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Presence of Morphine metabolites in human cerebrospinal fluid after intracerebroventricular administration of Morphine

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SUMMARY

After intracerebroventricular administration of morphine in four cancer patients, cerebrospinal fluid (CSF) was analyzed by two morphine radioimmunoassays (RIA), liquid chromatography (LC) and radioreceptor assay (RRA) to evaluate the presence of morphine metabolites. Immunoreactive morphine-like substances were detected by differential RIA's. The maximum concentrations of these compounds were achieved 3 hours after drug administration. These concentrations, according to the specificity of the antiserum, represent a mixture of several metabolites in which only morphine 3-glucuronide(M 3-G) and morphine 6-glucuronide (M 6-G) were identified by LC, and M 6-G by LC-RRA.These results confirm that brain is able to metabolize morphine to inactive (M 3-G) or more potent (M 6-G) derivatives.

INTRODUCTION

The presence of morphine metabolites in human blood after systemic or oral administration of morphine has been extensively described (1,2). Some of these metabolites bind to opiate receptors (3,4) and are pharmacologically active in animals (5,6,7) and in humans (8). The pharmacological activity of morphine metabolites strictly depends on their structural conformation. Briefly, 6-glucuronide or 6-sulfate metabolites of morphine are much more analgesic than morphine, while 3-glucuronide or 3-sulfate metabolites showed no analgesic effect (5).

The metabolism of morphine occurs in several organs, principally in liver (1). However, little is known about the metabolism of morphine within the central nervous system, although brain contains the enzymes which form sulfate (phenol-sulfotransferase) and glucuronide (UDP-glucuronosyltransferase) conjugates (9,10,11). The first observation of morphine brain metabolism was reported by Cardinale et al. who have described conjugated, acid hydrolysable forms of endogenous morphine and codeine in human CSF (12). Then, Wahlstrom et al. demonstrated the in-vitro formation of morphine glucuronides after incubation of morphine with human post-mortem brain tissues (13). We report here the in-vivo presence of morphine metabolites in human CSF after intracerebroventricular (ICV) administration of morphine in cancer patients.

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MATERIALS AND METHODS

Chemicals:

^{3}H-morphine (1.83 TBq/mmol) and 3H-DAGO (2.22 TBq/mmol) were obtained from Amersham. Morphine, normorphine, levallorphan and morphine 3-glucuronide were a gift from Francopia, Paris. Morphine 6-glucuronide was a gift from Laboratoires X-TEC, 91370 Verriéres-le -Buisson, β-glucuronidase from bovine liver, type B-10, was purchased from Sigma - Chimie, 38297 La Verpilliére. The haptens 3-carboxymethylnormorphine and 6-succinylmorphine were synthesized in this laboratory according to published procedures, and characterised by tandem

mass-spectrometry (MS/MS) (Laboratoire de Biophysique, Université Pierre et Marie Curie, Paris).

Patients:

Four male patients suffering from intractable pain caused by orofacial malignancies received intracerebroventricular hydrochloride morphine 0.4 ± 0.04 mg (expressed as base). Each patient gave informed consent before the protocol. Morphine was administered through an Ommaya reservoir implanted under local anesthesia with the catheter tip positioned in the frontal horn of the lateral ventricle at the level of the foramen of Monroe. For 36 hours prior to reservoir implantation no opiates were given and pain was controlled with non-opiate drugs. The ICV morphine administration was performed 24 hours later. Ventricular CSF (1ml) and peripheral blood (5ml) were simultaneously collected prior to ICV administration and at 1,3,6,12 and 24 hours thereafter. Following centrifugation, samples were stored at -20°C until analysis.

ASSAY METHODS: Radioimmunoassays (RIA):

Morphine CSF and plasma concentrations were measured by two RIA's. Total morphine ,i.e. unchanged morphine and morphine metabolites, was evaluated by RIA with an antiserum raised in rabbits with 6-succinylmorphine (6-SM) linked to bovine serum albumin (BSA) as an immunogen. 6-SM was prepared according to Simon et al. (14). Linkage of the succinyl moiety at the 6-OH position of morphine was confirmed by a positive phenol test of 6-SM and MS/MS analysis. This hapten was conjugated to BSA by the mixed-anhydride method of Wainer et al. (15). Immunization of five New Zealand white rabbits according to a standard protocol (16) gave anti- morphine antiserum during 2-10 weeks. For a chosen batch of antiserum, the association constant (Ka) was determined by Scatchard plot analysis. The cross-reactivity of this antiserum against several morphine-related compounds was calculated relative to morphine at the concentration sufficient to cause 50% displacement of labelled morphine.

Unchanged morphine was measured by a second RIA using antibodies generated in a goat against N-carboxymethylnormorphine (N-CMN) linked to BSA. The synthesis of N-CMN has been previously described by Gintzler et al. (17). Free and bound labelled morphine were separated at equilibrium (1 hour at laboratory (temperature) by ammonium sulphate precipitation at half saturation.

To assess recovery of morphine, 3 CSF blanks loaded with 6.25, 12.5, and 25 ng/ml morphine were assayed in triplicate with the two RIA's.

Quantitative recovery of morphine metabolites was also performed in blank CSF spiked with both M 3-G and M 6-G (10 ng/ml of each) 0.5 ml aliquots of CSF drawn at 3 hours were adjusted to pH 5.0, incubated at 35°C for 24 hours with 12,500 U of β-glucuronidase containing 10,000 units per mg solid, and assayed by the two RIA's.

Liquid chromatography (LC):

CSF samples were analyzed by LC: without extraction, samples were chromatographed by reverse-phase LC using a Merck analytical system with a photodiode array detector L-3000, a sample injector fitted with a 50 µl loop and a Lichrosorb RP-Select-B column (250x4 mm,5 µ m). The isocratic mobile phase was composed of 10% acetonitrile in water containing 1.5 mM 1 - heptanesulfonic acid adjusted to pH 3.5.

Under these conditions and at a flow rate of 0.9 ml/mn, standard morphine 3-glucuronide has a retention time of 3.7 min; morphine 6-glucuronide: 5.7 min; normorphine: 9.6 min and morphine: 11.1 min (Fig 2A). 15-min fractions were collected (Gilson FC 203 fraction collector), dried under nitrogen at 40°C and directly assayed by the two RIA's.

Radioreceptor assay (RRA):

Eluted LC fractions were evaporated to dryness under nitrogen. The resjdues were reconstituted in 0.2 ml of Tris-HCL buffer (50 mmol, pH 7.4) and assayed by RRA. The source of opioid receptors was total brain homogenate from Sprague Dawley rats (3 months, 250 g) after removal of the cerebellum. The brain was rapidly homogenized in 30 ml Tris buffer , then centrifuged at 49,000 g for 10 minutes. The pellet was resuspended in 30 ml Tris Buffer, then incubated at 37°C for 30 minutes in order to remove morphine-like peptides from their binding sites (18). The solution was recentrifuged and the pellet washed with 30 ml Tris buffer. Binding assays were performed with 500 µl Tris buffer, 25 µl ^{3}H- DAGO (1 nM), and 25 µl morphine for the standard curve, or 25 µl of the eluted LC fractions and 25 µl of levallorphan (1 µM) for the determination of non-specific binding, and finally 500 µl of the brain pellet diluted to 1/40. After incubation at 37°C for 60 minutes, bound and free 3H-DAGO were separated by filtration through Whatman GF/B filters using a Brandel cell-harvester system (Gaithersburgh, MD, USA). After washing the filters with 5 ml x 4 Tris buffer, the filters were placed in scintillation vials containing 5 ml of Filter-count TM LSC cocktail (Packard).

RESULTS

Radioimmunoassays

The K a values at equilibrium determined by Scatchard plot analysis were 4.10 9 M -1 for the 6-SM antiserum, and 2.10 9 M -1 for the N-CMN antiserum.

The 6-SM RIA procedure leads to a detection limit of 50 pg/tube. The standard curve was linear over a concentration range of 1.0 to 100 ng/ml (linear/Log plot). Within-day CV was 4.2 ± 2.8% and between-day CV was 5.0 ± 3.1%. The recovery was found to be 100 ± 2.5% for morphine, 99.0 ± 2.8% for morphine-6-glucuronide, and 81.0 ± 4.0% for morphine-3-glucuronide, against a morphine base standard curve.

The detection limit of the N-CMN RIA was 5 pg/tube, the standard curve was linear between 0.1 and 50 ng/ml. Intra-assay CV were 3.4- 5.5%, and inter-assay CV were 4.1-7.5%. The recovery of morphine with this RIA was 101 ± 1.8%.

The two antisera possess different specificities due to the stuctural differences of the antigens against which antibodies were raised: The 6-SM antiserum raised against the 6-succinyl morphine antigen binds the 6-glucuronide conjugate to the same degree as morphine itself (Table I). However, it has limited ability to discriminate structural changes around the 3-OH position; thus 3-O-glucuronide had high cross-reactivity with this antiserum (80%). Coupling of morphine to carrier protein on this side of the molecule does not give a good discrimination of 3-glucuronide and 6-glucuronide (19).

The specificity of the N-CMN antiserum (Table I) excludes interference from the main morphine metabolites and endogenous morphine like-peptides. This RIA was previously used for several morphine pharmacokinetic studies and was validated against liquid- chromatography (20,21).

Before morphine administration, plasma and CSF morphine concentrations determined by both RIA methods were consistently negative. After morphine administration, unchanged morphine concentrations were calculated as the total assay result using N-CMN RIA. The good sensitivity, accuracy and precision of the two RIA's allowed calculation of the concentrations of morphine metabolites (expressed as morphine equivalents) by substracting the amount of morphine measured with the N-CMN RIA from the result obtained with the 6-SM RIA. Figure 1 represents the time course of unchanged morphine and morphine metabolite concentrations in CSF of 4 patients.

The maximum concentration of morphine metabolites occurred 3 hours after morphine administration. At this time, the metabolite concentration represents from 1,5% to 3,6% of the morphine concentration in the four patients.It was 9% to 25% at 24 hours.

Despite the large concentrations of morphine in CSF, neither total morphine nor unchanged morphine were detected in plasma for 24 hours after administration.

Incubating CSF with β-glucuronidase induces an increase of 2% to 4% in morphine concentrations measured by the specific morphine RIA, and leads to the disappearance of a significant difference in concentrations between the two RIA's.

Table I: Percentage Cross-reaction relative to Morphine of Anti-morphine Antisera to Morphine Metabolites and Analogues. Cross-reactivity was calculated at concentrations sufficient to cause 50% displacement of labeled morphine from the antiserum.

	N-CMN antiserum %	6-SM antiserum %
Morphine	100	100
Morphine 3-glucuronide	<0.2	80
Morphine 6-glucuronide	<0.2	100
Normorphine	30	<0.2
Codeine	<0.2	84
β-endorphin	<0.05	<0.05
Leu-enkephalin	<0.05	<0.05
Met-enkephalin	<0.05	<0.05

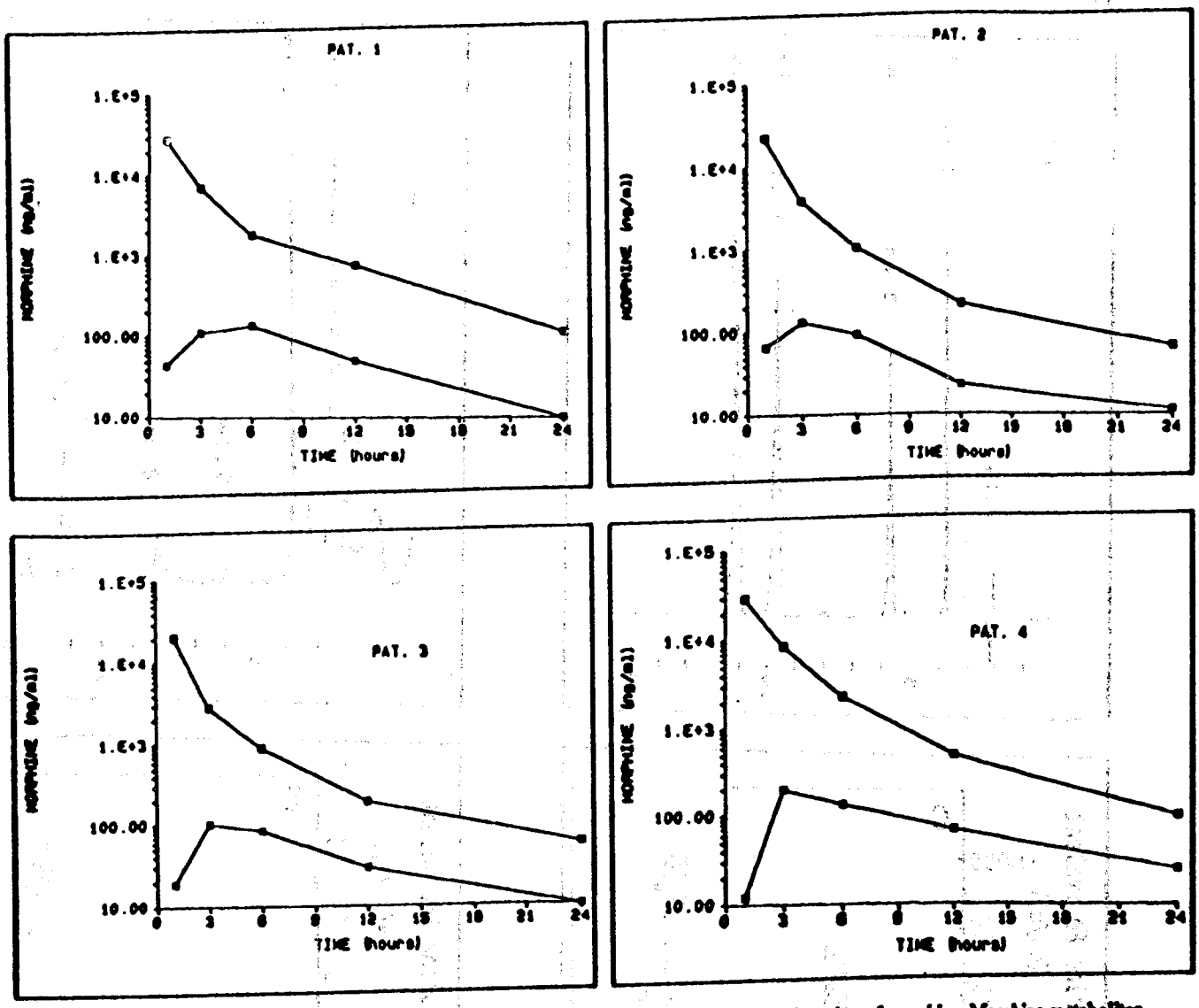

Figure 1: Unchanged morphine concentrations (■) in CSF of 4 patients after ICV administration of morphine. Morphine metabolites concentrations (°) expressed as morphine equivalents, were calculated by difference between values obtained from 2 morphine RIA's.

Liquid chromatography

After CSF analysis by LC (Fig 2B), eluted fractions were assayed by the two RIA's: Using the specific N-CMN RIA, only one immunoreactive peak was observed and was identified by its retention time as morphine. With the 6-SM RIA a peak was eluted at the same position as morphine and two other immunoreactive fractions at 3.7±0.2 min and 5.7±0.2 min were also found. These two immunoreactive fractions have the same retention time as morphine 3-glucuronide and morphine 6-glucuronide respectively (Fig 2C).

Radioreceptor assay

Saturation analysis of the opioid receptors with 3H-DAGO resulted in a B max of 46 fmol/mg protein, a K d of 4.7 nM for the labelled compound, and a Hill coefficient of 0.75. With a 25 µl morphine sample, sensitivity was of the order of 2.5 ng/ml.

LC eluates assayed by RRA revealed the presence of two fractions with an affinity to the opiate receptor: fraction 11-12 which corresponds to morphine (Fig 2D), and fraction 6 which correlates to the LC peak of morphine 6-glucuronide.

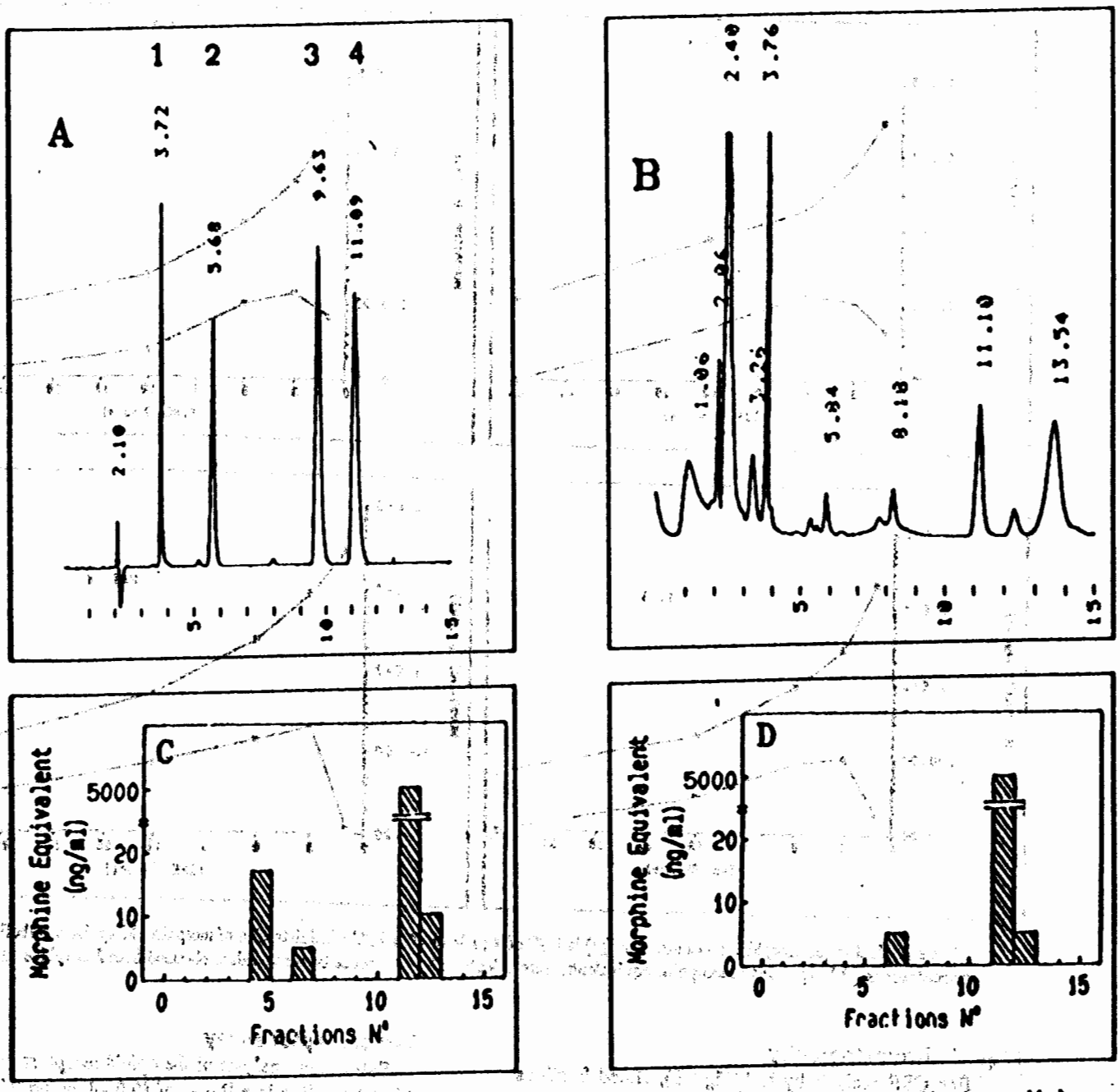

Figure 2: A: Chromatogram of opiate standards (1 = morphine 3-glucuronide, 2 = morphine 6-glucuronide, 3 = normorphine, 4 = morphine). B: Chromatogram of CSF (patient 4) drawned 3 hours after morphine ICV administration. C: Fractions of LC eluate obtained from a CSF, and analysed for immunoreactivity against 6-SM antiserum. D: Fractions of LC eluate obtained from a CSF, and analysed by RRA.

DISCUSSION

Similar use of two morphine antisera with different specificities has been described by several authors for detection of morphine and morphine-like compounds in animal tissues (22), or in human CSF (12). This procedure shows that morphine metabolite detection can be performed by differential radioimmunoassays when sensitivity and assay precision are adequate.

Morphine and morphine metabolites expressed as "total morphine" and detected by 6-SM RIA represents only a mixture of several morphine-related compounds that cannot be individually quantified because of their different cross-reactivity with this antiserum (Table 1).

The quantification of morphine metabolites by this method,is an approach that only reveals the presence of immunoreactive morphine-like compounds.

The major question is whether these immunoreactive morphine-like compounds detected in CSF are endogenous opiates (peptides or alkaloids) or morphine metabolites biosynthesized in the CNS from administered morphine. The detection of these compounds cannot be due to the cross-reactivity of the 6-SM antisera with unknown opioid peptides, because anti-morphine antisera do not react with any of the peptide conformations of opioid peptides (Table 1), as confirmed by several authors (22,23). Endogenous morphine and codeine have been reported as being present in femtomolar range concentrations in conjugated forms in human CSF (12), and functional adaptation to pain can increase the concentration of endogenous conjugated morphine (24). This possibility is excluded in this study because no morphine could be detected in CSF before drug administration. On the other hand, the absence of detectable total or unchanged morphine in plasma after ICV morphine administration makes the penetration of morphine metabolites into the CSF after hepatic metabolism unlikely.

The morphine metabolites revealed in human CSF in this study are probably M-3G and M-6G as demonstrated by i) the detection of two immunoreactive peaks by LC-RIA, ii) the increase in morphine concentrations after incubating CSF with β-glucuronidase, iii) the presence of a fraction in RRA which corresponds to the LC peaks of M-6G.

Formation of morphine metabolites in brain reaches a maximum 3 hours after morphine administration. This indicates a metabolite formation rate very much slower than in liver (13).

The relatively high concentrations of morphine metabolites calculated by differential RIA, do not correspond to M-3G and M-6G recoveries by LC-RIA or LC-RRA. This suggest that other morphine metabolites (ethereal sulfate morphine, glutathionyl-morphine ?) were probably included in these concentrations and cannot be individualized by LC.

Normorphine, an active metabolite, was not detected by LC-RRA, although N-dealkylation of morphine in animal brain has been described (25); Perhaps the concentration of normorphine in human CSF was too low to be detected by our procedures.

The results of this study confirm those obtained in vitro by Wahlström et al. (13): morphine is metabolized within the CNS. The concentration of morphine metabolites certainly depends on the route of morphine administration: the large concentrations of morphine obtained in ventricular CSF after ICV administration results in a maximum formation of metabolites.

The significance of brain morphine glucuronidation is of interest. As remarked by Ghersi-Egea et al.: "lipophilic drugs could remain within the brain almost indefinitely if they were not converted to more polar metabolites which are eliminated in the blood" (26). This is an attractive assumption because in this study no morphine could be detected in blood for 24 hours after ICV administration, which emphasizes the long persistence of morphine in cerebral tissues. However, the concentration of morphine metabolites was too low to be detected when distributed in the blood compartment.

The degree of morphine metabolite participation in analgesia and perhaps in opiate tolerance or dependence remains to be clarified in humans.

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