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3-O-Acetylmorphine-6-O-Sulfate: A Potent, Centrally Acting Morphine Derivative

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HOUDI, A. A., S. KOTTAYIL, P. A. CROOKS AND D. A. BUTTERFIELD. *3-O-Acetylmorphine-6-O-sulfate: A potent, centrally acting morphine derivative*. PHARMACOL BIOCHEM BEHAV 53(3) 665-671, 1996. — In view of the potent analgesia exhibited by the apparent structurally dissimilar morphine-6-O-glucuronide (M6G) and morphine-6-O-sulfate (M6S) conjugates of morphine, we have examined the effect of structural modification of M6S on analgesic activity, using the tail-flick test. Changes in the M6S structure were made that would affect the lipophilicity and polarity of the molecule. Subcutaneous (sc) and intracerebroventricular (ICV) administration of equimolar doses of morphine, M6S, 3-O-acetylmorphine-6-O-sulfate (M3A6S), 3-O-benzoylmorphine-6-O-sulfate (M3B6S), and 3-O-acetyl-N-methylmorphinium-6-O-sulfate (MM3A6S) were employed. M6S and M3A6S exhibited a longer duration of action and greater activity compared to morphine after SC and ICV administration. However, M3B6S and MM3A6S in doses equimolar to that of morphine were found to be inactive after both SC and ICV administration. In addition, M3A6S showed the highest potency in inhibiting electrically stimulated guinea pig ileum followed by M6S and M3B6S. Moreover, both M6S and M3A6S displayed a greater affinity than that of morphine to μ and κ_3 receptor sites in guinea pig brain homogenate. In contrast, the nonanalgesic compounds M3B6S and MM3A6S showed weak receptor binding ability compared to morphine. These results indicate that lipophilicity alone is not a determinant of analgesic activity in these novel morphine derivatives. These modified effects of morphine by the conjugations at the 3- and 6-position, appear to be due to their altered interactions with opioid receptors.

Morphine derivatives Analgesia Tail-flick test Central administration μ -Receptor
 δ -Receptor κ -Receptor

THE OPIATE analgesic morphine, when administered to humans, is converted by the liver into three major metabolites, viz. morphine-3-O-glucuronide (M3G), morphine-6-O-glucuronide (M6G), and morphine-3-O-sulfate (M3S) (9,19). M6G is found in the systemic circulation in concentrations exceeding those of morphine itself, after both parenteral (12,19) and oral administration (12,18). M6G is a very potent μ -receptor agonist (3) with a high affinity for both μ_1 and μ_2 receptors (1,14) and appears to cross the blood-brain barrier in spite of its high polarity compared to morphine (20). After examining the clinical activity of M6G in a group of six cancer patients, it has been concluded that the analgesic effect of administered morphine is due mainly to metabolically formed M6G rather than to morphine itself (11). Interestingly, the related conju-

gate, morphine-6-O-sulfate (M6S) was shown to exhibit more potent and longer acting analgesia than morphine itself in mice (2). On the other hand, M6S showed comparatively reduced competing potencies toward μ -receptors but enhanced delta-receptor affinity compared to parent compound morphine (10).

In view of the potent analgesia exhibited by the apparent structurally dissimilar 6-O-glucuronide and 6-O-sulfate conjugates of morphine, we have examined the effect of structural modification on antinociceptivity in the M6S molecule. Of particular interest was the effect of increasing lipophilicity on activity by esterification of the 3-hydroxy group and the effect of increasing polarity by conversion of M6S to its N-methylmorphinium betaine (see Fig. 1). As part of these struc-

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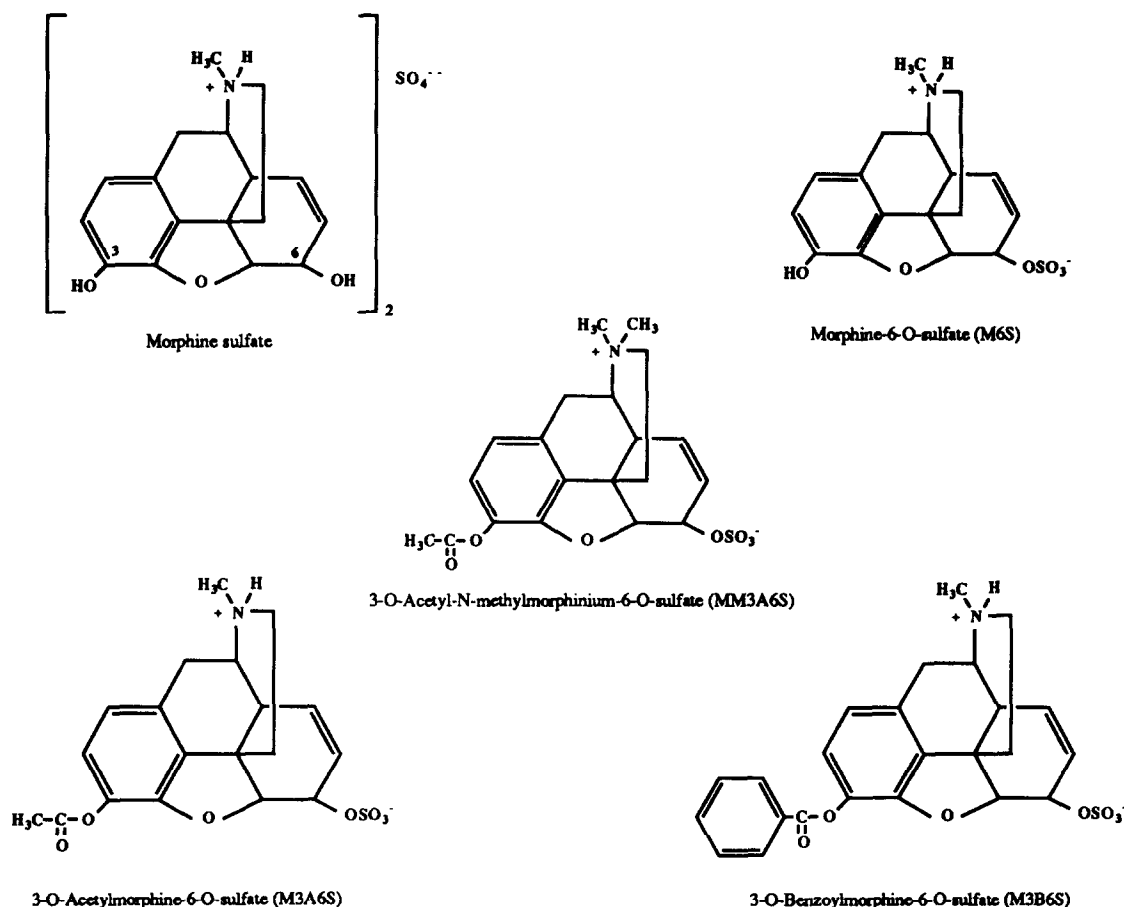


FIG. 1. Structures of morphine and morphine-6-O-sulfate derivatives.

ture-activity studies, we now report on the potent, centrally acting antinociceptive activity of the morphine derivative, 3-O-acetylmorphine-6-O-sulfate, in the rat.

METHOD

Animals and Surgery

The assessment of analgesic response by the tail flick test was carried out on male Sprague-Dawley rats (Harlan Industrial, Indianapolis, IN) weighing 270–310 g at the time of experimentation. Rats were housed individually in a room maintained at 24°C (constant temperature and humidity) with a 12 L : 12 D cycle for 1 week before use in the studies. Food and water were available ad lib.

Rats were anesthetized using equithesin (3 ml/kg) during the duration of the surgery. A stainless steel guide cannula (Plastic Products, Roanoke, VA) was implanted over the left lateral cerebral ventricle. The rat was positioned in a stereotaxic frame (David Kopf); stereotaxic coordinates were 0.8 mm posterior to (Lang Dental Manufacturing Co., Chicago, IL). A 28-gauge dummy cannula was kept in the implanted cannula, except during intracerebroventricular (ICV) injections. The rats were allowed to recover from surgery for 3–4 days prior to the experiments.

Antinociceptive Testing

The antinociceptive activity of the morphine analogs was evaluated by thermal stimuli, utilizing the tail-flick test of D'Amour and Smith (4). The radiant heat was focused on the tail tip of a male Sprague-Dawley rat, and the heat intensity was adjusted so that a control animal flicked its tail within 1.7–2.6 s of exposure. To prevent tissue damage in drug-treated animals, a latency cut-off time of 8 s was employed. Each rat served as its own control, because the latency to response was measured both before and after drug administration. Rats were acclimated to the tail flick test three times before the actual experiment was run. Measurements of analgesia, or antinociception were expressed as:

$$\% \text{ Maximum Possible Response (\% MPR)} = \frac{\text{Post-drug Latency} - \text{Base line Latency}}{\text{Cutoff Time (8.0 s)} - \text{Base line Latency}} \times 100$$

Experimental Protocol

On the day of the experiment, rats were placed in a restrainer for 15 min before actual testing began. Four baseline trials were carried out before drug administration, one every 5 min. Then, rats were removed briefly from their restrainers

for SC administration of drugs. The drug was dissolved or suspended (sonicated and vortexed) in sesame seed oil (Fisher Scientific Co.) prior to injection (1 ml/kg). Morphine [5 mg/kg (0.0175 mmol)] as free base was used. Equimolar doses of morphine derivatives to that of morphine were as follows: M6S (6.4 mg/kg); M3A6S (7.1 mg/kg); M3B6S (8.2 mg/kg); and MM3A6S (7.4 mg/kg). The tail-flick procedure was then continued.

For ICV administration, test compounds were dissolved in saline prior to administration. On the day of the experiment, 30 min before drug administration, animals were handled briefly to lower a drug-filled injector through the guide cannula into the lateral ventricle. The injector consisted of a segment of stainless steel tubing connected to a Hamilton Syringe by PE20 tubing. The rats were then placed in restrainers for 15 min prior to actual testing. Four base-line trials were done, one every 5 min, before drug administration. Microinjection of the drug solution (each treatment consisted of 4 μ l of solution per rat) was made over 1 min, using a preprogrammed syringe pump (Tracor, Atlas, Houston, TX). The tail-flick procedure was then continued at 3-min intervals for the first 30–45 min and then at longer time intervals (15–30 min) until analgesia was abolished. An 8-s cutoff time was used to prevent damage to the tail. For ICV drug administration, two doses of morphine were used; low dose morphine 0.236 μ g/rat (0.83 pmol μ mol/rat as free base), high dose morphine 23.6 μ g/rat (83 pmol/rat). Doses of morphine derivatives administered ICV were equimolar to the low dose of morphine and were as follows: M6S (0.22 μ g); M3A6S (0.25 μ g); M3B6S (0.29 μ g); and MM3A6S (0.26 μ g).

Successful ICV injection was confirmed by monitoring the movement of a small air bubble over a calibrated distance in the PE20 tubing during the drug administration. Additionally, at the conclusion of the experiment, each ICV treatment was verified by examining the cerebral ventricle after a 4 μ l fast green dye injection over 1 min, into the deeply anesthetized rat.

Receptor Binding

Hartley guinea pigs were decapitated and their brains were quickly removed and weighed. The brains were then homogenized in 50 mM Tris HCl, buffer pH 7.7, using a Polytron (~25 ml/brain). The homogenate was centrifuged at 40,000 \times g for 15 min, rehomogenized, and centrifuged. The final pellet was resuspended in Tris HCl, pH 7.7, at a final concentration of 6.67 mg original wet weight of tissue per ml, except for tissue prepared for (3 H)NalBzoH (naloxone benzoylhydrazine, NIDA) binding, which was resuspended in buffer containing 5 mM EDTA.

The following radioligands (~1 nM) were used to label the receptor binding sites indicated in parentheses: (3 H)DAMGO [D-Ala²,N-Me-Phe⁴,Gly ol⁵]-enkephalin, (μ), (3 H)DPDPE [D-Pen^{2,5}-enkephalin], (δ), (3 H)U69,593 (κ_1), (3 H)-NalBzoH (κ_2). The guinea pig brain suspension (1.8 ml) was incubated in 50 mM Tris HCl. Nonspecific binding was determined by incubating in the presence of 1 μ M of the cold unlabeled counterpart of each labeled ligand, except that 10 μ M \pm NalBzoH was used for the κ_2 assay. The samples were then filtered through glass fiber filters on a 48-well Brandel cell harvester. The filters were washed three times with 3 ml of buffer. Filters were incubated overnight with 5 ml of scintillation cocktail before counting.

Results have been reported in terms of IC₅₀ (concentration of test compound that produces 50% inhibition of labeled

ligand binding). The K_i (Inhibitory Dissociation Constant) values are derived from the following equation: $K_i = IC_{50}/1 + [L]/K_d$. The K_d values were obtained by computer analysis of detailed self-inhibition curves for each of the labeled ligands (L), using the curve-fitting program LIGAND.

In Vitro Functional Assays

Guinea pig ileum (GPI) preparation. Male Hartley guinea pigs (350–400 g wt.) were decapitated and their small intestines were removed; about 20 cm of the terminal ileum was discarded. The longitudinal muscle with the myenteric plexus attached was gently separated from the underlying circular muscle by the method of Paton and Vizi (13). The muscle strip was mounted in an 8 ml water-jacketed organ bath containing Krebs-bicarbonate solution of the following composition: 118 mM NaCl, 2.5 mM CaCl₂, 4.7 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄ and 11.5 mM glucose. The tissues were kept at 37°C and bubbled with 5% CO₂ in oxygen. An initial tension of 0.6–1.0 g was applied to the strips. The muscle strip was stimulated for 60 min before the start of each experiment.

Field electrical stimulation was delivered through platinum wire electrodes positioned at the top and bottom of the organ bath and kept at a fixed distance apart (3.5 cm). The upper electrode is a ring of 4 mm diameter. The parameters of rectangular stimulation were as follows: supramaximal voltage, 1 ms impulse duration at a rate of 0.1 Hz. A Grass S-88 electrostimulator was used for stimulation. The electrically induced twitches were recorded using an isometric transducer (Metrigram) coupled to a Grass 7D multichannel polygraph.

Mouse vas deferens (MVD) preparation. The vas deferens from Swiss-Webster mice (30–35 g) were prepared as described by Hughes et al. (6). The tissues were mounted on organ bath containing 8 ml of magnesium free Krebs solution at 31°C, which was bubbled with a mixture of oxygen and carbon dioxide (95:5). An initial tension of 150–200 mg was used.

Field stimulation parameters were modified slightly from those of Hughes et al. (6) as described by Ronai et al. (17); paired shocks of 100 ms delay between supramaximal rectangular pulses of 1 ms delay between supramaximal rectangular pulses of 1 ms duration were delivered at a rate of 0.1 Hz. A Grass S-88 electrostimulator was used for stimulation. The contractions were recorded using an isometric transducer (Metrigram) coupled to a Grass 7D multichannel polygraph.

Functional Activity

The agonist potencies of test compounds were determined from concentration-response curves and characterized by their IC₅₀ values. IC₅₀ is defined as the concentration of the agonist that causes 50% inhibition of the electrically induced contractions. To determine the site(s) at which the agonists acted, assays were conducted in the presence of site-selective antagonists. A shift of the dose-response curve to the right is indicative of activity at the antagonist bound site. CTAP (100 nM) (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂) and 20 nM nor-BNI [nor-binaltorphimine] were added to the GPI preparation to selectively block μ (7) and κ_2 (15) receptors, respectively; 1 nM naltrindole was added to the MVD preparation to selectively block δ receptors (16). This activity was characterized by dose ratios (DR). DR was calculated from the following equations:

$$DR = \frac{IC_{50} \text{ in the presence of antagonist}}{IC_{50} \text{ in the absence of antagonist}}$$

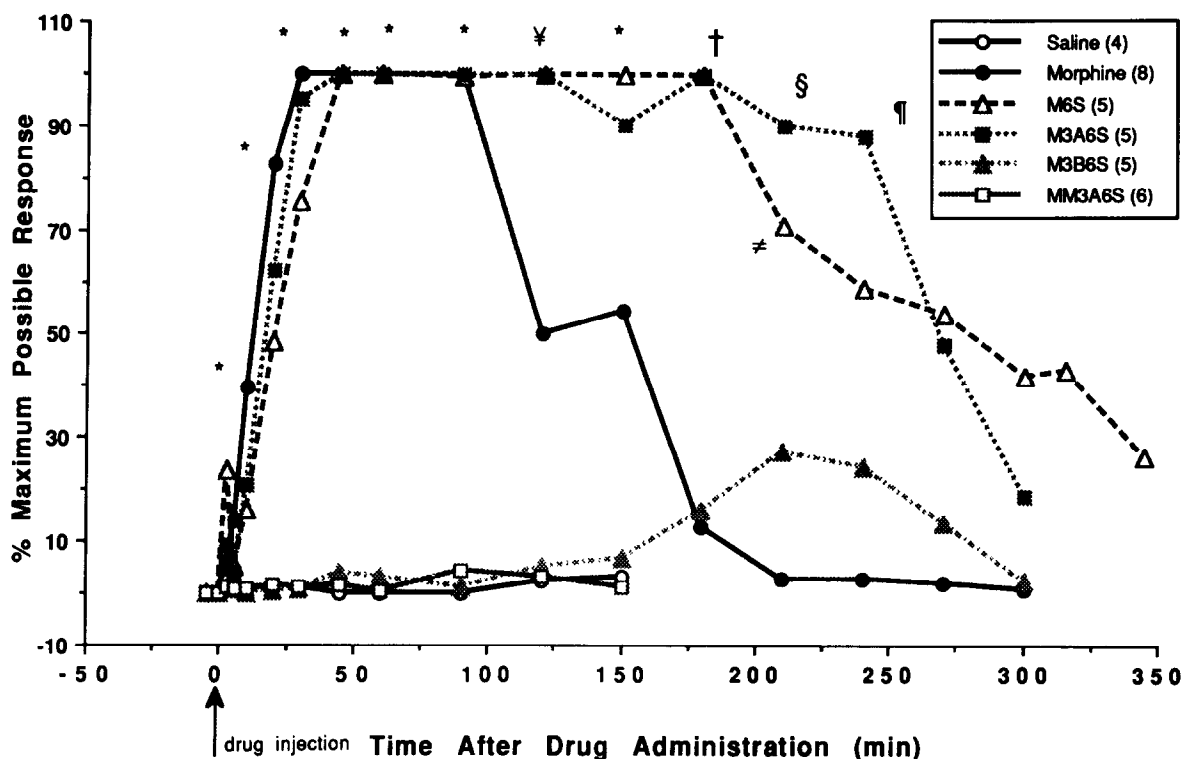


FIG. 2. Antinociceptive response to SC treatment with morphine. Antinociception is shown as the percent of maximum possible response in the tail-flick test. Free base morphine was used for SC administration (5 mg/kg, 0.0175 mmol). Equimolar doses of morphine derivatives were as follows: M6S (6.4 mg/kg); M3A6S (7.1 mg/kg); M3B6S (8.2 mg/kg); and MM3A6S (7.4 mg/kg) were administered subcutaneously. Pre-drug response latencies for saline, morphine, M6S, M3A6S, M3B6S, and MM3A6S were 2.15, 1.96, 2.37, 1.79, 1.95, and 2.11 s, respectively. Analysis of variance revealed a highly significant treatment by time interaction. *Represents a significant difference ($p < 0.005$) of morphine, M6S, and M3A6S from the corresponding response to saline-, M3B6S-, and MM3A6S-treated rats. †Represents a significant difference ($p < 0.0001$) of M6S and M3A6S from the corresponding response to morphine- and M3B6S-treated rats. ‡Represents a significant difference ($p < 0.0007$) of M6S and M3A6S from the corresponding response to morphine. §Represents a significant difference ($p < 0.0007$) of M3A6S from the corresponding response to M3B6S treated rats. ¶Represents a significant difference ($p < 0.004$) of morphine, M6S, and M3A6S from the corresponding response to morphine treated rats. Comparisons were made using two-way repeated measure ANOVA followed by Tukey's test. Numbers in parentheses represent the number of rats per group.

Chemicals

The compounds utilized in this study were: morphine sulfate salt (Mallinckrodt Inc., Paris, KY), morphine-6-O-sulfate (M6S), 3-O-acetyl morphine-6-O-sulfate (M3A6S), 3-O-benzoylmorphine-6-O-sulfate (M3B6S), and 3-O-acetyl-N-methylmorphinium-6-O-sulfate (MM3A6S). The sulfate esters were synthesized in our laboratories and their preparation is reported elsewhere (Crooks et al., manuscript in preparation); see Fig. 1 for the structural formula of these compounds. Equithesin was composed of chloral hydrate (2.13 g, Sigma Chemical Co., St. Louis, MO), magnesium sulfate (1.07 g, Fisher Scientific Co., Fairlawn, NJ), propylene glycol (14.1 ml, Fisher Scientific Co.), ethanol (3.8 ml, Curtin Matheson, Houston, TX), distilled water (24.6 ml), and sodium pentobarbital (7.5 ml, 64.8 mg/ml, Butler Co., Columbus, OH).

Statistical Analysis

The data are presented as mean \pm SEM for the given number of rats. Two-way repeated measure with interaction ANOVA followed by Tukey's post hoc test for comparisons

was utilized for analysis between groups treated with different drugs and tested for analgesia.

RESULTS

Antinociceptive Activity of Morphine-6-O-Sulfate Derivatives After Subcutaneous Injection

The antinociceptive activities of M6S, M3A6S, M3B6S, MM3A6S, and morphine, as determined by the tail-flick method following SC injection, are illustrated in Fig. 2. The onset of morphine (5 mg/kg, SC) analgesia started within 10 min of administration; maximal analgesic response was achieved within 30 min and was maintained for 60–80 min. This analgesic profile for morphine is similar to other published reports (5,8). An equimolar dose of M6S produced a maximal analgesic effect similar to morphine within 30 min after administration. However, the maximal analgesic effect lasted over 3 h. An equimolar dose of M3A6S produced an analgesic profile similar to that of M6S with respect to onset of action and maximal analgesic effect, which was maintained for 4 h. Interestingly, M3B6S at an equimolar dose, showed

only a gradual onset of weak analgesia over 100 min, which reached a maximum equivalent to only 27% of maximal analgesia over 4 h. The dipolar betaine, MM3A6S, was devoid of any analgesic properties when tested at an equivalent molar dose.

Antinociceptive Activity of Morphine-6-O-Sulfate Derivatives After Intracerebroventricular Administration

The above five compounds were also evaluated for analgesic activity by the tail-flick assay after administration by intracerebroventricular injection. These results are shown in Fig. 3. Two doses of morphine were initially employed. The lower dose of morphine (0.23 $\mu\text{g}/4 \mu\text{l}/\text{rat}$) failed to produce an analgesic effect. The higher dose (23.6 $\mu\text{g}/4 \mu\text{l}/\text{rat}$) produced a rapid analgesic response (6 min after ICV injection), and maximal analgesia was achieved within 10 min of administration and maintained for an additional 3 h. Administration of M6S at a molar dose equivalent to the lower dose of morphine produced a maximal analgesic effect, within 10 min, that was maintained for 70 min. M3A6S administered at an equivalent molar dose to M6S exhibited a similar profile,

maximal analgesia being established at 10 min and lasting for an additional 90 min. A similar dose of either M3B6A or MM3A6S afforded no analgesic response over the 100 min time period examined.

Receptor Binding

The results of binding studies using guinea pig brain homogenates are presented in Table 1. M6S and M3A6S displayed a greater affinity than that of morphine to mu and kappa₂ receptors. Both compounds were even more mu-selective than DAMGO, a mu-preferring peptide. M6S also had greater affinity than that of morphine to delta receptors. Both M6S and M3A6S bound kappa₁ sites weakly in guinea pig brain homogenate. In addition, M3A6S showed a better ability to discriminate between mu and delta sites than morphine ($K_i \text{ mu}/K_i \text{ delta} = 0.0052$ and 0.043 for M3A6S and morphine, respectively). In contrast, the K_i of the nonanalgesic compounds M3B6S and MM3A6S were $15\times$ and $14\times$ greater than that of morphine for mu-receptor site and exhibited much greater K_i s for delta, kappa₂ and kappa₁ sites.

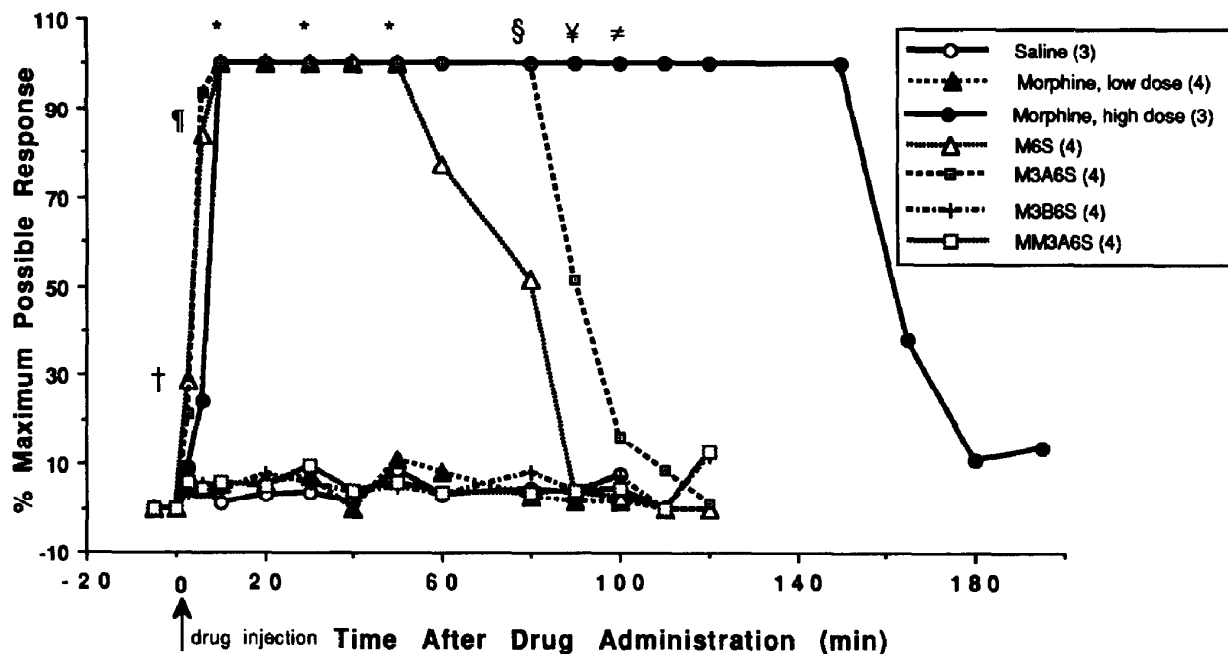


FIG. 3. Antinociceptive response to treatment with saline vehicle, morphine, and morphine derivatives. For intracerebroventricular drug administration, two doses of morphine were used; low dose morphine (0.236 $\mu\text{g}/\text{rat}$, 0.83 pmol $\mu\text{mol}/\text{rat}$ as free base) and high dose of morphine (23.6 $\mu\text{g}/\text{rat}$, 83 pmol/ $\mu\text{mol}/\text{rat}$). Doses of morphine derivatives administered intracerebroventricularly were equimolar to the low dose of morphine and were as follows: M6S (0.22 μg); M3A6S (0.25 μg); M3B6S (0.29 μg); and MM3A6S (0.26 μg). Antinociception is shown as the percent of maximum possible response in the tail-flick test. Predrug response latencies for saline, morphine low dose, morphine high dose, M6S, M3A6S, M3B6S, and MM3A6S were 2.05, 2.06, 1.69, 1.92, 2.62, 2.06, and 1.82 s, respectively. Analysis of variance revealed a highly significant treatment by time interaction. †Represents a significant difference ($p < 0.009$) of M6S from the corresponding response to saline-, M3B6S-, and MM3A6S-treated rats. ‡Represents a significant difference ($p < 0.0001$) of M6S and M3A6S from the corresponding response to morphine high dose-, saline-, M3B6S-, MM3A6S-, and morphine low dose-treated rats. *Represents a significant difference ($p < 0.0001$) of morphine high dose, M6S, and M3A6S from the corresponding response to MM3A6S-, morphine low dose-, M3B6S-, and saline-treated rats. §Represents a significant difference ($p < 0.0001$) of morphine high dose and M3A6S from the corresponding response to M3B6S, saline, MM3A6S and morphine low dose. ¶Represents a significant difference ($p < 0.001$) of morphine high dose from the corresponding response to M3B6S-, MM3A6S-, saline-, M6S-, and morphine low dose-treated rats. ≠ Represents a significant difference ($p < 0.004$) of morphine I from the corresponding response to MM3A6S, M3B6S, M3A6S, M6S, and saline treated rats. Comparisons were made using two-way repeated measure ANOVA followed by Tukey's test. Numbers in parentheses represent the number of rats per group.

TABLE 1
INHIBITORY EFFECT (K_i) OPIOIDS ON THE BINDING OF TRITIATED LIGANDS TO MU, DELTA,
AND KAPPA-SITES OF GUINEA PIG BRAIN HOMOGENATES

Cold Ligand	K_i (nM)*			
	[³ H]DAMGO mu	[³ H]DPDPE delta	[³ H]U69593 kappa-1	[³ H]NalBzoH kappa-3
DAMGO	1.1 ± 0.2	180.4 ± 16.0	1,841.3 ± 22	26.9 ± 0.9
DPDPE	>10,000	2.8 ± 0.4	>10,000	>10,000
U69593	692.0 ± 97.0	1,358.0 ± 118.0	0.7 ± 0.1	1,099.0 ± 0.2
NalBzoH	0.2 ± 0.0	1.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.07
Morphine	2.0 ± 0.3	50.0 ± 0.6	33.9 ± 9.0	13.9 ± 1.7
M6S	0.9 ± 0.0	18.0 ± 0.4	1,192.0 ± 697.0	6.3 ± 0.3
M3A6S	0.8 ± 0.0	307.8 (1)	1,165.0 ± 185.0	5.5 ± 1.2
M3B6S	36.9 ± 1.3	599.5 ± 150.0	>10,000	513.3 ± 15.0
MM3A6S	34.2 ± 12	841.8 ± 189.0	>10,000	400.1 ± 58.0

*Data represent the average of two experiments, each conducted in triplicate, unless indicated in parentheses.

In Vitro Functional Assays (Guinea Pig Ileum and Mouse Vas Deferens)

The effects of opioids standards and the synthetic derivatives of morphine on electrically stimulated contraction of guinea pig ileum (enriched in mu sites) and mouse vas deferens (enriched in delta sites) and their selectivity to opioid receptors are shown in Table 2. M6S, M3A6S, and M3B6S all inhibited electrically induced twitches of GPI. M3A6S showed the highest potency followed by M6S > M3B6S. MM3A6S showed no activity on GPI. All four compounds displayed weak activity in MVD assay. Based on their activities in both tissues, M3A6S and M6S exhibited more selective mu activity.

DISCUSSION

The data from this study clearly show that esterification of the 3-hydroxyl group of M6S with an acetyl group affords a morphine derivative that has enhanced duration of action as

M6S when administered via the SC route in the rat. Both M6S and M3A6S have a more prolonged duration of action than an equimolar dose of morphine given SC (Fig. 2). More importantly, when given ICV, M3A6S enhanced duration of action than M6S as an analgesic (see Fig. 3), and both M6S and M3A6S possessed greater activity than morphine via this route. Interestingly, the 3-O-benzoyl ester of M6S exhibited extremely weak antinociceptive activity via the SC route, and was essentially devoid of activity when administered ICV at doses equimolar to morphine used in this study. We attribute this result to metabolic factors, and propose that the activities of the two esters are related to their susceptibilities to in vivo enzymatic cleavage by esterases to M6S. Thus, the acetyl group in M3A6S would be expected to be rapidly cleaved in vivo, whereas the corresponding benzoate ester would be relatively resistant to esterolysis. Therefore, one may regard M3A6S as a prodrug form of M6S. However, both compounds M6S and M3A6S showed high affinity binding to mu

TABLE 2
INHIBITION OF ELECTRICALLY EVOKED TWITCHES IN GUINEA PIG ILEUM AND MOUSE VAS DEFERENS PREPARATION

Compound	Guinea Pig Ileum			Mouse Vas Deferens	
	IC ₅₀ (nM)	DR With* CTAP	DR With* Nor-BNI	IC ₅₀ (nM)	DR With* Naltrindole
DAMGO	8.3 ± 2.0 (13)	5.0 ± 0.3 (4)	1.7 ± 0.1 (6)	177.6 ± 134.0 (7)	0.9 ± 0.1 (4)
DPDPE	4,130.0 ± 870.0 (6)	5.8 ± 2.6 (3)	1.5 ± 0.3 (4)	4.11.0 ± 1.3 (80)	53.1 ± 17.6 (3)
U69593	1.7 ± 0.6 (12)	0.7 ± 0.1 (4)	363.0 ± 97.0 (7)	208.3 ± 139.0 (8)	0.4 ± 0.1 (4)
Morphine	24.8 ± 2.4 (4)	4.3 ± 0.1 (2)	1.1 ± 0.1 (2)	2,131.0 ± 904.0 (4)	0.9 ± 0.4 (4)
M6S	67.0 ± 12.1 (4)	5.0 ± 2.2 (2)	0.9 ± 0.1 (2)	No IC ₅₀ could be determined	
M3A6S	26.0 ± 5.3 (4)	5.4 ± 0.2 (2)	1.4 ± 0.1 (2)	No IC ₅₀ could be determined (Max. inhibition 30%)	
M3B6S	657.5 ± 130.0 (4)	3.9 ± 0.3 (2)	1.3 ± 0.3 (2)	No IC ₅₀ could be determined (max. inhibition 29%)	
MM3A6S	No activity	—	—	No IC ₅₀ could be determined (max. inhibition 20%)	

The number of observations are given in parentheses.

*CTAP (100 nM) and 20 nM nor-BNI were added to the GPI preparation to selectively block mu and kappa receptors, respectively. Naltrindole (1 nM) was added to the MVD preparation to selectively block delta receptors.

Dose Ratio (DR) = IC₅₀ in the presence of antagonist/IC₅₀ in the absence of antagonist.

opioid receptors in our in vitro binding assay using guinea pig brain homogenate.

The observation that both M6S and M3A6S are active with a rapid onset of antinociception after ICV and SC administration, strongly suggests that these morphine derivatives, like the 6-O-glucuronide conjugates, are capable of penetrating the blood-brain barrier after peripheral administration, in spite of their zwitterionic nature at physiological pH. However, further studies are necessary to determine if the analgesic properties of M6S and M3A6S are due to subsequent cleavage of the sulfate moiety, by sulfatases present in either the periphery or the central nervous system, to form morphine.

Addition of a sulfate group to the 6-position of morphine, as in M6S and M3A6S, seems to favor mu- and kappa-like activity, because such alteration displayed more selectivity for mu-receptors (threefold greater than morphine) and kappa₃ receptors (2.2-fold greater than morphine) in our receptor binding assay. Furthermore, M3A6S showed better ability to discriminate between mu- and delta-sites than did morphine or even DAMGO, a mu-preferring peptide, in receptor binding assay using guinea pig brain homogenate. This selectivity has also been supported by its high potency in GPI (enriched in mu-sites) and decreased potency in the MVD (enriched in delta-sites) preparations.

Increasing the polarity of the M3A6S molecule by quater-

nization of the tertiary amino group to form *N*-methylmorphinium-6-O-sulfate betaine (MM3A6S) abolished all antinociceptive activity following peripheral or central administration at doses equimolar to morphine used in this study. It seems that this dipolar derivative is unable to interact with CNS opioid receptors because it is inactive via the ICV route. In addition, it lacks activity in both GPI and MVD assays and shows weak binding affinity to opioid receptors in our receptor binding assay using guinea pig brain homogenate. These results indicate that a protonated tertiary amino group is a structural requirement for antinociceptive activity in morphine-6-O-sulfate derivatives.

These results indicate that lipophilicity alone is not a determinant of analgesic activity in these novel morphine derivatives. These modified effects of morphine by the conjugations at the 3- and 6-position, appear to be due to their altered interactions with opioid receptors.

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