N-demethylation of ethylmorphine in pregnant and non-pregnant women and in men: an evaluation of the effects of sex steroids

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- 1 The effects of oestrogens, testosterone, progesterone and medroxyprogesterone acetate (MPA) on the rate of *N*-demethylation of ethylmorphine (EM) to norethylmorphine (NEM) were studied in human adult liver microsomes.
- 2 *N*-Demethylase activity was found to be inhibited by progesterone and MPA to a similar extent while oestrogens and testosterone had no or negligible effects.
- 3 These findings prompted us to measure the *N*-demethylation of EM in relation to serum progesterone concentration *in vivo* in three groups of volunteers with large physiological differences in their endogenous levels of progesterone, i.e. i) pregnant women, ii) non-pregnant ovulating women and iii) men.
- 4 The metabolic ratio (MR_P) of EM to NEM in plasma 60 min after dosage and the corresponding ratio in urine sampled for 6 h (MR_{U,1}), measured on two occasions 14 days apart were used to reflect intraindividual variation in the rate of *N*-demethylation.
- 5 The average difference in MR_P and MR_{U,1} between the two occasions was similar in all groups. However, the variability in MR_P between individuals within a group was significantly higher in ovulating women than in men, but this had no relation to the serum concentrations of progesterone or oestradiol.
- 6 The cumulative 12 h urinary excretion of EM, NEM and morphine (MO) after hydrolysis with β -glucuronidase was about 46%. There was no difference in the metabolic ratio of EM to NEM and its conjugate(s) in the urine between the luteal and the follicular phases. Our findings suggest that the menstrual cycle does not influence the rate of *N*-demethylation of EM.

Keywords ethylmorphine menstrual cycle microsomes pregnancy progesterone

Introduction

Ethylmorphine (EM) is used as a model substrate for *in vitro* studies of *N*-demethylation (Takemori & Mannering, 1958) and oestradiol and testosterone are known to inhibit this process in rat liver microsomes (Tephly & Mannering, 1964). The *N*-demethylation of EM to norethylmorphine (NEM) has been found to be catalyzed by the cytochrome P450 3A enzyme subfamily (Elshourbagy & Guzelian, 1980; Ladona *et al.*, 1989; previous nomenclature P450HLP and P450III_A, see Nebert *et al.*, 1989, 1991). In view of the important role of cytochrome P450 3A in the hydroxylation of oestradiol (Ball *et al.*, 1990) and testosterone (Guenrich *et al.*, 1986) and of our finding that progesterone

inhibits EM *N*-demethylation in human foetal liver microsomes (Ladona *et al.*, 1989), we considered it of interest to investigate possible metabolic interactions between sex steroids and EM in man.

Our study was based on the hypothesis that the cytochrome P450 catalyzed *N*-demethylation of EM may be affected *in vivo* by endogenous sex steroids, in particular progesterone. *In vitro* experiments described here revealed a marked inhibitory effect of progesterone on the *N*-demethylation of EM in human adult liver microsomes. This prompted us to investigate the *N*-demethylation of EM *in vivo* in three groups of subjects with large differences in their endogenous

levels of progesterone. The effect of progesterone on the *N*-demethylation of EM was evaluated by determination of drug:metabolic ratios in plasma and in urine.

Methods

Chemicals

Ethylmorphine hydrochloride was obtained from the Hospital Pharmacy. Progesterone, oestrone, oestriol, 17- β -oestradiol, testosterone, MPA, β -glucuronidase (EC 3.2.1.31; from *Helix pomatia*) and aryl-sulphatase (arylsulphate sulphohydrolase; EC 3.1.6.1; type 5 from *Patella vulgata*) were from Sigma Chemical Co. (St Louis, Mo, USA).

Liver microsomes

Specimens of histologically normal liver tissue were obtained from three adults undergoing surgery. Specimen 1 was taken at a partial liver resection due to a malignant melanoma in a 60-year-old woman. Specimens 2 and 3 were obtained from healthy organ donors; a 63-year-old male and a 40-year-old female. The isolation of microsomes from the livers was performed as described by Pacifici *et al.* (1982). The inhibitory effect on the metabolism of EM of oestradiol, oestriol, oestrone, testosterone and MPA (0.05 and 0.5 mM of each steroid) and of progesterone (0.005, 0.05 and 0.5 mM) was investigated. The microsomes were preincubated with the steroids for 6 min, then EM (5 mM) was added and the combination was incubated for a further 15 min. The amount of NEM formed was measured by h.p.l.c. as described by Ladona *et al.* (1989).

In vivo study

Three groups of subjects were studied: 1) Nineteen women, aged 20–42 (mean 32) years, considered medically fit and with a history of regular menstrual cycles, were investigated during the menstrual period (days 2–7 of the menstrual cycle) and in the postovulatory phase (days 19–23). They were not taking oral contraceptives or other medication. Fourteen had ovulatory menstrual cycles, as documented by a rise in progesterone levels during the luteal phase (more than 25 nmol l⁻¹) and onset of menstruation at the expected time. Five did not ovulate (progesterone rise less than 10 nmol l⁻¹). 2) Six women, aged 17–43 (mean 25) years, who were pregnant in gestational week 12–21 were investigated before prostaglandin-induced legal abortion, and 28–35 days later. 3) Fifteen healthy, non-medicated men, 22–47 (mean 30) years, were investigated twice at an interval of 13–15 days.

All volunteers were given a single oral dose of 25 mg of EM in the morning. In the first six volunteers (five of the non-pregnant women and one man), blood samples were taken before and at 15, 30, 45, 60, 90, 120, 180, 240 and 360 min after the dose of EM. In the remaining subjects blood samples were collected before and at 60

min after the dose only (see below under 'Pharmacokinetic and statistical analysis'). In one of the female subjects the bioavailability of orally administered EM was estimated by comparing AUC values after both orally and intravenously administered drug.

Urine was collected in two time periods, at 0–6 and 6–12 h, in the non-pregnant females. We observed that most of EM and its metabolites was excreted during the first 6 h and that the urine collections (0–6 and 0–12 h) were equivalent with respect to the metabolic ratio of EM to NEM (ratio = 1.08, s.d. 0.24). Therefore, we restricted the urine sampling period to the first 6 h in the male group. The plasma and urine samples were stored at -20°C until analysis.

Serum samples were collected for the assay of progesterone and oestradiol. This was done on two occasions in the females, when ovulating and when not ovulating.

The study was approved by the Ethics Committee of the University Hospital of Uppsala. Informed consent was obtained from all volunteers.

Analytical methods

Plasma (1 ml) or urine (1 ml) was mixed with 3 ml 0.5 M ammonium sulphate buffer, pH 9.3, applied to a SEP-PAK C₁₈ cartridge which was washed with 20 ml 5 mM ammonium sulphate buffer, pH 9.1, and, thereafter, eluted with 3 ml 20% v/v acetonitrile in 20 mM sodium phosphate buffer, pH 6.9. The concentrations of EM, NEM and MO in plasma and urine were measured by the h.p.l.c. method of Ladona *et al.* (1989). All assays were carried out in duplicate.

To measure the total urinary excretion of EM and its metabolites in six of the ovulating women, urine samples were first subjected to enzymatic hydrolysis. Urine (1 ml) diluted in 1 ml 0.1 M sodium citrate buffer, pH 5.0, was incubated with β -glucuronidase (12,500 U ml⁻¹) at 37°C. The hydrolyzed urine was then processed as described above. In pilot experiments it was observed that hydrolysis caused an increased yield of EM and NEM, which reached a plateau between 24 and 48 h. Urine was therefore hydrolysed for 48 h.

Progesterone and oestradiol in serum were measured by radioimmunoassays (Amerlex-M Progesterone RIA kit, Amersham; Coat-a-Count Estradiol, DPC). The assays had limits of determination of 0.25 nmol l⁻¹ and 29 pmol l⁻¹, respectively. The coefficient of variation was 2–5% for the progesterone assay and 5–8% for the oestradiol assay.

Pharmacokinetic and statistical analysis

In the *in vivo* study the data from the first six volunteers were used to determine a metabolic ratio in plasma as a measure of the rate of *N*-demethylation of EM. A metabolic ratio in plasma (MR_P) was defined as the ratio of 60 min plasma concentrations $C(60)_{EM}/C(60)_{NEM}$ (see Results). We assumed that the maximum plasma concentrations reflect the AUC.

As further indices of *N*-demethylation, three urinary metabolic ratios (MR_U) were defined. First, the ratio of urinary recoveries of unconjugated EM and NEM,

calculated in all non-pregnant females and males was determined ($MR_{U,1}$). It was subsequently observed (see Results) that the substrate, EM, was also metabolised by at least two alternative metabolic pathways (*O*-demethylation and conjugation) and that the product, NEM, was also conjugated to some extent. We therefore attempted to define metabolic ratios which would correct for the possible interaction of those processes with the *N*-demethylation pathway. Thus, $MR_{U,2}$ was defined as the ratio of the urinary recoveries of unconjugated EM to total (unconjugated and conjugated) NEM. $MR_{U,3}$ was defined as the ratio of urinary recoveries of total (unconjugated and conjugated) NEM to the sum of total EM (unconjugated + conjugated) + morphine + morphine-3-glucuronide + total NEM (unconjugated + conjugated). $MR_{U,2}$ and $MR_{U,3}$ were only determined in the six ovulating women where enzymatic hydrolysis of the urine was performed.

Non-compartmental pharmacokinetic analysis of the EM concentration in plasma after oral and intravenous dosage was performed with a non-linear weighted least squares (weight $1/y$) regression analysis (Excel) computer program.

Data are given as mean and s.d. The variance ratio test (*F* distribution) was used to compare variances between groups for a 95% confidence interval.

Results

The effects of various steroids on the microsomal *N*-demethylation of EM are illustrated in Figure 1. Significant inhibition was observed with progesterone and MPA while the oestrogens and testosterone had negligible effect.

In the *in vivo* study in the first six volunteers maximum plasma concentrations of EM were observed at 60 min after dosage (Figure 2) and were closely correlated to the AUC ($r = 0.9$). The plasma concentration of NEM was similar between individuals; it reached a

Figure 2 Mean ($\pm$ s.d.) plasma concentrations of ethylmorphine (EM) and norethylmorphine (NEM) and the metabolic ratio (MR; EM/NEM) plotted over time (min) after oral administration of 25 mg EM ($n = 6$).

maximum value at 60 min and was stable thereafter for 180 min. The metabolic ratio of EM to NEM initially increased rapidly but was relatively stable between 30 and 90 min after the dose. These results justified the subsequent use of MR_P , as described above, as a measure of *N*-demethylation of EM.

The possible *in vivo* effects of progesterone on MR_P in an individual were evaluated after calculating the difference between MR_P measured during the follicular (low progesterone concentration) and luteal phases (high progesterone concentration). In males the corresponding difference between values obtained on two occasions, 2 weeks apart, was calculated. The average difference so calculated was similar in all groups. However, within groups interindividual variation was significantly higher in ovulating women than in men

Figure 1 The effect of sex steroids on the rate of *N*-demethylation of ethylmorphine (expressed as % of control) in liver microsomes from three human livers (mean $\pm$ s.d.).

Figure 3 a) The metabolic ratio of ethylmorphine (EM) to norethylmorphine (NEM) in plasma, 60 min after dosage in the four groups of volunteers [pregnant (Preg), ovulating (Ov) and non-ovulating women (Nov) and men]. Each symbol ($\circ-\circ$) represents the metabolic ratio (MR) values in plasma of one individual observed on two different occasions. b) The intraindividual differences in metabolic ratio (delta-MR) of EM to NEM in plasma at 60 min between the two study periods in the different groups. The variances (F) between the ovulating women and the men were compared at the 5% level.

(Figure 3). MR_P values in ovulating women were not related to the serum concentrations of progesterone or oestradiol (Figure 4).

The 6 h urinary recoveries of EM, NEM and MO were low, but similar in non-pregnant women and men (mean 9%; range 4–12% in both groups), and the relation between unconjugated EM and NEM in urine ($MR_{U,1}$) in these two groups as a whole did not differ (data not shown).

Because of the low urinary recovery of EM, NEM and MO we investigated the bioavailability of oral EM by also giving 25 mg EM intravenously to one volunteer. In this individual the systemic clearance was $9 \text{ ml min}^{-1} \text{ kg}^{-1}$ and the oral bioavailability was 28%. The 12 h urinary recovery of EM, NEM and MO was similar after oral (14%) and intravenous (12%) administration. However, it was observed that the urinary recovery was considerably increased after treatment of the urine with β -glucuronidase (oral 59% and intravenous 53%), indicative of substantial conjugation.

In six non-pregnant females in follicular and luteal phases, treatment of the urine with β -glucuronidase increased the urinary recovery of conjugated and unconjugated EM and metabolites five-fold ($n = 6$; mean 46%; Table 1). A high concentration of β -glucuronidase (12,500 U) and a long incubation time (48 h) were used, as the conjugates of EM were more resistant to hydrolysis than those of MO (Predmore *et al.* 1978) and of NEM (unpublished observations). As the β -glucuronidase preparation used is contaminated with arylsulphatase, we investigated whether incubation with corresponding amounts of this enzyme increased the recovery in two urine specimens. No such increase was found. About half (49–64%) of the recovered metabolites were identified as EM conjugate(s) and 8–35% as MO and MO-glucuronides. The *N*-demethylated

Figure 4 The metabolic ratio of ethylmorphine (EM) to norethylmorphine (NEM) in plasma plotted against progesterone (a) and oestradiol (b) concentrations in serum on days 19–23 of the menstrual cycle in the 19 non-pregnant volunteers. ● ovulating, ○ non-ovulating.

Table 1 Cumulative urinary excretion of ethylmorphine (EM), norethylmorphine (NEM) and morphine (MO) in 12 h urine samples from six women in the follicular and luteal phases. The amounts of EM and its metabolites are identified as the unconjugated (unconj) fraction before hydrolysis and as the conjugated (conj) fraction (defined as total amount minus the unconjugated fraction) after hydrolysis with β -glucuronidase. Three different metabolic ratios (MR_U) were calculated: $MR_{U,1}$ = [the unconjugated fraction of EM]: [the unconjugated fraction of NEM]; $MR_{U,2}$ = [the unconjugated fraction of EM]: [the unconjugated and conjugated NEM] and $MR_{U,3}$ = [NEM + NEM conjugates]: [total amount of EM, NEM, MO and their conjugates]

Subject		EM		NEM		MO		EM + NEM + MO		$MR_{U,1}$	$MR_{U,2}$	$MR_{U,3}$
		unconj (μ mol)	conj (μ mol)	unconj (μ mol)	conj (μ mol)	unconj (μ mol)	conj (μ mol)	unconj + conj (μ mol)	(%)			
1	luteal	1.8	18.7	2.0	1.9	0.6	12.0	36.9	47	0.90	0.47	0.10
	follicular	2.9	21.2	2.2	2.3	0.7	14.4	43.7	56	1.32	0.64	0.10
2	luteal	2.5	32.2	3.0	2.0	0.4	10.4	50.5	64	0.83	0.52	0.10
	follicular	1.7	9.2	1.2	0.4	0.2	4.2	16.9	21	1.42	1.09	0.09
3	luteal	3.6	20.3	3.1	2.1	0.5	5.2	34.7	44	1.16	0.69	0.15
	follicular	3.5	18.5	4.9	0.7	0.7	5.7	34.1	43	0.71	0.61	0.17
4	luteal	2.1	17.6	6.3	2.9	0.3	2.1	31.4	40	0.33	0.23	0.29
	follicular	2.7	21.7	5.7	4.1	0.5	2.4	37.2	47	0.47	0.21	0.26
5	luteal	2.3	17.8	4.4	0.3	0.5	4.7	29.9	38	0.52	0.48	0.16
	follicular	2.5	30.1	4.4	2.2	0.5	7.6	47.3	60	0.57	0.38	0.14
6	luteal	2.6	16.5	4.4	2.2	0.5	7.6	33.8	43	0.59	0.40	0.20
	follicular	2.3	20.8	4.4	2.2	0.5	7.6	36.8	47	0.52	0.19	0.18
<i>Mean values</i>												
	luteal	2.5	20.5	3.9	1.9	0.5	7.0	36.2	46	0.72	0.47	0.17
	follicular	2.6	20.3	3.8	2.0	0.5	7.0	36.0	46	0.84	0.52	0.16

product NEM and its conjugate(s) constituted a smaller fraction of the total recovery of metabolites (9–29%). There was some interindividual variability in metabolite recoveries but relatively small differences in individuals between follicular and luteal phases.

MR_U values calculated after hydrolysis with β -glucuronidase did not correlate with plasma concentrations of progesterone or oestradiol (data not shown).

Discussion

We have shown that progesterone and MPA, in contrast to oestrogens and testosterone, inhibit the *N*-demethylation of EM by human adult liver microsomes. *In vivo*, however, there were no differences in the plasma metabolic ratios between groups or within individuals. Thus, although there was an approximately 25-fold difference in serum progesterone concentration between the follicular and the luteal phase, no inhibition of the *N*-demethylation of EM was observed *in vivo* either during the high progesterone phase of ovulating women or during pregnancy.

The MR_P was used in this study to describe the *N*-demethylation of EM, although its justification as a measure of metabolic activity has not been validated. It was observed that the intraindividual variation of this parameter was greater in ovulating women than in men when repeated determinations were performed 2 weeks apart. There is no clear explanation for this observation but the indication is that several undetermined factors are able to influence the *N*-demethylation of EM.

Only a small fraction of the administered dose was identified in the urine as unconjugated EM, NEM or MO. After hydrolysis the recovery of EM increased

eight-fold, indicating that EM undergoes extensive conjugation, possibly to the 6-glucuronide. *N*-Demethylation of EM to NEM is only a minor pathway *in vivo*. Even after hydrolysis the total cumulative urinary recovery did not exceed 65% in any subject, indicating further metabolic pathways or routes of excretion.

No significant difference in *N*-demethylase activity, as determined by MR_P and $MR_{U,1}$, was detected between males and females. Most previous studies on sex-related differences in the pharmacokinetics of drugs have been made in rodents. The *N*-demethylation of EM in rat liver microsomes has been shown to decrease during gestation and return to non-pregnant levels by the fifth day postpartum (Symons *et al.*, 1982). The existence of male- and female-specific cytochrome P450 enzymes was reported by Kato (1974). The sex-specific forms are subject to differential induction by, for example, testosterone (Kamataki *et al.*, 1984) and oestrogens (Waxman *et al.*, 1985). As with other steroids, progesterone may act both as an inhibitor and an inducer of drug metabolism (Conney, 1967). Thus, it induced EM *N*-demethylase activity in liver microsomes in the rat (Ochs *et al.*, 1986) and mouse (Brown *et al.*, 1977). However, there are large species differences in P450 enzyme systems, and no convincing evidence has been presented that sex-specific P450 enzymes exist in man (Guengerich, 1989).

Our results emphasize the potential pitfalls of extrapolation from *in vitro* data to the conditions prevailing *in vivo*. Thus, although there was clear inhibition of EM *N*-demethylation *in vitro* by the two gestagens studied there was no relation between metabolic ratios of EM and plasma progesterone concentrations. Possible reasons for this discrepancy include the following: Firstly, the metabolic ratios in plasma that we have used, and which were in agreement with the metabolic

ratios in urine, may be poor indices of enzyme activity. Secondly, *in vivo* concentrations of progesterone at the enzyme site may be much lower than those used in our *in vitro* experiments. The unbound plasma concentration of progesterone in luteal phase and during pregnancy (1.1–3.4 nM) is in the order of 5000–500 000 times lower than that used in the microsome study (0.005–0.5 mM). The ratio of concentrations of EM and progesterone used in the *in vitro* experiments was, however, similar to that seen in plasma *in vivo*.

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