J. Pharm. Pharmacol. 1988, 40: 862–869
Received March 9, 1988

© 1988 J. Pharm. Pharmacol.

Enantioselective Metabolism During Continuous Administration of *S*-(−)- and *R*-(+)-Nicotine Isomers to Guinea-Pigs

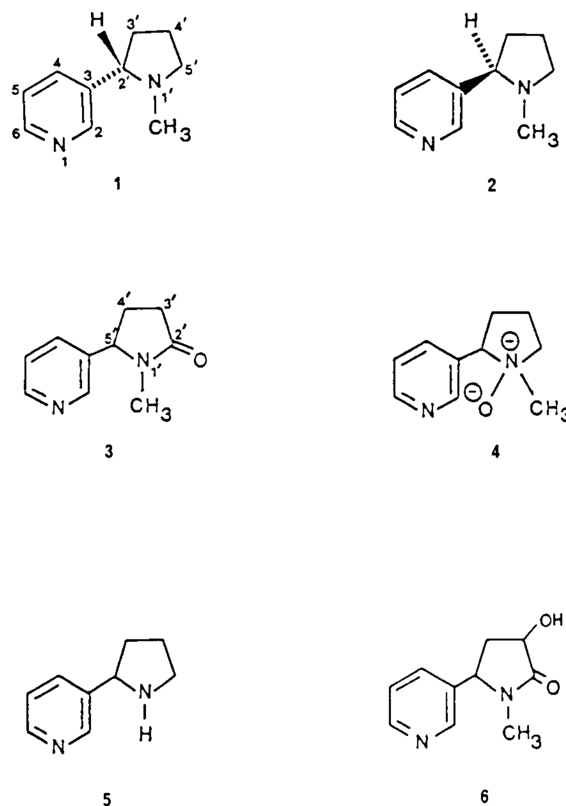
C. G. NWOSU, C. S. GODIN, A. A. HOUDI, L. A. DAMANI†, AND P. A. CROOKS

College of Pharmacy, Division of Medicinal Chemistry and Pharmacognosy, and Graduate Centre for Toxicology, University of Kentucky, Lexington, Kentucky 40536–0083, USA

Abstract—The *S*-(−)- and *R*-(+)-nicotine isomers were administered subcutaneously via Alzet osmotic pumps to male Hartley guinea-pigs (n = 5 with each isomer) over a 23-day period. Estimated dosage rate throughout the experiment was 0·6 mg^{-1}. Urine samples were collected over this time and the levels of urinary oxidative and *N*-methylated nicotine metabolites were measured by cation-exchange HPLC analysis. *S*-(−)-Nicotine formed only oxidative metabolites, whereas the *R*-(+)-isomer formed both oxidative and *N*-methylated metabolites. 3′-Hydroxycotinine and nicotine-1′-oxide were major metabolites of both enantiomers; cotinine and nornicotine were only minor metabolites. The major *N*-methylated metabolite of *R*-(+)-nicotine was *N*-methylnicotinium ion; *N*-methylcotininium ion and *N*-methylnornicotinium ion were also identified as metabolites of this nicotine isomer. Total *N*-methylated quaternary ammonium metabolites accounted for 15 to 20% of the administered dose of *R*-(+)-nicotine. An interesting enantioselective reduction in the percent of oxidative urinary metabolites formed from *S*-(−)-nicotine was observed over 23 days. This may indicate the enantioselective induction of an uncharacterized metabolic pathway for this nicotine isomer.

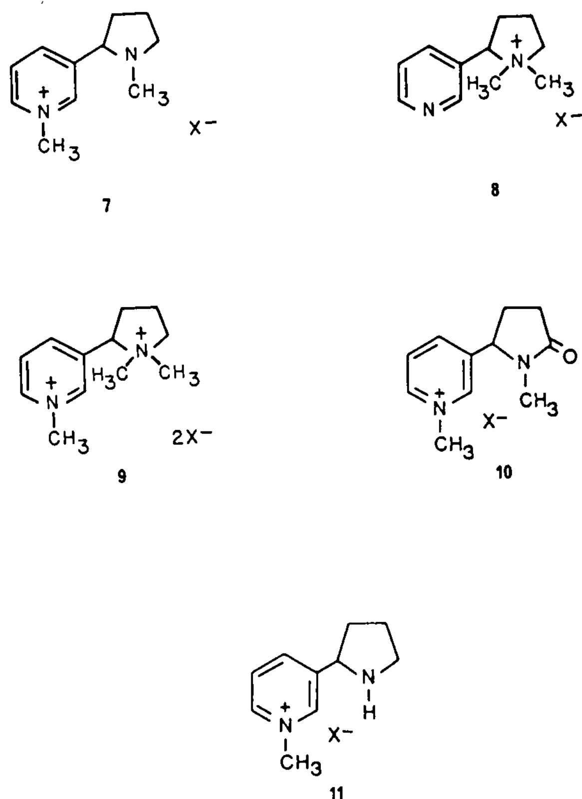
The metabolism of *S*-(−)-nicotine (1), the natural nicotine isomer present in tobacco, has been extensively studied over the last few decades (see Gorrod & Jenner 1975 and references cited therein). Cotinine (3) and nicotine 1′-*N*-oxide (4) have been reported to be "major" metabolites of *S*-(−)-nicotine in-vivo in a number of animal species, including man (Booth & Boyland 1970; Beckett et al 1971a,b). However, in most studies, those metabolites have not been quantified, and in studies where quantification was carried out they did not account for the complete dose of nicotine administered to the animal, indicating that a large percentage of the alkaloid is metabolized to, as yet, unidentified metabolites. Previous studies have indicated that racemization of *S*-(−)-nicotine during the course of tobacco smoking leads to the formation of significant quantities of *R*-(+)-nicotine (2)(4 − 12%) in mainstream cigarette smoke (Klus & Kuhn 1977). This has led to a renewed interest in the biotransformation of *S*-(−)- and *R*-(+)-nicotines, and recent studies in our laboratory have demonstrated that both qualitative and quantitative differences exist in the metabolism of these two enantiomers. For example, studies with [^{3}H-CH_3]nicotine enantiomers have indicated an enantioselectivity in the in-vivo *N*-methylation of nicotine in the guinea-pig (Cundy et al 1985). While both isomers were oxidized to cotinine and nicotine 1′-*N*-oxide, a significant fraction (∼50%) of the recovered radioactivity in the urine from experiments with each of the enantiomers was associated with unidentified polar metabolites (Cundy et al 1984, 1985).

The present investigation was undertaken to identify and quantify the polar urinary metabolites of nicotine enantiomers in male Hartley guinea-pigs, to obtain a more complete picture of the comparative biotransformation pathways for the two optical isomers, and also to examine the effect of the continuous infusion of each of the nicotine enantiomers on their urinary metabolite profiles.



Present address and correspondence to: P. A. Crooks, Southern Research Institute, 2000 Ninth Avenue South, PO Box 55305, Birmingham, Alabama, 35255-5305, USA.

† Visiting scientist from Chelsea Department of Pharmacy, King's College London (KQC), Chelsea Campus, Manresa Road, London SW3 6LX, UK.



Materials and Methods

Compounds

Gold Label triethylamine, analytical grade sodium acetate, sodium dodecylsulphate, Amberlite CG-50 resin, *S*-(−)-nicotine, and iodomethane were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). HPLC-grade methanol, HPLC-grade acetonitrile and sodium dihydrogen phosphate was purchased from Fisher Scientific (Pittsburgh, PA, USA). Commercial *S*-(−)-nicotine was vacuum-distilled before use. *R*-(+)-Nicotine was prepared from *R*,*S*-(±)-nicotine by the method of Bowman et al (1982). The optical purity of *R*-(+)-nicotine was determined to be >99% by polarimetric analysis on a Polyscience Model SR6 polarimeter, and by NMR spectroscopy using a chiral lanthanide shift reagent on a Varian XL 200 spectrometer (Crooks, Godin, Pool, unpublished observation). Nicotine 1′-*N*-oxide was prepared from *S*-(−)-nicotine by the method of Phillipson & Handa (1975), and consists of a mixture of the two possible diastereomers. *S*-(−)-Cotinine was a gift from Dr E. Bowman, Medical College of Virginia, Virginia Commonwealth University (Richmond, VA, USA). *R*,*S*-(±)-Nornicotine (5) was synthesized via the method of Jacob (1982). 3′-Hydroxycotinine (*cis*-isomer) (6) was synthesized essentially as described by Dagne & Castagnoli (1972), followed by epimerization to afford the corresponding *trans*-isomer. The iodide salts of the *N*-methyl (7, X = I), *N*′methyl (8, X = I) and *N*,*N*′-dimethyl (9, X = I) derivatives of the *R*-(+)- and *S*-(−)-nicotine enantiomers were prepared by the method of Seeman & Whidby (1976). *N*-Methylcotininium iodide (10, X = I) was prepared from *S*-(−)-cotinine and methyl iodide by the method of McKennis et al (1963). *N*-Methylnornicotinium iodide (11, X = I) was synthesized by the addition of iodomethane to a glacial acetic acid solution of *R*,*S*-(±)-nornicotine. All authentic standards were purified by recrystallization, vacuum-distillation or by preparative-HPLC, and fully characterized by chemical means.

Animals and drug administration

Animal experiments were carried out on three groups of five male Hartley guinea-pigs (728 ± 45g, age 26 weeks) (Hilltop Kennels, Campbellsville, KY, USA). Animals in group 1 received saline (control group), whereas those in groups 2 and 3 were administered *R*-(+)- and *S*-(−)-nicotine enantiomers, respectively. Solutions of the nicotine enantiomers were prepared by titrating aqueous solutions of each isomer (free base) to neutrality with concentrated HCl; these solutions were diluted with normal saline and filtered through a Type S filtration unit (Nalge Co., Rochester, NY, USA), to give solutions containing 400 mg mL^{-1} nicotine. Alzet Osmotic pumps, Model 2ML4 (Alza Corporation, Palo Alto, CA, USA) were loaded, either with saline, or with solutions of *R*-(+)- and *S*-(−)-nicotine enantiomers and equilibrated in sterile saline at 37°C for 4h before implantation. The animals were anaesthetized with sodium pentobarbitone (300 mg kg^{-1}) and the area above the scapula shaved and cleansed with Pharmadine (Sherwood Pharmaceutical Co., Makway, NJ, USA). An appropriately loaded osmotic pump was surgically implanted subcutaneously above the scapula in each of the animals. The incision was closed with silk, the animals allowed to recover from surgery (48h) and placed separately in a glass metabolism cage (Crown Glass Company, Somerville, NJ, USA) which incorporated an efficient urine and faeces separator. Urine samples were collected over 24h at various time periods during the 23-day experiment, and frozen (−20°C) until analysed.

HPLC analysis of nicotine metabolites

Standard calibration curves were generated for each of the nicotine metabolites as follows. Known amounts of each of the synthetic standards in distilled water were added to control guinea-pig urine to afford solutions with varying concentration ranges of each compound i.e.-nicotine 0·2–0·08 mg mL^{-1}, cotinine 0·2–0·8 mg mL^{-1}, nicotine 1′-*N*-oxide 0·2–0·8 mg mL^{-1}, nornicotine 0·02–0·08 mg mL^{-1}, 3′-hydroxycotinine 0·1–0·4 mg mL^{-1}, *N*-methylnicotinium ion (NMN) 0·06–0·23 mg mL^{-1}, *N*-methylcotininium ion (NMC) 0·012–0·48 mg mL^{-1}, *N*-methylnornicotinium ion 0·044–0·18 mg mL^{-1}. Each of these standard solutions were analysed twice (i.e., two replicate injections) by each of the three different HPLC systems (see later). The areas under the UV peaks were integrated, utilizing a reporting integrator (Hewlett-Packard Model 3390A), and area units (arbitary units) plotted against concentration (mg mL^{-1}). The entire calibration routine was performed three times (i.e., n = 3) with newly-weighed standards and control guinea-pig urine from different animals. In each case, linear reproducible calibration curves were obtained; regression analysis of the data (n = 6 for each concentration) gave a correlation coefficient of 0·99 in each case, with no significant inter- or intra-day variability. All urine samples used for calibration curves were centrifuged and/or filtered and 50 μL aliquots injected directly via a Rheodyne loop injector onto a

25 × 0·46 cm Partisil 10 SCX cation-exchange column (Whatman, Clifton, NJ, USA) with a 7 × 0·4 cm CSK-1 Whatman pellicular cation-exchange guard column. Analyses were carried out utilizing three different HPLC systems. In System 1 the mobile phase consisted of 0·3M sodium acetate–MeOH (70:30, v/v%), pH 4·5 with glacial acetic acid, at a flow rate of 2 mL min^{-1}. This system effected the separation of nicotine 1′-*N*-oxide (Rt = 8·1 min), nornicotine (Rt = 6·8 min), and nicotine (Rt = 10·3 min) in urine. However, it was not possible to use this system for the separation of cotinine and 3′-hydroxycotinine from the void peak (Rt = 2·4 min). The *N*-methylated derivatives were retained on the column during these analyses, and were periodically washed off the column using a mobile phase containing triethylamine (see System 3 below).

System 2 was a mobile phase of 0·3M sodium acetate–methanol (90:10, v/v%) for the analysis of the less cationic metabolites. This system effectively separated cotinine (Rt = 6·2 min) and 3′-hydroxycotinine (Rt = 4·1 min) from the void components. Nicotine, nicotine 1′-*N*-oxide, and nornicotine, had relatively long retention times in this system.

The identification and quantification of quaternary ammonium *N*-methylated metabolites of nicotine was achieved on a Partisil 10 SCX cation exchange column (25 × 0·46 cm) (Whatman) with a mobile phase of 0·3M sodium acetate–methanol (50:50) containing 2% v/v triethylamine, pH adjusted to 4·5 with glacial acetic acid (System 3), to elute these more strongly bound cationic species off the cation-exchange column. This system afforded a good separation of *N*-methylcotininium ion (Rt = 4·0 min)), *N*-methylnornicotinium ion (Rt = 7·5 min) and *N*-methylnicotinium ion (Rt = 9·6 min). The other cationic metabolites eluted in the void volume in this system.

Isolation and identification of 3′-hydroxycotinine and N-*methylcotininium salt as urinary metabolites of nicotine*

Isolation of the urinary metabolites having the same retention characteristics as authentic 3′-hydroxycotinine and NMC was achieved by preparative HPLC on a Magnum 9 Partisil-10 SCX column (50 × 0·9 cm) attached to a Partisil CSK1 pellicular cation-exchange guard column (14 × 0·4 cm). For the isolation of 3′-hydroxycotinine, the mobile phase of System 2 was used, whereas for the isolation of NMC, the mobile phase of System 3 was used. Desalting of the isolated metabolite fractions was carried out as previously described (Pool et al 1986). Structural analysis and identification of 3′-hydroxycotinine was carried out on a Finnigan 4000 GLC-mass spectrometer equipped with a glass jet separator, an INCOS data system, and utilizing a 3% OV methylsilicone capillary column. GLC separation was initially at 120°C followed by a temperature gradient of 20°C min^{-1} to 220°C. Analysis was carried out in the centroid positive ion mode. Structural identification of NMC was by two procedures. Firstly, the isolated metabolite was analysed by thermospray mass spectrometry on a Finnigan MAT 4600 mass spectrometer interfaced to a thermospray unit (Finnigan) and a Waters 6000 series pump, utilizing a Rheodyne 7125 loop injector. The liquid phase used in the thermospray unit was 0·1M ammonium acetate–methanol (50:50) at a flow rate of 1 mL min^{-1}. The vaporizer temperature was 150°C, and the aerosol temperature was 214°C. Secondly, sodium borohydride reduction of the metabolite in methanol–water was carried out, followed by GLC-mass spectrometric analysis of the more volatile reduction product (see Cundy et al 1985 for details of this procedure).

Results and Discussion

Three cation-exchange HPLC systems for the identification and quantitative analysis of nicotine metabolites have been developed (Fig. 1). System 1 proved to be satisfactory for the analysis of nicotine and its oxidative urinary metabolites, nicotine *N*′-1-oxide and nornicotine, while System 2 was required for the quantification of nicotine, cotinine and 3-hydroxycotinine. System 3 was also used for the quantification of *N*-methylated urinary metabolites of nicotine. The identity of both 3′-hydroxycotinine and *N*-methylcotininium salt were confirmed by mass spectrometric analysis after isolation by preparative HPLC. The former metabolite eluted with relatively little retention on Systems 1 and 3, and therefore was subjected to mass spectral analysis after isolation by preparative HPLC to determine its identity and purity (Fig. 2).

Isolation and identification of N-*methylcotininium salt as a urinary metabolite of nicotine*

N-Methylcotininium salt, although reported to be a urinary metabolite of nicotine in several studies (McKennis et al 1963; Cundy et al 1984, 1985), has never been fully identified by structural analysis. This isolated metabolite was therefore subjected to thermospray spectral analysis, and also to sodium borohydride reduction followed by GLC-mass spectral analysis of the more volatile reduction product (Fig. 3 and Scheme 1). The reduction procedure produced 1,4,5,6-tetrahydro-*N*-methylcotinine, as would be predicted from previous analogous studies (Cundy et al 1984; Sato & Crooks 1985) on *N*-methylnicotinium salt and *N*-methylnornicotinium salt, thus confirming the metabolite as *N*-methylcotininium salt.

Quantification of urinary metabolites of nicotine enantiomers during continuous nicotine administration

Fig. 4 provides data on the per cent recovery of total urinary metabolites from guinea-pigs that had been treated with either *S*-(−)-nicotine (A) or *R*-(+)-nicotine (B) at each of six equally spaced 24 h units over 23 days. Figs 4C and D represent the total percent of oxidative, and total percent of *N*-methylated metabolites, respectively, recovered in the urine after treatment with *R*-(+)-nicotine. Interestingly, based upon the amount of nicotine effluxed from the osmotic pump over the appropriate 24 h time units, there appears to be a continuous decrease in the total recovered urinary metabolites with increasing days of exposure to *S*-(−)-nicotine, but not with increasing days exposure to *R*-(+)-nicotine. Over days 2–3, 77% conversion of *S*-(−)-nicotine to oxidative metabolites in the urine was detected, whereas over days 22–23, only 43% conversion was observed. These data indicate that nicotine is probably not an inducer of hepatic oxidative enzymes, which is consistent with pre-

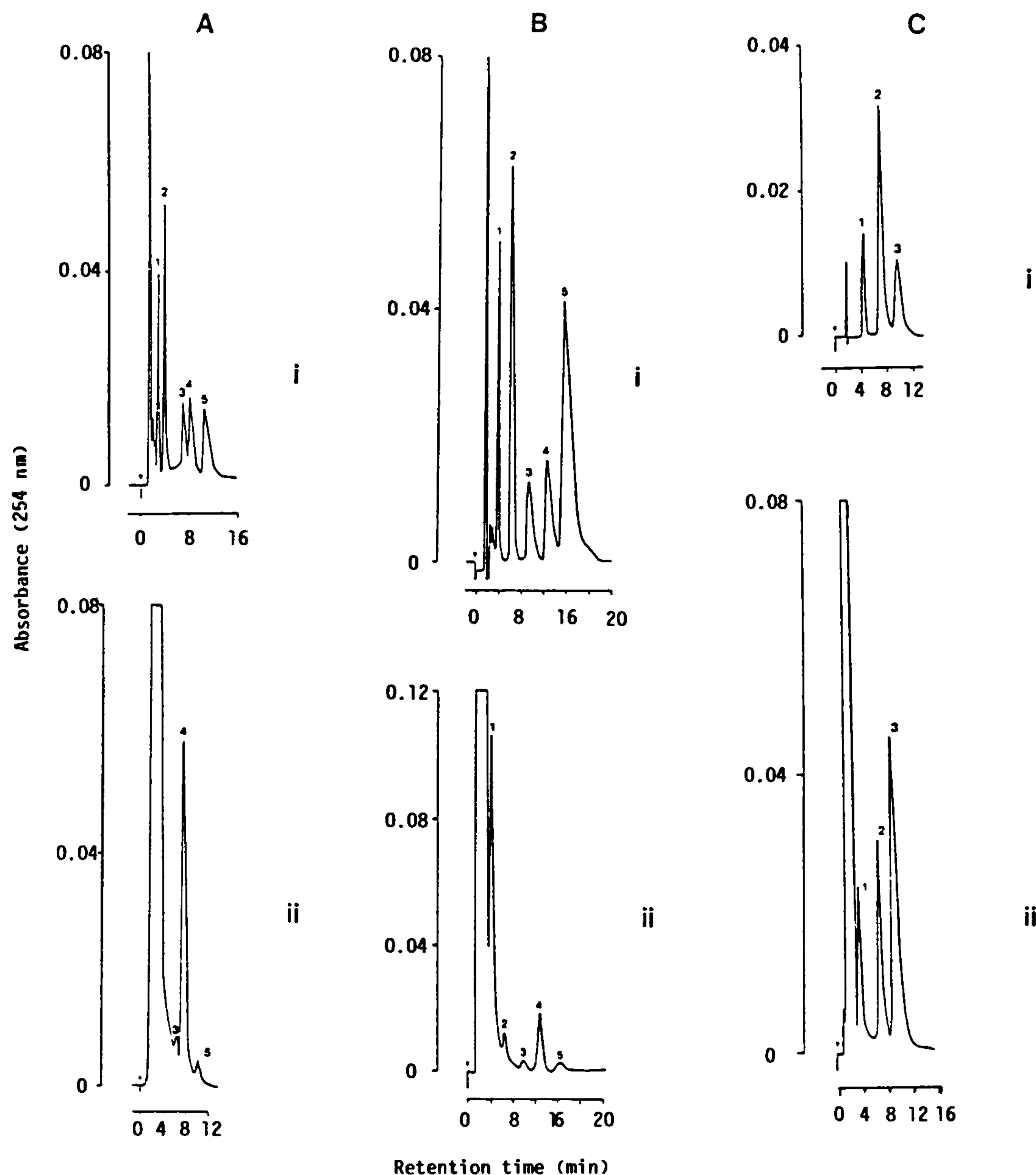


FIG. 1. Chromatograms obtained from the direct cation-exchange high performance liquid chromatographic analysis of a 24 h (14–15 day) urine sample obtained from a guinea-pig chronically dosed with *R*-(+)-nicotine. Panel A: HPLC analysis of authentic standards (i) and guinea-pig urines (ii) by System 1. Panel B: HPLC analysis of authentic standards (i) and guinea-pig urines (ii) by System 2. Panel C: HPLC analysis of authentic standards (i) and guinea-pig urines (ii) by System 3.

viously reported data (Graziano & Dorough 1984). In contrast, analysis of the urinary metabolites isolated from animals treated with *R*-(+)-nicotine gave relatively constant amounts of both oxidative and *N*-methylated metabolites throughout the 23 day analysis period. The amounts of the oxidative metabolites were relatively lower than those obtained from the *S*-(−)-isomer study, due to the former isomer also acting as a substrate for the *N*-methyltransferase pathway.

The enantioselective time-dependent decrease in oxidative metabolites observed in the urine of animals dosed with *S*-(−)-nicotine may be the result of an inducible, but as yet unidentified, pathway into which the oxidative products are channelled. At day 23 this pathway may be a major biotransformation route for *S*-(−)-nicotine, since less than 50% of the administered dose can be accounted for as urinary metabolites.

S-(−)-Nicotine metabolites

S-(−)-Nicotine, afforded only oxidative urinary metabolites over the 23 days studied. The percentage of a 24 h administered dose excreted in appropriately chosen 24 h urine samples over the 23 days, for each of the nicotine metabolites, is shown in Table 1. 3′Hydroxycotinine was the major metabolite in the urine of the *S*-(−)-nicotine-dosed guinea-pigs. The urinary levels of this metabolite decreased

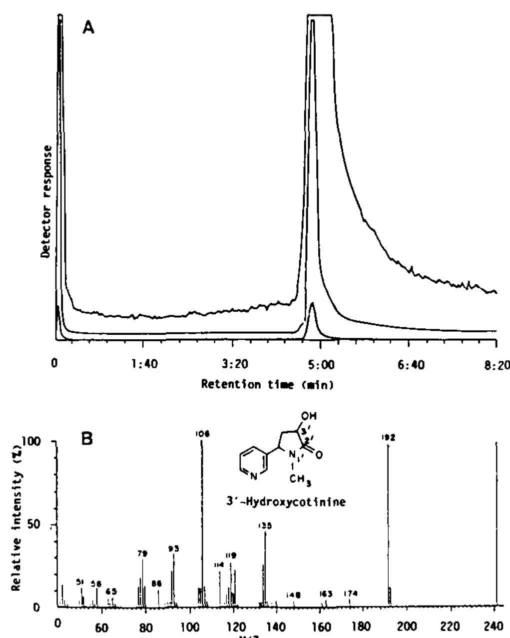
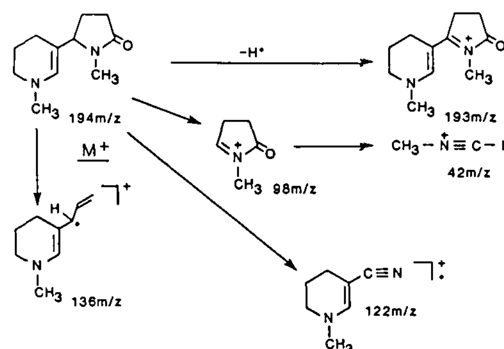


FIG. 2. Gas-liquid chromatogram (A) and mass spectrum (B) of 3'-hydroxycotinine isolated from the urine of *S*-(−)-nicotine-treated male Dunkin-Hartley guinea-pigs.

with increasing days exposure to constant *S*-(−)-nicotine infusion. On day 2 to 3, 3'-hydroxycotinine accounted for 43·29% of the total administered dose, while on day 22 to 23, this had decreased to 29·56%. This observation suggests an induction of an unknown pathway, which could reduce the production of this oxidative metabolite with time. 3'-Hydroxycotinine has not been previously reported as a major metabolite of *S*-(−)-nicotine, probably because the methods utilized in earlier studies (i.e. GLC) (Beckett et al 1971a) did not detect this metabolites due to its comparatively high polarity and low volatility. Obviously, more studies need to be done to identify and quantify other possible polar urinary metabolites of nicotine that may be eluting in the void peaks in this present study.



Scheme 1. Proposed electron impact mass spectral fragmentation pathways for 3,4,5,6-tetrahydro-*N*-methylcotinine, the sodium borohydride reduction product of *N*-methylcotininium salt.

Nicotine 1'-*N*-oxide was observed to be the second most important urinary metabolite of *S*-(−)-nicotine. Again, as was seen with 3'-hydroxycotinine, the amounts of this metabolite decreased with time; urinary levels at day 2 to 3, and day 22 to 23 were 28·55% and 12·53%, respectively, of the dose administered.

Cotinine is generally considered to be a major urinary metabolite of *S*-(−)-nicotine and urinary levels of this metabolite are often used as an indicator of plasma nicotine levels in smokers (Zeidenberg et al 1977; Matsukura et al 1979, 1984). However, in this study, cotinine was barely detectable as a urinary metabolite of *S*-(−)-nicotine in the continuously-dosed guinea-pig. This study, therefore, is consistent with the results from a previous radiolabelled study (Cundy et al 1985); both studies clearly show that cotinine is not a major urinary metabolite of nicotine in the guinea-pig. Nornicotine was also detected as a urinary metabolite of non-radiolabelled *S*-(−)-nicotine in continuously-dosed guinea-pigs. The amounts produced however, were low (ranging from 0·30% to 2·20% of the administered dose). This result is important, because in the radio-labelled studies previously described (Cundy et al 1985), it was not possible to detect this metabolite, since its

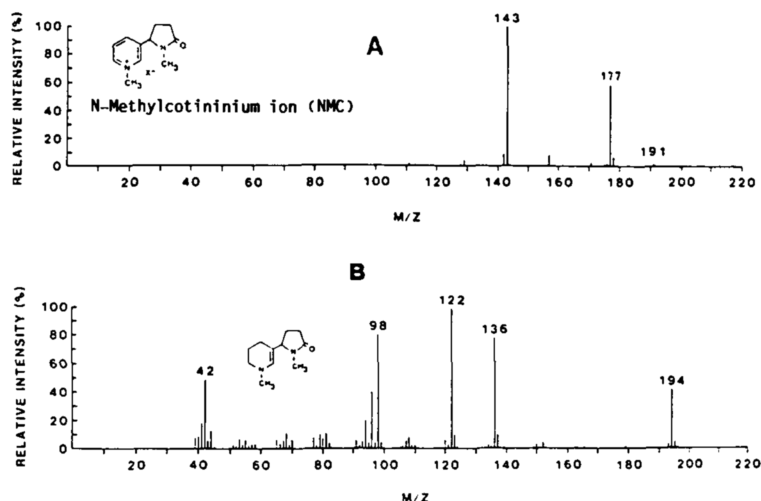


FIG. 3A. Mass spectrum of *N*-methylcotininium salt isolated from the urine of *R*-(+)-nicotine-treated male Dunkin-Hartley guinea-pigs.
B. Mass spectrum of the reduction product obtained after treatment of metabolically-formed *N*-methylcotininium salt with sodium borohydride in methanol-H_2O.

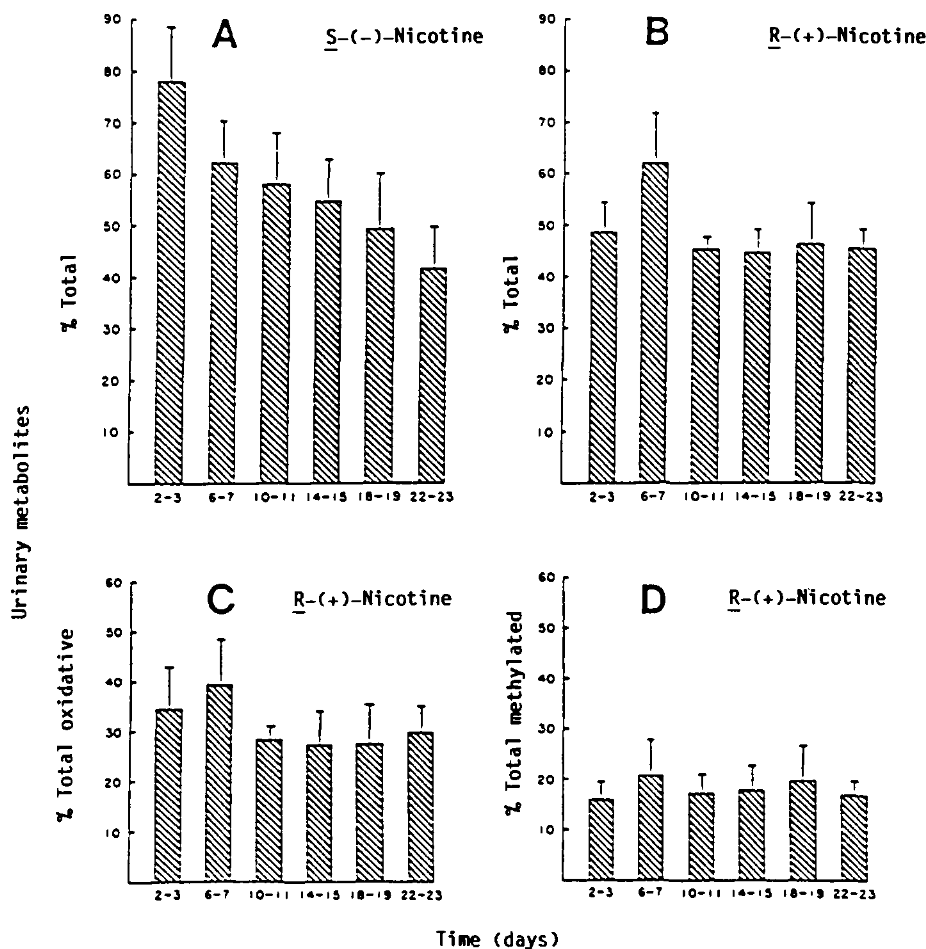


FIG. 4. Recovery of 24 h urinary metabolites with time.
A. Percent recovery of total identified urinary metabolites based upon dose displaced from osmotic pumps containing *S*-(−)-nicotine, in 24 h periods, over 23 days.
B. Percent recovery of total identified urinary metabolites based upon dose displaced from osmotic pumps containing *R*-(+)-nicotine, in 24 h periods, over 23 days.
C. Percent recovery of total oxidative urinary metabolites based upon dose displaced from osmotic pumps containing *R*-(+)-nicotine, in 24 h periods, over 23 days.
D. Percent recovery of methylated urinary metabolites based upon dose displaced from osmotic pumps containing *R*-(+)-nicotine, in 24 h periods, over 23 days.

formation results in the concomitant loss of the N'-CH_3 group that bears the tritium label.

Urinary *S*-(−)-nicotine levels were generally low (0·13% to 3·6% of the administered dose) in all the urine samples analysed. This finding is consistent with the previous data generated from the radiolabel study, and suggests that nicotine is rapidly and continuously metabolized in the chronically dosed guinea-pig.

R-(+)-Nicotine metabolites

Quantification of metabolites expressed as percentage of the 24 h dose excreted in appropriately chosen 24 h urine samples from guinea-pigs that had been continuously-dosed with *R*-(+)-nicotine, revealed that both oxidative metabolites (Table 2) and *N*-methylated metabolites (Table 3) were formed (see also Cundy et al 1985). 3′-Hydroxycotinine was also the major urinary metabolite in the *R*-(+)-nicotine-dosed guinea-pigs. However, in the latter study, the amounts were generally lower, probably because of the competing *N*-methylation pathway that operates for *R*-(+)-nicotine.

Nicotine 1′-*N*-oxide was the second most important urinary metabolite although urinary levels were lower than in the *S*-(−)-nicotine study, again because of the parallel pathway of *N*-methylation. Cotinine was barely detectable as a urinary metabolite of *R*-(+)-nicotine, which is consistent with the results from the radiochemical studies (Cundy et al 1985). Nornicotine was not detected as a urinary metabolite in the *R*-(+)-nicotine-dosed guinea-pig. Unchanged urinary *R*-(+)-nicotine levels were also barely detectable.

Quantification of the quaternary ammonium *N*-methylated urinary metabolites of *R*-(+)-nicotine in the continuously dosed guinea-pig revealed the following. *N*-Methylnicotinium ion was the major *N*-methylated urinary metabolite. Amounts of this metabolite were essentially constant from day 2 to 3 (7·38%) until day 22 to 23 (9·92%). *N*-methylnornicotinium ion was observed to be the second most abundant quaternary ammonium *N*-methylated urinary metabolite from the *R*-(+)-nicotine-dosed guinea-pig study. With the methodology used here, it is possible to detect this urinary metabolite which is not observed in

Table 1. Levels of oxidative metabolites of nicotine in urine samples from guinea-pigs dosed with *S*-(−)-nicotine, 12 mg/24 h, over a 23-day period.

Sampling time (days)	Percentage of 24 h dose excreted in 24 h urine samples as:				
	Nicotine[a]	Nornicotine[a]	Cotinine[a]	Nicotine *N*′-oxide[a]	3′-Hydroxycotinine[b]
2– 3	2·57±0·18[c]	2·20±0·08	n.d.[d]	28·55±10·57	43·29± 6·26
6– 7	0·13±0·01	0·30±0·08	0·51±0·01	27·98± 5·13	33·00± 2·45
10–11	n.d.	0·30±0·04	n.d.	22·15+ 2·50	35·92± 7·15
14–15	n.d.	n.d.	n.d.	21·96± 6·71	32·93± 5·76
18–19	3·6±2·12	0·30±0·03	0·38±0·03	17·22± 5·10	27·34± 5·76
22–23	n.d.	n.d.	0·51±0·07	12·53± 1·14	29·56±10·33

[a] analysis by cation exchange HPLC using Chromatographic System 1.
[b] analysis by cation exchange HPLC using Chromatographic System 2.
[c] results are means±s.e., n=5.
[d] not detected.

Table 2. Levels of oxidative metabolites of nicotine in urine samples from guinea-pigs dosed with *R*-(+)-nicotine, 12 mg/24 h, over a 23-day period.

Sampling time (days)	Percentage of 24 h dose excreted in 24 h urine samples as:				
	Nicotine[a]	Nornicotine[a]	Cotinine[a]	Nicotine *N*′-oxide[a]	3′-Hydroxycotinine[b]
2– 3	0·28±0·02[c]	n.d.[d]	n.d.	12·72±3·01	21·15±3·24
6– 7	n.d.	n.d.	n.d.	13·61±4·62	26·07±2·11
10–11	n.d.	n.d.	0·64±0·10	11·96±1·11	15·17±1·23
14–15	0·42+0·04	n.d.	n.d.	12·79±5·0	12·95±1·44
18–19	n.d.	n.d.	0·32±0·03	12·47±3·77	13·53±3·63
22–23	n.d.	n.d.	n.d.	11·02+1·24	17·16+2·76

[a] analysis by cation exchange HPLC using Chromatographic System 1.
[b] analysis by cation exchange HPLC using Chromatographic System 2.
[c] results are means±s.e., n=5.
[d] not detected.

Table 3. Levels of *N*-methylated metabolites of nicotine in urine samples from guinea-pigs dosed with *R*-(+)-nicotine, 12 mg/24 h, over a 23-day period.

Sampling time (days)	Percentage of 24 h dose excreted in 24 h urine samples as:		
	N-Methylcotininium ion[a]	*N*-Methylnornicotinium ion[a]	*N*-Methylnicotinium ion[a]
2– 3	1·35±0·06[b]	6·49±0·79	7·38±1·59
7– 7	1·29+0·03	7·11±1·38	12·40±3·18
10–11	1·29±0·09	5·66±0·31	9·98±1·97
14–15	1·53±0·21	7·94±1·21	9·10±0·07
18–19	1·29±0·12	7·04±0·76	10·94±4·06
22–23	1·24±0·03	6·28±0·05	9·92±0·52

[a] analysis of *N*-methylated metabolites was by cation-exchange HPLC using Chromatographic System 3.
[b] results are means±s.e., n=5.

radiotracer studies due to loss of the N'-C^3H_3 label of nicotine. *N*-Methylcotininium ion was produced in the smallest quantities of the three quaternary ammonium *N*-methylated urinary metabolites. There was no suggestion of enzyme induction in this pathway, since the levels of all three metabolites remained essentially constant throughout the whole time course of the study.

Under the conditions of this experiment, the total quaternary ammonium *N*-methylated metabolites accounted for 15 to 20%, while the total oxidative metabolites accounted for 28 to 36% of the total urinary metabolites of *R*-(+)-nicotine, based upon the dose of *R*-(+)-nicotine displaced from the osmotic pump over each of the 24 h periods studied. Thus, *N*-methylation appears to be a major route of metabolism for *R*-(+)-nicotine in the guinea-pig.

Conclusions

This study has revealed the following findings. Firstly, *S*-(−)-nicotine is metabolized differently from *R*-(+)-nicotine in the guinea-pig. Clearly there is an enantioselective metabolism that exists in the biotransformation of these two enantiomers. In the *S*-(−)-nicotine study, only oxidative urinary metabolites were formed, whereas, in the *R*-(+)-nicotine study, both oxidative and *N*-methylated urinary

metabolites were formed. The enantioselective *N*-methylation that operates for *R*-(+)-nicotine in the guinea-pig, occurs to the extent of 15 to 20% of the total metabolic activity, and therefore, is a major route of metabolism for this nicotine enantiomer. This value, obtained for the *N*-methylation of nicotine in this present study, is higher than the values (ca 12%) obtained from previous radiolabelled nicotine studies (Cundy et al 1985). Several factors may account for this observation. For example, since the *N*-methylnornicotinium ion cannot be observed as a metabolite in the radiotracer methodology, owing to loss of radiolabel, this may account for the detection of lower observable total *N*-methylated metabolites in these studies. Furthermore, the dose of nicotine used in the present studies is comparatively much higher than that used in the radiotracer studies, so there is a possibility that the oxidative pathway may become saturated, thereby funneling nicotine into the *N*-methylation pathway. However, dose-response studies need to be made to examine this in greater detail.

Secondly, the levels of oxidative urinary metabolites of *S*-(−)- and *R*-(+)-nicotine in continuously-dosed guinea-pigs were different, in that the *S*-(−)-nicotine oxidative levels were generally higher than those of *R*-(+)-nicotine. The levels of urinary oxidative metabolites of *S*-(−)-nicotine also decreased with time, while the *R*-(+)-nicotine urinary oxidative metabolites remained relatively constant throughout the study. The lower levels of oxidative urinary metabolite observed in *R*-(+)-nicotine-dosed guinea-pig urine is due to the parallel competing *N*-methylation pathway, which channels the *R*-(+)-nicotine away from the oxidative pathway. This study has also demonstrated that 3′-hydroxycotinine is a major urinary metabolite of both *S*-(−)- and *R*-(+)-nicotine.

Thirdly, the results of this study suggest that there may be an enantioselective induction of an unknown pathway involving the *S*-(−)-nicotine isomer. This may explain why lower levels of urinary oxidative metabolites of *S*-(−)-nicotine were observed with time, since induction of this unknown pathway would funnel *S*-(−)-nicotine into an alternative pathway than the oxidative pathway of metabolism. The unknown pathway could be a conjugation pathway, however, this needs to be established.

Acknowledgements
This study was supported by a grant (to PAC) from The Tobacco and Health Research Institute, Lexington, KY, USA.

References

Beckett, A. H., Gorrod, J. W., Jenner, P. (1971a) The analysis of nicotine-*N*-oxide in urine in the presence of nicotine and cotinine and its application to the study of in vitro nicotine metabolism in man. J. Pharm. Pharmacol. 23: 62–67

Beckett, A. H., Gorrod, J. W., Jenner, P. (1971b) The effect of smoking on nicotine metabolism in vivo in man. Ibid. 23: 55*S*–61*S*

Booth, J., Boyland, E. (1970) The metabolism of nicotine into two optically-active stereoisomers of nicotine-1′-oxide by animal tissues in vitro and by cigarette smokers. Biochem. Pharmacol. 19: 733–742

Bowman, E. R., McKennis, Jr., H. Martin, B. (1982) A convenient method for the preparation of racemic nicotine. Synth. Commun. 12: 871–879

Cundy, K. C., Godin, C. S., Crooks, P. A. (1984) Evidence of stereospecificity in the in vivo methylation of [^{14}C](±)-nicotine in the guinea-pig. Drug Metabol. Disposit. 12: 755–759

Cundy, K. C., Sato, M., Crooks, P. A. (1985) Stereospecific in vivo *N*-methylation of nicotine in guinea-pigs. Ibid. 13: 175–185

Dagne, E., Castagnoli, N. (1972) Structure of hydroxycotinine, a nicotine metabolite. J. Medicin. Chem. 15: 356–360

Gorrod, J. W., Jenner, P. (1975) The metabolism of tobacco alkaloids. Essays in Toxicology 6: 35–78

Graziano, M. J. Dorough, H. W. (1984) Effect of cigarette smoking on hepatic biotransformation in rats. Toxicol. Appl. Pharmacol. 75: 223–233

Jacob, P. (1982) Resolution of (±)-5-bromonornicotine: Synthesis of (*R*)- and (*S*)-nornicotine of high enantiomeric purity. J. Org. Chem. 47: 4165–4167

Klus, H., Kuhn, H. (1977) A study of the optical activity of smoke nicotines. Fachliche Mitt. Oesterr. Tabakregie 17: 331–336

McKennis, Jr., H., Turnbull, L. B., Bowman, E. R. (1963) *N*-Methylation of nicotine and cotinine in vivo. J. Biol. Chem. 238: 719–727

Matsukura, S., Taminato, T., Kitano, N., Seino, Y., Hamada, H., Uchihashi, M., Nakajima, H., Hirato, Y. (1984) Effects of environmental tobacco smoke on urinary cotinine excretion in nonsmokers. New England J. Med. 311: 828–832

Matsukura, S., Sakamoto, N., Seino, Y., Tamada, T., Matisyama, H., Muranaka, H. (1979) Cotinine excretion and daily cigarette smoking in habituated smokers. Clin. Pharmacol. Ther. 25: 555–561

Phillipson, J. D., Handa, S. S. (1975) Nicotine *N*-oxides. Phytochemistry 14: 2683–2690

Pool, W. F., Houdi, A. A., Damani, L. A., Layton, W. J., Crooks, P. A. (1986) Isolation and characterization of *N*-methyl-*N*′-oxonicotinium ion, a new urinary metabolite of *R*-(+)-nicotine in the guinea-pig. Drug Metabol. Disposit. 14: 574–579

Sato, M., Crooks, P. A. (1985) *N*-Methylnornicotinium ion, a new in vivo metabolite of *R*-(+)-nicotine. Ibid. 13: 348–353

Seeman, J. I., Whidby, J. F. (1976) The iodomethylation of nicotine: an unusual example of competitive nitrogen alkylation. J. Org. Chem. 41: 3824–3826

Zeidenberg, P., Jaffe, J. H., Kanzler, M., Levitt, M. D., Langone, J. J., Van Vunakis, M. (1977) Nicotine: cotinine levels in blood during cessation of smoking. Compr. Psychiatry 18: 93–101