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Isolation and stereochemical identification of a metabolite of naltrexone from human urine / Nithiananda Chatterjie, James M. Fujimoto, Charles E. Inturrisi, Sandra Reorig, Richard I.H. Wang, David V. Bowen, Frank H. Field, and Donald D. Clarke Department of Pharmacology, Cornell University Medical College (N.C., C.E.I.), Department of Pharmacology, Medical College of Wisconsin (J.M.F., S.R.), Veterans Administrative Center, Wood, Wisconsin (R.I.H.W.), Mass Spectrometry Service Laboratory, The Rockefeller University (D.V.B., F.H.F.), and Department of Chemistry, Fordham University (D.D.C.)

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ISOLATION AND STEREOCHEMICAL IDENTIFICATION OF A METABOLITE OF NALTREXONE FROM HUMAN URINE

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ABSTRACT

Pooled urine samples from patients receiving 100–200 mg of naltrexone per day orally were extracted; the basic (alkaloid) compounds derived were isolated by preparative thin-layer chromatography. The major metabolite of naltrexone was found to be an epimer of N-cyclopropylmethyl-14-hydroxy-7,8-dihydronormorphine wherein the 6-keto group of naltrexone had been reduced to yield the 6β-hydroxy epimer (an isomorphine). This conclusion was based on infrared, mass, and nuclear magnetic resonance spectra studies. Furthermore, the reduction product formed in vitro in a soluble chicken liver enzyme system from naltrexone and an in vivo metabolite of nalozone derived from the chicken were found to have the more commonly expected 6α-hydroxy orientation.

Naltrexone (EN 1639) is a narcotic antagonist which possesses a long duration of action and is currently under evaluation for the prevention of opiate dependence. Because of our interest in the comparative metabolism of a related narcotic antagonist, naloxone (1–3), we undertook the present study. The chemical difference between these compounds is that the alkyl moiety on the nitrogen atom is an allyl group for naloxone (1a) and a cyclopropylmethyl group for naltrexone (1b). The major metabolite of nalozone in man is nalozone-3-glucuronide (2); also a small amount of a 6-keto reduced product, N-allyl-14-hydroxy-7,8-dihydronormorphine-3-glucuronide, is formed (4).

In the rabbit, a similar predominance of nalozone-3-glucuronide is seen (1, 4); however, in the chicken, the dihydronormorphine glucuronide derivative is by far the predominant metabolite. Based on these past studies with nalozone we were surprised to find in the present study that in man a major metabolite of naltrexone was the product of reduction of the 6-keto group; furthermore, it was the 6β-hydroxy (2a) rather than the 6α-epimer (2b). This paper deals with the isolation and identification of this reduction product and gives further justification by way of nuclear magnetic resonance (NMR) spectral data for the 6β-OH configuration initially assigned to the metabolite by Cone et al. (5).

Materials and Methods

Source of Materials. Five gallons of pooled urine from patients receiving 100–200 mg of naltrexone orally per day were sent to the laboratory in Milwaukee (J.M.F.) through the cooperation of Dr. Richard B. Resnick of the Department of Psychiatry, New York Medical College. The urine was preserved for shipment with a layer of toluene. Naltrexone hydrochloride (EN 1639A) and nalozone hydrochloride were generously provided by Dr. Harold Blumberg, Endo Laboratories, Garden City, N.Y., and an authentic sample of EN 2260A, N-cyclopropylmethyl-14-hydroxy-7,8-dihydronormorphine hydrochloride was a gift from Dr. Everett L. May, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, Md. Reduced nalozone identical with EN 2265, N-allyl-14-hydroxy-7,8-dihydronormorphine, was made by us (N.C., C.E.I.) by reducing nalozone with NaBH4. The suffix A (e.g., EN 2260A) refers to the hydrochloride salt. When A is omitted, the free base is meant.

Thin-Layer Chromatography. Major analytical techniques have been described in previous publications (1, 6). The main system for separation of relatively less polar unconjugated metabolites and naltrexone itself consisted of a solvent system of benzene/hexane/diethylamine
(25:10:2, v/v) (BHD) used with Gelman Silica Gel SG sheets. In certain experiments, solvent system A (ethyl acetate/hexane/ethanol/ammonia, 60:25:14:1) was used on Analtech silica gel plates. Iodoplatinate reagent and Dragendorff’s reagent were used to visualize alkaloidal bases, and concentrated H₂SO₄ spray followed by charring on a hotplate was resorted to for detecting organic material.

Isolation of Metabolites from Human Urine. In initial attempts to isolate metabolites we used the Amberlite XAD-2 resin column procedure as dictated by experience in isolating conjugated metabolites of naloxone (1–3), naltorphine (7), morphine (8), and dihydromorphinone (9). It soon became evident, however, that most of the naltrexone metabolites were in unconjugated form and could be purified by solvent extractions as follows. After filtration by mixing with about 0.25 volume of diatomaceous earth, 1200 ml of urine in several 1-liter bottles were made alkaline by adding NaOH pellets until basic to litmus paper. Several grams of NaHCO₃ were then added to each bottle to make the pH about 8.5. This urine was extracted with a total volume of 1000 ml of ethyl acetate. The ethyl acetate phase was decanted and filtered through diatomaceous earth (a procedure which breaks emulsions). The ethyl acetate phase was evaporated under vacuum in a rotating flask apparatus to yield a moderately brown-colored residue. This material was dissolved in 150 ml of ethyl acetate and the mixture was extracted with 40 ml of 0.5 N HCl. The aqueous acid phase was separated and evaporated to dryness under reduced pressure. The residue dissolved in about 1 ml of methanol was streaked several times with a Cordis 50-μl applicator, on two 20 x 20-cm Gelman Silica Gel SG thin-layer chromatography (TLC) sheets. After developing (BHD¹ solvent) and drying, a thin vertical strip (½ in.) of the chromatogram was cut and sprayed with iodoplatinate reagent to locate alkaloids. On the basis of this localization, the sheets were cut horizontally into four sections, covering the following approximate range of RF values: 1.0–0.9, 0.9–0.7, 0.7–0.2, and 0.2–0.0. Each of the horizontal strips was eluted with liberal quantities of methanol (about 10–30 ml, the larger volume for the larger strips) delivered from a wash bottle. The corresponding fractions from the two chromatograms were pooled. On analytical TLC of each of the above four fractions, the RF = 0.7–0.2 material gave a single spot of RF = 0.3. It appeared that the broad band was obtained on the preparative plates because of overloading. No other organic material was perceptible in this major fraction. This material was used for mass and infrared spectral analysis. As a secondary study, the materials recovered in the streaked bands of RF = 1.0–0.9 and 0.9–0.7, both of which contained mixtures of alkaloids as well as other contaminants (seen as ultraviolet fluorescent spots), were pooled and streaked on plates as before. The plates were developed, cut into strips, and eluted with methanol. This procedure gave four separated alkaloids with RF of 1.0, 0.8, 0.7, and 0.5 in the BHD solvent system.

For purposes of NMR determination, an additional series of purification steps was carried out on the major RF = 0.3 fraction. The residue dissolved in HCl was precipitated with ammonium carbonate (pH = 9) from the aqueous layer. This precipitate was extracted into chloroform and subjected to preparative TLC on silica gel plates (Analtech) using solvent system A. This procedure gave a compound which showed RF = 0.49 (solvent system A). The material was extracted into chloroform and treated with Norit, filtered, and lyophilized to give a very small quantity of the free base (naltrexone metabolite). Multiple purifications of this material were carried out by dissolving in a minimum volume of chloroform and adding hexane and allowing the mixture to stand. The solid residues were washed and centrifuged to remove supernatant mother liquor. This procedure yielded a sample of approximately 0.5 mg of the free base, the NMR spectrum of which appeared to reveal a relatively pure compound, except for one absorption in the aliphatic region which was not readily accountable. For consistency, this naltrexone metabolite will be designated as the RF = 0.3 compound (referring back to the RF obtained in the BHD solvent system) or human metabolite.

In Vitro Enzyme System. An enzyme system from the soluble fraction of chicken liver homogenates (10) was used to reduce naltrexone in vitro. The incubation mixture consisted of the following: 31.6 mg of purified enzyme protein mixture, 290 μmol of glucose 6-phos-
phate; 0.5 μmol of NADP⁺; 5 units of glucose 6-phosphate dehydrogenase; 0.8 ml of 0.05 M KH₂PO₄/NaOH buffer, pH 7.4; and 40 μmol of naltrexone, all in a final volume of 3 ml. The purified protein mixture was prepared by making a 100,000 g supernatant fraction of a chicken liver homogenate, preparing a fraction which precipitated at 45–65% saturation with ammonium sulfate, and purifying this fraction further by DEAE-cellulose column chromatography. After incubation for 180 min, the mixture was adjusted to pH 8.5 with strong base and addition of NaHCO₃, and extracted with ethyl acetate. The ethyl acetate phase was reduced in volume and processed by preparative TLC as described above. This metabolite (with RF = 0.3 in BHD) was easily crystallized as the HCl salt and was called chicken metabolite of naltrexone.

An in vivo metabolite of naloxone isolated from chicken urine was included in this study (1). The N-allyl-14-hydroxy-7,8-dihydronormorphine-3-glucuronide was hydrolyzed in hydrochloric acid and the liberated free base form was isolated by extraction as above.

Further Analytical Procedures. For infrared (IR) analysis, methanol solutions of the materials were concentrated to KBr wicks (Wick-Stick, Harshaw Chemicals, Cleveland, Ohio). The tips of the wicks were made into microcubes. IR spectra were obtained with a Perkin-Elmer model 457 infrared spectrophotometer. Proton NMR spectra were run on a Varian XL-100 spectrometer in 5-mm sample tubes in a V-4405A probe. Fourier transform spectra were run with a Transform Technology TT-100 instrument using an NMR-800 computer and a TT-1010 Apulse transmitter. Ninety-degree tilt times for protons average 11 μsec. The Fourier transform spectra were plotted on the XL-100 recorder which was interfaced to the TT-100 instrument. The spectra of the free bases were obtained in CDCl₃. Electron ionization mass spectra reported here were obtained on a Dupont 21-492 mass spectrometer by The Rockefeller University Mass Spectrometry Service Laboratory, New York, N.Y.

Results and Discussion

The isolated materials with RF = 0.3, as discussed in the previous section, chromatographed like authentic EN 2260. The IR curves (not reproduced here) lacked absorption in the 5.8-μ region as did authentic EN 2260A. This result is in accord with Cone (11). The conclusion that the 6-keto group of naltrexone was reduced in both the human and chicken metabolites was thus in keeping with the metabolic reduction observed elsewhere (1, 9).

The mass spectral data given in table 1 helped establish the molecular weight of the human and chicken metabolites to be 343. A good relative order of intensities is seen between authentic EN 2260A and the RF = 0.3 metabolites. All 10 peaks of the known were present in the unknowns, so that the EN 2260A skeleton appeared to be present in the unknowns. Thus, the compounds have a chemical formula of C₂₈H₄₅NO₄. The data so far (IR and mass spectral) pointed clearly to a reduction of naltrexone to the alcohol.

In Table 1, the mass spectral data for the RF = 0.8 fraction indicated that unchanged naltrexone was present in the human urine sample. This fraction also appeared to be contaminated with caffeine because of a strong peak at m/e 194. The other fractions (RF 1, 0.7, and 0.5) did not yield mass spectral data which could be interpreted to indicate N-dealkylated products of either naltrexone or EN 2260 and were not examined further.

In order to arrive at the epimeric nature of the alcohol formed (human and chicken metabolites), we studied the proton NMR of these compounds. This portion of the study was initiated when one of us (N.C.) chemically reduced naloxone to obtain a compound which was found to be identical with EN 2265 as a result of the NMR study. This study gave rise to results which confirmed the epimeric nature of 6α-OH and 6β-OH compounds of various morphine derivatives (having 14-OH substitution). As seen in table 2, the chemical shifts of the C-5 and C-6 protons of the morphinan skeleton of various 14-OH derivatives are listed. We found that the 14-OH group did not seem to appreciably influence the chemical shift of the protons at C-6 in comparison with compounds that lacked the 14-OH group (12). Furthermore, in table 2, the chemical shifts of selected compounds of both the 14-hydroxy-7,8-dihydromorphine type and the isomorphine configuration (6β-OH) are given with respect to the 5β and 6β or 6α proton absorbances. It is seen in compounds having the 6α-OH group such as morphine, EN 2260, and EN 2265 that the 6β proton appears at somewhat lower fields than if it were a proton with 6α orientation (as in 6β-OH compounds). Comparison of the chemical shifts of the 5β and 6β protons of chemically reduced naltrexone and naloxone (EN 2260 and EN 2265, respectively) shows these compounds to be of similar structure (6α-OH orientation). EN 2260 exhibits a doublet centered at δ 4.67 (J = 4.5 Hz) due to the 5β proton and a multiplet between δ 4.38–4.16 due to the 6β proton. For EN 2265 the corresponding values are at δ 4.67 (doublet, J = 4.5 Hz) and δ 4.40–4.18 (multiplet), respectively. These chemical shift values are in good agreement with the values for the corresponding 5β and 6β protons of dihydrocodeine, which has the 6α-OH orientation although it lacks the 14-OH group (11). There is again good agreement between the
The intensities of 10 large peaks in each known spectrum were expressed as per cent of the base (largest) peak. Then the intensity of the peak in the unknown corresponding to the base peak in the known was set equal to 100% and the other nine intensities recalculated to this new base peak for the unknown.

<table>
<thead>
<tr>
<th>m/e</th>
<th>Intensity (EN 2260)</th>
<th>m/e</th>
<th>Intensity (Human)</th>
<th>m/e</th>
<th>Intensity (Chicken)</th>
<th>m/e</th>
<th>Intensity (Naltrexone)</th>
<th>m/e</th>
<th>Intensity (Human)</th>
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<tr>
<td>343</td>
<td>100</td>
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<td>8</td>
<td>288</td>
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<td>12</td>
<td>256</td>
<td>(9)</td>
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<tr>
<td>342</td>
<td>8</td>
<td>342</td>
<td>8</td>
<td>342</td>
<td>10</td>
<td>98</td>
<td>12</td>
<td>98</td>
<td>(51)</td>
</tr>
</tbody>
</table>

TABLE 2
Proton magnetic resonance spectra of various morphinans and metabolites of naltrexone isolated from human urine and chicken liver enzyme preparation, and a metabolite of naloxone isolated from chicken urine

Spectra were run in CDCl₃ solvent using tetramethylsilane as internal standard and are reported in δ values.

<table>
<thead>
<tr>
<th>Morphinan or Metabolite</th>
<th>5β-H</th>
<th>6α-H</th>
<th>6β-H</th>
</tr>
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<tbody>
<tr>
<td>EN 2260 (reduced naltrexone)</td>
<td>4.67</td>
<td>—</td>
<td>4.38-4.16</td>
</tr>
<tr>
<td>EN 2265 (reduced naloxone)</td>
<td>4.67</td>
<td>—</td>
<td>4.40-4.18</td>
</tr>
<tr>
<td>Human metabolite of naltrexone</td>
<td>4.52</td>
<td>3.68-3.38</td>
<td>—</td>
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<tr>
<td>Human metabolite of naltrexone</td>
<td>4.22</td>
<td>—c</td>
<td>—</td>
</tr>
<tr>
<td>Chicken metabolite of naltrexone</td>
<td>4.66</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Chicken metabolite of naltrexone</td>
<td>4.48</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Chicken metabolite of naloxone</td>
<td>4.67</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dihydrodihydroycodeine C (iso configuration)</td>
<td>4.48</td>
<td>3.66-3.38</td>
<td>—</td>
</tr>
<tr>
<td>Dihydrocodeine</td>
<td>4.58</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dihydrosisocodeine</td>
<td>4.37</td>
<td>3.63-3.42</td>
<td>—</td>
</tr>
</tbody>
</table>

a Solvent CDCl₃/CD₃OD.
b Spectra obtained on a 60-mHz spectrometer with CDCl₃ solvent by Dr. K. C. Rice, III.
c 6α-H absorbances under H-OD.

corresponding chemical shift values for the 5β and 6β protons of the in vitro chicken metabolite of naltrexone and the in vivo chicken metabolite of naloxone. These data clearly show that these compounds have the 6α-OH orientation. On the other hand, using the same form of reasoning it is clear that the naltrexone human metabolite should have the 6β-OH orientation because of the chemical shift value of δ 4.52 of the 5β-H doublet (J = 6 Hz) and the upfield shift of the 6α-H where absorbances between δ 3.68 and 3.38 are observed. These values would be similar to those expected of compounds with 6β-OH (6α-H) orientation.

Our assignment based on NMR considerations supports the 6β-OH orientation of the human metabolite of naltrexone (2α) and the 6α-OH orientation for the chicken metabolite. However, it must be said that in both cases the presence of the other epimer in amounts of 5% or less will go undetected. The result on the human naltrexone metabolite confirms the conclusion of Cone et al. (5). In addition, the present NMR analysis approach serves as a powerful basis for subsequent investigations involving species differences in stereochemical enzyme action on related 6-keto compounds.

Acknowledgments. We are thankful to Drs. Ulrich Weiss and K. C. Rice, III, for helping us arrive at the interpretation of the NMR spectra.
and for providing us with reference spectra. We are also thankful to Mr. Charles H. Strom and Mr. Jeffrey Shabanowitz for running Fourier transform NMR and mass spectra, respectively.

References