



2016

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### Recommended Citation

Bailey, Joan; White, Molly; Gordon, Kristen; Seddighi, Reza; and Cox, Sherry, "Determination of Fentanyl in Canine Plasma using HPLC-MS Detection" (2016). *Faculty Publications and Other Works -- Biomedical and Diagnostic Sciences*.  
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# Determination of Fentanyl in Canine Plasma using HPLC-MS Detection

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## ABSTRACT

A simple, easy, and accurate high-performance liquid chromatographic method for the determination of fentanyl concentrations in plasma samples has been developed and validated. Following a liquid extraction with acetonitrile, samples were separated by reversed-phase high-performance liquid chromatography (HPLC) on an XBridge C<sub>18</sub> column (2.1 x 50 mm, 3.5 μm) and detected by mass spectroscopy. The mobile phase was a mixture of water with 0.1% formic acid, and acetonitrile with 0.1% formic acid (90:10), with a flow rate of 0.6 ml/min. The procedure produced a linear curve over the concentration range of 0.1-25 ng/ml for fentanyl in canine plasma with a LOQ of 0.1 ng/ml. Intra- and inter-assay variability ranged from 2.6%-8.2% and the average recovery for fentanyl was 100%.



## INTRODUCTION

Fentanyl, *N*-(1-phenethyl-4-piperidyl) propionanilide, is a synthetic opioid which has a high affinity for μ-opioid receptors. Fentanyl injection and transdermal patches are used primarily in dogs and cats and have been shown to be useful for the adjunctive control of postoperative pain and in the control of severe pain associated with chronic pain, dull pain, and non-specific, widespread pain associated with cancer, pancreatitis, aortic thromboemboli, and peritonitis. Perioperative injectable fentanyl may also reduce the requirements for inhalational anesthetics during surgery, which could be particularly advantageous in patients with compromised cardiac function. Fentanyl levels have been determined using mass spec and ultraviolet detection in plasma, blood, urine, and saliva. Many extraction methods have been reported including solid phase extraction, and liquid-liquid extractions. This paper describes a simple, sensitive, and accurate method for extracting fentanyl from plasma samples using HPLC.

## MATERIALS and METHODS

### Reagents and Standards

Fentanyl was purchased from USP. Flurazepam, the internal standard, was purchased from Cerilliant. All reagent grade chemicals and solvents were purchased from Fisher Scientific. Water (18.2 megaohm) was obtained from a Barnstead Micropure Infinity ultrapure water system. Stock solutions of fentanyl and flurazepam (100 μg/ml) were prepared in methanol. Dilutions were prepared to produce working stock standards of 0.01, 0.1, and 1 μg/ml. The standards were stored at 4°C and were stable for a minimum of 6 months. Standard curves for plasma were prepared by spiking untreated plasma with fentanyl which produced a linear concentration range of 0.1 to 25 ng/ml. Spiked standards were treated exactly as plasma samples.

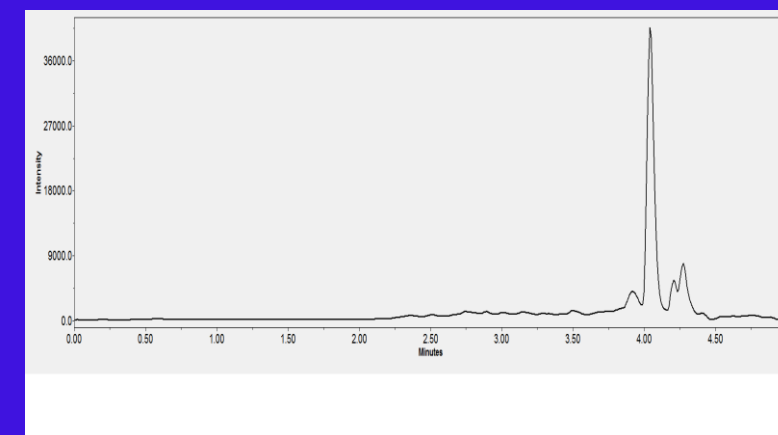
For stability testing, canine plasma (400 μL) was spiked with fentanyl at concentrations 0.3, 0.75, and 17.5 ng/ml. A 100 μL sample was pulled from this spiked plasma and analyzed immediately. The remaining spiked plasma was then frozen in an -80°C freezer, thawed, and another 100 μL sample was analyzed. This process was repeated for a total of three freeze-thaw cycles. The results were then compared to the initial analysis.

### Chromatographic Conditions

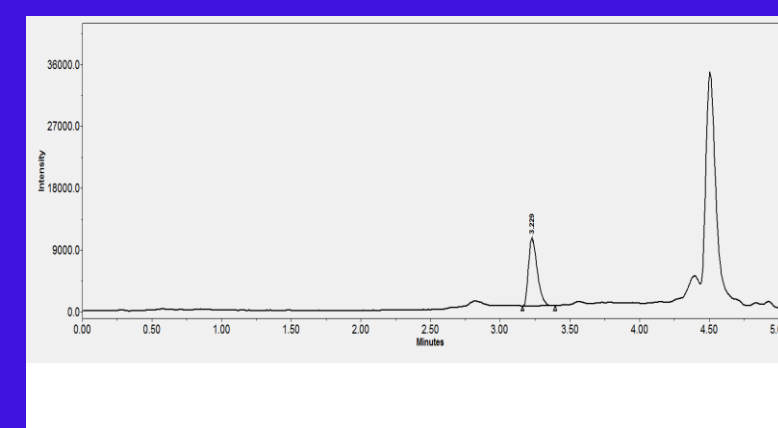
The analysis of fentanyl in plasma was conducted using reverse phase HPLC. The chromatography system consisted of a 2695 separation module and an Acuity QDa single-quadrupole mass detector (Waters). Separation was achieved on a Waters XBridge C<sub>18</sub> column (2.1 x 50 mm, 3.5 μm) preceded by a XBridge C<sub>18</sub> guard column.

**Figure 1**  
Chromatograms for Fentanyl in K9 plasma

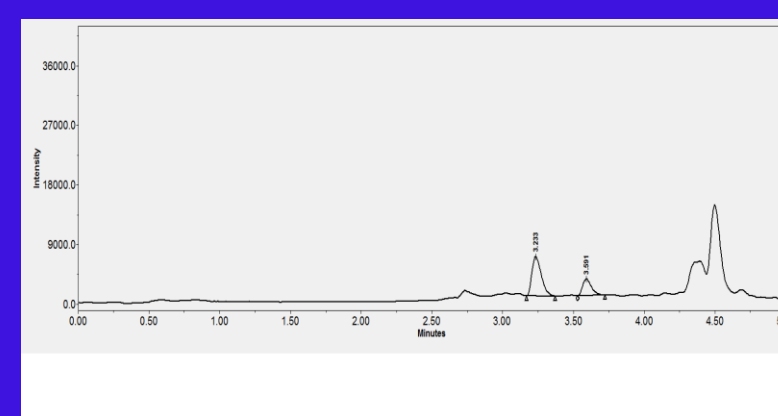
**A: Blank K9 plasma sample**



**B: Spiked 2.5 ng/ml K9 plasma standard**

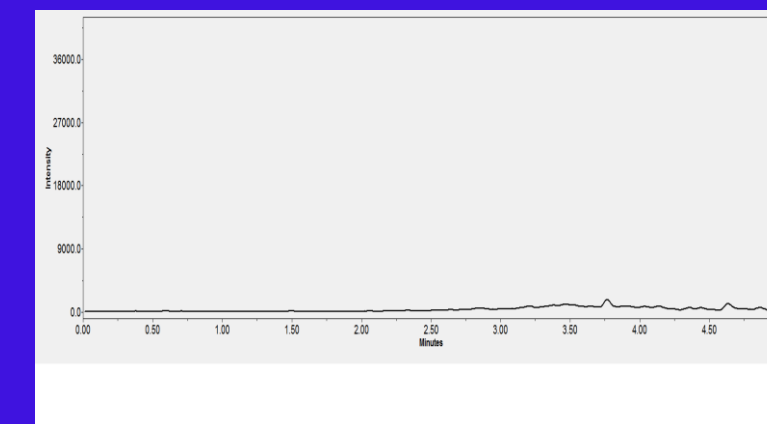


**C: Canine plasma sample Sherman 90 min after fentanyl administration**

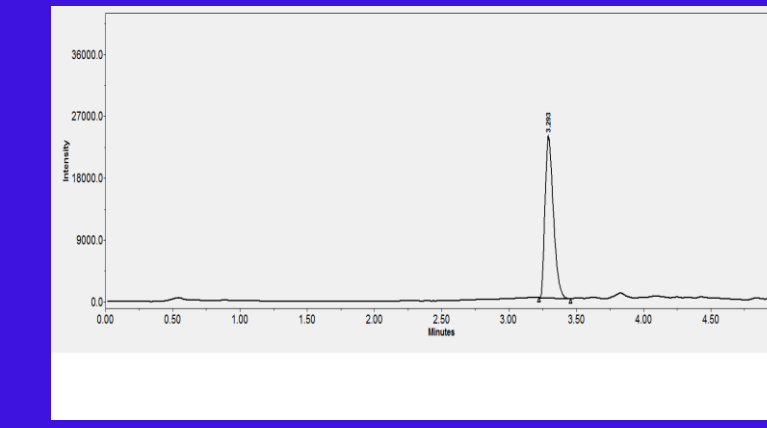


**Figure 2**  
Chromatograms for Flurazepam (I.S.) in K9 plasma

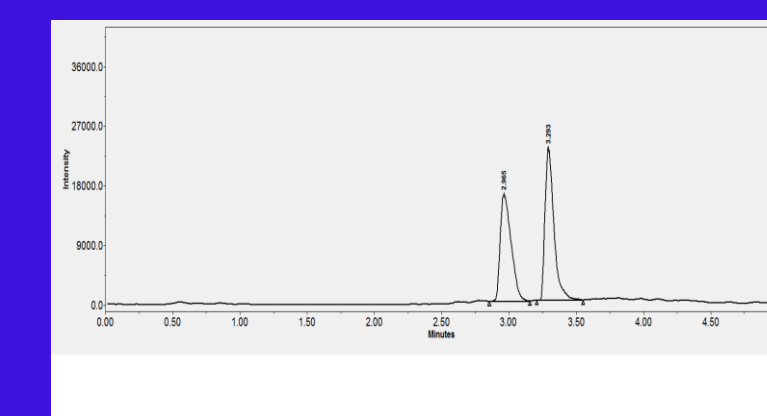
**A: Blank K9 plasma sample**



**B: Spiked 2.5 ng/ml K9 plasma standard**



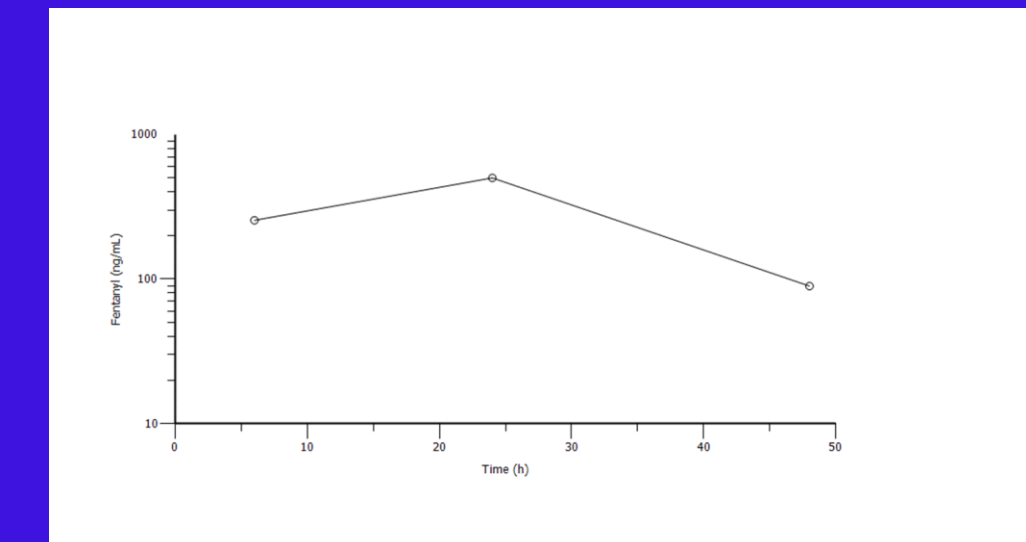
**C: Canine plasma sample Sherman 90 min after fentanyl administration**



**Table 3: Fentanyl freeze-thaw stability**

Conc.	1 <sup>st</sup> Thaw	2 <sup>nd</sup> Thaw	3 <sup>rd</sup> Thaw
0.3	91	91	127
0.75	86	94	97
17.5	97	102	99

**Figure 3: Pharmacokinetic profile of fentanyl in canine plasma**



## Chromatographic Conditions

The mobile phase was a mixture of (A): water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The mixture was pumped at a starting gradient of 90% A and 10% B and was adjusted to 10% A and 90% B over 4 min, and back to initial conditions over 4 min. The flow rate was 0.60 ml/min, and the column temperature was ambient which was 30°C. The compounds were detected by positive selected ion recording (SIR). The scan rate was 2 pts/s, gain 1, capillary voltage 0.8 kV, cone voltage 15, ion source temperature 150°C and probe temperature 600°C. Nitrogen was used as the nebulizing gas. Fentanyl was detected at 337.34 *m/z* and flurazepam was detected at 388.24 *m/z*.

## Sample Treatment

Fentanyl was extracted from plasma samples using a liquid extraction with acetonitrile. Previously frozen plasma samples were thawed and vortexed and 100 μl was transferred to a 7 ml glass screw top tube then 25 μl of internal standard (0.1 μg/ml flurazepam) added. One milliliter of acetonitrile was added and tubes were capped and vortexed for 60 s then centrifuged for 20 min at 1020 x g. The supernatant was removed and placed in a clean glass tube and evaporated to dryness with nitrogen gas. Samples were reconstituted in 200 μl of mobile phase and 50 μl injected into the HPLC system.

## RESULTS and CONCLUSIONS

Testing of blank and spiked canine plasma revealed no interferences for fentanyl or flurazepam. Figs. 1 & 2 show chromatograms of (A) blank plasma sample, (B) a 2.5 ng/ml spiked dog plasma standard and (C) a dog plasma sample after fentanyl administration. Retention times were 3.22 min for fentanyl and 3.28 min for flurazepam. The plasma peak ratio (area of fentanyl divided by the internal standard area) produced a linear curve for the concentration range 0.1-25 ng/mL with a correlation coefficient of >0.99 (Table 1), and having a LOD of 0.05 ng/mL and LOQ of 0.1 ng/ml. Intra- and inter-day assay relative standard deviation (RSD) for plasma spiked with fentanyl were used to determine accuracy and precision which ranged from 2.6% to 8.2% (Table 2). The precision was found to be below ± 15% for all quality control samples. The average recovery for fentanyl was 100%. Stability of fentanyl under freeze-thaw conditions was tested and resulted in an average 2% loss after three cycles (Table 3).

In conclusion, this HPLC method quantifies fentanyl from plasma by combining a liquid extraction procedure with mass spectroscopy. This analytical procedure was validated in terms of recovery, linearity, LOQ, precision, and accuracy. This method utilizes a small sample size of 100 μL making it potentially useful for small dogs, cats or other small animals. Fig 3 is a representative concentration-time profile from a fentanyl pharmacokinetic study.

**Table 1. Fentanyl assay linearity**

Assay linearity (n=5)		
	Mean ± STD	RSD (%)
Slope	0.1749 ± 0.0062	3.5
Y-intercept	0.0047 ± 0.0003	6.19
R <sup>2</sup>	0.9996 ± 0.0002	0.02

**Table 2. Intra-assay and Inter-assay accuracy and precision for fentanyl in plasma.**

Intra assay variability (n=5)		
Concentration (ng/ml)	Concentration measured (ng/ml) (mean ± SD)	RSD (%)
0.3	0.3 ± 0.02	6.6
0.75	0.73 ± 0.05	7
17.5	17.8 ± 0.58	3
Inter assay variability (n=5)		
Concentration (ng/ml)	Concentration measured (ng/ml) (mean ± SD)	RSD (%)
0.3	0.28 ± 0.02	7.5
0.75	0.77 ± 0.06	8.2
17.5	17.0 ± 0.44	2.6

SD, standard deviation; n, number of days or samples

