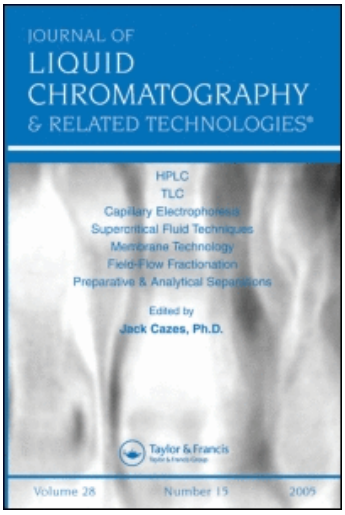

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Simultaneous Microassay of Alfentanil, Fentanil, and Sufentanil by High Performance Liquid Chromatography

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SIMULTANEOUS MICROASSAY OF ALFENTANIL, FENTANIL, AND SUFENTANIL BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A microassay for the simultaneous determination of alfentanil, fentanil and sufentanil by high performance liquid chromatography with ultraviolet detection in plasma and urine samples from newborn babies is reported. The HPLC analysis was performed with a commercially available 4 μm particle size reverse phase Waters Nova pak Cyano Column (8mm x 100 mm dimensions), guard pak cyano cartridges and a UV/VIS detector at 214 nm. Each run was completed within 10 min. The detection limits for the analysis were 0.15 ng, 0.12 ng and 0.25 ng for alfentanil, fentanil, and sufentanil, respectively with 50 μl injection. The respective retention times were 5.78 $\pm$ 0.29, 6.56 $\pm$ 0.32, and 7.08 $\pm$ 0.15 minutes for alfentanil, fentanil, and sufentanil. Applicability of this technique was evaluated in 6 newborn babies receiving constant I.V. infusion of fentanyl at 3.0 microgram/kg/h. Their plasma fentanyl concentrations were 3.04 $\pm$ 0.44 μg/L which correlated with adequate pain control. Microassay is relatively simple, rapid and precise and would be useful for therapeutic drug monitoring of these drugs in the newborn or in cases of limited sample volume.

INTRODUCTION

Fentanil, alfentanil, and sufentanil are semisynthetic opioid analgesics that are widely used as post-operative analgesics and as sedatives for patients requiring mechanical ventilation. They are also being used as premedications for surgery and other painful procedures. Clinically, these agents are well accepted as analgesics due to their high efficacy and safety margin. Alfentanil, fentanil, and sufentanil

provide adequate analgesia when administered intramuscularly or intravenously. These drugs exhibit important pharmacokinetic and pharmacodynamic differences in potency and rate of equilibrium between the plasma and the site of drug action.

Several chromatographic techniques, either for the single analysis of fentanil or the simultaneous analysis of fentanil and alfentanil or fentanil and sufentanil, have been previously reported (15,16). However, no studies have been performed, thus far, on the simultaneous analysis of these opiates in plasma and urine samples. Sensitive and selective assays for the determination of small concentrations of these analgesics in human plasma, urine and tissue samples utilized either radioassays (1,2), radioimmunoassay (3-6), gas liquid chromatography (7-11), thin layer chromatography (12), infrared and nuclear magnetic resonance (13,14) and high performance liquid chromatography with ultraviolet detection (15,16).

Radioassays, although very sensitive, are not suitable for routine monitoring of these drugs in patients because they require the use of radioactively labelled drugs. The RIA methods are very sensitive and sufficiently selective, but they are expensive and commercial anti-serum is not always available. Antisera have been prepared for each of the three drugs that show little cross-reactivity for the other fentanyl derivatives and known metabolites.

Among the chromatographic techniques commonly used, thin layer chromatography is simple but usually lacks sensitivity and selectivity, whereas gas chromatography, though more specific and reliable, is time-consuming because it usually requires derivatization of the opiates. Therefore, high performance liquid chromatography is the technique usually preferred due to its specificity, sensitivity and reliability.

These analgesics are increasingly used at present to provide relief from pain during anaesthesia in newborn infants although the methods available for pain measurement are limited. An analytical method with the sensitivity necessary to detect, quantitate and separate these drugs at the therapeutic concentrations are, therefore, extremely desirable. The widespread use of these potent drugs (alfentanil, fentanil and sufentanil) has created a need for chromatographic techniques to identify and quantitate low levels of these compounds in biological fluids. Due to the low levels being monitored, the method of detection must be free of endogenous interference or external contamination. This assay has been shown to be useful for therapeutic drug monitoring of these drugs in plasma and urine samples.

MATERIALS AND METHODS

Apparatus

The chromatographic separation was carried out at room temperature with a high-resolution on liquid chromatograph consisting of a Waters HPLC system model 510 HPLC pump, Waters 715 Ultrawisp autosampler, a Waters 994 UV-VIS detector with a variable wavelength. Peak height measurements of the drugs were integrated on a Waters model 820 integrator plotter. The separation column was a Nova pak reverse phase cyano column 8 mm x 100 mm (dimensions), with 4 μm particle size (Waters). PH-M-82 standard Ph meter, IEC centra-8R centrifuge, concentrator-Jouan RC 1010.

Reagents and Solutions

All chemicals used were of analytical grade unless otherwise stated. Sodium dihydrogen orthophosphate, sodium monobasic, orthophosphoric acid, sodium hydroxide, ammonium hydroxide (analytical grade), acetonitrile and N-Hexane (HPLC grade) were obtained from B.D.H. (Pooled, U.K.). Sigmacote (silanising reagent) and fentanil citrate were supplied by Sigma Chemical Company (U.S.A.). Alfentanil-Hydrochloride and sufentanil citrate were purchased from Janssen Pharmaceuticals, Belgium. Only HPLC-grade de-ionized water was used. The 15 ml screw cap culture tubes used were silanized prior to use to avoid drug adsorption on glass and all the glassware was rinsed prior to use with ethanol to remove substances interfering with chromatographic analysis.

The mobile phase consisted of a mixture of acetonitrile, phosphate buffer 5mM (Ph - 3.2) (70:30 V/V). The mobile phase was always filtered through a 0.45 μm filter and degassed under suction. Stock solutions of fentanil (50 μg/ml), alfentanil (500 μg/ml) and sufentanil (50 μg/ml) were prepared by dissolving appropriate amounts of the drugs in water. All stock solutions were stored at room temperature and were protected from prolonged exposure to light. These were used as a stock solution for the preparation of assay standards by serial dilution with plasma.

Plasma Samples and Patient Population

Drug free venous blood samples (1-2 ml) were collected from healthy human subjects receiving no medication. In addition, arterial blood samples (0.2 ml) taken from umbilical artery catheters were obtained from newborn infants receiving continuous intravenous infusion of fentanyl at 3.0 microgram/kg/h. Their birth weights (grams) and postnatal ages (days) were 3.8 $\pm$ 0.40 kg and 2-4

days, respectively. Blood was collected into plastic tubes containing lithium heparin and centrifuged to 10 minutes at 3000 rpm. Plasma was separated and stored at -80°C until the time of analysis.

Chromatographic Conditions

The chromatographic analysis was performed under isocratic conditions and at ambient temperatures, with the detector set at 214 nm with a sensitivity of 0.01 or 0.02 absorbance units (AUFS). The mobile phase was a mixture of acetonitrile, phosphate buffer 5mM, Ph - 3.2 (70:30 V/V). The flow rate was 2.5 ml/min with a pressure of 1200 psi and the run time less than 10 minutes.

Extraction Procedure

To 0.05 ml of standards or samples (plasma, urine) were pipetted into culture tubes containing 0.05 ml 4N NaoH, 0.1 ml acetonitrile and 500 μl of extraction solvent (n-Hexane). The mixture was vortexed for 30 seconds. All samples were centrifuged at 2000 rpm for 5 minutes. The organic phase was transferred into a glass tube and the solvent was evaporated under nitrogen at 30°C for about 10 minutes. The residue was reconstituted in 0.1 ml of the mobile phase and 50 μl injected into a column.

Calibration Curves for the Simultaneous Determination of Alfentanil, Fentanil, and Sufentanil in Standard Solutions

Ten working standards of alfentanil, fentanil and sufentanil were prepared in distilled water. The stock solutions were serially diluted with water by a factor of ten in 25 ml volumetric flasks for the preparation of the working standards. The resulting concentrations of the solutions were 3.0 - 500 ng/ml of the alfentanil free base, 2.5 - 150 ng/ml of the fentanil free base, and 5.0 - 200 ng/ml of the sufentanil free base. Five replicate injections of each of these standard mixtures were made. The peak height of alfentanil, fentanil and sufentanil were plotted separately as a function of the corresponding concentration of each compound.

Determination of Alfentanil, Fentanil and Sufentanil in Human Plasma and Urine

A set of calibration curves were constructed for the blood plasma and urine samples using the standard additions method. The samples were spiked with mixtures of alfentanil, fentanil and sufentanil at the different concentration levels (0,2.5,5.0,10.0,25,50,100,200 ng/ml). The extracted samples were used for HPLC analysis. Five replicate injections were made for each concentration level. The peak height of alfentanil, fentanil and sufentanil were plotted against the corresponding concentration of the drugs. The regression analysis shows good linear relationships between peak height and quantity of drug.

RESULTS AND DISCUSSION

The present paper describes an HPLC method for the simultaneous determination of alfentanil, fentanil and sufentanil in 50 μl blood plasma and urine samples. This method offers rapidity, very good separation of the drugs, satisfactory sensitivity with ultraviolet detection and good precision and accuracy. Using the described conditions, the analysis was completed within approximately 10 minutes with complete separation of these three drugs as shown in Fig. 1. The resolution factor between adjacent peaks was calculated and found to be 1.56 between alfentanil and fentanil and 1.04 between fentanil and sufentanil.

In the method described here, the Nova pak cyano columns were found to be suitable. Nova pak packing material has low surface activity (acidity) and is endcapped to minimize silanol interactions and

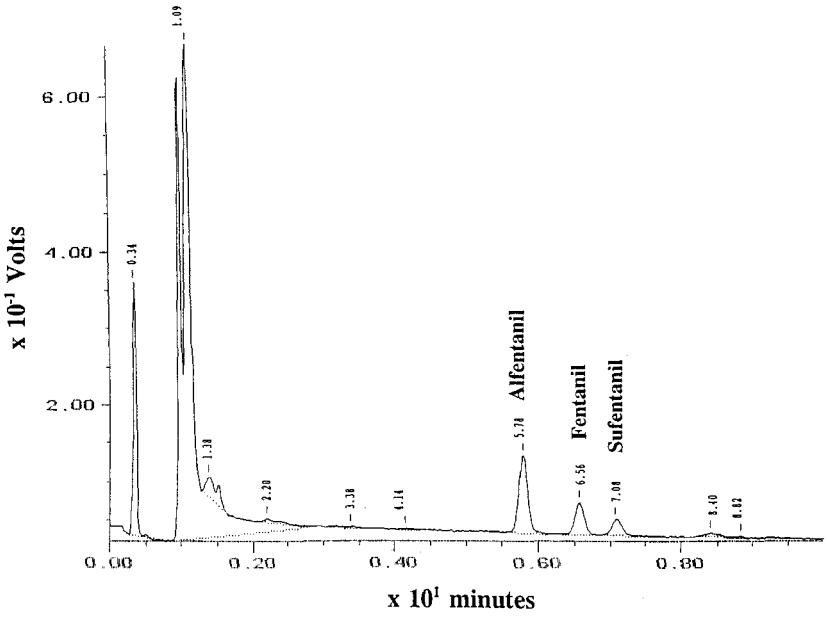

Figure 1

Chromatogram of a mixture of alfentanil, fentanil and sufentanil standard (10 ng/ml, 5 ng/ml and 5 ng/ml, respectively).

provide improved peak shape for basic molecules thereby reducing the need for mobile phase additives such as amine modifiers.

At the retention times of alfentanil, fentanil and sufentanil, no interferences from endogenous compounds were found in the chromatograms of extracted blood plasma and urine samples shown in Fig. 2 and 3. Since these opioids are usually used simultaneously with other drugs, the specificity for this assay has been tested in the presence of those medications that are commonly administered to patients. None of these medications - furosemide, morphine, calcium chloride, dobutamine, midazolam, ampicillin sodium, gentamicin, cefotaxime, diazepam, phenytoin, pavulon, and vitamin K - interfered with our method when they were added to blank plasma or urine samples.

Standard curves were linear and passed through the origin between the concentrations 3.0 - 500 ng/ml for alfentanil, 2.5 - 150 ng/ml for fentanil, and 5.0 - 200 ng/ml for sufentanil, respectively. The respective correlation coefficients were 0.996, 0.994 and 0.997 for these drugs, which indicate high precision. The lower limit of quantitation with a 50 μl sample size (injection) were 0.15 ng for alfentanil, 0.12 ng for fentanil, and 0.25 ng for sufentanil. The lower determination limits were 3.0 ng/ml, 2.5 ng/ml and 5.0 ng/ml for alfentanil, fentanil, and sufentanil, respectively. The retention times were 5.78 $\pm$ 0.29 minutes for alfentanil, 6.5 $\pm$ 0.32 minutes for fentanil, and 7.08 $\pm$ 0.15 minutes for sufentanil. The recovery determined at various concentrations for plasma and urine samples was in the range of 85.0 $\pm$ 4.08, 83.3 $\pm$ 3.84 and 78.2 $\pm$ 2.89%, for alfentanil, fentanil, and sufentanil, respectively.

Replicate analyses were conducted on four different concentrations (10,25,50, and 100 ng/ml) for each drug. Each concentration was replicated eight times under the same conditions in order to check the reproducibility and precision of the method. Inter- and intra-assay precision was determined by analysis of spiked samples on five consecutive days. Accuracy and reproducibility was calculated as the percentage difference between amount of drug added to drug-free plasma and amount of drug measured (Table 1). Coefficients of variation of the method were $< 10\%$. The experimentally determined concentrations agreed with the actual concentrations.

The proposed method is suitable for the simultaneous determination of alfentanil, fentanil and sufentanil in very small volumes of plasma and urine samples (50μl). The previously reported methods for the simultaneous determination of these drugs were based on 1 ml plasma samples and required longer

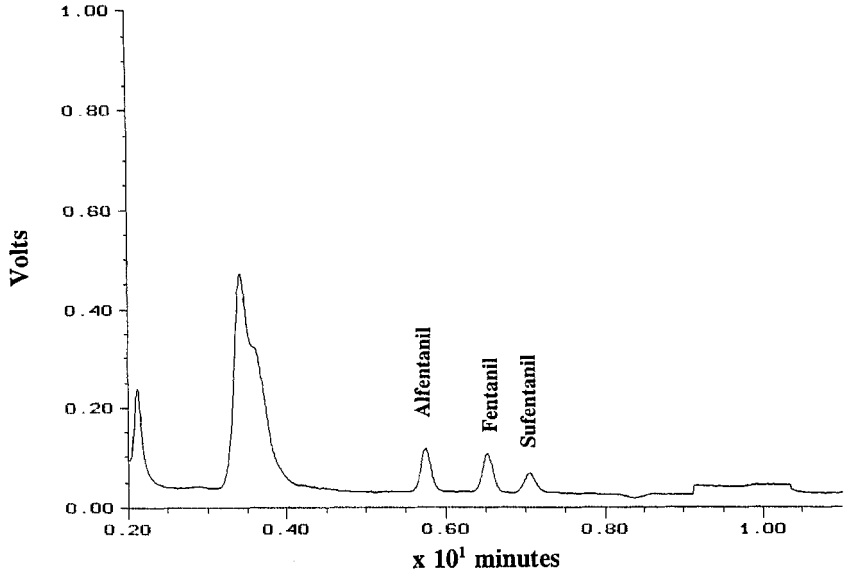

Figure 2

Chromatogram of extracted alfentanil, fentanil and sufentanil from blood plasma samples (5 ng/ml).

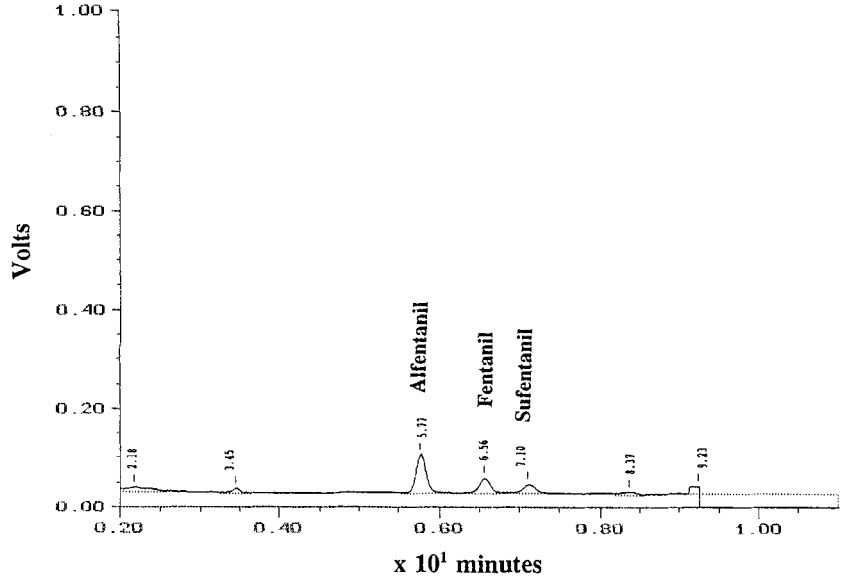

Figure 3

Chromatogram of extracted alfentanil, fentanil and sufentanil from urine sample (5 ng/ml, 2.5 ng/ml and 5.0 ng/ml, respectively).

TABLE 1

Reproducibility, Precision and Accuracy of the Method

Drug	Drug Concentration Injected (ng/ml)	Drug Concentration Found (ng/ml) *	Confidence Interval 95%	Coefficient of Variation (CV) %
Alfentanil	10.0	8.90 ± 0.39	8.70 - 9.10	4.50
	25.0	25.4 ± 0.55	25.16 - 25.65	2.17
	50.0	49.9 ± 1.30	49.91 - 50.05	2.60
	100.0	99.8 ± 1.50	99.68 - 99.83	1.50
Fentanil	10.0	9.75 ± 0.65	9.73 - 9.77	6.62
	25.0	25.9 ± 0.78	25.85 - 25.94	2.99
	50.0	48.4 ± 2.41	48.3 - 48.5	4.98
	100.0	100.3 ± 0.96	100.3 - 100.4	0.95
Sufentanil	10.0	9.41 ± 0.66	9.71 - 9.77	6.16
	25.0	25.5 ± 0.55	25.30 - 25.80	2.14
	50.0	50.5 ± 0.15	50.26 - 50.33	1.30
	100.0	100.2 ± 0.80	100.16 - 100.24	0.80

* Values are expressed as mean ± SD of 8 determinations. Volume injected was 50

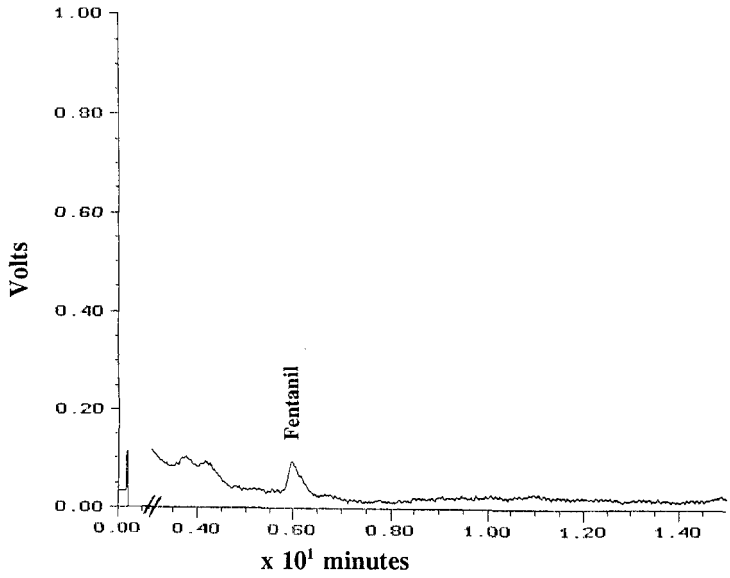

Figure 4

Chromatogram of fentanil from a newborn baby receiving a constant intravenous infusion of fentanil at 3.0 microgram/kg/hr. Fentanil concentration was 3.7 ng/ml.

extraction procedures (15,16). Kumar et al (16) have reported extraction of fentanil and alfentanil at Ph-3. However, as reported by Janicki et al (17) and supported by our own experiments, these drugs are hydrolyzed in acidic solutions by cleavage of propionic acid, thus reducing the sensitivity of the method.

We have used the proposed technique for the therapeutic drug monitoring of fentanyl in small, sick newborn babies receiving constant I.V. infusion of fentanyl 3.0 microgram/kg/h (Fig. 4). Their plasma fentanyl concentrations at these doses were 3.2 $\pm$ 0.22 ng/ml corresponding to an adequate pain control in these babies. We suggest that this microassay designed for the simultaneous determination of fentanyl, alfentanil and sufentanil be used in newborn and children or whenever there is limited sample volume.

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