A Sensitive Radioimmunoassay for Fentanyl

Plasma Level in Dogs and Man

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Summary. Antiserum to fentanyl was obtained in rabbits repeatedly injected with carboxyfentanyl conjugated to bovine serum albumin. Using the antiserum, a highly sensitive radioimmunoassay has been developed, based on the dextran-coated charcoal method. It proved possible to assay the drug directly in plasma, in amounts as small as 30 picogram in 0.5 ml. The antibody was highly specific for fentanyl and no cross-reaction was observed with its major metabolites. This sensitive and specific radioimmunoassay method was employed to determine fentanyl in plasma from six volunteers after an intravenous bolus of 0.2 mg, and in plasma from dogs treated both intravenously and subcutaneously with 0.02 mg/kg. The plasma level of fentanyl could be followed for up to 6 h after a therapeutic dose in dogs and man.

Key words: Fentanyl, radioimmunoassay, cross-reaction, plasma level, dog, man.

Fentanyl, N-phenyl-N-[1-(2-phenylethyl)-4-piperidinyl] propanamide, is a very potent and fast-acting narcotic analgesic, gaining wide clinical use in anaesthesia. It is used in combination with droperidol for the induction and maintenance of general anaesthesia in man (Holderness et al., 1963). So far, pharmacokinetic studies of fentanyl have been sparse because of lack of an analytical method suitable to measure the extremely small concentrations of the drug in plasma.

A radioimmunoassay for fentanyl has recently been described (Henderson et al., 1975). Antibodies specific to fentanyl were obtained by immunization of rabbits with a fentanyl-derivative chemically coupled to bovine γ-globulin. The clinical utility of that radioimmunoassay was greatly limited because its sensitivity was inadequate.

The present paper reports development of a very sensitive radioimmunoassay, capable of measuring subnanogram levels of fentanyl in plasma. It also describes an investigation on the specificity of the fentanyl antiserum. The value of the assay for pharmacokinetic studies was demonstrated by measuring the plasma concentration of fentanyl in man and dogs after a single therapeutic dose of the drug.

Materials and Methods

Specifically tritium-labelled fentanyl (specific activity 10 Ci/mM) was synthetized at I. R. E. (Fleurus, Belgium) according to a method developed in our laboratories. The purity was checked by thin-layer chromatography on Merck silica gel plates (HF 254) in chloroform/ethanol (95:5 v/v) as a solvent system (Rf 0.65); the radiochemical purity was 99%. It was stored as an ethanolic solution at −20 °C. Dextran-Grade D was purchased from Mann Research Laboratories, New York; bovine albumin (Cohn fraction V) from Sigma, St. Louis, Mo., Norit A-supra from C.M.I.-Codepa, Belgium, and Riafluor® from NEN-Chemicals, Belgium.

With the exception of naloxone hydrochloride (Endo Laboratories Inc., Garden City, N.Y.), all drugs and test compounds mentioned were originally synthetized and analyzed in the Janssen Research Laboratories, Beerse, Belgium.

Preparation of the Immunogen

The hapten, 4-oxo-4-{phenyl-[1-(2-phenylethyl)-4-piperidinyl] amino} butanoic acid, a fentanyl
analogue suitable for conjugation with macromolecules, was synthesized and coupled to bovine serum albumin (BSA) according to the method described by Henderson et al. (1975). Tracer amounts of tritium-labelled hapten were added to the reaction mixture. The reaction scheme is shown in Figure 1. From the specific activity of the conjugate, the degree of substitution was estimated to be 2 moles of hapten per mole of BSA.

**Immunization**

The protein conjugate was dissolved in phosphate-buffered saline (0.05 M), pH 7.4, at a concentration of 1 mg/ml, and emulsified with an equal volume of complete Freund's adjuvant. Two female New Zealand albino rabbits were immunized with 1.5 ml of the emulsion by 40 to 50 intradermal injections along both sides of the back. Beginning one month after the priming dose, four booster injections were given at intervals of about six to ten weeks. Blood was collected from the central ear artery 7 to 10 days after each booster injection and serum was tested for antibodies. The rabbits were killed 10 days after the last booster and the collected serum was pooled and stored at −20 °C.

**Assay of Fentanyl**

The procedure for immunoassay of fentanyl was essentially the same as that described for pimozide (Michiels et al., 1975). Antibody titers were determined by adding 0.2 ml of various dilutions of antisera to 0.5 ml phosphate buffer (pH 7.4) and 0.4 ng ³H-fentanyl (28000 dpm) in 0.05 ml 30% methanol/water (v/v). Incubations were carried out in 1.3 ml plastic tubes (Eppendorf) by continuous rotation (25 rev./min) for 2 h at room temperature. Thereafter, bound and free ³H-fentanyl were separated by selective adsorption on dextrancoated charcoal: aliquots of 0.2 ml of a suspension containing 500 mg activated charcoal, 50 mg dextran and 100 mg sodium azide per 100 ml phosphate buffer (pH 7.4) were added to the incubation mixture, vortexed and allowed to equilibrate at room temperature for 1 h with continuous rotation. The charcoal was removed by centrifugation at 10000 rev./min for 10 minutes (Microrapid, Hettich). The supernatant, containing antibody-bound ³H-fentanyl, was pipetted into a counting vial containing 10 ml of Riafluor, and the radioactivity was determined in a liquid-scintillation spectrometer (Packard Tri-Carb, Model 3380, 544 AAA).

Standard curves of fentanyl added to control plasma were obtained by incubation of increasing amounts of unlabelled fentanyl together with a fixed amount of ³H-fentanyl in the presence of 0.2 ml of a dilution of antisera, which bound almost 50% of the tritiated compound, as found by previous titration. Incubations were carried out as described for the antisera titration. The difference between the assay applied to plasma or to buffer concerned the quantity of dextran-coated charcoal. In order to keep blank values due to unspecific adsorption to plasma constituents within acceptable limits, a 2% w/v charcoal suspension was required.

Antibody-specificity was determined in buffer by measuring the inhibition of the antibody-fentanyl complex formation produced by increasing amounts, up to 1000 ng, of various structurally related compounds incubated with 0.2 ml of the appropriate dilution of antisera, in the presence of ³H-fentanyl 1 ng.

**Procedure in Man and Animals**

Six healthy volunteers, aged 21–35 years, were injected intravenously with fentanyl 0.2 mg after an overnight fast. Venous blood was collected in heparin before and from 2 min up to 6 h after administration. Plasma was separated by centrifugation and kept frozen until assayed.

Three mongrel dogs, weighing 22–24 kg, were treated subcutaneously and, one week later, intravenously with fentanyl 0.02 mg/kg. Blood samples were collected into heparin at intervals for up to 8 h.

Drug concentrations in the plasma samples were calculated from the degree to which an unknown