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BIOCHEMICAL BASIS FOR ANALGESIC ACTIVITY OF MORPHINE-6-GLUCURONIDE—I

PENETRATION OF MORPHINE-6-GLUCURONIDE IN THE BRAIN OF RATS*

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Abstract—To understand the potent analgesic action of morphine-6-glucuronide (M-6-G), which was reported previously to be a minor metabolite of morphine in several mammalian species, the penetration of this conjugate into the brain was investigated using ^{14}C-labeled compound. A similar study was also conducted with ^{14}C-morphine. These studies presented evidence that, although M-6-G was a highly polar conjugate, it can penetrate the blood brain barrier and react with the receptor of analgesic action without prior hydrolysis of the glucuronide linkage. It was further suggested that the lack of analgesic activity produced with morphine-3-glucuronide (M-3-G), a major metabolite of morphine, was attributable to its inability to react with the receptor, because it penetrates the brain as well as M-6-G.

ALTHOUGH Woods[1] isolated a metabolite of morphine in crystalline form and characterized it as morphine-3-glucuronide (M-3-G) in 1954, the question as to whether there was more than one form of conjugated morphine has not been resolved until recently. In previous papers[2,3] we reported for the first time that several mammalian species including man excreted morphine-6-glucuronide (M-6-G) as a minor urinary metabolite of morphine, along with the major metabolite M-3-G. It was further found that M-6-G, but not M-3-G, showed much stronger analgesic activity than morphine itself.[4] This finding seemed very interesting, because it has been believed that in general a polar metabolite such as a glucuronide (a) could not prenetrate into the brain to reach its site of action and (b) is quickly excreted, as exemplified in the study of meprobamate glucuronide by Emmerson *et al.*[5] Rapid excretion of injected glucuronide was also reported for phenyl glucuronide by Garton and Williams[6] and for *p*-nitrophenyl glucuronide by Kamata *et al.*[7]

The present investigation was undertaken (a) to obtain evidence for the penetration of M-6-G into the brain of rats and (b) to confirm the previous assumption that analgesia exerted after injection of M-6-G is not mediated by morphine which might be liberated *in vivo* from M-6-G, but by M-6-G itself.

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METHODS

Materials. Morphine hydrochloride was obtained from commercial sources. Reference standards of M-3-G and M-6-G were prepared by the method described previously.[8,9] *N*-methyl-^{14}C-labeled morphine hydrochloride (spec. act.: 4·20 μCi/mg) was kindly supplied by Dainippon Pharmaceutical Co., Ltd. and also was synthesized in this laboratory according to the method of Andersen and Woods[10] (spec. act.: 1·6 μCi/mg).

Preparation of ^{14}C-labeled M-6-G. To 10 mg of ^{14}C-morphine hydrochloride (spec. act.: 4·20 μCi/mg) was added 10 ml of 3·0 M phosphate buffer (pH 9·0) and the mixture was extracted five times with 10 ml of $CHCl_3$–isopropanol (3:1, v/v). The free base obtained after evaporation of the solvents from the above extract was synthetically converted to M-6-G, essentially by the method described in a previous paper.[9] The reaction scheme is shown in Fig. 1. ^{14}C-Acetyl morphine, which was

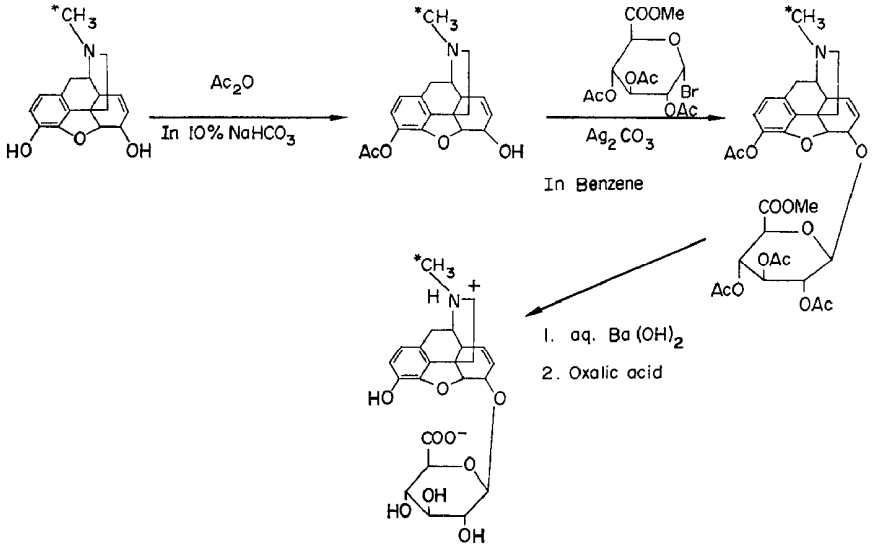

FIG. 1. Synthetic route for ^{14}C-M-6-G.

prepared by acetylation of ^{14}C-morphine with acetic anhydride in 10% $NaHCO_3$ solution, was dissolved in 1·5 ml of dry benzene and the solution was gently boiled. To this boiling solution was added a solution of 150 mg of methyl 2,3,4-tri-*O*-acetyl-1α-bromo-1-deoxy-D-glucopyranuronate in 3·0 ml of dry benzene, and 70 mg of freshly prepared dry Ag_2CO_3 was added slowly over a period of 3·5 hr. During this time, benzene was distilled off gradually and stirring was continued. The reaction mixture was filtered and filtrate was extracted four times with 10 ml of cold 0·5% HCl. The combined HCl extracts were then adjusted to pH 8·0 with $NaHCO_3$ and shaken four times with 20 ml of $CHCl_3$ to obtain the methyl acetyl derivative of ^{14}C-M-6-G. This product was dissolved in 10 ml of 0·43 N $Ba(OH)_2$. One hr later, the resulting hydrolysate was adjusted to pH 6·0 with 2 N oxalic acid and the solution was kept in a refrigerator for 1 hr. Precipitated barium oxalate was removed by filtration and the filtrate was purified by column chromatography using 10 ml of Dowex 50W-X4 (H-form). After the column was washed successively with 200 ml of H_2O and 50 ml of

ethanol, ^{14}C-M-6-G was eluted with 250 ml of 0·15 N NH_4OH, collecting in 10-ml fractions. The radioactive fractions (fractions 6–15) were combined and concentrated to about 10 ml. Trace amounts of contaminants of basic nature in this concentrate were removed by extraction four times with $CHCl_3$–isopropanol (3:1, v/v). The aqueous layer was evaporated to dryness under reduced pressure to yield 7·8 mg of ^{14}C-M-6-G (spec. act.: 2·16 μCi/mg), which was shown to be radiochemically pure by thin-layer chromatography using a solvent system of *n*-butanol–acetone–acetic acid–5% NH_4OH–H_2O (45:15:10:10:20, v/v).

Treatment of animals. Male rats of the Donryu strain weighing about 140 g were used in this study. ^{14}C-M-6-G and ^{14}C-morphine hydrochloride were dissolved in water to make a 1% solution, which was injected intraperitoneally in a single dose of 10 mg/kg and 13·18 mg/kg (10 mg/kg as free base), respectively, to each rat. At 45 min after drug administration, blood samples were withdrawn by cardiac puncture with a heparinized syringe, and the animals were then killed for excision of the brain, liver and kidneys after perfusion with physiological saline through the carotid artery.

Radioisotopic methods of analysis. The radioactivity of all samples was measured with an Aloka liquid scintillation spectrometer (model 502, Japan Radiation of Medical Electronics, Inc., Tokyo) and was corrected for quenching by an internal standard method using ^{14}C-toluene as the standard. Aliquots of blood (0·1 ml) and tissues (70 mg) were digested with 2 ml of hyamine hydroxide at 50° for 24 hr and decolored with a drop of 30% H_2O_2 and conc. HCl. The radioactivity in these solutions was determined with a toluene phosphor consisting of 4 g of 2,5-diphenyloxazole (PPO), 0·1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (POPOP) and toluene to make 1 l. The radioactivity on thin-layer chromatograms was obtained by extracting morphine or its glucuronides with H_2O from Silica gel and counting the radioactivity in a *p*-dioxane phosphor consisting of 60 g of naphthalene, 4 g of PPO, 0·2 g of POPOP, 100 ml of methanol, 20 ml of ethylene glycol and *p*-dioxane to make 1 l. The radioactivity of each fraction from Sephadex column chromatography of the plasma was also counted in the *p*-dioxane phosphor.

Identification of radioactive compounds in the brain of rats. After addition of 5 mg each of unlabeled morphine, M-3-G and M-6-G as carriers to an aliquot (1·5 g) of the brain sample of rats injected with ^{14}C-M-6-G described above, the mixture was homogenized in 9 volumes (13·5 ml) of 5% HCl. The homogenate was centrifuged at 10,000 *g* for 10 min, and the precipitate was homogenized and centrifuged two more times in 5 ml of 5% HCl. The combined supernatants (25 ml) were shaken twice with 15 ml of $CHCl_3$ to remove acidic and neutral lipids and the aqueous layer, after neutralization with $NaHCO_3$, was subjected to column chromatography using Dowex 50W-X4 (H-form, 42 ml) according to the methods described in previous papers.[2,3] After the column was washed successively with 200 ml of H_2O and 100 ml of ethanol, morphine and its glucuronides were eluted with 0·15 N NH_4OH. The Dragendorff reagent-positive fractions (fractions 12–17) were combined. It was concentrated to a small volume and subjected to the thin-layer chromatography described below. After development, 0·5 cm or 1·0 cm bands of Silica gel, corresponding to R_f 0·0 to 0·5, or R_f 0·5 to 1·0, respectively, were scraped from the plates and extracted three times with 5 ml of H_2O by shaking for 10 min each. The combined extracts were concentrated to a small volume *in vacuo*, and to this was added 10 ml of *p*-dioxane phosphor to count the radioactivity.

The brain of rats injected with ^{14}C-morphine hydrochloride was treated similarly after addition of 5 mg each of unlabeled morphine, M-3-G and M-6-G as carriers.

Estimation of plasma protein. Plasma protein was measured according to the method of Kalckar.[11] An aliquot (0·1 ml) of each fraction from Sephadex column chromatography of the plasma was diluted to 5·0 ml with water, and the protein concentration of this solution was then measured spectrophotometrically with a Hitachi spectrophotometer, type EPU-2A.

Thin-layer chromatography. This was conducted with Silica gel plates (Silica gel G, Merck: 0·25 mm in thickness), activated at 105° for 30 min. The solvent system used was *n*-butanol–acetone–acetic acid–5% NH_4OH–H_2O (45:15:10:10:20, v/v). Morphine and its glucuronides were visualized by spraying with Dragendorff reagent and also by counting the radioactivity.

RESULTS

Distribution of the radioactivity after injection of ^{14}C-M-6-G or ^{14}C-morphine into the rat. In order to compare the distribution of M-6-G with that of morphine in rat tissues, the radioactivities of brain, liver, kidney and blood of rats were determined at 45 min after the intraperitoneal injection of ^{14}C-labeled drug, when the induced analgesia was most potent. The results are summarized in Table 1.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY AFTER INJECTION OF ^{14}C-MORPHINE HYDROCHLORIDE AND ^{14}C-M-6-G*

Compound	Radioactivity (%/g tissue or ml blood)			
	Brain	Liver	Kidney	Blood
Morphine	0·028 ± 0·002	0·44 ± 0·10	2·36 ± 0·36	0·028 ± 0·003
M-6-G	0·0057 ± 0·0005	0·68 ± 0·29	0·91 ± 0·11	0·043 ± 0·005

* The results presented are averages ± S.D. of separate analyses performed on three adult male Donryu rats (body wt: about 140 g). The figures in the table are expressed as (dis/min in 1 g of tissue/total dis/min injected) ×100.

After injection of either ^{14}C-M-6-G or ^{14}C-morphine hydrochloride, a considerably low but significant level of the radioactivity was observed in the brain, although the radioactivity after injection of ^{14}C-M-6-G was only one-fifth of that after injection of ^{14}C-morphine hydrochloride. It is also noted that relatively high levels of radioactivity were present in the liver and kidneys after injection of ^{14}C-M-6-G, the quantities being comparable to the values obtained by the injection of ^{14}C-morphine hydrochloride. Furthermore, the radioactivity in the blood was found to be greater in the rats injected with ^{14}C-M-6-G than in the rats treated with ^{14}C-morphine hydrochloride. These results strongly suggested that M-6-G, although it is a highly polar compound, can penetrate into the brain as well as the other organs.

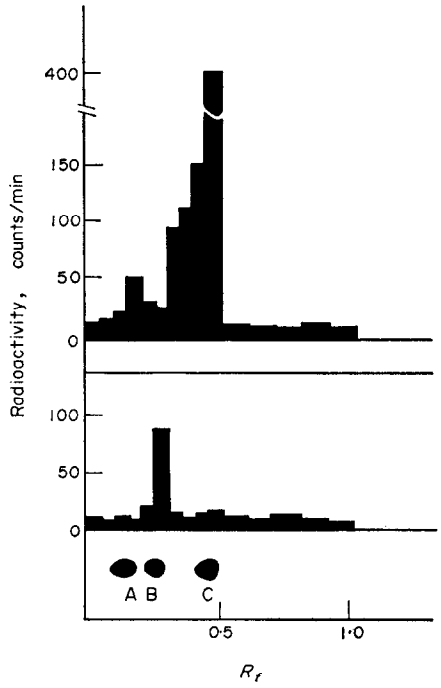

FIG. 2. Radio thin-layer chromatogram of extracts of the brain. Upper panel; ^{14}C-morphine hydrochloride (spec. act.: 4·2 μCi/mg); lower panel: ^{14}C-M-6-G (spec. act.: 2·16 μCi/mg). A = authentic M-3-G; B = authentic M-6-G; and C = authentic morphine. The solvent system used was *n*-butanol–acetone–acetic acid–5% NH_4OH–H_2O (45:15:10:10:20, v/v).

Identification of radioactive compounds in the brain of rats. Since it was not completely certain whether M-6-G was the only compound responsible for the radioactivity counted in the brain of rats injected with ^{14}C-M-6-G, thin-layer chromatographic examination was conducted with an aliquot of the brain sample used for the distribution study. The radiochromatograms obtained are shown in Fig. 2. When ^{14}C-M-6-G was injected, only one radioactive band was detected on the chromatogram. The R_f value of this compound was identical with that of authentic M-6-G. This undoubtedly indicated that M-6-G could penetrate the blood brain barrier. It should also be noted that M-6-G was rather stable, since no detectable amount of morphine was observed in the brain at 45 min after the injection of ^{14}C-M-6-G. On the other hand, the experiment with ^{14}C-morphine hydrochloride clearly revealed the existence of radioactive M-3-G, which was probably produced in the liver and then penetrated into the brain, together with unchanged morphine.

Identification of radioactive compounds in the plasma of rats injected with ^{14}C-morphine hydrochloride. Detection of M-3-G in the brain of rats at 45 min after injection of ^{14}C-morphine hydrochloride suggested that the blood circulating at this time contains M-3-G, since it seems reasonable that M-3-G should be produced mainly in the liver and then transferred to the brain. In order to prove this, a heparinized blood sample, withdrawn at 45 min after injection of ^{14}C-morphine hydrochloride, was centrifuged for 5 min at 1500 *g* and the plasma was collected. One ml of this plasma

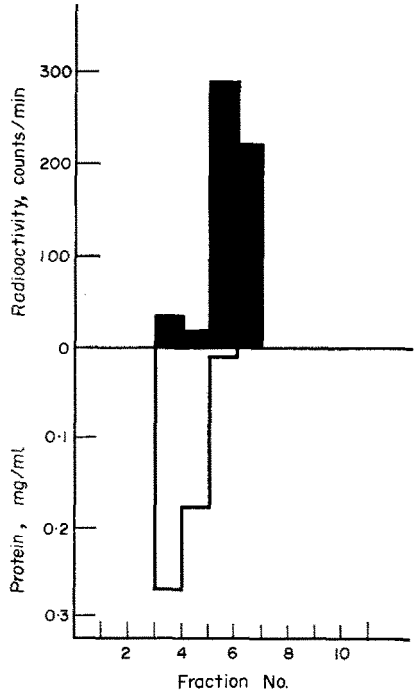

FIG. 3. Sephadex column chromatography of plasma of rats receiving ^{14}C-morphine hydrochloride (spec. act.: 1·6 μCi/mg).

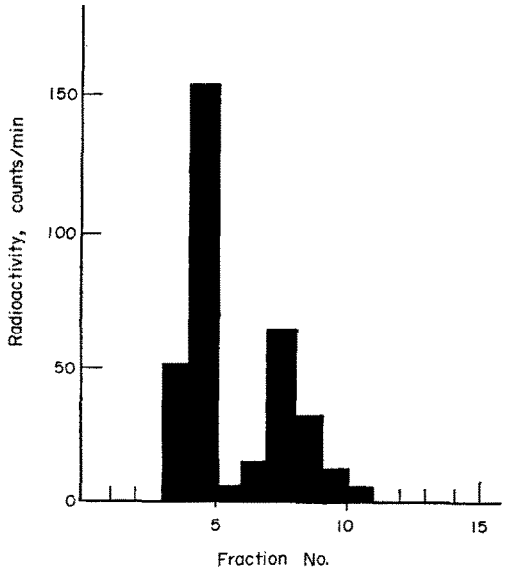

FIG. 4. DEAE-Sephadex A-25 column chromatography of fractions 6 and 7.

was first poured on a column of Sephadex G-25 (20 ml) and eluted with water at a flow rate of 2 ml/hr, collecting 2·0-ml fractions. As shown in Fig. 3, most of the radioactivity applied was eluted in fractions 6 and 7. In addition, a small amount of radioactivity appeared in fraction 4, which coincided with the position at which protein eluted. This fact suggested that a small amount of morphine and/or its metabolites might be bound to plasma protein.

Fractions 6 and 7 were combined and concentrated to 1·0 ml *in vacuo*, and then chromatographed on a column of DEAE-Sephadex A-25 (20 ml). The column was eluted with water at a flow rate of 2 ml/hr, collecting 2·0-ml fractions. Figure 4 shows the elution pattern of the radioactivity which was separated into two parts. Since it was confirmed that ^{14}C-morphine was eluted only in fractions 4 and 5 using the same chromatographic procedure, it was assumed that the radioactive peak observed in fractions 4 and 5 in Fig. 4 might correspond to morphine. This was further confirmed by radio thin-layer chromatography of the combined fractions of the above two peaks. The results showed that the former eluates (fractions 4 and 5) and the latter (fractions 7–10) consisted only of morphine and M-3-G respectively. It was also found that M-3-G accounted for about 30 per cent of the radioactivity in the plasma at 45 min after the intraperitoneal injection of ^{14}C-morphine hydrochloride.

DISCUSSION

Contrary to the hypothesis that formation of glucuronide or ethereal sulfate is one of the most important detoxication mechanisms for pharmacologically active substances, previous papers from this laboratory have reported that M-6-G[4] and morphine-6-sulfate,[12] although highly polar conjugates, induce potent analgesia. However, it was not undoubtedly shown whether the pharmacological activity of these conjugates was attributable to the conjugates themselves or to morphine which might be liberated from the conjugates. The present studies on distribution of M-6-G provide evidence that M-6-G penetrates the blood brain barrier and reacts with the receptor of analgesic action without prior removal of the glucuronic acid moiety. This was demonstrated by the fact that at 45 min after intraperitoneal injection of M-6-G, when the induced analgesia was greatest, essentially no free morphine, but only M-6-G could be detected in the brain. As shown in Table 1, the rate of penetration was much slower for M-6-G than for morphine. This presumably is the reason why M-6-G exerted much stronger analgesia by intracerebral injection than by the intraperitoneal route when compared with morphine.

It is also interesting to note that M-3-G can penetrate the blood brain barrier, as well as M-6-G. This is indirectly shown by the finding of significant amounts of M-3-G both in the brain and plasma of rats at 45 min after intraperitoneal injection of ^{14}C-morphine. The radioactivity determined to be M-3-G accounted for about 10 and 30 per cent of the total radioactivity in the brain and plasma, respectively. Similar results were also obtained by Misra *et al.*,[13] who reported that about 30 per cent of ^{14}C-compounds in the cerebral cortical hemispheres of the rat was present as bound morphine at 30 min after a single subcutaneous injection of 10 mg/kg of ^{14}C-morphine.

Muraki[14] recently reported that the uptake of M-3-G by the choroid plexuses *in vitro* was not performed by an active process, although it was definitely shown by Takemori and Stenwick[15] that morphine was transported actively by the choroid

plexuses. On the bases of these experiments *in vitro*, he speculated that M-3-G could not penetrate into the central nervous system from the systemic circulation. However, our present experiment *in vivo* strongly suggests that M-3-G, as well as M-6-G, is transported into the brain in a considerable amount (see Fig. 2). In addition to this, the previous findings[4] that M-3-G did not show any analgesic effect by either subcutaneous or intracerebral injection strongly suggest that M-3-G reaches but does not interact with the receptor of analgesic action in the brain.

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