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THE BIOSYNTHESIS OF [5'-^{14}C]COTININE AND OTHER RADIOLABELED NICOTINE METABOLITES

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SUMMARY

The present study describes the biosynthesis and isolation of the major radiolabeled nicotine metabolites formed using phenobarbitone (PB)-induced rabbit hepatic homogenates (10,000g fraction). The optimal incubation and extraction methods for cotinine formation from non-labeled nicotine were established. The biosynthesis and isolation of [5'-^{14}C]cotinine and other radiolabeled metabolites such as [2'-^{14}C]nornicotine and [4-^{14}C]-(3-pyridyl)-4-oxobutyric acid, from commercially available [2'-^{14}C]nicotine, were carried out using the developed methods. Cotinine was isolated using preparative silica gel TLC, whereas the other metabolites were obtained using a cation-exchange HPLC method.

This study showed that in addition to the two major metabolites (i.e. cotinine and nornicotine), 4-(3-pyridyl)-4-oxo-butyric acid, 3-hydroxycotinine, norcotinine, nicotine-1'-*N*-oxide and cotinine-1-*N*-oxide were also formed when PB-induced rabbit hepatic homogenates were used. Two further metabolites of unknown structure were detected. However, the isolation and further purification were only carried out on cotinine, nornicotine and 4-(3-pyridyl)-4-oxo-butyric acid.

Keywords: [2'-^{14}C]nicotine, [5'-^{14}C]cotinine, radio-HPLC

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INTRODUCTION

Nicotine metabolism is complex and its main metabolic pathways are well-established and have been reviewed[1-6]. The main metabolic pathways of (-)-nicotine metabolism are shown in Fig. 1. However, the enzymology involved in the further metabolism of many of the secondary nicotine metabolites and the formation of phase II metabolites has, to date, not been fully elucidated. In order to further study nicotine metabolic pathways and establish the enzymology involved, it was desirable to obtain the radiolabeled nicotine metabolites of interest such as cotinine, nornicotine and the acidic metabolites.

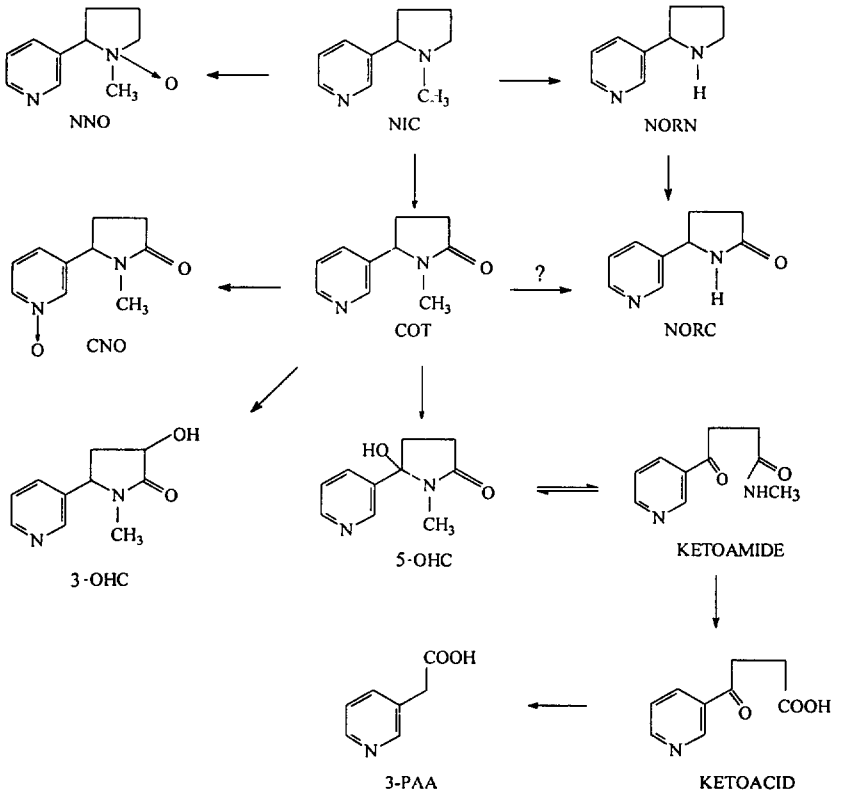

Fig. 1. The main metabolic pathways for the metabolism of (-)-nicotine in mammals.

(Note: certain intermediates have been omitted)

The use of radiolabeled nicotine metabolites should facilitate the analysis and provide evidence for the enzymology involved in some of the metabolic pathways. As the formation of cotinine from nicotine is a multi-step reaction, requiring both microsomal and cytoplasmic enzymes, a 10,000g hepatic preparation was used for this work. The present study describes (1) development of optimal *in vitro* incubation and extraction conditions for cotinine formation from nicotine, using phenobarbitone (PB)-induced rabbit hepatic homogenates; and (2) isolation of [5'-^{14}C]cotinine and some other radiolabeled nicotine metabolites formed *in vitro* from commercial [2'-^{14}C]nicotine.

EXPERIMENTAL

Materials

S-(-)-[Pyrrolidine-2'-^{14}C]nicotine-bi-(+)-tartrate salt, specific activity (58.8 mCi/mmol) and radiochemical purity (≥ 98%) was purchased from Chemsyn Science Laboratories, Kansas, USA. S-(-)-Nicotine (NIC) and 3-pyridylacetic acid (3-PAA) were purchased from Sigma Chem. Co., UK. S-(-)-Cotinine (COT), nornicotine (NORN), norcotinine (NORC), 3-hydroxycotinine (3-OHC), 5-hydroxycotinine (5-OHC), cotinine-1-*N*-oxide (CNO), 4-(3-pyridyl)-4-oxo-butyric acid (KETOACID), 4-(3-pyridyl)-4-oxo-*N*-methylbutyramide (KETOAMIDE) and nicotine-1'-*N*-oxide (NNO) were prepared according to established methods previously described[7-10]. Cocktail T (Scintran^{R}) was purchased from BDH Chem. Ltd., UK. Quickszint flow 302 was purchased from Zinsser Analytic, UK. Preparative silica gel TLC plates were prepared in our laboratories using Kieselgel 60 PF_{254} purchased from Merck Co., UK. A strong cation-exchange HPLC analytical column (Nucleosil SA 10 μm, 250 x 4.6 mm) was purchased from Phenomenex, UK.

HPLC Instrumentation

The HPLC system 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 1ml/min, was further connected to a Flow One Canberra Packard radiochemical detector (model A280), fitted with a 0.5 ml capacity radioactive flow cell for determination of radioactivity.

The HPLC analysis was performed using a strong cation-exchange HPLC analytical column (Nucleosil SA 10 μm, 250 x 4.6mm), with a guard column (100 x 4.6 mm) packed with pellicular cation-exchange material. Two isocratic HPLC mobile phase systems M1 and M2 were developed for the analysis and isolation of metabolites. Mobile phase M1 consisted of a mixture of sodium acetate buffer (0.2 M) and methanol (70:30 v/v) and triethylamine (0.02% v/v). The final pH of the mobile phase was adjusted to pH 4.5 using glacial acetic acid. M1 was developed specifically for the detection of nicotine metabolites. Mobile phase M2 consisted of a mixture of ammonium dihydrogen orthophosphate buffer (0.1M, 98% v/v), acetonitrile (1.9% v/v), triethylamine (0.1 % v/v); the final pH of the mobile phase was 5.5. This mobile phase M2 was used for the isolation of the nicotine metabolites that remained in the dichloromethane-extracted aqueous phase. A binary gradient mobile phase system was used for further purification of the metabolites in fraction (1) collected (see Fig. 9). This system consisted of two mobile phases i.e. (A) sodium acetate (0.1M, 99.5%) and triethylamine (0.5%), pH 4.5; and (B) mixture of mobile phase (A) and acetonitrile (10:1 v/v). The solvent gradient program was simplified to an initial 10 min linear gradient from 100% A to 95% A containing 5% B, followed by stepwise modification to 95% B, which was maintained for 40 min. Initial conditions were re-established by equilibrating the column with 100% A for 15 min. Throughout the 90 min run the solvent flow rate was 2 ml/min.

Determination of efficiency of radio-HPLC system

A stock solution of [2'-^{14}C]nicotine was prepared by taking [2'-^{14}C]nicotine bitartrate solution (20 μl) from the original purchased ampoule (6.4 mCi/ml in methanol solution), and making up to 500 μl with double distilled water. Aliquots (20 μl, 5.12 μ Ci) were taken from the stock solution and mixed with Cocktail T (5 ml) in polyethylene mini tubes inserted in glass scintillation vials and counted using the liquid scintillation counter (LSC, LKB 1209 Rackbeta Primo liquid scintillation counter) for comparison purposes.

Prior to analysis using the HPLC radiodetector, the HPLC column was conditioned for 30 min by passing mobile phase M1 through the HPLC-online radiodetector system for at least 30 min at a flow rate of 1.0 ml/min until equilibrium was reached within the analytical column. Quickszint flow 302 was used as it does not form a gel and has a high efficiency for radioactivity counting[11]. Quickszint flow 302 cocktail was pumped for 10 min at a flow rate of 2 ml/min. The HPLC eluent and the cocktail were mixed at the T-junction at a ratio of 1:3, respectively, producing a clear medium for radioactivity measurements. A background count of radioactivity was routinely performed in order to ensure that the HPLC column and the radiodetector flow cell were devoid of any radioactivity.

The same amount of the stock solution of [2'-^{14}C]nicotine (5.12 μCi), as for the previous LSC determination, was injected onto the radio-HPLC column. The cpm corresponding to nicotine, i.e. the area under the peak, was integrated using the A280 software provided for the radiochemical detector. The efficiency of the equipment was calculated as the ratio of cpm to the theoretical dpm.

Animal treatment and preparation of hepatic homogenates (10,000g fraction)

A male New Zealand White rabbit (approximately 2.5 kg) was supplied from King's College Animal Facility, London, U.K. The animal was administered 0.1% w/v

phenobarbitone (PB) in the drinking water for seven days and allowed free accesss to food. The procedure of induction with phenobarbitone (PB) was as described by McCoy *et al.*[12]. Prior to sacrifice, the animal was fasted for five hours and killed by cervical dislocation. The liver was removed, washed with isotonic potassium chloride solution, connective tissue removed, cut into small cubes and used to prepare homogenates and 10,000g preparation as described by Gorrod *et al.*[13]. The hepatic fraction was prepared at approximately 0.5 g original liver/ml. The hepatic homogenate preparation was then bubbled with nitrogen gas and stored in a Queue freezer at -80°C for later use. Prior to incubations, the cytochrome P450 content of the preparation was measured using the standard method[14].

Development of optimal incubation condition for non-labeled cotinine formation

Incubations were carried out in ten 25 ml Erlenmeyer flasks, each containing nicotine (3 μmol, 1 ml), cofactor solution (NADP, 1.57 mg; G-6-P, 3.04 mg; $MgCl_2$, 20 μmol, 6 μl and 0.2 M phosphate buffer, pH 7.4 to a final volume of 2 ml) and 10,000g PB-induced rabbit hepatic homogenate (0.5 g original liver/ml, 1 ml). The open-flask incubations were carried out, in duplicate, in a shaking incubator, at 37°C for up to 100 min using a reported method[15]. An extra 0.5 ml of the cofactor solution was added at 20 min and in succession, every 20 min, until 80 min incubation which was continued until 100 min. This meant that the incubation terminated at 20 min contained 2.0 ml of the cofactor solution and that terminated after 100 min had a cofactor volume of 4.0 ml. This process resulted in increased volume of incubates, increased cofactor used and allowed increased incubation time. Two flasks were removed from the incubator at each 20 min interval and the protein precipitated with 4N HCl solution (1 ml). The flasks were shaken and then centrifuged for 10 min at 4,000 rpm using a bench-top centrifuge (Centour, MSE). The resultant supernatant was adjusted to pH 10 with 4N NaOH solution and extracted with dichloromethane

(CH_2Cl_2, 3 x 5 ml). The organic phases were combined and tartaric acid solution (30 μ mol, 100 μl) was added before evaporation to dryness at 35°C, using a Vortex evaporator. The dried residues were reconstituted with double distilled water (50 μl). Aliquots (20 μl) of the concentrates were analysed using HPLC, coupled with a multiple-wavelength UV detector (Rapiscan, Severn Analytical) and monitored between 220 nm and 300 nm.

The effect of pH on the solvent extraction of cotinine

Stock solutions of nicotine (1 mg/ml) and cotinine (0.5 mg/ml) were prepared. Aliquots (100 μl) were taken from these solutions and placed in each of five screw-top tubes. The contents in each tube were separately adjusted to pH 3.5, 4.5, 5.0, 5.5, and 6.0 with 1M HCl. Subsequently, they were extracted, evaporated and analysed using the procedures described above.

Isolation of biosynthetic [5'-^{14}C]cotinine using TLC

Each ampoule as received from the supplier contained 1 mCi of [2'-^{14}C]nicotine bitartrate salt (2.85 mg of the salt equivalent to 1mg nicotine free base). An amount of radiolabeled nicotine (equivalent to 1 μmole of nicotine free base i.e 58.8 μCi) was diluted with water to 23 ml and 1ml of this solution was added to each of twenty-three 100 ml Erlenmeyer incubation flasks. The incubation contents (cofactor and homogenates used) of each flask were identical to those used previously for the non-labeled nicotine incubates. The maximum incubation time was set for 60 min. The flasks were capped throughout the experiment in order to prevent loss of radioactive nicotine into the atmosphere. Subsequently, the pH of the final incubates were adjusted to 5.5, and the contents were transferred to eighteen tubes and extracted with CH_2Cl_2 (3 x 5 ml). The organic phase from all the tubes was combined and divided between two test tubes and evaporated to dryness. Each tube containing the dried

residue was reconstituted with double distilled water (0.5 ml). The concentrates were applied to two preparative silica gel TLC plates (Kieselgel 60 PF_{254}, 20 x 20 cm, thickness 0.5 mm), as thin bands with authentic cotinine applied alongside. The plates were developed using a mobile phase consisting of butan-1-ol: acetone: glacial acetic acid: ammonium hydroxide solution (30%): water (70: 50: 18: 1.5: 60 v/v). The developed plates were air-dried in a fume-cupboard, and examined under UV light (254 nm). The band on the plate which had the R_f value corresponded to authentic cotinine was marked. The silica material was scraped off the plate and collected. Acetone (100 ml) was added to the collected silica material for the extraction of cotinine, the mixture well-stirred and filtered. The filtrate was evaporated to dryness using a Vortex evaporator, then reconstituted with double-distilled water (1 ml). An aliquot (10 μl) was analysed by radio-HPLC, using mobile phase M1, to check for purity and to determine the amount of radioactive cotinine obtained.

The aqueous phases, that remained after the dichloromethane extraction, were stored at 0°C until isolation of other radioactive metabolites present in the incubates (see Fig. 2).

Isolation of other radiolabeled nicotine metabolites

Nicotine metabolites that remained in the CH_2Cl_2-extracted incubation mixtures were further extracted using acetonitrile (CH_3CN). However, with this extraction method nicotine-1'-*N*-oxide formed was not extractable, and hence remained in the aqueous phase. Orthophosphoric acid (85%) was diluted with water (1:4), and aliquots (50 μl) were added to each of eighteen tubes containing the CH_2Cl_2-extracted aqueous phase (about 8 ml in each tube) and adjusted to pH 5.0 with 4N NaOH. Sodium chloride (2 g) was added to each tube and the metabolites were extracted with acetonitrile (4 x 5ml) by mixing on bench-top rockers for 20 min. The tubes were then centrifuged at 2,000 rpm for 10 min. The organic layers were collected, bulked and concentrated to 5

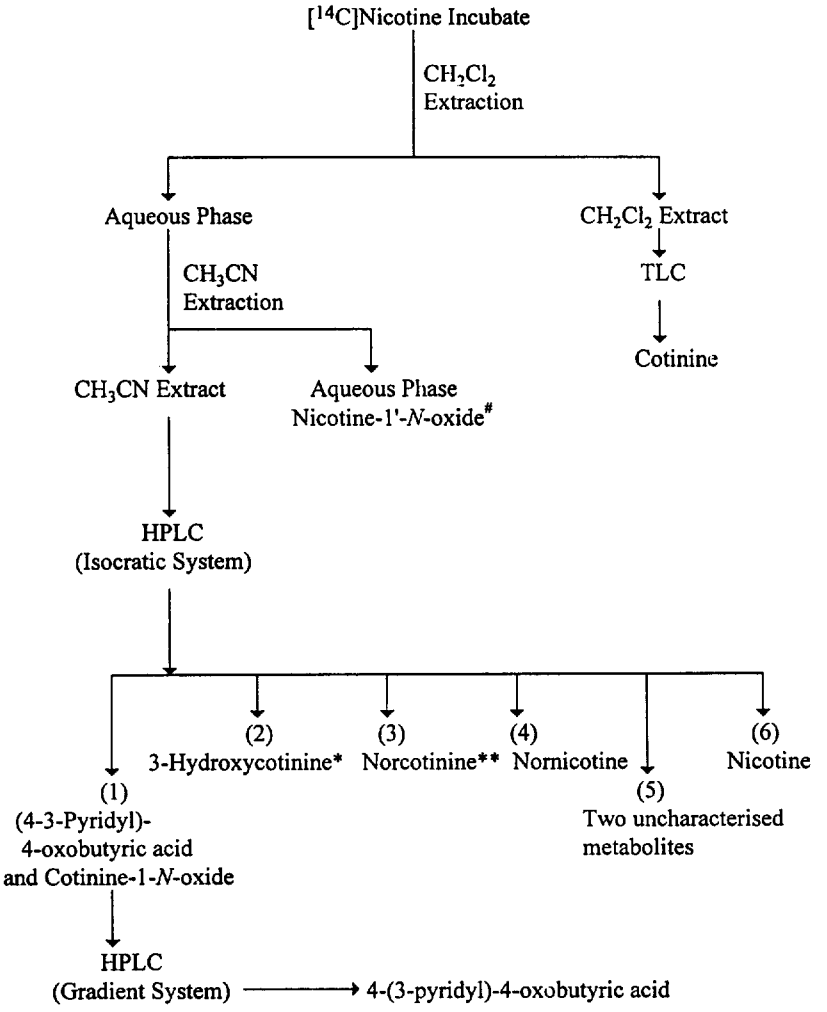

Fig 2. Separation and fractionation of [^{14}C]nicotine incubates of 10,000g PB-induced rabbit hepatic homogenates. # denotes some other nicotine metabolites may be present, * denotes a trace of cotinine-1-*N*-oxide was present, and ** a trace of cotinine was present.

ml under reduced pressure. The concentrated organic phases were transferred to a single screw-top tube and evaporated to dryness under a stream of nitrogen at 40°C in a water bath.

The dried extract was reconstituted with double-distilled water (1 ml). Metabolites present in the concentrate were isolated, by applying 100 μl aliquots, using the described HPLC system. The radiolabeled nicotine metabolites were separated using mobile phase M2. After identification by comparison of retention times with authentic standards, the various nicotine metabolites were collected in six fractions and monitored only by UV detection i.e. the eluent was not mixed with the Quickszint flow 302 cocktail. In order to obtain higher amounts of metabolites, a 200 μl injection loop was used instead of the 20 μl loop, which was used previously for routine analysis. The metabolites were collected and assigned as fractions 1 to 6. Some of the fractions were a mixture of two or more metabolites. Only those fractions that contained the major compounds of interest were further purified into individual compounds. Specifically, fraction 1 contained the most polar and acidic metabolites of nicotine. These metabolites could not be separated by the isocratic HPLC system using mobile phase M2 and therefore, the binary gradient HPLC system was used.

Separation of fraction (1) into individual radiolabeled compounds

The collected fraction (1), approximately 500 ml, was divided into Sovirel tubes and evaporated to dryness using a Vortex evaporator at 30°C. The dried residues were washed with absolute ethanol (2 x 5ml), and the ethanolic solution transferred into two Sovirel tubes. The tubes were centrifuged at 3,000 rpm using a bench-top centrifuge to remove inorganic salts derived from the mobile phase, and again evaporated to dryness. Subsequently, 5 ml of a mixture of acetonitrile and absolute ethanol (3:1) was added to each tube, mixed and combined. The solvent was further evaporated to dryness and reconstituted with double-distilled water (1.2 ml). The metabolites were isolated using the previously described binary gradient HPLC system. The fraction that had a component with a retention time that corresponded to 4-(3-pyridyl)-4-oxobutyric acid was collected.

Treatments on fractions 2 to 6

Fraction 2 (16-20 min) corresponded to a mixture of 3-hydroxycotinine, cotinine-1-*N*-oxide and some cotinine. Fraction 3 (21-27 min) consisted of norcotinine and some cotinine which was not completely extracted in the previous procedure. Fraction 4 (28-35 min), contained a metabolite which had an Rt that corresponded to that of authentic nornicotine, and fraction 5 (37-48 min) consisted of two radiolabeled peaks which were not further characterised. Fraction 6 (48-80 min) consisted of only unmetabolised nicotine. Fractions 2, 3 and 5 were not further separated into individual metabolites, but kept frozen for future use.

RESULTS AND DISCUSSION

The efficiency of the HPLC-radiodetector used was determined to be 85%. This efficiency factor was then taken into account in all further quantitative work.

Fig. 3 shows graphs of peak area ratio (PAR x100) of cotinine and nornicotine to unmetabolised nicotine versus incubation time. The rate of cotinine formation was initially linear, and only started to plateau after 60 min of incubation. It appeared that the formation of cotinine was saturable under these conditions, i.e. a further addition of cofactor and increased incubation time had no effect on the further generation of cotinine from nicotine. In the case of nornicotine formation, the rate of nornicotine formed was the same as that of cotinine in the first 20 min of incubation. However, in the next 20 min, the amount of cotinine formed was almost twice that of nornicotine. The formation of both cotinine and nornicotine from nicotine reached their maxima at 60 min of incubation under the present incubation conditions. Hence, an incubation time of 60 min was used for the later biosynthesis of radiolabeled cotinine and nornicotine. At this time, nicotine was predominantly metabolised to cotinine (27.5%) and nornicotine (15.9%). The ratio of cotinine to nornicotine formed was almost 2:1

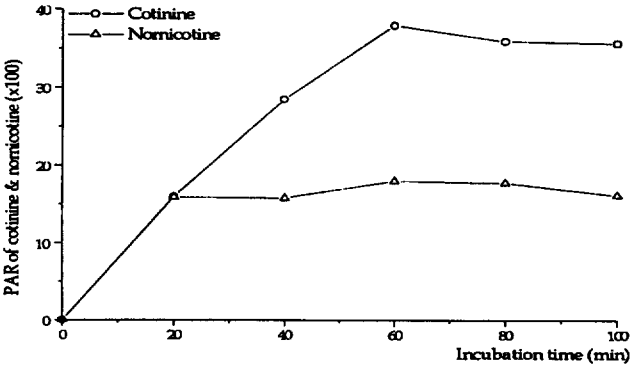

Fig. 3 Graphs of peak area ratios (PAR x100) of cotinine and nornicotine formed to unmetabolised nicotine versus incubation time using PB-induced rabbit hepatic homogenates.

in the incubates after 60 min. Using the incubation method of Seago and Gorrod[15,16] some 57% of the nicotine used as substrate, was either not metabolised or biotransformed to other metabolites quantitatively less important than cotinine (data not shown).

An HPLC chromatogram of six non-labeled authentic standards is shown in Fig. 4. HPLC analysis, using mobile phase M1, of the 60-min dichloromethane extracts revealed that, at least six metabolites of nicotine were formed. These metabolites corresponded to 3-hydroxycotinine (1, RT = 8.4 min), norcotinine (2, RT = 11.1 min), cotinine (3, RT = 12.6 min), nornicotine (4, RT = 23.4 min), nicotine-1'-*N*-oxide (5, RT = 28.5 min) and an uncharacterised compound (a, RT = 21.0 min), as shown in Fig 5). Only a trace of nicotine-1'-*N*-oxide was extractable using the dichloromethane extraction method. Since the main aim of the study was to collect cotinine, the further characterisation of compound (a) was not carried out.

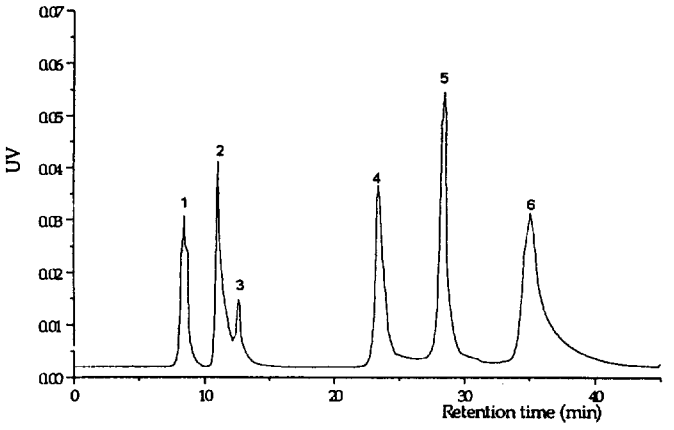

Fig. 4 HPLC chromatogram of five non-labeled authentic nicotine metabolite standards plus nicotine: 3-hydroxycotinine (1), norcotinine (2), cotinine (3), nornicotine (4), nicotine-1'-*N*-oxide (5) and nicotine (6), using mobile phase M1.

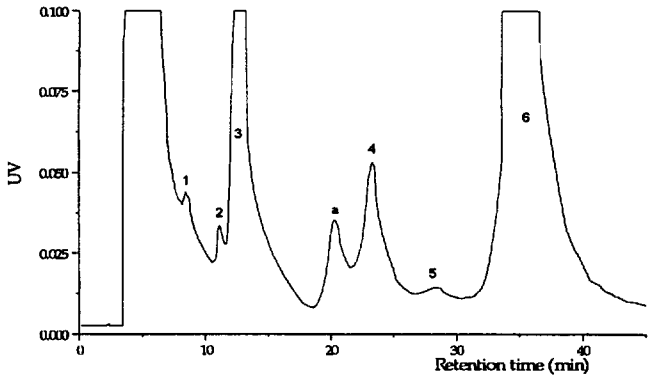

Fig. 5 HPLC chromatogram of non-labeled nicotine and metabolites present in dichloromethane extracts, using mobile phase M1, when PB-induced rabbit hepatic homogenates were used as enzyme source. a = an uncharacterised compound.

Further analysis of the eluent using a Rapiscan detector confirmed that cotinine and nornicotine were the major *in vitro* nicotine metabolites present in the DCM extracts. The UV spectra of these two metabolites and their authentic standards are shown in Fig. 6.

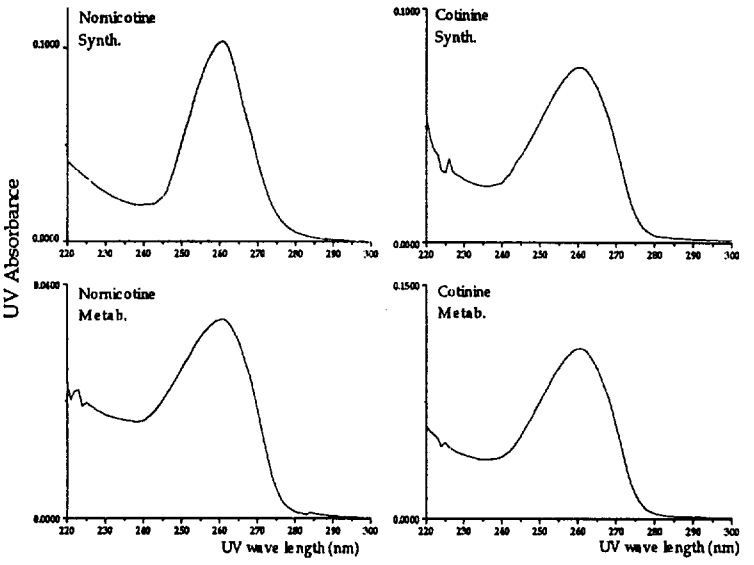

Fig. 6 Rapiscan UV spectra of non-labeled synthetic standards of nornicotine and cotinine (top) and metabolite peaks 3 and 4 (bottom).

HPLC analysis of the extracts after pH adjustment of authentic nicotine and cotinine solutions showed that pH 5.5 was the pH of choice for extraction of maximum amount of cotinine, and retained most of the nicotine in the aqueous phase (data not shown). For isolation of cotinine, thin-layer chromatography (TLC) was used, whilst in the case of other metabolites, quantitatively less than cotinine, a high-performance liquid chromatography (HPLC) was developed and used. The radio-HPLC chromatogram obtained from the cotinine purification procedures indicates that the TLC-collected radioactive compound was cotinine (Fig 7) as shown by R_f and UV spectra. However, a slightly broad tailing was observed which indicated that it was not absolutely pure. From the radio-HPLC chromatogram of radiolabeled cotinine collected (Fig. 7) it was estimated that it was about 85% pure. No further purification was carried out. The amount of radiolabel present in the cotinine obtained, was calculated to be approximately 15 μCi; this represents approximately 26% conversion of nicotine to cotinine.

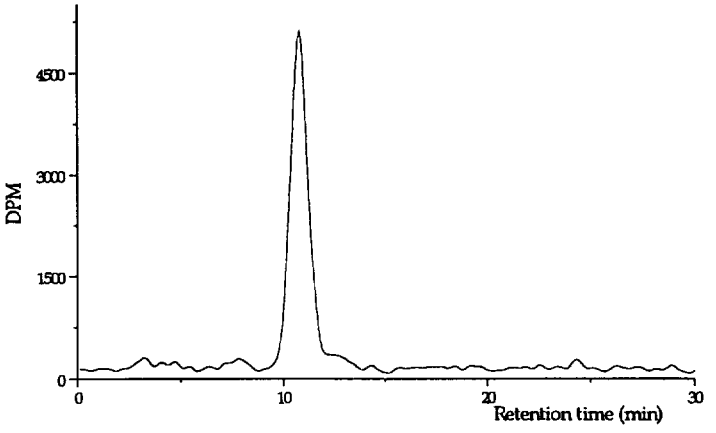

Fig. 7 Radio-HPLC chromatogram of radiolabeled cotinine isolated as a metabolite of nicotine, using mobile phase M1 as described in the text.

After extraction of cotinine, the bulked aqueous phase was further fractionated into six fractions using HPLC.The separation of fractions 1 to 6 was monitored only by using the UV detector, in order to avoid the contamination of collected fractions by the scintillant used for radio-detection (Fig. 8).

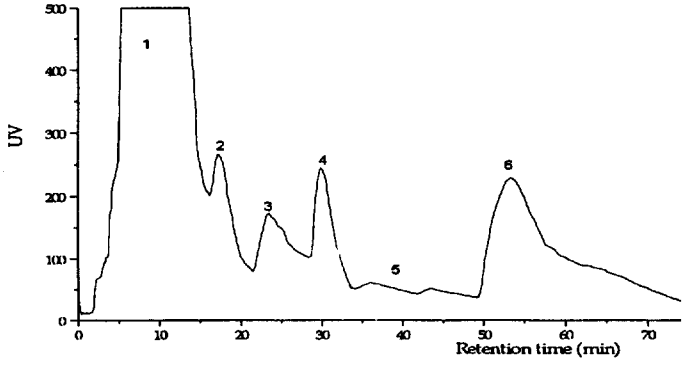

Fig. 8 UV-HPLC chromatogram of radiolabeled nicotine metabolites after the extraction of cotinine, collected as fractions (1-6), using mobile phase M2.

The UV- and radio-HPLC chromatograms of fraction 1 analysed using the described binary gradient HPLC system are given in Fig. 9 and show that other than 4-(3-pyridyl)-4-oxobutyric acid (with a Rt of 51.7 min), cotinine-1-*N*-oxide, norcotinine and cotinine are also present in the fraction. The calculated amount of radioactivity which corresponded to 4-(3-pyridyl)-4-oxobutyric acid was approximately 1 μCi. This represents approximately 2% conversion of nicotine to 4-(3-pyridyl)-4-oxobutyric acid. The UV- and radio-HPLC chromatograms of the isolated 4-(3-pyridyl)-4-oxobutyric acid are shown in Fig. 10.

The presence of cotinine and norcotinine could be due to contamination from the previous HPLC runs (see Fig. 9B). Since some cotinine is also present in fractions 1

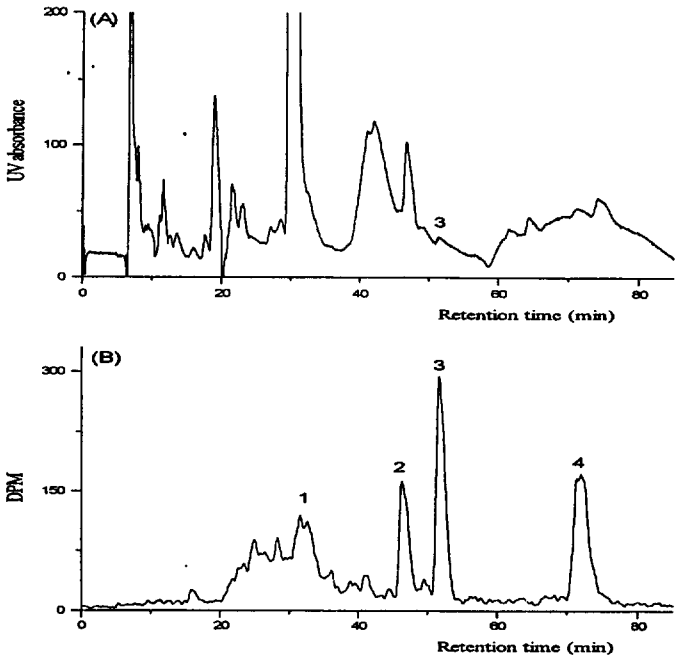

Fig. 9 UV- and radio-HPLC of fraction 1 before [4-^{14}C]-(3-pyridyl)-4-oxobutyric acid separation using the described binary gradient HPLC system: (A) UV-HPLC spectrum; (B) Radio-HPLC spectrum. Peak 1 = cotinine-1-*N*-oxide, 2 = norcotinine, 3 = 4-(3-pyridyl)-4-oxobutyric acid and 4 = cotinine.

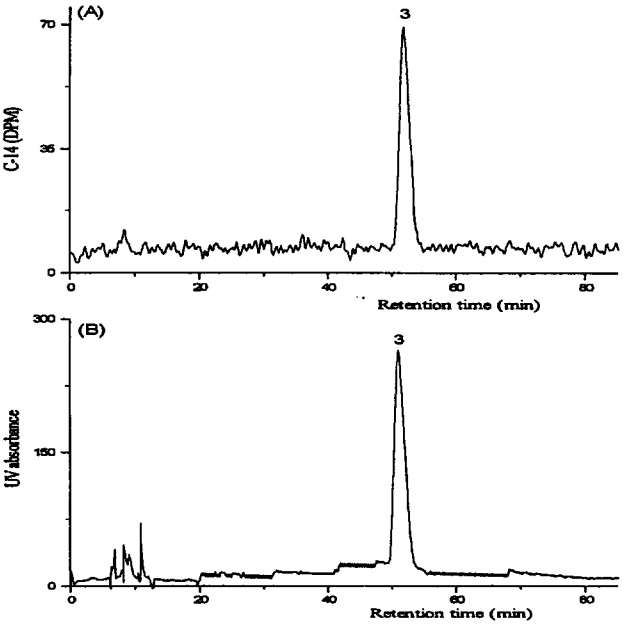

Fig. 10 Radio-HPLC chromatogram of [4-^{14}C]-(3-pyridyl)-4-oxobutyric acid (3) isolated from fraction 1 (A), and UV-HPLC chromatogram of authentic 4-(3-pyridyl)-4-oxobutyric acid (B).

and 3, this means that the true conversion of radiolabeled nicotine to cotinine is even higher than that calculated from the dichloromethane extract.

Fraction 2 (16-20 min) corresponded to a mixture of 3-hydroxycotinine, cotinine-1-*N*-oxide and a trace of cotinine.

Fraction 3 (21-27 min) consisted of norcotinine and and some cotinine which might not have been completely extracted in the dichloromethane extraction procedure.

Fraction 4 (28-35 min), which had a Rt that corresponded to that of authentic nornicotine, was collected using HPLC. It was shown to be relatively pure radioactive nornicotine (5.7 μCi). This represents approximately 10% conversion of nicotine to nornicotine. The radio-HPLC chromatogram of the radiolabeled nornicotine collected is shown in Fig. 11.

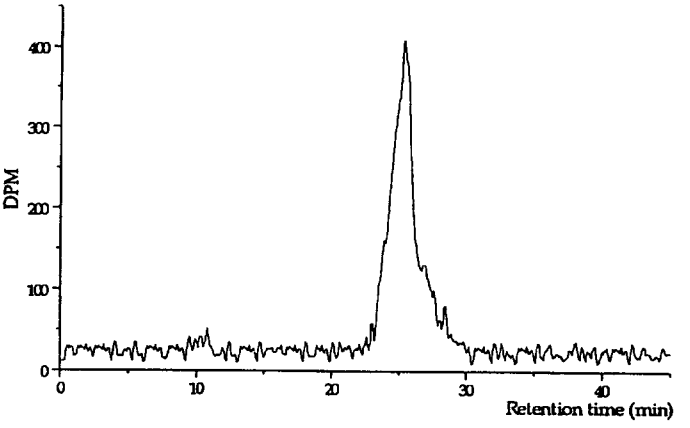

Fig. 11 Radio-HPLC chromatogram of radiolabeled nornicotine isolated from fraction 4.

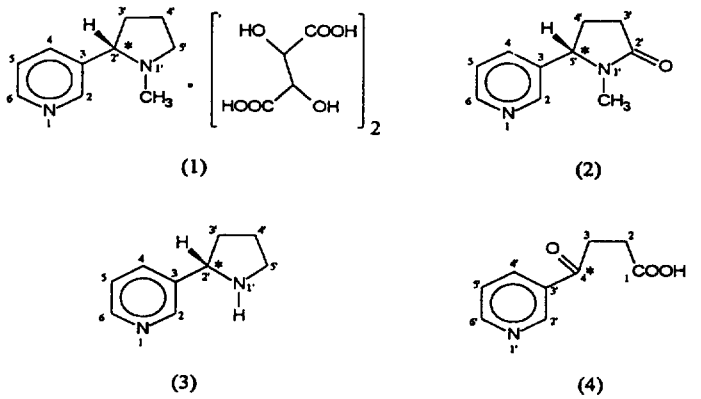

Fig. 12 The structures and position of radiolabel of S-(-)-[2'-^{14}C]nicotine bitartrate salt (1), S-(-)-[5'-^{14}C]cotinine (2), S-(-)-[2'-^{14}C]nornicotine (3) and [4-^{14}C]-(3'-pyridyl)-4-oxo-butyric acid (4).

Fraction 5 (37-48 min), consisted of materials which gave two peaks and was not further characterised, whilst fraction 6 (48-80 min) consisted only of unmetabolised nicotine. Fractions 2, 3 and 5 were not further separated into individual metabolites,

but kept frozen for future use. It is likely that these fractions contained 4-(3-pyridyl)-4-oxo-N-methylbutyramide (KETOAMIDE) as this a known metabolite of cotinine in the rabbit[8].

In this paper we present a relatively simple biosynthetic method of preparing radiolabeled metabolites of nicotine. Although chemical synthesis of [^{14}C]cotinine from [^{14}C]nicotine has been reported,[17] the biosynthetic preparations of S-(-)-[5'-^{14}C]cotinine, S-(-)-[2'-^{14}C]nornicotine and [4-^{14}C]-(3'-pyridyl)-4-oxo-butyric acid (4) have not been reported. The structures and position of radiolabel on nicotine and the isolated nicotine metabolites are shown in Fig. 11. The label on the 2'-position has proven to be useful, since further degradation of nicotine metabolites still provides radiolabeled products. The analytical methods developed provided a 'clean' and effective separation of the major nicotine metabolites. The purity of the compounds isolated was sufficient for other uses. [5'-^{14}C]Cotinine was used as a substrate for microsomal incubations [7]. The radiolabeled acidic metabolite was used in an attempt to elucidate the intermediate stages of the metabolism of nicotine-derived 4-(3-pyridyl)-butyric acid. Although *in vitro* metabolic studies of the 4-(3-pyridyl)-4-oxobutyric acid using various animal hepatic preparations have been carried out[18], the use of radiolabeled 4-(3-pyridyl)-4-oxobutyric acid avoids analytical problems associated with poor UV absorption of the acid metabolites such as 4-(3-pyridyl)-4-hydroxybutyric acid and 3-pyridylacetic acid. In this study, neither 4-(3-pyridyl)-butyric acid nor 3-pyridylacetic acid were formed from nicotine using PB-induced rabbit hepatic homogenates. These two nicotine metabolites would be expected to be present in fraction (1) if they were formed. Alternative pathways to the formation and degradation of norcotinine, as suggested by Gorrod[5,8] can now be evaluated by studying the metabolism of the isolated radiolabeled nornicotine and cotinine, since sensitive analytical procedures have now been developed for these radiolabeled

compounds. The methods developed should allow the isolation of other radiolabeled metabolites of nicotine and aid the establishment of the complete metabolic pathway of the ubiquitous xenobiotic.

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REFERENCES

1. Gorrod, J. W. and Jenner, P. - Essays in Toxicology, Academic Press, Orlando, FL, 6: 35 (1975)
2. Schievelbein, H. - Pharmac. Ther., 18: 233 (1982)
3. Nakayama, H. - Drug Metab Drug Interact. 6: 95 (1988)
4. Kyerematen, G. A. and Vesell, E. S. - Drug Metab. Rev. 23: 3 (1991)
5. Gorrod, J. W. - Nicotine and related alkaloids (J.W.Gorrod & J. Wahren) Chapman & Hall, London, 31 (1993)
6. Gorrod, J. W. - Nicotine : A case study in metabolic complexity, Eur. J. Drug Metab. Pharmacokinet. Special Issue, 5 (1995)
7. Aislaitner, G., Li, Y. and Gorrod, J. W. - Med. Sci. Res. 20: 897 (1992)
8. Li, Y. and Gorrod, J. W. - Xenobiotica, 24: 409 (1994)
9. Craig, J.C. and Purushothaman, K.K. - J. Org. Chem., 35: 1721 (1970)
10. Nguyen, T.L., Dagne, E., Gruenke, L., Bhargava, H. and Castagnoli, N. - J. Org. Chem., 46: 758 (1981)
11. Jahanshahi, M., Kajbaf, M. and Gorrod, J. W. - Progress in Pharmacology and Clinical Pharmacology, Verlag, Stuttgart, New York, 8: 41 (1991)
12. McCoy, G. D., DeMarco, G. J. and Koop, D. R. - Biochem. Pharmacol. 38: 1185 (1989)

13. Gorrod, J. W., Temple, D. J. and Beckett, A. H. - Xenobiotica 5: 453 (1975)

14. Omura, T. and Sato, R. - J. Biol. Chem. 239: 2370 (1964)

15. Seago, A. and Gorrod, J. W. - J. Pharm. Pharmacol. 39: 84 (1987)

16. Seago, A. and Gorrod, J. W. - Biological Oxidation of Nitrogen in Organic Molecules (J.W. Gorrod & L.A. Damani). Ellis Horwood, VCH Verlagsgesellschaft (1985)

17. Desai, D. H., Djordjevic, M. V. and Amin, S. - J. Labelled Comp. and Radiopharm., 29: 259 (1991)

18. Sai, Y. and Gorrod, J. W.- Drug Metab Drug Interact, 12: 93 (1995)

