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Serotonergic Neurotoxic Thioether Metabolites of 3,4-Methylenedioxymethamphetamine (MDMA, “Ecstasy”): Synthesis, Isolation and Characterization of Diastereoisomers

Nieves Pizarro^{†,‡}, Rafael de la Torre[‡], Jesús Joglar[§], Noriko Okumura[⊥], Ximena Perfetti[‡], Serrine S. Lau[†], and Terrence J. Monks^{†,*}

[†]Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona Health Sciences Center, Tucson, U.S.

[‡]Human Pharmacology and Clinical Neurosciences Research Group, Pharmacology Research Unit, IMIM-Hospital del Mar, Barcelona, Spain

[§]Biotransformation Group. Catalonia Institute for Advanced Chemistry (IQAC). Spanish Council for Scientific Research (CSIC). Barcelona, Spain

[⊥]Department of Chemistry, University of Arizona, Tucson, U.S.

Abstract

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is a synthetic recreational drug of abuse that produces long-term toxicity associated with the degeneration of serotonergic nerve terminals. In various animal models direct administration of MDMA into the brain fails to reproduce the serotonergic neurotoxicity, implying a requirement for the systemic metabolism and bioactivation of MDMA. Catechol-thioether metabolites of MDMA, formed via oxidation of 3,4-dihydroxymetamphetamine and 3,4-dihydroxyamphetamine (HHMA and HHA) and subsequent conjugation with glutathione (GSH), are selective serotonergic neurotoxicants when administered directly into brain. Moreover, following systemic administration of MDMA, the thioether adducts are present in rat brain dialysate. MDMA contains a stereogenic center, and is consumed as a racemate. Interestingly, different pharmacological properties have been attributed to the two enantiomers, (S)-MDMA being the most active in the central nervous system and responsible for the entactogenic effects, and most likely also for the neurodegeneration. The present study focused on the synthesis and stereochemical analysis of the neurotoxic MDMA thioether metabolites, 5-(glutathion-S-yl)-HHMA, 5-(N-acetylcysteine-S-yl)-HHMA, 2,5-bis-(glutathion-S-yl)-HHMA and 2,5-bis-(N-acetylcysteine-S-yl)-HHMA. Both enzymatic and electrochemical syntheses were explored, and methodologies for analytical and semi-preparative diastereoisomeric separation of MDMA thioether conjugates by HPLC-CEAS and HPLC-UV respectively were developed. Synthesis, diastereoisomeric separation, and unequivocal identification of the thioether conjugates

*To whom correspondence should be addressed: Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona Health Sciences Center, Tucson, AZ-85721, U.S. Phone: +1 (520) 626-9906. Fax: +1 (520) 626-2466. monks@pharmacy.arizona.edu.

Supporting Information Available: Formulae for the calculation of the diastereoisomeric separation, and figures illustrating HPLC-MS and HPLC-MS/MS spectra of 5-(GSyl)-(S)-HHMA; HPLC-MS and HPLC-MS/MS spectra of 5-(NAC)-(S)-HHMA; HPLC-MS spectra of 2,5-bis-(GSyl)-(R,S)-HHMA; and HPLC-MS and HPLC-MS/MS spectra of 2,5-bis-(NAC)-(R,S)-HHMA. This material is available free of charge via the Internet at <http://pubs.acs.org>.

of MDMA provide the chemical tools necessary for appropriate toxicological and metabolic studies on MDMA metabolites contributing to its neurotoxicity.

Introduction

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is a ring-substituted amphetamine that acts as a serotonergic neurotoxicant in several species, including non-human primates (1–4). The precise mechanisms associated with MDMA-mediated neurotoxicity are not fully elucidated, although systemic metabolism of MDMA likely contributes to the development of toxicity (5, 6).

The principal routes of MDMA metabolism include *N*-demethylation to 3,4-methylenedioxyamphetamine (MDA), and *O*-demethylenation to 3,4-dihydroxymethamphetamine (HHMA [*N*-methyl- α -methyldopamine]) (7). MDA can also be *O*-demethylenated to 3,4-dihydroxyamphetamine (HHA [α -methyldopamine]) and both HHA and HHMA are *O*-methylated in the 3 position of the benzene ring to give 4-hydroxy-3-methoxyamphetamine (HMA) and 4-hydroxy-3-methoxymethamphetamine (HMMA), respectively (8, 9). HHMA (and HHA) can undergo further oxidation to the corresponding *ortho*-quinones, which conjugate with glutathione (GSH) (10, 11) yielding 5-(glutathion-S-yl)-3,4-dihydroxymethamphetamine [5-(GSyl)-HHMA] and 2,5-bis-(glutathion-S-yl)-3,4-dihydroxymethamphetamine [2,5-bis-(GSyl)-HHMA] (12). The role of the GSH S-transferases (GST) in these reactions is unclear. Thus, although GST catalyzes the conjugation of some catecholamine derived *ortho*-quinones with GSH (13) GSTs are also irreversibly inhibited by quinones, and their GSH conjugates (14,15,16). In particular, human GSTs are inhibited by dopamine, α -methyldopa, and by their 5-S-glutathionyl conjugates (17). The GSH conjugates of HHMA undergo further metabolism to the corresponding mercapturic acids, 5-(*N*-acetylcystein-S-yl)-3,4-dihydroxymethamphetamine [5-(NAC)-HHMA] and 2,5-bis-(*N*-acetylcystein-S-yl)-3,4-dihydroxymethamphetamine [2,5-bis-(NAC)-HHMA], respectively (Figure 1).

In animal models, direct administration of MDMA or MDA (5, 18–21), or its metabolites HHMA (22) HMA, and HHA (23) fail to reproduce the neurotoxic effects observed after peripheral administration of MDMA. In contrast, direct injection of the catechol-thioether metabolites into the brain does produce a neurotoxic response similar to that observed following peripheral administration of the parent compounds (24–26). Moreover, neurotoxic metabolites of MDMA have been identified in rat brain following the peripheral administration of the drug (27,28). Thus, both the mono- and bis-GSH and NAC conjugates were detected in the striatum of rats administered MDMA (20 mg/kg subcutaneously). Moreover, a strong positive correlation was observed between the concentration of the NAC metabolites in striatal dialysate, and the severity of the neurotoxicity as assessed by determining striatal serotonin concentrations seven days after dosing with MDMA (27). The relative persistence of the mercapturates in brain (25,27) was predicted to lead to their accumulation in brain following multiple drug administration. This hypothesis was confirmed by Erives *et al* (28), who demonstrated a significant accumulation of the NAC

thioether metabolites in striatal dialysate following multiple administration of MDMA (4 × 20 mg/kg s.c. at 12h intervals).

For a complete understanding of MDMA-mediated neurotoxicity, the stereochemistry of the metabolism of MDMA needs to be taken into consideration. MDMA contains a stereogenic center at the carbon α to the amine, which is responsible for the existence of two enantiomers: (*R*)-MDMA and (*S*)-MDMA. MDMA is consumed as a racemate (a 50% mixture of its enantiomers), but each enantiomer has its own pharmacological properties. Thus, *in vitro* models have revealed that (*S*)-MDMA is more active than (*R*)-MDMA on the central nervous system (29, 30). Moreover, studies in animal models further revealed that (*S*)- rather than (*R*)-MDMA likely contributes to the serotonergic degeneration associated with MDMA consumption (31).

The stereogenic center of MDMA is preserved in its main metabolites (MDA, HHMA and HMMA) so they also all exist as a pair of enantiomers. Because the major metabolic pathway of MDMA in humans (*O*-demethylenation to HHMA) is mainly regulated by the enantioselective enzyme CYP2D6, (8, 32–34) various studies in humans have focused on the stereochemistry of this metabolic pathway. Such studies have revealed complexity in the pharmacokinetics of MDMA, MDA, HHMA and HMMA enantiomers (35–37). A thorough analysis of plasma and urine samples from healthy recreational users of MDMA (37) revealed that (*R*):(*S*) ratios for MDMA and MDA in both plasma (0–48 h) and urine (0–72 h) were >1 and <1, respectively. Ratios corresponding to HHMA and HMMA, close to unity, deviate from theoretical expectations, and are most likely explained by the non-linear pharmacokinetics of MDMA biotransformation to HHMA (8). These results were obtained from subjects typed as extensive metabolizers for CYP2D6. The relative rate of formation of specific enantiomers of the catechol metabolites in poor and ultrarapid metabolizers of this polymorphic enzyme is not known. HHMA is the precursor to the thioether adducts. Since larger amounts of *S*-HHMA relative to *R*-HHMA are formed after MDMA administration, a correspondingly larger amount of the (*S*)-*ortho*-quinones and their thioether adducts should also be formed. Since the biological activity of MDMA enantiomers is dissimilar, one would also predict that the thioether conjugates of the HHMA enantiomers would similarly display disparate biological activities. Both of the preceding assertions require the availability of pure reference substances for the development of analytical methodology and for the performance of animal studies. The current work was therefore conducted to provide the necessary tools for future *in vivo* experimentation.

In the present study we therefore explored enzymatic and electrochemical synthesis of the catechol thioether metabolites of MDMA and developed analytical and semi-preparative HPLC methods for the separation and unequivocal identification of the diastereoisomers of 5-(GSyl)-HHMA, 2,5-bis-(GSyl)-HHMA, 5-(NAC)-HHMA and 2,5-bis-(NAC)-HHMA. Interestingly, enzymatic and electrochemical approaches yielded mixtures with different diastereoisomeric ratios, which should be considered when interpreting toxicological data after thioether metabolite administration studies. With respect to the development of HPLC methods, both analytical and semi-preparative diastereoisomeric separation may be crucial tools to assist in providing a better understanding of MDMA-mediated neurotoxicity. Thus, semi-preparative separation would be required to obtain pure compounds for administration

in animal experiments designed to study discriminative effects of specific diastereoisomers, whereas analytical separation would be needed for the determination and unequivocal identification of MDMA thioether metabolites in biological fluids after MDMA administration.

Experimental Procedures

Chemicals

Sodium phosphate (monobasic, 99.0%), ammonium acetate (98.0%), citric acid (99.5 +%, ACS reagent), acetic acid (reagent plus™, 99%), trifluoroacetic acid (Reagent plus®, 99%), mushroom tyrosinase, glutathione reduced (GSH; 99%) and *N*-acetyl-L-cysteine (NAC, Sigma Grade) were all purchased from Sigma (St. Louis, MO). (*R*)-(-)- α -Methoxy- α -trifluoromethylphenylacetyl chloride (Mosher's reagent, (*R*)-MTPCl; 98% ee/GLC) and 1,1,1,3,3,3-hexamethyldisilazane (HMDS; 99.9%) were purchased from Aldrich (Steinheim, Germany). Methanol (HPLC grade) and HPLC-grade H₂O were provided by Fisher Scientific (Pittsburgh, PA) and acetonitrile (HPLC grade) was obtained through EMD (San Diego, CA). Bio-Rad (Hercules, CA) provided EDTA (disodium salt dihydrate, electrophoresis purity reagent) and octanesulphonic acid (sodium salt, anhydrous) was purchased from Acros (Geel, Belgium). HHMA·HBr was synthesized by the Synthetic Chemistry Facility Core of the Southwest Environmental Health Science Center (Department of Chemistry, University of Arizona, Tucson, AZ) through a procedure previously reported (34). The enantiomerically enriched (*S*)-HHMA (65.4% e.e.) used in this study was previously synthesized in the laboratories of IQAC-CSIC (Barcelona) as described (36).

Enzymatic Synthesis and Purification of 5-(GSyl)-HHMA

5-(GSyl)-HHMA was synthesized and purified as described for 5-(GSyl)-HHA by Miller and colleagues (21). In short, 60 mg mushroom tyrosinase (100 U mL⁻¹) was used in sodium phosphate buffer (100 mL, 50 mM, pH 7.4), to oxidize HHMA (10 mg, 0.04 mmol) to the corresponding *ortho*-quinone, which was subsequently reacted with GSH (40 mg, 0.13 mmol). Purification was performed by semi-preparative HPLC-UV (SCL-10A Shimadzu) (Kyoto, Japan) equipped with a C₁₈ column (Beckman Coulter, Ultrasphere ODS, 5 μ m $\times$ 10 mm i.d. $\times$ 25 cm) (Fullerton, CA). Solvents used were H₂O with 1% acetic acid (A) and MeOH (B) 90:10 at 2 mL min⁻¹ (λ = 280 nm). Several injections were required to finally obtain 1.24 mg of the product (2.55 μ mol, 4.6% reaction yield). After performing several batches of the reaction, the purified product was subsequently used for further separation into its diastereoisomers by HPLC-UV (see below).

Enzymatic Synthesis and Purification of 5-(NAC)-HHMA

5-(NAC)-HHMA was synthesized following the same procedure used for the synthesis of 5-(GSyl)-HHMA, but using *N*-acetylcysteine (NAC) (40 mg, 0.25 mmol) instead of GSH. In this case, the HPLC purification protocol required a (A):(B) ratio of 85:15 at 2 mL min⁻¹ (λ = 225 nm). The procedure was repeated several times and yielded 0.6 mg of product (1.75 μ mol, 3.2% reaction yield) which was used for subsequent separation into its diastereoisomers by HPLC-UV (see below).

Electrochemical Synthesis and Purification of 5-(GSyl)-HHMA, and Enantiomerically Enriched 5-(GSyl)-(S)-HHMA

Electrochemical synthesis was performed as described by Felim and colleagues (39). Briefly, the controlled potential electrolyses were carried out in an Advanced Electrochemical System instrument (PARSTAT 2273, Princeton Applied Research, Oak Ridge, Tennessee) in a cylindrical three-electrode divided cell. Synthesis of 5-(GSyl)-HHMA required a solution of HHMA·HBr (26.2 mg, 0.1 mmol) in 50 mL HCl (0.2N), which was oxidized under nitrogen at room temperature at a platinum mesh whose potential was fixed at +1.0 V versus Ag/AgCl. A platinum wire was used in the cathodic compartment (counter electrode). After the consumption of 2 electrons per molecule, 2 equivalents of GSH (62.8 mg, 0.2 mmol) were added to the yellow solution which first turned brown, and then subsequently turned almost transparent. The crude reaction mixture was immediately frozen at -80°C and lyophilized. The lyophilized residue was subsequently dissolved in water and purified using the same semi-preparative HPLC-UV system and chromatographic column employed for the enzymatic purification. Solvents used were H_2O with 1% trifluoroacetic acid (TFA, A) and $\text{H}_2\text{O}:\text{MeOH}$ (50:50) with 0.05% TFA (B) 70:30 at 3 mLmin^{-1} ($\lambda = 280\text{ nm}$). The reaction yielded 5-(GSyl)-HHMA (7.0 mg, $14.4\text{ }\mu\text{mol}$, 14.4% yield) and 2,5-bis-(GSyl)-HHMA (1.6 mg, $2.02\text{ }\mu\text{mol}$, 4% yield) as products. The same procedure, with a 65.4% enantiomerically enriched (S)-HHMA (31), was performed for identification purposes.

Electrochemical Synthesis and Purification of 5-(NAC)-HHMA, and Enantiomerically Enriched 5-(NAC)-(S)-HHMA

The synthesis of 5-(NAC)-HHMA was achieved following the above procedure by using 39.3 mg HHMA·HBr (0.15 mmol). Semi-preparative purification was performed by using the same chromatographic system and organic phase as previously described for solvent (A), but acetonitrile as organic phase for solvent (B) at a 93:7 ratio, and a 3 mLmin^{-1} flow rate ($\lambda = 225\text{ nm}$). Once lyophilized, 5-(NAC)-HHMA (3.8 mg, $11.1\text{ }\mu\text{mol}$, 7.4% yield) and 2,5-bis-(NAC)-HHMA (1.4 mg, $2.8\text{ }\mu\text{mol}$, 1.85% yield) were obtained as products. The synthesis was also performed using a 65.4% enantiomerically enriched (S)-HHMA as starting material.

Analytical HPLC-CEAS Separation of the Diastereoisomeric Pairs of 5-(GSyl)-HHMA and 5-(NAC)-HHMA

An equimolar mixture of 5-(GSyl)-HHMA and 5-(NAC)-HHMA was used to ascertain the analytical conditions required to achieve a good separation of both compounds in a single run, in order to perform analyses on biological samples. Initially, unsuccessful attempts to separate the 5-(GSyl)-HHMA and 5-(NAC)-HHMA diastereoisomers utilized HPLC C_{18} columns of 80 mm (HR-80, ESA Inc.), 250 mm (Ultrasphere ODS, Beckmann) and 500 mm (Ultrasphere ODS, Beckmann), C_8 HPLC columns (Aquapore RP-300, Alltech), a column specifically designed for analysis of amino acids and peptides (Macrosphere GPC, Alltech), and a polar column (ZIC-HILIC, Sequant). Finally, separation of 5-(GSyl)-HHMA and 5-(NAC)-HHMA diastereoisomeric pairs was obtained on a YMC ODS-AQ column (C_{18} AQ) (250 mm $\times$ 2.0 mm i.d. $\times$ 5 μm particle size (YMC Co. LTd, Kyoto, Japan). Separation was

performed using a HPLC (Shimadzu, SCL-10A) (Kyoto, Japan) equipped with a four-channel coulometric electrode array system (CEAS; ESA Inc., Chelmsford, MA) with electrode potentials set to +50, +150, +300, and +350 mV. Aqueous mobile phase consisted of 8 mM ammonium acetate, 4 mM citric acid, 54 μ M EDTA, 230 μ M octanesulfonic acid (pH 6.0), and the organic phase was methanol. The appropriate electrode potentials and mobile phases for the analysis of the diastereoisomeric mixtures have been previously optimized and reported in the literature (27). Electrode potentials were selected to provide optimum oxidation of the reduced analytes as they flow through the detector. The flow rate was 0.3 mLmin⁻¹ and a gradient from 0% to 5% of methanol was established over 70 min (maintained for a further 20 min) followed by an increase of methanol from 5% to 10% in 10 min, and maintaining this ratio for a further 30 min (see Figure 2a).

Analytical HPLC-CEAS Diastereoisomeric Separation of 2,5-bis-(GSyl)-HHMA

2,5-bis-(GSyl)-HHMA diastereoisomers were separated on a HR-80 column (C₁₈) (80 mm $\times$ 4.6 mm i.d. $\times$ 3 μ m particle size) (ESA Inc.). Optimal separation was performed at a 1 mLmin⁻¹ flow rate using 8 mM ammonium acetate, 4 mM citric acid, 54 μ M EDTA, 230 μ M octanesulfonic acid (pH 2.5) (A) and 12% methanol (B) in an isocratic mode for 50 min.

Analytical HPLC-CEAS Diastereoisomeric Separation of 2,5-bis-(NAC)-HHMA

The separation of 2,5-bis-(NAC)-HHMA diastereoisomers was achieved on a C₁₈ reverse-phase column (Beckman Coulter, Ultrasphere ODS, 25 cm $\times$ 4.6 mm I.D $\times$ 5 μ m particle size). Solvents used were H₂O with 1% TFA (A) and acetonitrile (B) 94:6 at 1 mLmin⁻¹.

Semi-preparative HPLC-UV Diastereoisomeric Separation of 5-(GSyl)-HHMA

Analytical HPLC-CEAS conditions for the diastereoisomeric separation of 5-(GSyl)-HHMA were extrapolated to perform a semi-preparative separation, using a HPLC equipped with an UV detector coupled to a YMC ODS-AQ column (YMC Co. LTd) (C₁₈ AQ) (250 mm $\times$ 10 mm i.d. $\times$ 5 μ m particle size). Aqueous and organic phases consisted of Milli-Q[®] grade water (pH 5.6) and methanol, respectively. The diastereoisomeric separation of 5-(GSyl)-HHMA (28 mg, 57.5 μ mol) was achieved at a flow rate of 0.9 mLmin⁻¹ and monitored at λ = 280 nm. The gradient consisted of 0% to 10% methanol over 60 min, holding at 10% for 20 min, and stepping from 10% to 20% of methanol over the next 60 min. UV absorbing peaks were collected, frozen at -80°C, and lyophilized, and resulted in the accumulation of 2.98 mg, (6.12 μ mol, 10.6% yield) of 5-(GSyl)-(S)-HHMA (diastereoisomer 1) and 1.45 mg (2.98 μ mol, 5.2% yield) of 5-(GSyl)-(R)-HHMA (diastereoisomer 2). Final products were analyzed by NMR and the data are in agreement with those previously reported (39, 25–27). HPLC-MS and HPLC-MS/MS analysis showed identical spectra to their corresponding mixtures (data not shown).

Semi-preparative HPLC-UV Diastereoisomeric Separation of 5-(NAC)-HHMA

The diastereoisomeric separation of 5-(NAC)-HHMA was performed with the same apparatus and buffers as those described above for 5-(GSyl)-HHMA. The solvent gradient consisted of 0% to 20% methanol over 30 min, and holding at 20% methanol for the next 50 min. The flow rate was 0.9 mLmin⁻¹ and λ = 225 nm. UV absorbing peaks were collected,

frozen at -80°C , and lyophilized. After purification of 8 mg of a diastereoisomeric mixture of 5-(NAC)-HHMA, 2.0 mg (5.84 μmol , 25% yield) of 5-(NAC)-(*S*)-HHMA (diastereoisomer 1) and 2.1 mg (6.13 μmol , 50% yield) of 5-(NAC)-(*R*)-HHMA (diastereoisomer 2) were recovered. Again, NMR and HPLC-MS, and HPLC-MS/MS analyses revealed identical spectra to their corresponding mixtures, confirming previously published data by our group and others (39,25–27) (data not shown).

Semi-preparative HPLC-UV Diastereoisomeric Separation of 2,5-bis-(NAC)-HHMA

The diastereoisomeric separation of 2,5-bis-(NAC)-HHMA was achieved on a C_{18} Ultrasphere ODS HPLC semi-preparative column (250 mm $\times$ 10 mm i.d. $\times$ 5 μm particle size; Beckman Coulter, Fullerton, CA). The aqueous phase consisted of H_2O + 1% TFA (A) with acetonitrile as the organic phase (B), 94:6 at 1 mLmin^{-1} ($\lambda=225$ nm). Because of the lower amounts of 2,5-bis-(NAC)-HHMA available, only semipreparative conditions for the diastereoisomeric separation could be developed. Further studies on semi-preparative yields for this diastereoisomeric pair separation will be performed in the future.

Enantioselective Analysis of HHMA by GC-MS

Unreacted HHMA obtained from both the HPLC-UV purification of 5-(GSyl)-HHMA and 5-(NAC)-HHMA was analyzed by GC (6890 N; Agilent Technologies, Wilmington, DA, USA) equipped with a mass selective detector (MS, 5973 Network, AT). Separation was performed on a cross-linked 5% phenylmethylsiloxane capillary column, (12 m, 0.22 mm i.d., 0.33 mm film thickness, Ultra 2, AT) after a two-step derivatization (40). First, the secondary amine of HHMA was derivatized with an enantiomerically pure Mosher reagent and a second derivatization step using hexamethyldisilazane was required to obtain the trimethylsilyl-derivatives of hydroxyl groups.

Results and Discussion

In contrast to MDMA and its major metabolites (HHMA, HMMA and MDA) which are present as pairs of enantiomers, thioether adducts of HHMA, 5-(GSyl)-HHMA, 2,5-bis-(GSyl)-HHMA, 5-(NAC)-HHMA and 2,5-bis-(NAC)-HHMA, have more than one stereogenic center in their structure, and therefore exist as a mixture of diastereoisomers. All of the HHMA thioether derivatives have in common the stereogenic center arising from MDMA, in the carbon atom α to the amine, but also possess stereogenic centers in the substituents at the 2 and/or 5 carbon positions of the benzene ring, where the covalent thioether bond is established following conjugation with either GSH (γ -glutamylcysteinylglycine) or *N*-acetylcysteine (NAC). Glycine (Gly) is an achiral aminoacid but cysteine (Cys), NAC and glutamate (Glu) are all chiral, with unique stereochemical configurations (*R* to Cys, *R* to NAC and *S* to Glu). Because of this, there are only two possible stereochemical combinations in all cases, which only differ at one stereogenic center (epimers). (Figure 1).

A diastereoisomeric study of MDMA thioether metabolites may take two different approaches: either synthesis of compounds as pure diastereoisomers or, alternatively, the synthesis of diastereoisomeric mixtures coupled to the appropriate analytical separation. For

the present study, we separated synthesized diastereoisomeric mixtures rather than synthesize pure diastereoisomers, since the synthesis of pure diastereoisomers requires the previous synthesis of enantiomerically pure precursors (*S*)-HHMA and (*R*)-HHMA, which are more difficult to synthesize than the racemate (36). Moreover, approaching the diastereoisomeric separation from an analytical rather than a synthetic perspective requires the development of analytical methodology capable of separating the diastereoisomeric pairs, which can subsequently be utilized in future research on the disposition of the diastereoisomers *in vivo*.

In the present study, 5-(GSyl)-HHMA, 5-(NAC)-HHMA, 2,5-bis-(GSyl)-HHMA and 2,5-bis-(NAC)-HHMA were synthesized as diastereoisomeric pairs. Synthesis of the different HHMA conjugates was achieved using either enzymatic or electrochemical approaches, with previously synthesized (*R,S*)-HHMA or enantiomerically enriched (*S*)-HHMA as starting materials. The enzymatic synthesis of 5-(GSyl)-HHMA and 5-(NAC)-HHMA was achieved following a protocol described by Miller and colleagues (25), with very low yields. This synthetic procedure also resulted in the formation of the corresponding *bis*-conjugates as by-products, but reaction required repeating several times in order to obtain just less than 1 mg of each *bis*-conjugate. In addition, the semi-preparative purification procedure described for these products (25), using 1% acetic acid and methanol, frequently eluted mixtures of the corresponding conjugate with its synthetic precursors, and required the performance of several re-purification processes. Electrochemical synthesis of 5-(GSyl)-HHMA and 5-(NAC)-HHMA (35) resulted in a 29% and 16% yield of the GSH and NAC adducts, respectively, together with minor amounts of the corresponding *bis*-conjugates as by-products of the reaction, a somewhat lower yield than that reported by Felim *et al.* (39) but still higher than that achieved *via* the enzymatic approach. Moreover, the subsequent purification process, using H₂O with 1% TFA and methanol or acetonitrile (39), permits the collection of pure conjugates in a single run.

HPLC-MS of the two lyophilized final products, 5-(GSyl)-HHMA and 2,5-bis-(GSyl)-HHMA, revealed single peaks that were confirmed by HPLC-MS/MS. Analysis revealed identical chromatograms for purified compounds obtained by enzymatic and electrochemical synthesis. The same analyses were performed for 5-(NAC)-HHMA and 2,5-bis-(NAC)-HHMA and again, equivalent chromatograms were obtained for compounds synthesized by enzymatic and electrochemical approaches (see supporting information). Furthermore, NMR analysis of 5-(GSyl)-HHMA and 5-(NAC)-HHMA unequivocally established their structures by comparison to previously reported data (39).

Because of the diastereoisomeric nature of the thioether adducts, their analysis does not require the use of chiral approaches, and their separation may be performed using conventional chromatography. Nevertheless, the separation of each pair of diastereoisomeric HHMA thioether conjugates remains challenging. In general, diastereoisomers with stereogenic centers located at nearby positions on the chemical structure are relatively easy to separate by conventional approaches. However, the stereogenic centers in the HHMA thioether adducts are, in all cases, several atoms apart, a situation that significantly increases the complexity of the analytical method. Thus, in order to separate these diastereoisomeric pairs, an equimolar mixture of 5-(GSyl)-HHMA and 5-(NAC)-HHMA was used to ascertain

the analytical conditions required to achieve a good separation of both compounds in a single run in order to carry out analyses in biological samples. Several different HPLC columns and a wide range of pHs and gradients were examined (see Experimental Procedures), with a YMC ODS-AQ analytical column (C₁₈ AQ) providing a good separation for both 5-(GSyl)-HHMA and 5-(NAC)-HHMA diastereoisomeric pairs in a single run. The analytical method developed for the diastereoisomeric separation of MDMA thioether adducts resulted in a separation (*R_s*, calculated with enantiomeric resolution formula, see supporting information) of 0.9 and 13.5 for 5-(GSyl)-HHMA (second vs. first eluting enantiomer) synthesized with the enzymatic and electrochemical approaches, respectively (Figure 2a). Because 5-(GSyl)-HHMA diastereoisomers were not baseline separated, the calculated d.e. for 5-(GSyl)-HHMA was an estimation. However, data for the 5-(GSyl)-HHMA obtained *via* enzymatic synthesis could be indirectly confirmed by the GC-MS analysis of unreacted HHMA (40) collected during the semi-preparative purification process, and which exhibited a 5.7% enantiomeric excess (e.e.) (*S* vs *R*) (no HHMA was collected when performing electrochemical synthesis purification). The e.e. result for unreacted HHMA may be useful to indirectly establish the elution order of 5-(GSyl)-HHMA diastereoisomers, but differences between both enantiomers (and diastereoisomers) are not sufficiently significant to obtain an unambiguous assignment, because diastereoisomeric separation is not baseline. Unequivocal assignment for the 5-(GSyl)-HHMA diastereoisomers was performed by analysis of a diastereoisomerically enriched pair, synthesized using 65.4% enantiomerically enriched (*S*)-HHMA (36) as starting material, resulting in 5-(GSyl)-(*S*)-HHMA and 5-(GSyl)-(*R*)-HHMA being the first and second eluted peaks, respectively (Figure 2b).

Diastereoisomeric separation of 5-(NAC)-HHMA by HPLC-CEAS showed baseline separation (*R_s* 1.5) (Figure 2a) and d.e. obtained were 22.7% and 5.0 % (second vs. first peak), for the enzymatic and electrochemically synthesized compounds, respectively. The e.e. of unreacted HHMA from the enzymatic synthesis of 5-(NAC)-HHMA (no HHMA was recovered from electrochemical synthesis), calculated by GC-MS, was 24.6% of (*S*)-HHMA, confirming the previously calculated d.e. of the corresponding adduct. Moreover, because data for the analysis of HHMA clearly suggests that more (*R*)-HHMA has reacted compared to the (*S*)-HHMA, the elution order of 5-(NAC)-HHMA diastereoisomers can be indirectly established as 5-(NAC)-(*S*)-HHMA prior to 5-(NAC)-(*R*)-HHMA,. Unequivocal assignment for each conjugate, obtained by analysis of the corresponding diastereoisomeric pairs synthesized using a 65.4% enantiomerically enriched (*S*)-HHMA (36) as starting material, confirmed the previous indirect assignment (Figure 2c).

Diastereoisomeric separation of 5-(NAC)-HHMA revealed that the d.e. of 5-(NAC)-HHMA diastereoisomers synthesized by enzymatic synthesis is higher than that obtained via electrolysis. Data on the relative enantioselective oxidation of HHMA enantiomers by mushroom tyrosinase were somewhat surprising. Stereospecificity in the oxidation of several *ortho*-diphenols (including Dopa and α -methyldopa) has been reported (41, 42), revealing that *K_m* values for the mushroom tyrosinase catalyzed oxidation of the *S*-isomers are higher than for the *R*-isomers, whereas transformation reaction constants [*V_{max}*] were similar for both substrates. We therefore expected a similar preference for the relative

mushroom tyrosinase-mediated oxidation of (*S*)-HHMA and (*R*)-HHMA (calculated with data obtained during the analytical separation of 5-(NAC)-HHMA by HPLC-CEAS). However, in contrast to the above referenced data, it appears that (*R*)-HHMA is a better substrate for mushroom tyrosinase-mediated oxidation than (*S*)-HHMA. In particular, the synthesis of diastereoisomerically enriched 5-(NAC)-(*S*)-HHMA confirmed the elution order of 5-(NAC)-HHMA diastereoisomers. The basis for this difference is unclear and warrants further studies.

Attempts to separate diastereoisomeric pairs of 2,5-*bis*-(GSyl)-HHMA and 2,5-*bis*-(NAC)-HHMA, using the same analytical conditions employed for the corresponding mono-conjugates, were unsuccessful. Moreover, the *bis*-GSyl and *bis*-NAC conjugates required totally different analytical conditions. Thus, HPLC-CEAS analytical separation of the diastereoisomeric pairs of 2,5-*bis*-(GSyl)-HHMA and 2,5-*bis*-(NAC)-HHMA were performed using HR-80 C₁₈ column (80 mm × 4.6 mm i.d. × 3 μm particle size) and an Ultrasphere ODS C₁₈ column (250 mm × 4.6 mm I.D × 5 μm particle size) respectively, achieving baseline separations in both cases (Figure 2d and 2e). For 2,5-*bis*-(GSyl)-HHMA, the d.e. was 9% and 1.3% (second vs. first peak) for the enzymatic and electrochemically synthesized compounds, respectively. For 2,5-*bis*-(NAC)-HHMA, the d.e. was 24.0% for the enzymatically synthesized compound and 2.5% for the adduct obtained by electrochemical synthesis, data similar to that obtained for 5-(NAC)-HHMA. Synthesis of diastereoisomerically enriched 2,5-*bis*-(GSyl)-(*S*)-HHMA and 2,5-*bis*-(NAC)-(*S*)-HHMA permitted the unequivocal elution order of their corresponding diastereoisomers to be established: 2,5-*bis*-(GSyl)-(*R*)-HHMA and 2,5-*bis*-(GSyl)-(*S*)-HHMA, first and second eluting peaks, respectively; and 2,5-*bis*-(NAC)-(*R*)-HHMA and 2,5-*bis*-(NAC)-(*S*)-HHMA, first and second eluting peaks, respectively.

Figure 2 illustrates the chromatographic separation of 50 μg/mL methanolic solutions of the corresponding diastereoisomeric pairs. The analysis of diastereoisomeric mixtures of the same compounds (27), using HPLC-CEAS with the same electrode potentials and buffers, is capable of quantifying 5-(GSyl)-HHMA, 5-(NAC)-HHMA, 2,5-*bis*-(GSyl)HHMA and 2,5-*bis*-(NAC)-HHMA in ranges from approximately 5 to 43 pmol/10 μL brain dialysate (125–4300 ng/mL) for each pair, which should be sufficient for work with the pure enantiomers. Limits of detection and/or quantitation for the pure enantiomers will be determined.

The HPLC-CEAS analytical conditions for the diastereoisomeric separation of the mono-conjugates, 5-(GSyl)-HHMA and 5-(NAC)-HHMA, were extrapolated to perform a semi-preparative separation by HPLC-UV in a YMC ODS-AQ column (Figures 3a and b), using the corresponding pure compounds obtained by enzymatic synthesis previously purified with a HPLC semi-preparative column, and verified by HPLC-MS. Semi-preparative separation of 5-(GSyl)-HHMA diastereoisomers resulted in a lower *R*s compared to the separation obtained analytically. In this case, the first quarter of the first peak, the last quarter of the second peak, and the intermediate sections were collected separately. The intermediate-eluting material was lyophilized and subjected to a further round of purification, and the subsequent purity of the peaks was estimated by HPLC-CEAS as approximately 100%. When these collected peaks were further analyzed by HPLC-CEAS, single peaks were now revealed. The products were also analyzed by HPLC-MS, HPLC-

MS/MS. (supporting information). Because NMR spectroscopy data of the diastereoisomeric mixtures revealed no differences, further efforts on the NMR analysis of pure diastereoisomers were not pursued. Semi-preparative separation of 5-(NAC)-HHMA epimers was baseline (Figure 3b) and the corresponding peaks could be appropriately collected. Purity of both diastereoisomers was confirmed by HPLC-CEAS, revealing 99.7% and 100% for 5-(NAC)-(S)-HHMA and 5-(NAC)-(R)-HHMA, respectively. Isolated diastereoisomers were also analyzed by HPLC-MS, HPLC-MS/MS, (supporting information), and again identical results were obtained for both pure diastereoisomers and their corresponding mixture.

Semi-preparative separation of 2,5-*bis*-(NAC)-HHMA was possible with a $R_s = 1.06$ and purity of collected peaks, as determined by HPLC-CEAS, being 66.3% and 100% for the first and second eluting peaks respectively. Limitations with the amount of available sample did not permit further repurification and analysis of the isolated diastereoisomers. Moreover, it was not possible to perform a semi-preparative separation of 2,5-*bis*-(GSyl)-HHMA diastereoisomeric pairs, mainly because the analytical conditions (ammonium acetate buffer with citric acid, EDTA, and octanesulfonic acid, pH 2.5) could not be optimized for the semi-preparative separation. Future efforts are focused on improving this methodology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Footnotes

CEAS	coulometric electrode array system
d.e.	diastereoisomeric excess
e.e.	enantiomeric excess
NAC	<i>N</i> -acetylcysteine
R_s	Separation
HHMA	3,4-dihydroxymethamphetamine (or <i>N</i> -methyl- α -methyldopamine)
HHA	3,4-dihydroxyamphetamine (or α -methyldopamine)
HMMA	4-hydroxy-3-methoxymethamphetamine

HMA	4-hydroxy-3-methoxyamphetamine
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine
5-(GSyl)-HHMA	5-(glutathion-S-yl)-3,4-dihydroxymethamphetamine
2,5-bis-(GSyl)-HHMA	2,5-bis-(glutathion-S-yl)-3,4-dihydroxymethamphetamine
5-(NAC)-HHMA	5-(N-acetylcystein-S-yl)-3,4-dihydroxymethamphetamine
2,5-bis-(NAC)-HHMA	2,5-bis-(N-acetylcystein-S-yl)-3,4-dihydroxymethamphetamine

References

1. Ricaurte G, Bryan G, Strauss L, Seiden L, Schuster C. Hallucinogenic amphetamine selectively destroys brain serotonin terminals. *Science*. 1985; 229:986–988. [PubMed: 4023719]
2. Commins DL, Vosmer G, Virus RM, Woolverton WL, Schuster CR, Seiden LS. Biochemical and histological evidence that methylenedioxymethamphetamine (MDMA) is toxic to neurons in rat brain. *J. Pharmacol. Exp. Ther.* 1987; 241:338–345. [PubMed: 2883295]
3. Ricaurte GA, DeLanney LE, Irwin I, Langston JW. Toxic effects of MDMA on central serotonergic neurons in the primate: importance of route and frequency of drug administration. *Brain Res.* 1988; 446:165–168. [PubMed: 2897228]
4. Ricaurte GA, McCann UD, Szabo Z, Scheffel U. Toxicodynamics and long-term toxicity of the recreational drug, 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy'). *Toxicol. Lett.* 2000; 112-113:143–146. [PubMed: 10720723]
5. Esteban B, O'Shea E, Camarero J, Sanchez V, Green AR, Colado MI. 3,4-Methylenedioxymethamphetamine induces monoamine release, but not toxicity, when administered centrally at a concentration occurring following a peripherally injected neurotoxic dose. *Psychopharmacology*. 2001; 154:251–260. [PubMed: 11351932]
6. Monks TJ, Jones DC. The metabolism and toxicity of quinones, quinonimines, quinone methides and quinone-thioethers. *Curr. Drug Metab.* 2002; 3:425–438. [PubMed: 12093358]
7. Lim HK, Foltz RL. In vivo and in vitro metabolism of 3,4- methylenedioxymethamphetamine in the rat: identification of metabolites using an ion trap detector. *Chem. Res. Toxicol.* 1988; 1:370–378. [PubMed: 2908769]
8. de la Torre R, Farre M, Ortuno J, Mas M, Brenneisen R, Segura J, Cami J. Nonlinear pharmacokinetics of MDMA ("ecstasy") in humans. *Br. J. Clin. Pharmacol.* 2000; 49:104–109. [PubMed: 10671903]
9. Segura M, Ortuno J, Farre M, McLure JA, Pujadas M, Pizarro N, Llebaria A, Joglar J, Roset PN, Segura J, de la Torre R. 3,4-Dihydroxymethamphetamine (HHMA). A major in vivo 3,4-methylenedioxymethamphetamine (MDMA) metabolite in humans. *Chem. Res. Toxicol.* 2001; 14:1203–1208. [PubMed: 11559034]
10. Hiramatsu M, Kumagai Y, Unger SE, Cho AK. Metabolism of methylenedioxymethamphetamine: formation of dihydroxymethamphetamine and a quinone identified as its glutathione adduct. *J. Pharmacol. Exp. Ther.* 1990; 254:521–527. [PubMed: 1974641]
11. Patel N, Kumagai Y, Unger SE, Cho AK. Transformation of dopamine and - methyl dopamine by NG 108-15 cells: formation of thiol adducts. *Chem. Res. Toxicol.* 1991; 4:421–426. [PubMed: 1912328]
12. Easton N, Fry J, O'Shea E, Watkins A, Kingston S, Marsden CA. Synthesis, in vitro formation, and behavioural effects of glutathione regioisomers of alpha-methyl dopamine with relevance to MDA and MDMA (ecstasy). *Brain Res.* 2003; 987:144–154. [PubMed: 14499958]

13. Baez S, Segura-Aguilar J, Widersten M, Johansson A-S, Mannervik B. Glutathione transferases catalyse the detoxication of oxidized metabolites (*o*-quinones) of catecholamines and may serve as an antioxidant system preventing degenerative cellular processes. *Biochem. J.* 1997; 324:25–28. [PubMed: 9164836]
14. Chang M, Zhang F, Shen L, Pauss N, Alam I, van Breemen RB, Blond SY, Bolton JL. Inhibition of glutathione S-transferase activity by the quinoid metabolites of equine estrogens. *Chem. Res. Toxicol.* 1998; 11:747–755.
15. Van Ommen B, Ploemen JH, Bogaards JJ, Monks TJ, Lau SS, van Bladeren PJ. Irreversible inhibition of rat glutathione S-transferase1-1 by quinones and their glutathione conjugates. Structure-activity relationship and mechanism. *Biochem. J.* 1991; 276:661–666. [PubMed: 2064604]
16. O'Sullivan SM, McCarthy RM, Vargo MA, Colman RF, Sheehan D. Chemical modification at subunit 1 of rat kidney Alpha class glutathione transferase with 2,3,5,5- tetrachloro-1,4-benzoquinone: close structural connectivity between glutathione conjugation activity and non-substrate ligand binding. *Biochem. Pharmacol.* 2006; 71(11):1629–1636. [PubMed: 16620786]
17. Ploemen JH, van Ommen B, De Haan A, Venekamp JC, van Bladeren PJ. Inhibition of juman glutathione S-transferases by dopamine, alpha-methyldopa and their 5-S-glutathionyl conjugates. *Chem. Biol. Interact.* 1994; 90(1):87–99. [PubMed: 8131222]
18. O'Shea E, Granados R, Esteban B, Colado MI, Green AR. The relationship between the degree of neurodegeneration of rat brain 5-HT nerve terminals and the dose and frequency of administration of MDMA ('ecstasy'). *Neuropharmacology.* 1998; 37:919–926. [PubMed: 9776387]
19. Molliver ME, O'Hearn E, Battaglia G, DeSouza EB. Direct intracerebral administration of MDA and MDMA does not produce neurotoxicity. *Soc. Neurosci. Abstr.* 1986; 12:1234.
20. Schmidt CJ, Taylor VL. Direct central effects of acute methylenedioxymethamphetamine on serotonergic neurons. *Eur. J. Pharmacol.* 1988; 156:121–131. [PubMed: 2463176]
21. Paris JM, Cunningham KA. Lack of serotonin neurotoxicity after intraraphe microinjection of (+)-3,4-methylenedioxymethamphetamine. *Brain Res. Bull.* 1992; 28:115–119. [PubMed: 1347247]
22. Zhao Z, Castagnoli N Jr, Ricaurte GA, Steele T, Martello M. Synthesis and neurotoxicological evaluation of putative metabolites of the serotonergic neurotoxin 2- (methylamino)-1-[3,4-(methylenedioxy)phenyl]propane[(methylenedioxy)methamphet-amine]. *Chem. Res. Toxicol.* 1992; 5:89–94. [PubMed: 1349835]
23. McCann UD, Ricaurte GA. Lasting neuropsychiatric sequelae of (+)-methylenedioxymethamphetamine (Ecstasy) in recreational users. *J. Clin. Psychopharmacol.* 1991; 11:302–305. [PubMed: 1684975]
24. Bai F, Lau SS, Monks TJ. Glutathione and *N*-acetylcysteine conjugates of α - methyldopamine produce serotonergic neurotoxicity. Possible role in methylenedioxy-amphetamine-mediated neurotoxicity. *Chem. Res. Toxicol.* 1999; 12:1150–1157. [PubMed: 10604863]
25. Miller RT, Lau SS, Monks TJ. Metabolism of 5-(Glutathion-S-yl)- α -methyldopamine following intracerebroventricular administration to male Sprague-Dawley rats. *Chem. Res. Toxicol.* 1995; 8:634–641.
26. Miller RT, Lau SS, Monks TJ. Effect of 5-(Glutathion-S-yl)- α - oxymethyldopamine on dopamine, serotonin and norepinephrine concentrations following intracerebroventricular administration to male Sprague-Dawley rats. *Chem. Res. Toxicol.* 1996; 9:457–465. [PubMed: 8839050]
27. Jones DC, Duvauchelle C, Ikegami A, Olsen CM, Lau SS, de la Torre R, Monks TJ. Serotonergic neurotoxic metabolites of ecstasy identified in rat brain. *J. Pharmacol. Exp. Ther.* 2005; 313:422–431. [PubMed: 15634943]
28. Erives GV, Lau SS, Monks TJ. Accumulation of neurotoxic thioether metabolites of 3,4-(+/-)-methylenedioxymethamphetamine in rat brain. *J. Pharmacol. Exp. Ther.* 2008; 324:284–291. [PubMed: 17906065]
29. Nichols DE, Lloyd DH, Hoffman AJ, Nichols MB, Yim GK. Effects of certain hallucinogenic amphetamine analogues on the release of [3H]serotonin from rat brain synaptosomes. *J. Med. Chem.* 1982; 25:530–535. [PubMed: 7086839]

30. Johnson MP, Hoffman AJ, Nichols DE. Effects of the enantiomers of MDA, MDMA and related analogues on [3H]serotonin and [3H]dopamine release from superfused rat brain slices. *Eur. J. Pharmacol.* 1986; 132:269–276. [PubMed: 2880735]
31. Schmidt CJ, Levin JA, Loumberg W. In vitro and in vivo neurochemical effects of methylenedioxymethamphetamine on striatal monoaminergic systems in the rat brain. *Biochem. Pharmacol.* 1987; 36:747–755. [PubMed: 2881549]
32. Tucker GT, Lennard MS, Ellis SW, Woods HF, Cho AK, Lin LY, Hiratsuka A, Schmitz DA, Chu TY. The demethylenation of methylenedioxymethamphetamine (Ecstasy) by debrisoquine hydroxylase (CYP2D6). *Biochem. Pharmacol.* 1994; 47:1151–1156. [PubMed: 7909223]
33. Kraemer T, Maurer HH. Toxicokinetics of amphetamines: metabolism and toxicokinetic data of designer drugs, amphetamine, methamphetamine, and their N-alkyl derivatives. *Ther Drug Monit.* 2002; 24:277–289. [PubMed: 11897973]
34. Kretz KL, Kovar KA, Schwab M, Zangert UM. Identification of human cytochromes P450 involved in the oxidative metabolism of "ecstasy" related designer drugs. *Biochem. Pharmacol.* 2000; 59:1563–1571. [PubMed: 10799653]
35. Fallon JK, Kicman AT, Henry JA, Milligan PJ, Cowan DA, Hutt AJ. Stereospecific analysis and enantiomeric disposition of 3,4-methylenedioxymethamphetamine (ecstasy) in humans. *Clin. Chem.* 1999; 45:1058–1069. [PubMed: 10388483]
36. Pizarro N, de la Torre R, Farre M, Segura J, Llebaria A, Joglar J. Synthesis and capillary electrophoretic analysis of enantiomerically enriched reference standards of MDMA and its main metabolites. *Bioorg. Med. Chem.* 2002; 10:1085–1092. [PubMed: 11836119]
37. Pizarro N, Farre M, Pujadas M, Peiro AM, Roset PN, Joglar J, de la Torre R. Stereochemical analysis of 3,4-methylenedioxymethamphetamine and its main metabolites in human samples including the catechol-type metabolite (3,4-dihydroxymethamphetamine). *Drug Metab. Dispos.* 2004; 32:1001–1007. [PubMed: 15319342]
38. Borgman RJ, Baylor MR, McPhillips JJ, Stitzel RE. Alpha-methyldopamine derivatives. Synthesis and pharmacology. *J. Med. Chem.* 1974; 17:427–430. [PubMed: 4830540]
39. Felim A, Urios A, Neudorffer A, Herrera G, Blanco M, Llargeron M. Bacterial plate assays and electrochemical methods: an efficient tandem for evaluation the ability of catecholthioether metabolites of MDMA ("ecstasy") to induce toxic effects through redox-cycling. *Chem. Res Toxicol.* 2007; 20:685–693. [PubMed: 17355154]
40. Pizarro N, Llebaria A, Cano S, Farre M, Segura J, de la Torre R. Method development for stereochemical analysis of MDMA and its main metabolites by GC-MS. *Rapid Commun. Mass Spectrom.* 2003; 17:330–336. [PubMed: 12569443]
41. Espin JC, Garcia-Ruiz PA, Tudela J, Garcia-Canovas F. Study of stereospecificity in mushroom tyrosinase. *Biochem. J.* 1998; 331:547–551. [PubMed: 9531496]
42. Marin-Zamora ME, Rojas-Melgarejo F, Garcia-Canovas F, Garcia-Ruiz PA. Stereospecificity of mushroom tyrosinase immobilized on a chiral and a nonchiral support. *J. Agric. Food Chem.* 2007; 55:4569–4575. [PubMed: 17488091]

Figure 1.
Chemical structures of (a) 5-(GSyl)-HHMA; (b) 5-(NAC)-HHMA; (c) 2,5-bis-(GSyl)-HHMA; and (d) 2,5-bis-(NAC)-HHMA.

Figure 2.

HPLC-CEAS chromatograms for the analytical resolution of (a) 5-(GSyl)-HHMA and 5-(NAC)-HHMA diastereoisomers (2.5 μg each pair approximately), respectively; (b) diastereoisomerically enriched 5-(GSyl)-(S)-HHMA (from diluted crude reaction), (c) diastereoisomerically enriched 5-(NAC)-(S)-HHMA (from diluted crude reaction), (d) 2,5-bis-(GSyl)-HHMA diastereoisomers (0.2 μg approximately); and (e) 2,5-bis-(NAC)-HHMA diastereoisomers (0.1 μg approximately).

Figure 3. HPLC-UV chromatograms for the semi-preparative resolution of (a) 5-(GSyl)-HHMA diastereoisomers (210 µg); (b) 5-(NAC)-HHMA diastereoisomers (210 µg); and (c) 2,5-bis-(NAC)-HHMA diastereoisomers (75 µg).