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RIA Opiates: Structure versus Reactivity

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ABSTRACT

Twenty-six different opiates were analyzed by radioimmunoassay (RIA) at five different concentrations. At attempt is made to relate structural differences to the affinity of the compounds for the Roche RIA morphine antibody. The effects of substituent placement on the morphine molecule are studied. As expected, the basic 5-ring opiate structure is essential for reactivity. Addition of an alkyl group to the oxygen in the 3-position increased affinity, but alteration of other key functional groups had a reverse affect.

INTRODUCTION

Radioimmunoassay procedures for morphine were developed several years ago by Roche [1-3]. Their RIA procedure using ^{125}I-morphine [3, 4] has been in use in our laboratory for about 4 yr to screen urine samples for the presence of opiate drugs. It has been found that codeine, morphine, and morphine glucuronide do not have the same degree of affinity for the morphine antibody [3, 4]. Some initial work has been done to determine the reactivity of other opiates and to identify structural factors affecting reactivity [3-6]. As might be expected, opiates closely related in structure to morphine will cross-react with the Roche morphine antibody [3, 7] whereas structurally unrelated com-

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pounds will not. In fact, modifications of the commercial Roche procedure [3] allow the detection of hydromorphone [8].

We have sought to do a more comprehensive study to determine the relationship between reactivity and structure. A total of 26 different substances (Table 1) containing various portions of the opiate structure were analyzed at several concentrations by RIA and correlations between structure and reactivity were determined. The effect on affinity of various alterations in the morphine structure is reported.

MATERIALS AND METHODS

Reagents

Morphine antibody reagent (Roche Diagnostics, Nutley, New Jersey)

^{125}I-morphine reagent (Roche Diagnostics)

Ammonium sulfate solution (saturated)

Opiates

All the opiates were obtained from Applied Science Laboratories (State College, Pennsylvania) with the following exceptions: (1) levallorphan, d-methorphan, and dextrorphan were obtained from Hoffmann-LaRoche (Nutley, New Jersey); (2) dihydrocodeine, oxymorphone, and levorphanol were obtained from The Theta Corp (Media, Pennsylvania); (3) hydromorphone was obtained from Elkins-Sinn, Inc. (Cherry Hill, New Jersey); (4) naloxone was obtained from Endo Labs, Inc. (Garden City, New York); (5) apomorphine was obtained from Eli Lilly & Co (Indianapolis, Indiana); and (6) heroin was obtained from United States Pharmacopeia, (Rockville, Maryland).

Opiate Standards

0.5, 1.0, 3.0, 5.0, and 10.0 μg/mL solutions of each opiate (Table 1), prepared fresh just prior to analysis.

Dibromo-opiate Standards

Two-tenths milliliter aliquots of 1 mg/mL solutions of codeine and morphine, respectively, were evaporated to dryness. To each residue was added 0.1 mL of methanol and 1 mL of bromine/carbon tetrachloride. The solutions were allowed to stand for several minutes, then were evaporated to dryness. The residue was reconstituted in 0.1 mL methanol and then was diluted to 100 mL with water.

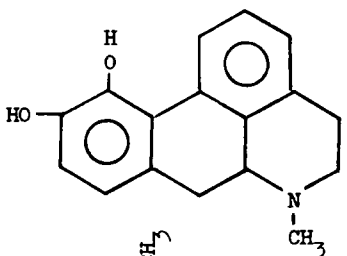
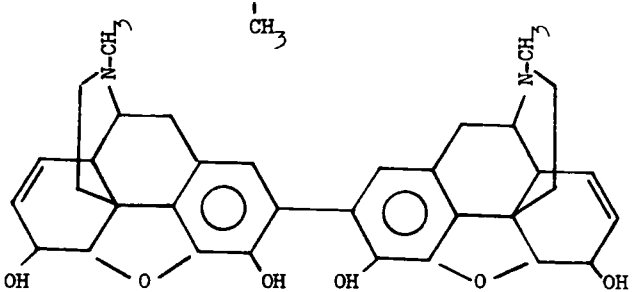
TABLE 1. Opiate Structures and Reactivity

Opiate	Group at position						cpm (0.5 μg/mL)	%
	3	4-5	6	7-8 bond	14	17 (N)		
Ethylmorphine	$CH_3–CH_2–O–$	–O–	HO–	Double	H–	$CH_3–$	3573	131
Codeine	$CH_3–O–$	–O–	HO–	Double	H–	$CH_3–$	3321	118
Morphine	HO–	–O–	HO–	Double	H–	$CH_3–$	2985	100
Dihydrocodeine	$CH_3–O–$	–O–	HO–	Single	H–	$CH_3–$	2821	91
Heroin	$CH_3–\overset{\overset{O}{\|}}{C}–O–$	–O–	$CH_3–\overset{\overset{O}{\|}}{C}–O–$	Double	H–	$CH_3–$	2608	80
Hydrocodone	$CH_3–O–$	–O–	O=	Single	H–	$CH_3–$	2576	78
Monoacetylmrophine	HO–	–O–	$CH_3–\overset{\overset{O}{\|}}{C}–O–$	Double	H–	$CH_3–$	2342	66
Thebaine	$CH_3–O–$	–O–	$CH_3–O–$	Single[a]	a	$CH_3–$	2105	53
Dihydromorphine	HO–	–O–	HO–	Single	H–	$CH_3–$	2091	52
Morphine-3-ethereal sulfate	$SO_4–$	–O–	HO–	Double	H–	$CH_3–$	2023	49
Hydromorphone	HO–	–O–	O=	Single	H–	$CH_3–$	1946	45
Morphine glucuronide	O-glucuronide	–O–	HO–	Double	H–	$CH_3–$	1900	42
Levorphanol (1)	HO–	H–, H–	H–	Single	H–	$CH_3–$	1502	21

(continued)

TABLE 1 (continued)

Opiate	Group at position						cpm (0.5 μg/mL)	%
	3	4-5	6	7-8 bond	14	17 (N)		
Codeine glucuronide	CH_3–O–	–O–	O-glucuronide	Double	H–	CH_3–	1445	18
Norcodeine	CH_3–O–	–O–	HO–	Double	H–	H–	1422	17
Nalorphine	HO–	–O–	HO–	Double	H–	CH_2=CH–CH_2–	1420	17
Levellorphan (l)	HO–	H–, H–	H–	Single	H–	CH_2=CH–CH_2–	1381	15
Oxycodone	CH_3–O–	–O–	O=	Single	HO–	CH_3–	1354	13
Normorphine	HO–	–O–	HO–	Double	H–	H–	1304	11
Oxymorphone	HO–	–O–	O=	Single	HO–	CH_3–	1224	6
Naloxone	HO–	–O–	O=	Single	HO–	CH_2=CH–CH_2–	1183	4
Morphine-N-oxide	HO–	–O–	HO–	Double	H–	CH_3–, O=	1164	3
Methorphan (d)	CH_3–O–	H–, H–	H–	Single	H–	CH_3–	1113	0
Dextrorphan (d)	HO–	H–, H–	H–	Single	H–	CH_3–	1108	0

Apomorphine		1107	0
Pseudomorphine		1102	0

[a]Double bonds 6-7, 8-14.

Apparatus

Disposable polystyrene tubes, 12 × 75 mm (Lancer)
Automatic pipetting station (Micromedic Model 24004)
Centrifuge: IEC Model K with 418 head
Gamma scintillation counter with printer (Packard 5160)
Micromedic receiving racks (blue)
Micromedic sample racks (red)
High speed automatic pipette (Micromedic Model 25004 F)

ANALYSIS PROCEDURE

Each opiate standard was assayed using the Roche RIA procedure [3]. A Micromedic automatic pipetting station was used to prepare all dilutions. Standards were analyzed 10 times at each of five concentrations to obtain an average count per minute.

The analysis was done as follows: Twenty-one sets of tubes were set up for each concentration of each opiate standard to be analyzed. Add about 1 mL of solution to the appropriate tube for each standard concentration. Using an automatic pipetting station add 0.1 mL of each solution to be analyzed from the above tubes, 0.2 mL of ^{125}I-morphine antigen, and 0.2 mL of morphine antibody to a set of tubes. Repeat this procedure 10 times to generate 10 reaction mixtures for each concentration of each standard to be analyzed. Incubate the reaction mixture at room temperature for 1 hr. After incubation add 0.5 mL of saturated ammonium sulfate to precipitate the protein complexes. Allow the reaction mixture to incubate at room temperature for 10 min. After incubation, centrifuge the tubes at 2700 rpm for 10 min. After centrifugation, use an automatic pipetting station to withdraw 0.5 mL of the supernatant fluid and add that supernatant fluid followed by 0.5 mL of distilled water to another tube for each tube of reaction mixture. Count the tubes of supernatant fluid for 1 min in a gamma scintillation counter. Average the 10 results obtained for each standard concentration.

INTERPRETATION OF RESULTS

The morphine RIA reaction is a competition reaction. The opiate in the solution being analyzed competes with the limited amount of ^{125}I-morphine antigen for the limited amount of morphine antibody. The resulting antigen-antibody and opiate-antibody complexes are precipitated by the ammonium sulfate. The amount of unreacted ^{125}I-morphine antigen which remains in solution is actually measured when an aliquot of the supernatant fluid is separated, diluted, and counted.

For morphine, if very little or none is present in the solution being analyzed, most of the antigen will be used up and little will be left in the supernatant fluid, resulting in a low count. If a large concentration of morphine is analyzed, a larger amount of antigen will be unreacted and left in the supernatant fluid, resulting in a higher count. Thus the larger the concentration of morphine in the solution being analyzed, the higher the count until a maximum is reached where further increases in concentration of morphine have little affect on the count produced.

For other opiates, if the opiate being analyzed is less reactive than morphine, a certain concentration of that opiate will not compete as effectively with the antigen for the antibody as would the same concentration of morphine, resulting in a lesser amount of unreacted antigen left in the supernatant fluid, yielding a lower count than would the same concentration of morphine. If the opiate is more reactive than morphine, it will compete very effectively with the antigen for the antibody, more effectively than would the same concentration of morphine. This results in a greater amount of unreacted antigen left in the supernatant fluid and yields a higher count.

RESULTS AND DISCUSSION

The structures of the 26 opiates studied are shown in Table 1. These structures can be compared to the percentages of cross-reactivity illustrated, as well, in Table 1.

In order for a molecule to have affinity for the morphine antibody, it must possess most of the aspects of the morphine pentacyclic structure (Fig. 1). Amphetamine-related drugs, barbiturates, benzodiazepines, caffeine, cocaine, diphenylhydantoin, ethinamate, glutethimide, meprobamate, methadone, methaqualone, nicotine, pentazocine, phencyclidine, phenothiazines, and propoxyphene, which have vastly different structures from morphine, were found to be totally unreactive up to 1 gm/L concentrations. The cross-reactivities of various opiates

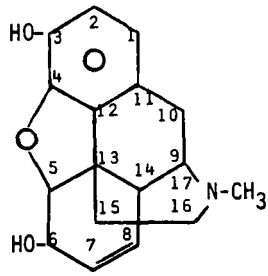


FIG. 1. Morphine.

(Table 1) indicate that while the basic pentacyclic structure is essential for high reactivity with the antibody, variations in structure affect affinity considerably.

The morphine antibody is a large protein molecule, generated in goats using a 3-carboxymethylmorphine-protein conjugate [1, 2]. The binding site of the antibody consists of a hydrophobic pocket with some few charged sites and hydrogen-bond donor or acceptor groups [9]. The chemical properties and size of the binding site depends on the functional groups of the hapten (3-carboxymethylmorphine), i.e., there will be complementary groups on the binding site for each functional group of the hapten. Therefore the antibody binding site would be expected to recognize the basic 5-ring morphine structure and specifically the various functional groups of the opiate nucleus, i.e., the 4,5-oxygen bridge, the 6-hydroxy group, the 7-8 double bond, the 14-hydrogen atom, and the 17 (N)-methyl group. The presence of these five groups is essential for strong reactivity.

Changes in the basic structure of morphine affect reactivity to varying extents. Specifically, changes at any position of the morphine molecule with the exception of the 3-position will reduce reactivity. The substitution of larger groups will prevent binding due to steric hindrance, while the substitution of a group of differing polarity or charge will reduce affinity because the antibody does not posses a complimentary group. In most cases with this opiate study, the groups substituted for those originally present on morphine differ both in size and chemical properties (Table 1).

The results shown in Table 1 and summarized in Table 2 illustrate that changes in the 2, 6, 14, 17 (N), 7-8 bond, and 4-5 oxygen bridge of morphine, codeine, or 3-carboxymethylmorphine reduce reactivity. Pseudomorphine (a 2,2-morphine dimer) in effect has the 2-hydrogen atom of morphine replaced with another morphine molecule connected at its 2-position. The replacement of the hydrogen atom with this large molecule with its complex chemistry virtually eliminates reactivity (Table 1).

Acetylation of the 6-hydroxy group (forming monoacetylmorphine), glucuronidation of the codeine 6-hydroxy group (forming codeine glucuronide), and oxidation of the 6-hydroxy groups of dihydrocodeine and dihydromorphine to oxo groups (forming hydrocodone and hydromorphone, respectively) all result in decreased affinity. As might be expected, replacement of the hydroxy group with the oxo group, which is similar in size but less polar and incapable of acting as a hydrogen-bond donor, results in a loss of affinity, to a small degree. However, replacement of the hydroxy group with the very large O-glucuronide group, which also has a much different chemistry, results in a greater loss of affinity (Table 1).

Hydrogenation of the 7,8-double bonds of codeine and morphine, yielding dihydrocodeine and dihydromorphine, respectively, slightly reduces affinity. This is expected since there are slight changes in the bond size, polarity, and geometry. The carbon-carbon single bond created is slightly longer and less polar. The geometry differs

TABLE 2. Summary of Factors Affecting Reactivity

Increase
A. Changes at 3-position
1. Replacing 3-hydrogen with 3-alkyl group
2. Lengthening of the 3-methyl group
Decrease
B. Changes in ring structure
1. Elimination of one of morphine's five rings
2. Connection of two opiate molecules at the 2-position
3. Conversion of the opiates that do not contain the tetrahydrofuran ring from a levorotatory form to a dextrorotatory form
C. Changes at 3-position
1. Replacement of 3-hydrogen with a 3-sulfate group
2. Replacement of 3-hydrogen with a 3-glucuronide group
D. Changes at the 6-position
1. Replacing a 6-hydroxy group with another group
2. Increasing size of 6-group
3. Replacement of the 6-hydroxy group with a 6-oxo group
E. Changes at 7-8 double bond
1. Adding hydrogen across the double bond
2. Adding bromine across the double bond
F. Changes at 14-position
1. Replacement of the 14-hydrogen with a 14-hydroxy group
G. Changes at 17(N)-position
1. Replacement of the 17-methyl group with a 17-hydrogen
2. Replacement of the 17-methyl group with a 17-allyl group
3. Addition of an oxide group to the nitrogen

greatly because the double bond contains two hydrogen atoms in the plane of the molecule, while the single bond contains four hydrogen atoms, two above the plane of the molecule and two below. Since hydrogen atoms are small atoms, these changes result in only a slight loss of reactivity. The addition of bromine atoms across this double bond greatly reduced the reactivity. Bromine atoms are much

larger and much more polar than hydrogen atoms, resulting in much more significant changes in the 7,8-position and causing a resulting greater loss of reactivity.

Replacement of the 14-hydrogen atom of hydrocodeine and of hydromorphine with a hydroxy group (forming oxycodone and oxymorphone, respectively) results in a great reduction in affinity. The hydroxy group is both much larger and much more polar than a hydrogen atom.

Replacement of the N-methyl (17-methyl) group of morphine with a hydrogen atom (forming normorphine) or an allyl group (forming nalorphine), or adding an oxide molecule (forming morphine-N-oxide) results in a great reduction in affinity. The hydrogen atom is smaller and more polar than the methyl group; the allyl group is larger and slightly more polar due to the double bond it contains; and the oxide molecule is much more polar due to its charge, and in combination with the methyl group is larger than the methyl group alone.

In sharp contrast to changes in the other positions of the opiate molecule, changes in the 3-position do not have as great an effect upon the reactivity and may even lead to increased reactivity.

The protein conjugate was attached to morphine at the 3-position for the preparation of the antibody, thus it is not surprising that changes in this position of morphine do not have as great an effect upon reactivity. Replacing the 3-hydroxy group of morphine with a 3-methoxy group (forming codeine) actually increases reactivity, and replacing the 3-methoxy group of codeine with a 3-ethoxy group (forming ethylmorphine) increases reactivity even more. This follows since these compounds are more closely related to the structure of the hapten used for production of the antibody, and thus a larger group would be more securely accommodated at this site. Even replacement of the 3-hydroxy group with a very large 3-O-glucuronide group (forming morphine glucuronide), which has a much different polarity, only slightly reduces reactivity (Table 1). This contrasts greatly with the large reduction of reactivity caused by replacing the 6-hydroxy group of codeine with a 6-O-glucuronide group (forming codeine glucuronide) (Table 1).

While the opiate 5-ring structure is essential for strong reactivity, removal of the 4,5-oxygen bridge (removal of the oxygen ring) does not necessarily eliminate reactivity entirely. Studies with four 4-ring opiates (levorphanol, levallorphan, dextrorphan, and methorphan) indicate that l-forms of these 4-ring opiates (levorphanol and levallorphan) retain some reactivity, whereas d-forms (dextrorphan and methorphan) have no reactivity (Table 1). The l-forms more closely resemble the spatial configuration of morphine which is an l-form also. It is interesting to note also that l-forms of the morphinan series have the analgesic and addictive properties of morphine, while d-forms do not [10].

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