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IDENTIFICATION OF PHASE I METABOLITES OF 3-METHYLINDOLE PRODUCED BY PIG LIVER MICROSOMES

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ABSTRACT:

A study was conducted to investigate qualitative and quantitative aspects of the phase I metabolism of 3-methylindole (3MI) by porcine liver microsomes. Microsomal suspensions were prepared from the liver of 30 intact (uncastrated) male pigs. Metabolites produced in microsomal incubations were identified and quantitated with HPLC-UV, HPLC-fluorescence, and UV-spectral analysis; liquid chromatography-mass spectrometry (LC-MS) and NMR were used for the identification of a metabolite for which a reference compound was not available. The results showed that seven major metabolites of 3MI are produced by porcine microsomes, three of which had already been identified in pigs (3-OH-3-methyloxindole, 5-OH-3-methylindole, and 6-OH-3-methylindole). The other four major 3MI metabolites identified were 3-OH-3-methylindolenine, 3-methyloxindole, indole-3-carbinol, and 2-aminoacetophenone. On average, the metabolite that was produced in larger amounts was 3-OH-3-methylindolenine (45.1%), followed by the two oxindoles 3-methyloxindole (27.9%) and 3-OH-3-methyloxindole (18.5%). Average percentage of production of 6-OH-3-methylindole was 4.9%, whereas indole-3-carbinol accounted for 2.7% of all metabolites produced; 2-amino-acetophenone and 5-OH-3-methylindole were the metabolites produced in lesser amounts (0.5 and 0.3%, respectively). Large interindividual differences in the rate of production of all metabolites were observed. This variation could be attributed to differences in the activity and/or level of expression of phase I biotransformation enzymes and this issue should be further investigated.

Skatole (3-methylindole, 3MI)[3] is a naturally occurring microbial metabolite produced from tryptophan in the gastrointestinal tract of ruminants (Yokoyama and Carlson, 1979), humans (Fordtran et al., 1964), and pigs (Jensen et al., 1995); 3MI is present in the feces of sheep, goats, cattle, pigs, and humans (Dehnhard et al., 1991).

3MI has been well established as a pneumotoxin in cattle (Carlson et al., 1972), sheep (Bradley et al., 1978), goats (Bradley and Carlson, 1980), horses (Turk et al., 1983), and rodents (Turk et al., 1984). Humans can be exposed to 3MI through intestinal absorption and by cigarette smoke. The toxic implications of this exposure have not been thoroughly assessed, but human enzymes activate 3MI to toxic intermediates (Ruangyuttikarn et al., 1991; Thornton-Manning et al., 1996). The toxicity of 3MI is species-, organ-, and even cell-specific. The most susceptible species are ruminants and horses, in which the target organ is the lung. In ruminants, type I alveolar epithelial cells and nonciliated bronchiolar epithelial (Clara) cells are the most susceptible (Huang et al., 1977; Bradley and Carlson, 1980); however, only Clara cells are affected in horses (Turk et al., 1983).

Even though doses of 3MI given to pigs have been unsuccessful in producing lung lesions (Carlson and Yost, 1989), 3MI has important implications for pig meat production. Intact male pigs (uncastrated pigs) are used for meat production in several countries, due to a better feed conversion, improved carcass leanness, and a better composition of fatty acids compared with castrated pigs (Bæk et al., 1995). However, 5 to 10% of the entire male pigs carry the so-called boar taint (a fecal-like odor liberated when the meat is cooked), and 3MI is one of the major contributors to boar taint (Bæk et al., 1995; Hansen et al., 1995). It is not known why only a small percentage of a given population of pigs accumulates 3MI to a level that can be detected by humans. One possible explanation for this difference could be individual differences in the metabolism of 3MI (Lundström et al., 1995).

The role of cytochrome P-450 enzymes in phase I metabolism of 3MI is well documented (Huijzer et al., 1989; Thornton-Manning et al., 1991, 1996; Squires and Lundström, 1997; Babol et al., 1998a), and the metabolic fate of 3MI in species susceptible to acute lung disease has been well characterized (Smith et al., 1993). However, our understanding of the metabolic pathways involved in 3MI biotransformation and elimination in pigs remains incomplete. Babol et al. (1998a) found that at least seven metabolites were produced by pig liver microsomes incubated with 3MI but only two metabolites could be identified. Formation of selected 3MI metabolites (so-called "F-1" and "MII") by pig liver microsomes was shown to be negatively correlated with fat levels of 3MI (Babol et al., 1998b). However, it is necessary to clarify both qualitative and quantitative aspects of 3MI biotransformation in pigs. The type of metabolites synthesized by the

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[3] Abbreviations used are: 3MI, 3-methylindole; I3C, indole-3-carbinol; 3MOI, 3-methyloxindole; HMOI, 3-hydroxy-3-methyloxindole.

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pig may be the same as those reported in other species; however, their relative concentrations (ratio) may vary quantitatively. The aim of the present work was to identify and quantitate the major phase I metabolites of 3MI produced in vitro by pig liver microsomes.

Materials and Methods

Chemicals. 3MI, indole-3-carbinol (I3C), indole-3-aldehyde, indole-3-carboxylic acid, 2-aminoacetophenone, and sulfatase type H-2 from *Helix pomatia* were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). The oxindoles 3-methyloxindole (3MOI) and 3-hydroxy-3-methyloxindole (HMOI) were synthesized according to the methods of Kende and Hodges (1982) and Skiles et al. (1989), respectively. Authentic 5-OH-3-methylindole and 6-OH-3-methylindole (in the form of 6-sulfatoxyskatole) were donated by Jens Hansen-Møller (Danish Meat Research Institute, Roskilde, Denmark). To obtain 6-OH-3-methylindole from 6-sulfatoxyskatole, the compound was hydrolyzed in a total volume of 0.5 ml of acetate buffer, pH 5.0, containing 90 U/ml of type H-2 sulfatase. Hydrolysis was conducted for 4 h in a shaking water bath at 40°C and then 0.5 ml of ice-cold acetonitrile was added both to stop the reaction and to precipitate the protein. After centrifugation at 7500 rpm for 15 min, 50 μl of clear supernatant was injected into the chromatograph, under the conditions described in Analytical Chromatography.

Preparation of Microsomes. Liver samples were taken from 30 intact male pigs obtained by back-crossing F3 European Wild Pig $\times$ Swedish Yorkshire boars with Swedish Yorkshire sows (Squires and Lundström, 1997). Liver samples were frozen in liquid nitrogen and stored at -80°C. For the preparation of microsomes, partially thawed liver samples were finely minced and homogenized with 4 volumes of 0.05 M Tris-HCl buffer, pH 7.4 (containing 0.15 M KCl, 1 mM EDTA, and 0.25 M sucrose) with an Ultra-Turax homogenizer (Janke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 10,000*g* for 20 min, and the resulting supernatant was centrifuged again at 100,000*g* for 60 min to obtain the microsomal pellet. The pellets were suspended in a 0.05 M Tris-HCl buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA, and 0.25 M sucrose to a final concentration of 20 mg protein/ml and stored at -80°C before analysis. Protein concentrations were determined by the method of Smith et al. (1985) with bicinchoninic acid protein assay reagents purchased from Pierce Chemical Co. (Rockford, IL) and BSA as standard.

Microsomal Incubations. Two milligrams of microsomal protein was incubated with 0.4 mM 3MI and 4 mM NADPH in 0.05 M sodium phosphate buffer, pH 7.4, containing 5 mM $MgCl_2$ and 1 mM EDTA for 30 min at 37°C (production of metabolites was determined to be linear over a range of 10–40 min). Incubation volumes were 0.5 ml. Reactions were started by the addition of NADPH after 3-min preincubation periods at 37°C and stopped with 0.5 ml of ice-cold acetonitrile. Incubations of all 30 samples were run in duplicate and for control incubations NADPH was omitted. After the addition of acetonitrile, the mixture was vortexed and centrifuged at 5000 rpm for 20 min. A 50-μl aliquot of the clear supernatant was analyzed by HPLC.

Analytical Chromatography. Analytical HPLC was done with a Spectra-Physics system (Spectra-Physics Anal., Fremont, CA) consisting of an SP8800 gradient pump, an SP8880 autosampler with a 50-μl injection loop, an SP Spectra 100 UV detector, and a Spectra System FL-2000 fluorescent detector. The HPLC method is a modification of a previously reported binary gradient system method (Bæk et al., 1995). 3MI and its metabolites were separated with a reversed-phase Prodigy ODS, 5 μm, 250 $\times$ 4.6 mm column (Phenomenex, Inc., Torrance, CA). The mobile phase consisted of two solvents, A (0.01 M potassium dihydrogen phosphate buffer, pH 3.9) and B (acetonitrile), with the following gradients: 0 min, 90% A; 6 min, 80% A; 12 min, 70% A; 18 min, 30% A; 25 min, 10% A; 26 min, 90% A; 35 min, 90% A. All gradients were linear and the flow rate was set at 1.2 ml/min. Absorbance was monitored at 250 nm; fluorescence was monitored at excitation and emission wavelengths of 286 and 350 nm, respectively. HPLC analysis for 3MI metabolites was conducted immediately after the incubations. Metabolites were identified by comparison of retention times and coinjection of standards (spiking the metabolite mixture with authentic standards).

Isolation and Purification of Metabolites by Preparative HPLC. To obtain a sufficient amount of metabolites to conduct UV-spectral analysis, a large-scale incubation (final volume of 4 ml) was performed, with the same concentrations of reactants as described above. Preparative HPLC was done with a Spectra-Physics SP8800 gradient pump (Spectra-Physics Anal.), a manual Rheodyne 7125 injector fitted with a 500-μl injection loop (Rheodyne Inc., Cotati, CA), and an SP Spectra 100 UV detector. The 3MI metabolites were separated with a reversed-phase Waters preparative HPLC C_{18} (10 μm, 300 $\times$ 7.6 mm) column (Waters Associates, Millipore Corp., Milford, MA). The mobile phase was the same as described above except that the flow rate was set at 3.0 ml/min. The peaks corresponding to the metabolites identified on the basis of their retention times as HMOI, I3C, 3MOI, and 2-aminoacetophenone were collected in enough amounts to determine their UV spectra. Purity of the collected fractions was verified by HPLC with the procedure described in Analytical Chromatography. One of the metabolites produced by pig liver microsomes could not be identified on the basis of comparison of retention times; this metabolite was named UV-1 due to its absorption in the far UV spectrum and the fact that it was the first metabolite that eluted from the column (Babol et al., 1998a). The peak corresponding to this metabolite, which eluted between 9.1 and 10.1 min, was collected after several 500-μl injections and subjected to HPLC-MS, ^{1}H NMR, and UV-spectral analysis.

UV Spectroscopy. UV spectra (200–300 nm) were recorded for the HPLC metabolites UV-1, HMOI, I3C, 3MOI, and 2-aminoacetophenone. UV spectra of available authentic standards also were recorded and compared with those of the isolated metabolites. Spectra were recorded on a model 4054 LKB Biochrom UV-Visible spectrophotometer (Pharmacia LKB Biochrom Ltd., Cambridge, UK). Because of their low levels of production, it was not possible to isolate the hydroxyskatoles in enough quantities to determine their UV spectra.

LC-MS of Metabolite UV-1. Metabolite UV-1 was analyzed by LC-MS under the following conditions: HPLC was performed with a Prodigy 5 ODS-2, 5 μm, 150 $\times$ 3.2-mm column (Phenomenex, Inc.) and water/acetonitrile (50:50) as mobile phase. The mobile phase was delivered by binary LC pumps (Hewlett Packard 1090 Series II/L; Palo Alto, CA). The eluent passed through a sample injection valve Rheodyne 7010 (Rheodyne Inc.) to an atmospheric pressure chemical ionization source configured with a corona discharge pin, at a flow rate of 0.7 ml/min. A sample volume of 20 μl was injected by an autosampler (Hewlett Packard 1090 Series II/L). MS detection was achieved with a VG Quattro II triple quadrupole mass spectrometer (Fisons UK Ltd., Altrincham, UK). Instrument control, data acquisition, and data processing were carried out with the MassLynx software package. Liquid nitrogen was used as a drying and sheath gas at flow rates of 200 and 50 l/h, respectively. The instrument was operated in the positive ion mode with an ion source temperature of 150°C, a corona discharge pin potential of +3.75 kV, and a cone voltage of 15V. The total ion chromatogram of LC-MS was obtained by scanning the first quadrupole from *m*/*z* 125 to 700 at a rate of 400 atomic mass units/s in full scan mode with interscan delay of 0.10 s. Data were acquired in continuum mode. The product-ion scan was performed by tandem mass spectrometry by transmitting the protonated molecular ion ($[M+H]^+$) through the first quadrupole into the second quadrupole containing ultrapure argon. The product-ion chromatogram was recorded by scanning the third quadrupole from *m*/*z* 50 to 450 in 1.0 s. The collision energy was varied between -20 and -50 eV to optimize fragmentation of the selected protonated molecular ion.

NMR Spectroscopy of Metabolite UV-1. UV-1 metabolite was isolated for NMR analysis with incubation conditions essentially as described above. However, these incubations contained 1 nmol of cytochrome P-450 content rather than 2 mg of total protein. UV-1 was separated from other microsomal 3MI metabolites by the HPLC conditions described above with a system consisting of an LDC Analytical Constametric 4100 solvent delivery module (ThermoQuest, Riviera Beach, FL), a Hewlett Packard 1040A diode array detector, and a Hewlett Packard 9000 series HPLC workstation (Hewlett Packard Co., Wilmington, DE). UV-1 was purified by HPLC and pooled from two identical incubations followed by concentration in a Savant Speed-Vac (Savant Instruments Inc., Farmingdale, NY). Concentration to dryness was not possible due to polymerization and degradation of unstable UV-1. Therefore, the sample was evaporated to a volume of 200 μl and reinjected on the HPLC for additional purification. In this case, however, the aqueous mobile phase consisted of 0.01 M dibasic potassium phosphate buffer, pH 9.0, in 99.9 atom % deuterium oxide. Due to the instability of UV-1 when it was evaporated to dryness, it was necessary to perform the final purification step in the NMR solvent, deuterium oxide. UV-1 was again collected and evaporated to a final

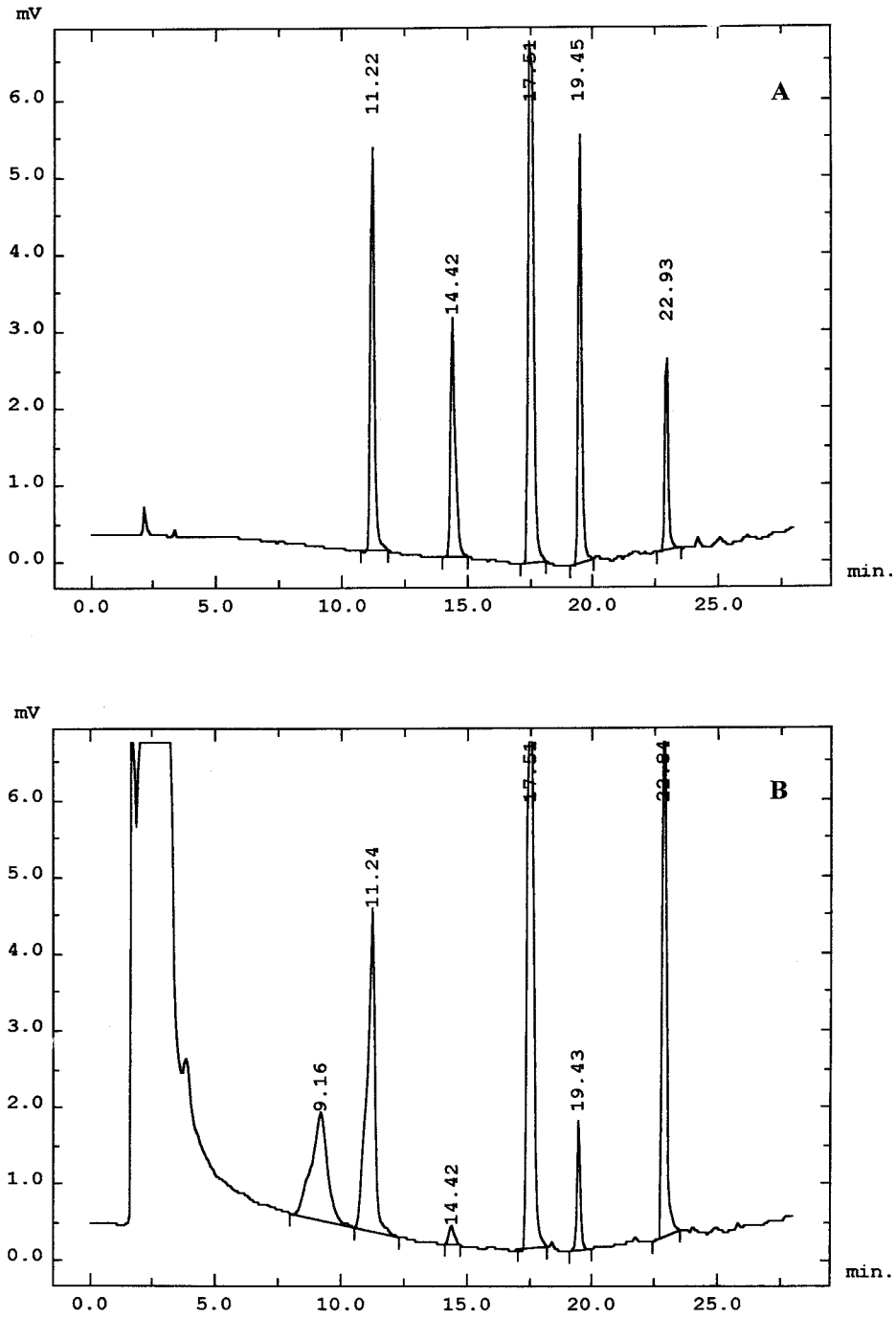

FIG. 1. *Chromatographic profile of the main five metabolites produced by pig liver microsomes as detected by UV absorption at 250 nm.*

Retention times correspond as follows: 9.16 min, UV-1; 11.24 min, 3-hydroxy-3-methyloxindole; 14.42 min, indole-3-carbinol; 17.51 min, 3-methyloxindole; 19.43 min, 2-aminoacetophenone; 22.84 min, parent compound (3MI). A, standard mixture containing 2 μg/ml of each metabolite. B, incubation mixture.

volume of 250 μl and directly added to the Shigemi NMR tube. The ^{1}H NMR spectrum was obtained in deuterium oxide with a Varian Unity Inova 600 MHz NMR (Varian Associates Inc., Palo Alto, CA).

Results

HPLC. None of the metabolites produced by pig liver microsomes coeluted with indole-3-carboxaldehyde or indole-3-carboxylic acid. However, metabolites that coeluted with HMOI, 3MOI, I3C, 2-aminoacetophenone, and the two hydroxyskatoles (5- and 6-OH-3-methylindole) were measured by UV and/or fluorescence detection. The oxindole metabolites (HMOI and 3MOI) and the pyrrole ring-opened metabolite (2-aminoacetophenone) were detected and quantitated by UV absorption because they do not fluoresce; I3C and the hydroxyskatoles were detected and quantitated by fluorescence detection. When microsomal incubations were spiked, all metabolites identified on the basis of their retention times, cochromatographed with their corresponding authentic standards. The chromatographic profile of a microsomal incubation and a standard mixture monitored by UV absorption at 250 nm is shown in Fig. 1.

UV Spectroscopy. The UV spectrum of the metabolites identified on the basis of their retention times on HPLC (HMOI, 3MOI, I3C, and

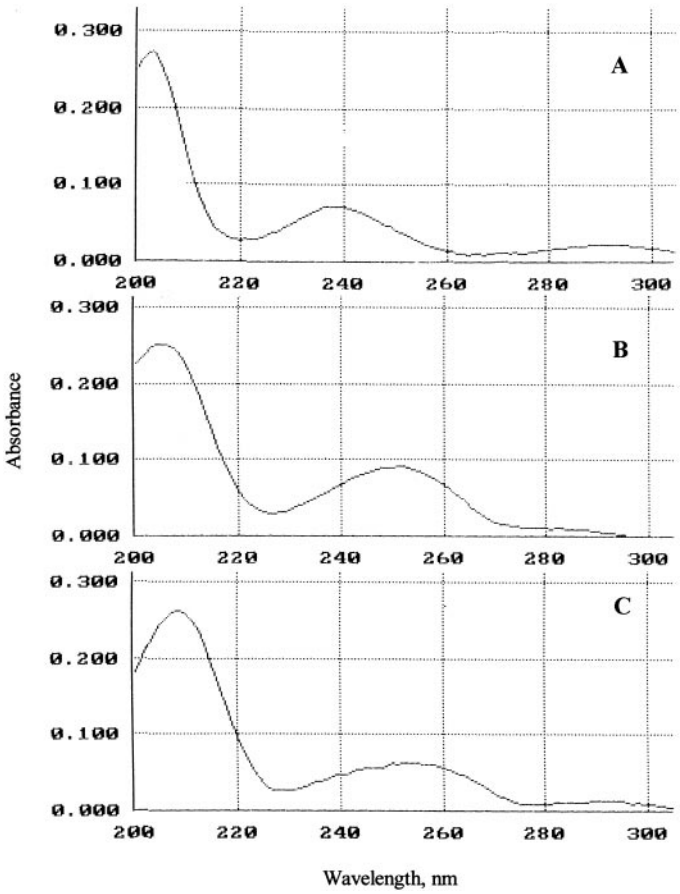

FIG. 2. *UV spectra of UV-1 metabolite (A) [λ_{max} (nm): 204, 238]; 3MOI (B) [λ_{max} (nm): 205, 252]; and HMOI (C) [HMOI: λ_{max} (nm): 208, 253].*

2-aminoacetophenone) were identical with those of authentic standards. Spectra of metabolites were recorded with water as solvent and the wavelengths of maximal absorption were as follows: HMOI, λ_{max} (nm): 208, 253; 3MOI, λ_{max} (nm): 205, 252; I3C: λ_{max} (nm), 221, 278; and 2-aminoacetophenone, λ_{max} (nm): 228, 257. The UV spectrum of 3-methylindole was λ_{max} (nm), 224, 281. The UV spectrum of UV-1 metabolite was λ_{max} (nm), 204, 238. The UV spectra of UV-1 was similar to the spectra of the oxindole metabolites 3MOI and HMOI as shown in Fig. 2. Changing the pH from 3 to 11 did not change the spectrum of UV-1; this lack of a bathochromic shift indicated that the unknown metabolite had no free phenolic group. Isolated UV-1 was kept in acetonitrile/water solution at room temperature and the solution was analyzed by HPLC at 7-day intervals for 6 weeks. After 6 weeks only ~25% of the original compound remained and it was observed that UV-1 was converted into 3MOI. The slopes of the linear regressions of 3MOI and UV-1 over time indicated that the molar response factor for UV-1 on HPLC-UV analysis was 2.95 times that of 3MOI.

Metabolite UV-1 Structural Data. The MS of isolated UV-1 produced a molecular ion at *m/z* 148 $[M + H]^+$ with major fragments at *m/z* 133 $[M - CH_3]^+$, 104 $[M - H_3C\text{-}C\text{-}OH]^+$, and 77 (protonated phenyl ring) (Fig. 3). The ^1H NMR spectrum of metabolite UV-1 is shown in Fig. 4. Assignments of the proton signals are provided, listed as chemical shift (multiplicity, integration, and assignment): δ1.4 (s, 3*H*, -CH_3); δ6.8 (d, 2*H*, H-5 and H-6); δ7.2 (d, 2*H*, H-4 and H-7); δ8.4 (s, 1*H*, H-2). The singlet at δ8.4 has been assigned to the proton at C-2 of 3-hydroxy-3-methylindolenine. This proton is attached to the sp^2 hybridized C-2, which is also deshielded by the adjacent nitrogen. Therefore, this proton is highly deshielded and appears downfield from all other protons in the proposed structure. At δ2.0 is a singlet corresponding to the methyl protons of contaminating acetonitrile. Due to the way in which the sample was purified, it was extremely difficult to remove all of the acetonitrile present in the HPLC organic phase.

In summary, seven metabolites of 3MI were found to be produced by pig liver microsomes: 3MOI, HMOI, 6-OH-3-methylindole (6-OH-3MI), I3C, 2-aminoacetophenone, 5-OH-3-methylindole (5-OH-3MI), and the metabolite that was named UV-1. When UV-1 was quantitated assuming a molar absorptivity 2.95 times greater than that of 3MOI, the total amount of nanomoles produced accounted for an average of 96.0% (range of 86.5–105.0%) of the 3MI molecules metabolized during the microsomal incubations. The rates of production of the seven metabolites identified in pig liver microsomal incubations are shown in Table 1. UV-1 metabolite was produced at the highest rate (750.7 pmol/mg protein/min), whereas 5-OH-3MI was produced at the lowest rate (5.1 pmol/mg protein/min). Large inter-individual differences were noted for the production rates of the same metabolite. For instance, UV-1 metabolite was produced at a rate of 1556.3 pmol/mg protein/min by the microsomes of one pig, whereas other microsomes produced this compound at a rate of 180.5 pmol/mg/protein/min (Table 1). The metabolite that was produced in larger amounts was UV-1, which, on average, accounted for 45.1% of all metabolites produced. The combined oxindoles accounted for 46.4% of the total metabolites: an average of 27.9% of the metabolites produced corresponded to 3 MOI, whereas 18.5% corresponded to HMOI. The other metabolites were produced in lesser amounts. 6-OH-3MI accounted for 4.9% of the metabolites, I3C accounted for 2.7%, and 2-aminoacetophenone and 5-OH-3MI accounted for only 0.5 and 0.3% of the metabolites, respectively. The chemical structures and percentages of production of these metabolites are shown in Fig. 5.

Discussion

Only three phase I metabolites of 3MI had been identified previously in pigs: HMOI and the hydroxyskatoles 5-OH-3MI and 6-OH-3MI. HMOI had been found in pig plasma and urine (Baek et al., 1997) and pig liver microsomal incubations (Babol et al., 1998a); 6-OH-3MI had been detected both in pig serum (Bæk et al., 1997) and pig liver microsomal incubations (Babol et al., 1998a), whereas 5-OH-3MI had only been reported to be present in pig serum (Bæk et al., 1997). In this study, all three metabolites were detected in the microsomal incubations and the production of four new metabolites is reported.

One of the pathways of 3MI biotransformation identified in species such as goats, mice, and rats is the formation of oxindole derivatives: 3MOI and HMOI (Frydman et al., 1972; Smith et al., 1993). On average, 46.4% of the metabolites produced by pig liver microsomes in this study corresponded to these two oxindole derivatives; this finding indicates that the oxidole pathway is quantitatively very important in the pig. 3MOI had been identified in rat liver microsomal incubations (Frydman et al., 1972), in goat lung and liver microsomal incubations (Huijzer et al., 1987), and in the urine of goats (Hammond et al., 1979). One of the metabolites observed in pig microsomal incubations by Babol et al. (1998a) was named "UV-3", and the results of the present study indicate that this metabolite corresponds to 3MOI. The other oxindole derivative of 3MI, HMOI, had already been isolated from the urine of pigs dosed with 3MI (Bæk et al., 1997) and was reported to be produced by pig liver microsomes (Babol et al., 1998a). HMOI is also a major urinary metabolite produced by mice

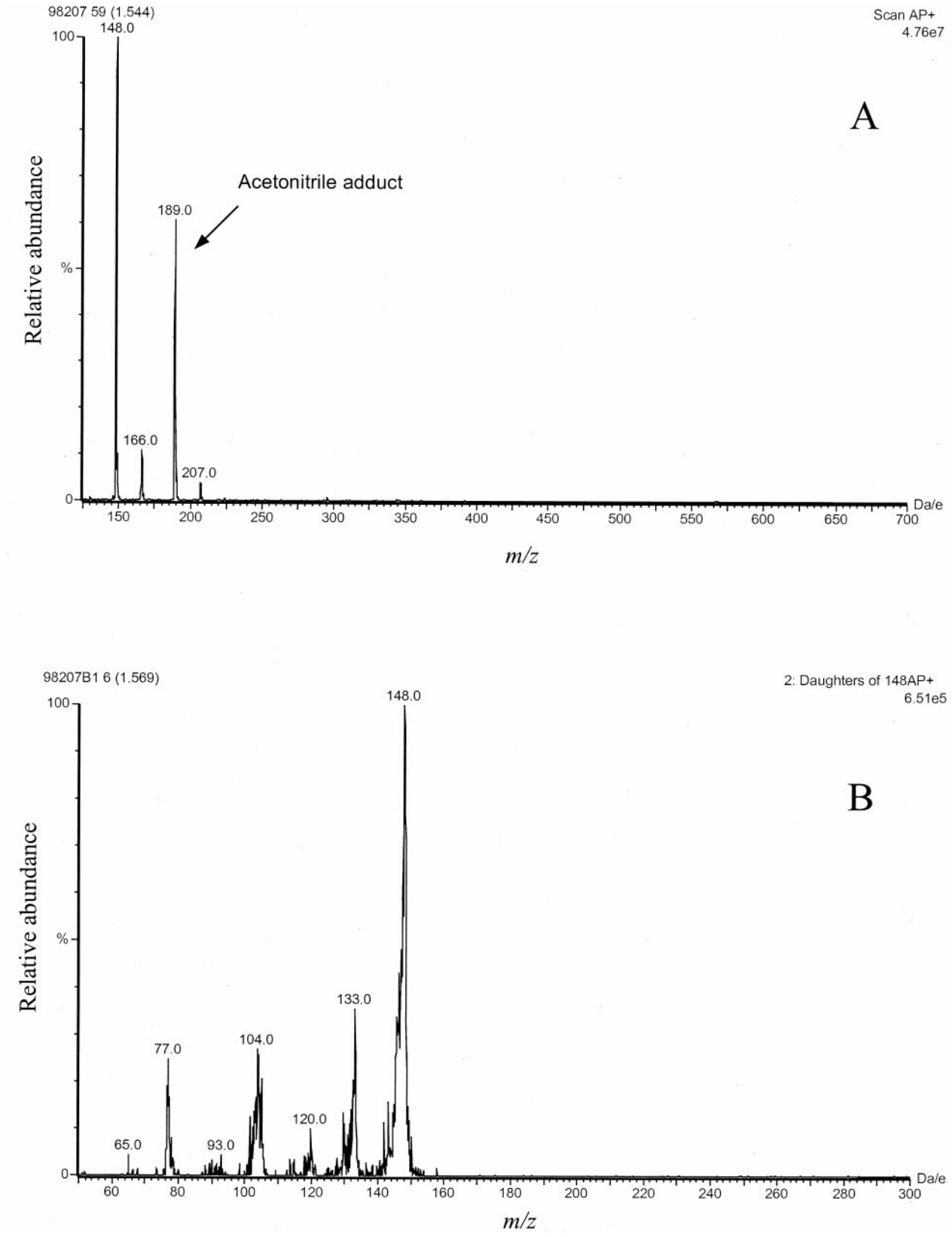

FIG. 3. *A, LC-MS spectrum of metabolite UV-1. B, MS-MS spectrum of daughter ion of m/z 148.*

dosed with radiolabeled 3MI (Skiles et al., 1989). Additionally, it has been found in the urine of humans (Albrecht et al., 1989) and goats (Smith et al., 1993). Interestingly, in the present study, pig liver microsomes produced large amounts of both oxidole derivatives 3MOI and HMOI. In other species studied, one of these metabolites predominates. In goats, production of 3MOI predominates (Hammond et al., 1979), whereas in mice it is HMOI that predominates (Smith et al., 1993).

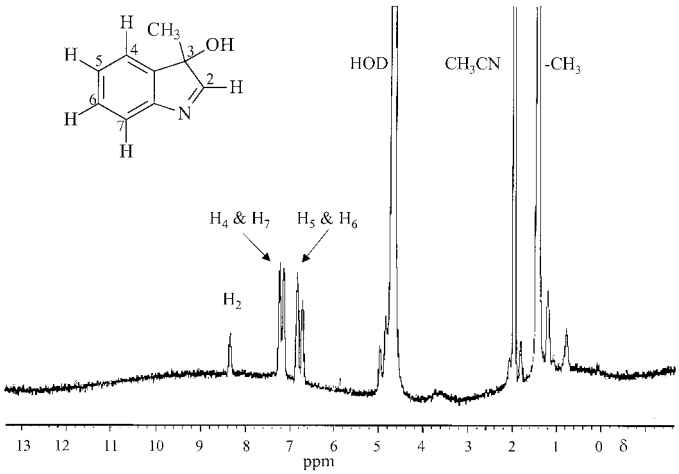

FIG. 4. *^{1}H NMR spectrum of metabolite UV-1.*

TABLE 1

Rate of production of 3MI metabolites by pig liver microsomes

Metabolite	Rate of Production ± S.D.	Minimum	Maximum
	pmol/mg protein/min		
UV-1	750.7 ± 414.5	180.5	1556.3
3MOI	420.9 ± 118.1	234.4	700.8
HMOI	272.4 ± 91.6	118.9	516.5
6-OH-3-Methylindole	58.4 ± 47.2	n.d.[a]	213.7
I3C	37.1 ± 15.8	12.1	85.7
2-Aminoacetophenone	7.8 ± 2.4	3.4	12.7
5-OH-3-methylindole	5.1 ± 5.8	0.7	27.3

$n = 30$.
[a] n.d., not detected.

The 3-methyl group of 3MI may be oxidized to the alcohol, aldehyde, and carboxylic acid functions (Hammond et al., 1979). In the present study, only the alcohol function of the 3-methyl group (indole-3-carbinol) was found to be produced by pig liver microsomes. This metabolite exhibits strong fluorescence and also absorbs in the UV and even though it had been previously reported to be produced by pig microsomes (named F-1 by Babol et al., 1998a), its structure was unknown. It is important to note that further metabolism of the alcohol function of indole-3-carbinol could possibly be catalyzed by alcohol dehydrogenase; if this is true, then the product of this reaction, indole-3-carboxaldehyde, would not be produced in microsomal incubations.

Hydroxylation of the aromatic ring of 3MI can occur at any of the carbons 4, 5, 6, or 7; however, the experimental evidence indicates that hydroxylation at positions 5 and 6 predominates. In 1962, Jepson and coworkers showed that rabbit liver microsomes hydroxylate tryptamine, indole acetic acid, and related indoles to their corresponding 6-hydroxy derivatives. The microsomal system required NADPH and oxygen and did not form 5- or 7-hydroxyindoles (Jepson et al., 1962). Mahon and Mattok (1967) analyzed the urine of 10 normal human subjects and found that all samples contained 6-hydroxyskatole and nine had the 5-isomer, although its excretion rate was ~50% of the 6-isomer; 7-hydroxyskatole was detected in three of the samples but its excretion rate was only 5% of the 6-isomer. None of the subjects excreted 4-hydroxyskatole (Mahon and Mattok, 1967). Bæk et al. (1995) found conjugates of both 5-OH-3MI and 6-OH-3MI in pig serum. In the present study, the average rate of production of 6-OH-3MI was ~11 times greater than the production of the 5 isomer, indicating that hydroxylation at position C6 predominates.

Frydman et al. (1972) found two pyrrole ring-opened metabolites produced after incubation of 3-MI with rat liver microsomes. The two compounds were identified as 2-formamidoacetophenone and 2-aminoacetophenone; a total of 33% of the metabolites formed corresponded to 2-formamidoacetophenone, 12% to 2-aminoacetophenone, and 5% to 3MOI. In the present study, 2-aminoacetophenone was found to be produced by all liver samples analyzed at an average percentage of 0.5%, which is much lower than the percentage reported for rats (Frydman et al., 1972). No previous reports of 2-aminoacetophenone production from 3MI metabolism by pigs were found in the literature.

The ^{1}H NMR, LC-MS, and UV-spectral characteristics of metabolite UV-1 indicate that this compound corresponds to 3-hydroxy-3-methylindolenine. UV-1 was found to be an unstable compound, intermediate between 3MI and 3MOI. The fact that UV-1 was converted into 3MOI suggested that this compound could be a precursor of 3MOI, possibly 2,3-epoxy-3-methylindolenine, the structure of which was postulated by Smith et al. (1993) or, most likely, its ring-opened product, 3-hydroxy-3-methylindolenine (Skordos et al., 1998a,b). The molecular weight of the compound (147) and its fragmentation pattern were compatible with the epoxyde or the imine (Fig. 3), but the UV spectrum, with a λ_{max} at 238 nm (Fig. 2) was more consistent with the imine structure. The molecular weight of 147 also could correspond to an aromatic phenolic metabolite of 3MI; however, when the UV spectrum of isolated UV-1 was taken under different pHs, it did not show the typical bathochromic shift observed in phenolic indoles. Furthermore, the fact that the UV spectrum of metabolite UV-1 was very similar to that of 3MOI and HMOI (Fig. 2) indicated that metabolite UV-1 could be structurally related to any of the two oxindoles; these metabolites, in which the pyrrol ring is oxidized at the 2-carbon position, show very different spectra than 3MI, or other metabolites such as I3C, 2-aminoacetophenone, or the hydroxyskatoles. Finally, the ^{1}H NMR spectrum of UV-1 (Fig. 4) was consistent with the assignment of this metabolite to 3-hydroxy-3-methylindolenine.

The results of the present study indicate that seven major metabolites of 3MI are produced by pig liver microsomes in vitro. In quantitative terms, the main pathway of phase I biotransformation of 3MI by pig liver microsomes appears to be the formation of oxindole derivatives and the formation of 3-hydroxy-3-methylindolenine. Differences in the metabolic fate of 3MI among species could explain the difference in species susceptibility to 3MI-induced lung toxicity. The extensive metabolism of 3MI to oxindole derivatives may explain the lack of pneumotoxicity exhibited by pigs and reported by Carlson and Yost (1989). The electrophilic metabolite 3-methylene-indolenine, which is the putative reactive metabolite of 3MI produced by cytochrome P-450 enzymes, is a precursor of I3C in lung microsomal incubations and susceptible species form I3C in appreciable amounts (Skiles and Yost, 1996). In the present in vitro study, <3% of the metabolites produced by pig liver microsomes corresponded to I3C, which also may explain the lack of susceptibility of pigs to suffer from 3MI-induced lung lesions. Large interindividual differences in the rate of production of metabolites were observed. These differences in phase I metabolism could be due to individual differences in cytochrome P-450 enzymes and this issue should be further investigated. It was previously reported that CYP2E1 plays a role in the metabolism of 3MI in the pig (Squires and Lundström, 1997; Babol et al., 1998a), but the role of other isoenzymes remains to be determined. Babol et al. (1998b) reported sulfation and glucuronidation of some 3MI metabolites produced by pig liver microsomes. However, more studies are needed to determine the complete phase II metabolism of the different metabolites of 3MI identified in the present study.

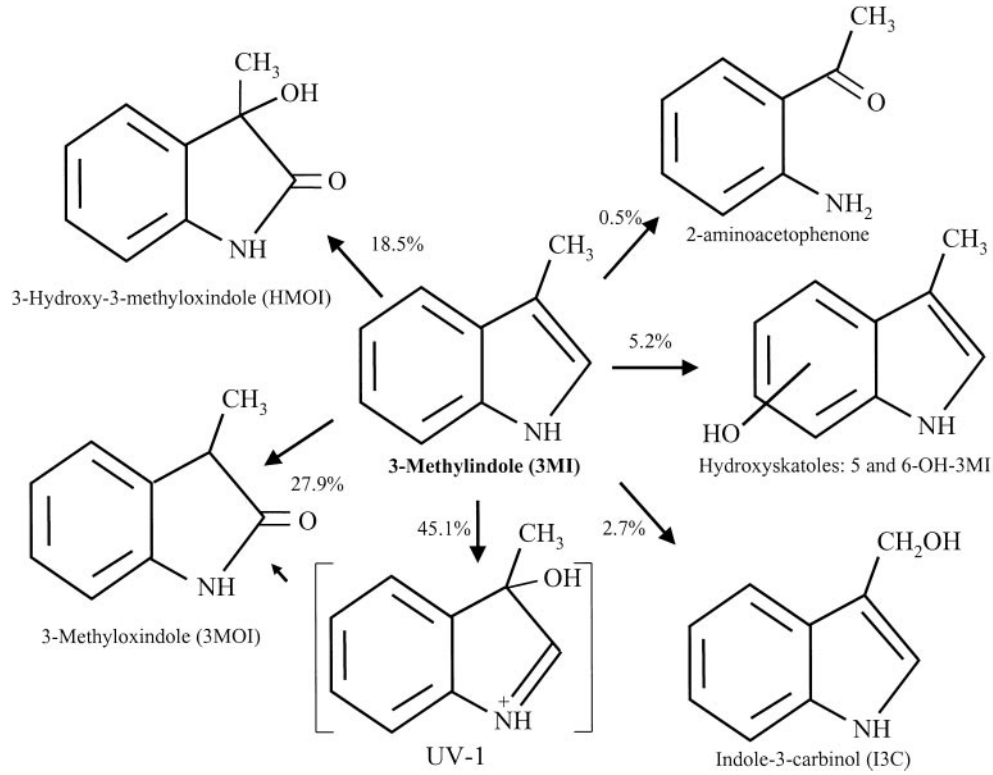

FIG. 5. *Chemical structures and percentages of 3MI metabolites produced by pig liver microsomes.*

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