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Note

Rapid extraction and measurement of morphine and opiate antagonists from rat brain using high-performance liquid chromatography and electrochemical detection

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A major site of action of opiates is the central nervous system. Following parenteral administration, these drugs penetrate the blood–brain barrier and enter the brain in concentrations high enough to induce the classic opiate effects having a central component (*e.g.*, antinociception, respiratory depression, constipation, etc.). Hence, in mathematical treatments of the relation between drug concentration and observed effect, brain concentration is preferred over administered dose[1,2].

Morphine concentration in brain tissue is often determined by first isolating the drug using a liquid–liquid extraction technique[3] and then quantifying the results using one of several available methods such as thin-layer chromatography, spectrofluorometry, immunoassay, electron capture or mass spectrometry[4,5].

A simplified extraction procedure has been reported for morphine and opiate antagonists[6] in which brain homogenate is adjusted to pH 8.9 by glycine buffer and then suspended on a polar matrix for exposure to the extracting solvent. This method is highly efficient and considerably more rapid than older techniques.

Recently, several authors have shown that solutions or blood extracts containing morphine or certain other opiate agonists and antagonists can be analyzed using high-performance liquid chromatography (HPLC) and electrochemical detection[7–9].

The present report describes a protocol by which the prototype opiate agonist, morphine, and antagonist, naloxone, can be rapidly extracted from rat brain and measured by HPLC with electrochemical detection. By combining and modifying the previous extraction and measurement procedures for compatibility, we found the sensitivity of this process to be sufficient to detect nanogram levels of morphine and naloxone in specific brain regions of rats.

MATERIALS AND METHODS

Extraction

Whole brains or brain sections from male Sprague-Dawley rats were homogenized by Teflon® pestle tissue grinder or sonicated (Bronwill, Rochester, NY, U.S.A.) in 1–4 ml of borate buffer (0.05 *M* boric acid, 0.043 *M* sodium borate) at pH 8.9. Borate buffer was found to be less reactive than glycine buffer at the electrochemical detector. Naltrexone (400 ng) was added to the buffered homogenate to serve as the

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internal standard. This mixture was then placed into extraction columns using Eppendorf pipettes. The extraction columns consisted of an inert hydrophilic material of large surface area, prepacked in disposable polypropylene barrels (ClinElut®, Analytichem International) to which we attached metal valves.

Two minutes after adding the homogenate, spiked with naltrexone, to the extraction columns, 30 ml of solvent was added. Ethanol–chloroform (10:90) is the solvent of choice for morphine, chloroform alone for naloxone[3,6]. The extracting solvent was then drained from the columns into tapered centrifuge tubes. Two additional 2-min exposures to solvent (10-ml volumes each) were followed by a 10-ml wash by solvent. Typically, some solvent remained in the extraction columns.

Special precaution was taken to ensure that solvent only contacted chloroform-resistant plastic, glass or stainless steel. Contact with other materials resulted in unwanted peaks on the chromatogram. Similarly, all glassware was siliconized (Siliclad®; Clay Adams, Parsippany, NJ, U.S.A.) before use to minimize the known problem of morphine adsorption.

The extraction solvent was evaporated from the centrifuge tubes by placing the tubes in a 55°C water-bath and passing filtered air or nitrogen over the open ends. The small amount of residue remaining after evaporation, pale yellowish green in color, was used immediately or refrigerated in the evaporation tubes at 5°C until analyzed. For the analysis, 100 μl of HPLC-grade methanol was added to the tubes using an Eppendorf pipette. The tube was then gently shaken for approximately 1 min, after which samples were injected by Hamilton syringe (No. 705) into the 20-μl sample loop of the HPLC apparatus.

Apparatus

The method used for measuring morphine concentration was adapted from previous reports[7,9]. The HPLC equipment was a Bioanalytical Systems (West Lafayette, IN, U.S.A.) Model LC-303 using a Waters M-45 solvent delivery system, Rheodyne sample injector (Model 7125), 20-μl sample loop (Model 7022), LC-3 amperometric detector and glassy carbon electrode. The reverse-phase column (RSil C18HL, 10 μl) had dimensions 25 cm × 1/4 in. O.D. × 4.6 mm I.D. (Alltech, Deerfield, U.S.A.). A potential of +0.6 to +0.8 V across the electrochemical detector provided good peak height, peak shape and signal-to-noise ratio.

Mobile phase

The mobile phase consisted of methanol–water (25:75) containing 50 m*M* tetramethylammonium hydroxide and adjusted to pH 6.1 with phosphoric acid. All chemicals were of HPLC grade and the solution was degassed and filtered (2 μm) before use. The mobile phase was delivered at a rate of 1.5 ml/min at ambient room temperature. Back pressure at this flow-rate was approximately 1500 p.s.i.

Drug standards

Morphine sulfate (Mallinckrodt, St. Louis, MO, U.S.A.), naloxone hydrochloride (Endo Labs., Garden City, NY, U.S.A.) and naltrexone hydrochloride (Endo Labs.) standards were made by dissolving the appropriate salts in HPLC-grade water.

RESULTS AND DISCUSSION

Shown in Fig. 1 are typical chromatograms of rat brain homogenate to which measured amounts of morphine sulfate, naloxone hydrochloride and naltrexone hydrochloride were added. Retention times for morphine, naltrexone and naloxone were approximately 5 min, 6.5 min and 8 min, respectively. Consistent with previous reports, the sensitivity of the electrochemical detector is about four times greater for morphine than for naloxone or naltrexone. As can be seen, at this sensitivity there are relatively few unwanted peaks or interferences with the peaks corresponding to the drugs of interest. At very high sensitivity (10 nA/V, 0.1 V full scale) a peak interfering with morphine (but not naltrexone or naloxone) was sometimes detected. The appearance of this peak was most pronounced when large amounts of tissue in proportion to chloroform were added to the extraction columns. Best results were obtained when tissue homogenate penetrated the column material less than about one fourth of its total length. Pilot studies indicated that per cent recovery was greater than 80% even under poor extraction conditions. Under optimal conditions, recovery would be expected to approach the high values originally reported by Sprague and Takemori[6].

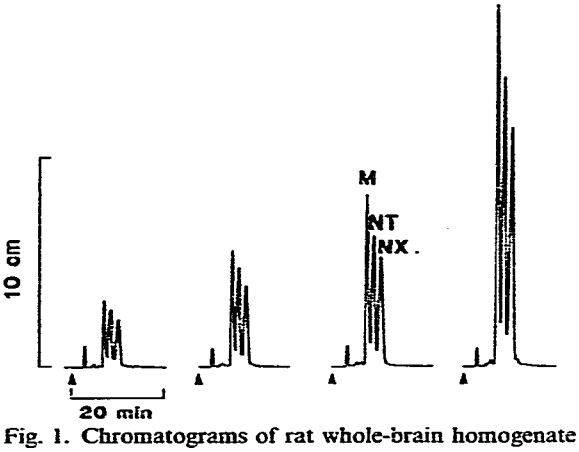
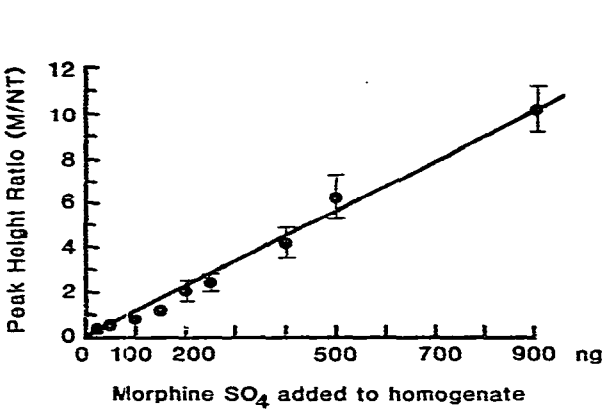


Fig. 1. Chromatograms of rat whole-brain homogenate showing changes in peak height ratios with increasing concentrations of morphine (M), naltrexone (NT) and naloxone (NX) added to the homogenate. Morphine sulfate concentrations are 200, 400, 500 and 900 ng. Naloxone and naltrexone concentrations are four times the concentration of morphine in each case. Chromatograms shown are from a column that had been used in several hundred analyses. With a new column, separation of the three drugs is more complete, with no overlap. Flow-rate = 1.5 ml/min; 10 nA/V, 1 V full scale; +0.6 V; 1400 p.s.i. Arrows indicate time of injection; 10 cm is approximately 5 nA.

For quantifying the amount of morphine present in brain tissue, the ratio of the height of the morphine peak to the height of the naltrexone peak was found to be convenient and reliable. The calibration curve for this measure was obtained by adding known amounts of morphine sulfate and naltrexone hydrochloride to the brain homogenate prior to extraction. The results for morphine are shown in Fig. 2. There is a good linear relationship ($r = 0.96$) between the amount of morphine sulfate added to the homogenate and the ratio of morphine to naltrexone peak heights. The equivalent calibration curve for naloxone is shown in Fig. 3.



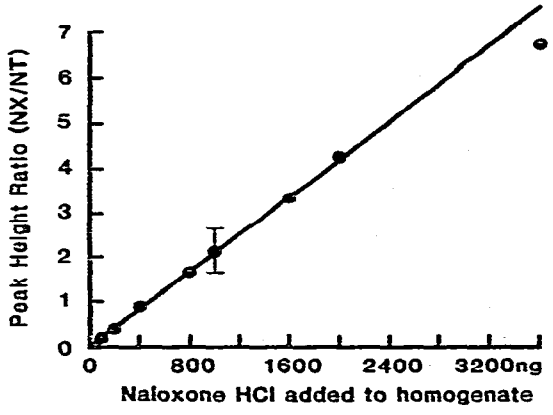


Fig. 2. Relationship between measured peak height ratio (morphine sulfate to naltrexone hydrochloride) and amount of morphine sulfate added to whole-brain homogenates of untreated rats. M = Morphine sulfate; NT = naltrexone hydrochloride. The data points represent the mean (± S.E.M.) of at least four runs. Where not shown, error is within data point symbol.

Fig. 3. Relationship between measured peak height ratio (naloxone hydrochloride, NX, to naltrexone hydrochloride, NT) and amount of naloxone hydrochloride added to whole-brain homogenate of untreated rats.

The procedure described in this paper was applied to measuring morphine levels in specific brain regions following subcutaneous (s.c.) administration of the drug to rats. The regions were dissected by the method of Glowinski and Iversen[10] at various time intervals following drug administration. A representative chromatogram of brainstem tissue is shown in Fig. 4. The other brain regions showed a similar pattern. Preliminary results from rats given morphine sulfate 60 min prior to testing show 223 ± 25 (S.D.) ng/g, 765 ± 206 ng/g and 1085 ± 293 ng/g of morphine sulfate present in brainstem following s.c. administration of 7.5, 35 and 55 mg/kg, respectively.

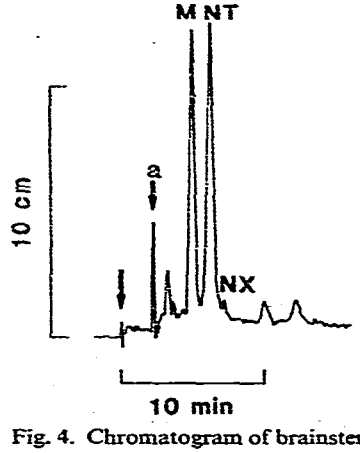


Fig. 4. Chromatogram of brainstem tissue from a rat that received 7.5 mg/kg morphine sulfate s.c. 60 min before and 0.5 mg/kg naloxone hydrochloride s.c. 30 min before testing. Flow-rate = 1.5 ml/min, 10 nA/V, 0.1 V full scale; +0.6 V; 1400 p.s.i. Unlabelled arrow indicates time of injection. Arrow labelled "a" indicates peak produced by solvent front; 10 cm is approximately 5 nA.

Morphine was detected in brain regions following s.c. doses as low as 2.5 mg/kg given 60 min before testing. Naloxone was not as readily detected at the subcutaneous doses normally used (0.05–0.25 mg/kg), hence brain levels were determined by pooling the extractions of several rat brains, by extrapolation from results of higher-than-normal administered doses, or by using the less convenient +0.8 V potential.

Morphine metabolites were not systematically examined in the present study. Normorphine can be separated and detected by HPLC with electrochemical detection, as has previously been reported[9]. Morphine-3-glucuronide analysis has also been described[9]. In a pilot study to measure morphine glucuronide, brain homogenates of rats pretreated with 80 mg/kg morphine sulfate s.c. were incubated with β-glucuronidase (Glucurase™; Sigma, St. Louis, MO, U.S.A.) at 37°C for 20 h and then extracted and measured as usual. Comparison with non-incubated homogenate samples showed a 20% increase in the ratio of morphine to naltrexone peak heights, indicating the presence of morphine glucuronide in amounts consistent with determinations using other methods[11].

In conclusion, the procedure described in the present report appears to offer a rapid and effective means to measure morphine and opiate antagonist levels in brain tissue. Application of this procedure to other types of tissue is presently being investigated.

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