REFERENCES

(1) R. D. Bulbrook, J. L. Hayward, and C. C. Spicer, *Lancet*, **ii**, 395 (1971).

(2) D. P. Rose, P. Stauber, A. Thiel, J. J. Crawley, and J. R. Milbrath, *Eur. J. Cancer*, **13**, 43 (1977).

(3) J. H. H. Thijssen, J. Poortman, and F. Schwarta, *J. Steroid Biochem.*, **6**, 729 (1975).

(4) A. Schwartz, G. Hard, L. Pashko, M. Abou-Gharbia, and D. Swern, *Nutr. Cancer*, **3**, 46 (1981).

(5) A. G. Schwartz and R. H. Tannen, *Carcinogenesis*, **2**, 1335 (1981).

(6) J. W. Nyce, P. N. Magee, G. C. Hard, and A. G. Schwartz, in press.

(7) L. Pashko, A. G. Schwartz, M. Abou-Gharbia, and D. Swern, *Carcinogenesis*, **2**, 717 (1981).

(8) P. A. Marks and J. Banks, *Proc. Natl. Acad. Sci., U.S.A.*, **46**, 447 (1960).

(9) G. W. Oertel and I. Rebelein, *Biochim. Biophys. Acta*, **184**, 459 (1969).

(10) C. R. Dworkin, S. D. Gorman, L. L. Pashko, V. J. Cristafalo and A. G. Schwartz, in press.

(11) G. W. Oertel, *Z. Hoppe-Seylers Physiol. Chem.*, **343**, 276 (1966).

(12) J. F. King and M. Aslam, *Synthesis*, **1980**, 285.

(13) J. P. Flatt, *J. Lipid Res.*, **11**, 131 (1970).

(14) M. Abou-Gharbia, L. Pashko, A. Schwartz, and D. Swern, *J. Pharm. Sci.*, **70**, 1154 (1981).

(15) G. I. Tesser and I. C. Balvert-Geev, *J. Peptide Protein Res.*, **7**, 295 (1975).

(16) V. A. Ziboh, M. A. Dreize, and S. L. Hsia, *J. Lipid Res.*, **11**, 346 (1970).

Nalmefene: Radioimmunoassay for a New Opioid Antagonist

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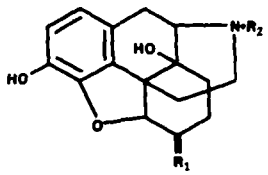
Abstract □ A specific radioimmunoassay (RIA) has been developed for the quantitation of a new opioid antagonist, nalmefene, in human plasma. The method employs a rabbit antiserum to an albumin conjugate of naltrexone-6-(*O*-carboxymethyl)oxime and [^{3}H]naltrexone as the radioligand. Assay specificity was achieved by extraction of nalmefene from plasma at pH 9 into ether prior to RIA. The procedure has a limit of sensitivity of 0.2 ng/mL of nalmefene using a 0.5-mL sample of plasma for analysis. The intra- and interassay coefficients of variation did not exceed 5.6 and 11%, respectively. The specificity of the RIA was established by demonstrating excellent agreement (r = 0.99) with a less sensitive and more time consuming HPLC procedure in the analysis of clinical plasma samples. The use of the RIA for the pharmacokinetic evaluation of nalmefene is illustrated with plasma concentration profiles of the drug in humans following intravenous and oral administration.

Keyphrases □ Nalmefene—human plasma, RIA, compared with HPLC □ RIA—compared with HPLC, nalmefene, human plasma

Nalmefene [17-(cyclopropylmethyl)-4,5α-epoxy-6-methylenemorphinan-3,14-diol] is a potent, orally active narcotic antagonist (1) which is undergoing clinical evaluation in humans. The drug is a structural analogue of naltrexone with an exocyclic methylene group in the 6-position. Hahn *et al.* (1) indicated in preliminary pharmacology studies that orally administered nalmefene was about 50 times more potent than naloxone in its ability to antagonize the antinociceptive activity of an ED_{95} dose of morphine in either the mouse hot-plate or tail-clip procedures. Furthermore, nalmefene was 16 times more potent than naloxone in precipitating narcotic withdrawal in morphine-dependent rhesus monkeys. Recently, nalmefene has been shown to decrease the food and water intake and weight gain in obese and lean Zucker rats, lending further support to a possible role for opioids in the control of food intake (2).

The present study was undertaken to develop a simple and specific radioimmunoassay (RIA) for nalmefene which would provide greater sensitivity and ease of operation than a recently reported high-performance liquid chromatographic (HPLC) procedure (3). It was hoped that the RIA might be utilized in a wide variety of human tolerance and pharmacokinetic studies that are under way with nalmefene.

The initial approach taken in the development of the RIA was to produce an antiserum which would cross-react almost equally well with nalmefene and naltrexone. In this way, high specific activity, commercially available [^{3}H]naltrexone could be used as the radioligand for the assay. Furthermore, the hapten which was likely to elicit the production of such an antiserum could be readily prepared from naltrexone in a fashion similar to the recently reported synthesis of naloxone-6-(*O*-carboxymethyl)oxime (4).



Nalmefene, R_1 = CH_2, R_2 = CH_2—◁
Naltrexone, R_1 = O, R_2 = CH_2—◁
Naloxone, R_1 = O, R_2 = $CH_2CH{=}CH_2$
Hapten, R_1 = N—O—CH_2COOH, R_2 = CH_2—◁

EXPERIMENTAL SECTION

Preparation of Immunogen and Antibody Production—The hapten, naltrexone-6-(*O*-carboxymethyl)oxime[1], was covalently coupled to bovine serum albumin using the mixed anhydride procedure of Erlanger *et al.* (5). The resulting conjugate was dialyzed against 0.05 M $NaHCO_3$, then water, and was then isolated by lyophilization. UV analysis of the immunogen indicated that ~17 moles of hapten were covalently coupled to 1 mol of albumin.

Two New Zealand White rabbits were immunized intradermally and boosted intravenously with the immunogen as previously described by Dixon and Crews (6). The antiserum with the highest titer of antibodies to nalmefene, obtained following the second booster immunization, was used for all subsequent studies. The antiserum was divided into 1-mL aliquots, lyophilized, and stored at −20°C.

Radioimmunoassay Procedure—Plasma samples (0.1–0.5 mL) containing

[1] Synthesized according to the procedure reported for naloxone-6-(*O*-methyl)oxime (4). The NMR, IR, and mass spectra were compatible with the proposed structure.

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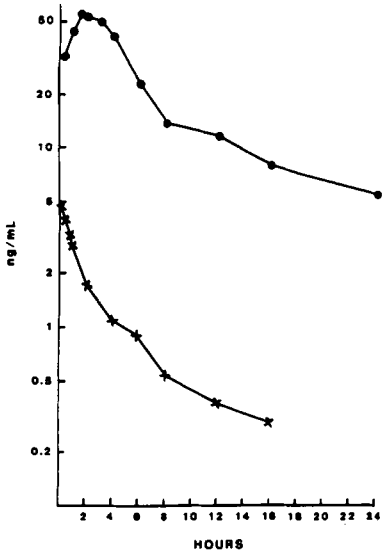

Figure 1—*Plasma concentrations of nalmefene following an intravenous injection (×) of 0.5 mg of nalmefene hydrochloride and an oral dose (●) of 64 mg of nalmefene hydrochloride.*

either known or unknown concentrations of nalmefene were diluted with 0.1 mL of 1 M borate buffer (pH 9) and extracted by vortexing with 2.5 mL of ether in a 12 × 75 mm disposable glass culture tube. Following brief centrifugation, the tubes were immersed in a shallow bath of 2-propanol–dry ice and the aqueous phases were frozen. The organic phase was decanted into a 12 × 75 mm tube and the ether was evaporated using a water bath at 50°C.

Each residue was dissolved in 0.1 mL of methanol and diluted with 0.4 mL of assay buffer[2]. Duplicate 0.2-mL aliquots of each extract were added to 12 × 75 mm tubes followed consecutively by 30,000 dpm of [^{3}H]naltrexone[3] in 0.2 mL of buffer and 0.2 mL of antiserum diluted 1:10,000 with buffer. After incubation at 4°C for 30 min, 0.5 mL of a well stirred suspension of dextran-charcoal[4] at 4°C was added to each tube, the contents were briefly vortexed and then allowed to stand at 4°C for ~10 min. The tubes were centrifuged at 4°C for 10 min at 1200×*g* and each supernatant, containing the antibody-bound radioligand, was decanted into a containing vial. After the addition of 10 mL of scintillation fluid[5], the vial was assayed in a liquid scintillation counter.

A calibration curve was generated using a four-parameter logistic curve-fitting program for a desk-top calculator[6] (7). Both the plasma standards and unknown samples were taken through the entire extraction and RIA procedure.

RESULTS AND DISCUSSION

Radioimmunoassay Characteristics—Depending on the degree of sensitivity required, two different ranges of calibration curves were used. The more sensitive assay ranged from 0.2 to 8 ng/mL using a 0.5-mL sample of plasma for analysis, while the other used a 0.1-mL sample and ranged from 2 to 100 ng/mL. In both instances, the logit-log plots were linear and the interday coefficients of variation ($n = 6$) for both standard curves did not exceed 11% for any individual standard (range, 2.4–11%). Linear regression analysis of the mean concentrations of nalmefene found *versus* the amount of nalmefene present in each plasma standard ($n = 6$) gave correlation coefficients of 0.999 and slopes within 6% of unity for both calibration curves. If necessary, unknown plasma samples with concentrations of nalmefene >100 ng/mL were appropriately diluted to bring the concentrations within the range of the 2–100-ng/mL calibration curve.

Using dextran-charcoal for the separation of antibody-bound and free radioligand, ~50% of the [^{3}H]naltrexone was bound in the presence of diluted antiserum alone while the nonspecific binding (no antiserum) was ~4%. The recovery of nalmefene from plasma using the present extraction procedure was 85 ± 4%; however, since both the standard and unknown plasma samples were handled in an identical fashion, no correction for recovery was necessary.

Assay Specificity—As mentioned previously, nalmefene and naltrexone were expected to cross-react about equally well with the antiserum, thereby allowing the use of commercially available [^{3}H]naltrexone as the radioligand for the quantitation of nalmefene. Cross-reactivity studies confirmed these expectations in that nalmefene exhibited an apparent cross-reactivity of 90% relative to naltrexone. At the same time 6β-naltrexol, the major metabolite of naltrexone found in plasma (8), cross-reacted 40%. The latter observation is not surprising since little antigenic specificity can be expected at the 6 position where the hapten was coupled to albumin. Thus, this antiserum would be of little value for use in a specific RIA for naltrexone due to the extensive cross-reactivity of 6 β-naltrexol. Naloxone, which only differs from naltrexone by having an *N*-allyl function, cross-reacted 20% with the antiserum, an indication that the *N*-cyclopropylmethyl group on the hapten was not a potent antigenic determinant.

During the initial developmental stages of the RIA, the apparent plasma concentrations of nalmefene in clinical samples were considerably higher when the plasma was assayed directly than following the extraction procedure outlined above. This was probably due to high concentrations of nalmefene glucuronide, in the presence of unconjugated nalmefene, cross-reacting to some extent with the antiserum. Therefore, the extraction step became a critical component of the assay procedure since nalmefene glucuronide would not be extracted into ether at alkaline pH.

A definitive and rigorous evaluation of the specificity of the RIA was made by comparing it with a recently established HPLC procedure (3). Clinical plasma samples obtained from four subjects who had received single oral doses of nalmefene hydrochloride (32 or 64 mg) were assayed by both analytical procedures and the joint determinations ($n = 21$) were subjected to linear regression analysis (RIA = y). Over a concentration range of 4–58 ng/mL, the correlation coefficient, regression-line slope, and y-intercept were 0.998, 0.996, and 0.38, respectively, which demonstrated excellent agreement between the two procedures and validated the specificity of the RIA for the quantitation of intact nalmefene in clinical plasma samples.

Nalmefene Plasma Concentrations in Humans—To illustrate the utility of the RIA, some typical plasma concentration profiles of nalmefene in subjects who had received various intravenous and oral doses of the drug are shown in Fig. 1.

Following intravenous administration of 0.5 mg of nalmefene hydrochloride, the plasma concentrations of the intact drug declined rapidly over the first 2 h, followed by a slower elimination phase up to 16 h, at which time the plasma concentration approached the limit of sensitivity of the assay (0.2 ng/mL). For the subject who received a 64-mg dose orally in solution, a peak plasma concentration of 55 ng/mL was reached 1.5 h after dosing and declined to 6 ng/mL after 24 h.

In conclusion, a simple and specific RIA for the quantitation of nalmefene in human plasma has been developed and validated by comparison with an established HPLC procedure. The RIA provides adequate sensitivity to obtain pharmacokinetic profiles of the drug following both intravenous and oral administration to humans. By virtue of its simplicity, the assay can facilitate the analysis of the ever increasing number of plasma samples which are being generated during new drug development studies.

REFERENCES

(1) E. F. Hahn, J. Fishman, and R. D. Heilman, *J. Med. Chem.*, **18**, 259 (1975).

(2) C. L. McLaughlin and C. A. Baile, *Pharmacol. Biochem. Behav.*, **19**, 235 (1983).

(3) R. Dixon and J. Hsiao, *Res. Commun. Chem. Pathol. Pharmacol.*, **42**, 449 (1983).

(4) E. F. Hahn, R. Lahita, M. J. Kreek, C. Duma, and C. E. Inturrisi, *J. Pharm. Pharmacol.*, **35**, 833 (1983).

(5) B. F. Erlanger, F. Borek, S. M. Beiser, and S. Lieberman, *J. Biol. Chem.*, **234**, 1090 (1959).

(6) R. Dixon and T. Crews, *J. Anal. Toxicol.*, **2**, 210 (1978).

(7) M. L. Jaffe, P. J. Munson, and D. Rodbard, "Four Parameter Logistic Curve-Fitting Program for the TI-59 Calculator," National Institute of Child Health, NIH, Bethesda, Md., 1979.

(8) M. E. Wall, D. R. Brine, and M. Perez-Reyes, *Drug Metab. Dispos.*, **9**, 369 (1981).

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[2] Tris buffer (0.05 M) at pH 7.4 containing 0.1% gelatin (Knox) and 0.1% sodium azide.

[3] Naltrexone [15,16-^{3}H], specific activity 34.8 Ci/mM, was purchased from New England Nuclear, Boston, Mass. and stored in methanol at −20°C.

[4] Assay buffer containing 0.05% (w/v) dextran T-70 (Sigma) and 0.5% (w/v) charcoal (Schwartz-Mann) was stored at 4°C.

[5] Aquasol; New England Nuclear.

[6] TI-59; Texas Instruments, Lubbock, Tex.