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Metabolism of imipramine *in vitro* by isozyme CYP2D6 expressed in a human cell line, and observations on metabolite stability

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ABSTRACT

A metabolism study of imipramine (IMI) has been conducted *in vitro* with commercially available human CYP2D6 isozyme expressed in a human AHH-1 TK +/− cell line. This enzyme system catalyzed the anticipated ring-oxidative biotransformation of IMI to 2-hydroxyimipramine (2-OH-IMI). In addition, however, the human CYP2D6 isozyme preparation was found to be unequivocally involved in the N-dealkylation of IMI to desmethylimipramine (DMI). 2-Hydroxydesipramine was also identified as a trace metabolite of IMI, but no 10-hydroxyimipramine was detected. The 2-OH-IMI metabolite was unstable and disappeared from metabolic solutions on standing. A procedure involving the O-acetylation of 2-OH-IMI was developed to minimize this decomposition. When an authentic sample of 10-OH-IMI was subjected to the same acetylation procedure, it was partially dehydrated to 10,11-dehydroimipramine, but also underwent unexpected degradations to two other products in which the dimethylaminopropyl side-chain was deaminated. Plausible structures for these two decomposition products are suggested from their gas chromatographic–mass spectrometric behaviour.

INTRODUCTION

Hepatic drug metabolism studies in humans are routinely conducted *in vitro* using fortified liver homogenate supernatants or microsomes prepared from them. While studies with homogenates, supernatants and microsomes supply information on overall metabolic processes, they generally provide only limited information on the specific enzyme(s) involved in most metabolic pathways. This limitation prompted Von Bahr *et al.* [1] to use purified cytochrome P450 isozymes in drug metabolism studies despite the requirement for large amounts of fresh liver and the tedious processes involved in the separation and purification of the isolated isozymes.

The metabolism of the antidepressant imipramine (IMI) in humans is well documented. Using

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human microsomes, Zeugin *et al.* [2] found that IMI was metabolized mainly by N-dealkylation to desmethylimipramine (desipramine, DMI). 2-Hydroxyimipramine (2-OH-IMI) was also isolated in significant amounts and 10-hydroxyimipramine (10-OH-IMI) was a trace metabolite. For structures see Fig. 1. Surprisingly, the major human *in vivo* metabolite of IMI, *i.e.* 2-hydroxydesipramine (2-OH-DMI), was not observed. These authors also found that metabolically formed 2-OH-IMI disappeared from metabolite solutions with the passage of time and attributed this to the presence of oxidants in some batches of solvents used in assay procedures. The *in vitro* metabolism of IMI has also been investigated in a homogenate of COS-1 cells in which human CYP2D6 (cytochrome P450IID6) was expressed [3]. Low yields of DMI and 2-OH-IMI were isolated together with trace quantities of 10-OH-IMI. In both studies, a high-performance liquid chromatographic (HPLC) procedure was used to quantify metabolites.

CYP2D6, expressed in human AHH-1 TK+/− cells, has recently become commercially available [4], and we are currently investigating its utility in drug metabolism studies with IMI. We have used combined gas chromatography–mass spectrometry (GC–MS) in our study because of our experience with the technique in the metabolism of trimipramine [5] and because it offers a better opportunity than HPLC to identify metabolites.

Our initial results indicated that the major observed metabolic reaction of IMI was its ring hydroxylation to 2-OH-IMI. A significant quantity of DMI was also formed, but no 10-OH-IMI was detected and only a trace amount 2-OH-DMI was produced. At the low concentration of substrate used, there was significant decomposition of the phenolic metabolite 2-OH-IMI, and linear calibration graphs could not be obtained. We now describe a GC–MS analytical procedure that we have developed to overcome these difficulties. The procedure is capable of quantifying low nanogram amounts of 2-OH-IMI, DMI and IMI in biological systems.

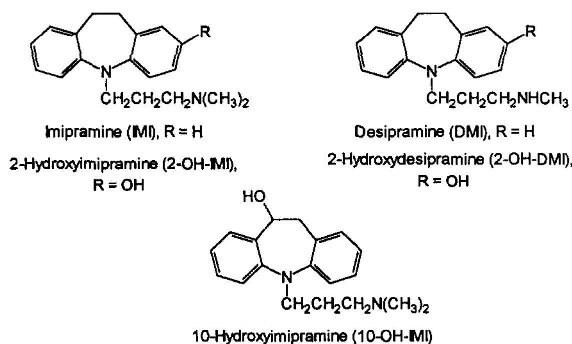

Fig. 1. Structures of imipramine and its metabolites.

EXPERIMENTAL

Purified human CYP2D6 isozyme

Human CYP2D6 microsomal protein and control microsomal protein were purchased from Gentest Corporation (Woburn, MA, USA). The CYP2D6 microsomal product was derived from a human AHH-1 TK+/− cell line which was transfected with complementary DNA that encoded human *CYP2D6*. The parent AHH-1 TK+/− cell line contains a low level of native human cytochrome P450 activity so cells that were not transfected were used as a control in all experiments involving transfected human cells. The commercial products were used as supplied.

Chemicals

Imipramine hydrochloride was purchased from Aldrich (Milwaukee, WI, USA). Desipramine hydrochloride, 2-hydroxyimipramine hydrochloride, 2-hydroxydesipramine oxalate and 10-hydroxyimipramine base were gifts from Ciba-Geigy (Mississauga, Canada). Iprindole (IPR) hydrochloride was a gift from Wyeth (Taplow, Maidenhead, UK). Other chemicals were purchased from various sources: acetic anhydride, analytical grade (Fisher Scientific, Ottawa, Canada); potassium hydrogencarbonate, analytical reagent (BDH, Toronto, Canada); NADP sodium salt from yeast (Terochem, Edmonton, Canada); D-glucose-6-phosphate, monosodium salt and glucose-6-phosphate dehydrogenase type XII from Torula yeast (Sigma, St. Louis, MO, USA).

Sample preparation

Solutions of the components of the NADPH-generating system were prepared as follows: 20 $\mu g/\mu l$ $NADP^+$ in 0.1 *M* potassium phosphate buffer (pH 7.4); 20 $\mu g/\mu l$ glucose-6-phosphate in the same buffer; 6.7 $\mu g/\mu l$ $MgCl_2 \cdot 6H_2O$ in distilled water. Glucose-6-phosphate dehydrogenase solution was used as supplied. A stock solution of internal standard (IPR) in distilled water, containing IPR hydrochloride equivalent to 10 mg/l of IPR base, was prepared.

Metabolism studies were conducted in KIMAX glass culture tubes (100 mm × 16 mm O.D.) purchased from Fisher Scientific.

Imipramine. A solution of IMI · HCl (40 μM) and CYP2D6 microsomal protein (0.5 mg) in potassium phosphate buffer (780 μl) was preincubated in a culture tube at 37°C for 5 min. Metabolism was started by adding NADPH-generating system [50 μl of $NADP^+$ solution, 50 μl of glucose-6-phosphate solution, 100 μl $MgCl_2 \cdot 6H_2O$ solution and 20 μl (equivalent to 1 U) of glucose-6-phosphate dehydrogenase], and incubation was continued at 37°C for 30 min in a shaking water bath in air. Metabolism was stopped by rapid cooling of the culture tube in ice, quickly followed by addition of internal standard solution (100 μl) and potassium bicarbonate (350 mg).

Control. The metabolism reaction described above for imipramine was repeated except that control microsomal protein derived from a human AHH-1 TK+/− cell line that had not been transfected with a complementary DNA was used instead of microsomal protein from the CYP2D6-containing cell line.

Acetylation

Acetic anhydride (300 μl) was carefully added to both basified metabolic IMI and control solutions, obtained as described immediately above, and when effervescence stopped, each mixture was extracted three times with 3-ml volumes of a mixed organic solvent (methylene chloride–diethyl ether, 11:14, v/v). Each combined organic extract was evaporated under a stream of nitrogen and dried extracts were separately reconstituted in toluene (25 μl). Aliquots (1 μl) were used for GC analyses.

Instrumental analysis

All GC analyses were performed on an HP 5730A gas chromatograph (Hewlett Packard, Avondale, PA, USA) equipped with a nitrogen–phosphorus (NP) detector. A DB-17 fused-silica capillary column (20 m × 0.32 mm I.D; 0.5 μm film thickness) was used for the separation of analytes. The initial column temperature of 180°C was held for 2 min, then increased to 280°C at a rate of 4°C/min and maintained at 280°C for 20 min. Ultrapure helium (Union Carbide, Edmonton, Canada) was used as carrier gas at a flow-rate of 1 ml/min. The injector and detector temperatures were 260 and 310°C, respectively. Chromatograms were recorded using an HP 3396A integrator; peak areas were measured.

The mass spectra (for confirmation of structures of metabolites) were recorded in the electron-impact mode (ionization voltage 50 eV) using a VG-7070E mass spectrometer (VG Instruments, Manchester, UK) coupled to a Varian Vista 6000 gas chromatograph (Varian Instruments, Sunnyvale, CA, USA). Mass spectra were automatically corrected for background. The GC column and temperature programmes used for separation of analytes were the same as those used in the GC–NP detection (GC–NPD) experiments. Helium was the carrier gas at a flow-rate of 3 ml/min.

RESULTS

Incubation of IMI with human CYP2D6 isozymes fortified with appropriate cofactors produced 2-OH-IMI and DMI in significant quantities and trace amounts of 2-OH-DMI. The structures of these metabolites were confirmed by comparing their GC retention times with those of authentic reference samples of 2-OH-IMI, DMI and 2-OH-DMI, by interpretation of their mass spectra and by comparing them with published spectra [6]. Quantitative analyses of these metabolites and their rates of formation will be described in a later manuscript. No 10-OH-IMI

was detected; the GC behavior of an authentic sample of this metabolite was determined to confirm the absence of 10-OH-IMI from the metabolism reaction. The GC analysis of a pure sample of 10-OH-IMI actually produced two peaks. Further comments on this observation are provided later. When extracts containing IMI, 2-OH-IMI, DMI and internal standard (IPR) were stored for two weeks at 4°C, then rechromatographed, the peak due to 2-OH-IMI was absent from the GC trace. During the two-week interval, the 2-OH-IMI had completely decomposed whereas the peaks due to IMI, DMI and IPR were unaffected. Attempts were made to prevent the decomposition of 2-OH-IMI. Addition of ascorbic acid, an antioxidant, to the solution prior to storage had no effect on the stability of 2-OH-IMI. Purging the extract with nitrogen prior to its storage was also unsuccessful. This analytical problem was solved by immediately acetylating the metabolism solutions with acetic anhydride at room temperature at the end of the incubation period. A preliminary experiment was conducted on authentic samples of IMI, DMI, 2-OH-IMI and 2-OH-DMI, and also on the internal standard IPR, to determine the stability of each drug or metabolite during the acetylation procedure and the extraction efficiency of the acetylated metabolites. This preliminary experiment also provided GC retention times (t_R) of authentic acetylated metabolites (Fig. 2). Structures of all acetylated products were confirmed by comparisons of their mass spectra with literature spectra [7]. Additional non-interfering peaks were routinely observed in the GC traces of the extract after the acetylation procedure (Fig. 2) and all are attributable to other components in the metabolism solution that were not derived from IPR, IMI or its metabolites. During actual metabolism studies (Fig. 2), these additional peaks were also observed in GC traces of most extracts.

Confirmation that 10-OH-IMI was not a metabolite of IMI in the human CYP2D6 microsomal preparation required a knowledge of the GC properties of an authentic sample of 10-OH-IMI, before and after its acetylation using the conditions employed in the IMI metabolism experiments. When underivatized 10-OH-IMI was analyzed by combined GC–MS, two GC peaks were observed (corresponding to peaks A and B in Fig. 3) with t_R values of 10.80 and 7.35 min, respectively. The mass spectra of both were recorded (Fig. 4) and interpreted (Fig. 5). This revealed that peak A was 10-OH-IMI, while the earlier-eluting peak B was the dehydrogenated product, 10,11-dehydroimipramine. Some dehydration had occurred "on-column". When an extract containing acetylated 10-OH-IMI was analyzed by GC–MS, no fewer than five peaks were observed (Fig. 3). The structures of three of these

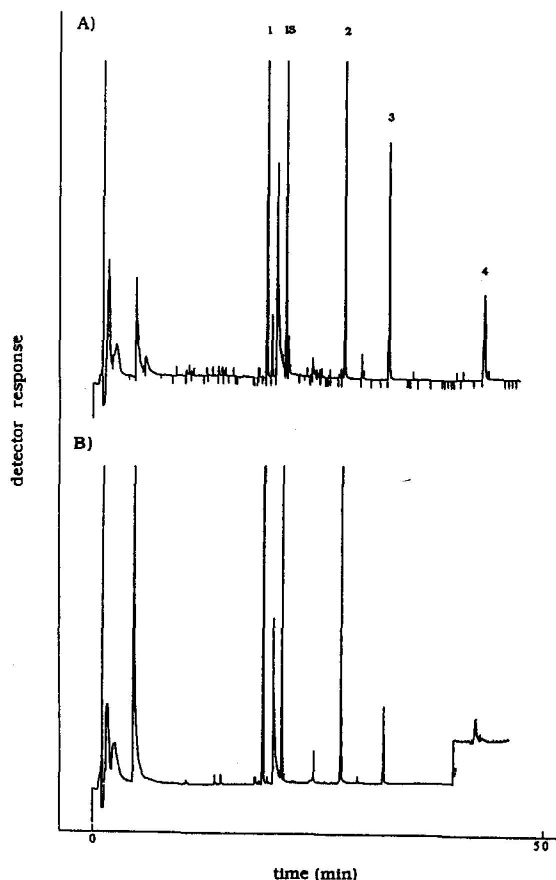

Fig. 2. GC–NPD traces of (A) an acetylated extract of an incubation solution containing authentic samples of imipramine (peak 1, t_R = 19.13 min), iprindole (internal standard, IS, t_R = 21.23 min), 2-acetoxyimipramine (peak 2, t_R = 27.73 min), N-acetyldesipramine (peak 3, t_R = 32.73 min) and 2-acetoxy-N-acetyldesipramine (peak 4, t_R = 43.56 min) and (B) an acetylated extract of an imipramine metabolism solution.

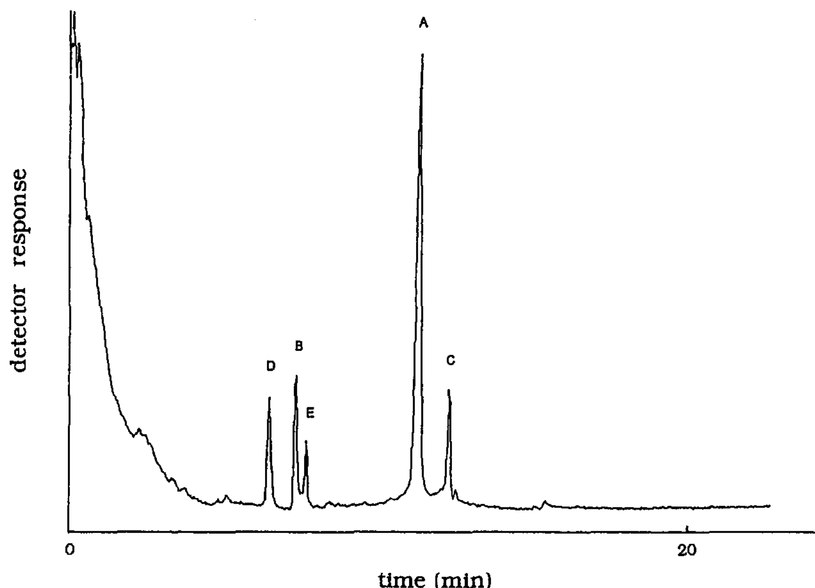

Fig. 3. GC trace obtained during the GC–MS analysis of an authentic sample of 10-OH-IMI which had been subjected to acetylation. Peaks: D = N-(1-propenyl) analogue (IVa) of 10-OH-IMI (t_R = 6.60 min); B = 10,11-dehydroimipramine (II) (t_R = 7.35 min); E = N-(1-propenyl) analogue (IVb) of 10-acetoxyimipramine (t_R = 7.68 min); A = 10-OH-IMI (Ia) (t_R = 10.80 min); C = 10-acetoxyimipramine (Ib) (t_R = 11.67 min).

five components were readily deduced from their mass spectra (Fig. 4) to be 10-OH-IMI (peak A), 10-acetoxyimipramine (peak C; t_R 11.67 min) and 10,11-dehydroimipramine (peak B). Fragmentation pathways compatible with these structures and common to all of them are provided in Fig. 5.

When the spectra of peaks D (t_R 6.60 min) and E (t_R 7.68 min) were examined, it was immediately apparent that both products were structurally related to 10-OH-IMI. An inspection of these mass spectra (Fig. 4) revealed that analyte E (MW 293) was the acetylated derivative of analyte D (MW 251). The presence of fragment ions of *m/z* 251, 233, 220, 210, and 192 in the spectrum of peak D were clearly structurally closely related to ions of *m/z* 251, 233 and 232, 218, 207 and 206, and 193 in the spectrum of 10-OH-IMI (peak A). Also, the absence of ions of *m/z* 58 and 85 revealed that the $CH_2CH_2CH_2N(CH_3)_2$ side-chain of analytes A, C and B had been modified in the formation of peaks D and E from A. The odd mass of the molecular ion of peak D indicated the presence of a single N atom (the ring N) in this degradation molecule. Conversion of A to analyte D requires the expulsion of a fragment of 45 a.m.u. that contains an N atom; this can only be $(CH_3)_2NH$. An initial conclusion from these data was that analytes D and E possessed structures IIIa and IIIb (Fig. 6), respectively, but further reflection revealed that this could not be true. If the structure of analyte D was IIIa, the initial fragmentation in its mass spectrum would be the expulsion of H_2O to produce an ion of *m/z* 233, identical to the ion of that mass in the spectra of A, C and B. This ion should fragment further exactly as depicted in Fig. 5 to ions of *m/z* 218, 207 and 206, but these fragmentation pathways were not observed. Also incompatible with analytes D and E possessing structures IIIa and IIIb is the abundance of the ion of *m/z* 192 which is the base peak in both spectra (Fig. 4) whereas it is of low abundance in the spectra of A, C and B. The only feasible explanation of these data is that analyte D is an isomer of IIIa that differs only in the nature of the C_3H_5 substituent on the ring N. Two possibilities are a CH=CH–CH_3 side-chain and a cyclopropyl group. If the side-chain was

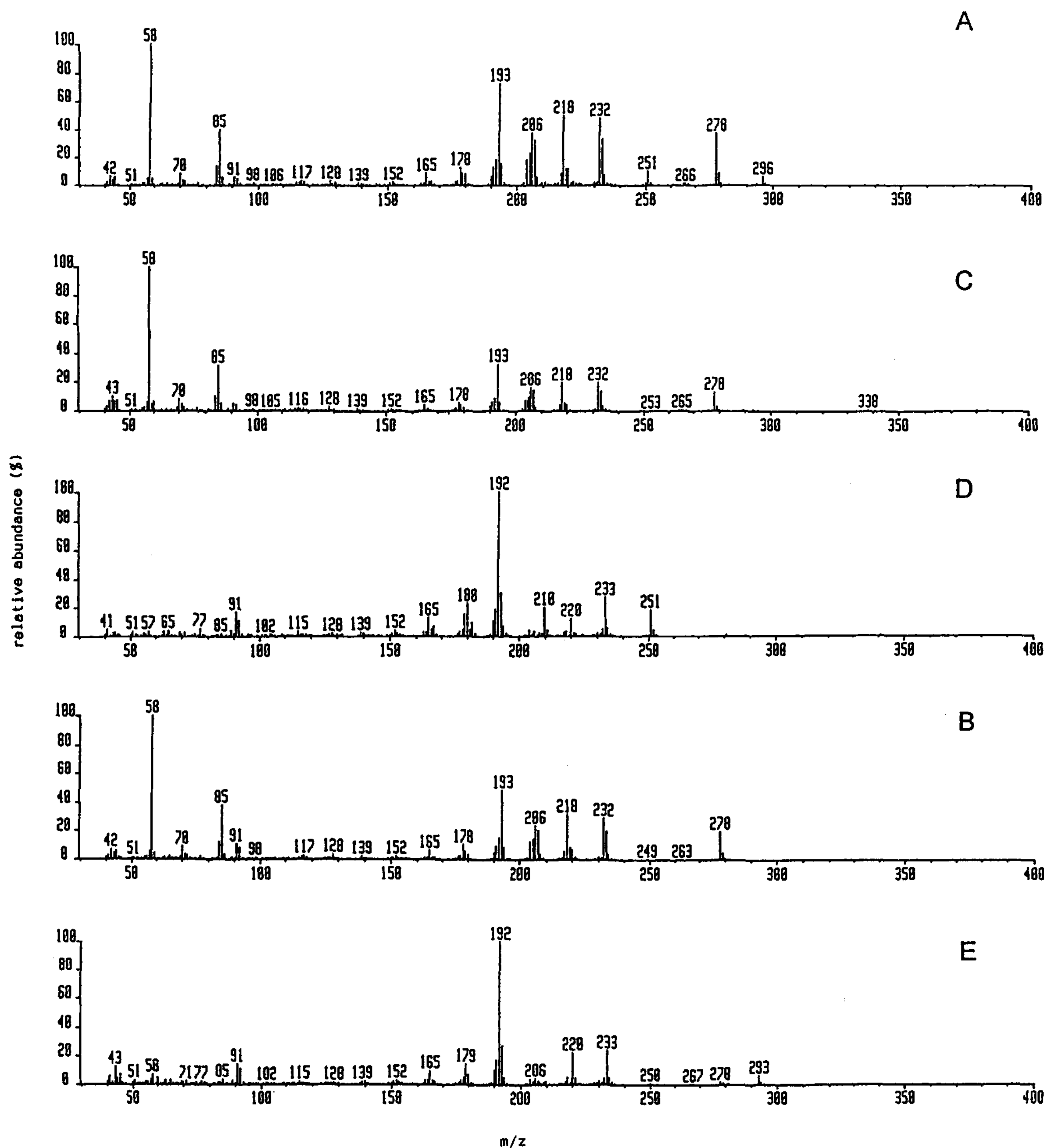

Fig. 4. Mass spectra of acetylated 10-OH-IMI (peak A in Fig. 3) and its degradation products (peaks B–E in Fig. 3).

CH=CH–CH_3, analytes D and E would possess structures IVa and IVb (Fig. 6), respectively. The compatibility of structure IVa with the mass spectrum of analyte D is indicated in Fig. 7. It is apparent from these observations that alicyclic compounds such as 10-OH-IMI undergo chemical degradation during the relatively mild processes of aqueous acetylation and subsequent GC.

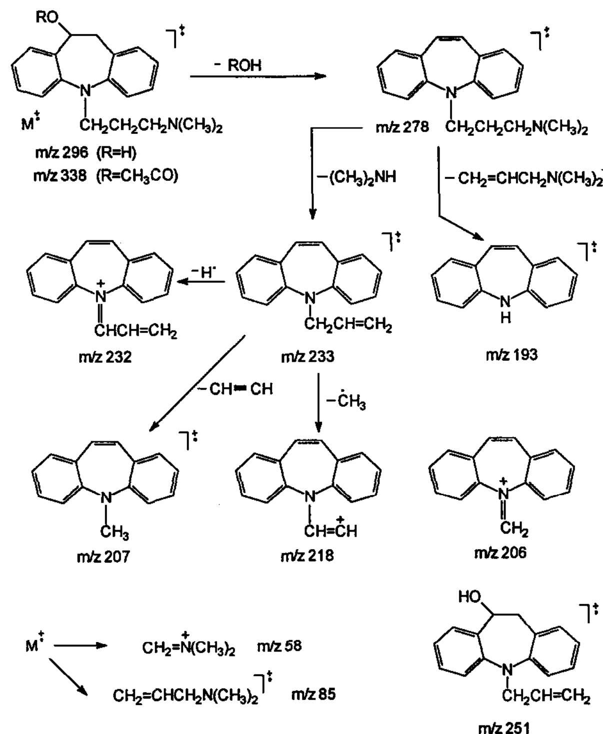

Fig. 5. Common fragmentation ions in the mass spectra of 10-hydroxyimipramine (MW 296; peak A in Fig. 3), 10-acetoxyimipramine (MW 338; peak C in Fig. 3) and 10,11-dehydroimipramine (MW 278; peak B in Fig. 3). (The ion of *m/z* 206 may arise from either of ions *m/z* 278 or 233. The ion of *m/z* 251 is present only in the spectrum of A in Fig. 4).

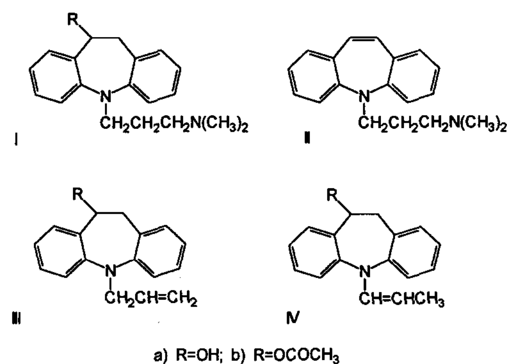

Fig. 6. Structures of the products obtained when an authentic sample of 10-OH-IMI (Ia) was acetylated and analyzed by GC. The products Ib, II, IVa and IVb are identified in Fig. 3. Structures IIIa and IIIb were also possible degradation products of Ia during acetylation but were rejected in favour of the isomeric structures IVa and IVb, respectively (see text).

DISCUSSION

This study has shown that a human CYP2D6 microsomal product prepared from the human AHH-1 TK+/− cell line is capable of catalyzing C_2-ring hydroxylation and N-dealkylation of IMI, but not 10-hydroxylation of IMI. It also catalyzes the 2-hydroxylation of DMI, but only to a very small extent.

Previous investigations have shown that 2-OH-IMI and 2-OH-DMI are major *in vivo* metabolites of IMI in humans and that the latter metabolite is formed mainly from the primary metabolite, DMI. The metabolism of IMI *in vitro* produces different results. When Zeugin *et al.* [2] and Brøsen *et al.* [3] investigated the biotransformation of IMI with human liver microsomes, the products identified were 2-OH-IMI and DMI, and a small amount of 10-OH-IMI, but no 2-OH-DMI was detected. Metabolism of IMI by the microorganism, *Mucor griseocyanus*, also produced DMI, 2-OH-IMI and 10-OH-IMI, but no 2-OH-DMI [8].

When we commenced this investigation of the *in vitro* metabolism of IMI with CYP2D6 isozyme expressed in a human AHH-1 TK+/− cell line, the anticipated result was that oxidation of IMI to 2-OH-IMI would be the major metabolic pathway observed since this biotransformation sequence is known to be catalyzed by CYP2D6 [3,9,10]. We also expected small amounts of 10-OH-IMI in view of the investigations by others that have just been described. No N-dealkylation to DMI and 2-OH-DMI was expected because the N-dealkylation metabolic pathway is generally not associated with the CYP2D6 isozyme. Biotransformation of IMI to 2-OH-IMI was observed but no 10-OH-IMI was detected. Somewhat surprisingly, N-demethylation of IMI to DMI occurred consistently, although, based on GC peak areas, to a lesser extent than 2-OH-IMI formation. Studies on the quantification the 2-OH-IMI and DMI formed are currently in progress. That metabolic formation of DMI was indeed catalyzed by CYP2D6 can be inferred from the observation that it was present in incubates containing the CYP2D6 microsomal protein, but

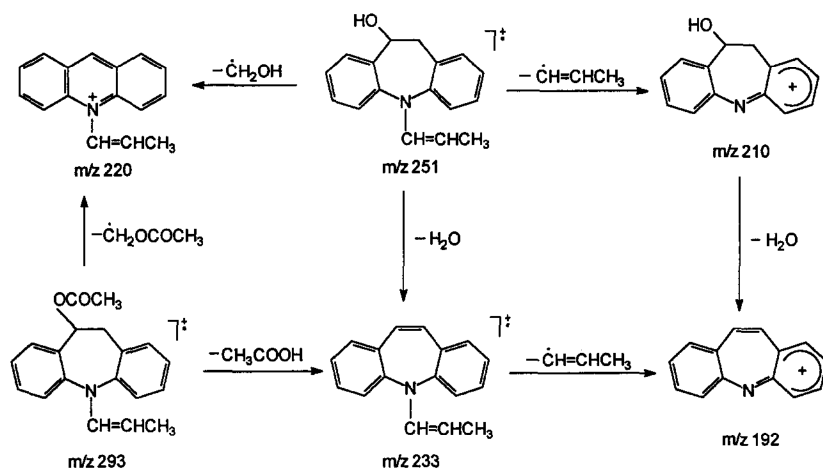

Fig. 7. Proposed mass spectral fragmentation pathways of N-(1-propenyl) analogues (MW 251; peak D in Fig. 3) and its O-acetyl derivative (MW 293; peak E in Fig. 3) of 10-hydroxyimipramine (Ia) formed as decomposition products during GC–MS analysis of acetylated Ia.

was absent from incubates containing microsomal product derived from the human cell line that lacked CYP2D6.

This study has overcome to a great extent the problem of the instability of 2-OH-IMI in biological media by the performance of a rapid acetylation of the metabolism mixture at the conclusion of the metabolic reaction. The acetylation was conducted in the aqueous medium of the metabolism solution. Previous investigators [11] have employed acetylation in the isolation of IMI and its metabolites from biological samples, but the method they employed was a tedious one in which phenolic bases were separated from non-phenolic bases, then dried before being acetylated with acetic anhydride in dry pyridine for a 3-h period. An unexpected observation was made during the development of the acetylation technique for the analysis of metabolites. A pure sample of 10-OH-IMI partially underwent a combination of O-acetylation, 10,11-dehydrogenation and side-chain modification to produce four degradation products, and thus complicating the analytical technique. Fortunately, 10-OH-IMI was not detected as a metabolite of IMI in this study, but in other investigations that require quantitation of 10-OH-IMI, the acetylation technique could not be used.

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