Pharmaceutical Research, Vol. 7, No. 11, 1990

Technical Note

Morphine Impurity with Opioid Activity Is Identified as 10α-Hydroxymorphine

Hassan Farsam,[1,3] Steven Eiger,[1] Jelveh Lameh,[1] Ahmad Rezvani,[1] Bradford W. Gibson,[2] and Wolfgang Sadée[1,2]

Received January 31, 1990; accepted May 3, 1990

KEY WORDS: morphine, hydroxylated; 10α-hydroxymorphine; morphine, impurity; morphine, hydroxylated, activity in guinea pig ileum; opioid receptor binding, morphine impurity.

INTRODUCTION

We have previously demonstrated the presence of a novel possibly active morphine metabolite in patient sera after morphine administration (1). This polar metabolite was distinct from all other known morphine metabolites, and in HPLC serum profiles it represented the only major metabolite fraction with affinity to the μ opioid receptor (1). Subsequent analysis of commercial morphine samples revealed a minor impurity which was also active in the radioreceptor assay and had an identical HPLC retention time as the *in vivo* metabolite (1).

We report here that the small morphine impurity is a hydroxylated morphine with pharmacological properties comparable to that of morphine. The structure of 10α-hydroxymorphine is suggested by spectral data.

EXPERIMENTAL

Materials

Morphine sulfate was purchased from Mallinckrodt, St. Louis, Missouri. The following radioactive tracers were used: ^{3}H-morphine, 40 Ci/mmol (from New England Nuclear, Wilmington, Delaware); ^{3}H-[D-Ala, N-Me-Phe, Gly^5-ol]-enkephalin (^{3}H-DAGO), specific activity 50 Ci/mmol; and ^{3}H-D-Pen^2, D-Pen^5]-enkephalin (^{3}H-DPDPE), specific activity 43 Ci/mmol (from Amersham, Inc., Arlington Heights, Illinois). Unlabeled DAGO and DPDPE were obtained from Peninsula Lab, Palo Alto, California.

Apparatus

Mass spectra were taken on a Kratos MS50S double focusing mass spectrometer equipped with a cesium ion source as previously described (2). Approximately 1–2 μg of the morphine impurity was dissolved in a 1:1 mixture of glycerol:thioglycerol with 0.1% trifluoroacetic acid and analyzed by liquid secondary ion mass spectrometry (LSI/MS) in the positive ion mode. A scan speed of 100 sec/decade was used, and the spectra were acquired on a Gould electrostatic recorder. To obtain the precise mass of the morphine impurity and its fragment ions, electron impact spectra were taken on a AEI-Kratos MS9 mass spectrometer operating at a resolution of approximately 5000. The mass scale was calibrated with perfluorokerosene, and the corresponding masses and elemental compositions are reported with deviations from their calculated values in parts per million (ppm). NMR spectra were run in d_6-DMSO on a GE 500 NMR spectrometer, with deuterated TMS as internal standard. UV spectra were recorded on a Beckman DU 64 spectrophotometer.

Methods

Isolation of the Morphine Impurity

Morphine sulfate, 10–20 m*M*, dissolved in the HPLC eluent (1% methanol, 0.01% acetic acid, in water), was repeatedly injected in volumes of up to 250 μl, onto an analytical μBondapak C_{18} reverse-phase column in a Waters Model 440 HPLC apparatus. The eluent flow rate was 2 ml/min, at ambient temperature, and UV absorbance was monitored at 280 nm. The peak eluting at 4.0 min (morphine, 6.8 min) was collected, and the collected fractions were combined to yield pools containing approximately 50 μg of the compound, calculated on the assumption of equal absorptivity with morphine at 280 nm. The combined sample was then concentrated in a rotatory evaporator under reduced pressure and lyophilized. Analysis of the residue by HPLC showed a single UV absorbing peak at the expected retention time, and the lyophilized material was therefore used directly for further analysis. For mass spectrometry, however, a second HPLC separation under the same conditions was required to allow efficient vaporization-ionization in the ion source. Because of the very small quantity of the impurity in commercial morphine samples, upscaling of the iso-

[1] Department of Pharmacy,
[2] Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143.
[3] Current address: College of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

0724-8741/90/1100-1205$06.00/0

lation procedure to obtain a crystalline product was not attempted in this study.

Opioid Receptor Assays

Incubations with rat brain homogenates were performed as described earlier (1). The tracers were 0.2 n*M* ^{3}H-DAGO and 0.5 n*M* ^{3}HDPDPE, to label μ and δ sites, respectively. When titrated against unlabeled DAGO and DPDPE, the IC_{50} values were 0.3 n*M* for DAGO and 5 n*M* for DPDPE, consistent with the expected affinities of the two peptides at μ and δ sites, respectively. Morphine and the isolated contaminant (10^{-10} to 10^{-6} *M*) were incubated with the tracers for 20 min, and tracer binding was determined by filtration (1).

Guinea Pig Ileum Preparation

Male guinea pigs weighing 300 to 400 g were used, and the longitudinal muscle with attached mysenteric plexus was prepared as described previously (3). The inhibitions of the electrically induced twitches of the strips by morphine and the isolated impurity were measured (3).

RESULTS AND DISCUSSION

An HPLC chromatogram of a commercial morphine sulfate sample is shown in Fig. 1. The UV spectrum of the minor peak at 4.0 min, taken in the eluent, was identical to that of morphine, with a maximum at 280 nm. All subsequent calculations of the amounts of this minor component are based on the assumption of equal absorptivity with morphine at 285 nm. Using this criterion, the impurity accounted for only approximately 0.1% of the morphine sample. Different batches of morphine (including a more than 10-year-old sample stored in the laboratory) gave similar yields, as did the

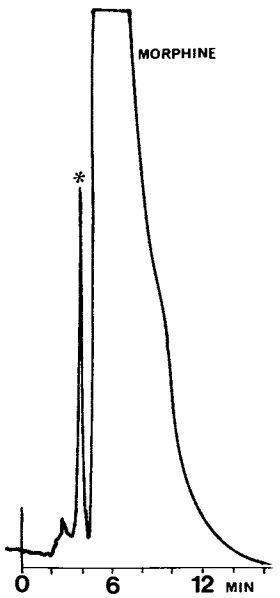

Fig. 1. HPLC record of a morphine sulfate sample (250 μl, 20 m*M*, in water) injected onto the reverse-phase column. Absorbance, 280 nm; range, AUFS 0.05. (*) Morphine impurity, ~0.1%.

^{3}H-labeled morphine (500,000 dpm injected), as determined by liquid scintillation analysis of HPLC eluent fractions.

Because of the presence of a sizable HPLC metabolite fraction in patients' sera after morphine treatment with an identical retention time to the impurity and with a high affinity to the μ opioid receptor (1), we first tested the pharmacological activity of the isolated morphine impurity. Its opioid receptor binding properties were virtually identical to those of morphine, with IC_{50} values ranging in different experiments from 1.5 to 3 n*M* against ^{3}H-DAGO at the μ site (morphine, 2–3 n*M*) and 60 to 80 n*M* against ^{3}H-DPDPE at the δ site (morphine, 70 to 150 nM) (two experiments). Further, morphine and the impurity were tested for inhibition of electrically induced guinea pig ileum contraction (3). Both compounds had similar activities, in several exploratory experiments. On the basis of one dose–response study ranging from 10^{-8} to 10^{-5} *M*, identical EC_{50} values of 0.3 μ*M* were observed for morphine and the impurity, with equal maximum twitch inhibition of 70%. Therefore, the impurity compound appears to be an opioid agonist with similar pharmacological properties to morphine. Since both the morphine impurity and the morphine-related metabolite in patient sera share affinity to the μ opioid receptor and have equal HPLC retention times, they may be identical compounds; however, the material observed *in vivo* is likely to be derived from metabolism, as it accounts for up to 30% of morphine radioreceptor activity in serum (1), whereas the impurity accounts for only ~0.1% of the injected morphine.

Because of these pharmacological results, we analyzed the structure of the morphine impurity. Liquid secondary ion mass spectrometry of this derivative in the positive ion mode gave an abundant protonated molecular ion (MH^+) at *m/z* 302 (MW = 301), suggesting the presence of one additional oxygen to the morphine structure (MW = 285). High-resolution electron impact mass spectrometry gave a molecular formula of $C_{17}H_{19}N_1O_4$ for M^+ with an error of less than 0.5 ppm (measured *m/z*, 301.1315; calculated, 301.1314), consistent with the insertion of one oxygen atom into morphine. Further, the UV spectrum identical to that of morphine suggested a hydroxylated morphine derivative. Morphine *N*-oxide could be excluded because the morphine impurity was eluted earlier from the HPLC column than authentic morphine *N*-oxide (1).

The nmr spectrum of morphine (for comparison) and of 30 μg of the impurity gave the following resonances in d6-DMSO: morphine—[δ (J, in Hz)] 6.495 (H-1,d,8), 6.40 (H-2,d,8), 5.59 (H-7,d,9.5), 5.24 (H-8,d,9.5), 4.73 (H-5,d,6); morphine impurity—6.73 (H-1,d,~10), 6.63 (H-2,d,~10), 5.57 (H-7,d,~14), 5.32 (H-8,d,~14), 4.785 (H-5,d,~7), and one additional peak at 4.98 (s). Because of the small amount of compound and residual contaminants from the HPLC eluent fractions, the higher-field portion of the nmr spectrum gave only poorly resolved peaks and could not be assigned. Peak assignments for morphine were taken from Ref. 4. The presence of the aromatic C-1 and C-2 proton and the olephinic C-7 and C-8 protons rules out hydroxylation or epoxidation at these sites. The sharp singlet at δ 4.98 ppm which was not present in the spectrum of morphine is consistent with the resonance expected from the 10β proton if hydroxylation occurred at 10α, because (1) a large downfield shift is expected for the 10β-H with adjacent aromatic and

OH substituents, and (2) no coupling should occur with the $C_{9\alpha}$-H with a tetrahedral angle of approximately 90° (5). Indeed, the spectrum of the morphine impurity was nearly identical over the observed range to that of 10α-hydroxydoceine previously published by Krowech *et al.*, with the 10β-H singlet appearing at 5.25 ppm (in $CDCl_3$) (4).

Further evidence for the relative position of the inserted oxygen close to the nitrogen of morphine is provided by the EI fragmentation pattern of the morphine impurity, in comparison with the mass spectra of morphine and 10α-hydroxycodeine (Table I). The most prominent peak in the spectra of both the impurity and 10α-hydroxycodeine, but not morphine, was *m/z* 112, with a molecular formula of $C_6H_{10}NO$ (1 ppm mass deviation), which contains both the piperidine nitrogen and one closely positioned oxygen. Also, major fragments in both the impurity and 10α-hydroxycodeine are at *m/z* 126, 112, and 91, a pattern that is quite different from that in the morphine spectrum. We therefore assign the structure of 10α-hydroxymorphine to the impurity in morphine samples (Fig. 2). Final structure confirmation will require the isolation and crystallization of larger quantities of the impurity and independent synthesis of 10-hydroxymorphine as described by Rapoport and Masamune (6).

Previously, morphinan alkaloids have been shown to undergo oxidation mostly in position C-10, and 10-oxomorphine was found as an impurity of morphine (7). Hence, 10-hydroxymorphine could have arisen by reduction of 10-oxomorphine or by oxidation of morphine.

Table I. Mass Spectra, Obtained by Electron Impact Ionization of Morphine, 10α-Hydroxycodeine, and the Morphine Impurity[a]

	% intensity of base peak		
m/z	Morphine	10α-Hydroxycodeine	Morphine impurity (elemental formula)
315	—	100	—
301	—	—	100 ($C_{17}H_{19}NO_4$)
285	100	—	—
283	7	—	55 ($C_{17}H_{17}NO_3$)
215	33	2	5
186	4	—	44 ($C_{12}H_{10}O_2$)
162	37	3	14
126	2	8	43 ($C_7H_{12}NO$)[b]
124	23	4	22
112	3	21	80 ($C_6H_{10}NO$)
91	9	8	42

[a] Only major fragment ions are provided. 10α-Hydroxycodeine was considerably more resistant to electron impact-induced fragmentations, yielding fragment ion of generally lower intensity. The calculated molecular formulas for the morphine impurity were within 2 ppm of the measured values, except where indicated.

[b] An 8-ppm mass deviation from theoretical value.

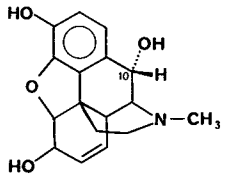

Fig. 2. The proposed structure of the morphine impurity, 10α-hydroxymorphine.

The potential significance of the morphine impurity is as follows. First, 10α-hydroxymorphine may represent an active metabolite of morphine. In their studies on the hepatotoxicity of morphine, Correia and colleagues (4) have proposed oxidation to occur at the C-10 position, followed by conjugation with glutathione in the 10α position, although 10-hydroxymorphine was never isolated. Metabolic reduction of 10-oxomorphine must also be considered. Second, the hydroxylated morphine impurity may represent a drug in its own right with pharmacological properties similar to that of morphine. Yet because of its higher polarity as judged by earlier elution from reverse-phase HPLC columns, it may be more efficiently trapped in the CNS upon spinal injection or it may display different kinetics and target organ selectivity upon iv administration because of an altered capacity to cross the blood–brain barrier. Surprisingly, however, the CH_2Cl_2/H_2O partition coefficient of morphine and 10-hydroxymorphine were shown to be similar (5). Third, morphine substitution in the C-10 position may lead to new active derivatives.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Research Grant DA 04166 from the National Institutes on Drug Abuse, Rockville, Maryland.

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