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DISPOSITION OF NALOXONE: USE OF A NEW RADIOIMMUNOASSAY

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ABSTRACT

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Understanding of the pharmacology of the narcotic antagonist naloxone has been limited by the lack of a convenient and sensitive method of assay. A radioimmunoassay for naloxone has been developed and is described. It is applicable for drug analysis in either serum or brain. The limit of sensitivity of the assay was 0.1 ng. Naloxone glucuronide, noroxymorphone (nor-naloxone) and morphine were not recognized by the antibody whereas naltrexone and 6-hydroxynaloxone were able to displace naloxone-^{3}H from the antibody. The assay was of sufficient sensitivity to follow the serum levels of naloxone in man for up to 2 hours after an i.v. injection of 0.4 mg. In animal studies, the biologic half-lives of naloxone or morphine (5 mg/kg) were compared after s.c. injection in rats. The peak serum levels A (1 μg/ml), time to peak serum levels ($< \frac{1}{2}$ hour), and serum half-life (40 minutes) were comparable. However, the brain entry and egress of the two compounds differed markedly. Peak brain levels of naloxone occurred within 15 minutes and had declined by 50% within 1 hour, whereas the peak brain levels of morphine were sustained for up to 2 hours. At peak serum levels, the brain/serum ratio for morphine was 0.1 whereas for naloxone it was 15 times greater. We suggest the high brain/serum ratio of naloxone contributes to its potency whereas the rapid egress from the brain is important in the short duration of action of naloxone.

Naloxone is a potent and specific antagonist of narcotic analgesics (Blumberg *et al.*, 1965; Fink *et al.*, 1968). Its usefulness includes both the treatment of narcotic overdose and prophylaxis of narcotic abuse (Zakes *et al.*, 1971). The short duration of action of naloxone can limit its utility as a narcotic antagonist (Hasbrouk, 1971; Evans *et al.*, 1974; Johnstone *et al.*, 1974). The explanation for this short duration of action is lacking. Studies on the disposition of naloxone have been hampered by insufficient sensitivity of assays in analyzing the very low tissue and fluid drug concentrations which occur *in vivo*.

In these studies, we describe the serum and brain diposition of naloxone in rats in comparison with that of morphine. Naloxone was measured by a new rapid, sensitive and specific radioimmunoassay. The applicability of the assay for measurement of naloxone in human serum is also shown.

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Materials and Methods

Preparation of immunogen and antibodies. The naloxone immunogen was linked to the protein carrier bovine serum albumin (BSA) with ethyl-N-carbamyl-cyanomethyl-acetimidate (DAZ reagent). The DAZ reagent (30 mg) was dissolved in 1 ml of water, 30 mg of BSA and 2 mg of 0.5 M sodium bicarbonate solution were added and the solution was stirred for 48 hours at 4°C and dialyzed extensively against phosphate-buffered saline. The BSA derivative solution was placed in an ice bath and diazotized by the addition of 3 ml of 4% sodium nitrite and 3 ml of 1 N HCl were added. Thirty seconds were allowed for diazotization and then 3 ml of a 2% ammonium sulfamate solution were added and the solution was mixed for another 30 seconds. Twenty milligrams of naloxone hydrochloride were dissolved in 2 ml of 0.5 M sodium acetate buffer, pH 4.8, and were added to the above mixture and the solution was stirred for 10 minutes maintaining the pH at 4.8 with several drops of 1 N NaOH. The pH was then adjusted to pH 7.4 with 0.5 N NaOH. The mixture was kept at 4°C for several hours and centrifuged to remove insoluble residues and then dialyzed against distilled water for 48 hours. The solution was adjusted with buffered saline to a final concentration of 1 μg/ml as protein. Male, 2 kg, New Zealand Albino rabbits were injected every month for 6 months and then every other month with 0.2 mg of naloxone immunogen in a 50% emulsion of complete Freund's adjuvant.

Immunoassay of naloxone. Ten to 200 μl of serum containing naloxone were assayed undiluted or, when serum naloxone concentration exceeded 1.0 μg/ml, were diluted with buffered saline. Aliquots of serum were added to tubes containing 20 μl of naloxone-^{3}H (1000-1500 cpm), 100 μl of rabbit antiserum diluted with buffered saline and 250 μl of buffered saline. Depending on the assay, the naloxone antisera had been diluted 1:75 (for animal studies) or 1:500 (for human studies). Samples containing less than 200 μl of serum required the addition of rabbit serum to make a total of 200 μl of serum in each sample. Final sample volumes were 570 μl.

For analysis of naloxone in the brain, samples were homogenized in 4 volumes of 0.01 N HCl and centrifuged at 30,000 × *g* for 10 minutes. The total supernatant volume was measured, and a 200-μl aliquot was assayed. Samples with concentrations of naloxone in excess of 5 μg/g may be diluted with 0.01 N HCl. The sample was added to a tube containing 100 μl of rabbit serum, 20 μl of naloxone-^{3}H (1000-1500 cpm), 100 μl of rabbit antisera (1:75 dilution) and 100 μl of 0.05 N sodium phosphate buffer, pH 7.3.

All tubes were incubated at room temperature for 60 minutes and then 0.5 ml of saturated ammonium sulfate solution was added. Fifteen minutes were allowed for the proteins to precipitate. Samples were centrifuged at 3000 × *g* at 4°C for 20 minutes and the supernatant fluid was discarded. The precipitate was washed twice with 0.5 ml of 50% saturated ammonium sulfate and the supernatant fluid was discarded after each centrifugation. The remaining precipitate was dissolved in 0.5 ml of Protosol and rinsed into a liquid scintillation counting vial with three 4-ml washes of liquifluor scintillation fluid. Radioactivity was determined by liquid scintillation counting.

Standard curves were obtained by adding known amounts of naloxone to either buffered saline (aqueous), brain homogenate, or serum. The standard curves obtained from different fluids were fairly close to each other as shown in figure 1. These data were obtained with a 100-μl aliquot of a 1:75 antibody dulution. Continued immunization of the rabbits resulted in a high titer antiserum and subsequently allowed a 10 fold increase in sensitivity which was obtained by using a 1:500 antibody dilution. The higher titer antisera was utilized for studies of naloxone in a human subject. The variability of duplicate determinations was 10% or less. The limit of sensitivity for this assay was about 2 ng of naloxone per g of brain and 0.5 ng/ml of serum. The absolute limit of sensitivity was about 0.1 ng.

Sample collection. Male Sprague Dawley rats, 250 to 300 g, received 5 mg/kg of either naloxone or morphine s.c. Blood was collected after decapitation and allowed to clot, and the serum was separated by centrifugation, was frozen and analyzed within 48 hours. Naloxone was also given i.v. to a healthy male human volunteer in a dose of 0.4 mg/70 kg. Venous

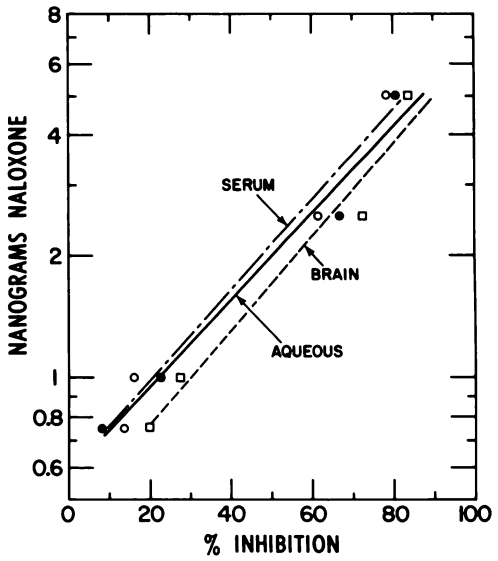


FIG. 1. Inhibition of binding of naloxone-^{3}H to rabbit antiserum by nonradioactive naloxone in different fluids. Antisera was diluted 1:75 with phosphate-buffered saline. The lines were fit by the method of least squares.

blood samples were drawn at regular intervals for 2 hours. The serum was separated and frozen. Serum rather than plasma was utilized in all studies since heparin may interfere with the naloxone immunoassay.

Drugs. Naloxone-^{3}H (1 mc/14 ng), dihydromorphine-^{3}H (1 mc/6.2 ng), Protosol and Liquifluor were obtained from New England Nuclear Corporation Boston, Mass. Naloxone and naltrexone were gifts from Endo Laboratories, Inc., Garden City, New York. Buffered saline is 0.01 M sodium phosphate. Morphine was measured by a specific radioimmunoassay (Spector, 1971) as previously described (Berkowitz, *et al.*, 1974).

Results

Naloxone antibody specificity. The concentration of naloxone and its structural congeners which displace 50% of the labeled naloxone from the antibody is shown in figure 2. Two nanograms of naloxone in the sample displaced the naloxone-^{3}H 50% and 4 ng of the reduced metabolite EN-2265 resulted in similar displacement. Removal of the alkyl chain from the nitrogen noroxymorphone, EN-3169 resulted in failure of the antibody to recognize the molecule. Naloxone glucuronide was also poorly recognized and required a 100-fold increase in concentration for equivalent displacement. Similarly, morphine did not compete with naloxone for binding to this antibody. The N-cyclopropylmethyl analog of naloxone (EN-1639 or naltrexone) was recognized by the antibody and required 4 ng for 50% displacement.

Comparative disposition of naloxone and morphine in rats. The brain and serum concentrations of morphine or naloxone were compared after subcutaneous injection (5 mg/kg). Both compounds were rapidly absorbed with peak serum concentrations occurring within 15 minutes (fig. 3). The serum half-life for both morphine and naloxone during the first four hours was about 40 minutes. There was a marked difference in the brain serum ratios of the two compounds. At peak serum levels, the ratio for morphine was 0.1 whereas for naloxone it was 15-fold greater, about 1.5. As the morphine serum levels declined, the brain serum ratio approaches 0.5 whereas that for naloxone remained between 1.5 to 2. Thus, at equivalent serum levels for both drugs, there was at least 3 to 4 times more naloxone in the brain. Perhaps the major difference between the brain disposition of the two compounds is that the peak brain levels of morphine were sustained for up to 2 hours whereas the naloxone brain levels had declined by 50% within 1 hour.

NALOXONE ANTIBODY SPECIFICITY

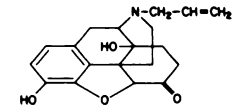
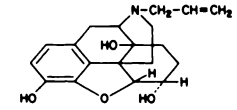
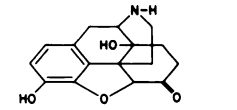
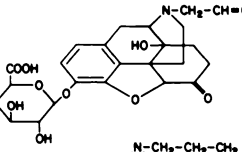
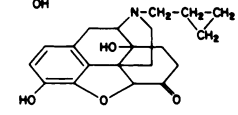
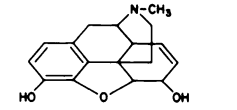
DRUG	STRUCTURE	CONCENTRATION INHIBITING NALOXONE-H^3 BINDING TO Ab by 50% (NANOGRAMS)
NALOXONE		2
EN-2265		4
EN-3169		>40,000
NALOXONE GLUCURONIDE		240
NALTREXONE		4
MORPHINE		>40,000

FIG. 2. Naloxone antibody specificity.

Serum levels of naloxone in man. The sensitivity of the radioimmunoassay appeared sufficient for the measurement of naloxone in human serum. Naloxone (0.4 mg) was injected i.v. into a healthy volunteer subject and blood samples were collected from the opposite arm over a 2-hour period. Within 2 minutes, blood levels had declined to 0.015 μg/ml of serum (fig. 4). Blood levels continued to fall rapidly for 20 minutes and then declined with a half-life of about 57 minutes between the 20 and 120-minute intervals.

Discussion

Naloxone is metabolized by glucuronide conjugation, N-dealkylation and reduction of the 6-ketone group (Fujimoto, 1969a, b; Weinstein, *et al.*, 1971, 1973). The major metabolite is naloxone-3-gluocuronide. Since this molecule is poorly recognized by the antibody, it is unlikely

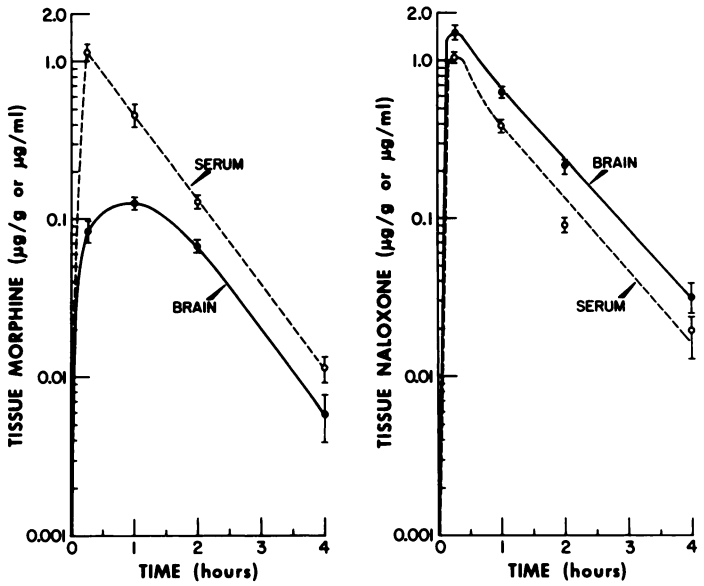


FIG. 3. Comparative brain and serum disposition of naloxone and morphine in rats after a dose of 5 mg/kg s.c. Results are the average ± S.E. of three to five rats at each time interval.

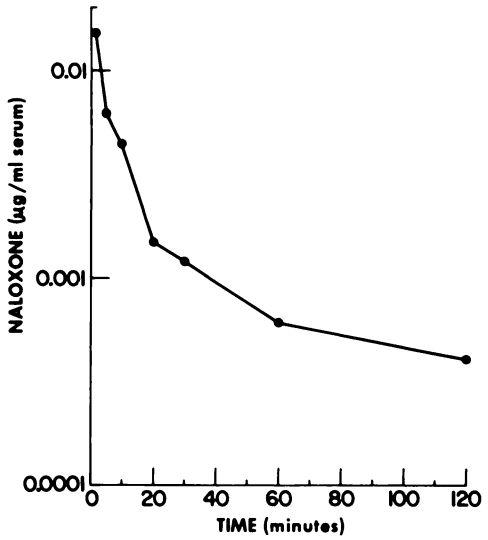


FIG. 4. Serum levels of naloxone in man. Naloxone was injected i.v. at a dose of 0.4 mg into a male volunteer and blood samples were collected from the opposite arm for the next 2 hours.

to be an interfering compound in brain or serum. N-dealkylated naloxone, noroxymorphone, cannot interfere with this assay either since it would have to be present in excess of 20,000-fold. Naloxone may have a pharmacologically active metabolite in the form of its reduced surrogate EN 2265 (Dayton and Blumberg, 1969). However, EN 2265 is not a major metabolite in any species except the chicken and pigeon (Fujimoto, 1969a,b) and while it has been reported in man only small amounts of EN 2265 were found and this was as a glucuronide conjugate (Weinstein *et al.*, 1971). Presently, we cannot exclude EN 2265 as an interfering compound in the rat; however, it is unlikely to be a major contaminant. In man the likelihood that EN 2265 is being measured in the serum is remote.

Morphine is not recognized by the naloxone antibody and the present assay may therefore be used in the future to measure naloxone under conditions where morphine is also administered. It is of interest that the antibody formed against the morphine immunogen, carboxymethyl morphine-bovine serum albumin, does not recognize naloxone (B. Berkowitz and S. Spector, unpublished observation). Clearly, the nitrogen substituent of naloxone and morphine (Koida *et al.*, 1974; Spector, 1971) is a major determinant of antibody specificity. It is tempting to raise the question as to whether these antibodies in any way resemble narcotic or narcotic antagonist receptors in the mammalian brain.

One objective of this study was to determine an adequate methodology for the measurement of naloxone. However, several observations on

the comparative disposition of subcutaneously administered naloxone and morphine merit discussion. Naloxone is both potent and short acting. The present studies indicate that its entry into and egress from the brain are important factors in its potency and duration. After a dose of 5 mg/kg, s.c., of naloxone or morphine, the peak serum levels, time to peak serum levels and serum half-life (40 minutes) were quite similar. The naloxone data are in agreement with the recent report of Misra and Colleagues (1974) in which a half-life of about $^1/_2$ hour was reported for naloxone ^{14}C in rat plasma.

Naloxone achieves a markedly higher brain to serum ratio than does morphine. Moreover, 15 minutes after morphine injection only 0.02% of the dose (as the base) is present in the brain whereas for naloxone 0.25% is in the brain, more than 10 times as much. Equally striking is the difference between the two compounds in terms of retention within the brain. In confirmation of previous studies (Miller and Elliot, 1955; Kupferberg and Way, 1963), we have found that the brain levels of morphine are sustained for at least an hour. The concentration of naloxone is not sustained and declines by 50% within an hour. The rapid decline in the brain levels of naloxone is consistent with and may well contribute to its short duration of action. If one wishes to maintain brain levels of naloxone, multiple dosing is required.

Retrospectively, these results should not have been surprising since pentazocine (Berkowitz and Way, 1971) as well as nalorphine (Woods, 1957), both of which have also had allyl or dimethylallyl chains on the nitrogen atom, also leave the brain more rapidly than morphine. If longer acting narcotic antagonists are desired, one suggestion arising from these studies is that molecules which are retained in the brain for a longer period of time should be investigated. We are presently utilizing the radioimmunoassay to determine the disposition of naloxone after i.v. or oral administration and to determine whether the prolonged action of naltrexone (Resnick *et al.*, 1974) stems from a prolonged brain retention. Should naltrexone not remain in the brain longer than naloxone, the possibility that its prolonged action is due to pharmacologically active metabolites (Cone *et al.*, 1974) will become more likely.

Only one report has appeared on the disposition of naloxone in human plasma (Fishman *et al.*, 1973). These investigators examined the disposition of labeled naloxone and found a plasma half-life of 70 to 90 minutes in two subjects. The radioimmunoassay which has been described is of sufficient sensitivity to follow serum naloxone for 2 hours at doses which are used in man, requires only 0.2 ml of serum for analysis, but does not require the injection of radioactive naloxone. During the 20-minute to 2-hour interval following naloxone administration, we found a serum halflife of about 60 minutes in the subject we examined. Based on the parallel fall of brain and serum naloxone levels in rats, it is probable that brain levels are also rapidly falling in man. Indeed naloxone (0.4 mg/70 kg) may only be effective for 45 (Evans *et al.*, 1974) to 79 minutes (Longnecker *et al.*, 1973) in man.

In summary, the radioimmunoassay which has been described is of sufficient sensitivity and specificity to measure naloxone conveniently in both animals and man. We are currently examining the disposition and pharmacokinetics of naloxone in man. The entry and egress of narcotic antagonists into and out of the brain appear to be important factors in their action.

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