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The absorption, distribution, metabolism and excretion study of radiolabelled meloxicam in sheep following trans-mucosal delivery

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Abstract

Meloxicam is a nonsteroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic effects. The objectives of the study were:

- To determine the rates and routes of excretion of [^{14}C]meloxicam and its metabolite(s);
- To determine the tissue distribution of radioactivity;
- Where residue levels dictated, to examine the profiles of radioactivity in samples using HPLC, with on- or off-line radioactivity measurement, and TLC;
- Where residue levels dictated, to determine the structural identity of the major metabolites to help elucidate the biotransformation pathway.

To satisfy the registration requirements for a new veterinary product for use in a food producing species, a metabolism study was conducted in the target species. The sheep is the target species for the proposed formulation. The oral transmucosal route was used as this is the route of exposure. The safety of the proposed dose rate in sheep was confirmed in a target animal safety study. The selected dose rate was based on the proposed dose rate.

Metabolism studies are used to assess the fate of the chemical in target animals and to assess the nature and disposition of chemical residues in food-producing animals.
The study investigated the nature and quantity of residues in liver, kidney, muscle, fat, and the application site (masseter muscle) obtained from male and female sheep following buccal administration of meloxicam.

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.

Metabolism studies in the target animal should permit an assessment of the quantity and nature of residues in food derived from animals treated with a veterinary drug, and should provide data on:

- the depletion of residues of concern from edible tissues of treated animals at varying times after drug administration
- the individual components, or residues, that comprise the residues of concern in edible tissues
- the residue(s) that can serve as a marker for analytical methods intended for compliance purposes (that is the monitoring of appropriate drug use)
- the ratio of marker residue to total radioactive residues
- the identification of a target tissue or tissues.

The GLP (Good Laboratory Practice) study described in this report investigated the disposition of meloxicam in sheep following a single buccal administration of a radiolabelled meloxicam formulation (10 mg [^{14}C]meloxicam/mL) at the maximum proposed dose rate of 1 mg meloxicam per kg bodyweight.

The study was conducted in accordance with the appropriate test facility standard operating procedures and in compliance with national and international standards such as: The UK Good Laboratory Practice Regulations; OECD Principles of Good Laboratory Practice; OECD Guidelines for the Testing of Chemicals, Metabolism in Livestock; EC Commission Directive 2004/10/EC; VICH GL46; and the APVMA data guideline on metabolism and kinetics.

Sixteen sheep (eight male and eight female) were given a single administration of [thiazole-2-^{14}C] meloxicam at the nominal dose rate of 1 mg/kg bodyweight (bw). Urine, faeces and cage wash samples were collected daily. One group of four sheep (two male and two female) were killed at each of the following time points: 4 hours, 4 days (96 hours), 8 days (192 hours) and 14 days (336 hours). Tissues were taken *post mortem* for quantification and analysis.
Analysis of meloxicam in ovine tissues was performed using a validated liquid chromatography – tandem mass spectrometry (LC/MS/MS) method. Residue identification using accurate mass full

scan and product ion analyses was carried out on selected sheep tissue, urine and faeces samples to screen for the presence of 'typical' or 'predicted' metabolites.

The study showed that the majority of the mean administered radioactivity was excreted in the faeces and urine.

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- Radiosynthesis of [thiazole-2-^{14}C]meloxicam – Quotient Bioresearch (Radiochemicals) Ltd.
- HPLC method transfer and GLP compliant analysis of [thiazole-2-^{14}C]meloxicam – Quotient Bioresearch (Rushden) Ltd.
- In-life, tissue analysis and metabolite analysis – Quotient Bioresearch (Rushden) Ltd.
- Study consultant Dr Merete Holm DVM – Merete Holm Veterinary Consultancy

1. Introduction

This report summarises the GLP metabolism study carried out to investigate the disposition and pharmacokinetics 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.[1]

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.

Metabolism and toxicokinetic or pharmacokinetic studies are submitted to facilitate the human-food-safety evaluation of veterinary drug residues that help to ensure that food derived from animals that have been treated with a veterinary product is safe for human consumption.

Ref. 1 https://pubchem.ncbi.nlm.nih.gov/compound/meloxicam#section=Top

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

MLA is committed to investing in top quality scientific research, performed by suitably qualified, experienced and registered researchers and organisations. In experiments that involve livestock, MLA acknowledges that such research will first need to be assessed, and if deemed relevant, approved by a recognised Animal Care and Ethics Committee (AEC). The responsibility for obtaining AEC approval lies with the researcher. MLA has in the past not specifically asked for evidence that such AEC approval had indeed been obtained.

4.1 Animal Phase

Sixteen sheep (eight male and eight female) were given a single administration of [thiazole-2-^{14}C]meloxicam at the nominal dose rate of 1 mg/kg bodyweight (BW). Dose volumes were calculated based on the animals' individual bodyweight on the day of treatment. The dose was administered into the sulcus between the molar teeth and inside of the cheek. Urine, faeces and cage wash samples were collected daily. One group of four sheep (two male and two female) were euthanased at each of the following time points: 4 hours, 4 days, 8 days, and 14 days. Tissues were taken *post mortem* for quantification and analysis.

Urine, cooled by solid carbon dioxide, was collected into individual, pre-weighed containers. At each collection time point, urine samples were pooled per sex, by group. After pooling, the weight of each urine sample was recorded. Triplicate weighed aliquots were taken for direct quantitative radiochemical analysis (QRA). Urine samples were retained at approximately -20 °C.

Faeces were collected into individual, pre-weighed containers. At each collection time point, faeces samples were pooled per sex, by group. After pooling, the weight of each faeces sample was recorded. Each faeces sample was homogenised with an appropriate amount of water until a consistent slurry was produced and a homogenate weight was taken. Five aliquots of each homogenate were then weighed into separate combustion cones which were combusted prior to liquid scintillation counting (LSC). Faeces samples were retained at approximately -20 °C.

Cage washes were collected into individual, pre-weighed containers. At each collection time point, cage washes were pooled per sex, by group. After pooling, the weight of each cage wash sample was recorded. Triplicate weighed aliquots were taken for QRA. Cage wash samples were stored at ambient temperature.

Serial blood samples were taken from Group 5 (14-day) sheep, for measurement of total radioactivity at the following times: Pre-dose, 0.25, 0.5, 1, 1.5, 2, 3, 4, 8, 12, 24, 48, 72 and 96 hours post-dose. A subsample of each whole blood sample was retained at approximately +4 °C pending QRA and a further aliquot of each whole blood sample was retained. The remaining whole blood samples were centrifuged for harvesting of plasma, and the blood cells were discarded. Plasma samples were stored at -20 °C pending analysis.

Immediately prior to sacrifice, whole blood samples were taken from each animal into lithium heparin tubes. Sheep were euthanased by lethal injection. Each tissue type was trimmed of any extraneous tissue. For each tissue sample type, a pooled sample per group per gender was generated. The weights of each tissue sample were recorded. Following removal of the sheep from the metabolism cage, a cage wash was performed and the sample was retained for analysis. In addition, one male and one female sheep (Group 1 - control), which were not administered with the test item, were euthanased by lethal injection and the collected tissues were retained, pending combustion analysis.

4.2 Analytical phase

4.2.1 Description of the quantitative radiochemical analysis (QRA)

Quantitative analysis of samples was performed by radioactive measurements such as liquid scintillation counting (LSC) and microplate scintillation counting (MSC). Both analytical techniques have been fully validated by Quotient Bioresearch (Rushden) Ltd.

Liquid Scintillation Counting
Aliquots were mixed with scintillant and analysed using a liquid scintillation counter. Limits of quantification were calculated for measurements of total radioactivity in each sample as two times the background (blank) value, as disintegrations per minute (dpm).

Fraction Collection and Microplate Scintillation Counting (Off-Line Radiodetection)
Due to lower levels of radioactivity in some samples, extracts were analysed with on-line fraction collection and off-line radiodetection. Fractions were collected into 96-well plates every 13 seconds for the duration of the analytical run (equivalent to *ca.* 14.7 second fractions). The 96-well plates were evaporated to dryness overnight in a sample evaporator. Radioactivity was counted using a Packard TopCount NXT Counter.

Oxidation Prior to LSC
Samples were combusted using an automated sample oxidizer and any $^{14}CO_2$ produced was trapped and then mixed with the scintillant prior to LSC. Combustion efficiency was determined at the start of each run.

Radioactivity measurements were corrected for counting efficiency using an external set of sealed quench standards. Representative blank specimen values were subtracted from the specimen count rates to give net dpm per specimen

4.2.2 Preparation of standards and samples

Standard Preparation.
A reference standard of unlabelled meloxicam was used in the study. A solution of unlabelled meloxicam was prepared in methanol:water. This solution was used to optimise the mass spectrometer source conditions and MS and MS/MS conditions for the parent compound for qualitative analyses.

Preparation of Urine and Faeces Samples
The sheep urine and faeces samples were analysed as supplied. Samples were removed from the freezer and allowed to thaw in a refrigerator before being vortex mixed thoroughly. An aliquot of urine and of faeces extract was transferred to a clean glass auto sampler vial for analysis.

Preparation of Liver Extracts

The sheep liver extracts were removed from the freezer and allowed to thaw in a refrigerator before being vortex mixed thoroughly. Due to high organic content, some liver extracts were diluted prior to analysis to avoid any potential solvent effects. An aliquot of liver extract was transferred to an Eppendorf tube and deionised water was added to give a 4-fold dilution. The sample was vortex mixed thoroughly and centrifuged at 13,000 rpm for 5 minutes at 4 °C. An aliquot of diluted sample was transferred to a clean glass auto sampler vial for analysis.

Preparation of Other Tissue Extracts

Sheep tissue extracts were removed from the freezer and allowed to thaw in a refrigerator before being vortex mixed thoroughly. Due to the high organic content, the tissue extracts were diluted prior to analysis to avoid potential solvent effects. An aliquot of tissue extract was transferred to an Eppendorf tube and deionised water was added to give a 4-fold dilution. The sample was vortex mixed thoroughly and centrifuged at 13,000 rpm for 5 minutes at 4 °C. An aliquot of diluted sample was transferred to a clean glass auto sampler vial for analysis.

4.2.3 Extraction/fractionation/separation/isolation of the residues

Extraction Procedures

Aliquots of tissue samples were extracted using a range of solvents (e.g. acetonitrile, acetonitrile:water and dichloromethane). The solid and liquid phases were then separated by centrifugation and decanting allowing radio-assay of the solid and liquid phases to be carried out by combustion/LSC and LSC, respectively.

Concentration of Extracts

Where necessary, extracts were concentrated using rotary evaporation, carried out in a water bath at ambient temperature and/or by concentrating under nitrogen gas.

Liquid-Liquid Partitions

Liquid-liquid partitions were carried out between two immiscible solvents; dichloromethane or ethyl acetate and aqueous solutions, from fat extracts. Appropriate volumes of each of the phases were mixed and shaken. The phases were then separated and the partition repeated as required.

Enzyme Hydrolysis

Protease digestion of tissue debris, remaining after solvent extraction, was performed using enzyme from *Streptomyces griseus*. A buffer was added to each debris sample and then equilibrated overnight. Protease was added to each sample and incubated for 24 hours. An additional aliquot of protease was added to each sample and incubated for a further 24 hours. Following incubation, acetonitrile was added to denature the enzyme and stop the reaction. The residual solid material was removed by centrifugation and rinsed with acetonitrile, the radioactivity was determined by LSC.

Lipase Hydrolysis

Lipase digestion of tissue debris, remaining after solvent extraction and subsequent protease hydrolysis, was performed using lipase enzyme from porcine pancreas. A buffer was added to each remaining debris sample and mixed. An aliquot of a solution of sodium dodecyl sulphate (SDS) was added, mixed and allowed to equilibrate overnight. Lipase was added to each sample and incubated for 24 hours. An additional aliquot of lipase was added to each sample and incubated for a further 24 hours.

Following incubation, acetonitrile was added to denature the enzyme and stop the reaction. The residual solid material was removed by centrifugation and rinsed with acetonitrile. The radioactivity

in the solution was determined by LSC. The radioactivity in the dry solid was determined by LSC analysis following solubilisation.

4.2.4 Residue identification

Residue identification was performed using high resolution accurate mass LC-MS/MS with on-line radiodetection. Samples were analysed using both positive and negative ion modes.

A reference standard of unlabelled meloxicam was provided for use in the study. A solution of unlabelled meloxicam was prepared in methanol: water. This solution was used to optimise the mass spectrometer source conditions and MS and MS/MS conditions for the parent compound for qualitative analyses. Optimisation was carried out using collision induced dissociation and high-energy collision induced dissociation to determine the optimum fragmentation method and normalised collision energy.

The chromatographic method developed for the study used an aqueous ammonium acetate/acetonitrile gradient and an YMC-Pack ODS-AQ column. Accurate mass full scan and product ion spectra were obtained for the reference standard of unlabelled meloxicam following chromatographic analysis. Where possible, major characteristic fragment ions were assigned and structures proposed. A reference standard of meloxicam was analysed prior to or during each batch of samples to provide a marker for retention times.

Residue identification using accurate mass full scan and product ion analyses was carried out on selected sheep tissue, urine and faeces samples to screen for the presence of 'typical' or 'predicted' metabolites. Samples were screened for components corresponding to the supplied reference standard, potential hydroxylated, N-dealkylated or acid metabolites, components formed by cleavage of the amide bond or ring-opening of the thiazole moiety and/or conjugated metabolites. Full scan techniques were used to attempt to identify any additional unassigned components by direct comparison with the [^{14}C]-radiochromatogram.

Where necessary, interferences were removed from the spectra by processing with mass defect filtering (MDF). Accurate mass full scan and product ion data were used to provide confirmation of the elemental formulae of potential metabolites and to facilitate interpretation of the fragmentation data.

A cross-matrix comparison was carried out on tissue, urine and faeces samples. Samples were analysed in positive and negative ion modes using accurate mass full scan with targeted data dependent on specific product ion data. The retention times, accurate mass extracted ion chromatograms and product ion data (where available) were used to confirm the presence of [^{14}C]meloxicam and any additional drug-related components identified during residue identification.

5. Results

The majority of the mean administered radioactivity was excreted in the faeces and urine.

The highest residues were observed in the liver at each time point. The highest residue concentration was found in the 4 hour female liver pool. Residue concentrations in the kidney were comparable to those in the liver at 4 hours, but declined more rapidly. Residue concentrations in both the liver and kidney were ≥ 0.01 mg/kg after 14 days (336 hours). Residue concentrations in muscle and fat were low and only exceeded the 0.010 mg/kg threshold at the 4 hour time point.

In tissue samples where the total residue was ≥ 0.010 mg/kg, liquid-liquid extraction techniques were employed to extract the residue. Once extracted, samples were pooled appropriately prior to chromatographic analysis with LC-MS/MS identification.
Parent meloxicam was considered to be the most abundant residue, and it was detected in all matrices (liver, kidney, muscle, fat, and application site) for both male and female sheep. As a result, meloxicam was selected as the marker residue.
The marker residue (meloxicam) was present at significant concentrations in all tissues, for both sexes, at the initial sampling time point of 4 hours post dose administration. The marker residue was significantly depleted in all tissues by Day 3 post dose. The last time point at which the marker residue was observed was Day 8 post dose in the male liver sample. By Day 14 post dose administration, meloxicam was fully depleted.

There was no bioaccumulation of the marker residue, meloxicam, during the study. Meloxicam may have been eliminated in the excreta and/or metabolised over time.

The next most abundant residue resulting from biotransformation of meloxicam and identified in all tissue samples was the oxoacetic acid metabolite of meloxicam. Absolute residue levels of this metabolite were highest in the female liver. This residue was also above the 0.050 mg/kg threshold in the male kidney, male and female liver and the female muscle.

Other biotransformation products included cyclic urea metabolite of meloxicam (observed in all matrices except the female fat sample) and N-formyl urea metabolite of meloxicam (observed in all matrices).

Other identified residues included four mono-hydroxy metabolites of meloxicam (two of these could possibly have been the N-oxide). These metabolites were generally present in most tissues, but were less prevalent in fat.

The acid metabolite of meloxicam was only observed in the female liver samples.