Pharmaceutical Research, Vol. 4, No. 1, 1987

Report

Improved Radioreceptor Assay of Opiate Narcotics in Human Serum: Application to Fentanyl and Morphine Metabolism

Viktorya Levi,[1] James C. Scott,[2] Paul F. White,[2] and Wolfgang Sadée[1,3]

Received March 5, 1986; accepted September 10, 1986

A recently developed radioreceptor assay (RRA) (1) that employs ^{3}H-naloxone and rat brain membrane homogenates was improved two ways. First, the brain membranes were preincubated in the presence of sodium ions, and second, manganase-II ions were added to the sample incubations. These changes enhanced the assay sensitivity and reproducibility with stored membrane preparations and reduced the effects of serum constituents (Na^+) on ligand–receptor binding. Patient sera were assayed by radioimmunoassay (RIA) and RRA after fentanyl administration and by high-performance liquid chromatography (HPLC) and RRA after morphine administration. The results with both fentanyl assays were comparable, and no fentanyl metabolites were detectable by RRA after HPLC of serum extracts. In contrast, preliminary results with the HPLC-RRA procedure suggest the presence of an active morphine metabolite of unknown structure in sera obtained from patients on morphine therapy.

KEY WORDS: opiate narcotics; radioreceptor assay; human serum; fentanyl; morphine; metabolism.

INTRODUCTION

The application of radioreceptor assays (RRA) to the analysis of drugs in biological fluids carries a number of advantages, e.g., any active metabolite should also be detectable. Further, assay sensitivity is inherent to RRA, as the more potent drugs that are present at low plasma levels also display a high receptor affinity. These features are particularly attractive for the assay of opiate narcotic agonists and antagonists, since many analogues are clinically used and they are extensively metabolized. We have recently described an RRA for opiates that is highly sensitive and sufficiently reproducible for pharmacokinetic studies (1). However, the assay was limited by the effects of serum on the ligand–receptor binding process, so that only small serum samples (10 μl) can be directly assayed without drug extraction. The procedure presented here circumvents these problems and can therefore serve as a rather simple, sensitive, and rapid general assay for opiate narcotic drugs in biological specimens.

EXPERIMENTAL

Radioreceptor Assay (RRA)

The details of the RRA procedure, including the source of materials and chemicals, have been published earlier (1). Briefly, ^{3}H-naloxone (0.65 n*M* final concentration), a tracer with selectivity to the μ opiate receptor, is incubated at 30°C with 0.5 ml of a rat membrane homogenate and ≤50 μl serum. After 20 min, the incubate is forced through GF/B filters presaturated with unlabeled naloxone (10^{-4} *M*), and the washed filters are placed in counting vials to determine the ^{3}H content by liquid scintillation counting. Standard curves (0.1 to 10 ng fentanyl or 0.3 to 15 ng morphine per incubation) were fitted by a computerized least-squares regressions program (NIH PROPHET) (2) to Eq. (1) as previously described:

$$\text{logistic } (X) = B_{max} - \frac{B_{max} - B_{min}}{1 + [IC_{50}/X]^N} \quad (1)$$

where X is the amount of opioid drug (ng per sample), B_{max} is the binding of ^{3}H-naloxone (dpm) in the absence of opioid drug, B_{min} is the binding of ^{3}H-naloxone (dpm) in the presence of saturating doses of opioid drug, IC_{50} is the amount of opioid drug (ng per sample) that reduces specific tracer binding ($B_{max} - B_{min}$) by 50%, and N is the slope factor.

Preparation of Membrane Homogenates from Rat Brain

Male Sprague–Dawley rats weighing 120 to 140 g were decapitated, and the brain was rapidly removed. The cerebellum was discarded, and the brain was weighed and homogenized for 20 sec in 30 ml ice-cold 50 m*M* 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* (lower centrifugation speeds are also feasible, e.g., 10,000*g*), and the membrane pellet was resuspended in the Tris–HCl buffer containing 100 m*M* NaCl. The suspension was kept for 30 min in an ice/water bath followed by another centrifu-

[1] Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143.

[2] Department of Anesthesia, Stanford University School of Medicine, Stanford, California 94305.

[3] To whom correspondence should be addressed.

0724-8741/87/0200-0046$05.00/0

gation (15 min, 40,000*g*). The resulting pellet was again resuspended in Tris–HCl buffer (without NaCl), then centrifuged, and the final brain membrane pellet was stored at −25 to −70°C. The resulting membrane pellet can be stored for at least 1 week with minimal loss of tracer binding capacity. For analysis, the thawed membrane pellet was resuspended by brief homogenization for 5 sec in 60 vol (relative to the original brain wet weight) of ice-cold Tris–HCl buffer (50 m*M*, pH 7.6) containing 1 m*M* $MnCl_2$. The resulting membrane suspension should be used for analysis within 2 hr or less.

Human Serum Samples

Blank sera were obtained from the Clinical Chemistry section of the Department of Laboratory Medicine at UCSF. Serum samples from patients receiving either fentanyl or morphine under approved human experimentation protocols were obtained in the Department of Anesthesia, Stanford Medical Center.

High-Performance Liquid Chromatography (HPLC) Separation of Fentanyl and Morphine Metabolites

Reverse-phase HPLC separations were carried out as previously described for fentanyl (1) and morphine (3).

Reference Assays for Fentanyl and Morphine in Serum

Fentanyl serum levels were measured by the radioimmunoassay of Michiels *et al.* (4), as modified by Schütter and White (5). Morphine levels were determined by an HPLC procedure with electrochemical detection that was derived from the method published by Wallace *et al.* (6).

RESULTS AND DISCUSSION

The opiate receptor binding process is highly sensitive to a number of regulatory factors. In particular, the presence of Na^+ ions in excess of 2 to 5 m*M* dramatically reduces the affinity of μ agonists, while it increases the binding of an antagonist such as naloxone (7). Divalent cations, most potently Mn^{2+}, have opposite effects on agonist and antagonist binding and were found to counteract the effects of Na^+ (8). Since serum contains a high concentration of Na^+ (120 m*M*), we tested the effect of adding 1 m*M* $MnCl_2$ to the final RRA incubation mixtures. Further, the rat membrane homogenates were preincubated for 30 min at 0°C with 100 m*M* NaCl in order to test whether such preincubation could minimize the effects of serum Na^+ on opiate receptor binding in the final incubate. Exposure of brain membranes both to 0°C plus NaCl and to Tris buffer alone at 30°C is commonly employed to unmask a maximum number of opiate binding sites.

The effects of Na^+ preincubation, the addition of Mn^{2+}, and the presence of serum (10 and 50 μl) were tested by running standard curves with morphine and fentanyl and estimating the IC_{50} values under each condition (Table I). The preincubation of the membrane with Na^+ at 0°C did not significantly affect the affinity of morphine in the presence of a small serum volume; however, it prevented an increase in the morphine IC_{50} from 5 to 6.4 ng/sample that occurred with 50 μl serum when the membranes were preincubated with Tris buffer alone at 30°C. Further addition of Mn^{2+} to the assay incubation lowered the IC_{50} and, thereby, increased the assay sensitivity by more than twofold, and effects of serum on the IC_{50} values were minimal.

We also tested the binding of the antagonist tracer ^{3}H-naloxone under the various conditions. The addition of serum (≤50 μl) to the assay incubate increased tracer binding by 20 to 30%, when membranes were preincubated at 30°C without NaCl (Table II). This effect was abolished with the NaCl preincubation, while the addition of $MnCl_2$ had only minimal effects on ^{3}H-naloxone binding under these conditions (data not shown). Serum volumes above 50 μl reduced ^{3}H-naloxone binding and, therefore, cannot be employed in the assay without organic solvent extraction of the drug before analysis.

Table I. Parameters of Standard Curves of Morphine and Fentanyl Under Different Assay Conditions

Type of standard curve[a]	Morphine		Fentanyl	
	IC_{50}[b]	N[c]	IC_{50}	N
(a) 0 μl serum				
$-Na^+$ $-Mn^{2+}$	5.1 ± 2.0[d]	0.8	2.9 ± 0.3	1.0
(b) 10 μl serum				
$-Na^+$ $-Mn^{2+}$	4.8 ± 0.8	0.78 ± 0.09	—	—
$+Na^+$ $-Mn^{2+}$	4.1 ± 0.5	0.75 ± 0.06	—	—
$-Na^+$ $+Mn^{2+}$	2.4 ± 0.2	0.97 ± 0.09	—	—
$+Na^+$ $+Mn^{2+}$	2.2 ± 0.2	0.84 ± 0.06	0.93 ± 0.09	0.86 ± 0.06
(c) 50 μl serum				
$-Na^+$ $-Mn^{2+}$	6.4 ± 1.4	0.91 ± 0.15	—	—
$+Na^+$ $-Mn^{2+}$	5.1 ± 0.8	0.64 ± 0.06	—	—
$-Na^+$ $+Mn^{2+}$	3.4 ± 0.3	0.91 ± 0.07	—	—
$+Na^+$ $+Mn^{2+}$	2.6 ± 0.2	0.85 ± 0.06	1.4 ± 0.2	0.73 ± 0.06

[a] Membranes were preincubated either at 30°C for 30 min in Tris buffer alone ($-Na^+$) or at 0°C for 30 min in Tris buffer + 100 m*M* NaCl ($+Na^+$). Note that the final assay incubation did not contain any added NaCl in either case. The final assay incubation contained either no Mn^{2+} ($-Mn^{2+}$) or 1 m*M* $MnCl_2$ ($+Mn^{2+}$).

[b] Nanograms of opiate per sample (~0.55-ml incubation).

[c] Slope factor, Eq. (1).

[d] ±SD ($N > 3$).

Table II. Effect of Serum on ^{3}H-Naloxone Binding After Preincubation of Brain Membranes with Tris Buffer Alone at 30°C (−NaCl) or with NaCl at 0°C (+100 m*M* NaCl)

	Total binding (% control)	
Serum (μl)	−NaCl	+100 m*M* NaCl
0	100	100
10	123	107
20	127	104
50	129	92
100	104	83

In summary, the preincubation of rat brain membranes with NaCl at 0°C and the addition of 1 m*M* $MnCl_2$ provide a general RRA for opioid narcotic drugs in serum samples with affinity to the μ receptor. Of 15 samples from different patients analyzed, none showed a significant depression of ^{3}H binding (<10% reduction of control binding in the presence of a 50-μl control serum). We previously noted significant contaminations in several serum samples (1) that reduced tracer–receptor binding and also appeared in HPLC eluate fractions. However, this was not encountered with any of the patient sera analyzed with the improved RRA procedure, and the reason for this earlier interference (probably from exogenous nonbiological sources) was therefore not clarified. Sensitivity with direct serum assay (50 μl) is of the order of 3 ng fentanyl/ml serum and 6 ng morphine/ml serum. Higher sensitivity can be achieved either by drug extraction from serum (1) or with the use of tracers of higher specific activity (^{125}I-labeled opioid peptides) and smaller incubation volumes. The coefficient of variation is essentially unchanged for the modified assay when compared to the original method (1) (CV, 10 to 15%). The RRA as presented requires the sacrifice of an experimental animal to obtain a receptor source. This can now be circumvented, as we have recently identified the first neuroblastoma cell line to express the μ receptor, human SK-N-SH cells, which can be grown *in vitro* as an alternative receptor source (9).

Fentanyl Disposition and Metabolism

Fentanyl serum levels were measured in two patients over 20 hr following drug administration. The comparison between fentanyl levels obtained with the radioimmunoassay (RIA) (5) and the RRA for fentanyl in patient sera (Fig. 1) reveals only small differences that may be accounted for by the technical variance of the assay procedure. While previous comparison between RIA and RRA was carried out for only 30 min after the dose (1), the present data, collected over a much longer time period, again show no evidence for the accumulation of RRA active fentanyl metabolites. This result was confirmed by analyzing fentanyl serum samples with the HPLC-RRA combination. The only detectable RRA peak coeluted with unchanged fentanyl in several serum samples from two different patients at 10 to 30 min after the dose (Fig. 2). Therefore, there is no evidence for the accumulation of any pharmacologically active fentanyl metabolite in patients.

Morphine Metabolism

Figure 3 compares morphine levels in patient sera, as determined by the HPLC assay with electrochemical detection (6) and by RRA. It is evident that the RRA gave consistently higher values than the HPLC method, which suggests the possible presence of active metabolites that were undetectable by the HPLC assay. The latter is thought to be specific for morphine, although the coelution of a morphine metabolite with the parent drug cannot be excluded. To detect the possible presence of an active morphine metabolite, therefore, sera from patients on morphine therapy were also analyzed by the HPLC-RRA combination, and the results are given in Fig. 4. While several active metabolites are

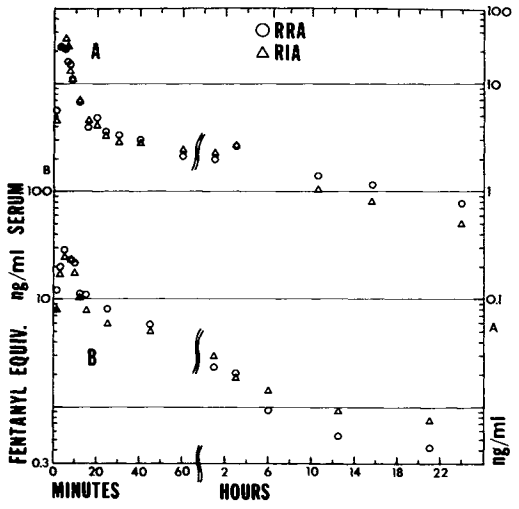

Fig. 1. Time course of fentanyl serum concentrations measured by RIA (5) and by RRA. An extraction procedure (1) was performed with 300 μl serum, when fentanyl levels were below 2–3 ng/ml. (A) Patient 1, 67 years old, 104 kg, received a fentanyl iv infusion of 150 μg/min over 5 min (7.2 μg/kg). (B) Patient 2, 33 years old, 104 kg, received a fentanyl infusion of 150 μg/kg over 9 min (13 μg/kg).

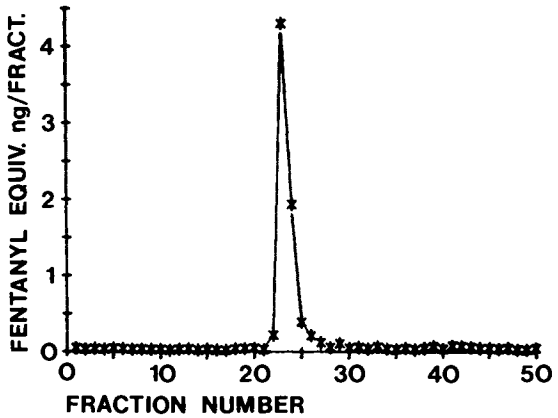

Fig. 2. HPLC-RRA record obtained from a serum extract from patient 2 (10 min after end of fentanyl infusion). Serum (0.3 ml) was mixed with acetonitrile (0.45 ml), and the mixture was vigorously agitated, then centrifuged. An aliquot of the supernatant was injected onto the HPLC column (1). Fractions (four per minute) were collected, concentrated in a stream of nitrogen, and analyzed by RRA. The peak coeluted with authentic fentanyl and was absent in control serum. Note the absence of any interfering material, as opposed to results with a similar extract published previously (1).

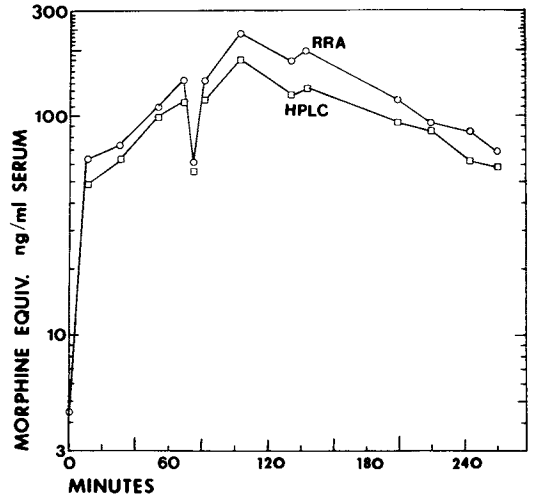

Fig. 3. Morphine serum levels determined by HPLC (6) and by RRA (using 5 μl serum per assay). This 68-year-old patient (63 kg) received a total dose of 52 mg morphine. The initial bolus dose equaled 7 mg, followed by the infusion of 45 mg over 225 min during an operation.

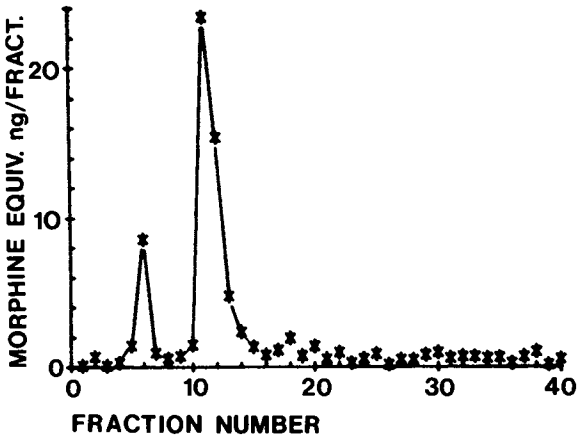

Fig. 4. HPLC-RRA record obtained from a serum extract from a patient receiving morphine during an operation (53 years old, 60 kg, 15-mg initial bolus dose followed by the infusion of 36 mg over 4 hr). The serum sample was drawn 160 min after initiation of the morphine infusion, and the morphine level, measured by RRA and HPLC (6), was 260 and 180 ng/ml, respectively. The reverse-phase HPLC method published in Ref. 3 was used for the HPLC-RRA, and serum extracts were obtained by protein precipitation with acetonitrile as described in the legend to Fig. 2. Authentic morphine coeluted with fractions 11–13 (four fractions per minute were collected), and no RRA peaks were apparent in blank sera. The relative peak size of the metabolite peak reflects the lower range of metabolite/parent drug ratio observed in five different patients.

known, e.g., normorphine, none of these was shown to accumulate to a significant extent in human blood (10). However, the HPLC-RRA revealed the presence of a major morphine metabolite with an affinity to the opiate receptor (Fig. 4). This fraction was found in each plasma sample from the five morphine-treated patients studied. Its elution time was different from that of any of the known active morphine metabolites (e.g., normorphine, dihydromorphinone). RRA peak height ratios for metabolite fraction/unchanged morphine range from 0.3 to 0.6. Therefore, the combined application of HPLC and RRA revealed the presence of a major metabolite with considerable opiate receptor binding affinity. The chemical identity of this potentially active metabolite remains to be elucidated. It should be noted, however, that we have recently detected the presence of a small impurity (0.2%) in commercial morphine samples, which eluted with a similar retention time as the RRA active species isolated from human serum and which also bound to the opiate receptor. Whether this morphine impurity contributes *in vivo* to the RRA activity in serum remains to be investigated.

ACKNOWLEDGMENTS

This study was supported by Public Health Research Grants DA 01095 from the Institute on Drug Abuse and AG 03104 from the Institute on Aging, NIH, HEW.

REFERENCES

1. J. Grevel, J. Thomas, M. L. Richards and W. Sadée. *Pharm. Res.* 1:209–214 (1984).
2. N. H. G. Holford. In H. M. Perry (ed.), *Prophet Public Procedures Notebook,* Beranek and Newman, Cambridge, Mass., 1982, pp. 4-89–4-126.
3. K. B. Mullis, D. C. Perry, A. M. Finn, B. Stafford, and W. Sadée. *J. Pharmacol. Exp. Ther.* 208:228–231 (1979).
4. M. Michiels, R. Hendriks, and J. Heykants. *Eur. J. Clin. Pharmacol.* 12:153–158 (1977).
5. J. Schütter and P. F. White. *Anesthesiology* 61:315–320 (1984).
6. J. E. Wallace, S. C. Harris, and M. W. Peek. *Anal. Chem.* 52:1328–1330 (1980).
7. C. B. Pert and S. H. Snyder. *Mol. Pharmacol.* 10:868–879 (1974).
8. W. Sadée, A. Pfeiffer, and A. Herz. *J. Neuro Chem.* 36:659–667 (1982).
9. V. C. Yu, M. L. Richards, and W. Sadée. *J. Biol. Chem.* 261:1065–1070 (1985).
10. W. Sadée and G. C. M. Beelen. *Drug Level Monitoring,* John Wiley & Sons, New York, 1980, pp. 343–352.