

Identification of Metabolite Biomarkers of the Designer Hallucinogen 25I-NBOMe in Mouse Hepatic Microsomal Preparations and Human Urine Samples Associated with Clinical Intoxication

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'NBOMe' (dimethoxyphenyl-*N*-[(2-methoxyphenyl)methyl]ethanamine) derivatives are a new class of designer hallucinogenic drugs widely available on the Internet. Currently, 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe) is the most popular abused derivative in the USA. There are little published data on the absorption, metabolism and elimination of 25I-NBOMe, or any of the other NBOMe derivatives. Therefore, there are no definitive metabolite biomarkers. We present the identification of fifteen 25I-NBOMe metabolites in phase I and II mouse hepatic microsomal preparations, and analysis of two human urine samples from 25I-NBOMe-intoxicated patients to test the utility of these metabolites as biomarkers of 25I-NBOMe use. The synthesis of two major urinary metabolites, 2-iodo-4-methoxy-5-[2-[(2-methoxyphenyl)methylamino]ethyl]phenol (2-*O*-desmethyl-5-I-NBOMe, M5) and 5-iodo-4-methoxy-2-[2-[(2-methoxyphenyl)methylamino]ethyl]phenol (5-*O*-desmethyl-2-I-NBOMe), is also presented. Seven phase II glucuronidated metabolites of the *O*-desmethyl or the hydroxylated phase I metabolites were identified. One human urine sample contained 25I-NBOMe as well as all 15 metabolites identified in mouse hepatic microsomal preparations. Another human urine sample contained no parent 25I-NBOMe, but was found to contain three *O*-desmethyl metabolites. We recommend β -glucuronidase enzymatic hydrolysis of urine prior to 25I-NBOMe screening and the use of M5 as the primary biomarker in drug testing.

Introduction

The identification and analysis of designer drug metabolites is crucial to the assessment of drug exposure (1). In general, these metabolites are formed only *in vivo* and are not available as analogs on the illicit drug market. Their presence in biological samples such as urine and hair positively confirms exposure to the parent drug. Also, analysis of drug metabolites reduces the likelihood of false negatives when the parent drug is not detected (2). Metabolites of many drugs of abuse can be detected in urine for days and sometimes weeks after exposure (3). Metabolites may be more abundant in biological matrices than the parent drug. Therefore, it is essential to consider drug metabolites as biomarkers for drugs in clinical and forensic toxicological analyses. 'NBOMe' (dimethoxyphenyl-*N*-[(2-methoxyphenyl)methyl]ethanamine) derivatives are a new class of designer hallucinogenic drugs widely available on the Internet. Adverse reactions to NBOMe derivatives include violent, bizarre and/or self-destructive behaviors (4–11). Currently, the most abused NBOMe derivative in the USA is 25I-NBOMe (2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine) (Figure 1) (12). Unfortunately, there are little published data on the absorption, metabolism

and elimination of 25I-NBOMe, or any of the other NBOMe derivatives. To the authors' knowledge, only a single case report has addressed NBOMe metabolites. Stellpflug *et al.* (6) described the intoxication of an 18-year-old woman after administration of 25I-NBOMe blotter paper. They suggested the possible structure of three *O*-desmethylated-25I-NBOMe compounds, which were apparently present only as glucuronidated conjugates and 2,5-dimethoxy-4-iodophenethylamine (2C-I) urinary metabolites. Their tentative identification was based on enzyme hydrolysis and mass spectrometric data. They lacked primary reference material to compare with their mass spectral data to produce a more definitive identification.

To establish appropriate urinary biomarkers for 25I-NBOMe exposure, we determined its metabolism in mouse hepatic microsomal preparations. We then tested for the presence of these metabolites in human urine samples from Emergency Department 25I-NBOMe-intoxicated patients. As part of this work, we synthesized two of the *O*-desmethylated-25I-NBOMe metabolites, with structural elucidation by NMR, for use as a reference material. The identification of fifteen 25I-NBOMe metabolites, including glucuronides, is presented.

Experimental

Reagents

The primary reference materials 25I-NBOMe hydrochloride salt, *N*-(2-trideuterated-methoxybenzyl)-2,5-dimethoxy-4-iodophenethylamine (25I-NBOMe-*d*₃) hydrochloride salt and 2-(2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine (25H-NBOMe) were obtained from Cayman Chemical (Ann Arbor, MI, USA). 2C-I in methanol was obtained from Cerilliant Corporation (Round Rock, TX, USA). 2-((2-(4-iodo-2,5-dimethoxyphenyl)ethylamino)methyl)phenol (25I-NBOH) hydrochloride in powder form was purchased from BC Distribution (Quebec, Canada). 2-Iodo-4-methoxy-5-[2-[(2-methoxyphenyl)methylamino]ethyl]phenol (2-*O*-desmethyl-5-I-NBOMe, M5) and 5-iodo-4-methoxy-2-[2-[(2-methoxyphenyl)methylamino]ethyl]phenol (5-*O*-desmethyl-2-I-NBOMe, M6) were prepared in house based on the previously published synthesis of 25I-NBOMe (13). Several milligrams of M5 and M6 of 97% purity were produced. Aluminum chloride, ammonium acetate, boron trichloride, dichloroethane, 2,5-dimethoxyphenylethylamine, dimethyl sulfide, Dulbecco's phosphate buffered saline (DPBS), *Helix pomatia* β -glucuronidase (100,000 units), iodine monochloride, 2-iodopropane, isopropanol, lithium aluminum hydride, 2-methoxybenzaldehyde, nitromethane, potassium carbonate,

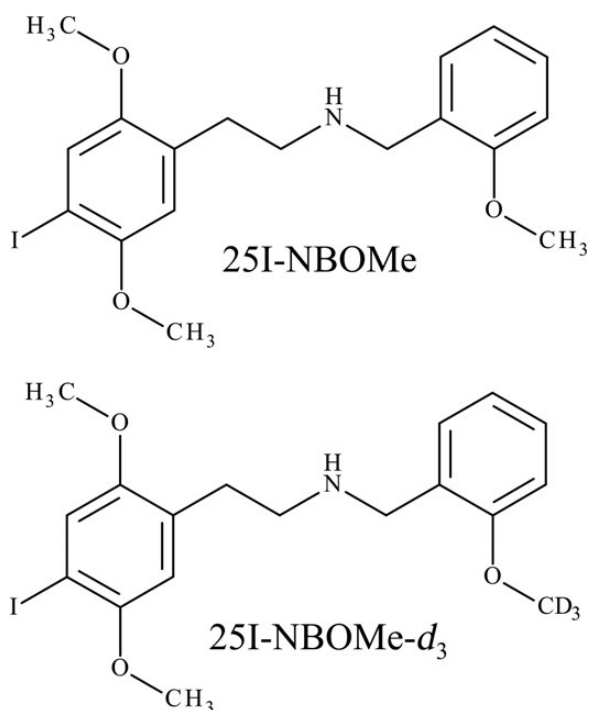


Figure 1. Structure of 25I-NBOMe and 25I-NBOMe-*d*₃.

reduced nicotinamide adenine dinucleotide phosphate (NADPH), sodium thiosulfate, sodium cyanoborohydride, sucrose, tetrahydrofuran (anhydrous) and uridine 5'-diphospho-glucuronic acid (UDPGA) were purchased from Sigma Aldrich (St Louis, MO, USA). Acetic acid, acetone, ammonium acetate, *n*-butyl chloride, chloroform, deionized (DI) water, dichloromethane, *N,N*-dimethylformamide, ethanol, ethylenediamine tetraacetic acid (EDTA), ethyl acetate, ethyl ether, glycerol, hexane, hydrazine, hydrochloric acid, magnesium chloride, methanol, nitric acid, 10% palladium on carbon, phthalic anhydride, potassium chloride, potassium phosphate buffer, silica gel, sodium hydroxide, sodium iodide, sodium nitrite, sodium sulfate and Tris-HCl were purchased from Fisher Scientific (Pittsburgh, PA, USA). Heptafluorobutyric anhydride (HFBA) and bis(trimethylsilyl) trifluoroacetamide + 10% trimethylchlorosilane (TMS) were purchased from Regis® Technologies, Inc. (Morton Grove, IL, USA). 2-Hydroxy-5-methoxybenzaldehyde was purchased from VWR International, LLC (Radnor, PA, USA). All reagents were ACS grade or better. The Bradford Protein Assay kit was purchased from Bio-Rad (Hercules, CA, USA). Male C57/BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Medical grade nitrogen was purchased from National Welders Supply Company (Richmond, VA, USA).

Synthesis of M5

A mixture of 2 g of 2-hydroxy-5-methoxybenzaldehyde, 3.25 mL of 2-iodopropane, 3.6 g of potassium carbonate and 10 mL of dimethylformamide was mixed at 70°C for 24 h. This mixture was then cooled to room temperature. Water was added. The mixture was extracted with ethyl acetate and washed with saturated sodium carbonate aqueous solution. The ethyl acetate layer was dried with sodium sulfate and the solvents were evaporated leaving a clear oil, 2-isopropoxy-5-methoxybenzaldehyde.

A mixture of 1.94 g of 2-isopropoxy-5-methoxybenzaldehyde, 20 mL of acetic acid, 1.93 g of ammonium acetate and 3.8 mL of nitromethane were mixed at 100°C overnight. The reaction was cooled to room temperature and poured into water after which the pH was adjusted to 7.0 with 2 M sodium hydroxide. The resulting aqueous solution was extracted with ethyl acetate and dried with sodium sulfate. After evaporating the solvent, the residue was purified by flash chromatography (silica gel, 1 : 4 hexane : dichloromethane) to give a yellowish solid, (*E*)-1-isopropoxy-4-methoxy-2-(2-nitrovinyl)benzene. The yellow solid was suspended in 20 mL of tetrahydrofuran and 672 mg of lithium aluminum hydride was added. The mixture was mixed at 65°C for 4 h and then cooled to room temperature. About 0.5 mL of water followed by 1 mL of 15% sodium hydroxide aqueous solution was added, followed by 3 mL of water. The precipitated white solid was filtered and the solvent was evaporated under vacuum. The residue was dissolved in 1 M hydrogen chloride acid and washed with ether. The acidic extract was basified with 5 M sodium hydroxide and extracted with ether, washed with water and dried over sodium sulfate. After removing solvent, the residue was dissolved in 20 mL of acetic acid. Eight hundred and eighty-eight milligrams of phthalic anhydride were added, and the solution was refluxed for 4 h and then cooled to room temperature. After evaporating the solvent, the residue was purified by flash chromatography (silica gel, 1 : 2 hexane : dichloromethane to 1 : 3 hexane : ethyl acetate) to yield a brown oil. The brown oil was dissolved in 8 mL of acetic acid and cooled to 10°C. A solution of 503 mg of iodine monochloride in 2 mL of acetic acid was added slowly while stirring vigorously. After 6 h, the solution was poured onto ice, and excess saturated thiosulfate solution was added. The aqueous solution was extracted with dichloromethane, washed with water and dried with sodium sulfate. After evaporating the solvent, the residue was purified by flash chromatography (silica gel, 10 : 1 hexane : acetone) resulting in a brown oil. The brown oil was dissolved in 30 mL of ethanol and then 1 mL of hydrazine was added, refluxed for 5 h and cooled to room temperature. After removing solvent under vacuum, the residue was purified by flash chromatography (silica gel, 20 : 1 dichloromethane : methanol) to yield 2-(4-iodo-2-isopropoxy-5-methoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine.

Two hundred and seventy milligrams of 2-(4-iodo-2-isopropoxy-5-methoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine, 95 mg of 2-methoxybenzaldehyde and 46 µL of acetic acid were added to 10 mL of dichloroethane, and stirred at room temperature for 1 h. Then, 64 mg of sodium cyanoborohydride was added and stirred continuously at room temperature overnight. Water was then added, and the mixture was extracted with dichloromethane and dried with sodium sulfate. After evaporating solvent, the residue was purified by flash chromatography (silica gel, 30 : 1 dichloromethane : methanol) to yield 2-(4-iodo-2-isopropoxy-5-methoxyphenyl)ethanamine.

One hundred and twenty-five milligram of 2-(4-iodo-2-isopropoxy-5-methoxyphenyl)ethanamine was added to 10 mL of dichloromethane and cooled to -78°C. A solution of 1 M boron trichloride in 0.412 mL of dichloromethane was added dropwise. The reaction mixture was allowed to warm to room temperature and was stirred overnight. Ten milliliters of methanol were added dropwise and stirred at room temperature for 5 h. The solvent was then removed under vacuum and

dichloromethane was added to the residue. It was filtered and the white solid precipitate, 2-*O*-desmethyl-5-*I*-NBOMe hydrochloride salt, was collected (Figure 2). The structure of this material was established by NMR analysis.

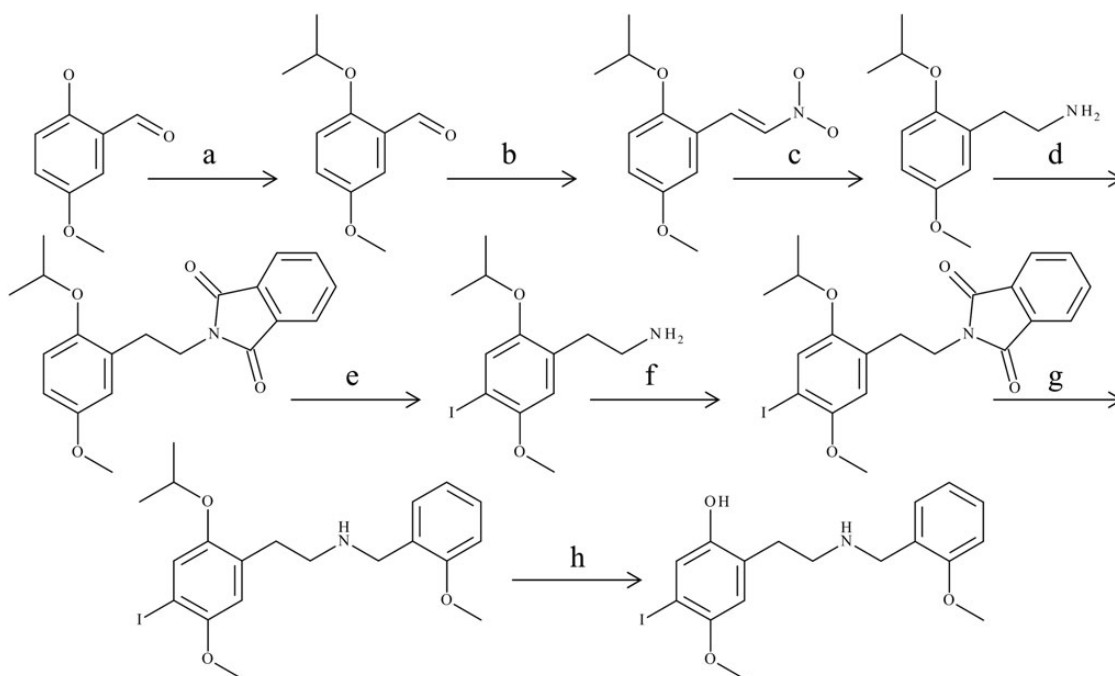
Synthesis of M6

A mixture of 1.84 mL of 2,5-dimethoxyphenylethylamine, 2.44 g of phthalic anhydride and 20 mL of acetic acid was stirred and refluxed for 8 h. After the mixture cooled to room temperature, 1 mL of nitric acid was added and mixed for 1 h. The precipitate was filtered, collected and washed with water to yield 2-(2,5-dimethoxy-4-nitrophenethyl)isoindoline-1,3-dione. The precipitate was dissolved in 50 mL of chloroform with 1 g of aluminum chloride; the reaction mixture was refluxed for 5 h. The mixture was cooled to room temperature and mixed with 1 M hydrochloric acid, extracted with dichloromethane and dried with sodium sulfate. The resulting residue was purified by flash chromatography (silica gel, dichloromethane) to yield 2-(2,5-dimethoxy-4-nitrophenethyl)isoindoline-1,3-dione.

A mixture of 1.5 g of 2-(2,5-dimethoxy-4-nitrophenethyl)isoindoline-1,3-dione, 1.1 mL of 2-iodopropane, 1.2 g of potassium carbonate and 20 mL of dimethylformamide was mixed at 65°C overnight. The mixture was cooled to room temperature and the solvent was removed under vacuum. Water was added to the residue, and the precipitate was filtered, washed with water and dried under vacuum to give a yellow solid, 2-(5-isopropoxy-2-methoxy-4-nitrophenethyl)isoindoline-1,3-dione. A mixture of 1.5 g of 2-(5-isopropoxy-2-methoxy-4-nitrophenethyl)isoindoline-1,3-dione, 150 mg of 10% palladium on carbon and 100 mL of methanol was mixed under hydrogen for 24 h.

The mixture was filtered to remove the palladium on carbon and then concentrated under vacuum to give a yellow solid. The yellow solid was dissolved in 20 mL of tetrahydrofuran and 10 mL of 4 M hydrochloric acid, and 0.41 g of sodium nitrite was added. The mixture was stirred at room temperature for 2 h and then, 0.884 g of sodium iodide was added. The mixture was refluxed for 3 h and then diluted with ethyl acetate and water. The organic layer was washed with sodium thiosulfate and brine, dried with sodium sulfate and evaporated. The residue was purified by flash chromatography (silica gel, 2:1 dichloromethane:hexane) to yield 2-(4-iodo-5-isopropoxy-2-methoxyphenethyl)isoindoline-1,3-dione. The residue was added to 20 mL of ethanol and 0.22 mL of hydrazine. The solution was refluxed for 5 h and cooled to room temperature. The solvent was removed under vacuum, and chloroform was added to the residue, the filtrate was concentrated under vacuum and the residue was dissolved in 10 mL of dichloroethane. Ninety-three milligrams of 2-methoxybenzaldehyde and 0.04 mL of acetic acid were added. The solution was mixed at room temperature for 1 h and then 64 mg of sodium cyanoborohydride was added. The mixture was then stirred at room temperature overnight. Water was then added. The mixture was extracted with dichloromethane and dried over sodium sulfate. After evaporating the solvent, the residue was purified by flash chromatography (silica gel, 30:1 dichloromethane:methanol) to yield 2-(4-iodo-5-isopropoxy-2-methoxyphenethyl)-*N*-(2-methoxybenzyl)ethanamine.

One hundred and forty-three milligrams of 2-(4-iodo-5-isopropoxy-2-methoxyphenethyl)-*N*-(2-methoxybenzyl)ethanamine was added to 10 mL of dichloromethane and was cooled to -78°C. A solution of 1 M boron trichloride in 0.62 mL of dichloromethane



Reagents and conditions: (a) 2-iodopropane, K_2CO_3 , DMF, 70°C; (b) nitromethane, NH_4OAc , AcOH, 100°C; (c) LAH, THF, 65°C; (d) phthalic anhydride, AcOH, reflux; (e) ICl, AcOH; (f) N_2H_2 , EtOH reflux; (g) 2-methoxybenzaldehyde, AcOH, $NaCNBH_3$, DCE; (h) BCl_3 , DCM

Figure 2. Synthesis of M5.

was added dropwise. The reaction mixture was cooled to room temperature and stirred overnight. Ten milliliters of methanol were added drop wise, and stirred at room temperature for 5 h. The solvent was then evaporated under vacuum and dichloromethane was added to the residue. It was filtered and the white solid precipitate, 5-O-desmethyl-2-I-NBOMe hydrochloride salt, was collected (Figure 3). The structure of this material was established by NMR analysis.

Analysis of primary reference material

The purity of the following reference materials: 25I-NBOMe, 25I-NBOMe-*d*₃, 25H-NBOMe, M6, M5, 25I-NBOH and 2C-I were analyzed by the UPLC–MS–MS methods described below using retention time and the ion spectra. 25I-NBOMe and 25I-NBOMe-*d*₃ reference materials were also checked for impurities prior to the incubation with the mouse hepatic microsomal preparations.

Animals

Livers for microsomal drug metabolism preparations were obtained from 18-month-old male C57/BL/6 mice with an average weight of 36.1 g. The mice were allowed free access to food and water, and housed in a temperature (20–22°C) and humidity-controlled facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). They were housed four per cage and maintained on a 12 : 12 light/dark cycle (lights on at 600 EST). All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Preparation of hepatic microsomes

Mouse hepatic microsomes were isolated and prepared as previously described (14). In brief, the mice were deeply anesthetized

via 3% isoflurane-containing oxygen and humanely sacrificed by cervical dislocation. The peritoneal cavity was then opened and the liver perfused *in situ* with cold DPBS. The livers were then removed, and fat and debris were trimmed away. The collected livers were weighed and 1.12–1.33 g aliquots were homogenized with 8 mL of sucrose in a cold homogenization tube fitted with a drill-driven Teflon pestle. Homogenates were centrifuged for 15 min at 8,000 g at 4°C. The supernatant was transferred to a clean tube and re-centrifuged for 15 min at 18,500 g. The supernatant was then transferred to an ultracentrifuge tube and centrifuged for 45 min at 205,000 g. The lipid layer was removed and the cytosolic fraction was decanted and saved frozen. The microsomal pellet in the bottom of the tube was rinsed with 200 µL of re-suspension buffer (0.1 M Tris–HCl pH 7.4, 1 mM EDTA and 20% glycerol) and then resuspended in 2.0 mL of resuspension buffer. The protein concentration was determined using the Bradford Protein Assay kit.

Incubation with hepatic microsomes

The assay medium used for the incubation consisted of 167 µg total protein of pooled mouse hepatic microsomal preparation and 50 mM Tris–HCl buffer, pH 7.4, 150 mM potassium chloride and 10 mM magnesium chloride. Thirty-five micrograms of 25I-NBOMe or 25I-NBOMe-*d*₃ dissolved in 20 µL of DMSO were added to 1 mL of the assay medium. Phase I metabolism was initiated by the addition of 0.4 mM freshly prepared NADPH. Samples of the medium assay with and without the drug added were incubated at 37°C in a water bath for 60 min. Phase II metabolism was initiated by placing 500 µL of each reaction in a new tube and adding 10 µL of UDPGA. The mixture was allowed to incubate for an additional hour at 37°C. The resultant metabolites were isolated in ultrafiltrates using 30 kDa centrifugal filters (EMD Millipore, Billerica, MA, USA). Aliquots of the phase II ultrafiltrates were further reacted by adding 400 µL of

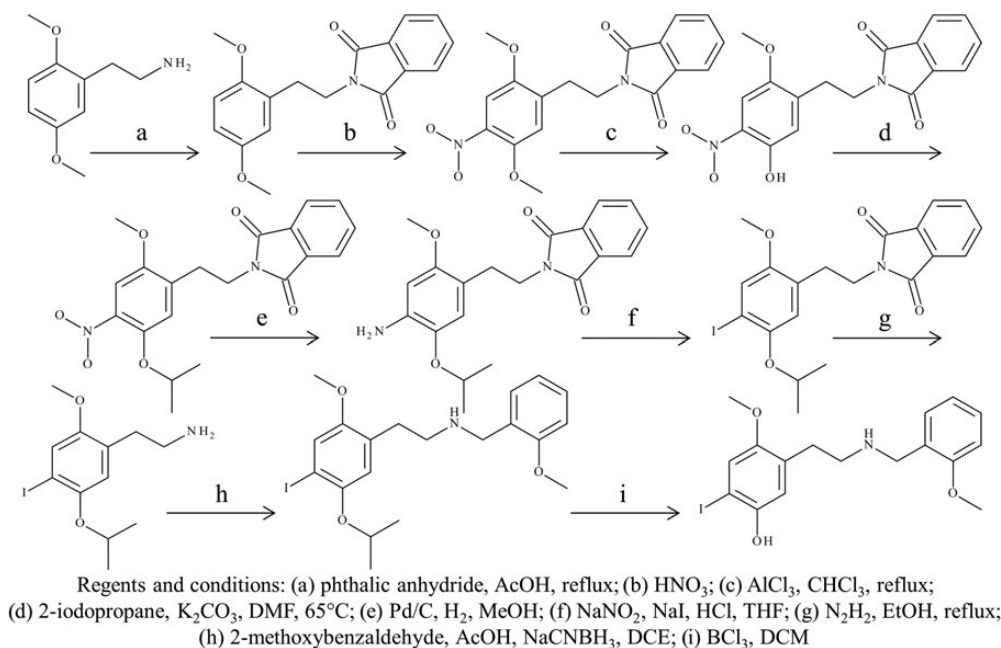


Figure 3. Synthesis of M6.

β -glucuronidase solution. These samples were then mixed, capped and heated at 55°C for 2 h. The phase I, pre- and post-phase II enzymatic hydrolyzed ultrafiltrates were analyzed by the UPLC–MS–MS and GC–MS methods described here in.

Human urine specimens

Two patient urine specimens previously tested for 25I-NBOMe by the VCU Department of Pharmacology and Toxicology's Mass Spectrometry Laboratory using HPLC–MS–MS procedures (15, 16) were re-analyzed for 25I-NBOMe metabolites identified in the mouse microsomal preparations. One urine specimen was from a 20-year-old male and was collected the day after suspected 25I-NBOMe use. It was found to contain 230 pg/mL of 25I-NBOMe. The other urine was from a 28-year-old male suspected of 25I-NBOMe overdose. This urine was collected 13 h post admittance to the Emergency Department; no parent 25I-NBOMe was detected.

Two sets of 1.0 mL aliquots of the clinical urine specimens were prepared. One set of aliquots received no sample pretreatment prior to extraction, whereas the second set was subjected to enzymatic hydrolysis to free apparent glucuronidated metabolites. To this second set, 400 μ L of β -glucuronidase solution was added to each aliquot. These samples were then mixed, capped and heated at 55°C for 2 h. The samples were then cooled to room temperature. Both the unhydrolyzed and hydrolyzed samples were extracted using Clean Screen ZSDUA020 SPE columns (United Chemical Technologies, Bristol, PA, USA) as previously described (16). In brief, to each sample, 500 pg of 25I-NBOMe- d_3 (ISTD) was added followed by the addition of 1 mL of 100 mM phosphate buffer (pH 6). The samples were then mixed for 5 min and centrifuged for 10 min at 1,500 g. The SPE columns were conditioned with 3 mL of methanol followed by 3 mL of DI water and finally 1 mL of 100 mM phosphate buffer (pH 6). The samples were added to the columns and aspirated under gravity. The columns were then washed with 3 mL of DI water followed by 1 mL of 100 mM acetic acid and finally 3 mL of methanol. The columns were dried under vacuum. 25I-NBOMe, its metabolites and ISTD were then eluted with 3 mL of 78 : 20 : 2 dichloromethane : isopropanol : ammonia (v : v : v). One hundred microliters of 1% HCl in methanol (v : v) and 200 μ L of DI water were added to the eluate. The samples were evaporated under nitrogen leaving $\sim$ 200 μ L of DI water. This solution was transferred to auto-sampler vials for UPLC–MS–MS analysis.

Methods

UPLC–MS–MS analysis

The metabolites of 25I-NBOMe and 25I-NBOMe- d_3 were identified in phase I and II mouse hepatic microsomal ultrafiltrates and in the human urine samples using a Waters Acquity Xevo TQD UPLC–MS–MS system with MassLynx version 3.5 software (Milford, MA, USA). The electrospray ionization probe was operated in both positive and negative ion modes. The source temperature was set at 150°C. The cone was at 34 V and had a flow rate of 100 mL/min. The ion spray voltage was 4.00 kV, with the ion source desolvation gas flow rate of 650 mL/min. The collision energy was set at 20 eV. The acquisition mode was either in positive or negative and had a scan range of 50–800 m/z .

Chromatographic separation was performed on a Selectra[®] PFPP column, 100 $\times$ 2.1 mm, 3.0 μ m (United Chemical Technologies, Bristol, PA, USA). The mobile phase consisted of A: water with 10 mM ammonium formate and B: methanol with 10 mM ammonium formate. The following gradient was used: 0.0–7.0 min starting at 5% B, with a linear gradient to 95% B, holding for 1.5 min and then returning at 9.0 min to 95% B. An injection volume of 5 μ L was used with a mobile phase flow rate of 0.4 mL/min and a total run time of 10 min.

GC–MS analysis

The metabolites of 25I-NBOMe and 25I-NBOMe- d_3 were further identified by GC–MS analysis.

Three 100 μ L aliquots of the phase I mouse hepatic microsomal ultrafiltrates and aliquots of methanolic solutions of primary reference material were dried under a stream of nitrogen and prepared for analysis by three different methods. The first set of aliquots was reconstituted in ethyl acetate and placed in auto-sampler vials. The second set of aliquots was derivatized at 75°C for 1 h with 50 μ L of HFBA and *n*-butyl chloride, dried down under nitrogen, reconstituted with 50 μ L of ethyl acetate and placed in auto-sampler vials. The third set of aliquots was derivatized at 75°C for 1 h with 50 μ L of TMS and 50 μ L of ethyl acetate in auto-sampler vials. All the samples were then analyzed using a Shimadzu multidimensional gas chromatography mass spectrometer QP-2010 Ultra with EI ionization (Shimadzu Scientific, Inc., Columbia, MD, USA) equipped with a Dean's switch. The separation was performed on Rtx-5 (20 m $\times$ 0.18 mm i.d. $\times$ 0.2 d.f.) and Rtx-50 (10 m $\times$ 0.18 mm i.d. $\times$ 0.2 d.f.) analytical columns (Restek Corporation, Bellefonte, PA, USA). The inlet temperature and transfer temperature were 250 and 280°C, respectively. The initial oven temperature was 70°C with a hold time of 0.1 min, then increased to 320°C at 20°C/min and held for 2.0 min. The compound mass spectra were collected in scan mode with a mass range of 40–650 m/z . The total run time was 15.5 min.

Results

Initial analysis by the described UPLC–MS–MS method of the 25I-NBOMe and 25I-NBOMe- d_3 primary reference material detected a small amount of 25H-NBOMe. This was confirmed by comparison against the 25H-NBOMe primary reference material. Since 25H-NBOMe was detected in the 25I-NBOMe primary reference material, its presence in the microsomal ultrafiltrates may be the direct result of this impurity. Also, dehalogenation of an aromatic ring system is not a reported human metabolic pathway. Therefore, 25H-NBOMe was not considered a genuine biomarker for 25I-NBOMe exposure.

UPLC–MS–MS analysis of the phase I mouse hepatic microsomal ultrafiltrates resulted in the identification of eight metabolites and the parent, 25I-NBOMe (Figure 4) or 25I-NBOMe- d_3 (Figure 5). 25I-NBOH, an *O*-desmethyl metabolite (M1), and 2C-I, an *N*-debenzylated metabolite (M2), were identified by comparing them with the primary reference material. Two additional *O*-desmethyl metabolites of 2C-I, 5-(2-aminoethyl)-2-iodo-4-methoxy-phenol (5-OH-2C-I) and 2-(2-aminoethyl)-5-iodo-4-methoxy-phenol (2-OH-2C-I) were also detected in the phase I hepatic microsomal ultrafiltrates as metabolites of 25I-NBOMe. These metabolites were confirmed by comparing their molecular

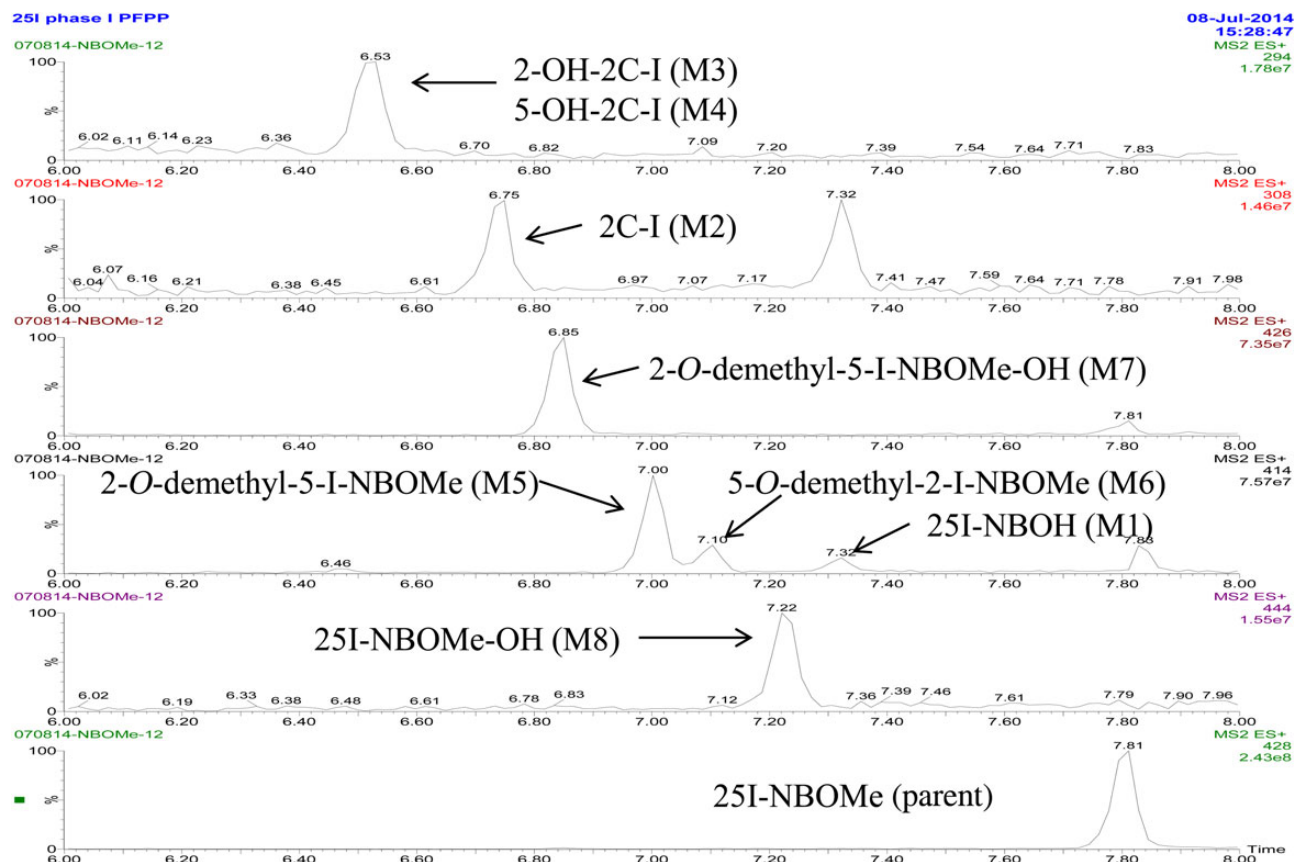


Figure 4. The molecular ions of each 25I-NBOME metabolite detected in phase I mouse hepatic microsomal ultrafiltrates using UPLC–MS–MS.

ions with previously published 2C-I metabolism data (2). These compounds were not chromatographically separated and were designated as M3 and M4. M3 and M4 were present in microsomal ultrafiltrates and human urine in minute concentrations; thus, they were not considered good candidates for 25I-NBOME biomarkers and their separation was not important for the goal of this study.

Three of the eight metabolites from the microsomal phase I studies had molecular ions of $M+1$ m/z and fragment ions at 121 and 91 m/z for 25I-NBOME ultrafiltrate and molecular ions of $M+3$ m/z with fragment ions at 124 and 92 m/z for 25I-NBOME- d_3 ultrafiltrate (Table I). Two *O*-desmethyl metabolites produced molecular ions at 414 m/z for 25I-NBOME and 417 m/z for 25I-NBOME- d_3 (Table I). The fragmentation of these *O*-desmethyl metabolites demonstrated that the methoxy group of the *N*-methoxybenzyl group was not demethylated. The three deuterium atoms in 25I-NBOME- d_3 are located on the methoxy group in the two-position of the *N*-methoxybenzyl group, not the 2,5-dimethoxy-4-iodophenethylamine part of the molecule (Figure 1). The structural identification of these desmethyl metabolites and the ring position of the methoxy group undergoing *O*-demethylation were determined by comparing retention times and mass spectra against our newly synthesized primary reference materials. These metabolites were designated M5 and M6 (Figure 6). M5 was chromatographically separated from M6, which has the same molecular weight and produces the same daughter ions. Another desmethyl metabolite appeared to be the product of a two-step process, *O*-desmethylation on

di-methoxy-iodo phenethyl ring and alkyl methylation of the ethyl amine chain. This metabolite had a molecular ion at 426 m/z and fragment ions at 121 and 91 m/z for 25I-NBOME and a molecular ion at 429 m/z with fragment ions at 124 and 92 m/z for 25I-NBOME- d_3 . Additionally, GC–MS analysis of the underivatized and derivatized phase I mouse hepatic microsomal ultrafiltrates of 25I-NBOME and 25I-NBOME- d_3 was performed to aid in the possible determination of the chemical structure of this metabolite. The metabolite was able to form both TMS and HBFA derivatives. Based on the electron impact spectra of the TMS and HFBA derivatives (Figure 7) and the observation that this metabolite was able to form a glucuronide metabolite, it was possible to postulate a chemical structure of 2-[(*E*)-2-[(*E*)-(hydroxy-2-methoxy-phenyl)methyleneamino]vinyl]-5-iodo-4-methoxy-phenol (2-*O*-desmethyl-5-NBOME-OH). This is proposed metabolite M7 (Figure 6). These three *O*-desmethyl metabolites (metabolites M5, M6 and M7) were present in the highest abundance in the mouse hepatic microsomal ultrafiltrates.

An additional metabolite containing a molecular ion at +16 m/z greater than both that of 25I-NBOME and the 25I-NBOME- d_3 is consistent with the addition of a hydroxyl group in the 2-methoxyphenyl ring. This designated metabolite M8, 25I-NBOME-OH, was likely the product of a common phase I metabolic pathway of aromatic ring hydroxylation. It was not an abundant metabolite.

UPLC–MS–MS analysis of the phase II hepatic microsomal ultrafiltrates of 25I-NBOME and 25I-NBOME- d_3 resulted in the detection of glucuronide conjugates of seven of the eight

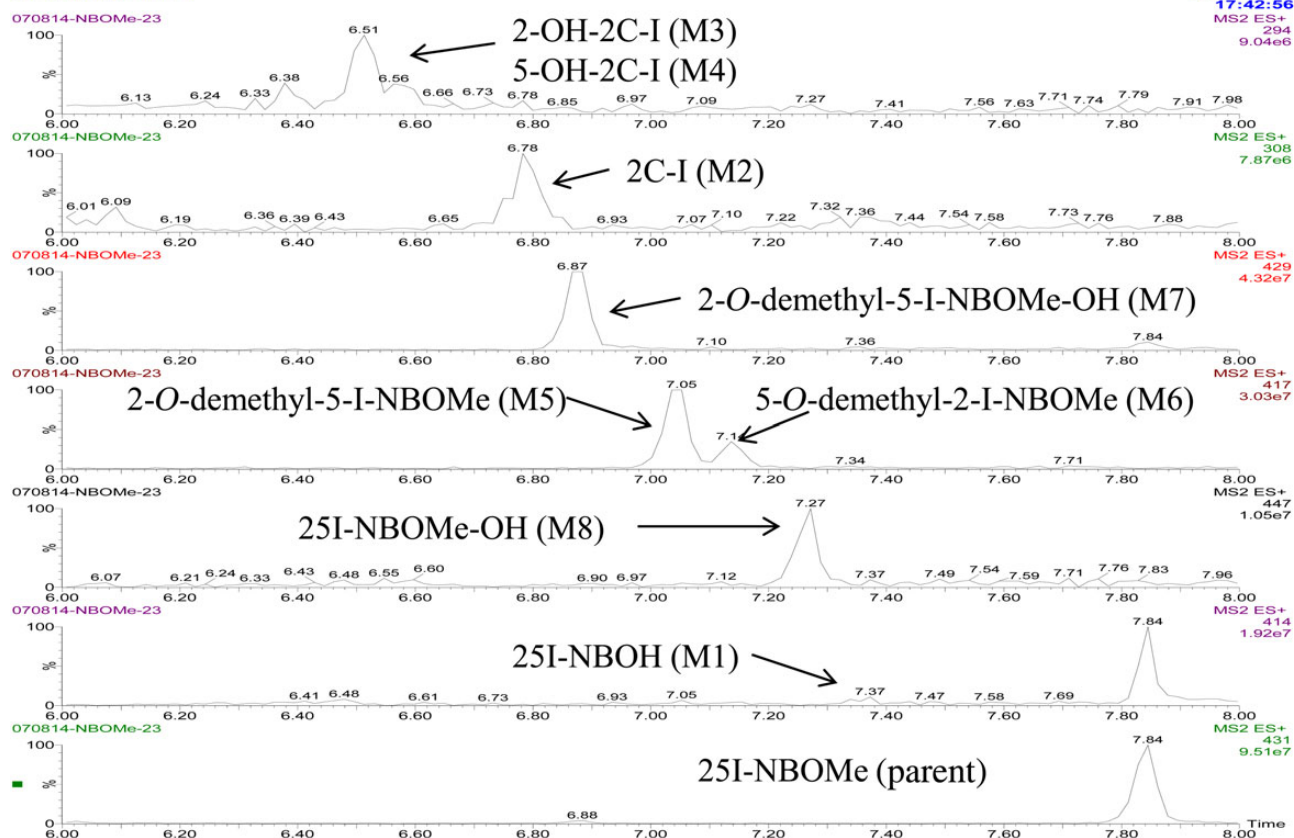


Figure 5. The molecular ions of each 25I-NBOMe- d_3 metabolite detected in phase I mouse hepatic microsomal ultrafiltrates using UPLC–MS–MS.

Table I

Phase I Metabolites Determined by UPLC–MS–MS

Metabolites	Name	Retention time (min)	Molecular ion (m/z)	Fragment ions (m/z)
M3–M4	2-OH-2C-I and 5-OH-2C-I	6.53	294	161 181
M2	2C-I	6.75	308	276 291 91
M7	2- <i>O</i> -desmethyl-5-I-NBOMe-OH	6.85	426	121 91
M5	2- <i>O</i> -desmethyl-5-I-NBOMe	7.00	414	121 91
M6	5- <i>O</i> -desmethyl-2-I-NBOMe	7.10	414	121 91
M8	25I-NBOMe-OH	7.22	444	137 107
M1	25I-NBOH	7.32	414	107 291
Parent	25I-NBOMe	7.81	428	308 121 91 272

phase I metabolites, M9–M15 (Table II). These glucuronidated metabolites were likely formed on the hydroxy metabolites produced in phase I reactions, metabolites M1, M3–M8. All of these metabolites produced a major fragment ion that had a loss of 176 m/z , consistent with the loss of a glucuronide acid group. These metabolites were confirmed by performing enzymatic hydrolysis on the mouse hepatic microsomal ultrafiltrates, which

resulted in the loss of the phase II glucuronide metabolites and the appearance of the unconjugated phase I metabolites.

No parent drug or metabolites were detected in the drug-free mouse hepatic microsomal ultrafiltrates by either the UPLC–MS–MS or GC/MS methods described. Other 25I-NBOMe metabolites from mouse hepatic microsomal preparations investigated but not detected include the *O*, *O*-bis-desmethyl and *O*-desmethyl hydroxy metabolites. Through the process of methylation, sulfation and acetylation other potential metabolites could be formed. These metabolites were not formed in the mouse hepatic microsomal as the cofactor of *S*-adenosyl methionine, 3'-phosphoadenosine-5'-phosphosulfate and acetyl coenzyme A were not added. These metabolites may form in low concentrations, but were not detectable in the human urine specimens using the described methods.

Without enzymatic hydrolysis prior to testing, all 15 glucuronidated and non-glucuronidated metabolites identified, plus the parent drug, 25I-NBOMe, were detected in one of the human urine specimens. However, all of the non-glucuronidated metabolites were detected in trace amounts only. After β -glucuronidase enzymatic hydrolysis of the urine, none of the glucuronide metabolites were detected, while all of the previously non-glucuronidated metabolites were detected in greater abundance. In the 20-year-old male's urine specimen, the *O*-desmethyl metabolites M1, M5, M6 and M8, as well as 2C-I, (M2) were detected in greater abundance than in the parent drug. The *O*-desmethyl metabolites M5 and M6 were present in high abundance, with M5 at a higher abundance than M6. In the

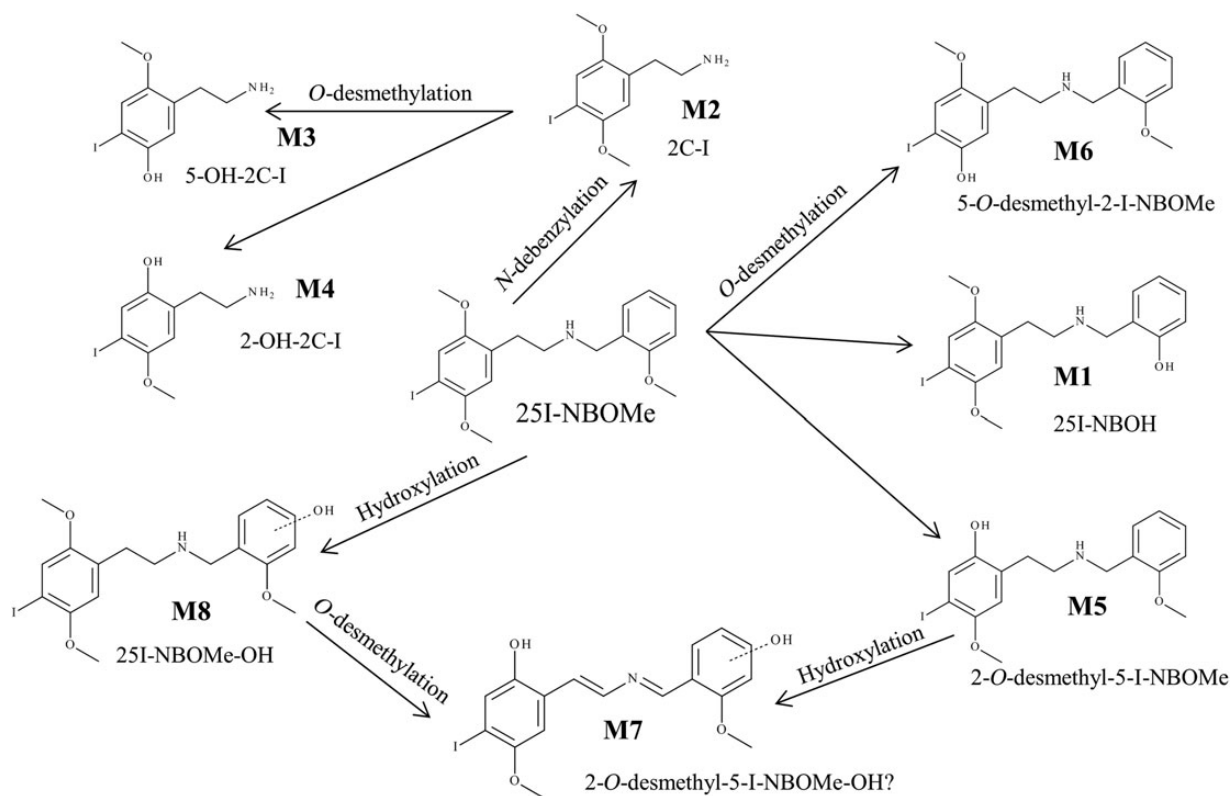


Figure 6. The metabolic pathways and chemical structures of the proposed 25I-NBOMe phase I metabolites.

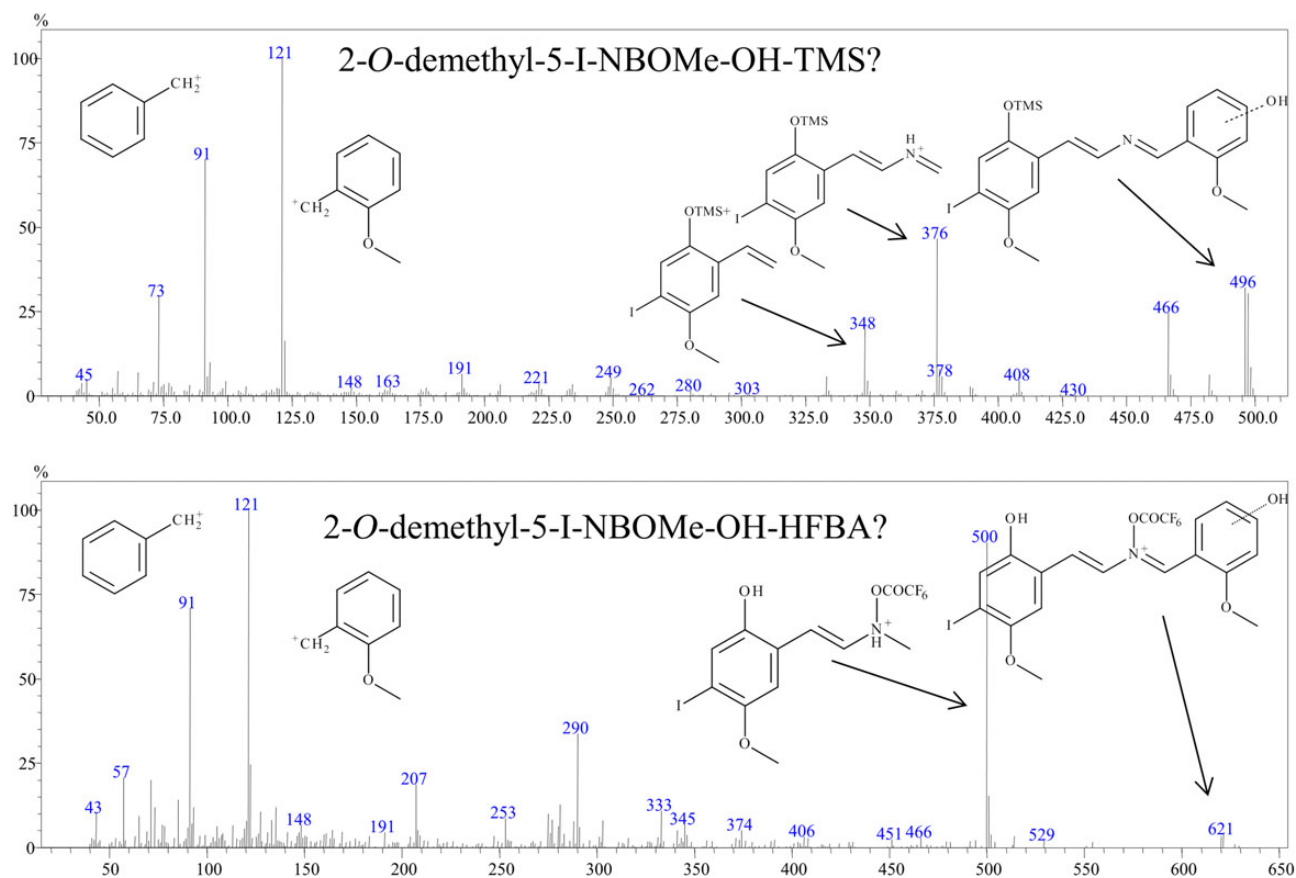


Figure 7. The electron impact fragments formed by the HFBA and TMS derivatives used to postulate a chemical structure of 2-O-desmethyl-5-I-NBOMe-OH (M7).

Table II
Phase II Metabolites Determined by UPLC–MS–MS

Metabolites	Name	Retention time (min)	Molecular ion (<i>m/z</i>)	Fragment ions (<i>m/z</i>)
M10–M11	2OH-2C-I-glu and 5OH-2C-I-glu	4.30	445	294
M12	2- <i>O</i> -desmethyl-5-I-NBOMe-glu	4.70	590	121
				414
M14	2- <i>O</i> -desmethyl-5-NBOMe-OH-glu	4.75	602	121
				426
M13	5- <i>O</i> -desmethyl-2-I-NBOMe-glu	4.90	590	121
				414
M15	25I-NBOMe-OH-glu	5.43	620	444
M9	25I-NBOH-glu	5.74	470	308
				414

other human urine specimen, no parent 25I-NBOMe was detected. Following enzymatic hydrolysis of the glucuronides, the four *O*-desmethyl metabolites M1, M5, M6 and M8 were the only metabolites detected in this specimen.

Discussion

In 12 of 17 previously reviewed case reports of 25I-NBOMe intoxication, the parent drug was detected in urine to confirm exposure (17). 25I-NBOMe was quantified in urine specimens from only 5 of these 12 cases yielding concentrations of 1.9–36 ng/mL. 25I-NBOMe exposure was confirmed in the remaining five cases by detection in serum. Parent 25I-NBOMe was also detected in one of our two patient urine specimens. Absent external contamination, it would appear that the parent drug may be a reliable biomarker, particularly in urine testing scenarios following recent drug use (e.g., in Emergency Department toxicological, DUID, ‘for cause’ workplace testing). However, parent 25I-NBOMe was not detected in one of the two patient urine specimens in this study. This specimen was collected 13 h post admission to the Emergency Department. The interval between drug administration and specimen collection is unknown. Variables such as administered dose and differences in absorption, distribution, biotransformation and elimination may account for the lack of parent drug in this urine. Therefore, parent 25I-NBOMe may not be the best urinary biomarker in drug abuse treatment programs, pre-employment drug testing, pain compliance management testing and other drug abuse testing arenas.

2C-I (M2) and its *O*-desmethyl metabolites (M3 and M4) were detected in one of the two patient urines in this study. It was also reported as a urinary metabolite of 25I-NBOMe by Stellpflug *et al.* (6) and identified with 25I-NBOMe in an additional four of seven urine specimens from intoxication patients (17). 2C-I and its metabolites were present in varying abundances in both hydrolyzed and unhydrolyzed urine samples. Like 25I-NBOMe, 2C-I is a designer hallucinogen subject to abuse (12). Therefore, 2C-I and its metabolites are not specific for 25I-NBOMe use. In their case report, Stellpflug *et al.* (6) had also reported 25H-NBOMe as a urinary metabolite of 25I-NBOMe. However, we found 25H-NBOMe as a trace contaminant in our commercial primary reference material, and it has also been identified as an impurity on 25I-NBOMe blotter paper available on the illicit drug market (18). Dehalogenation, such as loss of iodine, from an aromatic ring system has not been reported as a drug metabolism pathway in man. Therefore, we do not consider 25H-NBOMe as a true metabolite of, nor a urinary biomarker for 25I-NBOMe exposure.

In their case report, Stellpflug *et al.* (6) also suggested three likely conjugated *O*-desmethyl-NBOMe metabolites which would correspond to the herein identified metabolites M1, M5 and M6. They did not detect these metabolites in their patient urine specimen prior to chemical or enzymatic hydrolysis, suggesting phase I *O*-demethylation prior to conjugation by phase II glucuronidation or sulfation before excretion in the urine. In their case report, no authentic metabolite reference material was available to confirm the identity of the metabolites. The mass spectral abundances of these metabolites were estimated to be greater than that of the parent 25I-NBOMe. Our identification of M1, M5 and M6 metabolites in mouse hepatic microsomal preparations and in the human urine specimens supports their suggested metabolite identification. Additionally, we found in our first patient urine that M5 and M6 were hundreds of times greater in peak abundance than parent 25I-NBOMe. In the second patient specimen, where no parent drug was present, only M1, M5, M6 and M8 were detected.

The least abundant of the three *O*-desmethylated metabolites was M1 (25I-NBOH). 25I-NBOH, like 2C-I, is sold as a designer hallucinogen and would not serve well as a lone biomarker for 25I-NBOMe exposure. M7, suggested structure 2-*O*-desmethyl-5-I-NBOMe-OH, may be formed through the hydroxylation of M5 or *O*-desmethylation of M8 (25I-NBOMe-OH). In first patient urine, M7 displayed greater peak area abundance than parent 25I-NBOMe, but had less peak area abundance than M5 in both urines. As the identification of this metabolite is speculative, it is not given consideration as a 25I-NBOMe biomarker at this time. M8 (25-NBOMe-OH) was detected in only trace abundance in the second patient urine, particularly when compared with M5 and M6. Therefore, M8 may be a poor candidate as a biomarker for 25I-NBOMe exposure. Given the available data, it appears that the *O*-desmethyl 25I-NBOMe metabolites (M5 and M6) may be optimal biomarkers for detection of 25I-NBOMe use. They apparently remain in urine in readily detectable concentrations longer than the parent compound and in greater abundance than the other 25I-NBOMe metabolites.

Conclusion

The analysis of mouse hepatic microsomal preparations was an effective technique used to identify eight phase I and seven phase II urinary metabolites of 25I-NBOMe as possible metabolites in man. The analysis of two 25I-NBOMe intoxication human urine specimens using the information gained from the mouse hepatic microsomal preparations and from the literature yielded several potential urinary metabolite biomarkers for 25I-NBOMe exposure. With the exception of 2C-I, all identified 25I-NBOMe phase I metabolites undergo significant phase II glucuronidation. Therefore, in urine drug testing scenarios, β -glucuronidase enzymatic hydrolysis of the urine samples is recommended prior to 25I-NBOMe screening. Given the present study, M5 and M6 are the best urinary biomarkers for detection of 25I-NBOMe use. Additionally, neither is presently available on the illicit drug market as a designer hallucinogen. As M5 was present in urine at greater abundance than M6, it is recommended that M5 be the primary biomarker sought in urine screening for 25I-NBOMe use.

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