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Metabolism of Drugs. LXIX.¹⁾ Studies on the Urinary Metabolites of Morphine in Several Mammalian Species

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In the previous study of this series, morphine was shown to be metabolized not only to morphine-3-glucuronide, but also to morphine-6-glucuronide in rabbits.³⁾ The present study have demonstrated that this is common in rabbits, guinea pigs, rats, mice and human.

As a minor metabolite of morphine, normorphine was also detected in the urine samples of these species by thin-layer chromatography. However, the excretion of this metabolite into the urine of guinea pigs given morphine was not conclusive, since the similar spot was also observed on the thin-layer chromatogram of the urine extract from untreated animals.

Quantitative estimation of major metabolites of morphine in the urine of rabbits and guinea pigs indicated that most of the dose was accounted for as conjugated morphine, but in rats morphine was excreted mostly as free morphine and in lesser amount as the conjugates in 24 hr urine.

Morphine has been used as one of the most potent analgesics for many years and its metabolic studies have been performed extensively by a number of workers using various animal species and humans. It has been thus established that morphine undergoes conjugation, N-demethylation and O-methylation to form morphine conjugates, normorphine and codeine, respectively. Among these metabolites, a major one, morphine glucuronide, has been isolated for the first time from the urine of dogs by Woods⁴⁾ and later from the urine of human addicts by Fujimoto and Way.⁵⁾ They have characterized this glucuronide to be morphine-3-glucuronide. Recently the present authors succeeded in the chemical synthesis of morphine-3- and -6-glucuronides⁶⁾ and have elucidated the structure of morphine glucuronide, which had been isolated from the urine of rabbits, to be morphine-3-glucuronide.³⁾ In addition, morphine-6-glucuronide has been demonstrated for the first time to be a minor urinary metabolite of morphine in rabbits.³⁾ Almost simultaneously with our work, Fujimoto and Haarstad have reported the isolation of morphine-3-sulfate from the urine of the chicken and cat.⁷⁾

In general, these conjugates of pharmacologically active substances have been believed to be the detoxicated metabolites. In fact, morphine-3-glucuronide, the major metabolite of morphine in several mammalian species, was reported previously to have no analgesic activity by Woods.⁴⁾ Contrary to this concept, morphine-6-glucuronide, the minor metabolite of morphine in the rabbit was recently shown to be a quite potent analgesic.⁸⁾ It, therefore,

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- 2) Location: *Katakasu, Fukuoka*.
- 3) H. Yoshimura, K. Oguri and H. Tsukamoto, *Biochem. Pharmacol.*, **18**, 279 (1969).
- 4) L.A. Woods, *J. Pharmacol. Exptl. Therap.*, **112**, 158 (1954).
- 5) J.M. Fujimoto and E.L. Way, *J. Pharmacol. Exptl. Therap.*, **121**, 340 (1957).
- 6) H. Yoshimura, K. Oguri and H. Tsukamoto, *Tetrahedron Letters*, **1968**, 483; *idem*, *Chem. Pharm. Bull. (Tokyo)*, **16**, 2114 (1968); *idem*, *ibid.*, **18**, 209 (1970).
- 7) J.M. Fujimoto and V.B. Haarstad, *J. Pharmacol. Exptl. Therap.*, **165**, 45 (1969).
- 8) O. Kamata, S. Watanabe, S. Ishii, S. Ueki, K. Oguri, S. Îda, H. Yoshimura and H. Tsukamoto, Abstract of Papers, 89th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April 1969, p. 443.

seems worthwhile to examine whether the mammals other than the rabbit also metabolize morphine to morphine-6-glucuronide as well as the 3-isomer.

In the present paper utilizing synthetic samples of two morphine monoglucuronides as reference standards, a study especially designed for identification of the conjugated metabolites of morphine in the urine of several mammalian species including a human will be described. The quantitative estimation of urinary metabolites (free and conjugates) will also be reported.

Method

Materials—Morphine hydrochloride and normorphine were obtained from commercial source with permission of the Government. Reference standards of morphine-3- and -6-glucuronides were prepared by the method previously described.⁶⁾

Administration of Morphine—Morphine hydrochloride was dissolved in H₂O and injected subcutaneously into male rabbits and male guinea pigs at a dose of 13.18 mg/kg (equivalent to 10 mg of free base /kg) for both identification and determination of urinary metabolites. The drug was injected similarly into male rats (Wistar strain) and female mice (dd strain) at a dose of 39.54 mg/kg (equivalent to 30 mg of free base /kg) for identification or 26.36 mg/kg (equivalent to 20 mg of free base /kg) for determination of the metabolites, respectively. In each metabolic cage, one rabbit, one guinea pig, two rats or ten mice were housed and the urine samples were collected for 24 hr after injection. The urine of a male patient, who was injected three doses of 10 mg of morphine hydrochloride during 23 hr, was collected for 35 hr after the first injection.

Sample Preparation for Thin-Layer Chromatography—Prior to performance of thin-layer chromatography, each urine sample was purified according to the previously reported method³⁾ which consisted of three steps: adsorption on charcoal, chromatographic separation on columns of cationic and anionic exchange resins. For example, 25 ml of the rat urine was shaken for one hr with 2 g of charcoal and then centrifuged for 3 min at 3000 rpm. The aqueous layer was washed twice with 20 ml of H₂O. Unchanged morphine and its metabolites adsorbed on charcoal were extracted 3 times with 10 ml of AcOH by shaking for 30 min.

The extract, after evaporation of AcOH *in vacuo*, was dissolved in 20 ml of H₂O, and the solution was poured onto a column of Dowex 50W-X 8 (H-form, 40 ml). After the column was washed successively with 200 ml of H₂O, 100 ml of EtOH and again 200 ml of H₂O, unchanged morphine and its metabolites were eluted with 0.15N NH₄OH collecting in 20 ml of fractions. The fractions 5—12 which were indicated to be positive to Dragendorff reagent were combined and concentrated to 20 ml. The solution was adjusted to pH 9.0 with NH₄OH and shaken 3 times with 10 ml of CHCl₃-iso-PrOH (3:1, v/v) to remove free bases. This extract, after evaporation *in vacuo*, was dissolved in a small volume of MeOH and submitted to thin-layer chromatography for examination of unconjugated metabolites.

The aqueous phase was then evaporated to dryness *in vacuo* and the residue was dissolved in 10 ml of H₂O. This solution was adjusted to pH 10.0, poured onto a column of Dowex 1-X 2 (formate form, 20 ml) and eluted with 0.01N formic acid, collecting in 10 ml of fractions. The fractions 3—5 which were shown to be positive to Dragendorff reagent were combined and evaporated the solvent *in vacuo*. The residue was dissolved in a small amount of H₂O and submitted to thin-layer chromatography for examination of conjugated metabolites. The urine samples of other species were also treated similarly as above.

Thin-Layer Chromatography—Thin-layer chromatography was carried out by use of silica gel plates (Silica gel G, Merck), 0.25 mm thick, activated at 105° for 30 min. The solvent systems employed were A: *n*-BuOH-acetone-AcOH-5% NH₄OH-H₂O (45:15:10:10:20, v/v) and B: EtOH-dioxane-benzene-conc. NH₄OH (5:40:50:5, v/v, upper layer). The metabolites were detected by spraying the plates with Dragendorff reagent when the solvent system A was used or potassium platinum iodide reagent when the solvent system B was used. The *R_f* values of authentic samples are listed in Table I.

TABLE I. *R_f* Values for Thin-Layer Chromatograms of Authentic Samples

Compounds	System A	System B
Morphine		0.30
Normorphine		0.10
Morphine-3-glucuronide	0.15	
Morphine-6-glucuronide	0.25	

solvent system A: *n*-BuOH-acetone-AcOH-5% NH₄OH-H₂O (45:15:10:10:20, v/v)
 solvent system B: EtOH-dioxane-benzene-conc. NH₄OH (5:40:50:5, v/v, upper layer)

Sample Preparation for Gas Chromatography—Each urine sample was divided into two parts. One half part was used for estimation of free morphine. To this urine was added 3.0M phosphate buffer, pH 9.0 (an equal volume to that of the urine), and 40% NaHSO₃ solution (1 ml to 50 ml of urine), and the solution was extracted continuously for 3 hr with CHCl₃.

Another half of the urine was used for estimation of conjugated morphine. After the urine was made 15% HCl concentration by adding appropriate volume of conc. HCl, and to this was added 40% NaHSO₃ solution (1 ml to 50 ml of urine), it was heated on a boiling water bath for 30 min to hydrolyze conjugated metabolites. The recovery of morphine from two morphine mono-glucuronides was confirmed to be nearly quantitative.^{3,9)} The liberated morphine was extracted with CHCl₃ similarly as described above.

The solvent of the extracts of either unhydrolyzed or hydrolyzed urine sample was evaporated *in vacuo* after drying over anhyd. Na₂SO₄, and the residues were dissolved in 1 ml (the rabbit) or 0.5 ml (the guinea pig and rat) of MeOH and submitted to gas chromatographic analysis.

Gas Chromatography—The instrument used was a Shimadzu Model GC-1C Gas Chromatograph equipped with hydrogen flame ionization detector (dual column and differential flame type). The column was a glass U-shaped tube (4 mm × 2.625 m). The column packing was 1.5% OV-1 (the rabbit and rat urines) or 1.5% OV-17 (the guinea pig urine) on Shimalite W (80—100 mesh), which was pretreated with hexamethyldisilazane. The column of 1.5% OV-1 could also be used for the guinea pig urine. The column temperature was maintained at 230°, the sample chamber temperature at 250° and the detector cell temperature at 235°. Nitrogen was used as a carrier gas with a flow rate of 30 ml/min. A MeOH solution of the urine extracts was injected with a Hamilton microsyringe. The usual sample size was 2 μl (the rabbit and guinea pig urine) or 4 μl (the rat urine) corresponding to 3 to 5 μg of morphine. The retention time of morphine was about 3.7 min (OV-1) or 8.8 min (OV-17). The amount of morphine in the samples was calculated from the standard curve by measuring the peak height. The standard curve was made by running through the same extraction procedure using authentic morphine.

Result

Identification of Conjugated Metabolites

A typical thin-layer chromatogram of the purified urine samples obtained from several species (the solvent system A was used) is shown in Fig. 1.

There were demonstrated two Dragendorff positive spots in all of the samples; one spot located around *R_f* 0.15 showed the same mobility as that of morphine-3-glucuronide, and the other, a faint spot located around *R_f* 0.25 was corresponded to morphine-6-glucuronide. Only the latter spot was visualized as a blue color by spraying with Folin-Ciocalteu reagent, suggesting that this metabolite should possess unblocked phenol group.

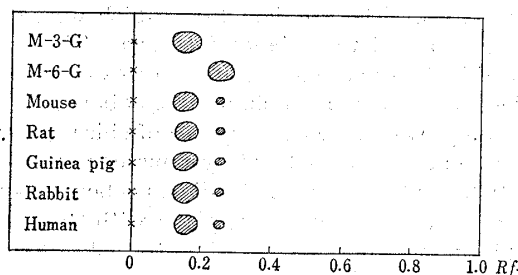


Fig. 1. Thin-Layer Chromatogram of Urinary Conjugated Metabolites of Morphine in Several Mammalian Species

The solvent system of *n*-BuOH-acetone-AcOH-5% NH₄OH-H₂O (45:15:10:10:20) was used, and spots were visualized by Dragendorff reagent.

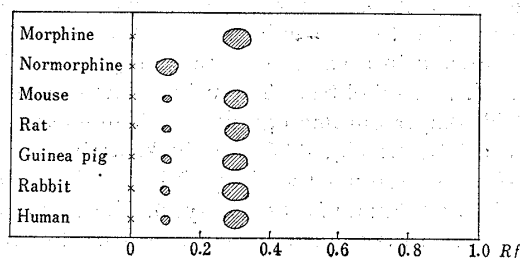


Fig. 2. Thin-Layer Chromatogram of Urinary Unconjugated Metabolites in Several Species

The solvent system of EtOH-dioxane-benzene-conc. NH₄OH (5:40:50:5) was used, and spots were visualized by K₂PtI₆ reagent.

In order to confirm that above two metabolites were both glucuronide, the purified urine sample from each species was spotted in a line on two silica gel plates (20 × 20 cm) and then developed with the solvent system A. After visualizing one side of the each plate with Dra-

9) H. Yoshimura, K. Oguri and H. Tsukamoto, *Chem. Pharm. Bull.* (Tokyo), **14**, 62 (1966).

gendorff reagent, the bands corresponding to morphine-3- and -6-glucuronides were separately scraped off of the remaining side into centrifuge tubes, and the metabolites on silica gel were extracted 3 times with 10 ml of H₂O. Both metabolites were shown to be positive to a naphthoresorcinol test.

Identification of Unconjugated Metabolites

Using the organic solvent extracts obtained in sample preparation for thin-layer chromatography or the continuous chloroform extracts of unhydrolyzed urines for gas chromatographic analyses, unconjugated metabolites were examined by thin-layer chromatography with the solvent system B. A typical chromatogram is shown in Fig. 2.

It seems likely from this chromatogram that all of the species examined excrete a small amount of normorphine (*Rf* 0.1) as well as unchanged morphine (*Rf* 0.3). In guinea pigs, however, a control sample (extract of the urine excreted before medication) showed also a Dragendorff positive spot, *Rf* value of which was same to that of normorphine, and in a human, the control urine was not obtained. Therefore, excretion of normorphine in these two species was not conclusive. Although free morphine in above extracts was also identified definitely by gas chromatography, further confirmation of normorphine by gas chromatography was without success, since the sensitivity of our gas chromatographic procedure was too low to detect a very small amount of normorphine excreted into the urine.

Quantitative Estimation of Major Metabolites

Representative results of gas chromatographic estimation of two major metabolites of morphine, excreted into 24 hr urines of rabbits, guinea pigs and rats injected morphine are summarized in Table II. In this Table, the amount of conjugated morphine were calculated from the difference between the amount of morphine in hydrolyzed samples and those in non-hydrolyzed one. Since excretion of morphine-6-glucuronide was much less than that the 3-isomer, as judged from the thin-layer chromatography, and also since conjugated metabolites excreted into urine consisted only of two morphine mono-glucuronides, the amount of conjugated morphine actually indicate those of morphine-3-glucuronide. The quantitative estimation of a minor metabolite, normorphine, was omitted in the present study because of its low sensitivity to this gas chromatographic condition.

TABLE II. Excretion Percentages of Metabolites of Morphine into 24 Hr Urines of Several Mammalian Species

	Dose (mg/kg)	Number of animals used	Unchanged morphine (%)	Mean value	Conjugated morphine (%)	Mean value	Total recovery (%)	Mean value
Rabbit	10	1	9.3		58.4		67.7	
		1	7.4	7.6	61.5	60.7	68.9	68.4
		1	6.3		62.3		68.6	
Guinea pig	10	1	10.9		26.3		37.2	
		1	8.2	9.9	34.1	31.6	42.5	41.5
		1	10.8		34.3		45.1	
Rat	20	2	19.9		3.1		23.0	
		2	18.9	19.5	4.0	3.5	22.9	23.0
		2	19.7		3.4		23.1	

As can be seen in Table II, the major fraction of the dose was accounted for as conjugated morphine in rabbits and guinea pigs, whereas in rats a large parts of the injected morphine was excreted as free morphine, but not as conjugates. In addition, it should be noticed that

the recovery in 24 hr urine was especially low in rats. This suggested that the biliary excretion might participate an important role in the metabolism of morphine in rats.

Discussion

It has been suggested for a long time that conjugation with glucuronic acid would be a major pathway for the detoxication of morphine. Convincing evidence leading to this conclusion was furnished by Woods⁴⁾ who was the first to report the isolation of conjugated morphine in crystalline form from the urine and bile of dogs. Subsequently, the identical conjugate was isolated from the urine of human addicts by Fujimoto and Way.⁵⁾ This conjugated morphine, although isolated in a limited amount, was characterized to be morphine-3-glucuronide possessing a zwitterion structure from its physical and chemical properties.^{4,5)} The pharmacological examination on this metabolite showed no analgesic activity.⁴⁾ Recently Oka¹⁰⁾ reported also the isolation of morphine-3-glucuronide in a better yield from the urine of dogs. Since then, however, no one has still answered to the question whether there are more than one conjugated forms of morphine.

Quite recently the present authors³⁾ isolated pure, crystalline morphine-3-glucuronide in an excellent recovery from the urine of rabbits given morphine developing a new purification method. The structure of this metabolite was unequivocally proved comparing the metabolite with authentic sample which was synthesized chemically. At the same time, it was demonstrated that a small amount of morphine-6-glucuronide was excreted along with the major metabolite, morphine-3-glucuronide, into the urine of rabbits. Almost simultaneously with this work, Fujimoto and Haarstad isolated morphine-3-sulfate as a major metabolite of morphine from the urine of the chicken and cat.⁷⁾

To these findings that a major site of conjugation in morphine molecule was a phenolic group on C₃, the present study using several species including human added further knowledge that alcoholic group on C₆ was a minor site of conjugation. Excretion ratio of morphine-6-glucuronide to the 3-isomer in rabbits was about one hundredth assuming from the amount isolated from 24 hr urine.³⁾ On the basis of thin-layer chromatographic results, it was also suggested that this ratio in guinea pigs and human were nearly same as that in rabbits, but smaller in rats and mice (see Fig. 1). Although morphine-6-glucuronide was shown to possess a potent analgesic activity,⁸⁾ it does not seem to contribute in any extent to the action of morphine *in vivo*, considering from the amount excreted into the urine.

There have been numerous studies concerning with renal excretion of morphine made on various species.¹¹⁾ The results have shown that most of a given dose of morphine is excreted in the urine as conjugates with certain quantitative differences. As indicated in Table II, the present result on estimation of free and conjugated morphine in the urine of rabbits and guinea pigs also coincided with these results. Rats, however, excreted more free morphine than the conjugates and indicated especially low recovery (about a quarter of the dose) in 24 hr urine. This discrepancy seems likely to due to the biliary excretion of the conjugates. Abou-El-Makarem, *et al.* has reported that the rat is the good biliary excretor of the high molecular compound.¹²⁾

Liver microsomes prepared from rabbits, guinea pigs, rats¹³⁾ and mice¹⁴⁾ have been known to be capable of catalyzing N-demethylation of morphine and there has been chromatographic

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13) J. Axelrod, *J. Pharmacol. Exptl. Therap.*, **117**, 322 (1956).

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evidence suggesting normorphine formation *in vivo* in rats.¹⁵⁾ The present study suggested also the excretion of this demethylated metabolite in the urine of rabbits, rats and mice given morphine by thin-layer chromatography. This metabolite, however, could not be detected by gas chromatography. Therefore, an assessment of this finding must await further studies.

Acknowledgement The authors wish to thank Miss E. Hasegawa for her excellent technical assistance in this research.

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