Biochemical Pharmacology, Vol. 46, No. 6, pp. 961–968, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

DIFFERENT BIOTRANSFORMATION OF MORPHINE IN ISOLATED LIVER CELLS FROM GUINEA PIG AND RAT

TOR A. AASMUNDSTAD,* ÅSE RIPEL, EGIL BODD, ANDERS BJØRNEBOE and JØRG MØRLAND

National Institute of Forensic Toxicology, PO 16 Gaustad, N-0320 Oslo 3, Norway

(*Received* 16 *February* 1993; *accepted* 24 *June* 1993)

Abstract—The biotransformation of morphine was characterized in freshly isolated parenchymal and non-parenchymal liver cells from rats and guinea pigs in suspension culture to establish an *in vitro* model for morphine metabolism. Liver cells were prepared by a collagenase perfusion technique, and separated by differential centrifugation. Morphine metabolism was investigated at different concentrations (1, 5, 100 and 200 μM). Samples were taken repeatedly during 2–4 hr of incubation, and subsequently analysed on a HPLC system employing both UV and electrochemical detection. In suspensions of hepatocytes from both animal species morphine-3-glucuronide (M3G) was the major metabolite of morphine, and was formed at comparable rates at all concentrations examined. Guinea pig hepatocytes in addition produced considerable quantities of morphine-6-glucuronide (M6G), whereas this metabolite was detected only intracellularly in minor quantities in rat hepatocytes. The ratio between the two morphine glucuronides (M3G/M6G) in suspensions of guinea pig hepatocytes was approximately 4:1. N-Demethylation of morphine was more pronounced per mg cell protein in rat hepatocytes compared to guinea pig cells. Metabolic activity towards morphine was not detected in non-parenchymal cells of the two species. The morphine glucuronidation pattern found in guinea pig hepatocytes resembles to a greater extent than that found in rat hepatocytes the pattern found in *in vivo* studies of humans. It was concluded that isolated guinea pig parenchymal cells appeared to be a promising *in vitro* system for studies of morphine glucuronidation, and to observe metabolism in general.

Morphine is the drug of choice in a variety of conditions with severe pain [1]. Progress in the field of opiate receptor research has provided valuable knowledge about this drug [2–4], but still relatively little is known about the pharmacokinetics of morphine and in particular its metabolites. In man, morphine-3-glucuronide (M3G†) is the major metabolite [5–7], but significant amounts of morphine-6-glucuronide (M6G) [5–7] are also formed in addition to some morphine-3,6-diglucuronide, normorphine (NM), normorphine-6-glucuronide (NM6G) and morphine-3-ethereal sulphate [6]. More than 40 years ago Casparis [8] showed that M6G is a potent analgesic. During recent years this has been further elucidated [9–13], proving that M6G has analgesic activity also in man [14, 15]. Furthermore, the major metabolite M3G, might antagonize the analgesic effect of morphine and M6G in animals [16, 17]. Recent papers also address this potential of M3G in man [13, 18, 19]. Thus, the possibility exists that altered morphine glucuronidation might influence the pharmacological effect of morphine.

Rat hepatocytes and rat liver microsomes have been widely used to study morphine metabolism in detail [20, 21]. A major drawback in such studies is the lack of M6G formation in the rat [22, 23]. *In vivo* studies have demonstrated that guinea pigs form M6G after morphine administration, and in this respect might demonstrate more human-like metabolism of morphine [23]. Recently, Lawrence *et al.* [24] confirmed the ability of guinea pigs to glucuronidate morphine in the 6-position, using liver homogenates.

Isolated hepatocytes have a possible advantage over microsomes and liver homogenates since intact cells possess a more complete drug metabolizing sequence [25]. Furthermore, isolated cells might also express metabolic capacity in a fashion which is more similar to *in vivo* conditions. Guinea pig hepatocytes are prepared almost as conveniently as isolated rat hepatocytes [25]. Since there are no published studies on morphine metabolism in isolated guinea pig hepatocytes, we wanted to compare morphine metabolism in isolated liver cells from guinea pigs with that of rat liver cells.

MATERIALS AND METHODS

Animals. Male Wistar rats, (250–300 g) (Møllegaard Hansens Avlslaboratorier A/S, Ejby, Denmark) and male Duncan Hartley guinea pigs (250–300 g) (Charles River, Germany) were maintained on a 12 hr light/dark cycle and allowed free access to standard pelleted food (Arex Møllesentralen, Oslo, Norway) and water. The animals were fasted overnight before the experiment.

Materials. M3G, M6G, NM, UDP-glucuronic acid (sodium salt), Pronase and Collagenase (type I), bovine serum albumin (fraction V, defatted), *N*-2-

* Corresponding author. Tel. (47) 22142190; FAX (47) 22143360.

† Abbreviations: M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; NM, normorphine; NM3G, normorphine-3-glucuronide; NM6G, normorphine-6-glucuronide; K_{el}, elimination rate constant; NP, non-parenchymal; IC, intracellular; EC, extracellular; ACN, acetonitrile.

hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid (HEPES) and β-glucuronidase (limpet *Patella vulgata* type L-II) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Morphine hydrochloride was purchased from Norsk Medisinaldepot (Oslo, Norway). Nycodenz [5-(*N*-2,3-dihydroxypropylacetamido)-2,4,6-tri-iodo-*N,N''*-bis(2,3-dihydroxypropyl) isophthalamide], density: 1.150 ± 0.002 g/mL (20°), osmolality: 290 ± 15 mOsm, was purchased from Nycomed Pharma, Norway. Acetonitrile (Fisons, U.K.) was HPLC grade. All other reagents were analytical grade, and used as received.

Cell isolation. Hepatocytes were isolated according to a modification of the Berry and Friend method [26] as described by Berg and Mørland [27]. In summary, the liver was removed from the animal and perfused *in vitro*, first with a Ca^{2+}-free solution for 5 min, and then with a solution containing 4 mM Ca^{2+} and 0.05% (w/v) collagenase. Heparin was not injected during the operative preparation of the liver. Vigorous mixing of the perfusion medium with 95% O_2 and 5% CO_2 was maintained throughout perfusion to keep the pH at 7.45 and pO_2 above 400 mmHg (in the influent medium). The liver started to fall apart after 6–10 min of perfusion with the collagenase solution, and the cell suspension was obtained by shaking the liver gently in the incubation medium. The cell suspension was filtered through nylon mesh (pore size 60 μ), incubated with shaking for 20 min at 37° and centrifuged at about 35 *g* for 2 min. This centrifugation was repeated twice, and then the viability and total cell count were determined.

Non-parenchymal (NP) cells were prepared by three alternative methods. In one, the centrifugation method was slightly modified from Nilsson and Berg [28]. The liver was disrupted at 0–5° after the collagenase perfusion, and centrifuged at 35 *g* for 2 min (all centrifugation steps were carried out at 5°). This was repeated once for the supernatant, which was then centrifuged at 530 *g* for 4 min. The pellet consisting of NP cells was washed and centrifuged once at 70 *g* for 2 min to pellet out contaminating hepatocytes, and this supernatant was then centrifuged at 530 *g* for 4 min. In the second method (pronase method) the primary suspension (after the collagenase perfusion) was incubated with 0.30% (w/v) Pronase for 60–90 min at 37° [29]. The suspension was subsequently centrifuged at 530 *g* for 4 min. In the third method (elutriation method) the cells were treated as described in the centrifugation method, and the pellet was then resuspended in 10 mL of the incubation medium. This fraction was elutriated as described by Blomhoff *et al.* [30]. In summary, a JE-6 elutriator rotor (Beckman Instruments, Palo Alto, CA, U.S.A.) equipped with a standard chamber was used in a J-21 type Beckman centrifuge. Cells were introduced into the elutriation system in a minimal salt solution containing 1% bovine serum albumin. The cells were washed into the chamber at a flow rate of 11.3 mL/min. The resulting NP cell pellets from all methods were resuspended in 10 mL of the incubation medium, and mixed with 30 mL density-gradient Nycodenz. An additional 8 mL of incubation medium was layered on top of this, and centrifuged at 2250 *g* for 15 min with careful acceleration and deceleration. The NP layer was removed by Pasteur pipette, and centrifuged at 530 *g* for 5 min. The pellet was resuspended in incubation buffer, and examined by light microscopy. Only preparations exhibiting more than 90% viable cells (hepatocytes or NP) as measured by the Trypan blue exclusion test were used.

Cell incubation. Hepatocytes (1.6×10^6 cells/mL, total vol. 60 mL in 1000 mL flasks) and NP cells (5×10^6 cells/mL, total vol. 20 mL in 250 mL flasks) were incubated in a medium containing NaCl (140 mM), KCl (5 mM), $MgSO_4$ (0.8 mM), $CaCl_2$ (2 mM), HEPES (20 mM) and 2% w/v bovine serum albumin (pH 7.4). Incubation flasks were shaken continuously at 37°. Morphine hydrochloride was added to obtain concentrations of 1, 5, 100 and 200 μM. Duplicate flasks at each concentration were used. Five and 1.5 mL aliquots for hepatocytes and NP cells, respectively, were taken repeatedly during the incubation period for up to 4 hr. The aliquots were immediately centrifuged to separate cells and medium, and these were frozen separately in liquid nitrogen. Cells and supernatants were analysed for morphine and metabolites. Samples were stored at −70° until analysed. Control samples consisted of cells and medium without morphine, and medium and morphine without cells.

Preparation of microsomes. Liver cells were prepared as described above. Cell pellets were frozen (−80°) in water and thawed three times and then homogenized in 250 mM sucrose, 5 mM HEPES, pH 7.4 to give a 20% (w/v) homogenate. Homogenates were centrifuged at 750 *g* for 10 min, the resulting supernatants were centrifuged at 27,000 *g* for 15 min. These supernatants were finally centrifuged at 105,000 *g* for 1 hr. The pellets were resuspended in the same buffer and used the same day.

Microsomal glucuronidation assay. This was done according to Coughtrie *et al.* [31]. The incubation mixture consisted of 100 μL microsome suspension (approximately 20 mg protein/mL suspension), 50 μL 1 M Tris–HCl (pH 7.4), 50 μL 50 mM $MgCl_2$, 50 μL 5 mM morphine, 5 μL Lubrol PX (non-ionic detergent) and 195 μL distilled water. After 2 min preincubation in a waterbath (37°), the incubations were started by addition of 50 μL 30 mM UDP-glucuronic acid. The incubations were stopped after 15 min by heating to 95° for 2 min, then centrifuged at 16,000 *g* for 5 min in an Eppendorf microcentrifuge. Of the supernatants 75 μL were mixed with 75 μL acetonitrile, and cooled on ice for 10 min, before further centrifugation for 2 min to remove any precipitated protein. Samples (25 μL) of supernatants were subjected to HPLC analyses as described below.

Sample purification. Solid-phase extraction and sample injection modified after Svensson *et al.* [32, 33] were performed by an ASPEC (Automated Sample Preparation with Extraction Columns) robot (Gilson Medical Electronics, France). Sep-Pak light C18 cartridges (Waters, U.S.A.) were washed with 1 mL methanol and 1 mL water. One millilitre of sample was mixed with 1 mL 0.5 M ammonium

sulphate (pH 9.3), and then passed through the cartridge and subsequently washed with 2 mL 5 mM ammonium sulphate (pH 9.3), and 200 μL 10% ACN in 10 mM NaH_2PO_4 (pH 2.1). Morphine and its metabolites were eluted with 600 μL 10% ACN in 10 mM NaH_2PO_4 (pH 2.1). A 25 μL extract was injected into the HPLC system.

HPLC analysis. The HPLC analysis was an automated modification of the method described by Svensson *et al.* [32, 33]. The system consisted of an ASPEC robot (Gilson Medical Electronics, Villiers de Bel, France), HP-1050 isocratic pump (Hewlett Packard, Waldbronn, Germany), Spectra System UV2000 (Spectra Physics Analytical, San Jose, CA, U.S.A.) and ESA Coulochem model 5100A with ESA model 5010 analytical cell (ESA, Bedford, MA, U.S.A.). Integration was performed with HP-3396 Series II integrators (Hewlett Packard, Avondale, PA, U.S.A.) equipped with HP-9122C disc drives (Hewlett Packard, Boise, Idaho, U.S.A.). The column used was Spherisorb S3 ODS2 (10 cm × 4.6 mm) (Phase Separations Ltd, Deeside, U.K.). The mobile phase consisted of 24% ACN (v/v) with the ion-paring agent sodium dodecyl sulphate (4 mM) in a mixture of 10 mM NaH_2PO_4 (pH 2.1). The flow rate at 33° was 1.2 mL/min. The UV wavelength used was 210 nm. The electrochemical detector operated at electrode potentials of 0.23 and 0.30 V, and only the second electrode was used for quantification.

The calculations of intracellular concentrations of morphine and metabolites were based on the assumptions that cell protein constitutes 20% of the hepatocyte cell mass, and that the density of hepatocytes equals that of water.

Determination of NM glucuronide(s). Concentrations of 5, 100, 200 and 1000 μM of NM were incubated with 1.6×10^6 hepatocytes/mL from either guinea pigs or rats. During the incubations the NM concentration decreased, and an increasing peak located in front of the M3G position in the HPLC chromatogram was observed. In guinea pig incubations, a small peak located in front of the M6G position was also noted. These peaks disappeared after treatment with β-glucuronidase (30,000 IU/mL) for 5 hr at 37°, with a subsequent increase in NM. They were considered to represent normorphine-3-glucuronide (NM3G) and NM6G, respectively. Using morphine as substrate, only rat hepatocyte suspensions revealed any NM glucuronides, most probably NM3G. NM6G was not found after incubation with morphine. The concentration of the tentative NM3G was calculated by determining the difference in NM concentration before and after hydrolysis with β-glucuronidase. This method of quantification of NM3G was compared with calculation of the peak by using the standard curve for M3G. The difference obtained by these two approaches was 10–15%, and was regarded as acceptable. The concentrations given for the NM3G were obtained by employing the M3G standard curve method.

Protein determination. This was done according to the method described by Lowry *et al.* [34] and bovine serum albumin was used as standard.

Analytical recovery, sensitivity and variability. The analytical recoveries of M3G, M6G and NM were greater than 85%, while that of morphine was approximately 75%. The inter-assay variability of the automated extraction was less than 5% for all compounds. The limits of quantification for the electrochemical detector (M6G, NM and morphine) were 0.01 μM, and for the UV detector (M3G, M6G, NM and morphine) 0.10 μM. When the initial morphine concentration was 1 or 5 μM, M6G, NM and morphine were quantified by the electrochemical detector. At initial morphine concentrations of 100 or 200 μM, M6G, NM and morphine were quantified by UV detection. M3G and the tentative NM3G were quantified by UV detection.

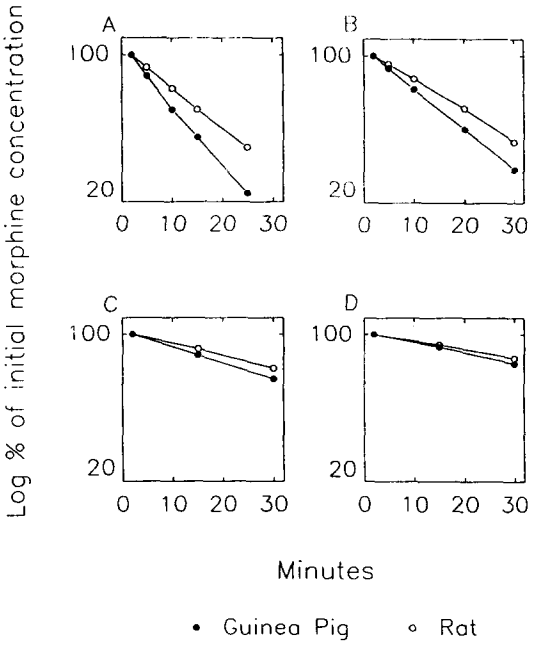

Fig. 1. The effect of substrate concentration on morphine metabolism in isolated guinea pig and rat hepatocytes. (A) 1 μM morphine, (B) 5 μM morphine, (C) 100 μM morphine, (D) 200 μM morphine. Values are medians of five separate experiments, except for the 100 μM experiments where N = 3 (guinea pig) and 2 (rat).

Calculation and statistical methods. All data were expressed as medians. The K_{el} of morphine was calculated from the slope of semilogarithmic plots using the formula: $K_{el} = -a \times \ln 10$, (where a is the slope of the line, in the equation: $y = ax + b$). Statistics were performed on a PC using Minitab statistical software. Significant differences were established at the $P < 0.05$ level using the Mann–Whitney test.

RESULTS

Cell viabilities for all cell types were above 90% after 120 min in all experiments, and above 80% after 240 min. Parenchymal liver cells from both guinea pig and rat metabolized morphine. Cells and supernatants were analysed separately, and the results were combined (media and cells) if no other

Table 1. Initial rate of formation (nmol/mg cell protein/min) of NM3G, NM, M3G and M6G at different morphine concentrations (μM) in isolated hepatocytes from guinea pig and rat

	Guinea pig				Rat			
	Initial concentration of morphine (μM)							
	1	5	100	200	1	5	100	200
NM3G	0*	0*	0	0*	0.63 (0.16–1.30)	4.5 (2.35–5.29)	18.2 (15.2–21.2)	16.0 (9.5–24.9)
NM	0*	0*	0	0*	2.9 (2.32–3.23)	13.1 (9.6–22.8)	58.3 (47.8–68.7)	101.9 (61.5–119.3)
M3G	7.2* (4.4–11.6)	21.9* (12.5–45.1)	71.6 (56.3–93.3)	81.1 (57.6–153.2)	1.7 (0.66–3.47)	8.7 (6.45–15.6)	65.3 (60.5–70.0)	95.1 (30.8–120.4)
M6G	1.9* (1.0–3.1)	5.4* (2.5–7.9)	17.1 (8.4–19.3)	18.1* (8.7–33.1)	0	0	0	0.6 (0–1.5)

Values are medians and ranges ( ) of five separate experiments, except the 100 μM experiments where N = 3 guinea pig and 2 (rat).

Initial rate is calculated from the first 10 min in the 1 and 5 μM, and the first 15 min in the 100 and 200 μM experiments, except for NM3G where the interval between 45 and 60 min is used.

* Denotes statistical significance between guinea pig and rat at the respective concentrations, $P < 0.05$.

information is given. Semilogarithmic plots of morphine concentration versus time were linear for all concentrations of morphine in both animal species (Fig. 1). The elimination rate constant of morphine (K_{el}) per milligram viable cell protein was calculated from the first 10 min in the 1 and 5 μM, and the first 15 min in the 100 and 200 μM experiments. $K_{el(guinea\,pig)} = 0.024$ and $K_{el(rat)} = 0.013$, $K_{el(guinea\,pig)} = 0.017$ and $K_{el(rat)} = 0.013$, $K_{el(guinea\,pig)} = 0.005$ and $K_{el(rat)} = 0.004$, $K_{el(guinea\,pig)} = 0.004$ and $K_{el(rat)} = 0.003$ in the 1, 5, 100 and 200 μM experiments, respectively. There was a higher initial rate of metabolism in the guinea pig hepatocytes compared to rat hepatocytes, but this difference was significant only in the 1 μM experiments ($P < 0.05$).

M3G was the major metabolic product of morphine in hepatocyte suspensions from both animals. The initial rate of formation of M3G (nanomoles per milligram cell protein per minute) was significantly higher in guinea pig than rat hepatocytes in the experiments with substrate concentrations of 1 and 5 μM ($P < 0.05$). In the 200 μM experiments, rat hepatocytes tended to form M3G faster than guinea pig cells, but the difference did not reach statistical significance (Table 1). The relative amount of morphine that was recovered as M3G at the end of the incubations (120, 180, 240 and 240 min in the 1, 5, 100 and 200 μM experiments, respectively) was dependent on the initial concentration of morphine (Table 2). Guinea pig cells formed appreciable quantities of M6G, but no NM was detected (Table 2). Rat hepatocytes failed to show any substantial formation of M6G, although this metabolite was detected intracellularly at all substrate concen-

Table 2. The relative amount (percentage of initial morphine concentration) of NM3G, NM, M3G, M6G and morphine in isolated hepatocytes from guinea pig and rat at 120, 180, 240 and 240 min after the addition of morphine in the 1, 5, 100 and 200 μM experiments, respectively

	Guinea pig				Rat			
	Initial concentration of morphine (μM)							
	1	5	100	200	1	5	100	200
NM3G	0*	0*	0	0*	18.7 (16.3–22.1)	32.6 (21.5–38.6)	20.1 (16.8–23.3)	4.1 (3.5–18.0)
NM	0*	0*	0	0*	11.0 (5.2–13.4)	5.4 (2.9–11.5)	10.4 (8.3–12.4)	22.5 (15.4–28.3)
M3G	41.7 (25.2–50.6)	38.1 (31.7–46.8)	22.5 (11.1–23.0)	8.5* (6.0–18.3)	34.6 (28.6–40.4)	32.6 (28.4–55.8)	37.4 (32.7–42.2)	22.7 (14.9–46.0)
M6G	11.2* (6.8–12.6)	10.2* (7.6–12.4)	5.7 (2.3–5.9)	2.1* (1.8–2.6)	0	0.4 (0.2–0.8)	0.6 (0.4–0.7)	0.6 (0.5–0.7)
Morphine	0 (0–2.1)	0 (0–2.5)	0.9 (0.5–1.1)	19.3* (13.4–20.1)	0 (0–1.3)	0.2 (0–0.8)	1.1 (1.0–1.2)	9.0 (4.1–12.0)

Values are medians and ranges ( ) of five separate experiments, except the 100 μM experiments where N = 3 (guinea pig) and 2 (rat).

* Denotes statistical significance between guinea pig and rat at the respective concentrations, $P < 0.05$.

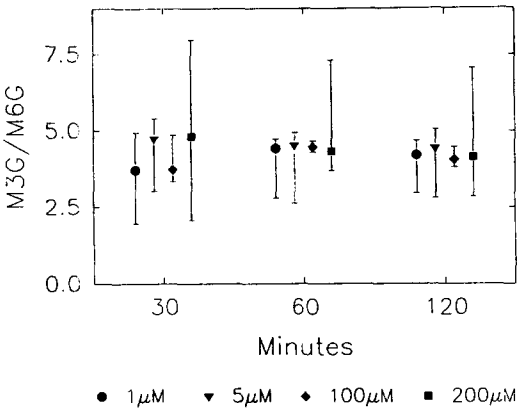

Fig. 2. The effect of substrate concentration and time on the M3G/M6G ratio in guinea pig hepatocytes. Values are medians and ranges of five separate experiments, except for the 100 μM experiments where N = 3.

trations, and also at low concentrations in the suspension medium of incubations after 5, 100 and 200 μM of morphine. In both species the glucuronides were found to be stable end-products, implying that the concentrations of these conjugates were unaltered after the total disappearance of morphine from the medium (1 and 5 μM experiment). In the 100 and 200 μM morphine experiments detectable amounts of morphine were present throughout the incubation period, and the concentration of M3G and M6G increased throughout the experimental period. The M3G/M6G ratio in guinea pig hepatocytes was neither significantly influenced by the initial morphine concentration, nor by time (Fig. 2).

N-Demethylation to NM was a major pathway in rat hepatocytes. The concentration of NM increased during the first part of the incubations, and reached a maximum shortly before morphine was below detection limit (1 and 5 μM experiments). After that, the concentration of NM decreased. Simultaneously, a peak located in front of M3G appeared in the chromatogram (Fig. 3). This unknown peak increased continuously throughout the incubation periods at all concentrations of morphine. The metabolite was believed to be NM3G. In the guinea pig experiments no NM or possible NM glucuronides were detected. The amount of detectable metabolites at the end of the experiments is given in Table 2.

The ratios between intra- and extracellular (IC/EC) concentrations of M, NM, M3G, M6G and NM3G for the 5 μM experiments are illustrated in Fig. 4. Comparable data were observed in the other experiments. Guinea pig and rat hepatocytes demonstrated a similar time-dependent pattern, but the absolute IC/EC ratios for M3G and M6G were significantly lower in guinea pig preparations ($P < 0.05$). The ratio of morphine increased throughout the incubation period, demonstrating that already at 2 min (first observation) morphine had entered the hepatocytes to such an extent that the IC exceeded the concentration in the incubation medium. The IC/EC ratios of M3G, M6G and NM3G decreased with time. The NM ratio displayed an initial decrease and then a subsequent increase in all but the 200 μM experiments, where the ratio increased throughout the incubation period.

Metabolic activity with regard to morphine was not detected in intact NP liver cells of either species, regardless of preparation technique (simple centrifugation, pronase treatment or centrifugal elutriation). The total amount of morphine added was recovered at the end of the incubations. Further investigation of homogenates and microsomes of NP cells showed no metabolic degradation of morphine. Homogenates and microsomes of hepatocytes from both guinea pig and rat exhibited a metabolic pattern of morphine metabolites similar to that found in isolated, undisrupted cells.

DISCUSSION

Guinea pig and rat parenchymal liver cells both metabolized morphine. In the guinea pig M3G was the major metabolic product, and the amount formed depended to some extent on the initial concentration of morphine. Guinea pig hepatocytes formed from four to five times as much M3G as M6G. M6G was however formed in appreciable amounts. The M3G/M6G ratio obtained is very close to that reported in humans [5, 7, 35]. Based on this finding, guinea pig hepatocytes should be more suitable than rat hepatocytes to use in the study of possible regulatory mechanisms involved in morphine glucuronidation. Based on the finding that the guinea pig M3G/M6G ratio in the present study is comparable to that reported both *in vivo* [23] and in liver cell homogenates [24], we conclude that the liver appears to be mainly responsible for the interspecies difference in 3- and 6-glucuronidation of morphine. This is of importance, since glucuronidation of morphine has been reported to occur extrahepatically in some species [36], and an extrahepatic formation of M6G in the guinea pig could have been responsible for the species difference. Sparse data are available about the possible regulation of morphine glucuronides, but Lawrence *et al.* [24] have demonstrated that in guinea pig liver cell homogenates some metal ions (Cu^+, Cu^{2+} and Cd^{2+}) inhibit the production of M3G without affecting glucuronidation in the 6-position. This supports the existence of UDP-glucuronosyltransferase isoenzymes responsible for the metabolism of morphine. Furthermore, this is in agreement with on-going studies on isolated guinea pig hepatocytes investigating a possible interaction between morphine and ranitidine in this laboratory [37]. The clinical relevance of the M3G/M6G ratio has not been elucidated in any detail, and the results so far are not conclusive [13, 16, 17]. The present study provides an *in vitro* model that can be used to examine the effects of factors on morphine glucuronidation that later may be tested in human studies.

Rat hepatocytes also formed large amounts of M3G, but virtually no M6G. This is in agreement with previous studies in rats [22, 23]. N-Demethylation to NM and subsequent conjugation to NM3G was also a major pathway in the rat. There is scarce data on

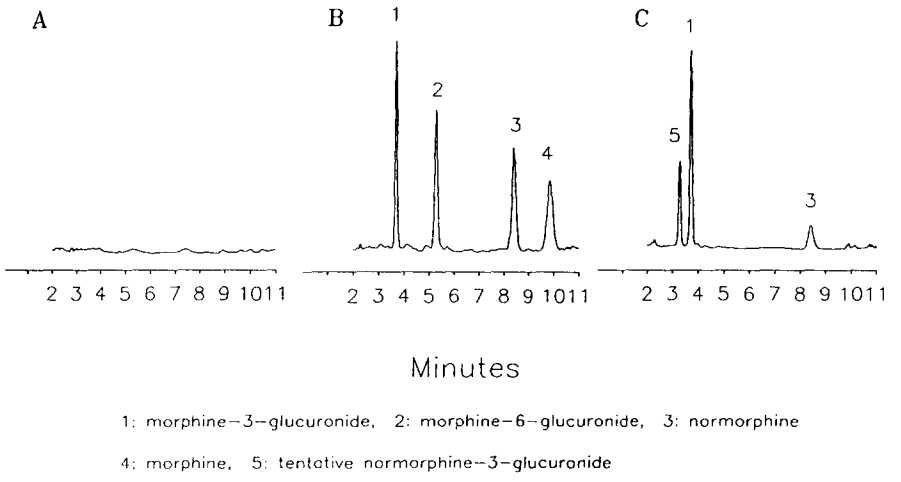

Fig. 3. Representative HPLC chromatograms (UV detection) of extracts from incubation media. (A) blank (rat hepatocytes), (B) 2.5 μM standards and (C) sample after 180 min of incubation of 5 μM morphine in suspension of rat hepatocytes.

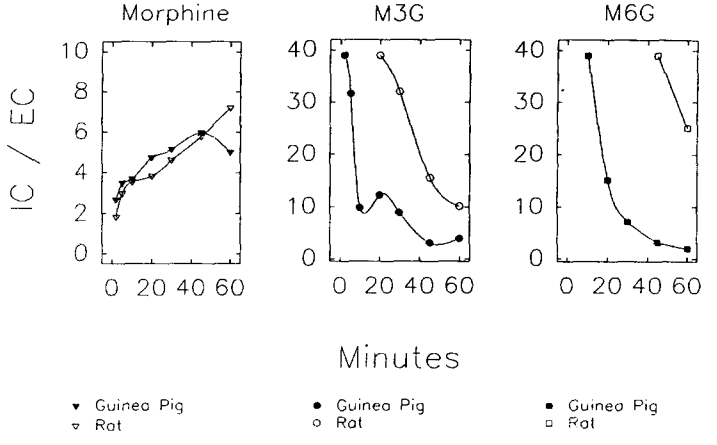

Fig. 4. IC/EC ratios of morphine, M3G and M6G concentrations in guinea pig and rat hepatocyte experiments against time. Starting concentration of morphine 5 μM. Values are medians of five separate experiments.

the N-demethylation pathway in humans, and it is therefore difficult to assess whether guinea pig or rat was closer to human metabolism in this respect. To what extent NM glucuronides are end-products after intake of morphine should be examined also in humans.

The observed difference in initial rate of metabolism between guinea pigs and rats was less with increasing morphine concentrations. This suggests that guinea pig hepatocytes might have glucuronosyltransferases with a higher affinity but lower capacity for morphine glucuronidation than rat hepatocytes. This is further supported by the relatively smaller contribution of glucuronidation to total morphine metabolism at higher concentrations of morphine in the guinea pig. In both species it was observed that the relative amount of morphine converted to detectable metabolites (M3G, M6G, NM and NM3G) was largely reflected by the initial rate of formation of the respective metabolites. The proportion of morphine transformed that could be detected as these metabolites decreased with increasing substrate concentration. This could be due to alternate metabolic pathways, such as formation of sulphates, glutathione conjugates, morphine-*N*-oxide and morphinone [6, 20, 38]. These pathways may have increased importance when the major metabolic pathway enzymes become saturated.

The ratio between the possibly antagonistic M3G and the pharmacologically active M6G in guinea pig hepatocyte suspensions was not significantly altered by substrate concentration. This is in concordance with studies of morphine glucuronidation in humans [35, 39].

The reported ratios between intra- and extracellular concentrations of morphine and its metabolites probably represent minimum values, since the cell pellets used for determination of intracellular concentrations contained small amounts of extracellular medium, diluting the intracellular concentrations. The lower IC/EC ratios for M3G and M6G in guinea pig could have several possible explanations such as lower intracellular binding capacity, lower energy charge or greater leakage from the hepatocytes. There was no indication that the guinea pig cell pellets contained more medium than rat cell pellets. Furthermore, the concentrations of M6G in the incubation buffers of the rat experiments were very low, and therefore the rat M6G IC/EC ratios should be regarded with caution. The present study does not permit conclusions concerning possible mechanisms responsible for the maintenance of these gradients. However, isolated hepatocytes are able to maintain drug concentration gradients across the cell membrane. The results might be of relevance with regard to *in vitro* studies in general, if intracellular concentrations typically are 20–50 times those usually found in blood. Thus, the use of drugs in the micromolar range in microsomal studies may expose the enzymes to concentrations comparable to intracellular levels in the liver *in vivo*.

The lack of metabolic activity towards morphine in NP cells could be a consequence of the analytical sensitivity, but major NP cell metabolism could be excluded. Our results are further in agreement with Oesch *et al.* [40] who observed activity in rat NP cells only after induction with Aroclor 1254.

In conclusion, the biotransformation of morphine in isolated guinea pig hepatocytes was found to resemble humans with regard to the pattern of morphine glucuronidation. Thus, guinea pig hepatocytes could be a promising *in vitro* system for the study of interactions with morphine glucuronidation.

The intracellular concentrations of morphine and its metabolites were found to exceed the concentration in the incubation medium up to 100 times.

Metabolic activity towards morphine was not detected in *non*-parenchymal liver cells from either guinea pig or rat.

Acknowledgements—This study was supported by grant No. 322.89/002 from The Norwegian Research Council for Science and Humanities; Medical Research Council, where T.A.A. is a research fellow.

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