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Narcotic Antagonist Activity of Several Metabolites of Naloxone and Naltrexone Tested in Morphine Dependent Mice[1] (38558)

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Naloxone and naltrexone are antagonists to the narcotic analgesics. Our interest in the metabolism of these two compounds has been stimulated by our observation of species differences in metabolic disposition. For naloxone (Fig. 1, I) we have that in man (1) and the rabbit (2), one metabolite formed is naloxone-3-glucuronide. In the chicken (2), the major metabolite is *N*-allyl-14-hydroxy-7,8 - dihydronormorphine - 3 - glucuronide where the 6-keto group of naloxone has been reduced. More recently, Cone *et al.* (3, 4) and we (5) have found that naltrexone (Fig. 1, II) is reduced in man to give *N*-cyclopropylmethyl - 14 - hydroxy - 7,8 - dihydronorisomorphine. The reduction of the 6-keto group yielded a product with a 6β-OH configuration. In the chicken, the reduction gave the 6α-OH product(5). Since all of the commercially available 6-OH morphine type compounds are of the α epimer form, the formation of the 6β-OH epimer as a metabolite is of particular interest in view of the possible pharmacologic activity that such products may possess.

The purpose of this paper is to present a method for enzymatically producing limited quantities of the 6β-OH epimers. The result of testing these compounds in morphine dependent mice for narcotic antagonist action is also described.

Methods. Biosynthesis of N-allyl-14-hydroxy-7,8-dihydronorisomorphine (III) and N-cyclopropylmethyl-14-hydroxy-7,8-dihydronorisomorphine (IV). The partial characterization of an enzyme system from the liver cytosol of rabbits has been described by Pollock and Fujimoto (6). This enzyme system used to reduce naloxone and naltrexone consisted of the following in each of four flasks: 290 μmole glucose-6-phosphate, 0.5 μmole NADP; 5 units glucose-6-phosphate dehydrogenase; 0.8 ml 0.05 *M* KH_2PO_4/NaOH buffer, pH 7.4; 40 μmole naltrexone or naloxone; 0.5 ml rabbit enzyme preparation; final volume 3 ml. The rabbit enzyme preparation was made by homogenizing 57 g of liver in 114 ml 0.05 *M* phosphate buffer, pH 7.9, taking all of the 100,000*g* supernatant and performing an ammonium sulfate fractionation. The protein fraction precipitating between 45 to 65% saturation with ammonium sulfate was isolated as a pellet; this pellet was suspended in 5 ml of 0.005 *M* phosphate buffer, pH 7, and used as stated above. After incubation of the flasks at 37° for 3 to 5 hr in a Dubnoff shaker, the mixture was adjusted to pH 8.5 (by adding sodium hydroxide solution until basic and dissolving about 1 g of $NaHCO_3$) and the alkaloids were extracted with ethyl acetate. The subsequent procedures for isolating the 6β-OH epimers were like those described for isolation of the 6α-OH epimers from an *in vitro* hepatic enzyme preparation of the chicken (5). About 6 mg each of III and IV were obtained as the hydrochloride salt.

Nuclear magnetic resonance spectra and gas chromatographic analysis. The NMR procedure and instrument used were those described previously (5) for characterizing the human and chicken metabolites.

For gas chromatography, a 1 or 2 mg/ml solution in water was made of each of the samples, 0.1 ml of this solution was brought to pH 8.5 by addition of solid $NaHCO_3$ and extracted with 0.2 ml ethyl acetate. A 0.1 ml sample of the ethyl acetate extract in a tube was evaporated to dryness under N_2. Twenty μl bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1 μl tetraphenylethylene

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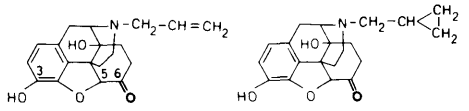

I NALOXONE II NALTREXONE

EN 2265 (6α-OH) EN 2260 (6α-OH)

III (6β-OH) IV (6β-OH)

FIG. 1. Formula of naloxone (I) and naltrexone (II) and their respective reduced metabolites, 6α and 6β-OH naloxone and 6α and 6β-OH naltrexone.

(TPE) 1 mg/ml in benzene, were added and silylation carried out for 30 min at room temperature. Samples of 0.5 μl of this mixture were injected into the gas chromatograph, a Perkin Elmer Model 900 equipped with a flame ionization detector. The column was 6 ft × ⅛ in. 10% QF-1 on Chromsorb Q 80–100 mesh. Conditions were: Oven 240°, injector 300° and manifold 260°. Flow rates for nitrogen, hydrogen and air were 55, 45 and 900 ml/min respectively.

Standard compounds. Naloxone, naltrexone, EN 2265A, EN 2260A (all as hydrochloride salts where the suffix A in the latter two compounds refers to the salt form) were obtained from Endo Laboratories, Garden City, NY. Near the end of our study, chemically synthesized 6β-OH congeners were provided as standards by Chatterjie *et al.* (unpublished material) for gas chromatographic comparisons with our biosynthetic material.

Test for narcotic antagonist activity in morphine pellet implanted mice. Male mice weighing 18–25 g were implanted subcutaneously with morphine pellets by the procedure of Way *et al.* (7). The pellets were obtained from the Private Formulae Co., St. Louis, MO and each pellet contained 75 mg of morphine base. After 72 hr, metabolites III and IV, naloxone (I), naltrexone (II), EN 2265A (*N*-allyl-14-hydroxy-7,8-dihydronormorphine, the 6α-OH epimer of III) and EN 2260A (*N*-cyclopropylmethyl-14-hydroxy-7,8-dihydronormorphine, the 6α-OH epimer of IV) were given intravenously into the tail vein. All compounds were dissolved in 0.9% sodium chloride and given in a volume no larger than 0.1 ml/10 g body wt. The solutions were warmed to about 37°. All doses are given in terms of the hydrochloride salts of the compounds. Immediately after the intravenous injection, the mouse was placed on a 30 × 30 cm platform. The jumping response as described by Way *et al.* (7) was used as an indication of narcotic antagonist activity. A response was recorded as being positive if the mouse jumped off the platform within 15 min. The ED50's and potency ratios were estimated by the method of Litchfield and Wilcoxon (8). The number of mice at each dose is given on the graph presenting these data.

Results and Discussion. The dose response curves for the ability of the compounds to produce jumping in 3-day morphine implanted mice are shown in Fig. 2. The respective ED50 and 95% confidence intervals are given in Table 1 as well as the relative potency ratio. All curves were shown to be parallel so that meaningful potency ratio relationships could be derived. Note that the parent compound naloxone, EN 2265A and the 6β-OH metabolite (III) have significantly different ED50 with the relative order of potency being naloxone > EN 2265A > III. In the naltrexone series, EN 2260A is also more potent than the 6β-OH epimer, IV. Thus, there is an interesting separation in activity of the α and β epimers for both naloxone and naltrexone series. Dayton and Blumberg (9) reported that in a variety of tests as a narcotic antagonist, EN 2265A was from one fifth to one tenth as potent as naloxone. In the present test, the potency of EN 2265A was about one ninth that of naloxone. Looking at the study of Eddy (10) it is interesting that in the series dihydromorphinone, dihydromorphine (which is 6α-OH) and dihydro-α-isomorphine (which according to the nomenclature used by Eddy refers to the 6β-OH compound), the most potent analgesic was dihydromorphinone followed by dihydromorphine and dihydro-α-isomorphine. Thus potencies for agonist and antagonist activities appear to be greater for the keto than the α and β-OH epimers. On the other hand for the series dihydrocodeinone, dihydrocodeine (the 6α-OH) and dihydro-α-isocodeine (the 6β-OH), the analgesic potency of the ketone was greatest but the 6α-OH compound (dihydrocodeine) was about one seventh the potency of the 6β-OH

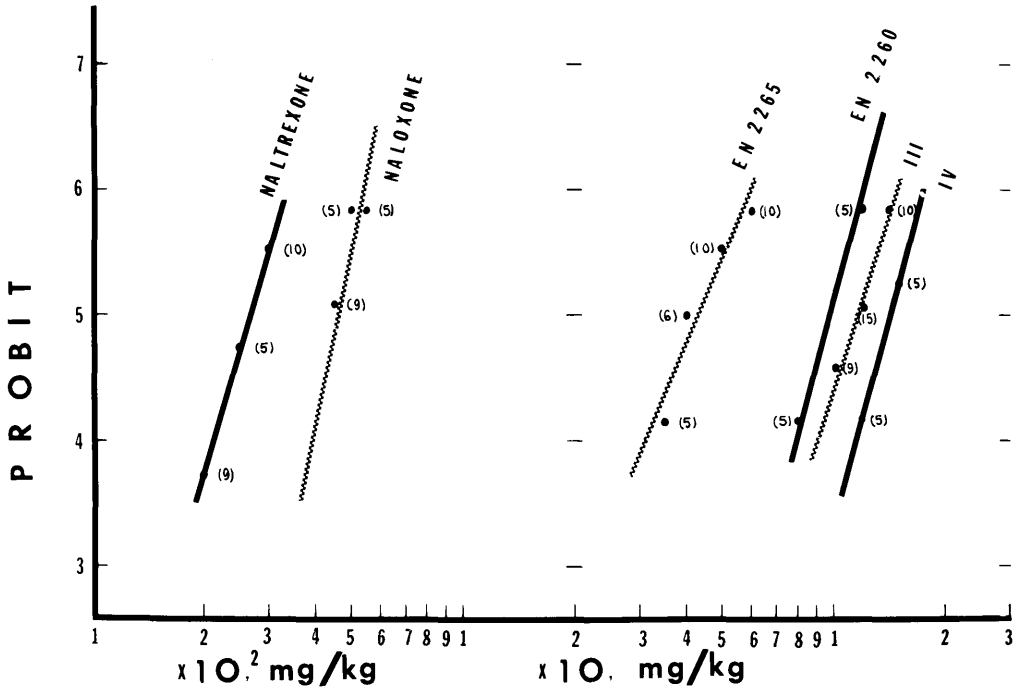

FIG. 2. Dose response relationship for jumping produced in morphine pellet implanted mice after intravenous administration of the compounds. The number in parentheses is the number of mice tested at a given dose.

TABLE I. ED50, POTENCY RATIO AND 95% CONFIDENCE INTERVAL OF NALOXONE, NALTREXONE, EN 2265, EN 2660, III AND IV in 3 DAY MORPHINE PELLET IMPLANTED MICE. FIGURES IN PARENTHESES ARE 95% CONFIDENCE INTERVALS. REFER TO FIG. 1 FOR STRUCTURE OF COMPOUNDS; ED50 ARE EXPRESSED AS mg/kg FOR THE HCl SALTS OF THE COMPOUNDS. FOR CALCULATING THE POTENCY RATIO THE ED50 OF THE LESS POTENT COMPOUND WAS DIVIDED BY THE ED50 OF THE MORE POTENT COMPOUND.

	ED50					
	0.046 (0.042–0.050)	0.026 (0.022–0.031)	0.43 (0.36–0.51)	1.14 (0.98–1.32)	0.99 (0.88–1.18	1.40 (1.18–1.67)
	Naloxone	Naltrexone	EN 2265	III	EN 2260	IV
Naltrexone	1.7 (1.4–2.1)					
EN 2265	9.4 (7.8–11.2)					
III	24.8 (21.0–29.2)		2.7 (2.1–3.3)			
EN 2260		37.5 (29.8–47.2)	2.3 (1.8–2.9)			
IV		53.0 (42.1–66.8)		1.23 (1.0–1.5)	1.41 (1.1–1.8)	

compound (dihydro-α-isocodeine) (10). Thus relative orders of potencies cannot be confidently extrapolated and it is necessary to actually test each of the α and β epimers for activity. Just as narcotic agonist activity is commonly associated with certain other pharmacologic actions and requires a variety of testing approaches, similarly narcotic antagonist activity cannot be fully assessed by a single test. Thus ability to induce jump-

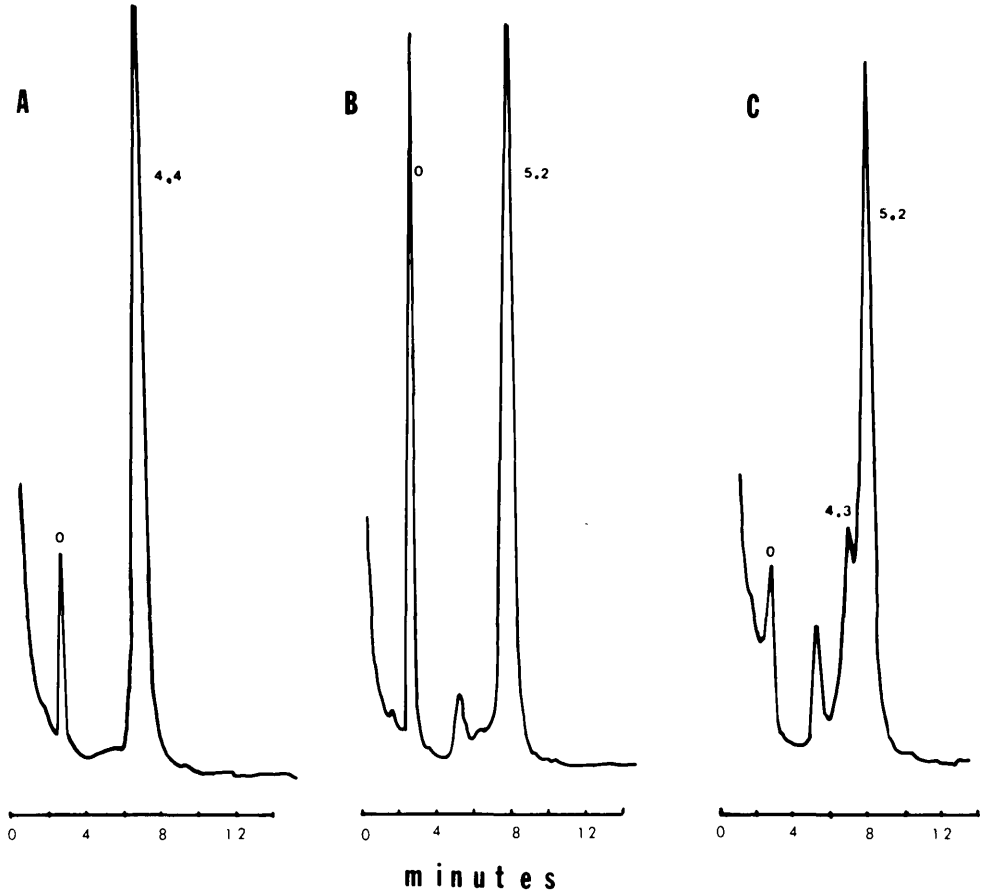

FIG. 3. Gas chromatogram of silylated (A) EN 2265 (6α-OH naloxone), (B) standard 6β-OH naloxone and (C) sample prepared by enzymatic process. The numbers on the peaks designate retention times in minutes relative to the interval standard, tetraphenylethylene, given a retention time of 0. Silylated naloxone had a retention time of 14.9 min relative to the interval standard.

ing in mice is but one of a number of tests and the potency relationship between compounds may change substantially with the test used. But, it has not been possible to do additional tests in the present study because of the limitation in amount of particularly the 6β-OH metabolites.

The NMR spectral data for metabolite IV indicated the 5β-H (doublet) appeared at δ 4.52 (J = 6 Hz) and 6α-H (multiplet) at δ 3.65–3.45. These chemical shift values were similar to and consistent with those found previously for the reduced naltrexone metabolite isolated from *human* urine. Thus, metabolite IV in the rabbit was *N*-cyclopropylmethyl - 14 - hydroxy - 7,8 - dihydronorisomorphine where the OH group at position 6 had a 6β-OH configuration. For metabolite III, the 6β-OH orientation was assigned by examining the NMR spectral data for the various morphine and isomorphine analogues published previously (Chatterjie *et al.*) in relation to the present data: The 5β-H showed signals at δ 4.50 (J = 6 Hz, doublet) and at δ 3.70–3.40 (6α-H, multiplet).

Figures 3 and 4 give the results of the gas chromatographic analysis of the biosynthetic samples compared to those for 6α and 6β-OH standards. The results indicate that both metabolites III and IV appear to have appreciable amounts of the 6α-OH compounds. Since this chromatographic method is not yet quantitative, the amount of 6α-OH compounds present cannot be stated (a rough guess might be 20%). Even though NMR is

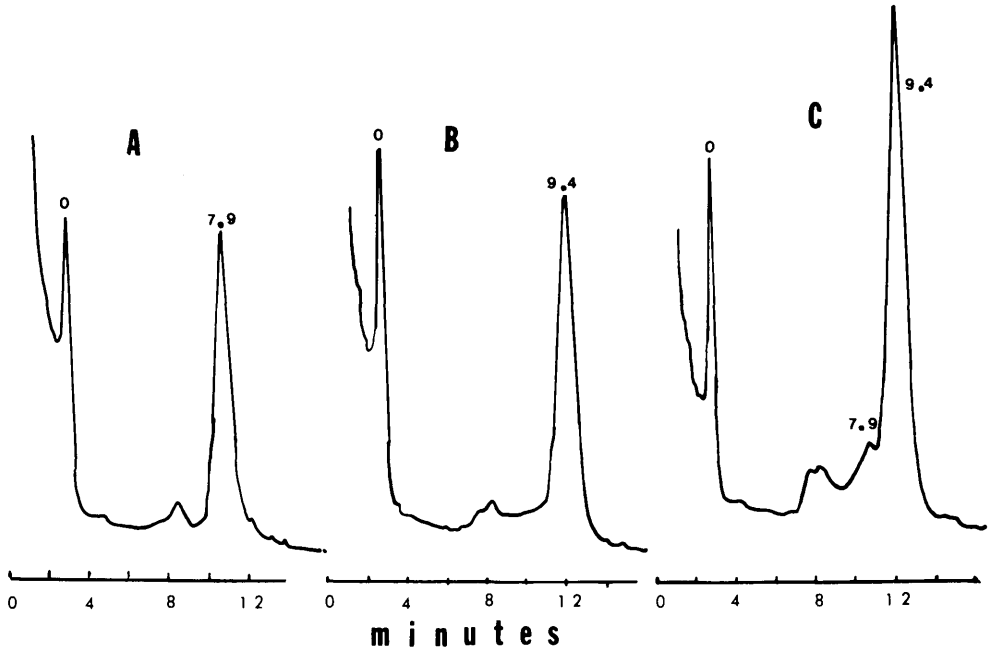

FIG. 4. Gas chromatograph of silylated (A) EN 2260 (6α-OH naltrexone), (B) standard 6β-OH naltrexone and (C) sample prepared by enzymatic process. Silylated naltrexone had a retention time of 24.1 min relative to the internal standard. Legend as in Fig. 3.

generally not as sensitive as gas chromatography, we feel that with the Fourier transform computerized spectra, the presence of any more than 5% 6α-OH compound in the samples should have been detected. Thus, this aspect remains an unresolved problem. It is possible that what apparently appears as the 6α-OH compound on the gas chromatograms is really something else and the NMR results reflect this possibility. The presence of this ambiguity means that the narcotic antagonist potencies given in Fig. 2 and Table 1 must be interpreted cautiously. While this study was in progress, Cone, Gorodetzky and Yeh (11) reported on the biosynthesis and isolation of 6β-OH naltrexone (same as our metabolite IV) from the urine of guinea pigs. Their material had 3% of the 6α-OH compound. Thus, in the animal species studied thus far, it has not been possible to obtain only the 6β-OH compound. Since Cone *et al.* were able to isolate 120 mg of material from the guinea pig urine, their procedure provides more material for biological testing. We should look to their future publication for results for comparison with ours.

As stated in the introduction, assessment of activity of the 6α and β reduction products of naloxone and naltrexone is important because these compounds are formed as metabolites in a relatively species specific manner. It is known from the work of Cone *et al.* (3) and Chatterjie *et al.* (5) that substantial amounts of 6β-OH naltrexone is produced after naltrexone administration in man. On the other hand, it appears that little 6β-OH naloxone is formed in man according to the work of Weinstein *et al.* (13) Fujimoto (1). From the present study, it seems unlikely that the formation of the reduced metabolites would contribute substantially to the narcotic antagonist action of the parent compounds. In considering the agonist action of naloxone in the pigeon, McMillan *et al.* (12) concluded that formation of EN 2265 probably contributed little to the action of naloxone.

Summary. A rabbit liver enzyme system was used to produce the 6β-OH reduced metabolites of naloxone and naltrexone. GC analysis indicated the presence of some 6α-OH metabolite in these samples. The narcotic antagonist activity of these 6β-OH

metabolite samples were compared to naloxone, naltrexone and standard 6α-OH naloxone (EN 2265A) and 6α-OH naltrexone (EN 2260A) using the jumping response of morphine pellet implanted mice. For the naloxone series, the potencies were: Naloxone > EN 2265A > 6β-OH naloxone. For the naltrexone series: Naltrexone > EN 2260A > 6β-OH naltrexone. The low potency of the reduced metabolites and the rapid onset of action of the parent compounds militate against the formation of these metabolites contributing substantially to the overall narcotic antagonist action of the parent compounds.

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1. Fujimoto, J. M., Proc. Soc. Exp. Biol. Med. **133,** 317 (1970).
2. Fujimoto, J. M., J. Pharmacol. Exp. Ther. **168,** 180 (1969).
3. Cone, E. J., Yeh, S. Y., and Gorodetzky, C. W., Pharmacologist **15,** 242 (1973).
4. Cone, E. J., Tetrahedron Letters **28,** 2607 (1973).
5. Chatterjie, N., Fujimoto, J. M., Inturrisi, C. E., Roerig, S., Wang, R. I. H., Bowen, D. V. Field, F. H., and Clarke, D. D., Drug Metab. Disp. **2,** 401 (1974).
6. Pollock, S. H., and Fujimoto, J. M., Pharmacologist **16,** 226 (1974).
7. Way, E. L., Loh, H. J., and Shen, F., J. Pharmacol. Exp. Ther. **167,** 1 (1969).
8. Litchfield, J. T., Jr., and Wilcoxon, F., J. Pharmacol. Exp. Ther. **96,** 99 (1949).
9. Dayton, H. B., and Blumberg, H., Fed. Proc **28,** 736 (1969).
10. Eddy, N. B., J. Pharmacol. Exp. Ther. **56,** 421 (1936).
11. Cone, E. J., Gorodetzky, C. W., and Yeh, S. V., Pharmacologist **16,** 225 (1974).
12. McMillan, D. E., Wolf, P. S., and Carchman, R. A., J. Pharmacol. Exp. Ther. **175,** 443 (1970).
13. Weinstein, S. H., Pfeffer, M., Schor, J. M., Indindoli, L., and Mintz, J., J. Pharm. Sci. **60,** 1567 (1971).

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