Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 9784–9788, December 1986
Neurobiology

Morphine and codeine from mammalian brain

(opioid/hypothalamus/immunoreactive morphine)

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Contributed by Avram Goldstein, August 13, 1986

ABSTRACT **Recently, we described the presence of six immunoreactive (ir) morphinans in bovine adrenal and hypothalamus and identified one as morphine [Goldstein, A., Barrett, R. W., James, I. F., Lowney, L. I., Weitz, C. J., Knipmeyer, L. L. & Rapaport, H. (1985) *Proc. Natl. Acad. Sci. USA* 82, 5203–5207]. We now report that ir morphinans corresponding to the previously reported peak 1 (morphine), peak 4, and peak 5 are consistently present in extracts of bovine hypothalamus and variably present in extracts of bovine adrenal and rat brain. We no longer detect the previously reported peaks 2, 3, or 6, and we have established that they were contamination artifacts. Peak 1 is coeluted with morphine in two distinct reversed-phase HPLC systems, as is peak 4 with codeine. We have purified peak 1 and peak 4 compounds from bovine hypothalamus and determined their identities by gas chromatography/mass spectrometry (GC/MS): peak 1 is confirmed to be morphine and peak 4 is codeine.**

Since the experiments of Davis and Walsh (1) and others (2) suggested a possible biosynthesis of morphine in mammalian brain, a number of investigators have used morphine radioimmunoassays (RIAs) to probe animal tissues or products for morphine-like substances (3), and several groups have reported positive findings (4–15). Recently, we reported (15) the detection of six immunoreactive (ir) morphinans in bovine hypothalamus and adrenal, purified four to apparent homogeneity, and determined the identity of one (peak 1) as morphine by multiple criteria including proton NMR spectroscopy. Subsequently, GC/MS and proton NMR spectroscopy identified the other three compounds and enabled us to determine that they were almost certainly artifacts of laboratory contamination (unpublished observations). The experiments reported here were performed in order to establish the existence and identity of ir morphinans from mammalian brain.

MATERIALS AND METHODS

Materials. [*methyl*-^{3}H]Morphine was from New England Nuclear; [*ring*-^{3}H]morphine, [*O-methyl*-^{3}H]codeine, and Na^{125}I, from Amersham; morphine sulfate, from S. B. Penick (Lyndhurst, NJ); and codeine phosphate, from Burroughs Wellcome (Research Triangle Park, NC). Other reagents were obtained from Sigma or Baker.

Morphine RIA. Morphine antisera 937 and S17 were donated by Syva (Palo Alto, CA). Antiserum preparation, assay methods, data analysis, and the contrasting specificities of the two morphine RIAs have been described (15). The immunoreactivity ratio is defined as the ir-morphine equivalent determined using antiserum 937 divided by that determined using antiserum S17 (15).

Sequential Blank Procedure. All analytical and preparative purification experiments (see below) were controlled as follows. Using glassware, reagents, and columns sequestered exclusively for this work, a tissue-less extraction was performed with an appropriate volume of extraction solvent. This blank sample was carried through all subsequent steps of the purification. Each tissue extract followed behind its own sequential blank in the same glassware and columns.

Analytical Purification. For the experiments shown in Fig. 2, approximately 500-g samples of frozen bovine hypothalami, bovine adrenals, or rat brains [Pel-Freez or J.R. Scientific (Woodland, CA)] were partially purified as described (ref. 15, as for bovine hypothalamus) through extraction, centrifugation, volume reduction (rotary evaporator bath temperature 35°C), phase partition, and analysis on "HPLC-C", a reversed-phase system using a linear gradient of acetonitrile in trifluoroacetic acid (samples were dissolved in 500 μl of water for injection).

Double-System HPLC Coelutions. Fractions containing ir morphinans from analytical purification experiments were pooled, lyophilized, dissolved in 200 μl of 0.1 M HCl containing ring-labeled [^{3}H]morphine or [^{3}H]codeine, and subjected to repeat analysis on HPLC-C. Samples from appropriate fractions were taken for liquid scintillation counting and RIA (antiserum 937). Fractions containing the peak of immunoreactivity and radioactivity were pooled, lyophilized, dissolved in 200 μl of 0.1 M HCl, and similarly analyzed on "HPLC-M2" (see below).

Preparative Purification. Two batches of 600 frozen bovine hypothalami (J.R. Scientific) were processed independently by the following sequence of steps.

Extraction and phase partition. An acetic acid/ethanol extraction method was used for efficient early volume reduction; results were the same as with hot acetic acid extraction. Batches of 50 hypothalami were partially thawed and homogenized (23°C) in a Waring blender in 3 ml per g of wet weight (2.5 liters) of 95% ethanol containing 1% (vol/vol) glacial acetic acid and 0.1 mM L-ascorbic acid. After centrifugation (10,000 × *g*, 10 min), the pooled supernatants from six such homogenates (15 liters) were concentrated 30-fold in a rotary evaporator (bath at 60°C). The concentrate was adjusted to pH 9.0 with NH_4OH and extracted twice with equal volumes of 10% 1-butanol in chloroform. The pooled organic phases were back-extracted with an equal volume of 0.1 mM L-ascorbic acid in 0.1 M HCl. The pH of the back-extract was adjusted to approximately 5 with NH_4OH, and the volume was reduced to 50 ml by rotary evaporation (bath at 35°C). The pH was adjusted to 9.0, another round of phase partition was performed as above (at 10% of the original volume), and the final aqueous back-extract was lyophilized. The dried material from two sets of 300 hypothalami was dissolved and pooled in 25 ml of water. This

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Abbreviation: ir, immunoreactive.

phase-partition procedure was also used as required for desalting after ion-exchange steps.

Cation-exchange chromatography. A CM-Sephadex C-25 (Pharmacia) column (20 × 2.5 cm) was used; elution was at 4 ml/min with a linear gradient of NaCl to 0.5 M (1-liter gradient, 11 ml per fraction). Best results were obtained by recycling ir material through CM-Sephadex twice. The ir material was then dissolved in 2 ml of 1 M NH_4OH in preparation for anion-exchange chromatography.

Anion-exchange chromatography. A QAE-Sephadex A-25 (Pharmacia) column (20 × 1 cm) was used; elution was at 40 ml/hr with a linear gradient of NaCl to 0.4 M in 1 M NH_4OH (120-ml gradient, 2 ml per fraction). Two ir peaks were obtained, one that was not retained (peak 4) and one that was eluted at 0.15 M NaCl (peak 1). These were processed separately through the remaining steps.

HPLC-M2. A Waters reversed-phase C_{18} μBondapak column (3.9 mm × 30 cm) was used; elution was with a 20-min linear gradient of 50–90% methanol in 30 mM NH_4OH, pH 8.5 (1.5 ml/min, 0.4 min per fraction). Absorbance was monitored at 280 nm and 297 nm.

HPLC-C. This was as described (15), at pH 2.5. For this final step, HPLC runs were performed in the following order: (*i*) a solvent blank, 500 μl of water; (*ii*) sequential blank (see above); (*iii*) ir peak from tissue extract; (*iv*) an external standard of morphine or codeine at about the same concentration as the ir peak from tissue.

GC/MS. Appropriate fractions from the final HPLC-C step for each of the four types of sample described above were pooled, lyophilized, dissolved in 100 μl of water, transferred to glass capillary tubes (90 mm × 1 mm i.d.) in 5-μl increments as necessary, and dried at low pressure (Vacuum Concentrater, Savant). Trifluoroacetyl derivatives were prepared by the addition of *N*-methylbis(trifluoroacetamide) (5 μl, Pierce) to each capillary tube, which was then sealed by flame and incubated at 80°C for 1 hr (peak 1 and morphine) or 2 hr (peak 4 and codeine). Samples were run on the GC/MS system (HP 5985, Hewlett-Packard; quadrupole filter, electron-impact mode, 70 eV) in the order listed above for the final HPLC-C step. GC components and conditions were as follows. Column: fused silica capillary, bonded phase, medium polarity, 30 m × 0.255 mm, film thickness 0.25 μm (DB-5, J & W Associates). Injection port and transfer line: 250°C. Column conditions: 180°C for 1 min, increasing to a plateau at 250°C at a rate of 20°C/min, in a total run time of 20 min. Carrier gas: He.

High-Resolution GC/MS. The elemental composition of the trifluoroacetyl derivative of the material comprising peak 1 was determined using a Varian 3700 gas chromatograph (column specifications and GC conditions as described above) coupled with a VG ZAB-SE high-resolution mass spectrometer (static resolution 8000; scan rate 2 sec/decade; mass range 500–300; reference PFK). Seven mass spectra were obtained over a single GC peak.

RESULTS

The purification to apparent homogeneity of compounds designated peaks 2, 3, and 6 from bovine adrenal was reported previously (15). Chemical conversion data, immunoreactivity ratios, chromatographic behavior, proton NMR spectroscopy (kindly performed by H. Rapoport, University of California, Berkeley). GC/MS, and synthesis of model compounds for comparison studies (data not shown) have enabled us to make an unambiguous structural assignment for each (Fig. 1). Because of their biochemically implausible features we became suspicious that they were artifactual; a

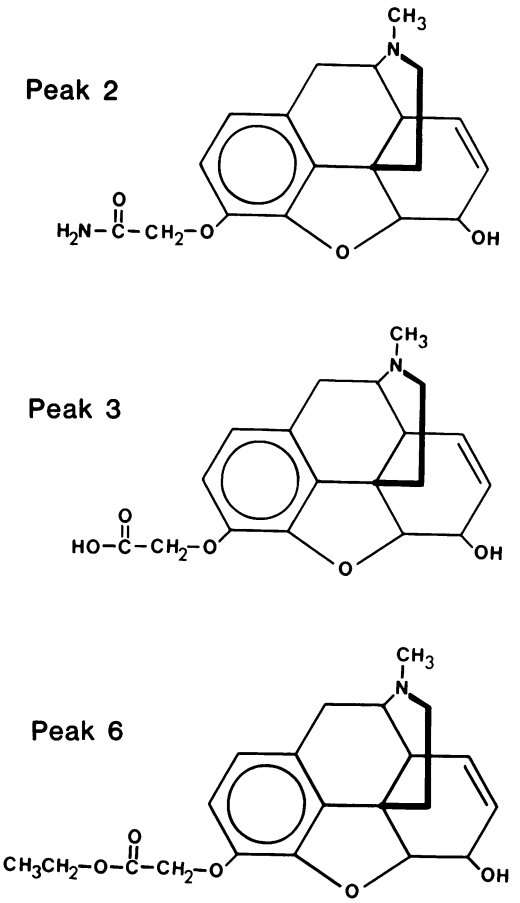

FIG. 1. Structures of compounds comprising peaks 2, 3, and 6 as determined by GC/MS and proton NMR. These compounds were contaminants in our earlier investigations (15).

search of MEDLINE[§] and earlier National Library of Medicine data bases revealed only a single biological example of carboxymethylation of a phenolic hydroxyl group (rifamycin B, ref. 16). Subsequently, we were able to identify a crude preparation of 3-*O*-carboxymethylmorphine that had been used in our laboratory. Reversed-phase HPLC analysis of this material revealed ir-morphinan peaks, some corresponding in elution position to peaks 3 and 6, as well as others; peak 2 was shown to be the amide derived from peak 6 by ammoniolysis. Study of our laboratory notebook entries for 1984 suggested sporadic episodes of contamination that persisted for at least 5 months, which by chance were not detected in blank runs. Because of the consistent finding of multiple ir morphinans in brain extracts prior to the introduction of carboxymethylmorphine into the laboratory, we suspected that the considerable variability in content and pattern of ir morphinans might reflect episodic contamination superimposed on native morphinans in the extracts.

We therefore instituted the more stringent sequential-blank procedure described in *Materials and Methods*, in which a tissue-less extract precedes each tissue extract throughout the purification process in sequestered glassware, reagents, and columns. Fig. 2 illustrates the results of partial purification and reversed-phase HPLC analysis of extracts of bovine hypothalamus, bovine adrenal, and rat brain. We have consistently found three ir morphinans in hypothalamus that correspond in immunoreactivity ratios and elution positions to the previously reported (15) peaks 1, 4, and 5. Although the relative abundances sometimes varied from that depicted,

[§]National Library of Medicine, MEDLINE, Bethesda, MD 20892.

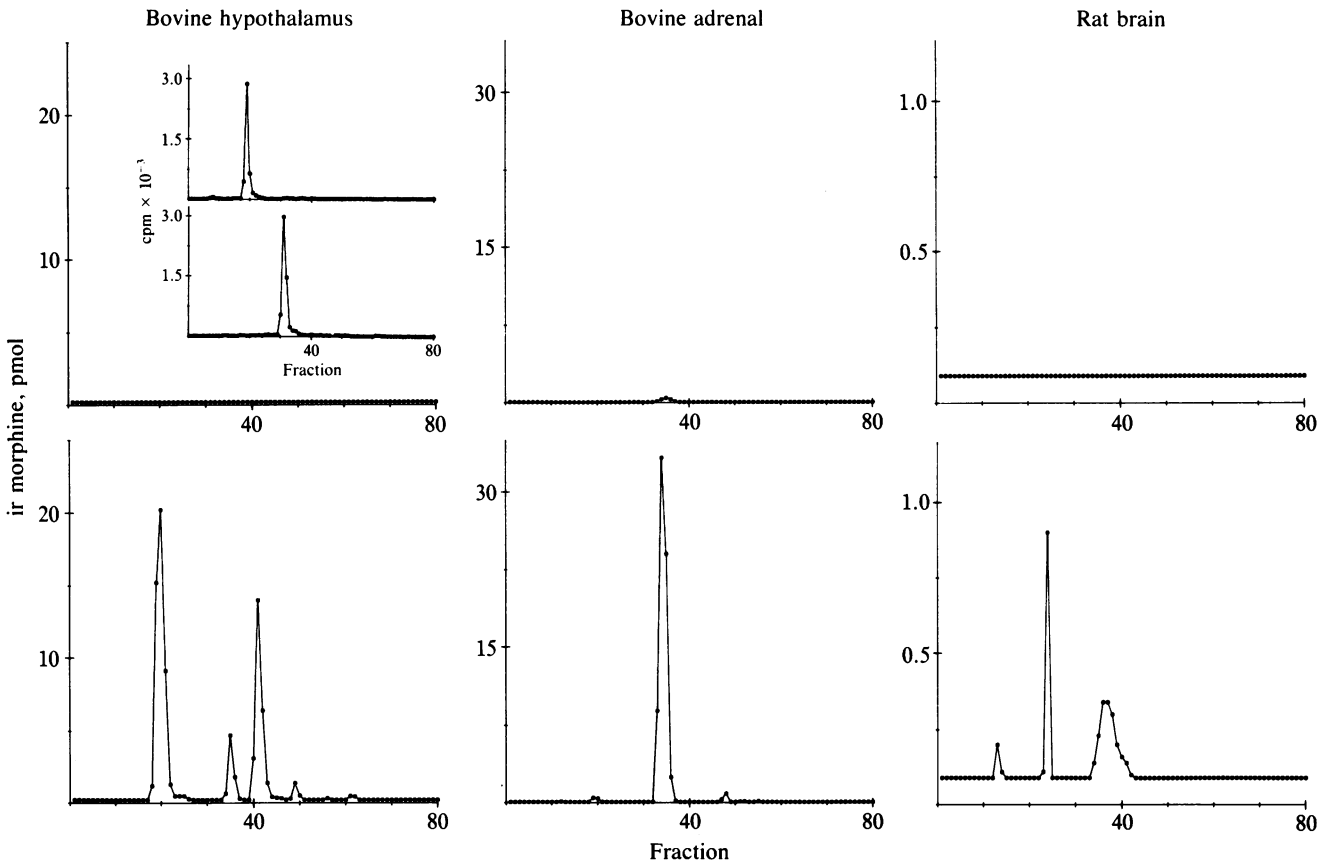

FIG. 2. HPLC profiles of ir morphinans (antiserum 937) from analytical purification experiments (see *Materials and Methods*). (*Upper*) Sequential blanks. (*Lower*) Tissue extracts. The quantity of material analyzed was equivalent (in wet weight) to extracts from about 15 g of bovine hypothalamus, 400 g of bovine adrenal, and 400 g of rat brain. (*Inset*) Separate experiments in which ring-labeled [^{3}H]morphine (upper tracing) or [^{3}H]codeine (lower tracing) internal standard was added to bovine hypothalamus homogenate at the outset of extraction.

total content (as ir morphine) has been consistently 5–25 pmol/g of wet weight. In bovine adrenal we have variably detected ir morphinans that correspond to peak 1 and peak 4, but in these experiments the levels are far lower (from <1 to 100 fmol/g of wet weight) than we reported before. In rat brain extracts we have also detected ir morphinans that correspond to peaks 1 and 4, and here also the levels are variable (from <1 to 200 fmol/g of wet weight). Since we adopted the sequential-blank procedure, no blank has contained immunoreactivity greater than that corresponding to 2% of the accompanying tissue extract, nor have we detected peaks 2, 3, or 6 in fifteen independent purification experiments.

When ring-labeled [^{3}H]morphine was added at the outset of the extraction of bovine hypothalamus, 26% was recovered

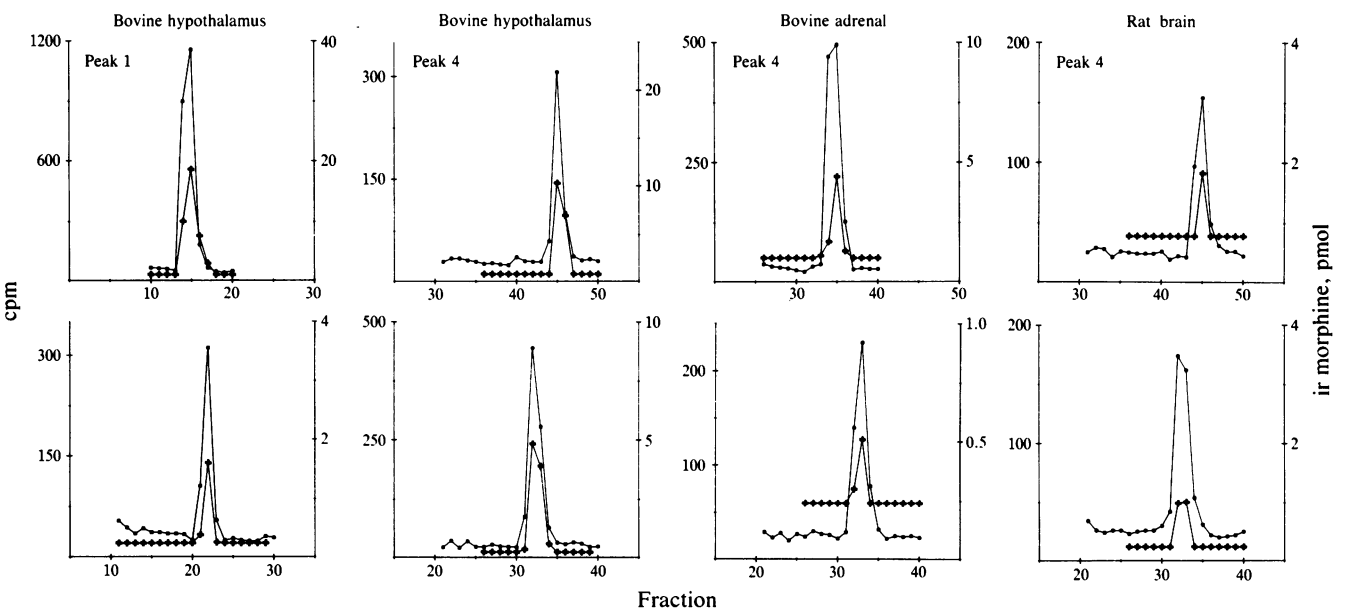

FIG. 3. Double-system HPLC coelutions (see *Materials and Methods*). (*Upper*) HPLC-C system. (*Lower*) HPLC-M2 system. For peak 1 experiment, ring-labeled [^{3}H]morphine was used; for peak 4 experiments, [^{3}H]codeine was used. Larger peaks, cpm; smaller peaks, ir morphine.

as a single peak at the elution position of authentic morphine (Fig. 2, *Upper Inset*). When the same experiment was performed with [*O-methyl*-^{3}H]codeine, 35% was recovered as a single peak at the codeine position (Fig. 2, *Lower Inset*).

With respect to HPLC elution position and immunoreactivity ratio, the ir morphinans corresponding to peaks 1 and 4 appeared to be identical to morphine and codeine, respectively. To test this hypothesis, we carried out HPLC coelutions (Fig. 3) as described above, using two HPLC systems that contrasted markedly in pH and solvent dielectric constant. Peak 1 material from bovine hypothalamus comigrated in both systems with internal standard [^{3}H]morphine; the quantity of peak 1 from adrenal and rat brain was insufficient for coelution studies. Peak 4 from bovine hypothalamus, bovine adrenal, and rat brain comigrated in both systems with internal standard [^{3}H]codeine.

In every step of the preparative purification procedure, peak 1 was eluted in the same position as morphine: fraction 53 on cation exchange, fraction 31 on anion exchange, fraction 24 on HPLC-M2, and fraction 27 on HPLC-C. Peak 4 was eluted at every step in the same position as codeine: fraction 50 on cation exchange, fraction 8 on anion exchange, fraction 32 on HPLC-M2, and fraction 43 on HPLC-C. Overall recovery of peak 1 from after the initial phase partition was 74% (75 nmol as ir morphine); that of peak 4 was 42% (7 nmol as ir morphine). Following the HPLC-C step, the criterion of sufficient purity for further structural studies was achieved for both ir morphinans—a single symmetrical peak of UV absorbance that coincided with a single symmetrical peak of immunoreactivity.

Fig. 4 shows the mass spectrum comparison (trifluoroacetyl derivatives) of peak 1 with morphine and peak 4 with codeine. The reconstructed ion chromatograms from GC/MS analysis of all solvent blanks and sequential blanks were free of peaks corresponding in m/z values to the major ions in derivatized morphine (m/z 477 and 364) or codeine (m/z 395 and 282) spectra (data not shown). In the peak 1 studies, the only significant peak in the total ion current had the GC retention time of authentic morphine. In the peak 4 studies, there were several peaks in the total ion current trace, one of which had the retention time of authentic codeine. In all studies, independent estimates of ir-morphinan quantity achieved by RIA, UV absorbance, and quantitative comparison of ion current with an external standard agreed within a factor of 3 (data not shown).

High-resolution GC/MS studies of the trifluoroacetyl derivative of peak 1 yielded a molecular ion of m/z 477.1009 ± 0.0016 (SEM) [calculated value for bis(trifluoroacetyl)morphine ($C_{21}H_{17}F_6NO_5$): 477.1011, M^+] and a major fragment ion of m/z 364.1173 ± 0.0011 (SEM) (calculated value for $C_{19}H_{17}F_3NO_3$: 364.1161, $[M - O_2CCF_3]^+$. The quantity of peak 4 material was not sufficient for high-resolution GC/MS.

We conclude that peak 1 is morphine and peak 4 is codeine.

DISCUSSION

Using stringent blank procedures, we have detected three ir morphinans consistently in extracts of bovine hypothalamus and variably in extracts of bovine adrenal and rat brain.

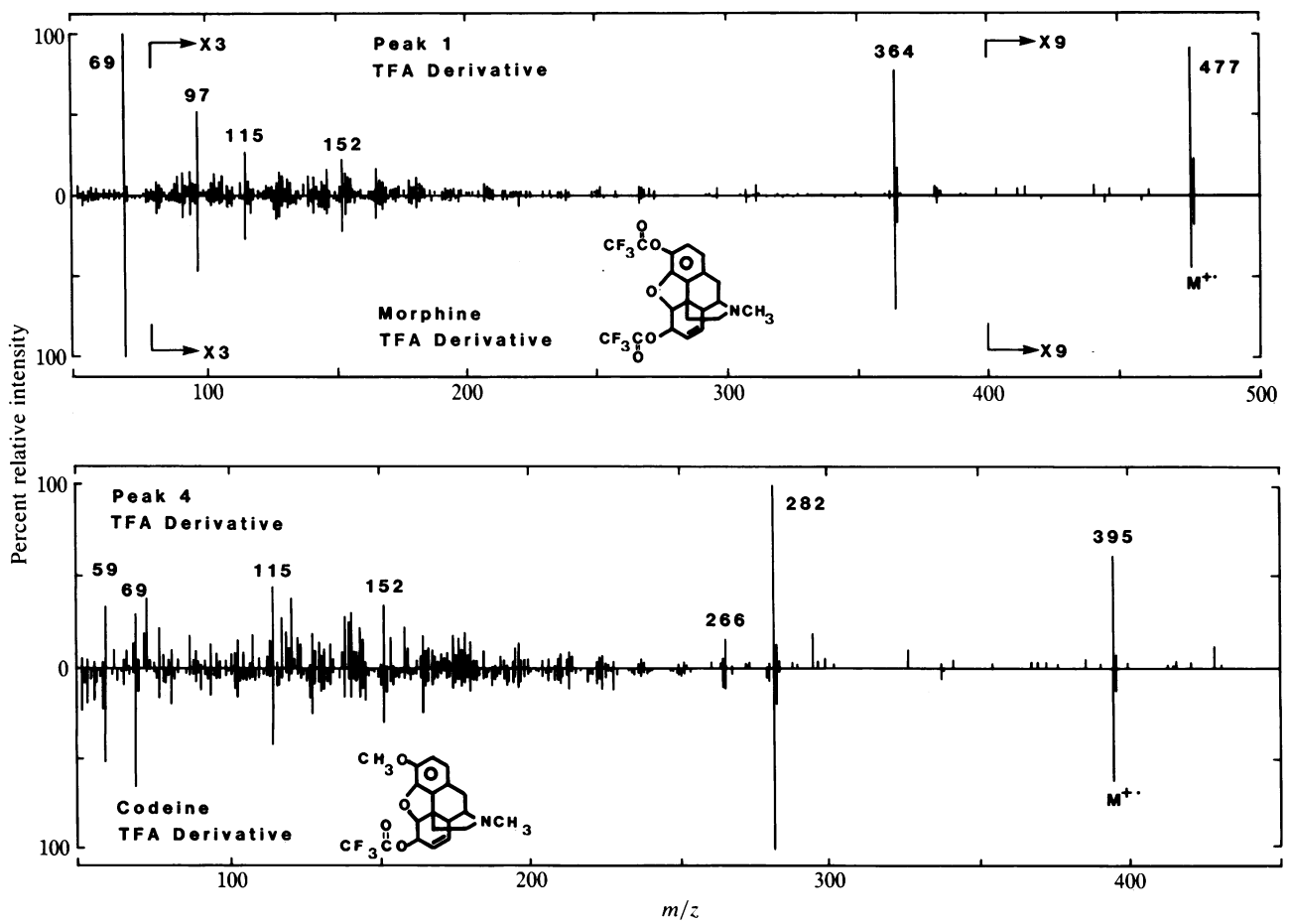

FIG. 4. Comparison of the mass spectra of the trifluoroacetyl (TFA) derivatives of peak 1 and morphine (*Upper*) and peak 4 and codeine (*Lower*). Within each panel, the mass spectrum of the purified ir morphinan is represented by the top tracing, and that of the standard by the inverted lower tracing. Standards were eluted from the final HPLC-C step and processed for GC/MS exactly as were the ir morphinans, as described under *Materials and Methods*.

These corresponded to our previously reported (15) peaks 1, 4, and 5. Immunoreactivity ratio, anion- and cation-exchange chromatographic behavior, and comigration with authentic standards in two reversed-phase HPLC systems indicated that peak 1 was morphine and peak 4 was codeine. Each was purified from bovine hypothalamus; GC/MS and elemental composition analysis from high-resolution GC/MS established that peak 1 is morphine, and GC/MS established that peak 4 is codeine. These results fully support our prior conclusion that "compounds closely related to morphine are present in bovine brain and adrenal" (15).

We have determined the structures of the compounds corresponding to our previously reported peaks 2, 3, and 6 from bovine adrenal and hypothalamus and have been able to identify a likely source of contamination that accounted for their presence. Since adopting in every experiment the stringent sequential-blank procedure, we have not detected peaks 2, 3, and 6, nor have we seen again the very high levels of immunoreactivity (nmol/g) we reported for bovine adrenal.

Although peak 4 had the same HPLC-C elution position as codeine, we argued in our previous paper (15) that it was not codeine because its immunoreactivity ratio was incompatibly low. In the present investigations we found that a small ir-morphinan component crossreactive only with antiserum S17 could be separated on further purification, following which peak 4 was indistinguishable from codeine in immunologic as well as chromatographic properties.

The stability of ring-labeled morphine in the extraction and purification procedure (Fig. 2 *Inset*) indicates that neither codeine nor the peak 5 material is derived artifactually from morphine. Although 35% of added *O*-methyl-labeled codeine was recovered intact, we would not have detected any 3-demethylated derivatives. The material in peak 5 has a relatively high immunoreactivity ratio and is more lipophilic than either morphine or codeine, properties that together are consistent with substitution at position 3 and/or 6; however, it is eluted much earlier than thebaine.

It is clear that morphine and codeine are present in extracts from mammalian brain. Codeine is known to be the immediate biosynthetic precursor to morphine in the opium poppy (17); the presence of both opiates in brain could indicate a similar relationship or merely reflect coexistence in an exogenous source. Two fundamental questions remain. (*i*) Are the morphinans in brain endogenous or exogenous? (*ii*) In either case, do they play a role in brain function?

We thank Patricia Lowery for outstanding technical assistance and Dr. E. F. Ullman (Syva) for providing antisera. Dr. H. Rapoport (University of California, Berkeley) kindly performed the NMR spectroscopy that aided in identifying the structures of the contaminant peaks 2, 3, and 6. K.F.F. and G.F. were supported in part by grants to Dr. J. D. Barchas from the National Institute of Mental Health (MH23861) and the National Institute on Drug Abuse (DA01207). This investigation was supported by Grant DA1199 from the National Institute on Drug Abuse to A.G.

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