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In vivo and *in vitro* formation of morphinone from morphine in rat

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1. Morphinone, a toxic metabolite, and its glutathione adduct (MO-GSH) were identified in the bile of rat after subcutaneous injection of morphine (25 mg/kg) by hplc procedures. The amounts of morphinone and MO-GSH excreted in the 12-h bile were 0·8±0·3 and 8·4±4·3% respectively.

2. The 9000 *g* supernatants of rat, guinea pig, rabbit, mouse, hamster and bovine livers produced morphinone from morphine in the presence of either NAD^+ or $NADP^+$. NAD^+ was a more efficient cofactor than $NADP^+$ except in the guinea pig which equally utilized both cofactors. With NAD^+ as cofactor, the amounts of morphinone formed in rat and guinea pig were 5·70 and 5·82 μmol/g liver/30 min respectively and were three-to-four times those in other species.

3. The enzyme activity responsible for formation of morphinone from morphine in the rat was almost exclusively distributed in the microsomal fraction, whereas guinea pig, hamster and bovine expressed the enzyme activity mainly in the cytosolic fraction. Rabbit and mouse gave higher activity in the cytosolic and microsomal fractions respectively, but other fractions of both species contained considerable activity.

4. The enzyme activities in male and female rat microsomes were characterized with respect to developmental pattern, kinetic parameters, pH dependency and susceptibility to inhibitors.

5. In conclusion the metabolism of morphine to morphinone in rat was confirmed by *in vivo* and *in vitro* experiments. It is also suggested that this pathway is a common route in morphine metabolism in several mammalian species.

Introduction

Morphinone, identified in the urine (Ishida *et al*. 1981) and the bile (Kumagai *et al*. 1987) of guinea pigs given morphine, is nine times more toxic than morphine (Nagamatsu *et al*. 1982a) and is a potent antagonistic of morphine (Nagamatsu *et al*. 1982b) in mouse. This metabolite was also found to block irreversibly naloxone binding in the mitochondrial-synaptosomal fraction of mouse brain (Nagamatsu *et al*. 1982b), to bind covalently to tissue macromolecules through sulphhydryl groups in mouse (Nagamatsu *et al*. 1983), and to cause hepatotoxicity in rat (Nagamatsu *et al*. 1986, Nagamatsu and Hasegawa 1992). These findings suggest that morphinone formed from morphine may affect morphine analgesia and has a potential role in morphine toxicity including appearance of tolerance, although much of this metabolite would be trapped by intracellular glutathione to form the morphinone–glutathione adduct (MO-GSH) and excreted readily into the bile as demonstrated in the guinea pig (Kumagai *et al*. 1990). It is therefore of interest and important to assess the *in vivo* and *in vitro* formation of morphinone including MO-GSH in various animals and man.

In the guinea pig, the formation of both morphinone and MO-GSH has been

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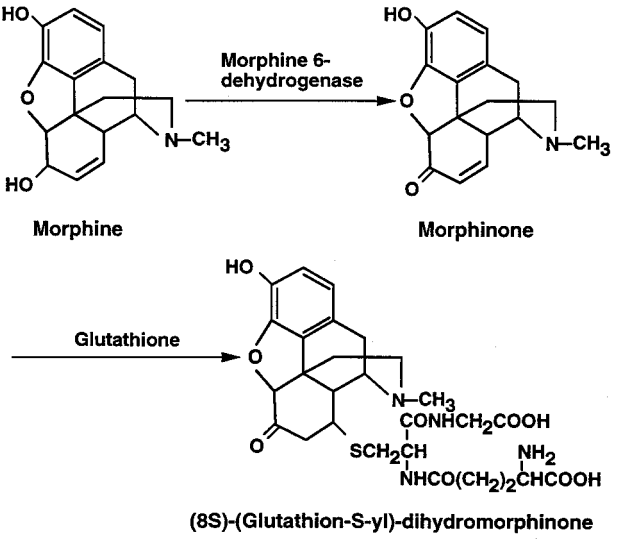

Figure 1. Morphinone formation and conjugation.

definitively demonstrated by *in vivo* and *in vitro* experiments (Kumagai *et al.* 1990), as shown in figure 1. Morphine 6-dehydrogenase (EC 1.1.1.218) catalysing the formation of morphinone from morphine in the liver of guinea pig has been purified a•nd extensively characterized (Yamano *et al.* 1985, 1986). Morphinone, having an α,β-unsaturated ketone group, non-enzymatically reacts with glutathione to form MO-GSH by a Michael addition under physiological conditions (Nagamatsu *et al.* 1982a) and glutathione *S*-transferase also participates in this reaction (Kumagai *et al.* 1990). MO-GSH isolated from the bile of guinea pig given morphine was identified as (8*S*)-(glutathione-*S*-yl)dihydromorphinone (Ishida *et al.* 1989). In the rat, the formation of morphinone and MO-GSH has been reported in isolated hepatocytes (Nagamatsu *et al.* 1986). In the mouse, the formation of MO-GSH has been postulated after incubation of cytosolic fractions with morphine in the presence of NAD^+ and glutathione (Nagamatsu *et al.* 1983). However, except in the guinea pig, the *in vivo* formation of morphinone and MO-GSH and the assessment of morphine 6-dehydrogenase activity in the livers of various species have not been reported.

In the current study, we describe the biliary excretion of morphinone and MO-GSH in the rat after subcutaneous (s.c.) administration of morphine, and compare the morphine 6-dehydrogenase activity in the livers of rat, guinea pig, mouse, rabbit hamster and bovine. Some properties of rat microsomal enzyme activity are also described.

Materials and methods

Materials

Morphine hydrochloride was obtained from Takeda Chemical Ind., Ltd (Osaka, Japan), pyridine nucleotides from Oriental Yeast Co., Ltd (Osaka, Japan), and steroids, naloxone hydrochloride and indomethacin from Sigma Chemical Co. (St Louis, MO, USA). Morphinone was synthesized by the method of Rapoport *et al.* (1957). Morphinone-mercaptoethanol adduct (MO-ME) and MO-GSH were synthesized as described previously (Yamano *et al.* 1985, Ishida *et al.* 1989). The purity of these three synthetic compounds (> 99%) were checked by hplc. All other chemicals used were of the highest grade available.

Animals

Male and female Wistar rats (3–12-weeks old, 40–400 g), male Hartley guinea pigs (400–500 g), male Japanese white rabbits (2–2·5 kg), male, ddY mice (10–15 g) and male golden hamsters (100–150 g) were fasted for 24 h before use, with water provided *ad libitum*. Bovine livers were obtained from a local slaughterhouse.

Determination of morphinone and MO-GSH in rat bile

Adult male Wistar rats (350–400 g) were anesthesized with diethyl ether and the bile ducts cannulated with polyethylene PE-10 tubing. Morphine (25 mg/kg) was injected subcutaneously after 1 h of surgery and the bile was collected every 1 h for 4 h and thereafter every 2 h for 8 h. The bile collected for 1 h before administration of morphine was used as a control. Each bile sample was adjusted to 2·5 ml with water, and divided into two portions to determine morphinone and MO-GSH formation separately. For morphinone analysis, one aliquot (1 ml) was added to 0·2 M sodium phosphate buffer, pH 7·4, containing 10 mM 2-mercaptoethanol to convert morphinone to the morphinone 2-mercaptoethanol adduct (MO-ME). The reaction mixture was poured onto a Sep-Pak C_{18} cartridge (Waters Division of Millipore Co., Milford, MA, USA). The cartridge was washed with 10 ml water and then eluted with 2 ml methanol. The eluate was used for quantification of morphinone by reversed-phase hplc on a column of YMC-Pak ODS-ALL (150 × 6 mm i.d., YMC Co., Kyoto, Japan) using a mobile phase consisted of 10 mM sodium phosphate buffer, pH 7·2/acetonitrile /methanol (50 : 45 : 5 by vol.) at a flow rate of 1·2 ml/min. The hplc apparatus consisted of a L-6200 Intelligent Pump, a L-4200 UV-VIS Detector and a AS-2000 Autosampler from Hitachi, Ltd (Tokyo, Japan). The peak area was determined on a Hitachi D-2500 Chromato-Integrator. The peaks were monitored for absorbance at 214 nm. Under these conditions, the retention time of MO-ME was 6·5 min. The mean recovery of morphinoe from bile was 93% (n = 3). The other aliquot of bile (1 ml), for MO-GSH analysis, was added to 0·2 M sodium phosphate buffer, pH 7·4, and poured onto a Sep-Pak C_{18}. The cartridge was washed with 1 ml water and then eluted with 3 ml 10 mM sodium phosphate buffer, pH 3·15/acetonitrile (3 : 1, v/v). The eluate was subjected to hplc on a Nova-Pak C_{18} cartridge (100 × 8 mm i.d., Waters Division of Millipore Co.) using a mobile phase consisted of 10 mM sodium phosphate buffer, pH 3·15, containing 1 mM sodium lauryl sulphate/acetonitorile (3 : 1, v/v) at a flow rate of 1·2 ml/min. The peaks were monitored for absorbance at 214 nm. Under these conditions, the retention time of MO-GSH was 12·0 min. The mean recovery of MO-GSH was 96% (n = 3).

In vitro *formation of morphinone from morphine in rat, guinea pig, rabbit, mouse, hamster and bovine livers*

Livers were homogenized in 2 vols 0·1 M sodium phosphate buffer, pH 7·4, with Poritron homogenizer (Kinematika GmbH, Luzen, Switzerland). The homogenate was centrifuged at 9000 *g* for 20 min and the resulting supernatant was further centrifuged at 105000 *g* for 60 min to sediment microsomes and a cytosolic fraction. The microsomal pellet was washed twice with 1·15% KCl. Each fraction was adjusted to a concentration of 0·33 g wet liver weight per ml with 0·1 M sodium phosphate buffer, pH 7·4. The incubation mixture consisted of morphine (3·3 mM), NAD^+ or $NADP^+$ (8·3 mM), enzyme fraction equivalent to 0·165 g wet liver) and 0·1 M sodium phosphate buffer, pH 7·4, in a total volume of 3 ml and was incubated at 37 °C for 30 min. Just before the reaction was terminated by immersing in a boiling water bath for 2 min, 30 μmol 2-mercaptoethanol was added to convert morphinone formed to MO-ME. The reaction mixture was treated with Sep-Pak C_{18}, and morphine was quantitated by hplc as described above.

Results

Biliary excretion of morphinone and MO-GSH in rat

Figure 2 shows a typical hplc profile of the bile extract obtained for morphinone analysis. After morphine administration, a peak at retention time of 6·5 min corresponding to authentic MO-ME was identified (figure 2B). This peak was not detected in the control bile (figure 2A). In this experiment, morphinone was converted to MO-ME by adding 2-mercaptoethanol to the bile before extraction as described in the Materials and Methods. When the extraction was carried out without addition of 2-mercaptoethanol, the peak with retention time of 6·5 min disappeared but a new peak with retention time of 7·5 min corresponding to morphinone emerged and the extract obtained after addition of glutathione instead

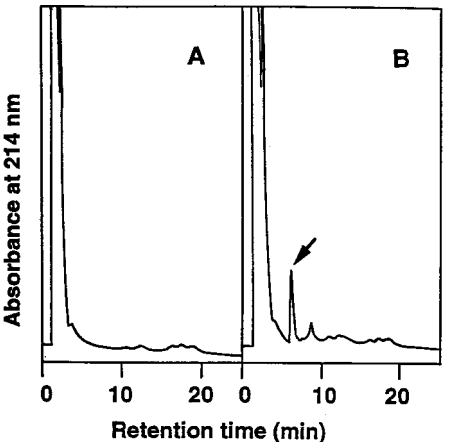

Figure 2. Identification of morphinone in bile. A portion of bile was added to 0·2 M sodium phosphate buffer, pH 7·4, containing 10 mM 2-mercaptoethanol to convert morphinone to morphinone 2-mercaptoethanol adduct (MO-ME) and then poured onto a Sep-Pak C_{18} cartridge. The extraction procedure and hplc conditions are described in the Materials and methods. (A) Control rat bile; (B) bile from rat given morphine (s.c. 25 mg/kg). Arrow indicates the retention time of MO-ME.

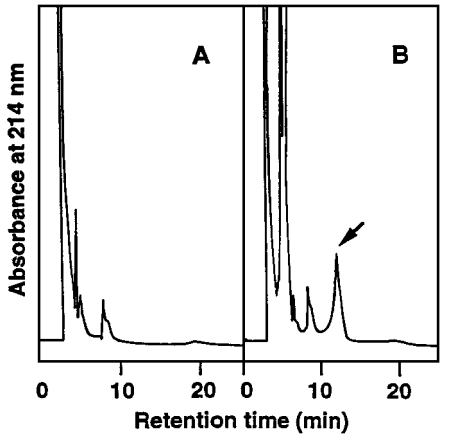

Figure 3. Identification of a glutathione conjugate of morphinone in bile. The extraction procedure and hplc conditions are described in the Materials and methods. (A) Control rat bile; (B) bile from rat administered morphine (s.c. 25 mg/kg). Arrow indicates the retention time of MO-GSH.

of 2-mercaptoethanol to the bile did not contain either peaks (data not shown). Thus, these findings indicate that the product with retention time of 6·5 min in figure 2B is MO-ME derived from morphinone.

Hplc chromatograms of the bile extract for MO-GSH analysis revealed a peak with retention time of 12·0 min corresponding to MO-GSH (figure 3B), which was not present in the control bile (figure 3A). Under the hplc conditions used, MO-GSH has been shown to be separated from morphine 3-glucuronide (M-3-G), which appeared at a retention time of 5·5 min as a large peak in figure 3B, morphine 6-glucuronide (M-6-G), morphine and other unconjugated metabolites (Kumagai *et al.* 1990). However, Correia *et al.* (1984a) isolated 10α-*S*-glutathionylmorphine after the incubation of morphine with liver microsomes prepared from the phenobarbital-treated rat in the presence of glutathione and NADPH and this

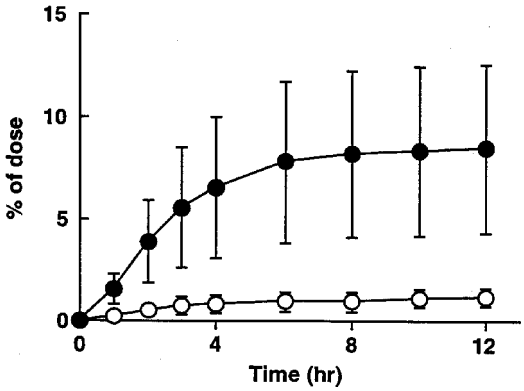

Figure 4. Cumulative biliary excretion of morphinone and MO-GSH in the adult male rat. A single dose of morphine (25 mg/kg) was administered s.c. to seven bile duct-cannulated rats. Morphinone (○) and MO-GSH (●) excreted in the bile were quantitated as described in the Materials and methods. Each value represents the mean ± SD from seven rats.

product, if formed in intact rat and excreted in bile, shows the same retention as MO-GSH on hplc. Therefore, we analysed the reaction products obtained after similar incubation conditions as reported by Correia *et al.* (1984a). This incubation procedure produced two unknown metabolites with retention times of 7·5 and 8·0 min in addition to normorphine (retention time of 14·0 min), but a peak was not observed at retention time of 12·0 min (chromatogram not shown). These two unknown metabolites were not formed in the absence of glutathione in the incubation mixture. These findings indicate that two glutathione conjugated metabolites, one of which probably is 10α-*S*-glutathionylmorphine, were formed during the incubation but were separated from MO-GSH on hplc. Accordingly, we conclude that the metabolite with retention time of 12 min in figure 3B is MO-GSH.

Figure 4 shows the cumulative biliary excretion of morphinone and MO-GSH after s.c. injection of morphine (25 mg/kg). The excretion of morphinone and MO-GSH occurred very soon after morphine injection and was complete after 8 h. The amounts of morphinone and MO-GSH excreted into the bile after 12 h were 0·8 ± 0·3 and 8·2 ± 4·3% of the administered dose (n = 7), respectively. Although the amounts of both metabolites excreted in the bile varied between individual rats, the amount of MO-GSH was 8–9 times that of morphinone in all rats.

In vitro *formation of morphinone from morphine in rat, guinea pig, rabbit, mouse, hamster and bovine liver*

Figure 5 illustrates the morphine 6-dehydrogenase activity responsible for the formation of morphinone from morphine in rat, guinea pig, rabbit, mouse, hamster and bovine liver using the 9000 *g* supernatant as the enzyme source. Morphinone formation was quantitated by hplc as the stable MO-ME derivative and the enzyme activity was expressed as μmol morphinone formed per g liver per 30 min. All species were capable of forming morphinone from morphine in the presence of either NAD^+ or $NADP^+$, although to differing extents. Guinea pig showed the highest activity with either NAD^+ or $NADP^+$ and the activities with either cofactor were similar. In the rat, the activity with NAD^+ was comparable with that in the guinea pig but $NADP^+$ supported about one-tenth of activity with NAD^+. Similarly,

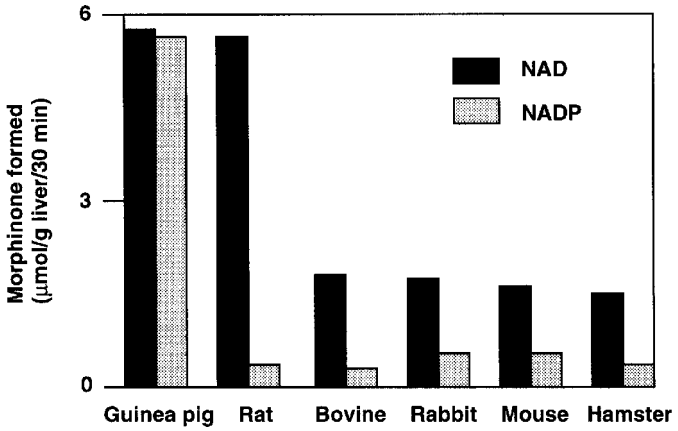

Figure 5. Morphinone formation by guinea pig, rat, bovine, rabbit, mouse and hamster liver 9000*g* supernatants. Incubation and quantification of morphinone formed were carried out as described in the Materials and methods. Data represent the mean of two-to-five animals except the mouse. Values for the mouse are the mean of three different preparations, each of 10 mice.

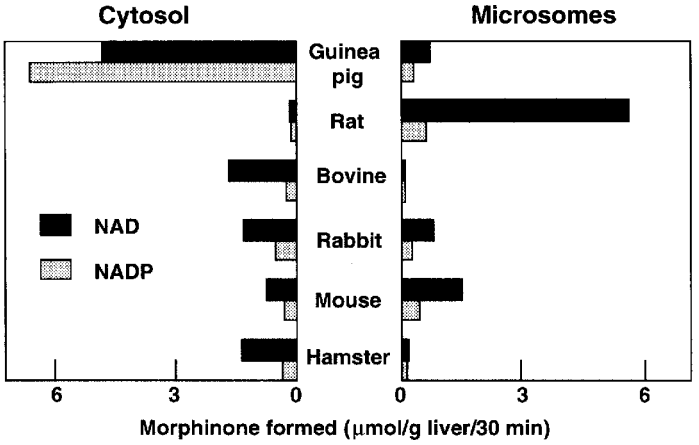

Figure 6. Subcellular distribution of morphine 6-dehydrogenase activity in guinea pig, rat, bovine, rabbit, mouse and hamster liver. Incubations and quantification of morphinone formed were carried out as described in the Materials and methods. Data represent the mean of two-to-five animals except mouse. Values for the mouse are the mean of three different preparations, 10 mice each.

the other four species exhibited a preference for NAD^+ over $NADP^+$ as cofactor. Comparing the activity with NAD^+, rat and guinea pig gave three-to-four times higher activity than bovine, rabbit, mouse and hamster.

The 9000 *g* supernatant was divided into the cytosolic and microsomal fractions and subcellular distribution of the enzyme activity was examined (figure 6). No consistent subcellular distribution of enzyme activity was observed. In rat, enzyme activity was almost exclusively distributed in the microsomal fraction, whereas the enzyme activities in the guinea pig, bovine and hamster were localized mainly in the cytosolic fraction. Rabbit and mouse exhibited higher activity in the cytosolic and microsomal fractions respectively, but both fractions of these two species seemed to contribute to the formation of morphinone from morphine in the liver.

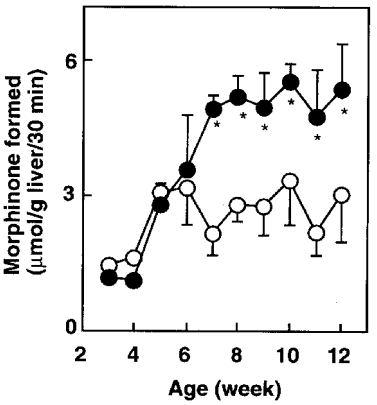

Figure 7. Developmental changes of morphine 6-dehydrogenase activity in male and female rat liver microsomes. Incubations using NAD^+ as a cofactor and quantification of morphinone formation were carried out as described in the Materials and methods. Values represent the mean ± SD of three-to-seven male (●) and female (○) rats. * Significantly different from the female rat ($p < 0{\cdot}05$).

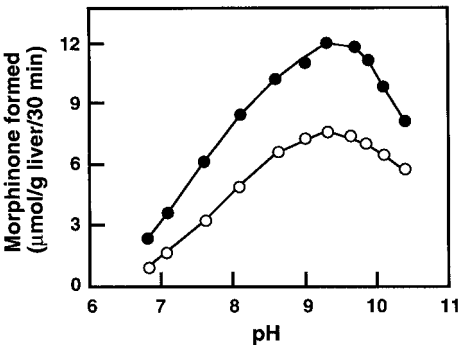

Figure 8. Effects of pH on morphine 6-dehydrogenase activity in male (●) and female (○) rat liver microsomes. Microsomes from either the adult male or female rat were incubated with morphine (3·3 mM) and NAD^+ (8·3 mM) in various buffers (pH 6·8–10·5) for 30 min at 37 °C. Buffers used were 100 mM sodium phosphate buffer, Tris-HCl buffer or glycine–NaOH buffer. Each value represent the average of duplicate determinations.

Developmental pattern of microsomal morphine 6-dehydrogenase activity in rat liver

Age-related changes of the enzyme activity with NAD^+ in the microsomal fractions of the male and female rat from 3 to 12 weeks of age are shown in figure 7. In the male rat, the enzyme activity at 3 weeks, which was about 25% that at 12 weeks, increased progressively up to 7 weeks of age and thereafter stayed at an almost constant level. The female rat showed a similar developmental pattern to the male until 5 weeks of age, but plateaued thereafter. Thus the enzyme activities in immature rats of both sexes were similar, but at adulthood the male showed 2–2·5 times higher activity than the female. Similar developmental patterns of enzyme activity with $NADP^+$ were observed (data not shown).

Properties of microsomal morphine 6-dehydrogenase activity in the adult rat

Since a sex difference in morphine 6-dehydrogenase activity was observed in the adult rat, we compared some properties between the adult male and female. Figure 8 shows the pH dependency of the enzyme activities in the adult male and female rat.

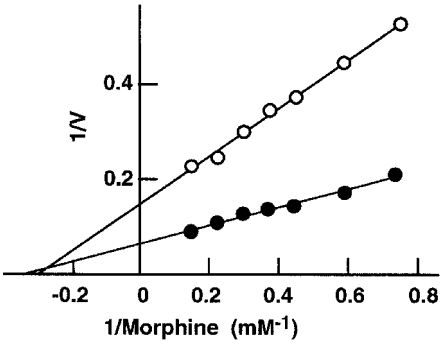

Figure 9. Lineweaver–Burk plots of morphine 6-dehydrogenase activity against morphine in male (●) and female (○) rat liver microsomes. Incubations were carried out as described in the Materials and methods, except that various concentrations of morphine (1·3–6·7 mM) were used. Velocity, v, is expressed as μmol morphinone formed/g liver/30 min.

Table 1. Inhibition of morphinone formation from morphine in adult male and female rat liver microsomes.

Inhibitor	Concentration (mM)	Inhibition (%) Male	Female
Pyrazole	1·0	2	9
Barbital	1·0	28	27
Quercetin	0·1	98	94
Naloxone	1·0	81	81
Indomethacin	1·0	78	77
Ketamine	1·0	31	23
Metyrapone	1·0	9	10
Testosterone	0·05	87	91
5α-Dihydrotestosterone	0·05	94	94
5β-Dihydrotestosterone	0·05	91	95
Androsterone	0·05	88	88
Epiandrosterone	0·05	93	90
Estradiol	0·05	85	93
Lithocholic acid	0·05	92	93
Chenodeoxycholic acid	0·05	54	51
Cholic acid	0·05	27	26

Incubations were carried out as described in the Materials and methods. Each value represents the average of duplicate determinations. The amounts of morphinone formed with male and female rat liver microsomes in the absence of inhibitor were 6·03 and 3·12 μmol/g liver/30 min respectively.

The maximum activity was obtained around pH 9·3 in both sexes, and the activity–pH curves were quite similar. K_m for morphinone formation were estimated by Lineweaver–Burk analysis and was determined to be 2·9 mM in the male adult and 3·3 mM in the female adult rat. V_{max} in the male was about twice that of the female (figure 9).

We finally compared the effects of various compounds on the enzyme activities in both sexes. As shown in table 1, all chemicals tested showed similar inhibitory effects on male and female enzyme activities. Androgens and estradiol inhibited enzyme activity by about 90% at 0·05 mM concentrations. Lithocholic acid, a monohydroxyl bile acid, was also a potent inhibitor, but the inhibitory potency of bile acid decreased as the number of hydroxyl groups in the molecule increased, at

least at the concentrations tested. Quercetin (inhibitor of aldo-keto reductase) almost completely inhibited at 0·1 mM, but pyrazole (inhibitor of alcohol dehydrogenase) and barbital (inhibitor of aldose and alehyde reductase) had little or no effect on the enzyme activity. These effects were similar to those previously reported with guinea pig morphine 6-dehydrogenase purified from the liver cytosol (Yamano *et al.* 1985). Naloxone and indomethacin inhibited about 80% at 1 mM.

Discussion

Metabolism of morphine has been extensively studied in various animals and man, and consequently a number of metabolites have been identified by *in vivo* and *in vitro* experiments (Milne *et al.* 1996 and references therein). Although the pattern of morphine metabolism is species-dependent, the main rout is commonly glucuronidation to form M-3-G. In rat, the amount of M-3-G excreted in the 24-h urine after morphine injection has been estimated to be 20–50% of the dose (Klutch, 1974, Yeh *et al.* 1977, Kuo *et al.* 1991), and 2–6 and 1·5–4% of dose have been also recovered as normorphine including its 3-glucuronide and dihydromorphinone respectively (Klutch 1974, Yeh *et al.* 1977). In addition to these latter metabolites, M-6-G, α-dihydromorphine, and mono- and dihydroxymorphine have been identified in the urine as minor metabolites (Yeh *et al.* 1979). In the present study, we identified both morphinone and MO-GSH in the bile of rat after s.c. administration of morphine (25 mg/kg) by hplc procedures using authentic standards for comparison and also demonstrated that morphinone and MO-GSH ($0{\cdot}8 \pm 0{\cdot}3$ and $8{\cdot}2 \pm 4{\cdot}3$% of dose respectively) were excreted in the 12-h bile samples. However, we could not detect neither morphinone nor MO-GSH in the urine (data not shown). These results imply that rat produced morphinone *in vivo* accounting for 3–13% of administered dose, and the majority (about 80–90%) was conjugated with glutathione and eliminated into the bile. This excretion is similar to that reported in the guinea pig (Kumagai *et al.* 1990), in which 10·6% of dose was excreted in the bile as total morphinone, of which about 90% was MO-GSH. The present study is the first case in which *in vivo* formation of morphinone and MO-GSH in rat has been demonstrated.

Nagamatsu *et al.* (1986) reported that isolated rat hepatocytes metabolized morphine to morphinone and MO-GSH in addition to M-3-G and normorphine. Although quantitative data were not presented, it is likely that M-3-G and MO-GSH are major metabolites by judging from the magnitude of the chromatographic peaks. On the other hand, Aasmundstad *et al.* (1993) reported that isolated rat hepatocytes formed large amounts of M-3-G, normorphine and normorphine 3-glucuronide (NM-3-G), but other metabolites (MO-GSH and morphinone) were not detected in their study. Evans and Shanahan (1995) also reported that isolated perfused rat livers metabolized morphine to M-3-G, normorphine and NM-3-G accounting for 73, 11 and 10% respectively of eliminated morphine, and the remaining 6% was due to unidentified metabolites. These latter two studies suggest that metabolism of morphine in rat liver is mainly proceeded by glucuronidation and *N*-demethylation. However, the amount of total morphinone (sum of morphinone and MO-GSH) excreted in the bile presented in this study was equal to or greater than that of total normorphine (sum of normorphine and NM-3-G) recovered in the urine (Klutch 1974, Yeh *et al.* 1977). This fact together with the result of Nagamatsu *et al.* (1986) indicates that the metabolism of morphine to morphinone may be a

route of secondary importance to the glucuronide formation in morphine metabolism. In this connection, we found that the rate of formation of morphinone was about four times that of normorphine in the same rat liver microsomes (data not shown).

We have also demonstrated that the livers of all species examined contained morphine 6-dehydrogenase activity (highest in guinea pig and lowest in hamster based on wet tissue weight). The rate of morphinone formation in rat liver was comparable with that in guinea pig liver, and three-to-four times that in mouse, rabbit, hamster and bovine livers. Surprisingly, it was found that the enzyme activity in rat liver was almost exclusively localized in the microsomal fraction, which was quite different from other species, and that rats exhibited a marked preference for NAD^+ over $NADP^+$ compared with other species.

We are currently purifying rat liver microsomal enzyme after solubilization by non-ionic detergent (octa-ethyleneglycol mono *n*-dodecyl ether). Although the rat enzyme has not yet been obtained as a homogeneous protein, morphine 6-dehydrogenase activity and 17β-hydroxysteroid dehydrogenase activity co-eluted with each other at all stages in the purification procedure, suggesting that the rat liver enzyme may be identical to 17β-hydroxysteroid dehydrogenase and physiologically involved in androgen metabolism. In this connection, strong inhibition by androgens in the present study is considered to be a result of their competition with morphine at the substrate binding site of the enzyme.

Sex-differentiated hepatic metabolism in rat has been described for a variety of drugs and steroids (Skett 1988). In the present study, it was found that morphine 6-dehydrogenase activities in the immature rat of both sexes were similar but, at onset of puberty to adulthood, male rat microsomes formed morphinone at about twice the rate of female rat microsomes. This sex difference was statistically significant but not pronounced compared with that in *N*-demethylation to normorphine in which the male rat showed about 15–20 times higher activity than the female (Blanck *et al*. 1990). In contrast, no sex difference in morphine 3-glucuronide formation has been reported (Rush *et al*. 1983). V_{max} for morphine 6-dehydrogenase activity of the male rat was about twice that of the female but the K_m toward morphine, pH dependency and inhibitor sensitivity were essentially the same in both sexes. These findings suggest that appearance of sex difference in morphine 6-dehydrogenase activity in rat may be due to the quantitative change of the enzyme, which would be regulated by endocrine but not due to the expression of sex-specific isozymes.

The administration of large doses of morphine to mouse or rat resulted in the depletion of glutathione in the liver (James *et al*. 1982, Nagamatsu *et al*. 1982a, Correia *et al*. 1984b) and potentiated the liver injury induced by acetaminophen and cocaine (James *et al*. 1982, Skoulis *et al*. 1989a). This decrease in glutathione content has been considered to be due to the action of morphine within the central nervous system via opioid receptors (James *et al*. 1988, Skoulis *et al*. 1989b) and/or due to the hepatic metabolism of morphine to reactive metabolites which attack hepatocellular glutathione and protein (Correia *et al*. 1984a, Nagamatsu *et al*. 1986, Eklow-Lastbom *et al*. 1986). Nagamatsu *et al*. (1986, 1992) provided convincing evidence for the involvement of hepatic metabolism of morphine to morphinone in morphine-induced hepatotoxicity using isolated rat hepatocytes. The formation of MO-GSH from intermediate metabolite, morphinone, occurred along with depleted cellular concentration of glutathione and with decreased viability of the hepatocytes, and morphinone–protein adducts were detected, particularly when the hepatocytes were

damaged. They also showed that the addition of naloxone decreased the formation of MO-GSH and prevented the morphine-induced hepatocyte toxicity. These effects of naloxone have been assumed to be due to the inhibition of the formation of morphinone. This assumption was evidenced by the finding that naloxone acted as a powerful inhibitor of rat liver microsomal morphine 6-dehydrogenase in the present study.

Other than morphinone, it has been postulated that the reactive intermediates generated by cytochrome P450-catalysed oxidation deplete glutathione content in the liver (Correia *et al*. 1984a, Eklow-Lastbom *et al*. 1986). Indeed, Correia *et al*. (1984a) isolated a morphine–glutathione adduct following the incubation of morphine with liver microsomes prepared from the phenobarbital-treated rat in the presence of glutathione and NADPH which was identified as 10α-*S*-glutathionylmorphine. We also detected two glutathione-conjugated metabolites produced by microsomes in the presence of NADPH and glutathione. However, these two metabolites formed *in vitro* do not appear to be excreted in the bile, since the peaks corresponding to these metabolites were not observed (figure 3B). Therefore, it is suggested that morphinone may be the main species responsible for the decrease in liver glutathione content.

In conclusion, the metabolism of morphine to morphinone occurs in the intact rat and contributes at least in part to the decrease of glutathione content in the liver through subsequent formation of MO-GSH. In addition, the fact that the livers of all species examined contained morphine 6-dehydrogenase activity suggests that this interesting metabolic pathway is a common route in mammalian species. The assessment of this metabolic pathway in man remains to be clarified, which is of clinical significance as morphine is often administered in large dose for analgesic purpose and concurrently with other drugs which may depend on glutathione for detoxification.

References

Aasmundstad, T. A., Ripel, Å., Bodd, E., Bjøneboe, A. and Mørland, J., 1993, Different biotransformation of morphine in isolated liver cells from guinea pig and rat. *Biochemical Pharmacology*, **46**, 961–968.

Blanck, A., Hansson, T., Näslund, B. and Rane, A., 1990, Sex differences and endocrine regulation of morphine oxidation in rat liver. *Biochemical Pharmacology*, **39**, 1820–1822.

Correia, M. A., Krowech, G., Caldera-Muonz, P., Yee, S. L., Straub, K. and Castagnoli, N. J., 1984a, Morphine metabolism revisited. II. Isolation and chemical characterization of a glutathionylmorphine adduct from rat liver preparations. *Chemico-Biological Interactions*, **51**, 13–24.

Correia, M. A., Wong, J. A. and Solvien, E., 1984b, Morphine metabolism revisited. I. Metabolic activation of morphine to a reactive species in rats. *Chemico-Biological Interactions*, **49**, 255–268.

Eklow-Lastbom, L., Moldeus, P. and Orrenius, S., 1986, On the mechanisms of glutathione depletion in hepatocytes exposed to morphine and ethylmorphine. *Toxicology*, **42**, 13–21.

Evans, A. M. and Shanahan, K., 1995, The disposition of morphine and its metabolites in the in-situ rat isolated perfused liver. *Journal of Pharmacy and Pharmacology*, **47**, 333–339.

Ishda, T., Kumagai, Y., Ikeda, Y., Yano, M., Toki, S., Mihashi, K., Fujioka, T., Iwase, Y. and Hachiyama, S., 1989, (8*S*)-(glutathione-*S*-yl)dihydromorphinone, a novel metabolite of morphine from guinea pig bile. *Drug Metabolism and Disposition*, **17**, 77–81.

Ishida, T., Yamano, S. and Toki, S., 1981, Detection of morphinone as a new metabolite of morphine in guinea pig urine. In *Advances in Endogenous and Exogenous Opioids*, edited by H. Takagi and E. J. Simon (Tokyo: Kodansha), p. 481.

James, R. C., Goodman, D. R. and Harbison, R. D., 1982, Hepatic glutathione and hepatoxicity: changes induced by selected narcotics. *Journal of Pharmacology and Experimental Therapeutics*, **221**, 708–714.

James, R. C., Wessinger, W. D., Roberts, S. M., Millner, G. C. and Paule, M. G., 1988, Centrally

mediated opioid induced depression of hepatic glutathione: effects of intracerbroventricular administration of mu, kappa, sigma, and delta agonists. *Toxicology*, **51**, 267–279.

KLUTCH, A., 1974, A chromatographic investigation of morphine metabolism in rats. Confirmation of *N*-demethylation of morphine and isolation of a new metabolite. *Drug Metabolism and Disposition*, **2**, 23–30.

KUMAGAI, Y., ISHIDA, T. and TOKI, S., 1987, Method for determination of morphinone in urine and bile of guinea-pig by high-performance liquid chromatography. *Journal of Chromatography*, **421**, 155–160.

KUMAGAI, Y., TODAKA, T. and TOKI, S., 1990, A new metabolic pathway of morphine; *in vivo* and *in vitro* formation of morphinone and morphinone–glutathione adduct in guinea pig. *Journal of Pharmacology and Experimental Therapeutics*, **225**, 504–510.

KUO, C. K., HANIOKA, N., HOSHIKAWA, Y., OGURI, K. and YOSHIMURA, H., 1991, Species difference of site-selective glucuronidation of morphine. *Journal of Pharmacobio-Dyanamics*, **14**, 187–193.

MILNE, R. W., NATION, R. L. and SOMOGYI, A. A., 1996, The disposition of morphine and its 3- and 6-glucuronide metabolites in humans and animals, and the importance of the metabolites to the pharmacological effects of morphine. *Drug Metabolism Reviews*, **28**, 345–472.

NAGAMATSU, K. and HASEGAWA, A., 1992, Covalent binding of morphine to isolated rat hepatocytes. *Biochemical Pharmacology*, **43**, 2631–2635.

NAGAMATSU, K., KIDO, Y., TERAO, T., ISHIDA, T. and TOKI, S., 1982a, Protective effect of sulfhydral compounds on acute toxicity of morphinone. *Life Sciences*, **30**, 1121–1127.

NAGAMATSU, K., KIDO, Y., TERAO, T., ISHIDA, T. and TOKI, S., 1982b, Effect of morphinone on opiate binding and morphine-elicited analgesia. *Life Sciences*, **31**, 1451–1457.

NAGAMATSU, K., KIDO, Y., TERAO, T., ISHIDA, T. and TOKI, S., 1983, Studies on the mechanism of covalent binding of morphine metabolites to proteins in mouse. *Drug Metabolism and Disposition*, **11**, 190–194.

NAGAMATSU, K., OHNO, Y., IKEBUCHI, H., TAKAHASHI, A., TERAO, T. and TAKANAKA, A., 1986, Morphine metabolism in isolated rat hepatocytes and implications for hepatotoxicity. *Biochemical Pharmacology*, **35**, 3543–3548.

RAPOPORT, H., BAKER, D. R. and REIST, H. N., 1957, Morphinone. *Journal of Organic Chemistry*, **22**, 1489–1492.

RUSH, G. F., NEWTON, J. F. and HOOK, J. B., 1983, Sex differences in the excretion of glucuronide conjugates: role of internal glucuronidation. *Journal of Pharmacology and Experimental Therapeutics*, **227**, 658–662.

SKOULIS, N. P., JAMES, R. C., HARBISON, R. D. and ROBERTS, S. M., 1989a, Depression of hepatic glutathione by opioid analgesic drugs in mice. *Toxicology and Applied Pharmacology*, **99**, 139–147.

SKOULIS, N. P., JAMES, R. C., HARBISON, R. D. and ROBERTS, S. M., 1989b, Perturbation of glutathione by a central action of morphine. *Toxicology*, **57**, 287–302.

SKETT, P., 1988, Biomedical basis of sex differences in drug metabolism. *Pharmacological Therapeutics*, **38**, 269–304.

YAMANO, S., KAGEURA, E., ISHIDA, T. and TOKI, S., 1985, Purification and characterization of guinea pig liver morphine 6-dehydrogenase. *Journal of Biological Chemistry*, **260**, 5259–5264.

YAMANO, S., NISHIDA, F. and TOKI, S., 1986, Guinea-pig liver morphine 6-dehydrogenase as a naloxone reductase. *Biochemical Pharmacology*, **35**, 4321–4326.

YEH, S. Y., KREBS, H. A. and GORODETTZKY, C. W., 1979, Isolation and identification of morphine *N*-oxide, α- and β-dihydromorphines, β- or γ-isomorphine, and hydroxylated morphine as morphine metabolites in several mammalian species, *Journal of Pharmaceutical Sciences*, **68**, 133–140.

YEH, S. Y., MCQUINN, R. L. and GORODETTZKY, C. W., 1977, Biotransformation of morphine to dihydromorphinone and normorphine in the mouse, rat, rabbit, guinea pig, cat, dog, and monkey. *Drug Metabolism and Disposition*, **5**, 335–342.