Identification of Selected Metabolites of Skatole in Plasma and Urine from Pigs

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During investigation of the kinetics of skatole in pigs, it was observed that some pigs, which are poor metabolizers of skatole (3-methylindole), are incapable of forming a metabolite named MII. This lead to the hypothesis that incidents of boar taint in Danish pigs could at least be due partly to polymorphism in one of the P-450 isoenzymes responsible for the oxidative metabolism of skatole. To further investigate the metabolism of skatole in pigs, selected metabolites of skatole were isolated and identified. Three major metabolites of skatole were isolated from urine from pigs given [^{14}C]-skatole (5 mg/kg). The metabolites were identified using mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy as 6-sulfatoxyskatole (MII), 3-hydroxy-3-methyloxindole (MIII), and the mercapturate adduct of skatole, 3-[(*N*-acetylcysteine-*S*-yl)methyl]indole (MX). MII and MIII are found both in plasma and urine, MX is only found in urine.

Keywords: *Skatole; 3-methylindole; metabolism; pig*

INTRODUCTION

Entire male pigs (uncastrated pigs) are used for meat production in several countries. The production in Denmark started about ten years ago mainly due to better feed conversion by male pigs and a better dietary composition of fatty acids in the meat. However, 5−10% of the entire male pigs carry the so-called boar taint. Meat carrying this boar taint is rejected by the consumer when heated, due to liberation of certain volatile substances with a repulsive smell.

In Denmark it is documented that the major contributor to boar taint in danish pigs is skatole (3-methylindole) (Mortensen and Soerensen, 1984; Bejerholm and Barton-Gade, 1993; Godt et al., 1995). Until now the reason for the boar taint, which is present in only some of the male pigs, has not been known exactly. In order to obtain additional knowledge of some of the causes of the diversity among the male pigs, the metabolism of skatole in the pig is being investigated.

Skatole is formed in the intestines of mammals by skatole-producing bacteria. The precursor of skatole is the amino acid tryptophan, which is transformed to indoleacetic acid with subsequent decarboxylation to skatole (Carlson and Breeze, 1984). Research in the regulation of the formation of skatole in the intestines has been performed (Jensen et al., 1995a,b).

During the investigation of the kinetics of skatole in pigs, it was observed that pigs, which are poor metabolizers of skatole, have diminished capability to form a metabolite named MII (Friis, 1993). Four different metabolites were seen in plasma and more than fifteen metabolites in urine could be detected when [^{14}C]skatole was used. In plasma from normal pigs, MII is the major metabolite of skatole. In poor metabolizers of skatole, the metabolite MIII is seen in larger amounts, probably to compensate for the diminished capability to form MII, and thus MIII becomes the major metabolite. These findings indicate that the formation of MII is an important metabolic pathway for skatole in pigs and lead to the hypothesis that the incidents of boar taint in Danish pigs are partly due to polymorphism in one of the P-450 isoenzymes that is responsible for the oxidative metabolism of skatole.

The aim of this study was to isolate and identify the major metabolites of skatole in pigs in order to contribute to the identification of the P-450 isoenzymes that are primarily responsible for the formation of MII and MIII. Furthermore, the aim was to develop an analytical method which could be used for the determination of the major metabolites of skatole in plasma samples from pigs in order to differentiate between pigs with varying skatole metabolism. The method is proposed for use in the selection of boars for breeding.

MATERIALS AND METHODS

Chemicals. Methanol, acetonitrile, and tetrahydrofuran were far-UV high-performance liquid chromatography (HPLC)-grade and were purchased from Lab-Scan, Dublin, Ireland. Potassium dihydrogen phosphate was analytical grade, purchased from Riedel-de Haën, Germany. Amberlite XAD-2 adsorbent was purchased from SERVA Feinbiochemica, Heidelberg.

Procedure and Apparatus. Three metabolites of skatole were isolated. Two metabolites, MII and MIII, were present in both plasma and urine. The third metabolite, a mercapturate adduct of skatole (MX), was found only in urine.

The metabolites are 6-sulfatoxy-3-methylindole (MII), 3-hydroxy-3-methyloxindole (MIII), and the mercapturate, 3-[(*N*-acetylcysteine-*S*-yl)methyl]indole (MX). For the isolation of MII, urine from a normal pig (extensive metabolizer of skatole, 5 months old, 84 kg) given [^{14}C]skatole was used. In order to obtain larger quantities of MIII, urine from a poor metabolizer (5 months old, 83 kg) was used for the isolation of MIII and MX.

An amount of 1.25 L of the urine containing the highest concentration of radioactivity preserved with 0.05% sodium

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S0021-8561(96)00586-9 CCC: $14.00

Table 1. Mobile Phases Utilized for the Purification of the Metabolites by Isocratic Preparative HPLC

metabolite	mobile phase in first purification step using preparative HPLC	retention time of metabolite	mobile phase in second purification step using preparative HPLC	retention time of metabolite
MII	tetrahydrofuran/0.01 M potassium phosphate buffer pH 6.0 (3/97)	11.6 min	acetonitrile/0.01 M potassium phosphate buffer pH 6.0 (12/88)	8.5 min
MIII	methanol/0.01 M potassium phosphate buffer pH 6.0 (30/70)	7.4 min	tetrahydrofuran/0.01 M potassium phosphate buffer pH 6.0 (12/88)	8.5 min
MX	methanol/0.01 M potassium phosphate buffer pH 6.0 (30/70)	9.6 min	methanol/acetonitrile/0.01 M potassium phosphate buffer pH 6.0 (9.5/6.5/84)	15.5 min

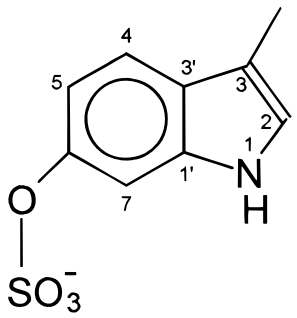

Figure 1. Molecular structure of 6-sulfatoxyskatole (MII).

azide was pumped (BIFOK F 08 peristaltic pump) onto a column (Pharmacia SR 25/100) with the dimensions 100 cm in length, 2.5 cm i.d., packed with Amberlite XAD-2, 0.3−1 mm particle size (p.s.). Before the application of the urine, the column was conditioned sequentially with 1 L methanol and 1 L of 0.01 M potassium dihydrogen phosphate buffer at pH 7.5. After the urine was pumped through the column, it was washed with 0.5 L of the above mentioned buffer. The flow was 3 mL/min.

Elution of the adsorbed compounds was performed by a gradient from 0 to 100% methanol during 10 h (two Waters 6000 HPLC pumps and a Waters 660 Solvent Programmer), and fractions of 25 mL were collected (LKB Fraction Collecter). The flow was 2.5 mL/min. Of each fraction 50 μL was analyzed by a Packard 200CA TRI-CARB Liquid Scintillation Analyzer.

For localization of the radioactive metabolites, selected fractions were analyzed by HPLC; Perkin Elmer Binary LC pump 250, Shimadzu SIL 9A auto-injector connected to a Spherisorb ODS-2 HPLC column, p.s. 5 μm, 125 mm × 4.6 mm i.d., a Shimadzu CR5A Chromatopac integrator, a Kontron 430 UV detector, and a Shimadzu RF 551 fluorescence detector. The radioactivity detector was attached to a Waters Maxima system equipped with an additional detector of fluorescence. Mobile phase A, 5% acetonitrile in 0.01 M potassium dihydrogen phosphate buffer, pH 6.5; mobile phase B, 90% acetonitrile, 10% water. Gradient: linear, 0−30% B in 20 min. The fractions containing the wanted metabolites were pooled in a 500 mL round flask, and the organic solvent was evaporated on a rotary evaporator. For the isolation of MII fractions 33−42 were chosen, and for the isolation of MIII and MX fractions 34−46 was chosen. Fractions were collected from the beginning of the gradient elution. For all three metabolites two purification steps using preparative HPLC were necessary to purify them for the identification by NMR and MS analysis. On the remaining aqueous solution from the evaporated pooled fractions (approximately 30 mL) the first preparative purification by HPLC was carried out by isocratic elution.

Table 1 shows the mobile phases for preparative HPLC used for the purification of the metabolites. For the purification of MII, the remaining aqueous solution was applied in portions of 500 μL on a preparative HPLC column (250 mm × 8 mm i.d.). For the purification of MIII and MX portions of 700 μL was applied on the column. The stationary phase was Spherisorb ODS-2, 5 μm p.s. The flow rate was 4 mL/min.

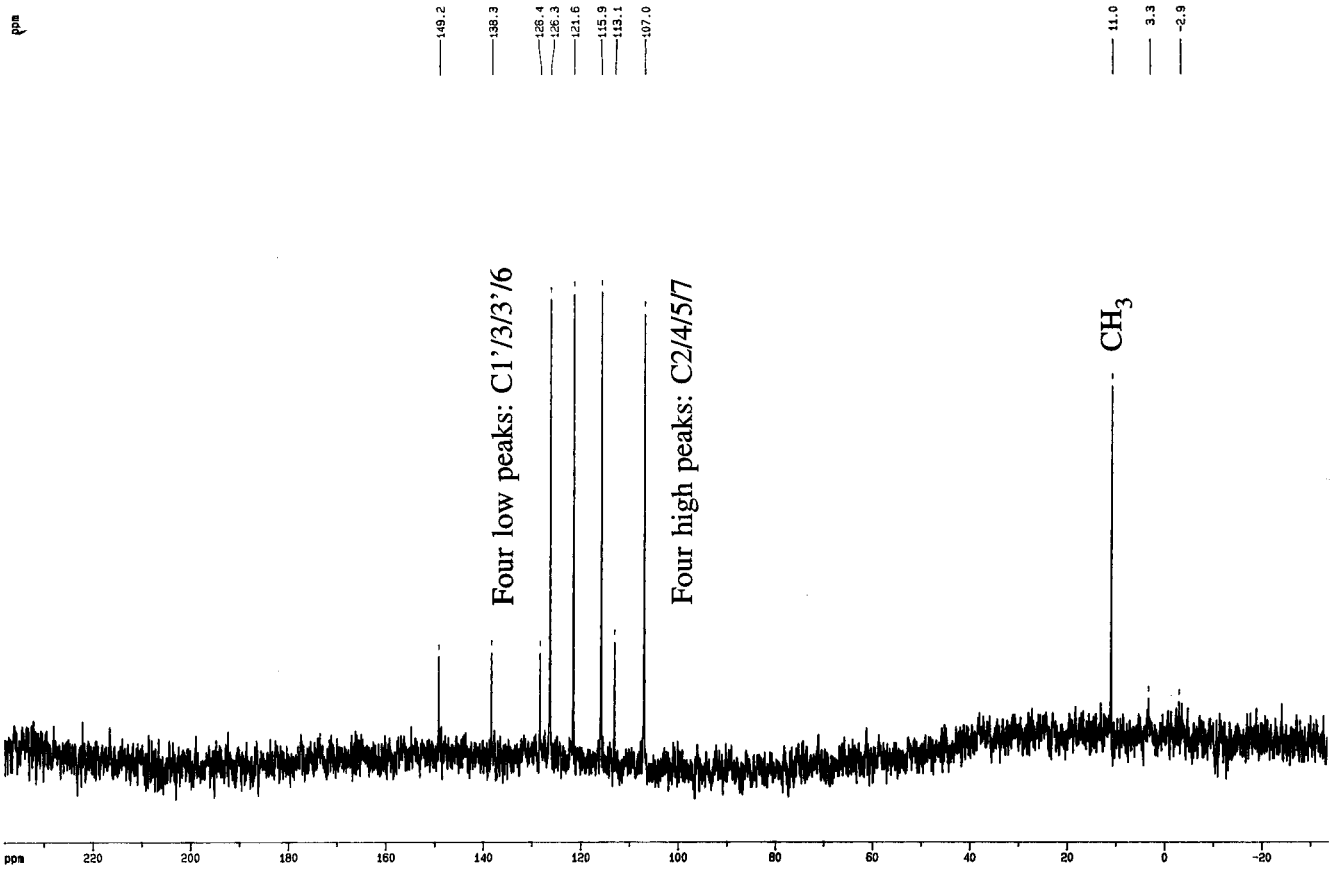

Figure 2. ^{13}C NMR spectrum of MII dissolved in deuterium oxide.

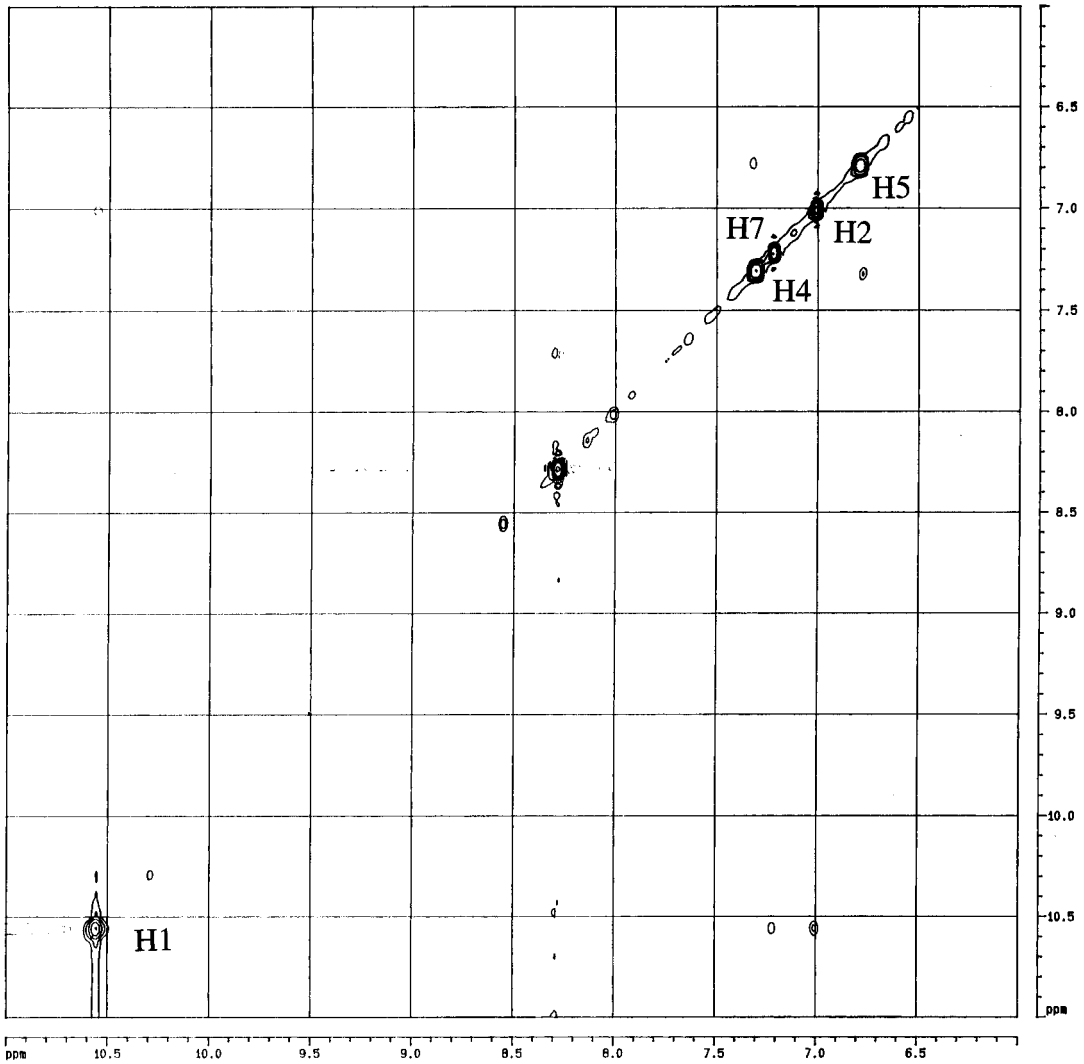

Figure 3. 2D NMR NOE spectrum of MII dissolved in dimethyl sulfoxide.

After evaporation of the organic solvent from the fraction containing the metabolite obtained from the preparative HPLC, the inorganic buffer from the mobile phases was removed by solid phase extraction. A polypropylene Econo column (diameter, 0.7 cm; height, 4.0 cm) filled with XAD-2, 0.3−1 mm p.s., resin was used. The column was subsequently conditioned with 20 mL of methanol and 50 mL of water. The aqueous solution of the metabolite was applied onto the column. The column was washed twice with 5 mL of water, and the pure metabolite was eluted with 3 × 5 mL of methanol. The procedure was monitored by HPLC analysis (above-mentioned analytical system). After evaporation of the methanol, the metabolites were ready for the spectroscopic analyses. Isolated were 5 mg of MII, 20 mg of MIII, and 14 mg of MX corresponding to 0.004 of mg MII/mL of urine, 0.016 mg of MIII/mL of urine, and 0.011 mg of MX/mL of urine.

RESULTS

Identification of the Metabolites. The NMR spectroscopic identification of the metabolites was performed using a Bruker AMX 400 WB NMR apparatus. The following conditions were used for 2D NMR. 2D nuclear Overhauser effect (NOESY) spectra were recorded using the pulse program supplied by Bruker (noesytp) in the phase sensitive mode (TPPI). A relaxation delay of 1.5 s was used in all cases. A pulse width of 13.8 μs corresponding to 90° and a mixing time of 300 ms (400 ms in one case) were used. 512 (474 in one case) spectra of 64 scans (56 in one case) and 1024 (real) data points were acquired for each. In all cases, the spectra were zero-filled to 1024 complex data points in both dimensions and a phase-shifted sinebell was applied prior to two-dimensional Fourier transformation in order to emphasize in-phase crosspeaks and suppress antiphase crosspeaks.

The mass spectrometric identification of the three metabolites was performed using a Finnigan TSQ-7000 MS apparatus with electron impact ionization (EI) on the solid probe. The molecular weight of MII was measured on a JEOL AX 505 W mass spectrometer. The metabolite was dissolved in glycerol and analyzed by direct inlet and ion mode FAB-ionization with xenon.

6-Sulfatoxyskatole (MII). From the ^{1}H NMR spectrum of MII (Figure 1) dissolved in deuterium oxide the following results can be derived, which indicated a hydroxylated skatole (chemical shift, multiplicity, integral value, coupling constant, and assignment): H1 is not visible in this spectrum because it is exchanged with deuterium; δ 7.05 (d, 1, J = 1.1 Hz, H-2); δ 2.18 (d, 3, J = 1.1 Hz, -CH_3), which indicates that the methyl group is unsubstituted and couples to H-2; δ 7.48 (d, 1, J = 8.6 Hz, H-4), which indicates the presence of *ortho* coupling only and not *meta* coupling and, thereby, a possibility of ring substitution in the 5- or 6-position; δ 6.93 (dd, 1, J = 8.6 and 2.1 Hz, H-5); δ 7.25 (d, 1, J = 2.1, H-7). A ^{13}C spectrum verified the above mentioned observations (Figure 2).

A 2D NOE NMR spectrum showed NOE's between the protons attached to the methyl group and to H2 and

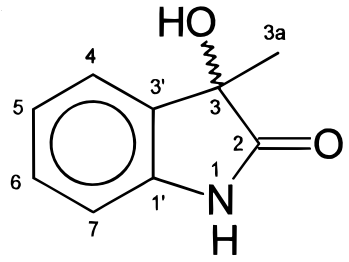

Figure 4. Molecular structure of 3-hydroxy-3-methyloxindole (MIII).

H4. H4 displays *ortho* coupling to another aromatic proton. This lead to the preliminary conclusion that MII was a 6-substituted skatole. To verify these findings, MII was dissolved in dimethyl sulfoxide (DMSO) and another 2D NMR NOE spectrum was obtained (Figure 3). It was observed that H1 showed an NOE to H2 and that H7 showed an NOE to H1 only.

The EI mass spectrum obtained on MII showed a base peak of 147, and the molecular weight of 6-hydroxyskatole is 147 g/mol. This indicated that MII could be 6-hydroxyskatole. However, further experiments treating the isolated metabolite with the enzyme preparation from *Helix pomatia* indicated that MII was a conjugate, and, as no other protons were observed, MII was most likely 6-sulfatoxyskatole.

After complete cleavage from the organic moiety by an enzyme preparation from *H. pomatia*, the sulfate part of the metabolite was identified and quantified by micellar electrokinetic capillary chromatography (MECC) employing a method intended for indirect detection of inorganic ions (Stahl, 1994). The capillary electrophoresis apparatus employed was a Waters Quanta 4000 equipped with a quartz capillary (uncoated, l. 57.4 cm, d. 50 μm) and a single-wavelength detector at 254 nm. Electrophoresis buffer: 5 mM chromate and 0.2 mM tetradecyltrimethylammonium bromide, pH 8.2. The electrophoresis voltage was −10 kV, and the injection was performed with 10 cm hydrostatic pressure for 20 s. The retention time of the sulfate ion was 5.6 min (tested by comparison with a sulfate standard and standard addition).

A FAB-mass spectrum of MII was obtained using a JEOL AX505 W mass spectrometer. The spectrum showed a large peak $[M - H]^-$ at m/z 226. The content of sulfur was confirmed by the presence of an isotope peak at m/z 228 (^{34}S). An accurate mass determination gave the result 226.0188, −6 ppm from the calculated 226.0174, which verified the molecular weight of a sulfatoxyskatole.

3-Hydroxy-3-methyloxindole. The ^1H NMR spectrum of MIII (Figure 4) dissolved in DMSO showed a spectrum indicating an oxindolic compound because the proton in position 2 was missing. Likewise it was observed that there was no ring substitution as the pattern of aromatic protons was typical for a 1,2-disubstituted aromatic spin system. Furthermore, a

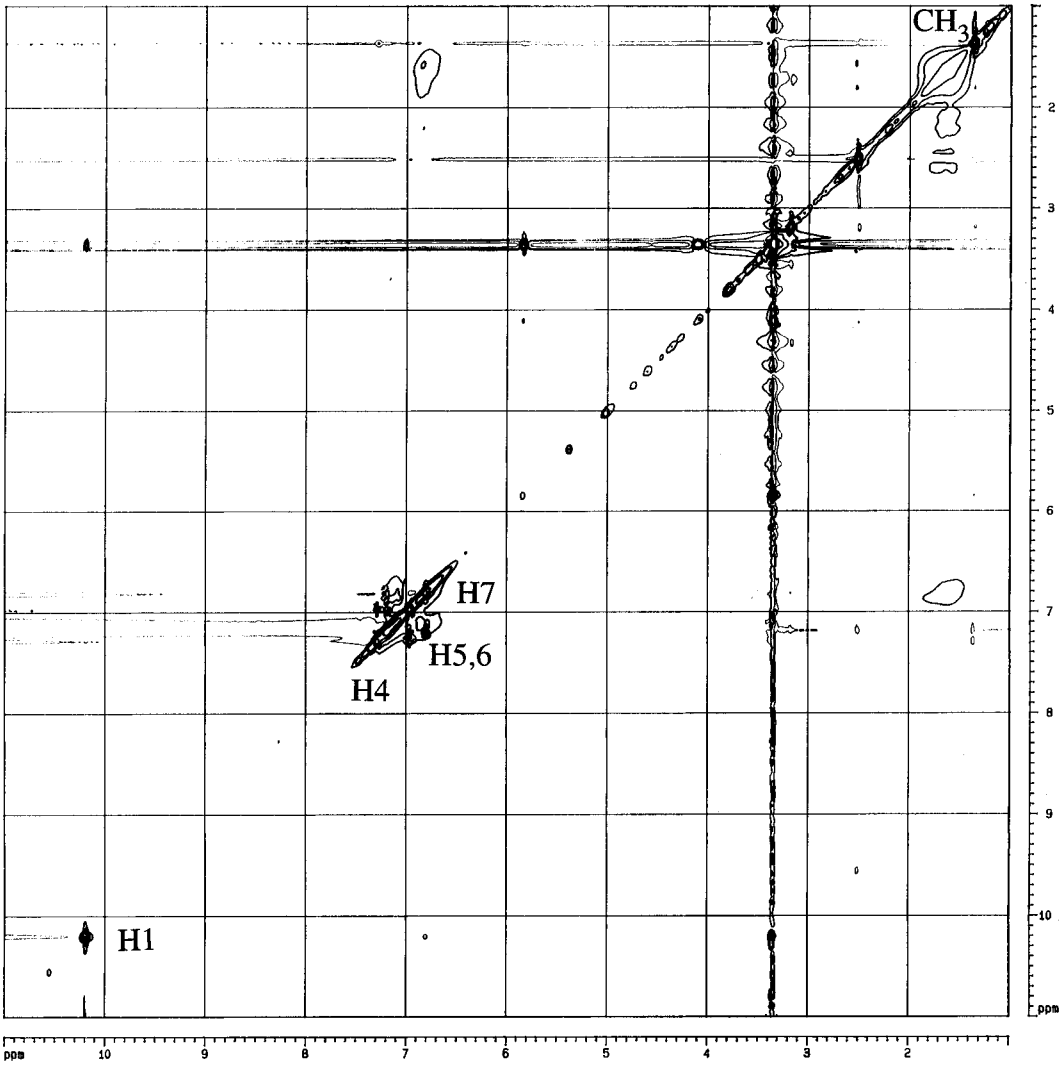

Figure 5. 2D NMR NOE spectrum of MIII dissolved in dimethyl sulfoxide.

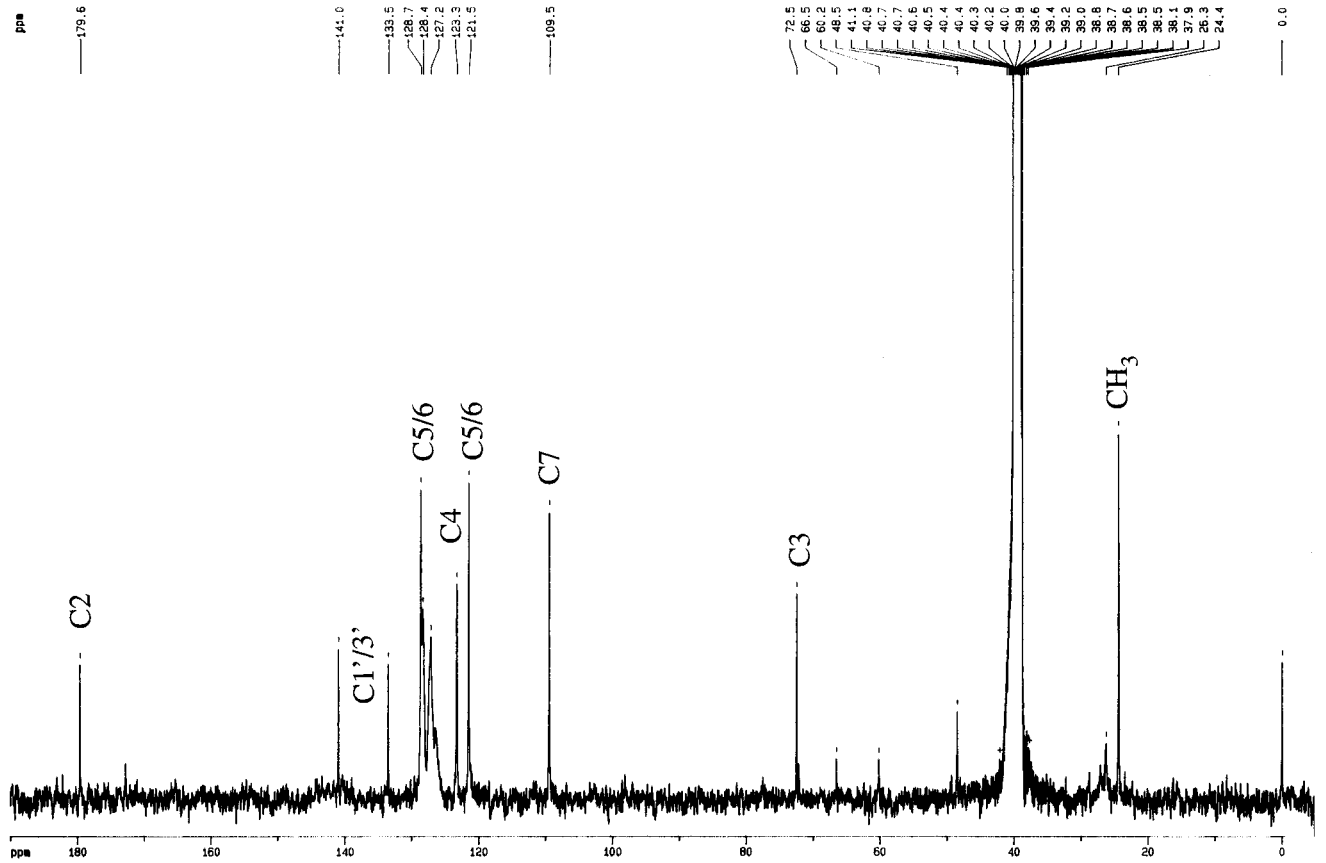

Figure 6. ^{13}C NMR spectra of MIII dissolved in dimethyl sulfoxide.

proton arising from a hydroxy group was observed: δ 10.20 (s, 1, H-1); δ 1.35 (s, 3, -CH_3); δ 7.28 (d, 1, $J = 7.3$ Hz, H-4). The identity of H-4 is confirmed as H-4 in the 2D NMR NOE spectrum shows an NOE to the methyl group (Figure 5). δ 6.95 and 7.19 (td, 1, $J = 7.5$ and 1.0 Hz, H-5 and H-6); δ 6.80 (d, 1, $J = 7.6$ Hz, H-7). The NOE spectrum (Figure 5) shows NOE between H-1 and H-7. δ 5.80 (s, 1, OH). No proton is observed in the 3-position, and thus this is the position of the hydroxy group. The chirality of the compound was not determined because of the small quantities isolated. A ^{13}C spectrum verified the above-mentioned observations (Figure 6). ^1H and ^{13}C spectra fit well with data from the literature (Skiles et al., 1989; Albrecht et al., 1989).

The molecular weight of 3-hydroxy-3-methyloxindole is 163 g/mol. The EI mass spectrum obtained on MIII showed peaks of m/z 120, 134, 148, and 163 as expected. The same ions were observed by Albrecht et al. (1989), who isolated this compound from urine from schizophrenics (sex not reported) and by Skiles et al. (1989), who isolated it from urine from male mice.

3-[(*N*-Acetylcystein-*S*-yl)methyl]indole (MX). ^1H NMR analysis of MX (Figure 7) dissolved in DMSO gave the following results: δ 10.85 (s, 1, indole-NH); δ 7.25 (d, 1, $J = 2.2$ Hz, H-2). The COSY spectrum shows an additional coupling to the indole-NH. δ 7.58 (dd, 1, $J = 7.4$, H-4); δ 6.96 (td, 1, $J = 7.0$ Hz, H-5); δ 7.05 (td, 1, $J = 7.0$ Hz, H-6); δ 7.32 (d, 1, $J = 8.0$ Hz, H-7). The COSY spectrum shows an additional coupling to the indole-NH. δ 3.85 (q, 2, $J = 8.0$ Hz, $J_{gem} = 21.3$ Hz, -CH_2-). In the NOE spectrum (Figure 8) -CH_2- shows NOEs to H-2, H-4, and the two β-protons. δ 2.86 (dd, 1, $J = 13.2$ Hz and 4.9 Hz, Cysβ_1); δ 2.69 (dd, 1, $J = 13.2$ Hz and 5.9 Hz, Cysβ_2). The NOE spectrum is showing NOEs between the β-protons and the -CH_2- group. δ 3.94 (q, 1, $J = 11.5$ and 5.9 Hz, Cysα); δ 7.35 (d, 1, $J = 6.9$ Hz, Cys-NH); δ 1.85 (s, 3, -CH_3).

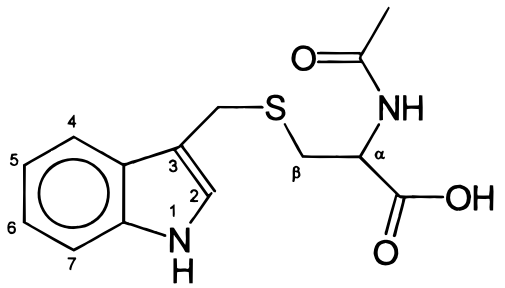

Figure 7. Molecular structure of 3-[(*N*-acetylcystein-*S*-yl)-methyl]indole (MX).

The molecular weight of 3-[(*N*-acetylcystein-*S*-yl)-methyl]indole (MX) is 292 g/mol. The EI mass spectrum obtained on the underivatized MX showed base peaks of m/z 130 and a small molecular ion at 292 (Figure 9). 130 is probably the skatole fragment. Skiles et al. (1991) isolated this compound from male rat urine. Even though Skiles methylated the compound before the mass spectrometric analysis, he obtained a large peak from the skatole fragment and only a small peak from the molecular ion.

DISCUSSION

The metabolism of skatole has been investigated for several years in different contexts, and a wide variety of metabolites have been found in different species. Species which have been thoroughly investigated in terms of the metabolism of skatole are those that are susceptible to skatole as a pneumotoxin (Skiles et al., 1989, 1991; Smith et al., 1993). Cattle, goats, and horses are particularly sensitive to this pneumotoxin, but the metabolism of skatole in mice and rats has also been investigated. Skatole can be metabolized by

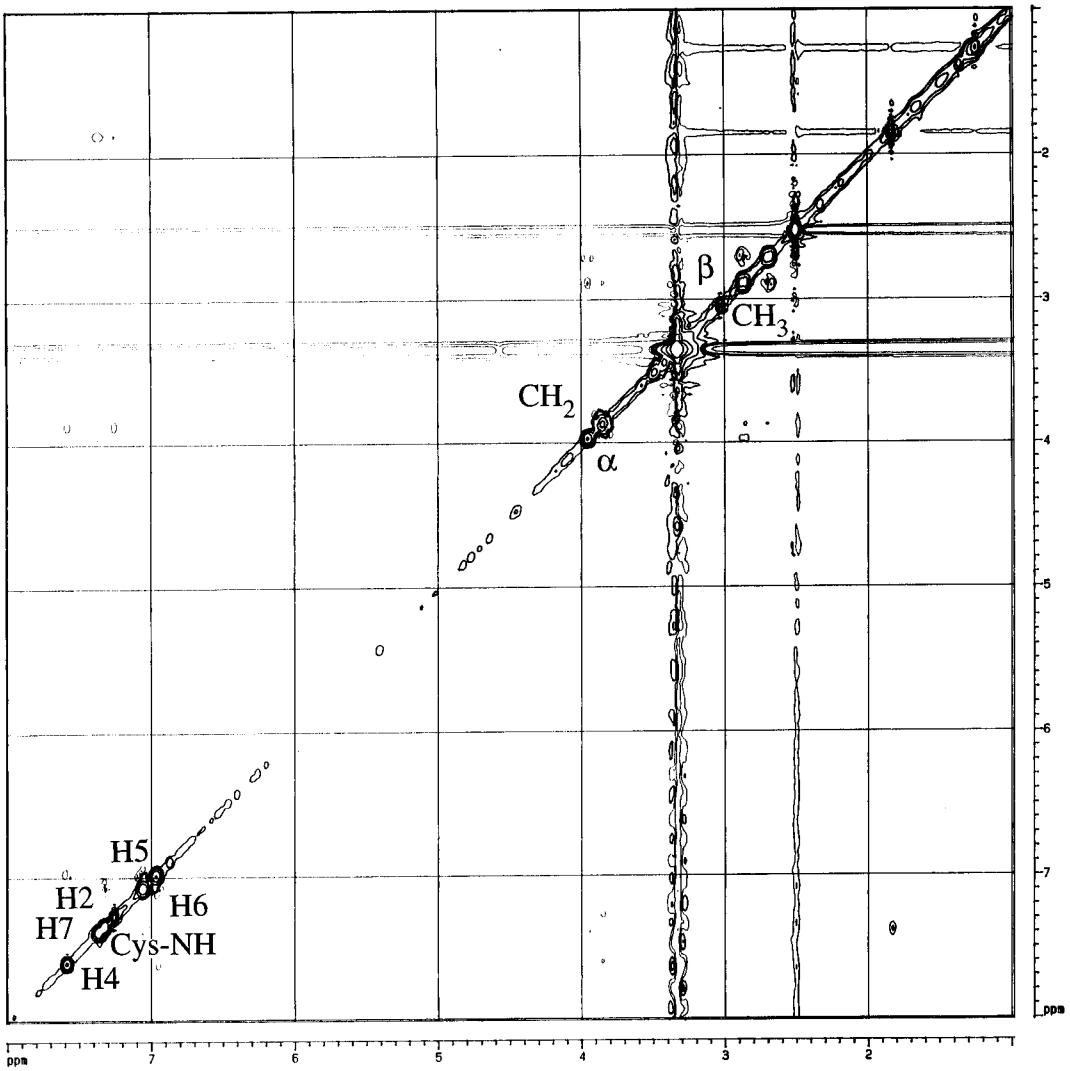

Figure 8. 2D NMR NOE spectrum of MX dissolved in dimethyl sulfoxide.

several pathways (Figure 10): by the formation of oxindoles (Skiles et al., 1989; Smith et al., 1993; Albrecht et al., 1989; Frydman et al., 1973) and aminoacetophenones (Frydman et al., 1973), by ring hydroxylations in the 5-, 6-, and 7-positions (Jepson et al., 1961; Mahon and Mattok, 1967), and by oxidation of the methyl group and conjugation with glutathione. Some of the metabolites are conjugated to sulfate or glucuronic acid (Skiles et al., 1989, 1991; Smith et al., 1993; Mahon and Mattok, 1967).

MII and MIII are the major metabolites of skatole seen in plasma from normal pigs of both sexes. MIII becomes the major metabolite seen in plasma in male pigs with dimished capability to form MII. MX is one of the major metabolites seen in urine from male pigs.

6-Sulfatoxyskatole (MII). The presence of 6-sulfatoxyskatole in urine from humans with certain mental disorders like schizophrenia was a matter of extensive research in the 1960s. 6-sulfatoxyskatole was found to occur more often in the urine of chronic schizophrenics (sex not reported) than in normal male controls (Nakao and Ball, 1960). However, no firm connection to the diseases was established.

Jepson et al. (1961) have shown that tryptamine and many other indoles are hydroxylated in the 6-position by liver microsomal hydroxylating system, neither 5- nor 7-hydroxy derivatives were formed. In liver microsomal preparations from rabbit (sex not reported), trace amounts of 6-hydroxyskatole were found after incubation with skatole. An amount of 4.5 μmol of 6-hydroxytryptamine/g of tissue/h was formed with incubation with tryptamine. The low amounts of 6-hydroxyskatole found were explained by its instability. Tryptamine injected into rats or rabbits (sex not reported) gave no 6-hydroxyindoles, but a definite excretion of acid-diazo-reacting material (probably a 6-hydroxyconjugate) followed the injection of 40 mg of tryptamine with a monoamine oxidase blockade into a rabbit. They concluded that it was not the monoamine oxidase in the liver that is responsible for the formation of the 6-hydroxyindoles but probably the mixed-function monooxidases (P-450). Human urine (sex not reported) gave an intense red color with acid diazo reagent following a large dose of indoleacetic acid. A part of the diazo-positive material was extractable into *n*-butanol at pH 2 and then into buffer at pH 7 following the addition of heptane. This material was identical with 6-hydroxyindoleacetic acid. It was stated that the formed conjugate probably was the 6-sulfatoxyindoleacetic acid. The same author claimed that many natural indolic compounds are substituted in the 6-position and that the enzymic 5-hydroxylation of tryptophan is a reaction of high specificity unrelated to the mechanism of the liver microsomal system. The 6-hydroxylating activity is limited to liver microsomes, while brain and other tissues are devoid of any activity.

Mahon and Mattok (1967) found 5-, 6-, and 7-sulfatoxyskatole in urine from 10 normal human subjects

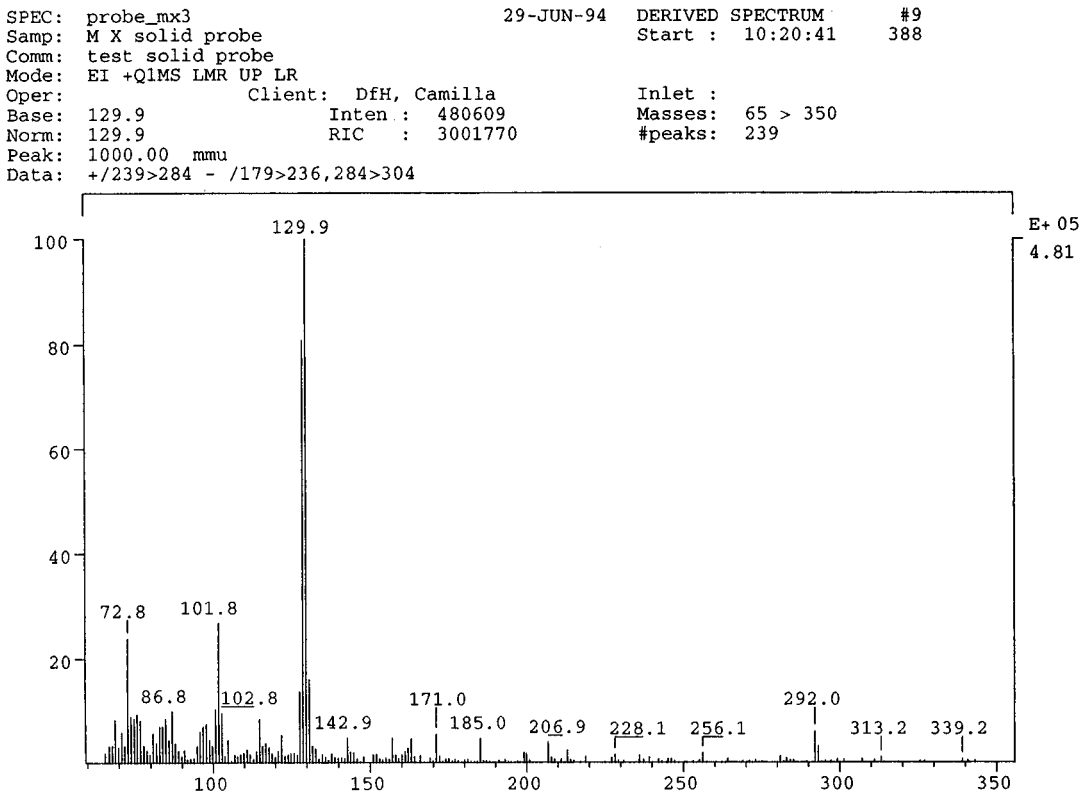

Figure 9. EI mass spectrum on MX (solid probe).

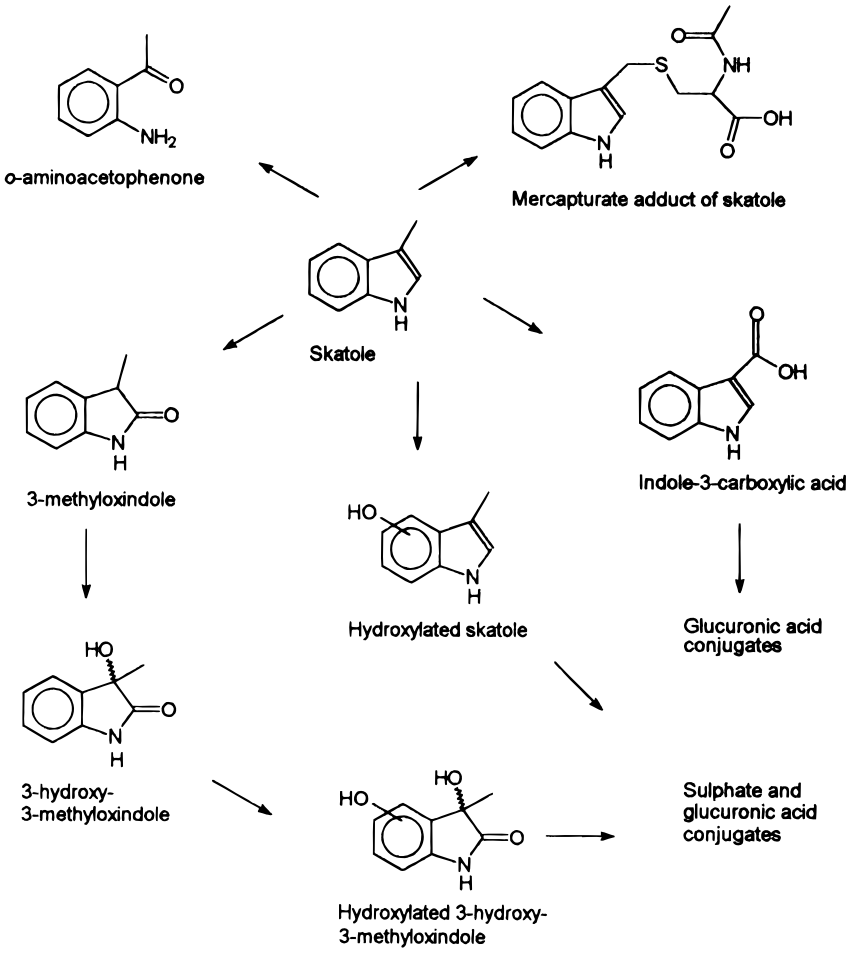

Figure 10. Metabolic pathways of skatole.

(sex not reported). The 6-isomer was by far the major sulfatoxyskatole present. 4-Sulfatoxyskatole was not found.

However, no examples of isolation of 6-hydroxyskatole or the sulfate conjugate of the compound, after direct administration of radioactive skatole, have been

reported in the literature. Furthermore it is observed that the metabolite is not found by Skiles et al. (1989, 1991) or by Smith et al. (1993) who have performed important studies of the metabolism of skatole in male, castrated goats, male mice, and male rats. It seems that the metabolite has only been found in substantial quantities in man until now.

The work of the identification of the polymorphic P-450 isoenzyme, which is responsible for the formation of 6-hydroxyskatole in pigs, is under publication.

3-Hydroxy-3-methyloxindole (MIII). Smith et al. (1993) isolated 4- or 7-, 5- or 6-hydroxylated and 3, 5-, or 6- dihydroxy-3-methyloxindole conjugated to sulfate or glucuronic acid in 20% of administered dose of [^{14}C]-skatole in goats and 15% in mice (only conjugates to glucuronic acid). The unconjugated 3-hydroxy-3-methyloxindole was found in 8% of the dose in male, castrated goats and 1.5% in male mice. Skiles et al. (1989) were the first to isolate this compound from a mammalian species. Because the isolated compound was chiral and consisted of a single enantiomer, he proposed that the formation of 3-hydroxy-3-methyloxindole must be enzymatic. It was not possible with the isolated MIII from our work to establish whether the compound was racemic or not. No indications in the literature were found regarding which P-450 isoenzyme could be responsible for the formation of oxindoles but it is proposed that the mixed-function oxidase system is responsible (Frydman et al., 1973). Albrecht et al. (1989) isolated this metabolite from urine from schizophrenics (sex not reported), but no relationship between the disease and the formation of the metabolite was established. The antimuscarine effect of the drugs leading to constipation is probably responsible for the formation of more significant quantities of skatole in the intestines.

3-[(*N*-Acetylcystein-*S*-yl)methyl]indole (MX). Skatole has been well established as a pneumotoxin in cattle, sheep, goats, horses and rodents. Glutathione plays a role in detoxication of skatole toxicity by trapping the skatole electrophilic intermediates formed by P-450 isoenzymes in the lungs, producing the glutathione adduct (Ruangyuttikarn et al., 1991). The human P-450 isoenzymes CYP2C8, CYP3A4, CYP2A6, and CYP2F1 were able to metabolize skatole to intermediates that became covalently bound to macromolecules (Thornton-Manning et al., 1991). Skiles and coworkers administered 3-methylindole to male, castrated goats, male mice, and male rats, and the urinary metabolites from these three species were analyzed by HPLC for the presence of the mercapturate that would be expected as the processed and excreted form of the 3-methylindole−glutathione adduct. 3-[(*N*-Acetylcysteine-*S*-yl)methyl]indole was identified in the urine from all three species and was isolated from rat urine for structural identification by NMR and MS. Male, castrated goats excreted 4.8% of the dose as the mercapturate adduct of 3-methylindole, and male mice and male rats excreted the dose as the mercapturate adduct in 2.6% and 7.3% respectively (Skiles et al., 1991). Now the metabolite is also found in male pigs, and it seems that the formation of the mercapturic acid conjugate of skatole is a major metabolic pathway in pigs. It is not possible with the available data to determine the exact extent to which skatole is metabolized to the mercapturate adduct, but the metabolite is found in substantial quantities in urine of pigs treated with skatole.

ABBREVIATIONS USED

MII, 6-sulfatoxyskatole; MIII, 3-hydroxy-3-methyloxindole; MX, 3-[(*N*-acetylcysteine-*S*-yl)methyl]indole; p.s., particle size; MS, mass spectroscopy; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; CZE, capillary zone electrophoresis; NOE, nuclear Overhauser effect; COSY, correlated spectroscopy (homonuclear couplings); EI, electron impact; FAB, fast atom bombardment.

ACKNOWLEDGMENT

We thank Jan S. Jensen, Ministry of the Interior, Emergency Management Agency, Chemical Laboratory, Denmark, for performing the MS−EI analysis of the three isolated metabolites and Carl E. Olsen, Department of Chemistry, Royal Danish Veterinary and Agricultural University, for performing the MS-FAB analysis of MII.

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Received for review August 5, 1996. Accepted December 5, 1996.[⊗]

JF9605862

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1997.