen GA, Morgan M, Jeszenka EV, Vessell ES. Nicotine metabolism in stumptailed macaques, *Macaca arctoides*. Drug Metab Dispos 1991; 19: 946-954.
8. Dagne E, Castagnoli N. Structure of hydroxycotinine, a nicotine metabolite. J Med Chem 1972; 15: 356-360.
9. Dagne E, Castagnoli N. Cotinine-N-oxide, a new metabolite of nicotine. J Med Chem 1972; 15: 840-841.
10. Craig JC, Purushothaman KK. An improved preparation of tertiary amine N-oxides. J Org Chem 1970; 35: 1721-1722.
11. Jacob P. Resolution of (±)-5-bromonornicotine. Synthesis of (R)- and (S)-nornicotine of high enantiometric purity. J Org Chem 1982; 47: 4165-4167.
12. Glenn DF, Edwards WB. Synthesis and mass spectrometry of some structurally related nicotinamides. J Org Chem 1978; 43: 2860-2871.
13. Gorrod JW, Temple DJ, Beckett AH. The metabolism of N-ethyl-N-methyl-aniline by rabbit liver microsomes: The measurement of metabolites by gas-liquid chromatography. Xenobiotica 1975; 5: 453-463.
14. Omura T, Sato R. The carbon-monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem 1964; 239: 2370-2378.
15. Lowry OH, Rosebrough HJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.
16. Li Y, Gorrod JW. N-Hydroxymethylnorcotinine, a new primary in vitro metabolite of cotinine. Xenobiotica 1994; 24: 409-415.
17. Aislaitner G, Li Y, Gorrod JW. In vitro metabolic studies on (-)-(S)-nornicotine. Med Sci Res 1992; 20: 897-899.
18. Hibberd AR, Gorrod JW. Comparative N-oxidation of nicotine and cotinine by hepatic microsomes. In: Gorrod JW, Damani LA, eds. Biological Oxidation of Nitrogen. Chichester: Ellis Horwood, 1985; 246-250.
19. Gorrod JW, Hibberd AR. The metabolism of nicotine-$\Delta^{1',5'}$iminium ion in vivo and in vitro. Eur J Drug Metab Pharmacokinet 1982; 7: 293-298.
20. McCoy GD, Howard PC, DeMarco GJ. Characterization of hamster liver nicotine metabolism. Biochem Pharmacol 1986; 35: 2767-2773.
21. McCoy GD, DeMarco GJ, Koop DR. Microsomal nicotine metabolism: A comparison of relative activities of six purified rabbit cytochrome P450 isozymes. Biochem Pharmacol 1989; 38: 1185-1188.
22. Damani LA, Pool WF, Crooks PA, Kaderlik RK, Ziegler DM. Stereoselectivity in the N'-oxidation of nicotine isomers by flavin-containing monooxygenase. Mol Pharmacol 1988; 33: 702-705.