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LC-MS/MS method for the simultaneous quantification of thiafentanil and naltrexone in bovine muscle, liver and kidney

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ABSTRACT

Thiafentanil is a μ-opioid agonist used for the chemical immobilisation of a variety of ungulate species and is antagonised by the administration of naltrexone. The potential for these ungulates to be hunted for consumption by humans or predators raises concerns of drug residues in animal tissues. No analytical method to quantify tissue residue concentrations of thiafentanil has been previously reported. This research developed an LC-MS/MS method to quantify thiafentanil in bovine muscle, and both thiafentanil and naltrexone in bovine liver and kidney matrices. The analytical method was applied to quantify tissue residues in samples collected from goats 1, 2, 3, and 6 days post thiafentanil administration. The assay was validated over the calibration range 6.25–200 ng/mg for thiafentanil in muscle, and 3.13–400 ng/mg for thiafentanil and 57.8–7400 ng/mg for naltrexone in liver and kidney. No residues above the lowest limit of quantification were detected in the injection site, *longissimus dorsi* muscle, liver or kidney samples collected from the goats. The reported analytical method and residue depletion data provide a foundation for future thiafentanil and naltrexone residue depletion studies in wildlife species.

1. Introduction

In wildlife veterinary practice, chemical immobilisation is an indispensable tool used to safely manage and handle captive and free-ranging wildlife species. Chemical immobilisation facilitates activities such as wildlife research studies, dehorning, translocation, fitting of tracking devices and medical treatments [1–3]. One of the more recently developed agonists used for chemical immobilisation and registered for use in wildlife in South Africa is thiafentanil [4]. Thiafentanil is a potent μ-opioid agonist that is quickly and completely antagonised by the opioid antagonist, naltrexone [5].

Thiafentanil is the immobilising agonist of choice for many ungulate species because of its high potency, short onset and duration of action as well as low risk of renarcotisation [1,4]. Numerous reports exist on the use of thiafentanil for chemical immobilisation of wildlife, predominantly in wild ungulate species [6–13].

Many ungulate species are hunted for game meat or released in reserves where there are predators, particularly in South Africa. Therefore, one of the concerns with administering thiafentanil to these animals is the potential for drug residues in edible tissues and, consequently, the potential for secondary intoxication. A suspected case thereof specifically involving thiafentanil has been reported in a captive mountain lion (*Puma concolor*) [14]. The consumption of game meat and the inclusion of wildlife species into the South African Meat Safety Act (Act No. 40 of 2000) as food-producing species has further raised concerns about drug residues in game tissues. To address these concerns, establishing the tissue concentrations of thiafentanil serves as a fundamental starting point for determining a suitable withdrawal time for this product.

For veterinary drugs administered to domestic food-producing animals, withdrawal times are well established to ensure meat safety for the consumer. In contrast, veterinary drug withdrawal times and pharmacokinetics are poorly established for wildlife species. Furthermore, withdrawal times are guided by threshold values such as maximum residue limits (MRLs). For many of the drugs administered to wildlife, and specifically for thiafentanil and naltrexone, these values have not

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been established. This is likely due to the variability in drug combinations, recommended doses and injection sites across numerous wildlife species, resulting in many of these drugs being administered in an off-label manner [15,16].

While tissue concentrations are unreported for thiafentanil, naltrexone tissue concentrations have been established in combination with various drugs for a few species. Naltrexone is rapidly cleared from tissues in rocky mountain elk (*Cervus elaphus nelsoni*) and white-tailed deer (*Odocoileus virginanus*) [17,18]. Animals were administered 50 mg and 25 mg naltrexone intramuscularly (i.m.), respectively. While naltrexone was detected in liver and muscle from an elk that died 12–24 hours post-administration and in liver samples collected three days post-administration, no residue was observed in elk after six days post-administration [17]. Similarly, no naltrexone residue was observed in muscle and liver samples collected from white-tailed deer 11 days post-administration [18]. Both studies had a detection limit of 0.01 ppm for naltrexone and detailed analytical methods were not reported.

To the best of the authors' knowledge, thiafentanil has only previously been detected in plasma and no analytical method to detect this drug in tissues has been published [19,20]. There is a need to develop an accurate and robust analytical method to determine concentrations of thiafentanil in tissues.

This research aimed to develop and validate a liquid chromatography tandem mass spectrometry (LC-MS/MS) method to detect thiafentanil in bovine muscle, and simultaneously detect thiafentanil and naltrexone in bovine liver and kidney matrices. Furthermore, a residue depletion study was implemented to quantify the tissue concentrations of these drugs at 1, 2, 3, and 6 days post thiafentanil administration using goats as a model species for ungulates.

2. Materials and methods

2.1. Chemicals and reagents

Reference standards for thiafentanil, naltrexone and the internal standard (ISTD), diprenorphine were provided as 1 mg/mL stock solutions in methanol by Wildlife Pharmaceuticals Pty. Ltd. (White River, South Africa). Formic acid was purchased from Fisher Scientific (Newington, NH, USA). LC-grade acetonitrile and methanol were purchased from ROMIL Pure Chemistry (Johannesburg, South Africa). A Milli-Q® water purification system coupled with a Synergy® system from Merck (Darmstadt, Germany) was used to supply ultrapure water. Blank bovine muscle, liver and kidney matrix was purchased from a butchery in Cape Town, South Africa.

2.2. LC-MS/MS conditions

The analytical method development was performed using a Shimadzu 8040 triple quadrupole-mass spectrometer connected to a Shimadzu Prominence Ultra-fast Liquid Chromatography (PUFLC) XR system (Kyoto, Japan). The mass spectrometer was operated in positive ionisation mode with source parameters including 250 °C DL temperature, collision-induced dissociation (CID) gas of 230 kPa, 4.5 V interface voltage and 400 °C heat block temperature. Multiple reaction monitoring (MRM) was used to detect analytes. The respective MRM transitions and retention times are reported in Table 1. Data were acquired using LabSolutions (Version 5.109) software and concentrations were quantified from the analyte to ISTD peak area ratio.

A Poroshell 120, EC-C18, 3.0 × 100 mm, 2.7 μm column manufactured by Agilent Technologies (Santa Clara, CA, USA) with a flow rate of 0.450 mL/min was used to achieve chromatographic separation of the analytes. Mobile phase A consisted of water containing 0.1 % formic acid and mobile phase B consisted of acetonitrile. A gradient elution program was carried out as follows: mobile phase B was increased from 15 to 95 % over 3.5 min, kept at 95 % B until 4 min, decreased from 95 to 15 % B until 5 min, and equilibrated at 15 % B until 8 min. The injection volume was set to 5 μL with a column oven temperature of 30 °C. The autosampler temperature was set at 15 °C and the autosampler needle rinse consisted of methanol and water (80:20, *v/v*). Additionally, during the gradient elution program, the flow from the column was diverted to waste for the time interval between 1.00 and 1.50 min. This step was implemented to minimise the amount of matrix co-eluents entering the LC-MS system.

Table 1
Exact masses, MRM transitions and retention times for thiafentanil, naltrexone and the internal standard, diprenorphine.

Compound	Exact mass (*m/z*)	Transitions (*m/z*)	Retention time (min)
Thiafentanil	416.17	417.00 > 113.05[a] 417.00 > 357.15[b]	2.665
Naltrexone	341.16	342.05 > 324.15[a] 342.05 > 270.00[b]	2.006
Diprenorphine (ISTD)	425.45	426.35 > 187.10[a] 426.35 > 372.20[b]	2.480

[a] Highest intensity product ion; quantifying ion.
[b] Qualifier ion.

2.3. Homogenisation of tissue matrices

Samples consisting of 100–500 mg of bovine muscle, liver or kidney were combined with water (1:2.5, *tissue weight: water volume*) and five 2.4 mm metal beads in 2 mL reinforced homogenising tubes supplied by United Scientific Pty. Ltd. (Cape Town, South Africa). These were then mechanically homogenised using a Bead Ruptor Elite NE486LL/A homogeniser supplied by Omni International (Kennesaw, GA, USA) with the Optimate ™ Bead Mill Operating System (Version 1.1). Samples were homogenised for four cycles on the meat pre-defined program (30 seconds at 6.00 m s^{-1}) with a one-minute dwell time between cycles. The homogenisation protocol was applied to STDs, QCs, blank, double blank and residue study samples.

2.4. Preparation of working stock solutions, calibration standards and quality control samples

Working solutions (WS) for the preparation of calibration standards (STDs) and quality control samples (QCs) were prepared from 1.00 mg/mL stock solutions in methanol. Volumetric 1:1 serial dilutions with methanol were performed to prepare six WS at concentrations ranging between 0.0150 and 0.480 μg/mL thiafentanil. These WS were used to prepare calibration STDs in muscle. Similarly, seven WS with concentrations ranging between 0.00750–0.960 μg/mL thiafentanil and 0.139–17.8 μg/mL naltrexone were used to prepare calibration STDs in liver and kidney.

Matrix-matched STDs and QCs for validation batches and quantitative analysis were prepared on the day of analysis by individually spiking 20 μL of the respective WS into 100 μL of the tissue homogenate. The final concentrations of STDs in bovine muscle were 2.50, 5.00, 10.0, 20.0, 40.0 and 80.0 ng/mL thiafentanil, corresponding to a final calibration range of 6.25–200 ng/mg of thiafentanil per milligram muscle. Final concentrations of QC lowest limit of quantitation (LLOQ), QC low (QCL), QC medium (QCM) and QC high (QCH) prepared in muscle matrix were 2.50, 6.00, 32.0 and 64.0 ng/mL of thiafentanil. The final concentrations of STDs in bovine liver and kidney were 1.25, 2.50, 5.00, 10.0, 20.0, 40.0, 80.0 and 160 ng/mL thiafentanil and 23.1, 46.3, 92.5, 185, 370, 740, 1480 and 2960 ng/mL naltrexone, respectively. The corresponding final calibration ranges of analyte per milligram tissue were 3.13–400 ng/mg of thiafentanil and 57.8–7400 ng/mg of naltrexone in liver and kidney matrices. Final concentrations of LLOQ, QCL, QCM and QCH prepared in liver and kidney matrices were 1.25, 3.60, 64.0 and 128 ng/mL thiafentanil and 23.1, 66.6, 1184 and 2368 ng/mL naltrexone, respectively.

2.5. Sample extraction procedure

STDs, QCs, blank, double blank and collected samples all underwent the same extraction procedure. Samples containing 100 μL of homogenised matrix, combined with either 20 μL of WS in the case of STDs and QCs, or 20 μL of blank methanol in the case of blank, double blank and collected samples, were extracted with 400 μL of ice-cold acetonitrile containing 600 ng/mL ISTD. Samples were vortex-mixed for 90 seconds at maximum speed and then centrifuged at 16,000×*g* at 23 °C for 10 min. The extract was then passed through an unconditioned Oasis PRiME HLB 3 cc (60 mg) extraction cartridge with no pre-treatment or washes as per the manufacturer's instructions. The collected extract was centrifuged at 16,000×*g* at 23 °C for a further 5 min and transferred to a 96-well plate for analysis.

2.6. Residue depletion study

To establish tissue residue concentrations after immobilisation with thiafentanil, sixteen healthy adult male goats (*Capra aegagrus hircus*) were used for this study. Health screenings were conducted on all individuals which included a physical examination, blood smear, faecal flotation, and blood chemistry panel. Animals were housed at Wildlife Pharmaceuticals Wildlife Research Facility, Mpumalanga, South Africa. The group was fed daily with water available *ad libitum*. Animals weighed between 20.0 and 27.0 kg.

Individuals were injected with 90 μg/kg of thiafentanil i.m. in the left *vastus lateralis* muscle. Immobilisation was reversed after 40 min with 10 mg of naltrexone for each milligram of thiafentanil, administered intravenously (i.v.). Thiafentanil and naltrexone were used as commercial formulations sold as Thianil (10 mg/mL) and Trexonil (50 mg/mL). These drugs were supplied by Wildlife Pharmaceuticals Pty. Ltd. (White River, South Africa).

At four separate time points, while immobilised with 1.96 mg etorphine administered i.m. in the right *vastus lateralis* muscle, four individuals were euthanised using a captive bolt and exsanguinated. Euthanasia was conducted at 1, 2, 3 and 6 days post thiafentanil administration. Muscle samples were collected from the *longissimus dorsi* muscle and the left *vastus lateralis* muscle making sure to include the needle trace from the thiafentanil injection site. Additionally, a cross-section of the liver and kidney was collected.

Tissue samples were subsampled and immediately frozen at −80 °C. Subsamples were weighed into 2 mL reinforced homogenising tubes to eliminate any sample loss during the heat inactivation and homogenisation steps described in Sections 2.7 and 2.3, respectively. Sample weights were between 113.6 and 295.2 mg for the injection site, 107.4–376.4 mg for the *longissimus dorsi* muscle, 143.5–389.9 mg for liver and 170.8–355.0 mg for kidney. These subsamples were exposed to a heat-inactivation treatment described below before being frozen at −80 °C and transported for analysis. Samples were received frozen at the laboratory.

2.7. Heat inactivation

This method was developed to be applied to samples collected from a foot-and-mouth disease (FMD) and bovine tuberculosis control zone. To inactivate any potential disease vectors, all samples collected from study animals were required to be heated to 70 °C for 30 min prior to transportation. This treatment was implemented by heating samples in a water bath or on a heating block set to 70 °C. This treatment was evaluated for each matrix during the development of the analytical method to determine any effect on the recovery, matrix effects, selectivity, sensitivity and stability of the analyte in the matrix.

2.8. Method validation

2.8.1. Linearity, intra- and inter-day validation

This method was separately validated for bovine muscle, liver and kidney according to the FDA and EMA guidelines for bioanalytical method validation [21,22]. Linearity, intra- and inter-day variability were assessed from three independent validation batches analysed on three different days. Accuracy and precision criteria according to FDA and EMA guidelines were followed for STDs, QCs and LLOQ [21,22]. Validation batches consisted of a matrix-matched calibration curve generated from STDs prepared in duplicate, and six replicates of each of the LLOQ, QCL, QCM and QCH. Calibration curves for muscle consisted of six STDs while calibration curves prepared in liver and kidney consisted of seven STDs.

Linearity was assessed by plotting the peak area ratio of the analyte to the internal standard against the nominal concentrations. Calibration curves fitted a quadratic regression with a weighting of $1/C^2$ (C = concentration) for thiafentanil and naltrexone.

2.8.2. Carryover, selectivity and sensitivity

Carryover was assessed from chromatograms of the blank and double blank samples analysed immediately after the highest STD to determine the presence of peak areas at the retention time of the analytes and ISTD, respectively. This was assessed in each matrix.

Blank matrix for each tissue type, collected from six different sources, was used to assess selectivity and sensitivity. Selectivity was evaluated by analysing chromatograms from blank samples for peak areas at the retention time of the analytes and ISTD. Sensitivity was assessed by analysing chromatograms from QCs prepared at the LLOQ to ensure the signal-to-noise ratio was ≥ 5.

2.8.3. Matrix effects, recovery and process efficiency

The matrix effects, recovery and process efficiency were evaluated in muscle, liver and kidney tissue matrices sourced from six different individuals. These parameters were assessed based on previously described methodology and were evaluated for both untreated and heat-inactivated samples [23].

The matrix effects were evaluated by drying down extracts from drug-free matrix (untreated and heat-treated) and reconstituting with an acetonitrile-water mixture (4:1, *v/v*) spiked with theoretical QCL, QCM and QCH concentrations accounting for the dilution steps during extraction. Regression slopes were generated from the peak area ratio of the analyte to ISTD in blank extracted matrix spiked post-extraction at QCL, QCM and QCH concentrations as described by Matuszewski et al. [23].

The recovery of the analytes was evaluated by comparing the peak area of the analyte responses from QCs prepared at low, medium and high concentrations spiked pre-extraction to the responses of blank extracted matrix spiked post-extraction. This ratio was presented as the percentage recovery and the mean recovery across the concentrations was reported. Process efficiency was assessed by comparing the peak area ratio of the analyte to ISTD of the QCs spiked pre-extraction to analytes in matrix-free injection solvent used for the post-extraction spiking of the matrix effects samples, representing 100 % recovery. This was presented as percentage process efficiency.

2.8.4. Stability evaluations

Stability evaluations in muscle, liver and kidney matrices were performed in six-fold at QCL and QCH concentrations. Freeze-thaw stability was evaluated by exposing QCs to one cycle of freezing at −80 °C for a minimum of 60 min, followed by thawing at room temperature for 60 min. The heat inactivation treatment was evaluated by heating QCs for 30 min at 70 °C. Benchtop stability was assessed by leaving freshly prepared QCs on bench at ~ 20 °C for two hours.

3. Results and discussion

3.1. Linearity, intra- and inter-day validation

Calibration curves demonstrated good linearity over the concentration ranges of thiafentanil in all matrices. The mean correlation coefficient (r^2 value) for thiafentanil from the validation batches was 0.998 in muscle and liver and 0.996 for kidney. Likewise, calibration curves prepared in liver and kidney demonstrated good linearity over the concentration range for naltrexone. The mean correlation coefficient (r^2 value) for naltrexone from the validation batches was 0.994 and 0.997 for liver and kidney, respectively. Representative chromatograms

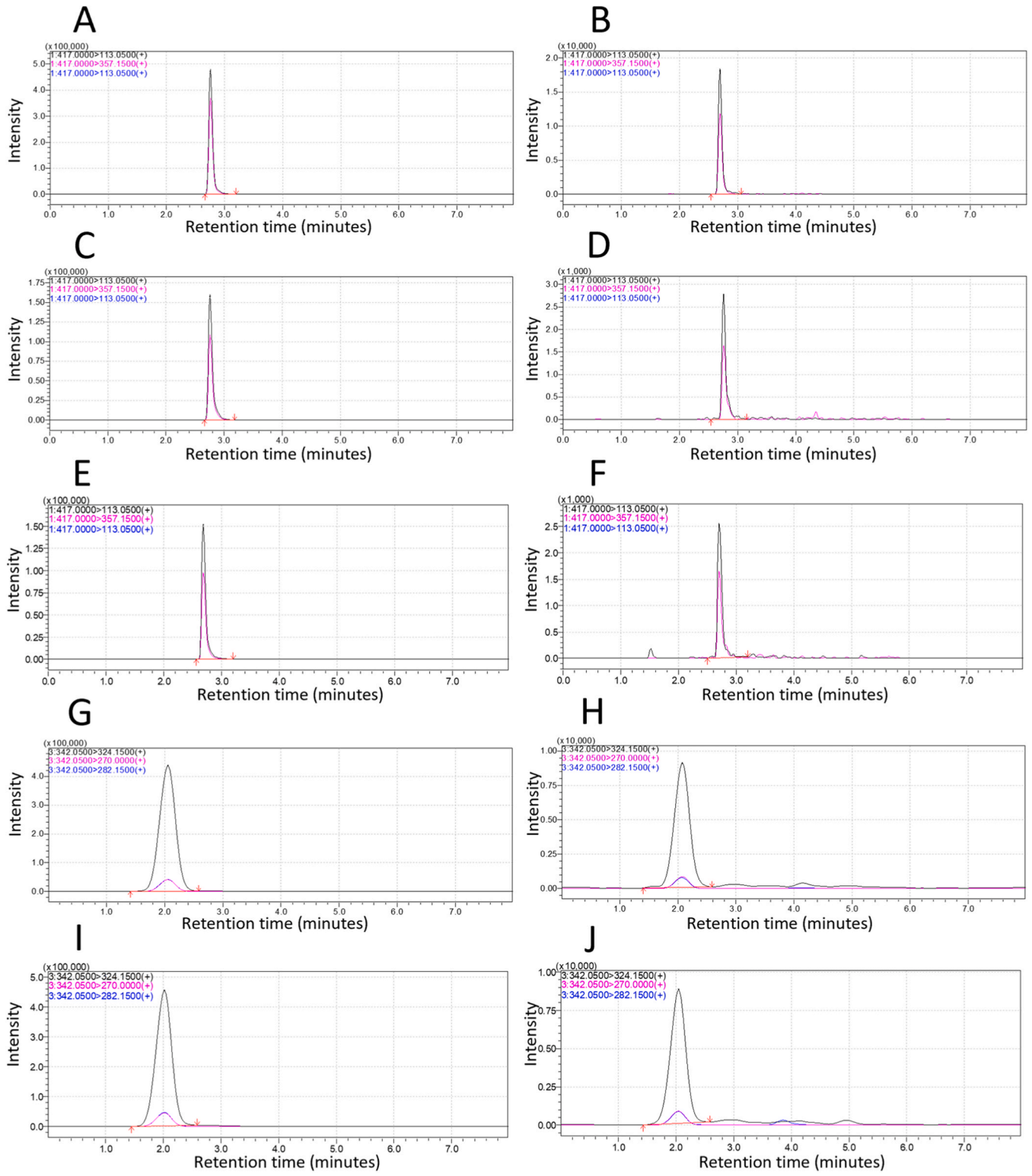

Fig. 1. Representative chromatograms for thiafentanil at the upper level of quantification (ULOQ, 64.0 ng/mL) and lowest level of quantification (LLOQ, 2.50 ng/mL) in muscle (A, B), thiafentanil at the ULOQ (128 ng/mL) and LLOQ (1.26 ng/mL) in liver (C, D) and kidney (E, F), respectively; and naltrexone at the ULOQ (2368 ng/mL) and LLOQ (23.1 ng/mL) for liver (G, H) and kidney (I, J), respectively.

at the LLOQ and upper level of quantification (ULOQ) in muscle, liver and kidney are represented in Fig. 1.

Intra- and inter-day accuracy and precision for QCs met criteria for thiafentanil in muscle, liver and kidney matrices and naltrexone in liver and kidney matrices (Tables 2 and 3). Intra-day accuracy for thiafentanil ranged between 95.9–107.9 %, 90.9–106.9 % and 91.9–102.8 % across validation batches for muscle, liver and kidney, respectively. Intra-day precision for thiafentanil was less than 7.6 %, 13.3 %, and 10.3 % for muscle, liver and kidney across validation batches, respectively. Inter-day accuracy for thiafentanil ranged between 101.0–104.5 % for muscle, 100.7–105.9 % for liver, and 90.8–98.6 % for kidney. Respective inter-day precisions for thiafentanil were $\leq$ 9.0 % for muscle, $\leq$ 10.7 % for liver except at the LLOQ where it was $\leq$ 16.0 %, and $\leq$ 10.4 % for kidney, except at the LLOQ where it was $\leq$ 16.3 % which is within the acceptable criteria for the LLOQ (accuracy 80–120 %).

Accuracy for naltrexone ranged between 91.3–111.8 % and 97.9–107.1 % across validation batches for liver and kidney, respectively. Intra-day precision for naltrexone was less than 10.6 % and 8.6 % for liver and kidney across validation batches, respectively. Inter-day accuracy for naltrexone ranged between 93.7–107.6 % for liver and 99.8–104.0 % for kidney. Respective inter-day precisions for naltrexone were $\leq$ 13.2 % for liver and $\leq$ 9.1 % for kidney.

3.2. Carryover, selectivity and sensitivity

No carryover was observed for thiafentanil, naltrexone or the ISTD in any of the matrices for both untreated and heat-activated samples. No interfering peaks at the retention time of the analytes and ISTD were observed, demonstrating that the method was highly selective for the analytes in all matrices and for untreated and heat-inactivated samples. Representative chromatograms for the selectivity assay have been included as supplementary material. The signal-to-noise ratio for the analytes in each matrix was $\geq$ 5 at the LOQ in untreated and heat-inactivated samples, which indicated suitable sensitivity for the method.

3.3. Matrix effects, recovery and process efficiency

A detailed summary of the matrix effects, recovery and process efficiency is provided in Table 4. The observed matrix effect in liver and kidney matrices was decreased during the heat inactivation step for both thiafentanil and naltrexone. Conversely, matrix effects were more pronounced for thiafentanil in muscle after heat inactivation. One of the challenges of drug residue analysis in tissues is the complex nature of these matrices and the extensive sample clean-up required due to co-extracted protein and fat components [24,25]. The matrix effects fell marginally outside of the acceptability criteria outlined by Matuszewski et al. [23] and could be improved by incorporating a stable isotopically labelled (SIL) ISTD for each analyte. Alternatively, a different cleanup approach, such as solid-phase-extraction (SPE), could be incorporated.

Recoveries for thiafentanil and naltrexone demonstrated high precision for both the untreated and heat-inactivated matrices, indicating that the method was suitable for simultaneously extracting both analytes from the matrices.

The process efficiency for thiafentanil demonstrated high precision ($\leq$15 %) in muscle, liver and kidney. The process efficiency of naltrexone also demonstrated high precision ($\leq$15 %) for liver but was marginally higher than 15 % for kidney. This could be attributed to the analytes interacting differently with co-eluted matrix components in different matrices. Furthermore, it could indicate that the ISTD compensates differently in the different scenarios.

3.4. Freeze-thaw, benchtop and heat inactivation stability evaluations

A summary of stability evaluations is provided in Table 5. QCs demonstrated suitable freeze-thaw stability for both analytes for one freeze-thaw cycle in all matrices. Analytes were stable on bench for up to

Table 2
Intra- and inter-day accuracy (%) and precision (%CV) of thiafentanil and naltrexone in bovine liver and kidney at the lowest limit of detection (LLOQ), QC low (QCL), QC medium (QCM) and QC high (QCH).

Analyte	QC concentration (ng/mL)	Intra-day (n = 6)								Inter-day (n = 18)			
		Day 1				Day 2				Day 3							
		Liver		Kidney		Liver		Kidney		Liver		Kidney		Liver		Kidney	
		Accuracy (%)	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)	Precision (CV%)
Thiafentanil	LLOQ–1.25	95.7	9.0	98.0	7.7	101.6	1.0	102.8	9.7	104.7	13.3	93.5	10.3	105.9	16.0	95.3	16.3
	QCL–3.60	97.9	9.2	98.9	7.0	106.9	3.9	98.1	8.9	101.6	8.6	100.3	7.7	101.6	10.7	98.6	10.4
	QCM–64.0	99.0	5.8	91.9	2.1	100.1	2.6	99.2	2.9	101.2	5.9	96.7	2.4	100.9	9.8	95.8	4.4
	QCH–128	90.9	6.4	93.9	5.5	103.2	4.6	97.8	3.1	99.1	10.3	98.7	5.1	100.7	10.4	90.8	7.5
Naltrexone	LLOQ–23.1	98.2	7.0	100.3	3.8	110.2	4.2	102.9	8.6	111.8	6.1	107.1	7.4	107.6	12.8	103.3	7.7
	QCL–66.6	102.9	8.0	96.9	1.7	105.3	6.3	102.8	7.1	104.1	7.7	101.7	6.6	102.2	13.2	104.0	9.1
	QCM–1184	94.0	4.0	97.9	4.5	101.3	5.4	102.1	5.8	98.7	7.7	99.7	4.4	99.4	10.3	99.8	5.5
	QCH–2368	92.5	10.6	101.3	5.3	98.7	3.1	102.9	5.4	91.3	4.2	100.2	6.5	93.7	11.4	101.5	6.0

Table 3
Intra- and inter-day accuracy (%) and precision (%CV) of thiafentanil in bovine muscle at the lowest limit of detection (LLOQ), QC low (QCL), QC medium (QCM) and QC high (QCH).

Analyte	QC concentration (ng/mL)	Intra-day (n = 6)						Inter-day (n = 18)	
		Day 1		Day 2		Day 3			
		Accuracy (%)	Precision (CV %)	Accuracy (%)	Precision (CV %)	Accuracy (%)	Precision (CV %)	Accuracy (%)	Precision (CV %)
Thiafentanil	LLOQ–2.50	102.8	6.4	104.1	7.6	102.8	6.9	103.9	7.5
	QCL–6.00	98.3	4.9	106.8	4.6	103.1	6.7	104.5	8.8
	QCM–32.0	99.5	3.9	100.5	4.9	99.6	7.5	101.0	7.2
	QCH–64.0	107.9	6.2	98.1	4.8	95.9	3.3	104.2	9.0

Table 4
Matrix effects, recovery and process efficiency for thiafentanil and naltrexone in muscle, liver and kidney matrices for untreated and heat-inactivated samples.

Matrix	Treatment	Matrix effects (%CV)		Recovery (%)		Process efficiency (%)	
		Thiafentanil	Naltrexone	Thiafentanil	Naltrexone	Thiafentanil	Naltrexone
Muscle	untreated	6.1	NA	103.9 (1.5% CV)	NA	135.9 (3.6% CV)	NA
	heat-inactivated	12.3	NA	102.0 (0.8% CV)	NA	144.1 (7.9% CV)	NA
Liver	untreated	5.5	6.9	67.8 (5.1% CV)	97.6 (4.0% CV)	107.9 (7.8% CV)	88.3 (4.2% CV)
	heat-inactivated	4.1	3.8	57.0 (0.9% CV)	50.9 (0.7% CV)	99.0 (4.8% CV)	91.2 (6.2% CV)
Kidney	untreated	7.4	7.6	81.6 (5.5% CV)	81.2 (7.6% CV)	163.2 (12.5% CV)	131.0 (16.5% CV)
	heat-inactivated	6.9	5.0	78.7 (1.6% CV)	75.0 (8.9% CV)	147.5 (11.5% CV)	114.8 (15.1% CV)

NA – not assessed

Table 5
Freeze-thaw, benchtop and matrix stability of thiafentanil and naltrexone in high (QCH) and low (QCL) quality control samples prepared from bovine muscle, liver and kidney.

Analyte	Matrix type	QC level	Concentration (ng/mL)	Freeze-thaw (one cycle)		Benchtop (two hours)		Matrix stability (heated to 70 °C for 30 min)	
				Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
Thiafentanil	Muscle	QCL	6.00	95.6	4.0	85.4	7.9	109.0	8.7
		QCH	64.0	88.3	7.5	95.6	4.4	99.6	7.1
	Liver	QCL	3.60	102.9	2.9	101.2	4.2	84.1	4.9
		QCH	128	96.0	3.2	100.6	7.5	86.3	7.8
	Kidney	QCL	3.60	109.8	5.1	102.8	5.1	68.4	21.5
		QCH	128	102.3	7.3	97.2	2.6	76.7	6.6
Naltrexone	Liver	QCL	66.6	101.8	6.5	104.1	7.4	95.7	5.4
		QCH	2368	97.6	5.9	93.5	9.7	90.1	11.4
	Kidney	QCL	66.6	98.9	7.9	108.6	3.8	91.0	12.6
		QCH	2368	96.8	3.2	100.8	4.8	100.9	3.4

two hours which allowed sufficient time to prepare an analytical batch of STDs, QCs and samples. The analyte stability in matrix after heat-inactivation demonstrated that the accuracy of thiafentanil in muscle QCs and naltrexone in all matrices was within the acceptable criteria (accuracy 85–115 %). However, the accuracy of thiafentanil in liver and kidney QCs indicated that a correction factor should be considered to account for the loss/degradation of thiafentanil due to the necessary heat-inactivation treatment.

3.5. Determination of analyte concentrations in tissue

The observed concentration during the analytical method development and validation was expressed as the weight of the analyte per millilitre of extract (ng/mL). This was converted to a weight per weight (ng/mg) unit to reflect the amount of drug in the tissue. This conversion was obtained by multiplying the observed concentration (ng/mL) by the volume of homogenising solvent (mL) that was added to the tissue. This value was then divided by the amount of tissue (mg) homogenised. The corresponding weight for weight concentration range for thiafentanil was 6.25–200 ng/mg in muscle and 3.13–400 ng/mg in liver and kidney. Similarly, for naltrexone the calibration range was 57.8–7400 ng/mg in liver and kidney.

3.6. Residue depletion study

The collected goat muscle, liver, and kidney samples were analysed using this method. No thiafentanil or naltrexone above the LLOQ was detected in the injection site, *longissimus dorsi* muscle, liver or kidney samples at any of the time points. However, trace amounts of naltrexone below the LLOQ (s/n $\geq$ 5) were observed in a liver sample collected on day three, which was in line with findings from rocky mountain elk [17]. If analyte concentrations were determined in the tissues, the application of a correction factor to account for the analyte loss/degradation of thiafentanil would have been considered. In future studies where heat-inactivation treatment cannot be avoided, assessments should be performed to ensure that a suitable correction factor is applied to account for any analyte loss/degradation in the matrix. The heat-inactivation treatment was unavoidable for this study due to

regulatory requirements for the geographical location in which the samples were collected and the FMD control measures in place. Another consideration for future work would be to consider conducting the study outside of FMD control zones to avoid necessary heat-inactivation treatment.

Withdrawal study guidelines recommend that from a total of 16 individuals, tissue samples are collected from four individuals at four appropriately distributed time points [26]. Since no tissue concentrations have previously been reported for thiafentanil, this presented a challenge when selecting sampling time points. Previous reports have recommended that, based on the rapid elimination exhibited by fentanyl in animal pharmacokinetic studies, a withdrawal time of at least 48–96 hours should be applied [27]. This, together with previously reported data for naltrexone, guided the final sampling time points for this study [17,18].

Withdrawal study guidelines also recommend that tissue samples are collected from the injection site, muscle, liver, kidney and peri-renal fat [26]. This study did not investigate analyte concentrations in peri-renal fat, but this should be investigated for future work considering the lipophilic nature of opioids [28].

3.7. Method limitations

Long-term stability of the analytes in muscle, liver or kidney was not assessed. While the stability of naltrexone has been assessed for up to 30 days at −70 °C in human plasma [29], the long-term stability of the analytes in tissue matrices should be evaluated. Autosampler stability was not assessed during this study, and there was no need for re-injection of samples. Plasma extracts have demonstrated autosampler stability for up to 24 hours for both compounds [19,30], however autosampler stability for extracts from the relevant tissue matrices should be assessed. This work evaluated one freeze-thaw cycle. This approach was taken as collected tissue samples were subsampled prior to freezing and therefore were only thawed once for analysis. Future work should consider evaluating three freeze-thaw cycles, given that samples may need to be reanalysed.

A further limitation of this study was that the WS used to prepare STDs and QCs were prepared from a single stock solution. The potency of thiafentanil presents safety concerns to laboratory staff. Therefore, to mitigate these concerns, only a limited quantity of a single stock solution was received. Previous studies have demonstrated that thiafentanil and naltrexone stock solutions were stable at 4 °C for eight months and 100 days, respectively [19,30].

The unestablished MRL for thiafentanil and naltrexone further presented the challenge of establishing a meaningful LLOQ for the analytical method. Therefore, a wide calibration range with as low an LLOQ as possible while still demonstrating accuracy and precision was applied. It is possible that an analytical method incorporating a concentration step could detect thiafentanil and naltrexone at lower concentrations.

Despite the analytical method application to goat tissue samples, the method was developed and validated in bovine matrices due to the local availability of these tissues. Matrix collected from different species could influence validation parameters and therefore was a limitation of the method. Future work should consider performing cross-validation for goat tissues to investigate whether validation parameters for thiafentanil and naltrexone are affected by matrix from different species.

Working with wildlife species presents numerous challenges, one of which is handling the animals in captivity. For wildlife that are unaccustomed to a captive environment, this can cause unnecessary stress and the potential for injury. Therefore, this study implemented the use of goats (*Capra aegagrus hircus*) as a model species for ungulates. Goats have proven to be an adequate model species for the pharmacokinetics of carfentanil in eland (*Taurotragus oryx*) [31,32]. This does, however, have limitations. While this study provides a foundation for future residue studies, a variety of factors could influence the pharmacokinetic parameters and persistence of drug residue in tissues. For example, drug absorption, metabolism and elimination can be influenced by species and drug combinations [16]. Thiafentanil drug combinations and dosages are known to vary widely between species, and while using goats as a model species for wild ungulates had practical advantages, this was a limitation of the study. Specific doses and combinations in different species should be further investigated to evaluate the suitability of goats as a model species.

Lastly, injecting thiafentanil using an i.m. approach was advantageous because of the control it offered over administering the entire dose into a specific muscle. However, future work should consider that remote delivery systems such as darts could cause more muscle damage compared to i.m. administration which can result in erratic drug elimination [16].

4. Conclusion

The analytical method described is the first reported method to detect thiafentanil in muscle and simultaneously detect thiafentanil and naltrexone in liver and kidney. This novel method implemented a wide calibration range with a low LLOQ with acceptable accuracy and precision. While more sensitive LLOQs are always desirable, this work provides a foundation for establishing tissue residue concentrations for thiafentanil and naltrexone in wildlife species. The matrix effects fell marginally outside of the acceptance criteria. However, these effects were considered acceptable in the novel context of this study.

The results from this study indicate the short time frame in which these residues are detectable in goat muscle, liver and kidney tissues. This data provides a starting point for further residue depletion studies in wildlife species in which these drugs are commonly administered.

Ethical considerations

Ethical approval was granted by the Murdoch University Animal Ethics Committee (permit number: R3301/20) and the Wildlife Pharmaceuticals Animal Ethics Committee (project number: WPAEC-2021-THIAPK-47-C).

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CRediT authorship contribution statement

Judith T. Christie: Writing – original draft, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mieghan Bruce:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Silke Pfitzer:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Liesel Laubscher:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Conceptualization. **Jacobus P. Raath:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Michael Laurence:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Tracy Kellermann:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Judith T Christie reports that financial support for the Murdoch University Strategic Partnership Scholarship was provided by Wildlife Pharmaceuticals Pty. Ltd. Liesel Laubscher and Jacobus P Raath report that they are employed by Wildlife Pharmaceuticals Pty. Ltd.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2025.116711.

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