Assay Methods for Sufentanil in Plasma

Radioimmunoassay Versus Gas Chromatography-Mass Spectrometry

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Background: The terminal pharmacokinetic parameters of sufentanil have, until now, been poorly characterized. This is probably because of the poor sensitivity or unreliability of the assay methods used. Radioimmunoassay (RIA) can be a very helpful assay method for sufentanil. However, before application to key pharmacokinetic studies, it requires adequate validation, e.g., by comparison with a method of proven sensitivity and specificity, such as gas chromatography-mass spectrometry (GC-MS).

Methods: Spiked control plasma samples and 135 plasma samples obtained from five patients receiving intravenous doses of 500 or 750 μg sufentanil, as a 10–20-min infusion, were analyzed by an improved, sensitive RIA and capillary GC-MS.

Results: Both techniques had comparable limits of quantitation (0.02 ng/ml). Between-day coefficients of variation in the 0.05–10-ng/ml concentration range were 8.5–10.5% for the RIA and less than 10% for the GC-MS method. The patient plasma concentrations determined by RIA (y) and GC-MS (x) showed a good agreement (y = 1.016x + 0.002) and a correlation coefficient of 0.97.

Conclusions: The results demonstrate the validity of the improved RIA method for the determination of sufentanil plasma concentrations. (Key words: Anesthetics, intravenous; sufentanil. Analgesics, opioid; sufentanil. Measurement techniques: gas chromatography-mass spectrometry; radioimmunoassay. Pharmacokinetics: plasma concentrations; sufentanil.)

SUFEANTANIL, N-[4-(methoxymethyl)-1-[2-(2-thienyl)-ethyl]-4-piperidinyl]-N-phenylpropanamide, is a potent opioid analgesic that is about five to ten times more potent than fentanyl.1,2 Plasma or serum concentrations in the analgesic range (0.05–0.3 ng/ml) are, therefore, extremely low, and much attention has been paid to the development and application of sensitive assay methods, including radioimmunoassay (RIA) and gas chromatography (GC) with either nitrogen-selective4 or mass spectrometric (MS)4 detection. The GC-MS method proved very sensitive, and the quantitation limit of the earlier-developed RIA method (0.10 ng/ml)5 has been improved fivefold by the application of an extraction step and the use of a radioligand with a higher specific activity. In this way, the improved RIA could already be successfully applied to analyze the low sufentanil concentrations after epidural or intrathecal sufentanil administration.5,6

Major discrepancies have been found between some pharmacokinetic studies with sufentanil. Bovill et al.7 reported a steady state distribution volume (Vdss) of 1.7 l·kg⁻¹ and a terminal elimination half-life of 2.7 h, whereas Hudson et al.8 found a much larger Vdss.
(8.7 1·kg⁻¹), accounting for a prolonged elimination half-life of 12.1 h. Poor characterization of the terminal pharmacokinetic parameters is most probably caused by inadequate assay sensitivity or the enlarged bias around the limit of quantitation. Therefore, we believed that it was important to thoroughly validate the improved RIA method and apply it to the analysis of spiked control plasma and plasma samples from treated patients. The samples were further analyzed by GC-MS and the assay results were compared. In this way, validated and reliable plasma concentration data are provided that may contribute to the elucidation of the true pharmacokinetics of sufentanil in patients.

Materials and Methods

Study Samples

Pooled blank plasma, obtained by plasmapheresis (Haemonetics, Braintree, MA) from untreated healthy volunteers, was spiked with sufentanil reference standard at different concentrations in the 0.05–10-ng/ml concentration range. Each of these spiked quality control (QC) samples was divided into 2.5–4.5-ml portions and frozen at −20°C until analysis. Patient samples were obtained from surgical patients, participating in a pharmacokinetic/pharmacodynamic study and given intravenous infusions of 250–1,500 μg sufentanil.## Blood was sampled for 48 h after sufentanil administration. The blood samples were anticoagulated with heparin and promptly centrifuged, and the separated plasma was frozen until analysis.

Radioimmunoassay Method

The spiked QC samples and patient samples were measured, employing the commercial Sufentanil Radioimmunoassay Kit (Janssen Biotech, Olen, Belgium). Aliquots of 1–2 ml of plasma were made alkaline with 1 ml 0.5 M sodium hydroxide, vortexed for a few seconds, and poured over 3-ml prepacked glass extraction columns (Extrelut, Merck, Darmstadt, Germany). After 5 min, the columns were eluted with 9 ml heptaneisoamyl alcohol (98.5:1.5, v/v), and the eluate was collected into 5-ml glass centrifuge tubes and evaporated to dryness under a gentle stream of nitrogen in a metal heating-block at 60°C.

The extraction residues were dissolved in 0.05 ml methanol and diluted with 0.5 ml of a 2% BSA-0.05 M phosphate buffer solution (pH 7.5). After vortexing for a few seconds and centrifugation at 2,500 rpm for about 2 min, 500-μl aliquots of the extracts were transferred to 1.5-ml polypropylene Eppendorf tubes, containing 0.08 ng of ³H-sufentanil (specific activity 54 Ci/mM, ca. 25,000 dpm) in 0.05 ml 30% methanol-water. After mixing, 0.1 ml of the diluted antiserum (1:3,300) was added and the samples were incubated by continuous rotation (25 rpm) at room temperature for 2 h. Bound and free sufentanil were separated by selective adsorption of the free ligand. For this purpose, 0.2-ml aliquots of a well stirred 2% dextran-coated charcoal suspension were added to the incubation mixtures and allowed to equilibrate under continuous rotation at room temperature for 1 h. The charcoal was precipitated by centrifugation at 8,000 g for 5 min (Microfuge, Heraeus, Osterode, Germany) and the supernatant fractions, containing the antibody-bound sufentanil fractions, were carefully aspirated and transferred to 5-ml scintillation vials. The contents were mixed with 4 ml of a scintillation cocktail (Pico-Fluor 30, Packard, Downers Grove, IL). Radioactivity (expressed as dpm) was measured for 2 min in a Packard Tri-Carb 1500 liquid scintillation analyzer equipped with a PC data acquisition and analysis system using a Packard SecuRia software package for RIA data reduction and quality control. Calibration curves were obtained by duplicate analysis of up to seven different calibration samples, a blank, and a zero, using a four-parameter logistic curve fitting approach, as described by Rodbard.⁹ In this approach, also the values for b₀ (response at zero dose) and NSB (nonspecific binding), which are considered as fixed constants in the usual logit-log method, are taken into account to calculate the best possible fit, especially at the extremes of the standard curve.

Gas Chromatography-Mass Spectrometry

Analysis of the plasma samples involved the specific extraction of sufentanil and its internal standard and subsequent analysis by capillary GC-MS. The extraction procedure corresponds to that reported earlier.⁷ Aliquots of 1 or 2 ml of plasma, spiked with 10 ng of internal standard (alfentanil), contained in 0.1 ml methanol, were alkalized with 1 ml 0.1 N sodium hydroxide (pH 15) and extracted twice with 3.5-ml aliquots of heptaneisoamyl alcohol (95:5, v/v). The combined organic layers were back-extracted with 4 ml 0.05 M sulphuric acid and, after alkalization of


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the latter phase with 0.15 ml of concentrated ammonia, reextracted twice with 2.5-ml aliquots of the extraction solvent.

After evaporation to dryness, the extraction residues were dissolved in 50 μl methanol, and 2- to 10-μl aliquots were injected through a solventless moving-needle injector (Alltech, Laarne, Belgium) onto a 25 m × 0.31 mm ID OV-101 capillary column (film thickness 0.17 μm, Hewlett-Packard) installed in a Hewlett-Packard Model 5985B quadrupole GC-MS system, operated in the 70 eV El-mode. Helium was used as the carrier gas at an inlet pressure of 6 psi. The column oven was programmed (30°C/min) from 250°C to 285°C. The injector, interface, and source temperatures were 290°C, 285°C, and 200°C, respectively. Ion source and analyzer pressures were 8 × 10⁻⁶ mmHg and 1 × 10⁻⁶ mmHg.

Standard curves were prepared by spiking blank human plasma with sufentanil at concentrations ranging from 0.020 to 20 ng/ml and with the internal standard at a fixed concentration of 10 ng/ml. After extraction and analysis as described above, the peak area ratios of the major fragment ion of the parent drug and its internal standard (m/z 289) were plotted against the sufentanil standard concentrations on a log-log scale.

Data Analysis
Within- and between-day accuracy and precision were calculated for each method using the independently prepared QC samples. The relationship between concentrations of sufentanil in patients as determined by GC-MS (used as independent variable because of superior precision) and by RIA (dependent variable) was studied by weighted linear regression analysis (weight = 1/x²) and evaluation of the 95% confidence limits of the slope and the intercept (SAS/STAT User’s Guide, Version 6.07, 1992, SAS Institute, Cary, NC). No data points were excluded from the analysis, and no samples were reassayed because of deviations from expected values.

Results

Radioimmunoassay Method
Using four-parametric logistic curve fitting, reproducible and linear (r > 0.999) RIA calibration curves were obtained in the range of 0.04–2.0 ng per assay tube. Corresponding binding levels were 80% at 0.04 ng and 15% at 2.0 ng/tube. With the extraction of 2-

ml volumes of plasma, sufentanil concentrations could be measured at a concentration as low as 0.020 ng/ml. Intra- and interassay coefficients of variation over a sufentanil concentration range of 0.056–9.75 ng/ml were 5.1–8.5% and 8.5–10.5%, respectively (table 1).

Gas Chromatography-Mass Spectrometry
Retention times of sufentanil and the internal standard were 3.2 and 4.1 min, respectively (fig. 1). The GC-MS standard curves were linear (r > 0.999) in the range from the quantitation limit (0.020 ng/ml) to over 20 ng/ml. At the lower limit of quantitation, the signal-to-noise ratio was at least 5 and the CV was below 20%. Average intra- and interassay coefficients of variation were less than 10%.

Methods Comparison
The sufentanil plasma concentrations in the patient samples (n = 135), as obtained by either RIA and GC-MS, are graphically represented in the correlation diagrams given in figs. 2 and 3. The expression of the weighted linear regression equation (fig. 2) was y = 1.016x + 0.002, with a correlation coefficient (r) of 0.97 (n = 135). The 95% confidence limits of the slope and the intercept ranged from 0.972 to 1.060 and from −0.008 to 0.011, indicating that the slope and the intercept were not significantly different from 1 and zero, respectively. In figure 3, the results are presented on a log-log scale to visualize the effect of a weighted regression.

Discussion
Although it was not the primary goal of this study to validate the GC-MS method for sufentanil, the GC-MS technique again proved its suitability for the very sen-

Table 1. Sufentanil QC Samples: Statistical Evaluation of the Analysis Results Obtained by Radioimmunoassay

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Within-day Precision</th>
<th>Between-day Precision</th>
<th>Overall Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CV</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>0.056</td>
<td>8.5</td>
<td>63</td>
<td>10.0</td>
</tr>
<tr>
<td>0.600</td>
<td>7.1</td>
<td>71</td>
<td>10.5</td>
</tr>
<tr>
<td>9.75</td>
<td>5.1</td>
<td>34</td>
<td>8.5</td>
</tr>
</tbody>
</table>

RE = relative error; N = number of replicates.
positive determination of sufentanil plasma concentrations. The lower quantitation limit of 0.020 ng/ml could be attained in a reproducible way by the application of the multistep plasma extraction and cleanup procedure, the solventless injection technique, and the very selective selected ion monitoring (SIM) mode.

The sensitivity of the originally described RIA was enhanced by the use of a tracer of a greater specific activity, i.e., 54 Ci/mM instead of the formerly used 15 Ci/mM. This, together with the fact that 2-ml plasma samples were submitted to a straightforward extraction step before the RIA, allowed us to reach a comparably lower limit of quantitation. The extraction procedure used is, of course, less critical for RIA than for GC purposes. The sample cleanup steps are not necessary, except in the case of tissue biopsies, and the single-step extraction procedure could be made even more time effective by the use of the sorbent extraction methodology. The method, as described herein, results in a low, but restricted, working concentration range. This range can be extended by the submission of 50-μl extracts to the RIA or even, as described in the manual provided with the RIA kit, by direct RIA of 50-μl aliquots of (eventually diluted) plasma.

The within- and between-day reproducibility of the assay using spiked QC samples proved to be adequate for both methods. For radioimmunoassays, however, this is not a guarantee that method bias does not exist because of possible matrix and metabolite interference problems. Therefore, as we did for fentanyl and alfentanil, real patient samples were analyzed by RIA and an alternative measurement technique of proven sensitivity and selectivity, e.g., GC-MS. The close agreement between the results obtained by both methods shows that, in the case of the sufentanil RIA, no such bias exists, and confirms the earlier reported specificity toward sufentanil.

Fig. 1. Chromatograms of plasma samples. (A) Control blank plasma spiked with 10 ng/ml of internal standard (IS). (B) Control plasma spiked with 0.050 ng/ml sufentanil (S) and 10 ng/ml of internal standard (IS). Sufentanil and internal standard retention times were 3.2 and 4.1 min, respectively.

Fig. 2. Correlation between sufentanil plasma concentrations as measured by radioimmunoassay (RIA) and by gas chromatography-mass spectrometry (GC-MS). The thick line represents the regression line; the thin lines enclose the 95% confidence interval for the individual values.
fentanyl metabolites. Moreover, the excellent correlation between the results confirms the good reproducibility of both methods.

Given that similarity, it is obvious that the RIA method, because of its higher sample throughput and the restricted availability of the sophisticated GC-MS equipment, remains the method of choice for pharmacokinetic studies. Nevertheless, we also feel obligated to emphasize that, in general, most of the assay variability consists of interlaboratory variability, and that analytical laboratories, irrespective of the methodology used, should always properly validate their assay methods, especially when measuring concentrations in the pg/ml range.\(^\text{13}\)

References