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Article

Determination of Thiafentanil in Plasma Using LC–MS

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Abstract

A new method of analysis has been developed and validated for the determination of thiafentanil in plasma. After protein precipitation, samples were separated on an XBridge BEH C_{18} column and quantified using mass spectrometry. The mobile phase was a mixture of water with 0.1% formic acid and acetonitrile with 0.1% formic acid (90:10). The standard curve ranged from 0.1 to 25 ng/mL. Intra- and Inter-assay variability for thiafentanil was less than 10%, and the average recovery was greater than 95%. The lower limit of quantification was 0.1 ng/mL. This is the first validated method for thiafentanil analysis in plasma.

Introduction

Opioid agonists are valuable for immobilization of many nondomestic species. Opioids such as etorphine and carfentanil are often preferred for their potency, fast action and ability to have their effects reversed. However, this class of drug is associated with adverse side effects such as excitement, muscle rigidity, regurgitation, bradypnea, abnormal blood pressure, hyperthermia and lactic acidosis (1).

Thiafentanil is a potent opioid that is a synthetic fentanyl derivative, structurally similar to sufentanil. It has a morphine-like analgesic mode of action and produces rapid immobilization following intramuscular injection. It is used for immobilization of captive minor species and free-ranging hoof stock and represents the next generation of opioid immobilizing agents. The restraint and immobilization of nondomestic ungulates has been extremely problematic and there continues to be a need for agents that will immobilize these animals quickly and safely (2). When compared to other opioids, thiafentanil has a much shorter induction time, by as much as 50%, while retaining agonist activity (3). In addition, recovery times after antagonization are also shorter (4). Because it has a shorter half-life, there is less incidence of renarcotization, which is the reoccurrence of opioid

effects after apparent antagonism. Renarcotization is dangerous for free-ranging wildlife because prolonged struggling during recovery could cause several life-threatening problems, such as hyperthermia and trauma (2). The shorter half-life also means that targeted animals may be handled and secured quicker than with other opioids, preventing problems with trauma, overheating and escapes by free-ranging wildlife.

A literature search revealed no published methods for the determination of thiafentanil in plasma. Therefore, the aim of this paper was to develop a simple, sensitive, specific and reliable method for determining thiafentanil concentrations using protein precipitation and mass-spectrometry detection.

Experimental

Instrumentation and reagents

The chromatography system consisted of an Acquity Arc system and an Acquity QDa single-quadrupole mass detector (Waters, Milford, MA). Separation occurred on an XBridge BEH C_{18} column (4.6 × 50 mm, 3.5 μm) preceded by a 3.5 μm BEH C_{18} guard column
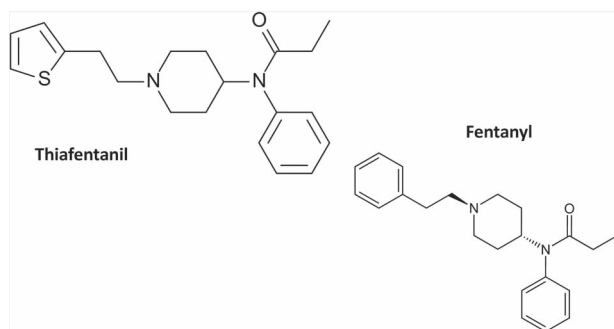
Figure 1. Structures of thiafentanil and fentanyl.

(3.9 × 5 mm). The mobile phase was a mixture of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile (90:10, v/v). The mixture was pumped at a starting ratio of 90% A and 10% B and then changes to 10% A and 90% B over 4 minutes and then returns to initial conditions over 3 minutes. The flow rate was 0.6 mL/min and the column temperature was 30°C. The compounds were detected by positive selected ion recording. The scan rate was 5 points/second, gain 10, capillary voltage 0.8 kV, cone voltage for thiafentanil 8, cone voltage for fentanyl 15, ion source temperature 150°C and probe temperature 600°C. Nitrogen was used as the nebulizing gas and maintained at 100 psi. Thiafentanil was detected at 417 m/z and fentanyl was detected at 337 m/z.

Thiafentanil (Figure 1) (Wildlife Pharmaceuticals Inc., Windsor, CO) was 98% pure. Fentanyl (Figure 1), which was the internal standard (99% purity) was purchased from US Pharmacopeia (Rockville, MD). All other mass spectrometry grade chemicals and solvents were purchased from Fisher Scientific (Pittsburg, PA). Water (18.2 megaohm) was obtained from a Barnstead Nanopure Infinity (Dubuque, IA) ultrapure water system.

Preparation of calibration standards

Five milligrams of thiafentanil or fentanyl were weighed and dissolved in methanol to produce stock concentrations of 100 μg/mL. Dilutions of the stock standards were prepared in methanol to produce 0.01, 0.1 and 1 μg/mL working stock solutions. Standards were aliquoted into 2 mL vials to prevent cross contamination and evaporation. All solutions were stored at 4°C. By comparing standard areas over time, it was determined that solutions were stable for a minimum of 8 months.

For preparation of calibration standards and quality control samples, appropriate volumes of stock solutions were placed in 13 × 100 mm glass tubes and evaporated with nitrogen then untreated plasma was added.

Sample preparation

Thiafentanil was extracted from plasma using a protein precipitation method. One hundred microliters of plasma was placed in a 13 × 100 mm tube followed by 5 μL of fentanyl (0.1 μg/mL) and then 1 mL of acetonitrile. Tubes were vortexed at high speed for 60 seconds and then centrifuged for 10 minutes at 1020 × g. The supernatant was removed and placed in a 13 x 100 mm glass tube and evaporated to dryness with nitrogen. Samples were reconstituted in 200 μL of mobile phase, vortexed and then placed in a total recovery chromatographic vial and 65 μL injected into the system.

Method validation

The method was validated according to the Guidelines for Bioanalytical Method Validation published by the Food and Drug Administration (5). Validation of the method was carried out using QC samples. All of the QC samples and calibration curves were prepared in a plasma matrix. The validation process looked at accuracy, precision, selectivity, sensitivity, reproducibility and stability.

Selectivity

Selectivity was determined by injecting blank plasma from six different deers to confirm no interfering peaks around the retention time of both thiafentanil and fentanyl, the internal standard (IS).

Calibration curve, linearity and quality control samples

The final concentrations for the calibration standard curve were 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 25 ng/mL. The calibration curve was constructed by using the ratio of the peak area of the analyte divided by the peak area of the internal standard versus the concentration and obtained on five different days. Linearity was assessed by linear regression analysis and expressed as the coefficient of determination (r^2). The standard deviations (SD) of the slope, intercepts and regression coefficient were calculated. The QC samples were prepared in a similar manner as the calibration standards at four different levels 0.3, 0.75, 3.5 and 17.5 ng/mL. The acceptance criterion for each back-calculated standard was 15% deviation from the nominal value except lower limit of quantification (LLOQ), which was set at 20%.

Accuracy, precision and recovery

The precision and accuracy of the assay were determined using QC samples of known thiafentanil concentrations (0.3, 0.75, 3.5 and 17.5 ng/mL), which were processed freshly each validation day. Five replicates of each QC were analyzed during the same day and on five different days, and the intra- and inter-assay means, SD and coefficient of variation were calculated. Recoveries were calculated as the measured concentrations divided by the expected concentrations and expressed as a percentage (5). The tailing factor was calculated by $A_s = W_{0.05}/2f$, where $W_{0.05}$ is the width of the peak at 5% height and f is the distance from the peak maximum to the leading edge of the peak height from the baseline (6).

Results

Selectivity

Endogenous components from the plasma did not interfere with the elution of the compounds of interest. Six different blank plasmas were used in the pre-validation process. Figure 2 shows chromatograms of a (A) blank plasma, (B) a 0.5 ng/mL spiked plasma standard and (C) a plasma sample from a deer 30 minutes after a 0.07 mg/kg dose via nonmetal dart administration. Retention times were 4.32 minutes for thiafentanil and 4.56 minutes for fentanyl.

Calibration curves, precision, accuracy and linearity

The plasma peak area ratio (area of thiafentanil divided by internal standard area) versus concentration was plotted and produced a linear curve for the concentration range used (0.1–25 ng/mL) with the correlation coefficients ranging from 0.9990 to 0.9998. The mean slopes, intercepts and r^2 values are reported in Table I. A typical linear equation for the plasma calibration curve was $y = 0.0682x + 0.0360$,
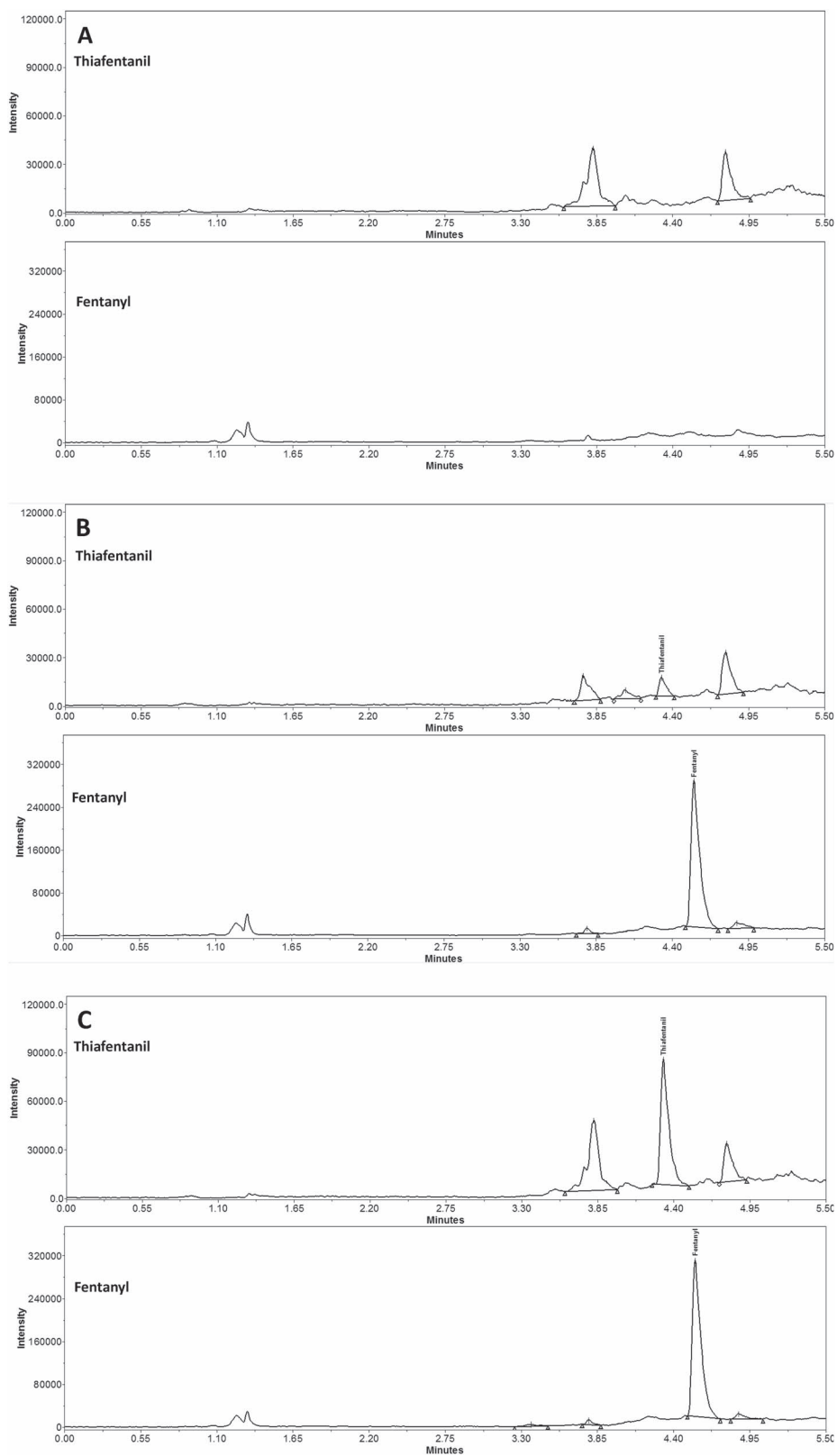
Figure 2. Chromatograms for a (A) blank plasma, (B) a plasma standard spiked with 0.5 ng/mL thiafentanil and IS, and (C) a plasma sample from a deer 30 minutes after 0.07 mg/kg via nonmetal dart administration.

Table I. Intra-assay accuracy, precision and assay linearity for thiafentanil in plasma

Intra-assay variability (n = 5)			
Concentration added (ng/mL)	Concentration measured (ng/mL) (mean ± SD)	RSD (%)	Accuracy (%) (mean ± SD)
0.3	0.36 ± 0.03	8.3	120 ± 9
0.75	0.72 ± 0.05	7.5	96 ± 7
3.5	3.3 ± 0.31	9.6	94 ± 5
17.5	18.6 ± 0.84	4.5	106 ± 5
Assay linearity (*n* = 5)			
	Mean ± SD	**RSD (%)**	
Y-Intercept	0.0360 ± 0.0010	2.7	
Slope	0.0682 ± 0.0011	0.5	
r^2	0.9990 ± 0.0005	0.05	

SD: standard deviation; *n*: number of samples; RSD: relative standard deviation

Table II. Inter-assay variability and recovery for thiafentanil in plasma (*n* = 5)

Concentration added (ng/mL)	Concentration measured (ng/mL) (mean ± SD)	RSD (%)	Accuracy (%) (mean ± SD)	Recovery (%)
0.3	0.31 ± 0.01	4.7	103 ± 5	100
0.75	0.77 ± 0.03	3.6	103 ± 3	109
3.5	3.5 ± 0.13	4.8	100 ± 4	103
17.5	17.6 ± 0.32	1.8	100 ± 3	99

SD: standard deviation; *n*: number of days; RSD: relative standard deviation

where *y* represents the peak area ratio of thiafentanil to internal standard and *x* represents the concentration of thiafentanil in nanograms per milliliter.

All values of accuracy and precision were within the recommended limits. Intra-assay precision ranged from 4.5 to 9.6%, while inter-assay precision ranged from 1.8 to 4.1%, respectively. These precision values are well below the set ±15% for all quality control samples as shown in Tables I and II. The intra-assay accuracy ranged from 94 to 120% and inter-assay accuracy ranged from 100 to 103%, respectively.

The thiafentanil peaks were slightly asymmetrical but the USP tailing factor was 1.52. Numbers ≤ 2 are considered acceptable.

Recovery, LLOQ and stability

The recovery of thiafentanil was determined by comparing the peak areas of extracted analytes with that of the directly injected standard solutions. The average recovery of thiafentanil from the four plasma QC concentrations ranged from 99 to 109%. The average recovery of fentanyl was 91%. The LLOQ was 0.1 ng/mL, which represents a peak approximately five times baseline noise. The LOD was 0.05 ng/mL, which represents a peak three times baseline noise. The baseline noise was calculated using the peak to peak method.

Testing of short-term stability of the quality control standards for plasma indicated that there was a 2% loss of drug after 24 h in the autosampler and 1% after 24 h at 4°C. Samples in our studies were stored at −80°C and thawed once for analysis. After one freeze-thaw cycle, there was no loss of thiafentanil.

Discussion

To the authors' knowledge, we have developed and validated the first method for the quantification of thiafentanil in plasma using reverse phase separation and mass spectrometry detection. We wanted to develop a simple, sensitive, straightforward method that would be effective with small volume samples. Several organic solvents and mixtures were tested during optimization of the extraction procedure including, methanol, ethyl acetate, hexane, chloroform and methylene chloride. All were found to produce much lower recoveries compared to acetonitrile. The amount of acetonitrile used for the extraction process was also investigated. Five hundred microliters, 1 and 1.5 mL of acetonitrile were used for the precipitation process and it was determined that the recovery decreased when using 0.5 mL and remained the same with the 1.5 mL, therefore, we elected to use 1 mL.

The recovery and limit of quantification are more than adequate for use in the determination of thiafentanil in plasma. If a lower LLOQ is required, a larger sample volume could be used. There was a 2% or less, sample loss after storage in either the autosampler or the refrigerator; therefore, if there were a power or equipment failure, samples could be reanalyzed. The validation indicates good intra- and inter-assay precision and accuracy within the concentration range used. The addition of fentanyl as an internal standard allows for the correction of intra- and inter-assay variability.

Conclusion

In conclusion, this analytical procedure was validated in terms of recovery, linearity, LLOQ, precision and accuracy. The procedure employs a low plasma volume, which could make it beneficial if the drug was used in smaller animals. The limit of quantification and recovery are more than adequate for use in quantitation studies. Our results indicate that this high performance liquid chromatography mass spectrometry procedure is a reproducible method that provides consistent quantification of thiafentanil in plasma. The addition of an internal standard allows for the correction of intra- and inter-assay variability. This method has been successfully applied to determine thiafentanil concentrations in plasma samples at this institution. This method should be applicable to other species.

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