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Determination of subnanogram concentrations of fentanyl in plasma by gas chromatography–mass spectrometry: comparison with standard radioimmunoassay

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Abstract

A method was devised to determine fentanyl plasma concentrations by GC–MS using selected-ion monitoring (SIM) with sufentanil as internal standard. This was compared with a commonly used commercial radioimmunoassay (RIA). Sample preparation for GC–MS involved basification of plasma then extraction using *n*-butyl chloride followed by concentration to dryness and reconstitution in toluene for chromatography. Using 1-ml plasma samples, the estimated limit of detection of fentanyl was 20 pg/ml. Blood samples for pharmacokinetic studies were split and assayed by GC–MS and RIA which had a limit of detection of 200 pg/ml. Pearson's r ($r=0.80$, $p<0.0001$) indicated the methods were highly correlated at all plasma concentrations. Owing to the greater sensitivity of the method, GC–MS is recommended over RIA for subnanogram determination of fentanyl in plasma.

Keywords: Fentanyl

1. Introduction

Fentanyl, N-phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]propanamide (Fig. 1), is a potent synthetic opioid. It is used in high doses ('anaesthetic doses') for inducing loss of consciousness in patients undergoing cardiac surgery because of its wide safety margin and its ability to produce loss of consciousness with ablation of the stress response to surgery without causing cardiovascular depression [1,2]. It is also used in low doses for the treatment of severe pain ('analgetic doses') where it is found to have a rapid onset of action. In comparison to morphine, 0.1 mg of fentanyl has essentially equivalent analgetic effect to 10 mg of morphine, but a shorter duration of action.

Comprehensive pharmacokinetic studies of fentanyl have proven difficult as the blood concentration of fentanyl from single or infrequent doses falls rapidly below the limit of detection of most assay procedures. The detection of lower levels of fentanyl from analgetic doses, however, is important to a full

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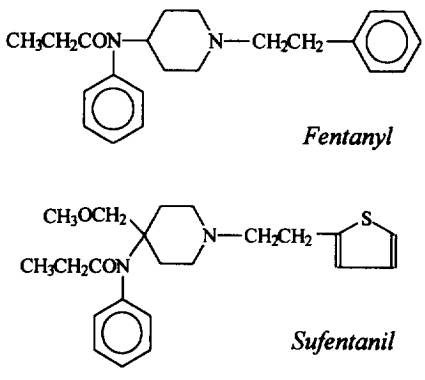

Fig. 1. Structure of fentanyl and sufentanil.

understanding of its pharmacokinetics [3,4]. A number of methods have been developed to measure fentanyl concentrations in biological fluids [5] with differing levels of sensitivity and usefulness in

[17] also reported a similar limit of detection using GC–NPD but their method was not suitable for a large throughput of samples. Moreover, the extraction solvent, benzene, was not suitable for rapid concentration and sample reconstitution in 10 μl solvent was too small a volume for the autosampler.

We have devised a new fentanyl assay method optimised for high sensitivity and throughput of samples. A one-step extraction technique with sufentanil as internal standard (Fig. 1) was used to give a high recovery from plasma. By using selected-ion monitoring (SIM), our method allowed a reduction in the number of extraction steps so that the instrument was used as the secondary clean-up step. The acknowledged disadvantage in using the instrument as a clean-up procedure is that the MS source must be cleaned more frequently. As the measurement in lower concentration samples was the most important

Sample vials, 4 ml (15×45 mm screw cap vials for use with the Waters WISP) and 2 ml (12×32 mm screw cap vials for use with the Shimadzu SIL-10A autosampler) were supplied by Alltech (Sydney, Australia); 200-ml glass inserts and wide-mouth screw-top autosampler vials were supplied by SGE Scientific (Melbourne, Australia) and Hewlett-Packard (Melbourne, Australia), respectively.

A Medos JAK evaporator was supplied by Dynavac Engineering (Sydney, Australia) and was used for concentration after the extraction procedure. The vacuum was achieved using a water aspirator and the temperature was held at 40°C.

2.2. Instrumentation

The analyses were carried out on a Hewlett-Packard 5890 series II Plus gas chromatograph equipped with a 7673 autosampler and a 5972 series mass selective detector (MSD) in EI mode (70 eV). High purity helium was used as the carrier gas at a constant flow-rate of 1.55 ml/min. The column was a 5% phenyl methyl siloxane capillary column (HP-5MS, Hewlett-Packard) 30 m×0.25 mm I.D. and a film thickness of 0.25 μm. The injector and detector temperatures both were maintained at 280°C. The oven temperature was programmed as follows: 150°C for 1 min after injection, increasing to 240°C at 50°C/min, then to 285°C at 10°C/min, finally holding at 285°C for 12.7 min (overall run time 20 min). The MS was operated in the selected-ion monitoring mode (SIM) using *m/z* 245 and 189 for fentanyl (where the abundance of 189 was 40% of 245), and *m/z* 289 and 290 for sufentanil (where the abundance of 290 was 20% of 289). Each ion had a dwell time of 40 ms.

2.3. Samples and standards

Blood samples were obtained from healthy human subjects before and after receiving fentanyl; these were placed in heparinized blood collecting tubes and centrifuged at 1500 *g* for 15 min. The plasma was stored at −20°C until analysis.

A stock solution of fentanyl citrate (equivalent to 50 μg/ml fentanyl base) was dissolved in 0.9% saline solution giving a concentration range of 1 to 250 ng/ml. Plasma standards were prepared by adding 0.9 ml drug-free plasma to 100 μl of aqueous working standard to give final concentrations 0.1–25 ng/ml.

The internal standard solution was prepared by the addition of 20 μl sufentanil citrate (equivalent to 50 μg/ml sufentanil base) and 2 μl of 1 mg/ml D_5-fentanyl citrate in 4 ml Milli-Q water.

2.4. Preparation of glassware

Glassware was siliconised by immersion in a 5% solution of SurfaSil in *n*-hexane for 10 min, then rinsing in acetone, followed by *n*-hexane and oven drying at 150°C.

2.5. Extraction and chromatography

Aliquots (1 ml) of plasma samples and plasma fentanyl standards were pipetted into a 4-ml vial containing 10 ml internal standard and made alkaline with 100 μl of 4 *M* sodium hydroxide; 2 ml *n*-butyl chloride was added and the mixture vortex mixed for 10 min followed by centrifuging at 2000 *g* for 5 min. The samples were frozen in dry ice for 15 min, the organic layer decanted into a 2-ml vial and evaporated to dryness under vacuum at 40°C. Extraction residues were reconstituted in 40 μl toluene, vortex mixed for 30 s, sonicated for 5 min, centrifuged for 1 min and transferred into a 200-μl siliconised glass insert. Of this final extract, 5 μl was injected into the GC–MS system via splitless mode.

2.6. Radioimmunoassay procedure

Fentanyl was quantitatively measured using the [^{125}I] Coat-a-Count fentanyl RIA kit by a commercial laboratory. The kit contained fentanyl antibody-coated tubes, [^{125}I]fentanyl, fentanyl standards and fentanyl controls.

Four plain polypropylene tubes were labelled to measure total counts (T) and nonspecific binding (NSB) in duplicate. Twelve fentanyl antibody-coated tubes were labelled A (maximum binding) through F in duplicate and standards A through F were prepared by adding 50 μl of the supplied standard to give a standard curve with concentrations of 0 (A), 0.25, 0.5, 1.0, 2.5 and 7.5 (F) ng/ml. Controls were prepared using 50 μl of the supplied control solution

for each control tube and 50 μl of plasma sample was added to each sample tube. The standards, controls and samples were then incubated for 1 h at room temperature, decanted until no residual droplets remained and then counted for 1 min in a gamma counter.

3. Results and discussion

In order to increase the sensitivity of this assay to that required for 'analgetic doses', several factors needed to be addressed. Silicone deactivation of all glass surfaces with which the fentanyl came into contact, including the glass injection port liner of the GC, decreased the potential for analyte adsorption. Initially D_5-fentanyl was added as an internal standard but subsequently sufentanil (Fig. 1) was used for this purpose. In the adopted method, D_5-fentanyl was added in high concentration and favourably competed with fentanyl for adsorption sites; sufentanil was chosen as the internal standard because it has similar physiochemical properties to fentanyl, had good peak shape and eluted only 30 s after fentanyl.

A 'blank' plasma sample from the same subject was run with each standard and sample set to verify possible contamination; none had measurable concentrations of fentanyl or sufentanil. Precautions such as using high purity solvents and cleaning solvent dispensers regularly aided in avoiding contamination.

The high concentration of sodium hydroxide added to basify the plasma samples minimised extraction of organic contaminants. In this way, a large cholesterol derivative peak, that was present in extracts when pH 9 borate buffer was used, was no longer found. The retention times for fentanyl and sufentanil were 7.9 and 8.5 min, respectively. A typical chromatogram from an extracted plasma sample is shown in Fig. 2 where the concentration of fentanyl was 1.4 ng/ml.

Extraction efficiency, determined by comparison of extracted plasma samples with extracted water samples, was 81%. The extraction efficiency was high due to minimising the number of extraction steps.

Precision, determined by six replicate analyses of plasma containing 25 and 0.1 ng/ml fentanyl, gave coefficients of variation of 3.8 and 12.5%, respectively. Accuracy determined by six replicate injections of the same extracted sample of 25 ng/ml, gave the coefficient of variation of 2.7%. The limit of detection, defined by a signal-to-noise ratio of greater than 3, was approximately 20 pg/ml. A method with similar performance to that presently described has recently been reported [20].

Samples and standards were quantified by the measurement of peak areas. Calibration curves from 0 to 25 ng/ml were linear, with all correlation coefficients being >0.99. A typical calibration curve is shown in Fig. 3 and this had a slope of 0.423 and correlation coefficient of r=0.9997. However, the equations for the standard curve were determined by weighted least squares using the reciprocal of concentration as the weight, because the majority of the

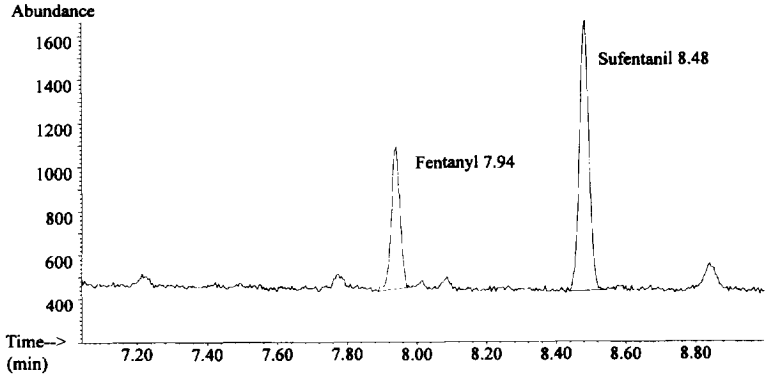

Fig. 2. Chromatogram showing resolution of fentanyl and sufentanil sampled 5 min after an intravenous dose of 100 μg fentanyl citrate.

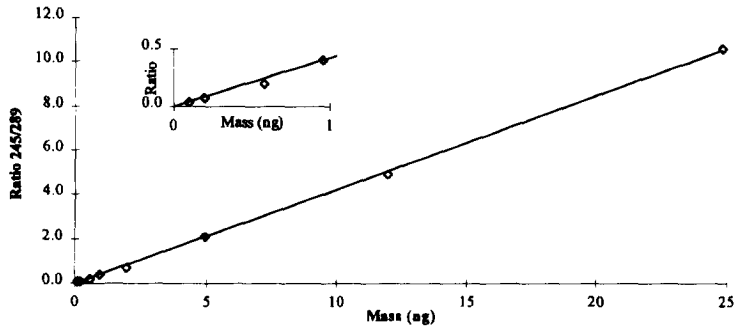

Fig. 3. Standard curve for fentanyl analysis. Insert: lower region of curve 0.1–1 ng/ml.

samples had concentrations <2 ng/ml. When the calibration curve in Fig. 3 was fitted, using the weighted least squares with reciprocal of concentration on the weight, the slope was 0.417 and correlation coefficient $r=0.9996$.

Application of this analytical method to a pharmacokinetic study produced a typical fentanyl concentration–time profile as shown in Fig. 4. Concentrations of fentanyl in peripheral venous blood after a single intravenous dose of 200 μg infused over 1 min, were measurable at least to 720 min.

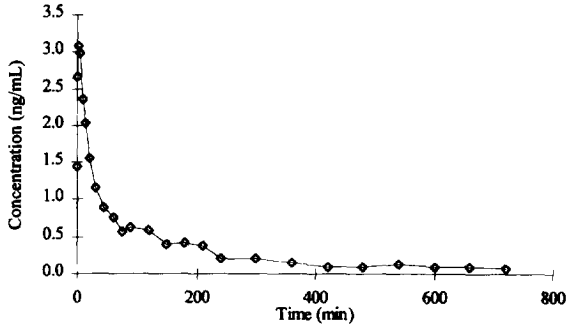

Fig. 4. Fentanyl plasma concentration; the profile after infusion over 1 min of 200 μg fentanyl citrate.

3.1. Radioimmunoassay and GC–MS comparison

Whereas the GC–MS method remained sensitive to 20 pg/ml, the RIA assay was sensitive only to 200 pg/ml. Owing to the differences in sensitivity, only 848 of the 1298 samples could be compared. The difference between the RIA and GC–MS assay of the same sample was calculated and is presented in Table 1 where positive values indicate that the GC–MS assay was greater than the RIA equivalent. Difference values were not normally distributed owing to the high assay values being quite discrepant. These values are mainly influenced by uncertainties in the calibration curves for methods at extreme concentrations which may be due to blood sampling vagaries. Thus 6 points were considered outliers (Table 1), leaving 842 samples for analysis.

GC–MS assay values were higher than RIA assay values for 474 of the 842 assays. A two-tailed paired dependant t-test indicated no significant differences

Table 1
Descriptive statistics for RIA and GC–MS fentanyl assays

	All data ($n=848$)			With 6 outlier points removed ($n=842$)		
	RIA (ng/ml)	GC–MS (ng/ml)	Difference (ng/ml)	RIA (ng/ml)	GC–MS (ng/ml)	Difference (ng/ml)
Max	930	3792	−2862	21.0	35.5	−14.5
Min	0.20	0.02	0.18	0.20	0.02	0.18
Mean	3.5	6.7	−3.2	1.0	1.2	−0.2
Median	0.6	0.7	0.03	0.6	0.7	0.02
SD	42	131	101	1.7	2.3	1.0

The calculated difference for median and standard deviation was determined by the average of difference values for each individual sample.

Table 2
Pearson's correlations for RIA and GC–MS when assays were analysed at different assay sizes

	Pearson's r	$p<$	n
RIA>10 ng/ml	0.77	0.002	13
RIA 1 to 10 ng/ml	0.76	0.0001	274
RIA 0.9 to 0.6 ng/ml	0.24	0.001	180
RIA 0.5 to 0.2 ng/ml	0.44	0.0001	381

between the RIA and GC–MS assays. Pearson's r indicated that, overall, there was a highly significant correlation ($r=0.931$; $p<0.0001$, $n=842$) between the two assay methods. There were also significant correlations between methods when assays were analysed in terms of stratified ranges of plasma concentrations (Table 2), although it was clear that the correlation deteriorated with decreasing concentrations.

The findings of this study indicate that RIA and GC–MS produce highly correlated fentanyl plasma concentrations over a large range of concentrations. However, the GC–MS method was more sensitive and generally produced higher assay values when compared to RIA. These differences became particularly important at very high and very low concentrations of fentanyl. The different assay methods were conducted in different laboratories and the authors acknowledge that there may have been more control over technique if the same laboratory had conducted both assay methods.

4. Conclusions

Our new GC–MS method has been used to process more than 2000 blood and plasma samples. All calibration curves have had a correlation coefficient of >0.99. Although methods have been previously reported in which the accuracy, precision and limit of detection are similar, sample preparation has been more complex and the volume of sample needed to achieve the low limit of detection has usually been larger. As our method required only a 1-ml plasma sample, it had a limit of detection two times more favourable than the GC–MS method reported by Caplan and Watts [16].

Both RIA and GC–MS produced similar results when used to determine fentanyl plasma concentrations. However, when choosing one method over another, the magnitude of the fentanyl concentration needs also to be considered. The GC–MS method described in this paper was more sensitive to the RIA method and would be more suitable for determination of subnanogram fentanyl concentrations. The GC–MS method allows more thorough description of the pharmacokinetics of fentanyl by better illustrating the duration of useful analgetic concentrations and describing the washout phase in more detail. For larger concentrations, either method appears acceptable and factors such as cost and time of the assay method should be considered.

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