MEAT & LIVESTOCK AUSTRALIA

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Pharmacokinetics, residue kinetics and residue depletion studies for lignocaine and bupivacaine in sheep and cattle

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Executive summary

The red meat industry needs access to pain relief products to ensure high standards of animal welfare are met during surgical interventions. Presently Minimum Residue Limits (MRL's), Withholding Periods (WHP's) and Export Slaughter Intervals (ESI's) are not available for the local anaesthetics lignocaine and bupivacaine in sheep and cattle, nor for the various metabolites produced when the drugs are broken down in the body.

Lignocaine and bupivacaine were administered to sheep and cattle in a pilot study to define the residue of these drugs. Highly sensitive QuEChERS- based HPLC-MS/MS methods were developed to measure parent drug and potential metabolites in plasma, urine and edible tissues. Disposition kinetics of both local anaesthetics and their metabolites were established. Parent molecule residues were detected in tissues up to 7 days post-administration of both local anaesthetics.

An important species difference in metabolism was discovered: cattle metabolise lignocaine primarily to 2,6-dimethylaniline (DMA, also called 2,6-xylidine) whereas DMA is only a minor metabolite in sheep. DMA is not a metabolite of bupivacaine in either species. These findings are important because DMA has been found to possess mutagenic and genotoxic properties, and is a genotoxic carcinogenic in rats.

For registration of lignocaine and/or bupivacaine a residue definition will need to be established for the purpose of calculating MRL's, WHP's and ESI's; results of the current study will help design appropriate protocols.

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1 Background

1.1 Local anaesthetics for use in sheep and cattle

1.1.1 Use and regulatory requirements

The red meat industry needs access to pain relief products to ensure high standards of animal welfare are met during surgical interventions. In particular the red meat industry needs access to the local anaesthetic molecules such as lignocaine (also called lidocaine) and possibly the longer-acting bupivacaine. Furthermore the projected increase in the use of pain relief products in whole herds or flocks increases the risk of residues appearing in food if appropriate withholding periods (WHPs) are not known. Although local anaesthetics are legal to use in veterinary and human medicine, the food safety aspects of their use are uncertain and the risk devolves back to the prescribing veterinarian.

To safeguard consumer health and export markets the Australian Pesticides and Veterinary Medicines Authority (APVMA) and other branches of the Federal Government have established guidelines and regulations about the presence of residues of veterinary chemicals in meat and offal. Parameters are established and safe WHPs between treatment and slaughter for food are set. In order to derive WHPs, the following data are needed for each chemical: No Observable Effect Level (NOEL) determined from chronic toxicity studies in laboratory animals, Allowable Daily Intake (ADI) for human consumption of the chemical and the Minimum Residue Limit (MRL) in various foods. Once the MRL is established, a WHP and Export Slaughter Interval (ESI) is established to ensure the MRL is not exceeded.

Presently MRL's, WHP's and ESI's are not available for lignocaine and bupivacaine in sheep and cattle, nor for the various metabolites produced when the drugs are broken down in the body. Although there are numerous data gaps regarding lignocaine, importantly the basic toxicology assessments have been done by the Office of Chemical Safety and ADIs set based on human safety data (Lowest Observable Effect Level, LOEL, rather than NOEL) with generous safety margins. The residue definitions (marker residue) for lignocaine and bupivacaine were set in Table 3 of the MRL Standard as parent molecule only, but were withdrawn in 2012. MRL's, WHP's and ESI's are not current for the two molecules. The European Agency for the Evaluation of Medicinal Products has prepared a MRL Summary Report (EMEA/MRL/584/99-FINAL) on lignocaine and their findings and assessment are relevant. Also within that report the lignocaine metabolite 2,6-xylidine (also called dimethylalanine, DMA) was identified as being mutagenic *in vitro* and has genotoxic characteristics *in vivo* (IARC 1993; Kirkland et al., 2012). In light of this information however, lignocaine is registered and continues to be used extensively in humans. In the European Union lignocaine is not registered for use in cattle but is used off-label (under Cascade rules).

Table 1. Established parameters for each local anaesthetic parent molecule

Parameter	Lignocaine	Bupivacaine
LOEL mg/kg/day	8.57[1]	1[2]
ADI mg/kg/day	0.009	0.001
MRL mg/kg	Temporary 0.02. Deleted table 1, Jan 2012. Currently Table 5[3] mulesing in wool bearing sheep	Temporary MRL 0.02 Deleted Table 1 Jan 2012. Currently Table 5[3] mulesing in wool bearing sheep
WHP		
ESI		
Residue definition	Table 3[4] Lignocaine, deleted Jan 2012	Table 3[4] bupivacaine, deleted Jan 2012

[1]Office of Chemical Safety. In human cardiac treatment, doses of 1-10 mg/kg are used. If 1 mg/kg bw is taken as a dose without adverse effects, and 35% bioavailability is assumed via the oral route, the corresponding oral dose can be estimated to be 3/0.35 =8.57 mg/kg bw. An ADI of 0.009 mg/kg bw was derived by applying a 1000-fold safety factor. The 1000-fold safety factor takes into account that 8.57 mg/kg bw represents a LOEL at the low end of the therapeutic range (10-fold), intraspecies variability (10-fold) and uncertainties due to an inadequate toxicological database (10-fold).

[2]Ofiice of Chemical Safety. i.v. infusion of a dose of 75 mg bupivacaine salt over 10 minutes to volunteers resulted in mean arterial plasma concentrations of approximately 5 µg base/mL; no signs of toxicity were recorded (Tucker and Mather, 1975). Therefore in the absence of adequate repeat dose toxicity studies, an ADI of 0.001 mg/kg bw was established using a LOEL of 1 mg/kg bw, and applying a 1000-fold safety factor. The 1000-fold safety factor represents uncertainties associated with the use of a LOEL (10-fold), intraspecies variation (10-fold), and an inadequate toxicological database (10-fold).

[3]Table 5 Agricultural and Veterinary Chemicals Code Instrument No. 4 (*MRL Standard*) 2012 lists uses of substances where MRLs are not necessary.

[4] Table 3 Agricultural and Veterinary Chemicals Code Instrument No. 4 (*MRL Standard*) 2012 Table 3 sets out the residue to which the MRL applies for each chemical compound

1.1.2 Lignocaine metabolism

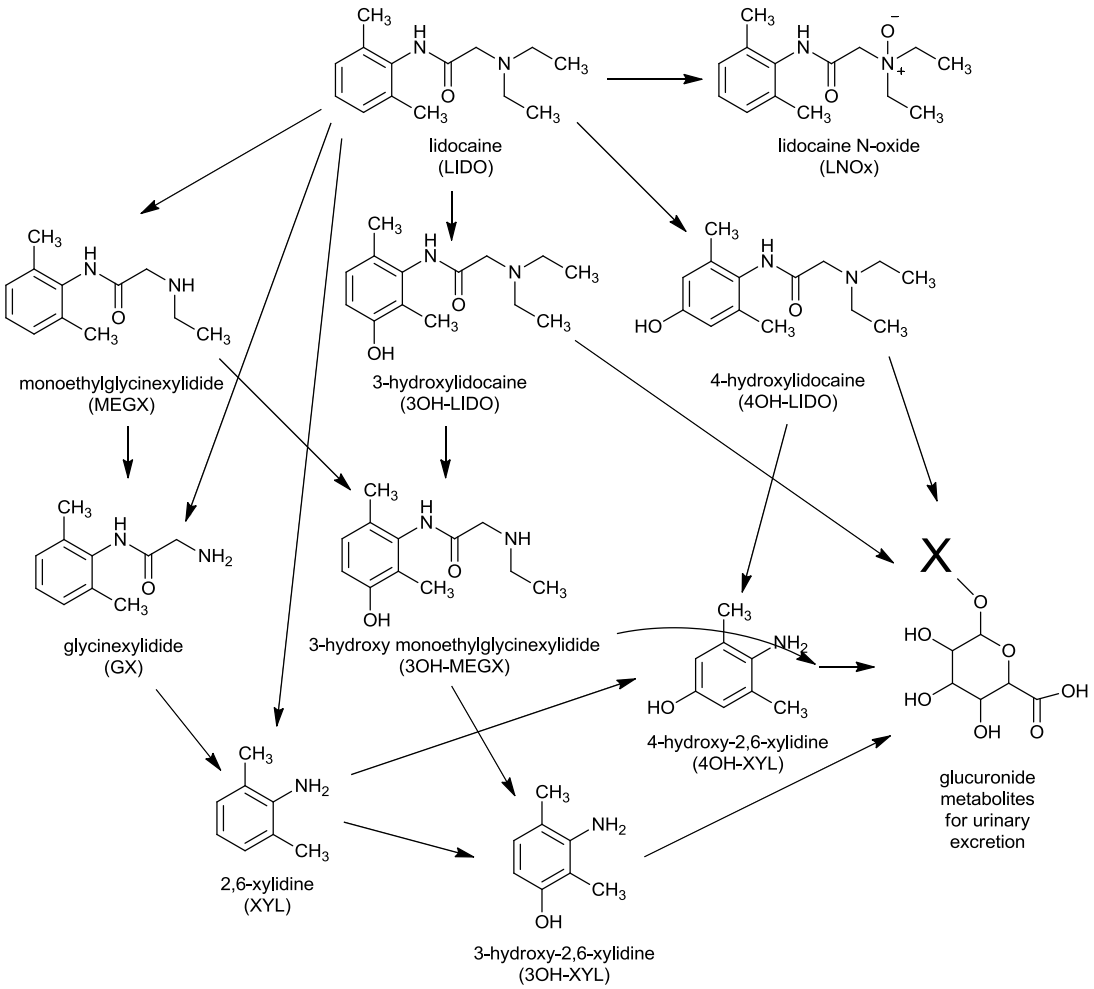

Fig. 1 Hypothetical scheme for the formation of metabolites of lignocaine

Table 2. Lignocaine metabolites (known prior to this study)

2,6-xylidine	(XYL)
Lidocaine-*N*-oxide	(LIDO-N-OX)
N-(*N*-ethylglycyl)-2,6-xylidine	(MEGX)
3-hydroxy-*N*-(*N*-ethylglycyl)-2,6-xylidine	(3-OH-MEGX)
N-glycyl-2,6-xylidine	(GX)
4-hydroxy-2,6-xylidine	(4-OH-XYL)
3-hydroxy-*N*-glycyl-2,6-xylidine	(3-OH-GX
3-hydroxy-lidocaine	(3-OH-LIDO)
3-hydroxy-2,6-xylidine	(3-OH-XYL)
4-hydroxy-lidocaine	(4-OH-LIDO)
Methylhydroxylidocaine	Me-OH-LIDO

Metabolite	Rat SD male ip[1]	Rat SD female po[2]	Rat SD in vitro [3,5]	Man in vitro[6]	Excretion Man in vivo [2, 4]	Cattle in vitro[5] in vivo[7]	Sheep Unknown
XYL	**Minor**	**Minor**	**Minor**[5only]			**Major**	
LIDO-N-OX						**Intermediate**	
MEGX	**Minor**	**Minor**	**Major**	**Major**		**Intermediate**	
3-OH-MEGX	**Major**	**Major**				**Minor**	
GX	**Minor**	**Minor**	**Minor**[5 only]			**Minor**	
4-OH-XYL	**Intermediate**	**Minor**	**Minor**[5 only]		**Major**	**Trace**	
3-OH-GX	**Minor**						
3-OH-LIDO	**Trace**	**Major**	**Minor**	**Minor**		**Minor**	
3-OH-XYL	**Trace**						
4-OH-LIDO	**Trace**					**Trace**	
Me-OH-LIDO			**Minor**[3only]				

[1]Coutts et al. (1987)
[2]Keenaghan and Boyes (1972)
[3] Oda et al. (1989)
[4]Tam et al. (1987)
[5] Thuesen and Friis (2012)
[6] Imaoka et al. (1990)
[7] Hoogenboom et al. (2015)

Table 2 indicates that the metabolites produced in rats, man and cattle are similar following exposure to lignocaine, the main difference being that 2,6-xylidine (XYL, DMA) is the major metabolite in cattle. Toxicological studies done in rats are relevant to cattle and presumably sheep and thence to man. The major and intermediate metabolites in cattle are known but there is no knowledge about the major metabolites in sheep. The residue definition has not been established and it is unlikely to be lignocaine parent alone, as it was before being withdrawn in 2012. The likely alternate residue definition would need to include DMA especially, along with other metabolites that have been identified in cattle, pigs and man. These molecules are all detectable and quantifiable using high resolution LC-MS/MS (Zheng et al, 2013; Alexson et al, 2002). Studies to confirm the full metabolite profile and residue definition following lignocaine treatment of sheep and cattle are needed.

In sheep the elimination half-life of lignocaine in blood following i.v. dosage of 0.5 – 2 mg/kg was between 42 and 62 minutes (Bloedow, Ralston and Hargrove; 1980). Following 5-10 mg/kg iv in non-pregnant sheep it was 31 minutes (Morishima et al; 1979). Following 4-5 mg/kg i.v. in ewes it was 38 min in non-pregnant ewes and 32 minutes in pregnant ewes (Santos et al; 1988).

In cattle following intranasal lignocaine the elimination half-life was 14 min (Dadak et al;2008). In dairy cows following 1.5 mg/kg i.v. the half-life was 1.5 hours and neither MEGX nor GX was detected in blood (<50ng/mL; Cox, Wilson and Doherty;2011). Holstein cows were given 4 mg/kg s.c. lignocaine and it had a half life in blood of 4.2 hours. Lignocaine was not detected (<2.0 ng/mL) after 10 hours in any animal. 3 of 9 animals still had detectable milk residues (>2ng/mL) 48 hours but not 60 hours after treatment (Sellers et al, 2009).

The residue kinetics in the edible tissues of sheep and cattle and the residue depletion from tissues in sheep and cattle is unknown. Once the residue definition is determined a residue kinetic study and a residue depletion study is required in sheep to establish the MRL, WHP and ESI. Studies would be conducted using the study design in the regulatory guidelines (apvma.gov.au/node/746). If sheep and cattle are shown to have a similar metabolic profile the need for a second species residue kinetic study in cattle is debatable, though it would be prudent to do so.

1.1.3 Bupivacaine metabolism

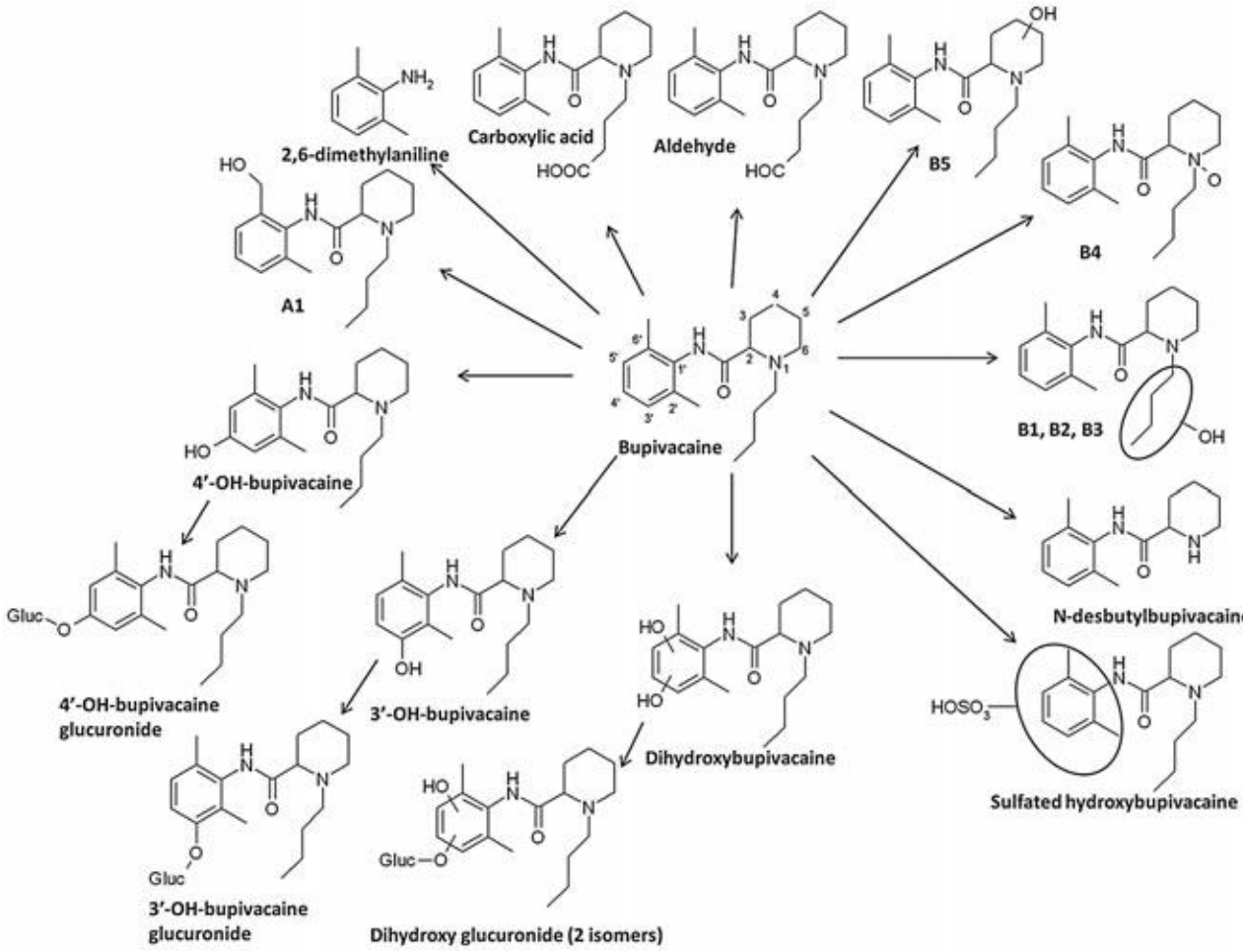

Fig. 2 Scheme of the different bupivacaine metabolites found in horses (Rydevik et al 2012)

Table 3. Bupivacaine metabolites (known prior to this study)

metabolite	Rat urinary in vivo po[1,2]	Man urinary iv[3, 6]	Rat iv[4]	Horse sc[5]	Cattle Unknown	Sheep Unknown
N-Desbutyl-bupivacaine. Pipecolylxylidine (PPX)	Present[1] Minor[2]	minor		present		
3'-Hydroxy-bupivacaine	Present[1] Major[2]	Minor[6]	major	present		
4-hydroxybupivacaine	Major[2]	Minor[6]		present		
N-Butylpipecolyl-2-amide	Present[1]					
(4, 5) mono-hydroxylated isomeres on the piperidine ring	Present[1]			present		
Pipecolic acid	Minor[2]					

[1]Dennhardt, Fricke, Stöckert (1978)
[2]Caldwell, Notarianni, Smith and Snedden (1977).
[3]Reynolds (1971) [4]Morishima HO et al (2000)
[5]Rydevik, Bondesson and Hedeland (2012)
[6]Zhang, Mitchell and Caldwell (1998)

The nature of the metabolites of bupivacaine in rats, man and horses are similar. However the identity of metabolites in ruminants is unknown. Therefore a study is required to identify the principle metabolites in sheep and cattle following s.c. treatment and to define the nature of the residue. What is evident from the study in horses (Rydevik et al. 2012) is that there are a multitude of potential metabolites of bupivacaine.

Bupivacaine is used extensively in human obstetrics, because of its long duration of action compared with other local anaesthetics. In sheep the half-lives of bupivacaine and lignocaine are approximately 90 minutes (Copeland et al. 2008) and 30 minutes (Santos et al. 1988), respectively. This difference is reflected clinically: in a metacarpal block in sheep, the duration of analgesia following administration of bupivacaine and lignocaine was 110 and 40 minutes, respectively (Lizzaraga et al. 2013). Bupivacaine pharmacokinetics have not been determined in cattle.

2 Project objectives

Presently MRL's, WHP's and ESI's are not available for lignocaine and bupivacaine in sheep and cattle, nor for the various metabolites as the drugs are broken down in the body. Although lignocaine is legal to use in veterinary and human medicine, the food safety aspects of their use are uncertain. The projected increase in the use of pain relief products in whole herds or flocks increases the risk of residues of pain relief products appearing in food unless appropriate WHPs are known. For the purpose of registration with APVMA, data showing the fate of the drug and its metabolites in blood, urine and the various edible tissues of the body is required in order to guide determination MRL's, ADI's and ESI's. This study is effectively a pilot study to define the residues of

lignocaine and bupivacaine by characterising metabolism and fate of the drugs in a small number of cattle and sheep; results of the current study will help design appropriate protocols for full residue definition studies towards APVMA registration of lignocaine and/or bupivacaine in sheep and cattle.

3 Methodology

3.1 Animals

3.1.1 Cattle

Six hereford yearling steers (288-360kg) were purchased from local farms.

On entry the steers were restrained in the metabolic crates and head collars fitted. Collars were made from foam rubber (4cm thick, 19cm wide, with an internal diameter of 38 cm) held by contact adhesive (suitable for foam rubber) and two cable ties. The collars were attached on each side of the animal's head collar, using baler twine.

Animals were acclimatised for 7 days in the metabolic crates. Animals were fed on a ration of milled roughage to firm faeces to aid collection and separation from urine. Steers were habituated to humans for one hour twice a day. Note: this was immensely valuable when it came to handling/sampling when placed in the metabolic crates.

On the day prior to administration of local anaesthetics steers were taken to a handling crate, collars were removed and a jugular catheter (Angiocath,15g x 13.3cm) placed aseptically and sutured to the skin. Catheters were flushed with heparinised saline then a connection-set (3mm ID x 150cm, Codan GmbH, Lensahn, Germany), primed with heparinised saline, was inserted through the foam collar at a dorsal mid-point, with a Buhner needle. The connection set was then pulled through the material of the collar to exit externally at the lateral midpoint, ipsilateral to the jugular. The catheter was then re-inserted just below that external exit point to continue through foam collar to re-exit internally in the middle of the ventral quadrant. A connection was made to the jugular catheter and the connection joint attached to the skin of the animal with acrylic adhesive. A loop was made in connection set between the jugular connecting joint and the collar (to allow some 'play') and attached to the skin with acrylic glue. Collars were placed over the steer's head and adjustment made to the connection-set to allow sufficient 'give' under collar for movement of the head, but not so much that excessive length could snag on rails, gate-hooks etc. The remainder of connection set was pulled through the collar and rolled up on dorsal aspect of collar, then placed in a small plastic bag attached to the collar by contact adhesive and duct-tape. This allowed 65-75 cm of 'free' for researchers to utilise during sample collection to allow for movement of animal (Fig 6.)

Foam collars were reattached, loosely, to head-collar with baler- twine to avoid rotation around the neck. Points of anchorage of baler twine through the foam collar were reinforced with duct tape to avoid 'pulling-through'. Animals were then taken to a metabolic crate, and tethered by a lead-rope connected to head-collar at a low point adjacent to the feed trough to allow access to food and water, and to enable standing-up and lying-down without the ability to turn around.

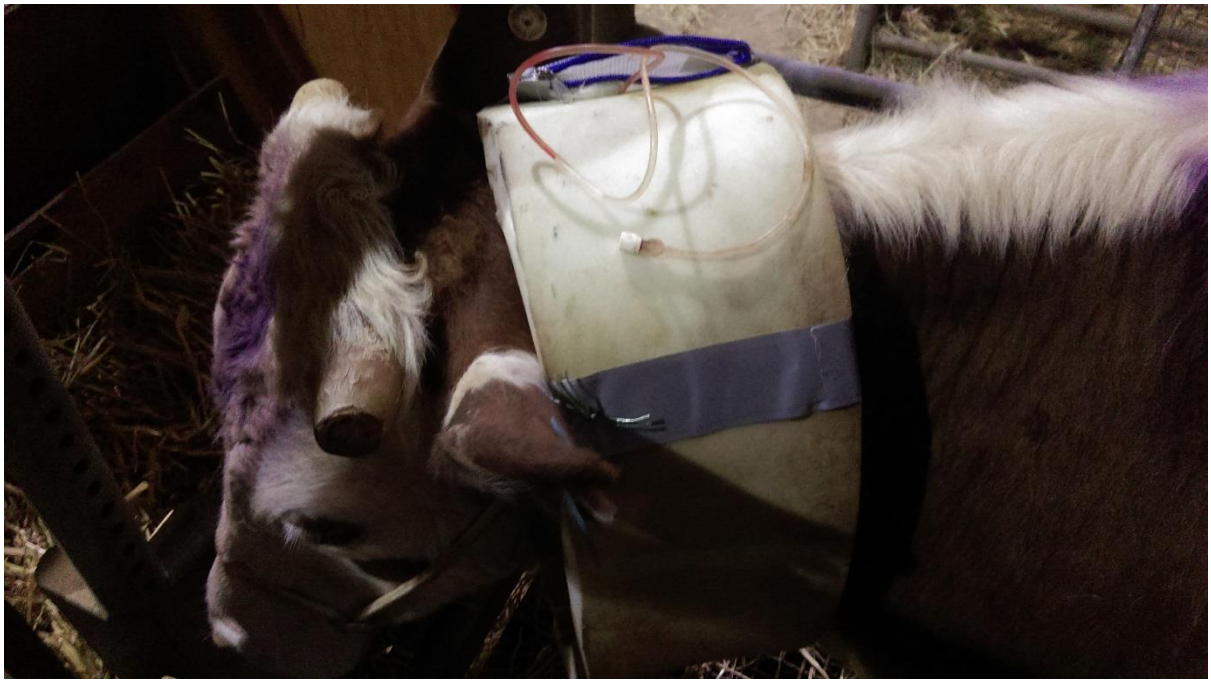

Fig. 6 Fabricated collar designed to ensure catheter security. The extension set (removed from its pouch) allows operator to draw blood samples "remotely".

3.1.2 Sheep

Six merino wethers (42.6-52.6kg) were purchased from a local farm. Sheep were placed in metabolic crates and acclimatised for 7 days, fed on a ration to firm faeces to aid collection and separation from urine.

On the day prior to administration of local anaesthetics sheep were restrained in the standing position, both jugular veins were clipped up and aseptically prepared. One jugular catheter (Angiocath16g x 13.3cm) was placed inserted into jugular vein and the catheter hub superglued to skin. The catheter was flushed with heparinised saline and stoppered. A stockinette "snood" was then placed over neck to secure the catheter further.

3.2 Administration of local anesthetics and sample collection

10 mg/kg lignocaine (Lignocaine 20, Ilium, Glendining, NSW, Australia) was administered subcutaneously to 3 sheep and 3 steers. Bupivacaine (Bupivacaine injection BP 0.5%, Pfizer, Perth, Western Australia) was given to 3 sheep and 3 steers at 5mg/kg. A lower dose for bupivacaine was used as bupivacaine is more potent, with a lower threshold for cardio- and neurotoxicity (Dickerson and Apfelbaum, 2014; DeRossi et al., 2010). 10mg/kg of bupivacaine was considered a welfare risk, with possible fatal outcome.

Blood samples for plasma were taken at 20 time points over 72 hours (Appendix 2.). Urine pools were collected at 6, 12, 18, 24, 36, 48 and 72 hours after treatment.

Two lignocaine steers and sheep and 2 bupivacaine steers and sheep were slaughtered at 72 hours using a captive bolt, followed by exanguination by transection of the carotid arteries and jugular

veins. The remaining 4 animals were slaughtered at 7 days post-aqdministration of local anesthetic. Triplicate samples of muscle, fat, kidney and liver were harvested and placed in -80º until analysis.

3.3 Sample preparation

Sample preparation was individualised for each matrix, based on QuEChERS principles (quick, easy, cheap, effective, rugged and safe).

3.3.1 Plasma

Plasma (2 mL) was spiked with the deuterated internal standard mix (20 μL) in a centrifuge tube (15 mL). Acetonitrile (5 mL) was added, the tube vortexed (1 min) to precipitate proteins and then centrifuged (2100 x *g*, 5 min). The contents of an EMR dSPE tube were transferred to a centrifuge tube (50 mL) containing ammonium acetate buffer (5 mL, 5 mM). The supernatant in the plasma/acetonitrile tube was decanted into the EMR tube, vortexed (1 min) and centrifuged (2100 x *g*, 5 min). The supernatant was decanted into an EMR Polish tube, vortexed, (1 min) and centrifuged (2100 x *g*, 5 min). The upper acetonitrile layer was transferred to a glass vial and evaporated under nitrogen (30 °C). The residue was resuspended in methanol (300 μL) and transferred to an autosampler vial for analysis by LC-MS/MS.

3.3.2 Urine

Urine was diluted up to 1000 fold in water due to high analyte concentrations. Total (glucuronide-bound + free) lidocaine metabolites were determined by adding urine or diluted urine (3 mL) to a glass vial and spiking with internal standard mix (20 μL). Potassium phosphate buffer (0.1 M, 1 mL) was added with β-glucuronidase (18,000 U/mL, 360uL) and the urine sample was incubated at 65 °C for 3 hours. After cooling, the solution was transferred to a centrifuge tube (15 mL) and treated the same as plasma samples. Free (unbound) lidocaine metabolites were determined using the same method as total metabolites with the exclusion of β-glucuronidase.

3.3.3 Muscle, liver and kidney

Muscle, liver and kidney tissue samples were chopped in a small food processor. Subsamples (2 g) were weighed into centrifuge tubes (15 mL) and spiked with the deuterated internal standard mix (20 μL). Acetonitrile (5 mL) was added and the tissue homogenised with an Ultra Turrax for 1 min (20,000 rpm), with the tubes kept in ice during homogenisation to minimise heating. Further processing of the sample was done with a probe ultrasonicator (FS-300N, Shenzhen XZB Instruments, China), on ice, for 10 seconds ON 10 seconds OFF, 90 seconds total, at 90% maximum output. After centrifugation, (2100 x *g*, 5 min), the supernatant was decanted into an EMR dSPE tube, which was purified and concentrated using QuEChERS using the same method as plasma samples.

3.3.4 Fat

Intra-abdominal fat was chopped in a small food processor. Subsamples (2 g) were weighed into centrifuge tubes (15 mL) and spiked with the deuterated internal standard mix (20 μL). Hexane (4 mL) was added and the tissue homogenised with an Ultra Turrax for 1 min (20,000 rpm). The sample

was then ultrasonicated, as per other tissues. Acetonitrile (5 mL) was added, vortexed and centrifuged (2100 x *g*, 5 min). The hexane layer was removed, and the fat plug punctured with a glass pipette. The acetonitrile layer was recovered and cleaned using QuEChERS.

3.4 Instrumentation

An Agilent Technologies 1200 Series LC with binary pump, degasser, column oven, autosampler, DAD (205 nm) and 6410 quadrupole tandem mass spectrometer was used for analysis. An Agilent Poroshell HPH-C18 (50 x 3 mm x 2.6 μm) column was used at 40 °C and the injection volume was 2 μL. The MS/MS used positive mode ESI in dynamic multiple reaction monitoring mode (dMRM). Dual ion transitions were used for each analyte while only a single transition was used for each deuterated internal standard. Ion transitions, retention times and parameters varied for each chemical and are shown in Table 4. Additional MS/MS parameters were gas temperature (350 °C), vaporiser temperature (250 °C), gas flow (10 L/min), nebuliser (30 psi) and capillary voltage (1500 V). A gradient elution was used and consisted of pure methanol (Solvent A), and ammonium bicarbonate (10 mM) in instrument grade water (Solvent B), which was pH adjusted to 8.5 using ammonium hydroxide (1 M). The gradient started with 10% solvent A, increased to 90% over 5 min, held for 2 min, and then returned to initial conditions over 0.1 min timeframe. The column was equilibrated for 2 min before the next injection, resulting in an overall run time of 9 min. Table 4 lists the MS/MS analytical parameters for each molecule.

Table 4 – MS/MS parameters for lignocaine (lidocaine), metabolites and internal standards used for analyte quantitation

Name	Abbrev	MW	Ion Transition	FV (V)	CE	RT (min)	IS used for calibration
4-hydroxy-2,6-xylidine	4OH-XYL1	137	138→123	110	17	2.37	XYL-D6
	4OH-XYL2		138→77	110	30		
3-hydroxy-2,6-xylidine	3OH-XYL1	137	138→123	110	17	2.73	XYL-D6
	3OH-XYL2		138→77	110	30		
glycinexylidide	GX1	178	179→122	100	9	3.28	GX-D6
	GX2		179→58	100	30		
	GX-D6	184	185→128	100	9		
2,6-xylidine	XYL1	121	122→105	100	16	3.31	XYL-D6
	XYL2		122→79	100	24		
	XYL-D6	127	128→111	100	16		
3-hydroxy mono-ethylglycinexylidide	3OH-MEGX1	222	223→58	100	14	3.43	MEGX-D5
	3OH-MEGX2		223→138	100	19		
Lidocaine N-oxide	LNOx1	250	251→86	110	18	3.45	LNOx-D10
	LNOx2		251→130	110	11		
	LNOx-D10	260	261→96	110	18		
monoethyl-glycinexylidide	MEGX1	206	207→58	90	14	4.30	MEGX-D5
	MEGX2		207→122	90	10		
	MEGX-D5	211	212→63	90	14		
4-hydroxylidocaine	4OH-LIDO1	250	251→86	105	19	4.84	3OH-LIDO-D5
	4OH-LIDO2		251→58	105	44		
3-hydroxylidocaine	3OH-LIDO1	250	251→86	105	19	4.99	3OH-LIDO-D5
	3OH-LIDO2		251→58	105	44		
	3OH-LIDO-D5	255	256→63	105	44		
lidocaine	LIDO1	234	235→86	106	17	5.49	LIDO-D10
	LIDO2		235→58	102	40		
	LIDO-D10	244	245→96	106	17		

Note: IS = internal standard, Prec Ion = precursor ion, Prod Ion = product ion, FV = fragmentor voltage, CE = collision energy, RT = retention time

4 Results

4.1 Overview of most significant finding

A major finding of this study is that cattle and sheep metabolise lignocaine very differently. In cattle, lignocaine is almost completely metabolised to DMA, with other metabolites being very much at minor/ trace levels in comparison. In contrast, sheep produce numerous minor metabolites (including DMA at plasma concentrations 25 x lower than those measured in cattle); there is no major metabolite per se. In both sheep and cattle, bupivacaine is not metabolised to DMA at all.

Considering that DMA is the potential carcinogenic/genotoxic molecule of concern, lignocaine use in sheep would appear to be a safer (with regards residues) option for pain control in the Australian flock. From a purely DMA health perspective, bupivacaine is a more attractive option, in both sheep and cattle.

4.1.1 Plasma

NOTE: Graphs below depict plasma concentrations out to 12 hours only as beyond this time point the concentration versus time curve appears to merge with the X-axis.

DMA is the major metabolite of lignocaine in cattle (Fig. 5).

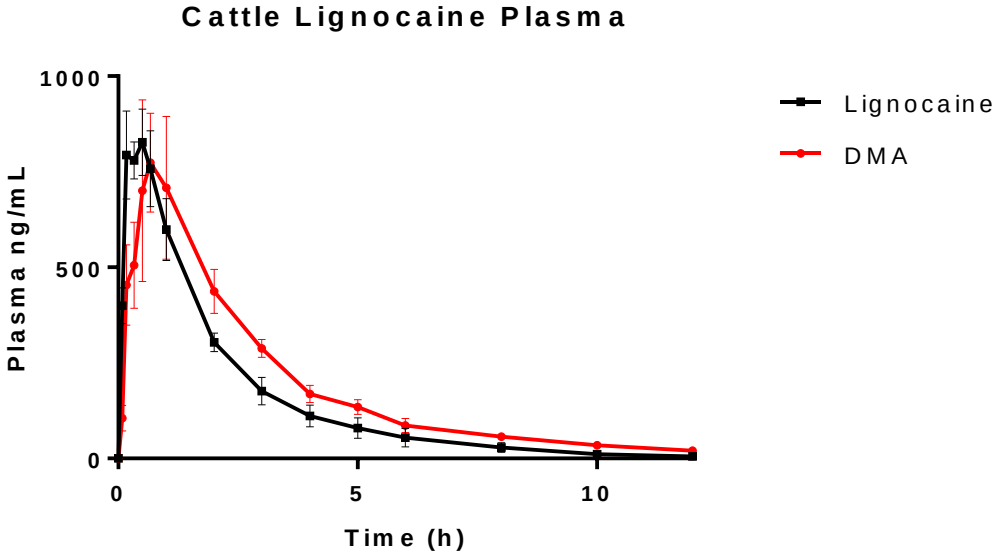

Fig. 5 Plasma concentration versus time in steers following lignocaine 10mg/kg subcutaneous injection. Lignocaine is almost exclusively metabilised to 2,6-dimethylaniline (DMA). *n* = 3 (mean ±SD)

Sheep metabolise lignocaine more rapidly than cattle and DMA is a minor metabolite of lignocaine in sheep (Fig. 6).

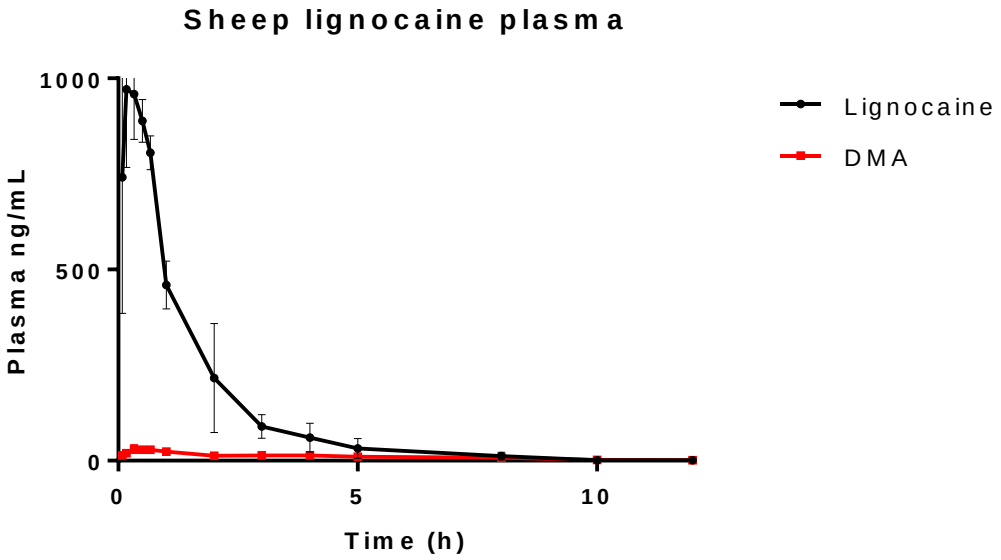

Fig. 6 Plasma concentration versus time of lignocaine and the minor metabolite DMA in sheep following lignocaine 10mg/kg subcutaneous injection. *n* = 3 (mean ±SD)

4-OH-DMA, LNOx and MEGX are minor metabolites of lignocaine in cattle (Fig. 7). 3-OH-lignocaine, 4-OH- lignocaine and 3-OH-DMA are trace metabolites.

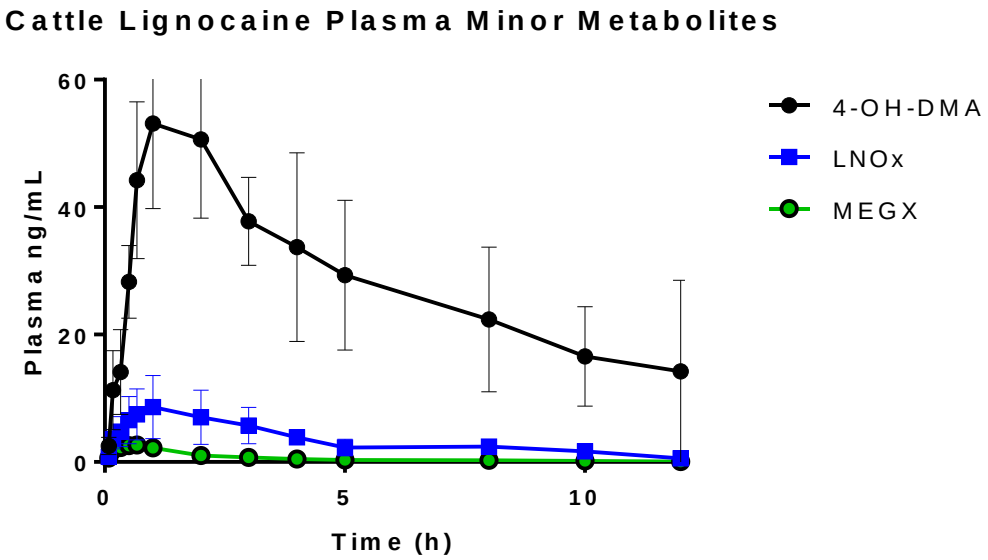

Fig. 7 Plasma concentration versus time of minor metabolites 4-OH-DMA, LNOx and MEGX in steers following lignocaine 10mg/kg subcutaneous injection. *n* = 3 (mean ±SD)

In contrast to cattle, sheep metabolise lignocaine extensively, with DMA being one of many minor metabolites (Fig 8).

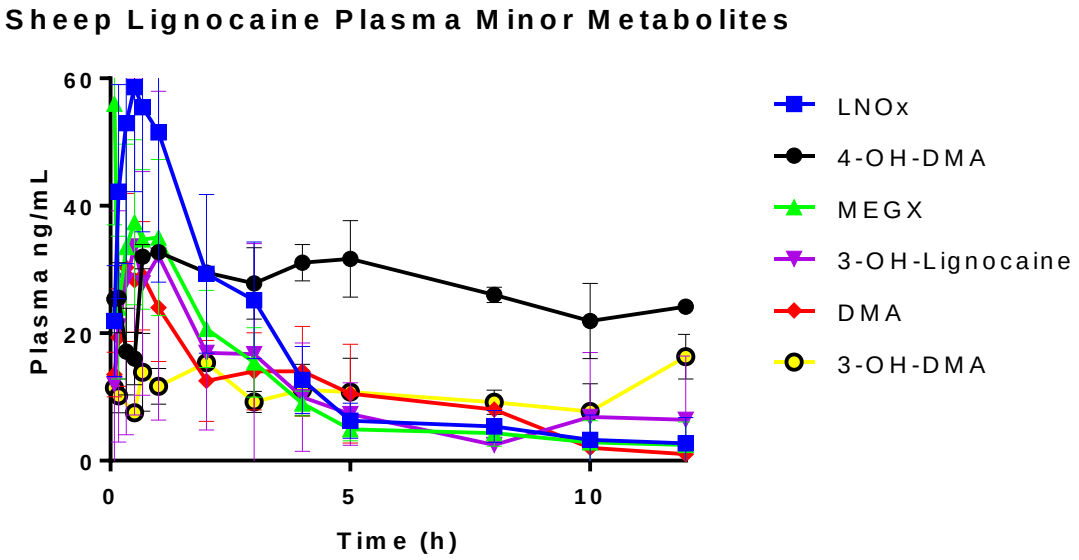

Fig. 8 Plasma concentration versus time of minor metabolites in sheep following lignocaine 10mg/kg subcutaneous injection. *n* = 3 (mean ±SD)

Pipecolylxylidine (PPX) is the major metabolite of Bupivacaine in cattle (Fig 9). Bupivacaine is not metabolised to DMA.

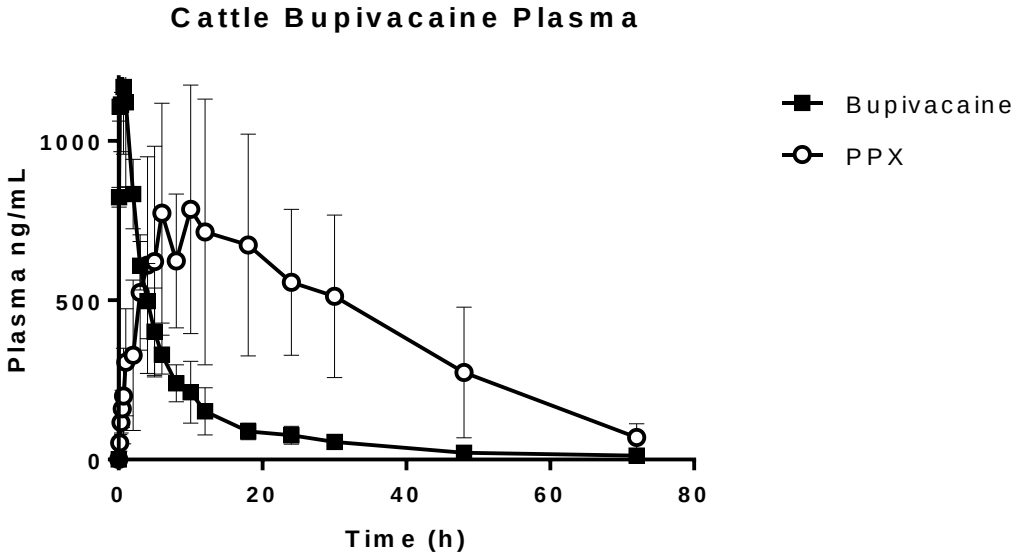

Fig. 9 Plasma concentration versus time in steers following bupivacaine 5mg/kg subcutaneous injection. Bupivacaine is almost exclusively metabolised to pipecolylxylidine (PPX). *n* = 3 (mean ±SD)

PPX is a minor metabolite of bupivacaine in sheep (Fig 10). BNOx is a trace metabolite. Bupivacaine is not metabolised to DMA.

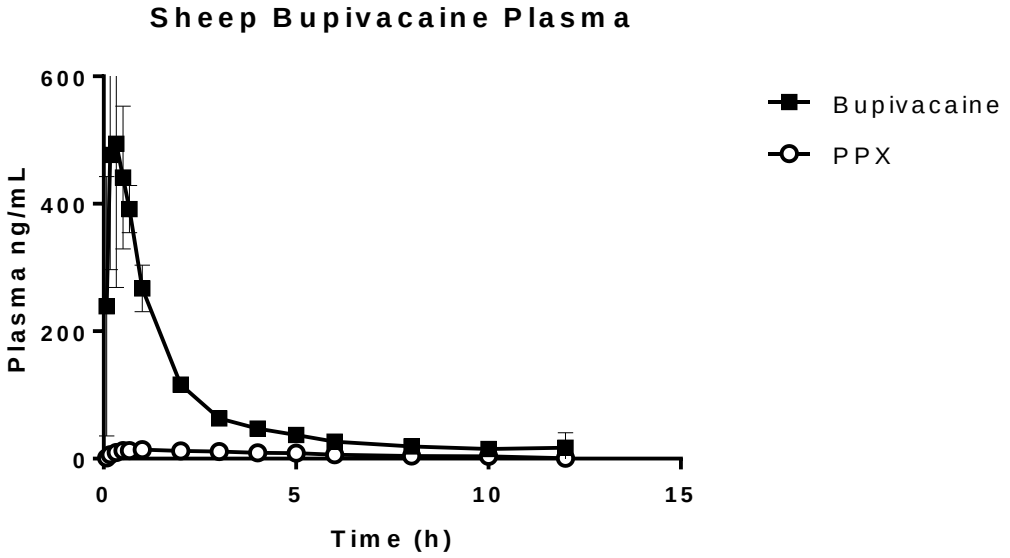

Fig. 10 Plasma concentration versus time in sheep following bupivacaine 5mg/kg subcutaneous injection. Pipecolylxylidine (PPX) is a minor metabolite. Bupivacaine is not metabolised to DMA. *n* = 3 (mean ±SD

4.1.2 Urine

In cattle, DMA constituted 98.7% (98.3-99.2) of the lignocaine metabolites recovered in urine. In sheep, DMA only constituted 3.6% (1.1-7.8), consistent with the much lower DMA concentrations found in sheep plasma, compared with concentrations in cattle plasma.

Percentage recovery of lignocaine and metabolites in urine was low in both cattle and sheep. In sheep the mean and range recovery was 9.0% (5.9-12.2) of the dose administered. In cattle the mean and range recovery was 12.2% (9.3-15.2). This level of recovery was consistent with the 8.4% (7.4-10.2) urinary recovery of lignocaine and metabolites after injection into cows (Hoogenboom et al., 2015). Because a deuterated 4-OH-DMA standard was not used, Hoogenboom et al. postulated that 4-OH-DMA may have been a major metabolite in urine. However, in that study concentrations of 4-OH-DMA in tissues was negligible, which suggests the “missing” lignocaine equivalents in urine is not 4-OH-DMA. In the present study 3-OH-DMA and 4-OH-DMA were present in low concentrations in urine.

In cattle, PPX constituted 98.6% (98.0-99.6) of the bupivacaine metabolites recovered in urine, the remainder being parent drug. Whereas in sheep, 99.99% of bupivacaine is excreted unchanged in the urine, with only a trace of PPX detected. Percentage recovery of bupivacaine and metabolites was much higher than for lignocaine, with a 30.8% (8.5-60.5) recovered in sheep and 100% recovery in cattle.

1. Tissue residues

At 72 hours post-administration of lignocaine in sheep, lignocaine, MEGX and GX residues were present in fat, kidney, muscle and liver. At 7 days post-administration of lignocaine in sheep, lignocaine (but no metabolites) was still detectable in fat, kidney, muscle and liver. The residues at 7 days post-administration were at extremely low levels, only detectable through optimisation of the EMR dSPE+polish sample processing for HPLC-MS/MS.

Table 5. Residues in sheep administered 10mg/kg lignocaine SC. *n* = 3 (ng/gm of tissue, mean ±SD). n.d. Not detected

LIGNOCAINE: SHEEP RESIDUES				
	Lignocaine		GX	
Tissue	72h	7 days	72h	7 days
Muscle	0.908 ± 1.065	0.159	0.167	
Fat (intra-abdominal)	0.629 ± 0.297	0.158	0.351	0.242
Fat (peri-renal	5.727 ± 7.216 (0.625, 10.830)	n.d.	0.158	n.d.
Liver	0.024 ± 0.001	0.014	0.568 ± 0.132	n.d.
Kidney	0.390 ± 0.225	0.016	0.588 ± 0.199	0.195
Brain	0.018 ± 0.009	n.d.	0.168 ± 0.100	n.d.

Table 6. Residues in cattle administered 10mg/kg lignocaine SC. *n* = 3 (ng/gm of tissue, mean ±SD). n.d. Not detected

LIGNOCAINE: CATTLE RESIDUES				
	Lignocaine		GX	
Tissue	72h	7 days	72h	7 days
Muscle	17.178 ± 19.319 (3.517, 30.838)	0.031	n.d.	n.d.
Fat (intra-abdominal)	1.189 ± 1.232	0.291	n.d.	0.303
Fat (peri-renal)	0.501 ± 0.026	0.044	n.d.	n.d.
Liver	0.009 ± 0.006	0.006	0.164 ± 0.071	n.d.
Kidney	0.850 ± 0.899	0.026	0.221	n.d.
Brain	0.118 ± 0.034	n.d.	0.183	n.d.

Fat appears to be the tissue in which residues of lignocaine and GX are highest, for both sheep and cattle. Fat is difficult to process for HPLC-MS/MS (Hoogenboom et al, 2015 collected fat but did not report processing thereof or results for this tissue). The sample preparation methodology for fat in this study was necessarily complex to achieve high sensitivity.

At 72 hours and 7 days post-administration of lignocaine in cattle, lignocaine was still detectable in fat, kidney, muscle and liver, again at extremely low levels.

At 72hours and 7 days post-administration of bupivacaine to sheep, bupivacaine was still detectable in muscle, fat,liver and kidney (Table 7.). Bupivacaine was the primary residue. BNOx was at trace levels in muscle. No DMA, 4-OH-DMA or 3-OH-DMA was detected in any tissue at either timepoint.

Table 7. Residues in sheep administered 5mg/kg bupivacaine SC. *n* = 3 (ng/gm of tissue, mean ±SD). n.d. Not detected

BUPIVACAINE: SHEEP RESIDUES				
	Bupivacaine		PPX	
Tissue	72h	7 days	72h	7 days
Muscle	13.612 ± 18.731	0.071	0.125 ± 0.102	0.012
Fat (intra-abdominal)	1.353 ± 0.597	0.148	0.018 ± 0.004	n.d.
Liver	0.083 ± 0.083	0.013	0.039 ± 0.027	0.016
Kidney	0.446 ± 0.341	0.040	0.050 ± 0.040	n.d.

At 72hours and 7 days post-administration of bupivacaine to cattle, bupivacaine was still detectable in muscle, fat,liver and kidney (Table 8.). PPX was the primary residue. No BNOx, DMA, 4-OH-DMA or 3-OH-DMA was detected in any tissue at either timepoint.

Table 8. Residues in cattle administered 5mg/kg bupivacaine SC. *n* = 3 (ng/gm of tissue, mean ±SD). n.d. Not detected

BUPIVACAINE: CATTLE RESIDUES				
	Bupivacaine		PPX	
Tissue	72h	7 days	72h	7 days
Muscle	5.079 ± 5.467	0.299	18.304 ± 12.529	0.276
Fat (intra-abdominal)	22.723 ± 28.348	1.486	0.774 ± 0.581	0.119
Liver	5.125 ± 4.760	0.047	101.323 ± 66.608	1.369
Kidney	6.699 ± 7.776	0.041	53.156 ± 9.706	1.600

5 Discussion

In cattle, lignocaine is almost exclusively metabolised to DMA, the chemical of concern to human health, being recognised as being mutagenic *in vitro* and having genotoxic characteristics *in vivo* (IARC 1993; Kirkland etal., 2012). The current EU 14 day milk withhold for lignocaine in cattle is supported by this study, although a 7 day withhold would still be a very conservative approach. Two weeks of milk production following a cow caesarean represents a significant financial loss to the producer. A 28 day meat withhold appears excessive, based on the findings of this study. Pursuing lignocaine through full APVMA registration for use in cattle (at least in dairy) may not be advisable.

In sheep, DMA is only a minor metabolite of lignocaine, with peak plasma concentrations being at least 25-times lower than those found in cattle. From 48 hours post-administration of lignocaine 10mg/kg (more than twice the usual dose) no DMA was detected in plasma or urine of sheep. If tissue residues reflect plasma profiles then lignocaine may be a strong candidate for APVMA registration for use in sheep.

DMA was not detected in any tissues in the 3 day and 7 day post-administration killed sheep and steers. To date, the only other study to investigate lignocaine (and its metabolites) in tissues (from dairy cows) was conducted by Hoogenboom et al, 2015. In that study cows were killed at 48 hours and no DMA was detected in liver and was only present in very low levels in other tissues (apart from the injection site).

The most likely explanation for the low urine recovery of the administered dose is drug binding to faecal contaminants in the urine pools, especially undigested plant material. Although urine recovery using the metabolic crates is virtually 100%, a degree of faecal contamination of the urine is unavoidable. Due to welfare considerations the animals were only loosely tethered in the crates, sufficient to prevent turning around but allowing lateral and back and forth freedom to move. More restrictive tethering, forcing an animal to stand for the full 72 hour period in which blood and urine samples were collected, was considered unethical. This welfare consideration meant that animals had a degree of freedom to move within the confines of the crates; consequently faeces could be deposited on the mesh covering the urine catchment area.

Lignocaine and bupivacaine, and their respective metabolites are all amines with pKa's of between 7 and 9. Within the typical pH range of ruminant urine approximately 50% of the drug molecules would be ionised and therefore able to bind faecal material. Net binding would be expected to increase further as equilibrium was established.

For the purposes of establishing a definitive mass balance of lignocaine, animals would need to have indwelling urinary catheters placed. This would necessitate using ewes and heifers. Indwelling urinary catheters need to be carefully maintained and animals' movement restricted. Because animals had to be able to lay down in this study, indwelling urinary catheters (in ewes and heifers) would have been impractical and prone to failure.

Further work could possibly include analysis for the many potential DMA metabolites, beyond 3-OH- and 4-OH-DMA. 4-OH-DMA is also inherently unstable, and has a multitude of metabolites of its own, many of which are increasingly reported as the actual genotoxic molecules. In rats DMA is metabolised to a number of molecules which can form adducts with DNA, conferring these "metabolites of a metabolite" with carcinogenic potential (Tydén et al., 2004). It is possible that some of the "missing" lignocaine profile is composed of these. Many of these metabolites are unstable or unavailable as standards and would need to be custom synthesised and stability assessed. It would be prudent to explore the faecal contamination cause first before embarking on this very expensive path.

The mass balance for bupivacaine was in stark contrast for that of lignocaine. 30.8% of bupivacaine dose was recovered in the urine of sheep and 100% recovered in the urine of cattle. Part of the high recovery is probably due to bupivacaine's metabolism; in sheep bupivacaine is excterd unchanged and in cattle it is metabolised almost exclusively to PPX. Both bupivacaine and PPX are stable molecules and are unlikely to be affected by the temperatures of β-glucuronidase incubation

involved in urine sample preparation. In contrast, many of the metabolites of lignocaine are volatile and realtively unstable.

6 Conclusions/recommendations

In cattle and sheep, DMA is not a metabolite of bupivacaine. For this reason alone bupivacaine is the ideal candidate for registration as the food safety concern of DMA is bypassed. Bupivacaine has the added advantage of a longer duration of action (which has welfare implications) and its extensive use in human obstetrics means that safety in humans has been thoroughly investigated. The human registered formulation of bupivacaine is expensive, however bupivacaine is not inherently more difficult to manufacture than lignocaine; it should be possible to produce a relatively inexpensive veterinary formulation for use in both sheep and cattle in Australia..

Cattle almost exclusively metabolise bupivacaine to pipecolylxylidine (PPX). In humans PPX is the major metabolite of bupivacaine; PPX is then hydroxylated then forms glucuronide conjugates (Gantenbein et al, 2000). There is no mention in the literature of PPX toxicity. If PPX is not a potentially carcinogen/genotoxin then APVMA registration may be possible, especially with PPX being the “sole” metabolite making a mass balance simpler to present. Sheep excrete bubivacaine unchanged in the urine. Pursuing bupivacaine registration for use in sheep is strongly advised to be the priority.

In sheep lignocaine has a terminal half-life of 40 minutes. Bupivacaine has a half-life of 1-2 hours, offering a significant advantage in duration of analgesia, which has obvious welfare benefit. The onset of action is slightly slower than for lignocaine, however not sufficient to be ahindrance to its use clinically and in the field. The introduction of a local anaesthetic with a duration of analgesia at least as twice as long as the currently used lignocaine would be most welcomed. Bupivacaine has the added advantage of not being metabolised to 2,6-xylidine, thereby bypassing the residue human dafety concerns concerns of this potentially carcinogenic metabolite. That bupivacaine is injected into tens of thousands of pregnant women every day attests to its human safety.

Pursuing the registration of bupivacaine for use in cattle and sheep is advised. Such an approach in Australia, if successful, is likely to be adopted world-wide.

7 Bibliography

1. Alexson, Diczfalusy, Halldin and Swedmark (2002). Involvement of liver carboxylesterases in the in vitro metabolism of lidocaine. *Drug metabolism and disposition* 30:643–647,
2. Bloedow, Ralston and Hargrove (1980). Lidocaine pharmacokinetics in pregnant and nonpregant sheep. *J Pharmaceutical Sc*, 69:32-37
3. Caldwell, Notarianni, Smith and Snedden (1977). The metabolism of bupivacaine in the rat. *Brit J Anaesth* **61**:135P
4. Copeland, Ladd, Xiao-Qing and Mather (2008). The effects of general anaesthesia on whole body and regional pharmacokinetics of local anesthetics at toxic doses. *Anesth Analg.* 106:1140-9
5. Coutts, Torok-Both, Chu, Tam, Pasutto (1987). In vivo metabolism of lidocaine in the rat: isoloation of urinary metabolites as pentaflurobenzoyl derivatives and their identification by combined gas chromatography-mass spectrometry. *J Chromatogr* 421:267-280.
6. Cox, Wilson and Doherty (2011) Pharmacokinetics of lidocaine after intravenous administration to cows *J. vet. Pharmacol. Therap*. 35, 305–308
7. Dadak, Franz, Jager, Tichy, Baumgartner, Mosing (2008) Pharmacokinetics and clinical efficacy of lidocaine in cattle after intranasal administration during rhinotracheobronchoscopy. *J. vet. Pharmacol. Therap* 32, 300–302,
8. Dennhardt, Fricke, Stöckert (1978) Metabolism and distribution of bupivacaine-experiments in rats I Methods and metabolism. *Der Anaesthesist* **27**(7):59-64
9. DeRossi, Verede-Selva, Bertoni, Ruzzon and Silva-Neto (2010). Lignocaine versus upivacaine for flank anaesthesia using multiport catheters via a caudal epidural approach in cattle. *Australian Vet. J.* 88:362-367.
10. Dickerson and Apfelbaum (2014). Local anesthetic systemic toxicity. *Anesth. Surg. J.* 34(7): 1111-1119.
11. Fricke Stöckert (1978) Metabolism and distribution of bupivacaine-experiments in rats. II. Distribution and elimination Der Anaesthesist 27(10):86-92
12. Fricke Stöckert; 1978 Metabolism and distribution of bupivacaine-experiments in rats. II. Distribution and elimination Der Anaesthesist 27(10):86-92
13. Keenaghan and Boyes (1972). The tissue distribution, metabolism and excretion of lidocaine in rats, guinea pigs, dogs and man. *J Pharmacol Exp Ther* 180:454–463.
14. Hoogenboom, Zuidema, Essers, van Vuuren, van Wikselaar, van Eijkeren, Mengelers, Zeilmaker and Bulder (2015) Concentrations of dimethylalanine and other metabolites in milk and tissues of dairy cows treated with lidocaine. *Food Additives and contaminants: Part A* 32:1256-1264.
15. Imaoka, Enomoto, Oda, Asada, Fujimori, Shimadam, Fujita, Guengerich and Funae (1990). Lidocaine Metabolism by Human Cytochrome P-450s Purified from Hepatic Microsomes: Comparison of Those with Rat Hepatic Cytochrome P-450s *J Pharmacology Exp (20Therapeutics* 255:3;1385-1391.
16. IARC. 1993. Monographs on the evaluation of carcinogenic risks to humans, 2,6-dimethylaniline (2,6-xylidine). Lyon: World Health Organisation, International Agency for Research on Cancer. Vol. 57. 323-325.
17. Kirkland,Ballantyne, Harlfinger, Will, jahnel, Kraus and van Dorp (2012). Further investigations into the genotoxicity of 2,6-xylidine and one of its key metabolites. Reg Toxicol Pharmacol. 62:151-159.
18. Lizarraga I, Janovyak E, Beths T (2013). Comparing lidocaine, bupivacaine and a lidocaine-bupivacaine mixture as a metacarpal block in sheep. *Vet J.* 197:515-8
19. Mather Huang, Veering, Pryor (1998). Systemic and Regional Pharmacokinetics of Levobupivacaine and Bupivacaine Enantiomers in Sheep *Anesth Analg*;86:805-11)

20. Morishima, Finster, Pedersen, Fukunaga, Ronfeld, Vassallo, Covino (1979). Pharmacokinetics of lidocaine in fetal and neonatal lambs and adult sheep. Anesthesiology 50:431-436.
21. Morishima, Ishizaki, Zhang, Whittington, Raymond Suckow, Cooper (2000). Disposition of bupivacaine and its metabolites in the maternal, placental, and metal compartments in rats *Anesthesiology.* **93**(4):1069-74
22. Oda, Imaoka, Nakahira, Asada, Fujimori, Fujita and Funae Y (1989) Metabolism of lidocaine by purified rat liver microsomal cytochrome P-450 isozymes. *Biochem Pharmacol* 38:4439–4444.
23. Reynolds (1971) Metabolism and excretion of bupivacaine in man: a comparison with mepivacaine. *Br. J. Anaesth.* **43**(1):33-37
24. Rydevik, Bondesson, Hedeland (2012) Structural elucidation of phase I and II metabolites of bupivacaine in horse urine and fungi of the Cunninghamella species using liquid chromatography/multi-stage mass spectrometry. *Rapid Commun Mass Spectrom.* **26**:11:1338-46
25. Santos Pedersen, Morishima, Finster, Arthur, Covino (1988). Pharmacokinetics of Lidocaine in Nonpregnant and Pregnant Ewes. Anesth Analg .67 1154-8
26. Sellers G, Lin HC, Riddell MG, Ravis WR, Duran SH, Givens MD (2009). Pharmacokinetics of lidocaine in serum and milk of mature Holstein cows. *J. vet. Pharmacol. Therap*. 32, 446–450.
27. Tam YK, Tawfik SR, Coutts RT, Gray MR, and Wyse DG (1987) High-performance liquid chromatography of lidocaine and nine of its metabolites in human plasma and urine. *J Chromatogr* 423:199–206.
28. Thusen and Friis, 2012 Thuesen and Friis (2012). The in vitro metabolism of lidocaine in pig cattle and rat. *J vet Pharmacol Therapeutics* 35 (suppl3);157.
29. Tydén, Tjälve and Larsson (2004). Metabolic activation of 2,6-xylidine in the nasal olfactory mucosa and the mucosa of the upper alimentary and respiratory tracts in rats. *Toxicol Sciences.* **81**:263-272
30. Zheng Mi, Li, Chen (2013) Determination of Xylazine and 2,6-Xylidine in Animal Tissues by Liquid Chromatography—Tandem Mass Spectrometry *Journal of Food Science* 78:955-959.
31. Santos Arthur,. Lehning, Finster, 1997). Comparative Pharmacokinetics of Ropivacaine and Bupivacaine in Nonpregnant and Pregnant Ewes. *Anesth Analg* 85:87-93
32. Zhang, Mitchell and Caldwell (1998) The application of capillary gas chromatography-selective ion mass spectrometry for the separation, identification and quantification of phenolic bupivacaine metabolites from human urine. *Journal of Pharmaceutical and Biomedical Analysis* **17**:1139–1142