ABSTRACT:

Twenty-one healthy Swedish Caucasian volunteers, representing different groups with 0–13 functional cytochrome P450 (CYP) 2D6 genes, were given a single oral dose of 20 mg of debrisoquine. The hypothesis of further oxidation of the main metabolite, (S)-4-hydroxydebrisoquine, in subjects with multiple CYP2D6 genes was tested by screening the 0–8-hr urine samples for dihydroxylated metabolites of debrisoquine with protonated molecular ions at m/z 208, using LC/MS. Three peaks were detected in a subject with 13 functional CYP2D6 genes. One compound was identified as dihydroxylated debrisoquine (presumably with hydroxylation at position 4 plus one of the positions in the aromatic ring). This metabolite had not been previously demonstrated in humans and was detected only in this subject. The other two compounds, which were measurable in various amounts in all subjects investigated, were identified as 2-(guanidinoethyl)phenylacetic acid and 2-(guanidinoethyl)benzoic acid. They had been previously detected in the urine of humans, dogs, and rats. They were distinguished by acid-catalyzed deuterium exchange of the hydrogens at the α-position, with respect to the carboxylic acid group, of the former but not the latter acid. The acids are formed by 3- and 1-hydroxylation of debrisoquine, respectively, followed by ring opening to aldehydes, which are further oxidized to acids. Strong Spearman rank correlations between debrisoquine products of 1- or 3-hydroxydebrisoquine and debrisoquine/4-hydroxydebrisoquine ratios (rS = 0.97 and rF = 0.96, respectively), using the intensity of the peaks of the reconstructed ion-current chromatograms, clearly showed that both hydroxylation steps are catalyzed by CYP2D6. Because reference compounds for the two acids were not available, the absolute quantities could not be determined.
CYP2D6 genes. Group 0 consisted of five subjects without any functional CYP2D6 gene (so-called PMs). Groups 1 and 2 consisted of five subjects each with one and two active genes, respectively. In group 3 was one individual with four active CYP2D6 genes and four individuals with three active CYP2D6 genes. In addition, one subject with 13 functional genes participated. Aliquots of 10 ml of urine were frozen and stored at −20°C until analysis. The urine samples collected 0–8 hr after drug intake were used for analysis in this study.

**Sample Preparation.** A solid-phase extraction column containing 500 mg of C18 sorbent in a 3-ml Sep-Pak column from Waters Millipore Corp. (Marlborough, MA) was conditioned with 3 ml of methanol and 3 ml of 0.1% PFPA in water. The urine sample (1 ml) was acidified with 0.1 ml of 10% PFPA in water and applied to the column. Salts were washed out with 3 ml of 0.1% PFPA in water, and the analytes were eluted with 3 ml of methanol, into a new test tube. The methanol was evaporated under nitrogen, and the sample was reconstituted in 0.15 ml of 0.1% PFPA in water. Samples were then filtered through a 0.45-μm nylon centrifuge-filter (Alltech Associates, Deerfield, IL). The metabolites studied were very well retained by reverse-phase material, and an organic modifier was required to elute the analytes during LC. Extraction efficiency was not tested. However, a good correlation between the previously determined MR for debrisoquine (Dalén P, Dahl M-L, Eichelbaum M, Bertilsson L, and Wilkinson G, submitted for publication) and the debrisoquine intensity/4-hydroxydebrisoquine intensity ratio (“4-hydroxylation index”) indicates consistent extraction efficiency.

**LC/MS.** The LC system consisted of a binary high-pressure gradient system with model LC10AD pumps (Shimadzu, Kyoto, Japan), a Valco injector with a 5-μl loop, and a reverse-phase column (1 × 150 mm) packed with 5-μm Supelcosil LC-ABZ particles (Supelco, Bellefonte, PA). The column effluent was connected to a six-port valve (Valco model C2) with 0.25-mm (i.d.) connections. One of the adjacent connections was diverted to waste and the other was connected to the ion source of the mass spectrometer by a fused silica capillary (75 μm i.d. × 300 mm). The mass spectrometer (model Q-ToF; Micromass, Manchester, UK) was equipped with an electrospray ion source operated at 70°C. The analytes were eluted, at a flow rate of 50 μl/min, by a linear gradient from 2% acetonitrile/97.95% water/0.05% PFPA to 40% acetonitrile/59.95% water/0.05% PFPA in 20 min. The column was cleaned with acetonitrile and reconditioned with 2% acetonitrile for 10 min between injections. The column effluent was directed to the ion source from 7 to 20 min after injection during analysis, to reduce contamination of the ion source. Analysis of debrisoquine and its metabolites was performed by integration of reconstructed ion-current chromatograms of the protonated molecular ions of interest. The Q-ToF instrument is constructed with a quadrupole for selection of precursor ions, followed by an orthogonal time-of-flight mass analyzer for registration of product ions after collision-induced dissociation (MS/MS). Mass spectra were recorded by the time-of-flight mass analyzer when the quadrupole was set to transmit all ions formed. Mass spectra were recorded with a resolution of approximately 5000, with a frequency of 30 spectra/min. The range of masses scanned for the total-ion chromatogram was m/z 100–600. Product-ion spectra were produced with the quadrupole set to unit resolution, and the collision gas (argon) was adjusted to yield a pressure of 50 nbar in the mass analyzer. The collision energy was set to 20 V.

**Chemical Experiments.** In experiment 1, LC/MS and MS/MS were conducted after the water in the mobile phase had been exchanged with deuterium oxide, to analyze exchangeable hydrogens bound to the heteroatoms. In experiment 2, a urine extract was acetylated with acetic anhydride under conditions selective for aromatic hydroxyl acetylation (acetic anhydride in 0.9 M aqueous sodium hydrogen carbonate). In experiment 3, a solid-phase extract of a urine sample was treated with 6 M deuterium chloride in deuterium oxide at 110°C for 3 hr, for exchange of the hydrogens in the α-position with respect to a carboxylic acid group.

**Statistical Analysis.** The Spearman rank correlation test was used to compare the relationship between the debrisoquine intensity/4-hydroxydebrisoquine intensity ratio and the debrisoquine intensity/product of 3- or 1-hydroxydebrisoquine intensity ratio; p values of ≤0.05 were regarded as statistically significant.
Results

Debrisoquine and its metabolites were extracted from the urine by a nonselective, solid-phase extraction procedure, mainly to desalt and concentrate the analytes before analysis by LC/MS. The urine was analyzed by LC/MS for determination of the molecular masses of possible metabolites, using LC separation on a Supelcosil LC-ABZ column. The analysis was repeated in the MS/MS mode for registration of product-ion spectra of the protonated molecular ions, to provide more structural information.

An LC chromatogram for the urine extract from the subject with 13 CYP2D6 genes is shown in fig. 1, together with reconstructed ion-current chromatograms of the positive ions \([M+H]^+\) of \(m/z\) 176 (debrisoquine), \(m/z\) 192 (monohydroxylated debrisoquine), and \(m/z\) 208 (dihydroxylated debrisoquine). The detection of debrisoquine (retention time, 15.9 min) and 4-hydroxydebrisoquine (retention time, 12.2 min) was verified by comparison of retention times and product-ion spectra with those of the reference compounds.

The reconstructed ion-current chromatogram for \(m/z\) 208 (dihydroxylated debrisoquine) showed one small peak at 11.0 min (I) and two larger peaks at 13.2 (II) and 16.1 (III) min (fig. 1). Peak I was tentatively identified as dihydroxylated debrisoquine from its product-ion spectrum. The compound was fragmented by characteristic losses of water and ammonia, followed by the loss of carbodiimide. Hydroxylation occurred at position 4 and at one of positions 5–8 in the aromatic ring. The aromatic hydroxylation was verified through experiment 2, which led to acetylation of all aromatic monohydroxylated metabolites (Idle et al., 1979). The product-ion spectra of the larger peaks II and III were different from the spectrum of peak I, because of very weak or absent fragment ions resulting from the loss of water and ammonia. A common fragment ion was observed at \(m/z\) 60, which was missing in the spectrum of peak I. Both compounds (II and III) showed prominent ions at \(m/z\) 149 [after loss of \((NH_2)_2C==NH\)]. The base peak for compound II was \(m/z\) 121, which was formed from \(m/z\) 149 by the loss of CO. The second largest peak for compound III was \(m/z\) 131, which was formed by the loss of urea and ammonia. These results suggested that metabolites II and III had been formed by hydroxylation at position 3 or 1, respectively, followed by ring opening to aldehydes, which were further oxidized to the compounds 2-(guanidinomethyl)phenylacetic acid and 2-(guanidinoethyl)benzoic acid (fig. 2). Among five EMs, Idle et al. (1979) detected these polar metabolites in varying amounts (0–15.1% of the administered dose), sometimes even exceeding the amount of 4-hydroxydebrisoquine.

Experiment 1 was conducted for verification of the structures of compounds I–III with the reconstructed ion-current chromatogram for \(m/z\) 208, in which all five protons were exchangeable by deuterium oxide, as expected. Experiment 3 was conducted to differentiate
between metabolites II and III, because it was not clear from their product-ion spectra whether the metabolite with the stronger response at m/z 208 (metabolite III) had been formed after 3- or 1-hydroxylation. LC/MS and LC/MS/MS analysis of the deuterium chloride-treated sample demonstrated that compound II (fig. 1) had partially (40%) exchanged both hydrogens with deuterium at the α-position with respect to the carboxylic acid. The mass of the compound that had exchanged both hydrogens was shifted from m/z 208 to m/z 210, and all fragment ions containing hydrogens at the α-position were shifted 2 Da higher in molecular mass. Metabolite III lacks hydrogens at the α-position, and no deuterium exchange was detected. According to these analyses, the metabolic scheme of debrisoquine shown in fig. 2 can be proposed.

Ratios of debrisoquine intensity/II, III and 4-hydroxydebrisoquine intensity were calculated from the reconstructed ion-current chromatograms for m/z 176, 192, and 208 and correlated using the Spearman rank correlation. One must be aware that these ratios are different from the debrisoquine/4-hydroxydebrisoquine MR used in the normal phenotyping procedure, when proper reference compounds are used for quantification. Here the ratios between ion currents were used for measurements, because reference compounds for metabolites II and III were not available. Fig. 3 shows the correlation between the debrisoquine intensity/2-(guanidinoethyl)benzoic acid intensity ratio ("1-hydroxylation index") and the debrisoquine intensity/4-hydroxydebrisoquine intensity ratio ("4-hydroxylation index") (r s = 0.965). Fig. 4 shows the correlation between the debrisoquine intensity/2-(guanidinomethyl)phenylacetic acid intensity ratio ("3-hydroxylation index") and the debrisoquine intensity/4-hydroxydebrisoquine intensity ratio (r s = 0.957). This shows that CYP2D6 catalyzes not only the S-4-hydroxylation of debrisoquine but also the 1- and 3-hydroxylations, as proposed in fig. 2. Correlation between the debrisoquine intensity/4-hydroxydebrisoquine intensity ratio, gained from the reconstructed ion-current chromatograms for m/z 176 and 192, and the debrisoquine/4-hydroxydebrisoquine MR (Dalen P, Dahl M-L, Eichelbaum M, Bertilsson L, and Wilkinson G, submitted for publication) yielded an r s value of 0.971.

Discussion

This study has shown that not only the 4-hydroxylation of debrisoquine but also the 1- and 3-hydroxylations of debrisoquine are catalyzed by CYP2D6. The decreasing urinary recovery of a given dose of debrisoquine with increasing numbers of active CYP2D6 genes among 21 healthy Swedish volunteers, as reported by Dalén et al. (Dalen P, Dahl M-L, Eichelbaum M, Bertilsson L, and Wilkinson G, submitted for publication), prompted us to analyze the 0–8-hr urine samples from those individuals. This urine fraction contains the greatest amounts of debrisoquine and its metabolites, as shown by HPLC analysis (Dalén P, Dahl M-L, Eichelbaum M, Bertilsson L, and Wilkinson G, submitted for publication). Further metabolism of the main metabolite, (S)-4-hydroxydebrisoquine, by CYP2D6 in subjects with multiple copies of the CYP2D6 gene was proposed, because neither glucuronidated nor sulfated metabolites had been detected in urine (Idle et al., 1979; Allen et al., 1975, 1976). The aromatic ring and positions 1, 3, and 4 were possible oxidation sites, and oxidation would result in dihydroxylated metabolites with an increase from m/z 192 (4-hydroxydebrisoquine) to m/z 208 (fig. 2).

Debrisoquine and 4-hydroxydebrisoquine were readily identified from their product-ion spectra and retention times, compared with the reference compounds (fig. 1), using a LC/MS system and preanalytical solid-phase extraction. The reconstructed ion-current chromatogram for m/z 208 for dihydroxylated metabolites from the subject with 13 functional CYP2D6 genes showed three peaks, with retention times of 11.0, 13.2, and 16.1 min (fig. 1). Peak I was very small and not detectable in any of the subjects from the other genotype groups. It was identified as dihydroxylated debrisoquine through its fragmenta-
tion pattern and through aromatic acetylation, which proved the location of the hydroxy group at the aromatic ring. This metabolite has not been previously reported for humans (Idle et al., 1979; Allen et al., 1975, 1976). Because a person with 13 active CYP2D6 genes metabolizes debrisoquine very rapidly to (S)-4-hydroxydebrisoquine, a further oxidation step at the aromatic ring might occur. In subjects with lower CYP2D6 activities, such high concentrations of (S)-4-hydroxydebrisoquine might never be reached.

The other two peaks (II and III, m/z 208) (fig. 1) were detectable in all subjects studied. The intensity of the ion-current chromatograms decreased with declining numbers of active CYP2D6 genes. The fragmentation patterns, together with several chemical experiments, showed that peak II was formed by 3-hydroxylation of debrisoquine, followed by ring opening and further oxidation (presumably by aldehyde dehydrogenase) (fig. 2) to 2-(guanidinoethyl)phenylacetic acid; peak III was formed by 1-hydroxylation of debrisoquine, ring opening, and further oxidation of the aldehyde to 2-(guanidinoethyl)benzoic acid (fig. 2). These two metabolites had been previously detected in the urine of rats, dogs, and humans (Idle et al., 1979; Allen et al., 1975, 1976) and classified as minor metabolites of debrisoquine. Allen et al. (1975, 1976) studied one human subject and detected 10–15% of the given dose as acidic metabolites in the 0–24-hr urine samples. The metabolites were identified as 2-(guanidinomethyl)phenylacetic acid and 2-(guanidinoethyl)benzoic acid. Determination of the phenotype of that subject was not possible. Idle et al. (1979) studied five different human subjects, all EMs according to their debrisoquine MR (0.78–10.1). The amounts excreted as polar metabolites in the 0–24 hr urine samples varied greatly among the subjects, from 0 to 15.1% of a given dose, sometimes even exceeding the amount of excreted 4-hydroxydebrisoquine. In the present investigation, the 1- and 3-hydroxylations of debrisoquine seem to be as important as the 4-hydroxylation of debrisoquine, based on the intensities of the peaks in reconstructed ion-current chromatograms. The metabolites could, however, not be quantified without reference compounds, because of possible differences in ionization efficiencies.

To investigate which enzyme is involved in the 1- and 3-hydroxylation of debrisoquine, a Spearman rank correlation was performed to correlate the debrisoquine intensity/2-(guanidinoethyl)benzoic acid ratio (3-hydroxylation index) and the debrisoquine intensity/4-hydroxydebrisoquine intensity ratio (4-hydroxylation index). Correlation coefficients were \( r_S = 0.965 \) for 1-hydroxylation and \( r_S = 0.957 \) for 3-hydroxylation. The subjects with 0, 1, and 13 functional genes could be clearly separated from the groups of subjects with two or three functional genes. This indicates the involvement of CYP2D6 in both metabolic steps. The strong correlation \( (r = 0.971) \) between the debrisoquine intensity/4-hydroxydebrisoquine intensity ratio and the debrisoquine MR (Dalén P, Dahl M-L, Eichelbaum M, Bertilsson L, and Wilkinson G, submitted for publication) confirms the good agreement between the quantitative HPLC analysis and the determination of the intensities of ion-current chromatograms used in this study. It remains to be clarified whether the formation of 1- and 3-hydroxydebrisoquine by CYP2D6 is consistent with the proposed enzyme model (Koymans et al., 1992; Strobl et al., 1993).

In this study, it was shown that, in addition to the (S)-4-hydroxylation of debrisoquine, two other metabolic reactions of debrisoquine, i.e. 1- and 3-hydroxylation, are catalyzed by CYP2D6. It remains unclear which of the metabolites formed is of greater importance, although comparisons of the intensities of the peaks in the reconstructed ion-current chromatograms indicate greater amounts of the 1- and 3-hydroxylated metabolites than proposed earlier by Allen et al. (1975, 1976) and Idle et al. (1979). Further investigations using
standards for the acidic metabolites are necessary to clarify the situation.

References


