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6-Acetylmorphine: A natural product present in mammalian brain

(opioid/immunoreactive morphine/hypothalamus/GC/MS)

CHARLES J. WEITZ*†, LOUISE I. LOWNEY*, KYM F. FAULL‡, GOTTFRIED FEISTNER‡, AND AVRAM GOLDSTEIN*†

*Addiction Research Foundation, Palo Alto, CA 94304; †Department of Pharmacology, Stanford University, Stanford, CA 94305; and ‡Nancy Pritzker Laboratory for Behavioral Neurochemistry, Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine, Stanford, CA 94305

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ABSTRACT **Recently, we described three substances in bovine hypothalamus, adrenal, and rat brain recognized by antisera raised against morphine, and we identified one as morphine and another as codeine by GC/MS. We now report the identification of the third immunoreactive (*ir*) morphinan from bovine brain as 6-acetylmorphine by chemical conversion to morphine, GC/MS, and high-resolution mass measurement. 6-Acetylmorphine has not previously been described as a natural product in plants or animals, but it has long been known as the metabolite in part responsible for the biological properties of heroin. However, we have excluded slaughterhouse or laboratory contamination by any morphinan as well as derivation from the morphine in tissues during our procedures. 6-Acetylmorphine is known to be more potent than morphine *in vivo* chiefly by virtue of its greater penetration into the central nervous system. Should morphinans prove to have physiological functions in animals, the properties of 6-acetylmorphine make it ideal for fulfilling the role of a peripheral-to-central hormone.**

Early work suggesting the possibility of morphine biosynthesis in mammalian brain (1) stimulated investigation of mammalian tissues with RIAs for morphine (2) and led to the detection of immunoreactive (*ir*) substances (3–7). Recently, we described three *ir* morphinans from mammalian brain and adrenal and identified one as morphine and another as codeine by GC/MS (8); this was subsequently confirmed for rat central nervous system (9). The possibility that morphine and codeine are endogenous in mammals is suggested by our recent demonstration that mammalian liver (but not brain or adrenal) can synthesize the morphine ring structure (10), carrying out the same critical step as does the opium poppy (11, 12). We now report the identification of the third *ir* morphinan, previously designated "peak 5" (8), as 6-acetylmorphine.

MATERIALS AND METHODS

Materials. [*methyl*-^{3}H]Morphine was from New England Nuclear; [*ring*-^{3}H]morphine was from Amersham; synthetic 3-acetylmorphine and 6-acetylmorphine standards were a gift from A. Allen (U.S. Drug Enforcement Administration). Other reagents were from Baker or Sigma.

Morphine RIAs. Anti-morphine antisera 937 and S17 were provided by Syva (Palo Alto, CA). A detailed description of preparation of antisera, assay conditions, analysis of RIA data, and the differing specificities of the two RIAs has appeared elsewhere (7).

Analytical Purification and Controls. For the experiments illustrated in Fig. 1, a blank "extraction" and "purification" was carried out first, followed by tissue extraction and purification with the same glassware and columns. Frozen bovine hypothalami (24 g, wet weight; J. R. Scientific, Woodland, CA) were homogenized in 0.1 mM L-ascorbic acid in 95% ethanol (3 ml/g, wet weight; 23°C) to which 1.2×10^5 cpm of [*ring*-^{3}H]morphine (14 Ci/mmol; 1 Ci = 37 GBq) had been added. After centrifugation (10,000 × *g*; 10 min), the supernatant was concentrated 60-fold by rotary evaporation (bath, 35°C), diluted 1:10 with water, and adsorbed to a C_{18} cartridge (Waters, Sep-Pak), which was then washed with trifluoroacetic acid (5 mM; 3 ml). The flow-through and trifluoroacetic acid wash fractions were pooled and the process was repeated twice. Elution from the three cartridges was with 50% acetonitrile in 5 mM trifluoroacetic acid (1 ml). The eluates were pooled, reduced in volume by N_2 gas, and lyophilized. The sample was dissolved in trifluoroacetic acid (5 mM; 1 ml) for analysis by HPLC-C (linear gradient of acetonitrile in trifluoroacetic acid; see ref. 7).

Peak 5 Conversion Studies. For the experiments described in Fig. 2, partially purified peak 5 (see Fig. 1; 45 pmol of *ir* morphine equivalents, antiserum 937) was incubated in water, NH_4OH (1 M; pH 11.8), NaOH (3.5 mM; pH 11.8), or HCl (1 M) (final vol, 200 μl; 20 hr; 23°C). After incubation, nonacidic samples were acidified to pH 1–2 by addition of HCl, and each sample was analyzed by HPLC-C. The *ir* peaks were collected and lyophilized. Samples were dissolved in 5 mM trifluoroacetic acid (500 μl) containing an internal standard of [*ring*-^{3}H]morphine (4×10^4 cpm; 14 Ci/mmol); half of each sample was analyzed in HPLC-C and half was analyzed in HPLC-M_2 (linear gradient of methanol in ammonium hydroxide; see ref. 8, lower recoveries than with HPLC-C are typical).

Preparative Purification. Peak 5 was purified from 1750 frozen bovine hypothalami (28 kg, wet weight) by the following sequence of steps. Batches of 50 were homogenized in 0.1 mM L-ascorbic acid in 95% ethanol (3 ml/g, wet weight; 23°C). After centrifugation and volume reduction (see above), five concentrated extracts (pH 5) were pooled. Samples were defatted by extracting twice with an equal volume of 10% 1-butanol in chloroform, the organic phases were pooled and back-extracted with an equal volume of 0.1 M HCl, the back-extract was pooled with the original aqueous phase, and the volume was reduced to 150 ml by rotary evaporation. The remaining hypothalami were processed as described above, and the defatted concentrated extracts were pooled. The pellets from the centrifugation step were reextracted (3 vol/vol) and processed in the same manner as the original homogenates, and the resulting defatted extract was pooled with the original extract (total vol, 1.5 liters), which was subsequently processed as two separate samples. Each sample was adjusted to pH 9.0 by addition of NaOH and adsorbed to an XAD-2 column (7) (10 mM sodium carbonate; 1 liter/hr); under these conditions (2-hr exposure to pH 9 in aqueous

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Abbreviations: *ir*, immunoreactive; EI, electron impact; CI, chemical ionization.

solution), the peak 5 material is stable. The column was washed with sodium carbonate buffer (500 ml) and eluted with 95% ethanol (1.5 liters). The eluate was dried by rotary evaporation and dissolved in water (350 ml). After applying the sample to QAE-Sephadex (7), the column was washed with water (350 ml; 100 ml/hr). The flow-through and wash fractions were pooled and applied to a CM-Sephadex column (7), which was washed with water (50 ml; 40 ml/hr), and eluted isocratically with 0.5 M NaCl (150 ml; 2 ml per fraction). The fractions containing *ir* morphinans were pooled, adjusted to pH 9.0 with NaOH, adsorbed to an XAD-2 column, and eluted with 95% ethanol (25 ml). The eluate was reduced in volume by a factor of 5 by rotary evaporation, diluted 1:5 with water, and lyophilized. The residue was dissolved in 5 mM trifluoroacetic acid (1 ml) and divided into two samples for purification by HPLC-C after first chromatographing a solvent blank (5 mM trifluoroacetic acid; 500 μl). Fractions containing the peak 5 material were pooled, corresponding blank fractions were pooled, and the samples were lyophilized. The blank and peak 5 samples were dissolved in 100 mM sodium phosphate buffer (pH 7.4), added to affinity resins (2 ml of resin in a final vol of 10 ml of buffer), incubated (4 hr; 23°C), and eluted (3 hr; 37°C) as described (7), and appropriate samples were pooled and lyophilized. Samples were dissolved in 5 mM trifluoroacetic acid (500 μl) and purified by HPLC-C, with blank purification preceding peak 5 purification. Absorbance was monitored at 280 nm. Fractions containing *ir* peak 5 were pooled, the corresponding blank fractions were pooled, and the samples were lyophilized.

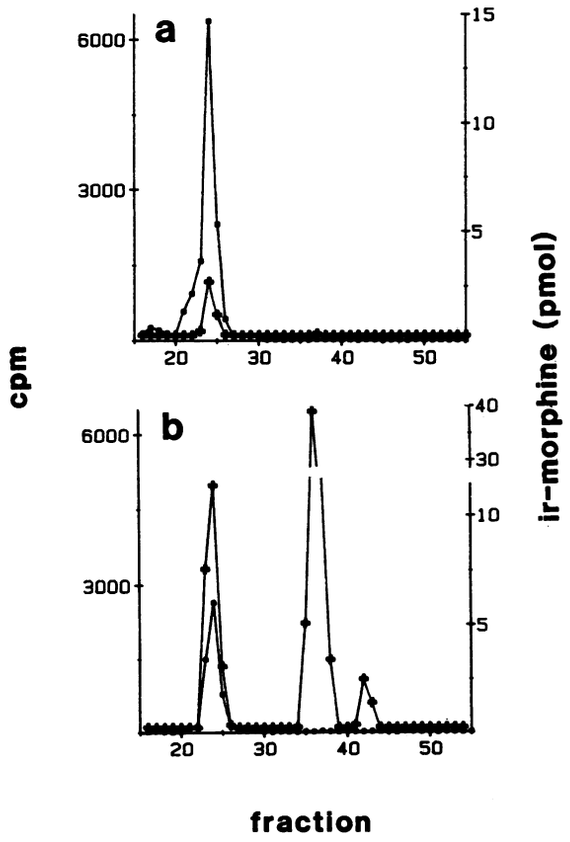

FIG. 1. HPLC profile of partially purified *ir* morphinans and radioactivity in a blank control extract (*a*) and a bovine hypothalamus extract (*b*) processed from the outset in the presence of [*ring*-^{3}H]morphine standard. ●, cpm; +, *ir* morphine equivalents determined with antiserum 937. The small peak of immunoreactivity under the peak of radioactivity in the control extract (*a*) is accounted for by the [^{3}H]morphine standard.

GC/MS. Samples were dissolved in water (200 μl), transferred to glass tubes, and dried under reduced pressure. Trifluoroacetic acid derivatives were prepared (1 hr; 75°C), and GC/electron impact (EI)/MS studies were performed as described (8), except that the column length was 15 m. For chemical ionization (CI) studies, the GC conditions were as described above and the reactant gas was methane (1 torr; 1 torr = 133 Pa). High-resolution mass measurement was as described (8), with the following exceptions: GC, 220°C isocratically; MS, static resolution 10,000 (10% valley), scan rate 4 sec/decade, five measurements over a single GC peak.

RESULTS

Reversed-phase HPLC analysis of partially purified extracts is illustrated in Fig. 1. The blank extract (Fig. 1*a*) that preceded the tissue extract through the same glassware and columns contained no detectable *ir* morphinan other than

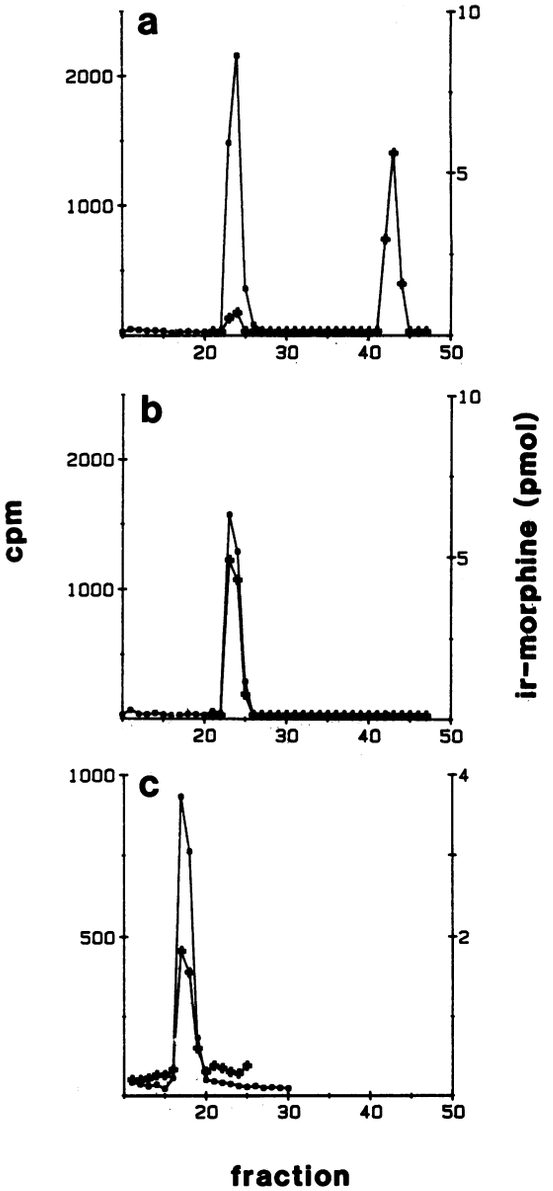

FIG. 2. Coelution in two HPLC systems of [*ring*-^{3}H]morphine standard and the *ir* product derived from peak 5 after a 20-hr incubation. (*a*) HPLC-C control after incubation in water. (*b*) HPLC-C after incubation in 1 M NH_4OH. (*c*) HPLC-M_2 incubation as for *b*. See Fig. 1 legend for symbols and explanation of immunoreactivity under cpm peak in control.

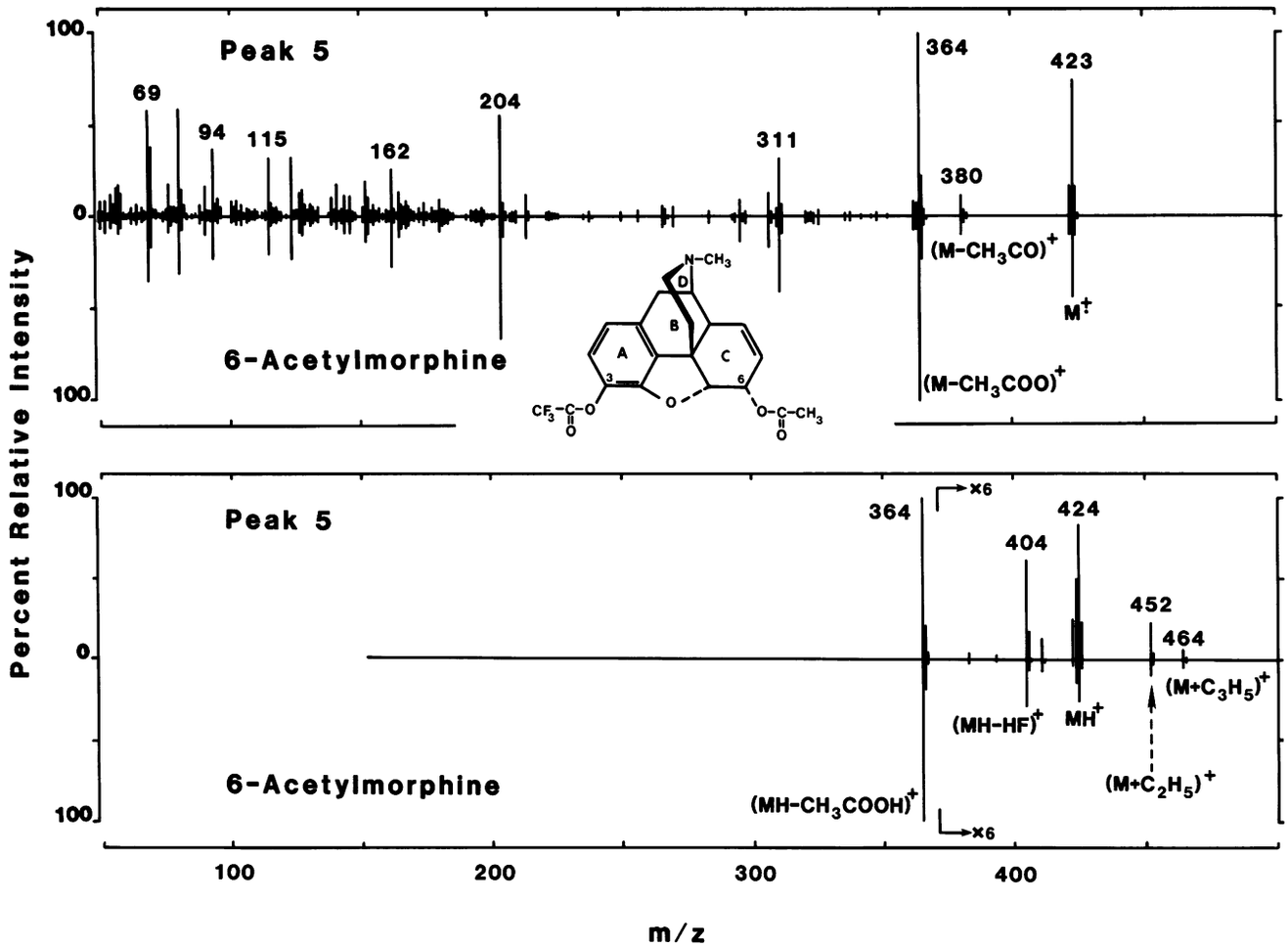

FIG. 3. Comparison of mass spectra of trifluoroacetic acid derivatives of purified peak 5 and synthetic 6-acetylmorphine after electron impact (*Upper*) and chemical ionization with methane (*Lower*). The mass spectrum of peak 5 is represented by the upright tracing, and that of the synthetic 6-acetylmorphine (trifluoroacetic acid derivative depicted) is represented by the inverted tracing. M, molecular ion. Postulated additions to or losses from the molecular ion are represented.

that due to the [*ring*-^{3}H]morphine standard added at the outset of processing. The bovine hypothalamus extract (Fig. 1*b*) contained three peaks of morphine immunoreactivity, corresponding to morphine (at fraction 24) and codeine (at fraction 36) as previously demonstrated, and to the unknown (peak 5, at fraction 42). Only background radioactivity (22–28 cpm) was detected in the fractions containing peak 5, demonstrating that it was not derived from morphine during the extraction and purification procedures. Had peak 5 been so derived, a peak of radioactivity containing at least 1000 cpm would be expected (assuming that peak 5 has a cross-reactivity with antiserum 937 not markedly greater than that of morphine; see below). All three peaks have been detected in 22 consecutive analytical experiments (including those described in ref. 8) in which the blank extracts contained <2% of the immunoreactivity found in the accompanying tissue extracts. Hypothalami used in these experiments were from five lots of frozen tissue obtained over a period of 3 years from suppliers in California and Arkansas. Hypothalami dissected from fresh brains obtained under direct observation by one of us at a local slaughterhouse gave the same results. When 15 individual hypothalami were dissected into six anatomical sections and the sections were separately extracted and analyzed by HPLC (blank-controlled as described above), every region was found to contain morphine, codeine, and peak 5 (in variable concentrations; data not shown), and thus all three compounds must have been distributed in the tissues prior to death. Taken together, these results exclude contamination as an explanation for the presence of the three *ir* morphinans.

In our earlier purification of morphine and codeine (8), peak 5 was selectively lost during alkaline phase partition. Because we could not determine from our data whether the peak 5 material was degraded to a non-*ir* form or converted into morphine or codeine, we performed the experiments shown in Fig. 2. After control incubation in water (Fig. 2*a*), peak 5 remained intact (at fraction 43). After incubation in 1 M NH_4OH, peak 5 was converted to an *ir* product that was coeluted with the [^{3}H]morphine internal standard in two HPLC systems (Fig. 2 *b* and *c*) and that acquired full cross-reactivity (data not shown) with antiserum S17 (with which peak 5 itself has no detectable crossreactivity). These data indicate that the product is morphine. Identical results were obtained after incubation of peak 5 in 3.5 mM NaOH or in 1 M HCl, whereas after incubation in 5 M acetic acid peak 5 remained intact (data not shown). The conditions under which peak 5 generated morphine suggested that it is an ester derivative of morphine; previous RIA data had suggested that it is a 3- and/or 6-substituted morphinan (8). Its elution position in HPLC-C (Figs. 1*b* and 2*a*), indicative of hydrophobicity greater than that of morphine, excluded a phosphate or sulfate ester but was compatible with a carboxylic acid ester of morphine.

After preparative purification, peak 5 was eluted from the final HPLC steps in each case as a single peak of UV absorbance (15 nmol total, assuming a molar extinction coef-

ficient equal to that of morphine) that corresponded to the peak of immunoreactivity (16 nmol *ir* morphine equivalents total; apparent crossreactivity, 93% compared with morphine; overall recovery, 64%). The blank HPLC samples showed only background absorbance in the peak 5 position and no detectable immunoreactivity (<1% of that in the tissue sample).

In preliminary GC/MS studies of peak 5 (trifluoroacetyl derivatives were used for all GC/MS experiments), the reconstructed ion chromatogram showed a predominant peak at a retention time of 8.87 min that was not present in the corresponding blank samples; from its EI and CI mass spectra we deduced a mass of 423 Da, consistent with a monoacetylmorphine. The EI fragmentation pattern was typical for a morphinan (13), and the presence of ions at m/z 311 (retro-Diels-Alder fragment) and at m/z 204 indicated that the unknown was a 6-acetylmorphinan. The elemental composition for the molecular ion was confirmed by high-resolution mass measurement [found, 423.1286 ± 0.0054 (SEM); calculated for $C_{21}H_{20}F_3NO_5$, 423.1294]. After the purification and initial GC/MS studies of peak 5 were completed, we obtained synthetic 3-acetylmorphine and 6-acetylmorphine standards and verified their identities by EI MS (solid probe) and proton NMR spectroscopy (500 MHz) in comparison with published spectra obtained under identical conditions (14, 15). The 6-acetylmorphine standard showed a GC retention time (data not shown) and EI and CI mass spectra identical to that of peak 5 (Fig. 3), whereas the 3-acetylmorphine standard generated a peak with a longer retention time and a different fragmentation pattern.

We conclude that peak 5 is 6-acetylmorphine.

DISCUSSION

Prior to this work 6-acetylmorphine had not been described as a natural product in plants or animals, so its identification in mammalian brain is unexpected. It has long been known as the metabolite in part responsible for the addicting and analgesic properties of heroin (synthetic 3,6-diacetylmorphine) (16–18). 6-Acetylmorphine penetrates into the central nervous system far more effectively than morphine itself (19) and after penetration is converted to morphine (16–18). It has not been described as a metabolite of morphine or codeine (16–18), but amounts as small as those found here would not have been detected with standard methods; furthermore, the typical extraction used to isolate nonpolar metabolites would likely hydrolyze 6-acetylmorphine.

No heroin or 6-acetylmorphine was ever present in the laboratory where the purification of peak 5 was carried out, and the results reported above exclude contamination at any point as an explanation for the presence of morphine, codeine, or 6-acetylmorphine in brain. The 6-acetylmorphine demonstrated in mammalian brain cannot therefore have any relationship to exogenous heroin. Presumably, it is derived from the morphine in tissues by O-acylation, which is known to occur in the biogenesis of acetylcholine and fatty acyl esters.

Should morphine prove to have a physiological function in the animal kingdom (20), the properties of 6-acetylmorphine make it ideal for fulfilling the role of a peripheral-to-central hormone. This speculation is especially attractive in light of our recent demonstration of morphinan synthesis by mammalian liver but not by brain (10, 21). Thus, it may be that manufacturers of heroin have unwittingly followed a chemical strategy already devised by evolution for delivering morphine to the brain. Further work will be required to establish an endogenous origin or a physiological function for any of the morphinans identified in mammalian brain.

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