Differences in the cytochrome *P*-450 isoenzymes involved in the 2-hydroxylation of oestradiol and 17 α -ethinyloestradiol

Relative activities of rat and human liver enzymes

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The metabolism of oestradiol and 17 α -ethinyloestradiol to their 2-hydroxy derivatives is an important determinant in their biological effects. In this work, we have investigated which rat or human cytochrome *P*-450 isoenzymes are involved in catalysing these reactions. Oestradiol 2-hydroxylation was catalysed by a wide variety of rat cytochrome *P*-450s from gene families P450IA, P450IIB, P450IIC and P450IIIA. Interestingly, 17 α -ethinyloestradiol, which only differs structurally from oestradiol at a position distant from the site of oxidation, was metabolized predominantly by members of the P450IIC gene subfamily. In order to establish which enzymes are responsible for the oxidation of these substrates in man, antibodies to rat liver cytochrome *P*-450 isoenzymes were used to inhibit these reactions in a panel of human liver microsomal fractions. Also, possible correlations between the proteins recognized by the antibodies and the 2-hydroxylation rate were determined. These experiments provide evidence that 2-hydroxylation of 17 α -ethinyloestradiol in man is catalysed by cytochromes from the P450IIC, P450IIE and P450IIIA gene families. In contrast, the major proteins involved in oestradiol metabolism are from the P450IA gene family, although members of the P450IIC and P450IIE gene families may also play a role. These data demonstrate that the differences in the capacity of rat *P*-450s to metabolize these substrates are also present in the comparable enzymes involved in man, and that a variety of factors will determine the rate of disposition of these compounds in man.

INTRODUCTION

The predominant route of oxidative metabolism of the endogenous steroid hormone oestradiol and of the oral contraceptive steroid 17 α -ethinyloestradiol in both rat and man is cytochrome *P*-450-dependent 2-hydroxylation to form the catechol oestrogens 2-hydroxyoestradiol and 2-hydroxyethinyloestradiol [1–5]. Since the compounds differ from each other only in the presence of the 17 α substituent, the question arises whether the same enzyme(s) metabolize both the endogenous and the synthetic oestrogens. Early publications on experiments in the rat referred to the catechol metabolites of both compounds being formed by the cytochrome *P*-450-dependent oestrogen hydroxylase [4–7]. Dannan *et al.* [8], however, were able to demonstrate in studies utilizing ten purified rat liver *P*-450s that three forms, which they designated *P*-450_{UT-A}, *P*-450_{BNF-B} and *P*-450_{ISF-G}, exhibited the highest 2-hydroxylase activities. In a separate study Ryan *et al.* [9] examined the catalytic activity of three purified rat *P*-450s and showed that *P*-450h (*P*-450_{UT-A}) had the highest activity. In the rat, therefore, there is some direct evidence for the involvement of different isoenzymes in oestradiol 2-hydroxylation. In man, Guengerich *et al.* [10] described the immunoinhibition of oestradiol 2-hydroxylase by an antibody to *P*-450_{NF} (P450IIIA1) in human liver microsomes. Similarly Guengerich [11] has also recently described the inhibition of 17 α -ethinyloestradiol 2-hydroxylation by this antibody, although purified *P*-450_{NF} had only relatively low catalytic 17 α -ethinyloestradiol 2-hydroxylase activity. While these data are consistent with *P*-450_{NF} being a major enzyme involved in both oestradiol and 17 α -ethinyloestradiol 2-hydroxylation, the potential involvement of other enzymes has not been explored thoroughly.

The aim of the present study was (1) to determine the activity of different *P*-450 forms purified from rats in the 2-hydroxylation

of oestradiol and 17 α -ethinyloestradiol and (2) to evaluate whether similar enzymes catalyse these metabolic reactions in man.

MATERIALS AND METHODS

Chemicals

Ethinyloestradiol, oestradiol, mestranol, ascorbic acid, DL-dilauryl phosphatidylcholine and NADPH were purchased from the Sigma Chemical Company Ltd., Poole, Dorset, U.K. The radiolabelled steroids [6,7-³H]oestradiol and 17 α -[6,7-³H]ethinyloestradiol were purchased from New England Nuclear, Stevenage, Herts., U.K. All other reagents were purchased from BDH Chemical Company Ltd., Poole, Dorset, U.K., and were of analytical grade.

Purified rat hepatic cytochromes *P*-450 and anti-(cytochrome *P*-450) antibodies

Rat hepatic cytochromes *P*-450, rabbit anti-(rat cytochrome *P*-450) antibodies and purified cytochrome *P*-450 reductase were isolated as previously described [12–14]. The antibodies to rat P450IIE1 and human P450IID were generously supplied by Dr. C. S. Yang (New Jersey School of Medicine and Dentistry, Newark, NJ, U.S.A.) and Dr. U. Meyer (Department of Pharmacology, Biocenter, University of Basle, Switzerland) respectively. The nomenclature used for the enzymes and comparison with other systems has been published previously [15]. Comparison with recent gene nomenclature [16] is given in Table 1.

Human livers

Human livers were obtained from renal transplant donors

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soon after clinical death. Consent for their removal was obtained from the donors' relatives and ethical approval was granted for their use in this study. Livers were stored as 10–20 g portions at –70 °C until use.

Oestrogen 2-hydroxylase activity of purified rat cytochromes *P*-450

Purified rat hepatic cytochrome *P*-450s were reconstituted and activity was determined. Incubations contained cytochrome *P*-450 (2 nmol), DL-dilauryl phosphatidylcholine (1 mg) and cytochrome *P*-450 reductase (10000 units). Components were mixed in approx. 50 µl of 10 mM-phosphate buffer (pH 7.4) and incubated for 5 min at 37 °C. Oestrogen 2-hydroxylase activity was determined by incubating reconstituted cytochromes *P*-450 with either oestradiol or ethinyloestradiol (25 µM; 1 µCi) and ascorbic acid (1 mM)/NADPH (0.6 mM) in 25 ml Erlenmeyer flasks. The total incubation volume was 3 ml in Tris/HCl buffer (20 mM; pH 7.4). The reaction, for 30 min at 37 °C, was initiated after a 2 min preincubation period by the addition of NADPH and terminated by extraction with diethyl ether (1 × 5 ml) following the addition of mestranol as internal standard (4 µg). The ether layer was then removed and evaporated to dryness. Catechol oestrogens were quantified by h.p.l.c. as described below.

Quantification of catechol oestrogens by h.p.l.c.

2-Hydroxy-oestradiol and -ethinyloestradiol were quantified by h.p.l.c. by a slightly modified method of Purba *et al.* [4]. Chromatographic separations were performed on a Spherisorb 5-ODS reverse-phase column (25 cm × 0.46 cm; HPLC Technology). Separations were performed at room temperature with a mobile phase of ammonium phosphate buffer (0.5% w/v; pH 3.0)/methanol at a flow rate of 1 ml/min. Metabolites were identified according to the retention times of authentic standards monitored by u.v. detection at 280 nm and quantified from the mestranol internal standard, by comparing peak height ratios. Eluate fractions were simultaneously collected at 30 s intervals and monitored for radioactivity by liquid scintillation spectrometry.

Enzyme activity of human hepatic microsomes and antibody inhibition studies

Human liver microsomes were prepared from a total of 16 human livers by differential ultracentrifugation as described previously [5]. Microsomal protein was determined by the method of Lowry *et al.* [17] and cytochrome *P*-450 by the method of Omura & Sato [18].

Oestrogen 2-hydroxylase activity was determined in incubations (45 min at 37 °C) containing microsomal protein (0.5 mg), oestradiol or 17 α -ethinyloestradiol (25 mM), ascorbic acid (1 mM) and NADPH (0.6 mM) in a total incubation volume of 3 ml. In immunoinhibition studies (six livers), microsomal protein (0.5 mg) was preincubated for 20 min either with specific antibody or with preimmune serum (2.5 mg of IgG). Enzyme activity was then determined as above and metabolites quantified by h.p.l.c.

Western blot analysis

Human liver microsomes (7.5 µg) were run on SDS/PAGE (9%) and then transferred to nitrocellulose [19] in 20 mM-sodium phosphate containing 20% methanol at 250 mA for 16 h. Filters were washed in 50 mM-Tris (pH 7.9) containing 250 mM-NaCl and 0.05% Tween 20 (TBST; 2 × 10 min) then blocked with low-fat dried milk powder (3%, w/v, in TBST). Polyclonal antibodies raised against purified rat *P*-450 isoenzymes UT1 isoenzymes UT1 (P450IIA1), PB1a (P450IIC6), PB3a (P450IIB1), PB2c (P450IIIA), MC1a (P450IA2) and P452 (P450IVA) were used at

a dilution of 1:500. Antibodies to rat P450IIE₁ and human P450IID were used in a similar way. Bound antibody was visualized using horseradish-peroxidase-linked donkey anti-(rabbit IgG) (1:1000; 1 h) and autoradiography, followed by ¹²⁵I-Protein A labelling (5 µCi in TBST; 1 h). This procedure considerably increased the sensitivity of the assay and also allowed visualization using the peroxidase substrate 4-chloro-1-naphthol if required. Relative cytochrome *P*-450 content was determined from the autoradiographs using a Joyce-Loebel scanning densitometer. Linearity was assessed from serial dilutions of the microsomal samples. Each gel also contained different amounts of the antigen being quantified. This enabled standard curves to be generated.

RESULTS

Ethinyloestradiol 2-hydroxylase activities of purified rat hepatic cytochrome *P*-450s are shown in Table 1. Interestingly, enzymes within the P450IIC gene sub-family, i.e. PB1a, PB1b and PB2a, had by far the highest activity, the activity of PB1a being almost 10-fold greater than that of *P*-450s from other gene families. PB1b had approximately half of the activity of PB1a (the structural differences between PB1a and PB1b are not clearly defined at this time; however, they both have identical *N*-terminal sequences to P450IIC6 [13]). The other member of this family with ethinyloestradiol 2-hydroxylase activity was the male-predominant form (P450IIC12; Levin form h). Intriguingly, the protein with highest activity towards 17 α -ethinyloestradiol (PB1a) had no activity towards oestradiol (Fig. 1); PB1b did however exhibit some activity. Almost all the other proteins tested from gene families P450IA (Fig. 2), P450IIB, P450IIC and P450IIIA had activity towards oestradiol. Indeed, for many of these proteins, the activities measured were similar. One point of interest was the large difference in activity of P450IIB2 (PB3b) and P450IIB1 (PB3a), as these proteins share 97% sequence identity at the amino acid level.

The above data indicate that many rat cytochrome *P*-450 forms can catalyse the 2-hydroxylation of oestradiol, but the

Table 1. Activity of purified rat hepatic cytochromes *P*-450

Enzyme activities were determined as described in the Materials and methods section. Values given are the average of 2 determinations. Gene nomenclature is according to Nebert *et al.* [16]

Purified enzyme	Gene nomenclature	17 α -Ethynyl-oestradiol 2-hydroxylase (nmol/min per nmol of <i>P</i> -450)	Oestradiol 2-hydroxylase (nmol/min per nmol of <i>P</i> -450)
PB1a*	P450IIC6	0.51	0.00
PB1b	P450IIC6	0.20	0.12
PB2a	P450IIC12	0.18	0.46
PB2c	P450IIIA†	0.06	0.30
PB3a	P450IIB1	0.01	0.04
PB3b	P450IIB2	0.05	0.65
MC1a	P450IA2	0.06	0.49
MC1b	P450IA1	0.05	0.47
No enzyme		0.00	0.00
No reductase		0.00	0.00
No NADPH		0.00	0.00
No enzyme or reductase		0.00	0.00

* It is not clear whether PB1a or PB1b or both are gene products of P450IIC6.

† No exact gene nomenclature is available.

Fig. 1. H.p.l.c. of incubations of purified PB1a isoenzyme with (a) ethinyl-oestradiol and (b) oestradiol

The retention times of the authentic parent steroids (B) and the 2-hydroxy metabolites (A) are shown.

Fig. 2. H.p.l.c. of incubations of purified PB3b isoenzyme with (a) ethinyl-oestradiol and (b) oestradiol

The retention times of the authentic parent steroids (B) and the 2-hydroxy metabolites (A) are shown.

oxidation of 17 α -ethinyloestradiol is more restricted to members of the P450IIC gene family. In order to establish the relative roles of different *P*-450s in the metabolism of these compounds in human liver, a series of *P*-450 antibodies was used as inhibitors of oestradiol and 17 α -ethinyloestradiol metabolism in human liver microsomal fractions. In addition, Western blot analysis using these antibodies was carried out to determine the relative concentrations of *P*-450 forms in a further ten samples and to establish whether the levels of specific isoenzymes correlated with the rate of oestradiol or ethinyloestradiol metabolism.

In a panel of 16 human livers, the 2-hydroxylation of oestradiol was up to 3-fold higher than that of 17 α -ethinyloestradiol (Table 2). The metabolism of oestradiol was also subject to larger individual variation (approx. 7-fold) relative to that of ethinyloestradiol (3-fold). Interestingly, the rates of metabolism of the two substrates correlated with each other and with the total cytochrome *P*-450 content (Fig. 3). Comparison of the relative cytochrome *P*-450 isoenzyme content determined by Western blot analysis showed significant correlations between oestradiol metabolism and the expression of P450IA and P450IIE proteins. Oestradiol metabolism also correlated with proteins in the P450IIC gene family (bands 2 and 3) (Table 3, Fig. 4). The metabolism of 17 α -ethinyloestradiol, however, was most highly correlated with the levels of P450IIC (band 2) and P450IIIA proteins. A correlation with P450IIE protein (band 2) was also observed; however, this may be fortuitous because of one sample with high activity (Fig. 5). Proteins of the P450IIA or P450IID and the P450IVA gene families did not appear to play a significant role in the metabolism of either substrate, as the level of expression of the proteins detected by Western blot analysis did not correlate with 2-hydroxylase activity.

In agreement with the findings with the purified rat liver proteins and the correlations described above, the antibody to P450IIC6 (PB1a) was an effective inhibitor of human liver 17 α -ethinyloestradiol 2-hydroxylase activity. The rate of metabolism was reduced to 65, 45, 39, 38, 11 and 49 % of control activities (Table 4). With oestradiol as substrate, however, this antibody

Table 2. Cytochrome *P*-450 oestradiol and 17 α -ethinyloestradiol 2-hydroxylase activities in 16 human livers

Human livers were obtained from renal transplant donors. Subject L8 had received phenobarbitone and phenytoin for 30 years. Subjects L10 and WT1 were heavy smokers. All other livers were from apparently normal individuals at time of death (mostly road traffic accidents).

Liver	Age	Sex	Cytochrome <i>P</i> -450 (nmol/mg)	Oestradiol 2-hydroxylase (nmol/min per mg)	17 α -Ethinyl-oestradiol 2-hydroxylase (nmol/min per mg)
L2	28	M	0.33	0.37	0.12
L3	35	M	0.37	0.59	0.20
L4	66	F	0.90	1.14	0.35
L5	60	M	0.41	0.62	0.22
L7	29	M	0.71	0.92	0.35
L8	46	F	0.87	1.03	0.45
L9	27	M	0.50	0.67	0.22
L10	54	M	0.38	0.75	0.24
L11	68	M	0.33	0.34	0.18
L13	38	M	0.36	0.67	0.17
L14	32	M	0.27	0.44	0.25
W1	17	F	0.46	0.77	0.34
WT1	18	M	0.38	0.61	0.19
C2	60	F	0.38	0.79	0.21
C3	53	F	0.24	0.15	0.15
B1	27	M	0.52	0.78	0.35

Fig. 3. Correlation of rates of ethinyloestradiol and oestradiol 2-hydroxylation (a) and of each activity with total cytochrome *P*-450 (b,c)

Table 3. Spearman's rank correlations between the metabolism of oestradiol and 17 α -ethinyloestradiol and the levels of *P*-450 forms in human liver

In certain cases, more than one band was recognized by the antibodies used. In the case of P450IIC this is consistent with the known expression of several members of this gene family in human liver. Also, the expressed recombinant P450IIC8 and P450IIC9 proteins reacted specifically with the antibody used and had the same M_r as bands 1 and 2 identified with the antibody by Western blot analysis. This was also the case for recombinant P450IIA1 (band 2), P450IIB1 and P450IIIA proteins. Significance is indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. N.D., not determined.

Antibody	Bands identified	Apparent M_r	Correlations	
			17 α -Ethynyl-oestradiol	Oestradiol
MC1a (P450IA2)	1	53 000	0.21	0.62**
UT1 (P450IIA1)	1	54 500	0.29	0.21
	2	51 000	0.48	0.54
PB3a (P450IIB1)	—	51 000	0.52	0.50
PB1a (P450IIC6)	1	54 500	0.43	0.37
	2	52 500	0.75**	0.58*
	3	51 000	0.49	0.61*
Debrisoquine (P450IID)	—	51 500	−0.05	−0.11
j (P450IIE1)	1	54 500	−0.10	−0.18
	2	51 000	0.62**	0.66**
PB2C (P450IIIA)	—	52 500	0.78***	0.50
P452 (P450IVA1)	1	N.D.	0.01	−0.09
	2	N.D.	0.37	0.31

Fig. 4. Correlation of rates of oestradiol 2-hydroxylation with the relative band intensity on Western blot analysis of (a) P450IIC band 3, (b) P450IIE band 2 and (c) P450IA2

Fig. 5. Correlation of rates of ethinyloestradiol 2-hydroxylation with the relative band intensity on Western blot analysis of (a) P450III A, (b) P450IIE band 2 and (c) P450IIC band 2

Table 4. Inhibition of ethinyloestradiol 2-hydroxylase activity in six human livers by anti-(cytochrome *P*-450) antibodies

Results are presented as a percentage of the control activity (i.e. without antibody); values are the averages of two determinations. PI, preimmune serum; N.D., not done.

Incubation	17 α -Ethinyloestradiol 2-hydroxylase activity (% of control)					
	L4	L5	L7	W1	L11	C3
No antibody	100	100	100	100	100	100
Anti-PB1a	65	45	39	38	11	49
Anti-PB2c	95	100	92	100	100	58
Anti-PB3a	88	75	91	100	100	74
Anti-MC1a	100	100	83	100	N.D.	N.D.
Anti-MC1b	88	94	90	97	88	79
PI	100	100	100	100	100	100

Table 5. Inhibition of oestradiol 2-hydroxylase activity in six human livers by anti-(cytochrome *P*-450) antibodies

Results are presented as a percentage of the control activity (i.e. without antibody); values are the averages of two determinations. PI, preimmune serum; N.D., not done.

Incubation	Oestradiol 2-hydroxylase activity (% of control)					
	L4	L5	L7	W1	L11	C3
No antibody	100	100	100	100	100	100
Anti-PB1a	75	60	77	76	71	51
Anti-PB2c	100	94	93	100	100	72
Anti-PB3a	99	80	71	91	86	75
Anti-MC1a	75	65	86	54	N.D.	N.D.
Anti-MC1b	82	26	55	21	50	39
PI	100	100	98	100	100	100

showed much lower inhibitory potency but did consistently give some inhibition, activities being reduced to 75, 60, 77, 76, 71 and 51 % of the control enzyme activity (Table 5). The antibody used has been shown to completely inhibit P450IIC-mediated metabolism of mephenytoin in human liver (L. M. Forrester, B. Meyer & C. R. Wolf, unpublished work).

An antibody to P450IA1 (MC1b) was an effective inhibitor of

oestradiol 2-hydroxylase activity with values reduced to 82, 26, 55, 21, 50 and 39 % of the control enzyme activity (Table 5). This is in agreement with the significant correlation between metabolism and the level of P450IA protein. However, this antibody had essentially no effect on 17 α -ethinyloestradiol 2-hydroxylase activity, which is consistent with the lack of activity of the purified rat proteins of this gene family towards this substrate. The antibody to P450IA2 (MC1a) also partially inhibited oestradiol metabolism. The antibodies to P450IIB1 and P450III A were not effective inhibitors of either oestradiol or 17 α -ethinyloestradiol metabolism. In the latter case, however, this may be because the antibody is a poor inhibitor of P450IIA-mediated reactions in human liver (S. E. Ball, L. M. Forrester, C. R. Wolf & D. J. Back, unpublished work).

DISCUSSION

In view of the structural similarity between oestradiol and 17 α -ethinyloestradiol, there is a surprising difference in the ability of cytochrome *P*-450 isoenzymes to metabolize these substrates in the 2-position. 17 α -Ethinyloestradiol was metabolized at a much higher rate by proteins from the P450IIC gene subfamily than by the other *P*-450s tested. In contrast, a variety of rat liver *P*-450s from different gene families had the capacity to metabolize oestradiol. The possibility that the P450IIC subfamily may be involved in ethinyloestradiol metabolism in man was confirmed by the findings that the proteins recognized by the antibody to P450IIC6 in human liver correlated with the level of ethinyloestradiol metabolism, and that the antibody was an effective inhibitor of metabolism of this substrate in the samples tested. In most of the microsomal samples inhibition was only partial, which indicates that other enzymes are also involved. This is in agreement with the findings of Guengerich [11], and our own findings were that proteins from the P450III A gene family also appear to be involved. The relative contribution of P450III A and P450IIC proteins still remains to be clearly established, as unfortunately the antibody to P450III A used was not inhibitory. Several enzymes in human liver therefore appear to have the capacity to metabolize 17 α -ethinyloestradiol.

The known effects of phenobarbitone on ethinyloestradiol metabolism [20] could be explained by the induction of the members of the P450IIC and P450III A families, as these proteins are induced by this compound in the rat. Also, the involvement of P450IIB cannot be precluded. Although some of the enzymes involved in ethinyloestradiol 2-hydroxylation also appear to have the capacity to metabolize oestradiol, e.g. members of the

P450IIC and P450IIE sub-families, there were clear differences in the relative roles of the different *P*-450s in the disposition of the endogenous oestrogen. For example, the antibody to P450IIC6 was a much poorer inhibitor of oestradiol than of ethinyl-oestradiol. Also, on the basis of antibody inhibition and the correlation between protein expression, proteins of the P450IA family appear to play an important role in oestradiol metabolism but not in ethinyl-oestradiol disposition. It is not clear which of the two known members of this gene family are involved, as both of the antibodies to P450IA1 and P450IA2 recognized the same protein in human liver. These data are consistent with the important finding that oestradiol metabolism is increased in cigarette smokers [21]. Pelkonen *et al.* [22] have demonstrated that cigarette smoking induces a form of *P*-450 in human liver that contains an epitope recognized by an anti-MC antibody. It has also been well established in a number of other studies that the rate of theophylline elimination is higher in cigarette smokers than in non-smokers [23], studies *in vitro* with human microsomes providing evidence of the involvement of two *P*-450 isoenzymes [24]. Campbell *et al.* [25] have implicated a polycyclic aromatic hydrocarbon-inducible *P*-450 in the metabolism of theophylline. Thus evidence is accumulating that smoking induces enzymes in the P450IA gene family which are at least partially responsible for the metabolism of 7-ethoxyresorufin, theophylline and oestradiol. Smoking does not affect the disposition of 17 α -ethinyl-oestradiol [26,27].

Although the relative contributions of specific *P*-450s to the metabolism of oestradiol and 17 α -ethinyl-oestradiol in human liver appear to be different, it is clear that several *P*-450 forms will metabolize these compounds. This will account for the general correlation with total *P*-450 levels and therefore also for the correlation between 2-hydroxylation of oestradiol and 17 α -ethinyl-oestradiol.

The specificity of *P*-450 isoenzymes in oestradiol and ethinyl-oestradiol 2-hydroxylation is intriguing. This is particularly the case for the proteins PB3a (P450IIB1) and PB3b (P450IIB2). These proteins are approximately 97% identical at both the protein and DNA levels [28], and have been shown previously to possess differential catalytic activity towards a number of other mono-oxygenase substrates. However, PB3a is invariably more active [14,29–30]. For example, Ryan *et al.* [31] reported that PB3b catalysed the metabolism of benzphetamine, testosterone at the 16 α -position, hexobarbital, 7-ethoxycoumarin and benzo[*a*]pyrene at rates which were 15–25% of those catalysed by PB3a. We have made similar observations for the metabolism of resorufin analogues [14]. However, in agreement with the present study, Ryan *et al.* [32] also found that PB3b has a much higher oestradiol 2-hydroxylase activity than PB3a.

The marked difference in selectivity of the purified cytochromes in oestradiol and 17 α -ethinyl-oestradiol 2-hydroxylation must be attributable to the D-ring 17 α -ethinyl substituent. Clearly, therefore, the steroid D-ring must play a role in substrate recognition, at least for some purified cytochromes. This has been shown to be the case for other steroid mono-oxygenase substrates which differ structurally only in the D-ring. For example, PB2a catalyses the 2-hydroxylation of progesterone (17 α -acetyl substituent), three times as rapidly as that of testosterone (17 β -hydroxyl substituent [33]). It is apparent, therefore, that structural modification of the D-ring can affect metabolism on the A-ring which is physically remote from the structural change. It should also be considered that 17 α -ethinyl-oestradiol has been shown to be a suicide substrate for cytochrome *P*-450 in both rat [34] and human [11] liver. For example, Guengerich [11] demonstrated a 25–30% loss of spectrally determined *P*-450 in

human liver microsomes in 60 min using a substrate concentration of 100 μ M. It is possible, therefore, that some in-activation of individual isoenzymes could take place during the 30 min of incubation in the present work. Further studies are needed on this particular point.

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