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3,4-DEHYDRODEBRISOQUINE, A NOVEL DEBRISOQUINE METABOLITE FORMED FROM 4-HYDROXYDEBRISOQUINE THAT IMPACTS THE CYP2D6 METABOLIC RATIO

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Running title: 3,4-Dehydrodebrisoquine impacts the CYP2D6 metabolic ratio

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ABBREVIATIONS: EM, extensive metabolizer; ftp, file transfer protocol; IM, intermediate metabolizer; P450, cytochrome P450; PM, poor metabolizer; UM, ultrarapid metabolizer; NS, not statistically significant; GCMS, gas chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; UPLC-TOFMS, ultra-performance liquid chromatography-coupled time-of-flight mass spectrometry; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy

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Abstract

Considerable unexplained inter-subject variability in the debrisoquine metabolic ratio (urinary debrisoquine/4-hydroxydebrisoquine) exists within individual *CYP2D6* genotypes. We speculated that debrisoquine was converted to as yet undisclosed metabolites. Thirteen healthy young volunteers, nine *CYP2D6*1* homozygotes (EMs) and four *CYP2D6*4* homozygotes (PMs) took 12.8 mg debrisoquine hemisulfate by mouth and collected 0-8 and 8-24 h urines, which were analyzed by GCMS before and after treatment with β -glucuronidase. Authentic 3,4-dehydrodebrisoquine was synthesized and characterized by GCMS, LC-MS/MS and ^1H NMR. 3,4-Dehydrodebrisoquine is a novel metabolite of debrisoquine excreted variably in 0-24 h urine, both in EMs (3.1-27.6% dose) and PMs (0-2.1% dose). This metabolite is produced from 4-hydroxydebrisoquine *in vitro* by human and rat liver microsomes. A previously unstudied *CYP2D6*1* homozygote was administered 10.2 mg 4-hydroxydebrisoquine orally and also excreted 3,4-dehydrodebrisoquine. EMs excreted 6-hydroxydebrisoquine (0-4.8%), 8-hydroxydebrisoquine (0-1.3%) but these phenolic metabolites were not detected in PM urine. Debrisoquine and 4-hydroxydebrisoquine glucuronides were excreted in a highly genotype-dependent manner. A microsomal activity that probably does not involve cytochrome P450 participates in the further metabolism of 4-hydroxydebrisoquine, which we speculate may also lead to the formation of 1- and 3-hydroxy-debrisoquine and their ring-opened products. In conclusion, this study suggests that the traditional metabolic ratio is not a true measure of the debrisoquine 4-hydroxylation capacity of an individual and thus may, in part, explain the wide intragenotype variation in metabolic ratio.

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Debrisoquine (3,4-dihydro-2(1*H*)-isoquinoline carboxamidine) sulfate was patented in the US by Hoffmann-La Roche in 1964 (Wenner, 1964) and immediately went into clinical trials as an antihypertensive agent (Talbot, 1965; Rosendorff et al., 1968; Somers et al., 1968; Blechman et al., 1969). Surprisingly, at the time of its launch, little, if anything was known about the metabolic disposition of debrisoquine. Workers at Hoffmann-La Roche UK (Allen et al., 1975) reported on a study in which ^{14}C -labeled debrisoquine was administered to rats (50 mg/kg) and a single hypertensive patient (2.6 mg, on top of 15 mg q.d.s. therapeutic dose). Debrisoquine was excreted unchanged in urine of both Man and rat, together with 4-hydroxydebrisoquine as the major metabolite, and traces of the phenolic metabolites 5-, 6-, 7-, and 8-hydroxydebrisoquine. In addition, both rats and humans excrete 10-15% of the dose as two ring-opened acidic metabolites, presumed to arise from hydroxylation of debrisoquine in positions 1 and 3 (Fig 1A). The nature of these metabolites was subsequently confirmed (Allen et al., 1976; Eiermann et al., 1998). For the rat, 70% of the administered radioactivity could be accounted for as debrisoquine and these seven aforementioned metabolites. In the single patient that was studied, 66% of the radioactivity was similarly accounted for (Allen et al., 1975).

It was noted that there was considerable variation (7-fold) in the extent of urinary excretion of unchanged debrisoquine in ten volunteers given 40 mg debrisoquine sulfate by mouth and that urinary debrisoquine excretion correlated positively with hypotensive response to debrisoquine (Angelo et al., 1975). This same group performed a second ^{14}C -debrisoquine study in four human volunteers, for which 71.3-76.7% of the radioactivity was recovered in the urine within 24 h (Angelo et al., 1976). These authors identified debrisoquine and 4-hydroxydebrisoquine in human urine, and tentatively identified the phenolic metabolites, the carboxylic acid metabolites,

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as previously reported (Allen et al., 1975), together with a dihydroxy metabolite, which was later reported by another laboratory (Eiermann et al., 1998). Of interest, was the report that one of the subjects excreted 60.8% of the dose as debrisoquine and only 5.4% as 4-hydroxydebrisoquine. This would correspond to a metabolic ratio (Mahgoub et al., 1977) (MR) of $60.8/5.4 = 11.3$. Subsequent studies showed this volunteer to be a phenotypic poor metabolizer (PM) (Mahgoub et al., 1977). The other three subjects in this study had MR values of 0.5-1.4, typical of what is regarded as extensive metabolizers (EMs) (Mahgoub et al., 1977). This was perhaps the first report containing evidence of the existence of two drug oxidation phenotypes, EM and PM.

There were little further qualitative insights added to the debrisoquine metabolism knowledge base at that time, with the exception of a single ^{14}C -debrisoquine study (Idle et al., 1979). These authors confirmed the excretion of debrisoquine, 4-, 5-, 6-, 7-, and 8-hydroxydebrisoquine, and also reported that neither the debrisoquine urea derivative nor *N*-hydroxydebrisoquine (see Fig 1A) were excreted in human urine after administration by mouth of 40 mg debrisoquine sulfate (Idle et al., 1979). However, none of these early studies accounted for the chemical nature of all the debrisoquine metabolites.

Subsequently, debrisoquine 4-hydroxylation took on a special significance as the first drug oxidation pathway to display genetic polymorphism (Mahgoub et al., 1977). This reaction is mediated by the polymorphic cytochrome P450 CYP2D6 (Gonzalez et al., 1988), but CYP1A1 may also play a role (Granvil et al., 2002). A wide variety of allelic variants of *CYP2D6* give rise to four phenotypes for CYP2D6-mediated metabolism -- ultrarapid metabolizer (UM), extensive metabolizer (EM), intermediate metabolizer (IM) and poor metabolizer (PM)

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phenotypes, which have been estimated to occur in 5-10%, 65-80%, 10-15%, and 5-10% of populations, respectively (Zanger et al., 2004). In addition to the archetypal polymorphically metabolized substrates, debrisoquine and sparteine, CYP2D6 substrates include at least 50 common pharmaceutical agents (Zanger et al., 2004), together with four endogenous 5-methoxyindolethylamines (Yu et al., 2003b), and also progesterone and tyramine (Niwa et al., 2004). PMs have an impaired hydroxylation capacity and this may lead to adverse effects (Yu et al., 2004; Zanger et al., 2004). The current practise for the identification of PMs is to screen for common *CYP2D6* inactivating alleles using PCR (Muller et al., 2003; Schaeffeler et al., 2003) or microarray-based methods (Chou et al., 2003). However, genotypic analysis provides only a forecast of the extent of metabolism of such well-studied test substrates as debrisoquine and sparteine. Part of the problem is that the UM, EM, and IM phenotypes are both broad and overlapping. Despite this, individual MR values for debrisoquine and sparteine are stable over many years (Crothers et al., 1986; Zanger et al., 2004), and phenotype testing with debrisoquine or sparteine still provides a cheap, reliable and direct evaluation of the CYP2D6 metabolic phenotype.

The factors that contribute to the intra-phenotype variance in CYP2D6-mediated metabolism are poorly understood. For example, in the case of polymorphic debrisoquine 4-hydroxylation, most homozygous extensive metabolizers have MR values in the range 0.1-1.0, corresponding to 50-91% 4-hydroxylation of the drug (Daly et al., 1991). The occurrence of multiple variant *CYP2D6* alleles explains the broad inter-phenotype variation in debrisoquine 4-hydroxylation, but does not account for the wide intra-phenotype variation in metabolism. Other explanations must be sought for this variability and thus the metabolism of debrisoquine was re-examined in

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both genotypic EM and PM subjects. In this study, a novel human urinary metabolite of debrisoquine, 3,4-dehydrodebrisoquine, was uncovered. The data also indicate that both debrisoquine and 4-hydroxydebrisoquine undergo glucuronidation. These novel metabolites could have a significant impact upon the debrisoquine metabolic ratio.

Methods

Chemicals. Debrisoquine hemisulfate was obtained from Kolbjørn Zahlse (Trondheim, Norway), 7-methoxyguanoxan from Professor Ann K. Daly (Newcastle, UK), and 4-, 6-, and 8-hydroxydebrisoquine from Professor Urs Meyer (Basel, Switzerland). 1,1,1,5,5,5-hexafluoro-2,4-pentanedione (hexafluoroacetylacetone) was purchased from Sigma-Aldrich (St. Louis, MO). All solvents were of HPLC grade.

Subjects. The study was approved by the ethics committee of the General Faculty Hospital Prague and the Office of Human Subjects Research, NIH, and was performed according to Good Clinical Practice Guidelines. All volunteers gave their written informed consent to participate in the study. The volunteers comprised 14 healthy white Caucasians of Czech or Slovak nationality, primarily students of Charles University 1st Faculty of Medicine, and were aged 23.5 ± 1.0 years (mean \pm SEM). They were selected according to *CYP2D6* genotype and availability from a pool of 250 volunteers that had been previously genotyped as EM or PM by the determination of *CYP2D6* alleles *2, *3, *4, *5, *6, *7, *8, *9, *10, *11, *12, *13, *14, *15 and gene duplications (Sachse et al., 1997). They comprised nine *1/*1 EM subjects (6M, 3F) and four *4/*4 PM subjects (3M, 1F), with mean BMI (\pm SEM) of 23.4 ± 0.7 (M) and 20.5 ± 1.8 (F). All subjects were normotensive with mean (\pm SEM) sitting blood pressures of $123 \pm 2/79 \pm 2$ (M) and $115 \pm 4/69 \pm 4$ (F). The volunteers were nonsmokers, with the exception of one female EM who smoked 5 cigarettes per day and one male PM who smoked 10 cigarettes per day. Alcohol consumption was 0-3 units/week for males and 0-2 units/week for females. The volunteers were drug-free for the previous month, with the exception of one male EM who had taken 800 mg ibuprofen at -7 days and 10 mg zolpidem (a *CYP3A4* substrate (Pichard et al., 1995)) at -10 days, and one female EM who had taken 1200 mg ibuprofen at -11/-12 days.

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Cigarette smoking, (Vincent-Viry et al., 2000) alcohol consumption, (Vincent-Viry et al., 2000) ibuprofen (Hamman et al., 1997) and zolpidem (von Moltke et al., 2002) administration are unlikely to alter debrisoquine metabolism by CYP2D6. However, the effect of these potential confounders on non-P450 metabolism is at present unknown.

Debrisoquine administration. After an overnight fast, each subject took a single tablet containing 12.8 mg debrisoquine hemisulfate, equivalent to 10 mg debrisoquine (gift of Professor Ann K. Daly, University of Newcastle, UK), taken from a newly opened bottle, from which 5 tablets had been taken, ground, suspended in water, shaken, centrifuged and assayed by GCMS (see below) to ensure the quantity and quality of their debrisoquine content. After a period of 2 h, volunteers were allowed food and soft drinks *ad libitum*. They collected bulked 0-8 and 8-24 h urines which were taken promptly to the Clinical Pharmacology Unit, General Teaching Hospital in Prague, measured, and 10 ml aliquots frozen at -20°C prior to transport of a single set of samples by courier on dry-ice to Bethesda for analysis. These samples were coded in such a way that they could not be identified in Bethesda with particular volunteers. The same urine samples were analyzed for creatinine excretion (mmol/24 h) and a blood sample taken for the determination of serum creatinine ($\mu\text{mol/L}$). Creatinine clearance (mL/s) and GFR (mL/s) were calculated using the Cockcroft-Gault formula (Cockcroft and Gault, 1976).

***In vitro* metabolism studies.** Long-term (24 h) incubations to obtain cytochrome P450 activity were carried out using pre-prepared human liver microsomes and rat liver microsomes under conditions we have previously described (Yu et al., 2003a). Both debrisoquine hemisulfate (7 $\mu\text{g/mL}$; 290 μM) and 4-hydroxydebrisoquine (20 $\mu\text{g/mL}$; 105 μM) were used as substrates. The

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incubations were assayed for their content of debrisoquine, 4-hydroxydebrisoquine and 3,4-dehydrodebrisoquine by GCMS with single ion extraction, as described below for urine samples.

Analysis of debrisoquine and its hydroxylated metabolites by gas chromatography-mass spectrometry (GCMS). The following calibration curves were constructed using blank human urine: debrisoquine hemisulfate and 4-hydroxydebrisoquine, from 0.2-20 $\mu\text{g/mL}$; 6- and 8-hydroxydebrisoquine, from 0.5-5.0 $\mu\text{g/mL}$. All urines were treated with β -glucuronidase to deconjugate phenolic metabolites, as follows: To urine (0.5 mL) was added 1M PBS (pH 4.8) and β -glucuronidase solution (54 μL ; Sigma-Aldrich, St. Louis, MO) to give a final pH of 5.0, and heated in a shaking water bath at 37°C for 24 h. Distilled water (0.44 mL) was then added and the samples with 1.00 mL volume were then treated like the diluted urine samples below. Standards and volunteer urines, with and without β -glucuronidase treatment, were treated with 1,1,1,5,5,5-hexafluoro-2,4-pentanedione (hexafluoroacetylacetone) (Sigma-Aldrich, St. Louis, MO) to create bis(trifluoromethyl)pyrimidine derivatives (Erdtmansky and Goehl, 1975) as follows: Duplicate urine samples or standards (0.5 mL) were diluted with distilled water (0.5 mL), unless already diluted after deconjugation, mixed with both saturated aqueous sodium hydrogen carbonate (0.2 mL) and internal standard solution (0.1 mL) containing 7 $\mu\text{g/mL}$ 7-methoxyguanoxan and then hexafluoroacetylacetone (200 μL) and HPLC grade toluene (1 mL) was added. The samples were heated in sealed septum-cap vials in a heating block at 90°C for 1 h, cooled, 1M sodium hydroxide (1 mL) added, vortexed for 10 s and centrifuged at 1800 g for 5 min. Upper organic layers were transferred to autoinjector vials and capped. Samples (1 μL) were injected in splitless mode from an Agilent 7683 Automatic Liquid Sampler into an Agilent 5890N Network GC system coupled to an Agilent 5973 Network Mass Selective Detector and a

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G1701 DA Enhanced ChemStation (Agilent, Palo Alto, CA). Chromatography was performed on a 0.25 μ m film thickness HP-5MS capillary column (28 m X 0.25 mm i.d.). Carrier gas was helium (linear velocity 39 cm/s) with a temperature program of 10 min at 120°C, then 10 deg/min to 250°C, then 20 deg/min to 300°C, then held at 300°C for 3 min (total run time 28.5 min). Injector and interface temperatures were 230 and 280°C, respectively. The mass spectrometer was operated in the EI mode (69.9eV). A mass range of 100-550 amu was scanned, only for the retention period of 16.8-24.0 min, and this permitted acquisition of 3.25 scans/s. Data sharing was accomplished by file transfer direct from the ChemStation to an ftp server in Prague, where the appropriate analytical software was located. Peaks were identified from the retention times and mass spectra of authentic standards. For quantitation, peak area ratios (ratio of peak area of analyte/peak area of internal standard) were calculated for all peaks of interest using single ion extracted chromatograms with the following ions: debrisroquine (347 m/z), 4-hydroxydebrisoquine (344 m/z), 3,4-dehydrodebrisoquine (344 m/z), 6-hydroxydebrisoquine (362 m/z), 8-hydroxydebrisoquine (362 m/z), and 7-methoxyguanoxan (internal standard) (379 m/z).

Analysis of conjugated metabolites by ultra-performance liquid chromatography-coupled time-of-flight mass spectroscopy (UPLC-TOF MS). Urine samples were mixed with the equal volume of acetonitrile and centrifuged at 14,000 rpm to remove particles and protein. A 200 μ L aliquot of the supernatant was transferred to an auto-sampler vial for UPLC-TOFMS analysis (Wilson et al., 2005). Urine samples (5 μ L/injection) were separated on a 50 X 2.1 mm ACQUITYTM 1.7 μ m C₁₈ column (Waters Corp, Milford, USA) using an ACQUITYTM UPLC system (Waters) with a gradient mobile phase comprised of A = 0.1% formic acid and B =

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acetonitrile containing 0.1% formic acid. A 0.6 mL min⁻¹ flow rate was maintained in a 10-min run. The eluent was directly introduced into the mass spectrometer. Mass spectrometry was performed on a Waters Q-TOF PremierTM operating in positive ion mode. The desolvation gas flow was set to 600 L h⁻¹ at a temperature of 350 °C with the cone gas set to 50 L h⁻¹ and the source temperature set to 120 °C. The capillary voltage and the cone voltage were set to 3000 and 60 V, respectively. Leucine-enkephalin was used as the lock mass (*m/z* 556.2771) for accurate mass measurement. In MS scanning, data were acquired in centroid mode from 100–950 *m/z*. As for MS/MS fragmentation of target ions, collision energy ranging from 15–30 V was applied. Following data acquisition, UPLC-MS chromatograms and spectra were further analyzed by MassLynx application software (Waters).

Synthesis and characterization of 3,4-dehydrodebrisoquine. 4-Hydroxydebrisoquine (6 mg; 31.4 μmol) was added to 18M sulfuric acid (100 μL; 1.8 mmol) and dimethylsulfoxide (200 μL) in a screw-topped vial and the mixture vortexed and then heated at 90°C for 20 min. An aliquot was taken, neutralized with saturated sodium hydrogen carbonate and derivatized with hexafluoroacetylacetone, as described above. GCMS analysis established the absence of 4-hydroxydebrisoquine at 19.60 min, but the appearance of a single large peak at 17.81 min (see Results). These observations were taken as evidence of near 100% conversion of 4-hydroxydebrisoquine to 3,4-dehydrodebrisoquine. The resulting reaction product was diluted and used to construct a calibration curve for 3,4-dehydrodebrisoquine from 0.46–4.6 μg/mL. For purposes of authentication of synthetic 3,4-dehydrodebrisoquine, iterative experiments were performed with 4-hydroxydebrisoquine and 18M sulfuric acid alone, and the products monitored by GCMS after derivatization with hexafluoroacetylacetone, in order to determine the best

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conditions for the isolation of solid product for further analysis. It was determined that 4-hydroxydebrisoquine (50 mg; 262 μ mol), when reacted with 1 ml of 18M sulfuric acid (18 mmol) at 35-40°C for 5 min, in the absence of DMSO, yielded sufficient off-white product (20 mg; 44% yield) after neutralization with saturated sodium hydrogen carbonate and sample clean-up using SepPak C18 cartridges (Waters Corp, Milford, MA). The methanol eluates were combined and reduced to dryness in a Speed Vac evaporating centrifuge (Savant Instruments, Farmingdale, NY). The resulting solid material was subjected to LC-MS/MS analysis as described above, and to ^1H NMR spectroscopy in D_2O using a Bruker Avance DPX 300 spectrometer.

Statistical Analyses. The 0-8h, 8-24h, and 0-24h urinary excretion of debrisoquine and its metabolites was expressed as mean \pm SEM. Interphenotype differences in the excretion of metabolites were evaluated statistically using the Mann-Whitney U test with one-tailed P values.

Results

Synthesis and characterization of 2(1*H*)-isoquinoline carboxamide (3,4-dehydrodebrisoquine). A typical single ion chromatogram (344 *m/z*) for the diluted and neutralized product of the reaction between 4-hydroxydebrisoquine and 18M sulfuric acid is displayed in Fig 2A, together with its mass spectrum (Fig 2B). A single large peak at 17.81 min was also observed in the total ion chromatogram with complete absence was of 4-hydroxydebrisoquine at 19.60 min, whose mass spectrum also contains a large 344 *m/z* ion (data not shown). These data were taken as evidence that 4-hydroxydebrisoquine had been quantitatively dehydrated under the conditions of the experiment. This product should be formed readily due to the resonance of the 3,4-double bond with both the aromatic ring and the guanidine nitrogen lone electron pairs. This mass spectrum (Fig 2B) is indicative of formation of 3,4-dehydrodebrisoquine. Moreover, the ¹H NMR spectrum of crude 3,4-dehydrodebrisoquine revealed two 7.6 Hz doublets at 6.5 and 6.2 ppm. A two-dimensional correlation spectroscopy (COSY) experiment confirmed that the two signals were coupled (Figure 2C). The chemical shifts, coupling constants and lack of further connectivity were consistent with protons occupying an isolated and disubstituted *cis* double bond such as that in 3,4-dehydrodebrisoquine. The chemical shifts are not consistent with H-3 and H-4 of a fully oxidized isoquinoline derivative which are expected to resonate further downfield. Furthermore, the ¹H NMR spectrum of the 4-hydroxydebrisoquine starting material showed no impurities and no signals between 4.7 and 7 ppm. Hence, the only plausible explanation for the coupled signals at 6.5 and 6.2 ppm is the formation of 3,4-dehydrodebrisoquine by the acid catalyzed dehydration of 4-hydroxydebrisoquine. In addition, the synthetic material yielded a mass spectrum by LC-MS/MS with a protonated molecular ion of mass 174.1043 (Fig 2D) that corresponded to an

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empirical formula of $C_{10}H_{12}N_3$ with an error of 5.1 ppm. In addition, two fragment ions of masses 157.0774 and 132.0780 were observed, that correspond to the neutral losses of ammonia and cyanamide, respectively. Finally, a fragment ion of mass 115.0692 was observed, corresponding to the neutral loss of ammonia from 132.0780 (Fig 2D). Taken together with the NMR evidence, these findings establish unequivocally that the synthetic material, that produces an ion of 344 m/z by GCMS, is 3,4-dehydrodebrisoquine.

GCMS assay of debrisoquine, 4-, 6- and 8-hydroxy-debrisoquine, and 3,4-dehydrodebrisoquine in urine. It should be stated at the outset that the peak on GCMS that we have characterized as the derivative of 3,4-dehydrodebrisoquine was not observed when authentic 4-hydroxydebrisoquine, or any other authentic debrisoquine metabolite, was derivatized with hexafluoroacetylacetone, showing that it is not formed as an artifact in the derivatization reaction. This peak was only detected in two situations, either when biological samples were analyzed or after the dehydration of authentic 4-hydroxydebrisoquine with 18M sulfuric acid. Additionally, LC-MS/MS analysis of urine from an EM subject gave the mass spectrum shown in Fig 2E. The protonated molecular ion of mass 174.1035 corresponds to protonated 3,4-dehydrodebrisoquine ($C_{10}H_{12}N_3$) with an error of 2.3 ppm. The fragmentation pattern of this ion is virtually identical to that for the synthetic material (Fig 2D).

Calibration curves for debrisoquine (A; retention time 17.46 min) and 4-hydroxydebrisoquine (retention time 19.60 min) were determined. The correlation coefficients (r^2) were 0.999 for both debrisoquine and 4-hydroxydebrisoquine and limits of sensitivity for debrisoquine and 4-hydroxydebrisoquine were 0.02 $\mu\text{g/mL}$ and 0.05 $\mu\text{g/mL}$, respectively. Interassay variation was

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in the range 3-5%. Calibration curves for 8-hydroxydebrisoquine (retention time 20.39 min) and 6-hydroxydebrisoquine (retention time 20.56 min) were determined. Coefficients (r^2) were 0.974 and 0.918 for 6-hydroxydebrisoquine and 8-hydroxydebrisoquine, respectively and the limit of sensitivity for both compounds was 0.2 $\mu\text{g/mL}$. Interassay variation was in the range 5-10%. Calibration curve for 3,4-dehydrodebrisoquine (retention time 17.36 min), and an unknown peak (retention time 17.52 min) that was always present in solutions of 3,4-dehydrodebrisoquine, except immediately after synthesis were determined (Fig 2). This unknown compound had an almost identical mass spectrum to 3,4-dehydrodebrisoquine. As will be seen, it was present in urine samples. The nature of this unknown compound is dealt with below. The relative concentrations of Peaks A and B were determined from the relative total ion abundances for each peak. The correlation coefficients (r^2) for both 3,4-dehydrodebrisoquine and its unknown derivative were 0.996 and the limits of sensitivity for 3,4-dehydrodebrisoquine and its unknown derivative were 0.4 $\mu\text{g/mL}$ and 0.06 $\mu\text{g/mL}$, respectively.

The nature of the unknown derivative of 3,4-dehydrodebrisoquine. An extracted ion chromatogram (344 m/z), with two peaks running at 17.33 min (Peak A) and 17.48 min (Peak B) in a solution of 5 $\mu\text{g/mL}$ authentic 3,4-dehydrodebrisoquine derivatized with hexafluoroacetylacetone, is displayed in Fig 3. Peak A corresponds to 3,4-dehydrodebrisoquine, the difference in retention time to the synthetic material shown in Fig 2 due wholly to analysis on different GC columns. Peak B has an almost identical mass spectrum dominated by the M-1 transition (loss of H radical) shown in Fig 2. No type of isomerism is possible for 3,4-dehydrodebrisoquine, neither around the 3,4-double bond nor the heterocyclic nitrogen atom. Therefore, Peak B must represent a precursor of 3,4-dehydrodebrisoquine and we propose that it

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is 3-hydroxydebrisoquine formed by the mechanism shown in Fig 1B, specifically via the equilibrium A and the addition of OH⁻ in reaction B. This relatively unstable carbinolamine, 3-hydroxydebrisoquine, is expected to dehydrate to give 3,4-dehydrodebrisoquine in the mass spectrometer (as the respective bis(trifluoromethyl)pyrimidine derivatives) via reaction C. Indeed, this reaction occurs to some extent for 4-hydroxydebrisoquine itself, which also has a prominent ion at 344 m/z. Therefore, it would appear that a proportion of synthetic 3,4-dehydrodebrisoquine is converted to 3-hydroxydebrisoquine *in vitro*.

Determination of debrisoquine metabolism in human volunteers. First, the 0-8 h and 8-24 h excretion of debrisoquine, 4-, 6-, and 8-hydroxydebrisoquine was determined, together with the excretion of 3,4-dehydrodebrisoquine (sum of Peaks A and B, see above). These determinations were performed on neat urine and on urine treated with β -glucuronidase. Table 1 shows the 0-8 h urinary excretion of debrisoquine, 4-hydroxydebrisoquine, and 3,4-dehydrodebrisoquine without β -glucuronidase treatment. As expected, EMs excreted significantly more 4-hydroxydebrisoquine (20.9 ± 3.0 % dose) than PMs (0.5 ± 0.2 % dose; U=36.0, P=0.0014) and PMs excreted significantly more debrisoquine (33.9 ± 2.1 % dose) than EMs (8.3 ± 1.0 % dose; U=36.0, P=0.0014) in this traditional phenotyping period (Mahgoub et al., 1977). Moreover, all EMs excreted 3,4-dehydrodebrisoquine as a metabolite of debrisoquine in urine (7.8 ± 2.2 % dose) and 3 of 4 PMs also excreted this metabolite (0.9 ± 0.4 % dose; U=35.0, P=0.0028). Table 2 shows that this pattern of metabolite excretion was mirrored in the 8-24 h urines also. 4-Hydroxydebrisoquine excretion (% dose) was significantly greater in EMs (7.2 ± 1.4) than in PMs (0.2 ± 0.1 ; U=36.0, P=0.0014) and debrisoquine excretion (% dose) was significantly greater in PMs (19.5 ± 5.3) than in EMs (3.7 ± 0.9 ; U=36.0, P=0.0014). The excretion of 3,4-

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dehydrodebrisoquine in the 8-24 h urine was 2.5 ± 0.9 % dose for EMs. Only 1 of 4 PMs excreted this metabolite in the 8-24 h urine. The inter-phenotype difference was barely statistically significant ($U=31.0$, $P=0.025$). Finally, Table 3 shows that, for the combined 0-24 h urine, debrisoquine metabolism displayed clear polymorphism based upon the excretion of debrisoquine itself (12.0 ± 1.5 % dose in EMs, 53.3 ± 7.0 % dose in PMs; $U=36.0$, $P=0.0014$), 4-hydroxydebrisoquine (28.1 ± 3.4 % dose in EMs, 0.7 ± 0.3 % dose in PMs; $U=36.0$, $P=0.0014$), and indeed for 3,4-dehydrodebrisoquine (10.3 ± 2.6 % dose in EMs, 1.0 ± 0.5 % dose in PMs; $U=36.0$, $P=0.0014$). This observation adds weight to the proposition that 3,4-dehydrodebrisoquine is formed from 4-hydroxydebrisoquine. Of intense interest is the observation that one EM subject excreted inordinately large amounts of 3,4-dehydrodebrisoquine. EM subject #6 excreted 23.6% of dose as 3,4-dehydrodebrisoquine in 0-8 h and 27.6% in 0-24 h. However, another subject (#3) excreted only 2.0% and 3.1% in 8 h and 24 h, respectively. These subjects are both homozygous for the *CYP2D6*1* allele.

The excretion of 6- and 8-hydroxy-debrisoquine was also determined, after deconjugation with β -glucuronidase. Unlike previous studies (Allen et al., 1975; Idle et al., 1979) that employed packed GC columns, in our case, on a relatively short and non-polar capillary column, these metabolites did not require further derivatization of the phenol group as methyl or trimethylsilyl derivatives. In all published gas chromatograms of the debrisoquine phenols (Allen et al., 1975; Idle et al., 1979), these metabolites eluted in the order 8-, 5-, 7-, and 6-hydroxy-debrisoquine. Although other peaks were observed to elute between the 8- and 6-hydroxy-debrisoquine peaks in our urine analyses, no attempt was made to assign these to 5- and 7-hydroxy-debrisoquine, in the absence of authentic standards. The excretion of 6-hydroxydebrisoquine was found to be 1.8

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± 0.5 and 0.1 ± 0.1 % dose in EM 0-8 h and 8-24 h urine, respectively (Table 4). The excretion of 8-hydroxydebrisoquine was found to be 0.5 ± 0.2 and 0 ± 0 % dose in the EM 0-8 h and 8-24 h urine, respectively. Neither phenolic metabolite was detected in any urine from a PM volunteer. The total 6- and 8-hydroxy-debrisoquine excretion for EMs in 0-24 h was 2.4 % dose, which was significantly greater than in PMs ($U=34.0$, $P=0.006$).

Finally, the concentration in all urine samples of debrisoquine and 4-hydroxydebrisoquine, both before and after β -glucuronidase treatment was determined for 24 h. In all duplicate urine samples we saw a small but consistent rise in these concentrations, which could not be explained by evaporation because the tubes were well sealed during incubation at 37°C. Moreover, concomitant elevation in 3,4-dehydrodebrisoquine was not observed. We interpret these findings as being due to conjugation of both debrisoquine and 4-hydroxydebrisoquine with glucuronic acid. In the case of debrisoquine, this must be as a β -glucuronidase-labile *N*-glucuronide. With 4-hydroxydebrisoquine, an *O*⁴-glucuronide would be formed. Glucuronide formation from secondary alcohols, including benzylic alcohols (such as 4-hydroxydebrisoquine) has been known for many years (Williams, 1959). Neither of these metabolites has been previously described (Allen et al., 1975; Idle et al., 1979). Definitive identification of *O*⁴-glucuronide was achieved using ultra-performance liquid chromatography-time of flight mass spectrometry (UPLC-TOFMS) that enables exact mass determination (Fig 4). Indeed, a 368.14 *m/z* ion, corresponding in *Mr* to the hydroxydebrisoquine *O*⁴-glucuronide, was found at 1.76 and 2.23 min of the chromatogram and upon fragmentation yielded a 192.1 *m/z* ion that corresponds to the *Mr* of hydroxylated debrisoquine. While it is assumed that this is the *O*⁴-glucuronide, glucuronides derived from other minor hydroxylated metabolites, i.e., 6-, 7-, and 8-hydroxydebrisoquine,

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cannot be excluded by this analysis. In any case, debrisoquine glucuronide accounted for 0.4 ± 0.2 , 1.5 ± 1.1 , 0.3 ± 0.1 , and 1.3 ± 0.8 % dose in EM 0-8 h, PM 0-8 h, EM 8-24 h, and PM 8-24 h urines, respectively. Interestingly, PMs excreted more of this glucuronide than EMs, but the difference did not reach statistical significance. 4-Hydroxydebrisoquine glucuronide accounted for 2.5 ± 0.9 , 0.1 ± 0.03 , 1.5 ± 0.5 , and 0.1 ± 0.1 % dose in EM 0-8 h, PM 0-8 h, EM 8-24 h, and PM 8-24 h urines, respectively. EMs excreted more of this glucuronide, in contrast to the debrisoquine glucuronide, and the difference was statistically significant in 0-8 h ($U=30.0$, $P=0.032$), 8-24 h ($U=32.5$, $P=0.01$), and 0-24 h ($U=33.0$, $P=0.01$) urines..

The biotransformation of debrisoquine to its various metabolites excreted in the 0-8 h urine is summarized in Fig 5. 3,4-Dehydrodebrisoquine (7.8 % dose) represented the second most abundant metabolite of debrisoquine detected in EMs, after 4-hydroxydebrisoquine (20.9 % dose). In PMs, debrisoquine glucuronide (1.5% dose) was the principal metabolite detected, more abundant than 4-hydroxydebrisoquine (0.5%). In most respects, debrisoquine is an unmetabolized drug in PM subjects, levels of determined metabolites being almost trivial. This insight may be at variance with a host of previous studies in various populations which reported greater 4-hydroxylation of debrisoquine in PMs.

Determination of 4-hydroxydebrisoquine metabolism in a single human volunteer. A 0-8 h urine was obtained from a single human volunteer, homozygous for the *CYP2D6*1* allele, who had taken 10.2 mg 4-hydroxydebrisoquine by mouth. This volunteer was not drawn from the panel who took debrisoquine. No peaks on GCMS analysis were found corresponding to debrisoquine, 6- or 8-hydroxy-debrisoquine. However, the % dose eliminated as unchanged 4-

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hydroxydebrisoquine was 32.7%. Moreover, 3,4-dehydrodebrisoquine accounted for 1.6% of the excreted dose in 0-8 h, similar to the findings after debrisoquine administration. The amount of this metabolite was not increased when urine was left overnight at 37°C. Incubation of 4-hydroxydebrisoquine under acidic conditions also did not result in formation of 3,4-dehydrodebrisoquine. These findings demonstrate that 4-hydroxydebrisoquine is converted into 3,4-dehydrodebrisoquine *in vivo*. The yield of 3,4-dehydrodebrisoquine was not as great as had been anticipated (7.8 ± 2.2 % dose in 0-8 h after debrisoquine administration, see Table 1), perhaps reflecting the polarity of 4-hydroxydebrisoquine and its poor penetration to sites of further metabolism. In addition, absorbed 4-hydroxydebrisoquine would be expected to be readily excreted by the kidney.

Formation of 3,4-dehydrodebrisoquine *in vitro*. Debrisoquine hemisulfate was incubated with both human and rat liver microsomes. Debrisoquine was converted to 4-hydroxydebrisoquine, as expected, with 6.6% and 2.1% substrate conversion in 24 h, by rat and human liver microsomes, respectively. However, debrisoquine was also converted to 3,4-dehydrodebrisoquine, with 6.3% and 4.4% substrate conversion in 24 h, by rat and human liver microsomes, respectively. Moreover, 4-hydroxydebrisoquine was converted to 3,4-dehydrodebrisoquine and a late running peak at 22.40 min, by both rat and human liver microsomes. The conversion of 4-hydroxydebrisoquine to 3,4-dehydrodebrisoquine in 24 h was 33% and 25.4% for rat and human liver microsomes, respectively. No metabolite was formed from boiled microsomes or in the absence of NADH or NADPH. The concentration of 3,4-dehydrodebrisoquine produced in these microsomal incubations with 4-hydroxydebrisoquine was sufficient, for both rat and human preparations, to obtain a full mass spectrum to confirm the identity of the 3,4-dehydrodebrisoquine peak (Fig 6). The production of 3,4-dehydrodebrisoquine from 4-

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hydroxydebrisoquine was dependent upon the presence of microsomes, and depended on either NADH or NADPH, suggesting involvement of a non-P450 enzyme, such as a dehydratase.

Modification of the metabolic ratio by 3,4-dehydrodebrisoquine formation from 4-hydroxydebrisoquine.

All observations point to the further metabolism of 4-hydroxydebrisoquine to 3,4-dehydrodebrisoquine by a dehydratase enzyme, which has yet to be identified. The true extent of 4-hydroxylation for individuals can therefore be better estimated by the summation of 4-hydroxydebrisoquine and 3,4-dehydrodebrisoquine fluxes. Fig 7A shows the correlation in EMs between the traditional metabolic ratio (Mahgoub et al., 1977) of % dose as debrisoquine/% dose as 4-hydroxydebrisoquine and a revised metabolic ratio of % dose as debrisoquine/(% dose as 4-hydroxydebrisoquine + % dose as 3,4-dehydrodebrisoquine). A strong and highly significant correlation was found ($r = 0.969$; $P < 0.001$) for which the revised metabolic ratio was 33% lower than the traditional metabolic ratio (paired Student's t test; $t = 3.43$, $P = 0.0089$). All homozygous EMs had a revised metabolic ratio < 1 . For PMs, who did not excrete much 3,4-dehydrodebrisoquine (0.9%), the effect on metabolic ratio was nevertheless considerable, with traditional and revised metabolic ratios of 111 ± 37 and 66.3 ± 43 , respectively (paired Student's t test; $t = 1.71$, $P = 0.19$ (NS)).

There was no statistically significant correlation found between debrisoquine 4-hydroxylation and 3,4-dehydrodebrisoquine formation in the nine subjects homozygous for *CYP2D6*1* (Fig 7B). This is not surprising since the formation of 3,4-dehydrodebrisoquine requires not only initial CYP2D6 activity to produce 4-hydroxydebrisoquine, but also a second enzyme activity,

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independent of CYP2D6, to desaturate the 3,4-carbon-carbon bond. This dehydratase activity may vary from person to person.

The question remains whether or not failure to account for the formation of 3,4-dehydrodebrisoquine in debrisoquine phenotyping tests employing 0-8 h urine collections, might have led to erroneous assignment of CYP2D6 phenotype. Many thousand of such tests have been performed and it is not possible to answer this question here based upon the study of only nine homozygous EMs, four PMs, and no heterozygotes. The consistent reduction by 33% of the metabolic ratio in our volunteers suggests that this may be a possibility. One way to answer this question would be in a large population study of debrisoquine metabolism *versus* CYP2D6 genotype.

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Discussion

Authentic 3,4-dehydrodebrisoquine was synthesized by dehydration of 4-hydroxydebrisoquine with concentrated sulfuric acid and characterized by GCMS, LC-MS/MS and ^1H NMR. *In vivo* studies with human volunteers revealed that 3,4-dehydrodebrisoquine is a novel metabolite of debrisoquine that is excreted in both the 0-8 h and 0-24 h urine after a single oral dose of 12.8 mg debrisoquine hemisulfate (10 mg debrisoquine free base). The excretion of this metabolite is highly variable, from 3.1-27.6% (10.3 ± 2.6) dose in homozygous EMs and 0-2.1% (1.0 ± 0.5) dose in PMs in the 0-24 h urine. 3,4-Dehydrodebrisoquine comprised over one-third of the measured debrisoquine metabolites in one individual (#6). In addition, the excretion of the two phenolic metabolites, 6-hydroxydebrisoquine and 8-hydroxydebrisoquine were determined but, taken together, they never exceeded 6.1% of the dose with a mean of $2.4 \pm 0.7\%$ in homozygous EMs. Interestingly, neither phenol was detected in any urine from the four PM volunteers. This finding is in stark contrast to earlier studies, in which phenols were reported in the urine of eight Caucasian PMs and four Ghanaian PMs (Woolhouse et al., 1979), and 0.6% and 0.4% dose of 6-hydroxydebrisoquine and 8-hydroxydebrisoquine, respectively, were reported in the 0-8 h urine of six Nigerian PMs (Mbanefo et al., 1980). It is possible that our findings reflect inter-ethnic variation, however, they are more likely to be due to the deployment in this study of the highly specific analytical tool of GCMS.

A second novel finding of the current study is the observation that both debrisoquine and 4-hydroxydebrisoquine appear to form β -glucuronidase-labile glucuronides, as confirmed by UPLC-ToFMS analysis of urine metabolites. Further, the pattern of glucuronide excretion was highly genotype-dependent, reducing the likelihood that it was due to artifact. A metabolic ratio

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constructed as % dose eliminated as debrisoquine glucuronide/% dose eliminated as 4-hydroxydebrisoquine glucuronide had a mean value of 0.2 for EMs and 15 for PMs.

Further studies revealed that 3,4-dehydrodebrisoquine is produced from 4-hydroxydebrisoquine, both *in vivo* and *in vitro*. Its formation *in vitro*, using both human and rat liver microsomes, was dependent on either NADPH or NADH, and was therefore probably not mediated by a cytochrome P450. Not only therefore is 3,4-dehydrodebrisoquine a major and variable metabolite, but its production may also depend upon the activity of a secondary enzyme system, possibly a dehydratase. Thus the intergenotype difference in excretion of 3,4-dehydrodebrisoquine may be largely due to the CYP2D6 polymorphism, while the considerable intragenotype variation in production of this metabolite may arise from intersubject variation in the 4-hydroxydebrisoquine desaturation reaction. A subject who has a high 4-hydroxydebrisoquine dehydratase activity, may excrete relatively little 4-hydroxydebrisoquine, compared to a subject (with the same *CYP2D6* genotype) who has a low 4-hydroxydebrisoquine dehydratase activity, and thus excretes relatively larger amounts of 4-hydroxydebrisoquine. Coupled with the contribution of CYP1A1 to debrisoquine 4-hydroxylation (Granvil et al., 2002), and the variation introduced by glucuronidation of debrisoquine and 4-hydroxydebrisoquine, described herein, we can begin to comprehend why the debrisoquine metabolic ratio is so highly variable within a single genotype such as *CYP2D6**1/*CYP2D6**1. The discovery in recent years of a large number (close to 90) *CYP2D6* variant alleles (<http://www.imm.ki.se/cypalleles/cyp2d6.htm>) has explained the existence of the CYP2D6 UM (ultrarapid metabolizer), EM, IM (intermediate metabolizer), and PM phenotypes and some of the ethnic variability associated with them. However, these genetic data have failed to explain

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why the archetypal drug debrisoquine is metabolized quite differently in persons with identical genotypes. Apart from other influential constitutional and environmental factors, such as age, diet, lifestyle, and liver and kidney blood flow, it is the operation of additional mechanisms, both metabolic and non-metabolic, that surely dictate the pattern of urinary metabolites of a drug such as debrisoquine. The effect of a second enzyme system on debrisoquine metabolism was not anticipated because debrisoquine has been very well studied with respect to the P450 system. It was not immediately apparent that the subject of investigation should be 4-hydroxydebrisoquine, in addition to debrisoquine, and only then was the participation of a second enzyme system, the 4-hydroxydebrisoquine 3,4-dehydratase, uncovered. The metabolism of debrisoquine in relation to the CYP2D6 polymorphism is now more completely understood.

Since the discovery of the debrisoquine 4-hydroxylation polymorphism almost three decades ago (Mahgoub et al., 1977), there have appeared a host of reports that correlated some aspect of drug disposition with numerical values of the debrisoquine metabolic ratio, with greater or lesser success, for example, with nortriptyline (Mellstrom et al., 1981; Woolhouse et al., 1984), amitriptyline (Mellstrom et al., 1986), phenformin (Oates et al., 1982), sparteine (Inaba et al., 1983), tolbutamide (Peart et al., 1987), and ifosfamide (Philip et al., 1988). It is now clear that the classical metabolic ratio, as used by these various investigators, may not have been the best tool with which to correlate the metabolism of a second substrate. However, these types of investigation have been superseded by genotype-based studies, which have their own advantages and disadvantages.

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The ring-opened metabolites of debrisoquine ([7] and [8] in Fig 1A) are believed to represent a significant proportion of debrisoquine metabolism in rats, dogs and humans (Allen et al., 1975; Allen et al., 1976; Eiermann et al., 1998). It was first proposed in 1976 that these two ring-opened metabolites were formed as a consequence of 1- and 3-hydroxylation of debrisoquine (Allen et al., 1976). More recently, this concept has been reinforced after the detection of the two ring-opened metabolites using LCMS (Eiermann et al., 1998). These authors also reported that the excretion of these two metabolites correlated highly, across a broad range of metabolic capacity, with debrisoquine 4-hydroxylation, as judged by the traditional metabolic ratio (debrisoquine/4-hydroxydebrisoquine). However, no-one has ever performed an experiment that demonstrated 1- or 3-hydroxylation of debrisoquine; simply detection of metabolites [7] and [8] (Fig 1A) is taken as evidence for these hydroxylation reactions. An alternative scenario, that is based both upon our observation of the formation of 3,4-dehydrodebrisoquine from 4-hydroxydebrisoquine and also some older literature, is proposed. Examination of the various published correlations between debrisoquine 4-hydroxylation and individual phenol metabolite formation, together with the data presented herein, is enlightening. The data presented in Tables 3 and 4 permits correlations to be made, within the homozygous *CYP2D6*1* genotype, between 4-, 6- and 8-hydroxylation of debrisoquine. The correlation coefficient (*r*) for 4-hydroxylation vs. 6-hydroxylation, 4-hydroxylation vs. 8-hydroxylation, and 6-hydroxylation vs. 8-hydroxylation are 0.059, 0.055, and 0.993, respectively. 4-Hydroxylation does not correlate with either 6- or 8-hydroxylation, even though all three reactions are CYP2D6-dependent (see Tables 1-4). Moreover, published data from 10 Nigerian EMs (Mbanefo et al., 1980) permits correlations to be made between 4-, 5-, 6-, 7-, and 8- hydroxylation of debrisoquine. The correlations with debrisoquine 4-hydroxylation and 6-, 7-, or 8-hydroxylation are virtually zero,

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and there is a weakly significant correlation with 5-hydroxylation ($r = 0.642$, $P < 0.05$). There is no reason to expect, therefore, 4-hydroxylation to correlate with 1- or 3-hydroxylation, should these latter reactions occur.

We propose that debrisoquine 4-hydroxylation also leads to 3,4-dehydrodebrisoquine in considerable amounts; as high as 27.6% of the dose was obtained in one subject. In addition, the possibility exists that 3,4-dehydrodebrisoquine is not only in equilibrium with the 3-dehydro-isoquinolinium compound in Fig 4, but also rearranges to the 1-dehydro-isoquinolinium compound, due to the resonance between the 1,2-double bond and the aromatic system and the guanidine lone pairs. As shown in Fig 1C, the desaturation of 4-hydroxydebrisoquine, subsequent rearrangement of the 3,4-dehydrodebrisoquine and reaction with hydroxyl ions, leads to 1- and 3-hydroxy-debrisoquine, which then become further oxidized to yield ultimately the two ring-opened metabolites. In this proposed scheme, all but the phenolic metabolites arise via 4-hydroxylation of debrisoquine by CYP2D6.

In summary, this study has shown that debrisoquine forms a novel metabolite, 3,4-dehydrodebrisoquine, both *in vivo* and *in vitro*, from 4-hydroxydebrisoquine by an unknown dehydratase activity. The existence of this new metabolite has several consequences. First, the total flux of 4-hydroxylation is, on average, at least 30% greater in *CYP2D6*1* homozygotes than previously thought. This has the effect of lowering the metabolic ratio, but does so in a predictable manner. In turn, this may have implications for studies where the metabolic ratio is correlated with the metabolism of a second drug, or indeed is used in case-control studies of cancer (Ayesh et al., 1984), Parkinson's disease (Barbeau et al., 1985), and other clinical entities.

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Second, the process of CYP2D6-mediated hydroxylation followed by desaturation, may not be restricted to debrisoquine. It is possible that this double reaction plays a role in sparteine metabolism. Finally, we propose a metabolic scheme whereby all the major metabolites of debrisoquine arise via the 4-hydroxylation pathway. Neither the extent of metabolism nor the ratio of metabolites to each other can be readily predicted from *CYP2D6* genotyping studies alone. Genotype determines an average metabolic performance, but the variability about this mean may be due to a range of factors, including alternative non-CYP2D6 metabolic pathways.

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Footnotes

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Legends for figures

Fig 1. (A) The known metabolic transformations of debrisoquine [1] showing the pathways of urea formation [2] and *N*-hydroxylation [3] that are thought not to occur, and the principal metabolites (*S*)-(+)-4-hydroxydebrisoquine [4], 5-, 6-, 7-, and 8-hydroxydebrisoquine [5], which are thought to be conjugated, an unidentified dihydroxy metabolite [6] thought to arise from 4-hydroxydebrisoquine, and a ring-opened phenylacetic acid derivative [7], together with a ring-opened benzoic acid derivative, which are thought to be formed by 3- and 1-hydroxylation of debrisoquine, respectively. (B) Conversion of 3,4-dehydrodebrisoquine to 3-hydroxydebrisoquine via equilibrium A and addition reaction B. Reaction C is proposed to occur for the bis(trifluoromethyl)pyrimidine derivatives in the mass spectrometer. (C) A proposed scheme for the formation of 1-hydroxydebrisoquine and 3-hydroxydebrisoquine and consequently the ring-opened amino acid metabolites of debrisoquine.

Fig 2. (A) Single ion chromatogram (344 *m/z*) of synthetic 3,4-dehydrodebrisoquine bis(trifluoromethyl)pyrimidine derivative. Note the absence of the starting material 4-hydroxydebrisoquine. (B) The mass spectrum of the 3,4-dehydrodebrisoquine derivative and fragmentation of the molecular ion (345 *m/z*) by loss of a H[•] radical to yield the fully aromatic and highly stable 344 *m/z* ion. (C) ¹H NMR spectrum of synthetic 3,4-dehydrodebrisoquine from δ 5.6 to δ 8.0 ppm showing two doublets (arrowed) that correspond to single protons at carbons C-3 and C-4. The two-dimensional COSY data show that these two protons are coupled only to each other. (D) LC-MS/MS of the synthetic 3,4-dehydrodebrisoquine showing the neutral losses that lead to the principal fragment ions. (E) LC-MS/MS of 3,4-dehydrodebrisoquine in

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the urine of an EM volunteer administered debrisoquine. The protonated molecular ion and all three principal fragment ions correspond to the ions in the synthetic material (Fig 2D, above).

Fig 3. Mass spectra of peaks A (17.33 min) and B (17.48 min) in a standard solution of 5µg/mL 3,4-dehydrodebrisoquine.

Fig 4. UPLC chromatogram and positive ion mass spectra of putative monohydroxylated debrisoquine glucuronides. Ions at eluting at 1.76 min and 2.23 min with the predicted molecular weight of monohydroxylated debrisoquine glucuronide (368.14) were extracted from the total ion chromatogram. Loss of the glucuronic acid moiety (-176) from these two parent ions in MS/MS fragmentation is shown in the respective inlaid panels .

Fig 5. Metabolic profiles in 0-8 h urine for nine *CYP2D6*1* homozygotes (open bars) and four *CYP2D6*4* homozygotes (filled bars) given 12.6 mg debrisoquine hemisulfate by mouth. Asterisks indicate metabolites showing statistical differences between genotypes. D = debrisoquine, 4-HD = 4-hydroxydebrisoquine, 6-HD = 6-hydroxydebrisoquine, 8-HD = 8-hydroxydebrisoquine, 3,4-DHD = 3,4-dehydrodebrisoquine, D-gluc = debrisoquine glucuronide, 4-HD-gluc = 4-hydroxydebrisoquine glucuronide.

Fig 6. Extracted ion chromatogram (344 m/z) of a 24 h incubation of 4-hydroxydebrisoquine with human liver microsomes showing the formation of 3,4-dehydrodebrisoquine and an unknown metabolite (upper panel). The identity of the 3,4-dehydrodebrisoquine product was confirmed by its typical mass spectrum (lower panel).

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Fig 7. (A) Correlation between the traditional 0-8 h metabolic ratio (MR; % dose as debrisoquine/% dose as 4-hydroxydebrisoquine) and a revised metabolic ratio (% dose as debrisoquine/(% dose as 4-hydroxydebrisoquine + % dose as 3,4-dehydrodebrisoquine) for nine *CYP2D6*1* homozygotes. (B) Lack of correlation between % dose excreted in 0-8 h urine as 4-hydroxydebrisoquine and % dose excreted in 0-8 h urine as 3,4-dehydrodebrisoquine for nine *CYP2D6*1* homozygotes.

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Tables

Table 1. Urinary excretion (0-8 h) of debrisoquine, 4-hydroxydebrisoquine, and 3,4-dehydrodebrisoquine (sum of Peaks A and B) in 13 human volunteers genotyped for *CYP2D6*. Debrisoquine (10 mg) was administered by mouth as debrisoquine hemisulfate (12.8 mg). Results are expressed as percent of dose excreted.

SUBJECT # (GENOTYPE)	DEBRISOQUINE	4-HYDROXY- DEBRISOQUINE	3,4-DIDEHYDRO- DEBRISOQUINE
#1 (*1/*1)	14.3	23.8	3.8
#2 (*1/*1)	6.7	30.0	10.0
#3 (*1/*1)	9.2	8.3	2.0
#4 (*1/*1)	7.9	19.0	10.1
#5 (*1/*1)	4.3	30.3	3.9
#6 (*1/*1)	8.0	31.1	23.6
#7 (*1/*1)	7.7	23.3	3.1
#8 (*1/*1)	6.6	12.9	6.7
#9 (*1/*1)	9.8	9.5	6.6
Mean ± SEM	8.3 ± 1.0	20.9 ± 3.0	7.8 ± 2.2
#10 (*4/*4)	39.2	1.1	2.1
#11 (*4/*4)	29.2	<0.15	0
#12 (*4/*4)	34.1	0.23	1.0
#13 (*4/*4)	32.9	0.5	0.6
Mean ± SEM	33.9 ± 2.1	0.5 ± 0.2	0.9 ± 0.4
Significance (P) (Mann-Whitney U test)	0.0014	0.0014	0.0028

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Table 2. Urinary excretion (8-24 h) of debrisoquine, 4-hydroxydebrisoquine, and 3,4-dehydrodebrisoquine in 13 human volunteers genotyped for *CYP2D6*. Debrisoquine (10 mg) was administered by mouth as debrisoquine hemisulfate (12.8 mg). Results are expressed as percent of dose excreted.

SUBJECT # (GENOTYPE)	DEBRISOQUINE	4-HYDROXY- DEBRISOQUINE	3,4-DIDEHYDRO- DEBRISOQUINE
#1 (*1/*1)	4.3	6.6	1.3
#2 (*1/*1)	1.1	3.6	2.7
#3 (*1/*1)	7.6	9.2	1.1
#4 (*1/*1)	1.0	2.1	0
#5 (*1/*1)	2.7	16.2	2.2
#6 (*1/*1)	1.7	6.2	4.0
#7 (*1/*1)	1.9	4.7	0
#8 (*1/*1)	6.0	11.2	8.9
#9 (*1/*1)	7.3	4.9	2.4
Mean ± SEM	3.7 ± 0.9	7.2 ± 1.4	2.5 ± 0.9
#10 (*4/*4)	33.8	0.3	0
#11 (*4/*4)	14.2	<0.2	0
#12 (*4/*4)	9.4	<0.08	0.4
#13 (*4/*4)	20.3	<0.2	0
Mean ± SEM	19.5 ± 5.3	0.2 ± 0.1	0.1 ± 0.1
Significance (P) (Mann-Whitney U test)	0.0014	0.0014	0.025

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Table 3. Urinary excretion (0-24 h) of debrisoquine, 4-hydroxydebrisoquine, and 3,4-dehydrodebrisoquine in 13 human volunteers genotyped for *CYP2D6*. Debrisoquine (10 mg) was administered by mouth as debrisoquine hemisulfate (12.8 mg). Results are expressed as percent of dose excreted.

SUBJECT # (GENOTYPE)	DEBRISOQUINE	4-HYDROXY- DEBRISOQUINE	3,4-DIDEHYDRO- DEBRISOQUINE
#1 (*1/*1)	18.6	30.4	5.1
#2 (*1/*1)	7.8	33.6	12.7
#3 (*1/*1)	16.8	17.5	3.1
#4 (*1/*1)	8.9	21.1	10.1
#5 (*1/*1)	7.0	46.5	6.1
#6 (*1/*1)	9.7	37.3	27.6
#7 (*1/*1)	9.6	28.0	3.1
#8 (*1/*1)	12.6	24.1	15.6
#9 (*1/*1)	17.1	14.4	9.0
Mean ± SEM	12.0 ± 1.5	28.1 ± 3.4	10.3 ± 2.6
#10 (*4/*4)	73.0	1.4	2.1
#11 (*4/*4)	43.4	<0.35	0
#12 (*4/*4)	43.5	<0.31	1.4
#13 (*4/*4)	53.2	<0.7	0.6
Mean ± SEM	53.3 ± 7.0	0.7 ± 0.3	1.0 ± 0.5
Significance (P) (Mann-Whitney U test)	0.0014	0.0014	0.0014

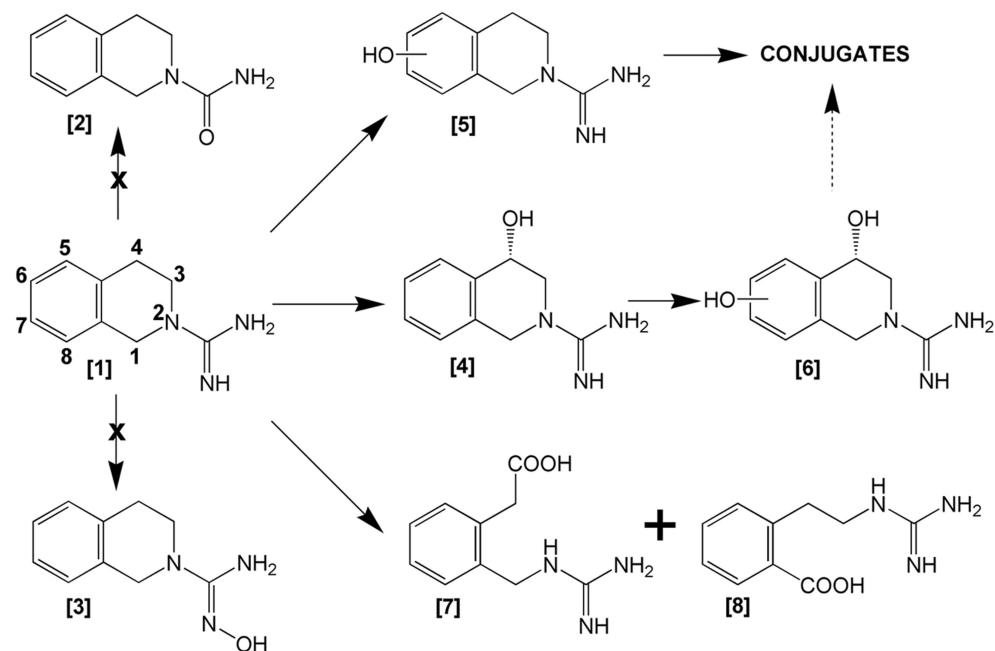
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Table 4. Urinary excretion (0-8, 8-24 and 0-24 h) of 6-hydroxydebrisoquine and 8-hydroxydebrisoquine in 13 human volunteers genotyped for *CYP2D6*. Debrisoquine (10 mg) was administered by mouth as debrisoquine hemisulfate (12.8 mg). Results are expressed as percent of dose excreted.

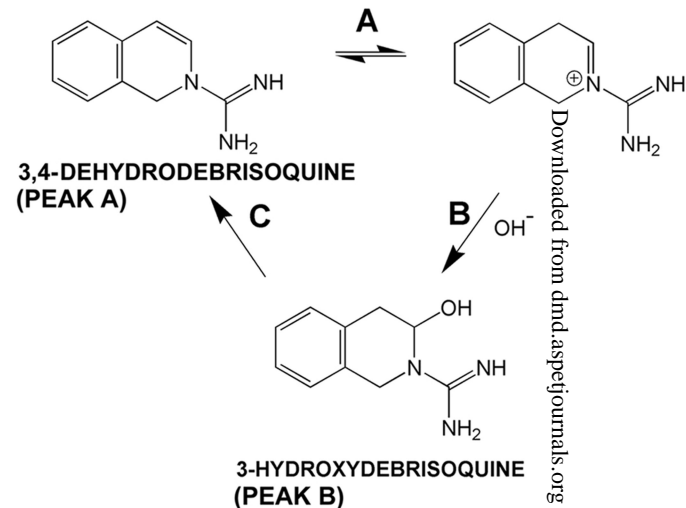
SUBJECT # (GENOTYPE)	6-HD 0-8 h	6-HD 8-24 h	6-HD 0-24 h	8-HD 0-8 h	8-HD 8-24 h	8-HD 0-24 h	6-HD + 8- HD 0-24 h
#1 (*1/*1)	4.1	0.7	4.8	1.2	0.1	1.3	6.1
#2 (*1/*1)	4.1	0	4.1	1.2	0	1.2	5.3
#3 (*1/*1)	0.1	0	0.1	0	0	0	0.1
#4 (*1/*1)	1.9	0.1	2.0	0.5	0	0.5	2.5
#5 (*1/*1)	0	0.1	0.1	0	0	0	0.1
#6 (*1/*1)	2.1	0	2.1	0.5	0	0.5	2.6
#7 (*1/*1)	2.5	0	2.5	0.7	0	0.7	3.2
#8 (*1/*1)	1.0	0.3	1.3	0.3	0	0.3	1.6
#9 (*1/*1)	0	0	0	0	0	0	0
Mean ± SEM	1.8 ± 0.5	0.1 ± 0.1	1.9 ± 0.6	0.5 ± 0.2	0 ± 0	0.5 ± 0.2	2.4 ± 0.7
#10 (*4/*4)	0	0	0	0	0	0	0
#11 (*4/*4)	0	0	0	0	0	0	0
#12 (*4/*4)	0	0	0	0	0	0	0
#13 (*4/*4)	0	0	0	0	0	0	0
Mean ± SEM	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Significance (P) Mann- Whitney U test	0.017	0.13 (NS)	0.006	0.038	0.41 (NS)	0.038	0.006

Figure 1

A



B



C

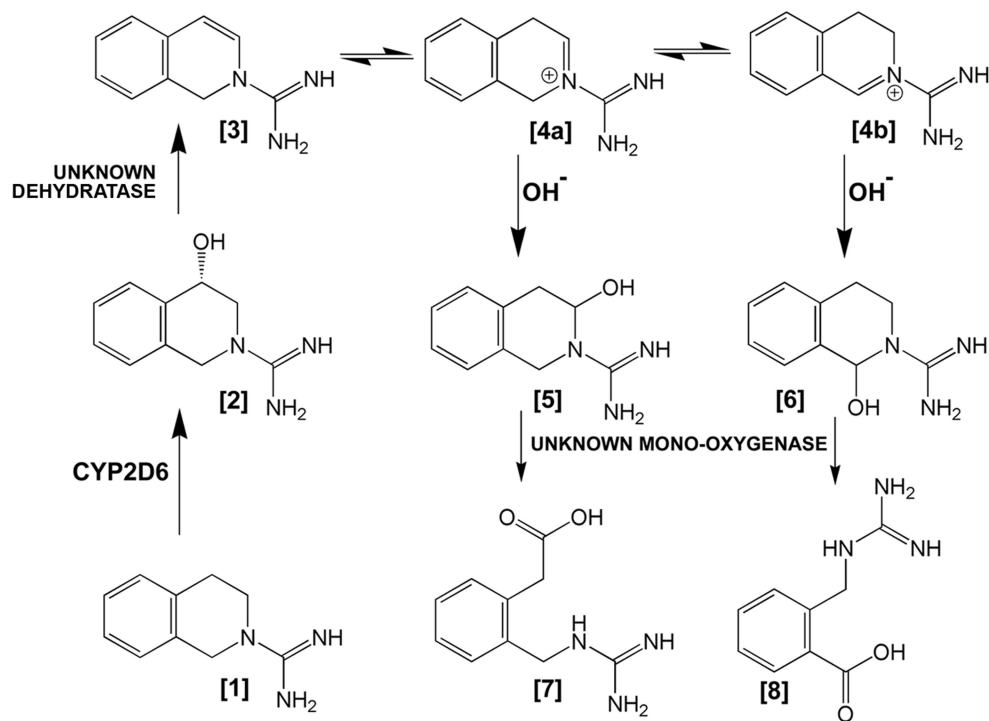
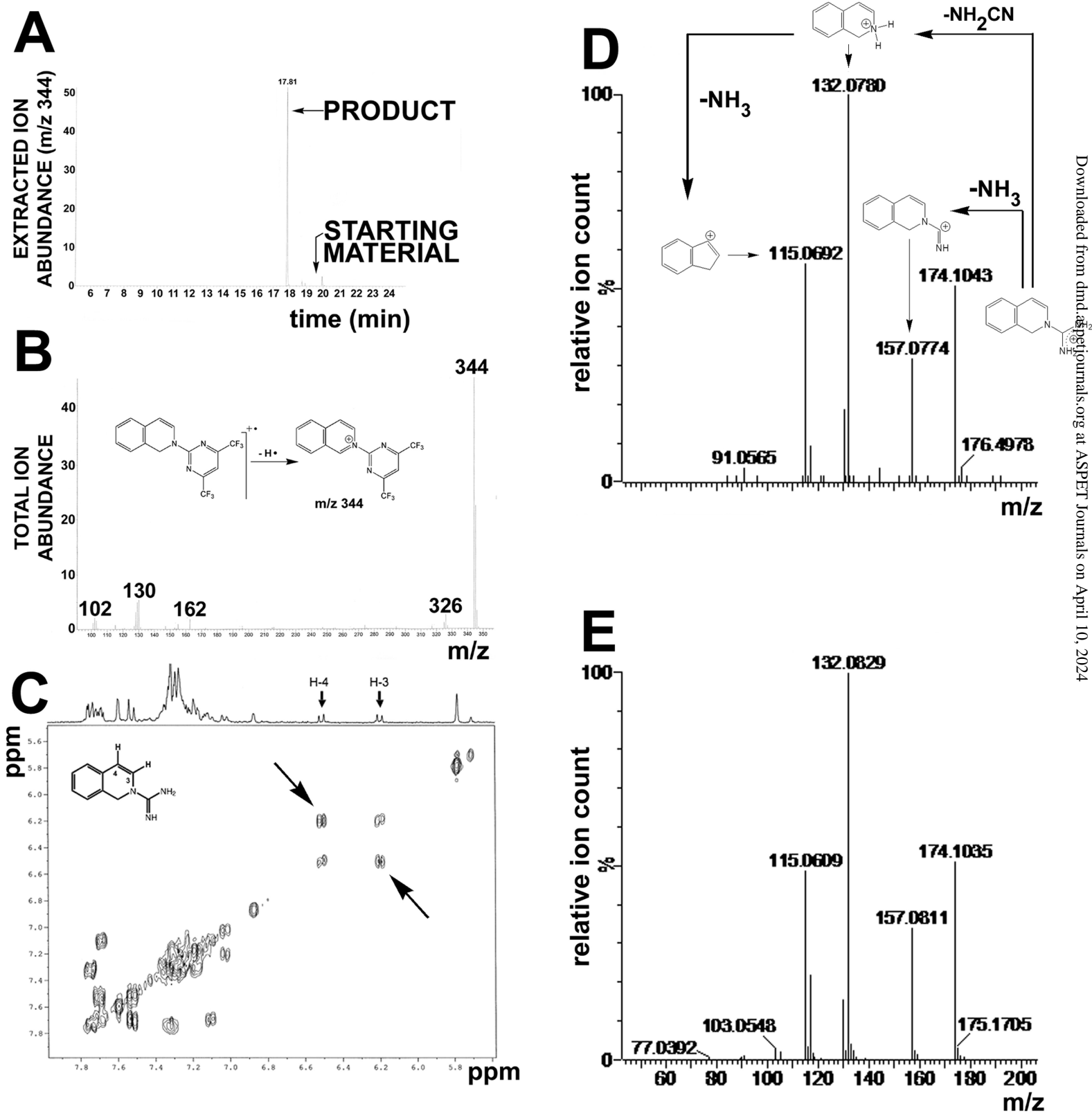
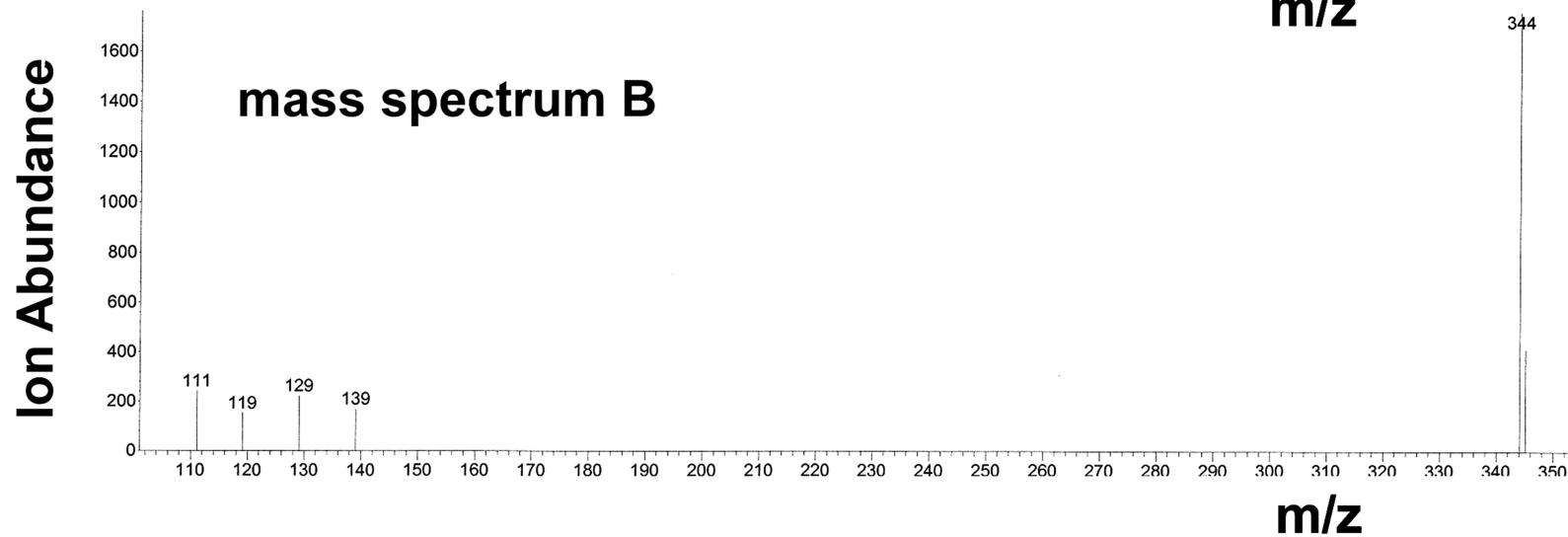
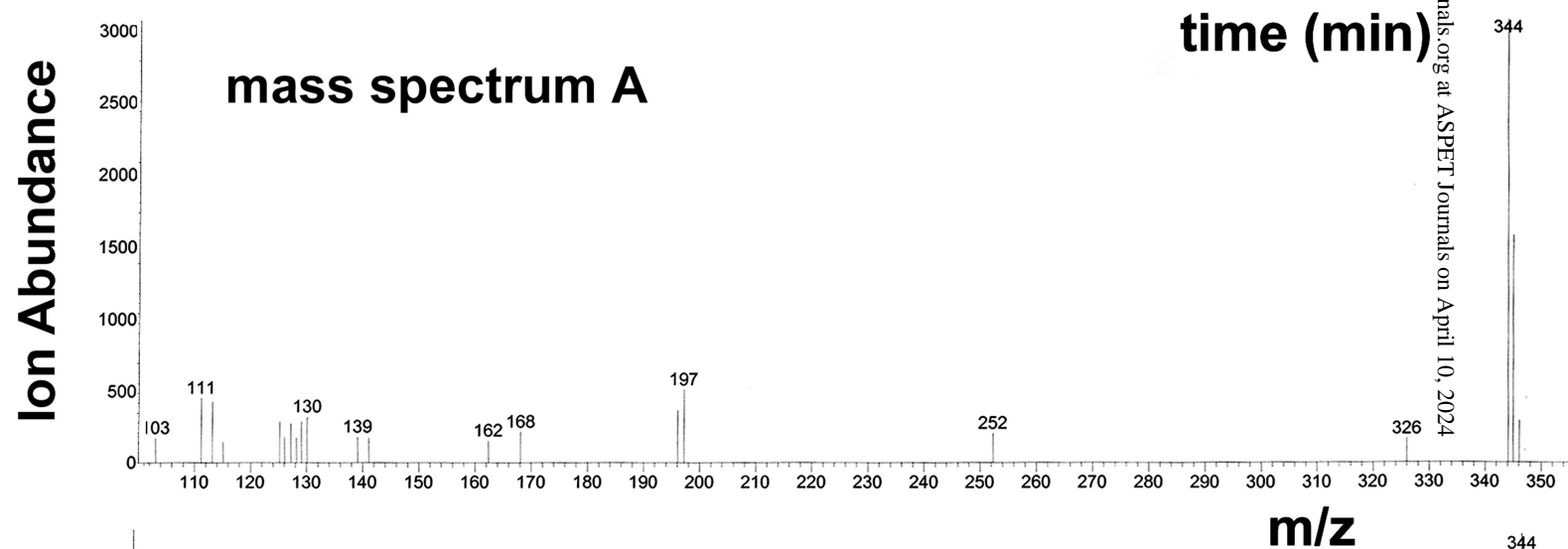
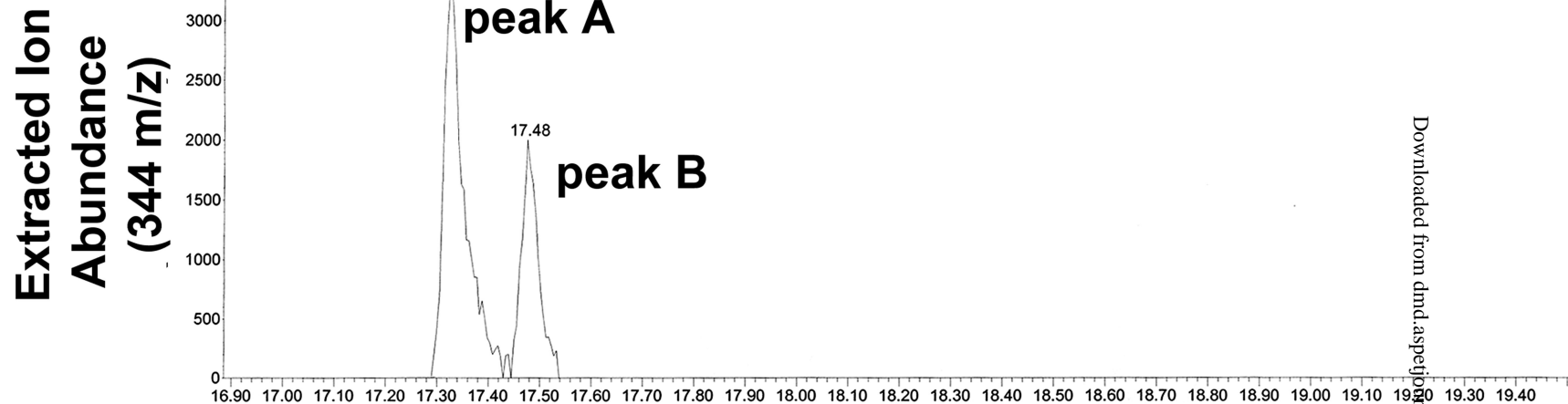
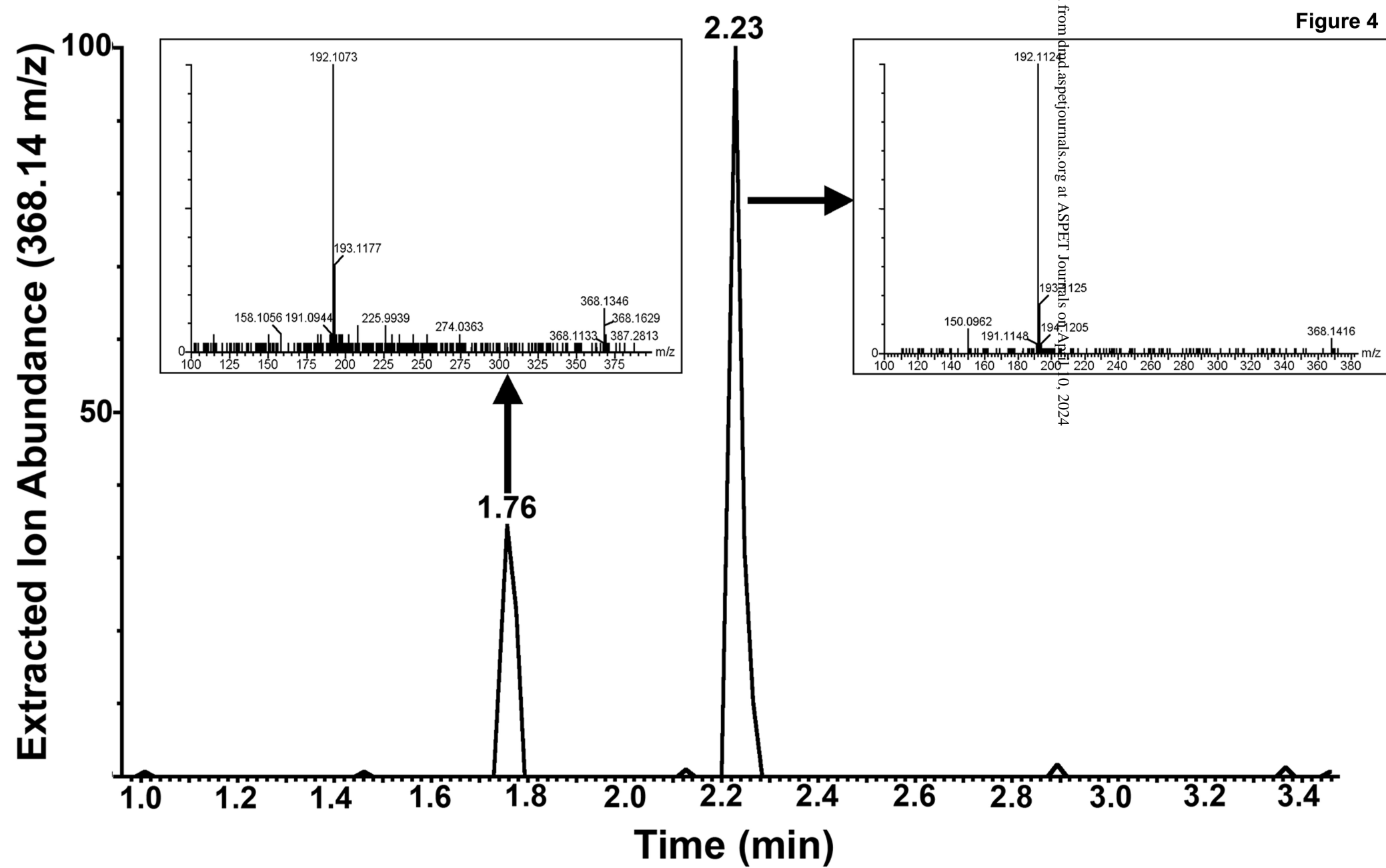
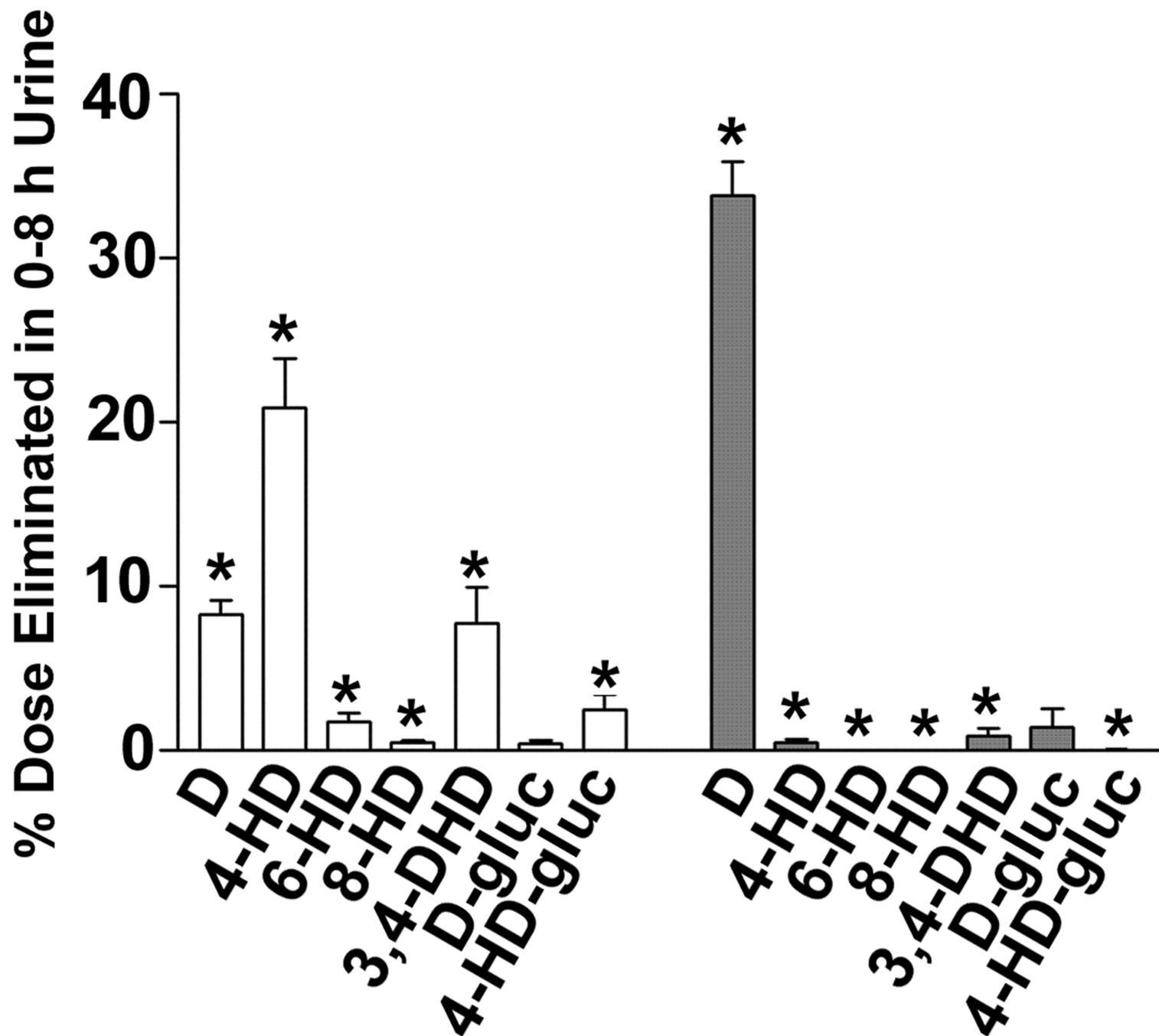


Figure 2







EM**PM**

4-hydroxydebrisoquine

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