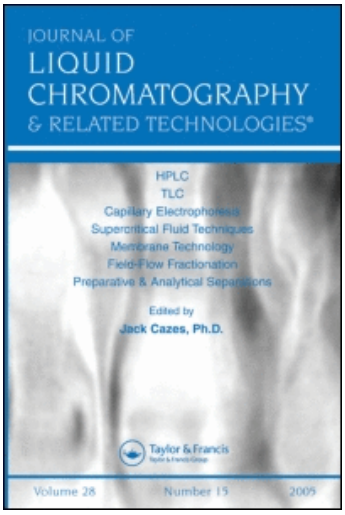

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High Performance Liquid Chromatography Microassay for the Simultaneous Determination of Fentanyl and Its Major Metabolites in Biological Samples

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MICROASSAY FOR THE SIMULTANEOUS DETERMINATION OF FENTANYL AND ITS MAJOR METABOLITES IN BIOLOGICAL SAMPLES

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ABSTRACT

A simple high performance liquid chromatographic (HPLC) procedure for the simultaneous determination of fentanyl and its wo major metabolites in biological samples was used in blood samples from 6 preterm sick newborn infants. A 8 mm x 100 mm, 4 μm cyano column, 0.05 m phosphate buffer adjusted at pH 3.2 and acetonitrile (50:50 V/V) as mobile phase, were used at a flow rate 2.5 ml/min. Each run was completed within 10 minutes. The detection limits for the analysis were 0.10 ng, 0.15 ng and 0.10 ng for norfentanyl, fentanyl and despropiofentanyl, respectively, with 50 μl injection for all the compounds at a signal-to-noise ratio of 3. The respective retention times were 4.62±0.10, 6.20±0.76 and 6.71±0.73 minutes for norfentanyl,

fentanyl and despropiofentanyl. Microassay is simple, rapid and precise. This assay may be used for the therapeutic drug monitoring in newborn babies.

INTRODUCTION

Fentanyl, N-phenyl-N-[1-(2-phenylethyl)-4-piperidinyl] propionamide, a potent and fast-acting narcotic analgesic, is widely used in neonatal anesthesia and intensive care. It is now the primary analgesic for both pediatric and adult cardiac surgery because of its wide margin of safety.[1,2,3,4,5] Due to its widespread use, studies requiring measurement of plasma fentanyl and its metabolites in biological fluids are increasing[6,7] and included such measurement as radioimmunoassay (RIA),[8-11] radioassays,[12,13] gas liquid chromatography (GLC),[14-18] thin layer chromatography (TLC),[19] infrared spectroscopy (IR),[20] nuclear magnetic resonance (NMR)[21] and high performance liquid chromatography (HPLC) with ultraviolet detection.[22,23]

In humans, fentanyl is metabolized primarily by N-dealkylation to norfentanyl (1-(2-phenethyl)-4-N-anilinopiperidine) (metabolite I) and by hydroxylation to despropiofentanyl (4-N-(N-propionyl-anilino)-piperidine) (metabolite II) respectively[25,26] as has been shown in rats.[24,25,26] Van Rooy et al.[16] were the first investigators to have worked on fentanyl and its two major metabolites, norfentanyl and despropiofentanyl, using GC-NPD in plasma of patients. Goromaru et al.[27] identified and quantitated fentanyl and its two major metabolites in human patients by GC-MS.

McClain and Hug[28] used paper chromatography to detect fentanyl and two metabolites in urine samples. Kintz et al.[17] reported simultaneous determination of fentanyl and its major metabolites using gas chromatography (GC) with nitrogen-selective detection.

Because of the high potency and low dosage of these compounds and small sample sizes available, extremely low (subnanogram) concentrations of drugs and their metabolites are present in biological specimens. There is a need to develop more and more sensitive, highly specific, analytical methods for drug monitoring, and its metabolites, in biological samples at therapeutic concentrations.

The aim of the present communication is to describe a suitable, highly specific and sensitive analytical method for determination of fentanyl and its

metabolites in body fluids for use in both clinical and research laboratories.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals used were of analytical grade. Sodium dihydrogen orthophosphate, sodium phosphate monobasic, orthophosphoric acid, sodium hydroxide, ammonium hydroxide, buffer solution pH 7.0, buffer pH 4.0, n-Hexane, acetonitrile (HPLC grade) were obtained from B.D.H. (Pooled, U.K.). Fentanyl was purchased from Sigma Chemical Company (U.S.A.). Norfentanyl (R U156) batch no. A0801 and despropiofentanyl (R 34853) batch no. V90799 were obtained from Janssen Pharmaceutical Company. Only HPLC-grade de-ionized water was used.

Equipment

HPLC instrumentation included Water 510 HPLC pump, 715 Ultrawisp autosampler, variable wavelength 994 UV/VIS detector and 820 inegrator plotter. A Waters 8 mm x 100 mm, 4 μm cyano column (Millipore Corporation, Milford, MA) was used. Additional equipment included a pH-M-82 standard pH meter, IEC Centra-8R centrifuge, and concentrator-Jouan RC - 1010.

Plasma and Urine Samples

Drug-free venous blood and urine were collected from six healthy adult human volunteers receiving no medications. In addition, arterial blood samples (0.2 ml) taken from umbilical artery catheters were obtained from six newborn infants receiving continuous intravenous infusion of fentanyl at 3.0 micrograms/kg/hour. Blood was collected into plastic tubes containing lithium heparin and centrifuged for 10 minutes at 3000 rpm. Plasma was separated and stored at -80°C until the time of analysis. Random urine samples were also collected in plastic bags and later stored at -80°C for the analysis of fentanyl and its metabolites.

Mobile Phase

The mobile phase consisted of a mixture of 50% acetonitrile and 50% phosphate buffer 0.05 M (pH 3.2) filtered through a 0.22 mm filter (Millipore) and degassed under suction.

Standard Solutions

Stock solutions of norfentanyl, fentanyl, and despropiofentanyl (50 mg/ml), respectively, were prepared by dissolving appropriate amounts of drug salts and metabolites. All stock solutions were prepared and stored at room temperature and protected from prolonged exposure to light. These were used as a stock solutions for the preparation of assay standards by serial dilution.

Chromatographic Conditions

The analysis of norfentanyl, fentanyl and despropiofentanyl was performed at room temperature (25°C) with the wavelength at 210 nm. The mobile phase was a mixture of acetonitrile and phosphate buffer (50:50 V/V) with a flow rate of 2.5 ml/min and a pressure of 1000 psi. The run time was less than 10 minutes.

Sample Preparations and Extraction Procedures

Extraction was performed in a silanized tube. Spiked plasma standards or sample plasma containing norfentanyl, fentanyl or despropiofentanyl (100 μl) in a silanized tube were added 50 μl, 5N NaOH, 100 μl acetonitrile and 600 μl of n-hexane. All samples were centrifuged at 2000 rpm for 5 minutes. The organic phase was transferred to a glass tube and the solvent was evaporated under nitrogen at 30°C for about 10 minutes. The residue was reconstituted in 100 μl of the acetonitrile and phosphate buffer (50:50 V/V) at pH 3.0 and 50 μl injected into a column.

Calculations

Standard curves and concentrations of norfentanyl, fentanyl and despropiofentanyl were calculated from peak heights. Calibration curves were constructed after the addition of known concentrations of norfentanyl, fentanyl

and despropiofentanyl to plasma and urine samples by linear regression analysis of peak height versus concentrations.

Recovery, Precision, Accuracy and Reproducibility

The precision and accuracy of the overall analytical procedure for the determination of norfentanyl, fentanyl and despropiofentanyl in plasma were assessed by processing spiked plasma samples (Table 1). The day-to-day precision and accuracy of the method were assessed by the repeated analyses of standard solutions of norfentanyl, fentanyl and despropiofentanyl over 5 days. Daily representative peak height for the three concentration levels tested was the mean value of five replicate injections. The inter- and intra-assay coefficient of variations of the method was < 10%.

Extraction recovery of the parent drug and its metabolites was estimated by comparison of peak height obtained from an extracted sample, containing a known amount of the compound. The peak height was obtained from direct

Table 1

Reproducibility, Precision and Accuracy of the Method

Drug	Amount Injected (ng/ml)	Drug Conc'n Measured (ng/mL)*	Confidence Interval 95%	Std Mean of Error**	Coeff of Variation (CV)%
Norfentanyl	10.0	8.66 ± 0.59	8.69 - 8.60	0.26	6.81
	25.0	25.44 ± 1.27	25.48 - 25.30	0.29	5.00
	50.0	49.40 ± 2.90	49.60 - 49.20	1.02	5.86
	100.0	100.40 ± 2.20	100.50 - 100.10	0.84	2.22
Fentanyl	10.0	9.70 ± 0.65	9.73 - 9.77	0.33	6.62
	25.0	25.90 ± 0.78	25.85 - 25.94	0.30	2.99
	50.0	48.40 ± 2.41	48.30 - 48.50	1.00	4.98
	100.0	100.30 ± 0.96	100.30 - 100.40	0.40	0.95
Despropio-	10.0	9.91 ± 0.86	9.97 - 0.85	0.29	8.68
fentanyl	25.0	25.00 ± 0.925	25.10 - 25.68	0.41	3.69
	50.0	48.50 ± 2.0	48.67 - 48.40	1.35	6.24
	100.0	99.80 ± 1.50	99.68 - 99.83	0.61	1.50

*Values are expressed as mean ± SD (n = 6 determinations). Volume injected was 50μL.

**Standard Mean of Error refers to the mean difference between the concentration injected and the concentration measured.

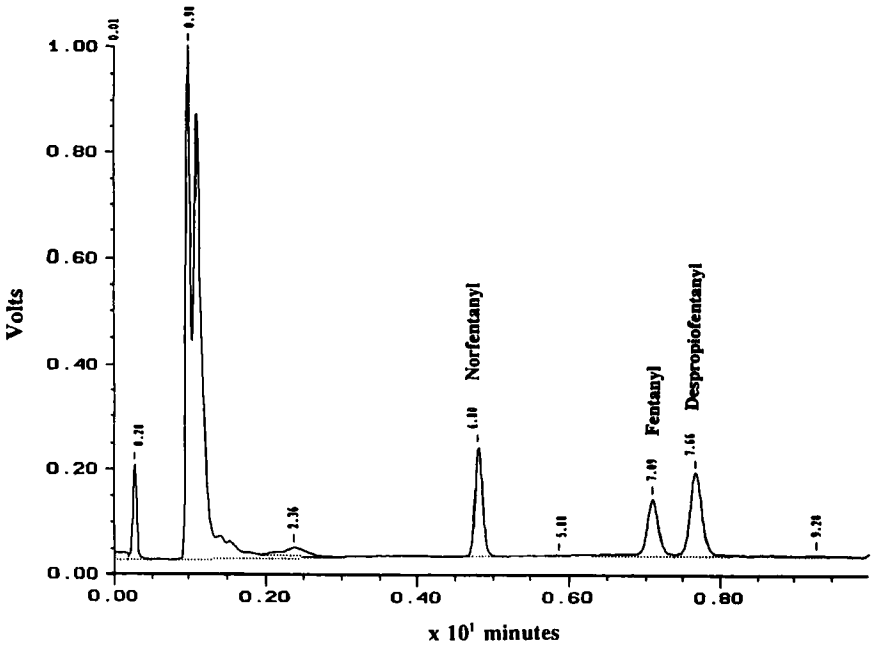

Figure 1. Chromatogram of Norfentanyl, Fentanyl and Despropiofentanyl from unextracted samples .

injection of a standard solution with different concentrations of the sample.

Statistical Analysis

Standard curves and concentrations of norfentanyl, fentanyl and despropiofentanyl were calculated from peak heights. Calibration curves, constructed after the addition of known concentrations of 1.0 to 200 ng/ml of norfentanyl, fentanyl and despropiofentanyl, were linear as analyzed by regression analysis of peak height versus concentrations. The correlation coefficients were 0.998, 0.994 and 0.997, for norfentanyl, fentanyl and despropiofentanyl, respectively.

RESULTS AND DISCUSSION

Using the chromatographic conditions for this method, the mixture of

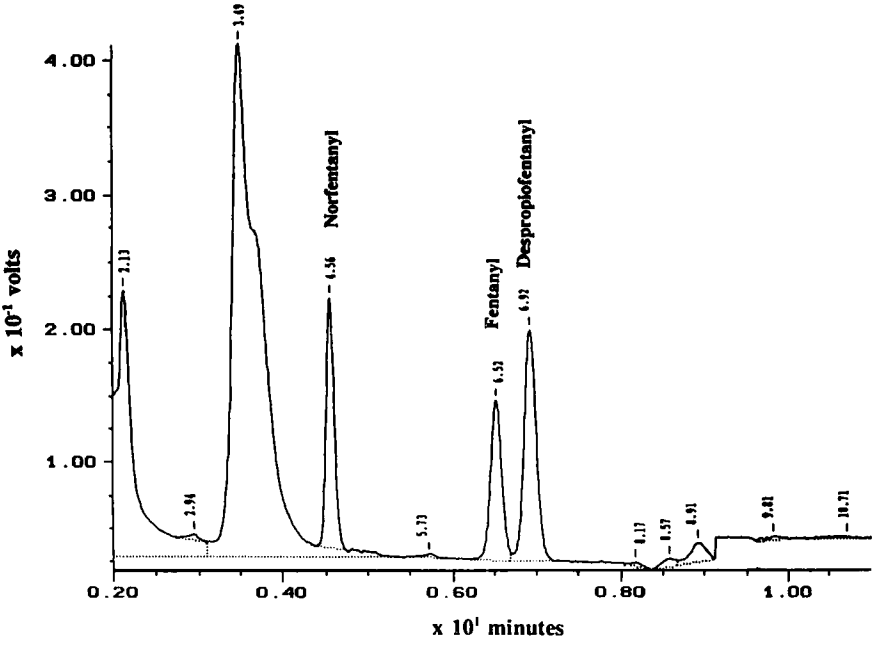

Figure 2. Chromatogram of blood sample spiked with Norfentanyl, Fentanyl and Despropiofentanyl from extracted samples

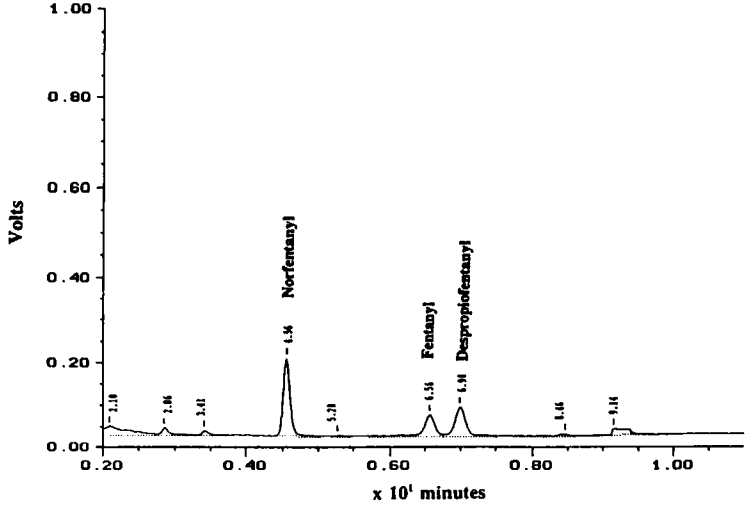

Figure 3. Chromatogram of urine sample spiked with Norfentanyl, Fentanyl and Despropiofentanyl from extracted samples

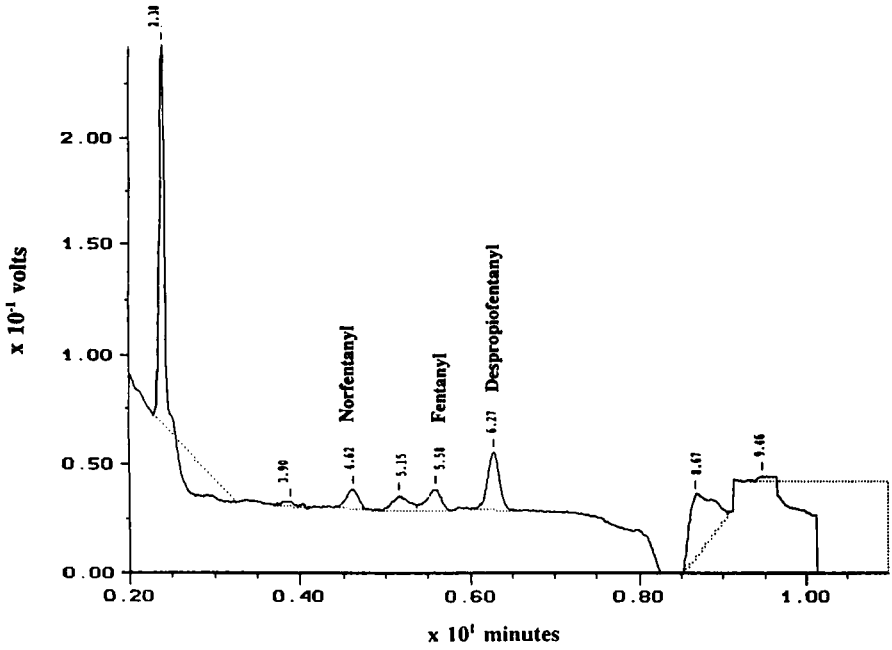

Figure 4. Chromatogram of Norfentanyl, Fentanyl and Despropiofentanyl from a newborn baby's blood sample, receiving a constant I.V. infusion of Fentanyl 3.0 microgram/kg/hour.

fentanyl and its major metabolites was separated in acetonitrile-phosphate buffer. The use of acetonitrile-phosphate buffer, pH 3.0, led to elution of all compounds. The application of acidic pH suppressed the ionization of acidic silanol groups, allowing the elution of drugs.

The chromatographic peaks of I, II, and III (norfentanyl, fentanyl and despropiofentanyl) were sharp and their retention times were 4.62±0.10, 6.20±0.76, and 6.71±0.73 minutes, respectively. The absolute peak heights of norfentanyl, fentanyl and despropiofentanyl were plotted against the concentration between 1.0 and 200 ng/ml of I (norfentanyl); 2.5 and 200 ng/ml of II (fentanyl); and 2.0 and 200 ng/ml of III (despropiofentanyl) respectively. The relationship was linear and passed through the origin. In the concentration range studied, the regression line was linear (r=0.998) with an intercept on the y-axis close to the origin. Results of the analysis of spiked serum for the determination of precision and accuracy of the method are given in Table 1.

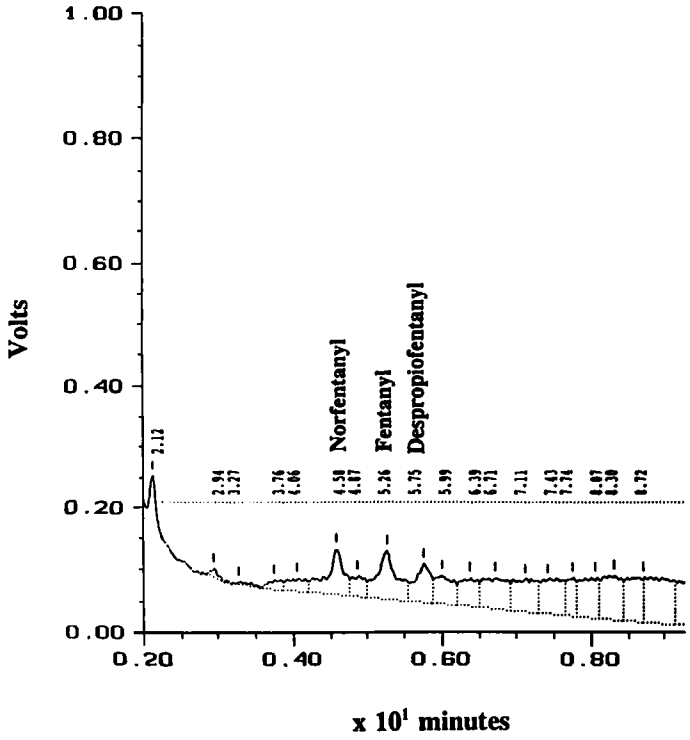

Figure 5. Chromatogram of Norfentanyl, Fentanyl and Despropiofentanyl from a newborn baby's urine sample, receiving a constant I.V. infusion of Fentanyl 3.0 microgram/kg/hour.

Using the described conditions, the analysis was completed within 10 minutes with complete separation of these three components. Figure 1 shows the chromatogram from the standard solutions. Figures 2 and 3 show the chromatograms from extracted blood and urine samples spiked with fentanyl and metabolites. Figures 4 and 5 show the chromatograms from the extracted blood and urine samples of a newborn baby. The resolution factor, between adjacent peaks, was calculated and found to be 4.28 between norfentanyl and fentanyl and 1.08 between fentanyl and despropiofentanyl. The extraction recoveries of all the compounds determined at all concentrations were 82.0±4.0, 83.3±3.84, and 79.5±3.20 % for norfentanyl, fentanyl and despropiofentanyl respectively for plasma and urine samples. There was no change in the peak height of the drug fentanyl and its two metabolites after

extraction by the described procedure. The inter- and intra-assay coefficent of variations of the method was < 10%. The precision of the method was evaluated in a blind study in the concentration range 10-100 ng/ml for fentanyl, norfentanyl and despropiofentanyl, respectively.

The specificity for this assay was tested by using those medications usually given to newborn infants that might be present in patient samples. Furosemide, morphine, calcium chloride, dobutamine, dopamine, midazolam, ampicillin, gentmicin, clafoxan, diazepam, phenytoin, pavulon, gentamicin, and vitamin K did not interfere with the measurement of norfentanyl, fentanyl and despropiofentanyl.

The method described is suitable for the simultaneous determination of norfentanyl, fentanyl and despropiofentanyl in a very small volume of plasma samples (50 ml). We have used the proposed technique for the therapeutic drug monitoring of fentanyl in small sick newborn babies receiving constant I.V. infusion of fetanyl 3.0 microgram/kg/h. Their plasma norfentanyl, fentanyl and despropiofentanyl concentrations at these doses were 2.8±1.2, 3.04±0.44, and 3.1±1.3 ng/ml, respectively. The proposed method is highly sensitive, precise and selective for fentanyl, norfentanyl and despropiofentanyl and is also rapid and simple to perform.

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