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METABOLISM OF THE CONTRACEPTIVE STEROID DESOGESTREL BY HUMAN LIVER *IN VITRO*

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Summary—The metabolism of the progestogen oral contraceptive desogestrel (Dg) has been studied *in vitro* using human liver microsomes. Metabolites have been separated using radiometric high performance liquid chromatography and identified by co-chromatography with authentic standards and by mass spectrometry. All the livers examined ($n = 6$) were able to form 3-keto desogestrel as the main identifiable metabolite and also the presumed intermediates 3α-hydroxydesogestrel (3α-OHDg) and 3β-hydroxydesogestrel (3β-OHDg). In addition, a large polar heterogenous peak was evident on the radiochromatograms which did not co-chromatograph with any known metabolites of desogestrel. Inter-individual variability in metabolite formation was seen. A number of drugs were examined for their propensity to inhibit desogestrel metabolism. Primaquine was the most potent tested having an IC_{50} value (inhibitory concentration reducing overall metabolite production by 50%) of 30 μM. Cimetidine, trilostane and levonorgestrel failed to inhibit at 250 μM.

With 3α-OHDg as substrate, 3α-hydroxysteroid dehydrogenase (3α-HSDH) activity was 1.0 ± 0.3 nmol min^{-1} mg^{-1} protein which was five times greater than the activity of the 3β-HSDH towards 3β-OHDg. Miconazole was the most potent inhibitor tested having IC_{50} values of 14 and 95 μM for 3α- and 3β-HSDH respectively. Surprisingly, trilostane was without inhibitory effect on either enzyme, which contrasts with other data involving 3β-HSDH in steroidogenic tissue. Our observations with trilostane may reflect tissue differences in the enzyme and/or differences in endogenous vs exogenous steroids (i.e. in the conversion of 3β-OHDg to 3-ketodesogestrel there is no requirement for isomerization). Kinetic parameters of 3α-HSDH were also determined.

INTRODUCTION

Desogestrel is an orally active progestogen which is metabolised to 3-keto desogestrel and it is this metabolite which mediates the progestogenic effects [1]. The bioavailability of 3-keto desogestrel was $76 \pm 22\%$ [2] after oral administration of desogestrel to 9 healthy volunteers who also received intravenous 3-keto desogestrel. The incomplete bioavailability of 3-keto desogestrel (range, 40–113%) may result from incomplete absorption and/or first-pass metabolism to inactive metabolites. The main sites of first-pass metabolism are gut wall and liver. In a recent report [3] we have shown the ability of the gut wall to metabolise desogestrel to the active metabolite as well as to other, unidentified, more polar metabolites. Viinikka[4] has previously demonstrated formation of 3-keto desogestrel by human liver homogenate. However, in the study of Viinikka only one liver was used, and as a result, any inter-individual variability in desogestrel metabolism was not taken into account. In order to form the active 3-keto metabolite, desogestrel must first of all undergo cytochrome *P*450-dependent hydroxylation at C-3 to give 3α-OH and/or 3β-OH desogestrel. In the present study we have investigated the oxidative metabolism of desogestrel in a number of human livers and examined the 3α- and 3β-hydroxysteroid dehydrogenase activity of human liver microsomes in order to establish the pathway of 3-keto desogestrel formation. In addition, we have investigated the effects of a range of known inhibitors of oxidative drug metabolism.

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Trivial names: Desogestrel; 13-ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-17-ol. 3-Keto desogestrel; 13-ethyl-17-hydroxy-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-3-one. 3α-hydroxy desogestrel; 13-ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yne-3α,17-diol. 3β-hydroxy desogestrel; 13-ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yne-3β,17-diol. 3α-hydroxy-5α-H-desogestrel; 13-ethyl-11-methylene-18,19-dinor-5α,17α-pregn-20-yne-3α,17-diol. 3β-hydroxy-5α-H-desogestrel; 13-ethyl-11-methylene-18,19-dinor-5α,17α-pregn-20-yne-3β,17-diol. 3-keto-5α-H-desogestrel; 13-ethyl-17-hydroxy-11-methylene-18,19-dinor-5α-17α-pregn-20-yn-3-one.Gestodene; 17-ethinyl-13-ethyl-17β-hydroxy,4,15-gonadien-3-one.

MATERIALS AND METHODS

Chemicals

[^{3}H]Desogestrel ([^{3}H]Dg; 4 Ci/mmol), [^{3}H]3-keto-desogestrel ([^{3}H]3-KDg; 25 Ci/mmol), desogestrel

(Dg), 3α-OH desogestrel (3α-OHDg), 3β-OH desogestrel (3β-OHDg), 3-keto desogestrel (3-KDg), 3α-OH, 5α-H desogestrel (3α-OH, 5α-HDg), 3β-OH, 5α-H desogestrel (3β-OH, 5α-HDg) were gifts from Organon, Oss, The Netherlands. Primaquine (PQ), nor-gestrel (Ng), miconazole (MC) and ethinyloestradiol (EE2) were all obtained from Sigma. Cimetidine (Cim) was a gift from Smith, Kline and French (Welwyn Garden City) and trilostane (Tri) was a gift from Sterling–Winthrop (Alnwick). All other chemicals were of Analar grade and obtained from BDH.

Human liver samples

Samples of histologically normal livers were obtained from kidney transplant donors. Ethical approval for the study was granted and consent to removal of the liver was obtained from the donors' relatives. Samples were transferred, on ice, to the laboratory within 30 min where they were sectioned into 10–20 g portions, placed in plastic vials and frozen in liquid nitrogen at −196°C. Liver was stored at −80°C until required.

Preparation of liver microsomes

Washed microsomes were prepared by the classical differential centrifugation technique. The tissue was roughly chopped with scissors and then ground in 1/15 M phosphate buffer, pH 7.4, containing 1.15% KCl, with an Ultra-Turrax device. A 25% homogenate was then produced using a Teflon in glass motor driven homogenisation device. The first microsomal pellets (105,000 *g* pellets) were resuspended in buffer and centrifuged to obtain the washed microsomes. Cytochrome *P*450 was assayed by the method of Omura and Sato[5], and the microsomal protein yield was determined by the method of Lowry *et al.*[6]. Microsomes were stored at −80°C until use, which was usually on the following day.

Microsomal incubation

Incubations at 37°C contained [^{3}H]Dg (0.2 μCi, 100 μM) or [^{3}H]3-KDg (0.2 μCi, 20 μM), KCl (1 mM), EDTA (1 mM), $MgCl_2$ (5 mM), microsomal protein (5 mg) and 1/15 M phosphate buffer (pH 7.4) to give a final volume of 2.5 ml. The reaction was initiated by the addition of NADPH (1 mM) and terminated at 5, 15, 30 or 45 min by extraction into diethyl ether. Some incubations also contained an alleged inhibitor (EE_2, Ng, PQ, Cim, MC or Tri; 25–250 μM). Dg and metabolites were analysed by radiometric HPLC as previously described [3].

In incubations with 3α-OHDg and 3β-OHDg the microsomal protein content was varied from 0.5 to 2.0 mg. The reaction was terminated at 11 min and internal standard (gestodene) added. Compounds were identified by u.v. detection at 214 nm and metabolite quantified by the peak height ratios method. Standard curves were prepared by adding known amounts of 3-KDg to microsomal incubations. The co-efficient of variation for 2.0 μg 3-KDg was 2.4% ($n = 6$). Inhibition and kinetic studies were carried out under initial velocity conditions. For inhibition studies the substrate (3α-OHDg and 3β-OHDg) concentration was 20 μM and the concentration of inhibitor (EE_2, MC, PQ or Tri) varied from 10 to 250 μM. For kinetic studies the substrate (3α-OHDg) concentration was varied from 4 to 20 μM and the inhibitor (EE_2, MC) concentration varied from $\frac{1}{4}$ to the IC_{50} value. For the determination of K_m and V_{max}, Lineweaver–Burk plots were constructed and kinetic parameters determined using the intercept of the regression line. The value of the apparent K_i was determined by the Dixon graphical method [7].

Mass spectrometry

Electron impact mass spectrometry was carried out using a VG Tritech TS250 mass spectrometer. Solid samples were analysed by direct probe insertion and data were acquired by full scanning acquisition over *m/z* 50–550 (scan rate 1 scan/s) via a VG 11/250 Data system.

RESULTS

The livers used in this study were obtained from 6 patients (3 male, 3 female) having an age range of 17–68 yr. The cytochrome *P*450 content of the microsomes was 0.45 ± 0.21 nmol/mg protein (range 0.27–0.72 nmol/mg protein).

Following microsomal incubation with [^{3}H]Dg, metabolite identification was by co-chromatography with authentic standards (Fig. 1) and by mass spectral analysis. Analysis of fractions 29–32 yielded diagnostic ions at *m/z* 324 $[M]^+$, *m/z* 295 $[M—CH_2CH_3]^+$, *m/z* 277 $[295—H_2O]^+$, *m/z* 267 $[295—CH_2CH_2]^+$, *m/z* 257 $[M—OC.CH{=}C{=}CH_2]^+$, consistent with the spectrum from authentic 3-KDg. The spectra obtained from fractions 24–28 and 33–35 contained diagnostic ions at *m/z* 326 $[M]^+$, *m/z* 308 $[M—H_2O]^+$, *m/z* 297 $[M—CH_2CH_3]^+$, *m/z* 279 $[308—CH_2CH_3]^+$, *m/z* 261 $[279—H_2O]^+$, *m/z* 255 $[308—CH_2{:}C(OH)C{\equiv}CHCH_3]^+$ and *m/z* 211 $[301—CH_2{:}C(OH)C{\equiv}CH\text{-}CH_2CH_3]^+$ consistent with the spectra from 3-OHDg. A large heterogenous peak (peak 1) of metabolites was observed with each of the livers; this did not co-chromatograph with any of the known metabolites of desogestrel. Mass spectral analysis of this peak did tentatively suggest the presence of mono-, di- and tri-hydroxylated metabolites of 3-KDg.

The rate of disappearance of desogestrel in microsomal incubations of six human livers is shown in Fig. 2. After 45 min, 76.2 ± 4.0% ($n = 6$; mean ± SD; range 69.5–81.1%) of the drug present in the medium was present as unchanged Dg. Figure 3 shows the formation of metabolites during a 45 min incubation in the six livers used. All the livers examined were able to form 3-KDg and its presumed precursors 3α-

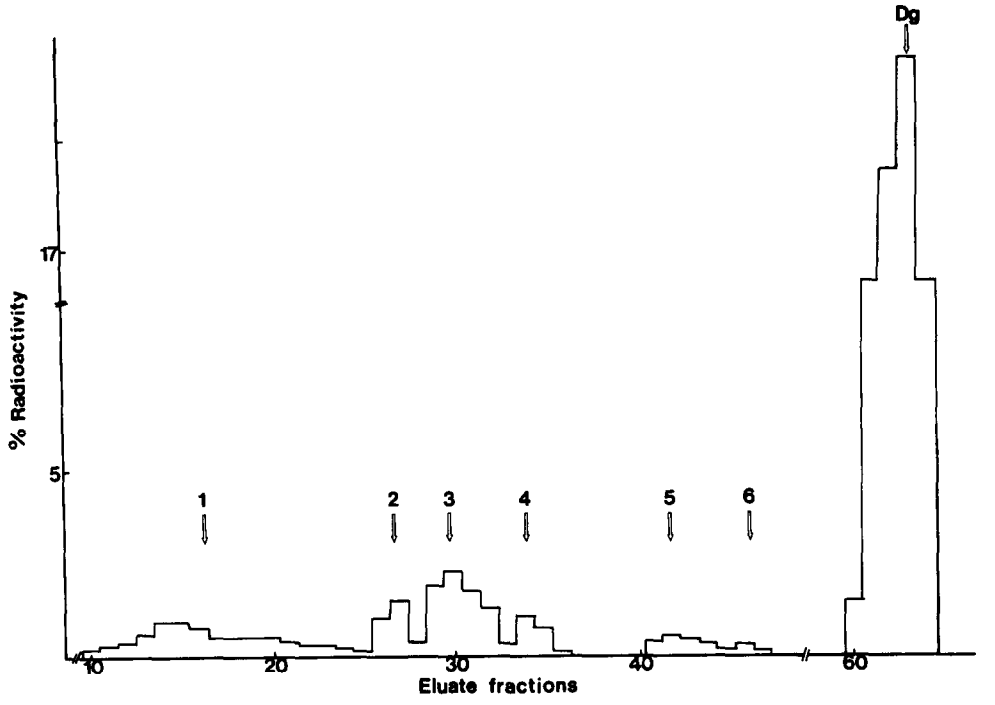

Fig. 1. A respresentative high performance liquid chromatogram of an ether extract of a microsomal incubation with [^{3}H]Dg. Peak assigment is by co-chromatography with authentic standards: 1 = unidentified metabolites, 2 = 3β-OHDg, 3 = 3-KDg, 4 = 3α-OHDg, 5 = 3α-OH, 5α-HDg, 6 = 3-K, 5α-HDg.

OHDg and 3β-OHDg. PQ was the most potent inhibitor of oxidative metabolite production causing 50% inhibition at a concentration of 30 μM (Table 1). EE_2 and MC were also found to inhibit the oxidative metabolism of Dg having IC_{50} values of 90 and 138 μM respectively. Cim, Ng and Tri failed to show any inhibition at 250 μM.

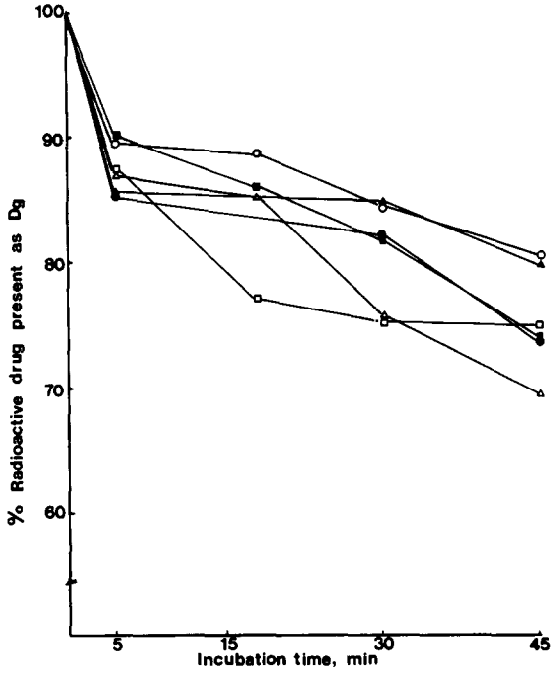

Fig. 2. Disappearance of [^{3}H]Dg from microsomal incubations of six human livers. ●——● Liver I, ▲——▲ Liver II, ■——■ Liver III, △——△ Liver IV, ○——○ Liver V, □——□ Liver VI.

When radiolabelled 3-KDg was incubated with microsomes, polar but mainly unidentified metabolites were seen (Fig. 4). Miconazole caused marked inhibition of 3-KDg metabolism (Fig. 4).

Optimal conditions for the measurement of 3α- and 3β-HSDH enzyme activities were chosen in order to maintain reaction in the linear portion of the curve with respect to both time and protein, i.e. an incubation time of 11 min with 0.5 mg protein per assay for 3α-HSDH and an incubation time of 10 min with 2.0 mg protein per assay for 3β-HSDH.

In the absence of inhibitor the 3α-HSDH activity was 1.0 ± 0.3 nmol min^{-1} mg^{-1} protein and the 3β-HSDH activity 0.2 ± 0.1 nmol min^{-1} mg^{-1} protein ($n = 4$), at a substrate concentration of 20 μM. MC was the most potent inhibitor of both dehydrogenases having IC_{50} values of 14 and 95 μM for 3α- and

Table 1. IC_{50} values of various inhibitors of desogestrel metabolism

Drug	IC_{50} (μM)
Ethinyloestradiol	90
Norgestrel	>250
Cimetidine	>250
Primaquine	30
Miconazole	138
Trilostane	>250

n = 4–6 livers. Each liver was analysed separately and the IC_{50} value calcutaled from the mean data.

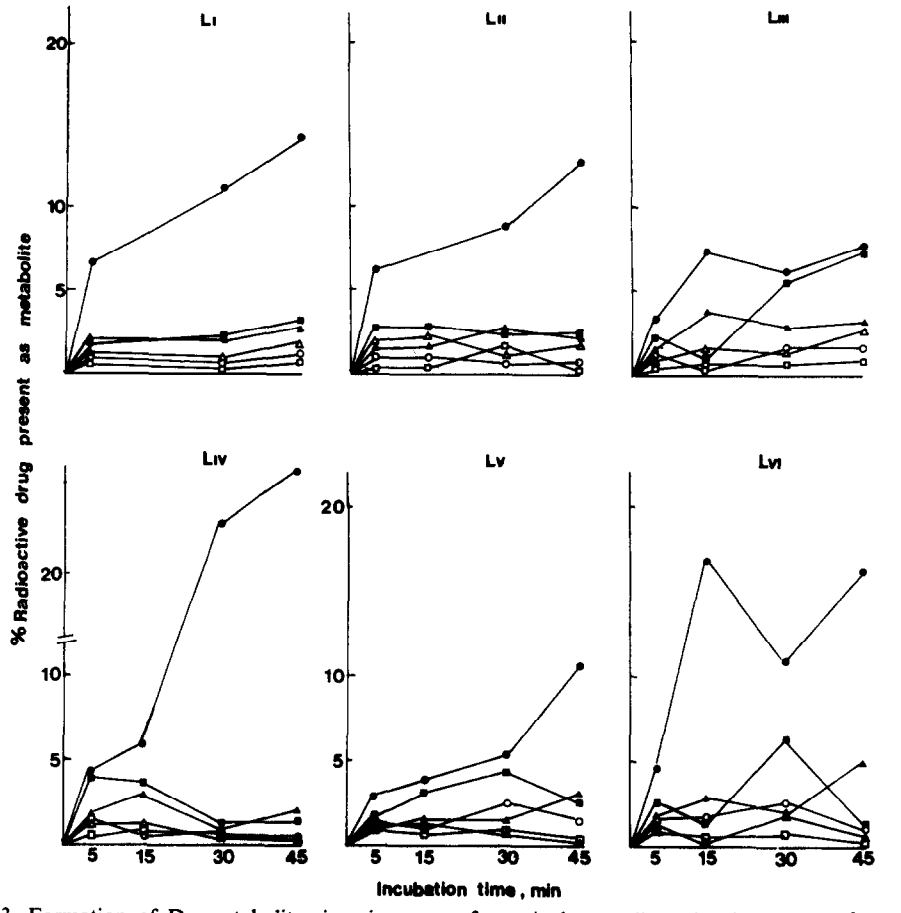

Fig. 3. Formation of Dg metabolites in microsomes from six human livers incubated with [^{3}H]Dg. ●——● Unidentified peak, ▲——▲ 3β-OHDg, ■——■ 3-KDg, ○——○ 3α-OHDg, △——△ 3-K, 5α-HDg, and □——□ 3α-OH5α-HDg.

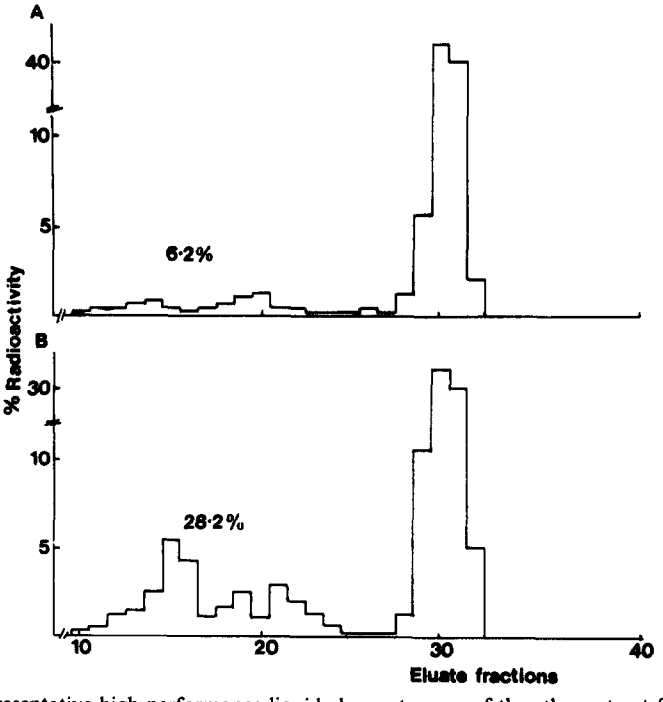

Fig. 4. A representative high-performance liquid chromatogram of the ether extract from a microsomal incubation of [^{3}H]3-KDg in the presence (A) and absence (B) of 50 μM miconazole.

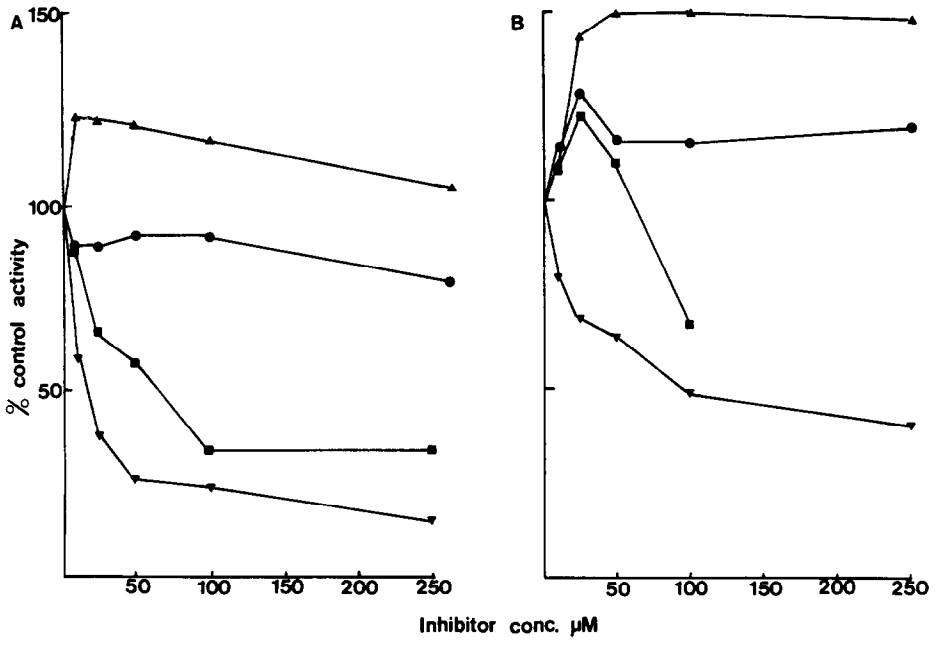

Fig. 5. Effect of various compounds on 3α-HSDH activity (A) and 3β-HSDH activity (B) of human liver microsomes. ▼——▼ miconazole; ■——■ ethinylestradiol; ●——● primaquine; ▲——▲ trilostane. Mean plots are shown with microsomes from four livers.

3β-HSDH respectively (Fig. 5). EE_2 also inhibited both activities whereas PQ only inhibited 3α-HSDH activity. Tri was without any inhibitory effect on either dehydrogenase.

Table 2 gives the enzyme (3α-HSDH) kinetic parameters in the presence of MC and EE_2. The K_m value for 3α-HSDH determined by regression analysis for Lineweaver–Burk plots was 4.4 μM and the V_{max} value 1.28 nmol min^{-1} mg^{-1} protein. MC was a fairly potent mixed non-competitive inhibitor ($K_i = 4.3$ μM) whereas EE_2 produced non-competitive inhibition with a K_i of 116 μM.

DISCUSSION

Desogestrel is an orally active progestogen, the activity of which is mediated by the 3-keto metabolite [1]. After oral administration Dg undergoes extensive first pass metabolism to the active metabolite [2, 8]. We have previously demonstrated that the biotransformation to 3-KDg readily takes place in the gut wall *in vitro*. Now, in this study we have shown that the liver also possesses the ability to convert Dg to the active metabolite. This finding is in agreement with the findings of Viinikka[4].

Table 2. Characterisation of inhibition of 3α-HSDH by EE_2 and MC

Inhibitor	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	K_i (μM)	Mode of inhibition
Control	4.4	1.28	—	—
EE_2	4.4	0.84	116	Non-comp.
MC	16.7	0.68	4.3	Mixed

To obtain these values for K_m and V_{max} the concentration of inhibitor was the IC_{50} value. The apparent K_i was calculated by the graphical method of Dixon[7], using variable inhibitor and substrate concentrations. Mean data from 4 livers.

Although there is less than a 2-fold variation in the overall extent of Dg metabolism (Fig. 2) there is much greater variation in the amounts of the individual metabolites (Fig. 3) formed in each of the six livers. This inter-individual variation is common for many drugs and is a result of various genetic [9, 10] and environmental [11] factors which influence the levels and activities of drug metabolising enzymes. In this study major contributory factors to the variation will be the age, sex and previous exposure to xenobiotics of the six donors. The age of the six donors ranged from 17–68 yr three being male and three female. Little, however, is known about their previous exposure to xenobiotics, although the donor of liver II had been receiving phenobarbitone, a known inducer of selected cytochrome *P*450 mediated oxidations for a number of years. There was no apparent increase in Dg metabolism in microsomes prepared from this liver suggesting that phenobarbitone may have no inductive effect on the enzymes responsible for Dg metabolism.

Prior to the formation of 3-KDg, Dg must first of all undergo hydroxylation at C-3. Ring hydroxylations are typically catalysed by the cytochrome *P*450 mixed function oxidase system [12]. Cytochrome *P*450 exists as a family of isozymes [13, 14] differing in the type of reaction they catalyse and in their substrate specificity. Hydroxylation of Dg at C-3 can lead to the formation of 3α-OHDg and/or 3β-OHDg. In the six livers studied (Fig. 3) both of these hydroxylated metabolites were formed, but there was a great deal of variation in the ratio of 3α/3β suggesting that the formation of these two isomers may be dependent upon different forms of cyto-

chrome *P*450, the levels of which vary between the livers used. Thus, the route of 3-KDg formation will be partly determined by the activity of the 3α-hydroxylase vs that of the 3β-hydroxlase.

Both 3α-OH and 3β-OH desogestrel were readily converted to the biologically active 3-keto desogestrel. However, there was a 5-fold difference in the relative activity of the two dehydrogenases (3α-HSDH > 3β-HSDH). In incubations with Dg as substrate (Fig. 3), 3β-OHDg was the major 3-hydroxylated metabolite found. This is consistent with a rapid turnover of 3α-OHDg to 3-KDg with much slower conversion of 3β-OHDg to the active metabolite, suggesting that, like the conversion of lynestrenol to norethisterone in the rabbit [15], the predominant route of conversion of Dg to 3-KDg is probably via the 3α-hydroxylated metabolite. Although the results from this study indicate that the 3β-pathway may only be a minor route of 3-KDg formation under control conditions, it may assume much greater importance if, for some reason, the 3α-pathway is perturbed.

The rate of metabolism of 3-KDg will also be a major factor in determining the levels of 3-KDg in the livers studied. 3-KDg is structurally very similar to the related progestogen Ng which undergoes extensive oxidative metabolism to a number of hydroxylated compounds [16, 17]. In all of the livers studied a large group of polar metabolites was observed which could not be identified as any of the known metabolites of Dg (Fig. 1). A similar group of metabolites was found following incubation of 3-KDg with microsomes (Fig. 4b). From preliminary mass spectrometry data we have postulated that these metabolites represent further hydroxylated metabolites of 3-KDg. Formation of such metabolites may explain the reduced bioavailability in some subjects [2, 8].

A number of drugs were tested as putative inhibitors of Dg metabolism. The most potent of these was the aminoquinoline anti-malarial drug PQ ($IC_{50} = 30\,\mu M$). PQ is a well documented inhibitor [18, 19] of cytochrome *P*450 mediated metabolism. Its mode of inhibition is by interaction with the oxidized form of cytochrome *P*450 [20, 21]. This relatively potent inhibition by PQ suggests that cytochrome *P*450-dependent hydroxylations are the rate limiting steps in Dg metabolism. The imidazole MC is known to be a very potent inhibitor of oxidative drug metabolism typically having IC_{50} values around $1\,\mu M$ [18]. In this system, however, MC was a relatively poor inhibitor of Dg metabolism having an IC_{50} value of $138\,\mu M$. Despite this poor inhibition of the overall oxidative metabolism of Dg it may be that MC is a potent inhibitor of individual steps in this complex pathway. Indeed, as can be seen from Fig. 5, MC was found to markedly inhibit the further metabolism of 3-KDg.

MC was also found to inhibit the microsomal 3α-HSDH and 3β-HSDH activities of human liver, although it was a less potent inhibitor of the latter having IC_{50} values of 14 and $95\,\mu M$ respectively. Again this inhibition of 3-HSDH activities may be a reflection upon the complexity of the overall metabolic pathway of Dg metabolism.

The other drugs tested as inhibitors of Dg metabolism were also investigated as possible inhibitors of 3α- and 3β-HSDH. In agreement with previous studies [22] EE_2 was found to inhibit both activities (Fig. 5). However, in contrast to the findings of other workers Tri [23, 24] had no inhibitory effect on 3β-HSDH and did, in fact, appear to cause some activation of the enzyme. Trilostane is a therapeutic agent which acts by inhibition of 3β-HSDH activity [25]. A number of studies have shown it to be a potent inhibitor in placental [23], ovarian [24] and adrenal [24] tissues in humans and rhesus monkeys. In this study Tri showed no inhibitory effect on either dehydrogenase activity at $250\,\mu M$.

This lack of inhibitory potency by Tri is worthy of note as it may be indicative of tissue differences in 3β-HSDH enzymes. We have concentrated our studies on the hepatic microsomal enzymes whereas previously published data have been from experiments carried out on steroidogenic tissue [23, 24]. Our results may suggest that the hepatic microsomal form of the enzyme is different from that found in the placenta, for example. In hormone producing tissues 3β-HSDH also possesses an isomerase activity [26] which catalyses the conversion of a Δ5 double bond to a Δ4 double bond, as can be seen from the structure of Dg (Fig. 6) there is no requirement for this isomerization step in the formation of 3-KDg. Thus, the difference between the 3β-HSDH of hepatic microsomes and that of steroidogenic tissue may reside in the lack of this isomerase activity in the hepatic enzyme. The lack of the isomerase activity may in some way alter the interaction of a drug such as trilostane with the enzyme.

The aminoquinoline anti-malarial PQ which was the most effective inhibitor of Dg metabolism had no inhibitory effect on either 3α- or 3β-HSDH; this is consistent with cytochrome *P*450-mediated hydroxylation being the rate limiting step in Dg metabolism.

Due to the low turnover of 3β-OHDg to 3-KDg it was not possible to determine any of the kinetic parameters for 3β-HSDH. Kinetic studies were, however, carried out on 3α-HSDH which was found to have a K_m value of $4.4\,\mu M$ and a V_{max} value of $1.28\,nmol\,min^{-1}\,mg^{-1}$ protein. Both EE_2 and MC inhibited the enzyme non-competitively, indicative of an interaction at a site other than the active site. However, at high concentrations of MC there was also an increase in K_m which suggests there is also some interference with binding of substrate to the active site.

The results of this study demonstrate the inter-individual variation in desogestrel metabolism and provide further evidence of the formation of the active metabolite by human liver microsomes. The

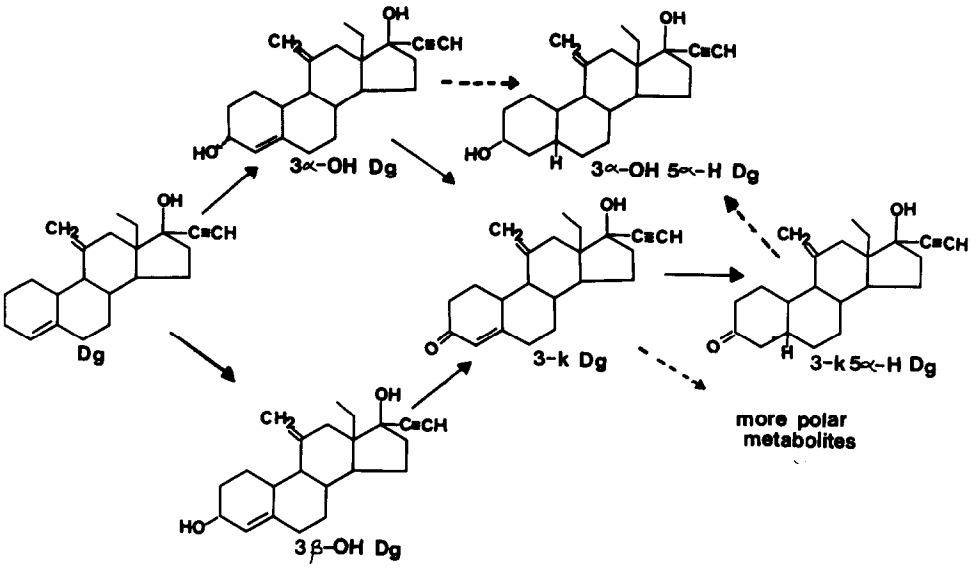

Fig. 6. The suggested metabolic pathways of Dg in human liver microsomes *in vitro*.

study also provides some insight into the pathways of Dg metabolism (Fig. 6). A number of drugs have been shown to exert some inhibitory effect on both the overall metabolism of Dg and on individual steps in the pathway of Dg metabolism.

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