Differential foetal development of the *O*- and *N*-demethylation of codeine and dextromethorphan in man

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- 1 Codeine and dextromethorphan were *N*-demethylated in human foetal liver microsomes at high rates which were close to the activities in adult livers. In contrast, foetal liver microsomes did not catalyze the *O*-demethylation of these drugs at mid-gestation.
- 2 The metabolic data were in accordance with the absence of P450 IID6 and the presence of P450 IIIA as determined by Western blotting with anti-human P450 IID6 (MAb 114/2) and anti-rat P450 IIIA (PCN 2-13-1/C2) monoclonal antibodies, respectively.
- 3 The inhibitory effects of midazolam and dehydroepiandrosterone support the contention that the *N*-demethylase is a human foetal form of the cytochrome P450 IIIA family. Consistent with this we found that blotting with the MAb PCN 2-13-1/C2, which recognizes an epitope specific for the P450 III family, correlated well with the *N*-demethylase activities.

Keywords cytochrome P450 IID6 and IIIA dextromethorphan codeine human foetal liver

Introduction

Dextromethorphan has received much attention as a model drug for the common genetic polymorphism of drug oxidation known as the debrisoquine/sparteine type polymorphism (Eichelbaum, 1984; Idle & Smith, 1979; Schmidt *et al.*, 1985). This monogenically inherited deficiency relates to the metabolism of several drugs in wide clinical use such as β -adrenoceptor antagonists, antiarrhythmics and antidepressants (Brosen & Gram, 1989). Six to 10% of individuals in the Caucasian population have a deficiency or an absence of the responsible enzyme termed P450 IID6 (Gonzalez *et al.*, 1988; Nebert *et al.*, 1989; Zanger *et al.*, 1988).

The defect is due to an autosomal recessive trait. Populations in Far East Asia such as the Chinese and the Japanese seem to have a lower frequency of the poor metaboliser (PM) phenotype (Horai *et al.*, 1989). Other populations, like Ghanaians, appear to have a metabolic pattern similar to Caucasians concerning debrisoquine metabolism, but the PMs of this population show high metabolic activities for sparteine (Eichelbaum & Woolhouse, 1985; Woolhouse, 1986). Recently, codeine was reported to be subject to this polymorphism (Chen *et al.*, 1988; Dayer *et al.*, 1988; Mortimer *et al.*, 1990; Yue *et al.*, 1989). In a series of extensive metabolisers (EM), morphine formation from codeine correlated well with the immunodetected protein in Western blots using a

monoclonal antibody (MAb) 114/2 against human P450 IID6 (Mortimer *et al.*, 1990).

The polymorphic reaction with dextromethorphan and codeine involves *O*-demethylation to dextrorphan (Pfaff *et al.*, 1983) and morphine (Dayer *et al.*, 1988), respectively. Both compounds are also *N*-demethylated, to 3-methoxymorphinan (3-MM) and norcodeine (NC), respectively; these reactions have a greater quantitative importance *in vitro* in human liver microsomes (Mortimer *et al.*, 1990). In codeine treated patients NC is the major oxidised metabolite in plasma (Persson *et al.*, 1991).

Recent studies strongly suggest that the *N*-demethylation is catalysed by a different enzyme than the *O*-demethylation, although this has not been thoroughly investigated. Our observation that the inter-individual variation in *O*-demethylation is different from that in the *N*-demethylation of codeine and dextromethorphan (Mortimer *et al.*, 1990) gives support to this contention.

The use of codeine and dextromethorphan as model substrates for *in vitro* studies of foetal liver drug metabolism offers the possibility of investigating the ontogenic expression of the *O*- and *N*-demethylation pathways. More than one enzyme reaction may thus be compared by simultaneous assays using the same substrate. To our knowledge there are no clinical studies in children regarding the debrisoquine/sparteine polymorphism.

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We (Ladona *et al.*, 1988) have recently reported the presence of high foetal liver concentrations of a P450 isoenzyme related to P450 IIIA3 (Watkins *et al.*, 1985) by using the MAb PCN 2-13-1/C2. The concentration of this protein in foetal liver microsomes, as measured by immunoblotting, correlated well with the rate of ethylmorphine *N*-demethylation (Ladona *et al.*, 1989). As codeine and dextromethorphan are structurally closely related to ethylmorphine, it was of interest to study these aspects of their *N*-demethylation.

The aim of this study was to compare the ontogenic development of *N*-demethylation with that of *O*-demethylation using codeine and other drugs as substrates.

We now demonstrate that these pathways develop differently in foetal livers in that only the *N*-demethylase activity is measurable in mid-gestation.

Methods

Chemicals

Reagents used for Western blot analyses were purchased from BIORAD. Dextromethorphan hydrobromide, dehydroepiandrosterone, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase were from Sigma (Saint Louis, MO, USA). Midazolam, quinidine, levomethorphan, levorphan, dextrorphan, 3-methoxymorphinan and propafenone were obtained from Hoffman La Roche (Switzerland). Cyclosporine A was obtained from Sandoz AG (Switzerland). Morphine base was obtained from the hospital pharmacy (Nord. Pharmacopea) as well as ethylmorphine and codeine. To remove minor impurities codeine was further purified on a preparative silica column (7.8 mm $\times$ 300 mm, uPorasil, Waters), eluted with 3.3% v/v methanol and 0.5% w/v triethylamine in chloroform at a flow rate of 3 ml min⁻¹. Norcodeine (NC) and norethylmorphine (NE) reference standards were prepared by demethylation of codeine and ethylmorphine, respectively, according to Rice (1975). The purity was controlled by using the same chromatographic system as described above as well as the chromatographic system used for measuring the dealkylation in the incubation system. All other chemicals used were of reagent or analytical grade from common commercial sources.

Tissue material and isolation of microsomes

Human liver specimens from 12 Caucasian fetuses (CaLM) were obtained at legal abortion for socio-medical reasons. Abortions were induced by prostaglandin instillation. Livers were excised and frozen at -70°C until assay. The study was approved by the Ethics Committee of the University Hospital. Eleven of the 14 foetal specimens were used in a previous study of ethylmorphine *N*-demethylation (Ladona *et al.*, 1989). All samples were from fetuses 14–24 weeks of age, whose mothers were not on any drugs during pregnancy.

Microsomes were isolated as described previously (Rane *et al.*, 1985). The microsomal pellets were resuspended in 0.25 M sucrose containing 50 mM Tris-HCl pH 7.4 and stored at -70°C until assay.

Liver microsomes were isolated from 10 aborted Han-Chinese fetuses (socio-medical indication, 18–27 weeks of gestation) obtained after informed consent from the women in China. They were prepared in a solution containing: 0.25 M sucrose, 3 mM MgCl₂ and 10 mM Tris-HCl, pH 7.4 buffer. After homogenization the homogenate was subjected to a two-step centrifugation (600 g and then 9000 g 10 min each) before ultracentrifugation at 105 000 g for 60 min. Pellets were resuspended in the same buffer and stored on dry ice during transportation.

Protein measurement was made according to Lowry *et al.* (1951) using bovine serum albumin (fraction V; Sigma Chem Co) as standard.

Codeine metabolism Liver microsomes were incubated with 170 mM Tris-HCl pH 7.4, 5 mM MgCl₂, 2.5 mM glucose-6-phosphate, 0.5 mM NADPH, and 0.2 units of glucose-6-phosphate dehydrogenase in a final volume of 0.1 ml; microsomal protein concentration was 1 mg ml⁻¹. The reaction was started by addition of NADPH and incubation was at 37°C for 30 min in a shaking bath. The *N*-demethylation of codeine was linear with protein concentrations between 0.5 and 2 mg ml⁻¹ and with time up to 40 min. Enzyme kinetics experiments were carried out with concentrations of codeine ranging from 0.5 to 5 mM. Based on these experiments a 5 mM codeine concentration was chosen for the assays with various inhibitors. The morphine *N*-demethylation pathway was investigated by incubation of morphine according to Rane *et al.* (1985).

H.p.l.c. measurement of norcodeine, morphine and normorphine The equipment used was an LKB pump 2150 with a Rheodyne injector and an LKB 2151 variable u.v. detector operated at 214 nm. The separation was carried out on a Nova Pak CN column, 5 mm i.d., operated in an RCM module (Millipore, Waters) and eluted with a mobile phase of 35% v/v acetonitrile in 0.02 M pH 6.9 phosphate buffer. The retention times for norcodeine, morphine, normorphine and codeine were 6 min, 7 min 25 s, 5 min, and 9 min 35 s, respectively. The recoveries of norcodeine and morphine were 82 and 75%, respectively. The coefficient of variation for norcodeine assay in incubation samples spiked with 20 ng was 14% ($n = 8$). The limit of assay for norcodeine and morphine was 10 ng ml⁻¹ using u.v. detection. To detect and monitor formation of morphine at concentrations below 10 ng ml⁻¹, an electrochemical detector (ESA Coulochem 5100 A) was used (Svensson, 1986). The amount of morphine that could be detected under these conditions was 100 pg. The coefficient of variation at 1.5 ng ml⁻¹ was 6% ($n = 6$).

Extraction of metabolites from microsomal incubation mixtures was as described previously for ethylmorphine metabolism (Ladona *et al.*, 1989). Briefly, 1 ml 1 M pH 10 bicarbonate buffer was added to the incubation mixture, which was extracted with 4 ml 15% (v/v) propanol in dichloromethane mixture for 10 min. Three ml of the organic phase were transferred to another tube and the solvent was evaporated at 40°C under nitrogen. The residue was dissolved in 50 μ l of the mobile phase and 20 μ l was injected.

When investigating normorphine formation (from morphine, norcodeine or norethylmorphine) the extrac-

tion procedure included use of a Sep-pak light cartridge (Waters). The sample was mixed with 3 ml 0.5 M, pH 9.3 ammonium sulphate buffer and eluted through the cartridge, which was washed with 20 ml 5 mM pH 9.1 ammonium buffer. Normorphine was eluted with 600 μ l 10% (v/v) acetonitrile in a 10 mM pH 2.1 phosphate buffer. The eluate (20 μ l) was injected on the h.p.l.c. system using the conditions described above. The retention time was 5 min 25 s. Recovery of normorphine was 90% and the limit of assay was 25 ng ml⁻¹. The coefficient of variation was about 20%.

H.p.l.c. measurement of norethylmorphine was performed according to Ladona *et al.* (1989). Ethylmorphine *N*-demethylation was assayed for comparative purposes according to Ladona *et al.* (1989). The same conditions as for codeine were applied and the detector wavelength setting was 210 nm.

Dextromethorphan, 3-methoxymorphinan and dextrorphan metabolism *In vitro N*-demethylation of dextromethorphan and its primary metabolites and h.p.l.c. analysis of the dextrorphan, 3-methoxymorphinan and 3-OH-morphinan metabolites were performed as described previously for adult human liver microsomes (Mortimer *et al.*, 1990). Incubations were performed as for codeine and ethylmorphine, with a microsomal protein concentration of 1 mg ml⁻¹ and 2 mM concentration of the substrate for 30 min at 37°C.

Inhibition studies Potential drug substrates of P450 IIIA (midazolam, cyclosporine A, quinidine) (Guengerich *et al.*, 1986; Kronbach *et al.*, 1988, 1989) were added at concentrations of 50 and 500 μ M to inhibit codeine *N*-demethylation. Propafenone, reported to be an inhibitor of P450 IID6 (Kroemer *et al.*, 1989; Siddoway *et al.*, 1987), was also tested at the above concentration range. Dehydroepiandrosterone, which inhibits ethylmorphine *N*-demethylation (Ladona *et al.*, 1989) was also tested as an inhibitor at these concentrations.

All drugs were dissolved in methanol and the final concentration was 2% in the incubates. Control incubates contained only 2% v/v methanol which did not inhibit activity by more than 5–10%. Inhibitors (or solvent) were preincubated with the microsomes for 6 min at room temperature. The reaction was started by addition of substrate and cofactors as described above. None of these drugs interfered in the h.p.l.c. analyses. The degree of inhibition was calculated from appropriate duplicate samples. All assays for the opioids were performed under conditions of linearity with time and protein according to Ladona *et al.* (1989).

Western blotting

Foetal liver microsomes were subjected to Western blotting as described previously (Ladona *et al.*, 1989). In order to immunodetect the presence of P450 IID6 in foetal liver microsomes (50 μ g/well) a monoclonal anti-human IID6 (114/2) antibody (MAb) was used. Additionally, Han-Chinese and Caucasian foetuses were checked with the MAb PCN 2-13-1/C2 for P450 IIIA. In 11 of the 14 Caucasian specimens, the immunodetected values were reported previously (Ladona *et al.*, 1989). Scanning

of the immunoblots was performed as described elsewhere (Ladona *et al.*, 1988).

Statistical calculations

Linear regression analysis and kinetic parameter estimation was done using an IBM PC and the program ENZFITTER (Elsevier Biosoft 1987). Correlations between enzyme activities and/or protein bands in Western blots were determined with the Spearman rank test.

Results

The proposed metabolic pathways of codeine, ethylmorphine and dextromethorphan are shown in Figure 1.

A summary of the various metabolic assays performed in different groups of liver specimens is given in Table 1. Upon incubation of codeine, dextromethorphan and ethylmorphine, the respective *N*-demethylated metabolites were formed at measurable rates in all investigated specimens (22 specimens). In contrast, there was no formation of the *O*-dealkylated metabolite (less than 3.3 pmol mg⁻¹ min⁻¹ for morphine and 180 pmol mg⁻¹ min⁻¹ for dextrorphan). These results were corroborated by Western blotting in which no P450 IID6 antigen was detected using a monoclonal anti-human P450 IID6 (114/2) antibody.

Incubations of the most active microsomal specimens with morphine (both enantiomers), norcodeine or norethylmorphine showed no further metabolism to normorphine at a substrate concentration of 2 mM.

Kinetic parameters were calculated for four foetal liver specimens (Table 2). The reactions with both substrates appeared to obey Michaelis-Menten kinetics, as

Table 1 Enzyme activities in different groups of foetal liver specimens. All activities are given as pmol mg⁻¹ protein min⁻¹

Enzyme activity	Caucasian		Chinese	
	Range (mean)	n	Range (mean)	n
Ethylmorphine <i>N</i> -demethylation	0–1278 ^a (377)	12	240–2220 ^d (697)	10
Codeine <i>N</i> -demethylation	304–1064 ^c (605)	5	125–1470 ^e (471)	10
Dextromethorphan- <i>N</i> -demethylation	0–163 ^b (47)	11		

^a Reported by Ladona *et al.* (1989).

Spearman rank test correlations:

^b dextromethorphan vs ethylmorphine, $r = 0.823$, $P = 0.002$, $n = 11$.

dextromethorphan vs codeine, $r = 0.90$, $P = 0.037$, $n = 5$.

dextromethorphan vs band intensity in Western blots, $r = 0.947$, $P = 0.001$, $n = 11$.

^c codeine vs band intensity in Western blots, $r = 0.90$, $P = 0.037$, $n = 5$.

^d ethylmorphine vs codeine, $r = 0.815$, $P = 0.004$, $n = 10$.

ethylmorphine vs band intensity in Western blots, $r = 0.745$, $P = 0.013$, $n = 10$.

^e codeine vs band intensity in Western blots, $r = 0.90$, $P = 0.0001$, $n = 10$.

Figure 1 Metabolic pathways of codeine, ethylmorphine and dextromethorphan. Pathways shown to be operating (—) or not operating (-----) in the human foetus are depicted, as well as pathways that have not been investigated conclusively (~~~~).

Table 2 Codeine, ethylmorphine and dextromethorphan *N*-demethylation enzyme kinetic parameters obtained in different foetal specimens

Specimens	Ethylmorphine <i>N</i> -demethylation			Codeine <i>N</i> -demethylation			Dextromethorphan <i>N</i> -demethylation		
	$V_{\max}$	K_m	$V_{\max}/K_m$	$V_{\max}$	K_m	$V_{\max}/K_m$	$V_{\max}$	K_m	$V_{\max}/K_m$
<i>Han-Chinese</i>									
ChLM-6	4680	0.46	10200	1290	0.29	4440	464	2.1	220
ChLM-2	1490	0.62	2400	888	0.93	955	n.i	n.i	n.i
<i>Caucasian</i>									
CaLM-1	1230	0.51	2410	4320	3.73	1160	n.i	n.i	n.i
CaLM-12	978	0.27	3620	348	0.28	1240	n.i	n.i	n.i

$V_{\max}$ = pmol $\text{mg}^{-1} \text{min}^{-1}$; K_m = mM; n.i = not investigated.

determined from Eadie-Hofstee plots (Figure 2). This indicated a single enzyme component responsible for these reactions, or several enzymes with similar K_m values. Table 2 summarizes the values of the kinetic parameters. The low number of specimens precluded any assessment of inter-ethnic variation.

The activities for ethylmorphine and codeine *N*-demethylation in Chinese specimens were 240–2220 and

125–1470 pmol $\text{mg}^{-1} \text{min}^{-1}$, respectively (Table 1, Figure 3). These activities were highly correlated with each other ($r = 0.815$) and also to the immunodetected PCN 2-13-1/C2 epitope ($r = 0.75$ and 0.90 , respectively), as shown in Figure 3.

In the investigated foetal Caucasian specimens the *N*-demethylation of dextromethorphan was catalyzed at rates up to 163 pmol $\text{mg}^{-1} \text{min}^{-1}$ (Table 1). This activity

Figure 2 Enzyme kinetics of the human foetal codeine (a) and ethylmorphine (b) *N*-demethylase. The figure shows a velocity vs substrate concentration curve and an Eadie-Hofstee plot (inset). Incubation was performed as described in **Methods**. Microsomes from a Chinese liver specimen were used.

also correlated well with the immunodetected epitopes ($r = 0.95$) and also with ethylmorphine enzyme activities reported in an earlier study (Ladona *et al.*, 1989), as shown in Figure 3.

Codeine *N*-demethylation was assayed in five Caucasian foetal liver specimens and varied between 304 and 1064 pmol mg⁻¹ min⁻¹. This activity correlated well with the immunodetected epitopes ($r = 0.90$) and with dextromethorphan *N*-demethylase activity (Table 1).

The MA b PCN 2-13-1/C2 lacks inhibitory capacity. Therefore, to study indirectly the possible participation of P450 IIIA in opioid *N*-demethylation, a series of reported substrates of this cytochrome, including cyclosporine A, midazolam and quinidine, were used as inhibitors in five specimens with high *N*-demethylation rates. Table 3 shows the inhibition of codeine *N*-demethylation by these agents in each microsomal preparation. Midazolam was strongly inhibitory in all tested specimens. Propafenone (racemic) had a significant inhibitory effect only at 500 µM concentration. The extent of inhibition was similar for both propafenone enantiomers (data not shown). It is not known if all of these compounds are metabolised in human foetal microsomes. As expected, dehydroepiandrosterone also inhibited the reaction in the specimen tested.

Discussion

The lack of measurable *O*-demethylase activity in prenatal human liver of both Caucasian and Chinese origin using codeine, ethylmorphine and dextromethorphan as substrates is of interest for various reasons. First, it clearly indicates that the two demethylation pathways at the 17 *N*- and 3 *O*- positions develop differently. The lack of morphine formation from codeine was confirmed using an improved analytical method with electrochemical detection which increased the sensitivity about one hundred times compared with u.v. fluorescence detection.

Our findings show that the two demethylation pathways are catalysed by different forms of cytochrome P450. The triggering factor(s) for development of the *O*-demethylase, i.e. the constitutive P450 IID6 (Gonzalez, 1989) is not yet in operation in mid-gestation. It would be of interest to study the expression of cytochrome P450 IID6 later in gestation or in the newborn infant. Such biological material has so far not been available. However, a recent report (Treluyer *et al.*, 1990) indicated that enzyme may be expressed at a later gestational stage in a minority of investigated specimens.

In contrast, the *N*-demethylase was consistently demonstrated in all but two liver specimens. Some livers contained immunoidentified P450 IIIA without appreciable *N*-demethylating enzyme activity (Figure 3). We do not know the reason for this observation but suspect it to represent the presence of apoprotein without activity. The latter enzyme(s) is probably of mandatory importance for the metabolism of steroid hormones of maternal,

Table 3 % inhibition of codeine *N*-demethylation by different drugs

Specimens	Midazolam		Cyclosporin A		Quinidine		Propafenone		Dehydroepiandrosterone	
	50 µM	500 µM	50 µM	500 µM	50 µM	500 µM	50 µM	500 µM	50 µM	500 µM
ChLM-2	55	72	29	27	n.m	n.m	17	48	48	67
ChLM-6	38.5	59	32	31	n.m	n.m	12	53.7	n.m	n.m
CaLM-1	42.4	99	n.m	n.m	0	76	n.m	n.m	n.m	n.m
CaLM-3	32.1	74	n.m	n.m	n.m	n.m	n.m	n.m	n.m	n.m
CaLM-4	32.3	62.9	n.m	n.m	17	68	n.m	n.m	n.m	n.m

n.m = not measured.

Figure 3 Correlation between ethylmorphine- and codeine- (a) or dextromethorphan- (b) *N*-demethylation rates. Figures c, d and e depict the *N*-demethylations of codeine, dextromethorphan and ethylmorphine, respectively, in relation to the epitopes detected by the monoclonal antibody PCN 2-13-1/C2 in Chinese (ChLM) or Caucasian (CaLM) human foetal liver specimens.

placental or foetal adrenal origin and thus develops at an early stage (Ladona *et al.*, 1989). Kitada *et al.* (1987) have demonstrated a P450 enzyme member active in the 16-hydroxylation of dehydroepiandro-sterone 3-sulphate, an estrogen precursor in the placenta. This enzyme was subsequently shown to be distinct from the IIIA3 and IIIA4 isoenzyme forms (Komori *et al.*, 1989).

The implications of the foetal lack of cytochrome P450 IID6 are unknown. In adults, the only known implication is in the oxidation of a limited number of drug substrates (Davies *et al.*, 1981; Gonzalez *et al.*, 1988; Meyer *et al.*, 1986). In some cases the deficiency of the IID6 isoenzyme leads to accumulation of the drug. Alternatively, the

metabolite in question may not be formed or impeded in its further elimination. Hence, in some situations the PM may be at increased risk of developing adverse drug reactions. Whether a similar foetal risk exists on exposure to P450 IID6 drug substrates *in utero* must remain a matter of speculation.

Our results demonstrate that it is impossible to identify foetuses that are PMs or EMs with codeine as a probe drug at this stage of gestation. The absence of *O*-demethylase activity by P450 IID6 is consistent with the lack of any measurable protein in Western blots using the MAb 114/2 which is specific for this isoenzyme (Mortimer *et al.*, 1990).

We have not proven that a P450III A member enzyme is responsible for the high foetal opioid N-demethylation activities found in human foetal liver. However, various pieces of information would support this contention. We found that the enzyme activities correlated well with the amount of MA b PCN 2-13-1/C2 detected epitope. The foetal N-demethylation rates studied were in the same range as those observed with adult liver specimens. That this enzyme belongs to the P450 family was further supported by its inhibition by midazolam and cyclosporine which are substrates of the P450 III A enzyme family (Kronbach *et al.*, 1979). Future studies with isolated cytochrome P450s or directed expression of the corresponding cDNAs in cell-expression systems will help confirm the metabolic pattern of the foetal enzyme form and establish its similarities or dissimilarities with the adult forms with regard to endogenous and exogenous substrates.

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