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Human liver morphine UDP-glucuronyl transferase enantioselectivity and inhibition by opioid congeners and oxazepam

[1]Agneta Wahlström, [2]Gian Maria Pacifici, [3]Björn Lindström, [4]Lena Hammar & Anders Rane

Division of Clinical Pharmacology, University Hospital, S-751 85 Uppsala, Sweden

1 Morphine uridine diphosphate glucuronyl transferase (UDP-GT) was studied in human liver microsomes. The (−)- and (+)-morphine enantiomers were used as substrates and inhibitors, such as oxazepam and various opioid congeners were employed to characterize the different glucuronidation pathways. The kinetics of the oxazepam inhibition were studied in the rat liver.

2 The overall glucuronidation of (+)-morphine was higher than that of (−)-morphine. The morphine congeners tested, potently inhibited the formation of (−)-morphine-3-glucuronide ((−)-M3G), except for normorphine and codeine. The formation of (+)-morphine-6-glucuronide ((+)-M6G) was potently inhibited by only dextromethorphan and (+)-naloxone. All drugs except normorphine inhibited the formation of (+)-M3G by 18–50%.

3 The metabolism of (−)-morphine to (−)-M3G was more sensitive to oxazepam inhibition than the formation of (+)-M3G from (+)-morphine in the rat liver.

4 The glucuronidation of natural morphine is subject to *in vitro* interaction with oxazepam and several opiate drugs. Our study supports the theory of more than one type of UDP-GT being involved in morphine glucuronidation.

Introduction

Enantioselectivity of morphine uridine diphosphate glucuronyl transferase (UDP-GT) was previously demonstrated in rats (Rane *et al.*, 1985). There is a preferential formation of morphine-3-glucuronide (M3G) from the (−)-enantiomer whereas morphine 6-glucuronide (M6G) is the major glucuronide formed from the unnatural, inactive (+)-enantiomer of morphine. This was demonstrated in human foetal (Gawronska-Szklarz *et al.*, 1985) and adult (Rane *et al.*, unpublished observations) liver as well as in rat liver (Rane *et al.*, 1985). In man, the overall glucuronidation of (+)-morphine exceeds that of (−)-morphine. In the rat the reverse is true.

[1] Author for correspondence.
[2] Present address: Instituto di Patologia Generale, Medical School, University of Pisa, 56100 Pisa, Italy.
[3] Present address: National Board of Health and Welfare, Section of Drugs, P.O. Box 607, S-751 24 Uppsala, Sweden.
[4] Present address: Department of Medical and Physiological Chemistry, Biomedical Center, P.O. Box 575, S-751 23 Uppsala, Sweden.

The glucuronidation of morphine in mammalian liver thus shows a complexity that was previously unknown. Not only is there an inherent substrate stereo- and site-selectivity in the rat liver, but there is also ample evidence that the glucuronidation of the two sites at the (+)-enantiomer is catalyzed by different enzymes. Thus, the ratio of (+)-M6G/(+)-M3G formation rates increases in phenobarbitone-treated rats (Rane *et al.*, 1985). Recent experiments in our laboratory (together with B. Burchell), utilizing chromatofocusing separation technique, are also indicative of the existence of at least two enzymes for the glucuronidation of the morphine enantiomers in the rat.

Morphine is increasingly used as a potent analgesic in cancer patients with severe chronic pain (Rane *et al.*, 1982). In view of the escalated and extremely high doses that are often employed (Säwe *et al.*, 1985) more knowledge about the mechanisms for its glucuronidation in the human liver is required. In such clinical situations anxiolytic drugs and other opioid congeners are often co-administered to the

patients (WHO, 1986). Therefore, their possible or confirmed metabolic interaction with morphine UDP-GT (Sanchez *et al.*, 1978; Pacifici & Rane, 1981; Pacifici *et al.*, 1986) have more than theoretical interest. Cyproheptadine, which is used as an antipruritic drug is also known to interfere with the metabolism of morphine (Porter *et al.*, 1975).

Our investigation was designed to study the metabolic interaction of oxazepam and several opiate-related drugs with the glucuronidation of the morphine enantiomers in human liver microsomes. The rat liver was also employed for studies of the kinetics of the oxazapam inhibition of UDP-GT.

Methods

Liver microsomes were isolated from male Sprague-Dawley rats (180–200 g) as described previously (Pacifici *et al.*, 1982). The human liver specimens were obtained from kidney transplant donors (H5–H8) or as *post-mortem* biopsies from geriatric patients (U50, U51, UAS2). One specimen (UAS1) was obtained at laparotomy because of liver lobe resection. The samples were frozen at −70°C until assay which was performed as described below. The tissue was minced and rinsed in ice-cold 0.3 M sucrose or 0.3 M sucrose with 0.05 M Tris-buffer (pH 7.4). Homogenization was performed in a Potter-Elvehjem glass-Teflon homogenizer. The homogenate was centrifuged at 9,000 *g* for 10 min at 4°C. The supernatant was further centrifuged for 60 min at 105,000 *g*, 4°C. The resulting microsomal pellets were resuspended in 0.3 M sucrose in 0.05 M Tris-buffer (pH 7.4).

Glucuronidation assay

The morphine glucuronidation assay was performed as described by Rane *et al.* (1985). Incubation mixtures (100 μl final volume) contained 0.17 M Tris-HCl buffer (pH 7.4), 8.3 mM $MgCl_2$, 3 mM morphine and microsomal suspension (to give a protein concentration of 1 to 2 mg ml^{-1} of incubation mixture) and 15 mM uridine diphosphoglucuronic acid (UDPGA).

The reaction was performed under conditions that were linear with respect to protein concentration and time. Incubations were carried out at 37°C for 12 min. The reaction was stopped by freezing the samples on dry ice in acetone.

The UDP-GT activity was calculated as the rate of glucuronide formation. The metabolite concentrations were determined by reversed-phase ion pair high performance liquid chromatography according to Svensson *et al.* (1982).

The kinetics of the metabolic inhibition are described as double reciprocal plots from which the inhibition constant (K_i) was also graphically estimated.

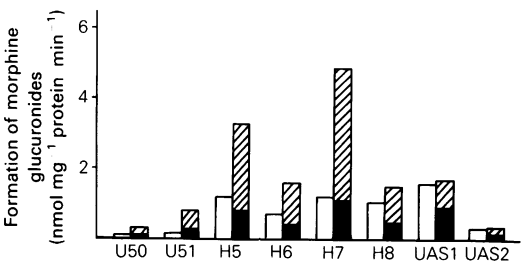


Figure 1 The overall glucuronidation rate of (+)- and (−)-morphine in 8 human liver microsomal preparations. The incubation mixtures contained 3 mM (+)- or (−)-morphine and 15 mM uridine diphosphoglucuronic acid. Incubations were carried out at pH 7.4 for 15 min at 37°C. Open columns represent the formation of (−)-morphine-3-glucuronide ((−)-M3G); solid columns, (+)-M3G and hatched columns, (+)-M6G.

The study was approved by the Ethics committee of the University Hospital.

(−)-Morphine-HCl (Nord. Pharmacopeia), nalorphine and codeine were obtained from the hospital pharmacy. (+)-Morphine and morphine-3-glucuronide were obtained from the National Institute on Drug Abuse (Rockville, MD, U.S.A.). (−)-Morphine-6-glucuronide was purchased from Ultrafine Chemicals (Salford, U.K.). Both of the naloxone enantiomers were obtained from Endo labs (Garden City, N.Y., U.S.A.) and oxazepam was obtained from Wyeth (Philadelphia, Pa, U.S.A.). Dextrorphan, dextromethorphan and 3-methoxymorphinan were obtained from Hoffman La Roche AB, Basel, Switzerland. UDPGA was purchased from Sigma Chemical Co (St. Louis, MD). All other chemicals were of analytical grade. Stock solutions and inhibition tests were performed as previously described (Pacifici & Rane, 1981; Rane *et al.*, 1985).

Non-linear regression using the Michaelis-Menten equation was done on an IBM PC with the aid of the programme ENZFITTER (Leatherbarrow, 1987).

Results

In human liver microsomes the overall glucuronidation of (+)-morphine was considerably higher than that of (−)-morphine in all but two cases (Figure 1). The rate of metabolism with (−)-morphine varied between 0.1 and 1.6 nmol mg^{-1} protein min^{-1}. With (+)-morphine the overall metabolic rate varied from 0.3 to 4.9 nmol mg^{-1} protein min^{-1}. (+)-Morphine was preferentially metabolized to

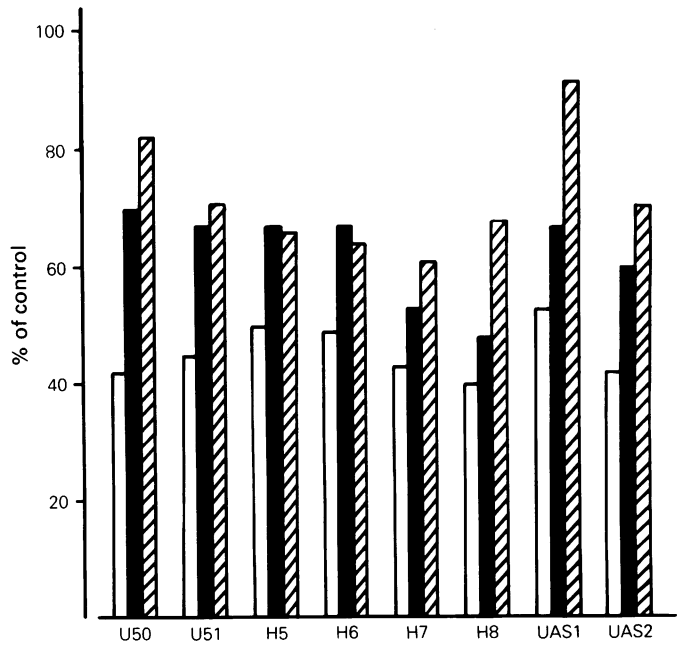


Figure 2 Uridine diphosphate-glucuronyltransferase activity with (−)- or (+)-morphine as substrate in the presence of 3 mM oxazepam in the incubation mixtures. The results from 8 human liver preparations are expressed as % enzyme activity compared to control activity obtained in the absence of oxazepam (= 100%) (see Figure 1). Open columns represent % control formation of (−)-morphine-3-glucuronide ((−)-M3G); solid columns, (+)-M3G and hatched columns, (+)-M6G.

(+)-M6G in all but two liver specimens and the average (+)-M6G/(+)-M3G ratio was 2.3 ± 0.87 (mean ± s.d.).

The UDP-GT in human liver microsomal preparations was also studied in the presence of oxazepam, which is a potent and competitive inhibitor of the enzyme (Pacifici & Rane, 1981). Oxazepam inhibited all three pathways (Figure 2) but the one for (−)-M3G formation pathway was more sensitive than the others in all eight liver specimens. In most cases the formation of (+)-M6G was least affected whereas the formation (+)-M3G displayed an intermediate inhibition in six of the eight livers.

In order to study the mechanisms for the inhibition we used rat liver microsomes, since the gross inhibition of morphine glucuronidation by oxazepam has previously been demonstrated in this species.

The oxazepam inhibition kinetics were studied in rat liver microsomes using five morphine and two oxazepam concentrations (Figure 3). These data were also applied to non-linear regression analyses using the Michaelis-Menten equation with or without correction terms for competitive inhibition. Table 1 gives the kinetic parameters thus obtained.

As seen in Table 1 the apparent K_M, in contrast to V_{max}, was changed in the presence of oxazepam. To obtain an idea of whether the inhibition was competitive or not, correction terms for competitive inhibition were included in the calculations. The corrected values for K_M were close to those obtained in the absence of oxazepam. This indicates that the inhibition by oxazepam of these metabolic pathways for morphine seems to be competitive.

Various congeners of morphine were used to study the possible differences in the inhibition patterns for the glucuronidation of the morphine enantiomers. For this purpose we included drugs (Figure 4) that represent opiate antagonists, antitussives as well as analgesics. In addition, two metabolites of dextromethorphan were studied. Some of the drugs have substituents at the nitrogen position (dextromethorphan, dextrorphan, codeine, nalorphine, naloxone) and some lack such a substituent (normorphine, 3-methoxy-morphinan).

The formation of (−)-M3G was potently inhibited by all the congeners tested, except for normorphine (Figure 5).

With (+)-morphine as substrate dextromethrophan and (+)-naloxone were the only drugs

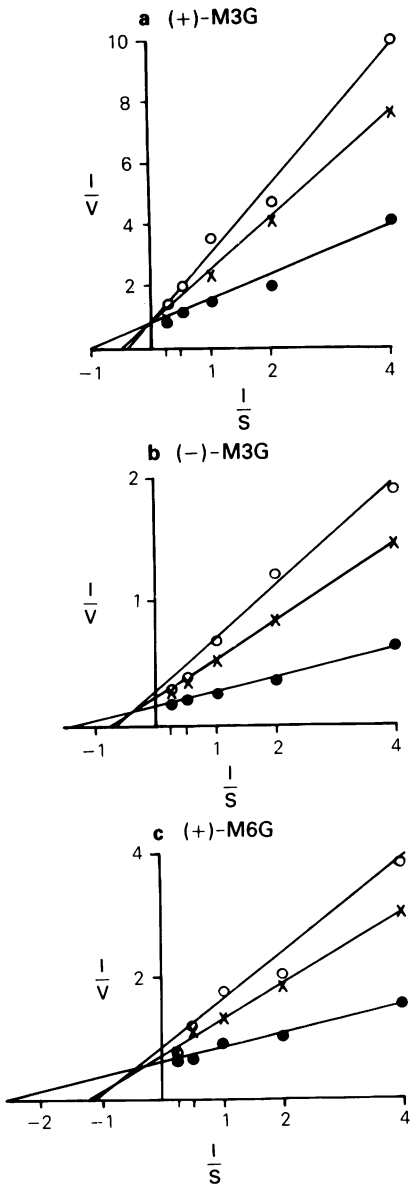


Figure 3 The inhibition of rat liver microsomal morphine glucuronidation by different concentrations of oxazepam (×) 0.15 and (○) 0.3 mM; (○) control glucuronidation. (a) Inhibition of the formation of (+)-morphine-3-glucuronide ((+)-M3G), (b) (−)-M3G and (c) (+)-M6G. The concentration of morphine was varied (0.25–4 mM) in the presence of 15 mM uridine diphosphoglucuronic acid. A double reciprocal plot of initial velocity versus the concentration of morphine is presented. Velocity is expressed as nmol of morphine conjugated min^{-1} mg^{-1} protein.

that potently inhibited the formation of (+)-M6G. In contrast to the other inhibitors tested these two drugs caused a similar degree of inhibition of the formation of (−)-M3G and (+)-M6G. All other drugs suppressed the reactions with (+)-morphine only moderately (Figure 5).

Discussion

Our results concern aspects of the human morphine metabolism that may have clinical implications. When studied *in vitro* the metabolic routes for morphine are affected by some drugs in common clinical use as well as other compounds. Our data indicate the existence of at least two types of UDP-GT catalyzing the glucuronidation pathways of (−)- and (+)-morphine.

There was a wide (10 fold) interindividual variation in the glucuronidation of the morphine enantiomers (Figure 1). These data confirm earlier findings of large pharmacokinetic differences between morphine-treated patients (Säwe *et al.*, 1985). Nevertheless, the relative inhibitory effect of oxazepam on the three pathways investigated did not differ much between individuals (Figure 2). The inhibition of the formation of (−)-M3G was always greater than that for the other pathways and ranged between 50 and 60%.

We have previously obtained evidence that there are two sites in (+)-morphine which are glucuronidated by separate enzymes in the rat liver (Hammar *et al.*, 1986) and, on the basis of similar results, also in human liver. Therefore, we investigated in detail the inhibition kinetics for oxazepam in rat liver, to find further support for the idea of the presence of more than one morphine UDP-GT. Such kinetic experiments could not be performed in the present study in human liver because of the limited supply of tissue material. However, the differential inhibition by oxazepam of morphine glucuronidation in human liver provides suggestive evidence of at least two types of morphine UDP-GT in man as well.

Various morphine congeners, including substrates as well as non-substrates of UDP-GT, were used as tools for further attempts to study the diversity of the enzymes. Some of these drugs are known inhibitors of the glucuronidation (Sanchez *et al.*, 1978). In addition they are often used concomitantly with morphine in patients with chronic cancer pain.

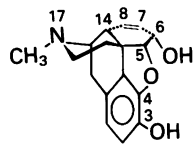
Three of the drugs lack a substituent in position 6 (Figure 4), but their patterns of inhibition were dissimilar. Dextromethorphan was, however, a strong inhibitor of the formation of (+)-M6G. Hence, we concluded that the 6-substituent is not mandatory for inhibition of this pathway since (+)-naloxone had a similar inhibitory pattern. Interestingly, there

Table 1 Estimated kinetic parameters for the glucuronidation of morphine in rat liver and its inhibition by oxazepam

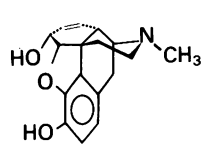
Oxazepam (mM)	V_{max} (nmol mg^{-1} min^{-1}) *Apparent*	*Correction for inh.*[2,3]	K_M (mM) *Apparent*	*Correction for inh.*[2,3]	K_i (mM)
(−)-M3G					
0	6.71		0.80		
0.15	5.12	5.12	1.65	0.98	0.22
0.30	5.50	5.50	2.68	1.10	0.21
(+)-M3G					
0	1.49		1.14		
0.15	2.09	2.10	4.66	1.35	0.06
0.30	1.21	1.21	2.96	1.21	0.21
(+)-M6G					
0	1.48		0.51		
0.15	1.25	1.25	1.34	0.81	0.22
0.30	0.95	0.95	1.12	0.81	0.81

[1] Estimates obtained by non-linear regression analyses using the Michaelis-Menten equation without correction for inhibitor.
[2] Estimates obtained by non-linear regression using the Michaelis-Menten equation with correction terms for competitive inhibition.
[3] $1 + I/K_i$, where I = inhibitor concentration and K_i the dissociation constant.

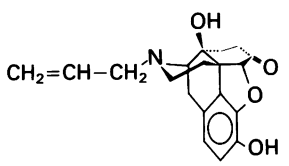
a



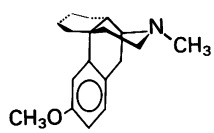
(−)-Morphine



(+)-Morphine



(−)-Naloxone



Dextrometorphan

b

Position 17	3	6		*Other changes*
$-CH_3$	−OH	−OH	Morphine	
−H	−OH	−OH	Normorphine	
$-CH_2CH=CH_2$	−OH	−OH	Nalorphine	
$-CH_2CH=CH_2$	−OH	=O	Naloxone	(1, 3)
$-CH_3$	$-OCH_3$	−OH	Codeine	
$-CH_3$	$-OCH_3$	−H	Dextromethorphan	(1, 2)
−H	$-OCH_3$	−H	3–Methoxymorphinan	(1, 2)
$-CH_3$	−OH	−H	Dextrorphan	(1, 2)

Figure 4 (a) The morphinan skeleton and (b) the various substituents used in positions 17, 6 and 3. In (b): (1) single instead of double bond between C7 and C8, (2) no oxygen between C4 and C5 and (3) OH in C14.

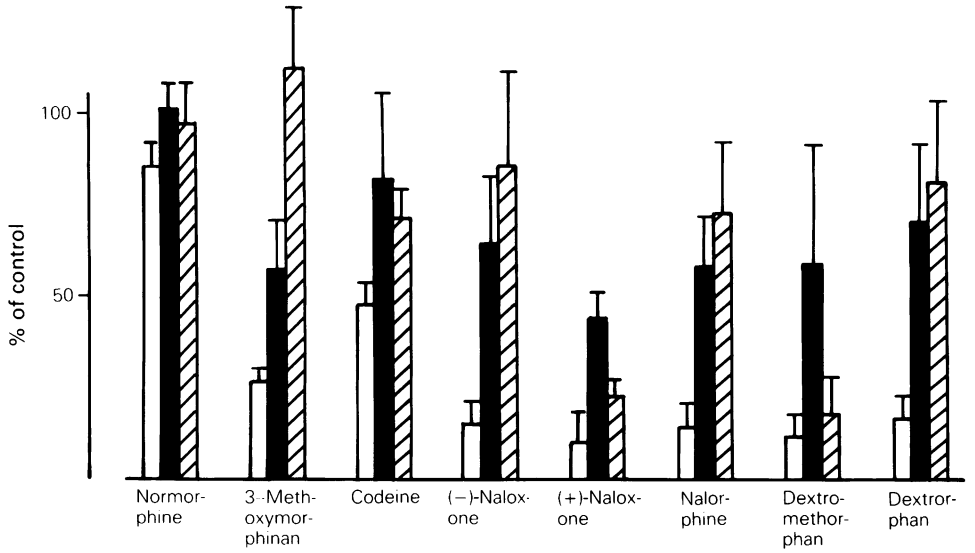


Figure 5 Uridine diphosphate-glucuronyltransferase activity with (−)- or (+)-morphine as substrate in the absence (control = 100%) or presence of 3 mM of various inhibitors. Each column represents the mean, based on 4 different experiments; vertical lines indicate s.d. Open columns represent % control formation of (−)-morphine-3-glucuronide ((−)-M3G); solid columns, (+)-M3G and hatched columns, (+)-M6G.

was an enantiomeric discrimination of the inhibition of (+)-M6G with naloxone, since only the (+)-enantiomer of naloxone had a pronounced effect on the formation of (+)-M6G.

We did not find any significant effect of the type of substituent in position 3 on the pattern of inhibition. Nor had the substituent in position 17 any decisive influence on the inhibitory pattern. On the other hand the lack of a 17-substituent as in the case of normophine and 3-methoxymorphinan left the (+)-M6G formation intact. This observation is in accord with the findings of Sanchez *et al.* (1978), who found that N-aklyl substituents were essential for inhibition of the overall glucuronidation of (−)-morphine in rabbit liver. However, among the drug molecules with N-substituents we found that only dextrorphan, nalorphine and naloxone exerted a potent inhibitory effect on the rate of formation of (−)-M3G.

The pronounced inhibitory effects of oxazepam and other benzodiazepines (Pacifici *et al.*, 1986; Rane *et al.*, 1986) is not clearly understood. This drug may serve as an alternative substrate inhibitor since it is known to be glucuronidated itself. The potentiating psychopharmacological effect of oxazepam on the pain experienced by cancer patients may partly be caused by an interaction *in vivo* at the metabolic site.

It must be emphasized that the concentrations of the investigated drugs used *in vitro* exceed by far the concentrations that are achieved *in vivo* in treated patients. Therefore, our data may only be indicative of interactions that may occur *in vivo* but indicate that further studies at clinically relevant concentrations are worthwhile.

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