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Note

Nicotine metabolites in the urine of smokers

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Nicotine in humans is almost exclusively derived from exposure to tobacco smoke, and the absorbed nicotine is cleared from the body either unchanged or in the form of a number of metabolites [1]. Determination of cotinine, a nicotine metabolite, has become widely used to assess exposure to tobacco smoke in humans, because it is thought to be the major nicotine metabolite.

Urinary nicotine metabolites produce coloured derivatives when subjected to the simple König reaction [2]. We have used this reaction as the basis of two simple methods for the assessment of exposure to tobacco smoke, which are more rapid and less expensive than cotinine estimations [2, 3]. Furthermore, we have developed a high-performance liquid chromatographic (HPLC) method to separate the coloured nicotine metabolite derivatives and have shown

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that the method detects nicotine, cotinine and at least five other metabolites in the urine of smokers [4]. One of these metabolites, which we call "metabolite 5", was present in substantially greater concentration than cotinine, accounting for ca. 40% of the total nicotine metabolite concentration compared with ca. 15% for cotinine. Further studies showed that metabolite 5 may be a useful marker of exposure to environmental tobacco smoke (passive smoking) [5, 6].

We describe here the isolation of metabolite 5 from the urine of tobacco smokers and its partial identification using HPLC, gas chromatography–mass spectrometry (GC–MS) and NMR spectroscopy.

EXPERIMENTAL

Reagents

Nicotine and cotinine were obtained from Sigma (Poole, U.K.) and 3-pyridylcarbinol from Aldrich (Gillingham, U.K.). *trans*-3′-Hydroxycotinine was obtained from Professor Adlkofer (Hamburg, F.R.G.). All other chemicals and HPLC-grade solvents were from Fisons (Loughborough, U.K.).

High-performance liquid chromatography

Nicotine metabolite concentrations were determined at each of the purification steps using the precolumn derivatization and HPLC method described previously [4], the only modification being the use of a Nova-Pak C_{18} column (4 μm, 15 cm$\times$3.9 mm I.D.) rather than the C_{18} μBondapak column (both from Waters Assoc., Milford, MA, U.S.A.).

In addition, the purified material was studied using a Perkin-Elmer Series 4 liquid chromatograph equipped with an ISS-100 autosampler, LC-95 UV–visible spectrophotometer and R100 recorder (all from Perkin-Elmer, Norwalk, CT, U.S.A.). Two different columns and mobile phase systems were used. A cyano column (Spherisorb CN, 5 μm, 20 cm$\times$4.6 mm I.D., Phase Separations, Queensferry, U.K.) was used with a gradient system consisting of 10% methanol in sodium acetate buffer, 60 m*M* (pH 4.18) at the beginning, and changing to 75% methanol in 15 min with a flow-rate of 0.8 ml/min. Alternatively, a μBondapak C_{18} column (10 μm, 30 cm$\times$3.9 mm I.D., from Waters Assoc.) was used isocratically with a mobile phase of 5% acetonitrile in water and a flow-rate of 1.5 ml/min. The injection volume was 20 μl, and the UV detection wavelength was set at 260 nm.

Gas chromatography–mass spectrometry

Peak identification was performed by GC–MS using a quadrupole instrument (gas chromatograph–mass spectrometer 5992A, Hewlett-Packard, Palo Alto, CA, U.S.A.). An SE-30 silica capillary column (25 m$\times$0.32 mm I.D.) was

used. The instrument was programmed from 60 to 110°C at 10°C/min, and operated at 2200 V and 70 eV.

NMR spectroscopy

The purified material was dissolved in ca. 0.5 ml of [$^2H_6$]acetone with a trace of tetra(methylsilane) (TMS). The spectra were run on a Bruker AM 250 (University of Joensuu, Joensuu, Finland) and a Jeol GSX 270 (University of Jyväskylä, Jyväskylä, Finland) spectrometers.

Extraction of urinary nicotine metabolites

Aliquots of ca. 15 ml of urine from 65 cigarette smokers were pooled. The 1-l urine pool was made alkaline with 50 ml of 20% sodium hydroxide and extracted three times (with 5-min mixing) in a separation funnel containing 500 ml of dichloromethane (DCM). The DCM extracts were discarded. The remaining alkaline water-phase was extracted three times (with 5-min mixing) with 500 ml of butanol–ethyl acetate (80:20, v/v). The organic phase was collected. This butanol–ethyl acetate extract was evaporated to dryness using a rotary evaporator and then dissolved in 10 ml of distilled water.

C_{18} column purification

The 10-ml water fraction was then applied to a primed C_{18} Sep-Pak (Millipore, Milford, MA, U.S.A.); priming was accomplished with the sequential addition of 5 ml of acetonitrile, 5 ml of methanol and 10 ml of distilled water. The 10-ml eluate from the column was collected, and the column was washed once with 5.0 ml of distilled water, twice with 3.0 ml of distilled water and finally with 5 ml of methanol.

The 10-ml eluate and the first 5 ml of water wash were combined, and pentanesulphonic acid and sodium hydroxide were added to yield concentrations of 20 and 240 m*M*, respectively. This mixture was extracted four times with 6 ml of DCM, and the organic extracts were discarded. The mixture was then also extracted six times with 12.5 ml of butanol. The butanol fractions were combined and evaporated to dryness under nitrogen, then redissolved in 2.0 ml of ethyl acetate–hexane (3:1, v/v).

Silica column purification

The 2.0-ml ethyl acetate–hexane fraction was than applied to a primed silica Sep-Pak cartridge; priming was accomplished with the sequential addition of 5 ml of methanol, 5 ml of acetonitrile, and 10 ml of ethyl acetate–hexane (3:1, v/v). After application the cartridge was washed with 10 ml of ethyl acetate–hexane (3:1, v/v), then with 10 ml of ethyl acetate. The nicotine metabolite 5 fraction was eluted from the cartridge by washing with two 5-ml portions of ethyl acetate–hexane (10:1, v/v) and twice with 5 ml of acetone. Finally the

TABLE I

RECOVERY OF THE METABOLITE 5 FRACTION THROUGH THE SUCCESSIVE ISOLATION AND PURIFICATION STEPS, AND THE RELATIVE PURITY WITH REGARD TO SEVEN NICOTINE COMPOUNDS AS ASSESSED BY THE HPLC METHOD [4]

Fraction/sample	Amount recovered (mg)	Recovery (%)	Relative purity (%)
Native	6.34	100	44.0
DCM extract	5.64	88.9	50.5
Butanol-ethyl acetate extract	3.99	62.9	85.8
C_{18} chromatography	3.82	60.3	87.6
Silica chromatography	3.33	52.6	87.8

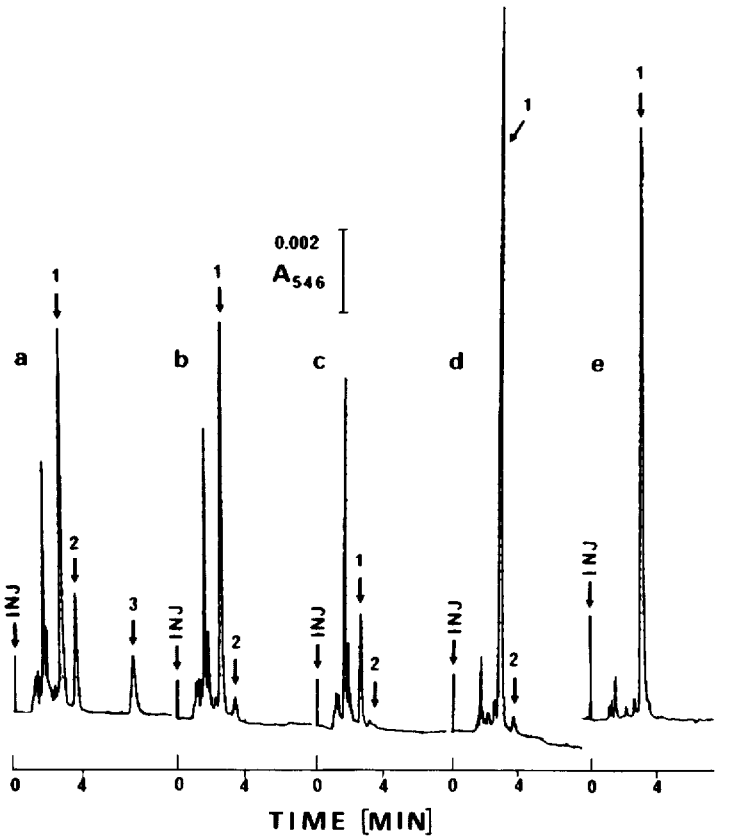

Fig. 1. HPLC profiles of the various isolation and purification steps for the urinary nicotine metabolite 5 fraction. Small aliquots from each of the steps were subjected to the pre-column derivatization procedure as described in the text. Notice that (a)-(c) are in the same scale. (a) Native urine sample; (b) DCM extract (remaining); (c) butanol-ethyl acetate extract (remaining); (d) butanol-ethyl acetate extract; (e) final purified metabolite 5 fraction from the silica column. Peaks: 1 = metabolite 5; 2 = cotinine; 3 = nicotine.

cartridge was washed with 5 ml of acetonitrile and methanol to elute the remaining materials.

We later showed that this fraction was contaminated with plasticizers, and so the following purification step was employed. A preparative silica glass column (14 cm×5 mm I.D.) was prepared in a Pasteur pipette by packing with Silica Woelm 100–200 μm Aktiv (70–150 mesh, Woelm Pharma, Eschwege, F.R.G.). Priming of the column was done as described above for silica Sep-Pak but using 10-ml solvent volumes. After application of the evaporated extract in ethyl acetate–hexane (3:1, v/v), the column was washed with 10 ml of the same solvent mixture followed by 15 ml of ethyl acetate. The nicotine metabolite 5 fraction was eluted in two 5-ml portions of ethyl acetate–acetone (3:1, v/v) followed by ethyl acetate–acetone (1:1, v/v), and finally the remaining material was removed by eluting with more polar solvents.

RESULTS

Table I and Fig. 1 show the efficiency of the various extraction and purification steps in isolating metabolite 5 from urine. The purity with respect to the other König-positive nicotine metabolites is also shown.

The initial extraction of alkaline urine using dichloromethane was shown to remove most of the nicotine and cotinine (Fig. 1). However, other metabolites were still present in appreciable amounts and ca. 90% of metabolite 5 remained

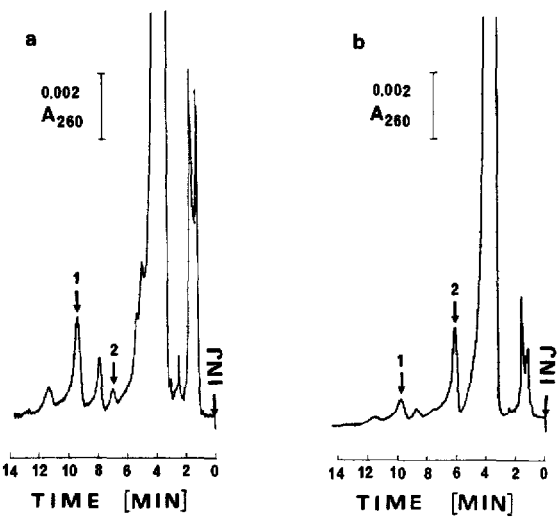

Fig. 2. HPLC profiles obtained using a reversed-phase column (μBondapak C_{18}). (a) Final metabolite 5 fraction; (b) same as in (a) but after addition of authentic 3-pyridylcarbinol. The elution positions of 3-pyridylcarbinol (2) and *trans*-3′-hydroxycotinine (1) are shown.

(Fig. 1, Table I). The butanol–ethyl acetate extraction step increased the relative purity of the metabolites to 86% and gave a 63% recovery.

The preparative chromatographic steps did not increase the relative purity of the isolated nicotine metabolite 5 fraction (Table I and Fig. 1). The steps were, however, effective in removing the majority of other interfering urinary compounds. The Sep-Pak preparative cartridges were found to release some plasticizers that interfered with the NMR and GC–MS analyses. These plasticizers were removed using the additional silica purification step on a glass column.

The final metabolite 5 fraction showed a single symmetric peak in our HPLC system with derivatization [4], but the two independent HPLC systems with UV detection yielded three peaks. Two of these peaks matched the authentic 3-pyridylcarbinol (3-PC) and *trans*-3′-hydroxycotinine (3-OH-Cot) standards; the third peak is unknown and possibly a nicotine metabolite (Fig. 2a).

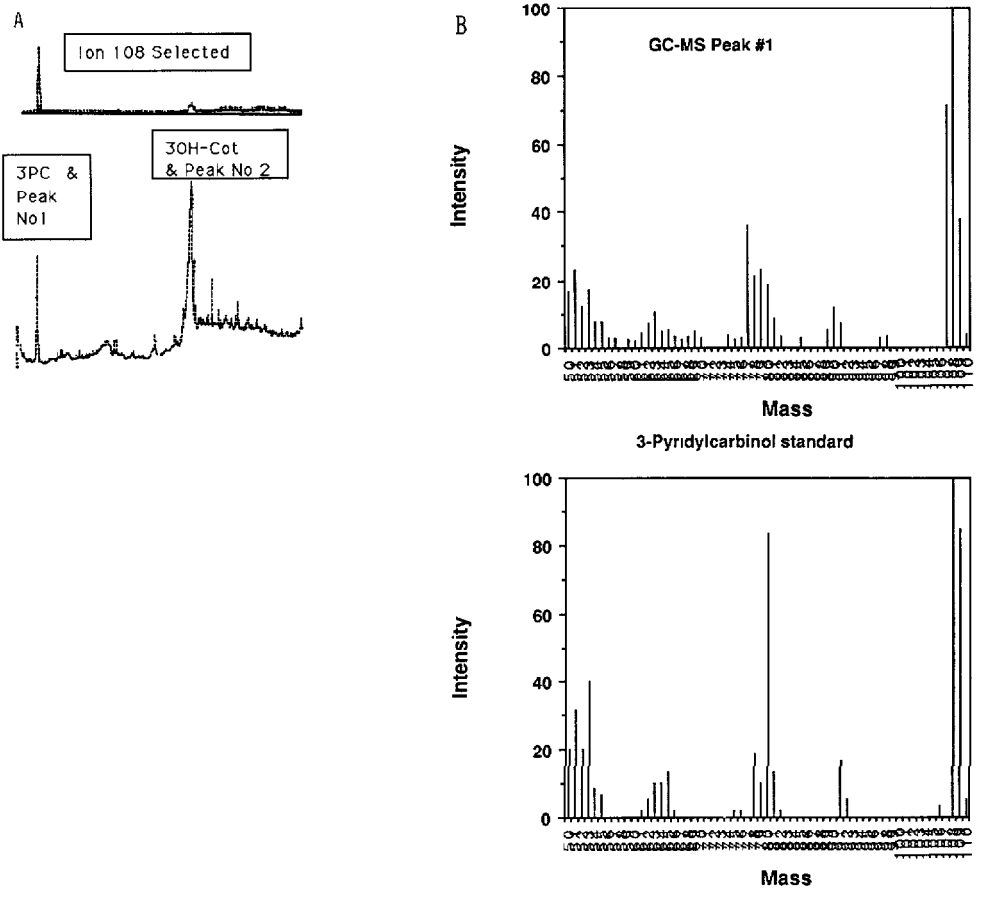

Fig 3

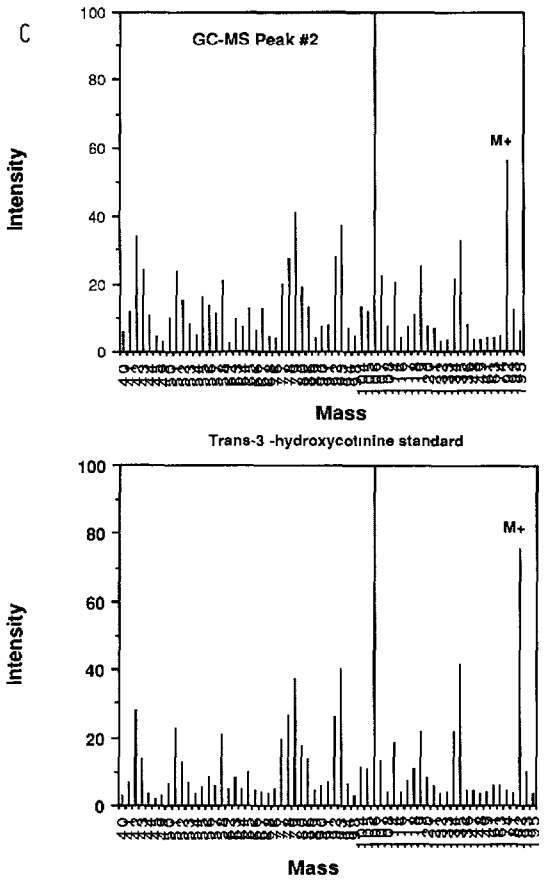

Fig. 3. GC–MS analysis of the final metabolite 5 fraction. (A) GC chromatogram obtained from an underivatized sample. The elution positions of 3-pyridylcarbinol (1) and *trans*-3′-hydroxycotinine (2) are shown. (B) Mass spectrum obtained for peak 1, together with the spectrum of the authentic standard of 3-pyridylcarbinol. (C) Mass spectrum obtained for peak 2, together with the spectrum of the authentic standard of *trans*-3′-hydroxycotinine.

The major UV-absorbing peak represents an impurity of the sample matrix or a plasticizer, which is not detected with the König reaction. The ratio of 3-PC to 3-OH-Cot was ca. 1:6 according to the UV absorption at 260 nm. When a 3-PC standard was added to the sample, the postulated 3-PC peak was increased (Fig. 2b).

The GC–MS analysis (Fig. 3) revealed two major fractions with similar retention times to the authentic standards of 3-PC and 3-OH-Cot (Fig. 3A). The fragmentation of standard 3-OH-Cot was identical with that of the latter peak

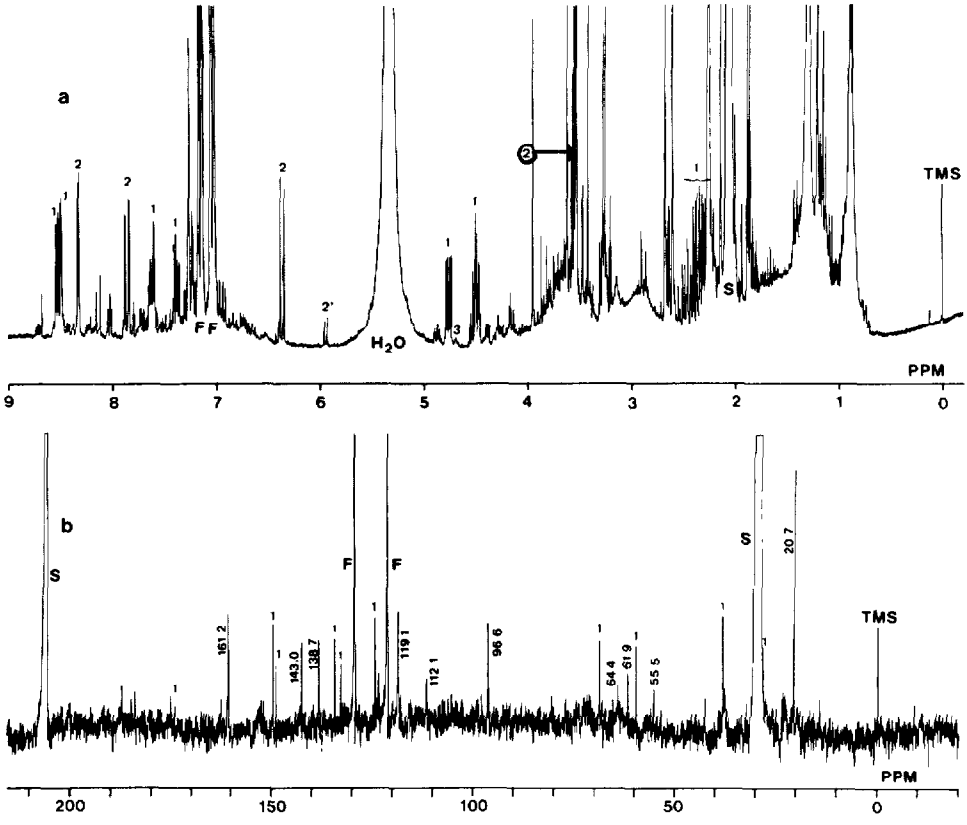

Fig. 4. (a) A 250-MHz ^1H NMR spectrum of the nicotine metabolite sample in [$^2H_6$]acetone at 310 K. (b) A proton noise-decoupled ^{13}C spectrum of the same sample (with Jeol GXS 270) at 310 K. The number of scans was 104 000, the pulse angle 70 and the pulse retention time 2.7 s.

(Fig. 3C). There were some differences in the fragmentation of the first peak when compared with that of 3-PC (Fig. 3B). These differences can tentatively be explained by the impurities of the sample matrix. Other candidates tested, e.g. the corresponding aldehyde, did not match in the GC elution or in the MS analysis.

The analysis of the ^1H NMR spectrum (Fig. 4a) was disturbed by the signals arising from solvent, phthalic acid or its derivatives (designated F) from the purification. The spectra of the main products (**1** and **2**) were picked up by decoupling experiments. The multiplet at 4.75 ppm is coupled with 0.2–0.5 Hz to the signals in the aromatic region and with large couplings to a multiplet at 2.2–2.5 ppm. Both the triplet at 4.5 ppm and the quartet at 4.75 ppm are coupled to the same multiplet at 2.2–2.5 ppm. The long-range couplings typical of a benzylic proton [7, 8] and a comparison of the chemical shifts with those of cotinine (an authentic sample) suggest that product **1** is 3′-hydroxycotinine.

Also the ^{13}C spectrum (Fig. 4b) shows signals with chemical shifts close to those reported for 3′-hydroxycotinine [9] in C^2HCl_3; the small differences were due to solvent effects.

The spectrum of **2** consists of four signals. The lowest field signal is clearly broadened due to couplings to ^{14}N and it is coupled with 0.2–0.5 Hz to the CH_3 signal at 3.53 ppm (seen better from another sample) and with 2 Hz to the signal at 7.85 ppm. The signals at 7.85 and 6.37 ppm are coupled with 9.6 Hz, which indicates, together with the proton chemical shifts, an incompletely conjugated aromatic system. The ^1H NMR spectrum (in $[^2H_6]$acetone) is very similar to the spectrum of 1,6-dihydro-1-methyl-6-oxo-3-pyridine carboxylic acid (in $[^2H_4]$methanol) [10]. A mass spectrum library search for a component suggested the corresponding carbonitrile (a possible precursor of **2**), the ^1H NMR spectrum of which [11], however, differs clearly from that of **2**. A compound of the same type (**2′**) was indicated by a signal at 5.95 ppm.

3-Pyridylcarbinol (**3**) is indicated by the very weak CH_2 proton signal at 4.7 ppm. The signal grew when 3-PC was added to the sample. The ratio 3-PC to 3-OH-Cot was ca. 1:40 according to this NMR analysis.

DISCUSSION

Our results provide independent data supporting those of Neurath et al. [12] 3-OH-Cot is one of the major nicotine metabolites in the urine of tobacco smokers.

Previous studies have concluded that 3-pyridylacetic acid was the terminal metabolite of nicotine degradation [1]. However, our study gives evidence of the presence of appreciable amounts of putative 3-PC which has a lower molecular mass and might be the terminal metabolite. Further work needs to be done to purify completely the various nicotine compounds in smokers' urine. These compounds are true nicotine metabolites since they cannot be found in non-smokers unless exposed to tobacco smoke [4–6].

The data presented here suggests that 3-OH-Cot may be quantitatively more important than 3-PC, accounting for ca. 80–90% of the metabolite 5 fraction. This finding would be in agreement with those of Neurath et al. [12] and Kyerematen et al. [13], but they have also shown that 3-OH-Cot has a short serum half-life [12, 13] which seems incompatible with our urinary studies of metabolite 5 [5, 6]. It is therefore possible that 3-PC may comprise a significant portion of metabolite 5. However, independent analytical methods for 3-PC and 3-OH-Cot need to be developed before the relative proportions of these two compounds in the urine of smokers can be confirmed. It will be important to determine which is quantitatively the more important since we have already shown that metabolite 5 estimations provide a sensitive marker of exposure to environmental tobacco smoke [6].

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