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Metabolism of Drugs. LXXII.[1] Synthesis of Nalorphine-3- and -6-glucuronide and Identification of Urinary Metabolites of Nalorphine in Rabbits

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Nalorphine-3- and -6-glucuronide were synthesized according to the method used in the synthesis of morphine glucuronides.[3a,b] These glucuronides were then utilized as reference standards for identification of the urinary metabolites of nalorphine in rabbits, and nalorphine-3-glucuronide was established as a major metabolite. No other metabolite could be detected in the 24 hr urine samples, except a small amount of unchanged nalorphine was detected. Isolation procedure of the urinary glucuronides adopted in the present and previous[4a,b] studies was confirmed to be very effective especially for the morphine alkaloids.

Nalorphine, the potent antagonist of morphine, is a derivative of morphine in which N-methyl group has been replaced by allyl group. Woods and Muehlenbeck[5] reported in 1957 that a major metabolic pathway of this drug in the dog and rat was the conjugation. Quite recently Fujimoto, *et al.*[6] isolated crystalline glucuronide from the urine of rabbits dosed nalorphine and characterized it to be nalorphine-3-glucuronide. Since then, however, this metabolite has not yet been synthesized. In the previous papers[3a,b] we reported the synthesis of morphine-3- and -6-glucuronide together with the synthesis of codeine glucuronide. These glucuronides were subsequently utilized as reference standards for identification of the urinary metabolites of morphine and codeine in several mammalian species.[4a,b] It is also noticeable that morphine-6-glucuronide was found to exert a potent analgesic activity in the rat and mouse.[7]

The present paper deals with the chemical synthesis of nalorphine-3- and -6-glucuronide and the identification of the urinary metabolites of nalorphine in rabbits applying the method similar to that described in the previous papers.[3,4]

Materials and Methods[8]

Synthesis of Nalorphin-6-yl-β-D-glucopyranosiduronic Acid (V)——A reaction scheme is shown in Chart 1 and detailed procedure in each step is described below.

1) 3-Acetylnalorphine (I): Nalorphine (1.1 g) was suspended in 100 ml of 10% $NaHCO_3$ solution, and to this suspension was added 5.5 ml of Ac_2O. At the end of evolution of CO_2 gas the mixture was poured

1) Part LXXI: H. Yoshimura, H. Shimeno and H. Tsukamoto, *Yakugaku Zasshi*, **90**, 1406 (1970).
2) Location: *Katakasu, Fukuoka.*
3) *a*) H. Yoshimura, K. Oguri and H. Tsukamoto, *Tetrahedron Letters*, **1968**, 483; *Idem*, *Chem. Pharm. Bull.* (Tokyo), **16**, 2114 (1968); *b*) *Idem*, *ibid.*, **18**, 209 (1970).
4) *a*) H. Yoshimura, K. Oguri and H. Tsukamoto, *Biochem. Pharmacol.*, **17**, 279 (1969); K. Oguri, S. Îda, H. Yoshimura and H. Tsukamoto, *Chem. Pharm. Bull.* (Tokyo), **18**, 2414 (1970); *b*) H. Yoshimura, M. Mori, K. Oguri and H. Tsukamoto, *Biochem. Pharmacol.*, **19**, 2353 (1970).
5) L.A. Woods and H.E. Muehlenbeck, *J. Pharmacol. Exptl. Therap.*, **120**, 52 (1957).
6) J.M. Fujimoto, W.M. Watrous and V.B. Haarstad, *Proc. Soc. Exptl. Biol. Med.*, **130**, 546 (1969).
7) O. Kamata, K. Shimomura, S. Ueki, S. Îda, K. Oguri, H. Yoshimura and H. Tsukamoto, *Tohoku J. Exptl. Med.*, submitted.
8) All melting points were uncorrected. Ultraviolet (UV), infrared (IR) and mass spectra were recorded with a Shimazu Model SV-50A, JASCO Model DS-301 and Japan Electron Optics Model JMS-O1SG, respectively.

onto ice–water and extracted with $CHCl_3$. The $CHCl_3$ layer was dried over anhyd. Na_2SO_4, and the solvent was evaporated to dryness to give 1.2 g of amorphous 3-acetylnalorphine (I). UV λ_{max}^{EtOH} 283 mμ (log ε: 3.28). No bathochromic shift occurred in the UV spectra with a change from acidic to basic condition. Neither 6-acetyl nor 3,6-diacetylnalorphine did produce in this acetylation process.

2) Methyl(3-acetylnalorphin-6-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (III): Amorphous 3-acetylnalorphine (1.1 g) was dissolved in 250 ml of dry benzene in a three necked flask which was protected from the moisture. To this boiling solution, a solution of 5.0 g of methyl 2,3,4-tri-O-acetyl-1α-bromo-1-deoxy-D-glucopyranuronate (II) in 100 ml of dry benzene and 3.0 g of freshly prepared dry Ag_2CO_3 were added little by little during about 25 hr. In the meantime, benzene was distilled off gradually, and stirring was continued. The formation of the reaction product (III) and disappearance of acetylnalorphine were occasionally examined by thin-layer chromatography (TLC), using $CHCl_3$–EtOH (4:1) as the solvent system and potassium platinum iodide reagent for colorlation. The solid in the reaction mixture was filtered off after 3-acetylnalorphine disappeared mostly and washed with benzene. The filtrate and washings were combined, and it was shaken with water to remove polar impurities. Benzene was then evaporated to dryness, and the residue was further dried over P_2O_5 under reduced pressure. To this dry residue was added 3 ml each of pyridine and Ac_2O, and the solution was allowed to stand for 6 hr at room temperature. It was then poured onto ice–water, and the mixture was extracted 3 times with $CHCl_3$. The $CHCl_3$ extracts were combined, concentrated to about 100 ml and extracted 3 times with 100 ml of ice-cooled 0.5% HCl solution. The combined acid layers were adjusted to pH 8.0 with $NaHCO_3$ and extracted 3 times with 200 ml of $CHCl_3$. The $CHCl_3$ layers were dried over anhyd. Na_2SO_4 and evaporated to dryness *in vacuo*. The resulting viscous residue was crystallized from EtOH and recrystallized from the same solvent to give colorless prisms, mp 181—183°. The yield was 1.0 g. *Anal.* Calcd. for $C_{34}H_{39}O_{13}N$: C, 60.98; H, 5.87; N, 2.09. Found: C, 61.06; H, 6.05; N, 2.17. UV λ_{max}^{EtOH} 287 mμ (log ε=3.27). IR ν_{max}^{KBr} cm^{-1}: 1770, 1754 (ester).

3) Nalorphin-6-yl-β-D-glucopyranosiduronic Acid (V): To 1.0 g of (III) which was suspended in 10 ml of abs. MeOH was added 4.5 ml of 1% solution of NaOMe in abs. MeOH. After allowing to stand over night, the reaction mixture was evaporated to dryness *in vacuo*. The residue (IV) was dissolved in 5.0 ml of 0.43N $Ba(OH)_2$ and left aside for 2 hr. The barium salt which precipitated on cooling was dissolved in 10 ml of H_2O and adjusted to pH 6.0 with 2N oxalic acid. The solution was kept in a refrigerator for 1 hr and barium oxalate precipitated was filtered off. The filtrate was evaporated to dryness *in vacuo*, and the residue was crystallized from water to colorless needles (crude V). This crude product was dissolved in 50 ml of water and passed through a column of 50 ml of Dowex 50WX4 (H-form). The resin was washed with 500 ml of water, and the glucuronide was then eluted with 0.15N NH_4OH, collecting in about 30 ml fractions. This chromatography was carried out in a cold room (3°). Combined fractions, possitive to Dragendorff reagent, were concentrated to about 20 ml, and a trace of basic impurities in this concentarte was extracted 3 times with $CHCl_3$–iso-PrOH (3:1). The aqueous layer was evaporated to dryness, and the residue was recrystallized from water to give 0.66 g of colorless needles (pure V), mp 249—252° (decomp.). *Anal.* Calcd. for $C_{25}H_{29}O_9N\cdot H_2O$: C, 59.40; H, 6.18; N, 2.76. Found: C, 59.41; H, 6.32; N, 2.87. UV $\lambda_{max}^{H_2O}$ 286 mμ (log ε=3.17); $\lambda_{max}^{0.1N\ NaOH}$: 299 m$\mu$ (log ε=3.40). IR ν_{max}^{KBr} cm^{-1}: 3470 (OH), 1610 (C=O). $[\alpha]_D^9$ −195° (c=0.5 in H_2O).

Synthesis of Nalorphin-3-yl-β-D-glucopyranosiduronic Acid (VIII)——A reaction scheme is shown in Chart 2. To a solution of 1.0 g of nalorphine (VI) in 3.0 ml of 2N NaOH was added a solution of 2.0 g of (II) in 6 ml of acetone, and the mixture was allowed to stand over night. It formed two layers and precipitated a considerable amount of unchanged nalorphine. With addition of 1 ml of 30% NaOH, this nalorphine was again dissolved under stirring. To this, a solution of 0.6 g of II in 2 ml of acetone was added and the mixture was allowed to stand over night. Such a procedure was further repeated 9 times more (a total of 8.0 g of II was used against 1.0 g of nalorphine). The reaction mixture was adjusted to pH 9.0 with AcOH and extracted 3 times with 50 ml of $CHCl_3$–iso-PrOH (3:1) to remove unchanged nalorphine. The aqueous layer was then diluted to 100 ml with water and passed through a column of 100 ml of Dowex 50WX4 (H-form). It was treated similarly to the purification of V. The fractions, possitive to Dragendorff reagent, were evaporated to dryness *in vacuo*. The residue was dissolved in a small amount of water and passed through a column of 50 ml of Dowex 1X2 (formate form). After the resin was washed with 500 ml of water, the glucuronide was eluted with 0.1N HCOOH, collecting in about 30 ml fractions. The fractions, possitive to Dragendorff reagent, were combined, and the solvent was evaporated to complete dryness *in vacuo*. The residue was recrystallized from hot water to give 50 mg of colorless needles (VIII), mp 248—251° (decomp.). *Anal.* Calcd. for $C_{25}H_{29}O_9N\cdot 3H_2O$: C, 55.44; H, 6.51; N, 2.59. Found: C, 55.75; H, 6.37; N, 2.57. IR ν_{max}^{KBr} cm^{-1}: 3360 (OH), 1632 (C=C), 1580 (COO$^-$). $[\alpha]_D^9$ −145° (c=0.1 in H_2O).

Thin-Layer Chromatography——It was carried out by use of silica gel plates, 0.25 mm thick (Silica gel G, Merck), which were activated at 105° for 30 min, unless otherwise mentioned. The solvent systems used were A) $CHCl_3$–MeOH (4:1), B) $CHCl_3$–MeOH–H_2O (65:35:10, lower layer) and C) n-BuOH–acetone–AcOH–5%NH_4OH–H_2O (45:15:10:10:20). Nalorphine and its derivatives on the chromatograms were visualized by spraying either potassium platinum iodide reagent (solvent system A) or Dragendorff reagent

(solvent system B and C). The *Rf* values of nalorphine and its derivatives in the three solvent systems are summarized in Table I.

Table I. *Rf* Values of Thin-Layer Chromatography of Nalorphine, Nalorphine Glucuronide and Their Derivatives

Compound	Solvent system A	B	C
Nalorphine (VI)	0.57	—	—
3-Acetyl nalorphine (I)	0.73	—	—
Methyl acetyl derivative of nalorphine-6-glucuronide (III)	—	0.91	—
Methyl acetyl derivative of nalorphine-3-glucuronide (VII)	—	0.87	—
Methyl derivative of nalorphine-6-glucuronide (IV)	—	0.50	—
Nalorphine-6-glucuronide (V)	—	0.15	0.34
Nalorphine-3-glucuronide (VIII)	—	0.10	0.25

solvent system: A, $CHCl_3$–EtOH (4:1); B, $CHCl_3$–MeOH–H_2O (65:35:10, lower layer); C, *n*-BuOH–acetone–AcOH–5% NH_4OH–H_2O (45:15:10:10:20)

Hydrolysis of Nalorphine Glucuronides with β-Glucuronidase——Each of two nalorphine glucuronides (1.3 μmole) was incubated at 37° for 24 hr in 3.0 ml of 0.2M acetate buffer (pH 5.0) with bovine β-glucuronidase (1 ml, the activity was estimated to be 7000 *p*-nitrophenylglucuronide units/1 ml).

Sample Preparation for Gas Chromatography——After hydrolysis with β-glucuronidase, the incubation mixture was adjusted to pH 9.0 with dil. NH_4OH, and nalorphine liberated was extracted 3 times with 5.0 ml of $CHCl_3$–iso-PrOH (3:1) by shaking for 3 min. The combined extracts were dried over anhyd. Na_2SO_4, and the solvent was evaporated to dryness *in vacuo*. The residue was dissolved in 2.0 ml of MeOH, which was divided into two portions and each half was used for gas chromatographic and mass spectral analyses, respectively.

Sample Preparation for Mass Spectral Measurement——One half of above MeOH solution (1.0 ml) was spotted in a line on a TLC (0.5 mm thick), authentic nalorphine being spotted on its end and developed with the solvent system A. After visualizing only the authentic nalorphine with potassium platinum iodide reagent, the band corresponding to nalorphine was scraped off of remaining side into a centrifuge tube, and the material was extracted with $CHCl_3$–iso-PrOH (3:1). From this extract the solvent was evaporated to complete dryness *in vacuo*. The residue was submitted to mass spectral measurement.

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 on Shimalite W (80—100 mesh). The column temperature was maintained at 230°, the sample chamber temperature at 250° and the detector temperature at 240°. Nitrogen was used as the carrier gas with a flow rate of 30 ml/min (2.5 kg/cm²). The amount of nalorphine in the samples was calculated from the standard curve by measuirng the peak height. The standard curve was made using authentic nalorphine by running through the extraction procedure same to that described above.

Administration of Nalorphine——Nalorphine hydrochloride was dissolved in water and was injected subcutaneously into male albino rabbits weighing 2.9 and 3.1 kg in a dose of 20 mg/kg as free base, so that a total of 120 mg of nalorphine base was injected. The urines excreted within 24 hr were collected, centrifuged and submitted to the purification procedure described below.

Purification of Nalorphine Glucuronide in Urine——The method used for this purification was essentially same to that described for isolation of morphine glucuronides.[4a,b] The urine sample (about 1 liter) was mixed with 70 g of charcoal and the mixture was stirred for 1 hr and then centrifuged. The aqueous layer was discarded and the charcoal was washed twice with 1 liter of water. Nalorphine and its metabolites adsorbed on charcoal was then extracted 3 times with 300 ml of AcOH by stirring for 30 min. After centrifugation the extract was pipetted out and filtered through filter paper to remove the finer charcoal particles. The solvent was evaporated to dryness *in vacuo* from above filtrate. The resulting residue was dissolved in a small amount of water and passed through a column of 150 ml of Dowex 50 WX4 (H-form). The column was washed successively with 1 liter of water, 500 ml of EtOH and 2 liters of water. The metabolites retained on the resin were eluted with 0.15N NH_4OH, collecting in 50 ml fractions. The chromatography was performed in a cold room (3°).

A considerably large amount of metabolite which gave a *Rf* value corresponding to synthetic nalorphine-3-glucuronide was eluted into fraction 8 and neighboring fractions, together with a small amount of unchanged nalorphine (see Fig. 1). The residues obtained after evaporation of the solvent from fraction 5—12 were combined, and it was dissolved in 70 ml of water. The solution was adjusted to pH 9.0 with dil. NH_4OH

and shaken 3 times with 150 ml of $CHCl_3$–iso-PrOH (3:1) to remove free nalorphine. The aqueous phase was evaporated to dryness *in vacuo*, and the resulting gum was dissolved in 80 ml of water. The solution was passed through a column of 80 ml of Dowex 1X2 (formate form) which was then washed with 1 liter of water. The metabolites were eluted with 0.2N formic acid, collecting into 50 ml fractions. Fractions 1—6, which were shown chromatographically to involve only one metabolite corresponding to nalorphine-3-glucuronide, were combined, and the solvent was evaporated to complete dryness *in vacuo*. Fine needles crystallized out from the residue were filtered and recrystallized from hot water to give 26 mg of colorless needles, mp 246—249° (decomp.).

Result

Synthesis of Nalorphine-6-glucuronide (V)

Chemical synthesis of nalorphine-6-glucuronide (V) was accomplished by using the same reaction scheme as was used for synthesis of morphine-6-glucuronide[3a] (see Chart 1).

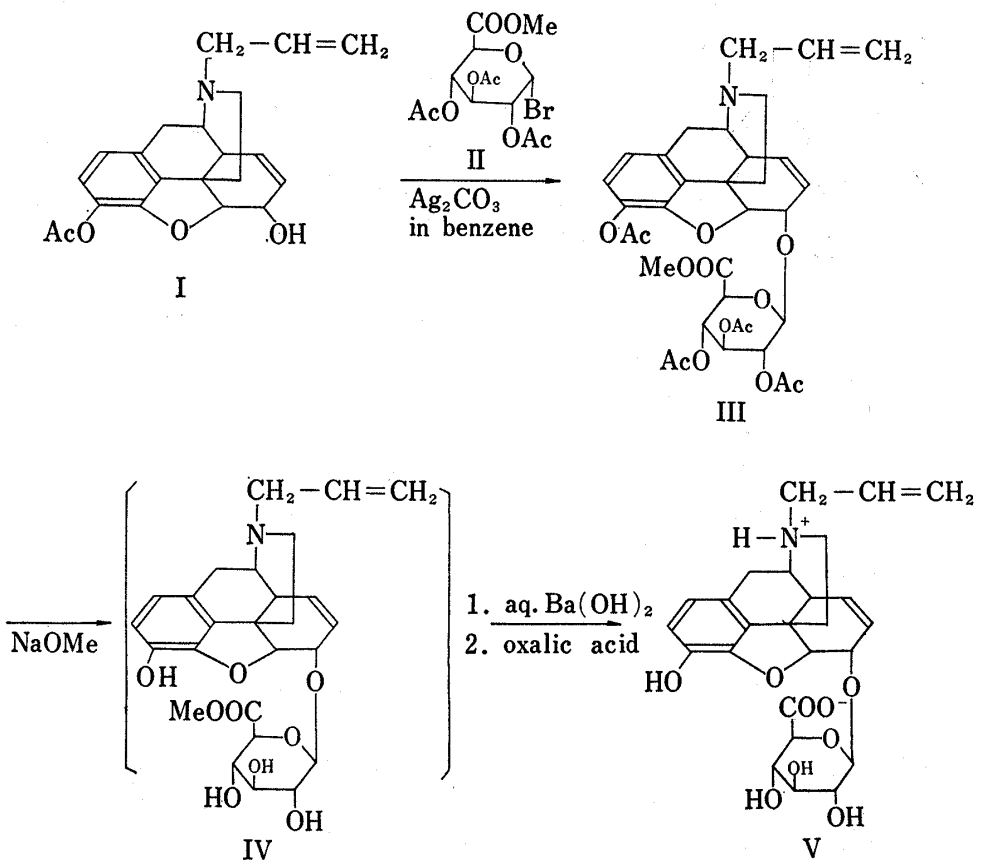

Chart 1

The starting material, 3-acetylnalorphine (I), could be prepared quantitatively by selective acetylation of nalorphine as well as 3-acetylmorphine. However, the condensation reaction of 3-acetylnalorphine with acetobromo derivative of glucuronic acid (II) proceeded more slowly than that of 3-acetylmorphine and consequently produced a considerable amount of by–products which prevent a desired product (III) fromcrystallizing. A major component of above by–products seemed to be a partially deacetylated derivative of acetobromo glucuronate (II) by TLC examination. Therefore prior to separating (III) from the by–product, the latter compound was converted to a fully acetylated compound which should not be extracted with hydrochloric acid solution in the subsequent separation process. The crystalline compound thus obtained was shown to have the proper structure of methyl acetyl derivative of nalorphine-6-glucuronide by the elemental analysis, and UV and IR absorption spectra.

Solvolysis of III with a catalytic amount of sodium methoxide afforded IV which was, without any purification, converted to V by alkaline hydrolysis. Elemental analysis of this compound was in a good accordance with monohydrate of nalorphine-6-glucuronide. Since a marked bathochromic shift was observed in the UV absorption spectra with a change from acidic to basic condition, compound V should possess an unblocked phenol group. The IR absorption spectrum showed a strong peak at 1610 cm^{-1} and a broad absorption band between 2800 and 2400 cm^{-1}, suggesting that it occurred as internal salt, same as morphine and codeine glucuronides.[3a,b] β-Configuration of the glucuronide linkage was supported by the optical rotation and also the fact that it was hydrolyzable with β-glucuronidase.

Synthesis of Nalorphine-3-glucuronide (VIII)

The method used for the synthesis of nalorphine-3-glucuronide (VIII) was essentially same as that of morphine-3-glucuronide which was reported previously.[3a]

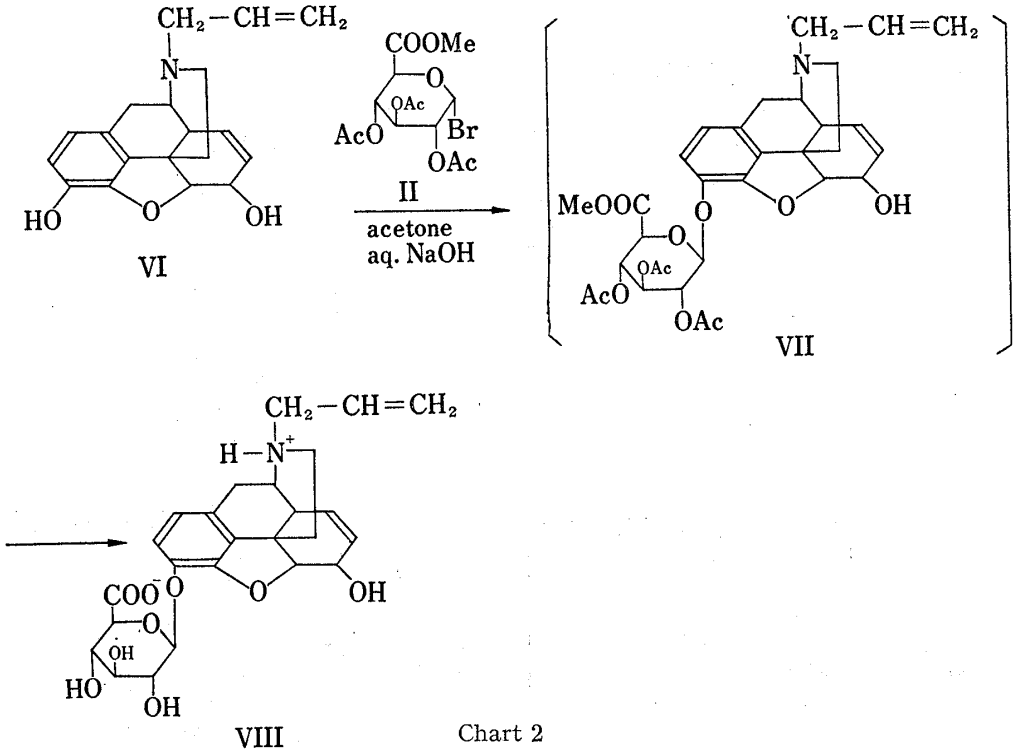

Chart 2

As shown in chart 2, acetobromo derivative of glucuronic acid (II) was selectively attacked by phenolic anion of nalorphine in the presence of sodium hydroxide, producing only nalorphine-3-glucuronide. Although the reaction proceeded very slowly and the yield was very poor, the process consisted of only one step.

The compound VIII showed the analogeous UV absorption spectrum as that of 3-acetylnalorphine and no bathochromic shift with a change from acidic to basic medium, indicating that it should be a phenolic glucuronide. The IR absorption spectrum revealed two strong bands at 1632 and 1580 cm^{-1} which were probably attributable to double bonds of aryl and allyl groups, and to carboxylic ion, respectively. As described below, this spectrum was completely identical with that of the glucuronide isolated from the urine of rabbits. Treatment of VIII with β-glucuronidase resulted in a stoichiometric formation of nalorphine. In addition, the data of elemental analysis and optical rotation lead to the conclusion that compound VIII must be nalrophin-3-yl-β-D-glucopyranosiduronic acid possessing zwitterionic structure.

Hydrolysis of Nalorphine Glucuronides with β-Glucuronidase

Two isomeric glucuronides of nalorphine described above were treated with bovine β-glucuronidase in acetate buffer (pH 5.0) at 37° for 24 hr, and liberated nalorphine was estimated by

gas chromatography. The result showed that both glucuronides were susceptible to hydrolysis with β-glucuronidase. The reaction rates, were however, different each other, being faster in 3-glucuronide than in 6-isomer. During 24 hr incubation, nalorphine liberated from 3-glucuronide was accounted for 95.3 per cent of theoretical yield, whereas that from 6-isomer was accounted for **70**.4 per cent. Similar relation between hydrolysis rate and glucuronide structure was also observed in the experiment on isomeric glucuronides of morphine.[3a)]

Nalorphine liberated from two glucuronides of nalorphine was identified not only by TLC, but also by mass spectral measurement. Samples for mass spectra were prepared by preparative TLC of the extracts from hydrolyzate of two glucuronides, and authentic nalorphine was also submitted to the same process. All of three samples showed the identical spectra possessing molecular ion peak at *m/e* 311.

Identification of Nalorphine Glucuronide isolated from the Urine of Rabbits

During the purification process of the urinary metabolites, only one spot was detected chromatographically except a much smaller spot corresponding to unchanged nalorphine. A typical chromatogram is shown in Fig. 1.

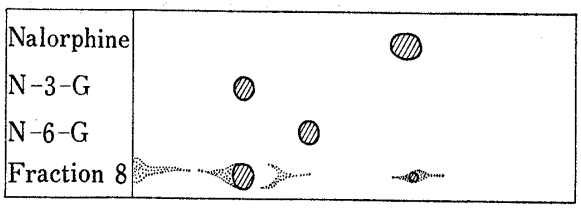

Fig. 1. The Thin-Layer Chromatogram of Fraction 8 obtained by Column Chromatography on Cationic Exchange Resin

N-3-G, nalorphine-3-glucuronide; N-6-G, nalorphine-6-glucuronide; Solvent system C was used.

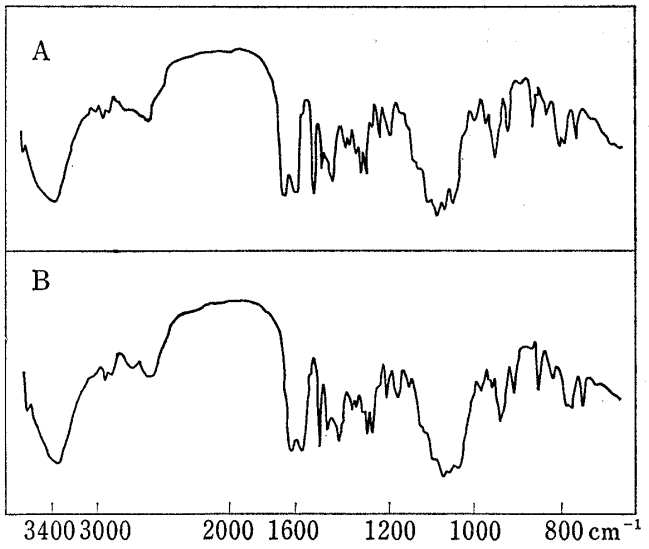

Fig. 2. Infrared Absorption Spectra of Nalorphine-3-glucuronide (KBr)

A: synthetic sample; B: biological sample

This metabolite was isolated as a pure, crystalline compound, mp 246—249° (decomp.), which showed the same mobility with synthetic nalorphine-3-glucuronide on TLC. The UV absorption spectrum ($\lambda_{max}^{H_2O}$ 284 mμ, log ε=3.17) and optical rotation ($[\alpha]_D^9$ −145°, *c*=0.1 in H_2O) were in a good agreement with those of synthetic sample. In addition, as can be seen in Fig. 2, the IR absorption spectra of both samples from synthetic and biological indicated that those were completely identical.

Possible occurrence of other conjugates, including nalorphine-6-glucuronide was carefully examined, but without success.

Discussion

The present study has established nalorphine-3-glucuronide as a major metabolite in the rabbit, and in addition, has demonstrated the general value of the isolation procedure of the urinary glucuronides. Fujimoto, *et al.* isolated for the first time nalorphine glucuronide in a crystalline form from the urine of rabbits, using Amberlite XAD-2 resin column and provided evidence that this metabolite should be nalorphine-3-glucuronide. The yield of the crystalline glucuronide, however, seemed very poor, and the purity of this material seemed also unsatisfactory, as they mentioned.[6)] The IR absorption spectrum presented in their paper[6)] was not

identical with ours although the renewed spectrum appeared in the subsequent paper[9] was almost same. The present study using anionic and cationic ion exchange resins for purification of the glucuronide afforded better yield of the metabolite with satisfactory purity. Finally by the synthesis of authentic sample in the present study Fujimoto's conclusion was reconfirmed.

In the previous papers,[4a,b] the authors reported that after subcutaneous injection of morphine, the rabbit, guinea pig, rat, mouse and man excreted a small amount of morphine-6-glucuronide along with a major metabolite, morphine-3-glucuronide. Analogous result was expected before the experiment, however, no metabolite other than nalorphine-3-glucuronide could be detected in the urine sample, except a small amount of unchanged nalorphine was confirmed.

Contrary to the generally accepted hypothesis that the glucuronide is a detoxicated metabolite, Kamata, *et al.*[7] recently found that morphine-6-glucuronide is a potent analgesic. The present success in the synthesis of nalorphine-6-glucuronide makes possible to examine its pharmacological effect.

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9) J.M. Fujimoto, *J. Pharmacol. Exptl. Therap.*, **168**, 180 (1969).