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METABOLISM OF NORGESTIMATE BY HUMAN GASTROINTESTINAL MUCOSA AND LIVER MICROSOMES *IN VITRO*

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Summary—The metabolism of the progestogen oral contraceptive norgestimate has been studied *in vitro* using human intestinal mucosa and human liver microsomes. Metabolites have been separated using radiometric high-performance liquid chromatography (HPLC) and identified by co-chromatography with authentic standards and by mass spectrometry.

Histologically normal colon was obtained from 6 patients undergoing various resections and the mucosa mounted between 2 perspex (Ussing) chambers. 2 h after addition of [^{3}H]norgestimate to the mucosal chamber, more than 95% of the radioactivity was present in that chamber. Metabolite analysis showed 38.1 ± 11.6% (mean ± SD; $n = 8$) of drug present was norgestimate, 49.2 ± 14.5% as 17-deacetyl norgestimate and 8.1 ± 4.5% as conjugated metabolites. Small amounts of 3-keto norgestimate, norgestrel and uncharacterized metabolites were found. Norgestimate was also metabolized by stomach tissue with 17-deacetyl norgestimate again being the main metabolite found.

Microsomes were prepared from 6 human livers. Metabolism was studied over a 5 h time-course in the absence and presence of NADPH. Deacetylation to 17-deacetyl norgestimate took place in the absence of the cofactor. In the presence of NADPH, after 5 h incubation only 30.5 ± 14.6% (mean ± SD) of steroid present was norgestimate. The major metabolite formed was 17-deacetyl norgestimate which accounted for 39.3 ± 20.5%. Less than 2% was present as 3-keto norgestimate but 10.0 ± 2.3% was identified as norgestrel and 15.5 ± 8.9% as uncharacterized metabolites. We also examined the microsomal breakdown of [^{3}H]17-deacetyl norgestimate. This was NADPH and oxygen dependent. Norgestrel and other metabolites were formed.

This study has demonstrated that norgestimate is rapidly deacetylated by both gut wall and liver. The deacetylated metabolite can then be further metabolized.

INTRODUCTION

Norgestimate(13-ethyl-17-acetoxy-18,19-dinor-17α-pregn-4-en-20yn-3 oxime; norgestrel-3-oxime-17-acetate) is a new synthetic progestational agent given in combination with ethinyloestradiol (EE_2) for oral contraception. The possibility has been raised, principally on the basis of studies carried out in rhesus monkeys [1], that metabolites of norgestimate and not norgestimate itself may be responsible for receptor binding. Sisenwine *et al.* [1] concluded that norgestimate was a pro-drug, which is rapidly deacetylated to form 17-deacetyl norgestimate. This metabolite can subsequently be hydrolyzed to norgestrel which may then undergo further metabolism. There is only a small published literature on the biotransformation of norgestimate in women. In one study [2] the urinary metabolites of norgestimate were monitored over a 2-week period following administration of [^{14}C]norgestimate. A number of metabolites were identified including norgestrel, tetrahydronogestrel and various hydroxylated metabolites. Some of the metabolites were conjugated.

The receptor binding affinity of norgestimate and some of its metabolites (3-keto norgestimate; 17-deacetyl norgestimate, norgestrel) has been investigated in rabbit uterine tissue [3]. All four steroids had affinity for the progesterone receptor indicating that if formed *in vivo* they may all contribute to the progestogenic potency

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Trivial names: Norgestimate: (+)-13-ethyl-17-acetoxy-18,19-dinor-17α-preg-4-en-20-yn-3-oxime; 3-keto norgestimate: (+)-13-ethyl-17-acetoxy-18,19-dinor-17α-preg-4-en-20-yn-3-one; 17-deacetyl norgestimate: (+)-13-ethyl-17-hydroxy-18,19-dinor-17α-preg-4-en-20-yn-3-oxime; norgestrel: (+)-13-ethyl-17-hydroxy-18,19-dinor-17α-preg-4-en-20-yn-3-one.

of norgestimate. However, following oral administration of norgestimate there was an increase in the level of HDL and the EE_2-induced increase in SHBG was maintained [4] suggesting a lack in androgenicity after norgestimate administration. This is supported by receptor binding and studies *in vivo* which have shown norgestimate to be virtually devoid of any androgenic activity [5]. Since levonorgestrel is inherently androgenic [6] it would appear that *in vivo* levonorgestrel is not making a major contribution to the response.

In the present study we have examined the metabolism of norgestimate by human gastric mucosa, using a technique (Ussing Chambers; Ussing and Zehran [7]) which we have previously used to study the metabolism of EE_2 [8] and desogestrel [9], and by human liver microsomes.

EXPERIMENTAL

Chemicals

[^{3}H]Norgestimate (sp.act. 44 Ci mmol^{-1}), norgestimate, [^{3}H]17-deacetyl norgestimate (sp.act. 40 Ci mmol^{-1}), 17-deacetyl norgestimate (norgestrel oxime), 3-keto norgestimate (norgestrel 17-acetate) and levonorgestrel were all gifts from Ortho Pharmaceuticals Corp., N.J., U.S.A All other chemicals were obtained from either Sigma Chemical Co. or BDH Chemicals, Poole, U.K.

Intestinal studies

Histologically normal colon or stomach were obtained from patients undergoing resections for localized tumours. The study was approved by the Mersey Regional Hospital Ethics Committee. Mucosal sheets were prepared from the colon samples and mounted between 4 pairs of perspex chambers as previously described [8]. In brief, while mounted on a glass tube, the muscularis externa was stripped off the mucosa and then following a longitudinal incision, the mucosal sheets were mounted between 2 perspex chambers. Fresh Krebs–Henseleit buffer (2.5 ml; pH 7.4) was added to each chamber, maintained at 37°C by water jackets. The buffer was continually aerated with 95% O_2 and 5% CO_2. The transmural potential difference was measured on an automatic multimeter (Phillips, Model PM 2521), this gave a measure of tissue viability [8]. Radiolabelled norgestimate (0.2 μCi; 200 ng; in buffer; radiochemical purity >97%) was added to each mucosal chamber. In preliminary experiments with tissue from 2 patients, aliquots (1 ml) of mucosal and serosal fluid were removed after 2 h for determination of drug and metabolites by HPLC. In further studies with tissue from 4 patients, aliquots (1 ml) were removed after 15, 30, 60 and 120 min from successive chambers to determine the time-course of metabolite formation.

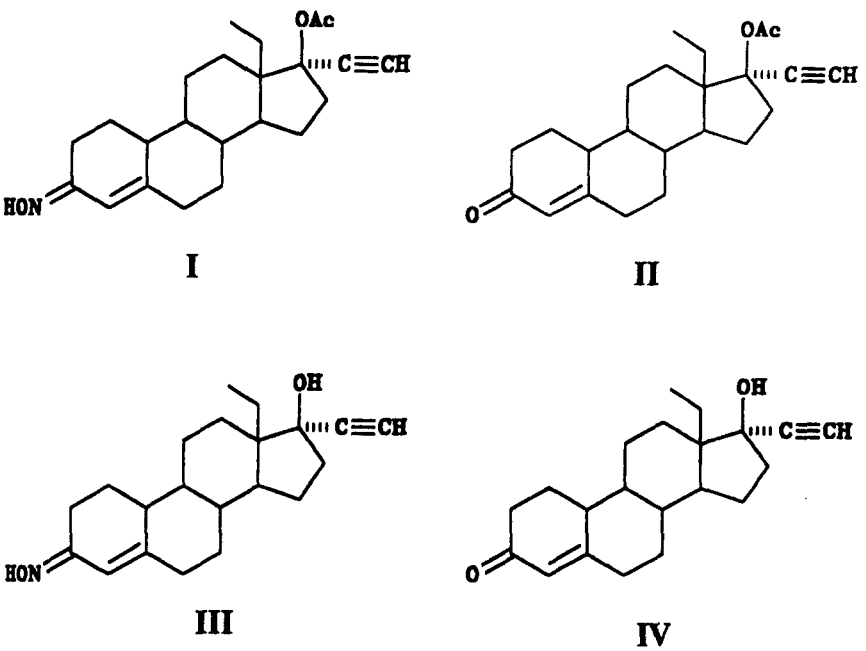

Fig. 1. Structures of norgestimate (I), 3-keto norgestimate (II), 17-deacetyl norgestimate (III) and norgestrel (IV).

In experiments with human stomach, after stripping off the muscle layers, the tissue was placed in a glass tube and buffer and radiolabelled norgestimate added. Incubation was at 37°C for 2 h. Aliquots (1 ml) were then removed for determination of metabolites.

Metabolite separation

Aliquots of mucosal fluid (1 ml), or buffer in stomach experiments, were extracted with diethyl ether (2 × 5 ml) for the determination of parent drug and unconjugated metabolites. Aliquots (400 μl) of the remaining aqueous phase were incubated for 3 h at 37°C with β-glucuronidase/sulphatase enzyme (*Helix pomatia*, Type H1; 100 units; Sigma) either in the presence or absence of the specific glucuronidase inhibitor glucarolactone (24 mg ml^{-1} in 0.1 M acetate buffer; pH 5.0). Control samples were incubated with acetate buffer. After incubation, steroid was extracted as above and the proportion of the ^{3}H extracted into ether determined. Extracts were evaporated to dryness and the residue resuspended in methanol (100 μl) for HPLC analysis.

HPLC analysis

A Spectra Physics SP 8700 solvent delivery system was used connected to an LKB 2122 Redirac fraction collector. Chromatographic separation was performed on a μBondapak cartridge (10 × 0.8 cm i.d., Waters, U.K.) housed in a radial compression module and protected by an on-line guard column. Samples were eluted using a mobile phase of methanol in water (70/30; v/v) at a flow rate of 1.5 ml min^{-1}. The following authentic standards were monitored by U.V. detection at 240 nM; norgestimate, 3-keto norgestimate, 17-deacetyl norgestimate and norgestrel.

Human liver studies

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 obtained from the donor's 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.

Washed microsomes were prepared as previously described [10]. Cytochrome *P*-450 was assayed by the method of Omura and Sato [11] and the microsomal protein by the method of Lowry *et al.* [12].

Microsomal incubations

Incubations were performed in 10 ml screw-top tubes at 37°C in a shaking water bath. Incubations contained [^{3}H]norgestimate or [^{3}H]17-deacetyl norgestimate (60 μM; 0.2 μCi), KCl (1 mM), EDTA (1 mM), $MgCl_2$ (5 mM), microsomal protein (2 mg) and 1/15 M phosphate buffer (pH 7.4) to give a final volume of 2.5 ml. These conditions were chosen to give adequate turnover of substrate for detection of metabolites. The reaction was initiated by the addition of NADPH (1 mM) which was added again at hourly intervals. The reaction was terminated by the addition of diethyl ether (5 ml). In some incubations, NADPH was omitted and the reaction initiated by the addition of microsomal protein. Following extraction the samples were evaporated to dryness and resuspended in methanol for HPLC analysis as described above.

In one series of experiments with 17-deacetyl norgestimate as substrate, the incubations were carried out under anaerobic conditions. This was achieved by degassing all the buffers and then bubbling nitrogen into them. Incubations were carried out in sealed tubes with a continuous stream of nitrogen.

Mass spectra analysis

After incubation, drug and metabolites were extracted into ether and separated by HPLC. The relevant fractions were collected in screw-top tubes and analyzed by probe inlet electron impact mass spectrometry (VG Tritech TS 250). Data were acquired by full scanning acquisition over m/z 50–500 (scan rate, 1 scan/s) via a VG 11/250 data system.

RESULTS

Intestinal studies

Good separation of the authentic standards, norgestimate, 3-keto norgestimate, 17-deacetyl norgestimate and norgestrel was obtained by HPLC with retention times of 17.5, 12.5, 8.5 and 6 min respectively.

The intestinal metabolism of norgestimate was studied in tissues obtained from a total of 6 patients. Initial studies were carried out with 8 pieces of tissue from 2 patients. Since only very low amounts of steroid were transported to the serosal chamber, norgestimate and

Table 1. Metabolism of norgestimate by human colon and stomach. Drug and metabolites were determined in the mucosal chamber (for colon) or incubation buffer (for stomach) 2 h after the addition of [^{3}H]norgestimate (0.2 μCi; 200 ng)

	% of drug present is					
	X	Ng	NgOx	NgAc	Ngmate	Conjugates
Colon	1.1 ± 1.3	2.4 ± 0.6	49.2 ± 14.5	0.3 ± 0.3	38.1 ± 11.6	8.1 ± 4.5
Stomach	0.9 ± 0.3	1.4 ± 1.7	20.3 ± 16.6	1.3 ± 0.8	75.0 ± 17.7	0

Values are mean ± SD of 4 separate pieces of tissue from 2 patients (i.e. $n = 8$).
X = uncharacterized metabolites. Ng = norgestrel. NgOx = norgestrel oxime (17-deacetyl norgestimate). NgAc = norgestrel acetate (3-keto norgestimate). Ngmate = norgestimate.

metabolites were analyzed from the mucosal chamber only (>95% radioactive steroid present). Metabolites were identified by co-chromatography with the authentic standards (mass spectral analysis was performed on metabolites obtained in liver microsomal incubations). After 2 h, 38.1 ± 11.6% ($n = 8$ chambers; mean ± SD) of drug was present as unchanged norgestimate, 49.2 ± 14.5% as 17-deacetyl norgestimate and 8.1 ± 4.5% as conjugated metabolites. Small amounts of 3-keto norgestimate, norgestrel and uncharacterized metabolites (possibly further metabolites of norgestrel) were found (Table 1). A representative HPLC profile from a 2 h incubation is shown in Fig. 2.

In tissues from the remaining 4 patients, norgestimate metabolism was studied over a 2 h time-course. Metabolism was time-dependent (Table 2). After 15 min, 68.3 ± 19.4% ($n = 4$) of drug was present as unchanged norgestimate, and this fell to 46.3 ± 18.4% by 2 h. 17-Deacetyl norgestimate was the major metabolite present in all samples at all time points with 41.5 ± 21.6% being present at 2 h. Low amounts of 3-keto norgestimate, norgestrel, other Phase I metabolites and conjugated metabolites were also present (Fig. 3).

Norgestimate was also metabolized by stomach tissue (Table 1) with 17-deacetyl norgestimate again being the main metabolite formed.

Liver microsomal studies

The livers used in this study were obtained from 6 patients (5 male; 1 female; age range 27–54). The cytochrome *P*-450 content of the

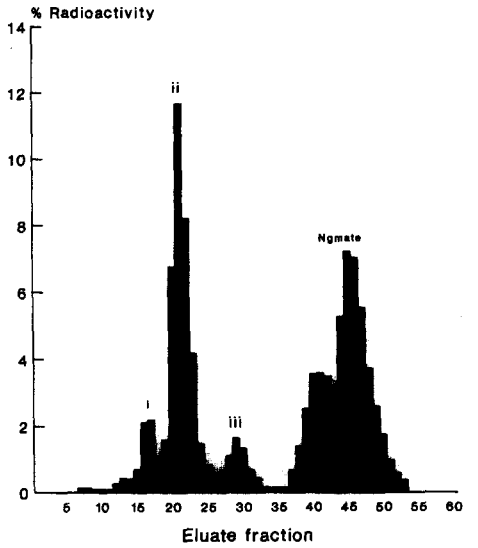

Fig. 2. A representative HPLC from an ether extract of mucosal fluid from a 2 h incubation of [^{3}H]norgestimate with human colon. Peak assignment is based on co-chromatography with authentic standards. (i) norgestrel; (ii) 17-deacetyl norgestimate; (iii) 3-keto norgestimate; Ngmate, norgestimate.

microsomes was 0.44 ± 0.19 nmol mg^{-1} protein. The metabolism of norgestimate in the absence and presence of NADPH is shown in Fig. 3. It is evident that the steroid undergoes metabolic breakdown even in the absence of NADPH. However, in the presence of the cofactor the metabolic profile is more complex. Metabolites were identified by co-chromatography and by mass spectral analysis. Fractions 21–24 (collected from the HPLC eluate) yielded diagnostic ions at m/z 327 $[M]^+$, m/z 311 $[M\text{-}O]^+$, m/z 282 $[311\text{-}C_2H_5]^+$, m/z 259 $[M\text{-}CH_2C(OH)C\text{-}CH]^+$ and m/z 214 $[282\text{-}CH_2C(OH)C\text{-}CH]^+$ consistent

Table 2. Time-course of the metabolism of norgestimate by human colon. Drug and metabolites were determined in the mucosal chamber* after the addition of [^{3}H]norgestimate (0.2 μCi; 200 ng)

Time	% of drug present					
(min)	X	Ng	NgOx	NgAc	Ngmate	Conjugates
15	3.6 ± 4.1	2.7 ± 1.3	16.0 ± 7.6	3.4 ± 2.1	68.3 ± 9.4	3.0 ± 1.3
30	2.1 ± 0.9	2.9 ± 1.0	23.5 ± 10.2	2.7 ± 1.0	63.7 ± 9.9	4.1 ± 1.9
60	2.1 ± 1.0	4.3 ± 1.6	30.1 ± 21.3	3.2 ± 2.2	51.8 ± 19.8	5.4 ± 3.2
120	1.2 ± 1.1	4.5 ± 1.6	41.5 ± 21.6	2.3 ± 2.3	46.3 ± 18.4	3.6 ± 2.5

Values are mean ± SD of tissue from 4 separate patients for each time point.
X = uncharacterized metabolites. Ng = norgestrel. NgOx = norgestrel oxime (17-deacetyl norgestimate). NgAc = norgestrel acetate (3-keto norgestimate). Ngmate = norgestimate.
*Greater than 95% of radioactivity was recovered from this chamber at each time point.

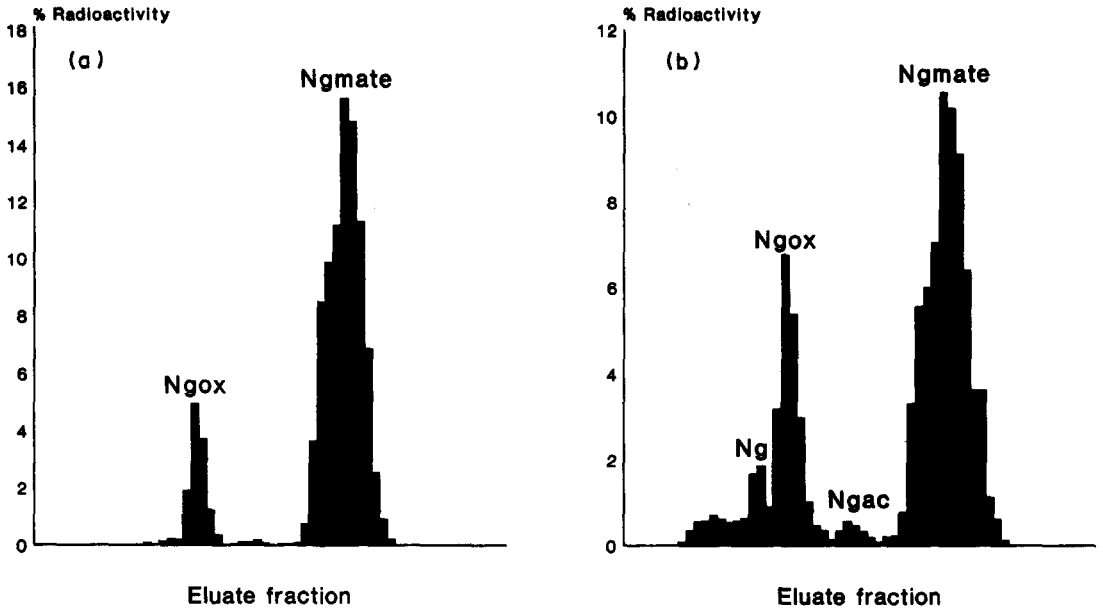

Fig. 3. Representative high-performance liquid chromatographs of ether extract of human microsomal liver incubations with [^{3}H]norgestimate in (a) absence and (b) presence of NADPH. Ngmate, norgestimate; Ngox, 17-deacetyl norgestimate (norgestrel oxime); Ng, norgestrel; Ngac, 3-keto norgestimate (norgestrel acetate)

with the spectra from authentic 17-deacetyl norgestimate. Fractions 16–19 yielded diagnostic ions at m/z 312 [M^+], m/z 294 [$M\text{-}H_2O$]$^+$, m/z 283 [$M\text{-}C_2H_5$]$^+$, m/z 265 [$283\text{-}H_2O$]$^+$, m/z 255 [$283\text{-}CO$]$^+$, m/z 245 [$M\text{-}C_4\text{-}OH_3$]$^+$ and m/z 215 [$283\text{-}CH_2C(OH)CH_2$]$^+$ consistent with the spectra from authentic 17-deacetyl norgestimate. A polar metabolite or group of polar metabolites were also formed which could not be identified as any of the available metabolites of norgestimate; they possibly represent further hydroxylation of norgestrel.

The metabolism of norgestimate was studied in 6 livers over a 5 h time-course (Fig. 4). After 5 h only $30.5 \pm 14.6\%$ (mean $\pm$ SD) of drug present was norgestimate. The major metabolite formed was 17-deacetyl norgestimate which accounted for $39.3 \pm 20.5\%$ (mean $\pm$ SD) of steroid present at 5 h. Less than 2% was present as 3-keto norgestimate but $10.0 \pm 2.3\%$ was identified as norgestrel and $15.5 \pm 8.9\%$ as uncharacterized metabolites.

The metabolism of 17-deacetyl norgestimate was very much dependent on the presence of NADPH and oxygen in the incubation (Fig. 5; Table 3). At 2 h, $60.9 \pm 20.2\%$ of the steroid present was 17-deacetyl norgestimate, $11.3 \pm 3.5\%$ norgestrel and $27.4 \pm 17.3\%$ uncharacterized metabolites.

There was a significant correlation ($r = 0.97$) between the extent of 17-deacetyl norgestimate metabolism and cytochrome P-450 content.

DISCUSSION

Norgestimate was extensively metabolized by the gastrointestinal mucosa. With tissue obtained from the colon the main metabolic reaction was deacetylation to give 17-deacetyl norgestimate. However, removal of the oxime moiety also occurred as seen by the presence of small amounts of 3-keto norgestimate and norgestrel. Deacetylation was also seen in tissue

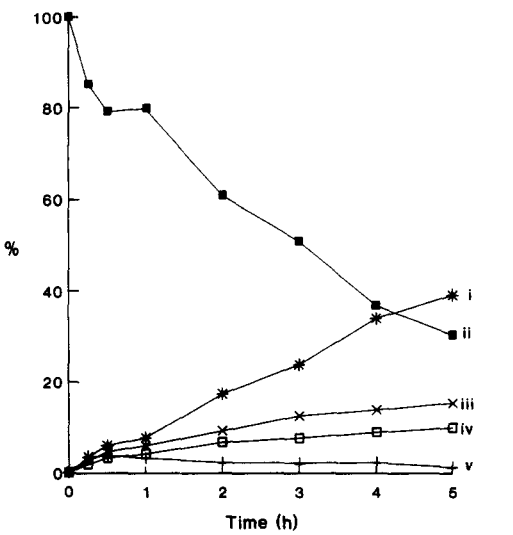

Fig. 4. Disappearance of norgestimate and appearance of metabolites in microsomal incubations with [^{3}H]norgestimate. Each point is mean data from 6 livers. (i) 17-deacetyl norgestimate; (ii) norgestimate (iii) uncharacterized metabolites; (iv) norgestrel; (v) 3-keto norgestimate (norgestrel acetate).

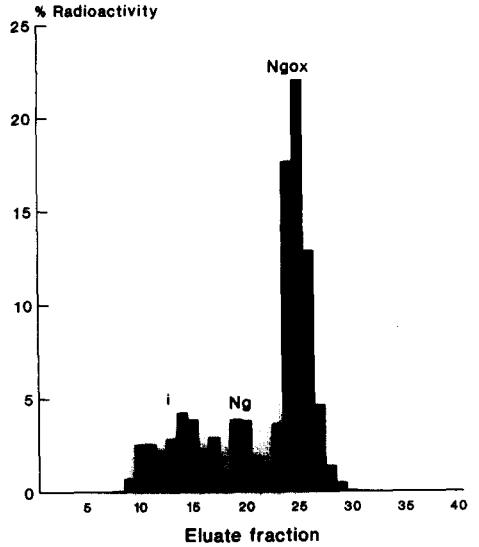

Fig. 5. A representative high-performance liquid chromatograph from an ether extract of human microsomal liver incubation with [^{3}H]17-deacetyl norgestimate in the presence of both O_2 and NADPH. It should be noted that when O_2 and NADPH were not present no metabolite formation was seen (figure not shown). (i) uncharacterized metabolites; Ng, norgestrel; Ngox, 17-deacetyl norgestimate (norgestrel oxime).

obtained from the stomach. The deacetylation reaction is typically catalyzed by esterase enzymes. Like other enzymes involved in xenobiotic metabolism, they exist as a family of distinct isoenzymes which exhibit overlapping substrate specificity [13], and the same substrate may be metabolized by a number of enzymes. Steroids are metabolized by the class of esterase enzymes termed carboxylesterases [14]. The same class of enzymes is responsible for the metabolism of other compounds such as acetylsalicylic acid [15, 16] and clofibrate [17]. Inoue *et al.* [18] purified carboxylesterases from both human hepatic tissue and intestinal mucosa and identified 2 esterases. The intestinal and hepatic forms were found to have similar substrate specificities.

Sisenwine *et al.* [1] have previously reported the plasma metabolite profile of norgestimate in rhesus monkeys in which norgestrel and hydroxylated metabolites of norgestrel were seen. Alton *et al.* [2] identified these as terminal metabolites in women. The present study indicates that the gut wall may be an important site for the initial breakdown of the drug (i.e. deacetylation) *in vivo*, but it is unlikely that quantitatively important further metabolism occurs at this sites. Hence we turned our attention to the liver.

Table 3. Metabolism of 17-deacetyl norgestimate by human liver microsomes over a 2 h period of incubation

Time (min)	% of steroid present in the incubate as X	Ng	NgOx
15	19.4 ± 13.1	9.2 ± 3.3	70.1 ± 16.7
30	20.7 ± 13.5	9.1 ± 3.3	68.7 ± 16.9
60	26.3 ± 15.2	11.8 ± 3.7	60.1 ± 19.3
120	27.4 ± 17.3	11.3 ± 3.5	60.9 ± 20.2

Values are mean ± SD from 6 livers.
X = uncharacterized metabolites. Ng = norgestrel. NgOx = norgestrel oxime (17-deacetyl norgestimate).

In the absence of NADPH, norgestimate underwent deacetylation by hepatic microsomes. The extent of hydrolysis of the acetate group was roughly comparable to that seen in the intestinal study, although comparisons are difficult to make because of the different conditions of the *in vitro* studies. Thus, 17-deacetylated norgestimate was the major metabolite identified in the microsomal preparation. Hepatic steroid esterases have been studied in a variety of species [14, 19] and have been shown to exist predominantly in the microsomal fraction.

In the presence of NADPH in the microsomal incubation, the metabolic profile was more complex, with evidence of norgestrel, and uncharacterized metabolite(s) (possibly further metabolites of norgestrel). Norgestrel itself has previously been shown to undergo both A and D ring oxidation [20, 21] to yield a variety of polyhydroxylated metabolites. A number of these hydroxylated metabolites of norgestrel have been identified in the plasma of rhesus monkeys and the urine of women [1, 2]. It should be noted that our microsomal incubations did not include UDPGA and therefore we were not able to observe the formation of glucuronide conjugates.

Since norgestimate undergoes extensive deacetylation in both gut and liver we finally focussed attention on hepatic metabolism of 17-deacetyl norgestimate. In the absence of oxygen, oximes have been said to undergo reduction to the corresponding hydroxylamine and amine [22] and in aerobic conditions to form the corresponding ketone. We found that 17-deacetyl norgestimate underwent virtually no metabolism when oxygen and/or NADPH was excluded. However, in the presence of both oxygen and NADPH there was fairly extensive metabolism. The implication from this is the possible involvement of cytochrome *P*-450 in the removal of the oxime moiety. Further evidence for this is the significant correlation between extent of metabolism and cytochrome *P*-450 concentration. Also we have recently shown (unpublished observations) that a

number of purified rat cytochrome *P*-450 isozymes metabolize norgestrel oxime to norgestrel and other metabolite(s).

In summary, the study has demonstrated that norgestimate is rapidly deacetylated by both gut wall and liver to form 17-deacetyl norgestimate. Such extensive deacetylation is also known to occur *in vivo*. It is likely that 17-deacetyl norgestimate undergoes further metabolism although a significant amount appears to remain intact as shown by the pharmacokinetics of the metabolite in plasma after norgestimate administration to women [23]. It is probable that the progestogenic effects of norgestimate are mediated by one or more metabolites with the most likely candidate being deacetylated norgestimate. This is supported not only by the *in vitro* and pharmacokinetics studies but also from clinical studies which have indicated norgestimate to be devoid of much androgenic activity [5] and hence suggesting that norgestrel does not make a major contribution to the response.

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