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NEUROTOXIC THIOETHER ADDUCTS OF MDMA IDENTIFIED IN HUMAN URINE AFTER ECSTASY INGESTION

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Running Title: Neurotoxic Thioether Adducts of MDMA In Human Urine

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Abbreviations: NAC, N-Ac-5-Cys, N-acetylcysteine; 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-methoxy-amphetamine; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; N-Ac-5-Cys-HHA or NAC-HHA, 5-(N-acetylcystein-S-yl)-3,4-dihydroxyamphetamine; N-Ac-5-Cys-HHMA or NAC-HHMA, 5-(N-acetylcystein-S-yl)-3,4-dihydroxymethamphetamine; N-Ac-5-Cys-O-Me-HHMA or NACME-HHMA, 5-(N-acetylcystein-S-yl)-methylesther-3,4-dihydroxymethamphetamine; LC-MS/MS, liquid chromatography tandem mass spectrometry; SRM, selected reaction monitoring; MBS, metabisulfite;

SMBS; Sodium metabisulfite; DTT, Dithiothreitol; CYP2D6, Cytochrome P450 2D6; COMT, catechol-O-methyl transferase, DL, detection limit; QL, Quantification limit.

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Abstract

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is a widely misused synthetic amphetamine derivative, and a serotonergic neurotoxicant in animal models and possibly humans. The underlying mechanism of neurotoxicity involves the formation of reactive oxygen species (ROS) although their source remains unclear. It has been postulated that MDMA induced neurotoxicity is mediated via the formation of bioreactive metabolites. Specifically, the primary catechol metabolites, 3,4-dihydroxymethamphetamine (HHMA) and 3,4-dihydroxyamphetamine (HHA), subsequently give rise to the formation of glutathione and *N*-acetylcysteine conjugates, which retain the ability to redox cycle, and are serotonergic neurotoxicants in rats. Although the presence of such metabolites has been recently demonstrated in rat brain microdialysate, their formation in humans has not been reported. The present study describes the detection of *N*-acetyl-5-cysteinyl-HHMA (NAC-HHMA) and N-acetyl-5-cysteinyl-HHA (NAC-HHA) in human urine of fifteen recreational users of MDMA (1.5 mg/kg) in a controlled setting. The results reveal that in the first 4 hours after MDMA ingestion ~0.002% of the administered dose was recovered as thioether adducts. Genetic polymorphisms in CYP2D6 and COMT expression, the combination of which are major determinants of steady state levels of HHMA and HMA, likely explain the inter-individual variability seen in the recovery of NAC-HHMA and NAC-HHA. In summary, the formation of neurotoxic thioether adducts of MDMA has been demonstrated for the first time in humans. The findings lend weight to the hypothesis that the bioactivation of MDMA to neurotoxic metabolites is a relevant pathway to neurotoxicity in humans.

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Introduction

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is a psychostimulant widely abused among young people. MDMA exhibits distinct pharmacological properties, collectively described as entactogenic which differentiates it from classical amphetamines (Nichols et al.,, 1986). MDMA produces an acute and long-term serotonergic neurotoxicity in rodents, primates and, possibly, in humans, with the severity of toxicity dependent upon on the dose and frequency of administration (Green et al., 2003). Such neurotoxicity is evidence by a decrease tryptophan hydroxylase activity (Stone et al., 1988), a reduction on serotonin content, a dose-dependent persistent decrease in the number of 5-HT transporter sites and 5-HT receptors in several regions of the brain (Ricaurte et al., 2000, Aguirre et al., 1995) and an impairment of central 5-HT function (Barrionuevo et al.,, 2000).

Oxidative stress, hyperthermia, excitotoxicity and various apoptotic pathways have been invoked as the underlying mechanism(s) of MDMA-induced neurotoxicity (Cadet et al., 2007). With respect to oxidative stress, various sources of reactive oxygen species (ROS) have been suggested, including the monoamine oxidase mediated metabolism of tyrosine/dopamine, which generates hydrogen peroxide as a by-product, and the oxidative metabolism of MDMA to redox active catechols. However, until recently, evidence supporting a link between MDMA metabolism and neurotoxicity has been primarily indirect (Monks et al., 2004; Jones et al., 2005). MDMA is metabolized via cytochrome P450 mediated *N*-demethylation to the active metabolite 3,4-methylenedioxyamphetamine (MDA). MDMA and MDA are both *O*-demethylenated, again via cytochrome P450, to 3,4-dihydroxymethamphetamine (HHMA, *N*-methyl-α-methyldopamine) and 3,4-dihydroxyamphetamine (HHA, α-methyldopamine), respectively (Fig. 1) (Maurer et al., 2000; de la Torre et al., 2004a). Since HHMA and HHA are both catechols, they can undergo further autooxidation to the corresponding *ortho*-quinones, which are readily conjugated with glutathione (GSH) to form glutathionyl adducts (Hiramatsu et al., 1990). The GSH adducts of HHMA and HMA appear to be transported into the brain via blood-brain-barrier GSH transporters, were they are subsequently metabolized to the corresponding N-acetylcysteine (NAC)

adducts (Bai et al., 2001). Direct injection of the GSH and N-acetylcysteine conjugates of HHMA or HMA into rat brain produces not only the acute neurobehavioral effects of the parent drug, but also its selective serotonergic neurotoxicity (Miller et al., 1996; Bai et al., 1999). Moreover, since multi-dose administration of MDMA is typical of drug intake during rave parties, the effects of multiple doses of MDMA on the concentration of catechol-thioether metabolites in rat brain were determined. The data revealed that thioether metabolites, especially the NAC conjugates, accumulate in rat brain following multi-dose administration of MDMA (Erives et al., 2008). The ability of these metabolites to generate reactive oxygen species and to arylate proteins, in combination with their ability to modulate the activity of proteins involved in the regulation of neurotransmitter transport (Jones et al., 2004), suggest they play an important role in the development of MDMA mediated serotonergic neurotoxicity.

Although catechol-thioether metabolites of MDMA have been identified in rat brain (Jones et al., 2005), there is no evidence for their formation in humans after MDMA exposure. GSH adducts are metabolized by γ-glutamyl transpeptidase (γ-GT) and subsequently by M-aminopeptidase to the corresponding cysteine conjugates, which are ultimately *N*-acetylated to form the *N*-acetylcysteine derivative (also referred to as the mercapturic acid). In humans, a non-invasive approach to demonstrate the formation of GSH adducts of HHMA and HHA after MDMA exposure would be the detection of the mercapturate derivatives in urine. In the present study, analytical methodology for the detection of MDMA-derived mercapturates in human urine has been developed, and applied to samples obtained from recreational users of MDMA (1.5 mg/kg, 75-100 mg dose) in a controlled setting. Finally, since enzymes regulating the formation (CYP2D6) and the inactivation (COMT) of catechol metabolites of MDMA in humans are highly polymorphic, the contribution of genetic variability to inter-individual differences in the urinary concentration of mercapturates has also been examined.

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Materials and Methods

Chemicals & Reagents. Ultrapure water was obtained using a Milli-Q purification system (Millipore, Molsheim, France). HPLC-grade acetonitrile, methanol, hydrochloric acid, sodium acetate, potassium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydroxide, trifluoroacetic acid, metabisulfite (MBS), dithiothreitol (DTT), formic acid, ammonia, ammonium formate and ammonium chloride were obtained from Merck (Darmstadt, Germany). EDTA, *N*-Acetyl-L-cysteine and mushroom tyrosinase (2033 U/mg) were obtained from Sigma Aldrich (St. Louis, MO). *N*-Acetyl-Lcysteine methyl ester was obtained from Fluka Biochemika (Riedel-de Haën, Seelze, Germany). Bond Elut PBA (phenylboronic acid-PBA 500 mg sorbent) columns were purchased from Varian (Harbor City, CA) and mounted on a Vac Elut vacuum manifold (Supelco, Bellefonte, PA).

Synthesis of N-acetylcysteine and N-acetylcysteine-methylester adducts of HHMA and HHA. *N* acetyl- 5-cysteinyl-HHMA (*N*-Ac-5-Cys-HHMA), *N*-acetyl-5-cysteinyl-HHA (*N*-Ac-5-Cys-HHA) and *N*-acetyl-5-cysteinyl-*O*-Me-HHMA (*N*-Ac-5-Cys-*O*-Me-HHMA, as IS) were synthesized following an experimental procedure similar to that described previously (Jones et al., 2005). Briefly, 3mM HHMA or HHA, 8 mM *N*-acetyl-L-cysteine or *N*-acetyl-L-cysteine methyl ester and 1016 UI of tyrosinase from mushroom (2033 units/mg) in 100 ml of 50 mM phosphate buffer, pH 7.4, previously degassed with argon were incubated for 30 min at room temperature. The reaction was quenched with 2 ml of 88% formic acid. The reaction mixture was concentrated by lyophilization, and the adducts were isolated by semipreparative reverse phase HPLC–UV (conditions at instrumentation section), Collected fractions were lyophilized, and the structure and purity of the compound were determined by RMN and HPLC-MS/MS. HPLC-MS/MS revealed a single compound with a molecular ion corresponding to each product synthesized: *N*-acetyl-5- cysteinyl-HHA (m/z 329), *N*-acetyl-5-cysteinyl-HHMA (m/z 343) *N*-acetyl-5-cysteinyl-*O*-Me-HHA (m/z 357). The molecular ion, once further fragmented, gives rise to several

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daughter ions, including those of *N*-acetylcysteine (m/z 161.9), *N*-acetylcysteine methyl ester (m/z 175.9), HHMA (m/z 182.9) and HHA (m/z 168.9).

Working Standards. Solutions of 1 mg/ml of *N*-Ac-5-Cys-HHMA, *N*-Ac-5-Cys-HHA and *N*-Ac-5- Cys-*O*-Me-HHMA (IS) were prepared in 10 methanol. Working solutions of 0.1, 1, 10 and 100 μg/ml of each compound were prepared by dilution of the corresponding 1 mg/ml stock solution.

Preparation of Calibration and Quality Control (QC) Samples. Calibration curves and QC samples were prepared by adding appropriate volumes of working solutions to test tubes, each containing 5 ml of drug-free urine. QC samples were prepared with solutions different from those used for the preparation of calibration curves. Final concentrations in the calibration curves were 4, 7, 15, 20, and 30 ng/ml of *N*-Ac-5-Cys-HHMA and *N*-Ac-5-Cys-HHA. Control urine samples containing appropriate analytes at different concentrations were prepared in drug-free samples in 5 ml aliquots. The concentrations of QC samples were as follows: 4, 12, and 26 ng/ml of both metabolites.

1H NMR spectra were obtained in MeOD solutions on a Varian Anova 500 and Unity 300 spectrometers. Chemical shifts are reported in ppm relative to the multiplet at 3.39 ppm of MeOD.

Analytical HPLC-UV. A LaChrom Pump L-7100 (Merck, Hitachi) with manual injector coupled to a UV LC-75 Spectrophotometric Detector (Perkin Elmer) was used, with a Lichrosphere RP-18 5μm (4 x 250 mm) column. The HPLC solvent system was a gradient and consisted of two phases: A: 0.1 % TFA in water and B: 20% water/ 80% acetonitrile with 0.095% TFA. The step linear gradient for elution was from 0 %B to 5 %B in 1 min, and from 5 %B to 60 %B in 30 min with a flow rate 1 ml/min, and the eluate was monitored at 215 nm.

Semipreparative HPLC-UV. A Waters LC 4000 HPLC system (Waters) coupled to an UV 4000 Series Spectrophotometric Detector (Merck, Hitachi) was used with a X-Terra MS C18 column (10µm, 19 x 250 mm). The same mobile phases and gradient as those described for the analytical HPLC-UV system were used for semipreparative HPLC, but with a flow rate of 12 ml/min.

High-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). Extracted urine samples were analyzed in a Micromass Quattro micro API triple quadrupole mass spectrometer (Waters) equipped with an ESI probe coupled to an Alliance HPLC system (Waters, Etten-Leur, The Netherlands). An Atlantis T3 3µm (2.1 x 20 mm) Column (Waters) was used. The mobile phase flow rate was 0.25 mL/min. A binary mobile phase was used: A)100% 0.1% formic acid in water , B)100% acetonitrile. The linear gradient for elution was: 0-10% B in 15 min. The electrospray ionization (ESI) was operated in positive mode and the ESI settings were as follows: capillary voltage, 3000 V; source temperature 120ºC; desolvation temperature, 420ºC; cone voltage, 20 V; cone gas and desolvation gas flow rates, 34 and 609 l/h, respectively. Initial screening of the samples was performed in positive mode in the first quadrupole based on full MS measurements between 50-500 m/z only (Q1 MS mode). Following the Q1 MS scan, selected reactions monitoring (SRM) was performed. The fragmentation channels monitored for [M+H]+ to product ions were, for *N*-Ac-5-Cys-HHMA: m/z 343 → 130, m/z 343 → 181, m/z 343 → 207; and m/z 343 → 130; for N-Ac-5-Cys-HHA: m/z 329 → 130, m/z 329 → 207, m/z 329 → 270; and for *N*-Ac-5-Cys-*O*-Me-HHMA (IS) m/z 357 → 144, m/z 357 → 181 and m/z 357 → 207. The maximum ionization time for each product ion scan was 2 sec with an isolation width of 0.9 m/z. Quantitative analysis was based on peak area ratios of the 343 and 329 amu ions relative to the internal standard ion at 357 amu. Each day of analysis, a 5 point calibration curve ranging from 3 to 30 ng/ml was performed in duplicate.

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Genotyping. Samples of 1 ml whole blood were taken for DNA extraction and CYP 2D6 genotyping (DrugMEt; Jurilab Ltd, Kuopio, Finland). The following allelic variants were determined: *1, *2, *4, *5, *9, *10, *35 and *41. As well as deletions (*3) and gene duplications (*1xn; *2xn)

The COMT Val/Met (rs4680) and P2 promoter (rs2097603) allelic variants were determined using the 5' exonuclease TaqMan assay performed using an ABI 7900HT Sequence Detection System (Real Time PCR) supplied by Applied Biosystem (Foster City, CA, USA). Primers and fluorescent probes for the Val/Met assay were obtained from Applied Biosystem by submission of polymorphic sequence of COMT gene to Assay-by-Design Service (p/n 4332728, COMT_V158MS1AG). According to assay design the primers have the following sequences: forward 5'- CCCAGCGGATGGTGGAT-3' reverse 5'-CAGGCATGCACACCTTGTC-3'. The reporter probes for the Real Time PCR reaction have the following sequence: FAM-TCGCTGGCGTGAAG-3', VICTTCGCTGGCATGAAG-3'. For the P2 promoter assay primers have the following sequences according to those previously published (Chen et al., 2004): forward 5'-GCCGTGTCTGGACTGTGAGT-3', reverse 5'-GGGTTCAGAATCACGGATGTG-3'. The reporter probes have the following sequence 6FAMAACAGACAGAAAAGTTTCCCCTTCCCA-3' and VICCAGACAGAAAAGCTTCCCCTTCCCATA-3'. Reaction conditions were those described in the ABIPRISM 7900HT User's Guide. Endpoint fluorescent signals were detected on the ABI 7900, and the data were analyzed using the Sequence Detector System software, version 2.1.

Subjects and dosing. The study was conducted in accordance with the Declaration of Helsinki (2000), approved by the local Institutional Review Board (CEIC-IMAS), and authorized by the Spanish Medicines Agency of the Spanish Ministry of Health. All subjects gave their written informed consent before inclusion in the study and were compensated for their participation.

Each subject was interviewed by a physician to exclude concomitant medical conditions, and underwent a general physical examination, routine laboratory tests, urinalysis, and 12-lead ECG. Subjects were interviewed by a psychiatrist (Psychiatric Research Interview for Substance and Mental Disorders for

DSM-IV, PRISM-IV) to exclude those with a history of or actual major psychiatric disorders (schizophrenia, psychosis, and major affective disorder). Fifteen individuals (11 males and 4 females) fulfilled the inclusion criteria and had a mean age of 26 years (range 19– 33), mean body weight of 69.6 Kg (range 54.2–91.2), and mean height of 181 cm (range 171–196). Subjects reported an average of 26 previous experiences (range 6 – 100) with MDMA. All but four were current smokers. None met criteria of abuse or drug dependence (except for nicotine dependence). All had previous experience with other psychostimulants, cannabis or hallucinogens. None had a history of adverse medical or psychiatric reactions after MDMA consumption. The subjects were phenotyped with dextromethorphan for CYP2D6 enzyme activity and all were categorized as extensive metabolizers (Schmid et al., 1985). All subjects received a single 1.5mg/kg dose of MDMA. MDMA was obtained from the Spanish Ministry of Health and MDMA soft gelatin capsules were prepared and supplied by the Department of Pharmacy of Hospital del Mar (Barcelona, Spain).

Sample Collection. Plasma samples were collected at 0, 0.3, 0.6, 1, 1.5, 2 and 4 hours after MDMA administration. Urine samples were collected before and after drug administration at 0 and 0 to 4 h time period. Urine was collected in refrigerated plastic containers covered with aluminum paper to prevent light exposure. Samples were immediately acidified with 0.5 ml 6M HCl, distributed in aliquots and stored at -20ºC until further analysis.

Concentrations of MDMA and metabolites in plasma and urine were determined by gaschromatography coupled to mass spectrometry following an analytical procedure described previously (Pizarro et al., 2002)

Preparation of Urine Sample for the Analysis of N-Acetyl-Cysteinyl Adducts. For the determination of *N*-Ac-5-Cys-HHMA and *N*-Ac-5-Cys-HHA, 5 ml of urine and 25 μL of the appropriate IS solution were transferred into 15 ml screw-capped glass tubes. Urine pH was adjusted to 9.5 with ammonium chloride buffer (pH 9.5). After vortex mixing, the sample was filtered through a 0.22 μm centrifuge filter

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at 3500 rpm for 5 min. Bond Elut PBA (500 mg) columns were conditioned by washing with 5 ml of ammonium chloride buffer (pH 9.5). Urine samples were applied to the column and forced to pass through. After application of the sample, the column was washed twice with 3 ml of H2O/MeOH (95/5). Analytes were then eluted with 2 ml of TFA 1M/MeOH (30/70), 20mM DTT. The eluate was then evaporated for 20 min under a stream of nitrogen in a water bath at 39°C to evaporate the methanol and then frozen and lyophilized. The dried extract was reconstituted in 100 μL of the mobile phase with 9 mM Na2S2O5 and 5mM EDTA as preservatives, transferred to a 0.22 μm centrifuge tube and centrifuged at 15000 rpm for 5 min. The supernatant was then transferred into 200 μL injection vials, and a 40 μL aliquot was injected into the LC–ESI-MS/MS system.

Validation Procedure. Validation of the method was performed according to a 4-day protocol Linearity was determined by checking different calibration curves (n = 10 in four consecutive days) at five different concentrations of *N*-Ac-5-Cys-HHMA (4, 7, 15, 20 and 30 ng/ml) and *N*-Ac-5-Cys-HHA (4, 7, 15, 20 and 30 ng/ml). Peak area ratios between *N*-Ac-5-Cys-HHMA or *N*-Ac-5-Cys-HHA and the internal standard (*N*-Ac-5-Cys-*O*-Me-HHMA) were used for calculations. A weighted (1/concentration) least-squares regression analysis was used (SPSS computer software package, version 12.0 for Windows; SPSS Inc., Chicago, IL). By quantifying a quadruplicate of the lower concentration of the calibration curves, we estimated the limits of detection (DL) and quantification (QL) as 3 and 10 standard deviations (S.D.) of the calculated concentrations, respectively. Intermediate precision was calculated with the relative S.D. of concentrations calculated for quality control samples (5, 12 and 26 ng/ml *N*-Ac-5-Cys-HHMA or *N*-Ac-5-Cys-HHA), and the interassay accuracy was the relative error of the calculated concentrations. Five replicates at three different concentrations of *N*-Ac-5-Cys-HHMA or *N*-Ac-5-Cys-HHA (4, 7, 15, 20 and 30 ng/ml) spiked in blank urine were used for the determination of intra-assay precision (expressed as coefficient of variation for specific added target concentrations) and accuracy (expressed as percentage error of concentration found as compared with target added concentrations). Inter-day precision and accuracy were determined in three different experimental days. Analytical recoveries were calculated by

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comparison between peak areas of the calibration samples analyzed with the normal procedure and those obtained after adding the same amounts of reference substances and IS to blank urine after extraction. Recoveries were analyzed at three different concentrations, 4, 15 and 30 ng/ml, using four replicates for each evaluated concentration.

The stability of *N*-Ac-5-Cys-HHMA and *N*-Ac-5-Cys-HHA in urine was evaluated in two freeze/thaw cycles. The test involved a comparison of replicate stability samples, which had been frozen and thawed two times with a fresh urine sample that had not been frozen.

Results

Sample Preparation

Since the catecholamine-like properties of HHMA and HHA conjugates makes them very unstable and sensitive to oxidation and decomposition, the whole process from sample collection to HPLC-MS/MS determination was performed as quickly as possible, keeping samples cold and protected from light during the entire procedure. Moreover, addition of preservatives was necessary to prevent oxidation. More precisely, EDTA prevents catalytic oxidation by metal ions, whereas SMBS acts as a reducing agent. Since these two preservatives interfere with our solid-phase extraction process, we utilized 20mM DTT as a preservative during the extraction and we added EDTA and SMBS to the samples after the extraction to further protect the analytes from oxidation during the remainder of the analysis.

Phenylboronic acid (PBA) has proven to be especially effective in the isolation of catecholamines from biological fluids since boronate groups have a high specificity for cis-diol containing compounds like catechols. We chose Varian Bond Elut PBA columns for SPE extraction, these columns are packed with phenylboronic acid covalently linked to a silica gel surface. The specificity of this sample preparation method is based on the difference in affinity for PBA between the catecholamines and potentially interfering compounds present in the sample matrix.

LC-MS/MS Analysis

The analytes were separated on an Atlantis T3 column which produces good separation of small polar compounds with good retention and selectivity. Quantitation by ESI-MS was performed in MRM mode to enhance sensitivity and precision, 3 fragment ions of high abundance were used for the detection of each compound. The full scan MS/MS spectra as well as the proposed fragmentation patterns for *N*-Ac-5-Cys-HHMA, N-Ac-5-Cys-HHA and *N*-Ac-5-Cys-*O*-Me-HHMA are shown in Fig. 2. A representative multiple reaction monitoring (MRM) chromatogram of the second lowest calibrator sample (7 ng/ml *N*-Ac-5-Cys-HHMA and *N*-Ac-5-Cys-HHA; and 5 ng/ml IS) is shown in Fig. 3. Quantitation was carried out by comparison of peak area ratio (analyte versus IS) with calibration curves obtained with spiked calibrators. We used the methyl ester analog of *N*-Ac-5-Cys-HHMA (*N*-Ac-5-Cys-*O*-Me-HHMA) as IS for both the HHMA and HHA adducts since these two compounds are structurally very similar, and adding the methyl ester analog of *N*-Ac-5-Cys-HHA as an IS would only add more complexity to the separation of these structurally similar compounds, without improving quantitation.

Method Validation

Standard curve plots for the analytes were linear in the range of tested concentrations (4-30 ng/ml). Intra- and inter-assay accuracy and precision results satisfactorily met current acceptance criteria in the validation of bioanalytical methods (see supplementary materials). Analytical recoveries (80.5% for *N*-Ac-5-Cys-HMA and 90.5% for *N*-Ac-5-Cys-HHMA) and calculated limits of detection and quantification (*N*-Ac-5-Cys-HMA 1.5 and 4.3 ng/ml; *N*-Ac-5-Cys-HHMA 4.3 and 12.9 ng/ml) were considered adequate for the purpose of the study.

The freeze/thaw stability test showed that neither *N*-Ac-5-Cys-HHMA, nor *N*-Ac-5-Cys-HHA were stable in urine during freeze/thaw cycles (data not shown).

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Analysis of *N*-Ac-5-Cys-HHMA and *N*-Ac-5-Cys-HHA in human urine after MDMA consumption

The optimized LC-MS/MS analysis was applied to urine samples from healthy recreational users of MDMA, who were given a single 1.5 mg/kg oral dose of MDMA. Urine samples were collected before and after drug administration at 0 and 0 to 4 hrs time period. Figure 4 shows MRM chromatograms of urine from a volunteer before (t=0) and 0-4 h after MDMA intake. Estimated adduct concentrations for this volunteer were 2.1 ng/ml and 7.2 ng/ml for *N*-Ac-5-Cys-HHA and *N*-Ac-5-Cys-HHMA respectively.

MDMA, MDA, HMMA and HMA were also determined in urine. Tables 1 and 2 and Fig. 5 summarizes urinary excretion of MDMA and its metabolites, including thioether adducts of HHMA and HHA.

There is a significant correlation between NAC-HHMA recovered in urine and the composite parameters MDMA-HMMA (ratio of urinary recoveries, 0-4 h, r=-7.27, $p<0.003$, n=14) (Fig 5a), MDA-HMA (ratio of urinary recoveries, 0-4 h, r=-5.69, $p<0.034$, n=14) but not with MDMA or HMMA taken alone.

The recovery of NAC-HHMA, was related marginally to the CYP2D6 score ($p<0.1$) (Fig 5c) and to the COMT genotype ($p<0.1$) (Fig 5b) of subjects. The recovery of NAC-HHMA was two-fold higher among met/met subjects when compared with the value for the val/val subjects (0.091±0.005 vs. 0.041±0.003 μmols/4h, n=4 for each genotype). A similar trend was observed for MDMA-HMMA and COMT genotype.

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Discussion

We have, for the first time in humans, identified and quantified catechol-thioether metabolites of MDMA (*N*-Ac-5-Cys-HHMA and *N*-Ac-5-Cys-HHA). The identification of MDMA mercapturates lends credence to the hypothesis that the metabolic bioactivation of MDMA has the potential to contribute to MDMA neurotoxicity in humans. Preliminary data on polymorphisms of genes involved in the metabolism of MDMA reveal that specific genotypic profiles may constitute risk factors for the development of neurotoxicity.

The fraction of the MDMA dose recovered as thioether adducts excreted in urine 4 hours after MDMA ingestion is ~0.002 %. About 1.6% of a dose of MDA (23 μmol; sc) is excreted in bile as 5-(glutathion-*S*-yl)-α-MeDA, within 5 h. This translates to ~50% of the dose of MDA that causes both neurobehavioral and neurochemical changes (Miller et al., 1996). Moreover, because polyphenolic-GSH conjugates and the corresponding cysteine and NAC conjugates are biologically reactive, quantitation of their biliary and urinary excretion likely represents a minimum estimate of *in vivo* formation. Indeed, following administration of 2-hydroxy-1-(glutathion-*S*-yl)-17β-estradiol to rats, only 15% of the dose was recovered in urine, and 5% in feces, several days after administration (Elce, 1972), and up to 96% of an infused dose of polyphenolic-GSH conjugates is retained in the animal in the *in situ* perfused rat kidney model (Rivera et al., 1994; Hill et al., 1994). These findings emphasize that quantitation of *N*-Ac-5-Cys-HHMA and *N*-Ac-5-Cys-HHA in urine likely underestimates the contribution of this metabolic pathway to MDMA disposition. It is also relevant to note that these metabolites are extremely potent serotonergic toxicants (Miller et al., 1997; Bai et al., 1999; Jones et al., 2005). As little as 7 nmol of *N*-Ac-5-Cys-HHMA is sufficient to produce decreases in striatal and cortical 5-HT concentrations when injected directly into the brain (Jones et al., 2005). Finally, multiple doses of MDMA result in the accumulation of *N*-Ac-5-Cys-HHMA and *N*-Ac-5-Cys-HHA in rat brain (Erives et al., 2008). Thus, the fraction of MDMA *excreted* in urine needs to be considered in the

context of the fraction of MDMA *converted* to *N*-Ac-5-Cys-HHMA and *N*-Ac-5-Cys-HHA in vivo, in combination with the neurotoxic potency of these metabolites.

The 0-4 h time period for the collection of urine samples was selected on the premise that CYP2D6 would remain active during this time period, and prior to complete inactivation. The low urinary recovery during the first 4 hours post ingestion could also be explained by the accumulation of these metabolites in the brain, as shown in rats (Erives et al., 2008), and by the fact that the kinetics of mercapturate formation and excretion is unknown, with larger recoveries possible over extended urine collection periods.

The fraction of the catechol metabolites converted to neurotoxic metabolites varies greatly between individuals exposed to similar doses of MDMA (Tables 1 and 2). This finding is somewhat expected, since the enzymes that participate in the formation (CYP2D6) and inactivation (COMT) of HHMA and HMA are highly polymorphic in the human population (de la Torre et al., 2004b). There is a significant correlation between *N*-Ac-5-Cys-HHMA recovered in urine and the ratio of MDMA:HMMA but not with MDMA or HHMA taken alone. Again, this is not unexpected since the formation of the *N*-Ac-5-Cys adduct depends on the availability of the intermediary catechol metabolite, which in turn is to some extent a balance between it's generation via *O*-demethylenation of MDMA (CYP2D6) and it's removal via COMT-mediated *O*-methylation to HMMA. In concordance with this view, the recovery of *N*-Ac-5-Cys-HHMA is also moderately correlated to the CYP2D6 score and the COMT genotype of subjects ($p<0.1$). All the subjects exhibited the same CYP2D6 phenotype (extensive metabolizers). The extensive metabolizer phenotype is explained by the combination of several allelic variants displaying different degrees of functionality (see Table 1, 2 and Figure 5c). In the present study the relevance of the CYP2D6 genetic polymorphism on *N*-Ac-5-Cys- adduct formation can only be partially delineated on the basis of different activity rates within each particular genotype. It would be necessary to include more extreme phenotypes (poor metabolizers, ultra rapid

metabolizers) to fully assess the contribution of the CYP2D6 polymorphism to *N*-Ac-5-Cys- adducts formation. However, the effect of genetic polymorphisms in CYP2D6 on thioether catechol adduct formation, and subsequent neurotoxicity, is likely to be moderate and possibly only relevant during the first hours post MDMA ingestion. This is because the majority of hepatic CYP2D6 is inactivated within an hour after a recreational dose of MDMA (Yang et al., 2006; O´Mathúna et al., 2008), and that when CYP2D6 is inhibited prior to MDMA intake with paroxetine, a potent mechanism based inactivator of this enzyme (Bertelsen et al., 2003), only a 30% increase of the AUC of MDMA in plasma is observed (Segura et al., 2005). Prolonging the urine collection after MDMA intake would assist in assessing the correlation (or lack thereof) between CYP2D6 polymorphism and *N*-Ac-5-Cys- adducts formation.

Considering the pattern of MDMA consumption often involves repeated doses, and that CYP2D6 MDMA induced inactivation gives rise to a phenomenon of phenocopying that converts subjects towards a more poor metabolizer phenotype, independent of their original genotype (O´Mathúna et al., 2008), we would expect genetic polymorphisms and activity of COMT to be a more important determinant of NAC adduct formation, and of susceptibility to MDMA-mediated neurotoxicity. Consistent with this view, administration of the COMT inhibitor entacapone 30 min prior to MDMA dosing to rats exacerbates the 5-HT depletion produced by MDMA, an effect not related neither to changes on core body temperature, since the MDMA induced hyperthermic response was not significantly altered, nor to the serum L-tyrosine levels, which where higher on the MDMA treatment rats than on the MDMA/entacapone treated ones. Suggesting that toxic MDMA catechol metabolites are responsible for the MDMA toxicity on the entacapone treated- rats (Goñi-Allo et al., 2008a). Moreover, while the HMMA and HMA plasma concentration were significantly lower in the entacapone-treated animals, plasma concentrations of HHMA and HHA were unchanged, suggesting that these compounds are cleared by alternative pathways (Goñi-Allo et al., 2008b), for example, thioether adduct formation. Taken together, these results supports the idea that COMT activity level may be relevant in terms of susceptibility to MDMA neurotoxicity.

The observed gender differences in the urinary excretion of MDMA and its metabolites can be

partly explained by the more rapid renal process in males, including clearance of drugs metabolized by CYP2D6 (Schawrtz et al., 2003). Further studies with female subjects are needed to draw any further conclusions on gender differences in MDMA metabolism. The urinary excretion of the catechols HHMA and HHA need to be estimated to determine whether there are gender differences in the fraction of these metabolites that are converted to thioether adducts.

In summary, we report the first published data on the urinary excretion of catechol-thioether metabolites of MDMA. The neurotoxicity of these metabolites is well established in rats. Since the metabolic pathways of MDMA in humans are similar to rats, with differences only in the relative kinetics of metabolism (21), it is likely that these metabolites are also present in human brain, and with the potential to achieve neurotoxic concentrations. The dosed administrated in this study (1.5 mg/kg, 75-100 mg) represents a typicall recreational single dose (Parrot et al, 2002), although it is not well established whether a single recreational dose is likely to produce long term serotonergic deficits in humans, it can be expected that multiple or regular use of MDMA in humans (the typical pattern of MDMA consumption) may lead to long-term serotonergic damage similar to that seen in animal studies. Thus, catechol-thioether metabolites of MDMA may contribute to MDMA neurotoxicity in humans. Factors that influence inter-individual differences in the formation of these adduct will be major determinants of the susceptibility of humans to MDMA neurotoxicity. In this respect, although much attention has been focused on the CYP2D6 polymorphism with respect to the generation of thioether adducts and neurotoxicity, polymorphisms in COMT appear to be more relevant. A more detailed examination on the influence of COMT polymorphism on NAC adducts formation is warranted. An assessment of thioether adducts in human plasma would also assist in understanding their pharmacokinetics, which would also be of relevance to a possible prediction of a neurotoxic response, especially in view of the fact that a critical threshold concentration of neurotoxic metabolites appears necessary to produce a permanent neurotoxic effect (5). The extent of metabolic bioactivation, modulated by environmental and genetic factors may be major a contributing susceptibility factor to MDMA neurotoxicity.

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Footnotes

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Figure Legends

Fig.1: Pathways involved in the metabolic disposition of MDMA in humans (information obtained in part from Maurer, H., et al., 2000). Major pathways are outlined with thicker lines.

Fig. 2: Full scan MS/MS spectra and the proposed patterns of fragmentation for (A) *N*-Ac-5-Cys-HHMA, (B) **N**-Ac-5-Cys-*O*-Me-HHMA (IS) and (C) *N*-Ac-5-Cys-HHA. Fragment ions used for detection and quantification of each compound are highlighted in grey

Fig. 3: LC-MS/MS chromatograms of (A) blank human urine, (B) human urine spiked with *N*-Ac-5-Cys-HHMA and *N*-Ac-5-Cys-HHA, 7 ng/ml each, and 5 ng/ml of IS

Fig. 4: LC-MS/MS chromatograms of urine samples from (A) a volunteer prior to MDMA intake (t=0), and (B) a 0-4 h sample from a volunteer ingesting 80 mg (*R*,*S*)-MDMA

Fig. 5: Correlation of COMT genotypes and CYP (2D6) scores with urinary *N*-Ac-5-Cys-HHMA excretion.

CYP score 1= subjects heterozygous for a wild type allele (*1 or *2) and a non-functional allele (*4), CYP score 2 = subjects bearing alleles partially functional (*9,*10 or *41), CYP score 3 = homozygous wild type (*1,*2 or *35).

Bars show means. Error bars show mean +/- 1.0 SE.

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Table 1: Urinary excretion of MDMA and its metabolites (Males)

				µmol excreted 0-4h (Male)						
Vol. code	**CYP2D6 genotype**	**MDMA dose(mg)**	**mg/kg dose**	**MDMA**	**MDA**	**HMMA**	**HMA**	***N*-Ac-5-Cys-HHMA**	***N*-Ac-5-Cys-HHA**	**Total**
11	*1/*4	90	1.4	33.7	0.94	9.9	1.1	0.0016	0.0002	36.8
12	*1/*10	80	1.5	12.8	0.65	12.3	1.1	0.0041	ND	21.652
13	*1/*2	100	1.4	9.4	0.75	26.3	1.5	0.0062	ND	14.161
14	*1/*9	100	1.1	4.5	0.37	6.2	0.77	0.0163	0.0040	7.082
15	*2/*41	90	1.5	9.7	0.76	7.1	1.4	ND	0.0012	8.684
16	*1/*2	100	1.4	3.3	0.58	19.9	1.3	0.0102	0.0025	8.973
18	*9/*10	100	1.2	2.7	0.30	2.9	0.52	0.0022	0.0017	4.117
20	*2/*4	100	1.4	25.1	0.67	11.5	0.51	0.0010	0.0004	28.473
21	*1/*2	100	1.5	10.9	0.50	16.5	0.95	0.0160	0.0036	23.680
22	*1/*2	100	1.5	45.2	2.25	9.8	1.7	0.0012	0.0028	54.293
23	*1/*2	80	1.4	68.3	3.04	16.8	3.5	0.0031	0.0055	90.568
			mean	20.5	0.98	12.66	1.3	0.006	0.002	53.952
			STD	6.29	0.25	2.03	0.2	0.002	0.001	8.826
		Recovery as % of the dose		**3.96**	**0.19**	**2.45**	**0.25**	**0.0012**	**0.0005**	**10.426**

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Table 2: Urinary excretion of MDMA and its metabolites (Females)

				µmol excreted 0-4h (Male)						
Vol. code	**CYP2D6 genotype**	**MDMA dose(mg)**	**mg/kg dose**	**MDMA**	**MDA**	**HMMA**	**HMA**	***N*-Ac-5-Cys-HHMA**	***N*-Ac-5-Cys-HHA**	**Total**
17	*2/*10	80	1.3	46.4	1.64	16.34	0.93	0.0042	0.0013	33.670
19	*1/*2	75	1.5	19.7	0.96	32.93	1.8	0.0088	0.0018	19.111
26	*1/*10	75	1.5	15.5	1.35	38.92	2.2	0.0049	0.0015	22.073
27	*1/*35	75	1.4	7.9	0.49	16.86	1.1	0.0091	0.0019	29.803
			mean	22.4	1.11	26.26	1.53	0.007	0.002	51.297
			STD	8.4	0.25	5.71	0.32	0.001	0.0002	14.654
			Recovery as % of the dose	**4.33**	**0.21**	**5.07**	**0.30**	**0.0013**	**0.0003**	**9.913**

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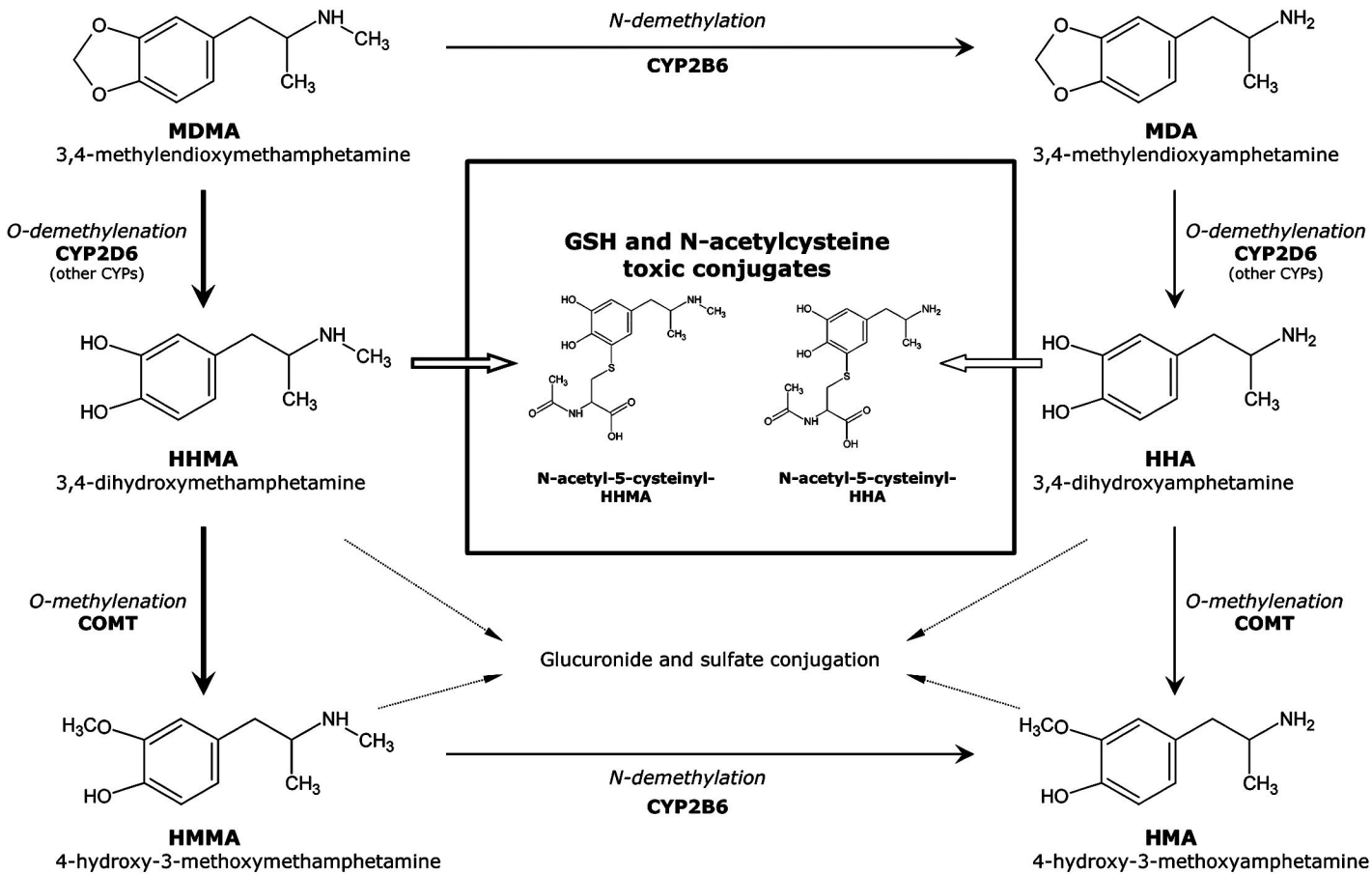

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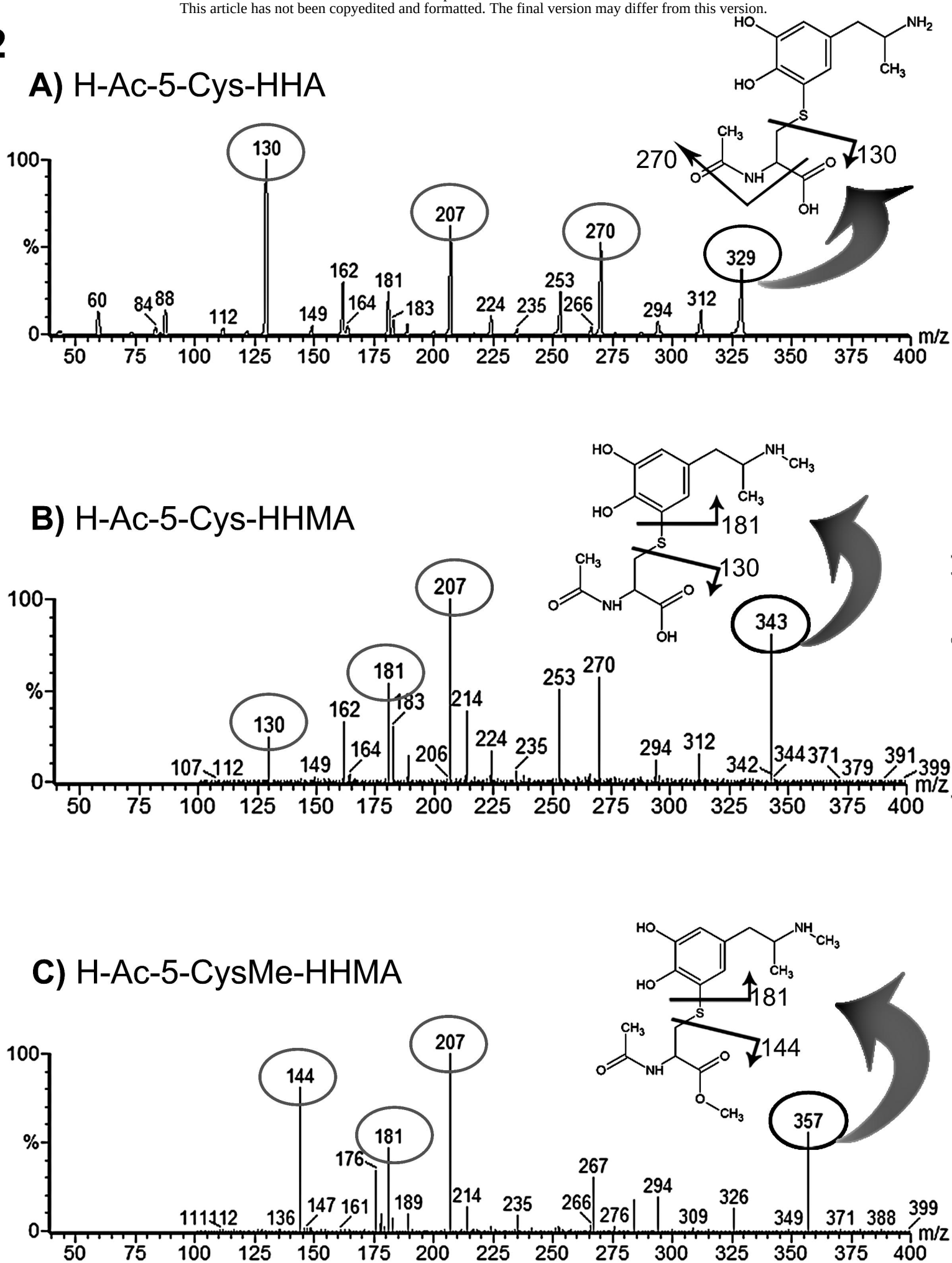

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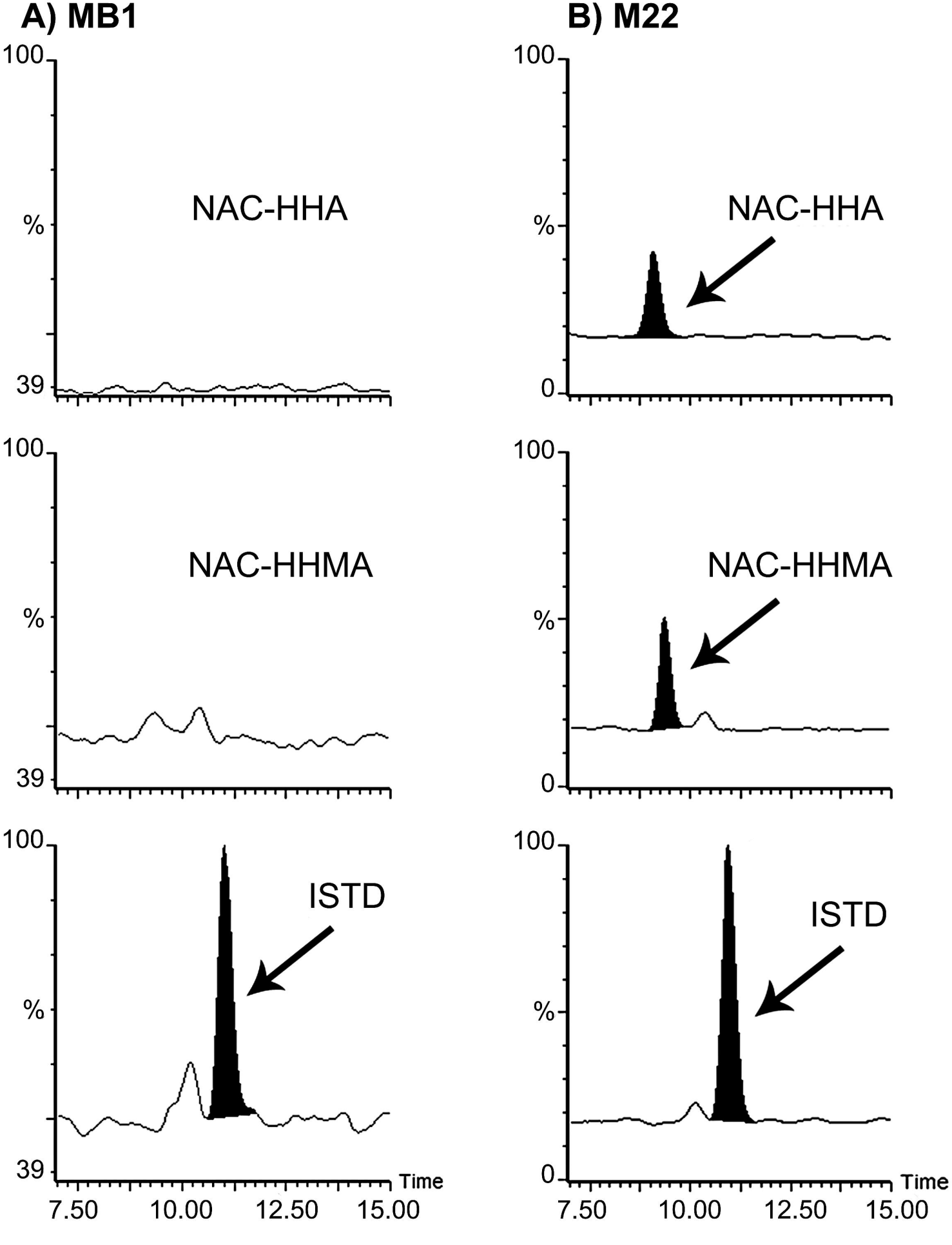

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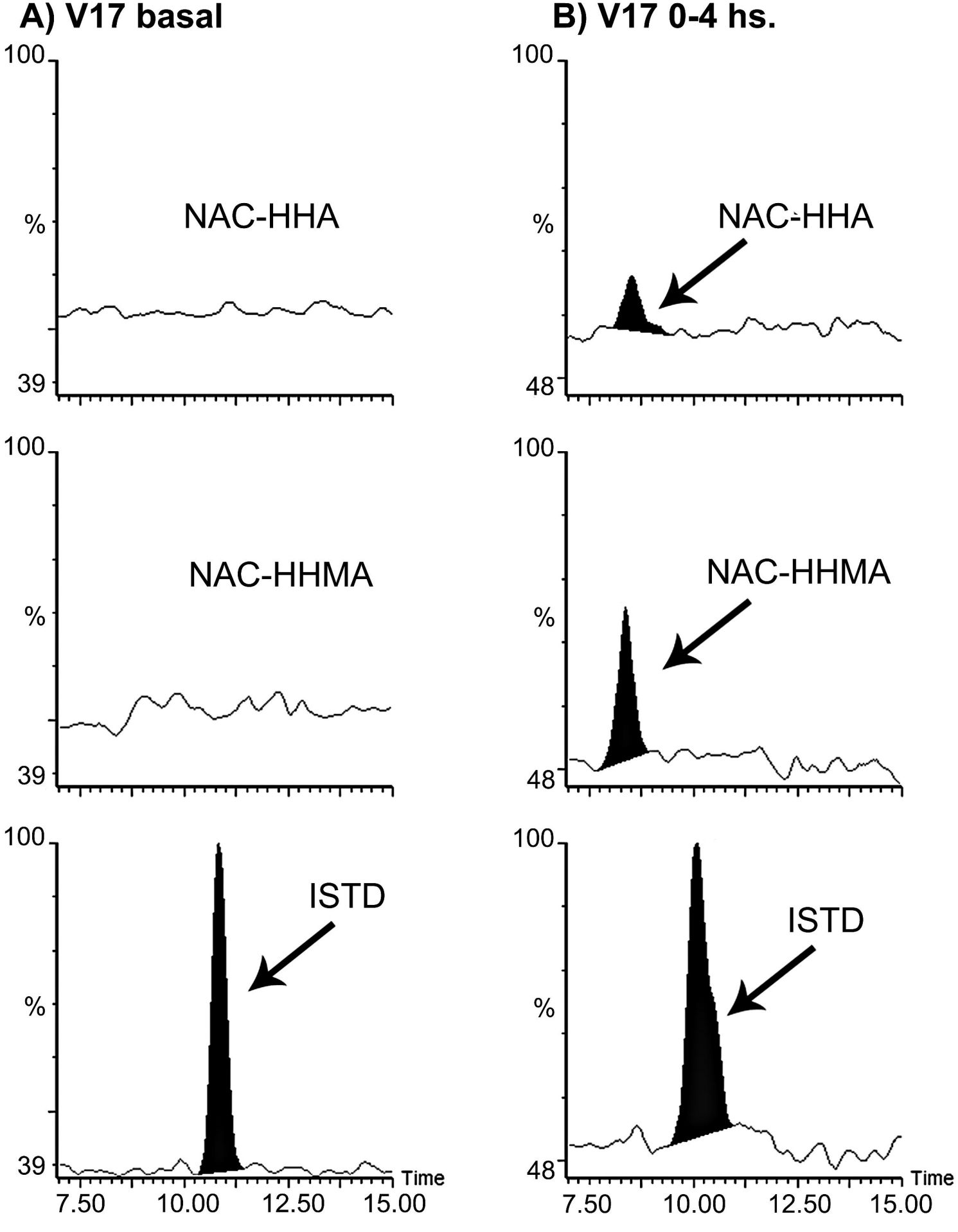

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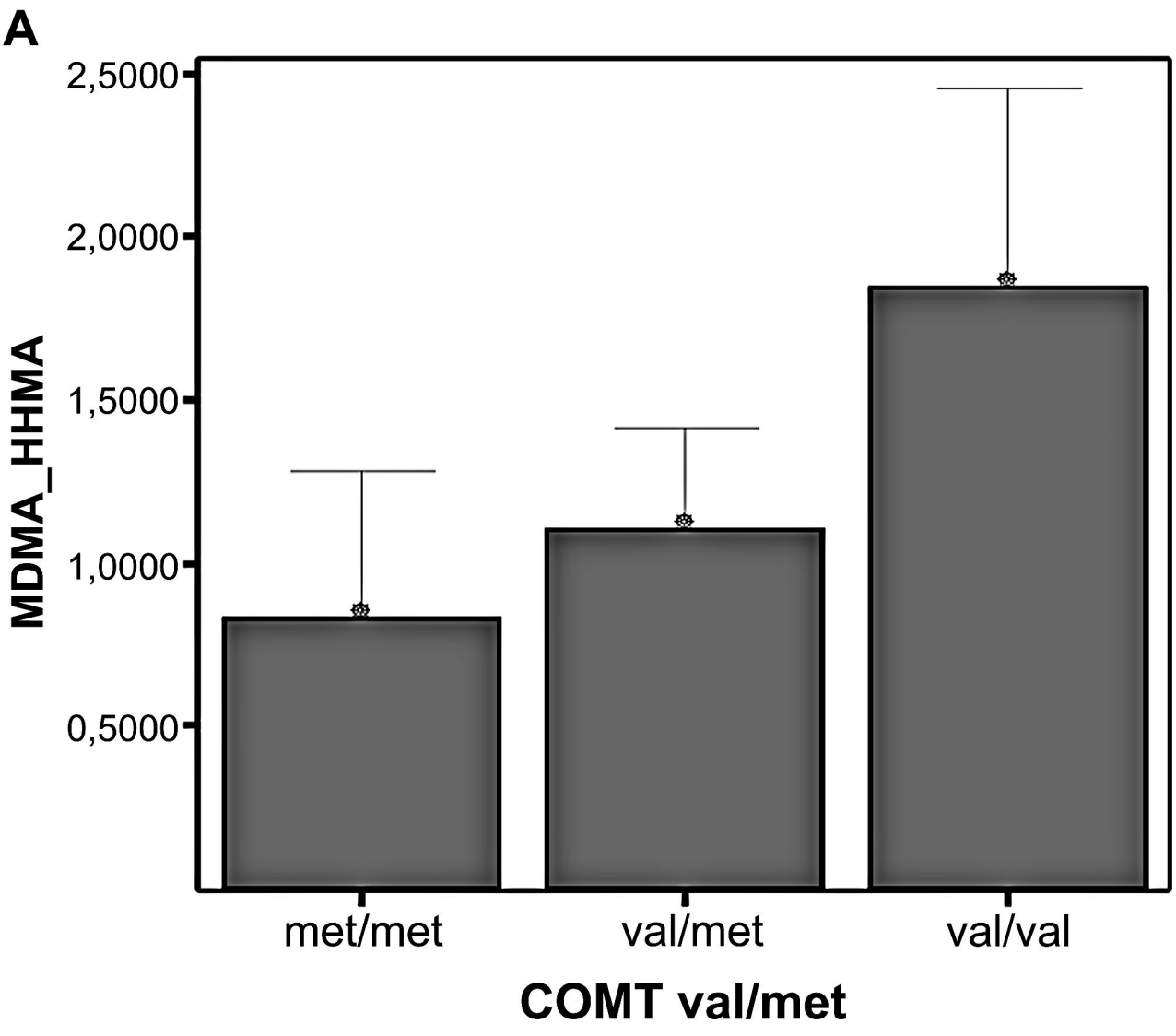

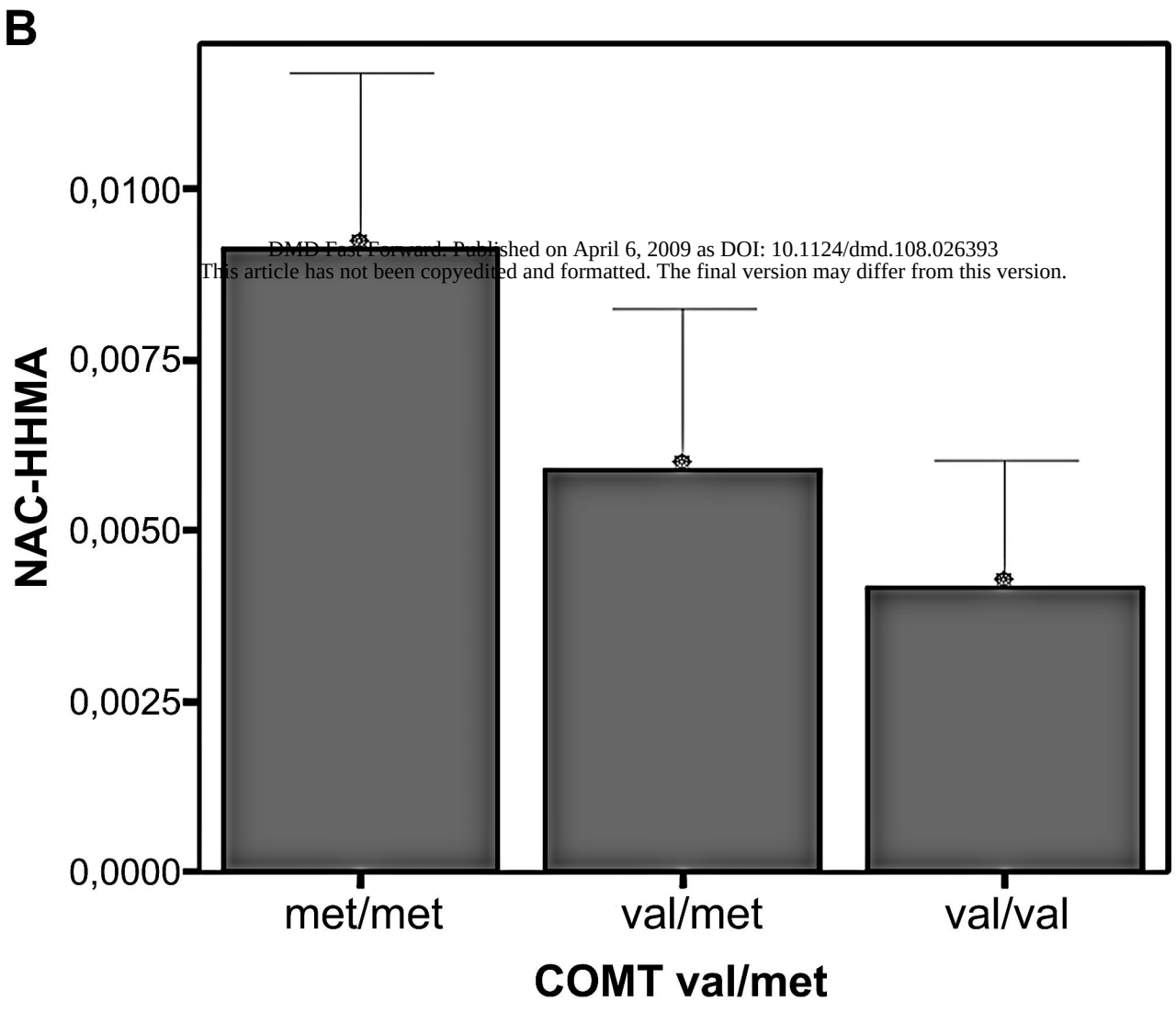

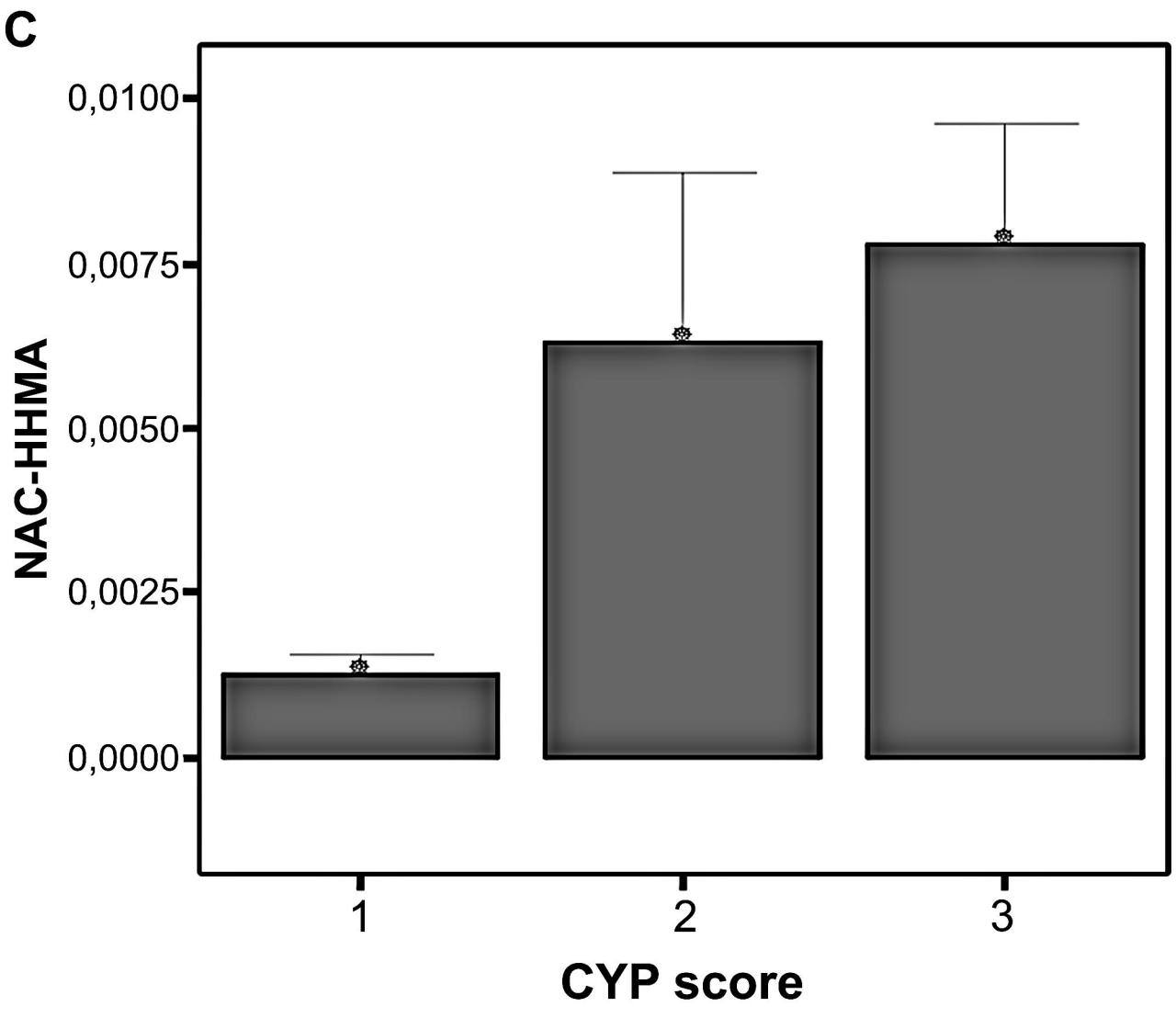

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