Assay Methods for Fentanyl in Serum: Gas-Liquid Chromatography Versus Radioimmunoassay

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In this study, two independent laboratories assessed the validity of the fentanyl radioimmunoassay (RIA) by measuring a series of spiked control serum samples and 429 serum samples from 20 patients receiving fentanyl for their anesthesia. Additionally, a gas-liquid chromatographic (GLC) method specific for the parent drug was also applied to the same serum samples. The RIA measurement of fentanyl by the two laboratories resulted in comparable values for both control samples and samples from patients in a range of 0.5–50 ng/ml. The GLC method agreed with both RIA measurements in the spiked control and patient samples. The authors' results demonstrate the validity of the RIA as a measurement technique for fentanyl in human serum samples. (Key words: Analgesics, narcotic: fentanyl. Anesthetics, intravenous: fentanyl. Measurement techniques: gas chromatography; radioimmunoassay.)

FENTANYL, N-phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]propanamide is the prototype of the 4-anilinopiperidine series of narcotic analgesics.[1] Serum concentrations between 0.5 and 20 ng/ml are generally accepted as providing adequate narcotic drug effect (*i.e.*, analgesia; respiratory depression; and anesthesia, regardless of concomitant administration of nitrous oxide) in humans.[2-4] Assay techniques reported for the measurement of fentanyl in biological samples include radiochemical assay,[5] radioimmunoassay (RIA),[6,7] radioreceptor assay,[8] and gas chromatography (GLC) using either flame-ionization, nitrogen-selective (NPD), or mass spectrometric (MS) detection.[9-12]

Using these methods, the pharmacokinetics of fentanyl have been extensively studied, but remarkable discrepancies have been found between the various studies.[4,13] Obviously, this variability can be attributed in part to assay reproducibility as serum concentrations of fentanyl are extremely low and at the limits of most analytical detection systems. Schüttler and White[14] reported high variability and substantial overestimation of plasma concentrations determined by RIA, whereas Phipps *et al.*[15] concluded that RIA was specific and accurate, yielding results equal to those obtained by GC-NPD. Because Phipps *et al.*[15] used only spiked human serum samples, they could not evaluate the possible detection (cross-reactivity) of fentanyl metabolites at low parent drug concentrations. If cross-reactive metabolites are present with low parent drug concentrations, then the measured concentration of fentanyl would be higher than the true concentration of the parent drug.

In our study, the validity of the RIA was further investigated by its application in two different laboratories to both spiked control samples and serum from patients given clinical doses of fentanyl. Furthermore, a GLC method was used to analyze the same samples. Comparison of the assay results should enable the validation of either analytical method and, hence, provide more reliable serum concentration data for the calculation of fentanyl pharmacokinetics.

Methods and Materials

STUDY SAMPLES

Two sample sets were examined. One sample set consisted of serum samples from healthy ASA Physical Status I or II surgical patients participating in a pharmacokinetic/pharmacodynamic study of fentanyl.[16] Each patient was given 0.5–1 mg of fentanyl as a rapid iv infusion 0.5–1 h before induction of anesthesia with thiopental. Frequent arterial blood samples were obtained to characterize the distribution/redistribution phase for the first 3 h. Central venous blood samples were obtained from 4–24 h to characterize the terminal elimination phase. For 2–4 h, general anesthesia was maintained with nitrous oxide/oxygen and enflurane, based on clinical need. Each blood sample was allowed to clot, promptly centrifuged, and frozen at −20° C until analysis.

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The second sample set consisted of spiked serum samples from four healthy volunteers not previously exposed to fentanyl. For each volunteer's serum, the following concentrations of fentanyl were prepared: 9.51, 6.34, 2.54, and 0.51 ng/ml. Each spiked sample, along with a serum blank, was divided into three 1-ml portions and frozen until analysis by one of the three procedures described hereafter.

All samples were first measured at Stanford University using RIA, then shipped frozen to Janssen Pharmaceutica, Beerse, Belgium, where they were remeasured using both RIA and GLC methods. The measurements at Janssen were performed "blindly," the analysts not knowing the values obtained at Stanford.

JANSSEN RIA

The patient and spiked serum samples were measured, employing the FEN-RIA-200 fentanyl radioimmunoassay kit (IRE, Fleurus, Belgium). Following the revised version of the manual associated with the RIA kit, the antiserum was added as the last step to the well-stirred mixture of labeled drug (^{3}H-fentanyl), sample (or standard), and BSA-buffer. The vials (Eppendorf, West Germany) were rotated for 2 h at room temperature. Thereafter, bound and free ^{3}H-fentanyl were separated by selective adsorption of the free ligand on dextran-coated charcoal that was added to the incubation mixture and allowed to equilibrate at room temperature for 1 h with continuous rotation. The charcoal was precipitated by centrifugation at 8,500 g for 5 min (Microfuge, Heraeus-Christ, Osterode, G.F.R.). The supernatants, containing the antibody-bound ^{3}H-fentanyl, were pipetted into 6-ml scintillation vials, containing 4 ml of a scintillation cocktail (Pico-Fluor 30, Packard Instruments, Downers Grove, IL). Radioactivity in each sample was then counted for 2 min in a liquid scintillation spectrometer (Prias, Packard Instruments) using the external standard technique for color and chemical quenching correction. Non-specific binding was determined by replacing the antiserum with an equal volume of BSA-buffer solution. Standard curves were obtained by adding increasing amounts of unlabeled drug to control human plasma. Weighted linear regression analysis of logit B/Bo *versus* log dose was used for calibration curve fitting and calculation of sample concentrations.

STANFORD RIA

Patient and spiked serum samples were assayed in a manner similar to that used by Janssen. Changes in instrumentation and methodology were as follows. The assay was performed in 1.5-ml polypropylene microcentrifuge tubes (Robbins Scientific, Mt. View, CA).

Dextran-coated charcoal (E & K Scientific, Saratoga, CA) was precipitated by centrifugation in an Eppendorf Model 5413 microcentrifuge (Eppendorf/Brinkman, Westbury, NY) at 8,500 g for 10 min. The resulting supernatants were added to 20-ml capacity scintillation vials (Packard Instruments) containing 15 ml of Aquasol (New England Nuclear, Boston, MA). Samples were counted on a Beckman LS-250 liquid scintillation counter (Fullerton, CA) for 10 min per sample. The calibration curve and sample calculations were completed on a progammable desk calculator, using an unweighted linear regression of logit B/Bo *versus* log dose. Color and chemical quenching correction was not performed on the samples.

GAS-LIQUID CHROMATOGRAPHY

Analysis of the samples involved the specific extraction of fentanyl and its internal standard from the serum and subsequent analysis by GLC on either packed or capillary columns. The extraction procedure corresponds to that reported for alfentanil and sufentanil.[17] The serum (1-2 ml), spiked with 50 ng of internal standard (alfentanil), contained in 0.1 ml of methanol, was alkalinized by addition of 0.5-1 ml of 0.1 M sodium hydroxide and was extracted twice with 4-ml aliquots of heptane-isoamyl alcohol (98.5:1.5, v/v). The organic phase was back-extracted with 3 ml of 0.05 M sulfuric acid and, after alkalinization of the latter phase with 0.15 ml of concentrated ammonia, re-extracted twice with 2.5-ml aliquots of the extraction solvent. After evaporation at 55° C under nitrogen, the extraction residues were dissolved in 0.05 ml of methanol and 1- or 2-μl aliquots were injected on the capillary or packed GLC column, respectively. The latter column (1 m × 2 mm i.d.), packed with 3% OV-17 on Supelcoport 80/100 mesh (Supelco, Bellefonte, PA), was installed in a Varian M 3700 gas chromatograph, equipped with a thermionic specific detector (TSD) containing an electrically heated ceramic-alkali bead. Temperatures for the column, injector, and detector were 280° C, 310° C, and 340° C, respectively. Nitrogen was used as the carrier gas at a flow rate of 20 ml/min. The fused silica capillary column (10 m × 0.32 mm i.d.), coated with Sil 19 CB (Chrompack, Antwerp, Belgium), was installed in a Vista 6000 gas chromatograph (Varian) equipped with a solventless falling-needle injector and a TSD. Column, injector, and detector temperatures were 235° C, 285° C and 290° C, respectively. Helium was used as the carrier gas at a flow rate of 5 ml/min.

Standard curves were prepared by spiking blank human serum with fentanyl at concentrations ranging from 0.25-50 ng/ml and with the internal standard at fixed concentrations of 25 or 50 ng/ml. For the con-

struction of calibration curves, these standards were extracted and chromatographed as described above, and the peak height ratios (fentanyl/internal standard) were plotted against the fentanyl standard concentrations on a log-log scale.

DATA ANALYSIS

Weighted linear regression was used to examine the relationship between concentrations of fentanyl in patient samples as determined by GLC (independent variable) and those determined by both RIA procedures (dependent variable). An extended least-squares non-linear regression (ELSFIT)¶ computer program was used for the linear regression. The variance of the regression relationship was related to the regression prediction using a power function.[18] From the regression analysis, the 95% confidence limits of the slope and intercept were calculated. If these limits of the intercept included zero, the regression was rerun, setting the intercept to zero.

For the spiked samples, one-way analysis of variance (ANOVA) at each spiked concentration was used to determine if the two RIA measurements differed significantly ($P = 0.05$) from those obtained with GLC.

Results

The RIA calibration curves for both laboratories were linear (r > 0.994) in the range of 0.05–4 ng per assay tube. Using 0.05–0.2-ml volumes of serum, fentanyl concentrations thus could be measured from the detection limit (0.25 ng/ml) to 80 ng/ml. Over the concentration range studied, mean intra- and interassay coefficients of variation were 6.0% and 6.9%, respectively.

All GLC calibration curves were linear (r > 0.9996) from the detection limit (0.25 ng/ml) to 50 ng/ml. Using 50 ng of internal standard per 1- or 2-ml sample, the mean expressions of the log-transformed calibration curves were $y = 0.0304\ x^{1.0024}$ and $y = 0.0298\ x^{1.0663}$ on the packed and capillary GLC column, respectively. Mean intra- and interassay coefficients of variation were 6.4% and 8.1%. Figure 1 shows two chromatograms of patient serum samples, analyzed by either packed- or capillary-column GLC. For some patients, receiving quinidine as a peri-operative co-medication, initial analysis of fentanyl on the packed GLC column failed because of inadequate separation from interfering sub-
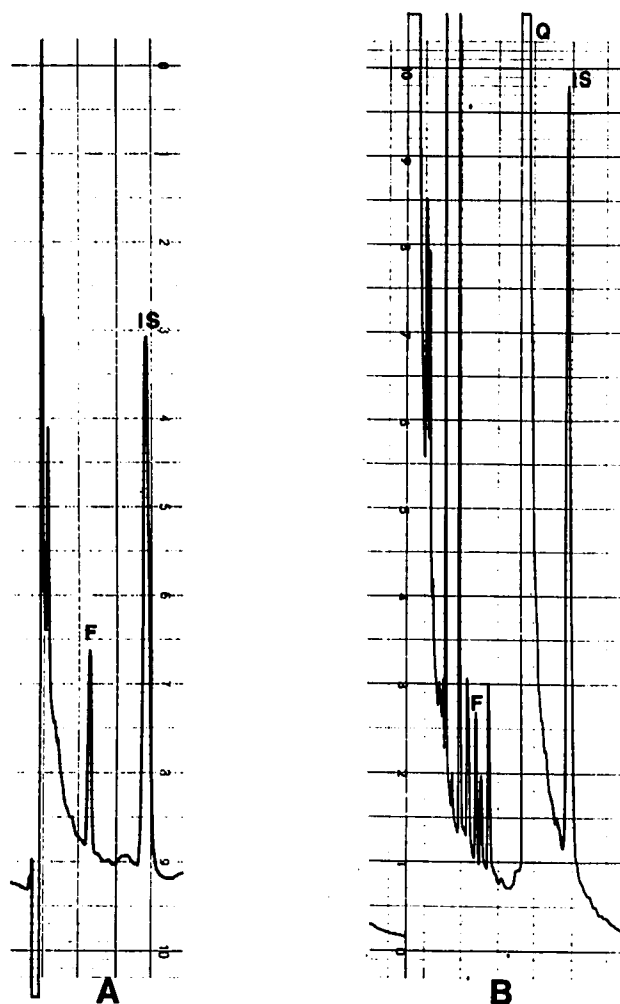
FIG. 1. Chromatograms of serum samples from patients given fentanyl obtained by gas-liquid chromatography on packed (A) or capillary (B) columns. Fentanyl (F) concentrations are 5.8 and 5.7 ng/ml; internal standard (IS) concentrations 25 and 50 ng/ml. Fentanyl retention times were 1.7 and 2.1 min, respectively. The capillary chromatogram was obtained from a patient given quinidine (Q).

stances. In such cases, serum samples needed to be reanalyzed on the capillary GLC column.

In the RIA results for the patient samples, the 95% confidence limits of the intercept term included zero (table 1). When the regression line was forced through the origin, the slope of the Janssen RIA was 1.0, whereas for the Stanford RIA, it was 0.97 with 95% confidence limits from 0.95–0.99. This indicates that the Janssen RIA was not statistically different from the GLC. The Stanford RIA regression slope differed significantly from 1.0, but resulted in only a 3% under-prediction when compared with the GLC results. The raw data and regression relationship for the Janssen RIA and GLC data are represented in figures 2 and 3.

TABLE 1. Comparison of RIA *Versus* GLC for Fentanyl Levels, Using Regression Analysis

Data Set	Slope (95% Confidence Limits)	Intercept·ng/ml (95% Confidence Limits)
Stanford RIA (n = 429)*	0.98 (0.96–1.00) 0.97 (0.95–0.99)	−0.05 (−0.11–0.01) Set to 0
Janssen RIA (n = 370)	1.0 (0.98–1.02) 1.0 (0.98–1.02)	−0.02 (−0.08–0.04) Set to 0

RIA = radioimmunoassay; GLC = gas-liquid chromatography; Stanford = Department of Anesthesia, Stanford University School of Medicine; Janssen = Janssen Pharmaceutica, Belgium.
* n = number of serum samples studied.

The scatter plots of the Stanford RIA were similar. Figure 3 clearly demonstrates that low fentanyl serum concentrations, as measured by RIA, were not overpredicted relative to the GLC results. When the spiked samples were compared at the four different fentanyl concentrations, there were no statistically significant differences in the mean values measured by GLC and the two RIA methods (table 2).

Discussion

The gas-liquid chromatographic technique we describe in this paper proved to be a valuable alternative method for measuring fentanyl serum concentrations. GLC was both accurate and reproducible, even at its detection limit (0.25 ng/ml). During the GLC analysis on packed columns of serum samples from some patients, however, we noted interference problems and
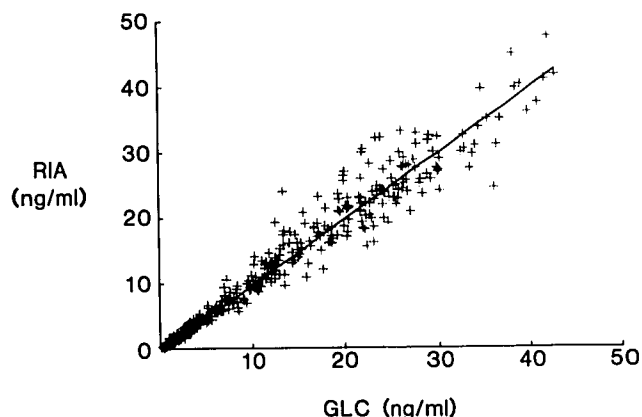

FIG. 3. Correlation between lower fentanyl serum concentrations (0–5 ng/ml) as measured by radioimmunoassay (RIA) and by gas-liquid chromatography (GLC). The solid line represents the slope of the regression line (table 1).
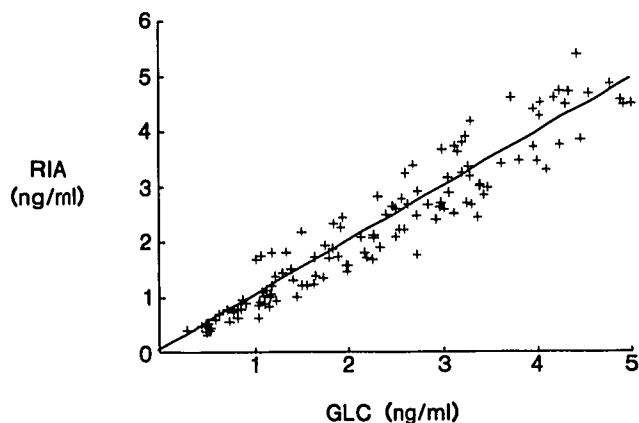
found, in comparison with RIA, drastically overestimated serum levels. Re-analysis of these samples on the capillary column could separate fentanyl completely from its interfering peaks (fig. 1) which, after GLC/MS, could be assigned to metabolites of quinidine, which indeed had been administered to some patients as a perioperative medication.

TABLE 2. Comparison of Assays of Human Serum with Known Amounts of Added Fentanyl

Actual Concentration (ng/ml)	Janssen GLC (ng/ml)	Janssen RIA (ng/ml)	Stanford RIA (ng/ml)
9.51	9.28 9.00 9.24 9.28	9.22 8.88 8.93 8.53	9.19 8.41 9.50
Mean ± SD	9.20 ± 0.13	8.89 ± 0.28	9.03 ± 0.56
6.34	6.08 6.18 6.32 5.87	6.08 6.20 6.18 6.35	6.01 5.22 6.45
Mean ± SD	6.11 ± 0.19	6.20 ± 0.11	5.89 ± 0.62
2.54	2.54 2.44 2.29	2.38 2.58 2.38	2.40 2.57 2.67
Mean ± S.D.	2.42 ± 0.13	2.45 ± 0.12	2.55 ± 0.14
0.51	0.63 0.50 0.66 0.66	0.54 0.54 0.48 0.57	0.63 0.62 0.59
Mean ± SD	0.61 ± 0.08	0.53 ± 0.04	0.61 ± 0.02

FIG. 2. Correlation between fentanyl serum concentrations (0–50 ng/ml) as measured by radioimmunoassay (RIA) and by gas-liquid chromatography (GLC). The solid line represents the slope of the regression line (table 1).

Janssen = Janssen Pharmaceutica, Belgium; Stanford = Department of Anesthesia, Stanford University School of Medicine; GLC = gas-liquid chromatography; RIA = radioimmunoassay.

The RIA results we obtained prove the validity of the method for the measurement of fentanyl serum concentrations. Detection limits of 0.050 ng per assay tube, in this case corresponding to 0.25 ng/ml, could easily be obtained in a reproducible way. The accuracy of the RIA methods is very satisfactory, as can be concluded from the analysis of the spiked controls, and there is no overestimation of the lowest concentration of patient or spiked control samples. The latter is, of course, important before one should apply a bioanalytical method to define the pharmacokinetics of a drug such as fentanyl. At the detection limit (0.25 ng/ml), the coefficient of variation (CV) was 14.7% for GLC and 14.2% for RIA. At the higher concentrations, the CV was much lower (about 5%) and independent of concentration for both assays. The increased variability at the detection limit has a significant impact on any pharmacokinetic analysis as the terminal half-life for low-to-moderate doses of fentanyl (5–15 μg/kg) will be determined at serum levels in the region of that detection limit. This may be a major source of the variability for the pharmacokinetic data reported for fentanyl.[4,13] Recently, the Association of Official Analytical Chemists examined the results of 50 interlaboratory collaborative studies that measured trace constituents in food.[19] For analytical techniques that measured concentrations of approximately 1 ng/ml, the CV of the assay methodology between laboratories was approximately 45%. In our comparison of only two laboratories, the measurement of fentanyl was much more precise than could be expected from the study cited above. A comparison of six to eight laboratories measuring fentanyl would be needed to determine the exact contribution of assay methodology to the reported variability pharmacokinetic data for fentanyl.

Of course, laboratories should thoroughly validate their methods before applying them. Phipps *et al.*[15,20] have examined the fentanyl assay methodology, but there are several inconsistencies in their data. In the description of their GLC method,[20] for which a detection limit in the lower pg/ml range is claimed, several discrepancies were found. The most obvious is the chromatogram representing a 20-pg/ml fentanyl peak giving a 10% of full-scale deflection next to an internal standard (alfentanil) peak representing a 100,000-fold (2 μg/ml) concentration, but giving only a four-fold deflection of the fentanyl peak. In a second paper,[15] they compare GLC and RIA for measuring fentanyl concentrations in plasma. The comparison, however, is based on spiked serum samples only, and, therefore, they could not evaluate the possible cross-reactivity of fentanyl metabolites. Moreover, at the median plasma concentration studied (10 ng/ml), the CV was 13.6% for RIA and 14.8% for GLC, about four times the CV

found by our methods at that concentration. These findings, together with the potential GLC interference problem discussed above and the favorable RIA results obtained in this study, allow us to conclude that the RIA may be at least as reliable as GLC. In any regard, further validation of an analytical method by comparison with other assays, as we did recently in comparing RIA and GLC/MS for the determination of plasma sufentanil levels,[21] as well as inter-laboratory studies, such as the present one and the studies we carried out earlier on alfentanil,** should be the prerequisite before applying any assay to study the pharmacokinetics of a compound. We further recommend that color and chemical quenching correction be performed for all fentanyl RIA measurement. Any degree of red blood cell hemolysis or alteration of serum protein concentration that can occur from the operative procedure or blood sampling can affect the liquid scintillation counting and alter the measured fentanyl concentration. This would be especially prominent during or after cardiopulmonary bypass. Color and chemical quenching correction was performed for the Janssen RIA, but not for the Stanford RIA. The serum samples used in the analysis had minimal hemolysis and alteration of serum protein concentration. Therefore, no effect was detectable in the Stanford analysis.

Lastly, the validation of both applied methods in the lower ng/ml-range, as well as their application by two independent laboratories to the serum samples of 20 surgical patients, should assist in deriving unambiguously the pharmacokinetic parameters of fentanyl.

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