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Radioreceptor Assay of Narcotic Analgesics in Serum

Joachim Grevel[1,2], Jeff Thomas[1], Mark L. Richards[1] and Wolfgang Sadée[1,3,4]

Received: February 1, 1984; accepted: April 5, 1984.

Abstract: A sensitive radioreceptor assay (RRA) to determine the serum concentrations of fentanyl, pentazocine and morphine was developed on the basis of the drug's competition with a labeled tracer (^{3}H-naloxone) for the membrane bound opioid receptor in rat brain homogenates. The binding data were computer-fitted to a standard curve by means of nonlinear least square regression. Sensitivity of the assay applied directly to serum samples without extraction was limited to approximately 3, 5 and 25 ng/ml for fentanyl, morphine and pentazocine, respectively, because of endogenous plasma constituents that interfere with the opioid receptor binding. With the use of petrol-ether extraction the sensitivity was improved to 0.3 ng/ml fentanyl and 3 ng/ml pentazocine (0.3 ml serum samples). No RRA-active metabolites were detectable after HPLC separation of serum from a patient treated with fentanyl. The plasma concentration time course of fentanyl in a patient, measured by RRA, was similar to that obtained by a radioimmunoassay (RIA). The RRA represents a general procedure for the detection of clinically used opioid analgesics and their active metabolites.

Numerous assay procedures have been published for individual opioid drugs (1). For example the fast-acting narcotic analgesic fentanyl can be detected in serum with a sensitive gas chromatographic assay with a nitrogen/phosphorus-specific detector (2). Most pharmacokinetic studies of fentanyl, however, have employed a radioimmunoassay (RIA) (3). While these assays may be specific for fentanyl in the presence of its metabolites, they fail to answer the question whether any pharmacologically active metabolites are present. Indeed, fentanyl metabolites are known to accumulate in serum to concentrations exceeding those of the parent drug (4). This problem is not limited to fentanyl, but applies to most other opioids as well. An opioid radioreceptor assay (RRA) could be applied to detect active drug metabolites as well as provide a valuable general assay method for comparison with other techniques.

The lack of a significant literature on this subject may be caused by a series of difficulties that impede the application of the RRA to the measurement of opioid serum levels. In addition to interference from serum constituents, the heterogeneity of the opioid receptor system must be considered. The only previous report (5) describing an RRA of opioids in serum failed to adequately overcome these difficulties because of the lack of receptor type selectivity of the tracer employed (^{3}H-buprenorphine). The present report describes a reliable RRA technique to measure serum levels of fentanyl, pentazocine and morphine and their potential active metabolites in the subnanogram range.

Materials and Methods

Chemicals and equipment. [N-Allyl-2,3-^{3}H]naloxone (specific activity 203.5 x 10^{10} s^{-1} $mmol^{-1}$ ≙ 55 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Fentanyl citrate was supplied by Janssen Pharmaceuticals (Beerse, Belgium), pentazocine by Sterling-Winthrop (Rensselaer, NY), and morphine sulfate was purchased from Mallinckrodt (St. Louis). Control serum was purchased in freeze-dried form from Sigma (St. Louis, MO). Glass fiber filters type GF/B were from Whatman (England). The HPLC equipment consisted of: pump M600A from Waters Ass. (Milford, MA), UV detector LC-15 from Perkin-Elmer (Norwalk, CT), column C-18 250 mm x 4.6 mm from Alltech (Los Altos, CA.). The liquid scintillation cocktail was Aquasol, New England Nuclear (Gardena, CA). The scintillation counter (LS 7800) was from Beckman (Irvine, CA).

[1]Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143
[2]Current address: Pharmaceutical Division, Development, Sandoz LTD., CH-4002 Basle
[3]To whom reprint requests should be addressed.
[4]This paper was independently reviewed outside the editioral office.

Serum extraction for fentanyl and pentazocine. Patient serum (300 µl or 50 µl + 250 µl water) was mixed with 25 µl or 5 µl 6 % NH_3, respectively, in a closed 1.5 ml centrifuge tube to give a pH of 9.8. 1.0 ml petrolether was added, and the tube was agitated vigorously until a gel was formed. Separation into two phases was achieved by freezing in dry ice and thawing. Finally, the petrolether phase was pipetted off, and the frozen aqueous pellet was washed once with 1.0 ml cold petrolether. The combined organic phases were collected in culture tubes and blown to dryness by a stream of nitrogen at room temperature. The tubes were sealed and stored until analysis.

Standard curves. Varying amounts of fentanyl (0.1, 0.3, 1.0, 3.0 and 10.0 ng), pentazocine (0.3, 1, 3, 10, 30 ng) and morphine (0.15, 0.5, 1.5, 5, 15, and 1500 ng) were added either directly to the brain membrane incubates or to control serum. Direct RRA assays were performed with 10 or 20 µl serum, while the extraction procedure for fentanyl and pentazocine was performed with 50 or 300 µl serum as indicated. Triplicates of each amount were measured for fentanyl and pentazocine, resulting in 18 data points per curve, while the morphine standard curve consisted of duplicates, except for a triplicate control value (0 ng drug). The standard curves were fitted by a computerized extended least square regression program (NIH, Prophet) (6).

Preparation of membranes from rat brain. Male Sprague-Dawley rats weighing 120–140 g were decapitated and the brain was rapidly removed. The cerebellum was discarded, and the brain was weighed and homogenized for 20 s in 30 ml ice-cold Tris-HCl buffer (pH 7.4) with a Brinkman blender (setting 6). The homogenate was centrifuged at 4°C for 15 min at 40 000 g, and the membrane pellet was resuspended in 30 ml of Tris-HCl buffer. The suspension was kept for 30 min at 30°C in order to increase the number of ^{3}H-naloxone binding sites (7) followed by another centrifugation (15 min, 40000 g). The washed membrane pellet was finally resuspended by brief homogenization for 5 s in 60 volumes (according to the original brain wet weight) of ice-cold Tris-HCl buffer. Each membrane preparation required a separate standard curve because of variations of the biological material. Furthermore, the preparation of the membrane homogenate should not be interrupted at any time. Membranes in the final 1:60 dilution should be used within 60 min. Storage of the ice-cold membrane preparation beyond 120 min resulted in deterioration of the receptor binding which is primarily detected as a loss of affinity (increase of the IC_{50}). The same damage occurred after shock freezing in liquid nitrogen.

Incubation with rat brain membranes and radiolabeled tracer. 500 µl of the brain membrane homogenate and 25 µl ^{3}H-naloxone tracer solution were added to the test tubes containing the serum extracts. Total radioactivity per tube was 40 000 dpm corresponding to a tracer concentration of 0.65×10^{-9} M. The tubes were incubated in a shaking waterbath at 25°C for 40 min. Separation of bound and unbound tracer was achieved by filtration through GF/B filters (2.5 cm), under vacuum suction. In order to prevent filter binding of ^{3}H-naloxone, 2 ml of an ice-cold solution of 10^{-4} M naloxone (unlabeled) in Tris-HCl buffer was applied on the filters immediately before adding the assay samples. This naloxone concentration reduced filter binding maximally and reduced the variability of the results. The assay tubes were emptied into the filter wells, the vacuum valves were opened, and the retained membranes were washed 3 times with 2 ml ice-cold Tris-HCl buffer. By handling each sample individually the disturbance of equilibrium during filtration and washing took no longer than 5 s and did not influence specific binding of ^{3}H-naloxone. The sampled filters were shaken for 3 h with 10 ml Aquasol scintillation fluid and assayed for ^{3}H-content in a liquid scintillation counter.

HPLC separation of serum extracts. A C-18 reversed phase HPLC column was equilibrated at room temperature with 60 % acetonitrile and 40 % aqueous 5 mM K_2HPO_4 (flow rate 4.0 ml/min, pressure 10342 x 10^3 Pa △, 1500 PSI). In contrast to NA^+, K^+ ions do not interfere with the subsequent RRA. 400 µl of either control serum or patient serum containing fentanyl and its metabolites were vigorously mixed with 600 µl of acetonitrile and centrifuged (5 min, 12,800 g). 900 µl of the supernatant was injected into the HPLC. The eluent was collected with a fraction collector into culture tubes at a rate of 4 fractions/min. The fractions were either evaporated under nitrogen at 40°C to complete dryness to be used for direct RRA or were reduced to 100–200 µl fluid. In the latter case the volume was then adjusted to about 300 µl with water, 10 µl of 1 % NH_3 was added, and extraction with petrolether was performed as described above. The dried or extracted HPLC fractions were assayed for reduction of ^{3}H-naloxone tracer binding. The reduction was calculated as ng fentanyl equivalent.

Results

Effect of serum on opioid receptor binding. The addition of serum to the rat brain membrane homogenate reduced opioid binding in a dose-dependent fashion. While 10 µl control serum had little or no effect on ^{3}H-naloxone binding, 50 µl serum reduced tracer binding by 40 to 70 %. At 25 µl serum in the 0.5 ml incubation mixture, a decrease of ~ 10 %^{3} H-naloxone binding was observed. Therefore, 20 µl serum appears to represent the maximum volume that can be added to the incubation mixture of 0.5 ml, thereby limiting the sensitivity of the assay. Addition of 20 µl serum had no measurable effect on the IC_{50} concentration of morphine. This was important to ascertain since Na^+ ions affect agonist binding negatively (8). Several attempts to prevent the negative effect of serum on ^{3}H-naloxone binding were unsuccessful. Neither heat inactivation of the serum at 70°C and 90°C for 10 min before incubation nor the addition of EDTA had any effect.

Of 10 randomly selected patient sera, obtained from the Clinical Chemistry Section, UCSF, 8 did not show a measurable reduction of ^{3}H-naloxone binding when 10 µl serum was added, and one serum sample reduced binding by 10 %. One sample that significantly reduced binding was later shown to be drawn from a patient receiving pentazocine. At 20 µl, only 5 patient sera did not affect ^{3}H-naloxone binding, 2 reduced binding by about 10 %, 2 by more than 10 %, and one sample again contained pentazocine. Therefore, use of the direct RRA should include measurement of control sera from the patient under study.

In order to avoid the serum assay interference and improve the assay sensitivity in particular for fentanyl and pentazocine, an extraction scheme was developed. A serum component that inhibits ^{3}H-naloxone binding was extractable into organic solvents, including ether, but it was not sufficiently lipophilic to be extracted into petrolether. The petrolether extract of serum (300 µl) reduced specific ^{3}H-naloxone binding by only 10 ± 4 % of control. However, both fentanyl and pentazocine

Table I. Parameters of standard curves of fentanyl, morphine and pentazocine, estimated by a computerized curve fitting program. Fentanyl and pentazocine were assayed after petrolether extraction, while morphine was assayed directly in serum samples. The Hill coefficient for fentanyl and pentazocine was N = 1.0 and 1.08, respectively, and that for morphine was N = 0.80. The IC_{50} (nM) value refers to the concentration in the 0.5 ml incubate, assuming full extraction recovery and lack of interference by serum components. VAR and PWR are parameters of the error model

Type of Standard Curve[a]	B_{max}[b] (DPM)	B_{min} (DPM)	I_{50} (ng/sample)	IC_{50} (nM)	VAR	PWR
Fentanyl extracted from control serum (300 µl)	4206 ±97	1101 ±113	0.51 ±0.032	3.6 ±0.2	2.45 ±0.47	1.036 ±0.046
Fentanyl added directly into test tubes containing an extract of control serum (300 µl)	4350 ±115	1160 ±130	0.43 ±0.045	3.0 ±0.3	2.61 ±2.2	1.029 ±0.037
Fentanyl added directly into clean test tubes	4778 ±161	1148 ±96	0.42 ±0.036	2.9 ±0.3	4.4 ±2.2	0.994 ±0.048
Pentazocine extracted from control serum (50 µl)	4683	870	5.50	38	2.74	0.992
Pentazocine added directly into test tubes containing an extract of control serum (50 µl)	4689	1031	3.06	21	6.07	1.003
Morphine added directly into clean test tubes	3061 ±433	695 ±134	0.73 ±0.28	5.1 ±2.0	4.6 ±2.5	1.02 ±0.02
Morphine assayed directly in 20 µl serum samples	2897 ±83	583 ±161	0.84 ±0.13	5.9 ±0.9	5.1 ±2.4	0.97 ±0.07

[a] The amounts of the three opioid drug added are given in the experimental section.

[b] Data represent the mean ± standard deviation (n = 3 independent sets of standard curves or more), except for pentazocine where only one experiment is reported.

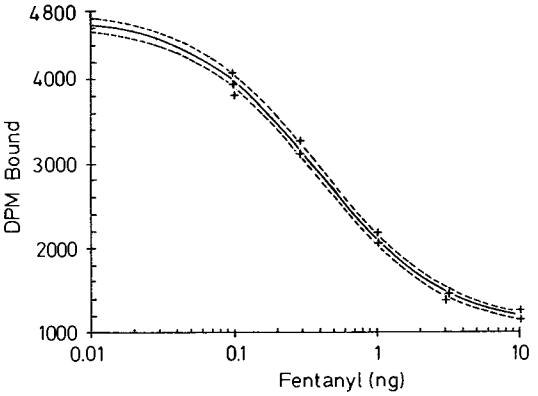

Fig. 1 Standard curve for the extraction of fentanyl from 50 µl control serum. The solid line was fitted by the extended least square regression program MKMODEL (6) using the logistic function shown in Eq. 1. The dashed lines display the S.D. associated with the measurement of the DPM values. The S.D. was calculated according to the error model shown in Eq. 2. The x-axis represents the amount (ng) of fentanyl per sample. To obtain serum concentrations, the values have to me multiplied x 20 (50 µl serum samples). + measured data; — fitted curve; --- ± S.D.

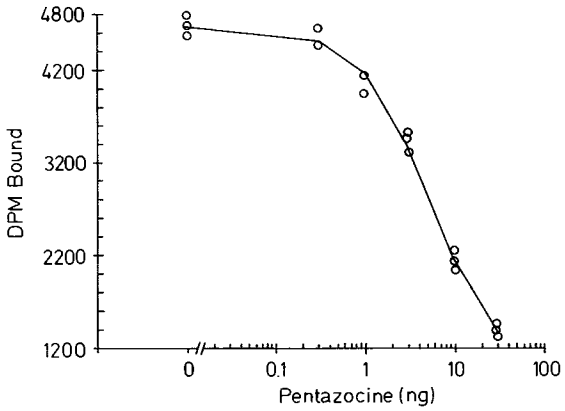

Fig. 2 Standard curve for the extraction of pentazocine from 50 µl control serum (see also legend, Fig. 1).

were readily extractable into petrolether with an overall assay recovery of 84 ± 7% and 57%, respectively (Table I, calculated from the reduction of the aparent IC_{50} values after extraction). Of 10 randomly selected patient sera, only one (300 µl serum containing pentazocine) reduced ^{3}H-naloxone binding by more than 10%.

Standard curves and data analysis. Typical standard curves for fentanyl, pentazocine and morphine are shown in Figs. 1–3. The fitted lines were generated by a computerized extended least square regression program (6). Fig. 1 includes a smoothed binding curve with the standard deviation throughout the assay range. The structural model consists of a logistic function (Eq. 1). It had been shown on empirical grounds, (9) that this function describes the binding data of an RIA as well as the complete law of mass action function, which accounts for different affinity constants of the radiolabeled tracer and the displacing drug. The logistic function also proved suitable for the RRA presented here.

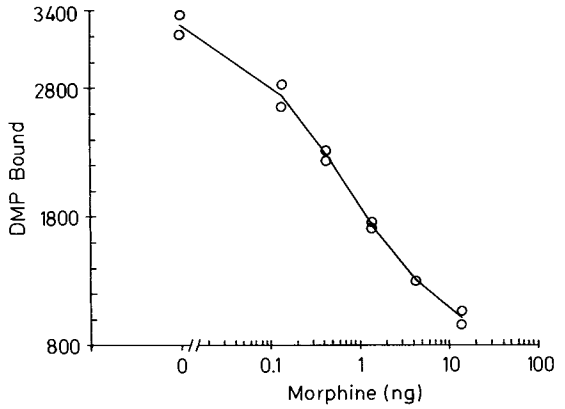

Fig. 3 Standard curve for the assay of morphine in 10 µl control serum samples without extraction. To obtain serum concentrations in ng/ml, multiply with x 100 (see also legend, Fig. 1).

$$\text{Logistic}(X) = B_{max} \quad \text{if } X = 0$$

$$= B_{max} - \frac{B_{max}-B_{min}}{1 + \frac{IC_{50}}{X}^{N}} \quad \text{if } X > 0 \quad \text{(Eq. 1)}$$

X amount of opioid drug in ng present
B_{max} binding of ^{3}H-naloxone as DPM in the absence of any fentanyl
B_{min} binding of ^{3}H-naloxone as DPM which cannot be further depressed by increasing amounts of opioid drug
IC_{50} amount of opioid drug in ng which displaces 50 % of the ^{3}H-naloxone binding between B_{max} and B_{min}
N Hill coefficient which is unity when no binding cooperativity occurs and a homogeneous population of binding sites is present.

The IC_{50} parameter reflects the sensitivity of the RRA. In the case of the standard curve in Fig. 1, the IC_{50} was 0.44 ng fentanyl. If a 50 µl serum sample is extracted, fentanyl concentrations between 1.5 and 50 ng/ml can be estimated with sufficient accuracy (coefficient of variation less than 20 %). Serum samples of 300 µl yielded similar standard curves with slightly increased IC_{50} values (0.51 ng, Table I). In this case, fentanyl levels between 0.3 and 10 ng/ml can be measured. These concentrations fall within the therapeutic range (4, 10). The analytical data obtained from various standard curves (with or without extractions) for fentanyl, pentazocine and morphine are summarized in Table I. Fentanyl displayed highest affinity, followed by morphine and pentazocine (IC_{50} 2.9, 5.1 and 21 nM, respectively). Reduction of the fitted IC_{50} values after extraction was used to calculate analytical recovery (see above). Because the shape of all calibration curves was similar, assay sensitivity is approximately proportional to the IC_{50} values. The Hill coefficient N was not significantly different from 1 for fentanyl and pentazocine, while it was lower than 1 for morphine (0.80 ± 0.14; n = 8).

The dashed curves in Fig. 1 show the standard deviation (S.D.) associated with the measurement of the bound tracer (DPM). In the case of fentanyl there was no detectable difference in the standard deviation whether 50 or 300 µl serum were extracted. The error model for the variance is shown in Eq. 2.

$$\text{Variance} = \text{Var}(Y^{PWR}) \quad \text{(Eq. 2)}$$

Y measured DPM value
VAR scaling parameter of the variance
PWR power parameter for Y

This error model avoids the imposition of arbitrary weighting functions (frequently PWR is set at 2); rather, it defines the error distribution and thus the weighting function on the basis of the actual data in the standard curve. The S.D. is calculated as the square root of the variance. For all the standard curves PWR was approximately 1 (Table I) accounting for a larger error in the presence of low amounts of opioid drug.

Reproducibility. The interassay variability is addressed in Table I as the S.D. of the structural parameters B_{max}, B_{min}, and IC_{50}. Standard curves of fentanyl were constructed from 6 different amounts assayed in triplicates. Omission of the third measurement at each of the 6 amounts resulted in a change of less than 3 % in the computer estimated parameter values. Reducing the number of observations further to only 3 different amounts assayed in triplicates changed the parameters less than 5 %. Thus, the standard curve can be reduced to 9 observations without loss of information about the binding properties of the membrane-bound receptor. The interassay variability is indicated graphically in Fig. 1 and was also determined by measuring 5 replicate samples of the opioid drug at different concentrations. For fentanyl, the coefficient of variation was 9.2 % at 1 ng/ml (300 µl serum extracted) and 14.7 % at 20 ng/ml (50 µl serum extracted). For morphine the coefficient of variation was 29 %, 16 % and 18 %, at 7, 20, and 70 ng/ml, respectively (20 µl serum direct assay).

Fentanyl serum concentration-time curves measured by RRA and RIA. Fig. 4 shows the serum concentration/time course of fentanyl administered as a brief i. v. infusion to a healthy volunteer. Each serum sample was assayed by an RIA (3) and by the RRA. Both curves display the rapid decline of fentanyl serum levels after the end of the infusion. The curve generated by RRA is slightly higher than that derived from RIA measurements. When the RRA results from this experiment were plotted (y-axis) against the RIA results (x-axis), linear regression gave a line with the slope of 1.142 (intercept 3.0 ng/ml) and a correlation coefficient of 0.946.

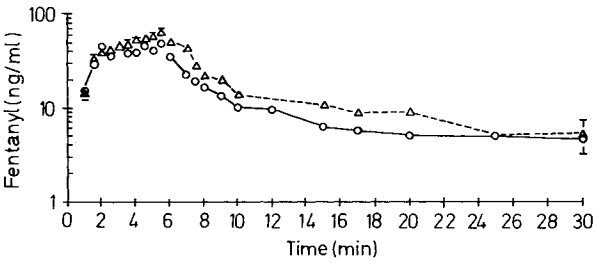

Fig. 4 Serum concentration-time course of fentanyl. A healthy volunteer (male, 57 years, 87 kg) received a brief i. v. infusion (150 µg/min, 5.5 min, total dose of fentanyl 825 µg). Blood samples (3–5 ml) were drawn every 0.5 min beginning 1 min after the start of the infusion. The sampling interval was increased after 8 min. Serum was generated by centrifugation (10 min, 1000 rpm), and each sample was assayed by RIA (0) and RRA (Δ) (± S.D.). Each concentration measured by RRA is the mean of six determinations derived from two independent experiments. The serum samples and RIA data were provided by D. Stanski, Stanford Univ., Palo Alto, California.

HPLC separation in combination with RRA of serum containing fentanyl and its metabolites. In order to address the question whether active metabolites of fentanyl are present in serum, we analyzed serum extracts by HPLC. Fig. 5 shows the HPLC eluent profile monitored by RRA. Direct assay of the eluent fractions displayed significant interfering substances present in control serum (panel A). In contrast, petrolether extraction of the eluent fractions eliminated all interfering peaks (panel B). When a sample of patient serum (drawn 3 h after i. v. injection) containing 8 ng fentanyl (measured by RRA) was separated by HPLC and analyzed by RRA after extraction into petrolether, only a single peak was detected (panel C). The retention time of this peak (4.75 min) corresponded to that of authentic fentanyl (4.7 min, 50 µg injected, UV 254 nm detection), and all of the drug present (8 ng) was recovered in this peak. When the HPLC fractions of the same patient serum were analyzed by RRA without extraction with

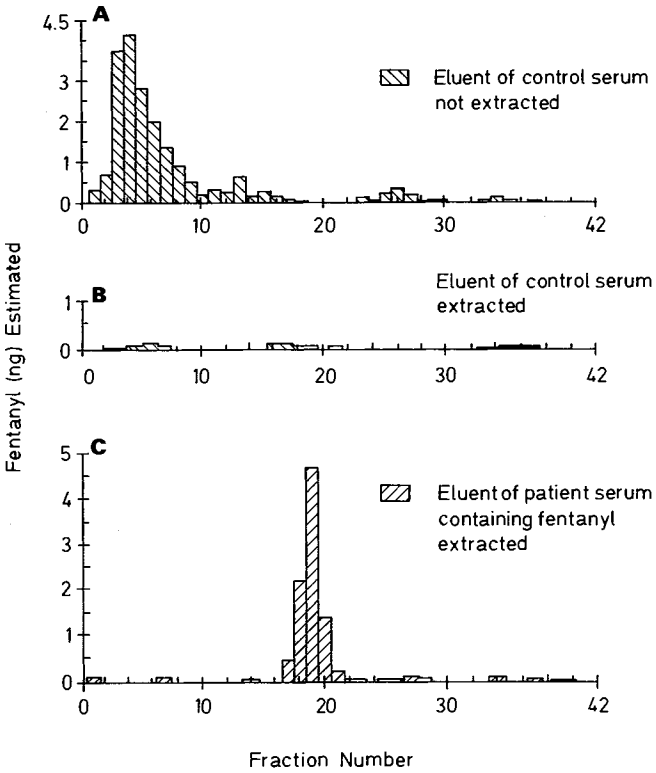

Fig. 5 HPLC separation of serum monitored by RRA. 400 μl of serum was precipitated with 600 μl of acetonitrile and the supernatant injected into the HPLC system. Fractions containing 1.0 ml of the eluent were collected every 15 s. After either evaporation (panel A) (direct assay) or extraction with petrolether (panel B and C) each fraction was tested by RRA. The reduction of ^{3}H-naloxone binding was calculated as ng fentanyl using a standard curve. **Panel A** shows the eluent profile of control serum without extraction. **Panel B** shows the eluent profile of the same control serum after eluent extraction with petrolether. **Panel C** displays the profile of a patient serum sample 3 h after i.v. injection. The serum sample contained 8 ng fentanyl and the fractions were extracted with petrolether prior to RRA.

petrolether (in order to detect nonextractable metabolites), the eluent profile was identical to that of control serum shown in panel A, with the exception of the fentanyl peak at 4.75 min (data not shown). Therefore, the HPLC-RRA failed to detect any potential metabolites with μ receptor affinity.

Serum concentrations of pentazocine and morphine. Two randomly selected sera that were obtained from a patient receiving therapeutic doses of pentazocine contained ~ 150 ng/ml and 400 ng/ml active pentazocine equivalent. Four serum samples collected at various times from a patient receiving 10 mg morphine sulfate s.c. were assayed by direct RRA using 10 μl (20 μl) serum. The results (mean of duplicates) in ng/ml were (20 μl results in parentheses): 1. 300 (277); 2. 81 (87); 3. 66 (76); 4. 19 (18). This patient's serum did not affect ^{3}H-naloxone and morphine binding at or below 20 μl added, and therefore, results with 10 and 20 μl samples were identical. These data show that the RRA is applicable to the assay of morphine and pentazocine at therapeutic concentrations. Comparison to other assays and detection of potential active metabolites will be performed in the future.

Discussion

There are only a few radioreceptor assays (RRA) for drug serum concentrations reported in the literature. Hunt et al. (11), Bilezikian und Mitarb. (12) and Creese and Snyder (13) described RRA's for benzodiazepines, propranolol, and antischizophrenic drugs, respectively. The only reported RRA for opioids (5) is of limited utility since the employed radioligand, ^{3}H-buprenorphine, is not selective (14) for the opioid receptor type (μ-site) which is primarily responsible for mediating the analgesic effect (15, 16). Consequently, ^{3}H-buprenorphine displacement curves by other opioids that bind with different affinities to the μ, δ, κ types of the opioid receptor tend to be shallow which results in an increased variability of the results. All reported RRA procedures appear to suffer from poor precision because of the greater sensitivity of the receptor molecules to changing assay conditions relative to that of antibodies employed in RIA, as an example. The major purpose of this study, therefore, was to select a suitable tracer, minimize variability and determine the nature of serum components that may interfere with the assay. Once these questions are solved, the RRA should in principle be applicable to all opioid narcotic analgesics with affinity to the μ type binding sites.

The radioligand used in the present report is ^{3}H-naloxone, an opioid antagonist which shows more than 10-fold selectivity for the μ receptor over the δ and κ binding sites (16, 17). The relative extent of ^{3}H-naloxone binding to μ over that to δ, κ opioid binding sites is enhanced at low tracer concentrations (0.65×10^{-9}M was used in this study). Therefore, the drugs or their metabolites detected by this RRA represent active opioid agonists or antagonists which interact primarily with the μ-receptor. This assumption applies to fentanyl which is known to be an opioid agonist highly selective for the μ-receptor (18). The Hill coefficient was, therefore, unity which means that the fentanyl-^{3}H-naloxone displacement curve is consistent with the law of mass action involving only one type of binding sites (μ) with no apparent cooperativity. The same was found for pentazocine (19) over the concentration range studied, although pentazocine may also interact with the κ type of binding sites. However, the Hill coefficient was below unity (N = 0.8) for morphine, which means that the morphine-^{3}H-naloxone displacement curve is somewhat more shallow than those of fentanyl and pentazocine. One possible explanation for the morphine binding behavior is that although morphine is thought to be μ-selective it also binds to the newly discovered λ type of binding sites (20) that are labeled to a small extent under the assay conditions. Therefore, the B_{min} of the morphine-^{3}H-naloxone curve is lower than that of fentanyl and pentazocine (Table I), neither of which bind to the λ sites (unpublished data). Nevertheless, the morphine-^{3}H-naloxone binding curve was suitable for a quantitative RRA, if the fitting program reflects systematic slope variations (individual fitting of the Hill coefficient N).

Potential serum components that interfere with the opioid RRA include metal ions (8, 21), lipids, or endogenous opioid peptides. The latter may be present in variable concentrations. The high sodium ion concentration in the serum (140 mM) is of primary concern, since Na^+ greatly reduces μ agonist affinity while enhancing the binding of ^{3}H-naloxone (8). This effect of Na^+ begins to appear between 1 to 5 mM with half maximum effect at 27 mM, but it is modulated by divalent cations such as Mg-II and Ca-II (8, 21). Therefore, serum dilutions of more than 10-fold may be required, when the opioid agonists are

assayed without extraction. We found that 20 μl serum in 0.5 ml incubate (25-fold dilution) had little effect on the receptor affinity of morphine. Divalent metal cations such as Cu-II, Zn-II and Fe-II may also cause interferences by destroying opioid ligand receptor binding (21). However, the addition of EDTA as a chelator had little effect on the serum interference of ^{3}H-naloxone binding. Heat denaturation equally was without effect, while interfering substances were readily extractable with diethylether, but not petrolether. Several extraction schemes are thus available to separate opioid drugs from these interfering serum component, if increased assay sensitivity is needed. The nature of the interfering components, e. g., opioid peptides or lipids, remains to be elucidated.

Fentanyl, morphine and pentazocine can be measured under the assay conditions in amounts of approximately 0.07 to 3 ng/sample, 0.10 to 15 ng/sample, and 0.5 ng to 30 ng/sample, respectively. With the use of 20 μl serum (that does not affect ^{3}H-naloxone binding), this results in an assay range of 3 to 150 ng/ml fentanyl, 5 to 750 ng/ml morphine, and 25 to 1500 ng/ml pentazocine. At least in the case of fentanyl, the therapeutic levels are at the lower limit of this range. Therefore, an extraction with petrolether was employed to concentrate the fentanyl sample and remove serum interferences. With the use of 300 μl serum, the fentanyl assay range was 0.3 to 15 ng/ml, which is sufficiently sensitive for pharmacological studies. The same extraction scheme was applicable to pentazocine, but not to morphine. Direct serum assays, however, should be sufficiently sensitive for most pharmacological studies with pentazocine and morphine.

RRA reproducibility. The precision of the measurements was mainly affected by two parameters, i. e., nonspecific, saturable binding of ^{3}H-naloxone to the glass fiber filters and the instability of the brain membrane homogenate sample. Suppression of saturable ^{3}H-naloxone filter binding with a 10^{-4} M naloxone solution, as reported previously (20), was essential to suppress excessive data scatter. Other ^{3}H-opiate tracers with μ selectivity are available that do not bind to the filters; however, ^{3}H-naloxone was chosen because of its widespread use and ready availability. Secondly, the use of freshly prepared membranes gave better signal to noise ratios and also less scatter. Despite these precautions, the opioid RRA ist not as precise as conventional RIA procedures, having a C. V. of between 10 and 20 %.

HPLC-RRA combinations. The combined use of RRA with a preceding HPLC separation provides a powerful analytical tool to screen for the presence of active metabolites. The utility of HPLC-RRA is demonstrated here with fentanyl as an example. Although it seems to be generally accepted that all metabolites of fentanyl are pharmacologically inactive (22), no thorough investigation has been published. Moreover, the complete metabolic pattern of fentanyl remains unknown. The HPLC separation of patient serum (≈ 3 h after the dose) monitored by RRA (Fig. 3) did not reveal any evidence for the presence of active fentanyl metabolites, either petrolether extractable or nonextractable. Furthermore, fentanyl levels in clinical samples from one patient measured by RRA and RIA (3) were similar. Since the RIA claims no interference by metabolites, this finding also argues against the presence of active metabolites. However, sera from further patients, sampled at later times after drug administration, should be analyzed by HPLC-RRA, before the absence of active fentanyl metabolites is confirmed.

In conclusion, the reported RRA procedure for narcotic analgesic drugs with affinity fot he μ type of opioid receptor sites is generally applicable to the assay of patient serum samples after therapeutic drug dosages.

Acknowledgements

Patient serum samples and fentanyl serum concentrations determined by RIA were kindly provided by Dr. D. Stanski, Stanford. This work was supported in part by Public Health Grants from NIH, AG031047 and from NIDA, DA 01095. J. Grevel was in part supported by the Alexander von Humboldt-Stiftung, Bonn, F. R. Germany.

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