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In vitro metabolism of lidocaine in subcellular post-mitochondrial fractions and precision cut slices from cattle liver

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ABSTRACT

In vitro models are widely used to study the biotransformation of xenobiotics and to provide input parameters to physiologically based kinetic models required to predict the kinetic behavior *in vivo*. For farm animals this is not common practice yet. The use of slaughterhouse-derived tissue material may provide opportunities to study biotransformation reactions in farm animals. The goal of the present study was to explore the potential of slaughterhouse-derived bovine liver S9 (S9) and precision cut liver slices (PCLSs) to capture observed biotransformation reactions of lidocaine in cows. The *in vitro* data obtained with both S9 and PCLSs confirm *in vivo* findings that 2,6-dimethylaniline (DMA) is an important metabolite of lidocaine in cows, being for both PCLSs and S9 the end-product. In case of S9, also conversion of lidocaine to lidocaine-N-oxide and monoethylglycinexylidine (MEXG) was observed. MEGX is considered as intermediate for DMA formation, given that this metabolite was metabolized to DMA by both PLCSs and S9. In contrast to *in vivo*, no *in vitro* conversion of DMA to 4-OH-DMA was observed. Further work is needed to explain this lack of conversion and to further evaluate the use of slaughterhouse-derived tissue materials to predict the biotransformation of xenobiotics in farm animals.

1. Introduction

Insight in the transfer of chemicals and their species-specific metabolites from feed to food is an important aspect of estimating human health risks related to animal derived products. To date, information on metabolites and transfer factors are generally derived from animal studies. However, this reliance on animal experimental results makes the availability of such information scarce (Leeman et al., 2007). An important step forward is the use of *in vitro* models from farm animals combined with physiologically based kinetic (PBK) models to simulate the absorption, distribution, metabolism, and excretion of chemicals in food producing animals (Lautz et al., 2019; Thiel et al., 2015). Such models can provide a mean to predict the concentrations in *e.g.* (muscle) meat, milk or eggs over time and at different feed contamination levels (Lautz et al., 2020; MacLachlan, 2009; MacLachlan, 2010; van Eijkeren et al., 1998). The use of *in vitro* and *in silico* chemical-specific data as input for the development of these models has gained increasing attention over the last years, especially in the field of human risk assessment (Paini et al., 2019; Tan et al., 2018). However, for farm animals this is not common practice yet (EFSA, 2020).

The use of slaughterhouse-derived tissue material provides opportunities to study biotransformation reactions in farm animals to obtain the required input parameters to PBK model development. Next to isolated hepatocytes and microsomes, precision cut liver slices (PCLS) can be used as an alternative method for the assessment of xenobiotic metabolism. The PCLS approach maintains the tissue architecture and functional heterogeneity in order to mimic the metabolic reactions that occur *in vivo* (Santi et al., 2002). Previously, the PCLS method was used to investigate the metabolism of several substances such as dehydroepiandrosterone, benzydamine, fenbendazole, triclabendazole, but also for drug-drug interactions (Maté et al., 2019; Maté et al., 2015; Rijk et al., 2012; Santi et al., 2002; Sivapathasundaram et al., 2004; Viviani et al., 2017). For lidocaine *in vivo* studies were previously performed in dairy cows, however, it is important weather the biotransformation reactions seen *in vivo* can be captured *in vitro* by PCLS and a subcellular post-mitochondrial fraction, the liver S9.

Lidocaine is an anesthetic drug used in dairy cows prior to a laparotomy for caesarean section or for repositioning a displaced abomasum,

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for which the drug is injected into the subcutaneous and muscle layers of the abdominal wall. Within the European Union there is no registration for lidocaine as local anesthetic in cows (EMEA, 1999). However, off-label use is permitted under the so-called Cascade rules (EC, 2001) with an off-label withdrawal period of 7 days for milk and 28 days for meat. The study by Hoogenboom et al. (2015), in which eight dairy cows were treated with lidocaine by injection in the abdominal muscles, revealed that levels of lidocaine and metabolites in milk and meat decrease rapidly after the application and that the off-label withdrawal times of 7 and 28 days for milk and meat, respectively, guarantee the absence of detectable levels of lidocaine and metabolites (including DMA) in meat and milk.

In mammalian species, lidocaine is mainly cleared *via* liver metabolism. Fig. 1 provides a schematic overview of the major metabolic pathways reported for different animal species (*e.g.*, cows, rats, dogs) and humans on the basis of both *in vitro* and *in vivo* studies (Delehant et al., 2003; Gan et al., 2001; Hoogenboom et al., 2015; Short et al., 1989). Among the different metabolites, 2,6-dimethylaniline (DMA) poses a concern as it is reported to possess carcinogenic and mutagenic properties (Duan et al., 2008; Skipper et al., 2009). This metabolite can be formed from lidocaine itself, or indirectly from another lidocaine metabolite, monoethylglycinexylidine (MEGX). DMA can be further converted to 4-OH-DMA which was shown to be the major metabolite in urine of cows (Hoogenboom et al., 2015). Important detoxification routes of lidocaine are hydroxylation to 4- and 3-OH-lidocaine, as well as N-oxidation to lidocaine-N-oxide (Delehant et al., 2003; Duan et al., 2008; Gan et al., 2001; Hoogenboom et al., 2015; Short et al., 1989). After phase I metabolism (Fig. 1), the hydroxylates metabolites undergo phase II metabolism by addition of a glucuronic acid moiety, which was confirmed in horse and sheep urine (Dirikolu et al., 2000; Doran et al., 2018).

The key question is whether insights in the metabolism and clearance can be obtained with an animal-experiment free approach. *In vitro*-based predictions of the fate of DMA in cows requires insights in the kinetics of the formation of DMA from lidocaine (from lidocaine itself or indirectly from MEGX as intermediate metabolite of lidocaine), and the further conversion of DMA into 4-OH DMA. These different metabolites have been detected in incubations with bovine hepatocytes and liver microsomes in a study by Thuesen and Friis (2012).

The goal of the present study was to explore whether *in vivo* observed biotransformation reactions of lidocaine in cows, as recently observed in an *in vivo* experiment with dairy cows (Hoogenboom et al., 2015), can be captured *in vitro* with slaughterhouse-derived precision cut liver slices (PCLS) and liver S9. An advantage of PCLSs is that the whole metabolic system is present, but problems might occur with diffusion of the chemical into the tissue layer (van Eijkeren, 2002). Diffusion is not rate limiting in incubations with S9, but this model may not capture the whole metabolic apparatus as this depends on the co-factors that are added (Gouliarmou et al., 2018). The possibilities of both *in vitro* models in providing information on the metabolism were therefore evaluated.

2. Materials and methods

2.1. Chemicals

HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)), sodium bicarbonate, glucose, lidocaine, MEGX (Monoethylglycinexylidide), DMA (2,6-dimethylaniline), potassium chloride and NADPH were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Tris (Tris(hydroxymethyl)aminomethane) and Williams-E medium (with Glutamax I, without phenol red) were purchased from Fisher Scientific (Landsmeer, The Netherlands). Lidocaine and lidocaine metabolite standards for LC-MS (lidocaine, 3-OH-lidocaine, 3-OH-lidocaine-D5, 4-OH-lidocaine, 4-OH-lidocaine-D10, 4-OH-DMA, lidocaine-N-oxide, MEGX-d5, DMA-d6) were purchased from Toronto Research Chemicals (North York, ON, Canada). Methanol was purchased from Actu-All (Oss, The Netherlands), ammonia solution (32%) from VWR International (Amsterdam, The Netherlands) and formic acid from Merck (Schiphol-Rijk, The Netherlands).

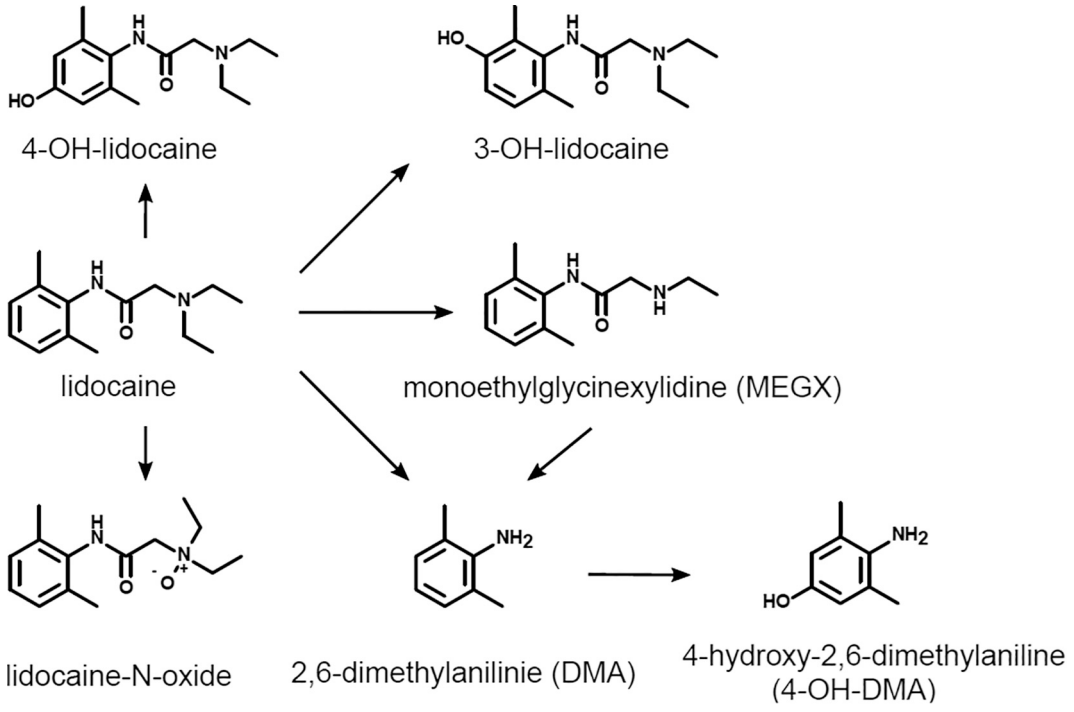

Fig. 1. Major metabolic pathways of lidocaine.

2.2. Preparation of Precision Cut Liver Slices (PCLSs) and S9 fractions of bovine liver

Fresh bovine livers from two cows were obtained from the slaughterhouse on two independent days. The caudate lobes were cut off immediately after the livers were removed from the carcasses. The tissues were perfused with ice cold oxygenated Krebs buffer (pH 7.4) containing 10 mM HEPES, 25 mM sodium bicarbonate and 25 mM glucose. A part of each lobe was cut in small pieces and snap frozen in liquid nitrogen for preparation of the S9-fraction (see below). The remaining parts were transported to the laboratory on ice in oxygenated Krebs buffer. The time between killing the animal and preparing PCLSs was approximately one hour. Cylindrical liver cores were prepared using a drill with a hollow bit. The cores were stored in ice-cold Williams-E medium with Glutamax I, without phenol red (WE) and without supplements. PCLSs were prepared using a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA) filled with ice-cold WE. Slices were 250 μm thick and the diameter was 8 mm. They were kept on ice in WE medium until use.

For the preparation of S9, frozen liver tissue (derived from liver material of one of the cows) was added to ice cold Tris/KCl buffer (50 mM Tris/HCl pH 7.4, 1.15% KCl; 2 mL per gram tissue) and homogenized in a stainless steel blender (Waring, Torrington, CT, USA). The homogenate was centrifuged 25 min at 9000*g* and 4 °C. The supernatant was collected, snap frozen in liquid nitrogen and stored at −80 °C. The protein concentration (52 mg/mL) was determined using the DC protein assay (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturers protocol.

2.3. Incubations with bovine precision cut liver slices

PCLSs were incubated in 6-well-culture plates (Corning, Schiphol-Rijk, The Netherlands). Each well contained 3 mL of WE-medium with Glutamax supplemented with 25 mM glucose. To saturate the medium with oxygen, the plates were incubated for 30 min in an O_2/CO_2 incubator at 37 °C, 80% O_2, 15% N, 5% CO_2 and rotated at 60 rpm. Each well contained three slices and plates were pre-incubated in the O_2-incubator for approximately 20 min. Incubations were started by adding the compounds to the wells. Slices were incubated with lidocaine, MEGX and DMA at concentrations of 1 μM from a 200 times concentrated stock in DMSO, and 0.5% DMSO as vehicle control. In addition, all compounds were incubated without slices to test the stability in WE-medium during the time course of the experiment. After 0, 15, 30, 45 and 60 min, 100 μL of incubation medium was transferred to a tube containing 100 μL ice cold methanol. The samples were vortexed thoroughly and put on ice. *T* = 0 samples were taken immediately after adding and mixing the compounds. Samples were stored at −80 °C. The weight of control slices was determined at the start and end of the experiment.

2.4. Incubations with bovine liver S9

Incubations with bovine liver S9 were performed in low-binding Eppendorf tubes. The incubation mixtures contained (final concentrations) 5 mM NAPDH, 5 mM glucose-6-phosphate, 2.5 mg S9 protein per mL and 1 μM lidocaine, MEGX or DMA, in Regensys A (100 mM sodium phosphate buffer pH 7.4, 33 mM KCl, 8 mM $MgCl_2$; Trinova Biochem, Giessen, Germany) at 37 °C. The substrates were prepared from stock solutions in DMSO (6.25 mM), diluted first to 1 mM in methanol/water (50:50) and then diluted to 1 μM in the incubation mixture. The final percentage of solvent was thereby 0.016% DMSO and 0.042% methanol. Aliquots of this mixture were added to the Eppendorf tubes and reactions were started after a pre-incubation period of 5 min at 37 °C by addition of the S9. After 10, 20, 40, and 80 min, 100 μL of sample was transferred to a tube containing 100 μL ice-cold methanol to stop the reaction. The samples were vortexed thoroughly and put on ice. T = 0 samples were prepared by adding 100 μL of ice-cold methanol to 95 μL of the incubation mixture prior to the addition of 5 μL S9. Blank incubations were carried out without S9. Samples were stored at −80 °C.

The intrinsic clearance (Clint) values were determined by plotting the natural logarithm (ln) of substrate concentrations against time. The slope of the linear part of these ln-transformed substrate depletion curves represents the elimination rate constants (k, min^{-1}). After calculation of the half-life of each compound ($t_{1/2}$ (min) = ln(2)/k (min^{-1})) and incubation volume (V (μL/mg) = 1000 / [liver S9] (mg/mL), CLint was calculated by: CLint (μL/min/mg protein) = V (μL/mg) * ln(2)/$t_{1/2}$ (min).

2.5. Sample and standard preparation for U-HPLC analysis

Samples from the liver slices and S9 experiments were thawed, mixed thoroughly and centrifuged at 20817 x*g* for 10 min. 80 μL of the clear supernatant was mixed with 50 μL water/NH_3 32% solution (100/0.51) and transferred to glass ultra-recovery LC-MS vials (Grace, USA). The final concentrations of the internal standards were 27 μg/L DMA and 5.4 μg/L for lidocaine, 3-OH-lidocaine, and MEGX. Standard dilution series of lidocaine, DMA, MEGX, 3-OH-lidocaine, 4-OH-DMA and lidocaine-N-oxide were prepared in matrix (*i.e.* the S9-incubation mixture containing buffer, S9, NADPH, and the equivalent percentage solvent, mixed 1:1 with cold methanol or, for PCLSs, incubation medium of PCLS with the vehicle control). Also, for these reference samples, 80 μL of solution was mixed with 50 μL water/NH_3 32% solution before analysis. The final concentrations of the standards ranged from 0 to 8 μg/L for lidocaine, MEGX, lidocaine-N-oxide, 3-OH-lidocaine, and 4-OH-lidocaine, and 0 to 40 μg/L for DMA and 4-OH-DMA. NH_3 was added to the samples for adequate analytical detection. 4-OH-DMA appeared to be unstable after addition of NH_3, but during the course of one set of analyses, the reduction in the concentrations was less than 10%, which was considered to be acceptable.

2.6. LC-MS/MS

Ultra-high-performance liquid chromatography (U-HPLC) was performed using detection by a Waters TQ-S mass spectrometer with electrospray source. Samples were kept at 10 °C in the autosampler and 5 μL of each sample was separated on an Aquity UPLC BEH C18, 1.7 μm, 2.1 × 100 mm column from Waters (Etten-Leur, The Netherlands). The mobile phases consisted of 0.02% formic acid in water (A) and 0.02% formic acid in MeOH (B). The flow rate was 0.3 mL/min. After injection, the composition of the mobile phase was linearly changed from 100% A to 100% B in 4 min, kept for 2 min at 100% B, followed by a linear decrease to 100% A in 0.5 min. These conditions were maintained for 6 min to stabilize the system prior to the next injection. The temperature of the column oven was 50 °C. Samples were measured in the positive mode and data were acquired in MRM mode (see Appendix I). The substrates (lidocaine, MEGX and DMA) of the incubations were monitored and quantified based on the standard reference compounds, as well as all potential metabolites depicted in Fig. 1.

3. Results

3.1. Metabolic conversion of lidocaine by bovine liver S9

Both the metabolic reactions that were expected to lead to formation DMA (from lidocaine and from MEGX) and the further metabolic conversion of DMA were explored in incubations with bovine liver S9 using NADPH as co-factor. Fig. 2 depicts these metabolic conversions, representing the results of 4 replicates obtained on two independent dates. Fig. 2A reveals that the concentration of lidocaine decreased from 0.70 to 0.28 μM during the 80 min incubation. Three metabolites were identified, as being DMA, MEGX and lidocaine-N-oxide. Of these, DMA represented 70, 67, 63 and 59% of the sum of these 3 metabolites at, respectively, 10, 20, 40 and 80 min. This was followed by MEGX with 7,

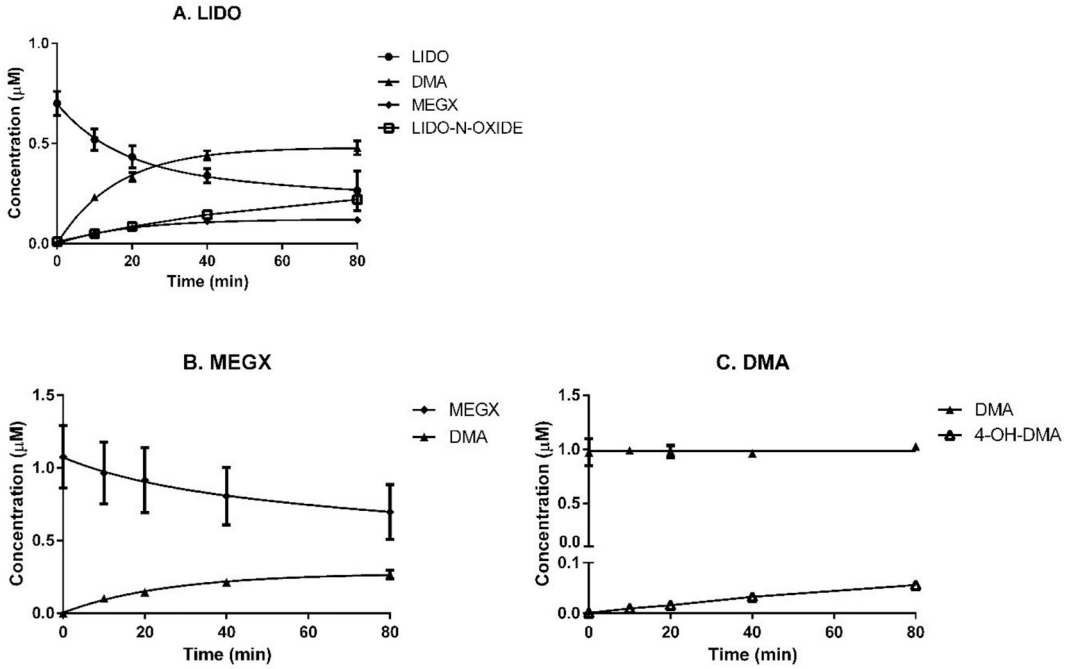

Fig. 2. Substrate depletion and metabolite formation as observed in incubations with lidocaine (A), MEGX (B), or DMA (C) with bovine liver S9 and NADPH as cofactor. Traces of 4-OH-DMA were formed in the incubations with DMA. Results from 4 replicates performed on two independent days (mean ± SD).

11, 16 and 17%, and lidocaine-N-oxide, with 7, 12, 21 and 32% at these 4 time points. Whereas the formation of DMA and MEGX levelled off after 40 min, that of the N-oxide further increased. Based on the linear part of the log transformed data of the incubation of lidocaine, a $t_{1/2}$ of 29 min was calculated, corresponding to an intrinsic clearance of lidocaine of 9.6 μL/min/mg S9 protein. Based on a protein content of 52 mg/mL S9 and starting from 1 g liver homogenized with 2 mL buffer, the S9 protein concentration would be 156 mg/g liver. This implies that the clearance of lidocaine would be 1.5 mL/min/g liver.

When incubated with MEGX, only DMA was detected (Fig. 2B). Based on the linear part of the log transformed data, a $t_{1/2}$ of 86 min was calculated, corresponding to an intrinsic clearance of 3.2 μl/min/mg S9

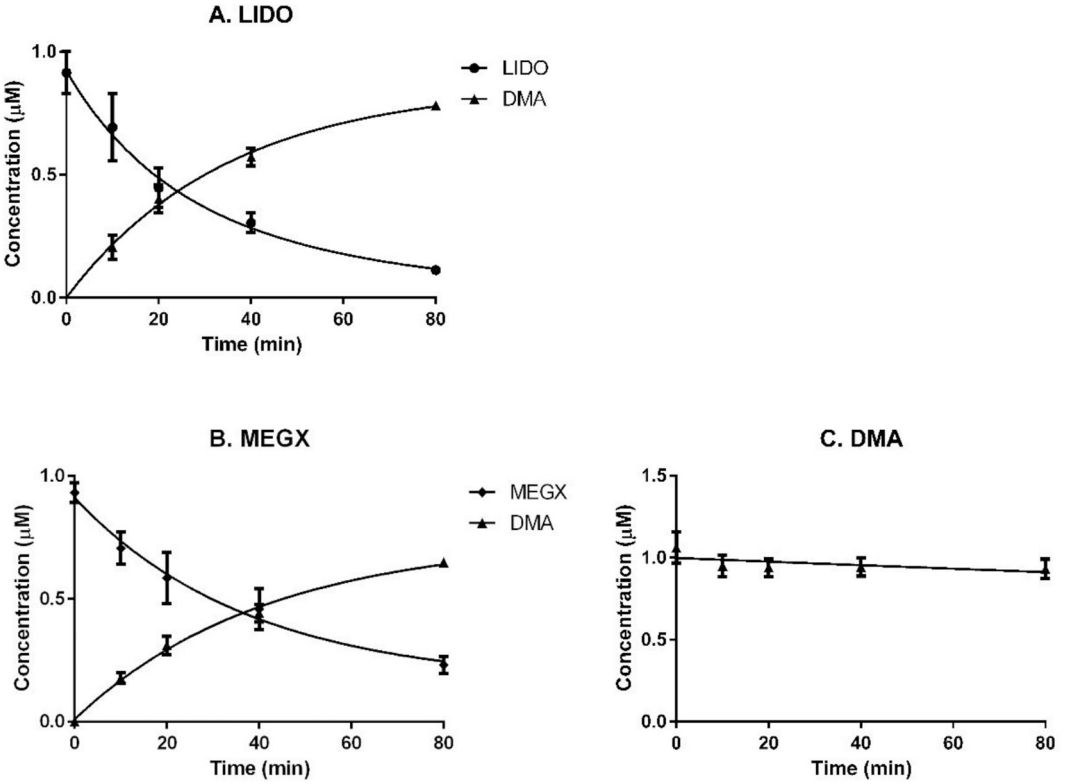

Fig. 3. Substrate depletion and metabolite formation as observed in incubations with bovine PCLSs with lidocaine (A), MEGX (B) or DMA (C). No conversion of DMA to 4-OH-DMA was observed. Results from 4 replicates performed on two independent days (mean ± SD).

protein corresponding to 0.5 mL/min/g liver. The incubations of DMA with bovine liver S9 revealed little conversion (Fig. 2C) and only a very small amount of 4-OH DMA was formed.

3.2. Metabolic conversion of lidocaine by bovine precision cut liver slices (PCLSs)

The results obtained after incubating bovine PCLSs with lidocaine, MEGX or DMA are shown in Fig. 3. In the case of lidocaine (Fig. 3A), DMA was observed but no MEGX or lidocaine-N-oxide, in contrast to the incubations with S9 (Fig. 2B), which is the result of a faster conversion of MEGX to DMA by the PCLSs (Fig. 3B). Based on the linear parts of the log transformed data, $t_{1/2}$ values of 19 and 40 min were calculated for, respectively, lidocaine and MEGX, corresponding to an intrinsic clearance of 2.0 and 0.9 mL/min/g liver slice, respectively. These are slightly higher but in a similar range as calculated for S9, being respectively 1.5 and 0.5 mL/min/g liver. Similar to the incubations with S9, no further conversion of DMA was observed, with even no traces of 4-OH-DMA in the case of incubations with DMA.

4. Discussion

The goal of the current study was to explore the potential of *in vitro* models to capture *in vivo* observed metabolite formation. The *in vitro* data obtained with both S9 and PCLSs confirm the *in vivo* findings that DMA is an important metabolite of lidocaine (at least partly formed *via* the intermediate metabolite MEGX). However, the subsequent conversion of DMA to 4-OH DMA did not take place in the *in vitro* incubations, whereas this latter metabolite was the major metabolite in the urine of treated cows (Hoogenboom et al., 2015). In plasma, milk and tissues this metabolite was detected at relatively low levels, as compared to lidocaine and DMA. Also MEGX and the N-oxide occurred in relative low levels. The *in vitro* data are in line with the latter result, also revealing little formation of MEGX and N-oxide. When S9 was incubated with MEGX, high concentrations of DMA were observed, which suggests that DMA is the major metabolite, and that it can be formed from lidocaine itself or indirectly *via* MEGX.

Given the lack of metabolic conversion of DMA to 4-OH-DMA in the incubations with S9 and PCLSs, the *in vitro* incubations did only partly mimic the *in vivo* situation, since in cows 4-OH-DMA was a major metabolite of lidocaine excreted in the urine (Hoogenboom et al., 2015). Also, in the study by Thuesen and Friis (2012) with bovine hepatocytes and microsomes, formation of 4-OH-DMA was reported. However, it is shown previously that this conversion is highly instable and that unknown cofactors could be missing (Parker et al., 1996). Multiple hypotheses were postulated and explored to explain the lack of conversion of DMA to 4-OH-DMA in the incubations with S9 and PCLSs. A key question rises whether the prepared PCLS and S9 contained sufficient cytochrome P450 activity. For humans the conversion of DMA to 4-OH-DMA has been reported to be catalysed by cytochrome P450 2E1 (Gan et al., 2001). Incubations with chlorzoxazone (a CYP2E1 marker substrate) revealed that relevant CYP2E1 activity was present in the prepared bovine liver S9 (Appendix II) and suggests that a lack of enzyme activity is not the underlying cause for lack of conversion of DMA to 4-OH-DMA. Another hypothesis could be that the reaction does not occur in the liver of the cow, but in other organs like the kidneys, which are involved in the excretion of 4-OH-DMA into the urine. Incubations with bovine kidney S9 revealed, however, no conversion of DMA to 4-OH-DMA (Appendix II).

A direct effect of DMA itself on the enzyme was also considered, particularly since a reduction in enzyme activity over time was observed during the incubations of lidocaine and MEGX with S9. These substrate depletion curves followed a bi-phasic decay, with initially a fast conversion of the substrates followed by a slow conversion. Such a bi-phasic decay is often a result of end-product inhibition (Jones et al., 2005), where one or more of the metabolites that are formed inhibit the enzyme activity, or by lability of the enzymes (Foti and Fisher, 2004). Given that DMA is the major metabolite of lidocaine and MEGX, it could be that this metabolite affects the enzyme and almost completely blocks enzyme activity when used as substrate, being a possible reason for the lack of formation of 4-OH-DMA. S9 and microsomes are in general more susceptible than higher tier systems such as primary hepatocytes and liver slices (Jones et al., 2005), which might explain why no bi-phasic decay was observed in the conversion of lidocaine and MEGX in the incubations with PCLSs. However, the possible inhibition by DMA cannot explain why Thuesen and Friis (2012) observed the formation of 4-OH-DMA by bovine hepatocytes and liver microsomes. The instability of the 4-OH-DMA metabolite is also considered, as this metabolite is highly unstable and very difficult to quantify in humans as well in other animals like sheep (Doran et al., 2018; Parker et al., 1996). Yet it is more likely that 4-OH-DMA is not formed in the incubation rather than that it is not picked up due its instability, particularly given that the concentration of DMA did not decrease during the incubation, suggesting that no metabolic conversion takes place.

Overall, further work is needed to explain the lack of conversion of DMA to 4-OH DMA and to use these types of experiments as input to PBK models. However, it can also be concluded that both slaughterhouse-derived bovine liver S9 and PCLSs can provide relevant insights in the conversion of lidocaine. Both techniques can provide fast and adequate information about the metabolism of chemicals in cattle. Integration of obtained *in vitro* metabolism of chemicals provides the basis to further develop quantitative *in vitro* to *in vivo* extrapolation models, which can be implemented in PBK models for animal risk assessment and ultimately limit *in vivo* testing in farm animal species.

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Declaration of Competing Interest

None.

Appendix I: TQS

	Chan Reaction	Dwell (secs)	Cone Volt	Col. Energy	Delay (secs)	Compound
1	122.00 > 77.00	0.015	30.0	20.0	Auto	DMA
2	122.00 > 105.10	0.015	30.0	15.0	Auto	DMA
3	128.00 > 111.20	0.015	30.0	15.0	Auto	DMA-d6
4	138.00 > 121.10	0.015	30.0	15.0	Auto	4-OH-DMA
5	138.00 > 123.10	0.015	30.0	15.0	Auto	4-OH-DMA
7	207.20 > 58.10	0.015	30.0	12.0	Auto	MEGX
8	207.20 > 122.20	0.015	30.0	12.0	Auto	MEGX

(*continued on next page*)

(*continued*)

	Chan Reaction	Dwell (secs)	Cone Volt	Col. Energy	Delay (secs)	Compound
9	212.20 > 63.20	0.015	30.0	13.0	Auto	MEGX-d5
10	235.10 > 58.10	0.015	30.0	30.0	Auto	LIDO
11	235.10 > 86.00	0.015	30.0	15.0	Auto	LIDO
12	245.20 > 96.20	0.015	30.0	18.0	Auto	LIDO-d10
13	251.20 > 58.10	0.015	30.0	35.0	Auto	3-OH-LIDO +4-OH-LIDO
14	251.20 > 86.10	0.015	30.0	18.0	Auto	3-OH-LIDO +4-OH-LIDO + LIDO-N-OXIDE
15	251.20 > 120.00	0.015	30.0	25.0	Auto	LIDO-N-OXIDE
16	256.20 > 91.20	0.015	30.0	20.0	Auto	3-OH-LIDO-D5

TQS: Capillary 2.5 kV; Cone voltage: 30; Source temperature: 150; Desolvation temperature: 600; Cone gas flow: 150 L/h; Desolvation gas flow: 800 L/h; Collison gas flow: 0.18 ml/min; Nebuliser gas flow: 7 bar.
The LC system and the mass spectrometer were both controlled by Masslynx 4.1. This software is also used for data acquisition.

Appendix II: Exploration of possible causes for the lack of conversion of DMA to 4-OH-DMA

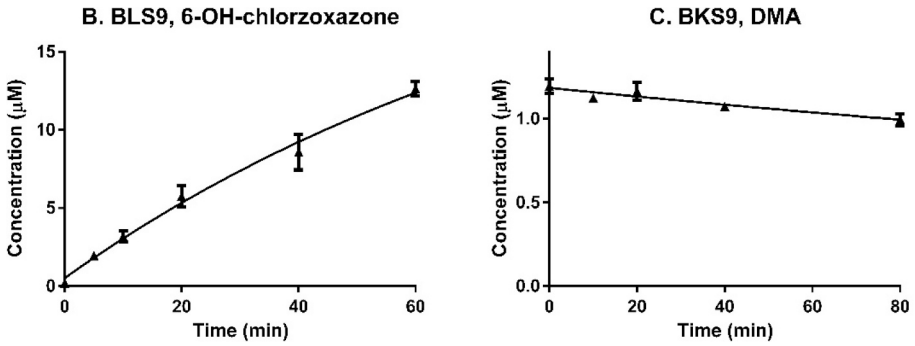

Figure SI1. Control incubations of chlorzoxazone with bovine liver S9 (B), and DMA with bovine kidney S9 (C), in the presence of NADPH as cofactor. Figure SI1B and SI1C are obtained from 4 replicates performed on two independent days (mean ± SD).

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