Studies on the Identification of Tobacco Alkaloids, their Mammalian Metabolites and Related Compounds by Gas Chromatography - Mass Spectrometry*

by

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INTRODUCTION

Substantial chemical changes are known to occur in the pyridine and pyrrolidine rings of nicotine, including almost complete degradation of the pyrrolidine ring, during fermentation and storage of tobacco (1-3) and during mammalian metabolism (4). Although some of the known routes in the mammalian metabolism of nicotine, including that in man, are well established (Schemes 1a and 1b), further inquiry into the detection and quantification of the numerous alkaloids involved was deemed important. A systematic gas chromatographic-mass spectrometric (GC-MS) study of a series of established nicotine metabolites was therefore undertaken to provide a basis for such an investigation. The results of these studies and of preliminary GC-MS experiments on the determination of nicotine in plasma, accomplished with the aid of multiple ion detection (MID) and deuterated nicotine as an internal standard, are here reported.

corresponding methyl esters (XIX, XXI, XXIII, XXVIII, XXX) were also prepared (CH_2N_2) and examined.

Gas Chromatography and Mass Spectrometry Conditions

GC-MS employing capillary columns was carried out, unless otherwise indicated, on an LKB 2091 mass spectrometer (ion source temperature: 270°C , electron energy: 70 eV) linked to a Varian 2700 instrument equipped with a capillary injector, a device for introduction of make-up gas, a splitter, and a flame-ionization detector. Helium was used as carrier gas in these determinations.

Mass spectra of the less volatile compounds were recorded using the direct inlet system.

GC was performed on a Varian 1700 or 2700 instrument, equipped with a flame-ionization detector. The temperature of the injector and the detector was maintained at 230°C . The packed glass columns were [i] 3% SE 30, [ii] 5% SE 52, and [iii] 8% Carbowax 20 M and 2% potassium hydroxide — all on Gas Chrom Q (100/120 mesh). The nitrogen flow rate was 30 ml/min.

High resolution GC was performed on the Varian 2700 instrument equipped with a capillary injector using either a glass capillary column (33 m, i. d. 0.4 mm) coated with Emulphor-O or a column (9.6 m, i. d. 0.2 mm) coated with OV-101. The nitrogen flow rates were 2.5 and 1.0 ml/min respectively, and the samples were injected on the capillary columns using a split ratio of 1:30.

The separations on the packed columns were carried out under isothermal conditions, while those on the capillary columns were performed using temperature programming. After 3 minutes at 125°C , the temperature was increased by 6° per minute to 200°C .

EXPERIMENTAL

Compounds

The compounds studied were synthesized or isolated as indicated by the references in Table 1. Of these compounds, the acids (XVIII, XX, XXII, XXVII and XXIX) possess less attractive gas chromatographic properties and in order to circumvent this problem the

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Scheme 1a. Abridged scheme for the mammalian metabolism of nicotine.

Scheme 1b. Abridged scheme showing ions and N-oxides implied in the mammalian metabolism of nicotine.

*Determination of Nicotine in Blood Plasma
by Mass Fragmentography*

To samples of heparin-treated blood plasma (1 ml) obtained from male smokers, 50 ng of 5-d₂-S-nicotine (5) (in 1 ml of water) was added as an internal standard. The sample was then adjusted to pH 10 by addition of 0.05 ml of N NaOH and extracted twice with 5 ml of n-hexane by mechanical shaking for 10 min. After centrifugation of the mixture, an aliquot (9 ml) of the combined hexane solutions was removed and concentrated under a stream of argon to a small residue which was adjusted to 25 µl by addition of methanol. A sample (1 µl) was removed for injection into an LKB 9000 instrument equipped with a glass column (1.7 m, i. d. 1 mm) packed with 8% Carbowax 20 M (2% KOH) on Gas-Chrom Q (100/120 mesh): helium carrier gas, 30 ml/min; injection port, 220° C; column oven, 142° C; separator, 255° C. The data system and instrumental control have been described previously (6).

RESULTS AND DISCUSSION

Prior to the present study, many gas chromatographic methods have been reported for the separation of nicotine and cotinine (7-10, 57). These tobacco constituents, of which nicotine is partly converted to cotinine during fermentation and storage of tobacco (2), are two of the most readily detectable compounds in urine and have been employed as possible indicators of exposure to tobacco smoke. Moreover, cotinine, like nicotine, is pharmacologically active and is known to lower the blood pressure in experimental animals (11) and to leave the blood at a much slower rate than nicotine after cessation of smoking (12-15). Assays of the cotinine content of biological fluids are receiving increased emphasis, but the many studies in which gas chromatography alone has been used for the determination of cotinine have not consistently mentioned necessary controls or specified conditions which would exclude the possible confusion of this compound with other bases such as norcotinine (XIII), hydroxycotinine (XIV) and alhydroxycotinine (XXVI). Since these mammalian metabolites of nicotine most often accompany cotinine in solvent extractions preceding gas chromatographic determinations, this constitutes a general problem, which was solved in the present study by the development of adequate conditions for gas chromatographic-mass spectrometric determinations. In particular, the use of capillary columns in combination with multiple ion detection (MID) employing a deuterated form of the compound under consideration as an internal standard is an advantage, since this procedure allows specific ion quantification of a number of nicotine metabolites at levels present in biological fluids.

The compounds covered in the present study are detailed in Table 1, which also includes relevant GC-data; the corresponding mass spectra are given in Figures 1 to 30.

Since reasonable stability or storage qualities were

deemed a prerequisite for including a compound in the present study, 4-(3-pyridyl)-4-methylaminobutyraldehyde, which is notorious for its instability, was excluded. This compound has a designated intermediary function in the mammalian degradation of nicotine (42, 43), especially when it is in the isomeric ring form 5'-hydroxynicotine or the related form nicotine-Δ^{1'}(5')-iminium ion (43). Myosmine (VI), β-nicotyrine (VII), anabasine (VIII) and methylanabasine (IX) were included, although of these four bases only β-nicotyrine has been implied — but not fully characterised (44) — as a nicotine metabolite. These minor tobacco alkaloids were included because of the increasing interest in the biological and pharmacological effects of the compounds and also because of various similarities in the mass spectral data. As can be seen, nicotine (Fig. 1a) and anabasine (Fig. 8) display molecular and base peaks at identical mass numbers, and cotinine (Fig. 10) and methylanabasine (Fig. 9) are also a comparable pair.

Although never shown to be a nicotine metabolite, nicotine-1,1'-dioxide (IV) was deemed of sufficient interest to be examined since both pyridine (25) and pyrrolidine (45) N-oxides have established roles in the metabolism of nicotine. 4-(3-Pyridyl)-4-oxobutyramide (XXV) was included because it has a possible intermediary function between norcotinine and 4-(3-pyridyl)-4-oxobutyric acid in the metabolism of nicotine.

Additionally, cis- and trans-metanicotine (30) were studied since metanicotine (46) may theoretically arise as a precursor in the mammalian metabolism of nicotine to dihydrometanicotine, 4-(3-pyridyl)-N-methylbutylamine (47). Metanicotine has also been reported as a tobacco alkaloid (48, 49), as a possible bacterial metabolite of nicotine (50) and as a component of cigarette smoke (51, 52), possibly arising through pyrolysis of nicotine (53). However, neither the geometry nor the relative distribution of the two isomers of metanicotine as they occur in nature or as a pyrolysis product of nicotine has been recorded. The two isomers are easily separated on an Emulphor-O capillary column, but the mass spectrometer cannot readily be used as the sole means of distinction, as the mass spectra of these isomers (Figures 16 and 17) are almost identical.

Of the present compounds (Table 1), neither the more acidic metabolites of nicotine (XVIII, XX, XXII, XXVII, XXIX) nor the thermally unstable quaternary ammonium compounds (II, XI) and N-oxides (III, IV, XII) could be studied by the GC-MS directly, although nicotine-1'-oxide could be chromatographed on the Emulphor-O capillary column without complete degradation. The quaternary ammonium compounds were examined only by the use of the direct inlet system, while the acids were converted to the methyl esters, for many of which appropriate GC-conditions have already been described (37). In preparing the esters, diazomethane was, however, preferred to the methanol-sulfuric acid previously used in the esterification, since a better ratio of methyl 4-(3-pyridyl)-4-hydroxybutyrate to the undesired 5-(3-pyridyl)tetrahydrofuran-2-one was obtained with diazomethane.

Table 1. Gas chromatographic data.

Compound		Retention times		Mode of recording MS	References
I a	S-(-)-Nicotine	6.0/85° C 5.3/135° C 5.0	A B C	GC-MS	5, 16
I b	5-d ₂ -S-(-)-Nicotine	4.7/142° C	B	GC-MS	5
II	S-(-)-Nicotine isomethiodide hydroiodide			Direct probe	17
III	S-(-)-Nicotine-1'-oxide ^a	10.6	C ^b	Direct probe	18, 19, 20
IV	S-(-)-Nicotine-1,1'-dioxide ^a			Direct probe	18, 20
V	S-(-)-Nornicotine	9.0/90° C 10.7	A C	GC-MS	5
VI	Myosmine	5.9/90° C	A	GC-MS	5
VII	β-Nicotyrine	5.0/100° C 13.2	A C	GC-MS	21
VIII	S-(-)-Anabasine	5.6/100° C 10.8	A C	GC-MS	5
IX	S-(-)-Methylanabasine	4.5/100° C 5.3	A C	GC-MS	22
X	S-(-)-Cotinine	5.8/130° C 7.0/213° C 23.2 3.2/170° C 5.4	A B C D E	GC-MS	5, 23
XI	S-(-)-Cotinine methiodide			Direct probe	17
XII	S-(-)-Cotinine-1-oxide			Direct probe	24, 25
XIII	S-(-)-Norcotinine	5.9/135° C 10.9/265° C 4.4/170° C	A B D	GC-MS	26, 27
XIV	(5S, 3R)-3-Hydroxycotinine	5.7/140° C 4.6/170° C	A D	GC-MS	25, 28
XV	Dihydrometanicotine	4.9/105° C 9.9	A C	GC-MS	29
XVI	trans-Metanicotine	4.3/110° C 13.5	A C	GC-MS	30
XVII	cis-Metanicotine	3.8/110° C 11.2	A C	GC-MS	30
XVIII	4-(3-Pyridyl)butyric acid			Direct probe	31
XIX	Methyl 4-(3-pyridyl)butyrate	6.8/95° C 12.6	A C	GC-MS	32, 33
XX	4-(3-Pyridyl)-4-oxobutyric acid			Direct probe	34
XXI	Methyl 4-(3-pyridyl)-4-oxobutyrate	4.3/125° C 19.1	A C	GC-MS	32
XXII	4-(3-Pyridyl)-4-hydroxybutyric acid ^c			Direct probe	32, 34
XXIII	Methyl 4-(3-pyridyl)-4-hydroxybutyrate ^c	5.9/140° C 19.0	A C	GC-MS	32
XXIV	5-(3-Pyridyl)tetrahydrofuran-2-one ^c	6.0/140° C 23.2 5.8	A C E	GC-MS	32, 34
XXV	4-(3-Pyridyl)-4-oxobutyramide	5.7/145° C	A	GC-MS	35
XXVI	Allohydroxycotinine ^c	5.6/140° C 6.8/213° C 3.0/170° C	A B D	GC-MS	25, 36, 37

Table 1 (contd.)

Compound		Retention times		Mode of recording MS	References
XXVII	3-Pyridylacetic acid			Direct probe	38, 39
XXVIII	Methyl 3-pyridylacetate	3.3/ 85° C 7.9	A C	GC-MS	39
XXIX	N-(3-pyridylacetyl)glycine			Direct probe	40, 41
XXX	Methyl N-(3-pyridylacetyl)glycinate	5.7/150° C	A	GC-MS	40, 41

A Retention times on the SE-30 column.

B Retention times on the Carbowax (KOH) column.

C Retention times on the Emulphor-O glass capillary column.

D Retention times on the SE-52 column.

E Retention times on the OV-101 glass capillary column.

a Mixture of R,S- and S,S-diastereoisomers.

b Partly decomposed into nicotine during gas chromatography.

c Mixture of enantiomers.

Allohydroxycotinine (XXVI), although weakly acidic and amphoteric, can be separated from the above acidic metabolites of nicotine by extraction with organic solvents and can even be chromatographed on a basic Carbowax column. However, it emerges simultaneously with cotinine from all of the three packed columns used in the present investigation, a problem which can easily be circumvented by employing the MID technique and monitoring mass numbers 98 for cotinine, 100 for 3-d₂-cotinine (5) and 192 for allohydroxycotinine. Refocusing on other masses further enhances the reliability of the quantifications.

Similarly, the emergence of norcotinine (XIII) and 3-hydroxycotinine (XIV) coincided on the SE-30 and SE-52 columns but focusing on m/e 162 (M⁺) for norcotinine, on m/e 192 (M⁺) for 3-hydroxycotinine and on the corresponding ions of suitable labelled internal standards would allow adequate quantification.

It may also be noted that cotinine and 5-(3-pyridyl)-tetrahydrofuran-2-one could not be separated on the Emulphor-O glass capillary column. These compounds were, however, easily separated on an OV-101 capillary column, which makes their determination possible in the event that a mass spectrometer is not available.

The mass spectra of nicotine, nornicotine, cotinine, anabasine, myosmine (5, 16), nicotine-1,1'-dioxide (18) and nicotine-1'-oxide (18), of which both cis and trans forms are generated metabolically from S-(-)-nicotine, have previously been studied in some detail. Moreover, mass spectrometric data have been presented for allohydroxycotinine, 5-(3-pyridyl)-5-hydroxy-1-methyl-2-pyrrolidone (25). In the present study, the spectra of allohydroxycotinine obtained by GC-MS and by direct inlet mass spectrometry were similar to those previously reported. The spectra do not, however, appear to provide direct evidence on the possible contributions of the ring (pyrrolidone) and open chain (ketoamide) tautomeric forms (4) of the compounds.

The GC data presented in this study form the basis for the development of reliable quantitative estimations of nicotine, its metabolites and related compounds. The use of a mass spectrometer and especially multiple ion detection further facilitates these estimations and may ultimately be of significance in checking various other quantification methods such as radioimmunoassays that are now being employed to some extent for the de-

termination of nicotine and its metabolites (14, 15, 54). Prior to the present investigation mass spectrometry has been used in only one study to confirm the identity of the nicotine peak in quantitative GC (9).

To illustrate the potentialities of GC-MS determinations of tobacco alkaloids in biological fluids along lines previously described for many other compounds (55), blood plasma was obtained from two smokers. In these preliminary experiments, deuterated nicotine was added as an internal standard to the heparin-treated venous blood, and an n-hexane extract of the plasma was subjected to GC-MS analysis.

The plasma from a heavy smoker (25 years of age, weight: 67 kg, average daily consumption: 20 cigarettes), who had just smoked four high-level nicotine cigarettes in 20 minutes, showed a nicotine level of 95 ng/ml as calculated from the relative peak heights of the base peak of nicotine at 84 mass units and the base peak of deuterated nicotine at 86 mass units (Fig. 1a). This very high nicotine level may be related to the fact that the subject inhaled very deeply while he smoked the four cigarettes. The plasma from the other smoker (44 years of age, weight: 80 kg, average daily consumption: 20 cigarettes), who had not smoked for 15 minutes before the blood sample was taken, contained nicotine at the level of 26 ng/ml (Fig. 1b). Both smokers used their own cigarette brands and the latter subject conformed to his regular smoking habits. The identity of the nicotine in the plasma samples was ascertained by the retention time, by recording the entire mass spectrum, and by refocusing on other mass units in the nicotine spectrum.

Further biological applications as well as mechanistic studies on the mass spectrometric fragmentation of the various alkaloids will be discussed elsewhere.

SUMMARY

Various pyridine compounds, either identified or implied as intermediates in the mammalian metabolism of nicotine or present in tobacco or tobacco smoke, have been studied by gas chromatography-mass spectrometry. Spectral and gas chromatographic data for these compounds, which provide a basis for further studies of pyridine compounds in plant and animal tissue, are given. The utility of these data in studies on nicotine

and its metabolites in body fluids from humans is illustrated by a determination of nicotine in the plasma from two smokers using quantitative mass fragmentography with the aid of deuterated nicotine.

ZUSAMMENFASSUNG

Verschiedene Pyridinverbindungen, die entweder als Zwischenprodukte im Nikotinstoffwechsel von Säugtieren identifiziert oder vermutet wurden oder im Tabak bzw. Tabakrauch vorhanden sind, wurden mit Hilfe von Gaschromatographie und damit gekoppelter Massenspektrometrie untersucht. Die massenspektrometrischen und gaschromatographischen Daten dieser Verbindungen, welche eine Grundlage für weitere Untersuchungen der Pyridinverbindungen in pflanzlichen und tierischen Geweben bieten, werden dargelegt. Die Verwendbarkeit dieser Daten bei der Untersuchung des Nikotins und seiner Metaboliten in den Körperflüssigkeiten des Menschen wird gezeigt anhand der Bestimmung des Nikotins im Blutplasma von zwei Rauchern unter Benutzung von deuteriertem Nikotin und der Verwendung quantitativer Massenspektrometrie.

RESUME

On a étudié à l'aide de chromatographie en phase gazeuse couplée à un spectromètre de masse différents composés pyridiniques, qui sont identifiés ou impliqués comme intermédiaires dans le métabolisme de la nicotine chez les mammifères ou présents dans le tabac ou la fumée de tabac. On a donné les résultats de spectrométrie et de chromatographie en phase gazeuse de ces composés, résultats sur lesquels peuvent s'appuyer des recherches plus avancées sur les composés pyridiniques dans le tissu végétal ou animal. On a illustré l'utilité de ces résultats par l'étude de la nicotine et de ses métabolites dans les liquides humains: on a déterminé la nicotine dans le plasma de deux fumeurs, par spectrométrie de masse quantitative à l'aide de nicotine marquée au deutérium.

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Figure 1. Mass fragmentogram of extract of plasma from smokers with deuterated nicotine as added internal standard.

The upper and lower traces represent *m/e* 84 (nicotine) and *m/e* 86 (deuterated nicotine) ion current respectively; the left hand part of the figure gives data for the first smoker (95 ng/ml) and the right hand part is for the second (26 ng/ml).

Figure 1a. Mass spectrum of S-(-)-nicotine (Ia).

Figure 1b. Mass spectrum of 5-d₂-S-(-)-nicotine (Ib).

Figure 2. Mass spectrum of S-(-)-nicotine isomethiodide hydroiodide (II).

Figure 3a. Mass spectrum of S-(-)-nicotine-1'-oxide (III) (direct probe).

Figure 3b. Mass spectrum of S-(-)-nicotine-1'-oxide (III) (GC-MS).

Figure 4. Mass spectrum of S-(-)-nicotine-1,1'-dioxide (IV).

Figure 5. Mass spectrum of S-(-)-nornicotine (V).

Figure 6. Mass spectrum of myosmine (VI).

Figure 7. Mass spectrum of β -nicotyrine (VII).

Figure 11. Mass spectrum of S-(-)-cotinine methiodide (XI).

Figure 8. Mass spectrum of S-(-)-anabasine (VIII).

Figure 12. Mass spectrum of S-(-)-cotinine-1-oxide (XII).

Figure 9. Mass spectrum of S-(-)-methylanabasine (IX).

Figure 13. Mass spectrum of S-(-)-norcotinine (XIII).

Figure 10. Mass spectrum of S-(-)-cotinine (X).

Figure 14. Mass spectrum of (5S,3R)-3-hydroxycotinine (XIV).

Figure 15. Mass spectrum of dihydrometanicotine (XV).

Figure 16. Mass spectrum of trans-metanicotine (XVI).

Figure 17. Mass spectrum of cis-metanicotine (XVII).

Figure 18. Mass spectrum of 4-(3-pyridyl)butyric acid (XVIII).

Figure 19. Mass spectrum of methyl 4-(3-pyridyl)butyrate (XIX).

Figure 20. Mass spectrum of 4-(3-pyridyl)-4-oxobutyric acid (XX).

Figure 21. Mass spectrum of methyl 4-(3-pyridyl)-4-oxobutyrate (XXI).

Figure 22. Mass spectrum of 4-(3-pyridyl)-4-hydroxybutyric acid (XXII).

Figure 23. Mass spectrum of methyl 4-(3-pyridyl)-4-hydroxybutyrate (XXIII).

Figure 24. Mass spectrum of 5-(3-pyridyl)tetrahydrofuran-2-one (XXIV).

Figure 25. Mass spectrum of 4-(3-pyridyl)-4-oxobutyramide (XXV).

Figure 26. Mass spectrum of allohydroxycotinine (XXVI).

Figure 27. Mass spectrum of 3-pyridylacetic acid (XXVII).

Figure 28. Mass spectrum of methyl 3-pyridylacetate (XXVIII).

Figure 29. Mass spectrum of N-(3-pyridylacetyl)glycine (XXIX).

Figure 30. Mass spectrum of methyl N-(3-pyridylacetyl)glycinate (XXX).