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METABOLISM OF DRUGS—LXII

ISOLATION AND IDENTIFICATION OF MORPHINE GLUCURONIDES IN URINE AND BILE OF RABBITS*

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Abstract—The isolation and identification of two morphine glucuronides in urine and bile of rabbits were studied. Charcoal adsorption and ion-exchange column chromatography with Dowex 50W-X8 and Dowex 1-X2 were effectively employed for the concentration and purification of the glucuronides. The major urinary metabolite was thus isolated in a crystalline form and was identified as morphine 3-glucuronide by comparison with a synthetic sample. It was also found by means of TLC that the same glucuronide was excreted in the bile as a major metabolite. Morphine 6-glucuronide could not be detected in either unfractionated urine or bile samples; however, after repeated purification of urinary extracts, this glucuronide was demonstrated for the first time to be a minor metabolite of morphine.

THE METABOLISM of morphine has been of particular interest pharmacologically and has been investigated extensively by many workers. However, among the various metabolites (conjugates, normorphine and codeine) known to date, only one metabolite has been isolated in a crystalline form. This isolation was first accomplished by Woods[1] from the urine and bile of a dog which had received morphine and later from the urine of a human addict by Fujimoto and Way.[2] The structure of this metabolite was demonstrated to be a phenolic monoglucuronide of morphine. Although this conjugate was supposed to be the major metabolite, the amounts isolated were rather small. The presence of other conjugates has also been postulated by several workers,[1–3] but conclusive evidence has not been obtained in any case except that of morphine 3-glucuronide mentioned above.

The present authors reported recently the first synthesis of codeine and morphine glucuronides.[4,5] In the present paper, utilizing these synthetic samples as reference standards, a study especially designed for the isolation and identification of the major metabolites of morphine in rabbits will be described.

METHODS

Materials. Reference standards of morphine 3- and 6-glucuronides were prepared by the method previously described.[4,5] The β-glucuronidase preparation was obtained from the preputial glands of adult female rats and was partially purified according to the method of Levvy *et al.*[6] The activity was shown to be 116,000 *p*-nitrophenyl glucuronide units/ml.[7]

* A part of this study has appeared in *Tetrahedron Lett.* 483 (1968).

Thin-layer chromatography (TLC). The following solvent systems and chromatoplates were employed. System A: *n*-butanol–acetone–acetic acid–5% ammonium hydroxide–water (45:15:10:10:20, v/v); silica gel G (Merck), 0·25 mm thick. System B: *n*-butanol–acetic acid–water (4:1:5 v/v; upper layer); MN-cellulose powder 300G (Macherey, Nagel & Co.), 0·25 mm thick. The chromatoplates were activated at 105° for 30 min (silica gel) or for 15 min (cellulose powder). Morphine and morphine glucuronides were detected by spraying the plates with Dragendorff reagent.

Estimation of morphine. The methyl orange method for the estimation of morphine described by Woods *et al.*[8] was modified as follows. To 2 ml of a solution containing 3–75 μg morphine in a 10-ml centrifuge tube was added 200 mg sodium bicarbonate and 0·1 ml acetic anhydride. The mixture was allowed to stand for 30 min with occassional stirring. After evolution of CO_2 ceased, 6 ml chloroform was added and the tube was shaken for 1 min by hand and for 5 min by machine. The mixture was then centrifuged for 2 min and the aqueous layer was removed. An aliquot of 5 ml of the chloroform layer was transferred to another tube containing 0·5 ml of methyl orange reagent, and the tube was shaken and centrifuged as described above. The aqueous layer was removed and an aliquot of 4 ml of the chloroform layer containing the dye complex was pipetted into a 10-ml tube and shaken with 4 ml of 0·5 N HCl for 1 min by hand. After centrifugation for 2 min, the optical density of the separated aqueous layer was measured at 515 mμ. The reagent blank usually gave a reading of about 0·002 against water.

Administration of morphine. Morphine hydrochloride was dissolved in water and was injected subcutaneously into four male albino rabbits weighing 2·5–3·5 kg in a dose of 13·2 mg/kg (equivalent to 10 mg free base/kg). At 3-day intervals, the above dose was repeated, so that a total of 1·0 g morphine base was injected over about a 1-month period. No appreciable decrease in body weight was observed during these treatments. The urine samples excreted over about 20 hr after each injection were collected, centrifuged and submitted immediately to the procedure of purification described below.

In the experiment designed for detection of the metabolites in bile, morphine hydrochloride was administered to a male albino rabbit subcutaneously in a single dose of 40 mg/kg. Four hr later the animal was stunned, exsanguinated and the gall bladder was removed.

Purification of morphine glucuronides in urine. Step 1: Adsorption on charcoal. A part (30 ml) of the urine sample, collected as described above, was mixed with 2g charcoal in a 50-ml centrifuge tube; the mixture was shaken for 1 hr and then centrifuged for 3 min. The aqueous layer was discarded by decantation and the charcoal was washed twice with 30 ml water. Unchanged morphine and the metabolites adsorbed on charcoal were then extracted three times with 10 ml acetic acid by shaking for 30 min. After centrifugation, the extract was collected and filtered through filter paper to remove the finer charcoal particles. The solvent was removed by evaporation from the clear solution under reduced pressure and the residue was dissolved in 10 ml water. This solution was stored at 4° until all of the urine samples were processed.

Step 2: Chromatography on a column of cationic-exchange resin. The combined extract from step 1 was diluted to 300 ml with water and passed through a column of 300 ml of Dowex 50W-X8 (H-form) in 1 hr. The chromatography was performed in a cold room at 0°. The column was further washed with 2 l. water and then with

400 ml ethanol. At this point, the resin was removed from the column and repacked, since many fine bubbles appeared in the column during the ethanol washing. The column was then washed with 2 l. water. Unchanged morphine and the glucuronides that were retained on the resin were eluted with 0·15 N ammonium hydroxide and collected in 200-ml fractions. As shown in Fig. 1, a metabolite which gave an R_f value equivalent to that of synthetic morphine 3-glucuronide was eluted into fraction 18 and neighboring flasks together with unchanged morphine. None of the other possible metabolites of morphine could be detected definitely in these fractions. The

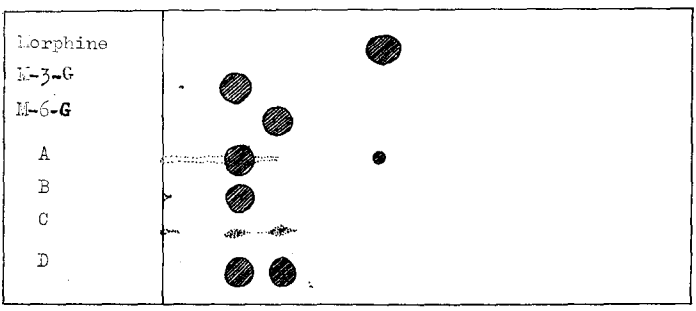

FIG. 1. The thin-layer chromatograms of the various fractions (System A). M-3-G, morphine 3-glucuronide; M-6-G, morphine 6-glucuronide; A, fraction 18 of step 2; B, fraction 2 of step 3; C, fraction 3 of step 3; D, fraction 7 of the rechromatography of fraction 3 of step 3 (see text).

residues obtained after evaporation of the solvent from fractions 14–23 were combined and were dissolved in 100 ml water. The solution was adjusted to pH 9·0 with ammonium hydroxide and shaken five times with 100 ml of chloroform–isopropanol (3:1, v/v) in order to remove free morphine. The aqueous phase was then evaporated to dryness under reduced pressure and the resulting gum was dissolved in 10 ml water containing a small amount of methanol. This solution was kept overnight in a refrigerator. Fine needles (M-1) which crystallized out from this solution were filtered and recrystallized from hot water to give colorless needles, m.p. 243–246° (decomp.). The yield was 640 mg.

Step 3: Chromatography on an anionic-exchange resin. The mother liquor remaining after purification step 2 was diluted to 50 ml with water and was passed through a column of 150 ml of Dowex 1-X2 (formate form). The column was washed with 1 l. of water and the adsorbed metabolites were eluted with 0·2 N formic acid. The eluates which were collected in 100-ml fractions, were evaporated to complete dryness under reduced pressure in order to remove traces of formic acid. From fraction 2, M-1 was further obtained as crystals and was recrystallized from water to give colorless needles, m.p. 243–246° (decomp.). The yield was 160 mg, and therefore the total quantity of pure M-1 obtained after the administration of 1 g morphine base amounted to 800 mg (step 2 plus step 3). On the thin-layer chromatogram of fraction 3 (see Fig. 1), another metabolite (M-2), which could not be detected before this step, was now observed together with the spot corresponding to M-1.

RESULTS

Indentification of M-1. M-1 had the same melting point 243–246°, (decomp.) and low water solubility as synthetic morphine 3-glucuronide. Thin-layer chromatography

with two different systems also showed that the properties of this metabolite corresponded with those of the authentic sample (Table 1).

Elementary analysis indicated that M-1 had the empirical formula $C_{23}H_{27}O_9N \cdot 2^1/2\ H_2O$*. (*Anal.* Calcd. for $C_{23}H_{27}O_9N \cdot 2^1/2\ H_2O$: C, 54·53; H, 6·37; N, 2·76.

TABLE 1. R_f VALUES OF THIN-LAYER CHROMATOGRAMS OF THE CONJUGATED METABOLITES OF MORPHINE

Compounds	System A*	System B*
Morphine 3-glucuronide	0·14	0·35
Morphine 6-glucuronide	0·22	0·45
M-1	0·14	0·35
M-2	0·22	0·45

* See text.

Found: C, 54·55; H, 6·39; N, 2·95.) These values very closely approximated those of the synthetic sample.[4,5] The ultraviolet absorption spectrum ($\lambda_{max}^{H_2O}$ 283 mμ, log $\varepsilon = 3{\cdot}23$) and optical rotation ($[\alpha]_D^{16} - 131°$, c = 0·5 in H_2O) were also in agreement with those of the synthetic sample ($\lambda_{max}^{H_2O}{}_{max}$ 283 mμ, log $\varepsilon = 3{\cdot}24$; $[\alpha]_{Di}^{28} - 132°$, c = 0·5 in H_2O). The infrared absorption spectrum of M-1 was found to be completely identical with that of the synthetic standard (Fig. 2). The strong absorption band at

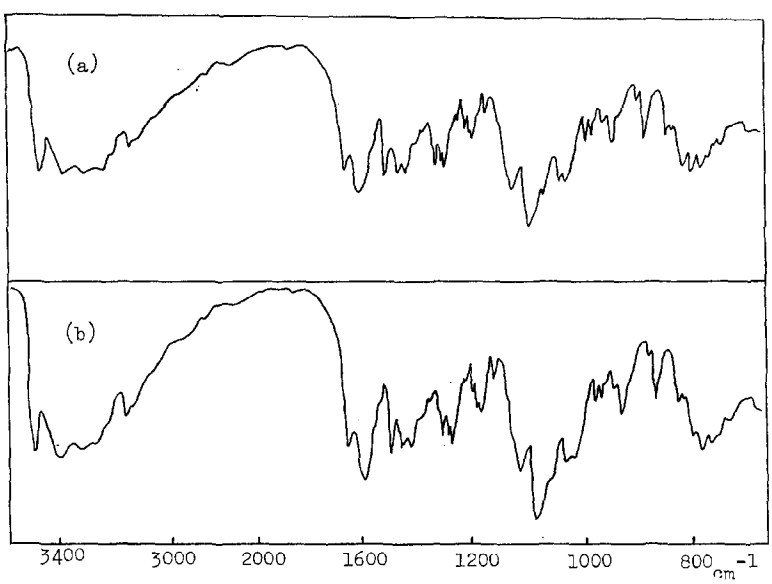

FIG. 2. Infrared absorption spectra of (a) synthetic morphine 3-glucuronide and (b) M-1 (KBr).

1596 cm^{-1} due to the carboxylate ion also indicated that this glucuronide had an ionized form that corresponded to that reported by Fujimoto and Way[2,9] and to our synthetic sample.[4,5]

* The sample was dried at 40° for 5 hr under reduced pressure.

On the basis of this evidence, it was concluded that the major metabolite was morphine 3-glucuronide. However, there were some differences between our observations and those of Woods[1] and of Fujimoto and Way.[2,9] Woods assigned the dihydrated formula to this glucuronide; our synthetic sample and metabolite corresponded more closely to a structure containing 2·5 moles of water. The infrared absorption spectra of our sample and that of Fujimoto and Way were essentially identical except that an extra peak at 1650 cm^{-1} was present in our sample. This peak was attributed to crystalline water, since it disappeared completely after drying the sample at 105° for 7 hr under reduced pressure.

Identification of M-2. As described in step 3 of the purification process, fraction 3 contained a very small amount of a new conjugated metabolite which was detected by thin-layer chromatography. The R_f values of this product in two chromatographic systems were equivalent to those of synthetic morphine 6-glucuronide (Table 1). This fraction was therefore further purified in order to isolate the metabolite in a crystalline form. To do this, the resinous gum obtained from fraction 3 was dissolved in 10 ml water. The solution was passed again through a column packed with 50 ml of Dowex 50W-X8 (H-form) and the column was washed with 300 ml water. The adsorbed metabolites were eluted with 0·15 N ammonium hydroxide and collected in 50-ml fractions. However, this rechromatography did not give a good separation of M-1 and M-2, since both were eluted in fractions 6–9. Fractions 6–9 were combined and the solvent was evaporated to dryness under reduced pressure. The residue was dissolved in 10 ml water; the solution was adjusted to pH 10 with ammonium hydroxide and passed through a column of 25 ml of Dowex 1-X2 (formate form). The metabolites were then eluted with 0·01 N formic acid and collected in 30-ml fractions. Fraction 7 was found to contain both metabolites M-1 and M-2; however, detection of M-2 by thin-layer chromatography became more definite than before (Fig. 1). The higher (M-2) and the lower (M-1) R_f values were equivalent to those of authentic morphine 6- and 3-glucuronides respectively.

The entire fraction was applied in a line on two silica gel plates (20 × 20 cm) and then developed with the solvent mixture of system A. After visualizing one side of the chromatogram with Dragendorff reagent, the band corresponding to M-2 was scraped off of the remaining side into a 10-ml centrifuge tube and the material was extracted twice for 30 min each with 10 ml and then 5 ml of water. In order to remove inorganic salts, the extract was treated with 2 g charcoal as described in purification step 1. The charcoal was washed twice with 30 ml water and the metabolite was extracted three times with 10 ml acetic acid. The combined extracts were filtered and the solvent was evaporated to dryness under reduced pressure, yielding 6·5 mg amorphous powder. Thin-layer chromatograms using both system A and B showed only one Dragendorff positive spot which had the same R_f value as that of authentic morphine 6-glucuronide. This amorphous compound still seemed to contain some inorganic material, but the major component was considered to be morphine 6-glucuronide from the following evidence: 1) it gave a positive naphthoresorcinol reaction, indicating that it was a glucuronide; and 2) the ultraviolet absorption spectra in water and in alkaline solution were identical to those of the authentic sample (M-2: $\lambda_{max}^{H_2O}$ 285 mμ, λ_{max}^{NaOH} 298 mμ; morphine 6-glucuronide: $\lambda_{max}^{H_2O}$ 286 mμ, λ_{max}^{NaOH} 298 mμ). The pH dependence of the spectrum indicated that there should be a free phenolic hydroxyl group in this metabolite. The glucuronide structure of this

amorphous product was further proved by enzymatic hydrolysis as follows. A solution of 1 mg of the amorphous product in 0·2 ml water was incubated with 0·1 ml of a preparation of β-glucuronidase and 0·1 M acetate buffer (pH 4·2) at 37° for 24 hr. In another pair of flasks, 1 mg saccharo-1,4-lactone, a specific inhibitor of β-glucuronidase, was added to the above incubation mixture. The reaction was stopped by addition of 100 mg sodium bicarbonate and the mixture was extracted three times with 5 ml chloroform–isopropanol (3:1, v/v). The combined organic phases were dried with anhydrous sodium sulfate and the solvent was evaporated under reduced pressure. The residue was then submitted to silica gel TLC in the solvent system of ethanol–dioxane–benzene–conc. ammonium hydroxide (5:40:50:5, v/v). Substantial release of morphine was confirmed in the experiment without inhibitor, whereas in the flask including the β-glucuronidase inhibitor only a trace of morphine could be detected.

The amount of this metabolite, morphine 6-glucuronide, excreted in urine was presumed to be very small. By calculation from the optical density of an aqueous solution at the ultraviolet absorption maximum of this metabolite, it was estimated to be about 5 mg at most in the amorphous mixture described above.

Detection of the conjugated metabolite of morphine in bile. The bile was removed from the gall bladder 4 hr after the injection of morphine and was submitted directly to TLC in system A. The chromatogram showed two spots having R_f values of 0·14 and 0·42. The spots of higher and lower R_f values corresponded to unchanged morphine and morphine 3-glucuronide respectively. It was also concluded from thin-layer chromatography that the major metabolite in bile was morphine 3-glucuronide. Morphine 6-glucuronide, however, was not detected in this unpurified bile.

Acid hydrolysis of morphine 3- and 6-glucuronides. Since a substantial amount of injected morphine was found to be excreted as two glucuronides and the detection and estimation of these glucuronides were usually based on the morphine liberated by acid hydrolysis, it was important to establish the rate of acid hydrolysis of both glucuronides. The optimum conditions for hydrolysis were therefore studied at various concentrations of hydrochloric acid. To a solution of either morphine 3-glucuronide (1·77 mg) or morphine 6-glucuronide (1·68 mg) in 0·5 ml water (these amounts were equivalent to 1 mg of morphine base) various concentrations of concentrated hydrochloric acid were added; these mixtures were then heated on a boiling water bath for 30 min. The mixtures were diluted to 50 ml with water and 1 ml of the solution was analyzed for the liberated morphine (Table 2).

TABLE 2. ACID HYDROLYSIS OF MORPHINE 3- AND 6-GLUCURONIDES*

Concentration of HCl (%)	Per cent of hydrolysis	
	Morphine 3-glucuronide	Morphine 6-glucuronide
5	38·5	15·0
10	87·5	42·5
15	99·5	66·3
20	99·5	75·0
25		82·5

* The samples were heated on a boiling water bath for 30 min.

It was found that morphine 3-glucuronide was hydrolyzed completely by heating in 15% hydrochloric acid for 30 min. On the other hand, the 6-isomer was more difficult to hydrolyze, even in 25% hydrochloric acid.

DISCUSSION

In the light of the present results, the use of charcoal and of chromatography on columns of ion-exchange resins were quite effective methods for purifying the urinary metabolites of morphine. The purification of morphine 3-glucuronide by adsorption on charcoal was first described by Fujimoto and Way.[2] Column chromatography on an ion-exchange resin was also employed for purification of apomorphine conjugates by Kaul *et al.*[10] In the present study, a good yield of pure morphine 3-glucuronide was thus obtained from the urine of rabbits given morphine. Morphine 6-glucuronide was also established as a minor metabolite in rabbit urine.

In an early report Thompson and Gross[3] suggested the existence of two conjugates of morphine, "easily hydrolyzable" and "difficulty hydrolyzable", in dog urine. Woods[1] also reported that two forms of conjugated morphine might be excreted in dog urine; one was a monoglucuronide which could be crystallized and possessed low water solubility, and the other was possibly an amorphous di-conjugated morphine of high water solubility. However, Fujimoto and Way[2] reported later that only one conjugated morphine derivative, morphine 3-glucuronide, was excreted in appreciable amounts as a urinary metabolite of morphine in humans.

In experiments with rabbits, about 45 per cent of administered morphine was recovered as the crystalline 3-glucuronide from the urine, and the same glucuronide was also detected as the main metabolite of morphine in the bile by thin-layer chromatography. In addition, it has been shown herein that morphine 6-glucuronide is excreted in the urine in very small amounts (about 0·3 per cent of the dose). The latter glucuronide was detected only in a repeatedly purified fraction of a urinary extract, but not in unpurified urine or in bile.

Recently Oka[11] reported a method for the isolation of a morphine glucuronide, which was demonstrated to be the 3-isomer, from dog urine by adsorption on charcoal and purification with column chromatography on Amberlite CG-50 and CG-400. However, the infrared absorption spectrum and the melting point (229–230°) of his sample, which were described in his report,[11] were not completely identical with those of our synthetic 3-glucuronide.[4,5]

It is also interesting to note that both morphine 3- and 6-glucuronides are surprisingly resistant to acid hydrolysis. Morphine 6-glucuronide was not hydrolyzed completely even in 25% hydrochloric acid when heated on a boiling water bath for 30 min. Although hydrolysis of the 3-glucuronide was more readily accomplished than that of the 6-isomer, it still required heating for 30 min in rather concentrated acid (15 per cent). This result also agreed with our previous work, which demonstrated that maximum liberation of morphine was observed when rabbit urine containing mainly the 3-glucuronide was refluxed for 30 min in 15% hydrochloric acid.[12] If any animal excretes more of the 6-glucuronide than of the 3-isomer, rather drastic conditions will be necessary for acid hydrolysis in estimating the morphine liberated.

The method for estimation of morphine adopted in the present study represents a slight modification of that of Woods *et al.*[8] Whereas morphine was converted to the *p*-nitrobenzoyl derivative prior to preparing the methyl orange complex in the original

study, 3-acetylmorphine, which could be prepared quantitatively from morphine by the method of Welsh,[13] was utilized in the present study. This modified method is simpler and affords more accurate results than the original method of Woods *et al.*[8]

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