Biosynthesis, Isolation, and Identification of 6β-Hydroxynaltrexone, a Major Human Metabolite of Naltrexone

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Abstract □ Chemical reduction of naltrexone is described in an attempt to synthesize 6β-hydroxynaltrexone. Only the epimer, 6α-hydroxynaltrexone, was produced. Pilot metabolic studies on naltrexone in the dog, rat, and guinea pig were made to determine which animal produced the greatest amount of 6β-hydroxynaltrexone. The guinea pig was selected and used to produce the metabolite. Isolation and purification methods are described, and spectral data are presented for structural confirmation of the metabolite.

Keyphrases □ Naltrexone—chemical reduction to 6α-hydroxynaltrexone, metabolism in dog, rat, and guinea pig □ 6α-Hydroxynaltrexone—synthesis *via* chemical reduction of naltrexone □ 6β-Hydroxynaltrexone—isolation and identification as naltrexone metabolite in guinea pig

Naltrexone (I) is extensively metabolized in humans at the C-6 carbonyl position to 6β-hydroxynaltrexone (*N*-cyclopropylmethyl-7,8-dihydro-14-hydroxynorisomorphine) (II), with the C-6 configuration isomeric to that of morphine (1). The role of this metabolite as a possible contributor in the pharmacological response of naltrexone was of interest. A suitable quantity of II was needed for pharmacological testing and standardization of analyses of biological fluids. Several chemical reduction procedures failed to produce the metabolite with the correctly oriented β-hydroxy group. An alternative route of preparation was employed using animals as the stereospecific reducing agent. This report details the preparation of 6β-hydroxynaltrexone.

EXPERIMENTAL

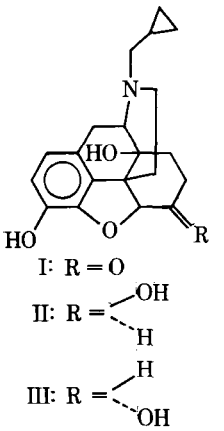
Instrumentation—*GLC*—A gas chromatograph[1] equipped with dual flame-ionization detectors and 2-mm × 1.83-m (6-ft) glass columns packed with 3% OV-17 on Gas Chrom Q, 60–80 mesh, was used. The detector and injector temperature was 280°. A temperature program was employed from 200 to 270° at a rate of 8°/min. The nitrogen carrier gas flow was 24 ml/min. The retention times of the internal standard, tetraphenylethylene, and the pentafluoropropionic anhydride derivatives of I, II, and III (two peaks) were 10.36, 5.81, 5.48, and 4.67 followed by 7.67 min.

IR—The IR spectra were obtained[2] as a KBr matrix (1%) with a 20-min scan length.

Mass Spectrometry—All mass spectra were recorded on a quadrupole GC–mass spectrometer[3]. The samples were introduced *via* the solid probe inlet, and the temperature was brought up gradually until the spectral pattern appeared. The ionizing potential was 70 ev, and the accelerating potential was 2.5 kv.

TLC Systems—*System I*—Glass fiber sheets[4] were developed with chloroform saturated with aqueous ammonia. The R_f values for I, II, and III were 0.62, 0.16, and 0.20, respectively.

System II—Silica gel G developed with benzene–diethylamine (4:1) was used. The R_f values for I, II, and III were 0.76, 0.35, and 0.35, respectively.



I: R = O

II: R = OH / H

III: R = H / OH

Attempted Chemical Synthesis of II—*Sodium Borohydride Reduction*—To 125 mg of I[5] in 25 ml of dioxane and 15 ml of methanol was added 250 mg of sodium borohydride in small portions. An immediate exothermic reaction was observed. The mixture was allowed to stir at room temperature for 2.5 hr. TLC analysis (System I visualized with potassium iodoplatinate spray) revealed that complete reduction had occurred. The solvent was evaporated under vacuum, and the residue was dissolved in 30 ml of 2 *N* NaOH and 20 ml of 40% (w/v) K_2HPO_4. The solution was extracted with chloroform, and the solvent was evaporated under vacuum. GLC analysis of the silylated residue (10% silylating reagent[6] in acetonitrile heated at 70° for 1 hr) confirmed that complete reduction to the epimer of II, *N*-cyclopropylmethyl-7,8-dihydro-14-hydroxynormorphine[5] (III) had occurred.

Sodium Amalgam Reduction—Compound I (200 mg) was reduced with 1.5 g of 5% sodium amalgam (Na-Hg) using the procedure reported by Blount *et al.* (2). The chloroform extract was analyzed by TLC (System I and visualized with potassium iodoplatinate spray) and GLC. The product of the reaction was unrelated to II or III and remained unidentified.

Iridium Tetrachloride Reduction—To a solution of iridium tetrachloride[7] (50 mg) in 1 ml of concentrated hydrochloric acid was added 40 ml of water followed by 11 ml of trimethylphosphite. Fifty milligrams of I in 40 ml of 2-propanol was added, and the mixture was refluxed overnight. After cooling, the mixture was made basic with 10 *N* NaOH and extracted with chloroform. Evaporation of solvent was followed by derivatization with pentafluoropropionic anhydride[8] (heated as a neat solution at 70° for 3 hr) in an acylation tube[9]. GLC analysis revealed that reduction to III was about 50% complete without a trace of II present.

Metabolic Studies on Animals—Pilot studies were performed on the dog, rat, and guinea pig to ascertain which species produced II as a metabolite of I. The animals were housed in stainless steel metabolic cages equipped with urine collectors. A single 50-mg dose of I was injected subcutaneously, and urine was collected periodically over 68 hr. The samples were filtered and refrigerated until analysis.

Two-milliliter aliquots of each collection were made basic (pH 9)

[1] Varian model 2700.
[2] Beckman model IR 18A.
[3] Finnigan model 1015D.
[4] Gelman I.T.L.C. type SG, Gelman Instrument Co., Ann Arbor, Mich.
[5] Provided by Endo Pharmaceutical Co., Garden City, N.Y.
[6] Regisil, Regis Chemical Co., Morton Grove, Ill.
[7] Pfaltz and Bauer, Inc., Flushing, N.Y.
[8] Pierce Chemical Co., Rockford, Ill.
[9] Regis Chemical Co., Morton Grove, Ill.

Table I—Urinary Excretion of Free and Conjugated Naltrexone and 6β-Hydroxynaltrexone in Guinea Pig and Dog

	Distribution in Urine[a]						
	Guinea Pig				Dog		
	0–2 hr	2–20 hr	20–44 hr	44–60 hr	0–5 hr	5–20 hr	20–96 hr
Naltrexone (I)	0.2	16.4	0.2	0.1	0.7	0.2	0.2
Conjugated naltrexone	1.2	58.8	1.6	0.2	161.3	24.8	0.9
6β-Hydroxynaltrexone (II)	—[b]	54.5	5.9	0.1	—[b]	—[b]	—[b]
Conjugated 6β-hydroxynaltrexone	—[b]	26.2	13.9	1.0	—[b]	—[b]	—[b]

[a] Values are reported in micrograms per milliliter for the collection period. [b] Not detectable.

with 2 *N* NaOH. One milliliter of 40% (w/v) K_2HPO_4, 0.5 g of sodium chloride, and 10 ml of chloroform were added, and the contents were shaken for 10 min. The aqueous phase was withdrawn and saved for acid hydrolysis of the conjugated drug and metabolites. The organic phase was transferred to a clean tube containing 3 ml of 2 *N* HCl and shaken for 10 min. The organic phase was discarded and the pH of the acid phase was adjusted to 9 and buffered with phosphate buffer. Ten milliliters of chloroform was added, and the contents were shaken for 10 min. The aqueous phase was aspirated and the organic phase was transferred to an acylation tube. The solvent was evaporated under nitrogen, and the residue was derivatized with pentafluoropropionic anhydride for 3 hr at 70°. Excess reagent was removed under nitrogen, and 100 μl of ethylene dichloride was added. The solution was analyzed immediately by GLC. Levels of I and II were estimated by absolute peak height based on the response of a known quantity of I.

The aqueous phase containing the conjugated drug and metabolites was hydrolyzed with excess concentrated hydrochloric acid in an autoclave for 0.5 hr at 115° and 1.27 kg/cm². After cooling, the solution was neutralized with solid potassium carbonate and extracted as described. The residue was derivatized with pentafluoropropionic anhydride and analyzed by GLC.

Isolation of II from Guinea Pig Urine—Ten white guinea pigs were injected subcutaneously twice daily with 25 mg of I in 0.5 ml of saline for 10 days. The urine was collected daily, filtered, and centrifuged, and the supernate was stored in the refrigerator.

The samples were extracted twice with chloroform (1.5 ml/ml of sample) following the adjustment of the pH to 9–9.5 with 10 *N* NaOH, buffering (40% K_2HPO_4, 1 ml for 7 ml of sample), and salting (sodium chloride, 1 g for each milliliter of sample). The combined organic extracts were centrifuged and dried over anhydrous potassium carbonate. The chloroform was removed under vacuum, and the residue was taken up in a small volume of solvent. The solution was applied to thin-layer plates and chromatographed (System II, 1000-μm layer). A 1-cm wide section of each side of the chromatogram was sprayed with potassium iodoplatinate for visualization of II. The band (R_f 0.27–0.50) corresponding to the region (R_f 0.35) where II was located from human urine was preparatively removed. The gel was washed with methanol, and the solvent was evaporated under nitrogen. The TLC isolation procedure was repeated, giving a brown oily residue. Further purification was necessary and was accomplished by solid–liquid adsorption chromatography.

The residue was fractionated into 70 10-ml fractions on a column of neutral alumina, 1.27 × 30.48 cm (0.5 × 12 in.), under gradient elution conditions using mixtures of benzene–chloroform and chloroform–methanol as the eluting solvents[10]. The fractionation procedure was monitored by TLC [System I visualized by spraying with potassium ferro-ferricyanide reagent (3) and observing under UV (254 nm)]. Fractions 54–64 [eluting solvent consisted of chloroform–methanol (4:1)] were combined and evaporated to a residue under nitrogen. The residue was extracted with chloroform, and the extract was treated with hydrogen chloride gas. A white solid precipitated immediately. The solvent was decanted, and the solid was dried under nitrogen. The solid was fractionally crystallized and recrystallized in a mixture of methanol–ether–heptane (9:1:1) to give fine, white needles of II. GLC and TLC (System I) analyses as described revealed II to contain about 3% III. The total yield of II was 187 mg (3.7% yield).

[10] The percent of chloroform in benzene was increased from 0 to 100 followed by increasing the percent of methanol in chloroform from 0 to 100 during the fractionation procedure.

RESULTS AND DISCUSSION

The complete lack of II in the chemical reduction of I prompted the consideration of a biosynthetic route as a means of preparing the metabolite. Pilot studies on the dog and guinea pig allowed estimates to be made of concentration levels of both free and conjugated I and II (Table I). Only minor amounts of free I were present in the urine of the dog, with a correspondingly high level of conjugated I in the early collections. The very active conjugation step (probably glucuronidation) along with an absence of II could account for the short biological half-life of naltrexone in the dog ($t_{1/2}$ = 2 hr) (4) as compared to humans ($t_{1/2}$ = 12 hr) (5).

The results of the study on the rat were inconclusive for the presence of II. Both free and conjugated I were detected in the

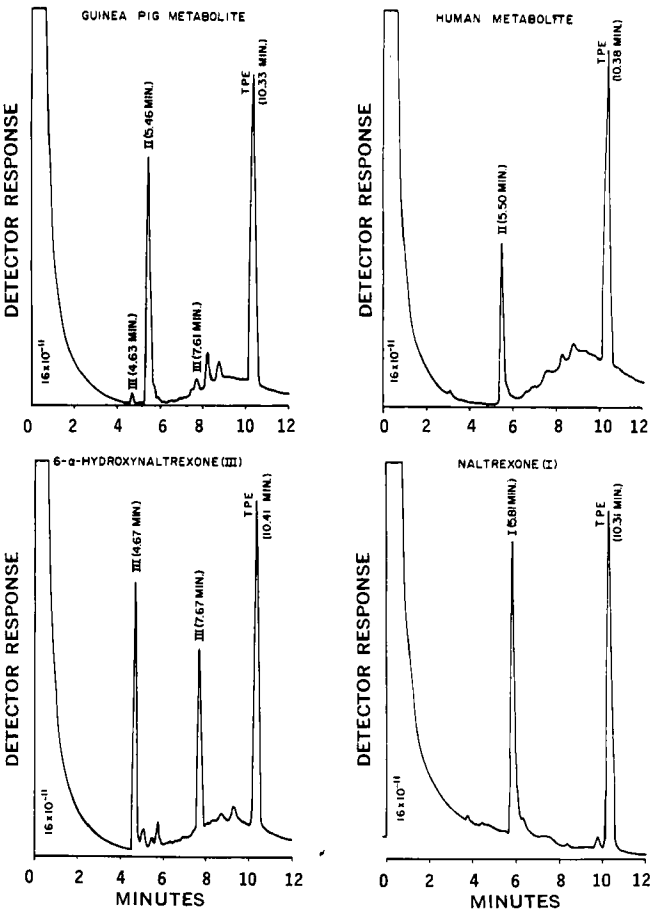


Figure 1—*Gas chromatogram of pentafluoropropionic anhydride derivatives of 6β-hydroxynaltrexone (II) isolated from the guinea pig and human (top) and 6α-hydroxynaltrexone (III) and naltrexone (I) (bottom). The internal standard was tetraphenylethylene (TPE).*

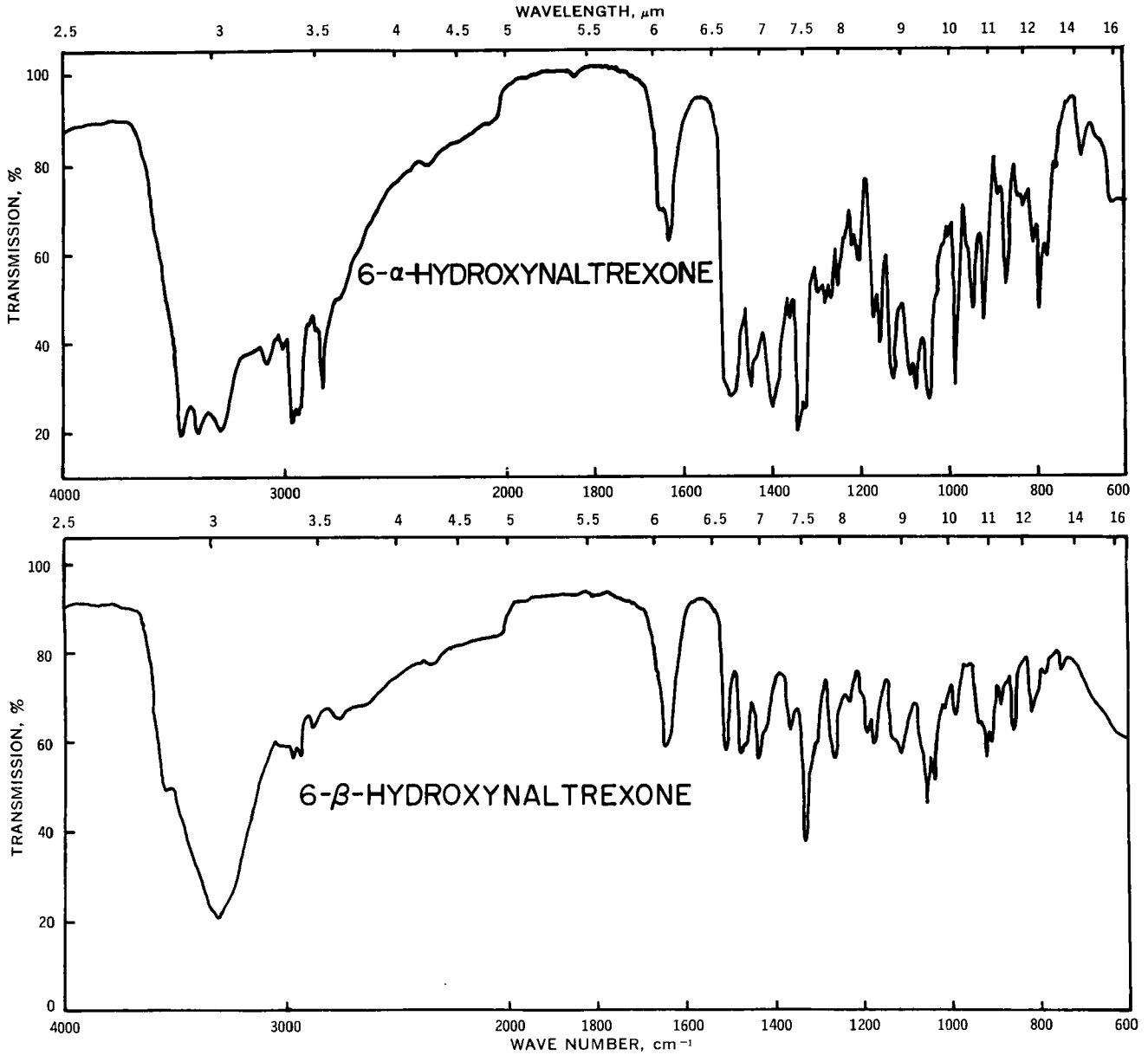


Figure 2—*IR spectra of 6α- and 6β-hydroxynaltrexone.*

urine. A small peak corresponding to the retention time of II was present in the gas chromatogram of unhydrolyzed rat urine extract. However, the relative concentration over background in the control sample made detection equivocal.

Free and conjugated I and II were present in the urine of the guinea pig. The level of free II was comparable to conjugated I while the level of free I was somewhat less than that of conjugated II. These results were encouraging, and use of the guinea pig as a preparative vehicle proved successful. Compound II was stereospecifically produced as a metabolite of I, isolated, and purified by chromatography to give pure crystalline needles of the hydrochloride.

The purity of II was evaluated by TLC (Systems I and II) and GLC (Fig. 1). The presence of about 3% III was confirmed by both techniques. A close separation of II and III can be achieved on TLC (System I) using small samples. Incomplete derivatization with pentafluoropropionic anhydride was characteristic for III and can be used beneficially for GLC identification. The lack of complete derivatization of III as opposed to II is probably explained by the lesser accessibility of the 6α-hydroxy group for derivatization. Inspection of Dreiding models of II and III indicated that the 6α-hydroxy group of III (axial position, chair form) is probably hydrogen bonded to the furan oxygen and that the 6α-hydroxy group is more sterically hindered by the surrounding ring system than that of II (equatorial position, chair form).

The origin of III in II was questioned as possibly being an artifact of the isolation procedure. To address this problem, the ratio of II to III was monitored through the purification process. Since the ratio remained essentially unchanged, it was concluded that III was a metabolite.

The GLC chromatograms of the pentafluoropropionic anhydride derivatives of I, II (obtained from human and guinea pig), and III are presented in Fig. 1. The internal standard, tetraphenylethylene, was included for comparison of relative retention times. The metabolite from humans (1) and guinea pig had the same retention time and are resolved from I and III when cochromatographed.

The IR spectra of the epimers, II and III, are shown in Fig. 2. The characteristic lack of the carbonyl band (1730 cm^{-1}) found in I is conclusive evidence for the reduction of the carbonyl bond. The fingerprint regions (910–1430 cm^{-1}) of II and III differ considerably and provide an extra means of identification of the metabolite.

The mass spectra of II and III are shown in Fig. 3. As expected, the pattern of fragmentation is quite similar. The loss of a hydroxy group ($M^+ - 17$) is typical for the reduced form, and other easily identifiable fragmentations are indicated. In addition, Fig. 3 con-

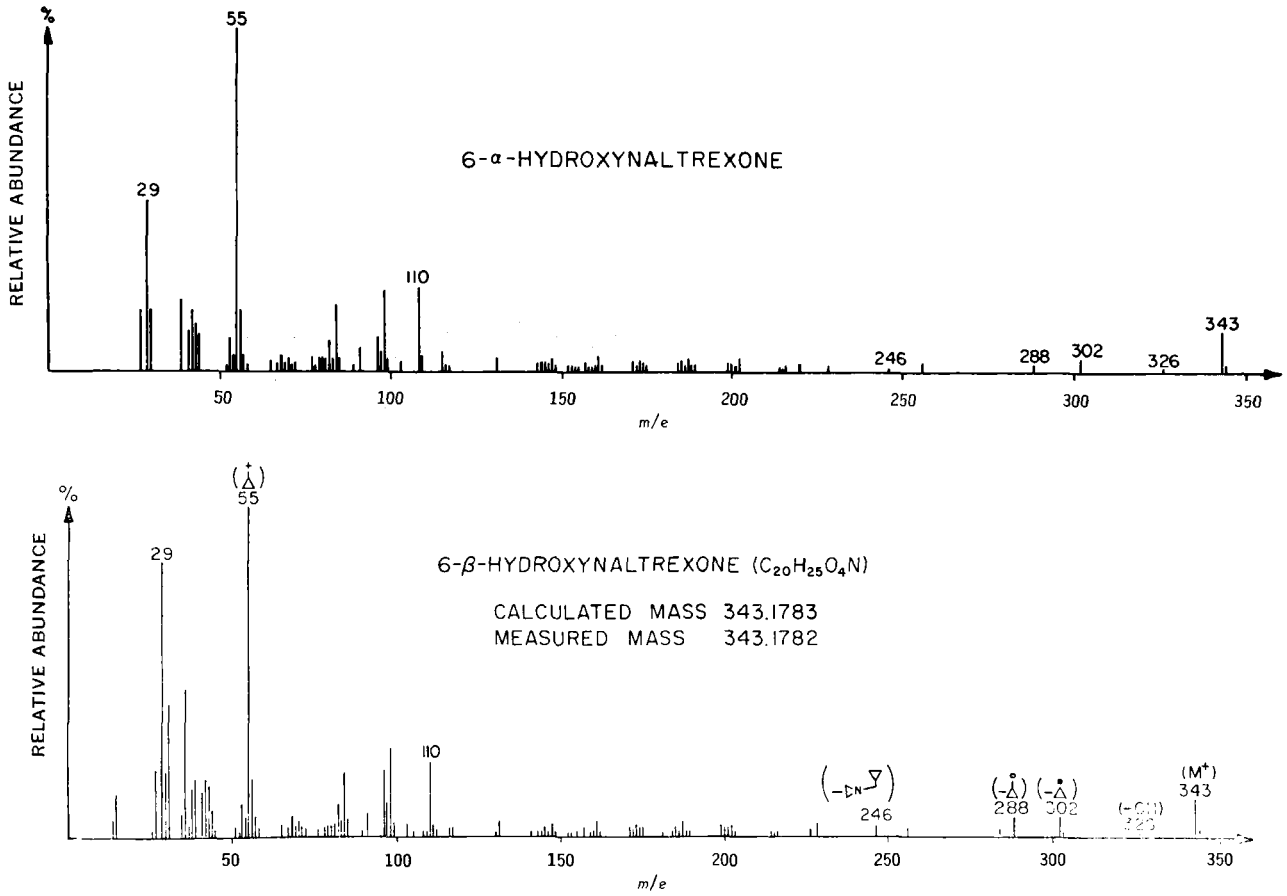


Figure 3—*Mass spectra of 6α- and 6β-hydroxynaltrexone.*

tains the high-resolution mass spectral analysis of the molecular ion of II at *m/e* 343 and confirms the structural assignment.

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