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Naloxone radioimmunoassay: an improved antiserum

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Naloxone is a potent opiate antagonist which has found clinical utility in the treatment of narcotic overdose. More recently the use of naloxone to identify the modulation of various systems by endogenous opiates (Sawynok et al 1979) has renewed interest in its pharmacokinetics in man and laboratory animals. Although a radioimmunoassay (RIA) procedure for naloxone was developed several years ago (Berkowitz et al 1975), both the antiserum and antigen are currently unavailable (Spector, personal communication). Since we required a sensitive and specific assay for naloxone in body fluids and tissues for various ongoing studies in our laboratory (Kreek et al 1983), we were prompted to prepare a new antiserum against the opiate antagonist.

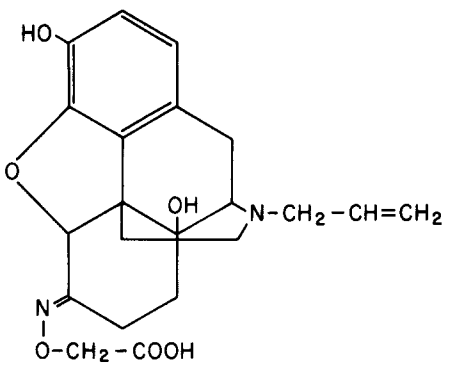
The major determinants of antibody specificity in the naloxone molecule that were of interest to us were the D-ring nitrogen function and the C-3 hydroxyl group. Since the C-6 carbonyl group in naloxone has been shown to lend itself readily to modification (Hahn et al 1975), we decided to produce an antibody which was raised against an antigen containing a substituent at C-6. This antibody was expected to exhibit little binding to other narcotic structures which could be present in the tissues and body fluids to be studied.

In this manuscript we describe the preparation and characterization of a new antiserum to naloxone. The cross reaction of the antiserum with various narcotic substances is examined and discussed. The use of the antiserum for the assay of naloxone in human serum is also reported.

Antiserum preparation. Carboxymethylamine hemihydrochloride (0·39 mmol, Aldrich Chemical Co.) and 2 M potassium hydroxide (0·5 ml) were added to a solution of naloxone (0.26 mmol) in ethanol (10 ml). The reaction was heated under reflux for 2 h, after which time the pH of the cooled solution was adjusted to 7 with 10% hydrochloric acid in methanol. The solvent was removed in-vacuo and the residue was purified by preparative thin layer chromatography on silica gel (Analabs, North Haven, CT) using chloroform–methanol–ammonia (60:30:5) as the solvent system. An infrared spectrum (KBr) of the isolated naloxone-6-(*O*-carboxymethyl) oxime derivative I (Fig. 1) showed that the C-6 ketone absorption was no longer present. The nuclear magnetic resonance spectrum (MeOH-d^4) showed an absorption for the added methylene group in the C-6 oxime derivative. The bovine serum albumin (BSA) derivative of compound I was prepared by a modification of the mixed anhydride procedure (Wainer et al 1972).

Naloxone-6-(*O*-carboxymethyl) oxime (0·12 mmol) was dissolved in dioxane (5 ml) and isobutylchloroformate (0·12 mmol, Aldrich Chemical Co.) and tributylamine (0·12 mmole, Eastman) were added. The reaction mixture was stirred for 30 min at about 18 °C after which a solution of BSA (2·4 μmol) in water (20 ml) and dioxane (20 ml) at pH 8·5 was added. The mixture was stirred at 5 °C for 4 h, and then dialysed for 48 h with water changes every 12 h. The antigen was isolated after removal of the water by lyophilization.

Rabbits were injected once intramuscularly with naloxone-6-(*O*-carboxymethyl) oxime-BSA (NLX-ag) in complete Freund's adjuvant (100 μg/0·5 ml). Two weeks after the first injection the rabbits received



Naloxone-6-(O-Carboxymethyl) Oxime

FIG. 1. Structural formulae of the naloxone hapten.

* Correspondence.

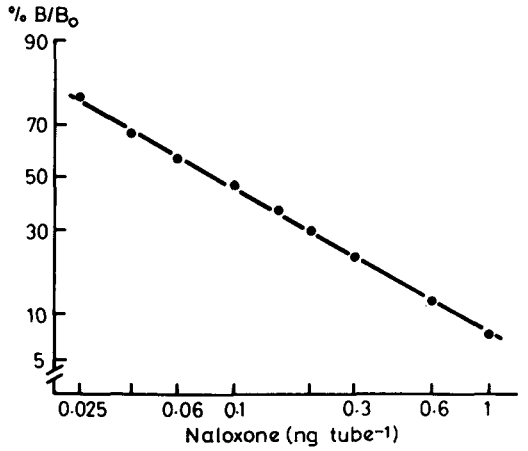


FIG. 2. Standard curve for a naloxone RIA (logit-log plot). Ordinate: logit B/B_0, Abscissa: amount of unlabelled naloxone per assay tube.

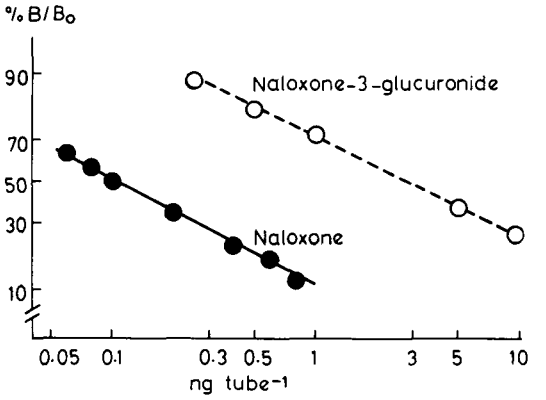


FIG. 3. Displacement of [^{3}H]naloxone from antiserum by unlabelled naloxone (●) or naloxone-3-glucuronide (○). Ordinate: logit B/B_0. Abscissa: amount of unlabelled ligand.

NLX-ag in incomplete Freund's adjuvant. These injections were given weekly for a total of four weeks. One week after the last injection the rabbits were bled. After a rest of four to six weeks the injection schedule was begun again.

Radioimmunoassay procedure. Tritiated naloxone (*N*-allyl-2,3-^{3}H) at a specific activity of 48 Ci mmol^{-1} (New England Nuclear, Boston, MA) was a gift of Dr G. Pasternak. Naloxone (Endo Laboratories, Garden City, NY) standards were prepared by serial dilution in 0·1% bovine serum albumin in saline and stored at 4 °C. All drug concentrations are given as the free base.

The antiserum is diluted with a phosphate (pH 7·2, 0·01 M) saline gelatin (0·1%) buffer (PSG) so that 100 µl will produce a final assay dilution of 1:2400. This antiserum dilution results in approximately 50% of the [^{3}H]naloxone being bound to the antibody in the absence of unlabelled naloxone (see B_0 in Calculations). The assay includes a 0·05 ml aliquot of a sample or standard, 30 000 d min^{-1} of [^{3}H]naloxone and PSG to a final volume of 0·3 ml. After incubation for 1 h at room temperature (20 °C), the assay tubes are transferred to an ice-water bath for 10 min. Separation of free and antibody bound ligand is achieved by the addition of a 0·2 ml aliquot of an iced, stirred suspension of 0·10% dextran and 1·0% charcoal in PSG. After an additional 12 min in the ice bath, the assay tubes are centrifuged for 15 min at 1500*g*. The clear supernatant is decanted into a 7 ml polyethylene vial containing 3 ml Liquiscint (National Diagnostics, Somerville, NJ). Each sample is counted for at least 5 min in a Beckman LS3100 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). An estimate of B_0 is obtained at the beginning, middle and end of each assay set. A change in B_0 of more than 5% caused the assay to be rejected.

RIA standard curve, precision and sensitivity. The standard curve and sample calculations are made using the radioimmunoassay (RIA) program (Clinical Lab and Nuclear Medicine Pac) supplied with the HP-97 electronic calculator (Hewlett-Packard Co., Cupertino, CA). The program performs calculations for a logit/log plot of RIA data using the equation:

$$\text{logit } B/B_0 = m \log X + b$$

where B is the average of duplicate counts min^{-1} in standards or samples containing unlabelled naloxone, B_0 is the average of triplicate counts min^{-1} in the absence of unlabelled naloxone (both numerator and denominator are corrected for non-specific binding); m is the slope, X the amount of unlabelled naloxone standard; and b is the point where the regression line intercepts the logit B/B_0 axis at log X = 1.

Fig. 2 shows a standard curve representing the mean of 10 replicate assays. The linear portion of the curve extends from 0·025 to 1·0 ng/tube. The mean slope is 2·30 ± 0·11 (s.d.) and the correlation coefficient 0·9985 ± 0·0006 (s.d.). The coefficient of variation averaged 5·4% for the 0·04 ng standard and 8·6% for the 1·00 ng assay standard. The mean interassay coefficient of variation for replicates of 180 naloxone containing plasma samples is 6·4%. With a sample volume of 0·05 ml and the standard curve shown in Fig. 2 the RIA has a lower limit of sensitivity of 0·5 ng ml^{-1} which is comparable to the value obtained for the antiserum raised against an antigen containing the carrier protein at C-1 in the naloxone molecule (Berkowitz et al 1975). The lower limit of sensitivity for the antiserum produced in our laboratories appears to be greater, because from Fig. 2 recognition may be extended to 0·025 ng/sample compared with 0·1 ng/sample for the previously reported antiserum. The titer of our antiserum is higher since the results in Fig. 2 were obtained at a dilution of 1:2400 compared to a dilution of 1:500 used by the other workers.

Table 1. Specificity of naloxone antiserum.

Compound	% Cross reactivity
Naltrexone	179
6α-Naloxol	43
6α-Naltrexol	34
6β-Naloxol	31
6β-Naltrexol	28
Naltrexone-3-glucuronide	6
Naloxone-3-glucuronide	4
Oxymorphone	0·8
Levallorphan	0·6
Oxycodone	0·2
Noroxymorphone	<0·1
Levorphanol	<0·1
Morphine	<0·1
Morphine-3-glucuronide	<0·1
(±)-Methadone	<0·1
Dextromethorphan	<0·1
Meperidine	<0·1
(+)-Propoxyphene	<0·1
(+)-Norpropoxyphene	<0·1

Specificity. Cross-reactivity (CR) was estimated by measuring the amount of a compound of interest required to displace [^{3}H]naloxone from the antibody. Percentage CR is calculated from:

$$\%CR = \frac{IN50}{IC50} \times 100$$

where IN50 is the concentration of naloxone that displaces 50% of the [^{3}H]naloxone bound to B_0 and IC50 is the concentration of the compound of interest required to achieve the same 50% displacement.

Table 1 shows the results of the cross-reactivity studies. Naltrexone, its 6-keto reduction products (6α-naltrexol and 6β-naltrexol) and the corresponding 6-keto reduction products of naloxone (6α-naloxol and 6β-naloxol) all showed significant CR. Naloxone-3-glucuronide, the primary urinary metabolite of naloxone in man and laboratory animals (Fujimoto 1969a, b) showed approximately a 4% CR, with a displacement curve for the glucuronide parallel to that of naloxone (Fig. 3). A number of commonly used or abused opioid-type analgesics including morphine, methadone, meperidine, propoxyphene and levorphanol did not show significant CR.

Plasma (heparinized) from normal (opioid-free) subjects, cancer patients receiving therapeutic doses of morphine, or opiate abusers receiving maintenance treatment with 1α-acetylmethadol did not show significant CR.

The C-6 position on the naloxone molecule was selected for linkage to the carrier protein and it was expected that this would generate antibodies which would not exhibit specificity for the C-6 function in ring B. The antibody binding site however should recognize the basic pentacyclic structure and more specifically the different functional groups which compromise the naloxone molecule including the 3- and 14-hydroxyl groups, the 4,5-oxygen bridge and the allyl group on the piperidine ring D nitrogen. The lesser specificity of the antiserum for C-6 derivatives was not of concern since the major metabolites of naloxone in man are the C-3 glucuronide and noroxymorphone, the product of *N*-dealkylation (Fujimoto 1969a; Weinstein et al 1971). Reduction of the C-6 carbonyl group has been reported in man, but only small amounts of β-naloxol were found mainly as the C-6 glucuronide conjugate (Weinstein et al 1971). The 6α-hydroxyl derivative has only been found to be a major metabolite in the chicken and pigeon (Fujimoto 1969b), and therefore it was felt that it would be unlikely to interfere in our assays.

Table 1 shows that the narcotic antagonist naltrexone which differs from naloxone only in the substitution on the piperidine ring D has a high affinity for the antibody. This suggests that the cyclopropylmethyl group, which is known (Gates & Montzka 1964) to be theoretically and chemically analogous to the ethylenic double bond, can fit into the binding site in the naloxone antibody normally occupied by the *N*-allyl group. The antibody is extremely sensitive to other substituents on the piperidine nitrogen since oxymorphone, which differs from naloxone only by an *N*-methyl group, is not recognized at all. Similarly, derivatization of the C-3 hydroxyl group (the 3-glucuronide of naloxone or naltrexone) drastically reduces affinity for the antibody. The 4,5-oxygen bridge and/or the 14-hydroxyl appear to be necessary for recognition since their absence (levallorphan) leads to poor affinity. An intact pentacyclic ring structure is necessary because methadone, meperidine, and dextropropoxyphene do not cross-react at all with the antibody.

Patient studies. Three patient studies were carried out in which the narcotic antagonist naloxone was administered intravenously be constant infusion. All patients were housed in a general clinical centre research unit and all studies were carried out after an initial stabilization period. Bloods were drawn from in-dwelling cannulae at multiple time periods during infusion and centrifuged at 4 °C. The plasma was separated and

Table 2. Pharmacokinetics of naloxone during constant intravenous infusion.

Study no.	Patient wt (kg)	C_{ss} (ng ml^{-1})	Clearance		
			litres h^{-1}	litres h^{-1} kg^{-1}	mg min^{-1} kg^{-1}
1	42·2	15·4	87·6	2·076	34·6
2	48·3	29·7	46·7	0·967	16·1
3	46·8	14·4	80·5	1·720	28·7

frozen at −45 °C until analysis. No haemolysis was observed and plasma samples were assayed directly without extraction. During the first hour, a dose of 1·6 mg h^{-1} was delivered by constant intravenous infusion. Thereafter from the beginning of the second hour through the 24th hour and in one case, through the 28th hour, naloxone was infused at a constant rate of 1·2 mg h^{-1}. The clearance of naloxone was calculated by the equation:

$$C_l = \frac{Ki}{C_{ss}}$$

in which Ki is equal to the infusion rate (in these three cases, 1·2 mg h^{-1}), C_{ss} is equal to the plasma concentration at steady state (as measured by the radioimmunoassay method described herein).

The results in Table 2 show that in two of the three patient studies the metabolic clearance rate (MCR) ranged from 80–90 litres h^{-1}. This clearance rate is similar to the report of Fishman et al (1973) in which a MCR of 90–100 litres h^{-1} was found in three studies following a single i.v. bolus injection of a tracer dose (less than 5 μg) of [^{3}H]naloxone. Table 2 also lists the steady state concentration of naloxone in the three patients. In a report by Ngai et al (1976), following intravenous injection of 0·4 mg naloxone the concentration of drug ranged from 10 ng ml^{-1} to 4·3 ng ml^{-1} at 5 min. The values in Table 2 are somewhat higher since in this study naloxone was administered by continuous infusion.

In a single patient study the apparent terminal half-life (t½) was determined from the plasma concentration of naloxone at the time of stopping a 24 h constant rate infusion and a plasma specimen obtained 4 h after the infusion was stopped. The t½ was found to be 2·2 h. In a single patient study Berkowitz et al (1975) determined the t½ of naloxone to be 60 min. This value was obtained using plasma specimens between 20 min and 2 h following a single i.v. bolus injection of naloxone (5 mg kg^{-1}). Similarly, Fishman et al (1973) found the t½ to range from 70–100 min in their studies.

In summary, the preparation of a new antiserum directed against naloxone is reported. We have characterized the antiserum with respect to sensitivity and cross-reaction, and described its use in a RIA for naloxone in human serum.

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