Xenobiotica
the fate of foreign compounds in biological systems

ISSN: 0049-8254 (Print) 1366-5928 (Online) Journal homepage: http://www.tandfonline.com/loi/ixen20

N-Hydroxymethylnorcotinine, a new primary in vitro metabolite of cotinine

Y. Li & J. W. Gorrod

To cite this article: Y. Li & J. W. Gorrod (1994) N-Hydroxymethylnorcotinine, a new primary in vitro metabolite of cotinine, Xenobiotica, 24:5, 409-415, DOI: 10.3109/00498259409043244

To link to this article: http://dx.doi.org/10.3109/00498259409043244

XENOBIOTICA, 1994, VOL. 24, NO. 5, 409–415

N-Hydroxymethylnorcotinine, a new primary *in vitro* metabolite of cotinine

Y. LI and J. W. GORROD

Chelsea Department of Pharmacy, King's College London, Manresa Road, London SW3 6LX, UK

Received 14 January 1994

1. *N*-Hydroxymethylnorcotinine; 5-(3′-pyridyl)-1-hydroxymethyl-pyrrolidone-2, was found as a new primary metabolite of cotinine *in vitro*.

2. *N*-Hydroxymethylnorcotinine was synthesized and characterized by gas chromatography-mass spectrometry, mass spectra, nuclear magnetic resonance and ultraviolet spectroscopy.

3. This new metabolite is formed by incubation of cotinine with hamster hepatic microsomes in the presence of NADPH and oxygen.

Introduction

Cotinine, like nicotine, can undergo both carbon and nitrogen oxidation. 3′-Hydroxycotinine (Dagne and Castagnoli 1972a), 5′-hydroxycotinine (Dagne *et al.* 1974), 4-(3-pyridyl)-4-oxo-*N*-methylbutyramide (McKennis *et al.* 1962), and cotinine-1-*N*-oxide (Dagne and Castagnoli 1972b), have been reported as primary metabolites of cotinine. Demethylcotinine occurs as a urinary metabolite of cotinine in dog (McKennis *et al.* 1959) (figure 1). Gorrod (1993) postulated that *N*-demethylation of cotinine proceeds via the corresponding hydroxymethyl compound, although there was no published evidence to support the existence of this intermediate. During investigations on the *in vitro* metabolism of cotinine with fortified hamster hepatic microsomal preparations, a new metabolite was detected in addition to cotinine-1-*N*-oxide, 4-(3-pyridyl)-4-oxo-*N*-methylbutyramide, and 3′-hydroxycotinine. The new metabolite has been isolated and on the basis of spectroscopic evidence and comparison with authentic material obtained by chemical synthesis, has been shown to be *N*-hydroxymethylnorcotinine.

Materials and methods

Materials

N-Hydroxymethylnorcotinine was synthesized as follows: to a stirred suspension of sodium hydride (500 mg, 60% dispersion in mineral oil, previously washed with dry ether 3 × 5 ml) in tetrahydrofuran (50 ml), a solution of demethylcotinine in tetrahydrofuran (50 ml) was introduced under nitrogen at 0–5°C over 30 min. The stirred reaction mixture was kept at 0–5°C for a further 30 min during which paraformaldehyde (1 g) was introduced dropwise in small portions. This reaction mixture was continuously stirred for 5 h, during which time it was allowed to rise to room temperature. Iced water (20 ml) was added and the aqueous solution was saturated with sodium chloride and extracted exhaustively with acetonitrile. The organic phase was dried overnight using sodium sulphate, the mixture filtered and the solution concentrated by rotary film evaporation (RFE) at 30°C. The desired compound was obtained by thin layer chromatography (tlc) using silica gel plates, thickness 1·0 mm and chloroform : methanol : concentrated ammonium hydroxide (8 : 1 : 1, by vol.) as mobile phase. *N*-Hydroxymethylnorcotinine and demethylcotinine were detected by UV analysis (254 mm) as two predominant fluorescent areas with $R_f = 0{\cdot}75$ and 0·85, respectively. The area corresponding to *N*-hydroxymethylnorcotine was collected, and the compound eluted with ethanol; evaporation of the solvent gave a light-yellow solid, melting point 84–86°C; elemental analysis calculated: C 62·5, H 6·3, N 14·6%; found: C 61·6, H 6·2, N 14·3%. The product was characterized by gc-ms, UV, ei-ms, and nmr.

0049–8254/94 $10·00

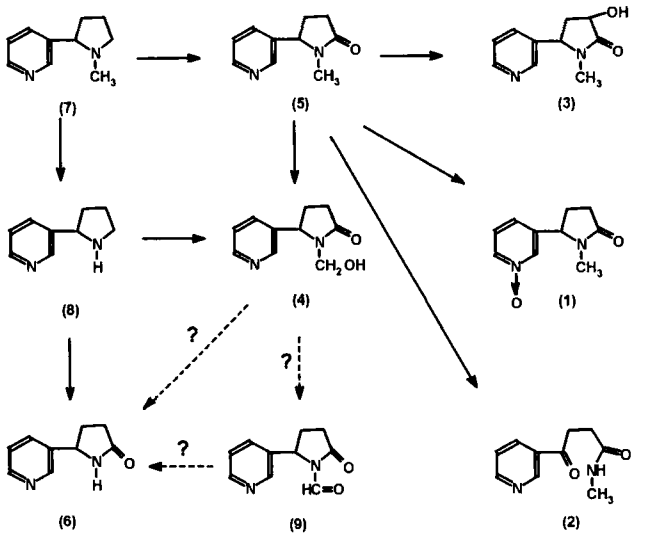

Figure 1. Metabolic pathways of nicotine and cotinine showing the new metabolite (4) and route to norcotinine. Figures in parenthesis are as mentioned in text and figures. ——→, established metabolic pathways; and - - - →, possible metabolic pathways.

Demethylcotinine was prepared in our laboratory as described previously (Aislaitner *et al.* 1992) using the method of Glenn and Edwards (1978). Nicotinamide adenine dinucleotide phosphate ($NADP^+$) and *D*-glucose 6-phosphate were purchased from Sigma Chemical Co. (Poole, UK). Glucose 6 phosphate dehydrogenase (G6PD), grade II from yeast (140 U/mg) was obtained from Boehringer Corporation Ltd (London, UK), magnesium chloride and ammonium dihydrogen orthophosphate were purchased from FSA Laboratory Supplies (Loughborough, UK). Sodium hydride, tetrahydrofuran and paraformaldehyde were obtained from Aldrich Chemical Co. (Dorset, UK).

Animals

Male Syrian golden hamsters (80–100 g) were provided by King's College Biological Services Facility. They were fed standard diet RM1(E) (W. Lillico, Manea, Cambridge, UK) and water *ad libitum*. Animals were deprived of food overnight and killed the next day by cervical dislocation.

Experimental procedure

Hepatic microsomes were prepared using the calcium precipitation method described by Schenkman and Cinti (1978) and resuspended in potassium phosphate buffer (0·2 M, pH 7·4) at a concentration equivalent to 1 g original liver/ml.

Preincubation of an NADPH-generating cofactor solution (1 ml) consisting of $NADP^+$ (1 μmol), G6P (5 μmol), G6PD (0·5 unit), magnesium chloride (10 μmol) was carried out at 37°C for 5 min in a shaking water bath prior to the addition of the hepatic microsomes (0·25 ml) and cotinine (1 μmol in 50 μl water). The final volume of the incubate was 1·5 ml. The incubation reaction was terminated at 30 min by placing the flasks on ice. The incubation mixture was saturated with 1 g sodium chloride, extracted with acetonitrile (3 × 2 ml), which was evaporated to dryness under a stream of nitrogen at 40°C. Metabolites were redissolved in 100 μl hplc mobile phase (see below) for analysis.

An isocratic hplc system described previously (Li *et al.* 1992) was modified for the analytical procedure of this study. Briefly, this system consisted of an LDC Analytical pump (Milton Roy, ConstaMetric 3000), a Nucleosil (10 FSA) cation exchange column (4·6 × 250 mm), and a CE 2112 UV detector (Cambridge, UK) coupled with an integrator (LDC/Milton Roy C1-10B). The hplc mobile phase consisted of acetonitrile and 0·1 M ammonium dihydrogen orthophosphate containing 0·07% (v/v) triethylamine (2:98, v/v, pH 5·8), flow rate 1 ml/min, and monitored at a wavelength of 260 nm. In order to ensure the purity of the compounds, hplc eluents were additionally monitored by a Rapiscan UV detector (Severn Analytical) capable of scanning different UV wavelengths simultaneously.

Isolation and identification of unknown metabolite

The combined eluents from the hplc column at retention times (t_R) between 14 and 16 mins were collected and concentrated to dryness by freeze drying. The residue was analysed by gc-ms after silylation

with trimethyl silylchloride (TMS) (Rose 1990). Authentic *N*-hydroxymethylnorcotinine was analysed according to the same procedure.

Results

The hplc chromatogram of the metabolic extracts after incubation of cotinine with hamster hepatic microsomes is shown in figure 2. In addition to cotinine-1-*N*-oxide (**1**, $t_R = 10{\cdot}1$ min), 4-(3′-pyridyl)-4-oxo-*N*-methylbutyramide (**2**, $t_R = 11{\cdot}8$ min), 3′-hydroxycotinine (**3**, $t_R = 14{\cdot}1$ min), and cotinine (**5**, $t_R = 26{\cdot}8$ min) a new metabolite (**4**, $t_R = 15{\cdot}1$ min) was observed. No metabolite having the chromatographic properties of demethylcotinine ($t_R = 23{\cdot}3$ min) was observed. The retention time of the new metabolite was identical to that of synthetic *N*-hydroxymethylnorcotinine described below. Furthermore, the peak height of the new metabolite was increased when authentic *N*-hydroxymethylnorcotinine was added to an incubation extract (data not shown). The ratio of the UV spectra for this latter

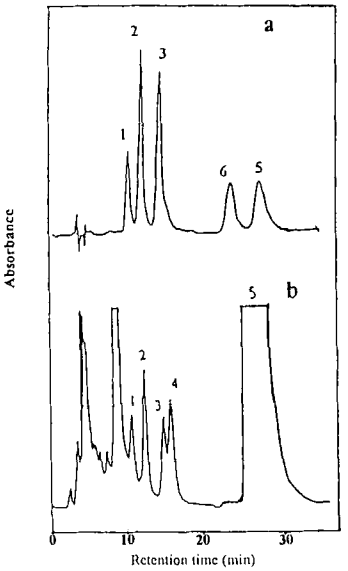

Figure 2. (**a**) Hplc chromatogram of authentic cotinine and its metabolites. (**b**) Hplc chromatogram of metabolic extract from incubation of cotinine with hamster hepatic microsomes. **1**, cotinine-1-*N*-oxide; **2**, 4-(3-pyridyl)-4-ono-*N*-methylbutyramide; **3**, 3′-hydroxycotinine; **4**, new metabolite; **5**, cotinine; and **6**, demethylcotinine.

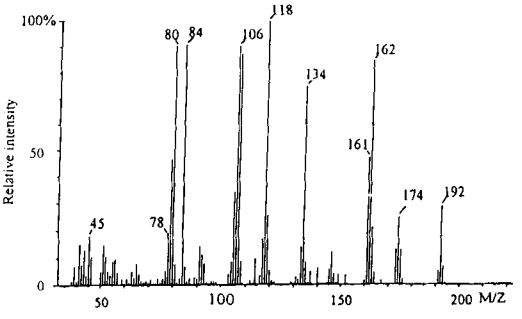

Figure 3. Ei-ms of *N*-hydroxymethylnorcotinine.

spiked mixture determined at two different time points for the same peak was $>0{\cdot}9$, confirming its identity as *N*-hydroxymethylnorcotinine.

The ei-ms of the chemically synthesized *N*-hydroxymethylnorcotinine is shown in figure 3. The molecular ion *m/z* 192, M-18, 174, M-CH_2OH; 161 and demethylcotinine 162 are readily observed. Treatment of the synthetic material or the isolated metabolite with TMS gave a derivative which was subjected to gc-ms, and the spectra are shown in figure 4. The peak at 264 is consistent with the trimethyl

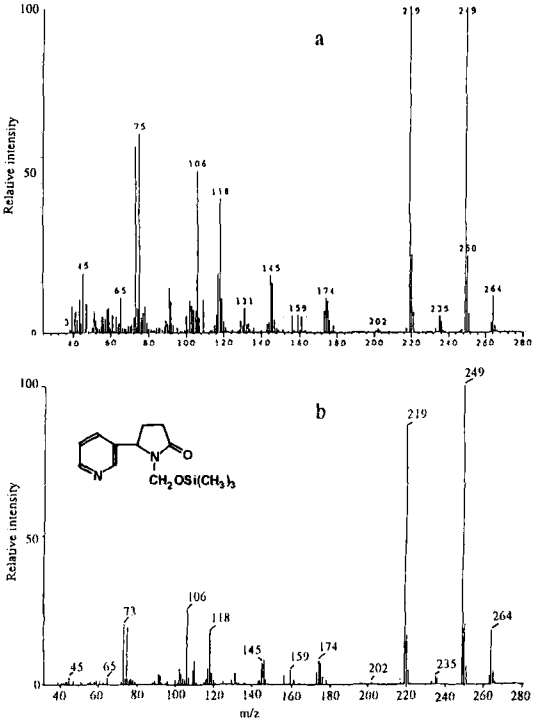

Figure 4. Gc-ms of (**a**) derivatized *N*-hydroxymethylnorcotinine isolated from hamster hepatic microsomes, and (**b**) authentic derivatized *N*-hydroxymethylnorcotinine.

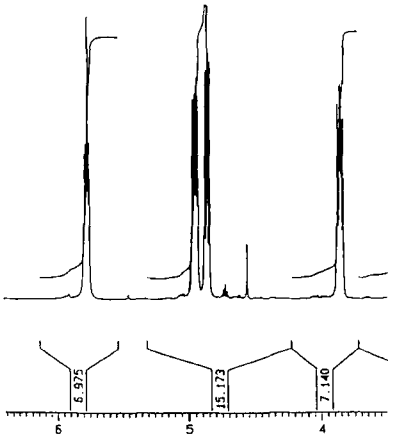

Figure 5. ^{1}H-nmr spectra (400 MH_3) of *N*-hydroxymethylnorcotinine (in DMSO).

silyl derivative of *N*-hydroxymethylnorcotinine. M-CH_3 = 249, M-$(CH_3)_3$ = 219 and M-90 peaks were also observed. Peaks at *m/z* 118 and 106 were observed for both the parent and the derivatized material.

The nmr spectrum of the synthetic *N*-hydroxymethylnorcotinine in DMSO is shown in figure 5. The signal due to the presence of the alcoholic proton is observed as a triplet at 5·78 ppm; this signal disappeared upon the addition of D_2O confirming the exchangeable nature of this proton. No signal corresponding to an *N*-methyl group was observed. The 5′-proton gave a triplet at 4·86 ppm. The methylene protons were observed as multiplets at 4·95 and 3·85 ppm due to restricted rotation at the N–CH_2OH bond and on treatment with D_2O these signals became doublets at 4·95 and 3·93 ppm. The spectrum is fully consistent with the proposed structure. *N*-Hydroxymethylnorcotinine was also formed when norcotinine was mixed with excess formaldehyde and allowed to stand at room temperature. After 10 days the required compound accounted for as much as 80% of the reaction mixture.

N-Hydroxymethylnorcotinine was characterized by UV λ^1_{max} = 209, λ^2_{max} = 261 (ε2818 in ethanol), λ_{min} = 236, in ethanol. The spectrum was slightly affected by the solvent and the Rapiscan spectra of the synthetic and biosynthetic *N*-hydroxymethylnorcotinine were identical when determined on column under the same conditions.

Discussion

During the course of studies on the enzymes involved in the metabolism of cotinine (**5**), a metabolite was recognized that did not have the characteristics of the known primary metabolites, i.e. cotinine-1-*N*-oxide (**1**), 3′-hydroxycotinine (**3**), 4-(3-pyridyl)-4-oxo-*N*-methylbutyramide (**2**) or demethylcotinine (**6**). This novel metabolite was isolated and characterized by comparison with synthetic *N*-hydroxymethylnorcotinine (**4**). *N*-Hydroxymethyl compounds (carbinolamines) are relatively rarely detected as metabolites due to their instability when formed from *N*-methylamines (Gorrod and Temple 1976). In the present case, the substrate, an amide, was converted to a carbinolamide, which is much more stable. The formation of *N*-hydroxymethylnorcotinine as a metabolite of cotinine raises questions about its role in the overall metabolism of nicotine, since cotinine is the major primary metabolite of this alkaloid (Gorrod 1993, and references therein). Despite the suggestion by Gorrod (1993) that *N*-hydroxymethylnorcotinine may be an intermediate in the formation of norcotinine, it is clear that *N*-hydroxymethylnorcotinine is formed *in vitro* under conditions where norcotinine is not detected. Indeed, whilst norcotinine has been reported as a metabolite of cotinine (McKennis *et al.* 1959) and nicotine *in vivo* (Bowman *et al.* 1959); it has not been unambiguously recognized *in vitro*. Stalhandske (1970) detected a metabolite of cotinine produced by mouse liver homogenates, which he suggested was either 4-(3-pyridyl)-4-oxo-*N*-methylbutyramide or norcotinine; his chromatographic methods could not distinguish between these two latter substances. Subsequent metabolic studies carried out in our laboratory using fortified hepatic microsomes from a variety of mammalian species have failed to detect a metabolite with the same chromatographic characteristics as the *N*-demethylated compound (Li *et al.* 1992, and unpublished observations). We have observed that nornicotine (**8**) is metabolized to norcotinine (**6**) (Aislaitner *et al.* 1992). The surprising stability of the *N*-hydroxymethylnorcotinine that we observed indicates that this is not converted, either chemically or biochemically, to the *N*-demethylated compound under the incubation conditions

used. This is in contrast with the instability of *N*-hydroxymethylcarbazole (Gorrod and Temple 1976) where both chemical and enzymic breakdown to carbazole was observed under similar incubation conditions. Similarly, stable *N*-hydroxymethyl compounds have been detected as metabolites of a number of *N*-methylcarbamates (Gorrod and Temple 1976 and references therein). It may be that the *N*-hydroxymethyl group [N-CH_2OH] is a substrate for alcohol dehydrogenase, generating the corresponding *N*-formyl [-N-CHO] compound (**9**). This pathway has been implicated in the conversion of the *N*-methyl side chain of aminopyrine to the *N*-formyl compound (Noda *et al.* 1976). This *N*-formyl compound would be expected to undergo hydrolysis to provide an alternative pathway to norcotinine (Sertkaya and Gorrod 1988). These interrelationships are shown in figure 1.

N-Hydroxymethyl compounds have been implicated as active metabolites from a number of drugs (Gorrod 1979) and in the covalent binding of amines to cellular macromolecules (Roberts and Warwick 1964), it may be that *N*-hydroxymethylnorcotinine provides an additional mechanism whereby 'nicotine' (**7**) is bound to macromolecules (Shigenaga *et al.* 1988).

During studies designed to recognize all the metabolites of nicotine in man (Kyerematen and Vessel 1991) and rat (Schepers *et al.* 1993) several metabolites remained uncharacterized; it may be that the novel metabolite that we now report or a conjugate or metabolite derived from it can account for certain of these observations.

Further studies on the formation and fate of this compound are in progress.

Acknowledgements

This work was supported by grants from the Fabriques de Tabac Réunies SA, (FTR), Neuchâtel, Switzerland. Y.L. acknowledges an award from the K. C. Wong Education Foundation. We wish to acknowledge the helpful discussions of Drs N. Y. Li and G. Aislaitner during the course of this work.

References

AISLAITNER, G., LI, Y., and GORROD, J. W., 1992, *In vitro* metabolic studies on (−)-(*S*)-nornicotine. *Medical Science Research*, **20**, 897–899.

BOWMAN, E. R., TURNBULL, L. B., and MCKENNIS, H., 1959, Metabolism of nicotine in the human and excretion of pyridine compounds by smokers. *Journal of Pharmacology and Experimental Therapeutics*, **127**, 92–95.

DAGNE, E., and CASTAGNOLI, N., 1972a, Structure of hydroxycotinine, a nicotine metabolite. *Journal of Medicinal Chemistry*, **15**, 356–360.

DAGNE, E., and CASTAGNOLI, N., 1972b, Cotinine *N*-oxide, a new metabolite of nicotine. *Journal of Medicinal Chemistry*, **15**, 840–841.

DAGNE, E., GRUENKE, L., and CASTAGNOLI, N., 1974, Deuterium isotope effects in the *in vivo* metabolism of cotinine. *Journal of Medicinal Chemistry*, **17**, 1330–1333.

GLENN, D. F., and EDWARDS, W. B., 1978, Synthesis and mass spectrometry of some structurally related nicotinoids. *Journal of Organic Chemistry*, **43**, 2860–2870.

GORROD, J. W., 1979, Toxic products during metabolism of drugs and foreign compounds. In *Drug Toxicity*, edited by J. W. Gorrod (London: Taylor & Francis), pp. 1–24.

GORROD, J. W., 1993, The mammalian metabolism of nicotine: an overview. In *Nicotine and related Alkaloids*, edited by J. W. Gorrod and J. Wahren (London: Chapman & Hall), pp. 31–43.

GORROD, J. W., and TEMPLE, D. J., 1976, The formation of an *N*-hydroxymethyl intermediate in the *N*-demethylation of *N*-methylcarbazole *in vivo* and *in vitro*. *Xenobiotica*, **6**, 265–274.

KYEREMATEN, G. A., and VESELL, E. S., 1991, Metabolism of nicotine. *Drug Metabolism Reviews*, **23**, 3–41.

LI, Y., LI, N. Y., AISLAITNER, G., and GORROD, J. W., 1992, *In vitro* metabolism of cotinine. *Medical Science Research*, **20**, 903–904.

McKENNIS, H., TURNBULL, L. B., BOWMAN, E. R., and SCHWARTZ, S. L., 1962, The corrected structure of a ketoamide arising from the metabolism of (−)-nicotine. *Journal of the American Chemical Society*, **84,** 4598–4599.

McKENNIS, H., TURNBULL, L. B., BOWMAN, E. R., and WADA, E., 1959, Demethylation of cotinine *in vivo*. *Journal of the American Chemical Society*, **81,** 3951–3954.

McMAHON, R. E., 1966, Microsomal dealkylation of drugs; substrate specificity and mechanism. *Journal of Pharmaceutical Science*, **55,** 457–466.

NODA, A., GOROMARU, T., TSUBONE, K. M., and IGUCHI, S., 1976, *In vivo* formation of 4-formylaminoantipyrine as a new metabolite of aminopyrine. *Chemical and Pharmaceutical Bulletin*, **24,** 1502–1505.

ROSE, M. E., 1990, Modern practice of gas chromatography/mass spectrometry, *VG Monographs in Mass Spectrometry*, **1,** 1–23.

ROBERTS, J. J., and WARWICK, G. P., 1964, Reactions of ^{3}H-labelled butter yellow *in vivo* and *N*-hydroxymethylaminoazobenzene *in vitro*. *Biochemical Journal*, **93,** 18–19.

STALHANDSKE, T., 1970, The metabolism of nicotine and cotinine by a mouse liver preparation. *Acta Physiologica Scandinavia*, **78,** 236–248.

SCHENKMAN, J. B., and CINTI, D. L., 1978, Preparation of microsomes with calcium. In *Methods in Enzymology*, edited by S. Fleischer and L. Packer (New York: Academic), **52,** 83–89.

SCHEPERS, G., RUSTEMEIER, K., WALK, R. A., and HACKENBERG, U., 1993, Metabolism of *S*-nicotine in noninduced and aroclor-induced rats. *European Journal of Drug Metabolism and Pharmacokinetics*, **18,** 187–197.

SERTKAYA, N. N., and GORROD, J. W., 1988, Metabolic cleavage of amide bonds. In *Metabolism of Xenobiotics*, edited by J. W. Gorrod, H. Oelschlager and J. Caldwell (London: Taylor & Francis), pp. 225–240.

SHIGENAGA, M. K., TREVOR, A. J., and CASTAGNOLI, N., 1988, Metabolism-dependent covalent binding of (*S*)-[5-^{3}H] nicotine to liver and lung microsomal macromolecules. *Drug Metabolism and Disposition*, **16,** 397–402.