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Metabolism of N,N-dialkylated amphetamines, including deprenyl, by CYP2D6 expressed in a human cell line

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1. Five N,N-dialkylated amphetamines, N-methyl-N-propargylamphetamine (deprenyl; DEP), N-benzyl-N-methylamphetamine (benzphetamine; BPA), N-allyl-N-methylamphetamine (AMA), N,N-diallylamphetamine (DAA) and N-methyl-N-propylamphetamine (MPA), were metabolized *in vitro* with a microsomal preparation from cells expressing human CYP2D6 to determine what influence the N,N-dialkyl substituents had on the extent of N-dealkylation and/or aromatic ring oxidation.

2. The results obtained from experiments with the first two substrates, DEP and BPA, were surprisingly different. Whereas DEP was N-demethylated and N-depropargylated by the CYP2D6 enzyme system, no metabolites were formed from BPA. Subsequently, it was determined that AMA, DAA and MPA also underwent CYP2D6-catalysed N-dealkylation. Both N-methyl- and N-allylamphetamine were identified as products of AMA metabolism; similarly, metabolism of MPA produced both N-methyl- and N-propargylamphetamine, and N-allylamphetamine was the sole metabolite of DAA.

3. No N,N-didealkylated product (i.e. amphetamine) was isolated from incubates of any of the five substrates, and none of the N,N-dialkylated substrates was metabolized to a ring-hydroxylated product.

4. Rates of these CYP2D6-catalysed reactions were dependent on the nature and degree of unsaturation of the N-substituents.

Introduction

Our interest in the involvement of CYP2D6 in the N-dealkylation of amphetamine derivatives (Bach *et al.* 1999) has been extended to include N,N-dialkylated derivatives. In a preliminary study, benzphetamine [(+)-N-benzyl-N-methylamphetamine; BPA] and (−)-deprenyl [(−)-N-methyl-N-propargylamphetamine; DEP] were metabolized with a fortified expressed CYP2D6 enzyme preparation using experimental conditions that had previously been employed successfully to metabolize other amphetamine derivatives and some tricyclic antidepressants (Coutts *et al.* 1994a, Bach *et al.* 1999). Surprisingly, whereas DEP was extensively metabolized to two major products in this system, no metabolites were produced from BPA. Others (Grace *et al.* 1994, Wacher *et al.* 1996) have since identified DEP's two CYP2D6-catalyzed metabolites as N-methylamphetamine (MA) and N-propargylamphetamine (PgA), but there was an appreciable difference in the relative amounts of both metabolites produced by recombinant CYP2D6 and CYP2D6-containing liver microsomes. PgA formation was favoured 13-fold over MA formation in the studies by Grace *et al.* (1994), but both metabolites were isolated by Wacher *et al.* (1996) in approximately equal quantities. Grace *et al.*

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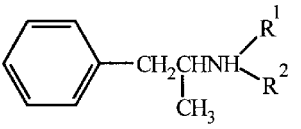


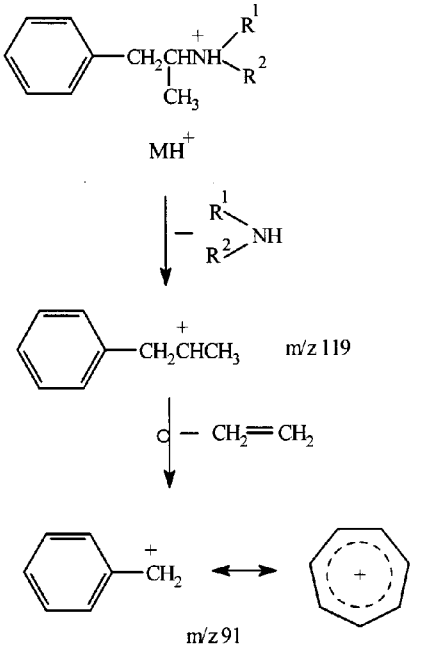
Compound	R^1	R^2
AA	H	CH_2-CH=CH_2
AMA	CH_3	CH_2-CH=CH_2
MPA	CH_3	$(CH_2)_2CH_3$
DEP	CH_3	CH_2-C≡CH
PgA	H	CH_2-C≡CH
DAA	CH_2-CH=CH_2	CH_2-CH=CH_2
DPgA	CH_2-C≡CH	CH_2-C≡CH
MA	H	CH_3
PA	H	$(CH_2)_2CH_3$
BPA	CH_3	$PhCH_2$

Figure 1. Structures of N,N-dialkylamphetamine analogues and N-dealkylated metabolites. Abbreviations are identified in the text.

(1994) described this metabolism of DEP as being an 'atypical' dealkylation, presumably because very few examples of CYP2D6-catalyzed N-dealkylation of basic drugs were known at that time. It is now recognized that many drugs, including amiflamine, amitriptyline, desmethylcitalopram, haloperidol, imipramine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), mianserin, tomoxetine, trazodone and venlafaxine, do undergo CYP2D6-catalyzed N-dealkylation (Coutts *et al.* 1994a, Coutts and Urichuk 1999), although in most instances CYP2D6's role in this important metabolic reaction is relatively minor.

Our observation that the N-demethylation and N-depropargylation of DEP were catalysed to some extent by CYP2D6, whereas no N-demethylation or N-debenzylation of BPA was catalysed by this enzyme, prompted the determination whether other N-methyl-N-alkylamphetamines could be N-demethylated and/or N-dealkylated by the catalytic action of CYP2D6. Three additional N,N-dialkylated amphetamines, N-allyl-N-methylamphetamine (AMA), N,N-diallylamphetamine (DAA) and N-methyl-N-propylamphetamine (MPA) were selected for this study. They were readily synthesized and their structures (figure 1) were confirmed by nuclear magnetic resonance (NMR) and electrospray mass spectrometry (ESMS; figure 2). N-allylamphetamine (AA), a potential metabolite of AMA and DAA, and DEP's metabolite, PgA, were also synthesized and similarly characterized. (−)-N,N-dipropargylamphetamine (DPgA) was isolated in low yield as a by-product in the synthesis of PgA, but its CYP2D6-catalyzed metabolism was not investigated at this time. Its mass spectrum was recorded for comparison with those of the other amphetamine derivatives (figure 2).

To determine whether the N-allyl and N-propyl analogues of DEP were suitable substrates for CYP2D6-catalyzed N-dealkylation, the *in vitro* metabolism of AMA, DAA, MPA, and also DEP and BPA, under identical conditions, was studied using CYP2D6 expressed in a human cell line. Since all of the compounds investigated,



	R^1	R^2
AA	$CH_2CH{=}CH_2$	H
AMA	$CH_2CH{=}CH_2$	CH_3
MPA	$CH_2CH_2CH_3$	CH_3
DEP	$CH_2C{\equiv}CH$	CH_3
PgA	$CH_2C{\equiv}CH$	H
DAA	$CH_2CH{=}CH_2$	$CH_2CH{=}CH_2$
DPgA	$CH_2C{\equiv}CH$	$CH_2C{\equiv}CH$

Compound	m/z	% Relative Abundance		
		MH^+	m/z 119	m/z 91
AA	176	100.0	36.1	63.0
AMA	190	100.0	19.9	29.8
MPA	192	100.0	13.5	18.0
DEP	188	100.0	35.2	53.1
PgA	174	79.1	43.8	100.0
DAA	216	100.0	20.9	32.2
DPgA	212	87.3	68.7	100.0

Figure 2. Characteristic fragment ions in the electrospray mass spectra of N-mono- and N,N-dialkylated amphetamines.

except DAA, are N-methylated and also N-alkylated with another moiety, to avoid confusion 'demethylation' has been used to indicate the loss of the methyl group, and 'dealkylation' to indicate the loss of the other moiety.

Materials and methods

Instrumentation

GC analyses were conducted using a DB-5 fused capillary column, 13 m × 0.25 mm i.d. × 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA) on a HP 5890 GC (Hewlett Packard, PA, USA) equipped with a nitrogen-phosphorus detector and a HP-3396A integrator. The carrier gas was ultrapure helium (Union Carbide, Edmonton, Canada) and its flow rate was adjusted to maintain a column head pressure of 10 psi. The make-up gas at the detector was a mixture of hydrogen (3 ml min^{-1}) and air (80 ml min^{-1}). The injector and detector temperatures were 260 and 310 °C respectively. Chromatographic peak areas were measured with a HP 3396A. GC conditions were: an initial column temperature of 110 °C held for 2 min and then raised by automatic temperature programming at 8 °C min^{-1} to 240 °C.

Electrospray mass spectrometric analyses were performed on Fisons Trio 2000. Samples were sprayed at 75 °C at a probe voltage of 3.5 KV.

NMR spectra were recorded on a Bruker 300 MHz instrument using $CDCl_3$ as a solvent and TMS as an internal standard

Chemicals

Amphetamine sulphate and 4-methoxy-N-methylamphetamine (MMA).HCl were gifts from the former Smith, Kline and French Laboratories (Philadelphia), and benzphetamine HCl was kindly supplied by Professor A. H. Beckett and Dr D. A. Cowan, King's College, London. N-(*n*-propyl) amphetamine.HCl and N-methylamphetamine.HCl were prepared by a reported method (Coutts *et al.* 1976, 1978). Other chemicals were purchased from various commercial sources: allyl bromide, 1-bromopropane, analytical grade trifluoroacetic acid (Aldrich Chemical Co., Milwaukee, WI, USA);

R-(−)-deprenyl.HCl (Research Biochemicals International, Natick, MA, USA); $NADP^+$ sodium salt from yeast, D-glucose 6-phosphate monosodium salt (G6P) and glucose 6-phosphate dehydrogenase (G6PD) type XII from *Tortula* yeast (Sigma Chemical Co., St Louis, MO, USA); potassium carbonate (K_2CO_3), methanol (CH_3OH), *n*-hexane, HPLC-grade acetonitrile (CH_3CN), isopropanol, dichloromethane (CH_2Cl_2), diethyl ether, toluene (BDH, Toronto, Canada). All solvents were distilled before use. The K_2CO_3 solution used was a 25% w/v aqueous solution. The buffer solution used in all experiments was 100 mM, pH 7.4.

Synthesis of potential substrates of CYP2D6 and their metabolites

(±)-*N-allylamphetamine* (*AA*) *and* (±)-*N,N-diallylamphetamine* (*DAA*). (±)-Amphetamine sulphate (500 mg; 2.71 mmol) was suspended in CH_3CN (3 ml) and basified with K_2CO_3 solution (25% w/v; 1.5 ml; 2.71 mmol). The CH_3CN solution was decanted into another flask. The residue of K_2SO_4 was washed with CH_3CN (2.5 ml × 2). To the combined organic solution, allyl bromide (117.45 μl; 1.36 mmol) was added, followed by K_2CO_3 solution (1.5 ml). The reaction mixture was left to stir at room temperature for 1 h during which time samples of the reaction mixture were examined by tlc using 10% CH_3OH in CH_2Cl_2 as the developing solvent. When the absence of allyl bromide was indicated, the reaction mixture was evaporated (rotatory evaporator) and the residue obtained was dissolved in water (40 ml). This solution was basified with K_2CO_3 solution (1.5 ml) and extracted with CH_2Cl_2 (5 ml × 3). The combined extract was evaporated to dryness and the product was purified by silica gel column chromatography, using 2% CH_3OH in CH_2Cl_2 as eluting solvent. Two products were obtained: (±)-N,N-diallylamphetamine (DAA; 24.80 mg) eluted first, followed by (±)-N-allylamphetamine (AA; 112.20 mg). Both were liquids. Total yield was 67.4%. AA was converted to a salt by treating a solution of it in diethyl ether with dry HCl gas, with cooling (solid CO_2 in acetone). The melting point of AA.HCl (recrystallized from ethyl acetate) was 168.5–169.5 °C. ^{1}H-NMR ($CDCl_3$) for AA, δ: 7.22–7.17 (m, 5H, **Ph**); 5.91–5.78 (m, 1H, allyl C**H**); 5.15–5.03 (m, 2H, terminal allyl C$\mathbf{H}_2$); 3.36–3.29 (m, 1H) and 3.24–3.16 (m, 1H) (allyl N-C$\mathbf{H}_2$); 3.00–2.90 (m, 1H, N-C**H**); 2.80–2.73 and 2.64–2.57 (two dd, 2H, $\mathcal{J}_{gem}$ = 10 H_z, $\mathcal{J}_{vic}$ = 5 Hz; C$\mathbf{H}_2$Ph); 1.07–1.05 (d, 3H, $\mathcal{J}$ = 5 Hz, CH-C$\mathbf{H}_3$).

The electrospray mass spectrum of AA was consistent with the proposed structure (figure 2).

When this reaction was repeated using excess allyl bromide (four equivalents relative to the quantity of amphetamine), a single product, DAA, was detected by tlc. The crude DAA was subjected to silica gel column chromatography using a mixture of ethyl acetate and *n*-hexane (1:4 v/v ratio) as developing solvent. The chromatographed product was converted to its HCl salt as described above in the synthesis of AA.HCl. (±)-DAA.HCl was obtained in 85% yield as a colourless solid, m.p. 162.0–163.0 °C when recrystallized from ethyl acetate. ^{1}H-NMR ($CDCl_3$) for DAA, δ: 7.25–7.14 (m, 5H, **Ph**); 5.88–5.75 (m, 2H, two allyl C**H**); 5.22–5.08 (m, 4H, two terminal allyl C$\mathbf{H}_2$); 3.22–3.06 (m, 5H, two allyl N-C$\mathbf{H}_2$ overlapping N-C**H**); 2.96–2.90 (dd, 1H, $\mathcal{J}$ = 8 Hz and $\mathcal{J}$ = 6 Hz; C$\mathbf{H}_2$Ph); 2.44–2.36 (dd, 1H, $\mathcal{J}$ = 8 and 3 Hz, C$\mathbf{H}_2$Ph); 0.95–0.93 (d, 3H, $\mathcal{J}$ = 5 Hz, CH-C$\mathbf{H}_3$).

The electrospray mass spectrum of DAA was consistent with the proposed structure (figure 2).

(±)-*N-allyl-N-methylamphetamine* (*AMA*). (±)-N-methylamphetamine.HCl (200.0 mg; 1.08 mmol) was suspended in CH_3CN (10 ml) and converted to its free base by the addition of K_2CO_3 solution (1 ml). To the base (isolated by the method used in the preparation of AA and DAA), an excess of allyl bromide (2.69 mmol; 233 μl) was added, followed by K_2CO_3 solution (1 ml). The resulting mixture was stirred at room temperature for 45 min at which time tlc monitoring of the reaction mixture indicated that > 90% of MA had been consumed. The residue obtained after solvent evaporation was dissolved in H_2O (10 ml) and basified with K_2CO_3 solution, then extracted into CH_2Cl_2. Chromatographic purification of the crude product on silica gel using 2.5% CH_3OH in CH_2Cl_2 as eluting solvent yielded (±)-AMA base (224.0 mg; liquid) in 92.1% yield. The base was converted to its HCl salt and recrystallized from ethyl acetate as a colourless solid, m.p. 130.5–132 °C. ^{1}H-NMR ($CDCl_3$), δ: 7.30–7.16 (m, 5H, **Ph**); 5.95–5.82 (m, 1H, allyl C**H**); 5.30–5.12 (m, 2H, terminal allyl C$\mathbf{H}_2$); 3.17–3.15 (d, 2H, allyl N-C$\mathbf{H}_2$); 3.02–2.95 (m, 2H, one H of C$\mathbf{H}_2$Ph overlapping N-CH); 2.45–2.37 (dd, one H of C$\mathbf{H}_2$Ph); 2.30 (s, 3H, N-C$\mathbf{H}_3$); 0.96–0.94 (d, 3H, $\mathcal{J}$ = 5 Hz, CH-C$\mathbf{H}_3$).

The electrospray mass spectrum of AMA was consistent with the proposed structure (figure 2).

(±)-*N-methyl-N-propylamphetamine* (*MPA*). This N-dialkylamphetamine was synthesized by a procedure similar to that used for the preparation of AMA. The amine and halide reagent used was (±)-N-methylamphetamine.HCl (1.38 mmol; 257.0 mg) and 1-bromopropane (2.76 mmol; 251.66 μl) respectively. After 1 day of stirring at room temperature, a tlc of the reaction mixture was developed with 7% CH_3OH in CH_2Cl_2. This showed that almost no reaction had occurred, so more 1-bromopropane (121 μl) and K_2CO_3 solution (1.02 ml) were added and the reaction continued for another day. The crude product (isolated as described in the preparation of AA and DAA) was chromatographed on a silica gel column, initially with 2%, and subsequently with 5% CH_3OH in CH_2Cl_2. Fractions were collected and examined by tlc. Those containing the desired product were eluted by 5% CH_3OH in CH_2Cl_2. Evaporation of the combined eluates gave MPA as a liquid in modest yield (40%). MPA.HCl salt,

prepared as described for AMA.HCl, was a colourless solid, m.p. 113.0–114.0 °C. ^{1}H-NMR ($CDCl_3$), δ: 7.32–7.27 (m, 2H of Ph) and 7.22–7.17 (m, 3H of Ph); 3.10–2.86 (m, 2H, one H of C**H**$_2$Ph overlapping N-C**H**); 2.46–2.37 (m, 3H, one H of C**H**$_2$Ph and 2H of N-C**H**$_2$CH$_2$CH$_3$); 2.32 (s, 3H, N-C**H**$_3$); 1.58–1.44 (m, 2H, C**H**$_2$CH$_3$); 0.95–0.89 (overlapping d and t, 6H, CHC**H**$_3$ and CH$_2$C**H**$_3$).

The electrospray mass spectrum of MPA was consistent with the proposed structure (figure 2).

(−)-*N-propargylamphetamine* (*PgA*). PgA was synthesized according to the method of MacGregor *et al.* (1988). The crude product obtained from a reaction of (−)-amphetamine sulphate (250 mg; 1.36 mmol) with propargyl bromide (75 μl; 0.86 mmol) was purified on a silica column (0.75 × 8 inches) using 2% CH_3OH in CH_2Cl_2 as eluting solvent. A small amount of DPgA as side product eluted first, followed by PgA as a liquid in 75% yield. NMR data of the PgA coincided with reported literature values. PgA.HCl and DPgA.HCl salts were prepared as described for AMA.HCl. Both products were colourless solids when recrystallized from ethyl acetate. PgA.HCl had a m.p. = 162.5–163.5 °C (m.p. reported by MacGregor *et al.* 158–160 °C). The melting point of DPgA.HCl was 166–167 °C. Their electrospray mass spectra were consistent with the proposed structures (figure 2).

Microsomal protein

Human CYP2D6 microsomal preparation used in this study was purchased from Gentest Corporation (Woburn, MA, USA). It was derived from a human AHH-1 TK+/− cell line transfected with complementary DNA that encoded human CYP2D6. Total protein content was 10 mg/ml in 100 mM potassium phosphate (pH 7.4) and CYP2D6 content was 260 pmol/mg protein. Control microsomes obtained from the same human cell line that had not been transfected with specific cDNA, were purchased from the same source.

NADPH-generating system

An NADPH-generating system was prepared by mixing freshly prepared stock solutions of $NADP^+$ (1.3 mM; 1 mg/ml), G6P (3.3 mM; 1 mg/ml), $MgCl_2.6H_2O$ (3.3 mM; 0.67 mg/ml) and G6PD (50 U/ml buffer) in a 5:5:10:2 volume ratio.

In vitro *microsomal incubation, extraction and derivatization*

Metabolism of N-allyl-N-methylamphetamine (*AMA*). An incubation mixture containing AMA (6.9 nmol), CYP2D6 (12.5 μl; 0.125 mg) and phosphate buffer to a volume of 97.5 μl was preheated at 37 °C for 5 min before the enzymatic reaction was started by an addition of NADPH-generating system (27.5 μl). The resulting mixture (125 μl) was incubated at 37 °C for 2 h. The reaction was terminated by cooling in an ice bath, followed by the addition of K_2CO_3 solution (100 μl). Internal standard (MMA; 0.7 nmol) was added and the mixture was extracted with a mixed organic solvent (2% v/v isopropanol in *n*-hexane; 2 × 3.5 ml) by vortexing vigorously for 1 min, shaking mechanically for 5 min and then centrifuging for 5 min at 2300 rpm in a Du Pont Sorvall GLC-2B General Laboratory Centrifuge (Mississauga, ON, Canada). The organic layer was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at room temperature. The residue was trifluoroacetylated under anhydrous conditions as described below, and the derivatized sample was analyzed by GC as described above.

An identical incubation with control microsomes instead of CYP2D6 microsomes was also performed for comparison.

Derivatization procedure

The dried residue obtained at the end of each metabolic reaction was mixed with trifluoroacetic anhydride (50 μl) and acetonitrile (25 μl) in a test tube which was capped tightly with a screw cap before its insertion in a metal heating module at 60 °C for 30 min. The reaction mixture was allowed to cool to room temperature before the tube was opened and its contents evaporated to dryness at room temperature under a stream of dry nitrogen. The residue was dissolved in toluene (30 μl) and aliquots (2 μl) were analyzed by GC.

Quantitative analysis of metabolites

Known but varying amounts of authentic standards of all substrates (1.58–22.15 nmol) and metabolites (0.09–1.05 nmol) and a fixed quantity of internal standard (0.7 nmol MMA) was added to a series of tubes containing the same amount of NADPH-generating system, control microsomes and substrate as indicated in incubation samples. Extraction and derivatization procedures were then applied to these tubes, and their trifluoroacetylated products were analyzed by GC. Peak area ratios of metabolite to internal standard were plotted against concentrations of metabolite to produce calibration curves. The concentration of each metabolite in incubation mixtures was determined from the equation of the straight line derived from its calibration curve.

Metabolism of N-methyl-N-propylamphetamine (MPA), N,N-diallylamphetamine (DAA), deprenyl (DEP) and benzphetamine (BPA). The incubation procedure described above was repeated, except that substrate AMA was individually replaced with MPA, DAA, DEP or BPA (6.9 nmol).

The metabolism of DEP was also conducted in the presence of quinidine (0.638 nmol), an inhibitor of CYP2D6.

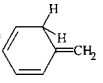
Results

The envisaged study required the synthesis of the substrates N-allyl-N-methylamphetamine, N,N-diallylamphetamine (a by-product of the synthesis of N-allylamphetamine) and N-methyl-N-propylamphetamine, and metabolites N-allylamphetamine and N-propargylamphetamine. The structures of these five compounds were confirmed by NMR and electrospray MS. Diagnostic ions (MH^+, m/z 119 and 91) were present in the mass spectra of all N-mono- and N,N-di-substituted compounds (figure 2). Most spectra also contained additional fragments of low abundance that were consistent with the structures from which they were derived. These fragment ions are identified in table 1.

When five N,N-dialkylated amphetamines (AMA, MPA, DAA, DEP, BPA) were individually incubated with the CYP2D6 isozyme fortified with appropriate

Table 1. Identities of minor fragment ions in the electrospray mass spectra of the synthesized N-mono- and N,N-dialkylated amphetamines.

Compound†	Fragment‡ (m/z; % rel. ab.)	Probable identity	
AA	84 (5.8%)	$CH_3CH{=}N^+HCH_2CH{=}CH_2$	(MH^+–C_7H_8)§
MPA	74 (7.8%)	$CH_3CH_2CH_2N^+H_2CH_3$	(MH^+–$C_6H_5CH{=}CHCH_3$)
DAA	174 (4.7%)	$C_6H_5CH_2C(CH_3){=}N^+HCH_2CH{=}CH_2$	(MH^+–$CH_3CH{=}CH_2$)
DAA	98 (7.2%)	$CH_2{=}CHCH_2N^+H_2CH_2CH{=}CH_2$	(MH^+–$C_6H_5CH{=}CHCH_3$)
DPgA	94 (17.9%)	$CH{\equiv}CHCH_2N^+H_2CH_2C{\equiv}CH$	(MH^+–$C_6H_5CH{=}CHCH_3$)

† Structures identified in Fig. 1; ‡ rel. ab., relative abundance; § C_7H_8= 

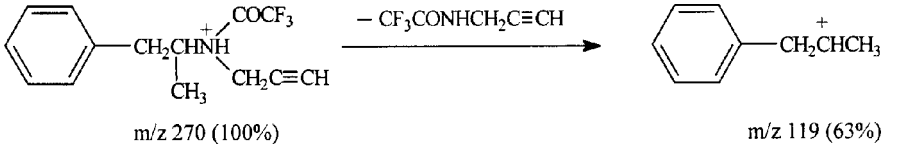


Figure 3. Diagnostic ions in the electrospray mass spectrum of the trifluoracetylated metabolite of deprenyl (PgA). Figures in brackets are % relative abundances.

Table 2. Yields of metabolites formed when various amphetamines were incubated with CYP2D6 and cofactors. Data are averages that differed by < 5% in duplicated experiments.

Substrate (6.9 nmol/incubation)	MA (pmol/incubation)	Nor metabolite (pmol/incubation)
AMA	98	279
MPA	44	73
DAA	(a)	594
DEP	624	1160
BPA	0	0

[a] Metabolism of DAA to MA is not possible.

cofactors, no ring hydroxylation of any of these substrates was observed. N-dealkylated metabolites, however, were detected in the incubation mixtures that contained AMA, MPA, DAA and DEP. AMA underwent two N-dealkylations to form the N-demethylation and N-deallylation products, AA and MA respectively. Similarly, CYP2D6-catalyzed metabolism of MPA produced MA and PA, and CYP2D6 promoted the production of MA and PgA from DEP.

In agreement with literature data (Grace *et al.* 1994, Wacher *et al.* 1996), DEP was metabolized by N-demethylation to nordeprenyl and by N-depropargylation to MA. As expected, these two metabolites were not observed when quinidine was included in the metabolism reaction. DAA was metabolized to AA, but no dideallylated product (amphetamine) was observed. The structures of these metabolites were confirmed by comparing their retention times with those of authentic reference samples of MA, PA and AA, and by interpretation of the mass spectrum of the N-demethylated metabolite of DEP after its trifluoroacetylation. This spectrum had two prominent ions of m/z 270 and 119, which are readily identified (figure 3). The quantities of metabolites formed are listed in table 2. No N-demethylated or N-dealkylated metabolites were detected in incubations of these substrates with control microsomes. This confirmed that CYP2D6 catalyzed these N-demethylations and N-dealkylations. Typical GC traces of derivatized incubation mixtures of DEP, AMA, MPA and DAA are shown in figure 4a and b.

Discussion

The anticipated result of this study on amphetamine derivatives was that the N,N-dialkylated amphetamines bearing an N-methyl substituent (AMA, MPA, DEP, BPA) would at least be N-demethylated since this pathway is known to be catalyzed partially by CYP2D6 (Coutts and Urichuk 1999). Ring hydroxylation was also expected because CYP2D6 can mediate the ring oxidation of other amphetamines such as N-*n*-butylamphetamine, N-ethylamphetamine and amphetamine (Bach *et al.* 1999). Three of the compounds investigated (AMA, MPA, DEP) were N-demethylated to varying extents (table 2) but, surprisingly, no CYP2D6-catalyzed N-demethylation or N-dealkylation occurred when BPA was incubated with CYP2D6 and none of the substrates was ring-hydroxylated. Aromatic ring-hydroxylated metabolites of the five dialkylated amphetamines (AMA, BPA, DEP, DAA, MPA) were not available to provide GC retention times of their O-acetylated derivatives and their mass spectra. However, it has been our experience that the GC retention times of the O-acetylated derivatives of many ring-hydroxylated drugs, including imipramine (Coutts *et al.* 1976, Su *et al.* 1993), trimipramine (Bolaji *et al.* 1993), methoxyphenamine, an amphetamine derivative (Coutts *et al.* 1994b), and N-alkylated amphetamines (Bach *et al.* 1999) are always in close proximity to those of the parent drug. If aromatic ring-hydroxylated metabolites had been formed in the present study, it probable that they would have been observed after acetylation in the GC traces and would have been readily identified by MS. No GC or MS evidence in favour of their presence was found.

The major structural difference in the four N-methylated substrates (AMA, MPA, DEP, BPA) is the nature of the N-alkyl groups (figure 1). These N-substituents appeared to influence the catalytic activity of CYP2D6 in the N-dealkylation pathway. Of the four alkyl groups, allyl ($CH_2CH{=}CH_2$), benzyl ($PhCH_2$), propargyl ($CH_2C{\equiv}CH$: and *n*-propyl ($CH_2CH_2CH_3$), the presence of

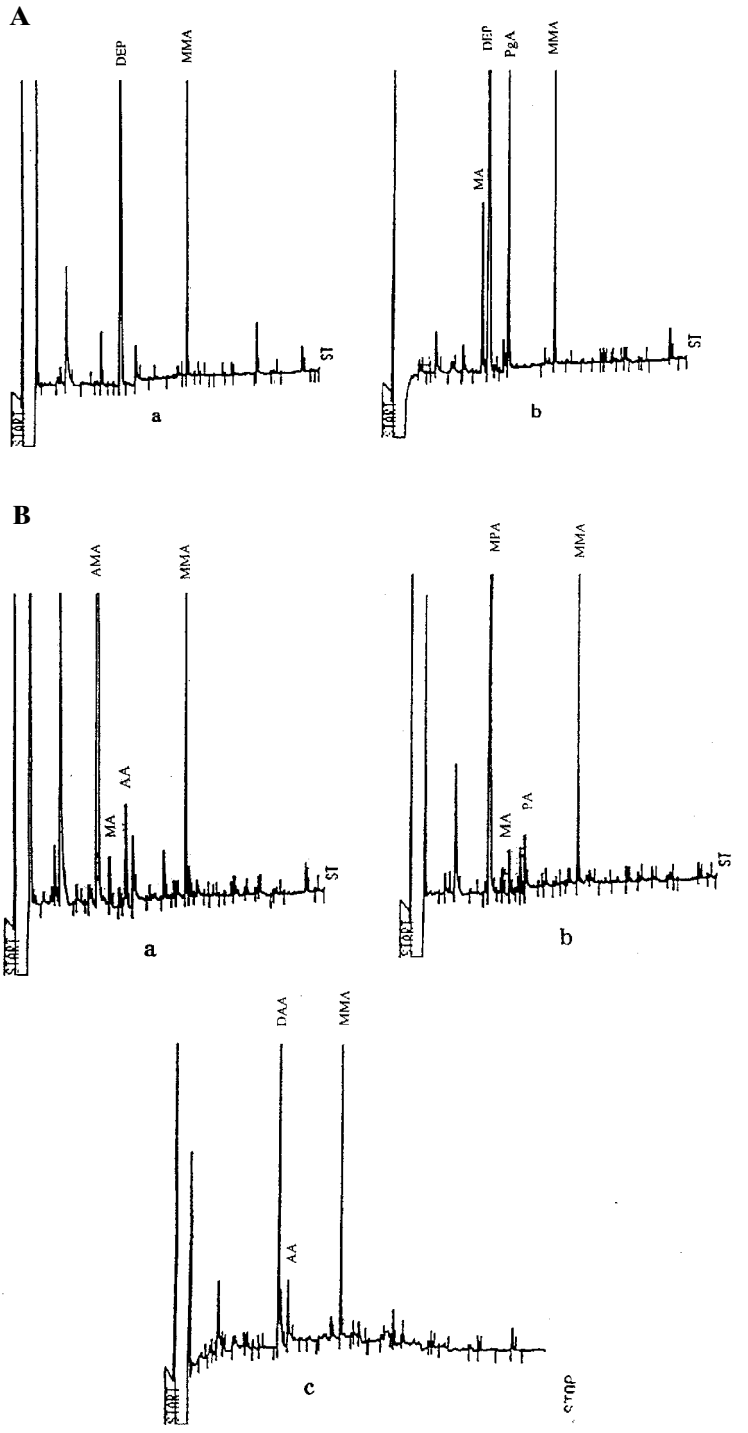


Figure 4. A. GC traces of trifluoracetylated dried extracts of DEP incubation with: (a) control microsomal protein; and (b) CYP2D6 enzyme preparation. MMA is the internal standard. B. (a) GC trace of trifluoroacetylated dried extract of AMA incubation with CYP2D6; (b) GC trace of trifluoroacetylated dried extract of MPA incubation with CYP2D6; (c) GC trace of trifluoroacetylated dried extract of DAA incubation with CYP2D6. Incubations were carried out under identical conditions. MMA is the internal standard.

the propargyl group had the greatest effect on CYP2D6 activity. Indeed, DEP was a very good *in vitro* substrate of CYP2D6 (table 2 and figure 4a). The two N-dealkylated metabolites of DEP (MA, nordeprenyl) were formed at different rates, indicating that CYP2D6 selectively mediated the cleavage of N-C bonds. When the propargyl group of DEP was replaced with an allyl group in AMA, the extent of N-dealkylation was much reduced, and when the propargyl group was replaced with an *n*-propyl group in MPA, a further reduction in the extent of N-dealkylation was observed. Finally, replacing the propargyl group with a benzyl moiety in benzphetamine resulted in a complete absence of CYP2D6-catalyzed metabolic N-demethylation or N-dealkylation. It appears that when the N atom of amphetamine contains both a 3-carbon substituent and a methyl group, the degree of unsaturation of the 3-carbon unit has an important influence on the extent of CYP2D6-catalyzed N-dealkylation. In addition, when the N-methyl group of AMA was replaced by an allyl substituent in DAA (which is N,N-diallylated), the extent of N-dealkylation was again increased (table 2). These findings suggest that the affinities of N-methyl, N-propyl, N-allyl and N-propargyl groups for CYP2D6 vary considerably.

In summary, this study has shown that human expressed CYP2D6 enzyme can catalyze the N-demethylation and N-dealkylation of four N,N-dialkylated amphetamines, namely AMA, MPA, DAA and DEP, but that it does not mediate the N-dealkylation of benzphetamine. Rates of these N-dealkylation reactions were clearly dependent upon the nature and extent of unsaturation of the N-substituents. Another noteworthy observation from this study was that none of the N,N-dialkylated substrates was ring-hydroxylated, in contrast to what was observed with N-monoalkylated amphetamines (Bach *et al.* 1999).

Results from the present study complemented those of previous investigators (Guengerich *et al.* 1986, Shet *et al.* 1993) who concluded that the N-demethylation of benzphetamine was catalyzed *in vitro* in humans by CYP3A4 and not by CYP2D6. Also, four of the five N,N-dialkylated amphetamines investigated were racemates. Only DEP was a single enantiomer. It would be of interest to extend the study to the enantiomers of all five N,N-dialkylated amphetamines to determine whether stereoselective metabolism is observed.

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