Validation of a LC-MS/MS method to simultaneously quantify thiafentanil and naltrexone in plasma for pharmacokinetic studies in wildlife

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ABSTRACT

Thiafentanil is a popular opioid agonist that is fully reversed by administering naltrexone. This agonist-antagonist combination is administered to a wide variety of wildlife species for chemical immobilisation, however plasma concentrations for thiafentanil remain unreported. This report describes a method that was developed and validated using human plasma and cross-validated for the analysis of goat plasma. Samples were extracted using a simple protein precipitation and analysed using LC-MS/MS. The assay was validated over the calibration range 4.38 – 1120 ng/mL for thiafentanil and 15.63 – 4000 ng/mL for naltrexone. The mean inter-day accuracies for QCs prepared in human plasma (n = 18) ranged from 94.8 – 103.8 % for thiafentanil and 94.8 – 95.9 % for naltrexone with corresponding precisions of 3.4 – 7.9 % and 2.8 – 11.4 %, respectively. The mean accuracies for QCs prepared in goat plasma (n = 6) ranged from 89.0 – 100.5 % for thiafentanil and 89.0 – 98.0 % for naltrexone with the associated precisions ranging from 7.1 – 11.6 % and 4.8 – 12.3 %, respectively. Both analytes were stable on bench for six hours and for three freeze–thaw cycles. The impact of heat-inactivation, necessary for the inactivation of potential foot-and-mouth disease, on analyte stability, matrix effect and recovery were evaluated, and a correction factor was established to determine the original analyte concentrations. The method was applied to pharmacokinetic samples collected from goats. The use of goats as a model species provides the first insight into the plasma concentrations of thiafentanil.

1. Introduction

Opioid agonists and their antagonists are essential tools for managing wildlife. These drugs allow for chemical immobilisation of wildlife, thereby facilitating safe restraint of these animals for relocation, medical treatment, and research. Opioid agonists provide rapid and relatively long-lasting immobilisation which is quickly and completely antagonised [1,2].

Thiafentanil is regarded as the next generation of opioid agonists, following etorphine and carfentanil [3]. It offers advantages over other potent opioid agonists because of its short onset of action allowing for quick handling of the immobilized animal, thereby reducing complications such as animals escaping [3,4]. Furthermore, thiafentanil is expected to be rapidly metabolised, reducing the likelihood of side-effects such as renarcotisation [3,5,6]. Thiafentanil is sold in a formulation with a high concentration (10 mg/mL), therefore, in conjunction with its potency, only a small dose is required enabling the drug to be easily administered intramuscularly through darts [1,3,7]. These attributes have resulted in the popularity of the agonist-antagonist combination of thiafentanil reversed with naltrexone in iconic African species such as black rhinoceros (Diceros bicornis), African buffalo (Syncerus caffer) and African elephant (Loxodonta africana), as well as a variety of antelope species globally [6,8–15].

Despite the extensive use for wildlife management, the pharmacokinetics of thiafentanil remain unreported. Establishing the pharmacokinetics could improve the clinical application of thiafentanil [16] and improve the safety of the chemical immobilisation for both the veterinarian and the animal. Furthermore, it could enable optimized drug dosing and later allow for allometric scaling for dose extrapolating between species [17].

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One of the contributing factors to this considerable lack of data could be due to the practical challenges of the intensive sample collection required to conduct a pharmacokinetic study, as thiafentanil is administered to wildlife species which are not always safe to manually restrain when not immobilised. Due to these challenges, model species have been used to conduct pharmacokinetic studies of other wildlife immobilising agents [1,18].

The lack of data could be further attributed to the limited published quantification methods as well as the unique challenges presented when working with ultra-potent opioids. For example, the high potency of thiafentanil necessitates analytical methods which are sensitive enough to detect low plasma concentrations of the drug. To the best of our knowledge, thiafentanil has only recently been quantified in plasma using liquid chromatography mass spectrometry (LC-MS) [19] and no published plasma concentrations exist. The study did apply the method to collected samples, however the observed concentrations remain unreported [19]. This method was limited as the matrix effect was not evaluated and although the selectivity of this analytical method was evaluated from deer plasma, it is unclear whether the entire method was developed from plasma sourced from this species. In comparison, LC-MS methods for naltrexone exist and concentrations have been well reported in humans. Additionally, the pharmacokinetics of naltrexone have been reported for both goats (Capra hircus) and eland (Taurotragus oryx) [1,18]. However, to the best of our knowledge no analytical method to simultaneously detect these drugs has been developed.

Control measures and precautions for the movement of biological samples out of foot-and-mouth disease (FMD) protection zones presents a further hurdle, especially when working within the South African context. Recommendations require blood products from FMD susceptible animals to be inactivated for any potential FMD virus by heating samples to a core temperature of 70 °C for 30 min [20]. Such a treatment should be evaluated for the effect it could have on the stability of analytes such as thiafentanil and naltrexone in plasma.

The first step towards investigating the pharmacokinetics of thiafentanil and naltrexone in wildlife is the development and validation of an analytical method which is sensitive enough to simultaneously detect both drugs. Collecting a sufficient volume of blank matrix for a full validation from a wildlife species poses significant challenges. For this reason, developing a method in human plasma and performing a simple cross-validation in plasma sourced from the species in which the study was conducted was deemed to be a viable approach that was fit for purpose. Furthermore, the simultaneous detection of both analytes decreases the total volume of plasma required for analysis, as well as decreases sample preparation time and analytical costs.

The aim of this paper was to develop and validate a simple and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to simultaneously quantify thiafentanil and naltrexone in plasma. This method also needed to be applicable and thoroughly assessed for the context in which it would be applied, therefore, the developed analytical method was cross-validated in goat plasma (Capra hircus) and the effect of heat-inactivation on the stability of the analytes was evaluated. This analytical method will allow for future investigation into the pharmacokinetics of this agonist–antagonist combination using goats as a model species.

2. Material and methods

2.1. Chemicals and reagents

Reference standards for thiafentanil, naltrexone and diprenorphine (internal standard; ISTD) were supplied by Wildlife Pharmaceuticals Pty, Ltd. (White River, South Africa). These were supplied individually as 1 mg/mL reference standards prepared in methanol. Water was purified using a Milli-Q® water purification system coupled with a Synergy® system (Merck KGaA, Darmstadt, Germany). LC grade acetonitrile and methanol were purchased from ROMIL Pure Chemistry (Johannesburg, South Africa) and formic acid was purchased from Fisher Scientific (New Hampshire, USA).

2.2. Ethical approval

Ethical clearance to conduct the pharmacokinetic study was approved by the animal ethic committees of Murdoch University (R3301/20) and Wildlife Pharmaceuticals (WPAEC-2021-THIAFK-47-C). Ethics exemption for the use of human plasma in bioanalytical method development was obtained by Stellenbosch University Health Research Ethics Committee (12 June 2019) and Murdoch University (2022/053).

2.3. LC-MS/MS conditions

The analytical method was developed on a Shimadzu 8040 triple quadrupole-mass spectrometer connected to a SHIMADZU Prominence Ultra-fast Liquid Chromatography (PUFLC) XR system (SHIMADZU, Japan). The system consisted of a LC-20ADXR solvent delivery system, Nexera XR SIL-20AXR autosampler and CTO-20A column oven. The mass spectrometer was operated in positive ionization mode with source parameters including 4.5 V interface voltage, 250 °C desolvation line temperature, 400 °C heat block temperature and collision-induced dissociation (CID) gas of 230 kPa. Analytes were detected in multiple reaction monitoring (MRM) mode and the transitions and retention times are described in Table 1. Concentrations were quantified from the analyte to ISTD peak area ratio. Peak areas were integrated using LabSolutions Version 5.109 software (Shimadzu Corporation).

Chromatography was achieved with a Poroshell 120, EC-C18, 3.0 x 100 mm, 2.7 µm column (Agilent Technologies). Mobile phase A consisted of water containing 0.1 % formic acid and mobile phase B consisted of acetonitrile. A gradient elution program with a flow rate of 0.450 mL/min was followed. 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 and autosampler temperature at 15 °C. The autosampler needle rinse consisted of methanol and water (80:20, v/v). MRM transitions and retention times for each analyte are summarized in Table 1.

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. Nine WS solutions for STDs were prepared in methanol to achieve concentrations of 0.219, 0.438, 0.875, 1.75, 3.50, 7.00, 14.0, 28.0 and 56.0 µg/mL for thiafentanil and 0.781, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0, 100 and 200 µg/mL for naltrexone. For QC samples, WS solutions containing 0.125, 0.500, 1.40 and 28.0 µg/mL of thiafentanil and 0.446, 1.79, 50 and 100 µg/mL of naltrexone were prepared.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Exact mass</th>
<th>MRM Transitions</th>
<th>Retention time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiafentanil</td>
<td>416.17</td>
<td>417.00 &gt; 113.05, 417.00 &gt; 252.05, 417.00 &gt; 357.15</td>
<td>2.66</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>341.16</td>
<td>342.05 &gt; 324.15, 342.05 &gt; 270.00, 342.05 &gt; 282.15</td>
<td>3.00</td>
</tr>
<tr>
<td>Diprenorphine (ISTD)</td>
<td>425.45</td>
<td>426.35 &gt; 187.10, 426.35 &gt; 408.15, 426.35 &gt; 372.20</td>
<td>2.48</td>
</tr>
</tbody>
</table>
prepared in methanol.

STDs and QCs for validation batches and quantitative analysis were prepared by spiking the respective WS into human plasma with lithium-heparin as anticoagulant not exceeding 5% of the total plasma volume. STDs and QCs were aliquoted into 50 µL aliquots and frozen at -80 °C until required. The final concentration of STDs in human plasma consisted of 4.37, 9.19, 17.5, 35.0, 70.0, 140, 280, 560, and 1120 ng/mL of thiafentanil and 15.6, 32.8, 62.5, 125, 250, 500, 1000, 2000, and 4000 ng/mL of naltrexone. Final concentrations for QC lowest limit of quantitation (LLOQ), QC low (QCL), QC medium (QCM) and QC high (QCH) consisted of 4.38, 12.5, 448, and 896 ng/mL of thiafentanil and 15.6, 44.6, 1600, and 3200 ng/mL of naltrexone, respectively.

2.5. Sample preparation

Samples were thawed at room temperature (20 °C) before being processed for LC-MS/MS analysis. All samples were extracted using a protein precipitation by adding 200 µL of ice-cold acetonitrile containing 600 ng/mL of ISTD to 50 µL plasma sample. The sample was vortex mixed at maximum speed for 90 s and then centrifuged at 23 °C for 10 min at 16 000 x g. The supernatant was then transferred to a 96-well plate for analysis.

2.6. Collection of pharmacokinetic samples

This analytical method was applied to heat-inactivated goat plasma samples collected as part of a pharmacokinetic study. The study consisted of a two-way cross-over study involving eight goats injected with 90 µg/kg of thiafentanil intravenously (i.v.) in the first event and intramuscularly (i.m.) in the second event. In both events the immobilisation was reversed with 10 mg of naltrexone administered i.v. for each milligram of thiafentanil. Samples were collected in lithium-heparin tubes at 3, 5, 7, 10, 15, 20, 30, 40, 45, 50, 60, 75, 90, and 120 min post thiafentanil administration. Samples were centrifuged at 1000 x g for 10 min and the plasma was collected. A 50 µL subsample was accurately pipetted into a 1.5 mL polypropylene tube in which the extraction was executed due to the necessary heat inactivation treatment. During analysis of samples, care was taken never to exceed the batch size of the validation batches.

2.7. Heat inactivation

This analytical method was developed to analyse pharmacokinetic samples collected from goats located in a FMD control zone. To inactivate any potential FMD contamination in the samples, samples were required to be heated to 70 °C for 30 min prior to transportation. Once transported to the analytical laboratory, these samples were then extracted and analysed using the developed analytical method. The heat inactivation treatment was evaluated during the validation process to determine any effect on the stability of the analyte in matrix, selectivity, sensitivity, matrix effect and recovery.

2.8. Method validation

2.8.1. Linearity, intra- and inter-day validation

The validation of this analytical method was performed in accordance with the guidelines for bioanalytical method validation [21,22]. Intra- and inter-day accuracy and precision, as well as linearity of the analytical method was assessed from three independent batches analysed on three different days. Curves were generated from nine STDs prepared in duplicate and six replicates of each of the LLOQ, QCL, QCM and QCH.

Linearity was assessed by plotting the ratio of the analyte peak area to the ISTD against the theoretical concentration. Calibration curves fitted a quadratic regression with a 1/C² and 1/C (C represented concentration) weighting for thiafentanil and naltrexone, respectively.

2.8.2. Cross-validation for goat plasma

Applicability of the developed method to goat plasma was assessed by performing a cross-validation using LLOQ, QCL, QCM and QCH prepared in drug-free goat plasma. These QCs were quantified from the calibration curve prepared in human plasma.

2.8.3. Dilution integrity

The integrity of diluted samples was assessed by preparing six QCs containing 2240 ng/mL and 8000 ng/mL of thiafentanil and naltrexone, respectively. These QCs were diluted 1:4 (v/v) post-extraction with analyte-free extracted plasma containing ISTD. A post-extraction dilution was evaluated due to the nature of the samples which originated in a FMD control zone and had to be heat inactivated prior to transfer to the site of analysis. Once the samples have been heat-inactivated, it is not possible to pipette them.

2.8.4. Carryover, selectivity and sensitivity

Carryover was assessed by inspecting the blank and double blank samples analysed immediately after the highest standard in each validation and analytical batch for peaks at the retention time of the analytes.

Human plasma sourced from six different individuals was used to assess both the selectivity and sensitivity of the method. These parameters were assessed for untreated and heat-inactivated samples. To assess selectivity, blank samples were analysed. Each chromatogram was visually inspected at the respective retention time of each analyte to check for co-eluting peaks. Sensitivity was assessed by spiking each plasma source at the LLOQ. Sensitivity was considered acceptable if the signal to noise ratio was greater than 5.

Furthermore, cross-talk experiments were performed by injecting the ULQ of thiafentanil (1120 ng/mL) and naltrexone (4000 ng/mL), as well as the working concentration of the ISTD (600 ng/mL), individually in 6-fold, as previously described [23].

2.8.5. Matrix effect, recovery and process efficiency

Matrix effects, process efficiency and recovery were assessed based on the methodology and acceptance criteria described by Matuszewski et al. [24]. These parameters were evaluated for untreated and heat-inactivated plasma samples sourced from six different human individuals. The matrix effect was evaluated from drying extract from blank plasma (untreated and heat-inactivated) and reconstituting it with an acetonitrile–water mixture (4:1, v/v) spiked with theoretical QCL, QCM and QCH analyte concentrations accounting for dilution steps during extraction. For each plasma source, a regression slope was generated from the ratio of the analyte to ISTD across the concentrations.

Recovery was assessed by extracting untreated and heat-inactivated QCL, QCM, and QCH samples prepared from each plasma source. The analyte peak area ratios were compared to the matrix effect QCs spiked post-extraction, as described above.

Process efficiency was calculated by comparing the average peak area ratio of analyte to ISTD from the QCs spiked pre-extraction (recovery samples, described above) to the corresponding pure stock solutions which were used to spike the matrix effect QCs post-extraction. The results are presented as the percentage process efficiency.

2.8.6. Stability evaluations

All stability evaluations were performed in six-fold at QCL and QCH concentrations. Freeze-thaw stability was evaluated by exposing QCs to three cycles of freezing at -80 °C for a minimum of 60 min, followed by thawing at room temperature for a minimum of 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 for six hours. The effect of freezing samples was evaluated by analysing QCs which had been frozen at -80 °C four days prior to analysis against freshly prepared calibration standards.
3. Results and discussion

3.1. Linearity, intra and inter-day validation

A summary of the intra- and inter-day accuracy and precision data for thiafentanil and naltrexone QCs prepared in human and goat plasma is presented in Table 2. For QCs prepared in human plasma intra- and inter-day accuracy ranged between 94.8 – 103.8 % for thiafentanil and 91.8 – 104.3 % for naltrexone, while the precision was ≤ 8.4 % for thiafentanil and ≤ 11.4 % for naltrexone. Intra- and inter-day accuracy of STDs was between 96.2 – 105.5 % for thiafentanil and between 96.2 – 106.7 % for naltrexone and precision was ≤ 11.4 % for thiafentanil and ≤ 10.1 % for naltrexone.

Calibration curves for thiafentanil and naltrexone yielded good linearity over the concentration ranges with mean correlation coefficients (R² values) of 0.9973 for thiafentanil and 0.9992 for naltrexone. Representative chromatograms at the LLOQ and upper limit of quantification (ULOQ) are represented in Fig. 1.

3.2. Cross-validation in goat plasma

The accuracy of QCs prepared in goat plasma ranged between 89.0 – 100.5 % for thiafentanil and 89.0 – 98.0 % for naltrexone while the precision was ≤ 11.6 % for thiafentanil and ≤ 12.3 % for naltrexone (Table 2). This cross-validation indicates that it is possible to measure these analytes accurately in goat plasma against calibration standards prepared in human plasma. Successful inter-species cross-validations on these analytes accurately in goat plasma against calibration standards prepared in human plasma.

3.3. Dilution integrity

Diluted QCs had an accuracy and precision of 102.7 % (5.6 % CV) for thiafentanil and 104.3 % (3.0 % CV) for naltrexone, respectively. This demonstrated dilution integrity should samples have concentrations greater than the ULOQ. Typically, samples are diluted with blank matrix and then extracted. Our approach had to be adapted since collected samples were from heat-inactivated goat plasma. Once heat-inactivated, goat plasma was difficult to pipette accurately, necessitating a dilution post-extraction with blank extracted matrix containing ISTD.

3.4. Carryover, selectivity and sensitivity

Representative chromatograms for thiafentanil and naltrexone in blank and double blank untreated QCs are shown in Fig. 1. No carryover for the analytes or ISTD was present in the blank and double blank of untreated and heat-inactivated samples, respectively. Furthermore, negligible cross-talk was observed between analytes when injecting the ULOQ of thiafentanil and naltrexone, as well as the working concentration factor was calculated from the ratio of average recoveries of untreated QCs to recoveries of heat inactivated QCs. The respective correction factors for thiafentanil and naltrexone were 1.60 and 1.74, respectively and were only applied to the heat-inactivated goat samples.

Mean process efficiency for thiafentanil and naltrexone in untreated QCs was 109.0 % (7.1 % CV) and 114.0 % (7.3 % CV), respectively. As expected, process efficiency was lower for heat-inactivated QCs, with a mean of 68.4 % (11.8 % CV) for thiafentanil and 67.9 % (10.9 % CV) for naltrexone.

These values indicate that our method has a high recovery and acceptable matrix effects for the anticipated application of the method. Furthermore, due to the consistent recovery across all concentrations tested a correction factor can be applied to the thiafentanil and naltrexone concentrations to compensate for analyte loss during heat-inactivation.

3.5. Matrix effect, recovery and process efficiency

The mean CV of the regression slopes for thiafentanil and naltrexone in untreated samples were 5.5 % and 4.3 %, respectively. Similarly, the mean CV of the regression slopes for heat-inactivated samples was 7.3 % for thiafentanil and 6.4 % for naltrexone. The increased matrix effect in the heat-inactivated samples could be improved with a stable isotopically labelled (SIL) ISTD. Unfortunately, a SIL ISTD is not available for thiafentanil, but could be included for naltrexone to reduce the observed increased matrix effects in the heat-treated plasma.

The mean recovery for thiafentanil was 95.9 % (1.3 % CV) and 91.9 % (4.7 % CV) for naltrexone in untreated QCs. For heat inactivated QCs, the mean recovery was 60.1 % (6.0 % CV) for thiafentanil and 54.6 % (3.4 % CV) for naltrexone. The high precision of the recovery observed across the high, medium and low QCs demonstrated that this method was well-suited for simultaneously extracting these analytes from both untreated and heat-inactivated matrix. Because of the difference in recoveries between untreated and heat-inactivated QCs, as well as the observed consistent recovery across concentrations, we applied a correction factor to the heat-inactivated goat samples to provide a true representation of the drug concentrations prior to heating. This correction factor was calculated from the ratio of average recoveries of untreated QCs to recoveries of heat inactivated QCs. The respective correction factors for thiafentanil and naltrexone were 1.60 and 1.74, respectively and were only applied to the heat-inactivated goat samples.

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3.6. Stability evaluations

Thiafentanil and naltrexone exhibited acceptable benchtop stability with accuracy between 94.9 – 106.0 % for thiafentanil and 98.9 – 102.9 % for naltrexone, with all CV less than 9.5 % (Table 3). Similar benchtop stabilities have been achieved for naltrexone in previous studies [27,28]. Analytes demonstrated acceptable freeze-thaw stability with accuracy between 95.0 – 99.3 % for thiafentanil and 98.9 – 103.4 % for naltrexone, with all CV less than 12.1 % (Table 3). Similar

The analytical method showed suitable sensitivity with a signal to noise ratio of greater than 5 in the LLOQ samples from 6 different plasma sources for both untreated and heat-inactivated samples (Fig. 1).

Table 2 Intra- and inter-day accuracy (%) and precision (%CV) of thiafentanil and naltrexone at the lowest limit of quantification (LLOQ), low (QCL), medium (QCM) and high (QCH) QCs prepared in human and goat plasma.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>QC Level</th>
<th>Concentration (ng/mL)</th>
<th>Intra-day (n = 6)</th>
<th>Inter-day (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Goat plasma</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Accuracy (%)</td>
<td>CV (%)</td>
</tr>
<tr>
<td>Thiafentanil</td>
<td>LLOQ</td>
<td>4.38</td>
<td>94.5</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>QCL</td>
<td>12.5</td>
<td>100.5</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>QCM</td>
<td>448</td>
<td>95.7</td>
<td>7.1</td>
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<tr>
<td></td>
<td>QCH</td>
<td>896</td>
<td>89.0</td>
<td>9.1</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>LLOQ</td>
<td>15.6</td>
<td>92.2</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>QCL</td>
<td>44.6</td>
<td>94.4</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>QCM</td>
<td>1600</td>
<td>89.0</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>QCH</td>
<td>3200</td>
<td>98.0</td>
<td>8.3</td>
</tr>
</tbody>
</table>
Fig. 1. Representative chromatograms from untreated (not heat inactivated) samples for thiafentanil at the ULOQ (A) and LLOQ (B), naltrexone at the ULOQ (C) and LLOQ (D), thiafentanil (E) and naltrexone (F) blanks, the ISTD in the double blank (G) and in a QC (H); and thiafentanil (I) and naltrexone (J) in heat-inactivated goat plasma samples.

Table 3
Summary of stability data of thiafentanil and naltrexone in high (QCH) and low (QCL) quality control samples prepared from human plasma.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>QC Level</th>
<th>Concentration (ng/mL)</th>
<th>Benchtop (Room temperature for six hours)</th>
<th>Freeze thaw (three cycles)</th>
<th>Stability at −80 °C for four days</th>
<th>Heat inactivation (70 °C for 30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Accuracy (%)</td>
<td>CV (%)</td>
<td>Accuracy (%)</td>
<td>CV (%)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td>Thiafentanil</td>
<td>QCL</td>
<td>4.38</td>
<td>94.9</td>
<td></td>
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<tr>
<td></td>
<td>QCH</td>
<td>3200</td>
<td>98.9</td>
<td></td>
<td>6.4</td>
<td>103.4</td>
</tr>
</tbody>
</table>
freeze–thaw stability has been reported for naltrexone [28]. This indicated that both analytes were stable on bench for up to six hours and for three freeze–thaw cycles.

Heat inactivated QCs without the application of a correction factor, had an accuracy of between 53.1 – 55.2 % for thiafentanil and 45.5 – 49.8 % for naltrexone, with precision ≤ 16.0 % for thiafentanil and ≤ 11.9 % for naltrexone (Table 3). It is evident that the necessary heat inactivation prior to transportation has an impact on the stability of the analytes. To correct for the loss of analyte when heat-inactivated, the correction factor determined during the recovery experiment was applied to the goat plasma samples collected for the pharmacokinetic study. Unfortunately, this heat inactivation step was unavoidable in the context of this study. For future studies, heat inactivation should ideally be avoided if possible. This could be done if samples are collected from animals that are not cloven hooved or are not located in a disease control area.

QCs remained stable when stored at −80 °C for four days. The accuracy for thiafentanil was between 97.1 – 97.9 % and 88.0 – 94.9 % for naltrexone with a precision of ≤ 11.8 % and ≤ 11.7 % for thiafentanil and naltrexone, respectively (Table 3).

Frozen QCs analysed against a freshly prepared calibration curve within a validation batch demonstrated accuracy between 91.9 – 97.9 % for thiafentanil and precision ≤ 11.8 % for all QCs except the LLOQ which had a precision of 17.5 %. Similarly, accuracy was between 88.8 – 94.9 % for naltrexone with precision ≤ 13.5 %. This met acceptable accuracy and precision criteria which indicated that freezing the samples had no effect on their concentrations and that STDs and QGs could be prepared in advance and stored at −80 °C until required.

3.7. Applicability to pharmacokinetic study

This method provided adequate sensitivity to detect thiafentanil in heat-inactivated goat plasma at concentrations above the LLOQ for up to 75 min in both the i.v. and i.m. administration events and naltrexone at concentrations above the LLOQ for up to 90 min post thiafentanil administration. Respective correction factors determined during recovery were applied to the concentrations detected and adjusted concentrations are reported. The highest concentration obtained for thiafentanil was 142 ng/mL in the i.v. event and 78.4 ng/mL in the i.m. event. Similarly, the highest concentration obtained for naltrexone was 268 ng/mL after the i.v. administration of thiafentanil and 102 ng/mL after the i.m. administration of thiafentanil. Plasma concentrations of naltrexone depleted quickly and reached the LLOQ much faster than anticipated in comparison to mean plasma concentrations previously reported for naltrexone detected in goat and eland which were > 10 ng/mL at three and four hours post carfentanil administration, respectively [1,18].

This method is suitable for the quantification of thiafentanil and naltrexone in plasma for pharmacokinetic studies. The detected concentrations of both analytes indicated that the ULOQ in future methods can be significantly reduced for similar dosing strategies. Due to no information on the anticipated concentrations for this drug combination, a broad calibration range was applied.

3.8. Method limitations

A limitation of this study was that WS for STDs and QCs were prepared from the same stock solution. Thiafentanil presents unique safety concerns to laboratory staff as it is an ultra-potent opioid with low doses required to produce a pharmacological effect. To mitigate risks while working with this analyte, a limited quantity of a single stock solution was received from Wildlife Pharmaceuticals Pty. Ltd, meaning that stock solution accuracy and stability results could not be generated. However, stock solution stability for thiafentanil and naltrexone have previously been reported for 8 months and 100 days at 4 °C, respectively [19,29]. A further limitation of this study is that long-term stability of thiafentanil and naltrexone in matrix at −80 °C should be evaluated beyond 4 days to mimic the length of storage of samples for pharmacokinetic studies. Naltrexone has previously been reported to be stable in human plasma for up to 30 days at −70 °C [28]. Although autosampler stability was not evaluated in this study, both analytes have previously been reported to be stable on-instrument at 4 °C for up to 24 h [19,27].

Although stability assessments were performed to evaluate the freeze–thaw stability, benchtop stability and the effect of freezing samples, these evaluations were only performed on QCs that were not heat-treated. Considering that the heat-treatment does have an effect on the observed concentrations of thiafentanil and naltrexone, future work should investigate these stability evaluations for heat-treated samples.

4. Conclusion

The agonist–antagonist combination of thiafentanil reversed with naltrexone is a valuable tool for wildlife immobilisation. However, to the best of the authors’ knowledge, the pharmacokinetics of thiafentanil in wildlife remain unknown. This report describes the development and validation of an LC-MS/MS method for the quantification of both analytes in human plasma using diprenorphine as ISTD. Furthermore, the method was successfully cross validated against QCs in goat plasma to ensure applicability to veterinary samples. For the pharmacokinetic study in goats, samples had to undergo an unavoidable heat inactivation step according to regulations governing the transport of samples from FMD control areas. However, this limitation is acknowledged, and as far as possible, mitigated by determining the impact of the heat inactivation on matrix effects, recovery and stability of the analytes. This has enabled the establishment of a correction factor to determine the original analyte concentrations within pharmacokinetic samples. As previously mentioned, in the South African context many wildlife species are found in FMD control zones, thus the implication of a heat inactivation treatment on analyte concentrations is an important consideration for future application of this method to wildlife species.

With the exception of matrix effects which fell marginally outside of acceptance criteria, the analytical method for the quantitation of thiafentanil and naltrexone met published FDA [22] and EMA [21] validation criteria and could be further optimized in future with the inclusion of a SIL ISTD for naltrexone. Given the challenges of working with heat-treated samples and highly potent pharmaceutical agents, this method was deemed to be acceptable within the context of its veterinary application. Despite challenges and limitations discussed, this is the first report of an analytical method for the simultaneous quantification of thiafentanil and naltrexone for application to a pharmacokinetic study. Furthermore, it is the first method to cross-validate the detection of these analytes in another species and evaluate the heat-inactivation treatment necessary in the veterinary context of the intended pharmacokinetic study.

CRediT authorship contribution statement

Judith T. Christie: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization, Project administration, Funding acquisition. Mieghan Bruce: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. Silke Pfitzer: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing. Liesel Laubscher: Conceptualization, Investigation, Project administration, Resources, Supervision, Writing – review & editing. Michael Laurence: Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing, Conceptualization. Tracy Kellermann: Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – review & editing.
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 financial support and equipment, drugs, or supplies were provided by Wildlife Pharmaceuticals Pty. Ltd. Liesel Laubscher reports financial support was provided by Wildlife Pharmaceuticals Pty. Ltd. Jacobus P Raath reports financial support was provided by Wildlife Pharmaceuticals Pty. Ltd. Judith T Christie reports a relationship with Wildlife Pharmaceuticals Pty. Ltd. that includes: funding grants. Liesel Laubscher reports a relationship with Wildlife Pharmaceuticals Pty. Ltd. that includes: employment. Jacobus P Raath reports a relationship with Wildlife Pharmaceuticals Pty. Ltd. that includes: employment. Judith T Christie reports financial support for the Murdoch University Strategic Partnership Scholarship was provided by Wildlife Pharmaceuticals Pty Ltd. Liesel Laubscher is employed as the Product Specialist at Wildlife Pharmaceuticals Pty Ltd. Jacobus P Raath is the Managing Director at Wildlife Pharmaceuticals Pty Ltd.

Data availability

Data will be made available on request.

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