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Review

A sensitive assay for the simultaneous measurement of alfentanil and fentanyl in plasma

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Abstract

A reversed-phase high-performance liquid chromatography method for the simultaneous determination of plasma concentrations of the narcotic analgesics alfentanil and fentanyl using papaverine hydrochloride as the internal standard is presented. Chromatographic separations were achieved with an Econosphere CN, 5 μm, 25 cm × 4.6 mm i.d. column and the effluent was monitored at 195 nm. The assay was linear over the clinically relevant plasma range of 2–2000 ng ml^{-1} for alfentanil and 2–100 ng ml^{-1} for fentanyl and has the sensitivity and specificity necessary to determine plasma concentrations of these compounds. Inter- and intra-day precision (RSD) for both compounds did not exceed 10% in these ranges. The assay procedure was utilized for pharmacokinetic studies of plasma concentrations in subjects receiving alfentanil and fentanyl during and after cardiac surgery. This will allow better elucidation of pharmacokinetic variables in this populace.

Keywords: Reversed-phase chromatography; Alfentanil; Fentanyl; Papaverine; Plasma

1. Introduction

Fentanyl, 1-(2-phenethyl)-4-*N*-(*N*-propionylanalino)piperidine, and alfentanil, *N*-[1-{2-(4-ethyl-4,5-dihydro-5-oxo-1H-tetrazol-1-yl)ethyl}-4-(methoxymethyl)-4-piperidinyl]-*N*-phenylpropanamide, are structurally similar narcotic analgesics with alfentanil having about one-third to one-seventh the potency of fentanyl [1]. Both drugs are commonly used as adjuncts or major anesthetis in surgery. Despite greater equianalgesic respiratory depression, fentanyl is more often used post-operatively for pain management than alfentanil. The total narcotic load in intensive care subjects having undergone supplemental alfentanil anesthesia may therefore be made

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up of both alfentanil and fentanyl. It was therefore important to develop an assay that could accurately quantify levels of both drugs simultaneously.

Gas chromatographic (GC) methods, particularly when coupled with mass spectroscopy, are sensitive but they are time-consuming due to the number of purification and derivatization steps required [2–8]. Radiochemical [9] and radioimmunoassay [10,11] methods suffer from a lack of selectivity particularly at clinically realistic levels of fentanyl (<10 ng ml^{-1}). This lack of selectivity may be partly responsible for the wide variability in kinetic parameters of fentanyl [1]. Enzyme-linked immunosorbent assay methods for fentanyl are sensitive and relatively simple but have low precision and show little cross reactivity with alfentanil [12,13]. High-performance liquid chromatography (HPLC) is robust and, in comparison with the aforementioned methods, is relatively inexpensive. Routine analysis is rapid as only back extraction into acid is required, compared with solvent evaporation in the purification step. This methodology is an improvement on a previous report [14] as it allows accurate simultaneous quantification without loss of sensitivity of either drug by the use of papaverine as an internal standard (IS). Papaverine was chosen due to structural similarity with both drugs (Fig. 1) and the presence of an ionizable nitrogen group.

2. Experimental

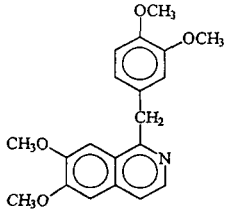
2.1. Materials

Alfentanil hydrochloride and fentanyl citrate powders were kindly supplied by Jansenn Cilag (New Zealand) and papaverine hydrochloride (3% w/v) was obtained from Astra Pharmaceuticals. HPLC-grade acetonitrile with 190 nm cutoff was purchased from Fisher Scientific and HPLC-grade water was obtained by distillation in glass and passage through a MilliQ purification system (Millipore Corporation, Bedford, MA). All other chemicals and reagents were of HPLC grade and analytical purity and were used as received.

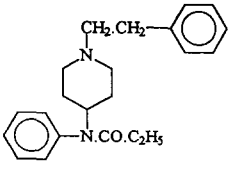
2.2. Chromatographic systems and mobile phase

The chromatographic analysis was performed with an LC-10AD solvent delivery module and an SPD-10AV UV–Vis detector. An SIL-10A autosampler was linked to an SCL-10A system controller and the output was interfaced via a CR501 chromatopac integrator (Shimadzu Scientific Instruments Inc., Columbia, MD). Separation was achieved with an Econosphere CN, 5 μm, 25 cm $\times$ 4.6 mm column (Alltech Associates Inc., Deerfield, IL) which was protected by a guard column (Cyano, Newguard 7 μm, 15 $\times$ 3.2 mm, Brownlee Laboratories, Santa Clara, CA). The mobile phase consisted of potassium dihydrogen phosphate (KH_2PO_4, pH 2.8, 0.01 M) adjusted with 85% orthophosphoric acid (H_3PO_4) and far-UV HPLC-grade acetonitrile (absorbance at 195 nm < 0.1) in a 65:35 v/v ratio.

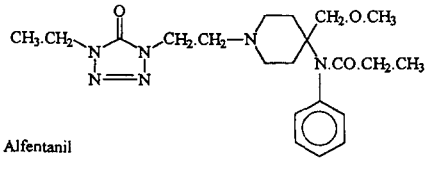
The mobile phase was filtered through a 0.45 μm filter (Millipore, Bedford, MA) and de-



Papaverine



Fentanyl



Alfentanil

Fig. 1. Chemical structures of fentanyl, alfentanil and the IS papaverine.

gassed (Medipure™ Helium, USP) before use. The system was operated at ambient temperature with a flow rate of 1.4 ml min^{-1} and the eluted compounds were monitored at 195 nm.

2.3. Sample collection and storage

After giving written informed consent a group of 20 patients received an alfentanil regimen consisting of a 400 $\mu g\ kg^{-1}$ bolus dose over 2 min followed by a maintenance infusion of 1 $\mu g\ kg^{-1}\ min^{-1}$ for the duration of surgery. Short infusions and/or bolus doses of fentanyl were given post-operatively as required for pain management. One subject received a fentanyl anesthetic regimen consisting of a bolus dose of 40 $\mu g\ kg^{-1}$ over 2 min and a maintenance infusion of 0.2 $\mu g\ kg^{-1}\ min^{-1}$ for the duration of surgery. At selected times pre-, intra- and post-operatively, 5 ml blood samples were drawn from a radial artery cannula and stored on crushed ice in lithium heparin tubes to prevent coagulation. Following separation by centrifugation plasma was either analyzed immediately or frozen at −72°C. Samples were stored in silanized glass tubes to prevent loss due to adsorption and stability tests were carried out over a 6 month period to ensure that storage had no deleterious effect on either compound. Stability was assessed by randomly placing quality control samples prepared at the start of the study into each analytical sequence. Quality control samples were prepared by spiking drug-free plasma with standards to final concentrations of 50 and 500 ng ml^{-1} alfentanil and 5 ng ml^{-1} fentanyl. Volumes of standard represented <5% of the total volume of quality control samples. Following vigorous mixing, 1 ml aliquots of the controls were stored under the same conditions as patient samples. These quality control samples were included with freshly prepared standards into every analytical sequence. The resultant data from controls and fresh standards were pooled and used in calculating precision and accuracy. The ambient stability in the autosampler was assessed for all concentrations of the calibration curve after 12 and 24 h.

2.4. Standard and sample preparation

Stock solutions of alfentanil and fentanyl were prepared by dissolving in methanol the mass of powder required to give a 1 mg ml^{-1} solution of the free base (1.57 mg ml^{-1} of fentanyl citrate and 1.13 mg ml^{-1} alfentanil hydrochloride). Stock solutions were diluted serially with HPLC-grade water to produce final concentrations of 5000, 500, 50 and 10 ng ml^{-1} for alfentanil and 500, 50 and 10 ng ml^{-1} for fentanyl. Two concentrations of the IS were prepared by diluting the stock solution (3% w/v papaverine hydrochloride) with HPLC-grade water to 15 $\mu g\ ml^{-1}$ and 1.5 $\mu g\ ml^{-1}$. All solutions were stored in a refrigerator at 6°C and tested daily for signs of degradation. Standards were prepared by addition of 50, 100 or 200 μl aliquots of fentanyl and/or alfentanil to 500 μl of blank plasma. The total volume was then adjusted to 1 ml by the addition of HPLC-grade water. Final concentrations for alfentanil were 2, 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng ml^{-1} and for fentanyl 2, 5, 10, 20, 50 and 100 ng ml^{-1}.

2.5. Sample extraction

Spiked plasma standards and patient plasma samples (1 ml) were vortexed gently for 30 s with 200 μl of potassium hydroxide (0.5 M) and 100 μl of IS. The appropriate strength of IS was used to reflect the concentrations in samples, i.e. 15 $\mu g\ ml^{-1}$ for intra-operative alfentanil samples and high standards and 1.5 $\mu g\ ml^{-1}$ for post-operative alfentanil, all fentanyl samples and low standards. 4 ml of the extractant (heptane:isoamyl alcohol, 98:2 v/v) was added and the mixture was shaken mechanically at 2 Hz (horizontally to allow maximum shear) for 10 min and then centrifuged at 420*g* for 15 min.

Approximately 3.8 ml of the upper organic layer was then transferred to a second set of specially adapted tubes (with a small nipple-like protrusion at the base), containing 200 μl of the back extractant (0.5 M KH_2PO_4, pH 2.8, adjusted with 85% H_3PO_4), and was shaken and centrifuged as before. The aqueous layer (up to 180 μl) was removed from the protrusion by syringe, transferred to an autosampler vial and 100 μl was

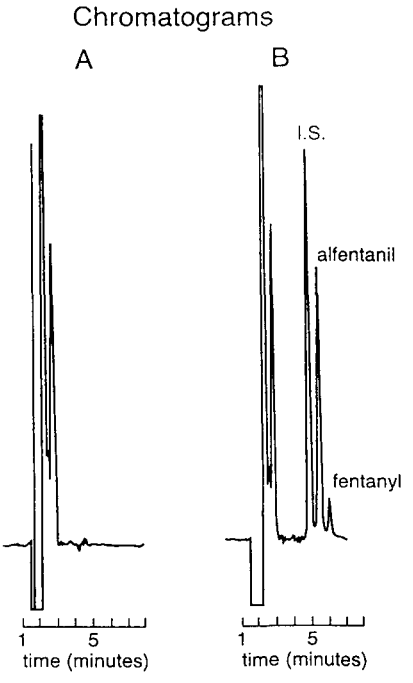


Fig. 2. Chromatograms of (A) a patient blank and (B) a post-operative sample in the same patient. Alfentanil = 101 ng ml^{-1} and fentanyl = 6.2 ng ml^{-1}.

injected onto the column. Aqueous standards in a similar low pH environment were injected at the beginning, middle and end of all analyses to determine if the compounds were stable at room temperature under these conditions.

2.6. Calibration curve

Integrated peak height and area ratios between the two compounds and the IS were determined and linear regression equations were used to find slopes, intercepts and correlation coefficients for both drugs. In order to keep drug:IS height and area ratios between 0.01 and 1.5, calibration curves for alfentanil were prepared over the ranges 2–200 and 100–2000 ng ml^{-1}. The lower strength IS was used over the 2–200 ng ml^{-1} range of alfentanil and for the fentanyl calibration curve (2–100 ng ml^{-1}).

2.7. Precision, extraction efficiency and limits

Intraday ($n = 5$) and interday ($n = 13$) variation was calculated by replicate analysis of spiked plasma at different concentrations of alfentanil and fentanyl and expressed as the relative standard deviation percentage (RSD%). The extraction efficiency of alfentanil, fentanyl and papaverine were determined by comparing peak heights of spiked standards with known amounts injected directly. Recovery was monitored daily for 10 days and calculated as the mean and sd % of all strengths. The limit of detection (LOD) was the lowest concentration that produced a peak signal-to-noise ratio of 3:1. The limit of quantitation (LOQ) was the lowest standard extract concentration that had an interday RSD of $<10\%$.

2.8. Chromatographic parameters

Chromatographic parameters as a means of quantifying the system were calculated by the following recognized equations [15].

Reduced retention time (t'_R)

$$t'_R = t_R - t_0$$

where t_R is the time from injection to peak maxima and t_0 is the time from injection to the first unretained peak of the solvent front (minutes).

Capacity factor (k')

$$k' = t'_R/t_0$$

Relative retention (α)

$$\alpha_{Alf} = t'_{R(Alf)}/t'_{R(Pap)} \quad \text{and} \quad \alpha_{Fent} = t'_{R(Fent)}/t'_{R(Pap)}$$

3. Results and discussion

3.1. Chromatographic conditions

Chromatograms of a patient blank and post-operative sample are shown in Fig. 2. Retention times for papaverine, alfentanil and fentanyl were 4.8, 5.3 and 6 min respectively. Increasing the ratio of phosphate buffer to acetonitrile (65:35 to 70:30) gave better resolution but no improvement in the linearity of the calibration curves for fentanyl and the lower strengths of alfentanil, while increasing overall run times by up to 60 s. There

Table 1
Alfentanil and fentanyl calibration curve parameters

Compound	Linear range (ng ml^{-1})	Regression equation y = peak height ratio x = concentration (ng ml^{-1})	r^2
Fentanyl	2–100	$y = 0.007 + 0.009906x$	0.988
Alfentanil	2–200	$y = -0.004 + 0.006971x$	0.991
Alfentanil	100–2000	$y = 0.018 + 0.000674x$	0.996

was an improvement in the linearity of the higher concentration ranges of alfentanil which was utilized when assaying intra-operative plasma concentrations. As only the first two peaks (IS and alfentanil) had to be resolved little time was lost.

3.2. Calibration curve

Integrated peak height and area ratios between the two compounds and the IS were calculated and slopes, intercepts and correlation coefficients were determined by an unweighted linear regression (PSI-Plot 4.0, Poly Software International, Salt Lake City, UT). Better correlation in all instances was observed for drug:IS height ratios than area ratios. Table 1 shows calibration curve parameters for fentanyl and the two ranges of alfentanil. Fentanyl, like alfentanil, was also linear over four orders of magnitude (2–2000 ng ml^{-1}) but it was impractical for our purposes to

Table 2
(a) Intraday reproducibility of the HPLC analysis ($n = 5$)

Compound	Theoretical plasma concentration (ng ml^{-1})	Experimental plasma concentration (ng ml^{-1}) Mean ± sd	RSD%
Alfentanil	5	4.74 ± 0.27	5.7
	20	19.78 ± 1.01	5.1
	200	203.6 ± 8.14	4.0
	1000	989.0 ± 34.62	3.5
Fentanyl	2	2.11 ± 0.16	7.7
	10	9.85 ± 0.67	6.8
	20	20.7 ± 0.87	4.2
	100	104.0 ± 4.37	4.2

(b) Interday reproducibility of the HPLC analysis ($n = 13$)

Compound	Theoretical plasma concentration (ng ml^{-1})	Experimental plasma concentration (ng ml^{-1}) Mean ± sd	RSD%
Alfentanil	5	4.86 ± 0.35	7.2
	20	19.38 ± 1.12	5.8
	200	204.7 ± 9.42	4.6
	1000	994.0 ± 45.72	4.6
Fentanyl	2	2.12 ± 0.19	8.9
	10	9.91 ± 0.87	8.8
	20	20.5 ± 0.98	4.8
	100	106.0 ± 4.66	4.4

Table 3
Stability of alfentanil and fentanyl samples. Mean ± sd recovery of quality control samples stored at −72°C over a 6 month period ($n = 5$)

Time (months)	Fentanyl (5 ng ml^{-1})	Alfentanil (50 ng ml^{-1})	Alfentanil (500 ng ml^{-1})
1	4.8 ± 0.2	51.0 ± 1.5	490 ± 30
2	4.8 ± 0.2	48.5 ± 2.4	505 ± 20
3	4.8 ± 0.3	49.5 ± 2.5	480 ± 20
6	4.7 ± 0.4	49.5 ± 2.2	485 ± 15

exceed 100 ng ml^{-1}, although this may be of use in stability studies. Standards containing both alfentanil and fentanyl (200 and 100 ng ml^{-1} respectively) showed no difference in drug:IS ratios compared with same-strength standards measured separately. When dealing with clinical samples of subjects who had received alfentanil anesthesia and fentanyl post-operatively, the higher IS strength was replaced by the lower IS strength for analyzing samples after fentanyl administration in the Intensive Care Unit. Papaverine was occasionally administered intermuscularly, or topically, prior to and during surgery, but plasma levels were too low to manifest themselves as significantly altered peak height ratios due to the far higher concentrations of the IS.

3.3. Precision

Intraday and interday accuracy and precision (Tables 2a and 2b) were calculated by the variance about the mean of data points in relation to the standard curve equation value. It was decided arbitrarily that a maximum of 10% interday variance was acceptable as the LOQ.

3.4. Stability

Frozen quality control samples tested over a 6 month period showed no sign of either degradation or loss. The stability data are presented in Table 3. For all concentrations no significant difference appeared between times 0, 1, 2, 3 and 6 months ($p > 0.05$). Refrigerated solutions were injected at intermittent strengths daily to test stability. No major changes in peak area or height (i.e. 95–105%) were observed over the time period of the study (6 months). Solutions adjusted with buffer to approximately pH 2.8 were injected at the beginning and end of each analytical sequence to assess the effect of low pH on stability within a given sample run. During a typical analysis of one subject's samples and standards ($n = 30$–35, 4.5–5.5 h), no alteration in peak height ratios or significant loss of individual peak heights or areas was discernible.

3.5. Extraction efficiency, chromatographic parameters and limits

Extraction efficiencies expressed as % recovery, chromatographic parameters t'_R, k' and α, and the LOD and LOQ are shown in Table 4. Incremental changes in the organic extractant composition from 96:4 v/v (heptane:isoamyl alcohol) through to 100% heptane, gave an optimal ratio of 98:2 v/v. Extraction efficiency of alfentanil was higher under more polar conditions (heptane:isoamyl alcohol, 96:4 v/v). The best balance between the volumes of extractant and back extractant buffer was found to be 4 ml and 0.2 ml respectively. Under these conditions percent recovery was similar between all three compounds over the ranges

Table 4
Extraction efficiency, normalized chromatographic parameters and limits of the assay procedure ($n = 13$)

Compound	% Recovery	t'_R (min)	k'	σ	LOQ (ng ml^{-1})	LOD (ng ml^{-1})
Papaverine	84.9 ± 3.6	3.4	2.43	–	–	–
Alfentanil	86.3 ± 4.1	3.9	2.79	1.15	2	0.25
Fentanyl	82.5 ± 5.1	4.6	3.29	1.35	2	0.25

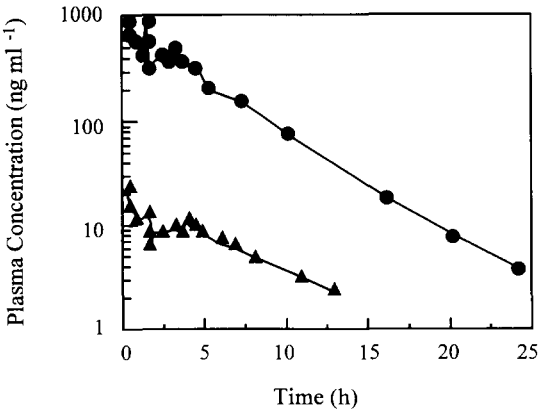


Fig. 3. Intra- and post-operative concentrations of alfentanil (●) and fentanyl (▲) in two different subjects.

covered. Effects of extractant and back extractant volumes and the pH and molarity on the extraction efficiency were studied. Extractant in volumes less than 2 ml became saturated at higher levels of alfentanil (>1000 ng ml^{-1}), resulting in decreased linearity of the calibration curve, while increasing the volume above 5 ml resulted in reduced recovery and sensitivity. The pH of the back extractant buffer was best when at least three pH units below the pK_a of all three compounds. A pH of 2.8 was chosen as it was the same as the pH of the aqueous portion of the mobile phase although the relative pH of the mobile phase was approximately 3.1.

Ionic strength of the back extractant was important and the best results were obtained at 0.5 M. Increasing the ionic strength above 0.5 M did not increase efficiency and precipitated buffer build-up on the operating system became unacceptable as did the risk of precipitation during analysis. The chromatographic parameters t'_R, k' and α were calculated based on the time t'_0 being the time to the negative peak and not the first positive unretained peak. The LOQ for both compounds was 2 ng ml^{-1} and the LOD was also the same in each case at 0.25 ng ml^{-1}. It was felt that an interday variance of greater than 10% was not acceptable, particularly for fentanyl, as this would have allowed too much error in calculations of pharmacokinetic variables. In the case of fentanyl 1 ng ml^{-1} was quantifiable, but it had a variance of approximately 17% which was excessive.

Intra- and post-operative plasma concentration–time profiles of two subjects anesthetized with fentanyl or alfentanil are shown in Fig. 3. Sporadic volatile assisted anesthesia in both patients was uneventful, indicating that the levels attained were satisfactory.

Accurate measurement of total narcotic loads in intensive care will help to establish post-operative recovery protocols for patients at risk of developing pain-induced cardiovascular disturbances.

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