Species Difference of Site-Selective Glucuronidation of Morphine

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Species difference in glucuronidation of morphine was studied using mice, rats, guinea pigs and rabbits *in vivo* and *in vitro*. Morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G) were determined by high-performance liquid chromatography. M-3-G was the major urinary metabolite of morphine in all these animal species. However, a remarkable species difference was observed in the urinary excretion of the M-6-G. Excretion ratios of the M-3-G to M-6-G were approximately 4:1 and 50:1 in guinea pigs and rabbits, respectively. The urinary excretion of M-6-G in mice and rats was too small to be determined. On the other hand, the ratios of uridine diphosphate-glucuronyltransferase (UDPGT) activities toward 3- and 6-hydroxyl groups of morphine in liver microsomes of mice, rats, guinea pigs and rabbits were approximately 300:1, 90:1, 4:1 and 40:1, respectively. Ratios of two morphine UDPGT activities in the liver microsomes of guinea pigs and rabbits, thus, reflected those of urinary excretion of morphine glucuronides.

Keywords — species difference; morphine; morphine-3-glucuronide; morphine-6-glucuronide; urinary excretion; uridine diphosphate-glucuronyltransferase; liver microsome

Introduction

Morphine is now increasingly used in the treatment of cancer pain. The metabolic studies of morphine have been performed extensively by a number of workers using various animal species. It has been established that morphine is metabolized mainly by glucuronidation.^{1,2)} Since morphine possesses two hydroxyl groups, it can be metabolized to two isomeric glucuronides. Morphine-3-glucuronide (M-3-G) has been found as the major metabolite of morphine, which loses the analgesic effect.²⁾ We previously detected another glucuronide, morphine-6-glucuronide (M-6-G), as a minor metabolite of morphine for the first time and found it to be a potent analgesic.^{3,4)}

Glucuronidation is catalyzed by hepatic microsomal uridine diphosphate-glucuronyltransferase (UDPGT),⁵⁾ and the heterogeneity of UDPGT has been elucidated.⁶⁾ In rat liver, UDPGT activities toward various substrates develop for different perinatal periods and are differently affected by various enzyme-inducers such as 3-methylcholanthrene and phenobarbital.⁷⁾ Several workers have reported purification of UDPGT from liver microsomes

of several animal species including human.^{8–15)} Moreover, complementary deoxyribonucleic acids (cDNAs) encoding rat liver UDPGTs have recently been cloned and their nucleotide sequences have been determined.^{16–23)} These isoenzymes are limited to UDPGTs with high activities toward xenobiotics such as 4-nitrophenol and 4-methylumbelliferone or UDPGTs toward endogenous substrates such as steroid hormones and bilirubin. A UDPGT toward morphine has been purified from liver microsomes of rats,¹³⁾ however, those of the other animal species have not yet been reported. Also, it is noteworthy that a great difference in analgesic activity exists between M-3-G and M-6-G, and it is interesting to characterize the UDPGT which catalyze two glucuronides.

Recent reports on morphine metabolism in humans suggest a possible contribution of M-6-G to morphine analgesia.^{24,25)} However, the urinary excretion or the formation of these glucuronides has not yet been determined quantitatively in various animal species.

In the present study, species difference in glucuronidation of morphine was examined *in vivo* and *in vitro* using mice, rats, guinea pigs and rabbits.

Materials and Methods

Materials — Morphine hydrochloride was purchased from Takeda Chemical Ind., Ltd. (Osaka, Japan). Uridine diphosphate (UDP)-glucuronic acid was purchased from Yamasa Shoyu Co. (Chiba, Japan). 4-Nitrophenol and 4-hydroxybiphenyl were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). M-3-G and M-6-G were prepared by the method previously described.^{26,27)}

Animals — Male ddY mice (21–29 g), male Wistar rats (165–200 g), male Hartley guinea pigs (310–560 g) and male New Zealand White rabbits (2.2–2.8 kg) were used in this study. Animals were housed in stainless-steel cages with water and standard laboratory chow *ad libitum*.

Administration of Morphine — Morphine hydrochloride was dissolved in physiological saline and injected subcutaneously at a dose of 10 mg base/kg. Urine samples excreted within 24 h after the injection were collected in vessels containing about 5 mg saccharo-1,4-lactone for 10 ml urine and stored at –20 °C until analysis.

Isolation of Metabolites — Morphine glucuronides in the urine were determined by the method of Svensson *et al.* as follows.²⁸⁾ Urine (1.0 ml) was mixed with 3.0 ml of 0.5 M ammonium sulfate adjusted to pH 9.3 with ammonia, and passed through a Sep-Pak C₁₈ cartridge. The cartridge was washed with 20 ml of 5 mM ammonium sulfate adjusted to pH 9.3 with ammonia, and then 0.5 ml of distilled water. Morphine and its metabolites were eluted with 3.0 ml of a 10% acetonitrile solution in 10 mM phosphate buffer (pH 2.1). The eluate was mixed with 3.0 ml of the 0.5 M ammonium buffer and submitted to a second Sep-Pak C₁₈ cartridge in the same way as the first one. Recoveries of both glucuronides were over 95% and that of morphine was approximately 90%. A part of the eluate (10–200 µl) was submitted to high-performance liquid chromatography (HPLC) as described below for the determination of metabolites.

Enzyme Preparation — The liver was removed from each of the animals, perfused with ice-cold physiological saline and homogenized with 5 volumes of buffer A (0.25 M sucrose, 25

mM Tris-HCl buffer, 25 mM KCl, 5 mM MgCl₂; pH 7.6) by using a glass-teflon homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min and the supernatant was preserved. The pellet was resuspended in 4 volumes of buffer A and centrifuged again at 9000 *g* for 20 min. The resultant supernatant was combined with the preserved supernatant and centrifuged at 105000 *g* for 60 min. The microsomal pellet was suspended in buffer B (0.15 M KCl, 25 mM Tris-HCl buffer; pH 7.6) for washing, centrifuged again at 105000 *g* for 60 min and resuspended in the buffer B at protein concentration of 5–10 mg/ml. Protein was determined by the method of Lowry *et al.* using bovine serum albumin as a standard protein.²⁹⁾

Assay of UDPGT Activity — Incubation medium consisted of microsomes (0.5 mg protein), 100 mM Tris-HCl buffer (pH 7.4), 10 mM MgCl₂, 0.05% Brij 58, 5 mM UDP-glucuronic acid and each substrate in a final volume of 0.5 ml. Concentration of morphine, 4-nitrophenol and 4-hydroxybiphenyl were 3.0, 2.0 and 0.5 mM, respectively. UDPGT activities toward morphine 3- and 6-hydroxyl groups were determined by measuring the amount of each glucuronide being separated by HPLC. 4-Nitrophenol and 4-hydroxybiphenyl UDPGT activities were assayed spectrometrically³⁰⁾ and fluorometrically,⁷⁾ respectively.

HPLC — The instrument used was Shimadzu Model LC-5A with a ultraviolet (UV) detector, Shimadzu Model SPD-2AM. The column was a Nova-Pak C₁₈ cartridge (8 × 100 mm) in a Waters RCM-100 compression module. Samples were loaded onto the column with a Shimadzu SIL-1A injector, and eluted with a mobile phase of 10 mM phosphate buffer, pH 2.1 containing 1 mM sodium dodecyl sulfate, acetonitrile and methanol (72:23:5). The flow rate was 1.2 ml/min. The wavelength of the detector was set at 220 nm. The amounts of M-3-G and M-6-G were calculated from the standard curve made by using each reference standards.

Results

Urinary Excretion of Morphine

Figure 1 shows typical chromatograms of the

Fig. 1. High-Performance Liquid Chromatograms of Morphine Metabolites in 24 h-Urine of Mice, Rats Guinea Pigs and Rabbits

samples from 24 h urine of mice, rats, guinea pigs and rabbits injected morphine s.c., and Table I summarizes the amounts of morphine glucuronides as percentage of dose excreted in 24 h urine. M-3-G was the major metabolite in the urine of mice and rats. However, the levels of M-6-G were too low to be measured. On the other hand, guinea pigs and rabbits administered morphine excreted considerable amounts of

M-6-G together with M-3-G. Excretion ratios of M-3-G and M-6-G were approximately 4:1 and 50:1 in guinea pigs and rabbits, respectively.

UDPGT Activity toward Morphine in Liver Microsomes

Morphine UDPGT activities in liver microsomes of mice, rats, guinea pigs and rabbits were assayed by HPLC described in Materials and Methods. The HPLC profiles of M-3-G and

TABLE I. Morphine and Its Glucuronides Excreted in the 24 h Urine after the Administration of Morphine

Metabolites	Percentage of dose			
	Mice (32)	Rats (4)	Guinea pigs (5)	Rabbits (3)
Morphine	36.8 ± 4.5	19.4 ± 1.9	15.2 ± 1.1	10.7 ± 2.0
M-3-G	30.8 ± 4.3	19.1 ± 3.6	19.6 ± 1.4	50.7 ± 4.8
M-6-G	N.D.	N.D.	4.3 ± 0.3	1.1 ± 0.1

Values are the means ± S.E.M. Values in parentheses represent number of animals. N.D.: not detected.

Fig. 2. High-Performance Liquid Chromatograms of Morphine Glucuronides Formed by Incubation with Liver Microsomes of Mice, Rats, Guinea Pigs and Rabbits

M-6-G formed by incubation with liver microsomes of mice, rats, guinea pigs and rabbits are shown in Fig. 2 and the specific enzyme activities are summarized in Table II. The ratios of UDPGT activities toward 3- and 6-hydroxyl groups of morphine in liver microsomes of mice and rats guinea pigs and rabbits were approximately 300:1, 90:1, 4:1 and 40:1, respectively.

These results fairly agreed with the results of the *in vivo* species difference in glucuronide formation. However, no correlation was observed between two morphine UDPGT activities and 4-nitrophenol or 4-hydroxybiphenyl UDPGT activities in these animal species.

TABLE II. UDPGT Activities toward Morphine, 4-Nitrophenol and 4-Hydroxybiphenyl in Liver Microsomes

Substrates	UDPGT activity (nmol/min/mg protein)			
	Mice (4)	Rats (4)	Guinea pigs (3)	Rabbits (3)
Morphine (3-OH)	25.4 ± 0.7	9.2 ± 0.8	3.4 ± 0.1	10.5 ± 1.6
Morphine (6-OH)	0.085 ± 0.002	0.103 ± 0.003	0.903 ± 0.218	0.237 ± 0.019
4-Nitrophenol	58.2 ± 1.7	36.7 ± 2.2	32.8 ± 3.0	70.0 ± 10.7
4-Hydroxybiphenyl	48.7 ± 0.9	27.2 ± 0.8	73.5 ± 3.4	64.9 ± 6.3

Values are the means ± S.E.M. Values in parentheses represent number of animals.

Discussion

Although morphine is used clinically as analgesic medicine, urinary excretion of two morphine glucuronides, M-3-G and M-6-G, have not yet been studied quantitatively in various animal species. In the present paper, urinary excretion and formation of M-3-G and M-6-G in mice, rats, guinea pigs and rabbits were determined by use of HPLC. A remarkable difference of urinary excretion and site-selective glucuronidation of morphine was observed in the animals.

In the *in vivo* experiment, excretion ratios of M-3-G and M-6-G were approximately 4:1 and 50:1 in guinea pigs and rabbits, respectively, while the amount of M-6-G was too small to be measured in the urines of mice and rats. Further clean-up of the urinary extracts was not performed for the determination of low level M-6-G in the urine of mice and rats. However, in our preliminary experiment, a very low amount of M-6-G was detected as the urinary metabolite of morphine in rats by further clean-up of the urine extract. Also, the ratios of UDPGT activities toward 3- and 6-hydroxyl groups of morphine in liver microsomes of mice, rats, guinea pigs and rabbits were 300:1, 90:1, 4:1 and 40:1, respectively. Species difference in the urinary excretion of morphine glucuronides were in fair agreement with that of two morphine UDPGT activities in liver microsomes of guinea pigs and rabbits. Low glucuronidation activity at the 6-position of morphine in the liver of mice and rats agreed also with the minimal excretion under estimation limit of M-6-G. This species difference may have originated from the altered multiplicity of UDPGTs among the animal species.

Rane *et al.* reported that the ratio of M-3-G and M-6-G in the plasma of Rhesus monkey dosed intravenously with morphine is 50:1, and ratio of two glucuronides formed by incubation with liver microsomes is also similar to that of plasma.³¹⁾ In humans, Yeh *et al.* have detected a fairly high excretion of M-6-G together with M-3-G and isolated 40 mg M-6-G against 600 mg M-3-G from the urine of healthy volunteers administered morphine.³²⁾ Säwe *et al.* have reported that the plasma level of M-6-G was relatively high in cancer patients treated with a high dose

of oral morphine, and ratios of M-6-G to morphine and M-3-G to M-6-G in plasma were approximately 3 and 9, respectively.^{33,34)} These reports suggest that the analgesic effect of morphine is attributable to M-6-G.

Puig and Tephly have purified UDPGT toward morphine 3-hydroxyl group from livers of female rats pretreated with phenobarbital.¹³⁾ This isoenzyme reacted with morphine but not with 4-nitrophenol, 4-hydroxybiphenyl, testosterone, androsterone, estrone, bilirubin or 2-aminophenol. The level of this enzyme in untreated rats, however, has not yet been reported. Studies on interspecies difference of purified UDPGT isoenzymes and the substrate specificity are very limited. In the present study, a significant difference in the site-selective glucuronidation of morphine was observed in the animals. We, therefore, assayed UDPGT activities toward 4-nitrophenol and 4-hydroxybiphenyl in the four animal species concurrently with morphine UDPGT activities. However, neither activity of 4-nitrophenol nor 4-hydroxybiphenyl UDPGT activities correlated well with morphine UDPGT activities. Also, we have reported previously no correlation between UDPGT activities toward codeine and 3-hydroxyl group of morphine.³⁵⁾ Boutin *et al.* have examined UDPGT activities toward sixteen substrates in mammalian liver microsomes and reported species difference of UDPGT activities and no correlation of the UDPGT activities in these animal species.³⁶⁾ The present results support the view that multiple isoenzymes of UDPGT are concerned in the formation of these glucuronides.

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