



# Final Report

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## A GLP tissue residue depletion study in sheep following oral / buccal administration of meloxicam

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## Abstract

Determination of the residue depletion profile of meloxicam was an essential part of the product development process as it allows a withholding period (WHP) and export slaughter interval (ESI) to be established to satisfy product registration requirements.

Forty sheep were allocated to 8 groups (six groups of 6 and two groups of 2) One of the groups of 2 acted as the untreated control group, the other contained treated spares. Sheep were treated with the test formulation according to individual bodyweight and sacrificed at pre-determined time intervals. Following euthanasia, muscle, liver, kidney, peri-renal fat and masseter muscle (treatment side) tissue samples were collected, processed, frozen and sent for analysis.

Analysis of meloxicam in ovine tissues was performed using a validated liquid chromatography – tandem mass spectrometry (LC/MS/MS) method. Sample preparation was based on acidic hydrolysis prior to neutralisation. Following addition of an internal standard and extraction into an organic solvent by salt-induced liquid-liquid partitioning the samples were analysed by LC/MS/MS.

The analytical methodology had a validated limit of quantitation (LOQ) of 5.0 µg/kg. A limit of detection (LOD) of 1.08 µg/kg, 1.26 µg/kg, 1.29 µg/kg, 1.29 µg/kg and 1.76 µg/kg was established for liver, kidney, muscle, masseter muscle and peri-renal fat respectively. The results showed that there were no detectable residues for meloxicam in any tissue matrix from Day 10 onwards post treatment.

## Executive Summary

Various forms of surgical husbandry procedures are necessary in sheep under good animal husbandry practices. Animal welfare, in particular pain management, following surgical husbandry procedures has evolved into a political issue that could impact the Australian sheep meat and sheep wool industries. While alternate technologies are developed to minimise invasive procedures, there will always be a need for non-steroidal anti-inflammatory drug (NSAID) therapy in sheep and currently, no NSAID is registered in Australia for use in this species. Any use of NSAIDs in sheep is 'off-label'. The additional issue is that NSAID therapy is currently administered by injection. This is undesirable from an on-farm occupational health and safety risk minimisation perspective.

Meloxicam, an oxicam derivative, is a selective cyclooxygenase-2 inhibitor and potent NSAID. It is used as a veterinary drug for dogs, cats, cattle, pigs and horses either as an injectable solution or oral suspension.

Buccal administration of specifically formulated medications can result in rapid absorption. Comparison of the bioavailability of oral and buccal meloxicam formulations administered to sheep showed that high serum levels of meloxicam were detected within 8 minutes of buccal dosing. These levels approximate reported therapeutic levels in other species.

A residue depletion study was required to establish a meat withholding period (WHP) and export slaughter interval (ESI) to satisfy product registration requirements. The GLP (Good Laboratory Practice) study described in this report determined the meloxicam tissue residue depletion profile in sheep following buccal administration of a meloxicam formulation (10 mg meloxicam/mL) at the maximum proposed dose rate of 1 mg meloxicam per kg bodyweight.

The study complied with national and international standards such as the OECD's Series on Principles of Good Laboratory Practice and Compliance Monitoring; APVMA RGL23 and VICH GL1 and GL49.

Forty sheep were randomly allocated to 8 groups (six groups of 6 and two groups of 2) One of the groups of 2 acted as the untreated control group, the other contained treated spares. Sheep were treated with the test formulation according to individual bodyweight at a dose rate of 1mg meloxicam/kg bodyweight and sacrificed at pre-determined time intervals. Following euthanasia, muscle, liver, kidney, peri-renal fat and masseter muscle (treatment side) tissue samples were collected, processed, frozen and sent for analysis.

Analysis of meloxicam in ovine tissues was performed using a validated liquid chromatography – tandem mass spectrometry (LC/MS/MS) method. Sample preparation was based on acidic hydrolysis prior to neutralisation. Following addition of an internal standard and extraction into an organic solvent by salt-induced liquid-liquid partitioning the samples were analysed by LC/MS/MS.

The analytical methodology had a validated limit of quantitation (LOQ) of 5.0 µg/kg. A limit of detection (LOD) of 1.08 µg/kg, 1.26 µg/kg, 1.29 µg/kg and 1.76 µg/kg was established for liver, kidney, muscle, masseter muscle and peri-renal fat respectively. The results showed that there were no detectable residues for meloxicam in any tissue matrix from (and inclusive of) Day 10 onwards post-treatment.

## 1. Introduction

This report summarises the animal and analytical component of a study investigating the residue depletion profile of meloxicam in a new formulation when administered as a buccal preparation in sheep. The study was commissioned by Troy Laboratories Pty Ltd.

## 2. Background

Various forms of surgical husbandry procedures are necessary in sheep under good animal husbandry practices. Animal welfare, in particular pain management, following surgical husbandry procedures has evolved into a political issue that could impact the Australian sheep meat and wool industries. While alternate technologies are being developed to minimise invasive procedures, there will always be a need for non-steroidal anti-inflammatory drug (NSAID) therapy in sheep. Currently, no NSAID is registered in Australia for use in sheep so any use of NSAIDs in this species is 'off-label'. An additional issue is that NSAID therapy is currently administered by injection. This is undesirable from an on-farm occupational health and safety risk minimisation perspective.

Buccal administration of specifically formulated medications can result in rapid absorption. Previously conducted feasibility studies compared the bioavailability of oral and buccal meloxicam formulations administered to sheep. Results showed that high serum levels of meloxicam were detected within 8 minutes of buccal dosing. These levels approximate reported therapeutic levels in other species. It was proposed that administering meloxicam to sheep via the buccal cavity could provide easy, quick, effective and safe pain management and inflammation control for sheep following surgical husbandry procedures such as castration and tail docking.

Meloxicam is an oxicam derivative and a NSAID, with anti-inflammatory, antipyretic and analgesic properties. Unlike traditional non-selective NSAIDs, meloxicam preferentially inhibits the activity of cyclooxygenase-2 (COX-2), resulting in a decreased conversion of arachidonic acid into prostaglandin precursors. The resulting decrease in prostaglandin synthesis is responsible for the therapeutic effects of meloxicam.<sup>1</sup>

Before a veterinary chemical product can be legally supplied, sold, or used in Australia it must be registered by the Australian Pesticides and Veterinary Medicines Authority (APVMA). As previously mentioned there are currently no NSAIDs registered for use in sheep. Various studies to support safety and efficacy, and to determine the pharmacokinetic/metabolic profile and residue depletion profile of meloxicam were required for registration of a product for use in sheep in Australia.

Determination of the residue depletion profile of animal health treatments allows the APVMA to set appropriate withholding periods (WHP) and export slaughter intervals (ESI) to protect both human health and agricultural trade.

Ref. 1 [www.cancer.gov/templates/drugdictionary/?CdrID=472195](http://www.cancer.gov/templates/drugdictionary/?CdrID=472195)

### **3. Objective**

The objective of this study was to determine the tissue residue depletion profile of meloxicam in sheep following buccal administration of a specifically formulated product at the recommended dose rate of 1 mg/kg bodyweight.

### **4. Methodology**

#### **4.1 Animal Phase**

Thirty-eight trial sheep were included in the study and were weighed, condition scored, and sex and breed recorded. Animals were sorted by breed, sex and then descending order of bodyweight. Two animals, with bodyweight closest to the group mean for their respective breed, were allocated to the untreated control group. Within each breed, the remaining thirty-six animals were sequentially blocked into groups of six and randomly allocated to treatment groups.

Dose volumes were calculated based on the animals' individual bodyweight on the day of treatment. The product was administered at a dose rate at 1 mg meloxicam per kg bodyweight. The dose was administered into the sulcus between the molar teeth and inside of the cheek.

All animals were observed at selection and treatment and at approximately 2 and 24 hours post-treatment. Each group of animals was observed again prior to, or at the point of, sacrifice. The two sheep from the untreated control group were euthanased and tissue samples collected prior to treatment of the other groups.

Duplicate tissue samples of muscle, kidney, liver, peri-renal fat and masseter (cheek) muscle (treatment side only) were collected from each animal at pre-defined time intervals. Tissue samples were processed prior to storage at -18°C. Masseter muscle samples were cryoprocessed prior to storage at -18°C.

All samples were dispatched frozen for analysis. Replicate 1 samples were packed with ice-bricks and an accompanying data-logger for same day delivery to the analytical laboratory. Samples for analysis were shipped in two batches, on Day 7 and Day 15 of the study. Replicate 1 samples were stored frozen at the analytical laboratory for 4 months after submission of results and after this time were destroyed. Replicate 2 samples will be stored frozen at the animal phase facility for a period of 12 months after the last sample collection time-point, after which they will be disposed of.

#### **4.2 Analytical Phase**

Blank sheep tissue samples were obtained from healthy sheep unrelated to the study and used as blank matrix for preparation of the standard curve, quality control (QC) samples, validation samples (VS) and blanks. Samples were received in two batches and all samples were confirmed frozen and accounted for.

Analyses were conducted on tissues that had been prepared as a powdered sample using cryogenic homogenisation with dry ice. Prior to analyses, test sample homogenates were removed from the freezer and immediately weighed into uniquely labelled centrifuge tubes for sample extraction.

Acidic hydrolysis was followed by neutralisation. After neutralisation the internal standard was added to all samples and this was followed by extraction with an organic solvent. Addition of inorganic and organic salts induced partitioning of the residues into the organic layer. An aliquot of the purified extract was then filtered into an auto-sampler vial for subsequent injection and analysis.

After extraction, meloxicam and the IS piroxicam were adequately resolved from each other and matrix interferences on an Acquity UPLC 50mm C<sub>18</sub> column using a gradient mixture of 0.1% formic acid in water and acetonitrile and a flow rate of 0.4mLmin<sup>-1</sup>. Run time was minimized to 4.5 minutes and the injection volume was 5µL. A tandem quadrupole detector was used with positive electrospray ionisation in multiple reaction monitoring (MRM) mode. Under these conditions the retention time of meloxicam was 2.20 minutes and that of piroxicam was 1.85 minutes, deemed suitable for the processing of adequate sample numbers by a single analyst to ensure minimum time between start and completion of the project.

Extracts were assayed with blank and QC samples spaced evenly throughout the run. QC samples were prepared by fortifying blank tissue with meloxicam, at selected concentrations within the linear range of the assay and extracted as described above. Matrix matched standards were used to generate the calibration curve used to quantitate the meloxicam in incurred samples.

Analytical methodology for the determination of meloxicam concentrations in tissues was validated according to VICH GL49 – Studies to evaluate metabolism and residue kinetics of veterinary drugs in food-producing animals: validation of analytical methods used in residue depletion studies. The following validation parameters were assessed:

- Selectivity
- Linearity
- Limit of detection (LOD)
- Limit of quantitation (LOQ)
- Accuracy and precision
- Matrix long-term frozen storage
- Robustness

### **Selectivity**

Selectivity is the ability of a method to distinguish between the analyte being measured and other substances that may be present in the sample matrix. Selectivity was assessed by comparison of chromatographic profiles of prepared samples with and without the addition of meloxicam. The assay was regarded as selective for meloxicam because the presence of any interfering peaks at the retention time of meloxicam contributed an equivalent area of less than 20% of the meloxicam peak in the lowest validation sample concentration (LOQ).

### **Linearity**

Linearity is the ability of a method, within a defined range, to obtain results that are directly proportional to the concentration of the analyte in the sample. System linearity was accepted where a statistical analysis gave a coefficient of determination of  $r^2 > 0.99$  using a minimum of five (5) standards. Residuals for calibration standards were  $\leq 20\%$  for all assays.

The calibration system was based on internal standardisation - where a known amount of a structurally similar reference compound was added to all samples and standards in identical proportions. A response ratio of the analyte to internal standard is produced following instrumental determination, which can partially offset variations in sample preparation and instrumental parameters. Piroxicam was selected as the internal standard as it has similar spectrochemical properties to meloxicam. Calibration curves were not forced through zero and 1/x weighting was applied to the calibration.

#### **Limit of Detection (LOD)**

The LOD is defined as the mean quantity of analyte, which gives a clearly discernible peak plus 3 times the standard deviation about that mean as a proportion of the analyte response at the lowest validation concentration. The estimated limit of detection for meloxicam in liver, kidney, muscle, masseter muscle and peri-renal fat were found to be 1.08 µg/kg, 1.26 µg/kg, 1.29 µg/kg, 1.29 µg/kg and 1.76 µg/kg respectively.

#### **Limit of Quantitation (LOQ)**

The LOQ corresponds to the lowest measured concentration of the target substance, which can be made with a specified accuracy and precision. The LOQ was determined from the standard curve as the lowest point where the precision of a standard was ≤ 25% and the percentage accuracy was between 60 – 120%. For meloxicam the lowest validation sample concentration in all tissues was 5.0 µg/kg and so this was the LOQ suggested.

#### **Accuracy**

The accuracy was determined by recovery experiments using fortified replicates of blank matrix. The accuracy is described as the closeness of agreement of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy was between 85 – 109% for all tissues at three (3) concentration levels. Results for accuracy were within the prescribed limits for each concentration range.

#### **Precision**

The precision of an analytical method describes the closeness in agreement between mutually independent test results. The precision under repeatability conditions is expressed as a relative standard deviation (%RSD).

Precision results ranged from 3.1 – 10.5% for all tissues at three (3) concentration levels. Results for precision were within the prescribed limits for each concentration range.

#### **Robustness**

Multiple replicates of sample matrix fortified with meloxicam, at two (2) concentration levels, were prepared on 24<sup>th</sup> April 2012 and frozen at -20°C. Three weeks later a set of samples was removed from the freezer and thawed at room temperature. These samples were assayed against a freshly prepared standard curve to assess the long-term frozen storage stability of meloxicam in each tissue matrix. Meloxicam residues were regarded as stable if meloxicam content was within the accuracy acceptance criteria. Percentage accuracy was between 88.7 – 104.6% for all tissues, at two (2) concentration levels. Results were within the prescribed limits for each concentration range.

Based on robustness investigations, the method was found to perform adequately when changes in the instrumental set-up were made or when consumables used for the application of the assay were varied.



The method performance was found to be well within the acceptance criteria when performed on different days over the duration of the analytical phase.

## Trial Samples

The animal phase of the trial commenced on 23<sup>rd</sup> April 2012. Tissue samples were dispatched to Advanced Analytical in two (2) batches. The first batch of samples was sent and received on 7<sup>th</sup> May 2012 and the second batch of samples was sent and received on 15<sup>th</sup> May 2012. Samples were confirmed as being frozen upon receipt and all samples were accounted for. All analyses were conducted within one month of sample collection and receipt.

Waters™ “Mass Lynx” software was used for instrument control, data logging, peak integration and raw data analysis for all samples. Quantities of meloxicam were calculated from standard curves based on a least squares regression of the respective peak area ratio of meloxicam versus piroxicam against the content of meloxicam in the standard. An internal calibration was generated to quantitate residue concentrations. Calibration curves were not forced through zero and a 1/x weighting was applied to the calibration.

The concentrations of meloxicam have been corrected for the weights of sample homogenate taken for analysis. Residue concentrations were presented as meloxicam in µg/kg. Results were not corrected for recovery.

All electronic information, including raw/processed data and chromatograms are maintained under unique identification (study number, assay number, sample ID) on computer. Back-up of electronic data was performed regularly during and on completion of the study.

On completion of the study the relevant laboratory notebooks, calibration details, sample storage temperature records, electronic copies, chromatographic data and a hardcopy of the analytical phase report were held under security in the Advanced Analytical Australia GLP Archive at North Ryde.

## 5. Results

Tissue samples were analysed for residues of meloxicam using a validated method. The analytical methodology had a validated limit of quantitation (LOQ) of 5.0 µg/kg. A limit of detection (LOD) of 1.08 µg/kg, 1.26 µg/kg, 1.29 µg/kg, 1.29 µg/kg and 1.76 µg/kg was established for liver, kidney, muscle, masseter muscle and peri-renal fat respectively.

There was no significant difference between group mean bodyweights prior to administration of the test item. The trial comprised of 6 groups of 6 animals and 2 groups of 2 animals. One group of 2 animals (Group 1) acted as the untreated control group.

The results showed that there were no detectable residues for meloxicam in any tissue matrix from (and inclusive of) Day 10 onwards post-treatment.

No adverse events occurred during the conduct of the study.

## **6. Conclusion**

This study investigated the residue depletion profile of meloxicam in sheep following buccal administration of a specifically formulated product. The dose rate was 1 mg/kg bodyweight and animals were treated according to their individual bodyweights.

No detectable residues for meloxicam were found in any tissue matrix from (and inclusive of) Day 10 onwards post-treatment. The formulation was found to be safe in sheep at the recommended dose rate.