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

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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-dihydroxymethamphetamine 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*-acetylcystein-*S*-yl)-HHMA, 2,5-bis-(glutathion-*S*-yl)-HHMA, and 2,5-bis-(*N*-acetylcystein-*S*-yl)-HHMA. Both enzymatic and electrochemical syntheses were explored, and methodologies for analytical and semipreparative 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 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,[1] ecstasy) is a ring-substituted amphetamine that acts as a serotonergic neurotoxicant in several species, including nonhuman 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*−*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-

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[1] Abbreviations: CEAS, coulometric electrode array system; d.e., diastereoisomeric excess; e.e., enantiomeric excess; NAC, *N*-acetylcysteine; Rs, separation; HHMA, 3,4-dihydroxymethamphetamine (or *N*-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.

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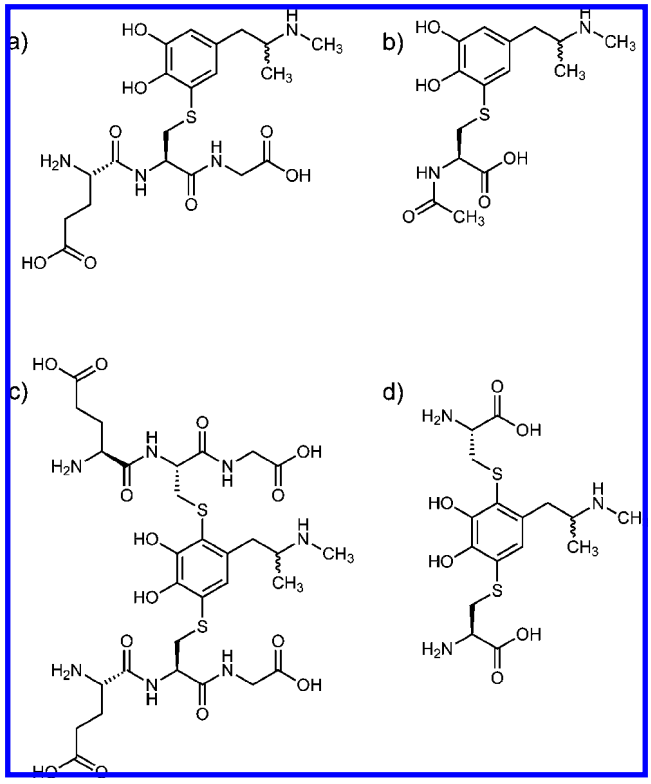

**Figure 1.** Chemical structures of (a) 5-(GSyl)-HHMA, (b) 5-(NAC)-HHMA, (c) 2,5-bis-(GSyl)-HHMA, and (d) 2,5-bis-(NAC)-HHMA.

(*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 the 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 7 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 12 h 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 the 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 nonlinear 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. Because 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. Because 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 semipreparative 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 semipreparative diastereoisomeric separation may be crucial tools to assist in providing a better understanding of MDMA-mediated neurotoxicity. Thus, semipreparative 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 $H_2O$ 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 (*38*). The enantiomerically enriched (*S*)-HHMA [65.4% enantiomeric excess (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 of 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 semipreparative HPLC-UV (SCL-10A Shimadzu) (Kyoto, Japan) equipped with a $C_{18}$ column (Beckman Coulter, Ultrasphere ODS, 5 $\mu$m × 10 mm i.d. × 25 cm) (Fullerton, CA). Solvents used were $H_2O$ with 1% acetic acid (A) and MeOH (B) 90:10 at 2 mL $min^{-1}$ ($\lambda$ = 280 nm). Several injections were required to finally obtain 1.24 mg of the product (2.55 $\mu$mol, 4.6% reaction yield). After several batches of the reaction were performed, 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 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 $min^{-1}$ ($\lambda$ = 225 nm). The procedure was repeated several times and yielded 0.6 mg of product (1.75 $\mu$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, TN) 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 of HCl (0.2 N), which was oxidized under nitrogen at room temperature at a platinum mesh whose potential was fixed at +1.0 V vs Ag/AgCl. A platinum wire was used in the cathodic compartment (counter electrode). After the consumption of 2 electrons per molecule, 2 equiv of GSH (62.8 mg, 0.2 mmol) was 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 semipreparative HPLC-UV system and chromatographic column employed for the enzymatic purification. Solvents used were $H_2O$ with 1‰ trifluoroacetic acid (TFA, A) and $H_2O$:MeOH (50:50) with 0.05‰ TFA (B) 70:30 at 3 mL $min^{-1}$ ($\lambda$ = 280 nm). The reaction yielded 5-(GSyl)-HHMA (7.0 mg, 14.4 $\mu$mol, 14.4% yield) and 2,5-bis-(GSyl)-HHMA (1.6 mg, 2.02 $\mu$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 of HHMA·HBr (0.15 mmol). Semipreparative 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 mL $min^{-1}$ flow rate ($\lambda$ = 225 nm). Once lyophilized, 5-(NAC)-HHMA (3.8 mg, 11.1 $\mu$mol, 7.4% yield) and 2,5-bis-(NAC)-HHMA (1.4 mg, 2.8 $\mu$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, 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 (HR-80, ESA Inc.), 250 (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 × 2.0 mm i.d. × 5 $\mu$m 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. The aqueous mobile phase consisted of 8 mM ammonium acetate, 4 mM citric acid, 54 $\mu$M EDTA, and 230 $\mu$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 mL $min^{-1}$, 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 was maintained at 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 ($C_{18}$) (80 mm × 4.6 mm i.d. × 3 $\mu$m particle size) (ESA Inc.). Optimal separation was performed at a 1 mL $min^{-1}$ flow rate using 8 mM ammonium acetate, 4 mM citric acid, 54 $\mu$M EDTA, 230 $\mu$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 $C_{18}$ reverse-phase column (Beckman Coulter, Ultrasphere ODS, 25 cm × 4.6 mm i.d. × 5 $\mu$m particle size). Solvents used were $H_2O$ with 1‰ TFA (A) and acetonitrile (B) 94:6 at 1 mL $min^{-1}$.

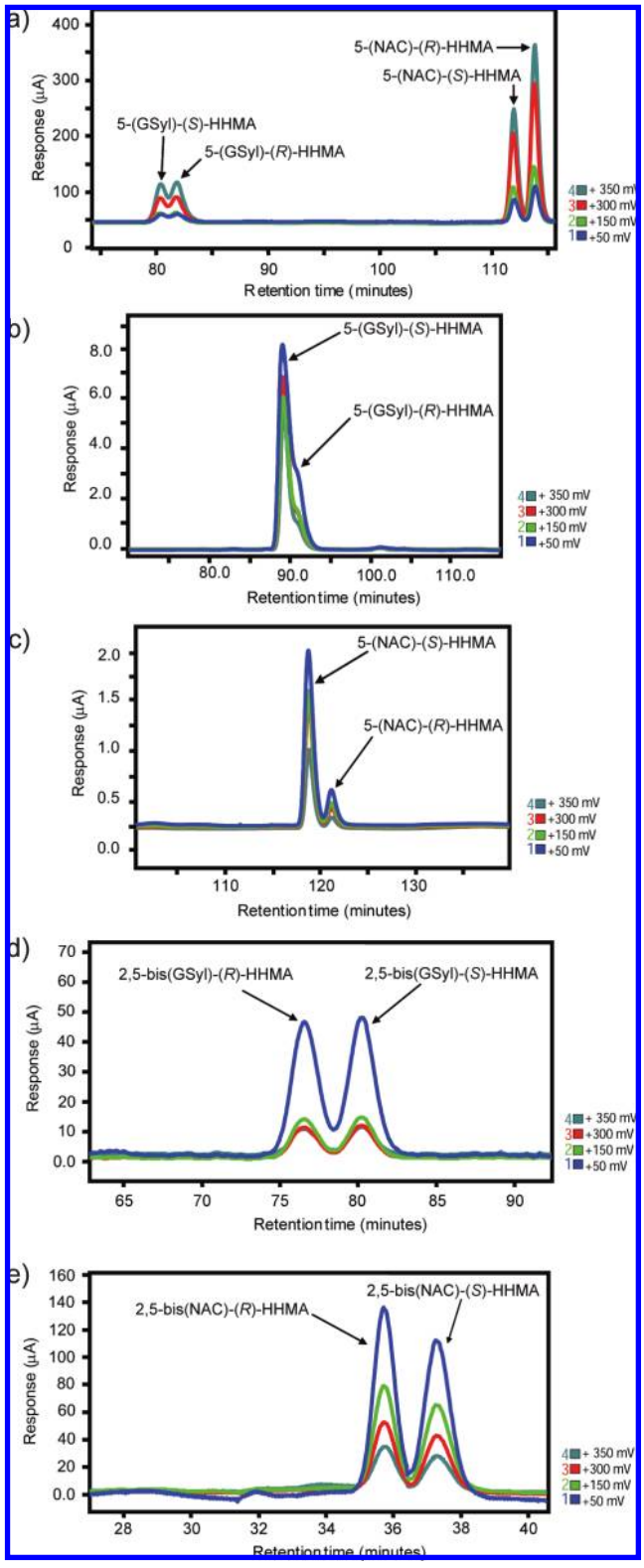


**Figure 2.** HPLC-CEAS chromatograms for the analytical resolution of (a) 5-(GSyl)-HHMA and 5-(NAC)-HHMA diastereoisomers (2.5 $\mu$g each pair approximately), respectively; (b) diastereoisomerically enriched 5-(GSyl)-(*S*)-HHMA (from diluted crude reaction); (c) diastereoisomerically enriched 5-(NAC)-(*S*)-HHMA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

**Semipreparative 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 semipreparative separation, using a HPLC equipped with an UV detector coupled to a YMC ODS-AQ column (YMC Co. LTd) ($C_{18}$ AQ) (250 mm × 10 mm i.d. × 5 $\mu$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 $\mu$mol) was achieved at a flow rate of 0.9 mL min$^{-1}$ and monitored at $\lambda = 280$ nm. The gradient consisted of 0−10% 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 $\mu$mol, 10.6% yield) of 5-(GSyl)-(*S*)-HHMA (diastereoisomer 1) and 1.45 mg (2.98 $\mu$mol, 5.2% yield) of 5-(GSyl)-(*R*)-HHMA (diastereoisomer 2). Final products were analyzed by NMR, and the data were 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).

**Semipreparative 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 0−20% methanol over 30 min and holding at 20% methanol for the next 50 min. The flow rate was 0.9 mL min$^{-1}$ and $\lambda = 225$ 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 $\mu$mol, 25% yield) of 5-(NAC)-(*S*)-HHMA (diastereoisomer 1) and 2.1 mg (6.13 $\mu$mol, 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).

**Semipreparative 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 semipreparative column (250 mm × 10 mm i.d. × 5 $\mu$m particle size; Beckman Coulter, Fullerton, CA). The aqueous phase consisted of $H_20$ + 1‰ TFA (A) with acetonitrile as the organic phase (B), 94:6 at 1 mL min$^{-1}$ ($\lambda$ = 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 semipreparative yields for this diasteroisomeric pair separation will be performed in the future.

**Enantioselective Analysis of HHMA by GC-MS.** Unreacted HHMA obtained from the HPLC-UV purification of both 5-(GSyl)-HHMA and 5-(NAC)-HHMA was analyzed by GC (6890 N; Agilent Technologies, Wilmington, DE) 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 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 byproduct, but reaction required repeating several times in order to obtain just less than 1 mg of each *bis*-conjugate. In addition, the semipreparative 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 repurification 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 byproduct 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 $H_2O$ 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 the 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, 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 to carry out analyses in biological samples. Several different HPLC columns and a wide range of pH values and gradients were examined (see the Experimental Procecures), with a YMC ODS-AQ analytical column ($C_{18}$ 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 (Rs, calculated with enantiomeric resolution formula, see the 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 diastereoisomeric excess (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 semipreparative purification process, 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 (Rs ≥ 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 suggest that more (*R*)-HHMA has reacted as 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% enantiomeri-

cally 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 monoconjugates, 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 was performed using HR-80 $C_{18}$ column (80 mm × 4.6 mm i.d. × 3 μm particle size) and an Ultrasphere ODS $C_{18}$ column (250 mm × 4.6 mm i.d. × 5 μm particle size), respectively, achieving baseline separations in both cases (Figure 2d,e). 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 as follows: 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 monoconjugates, 5-(GSyl)-HHMA and 5-(NAC)-HHMA, were extrapolated to perform a semipreparative separation by HPLC-UV in a YMC ODS-AQ column (Figure 3a,b), using the corresponding pure compounds obtained by enzymatic synthesis previously purified with a HPLC

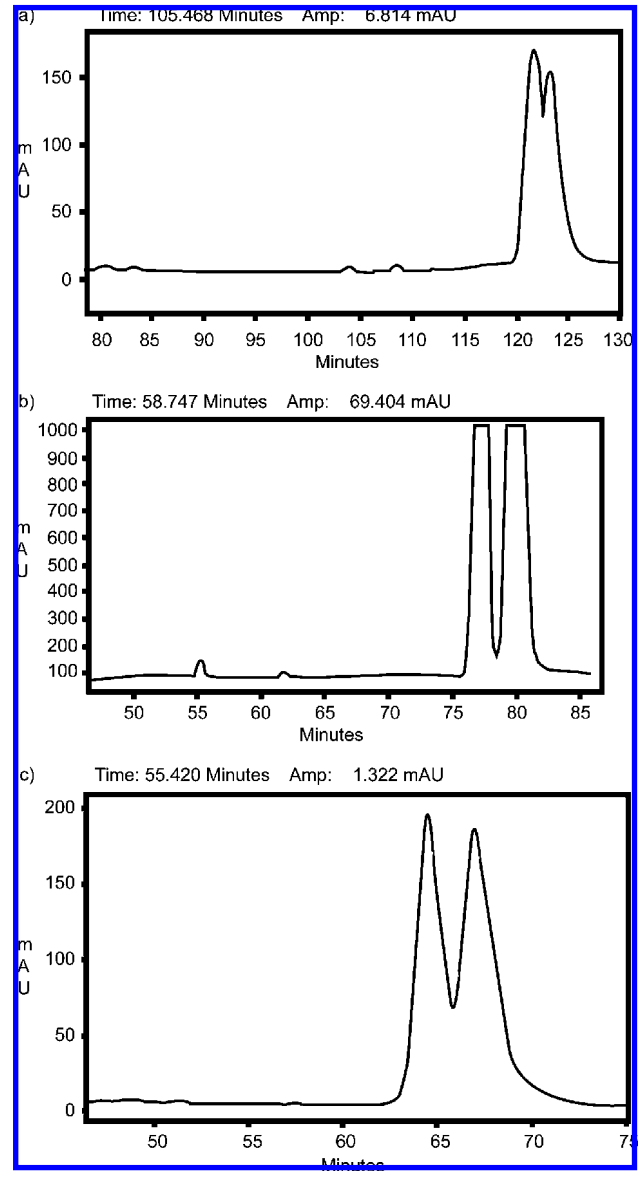


**Figure 3.** HPLC-UV chromatograms for the semipreparative resolution of (a) 5-(GSyl)-HHMA diastereoisomers (210 μg), (b) 5-(NAC)-HHMA diastereoisomers (210 μg), and (c) 2,5-bis-(NAC)-HHMA diastereoisomers (75 μg).

semipreparative column, and verified by HPLC-MS. Semipreparative separation of 5-(GSyl)-HHMA diastereoisomers resulted in a lower Rs as 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 and 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. Semipreparative 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 and HPLC-MS/MS (Supporting Infor-

mation), and again, identical results were obtained for both pure diastereoisomers and their corresponding mixture.

Semipreparative separation of 2,5-*bis*-(NAC)-HHMA was possible with a Rs = 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 semipreparative 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 semipreparative separation. Future efforts are focused on improving this methodology.

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**Supporting Information Available:** Formulas 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.



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